Povezanost izvanstaničnih molekula proteina toplinskoga šoka 70 i adenozin-trifosfata sa sustavnim upalnim odgovorom u pacijenata s kroničnom opstrukcijskom plućnom bolesti

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ASSOCIATION OF EXTRACELLULAR MOLECULES HEAT SHOCK PROTEIN 70 AND ADENOSINE TRIPHOSPHATE WITH SYSTEMIC INFLAMMATORY RESPONSE IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

DOCTORAL DISSERTATION

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Davor Rostuhar, iz Polarni san

SAŽETAK

Kronična opstrukcijska plućna bolest (KOPB) kompleksna je i heterogena bolest s kroničnom upalom. Upalni odgovor prisutan je na sustavnoj razini, a vidljiv je u obliku povećane koncentracije općih upalnih biljega poput C-reaktivnog proteina (CRP), fibrinogena (Fbg), ukupnog broja leukocita (lkc) te citokina čimbenika tumorske nekroze alfa (TNF)-α, interleukina (IL)-1β, IL-6 i IL-8. Izvanstanični protein toplinskoga šoka 70 (eHsp70) i izvanstanični adenozin-trifosfat (eATP) su molekularni obrasci oštećenja, čija se koncentracija u perifernoj cirkulaciji povećava prilikom upalnih imunosnih reakcija i oštećenja stanica.

Cilj ovog istraživanja bio je odrediti koncentracije eHsp70 i eATP-a u 137 pacijenata sa stabilnim KOPB-om i usporediti ih s koncentracijama u kontrolnoj skupini od 95 ispitanika te ispitati postoji li povezanost ovih parametara sa stupnjem plućne opstrukcije, simptomima i povijesti egzacerbacija te pušačkim statusom. Osim toga, kvantitativnom lančanom reakcijom polimerazom (qPCR, engl. *quantitative polymerase chain reaction*) određena je relativna razina ekspresije gena *HSP70*, *TLR2* i *TLR4* (dva receptora za eHsp70) te *P2X7R* i *P2Y2R* (dva receptora za eATP). Genotipizacija polimorfizama u genima *HSP70*, *TLR2* i *TLR4* provedena je PCR reakcijom na temelju alelne diskriminacije te je ispitana povezanost između odabranih polimorfizama s rizikom od KOPB-a.

Najznačajniji rezultati ovog istraživanja ukazuju na povezanost eHsp70 i eATP-a s KOPB-om, sa stupnjom plućne opstrukcije i progresijom simptoma. Također, koncentracije eHsp70 i eATP-a veće su u skupini zdravih pušača nego u zdravih nepušača što ukazuje na pojavu upalnih procesa prilikom izlaganja cigaretnom dimu prije pojave bolesti. Mrežnom analizom utvrđena je značajna povezanost IL-1β, IL-6, TNF-α, CRP-a, Fbg-a, eHsp70 i eATP-a u pacijenata s KOPB-om, a hijerarhijskom klasterskom analizom potvrđena je heterogenost i kompleksnost bolesti. Nadalje, na temelju analize logističkom regresijom, model sastavljen od IL-1β, eHsp70 i eATP-a mogao bi biti koristan kao pomoćni alat za prepoznavanje pacijenata s KOPB-om. Značajno povećanje genske ekspresije u pacijenata s KOPB-om u odnosu na zdrave ispitanike opaženo je za gene *HSP70*, *TLR2* i *P2Y2R*, dok je analiza polimorfizama ukazala da postoji povezanost između rizika od KOPB-a i polimorfizma rs6457452 u promotorskom dijelu gena *HSP70*.

eHsp70 i eATP biljezi su sustavnog upalnog odgovora u KOPB-u i dio su patogeneze KOPB-a, a u kombinaciji s IL-1β predstavljaju model s velikim predikcijskim potencijalom.

Promjene u ekspresiji gena *HSP70*, *TLR2* i *P2Y2R* iz uzoraka periferne cirkulacije te povezanost polimorfizma rs6457452 u promotorskoj regiji gena *HSP70* dodatno dokazuju da eHsp70 i eATP imaju značajnu ulogu u KOPB-u na sustavnoj razini te doprinose saznanjima o bolesti na razini gena u uzorku hrvatske populacije.

Ključne riječi

kronična opstrukcijska plućna bolest; sustavna upala; izvanstanični protein toplinskoga šoka 70; izvanstanični adenozin-trifosfat; genska ekspresija; polimorfizam jednog nukleotida

SUMMARY

Background: Chronic obstructive pulmonary disease (COPD) is a multicomponent heterogeneous disease characterized by irreversible decline in lung function caused by chronic inflammation and permanent airflow limitation. Among many risk factors, smoking is the greatest, but not all smokers develop COPD which indicates there are other very important both environmental and genetic risk factors. Inflammatory immune response is not only localized in lungs, yet it is considered that systemic inflammation have a great impact on the onset and progression of the disease. Systemic inflammation is characterized by increased concentrations of common inflammatory biomarkers C-reactive protein (CRP), fibrinogen (Fbg), total leukocyte count (lkc), and pro-inflammatory cytokines tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-8 in peripheral circulation.

Extracellular heat shock protein 70 (eHsp70) and extracellular adenosine-triphosphate (eATP) are known as damage-associated molecular patterns (DAMPs) whose concentrations in peripheral blood increase during inflammatory immune response and necrosis.

eHsp70 might have important immunomodulatory function in COPD. By engaging Toll like receptors (TLRs) 2 and 4, it promotes and prolongs chronic inflammation. It is believed that the sources of eHsp70 in peripheral circulation are peripheral blood mononuclear cells as well as epithelial cells. Similarly, eATP is secreted by activated macrophages, neutrophils, epithelial cells, and platelets. It is a ligand for purinergic receptors, among which P2X7R and P2Y2R are commonly investigated. When binding to P2X7R, eATP can induce the activation of nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome which leads to the maturation and release of IL-1β. On the other hand, binding to P2Y2R is associated with neutrophil chemotaxis and signal amplifying.

The aim of this study was to determine eHsp70 and eATP concentrations in patients with stable COPD and compare it with control group as well as to determine the association of eHsp70 and eATP with the stage of airflow limitation, sysmptoms burden and history of exacerbations and with smoking status. As COPD is a complex disease with genetic background, the relative gene expression of *HSP70*, *TLR2*, *TLR4*, *P2X7R* and *P2Y2R* was investigated in both groups. Moreover, association of single nucleotide polymorphims (SNPs) of *HSP70, TLR2* and *TLR4* with the risk of COPD was determined.

Common inflammatory biomarkers and cytokines are not COPD-specific parameters, yet their increase in peripheral circulation is associated with ongoing inflammation in COPD. Instead

of the determination of a single parameter, it is considered that a group of biomarkers might be better in stratifying COPD phenotypes based on clinical features. Therefore, the aim of the study was also to clarify the relations between the selected parameters in COPD patients.

Materials and methods: 137 COPD patients and 95 age- and sex-matched controls were included in the study. COPD diagnosis was confirmed by pulmonologists based on spirometry parameters, and the patients were subdivided into the groups according to the severity of airflow limitation as well as symptoms burden and history of exacerbations. Self-reported data about smoking were collected and included in the analysis. Blood samples were collected after overnight fasting by venepuncture of a large antecubital vein. Different tubes were used for the blood collection and samples were prepared as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines. eHsp70 concentration was determined in plasma using the AMP'D HSP70 high sensitivity enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Science, Farmingdale, NY, USA), while eATP was measured by luminescence ATPlite assay (Perkin Elmer, Waltham, MA, USA). Plasma was also used for cytokines determination which was performed using Platinum Procarta Plex Kit and ProCarta Plex High Sensitivity Luminex Kit (Thermo Fischer Scientific, Wlatham, MA, USA). For the gene expression analysis, RNA was isolated from buffy coat using the TRIzol/chloroform method. Following step was cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific, Waltham, MA, USA). Finally, commerical TaqMan Gene Expression Assays were used for the assessment of gene expression (Applied Biosystems, Foster City, CA, USA) by quantitative polymerase chain reaction (qPCR). Beta-2-microglobulin (*B2M*) and peptidylprolyl isomerase A (*PPIA*) were used as the endogenous controls for data normalization, and the $2^{-\Delta\Delta Ct}$ method was used for final calculation of the relative expression of target genes (*HSP70*, *TLR2*, *TLR4*, *P2X7R*, *P2Y2R*). DNA was extracted from blood cells by a standard salting-out procedure and the genotyping was performed by the Taqman allelic discrimination real-time PCR using the corresponding SNP Genotyping Assays (Applied Biosystems, Waltham MA, USA).

Most data were analyzed with MedCalc statistical software version 17.9.2. (MedCalc Software, Ostend, Belgium), while the SNPStats software was used for the assessment of Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD), and haplotype analysis.

Results: The concentrations of eHsp70 and eATP were increased in COPD patients compared to controls and they were significantly associated with disease severity which was assessed by

forced expiratory volume in the first second (FEV_1) (%) and symptoms burden assessed by COPD Assessment Test (CAT). This is the first study that showed the association of eHsp70 and eATP from peripheral blood with COPD severity, and the first study that successfully determined eATP in blood samples. Also, significant correlations of eHsp70 and eATP with multicomponent COPD clinical indices (eHsp70 with BODCAT, BODEx, CODEx, DOSE; eATP with ADO, BODCAT, BODEx, CODEx, DOSE) were observed as well as with diffusion capacity for carbon monoxide (DLCO). The effect of smoking on eHsp70 and eATP concentration was not consistently and clearly present. COPD patients showed to have increased eHsp70 and eATP concentrations compared to both non-smoking and smoking controls, but there was no difference in eHsp70 or eATP between COPD subject when subdivided based on self-reported smoking status. Still, there was an interesting observation of increased eHsp70 and eATP in control smokers in comparison to control non-smokers which indicates that smokers without airflow obstruction might be more susceptible to the eHsp70- and eATP-driven inflammation.

All cytokines (IL-1α, IL-1β, IL-6, IL-8 and TNF-α) were increased in COPD patients but did not show an association with the severity of airflow limitation or symptoms burden. Network analysis showed that inflammation assessed by cytokines, common inflammatory biomarkers, eHsp70 and eATP is more developed in control smokers than in control non-smokers, and even more developed in COPD patients. Moreover, different COPD clusters were identified when concentrations of selected parameters and clinical data were included in the hierarchical cluster analysis. Interestingly, three cluster groups with patients with the most severe COPD were identified, but they only shared increased eHsp70 and eATP, while concentrations of CRP, Fbg, lkc, IL-1β, IL-6 and TNF-α differed among the groups. Finally, a combination of IL-1β, eATP and eHsp70 was suggested as the best model for the identification of COPD patients.

When considering the genetic background of COPD patients from the study, the relative gene expression of *HSP70*, *TLR2* and *P2Y2R* was increased compared to controls, and the increase was not associated with COPD severity or smoking status. Amongst selected SNPs in *TLR2*, *TLR4* and *HSP70* genes, only rs6457452 in the promoter region of *HSP70* was associated with the risk of COPD and decreased gene expression of *HSP70* was detected in C/T carriers with COPD compared to COPD C/C carriers. Also, *HSP70* haplotype with the T allele of rs6457452 had a protective role in COPD. However, the results should be interpreted carefully due to a small number of participants included in the genetic analysis.

Conclusions: It is suggested that eHsp70 and eATP are biomarkers of systemic inflammation in COPD and it seems they might be useful as prognostic biomarkers, especially in a combination with IL-1β. Moreover, it seems that the burden of inflammatory response is reflected by the increase of eHsp70 and eATP in peripheral circulation, so they could be useful in identifying patients at severe stage of COPD and in therapy monitoring due to the association with ABCD classification. Finally, this thesis contributed with the data at genetic level in the Croatian population which could be useful for better understanding of the pathophysiological mechanisms underlying COPD.

Keywords

chronic obstructive pulmonary disease; systemic inflammation; extracellular heat shock protein 70; extracellular adenosine triphosphate; gene expression; single nucleotide polymorphism

SADRŽAJ

1. UVOD

1.1. Kronična opstrukcijska plućna bolest

1.1.1. Definicija i epidemiologija bolesti

Kronična opstrukcijska plućna bolest (KOPB) heterogena je bolest popraćena kroničnom upalom i ograničenim protokom zraka u dišnim putovima (1) te je jedna od vodećih uzroka smrtnosti u svijetu sa stalnim porastom oboljelih. KOPB predstavlja veliki javnozdravstveni problem i značajno povećava troškove zdravstvenog sustava. 56 % svih troškova liječenja bolesti dišnog sustava u Europskoj uniji otpada na KOPB (procijenjenih 38,6 milijardi eura) (1). Prema podacima Svjetske zdravstvene organizacije (WHO, engl. *World Health Organization*) iz 2019. godine, KOPB je na trećem mjestu uzročnika smrti u svijetu, odmah iza ishemijske bolesti srca i moždanog udara (2). Na godišnjoj razini radi se o 3,23 milijuna smrtnih slučajeva, a 90 % slučajeva vezano je uz države s niskim i srednjim prihodima (3). Ipak, zbog mnogih slučajeva koji su kasno dijagnosticirani ili uopće nisu prepoznati, ukupan broj oboljelih teško je utvrditi. Procijenjena prevalencija bolesti u svijetu za osobe starije od 40 godina je 13,1 %, a u Europi 12,4 % (4), te se smatra da će u budućnosti i dalje rasti zbog kontinuiranog izlaganja rizičnim čimbenicima i zbog starenja stanovništva s obzirom da se KOPB češće pojavljuje u starijih osoba u usporedbi s mlađom populacijom (5).

Kako bi se podigla svijest o ozbiljnosti problematike KOPB-a, krajem 1990.-ih godina osnovana je Globalna inicijativa za KOPB (GOLD, engl. *Global Initiative for Chronic Obstructive Lung disease*). GOLD čine najbolji stručnjaci iz područja respiracijske medicine, epidemiologije, socioekonomije, javnog zdravstva i zdravstvenog obrazovanja koji redovito revidiraju smjernice za prevenciju, dijagnozu, praćenje i terapiju KOPB-a na svjetskoj razini kako bi se objedinile najnovije spoznaje o bolesti sa svrhom prevencije bolesti i boljeg zbrinjavanja pacijenata (6).

Glavna karakteristika KOPB-a je progresivni ireverzibilni gubitak plućne funkcije koji nastaje zbog kronične upale u dišnom sustavu. Bolest može imati svoja stabilna razdoblja i razdoblja akutnih pogoršanja (egzacerbacije) koja najčešće nastaju kao posljedica infekcija u dišnom sustavu. Smatra se da na pojavnost i razvoj KOPB-a utječe niz dinamičkih i kumulativnih promjena i interakcija između gena i okoliša koje određuju razvoj pluća te njihovu funkcionalnost (7). Ipak, utvrđeno je da KOPB nije isključivo plućna bolest, već je i sustavna bolest koja zahvaća niz drugih organa.

1.1.2. Čimbenici rizika

Čimbenici rizika za razvoj KOPB-a su izloženost duhanskom dimu, zagađenje unutarnjih prostora izazvano izgaranjem bioloških materijala koji se koriste za kuhanje i grijanje, zagađenje vanjskog okoliša, profesionalni rad u industriji ruda, česte infekcije dišnog sustava u dječjoj dobi, spol i dob, socioekonomski status te genska podloga pojedinca i preosjetljivost dišnih putova.

Pušenje se smatra glavnim rizičnim čimbenikom, no samo oko 10 – 20 % pušača razvija KOPB (8). Poznato je da duhanski dim ima izravni utjecaj na funkciju plućnih stanica te djeluje upalno, citotoksično, mutageno i karcinogeno. Udisanjem duhanskog dima nastaju kisikovi radikali koji uzrokuju oksidacijski stres. Povišena koncentracija reaktivnih kisikovih spojeva (ROS, engl. *reactive oxygen species*) doprinosi inaktivaciji antiproteaza i aktivaciji metaloproteinaza matriksa (MMP, engl. *matrix metalloproteinase*) te smanjuje fagocitnu učinkovitost neutrofila i alveolarnih makrofaga. Također, unutar stanica dolazi do aktivacije transkripcijskih čimbenika koji su osjetljivi na redoks status čime se potiče pojačana ekspresija gena koji kodiraju za upalne citokine (9, 10).

Izloženost različitim česticama prašine, kemikalijama i parama na radnom mjestu može biti značajan čimbenik rizika za nastanak KOPB-a, posebno u državama slabije ekonomske moći. Osobe koje su izložene parama koje nastaju nakon izgaranja bioloških materijala, poput ugljena, slame, ostataka usjeva i drva, značajnije oboljevaju od KOPB-a. U ruralnim sredinama država srednje i niske platežne moći takvi izvori koriste se za grijanje i kuhanje, a s obzirom na podjelu poslova u tim zemljama, žene su značajnije izložene navedenom čimbeniku rizika nego muškarci iz istog područja ili žene iz razvijenijih zemalja (11).

Najbolje proučeni genski rizični čimbenik u KOPB-u je nasljedni nedostatak alfa-1 antitripsina (A1AT, engl. *alpha-1 antitrypsin*) koji je inhibitor serinskih proteaza. Urođeni nedostatak A1AT-a pojavljuje se do oko 5 % oboljelih od KOPB-a (12, 13). S obzirom da je A1AT proteazni inhibitor, u slučaju smanjene koncentracije ne dolazi do adekvatne inaktivacije neutrofilne elastaze zbog čega se plućno tkivo pojačano uništava.

Uočeno je da se KOPB češće pojavljuje kod starije populacije (14), a poznato je da starenjem dolazi do strukturnih promjena u dišnim putovima. Ipak, još uvijek nije razjašnjeno vodi li zdravo starenje samo po sebi do KOPB-a ili tek u starijoj životnoj dobi nastaje prepoznatljiva klinička slika KOPB-a zbog kumulativne izloženosti različitim rizičnim čimbenicima.

Prethodna istraživanja su ukazala da su KOPB i smrtnost od KOPB-a zastupljeniji u muškaraca nego u žena, no noviji podaci iz razvijenih država ukazuju na izjednačenost u tim podacima (15, 16). Smatra se da je djelomičan razlog taj što u suvremenom dobu žene češće konzumiraju duhanske proizvode nego što su to činile u prošlosti.

1.1.3. Dijagnostička procjena i klasifikacija bolesti

Dijagnostika KOPB-a provodi se prema GOLD smjernicama te uključuje prisutnost simptoma vezanih uz bolest (kronični produktivni kašalj, umor pri tjelesnim naporima, zaduha, kronično stvaranje ispljuvka), izloženost čimbenicima rizika i spirometrijsku potvrdu plućne opstrukcije određivanjem forsiranog izdisajnog volumena u prvoj sekundi (FEV_1, engl. *forced expiratory volume in the first second*) i forsiranog vitalnog kapaciteta (FVC, engl. *forced vital capacity*).

Iako je u današnjoj kliničkoj praksi spirometrijska potvrda prisutnosti plućne opstrukcije potrebna za postavljanje dijagnoze KOPB-a, sve se više naglašavaju nedostaci te metode. Spirometrijom se može procijeniti samo plućna komponenta bolesti, no KOPB nije isključivo plućna bolest, već može rezultirati nizom promjena u drugim organskim sustavima. Osim toga, simptomi KOPB-a mogu biti prisutni u pacijenata s normalnom spirometrijom ili pacijent može imati emfizem potvrđen slikovnom tehnikom, ali uz normalnu spirometriju (17). Zbog individualnih razlika tijekom rasta i razvoja, dio populacije može imati plućnu funkciju veću od očekivane putanje rasta što znači da bi pad plućne funkcije kod takvih pojedinaca morao biti puno veći kako bi se uopće mogao zabilježiti na temelju spirometrijskih kriterija (7). Stoga spirometrija nije najbolji pokazatelj sveopćeg zdravstvenog stanja pacijenata s KOPB-om i nedovoljan je dijagnostički kriterij za KOPB.

Osim spirometrije, mogu se vršiti dodatni testovi plućne funkcije, a to su test 6-minutnog hodanja, test progresivnog opterećenja, ergospirometrija ili testovi za procjenu intenziteta zaduhe. Tipični simptomi KOPB-a često se pripisuju „pušačkom kašlju“ pa se mnogi pacijenti koji su pušači vrlo kasno obraćaju za pomoć svom liječniku. S obzirom da je KOPB višekomponentna bolest, a spirometrija ne preslikava njenu heterogenost te je ograničena samo na određivanje plućne funkcije, procjena sveukupnog zdravstvenog stanja vrlo često uključuje više parametara. Nakon prvih prijedloga promjena smjernica iz 2013. godine, GOLD smjernice od 2017. godine uključuju promijenjenu kombiniranu procjenu bolesti gdje se FEV_1 koristi kao mjerilo opstrukcije, a podaci o težini simptoma i učestalosti egzacerbacija u prethodnoj godini s ili bez hospitalizacije koriste se za tzv. ABCD klasifikaciju (engl. *ABCD Assessment Tool*) (6). Prema GOLD smjernicama FEV_1 se mjeri nakon primjene bronhodilatatora te se na temelju izmjerene vrijednosti FEV_1 pacijentima pridružuje jedan od ukupno četiri stadija (GOLD 1 – 4) KOPB-a kao što je prikazano u Tablici 1. Za objektivno određivanje ABCD skupina koristi se modificirani upitnik Vijeća za medicinska istraživanja

(mMRC, engl. *modified Medical Research Council*) koji služi kao modificirana skala zaduhe ili noviji i sveobuhvatniji upitnik za procjenu simptoma KOPB-a (CAT, engl. *COPD Assessment Test*). Uz mMRC ili CAT upitnike, za ABCD klasifikaciju nužan je i podatak o broju prethodnih egzacerbacija. Na temelju postavljenih kriterija iz GOLD smjernica prikazanih na Slici 1, pacijentima s KOPB-om pridružuje se jedna od četiri (GOLD A - D) skupine.

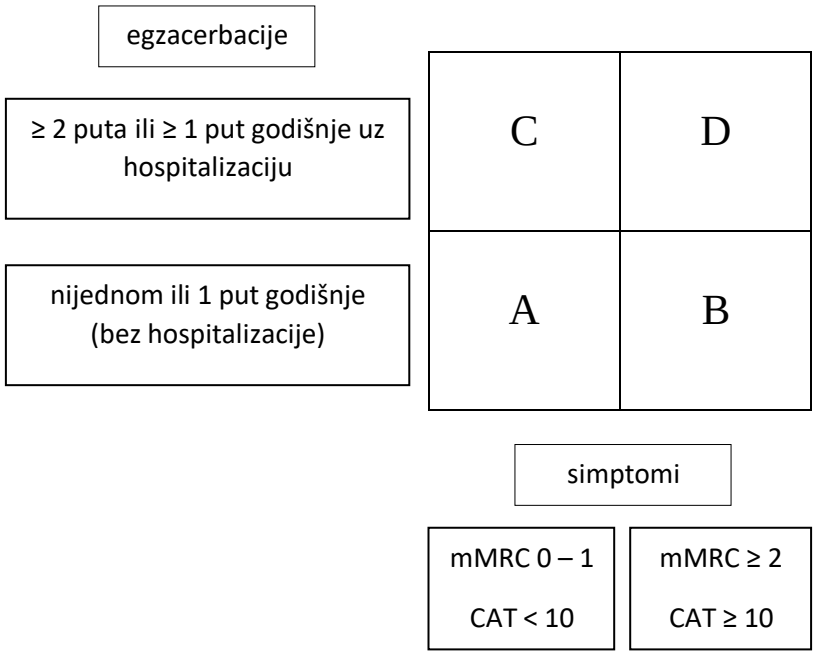
Tablica 1. Klasifikacija pacijenata s KOPB-om prema GOLD stadijima.

klasifikacija pacijenata s KOPB-om prema razini plućne opstrukcije s obzirom na FEV_1		
stadij KOPB-a		FEV_1 (% predviđenog)
GOLD 1	blagi KOPB	≥ 80
GOLD 2	umjereni KOPB	50 – 79
GOLD 3	teški KOPB	30 – 49
GOLD 4	vrlo teški KOPB	< 30

KOPB – kronična opstrukcijska plućna bolest; FEV_1 – forsirani izdisajni volumen u prvoj sekundi; GOLD – Globalna inicijativa za KOPB.

Preuzeto iz: Global Initiative for Chronic Obstructive Lung Disease (GOLD), 2022.

Upitnik koji se još može primijeniti za procjenu zdravstvenog stanja pacijenata s KOPB-om je SGRQ-C (engl. *St. George's Respiratory Questionnaire*), no ustanovljeno je da je takav upitnik kompleksniji za upotrebu u kliničkoj praksi.



Slika 1. ABCD klasifikacija pacijenata s KOPB-om na temelju procjene težine simptoma i povijesti o egzacerbacijama.

KOPB – kronična opstrukcijska plućna bolest; mMRC – modificirani upitnik Vijeća za medicinska istraživanja; CAT – upitnik za procjenu simptoma KOPB-a.

Preuzeto iz: Global Initiative for Chronic Obstructive Lung Disease (GOLD), 2022.

Dodatni indeksi procjene koji se mogu koristiti u kliničkoj praksi su BODEx, BODCAT, CODEx, ADO i DOSE. BODEx izmijenjena je inačica BODE indeksa koji uključuje procjenu uhranjenosti pomoću indeksa tjelesne mase (BMI, engl. *body mass index*), razinu opstrukcije, razinu zaduhe i fizičku sposobnost pacijenta (engl. *BMI, airflow obstruction, dyspnoea, exercise capacity*), a za koji se utvrdilo da ima bolju predikcijsku vrijednost u procjeni preživljenja i stadija bolesti uspoređujući s FEV_1 (18, 19). Fizička sposobnost pacijenta u BODE indeksu procijenjena je na temelju 6-minutnog testa hoda koji nije praktičan za primjenu u primarnoj zdravstvenoj zaštiti pa je ta varijabla zamijenjena brojem egzacerbacija tijekom prethodnih godinu dana i tako je nastala praktičnija verzija indeksa – BODEx (engl. *BMI, airflow obstruction, dyspnoea, previous exacerbations*). Nadalje, izmjenom BODE indeksa kreiran je BODCAT (engl. *BMI, airflow obstruction, dyspnoea, COPD Assessment Test*) u koji se umjesto procjene fizičke sposobnosti pacijenta uvrštava podatak o težini simptoma procijenjenog pomoću CAT upitnika. CODEx (engl. *Charlson's comorbidity index,*

airflow obstruction, dyspnoea, previous exacerbations) je također inačica nastala iz BODE indeksa koja u obzir uzima podatak o komorbiditetima u pacijenata s KOPB-om. Ustanovljeno je da CODEx može poslužiti kao dobar prediktor mortaliteta i budućih egzacerbacija u pacijenata s KOPB-om (20, 21). ADO je indeks koji uključuje dob, razinu zaduhe i razinu opstrukcije (engl. *age, dyspnoea, airflow obstruction*). Vrlo se lako primjenjuje te je dobar prediktor budućih egzacerbacija i hospitalizacija (22) kao i DOSE koji uključuje razinu zaduhe, razinu opstrukcije, status pušenja i egzacerbacije tijekom prethodnih godinu dana (engl. *dyspnoea, airflow obstruction, smoking status, previous exacerbation*). Također, DOSE je pokazao povezanost sa smrtnosti u pacijenata s KOPB-om te može poslužiti kao dodatni alat u kliničkoj procjeni prognoze bolesti (23).

Difuzijski kapacitet pluća (DLCO, engl. *diffusion capacity for carbon monoxide*) je parametar koji se najčešće određuje sa svrhom evaluacije zaduhe, a definira se kao količina ugljikova monoksida koja prolazi kroz alveokapilarnu membranu tijekom 1 minute pri razlici tlakova od 1 kPa. Pad vrijednosti DLCO-a povezuje se s pojavom emfizema u kojem je površina alveola puno manja pa je smanjena mogućnost prijenosa ugljikova monoksida iz prostora alveola preko alveokapilarne membrane (24).

1.1.4. Patogeneza, patofiziologija i patologija

1.1.4.1. Patogenetski čimbenici

Najznačajniji patogenetski čimbenici u razvoju KOPB-a su kronična upala, oksidacijski stres, neravnoteža između proteaza i antiproteaza, apoptoza i ubrzano stanično starenje.

Upalni odgovor smatra se središnjim patogenetskim procesom u KOPB-u. On pretežito uključuje komponente urođene imunosti dišnog sustava, a to su barijera cilindričnog epitela s trepetljikama, vrčaste stanice koje izlučuju sluz, antimikrobni peptidi, proteini komplementa i stanice koje cirkuliraju u dišnom sustavu, a čiji produkti uzrokuju oštećenje plućnog tkiva i onemogućavaju odvijanje mehanizama cijeljenja. Stanice koje sudjeluju u upalnim procesima urođenog imunosnog odgovora u KOPB-u pretežito su neutrofili i aktivirani alveolarni makrofagi koji otpuštaju različite citokine, kemokine i kemoatraktante. Također, epitelne stanice vrlo su bitan dio imunosnog odgovora u KOPB-u. Prilikom njihovog oštećenja, dolazi do skvamozne metaplazije, a time se remeti mukocilijarna zaštita u dišnom sustavu. Osim urođene imunosti, u patogenezi kronične upale sudjeluju i komponente stečenog imunosnog odgovora, CD4+ i CD8+ T-stanice, regulacijske T-stanice i B-stanice. CD4+ i CD8+ T-stanice te B-stanice nakupljaju se u alveolarnom i bronhijalnom tkivu. CD4+ stanice potiču autoimuni odgovor u plućnom tkivu, dok CD8+ stanice otpuštaju citotoksične enzime poput perforina i granzima B koji uzrokuju citolizu i apoptozu alveolarnih epitelnih stanica. Također, CD8+ stanice otpuštaju upalni citokin čimbenik tumorske nekroze alfa (TNF-α, engl. *tumour necrosis factor alpha*) (25 – 28).

Glavna poveznica između urođene i stanične imunosti su dendritične stanice smještene na bazalnoj membrani dišnog epitela. Dendritične stanice prezentiraju udahnute antigene B- i T-stanicama i na taj ih način aktiviraju (29).

Dodatno, upalne reakcije mogu biti potaknute dominantnim rizičnim čimbenikom – izlaganjem duhanskom dimu koji je izvor oksidansa poput reaktivnih kisikovih i reaktivnih dušikovih spojeva. Zbog svoje nestabilnosti takvi spojevi mogu poticati oksidaciju, odnosno oštećenje strukture proteina, lipida i deoksiribonukleinske kiseline (DNA, engl. *deoxyribonucleic acid*) čime se potiče i pojačava upalni odgovor (30). Osim iz dima cigareta, izvor egzogenih oksidansa mogu biti različiti okolišni zagađivači. Također, oksidansi se mogu otpuštati iz aktiviranih upalnih stanica u dišnom sustavu (epitelne stanice, neutrofili,

makrofagi) i tako doprinositi oksidacijskom stresu. Radi održanja ravnoteže, vrlo je bitna aktivnost plućnog antioksidacijskog sustava koji predstavlja zaštitu od endogenih i egzogenih oksidansa. Superoksid-dismutaza, katalaza i glutation-peroksidaza neki su od enzima koji djeluju zaštitno, a kada te zaštite nema dolazi do povećane sekrecije mukusa u dišnim putovima i inaktivacije antiproteaza te pojačane aktivnosti transkripcijskog jezgrinog čimbenika kappa B (NF-κB, engl. *nuclear factor kappa B*) i ekspresije gena uključenih u upalne procese (30, 31).

Osim neravnoteže između oksidansa i antioksidansa, bitna komponenta bolesti je neravnoteža između proteaza i antiproteaza koja može nastati zbog urođenog nedostatka A1AT-a ili zbog povećanog stvaranja proteolitičkih enzima. Zbog oštećenja plućnih alveola, nedostatak A1AT-a najčešće se klinički manifestira u obliku panacinarnog emfizema te se bolest ranije razvija u pušača u usporedbi s nepušačima (12). ROS-ovi su značajan uzrok poremećaja proteolitičke ravnoteže pa tako cigaretni dim ili neki drugi izvor ROS-ova može biti uzrokom inaktivacije antiproteaza. Također, već spomenutom aktivacijom upalnih stanica dolazi do povećanog otpuštanja niza proteaza poput neutrofilne elastaze, MMP-ova, katepsina i proteinaze-3.

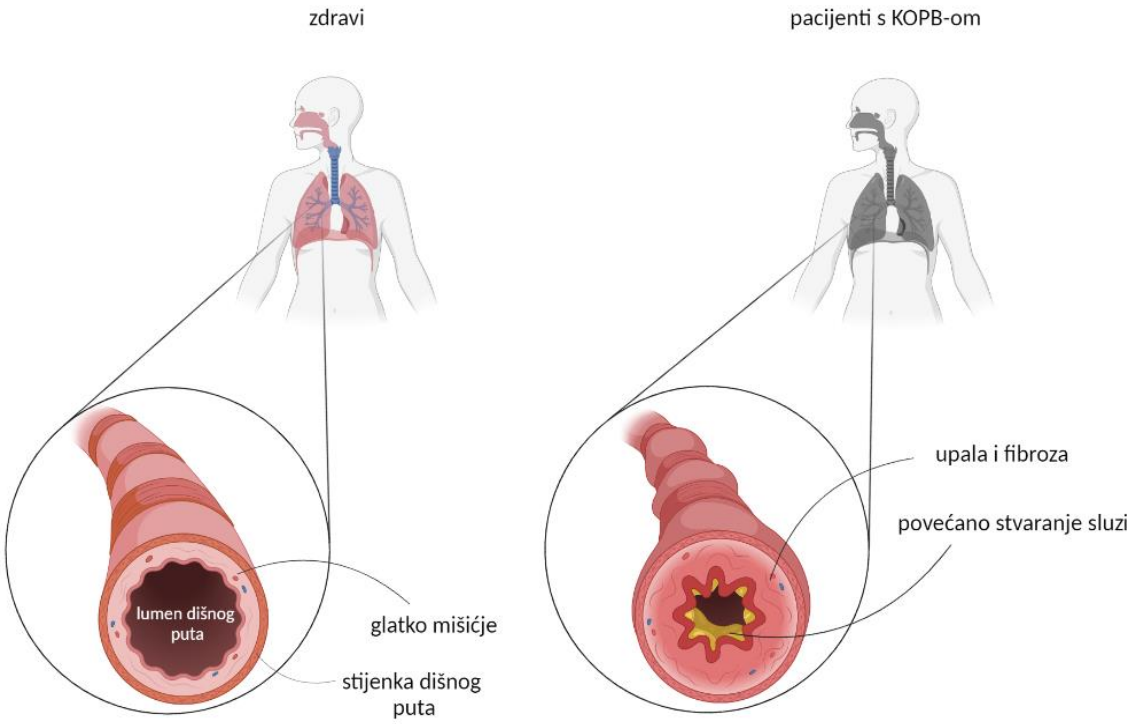
Apoptoza je regulirani proces stanične smrti pomoću kojeg se uklanjaju neželjene, oštećene ili stare stanice, a može biti uzrokovana različitim signalima, npr. oksidacijskim stresom. Bitni čimbenici apoptoze su kaspaze koje imaju proteazno djelovanje. Smatra se da se u KOPB-u pojavljuju neravnoteža između apoptoze i proliferacije stanice te usporeno uklanjanje apoptoznih stanica iz pluća koji doprinose razvoju kronične upale (32).

U novije vrijeme, velika pažnja daje se staničnom starenju kao bitnom mehanizmu u kroničnim plućnim bolestima. Smatra se da je starenje ubrzano u kroničnim bolestima, a pritom su neki od biljega starenja skraćene telomere, disfunkcija mitohondrija, smanjena proteostaza i dr. Ubrzano stanično starenje se pojavljuje zbog izlaganja stanica stresu, a alveolarne epitelne i endotelne stanice, kod kojih se odvija proces ubrzanog staničnog starenja, akumuliraju se u pacijenata s KOPB-om (33, 34).

1.1.4.2. Patološke promjene

Patološke promjene u KOPB-u uključuju hipersekreciju sluzi što može izazvati kronični bronhitis, oštećenje tkiva koje vodi do razvoja emfizema, nemogućnost popravka i obrane što uzrokuje upalu malih dišnih putova te fibrozu koja može rezultirati bronhiolitisom

(31, 35). Hipersekrecija sluzi nastaje zbog povećanog broja vrčastih stanica i hipertrofije bronhijalnih submukoznih žlijezda. Sluz koja nastaje ima promijenjen proteinski sastav, dolazi do smanjene glikozilacije mucina, povećane kiselosti i smanjene koncentracije antimikrobnih peptida te vodi do razvoja kroničnog kašlja.



Slika 2. Opstrukcija dišnih putova.

Napravljeno pomoću https://biorender.com/

Nadalje, plućna opstrukcija, odnosno pad vrijednosti FEV_1, nastaje zbog upalnih procesa i remodeliranja dišnih putova, gubitka elastina i oštećenja alveolarnih struktura (Slika 2) (30, 31, 35).

Uz neutrofile i makrofage, epitelne stanice dišnih putova i alveola izvor su upalnih medijatora u KOPB-u. Trajna upala u dišnom sustavu potiče uništavanje plućnog parenhima i fibrozu malih dišnih putova što dovodi do povećanja alveolarnog prostora i stanjivanja dišnih putova (36, 37). Stoga, prilikom izdisaja, ne dolazi do potpunog pražnjenja pluća, već se povećava mrtvi prostor koji ne sudjeluje u procesu disanja, a sve navedeno dovodi do smanjene ventilacije i poremećaja u izmjeni plinova. Smanjeni protok zraka doprinosi smanjenoj stopi

izmjene plinova, a zbog toga je slabiji dotok krvi bogate kisikom u tkiva (hipoksemija) i povišen parcijalni tlak ugljikova dioksida u arterijskoj krvi (hiperkapnija). Trajnije posljedice ovih procesa mogu biti pojava plućne hipertenzije te mnoge druge sustavne promjene.

1.1.5. Terapija KOPB-a

Cilj liječenja pacijenata sa stabilnim KOPB-om je ublažiti simptome, spriječiti daljnji razvoj bolesti i pojavu akutnih pogoršanja te unaprijediti sveukupno zdravstveno stanje. KOPB je kroničnog i progresivnog tijeka, a osim toga značajne karakteristike bolesti su njena heterogenost i kompleksnost što predstavlja veliki izazov u liječenju pacijenata i naglašava potrebu za personalizacijom terapijskog pristupa. Pritom je vrlo bitan dio procjene zdravstvenog stanja pacijenata ABCD klasifikacija iz GOLD smjernica jer se podaci o uznapredovalosti simptoma i povijesti o egzacerbacijama koriste u kliničkoj praksi za odabir terapije. Osim toga, velika pažnja daje se i što ranijem otkrivanju bolesti, a u tome veliku ulogu imaju stalna istraživanja o potencijalnim biljezima koji bi olakšali prepoznavanje pacijenata s KOPB-om i omogućili ranu terapijsku intervenciju (1, 38).

Prestanak pušenja je najbitnija nefarmakološka mjera u liječenju KOPB-a koja ima značajan učinak na poboljšanje simptoma i usporavanje daljnjeg tijeka bolesti te usporavanje pada plućne funkcije (39). Ostale bitne nefarmakološke mjere su cijepljenje, fizička aktivnost i plućna rehabilitacija.

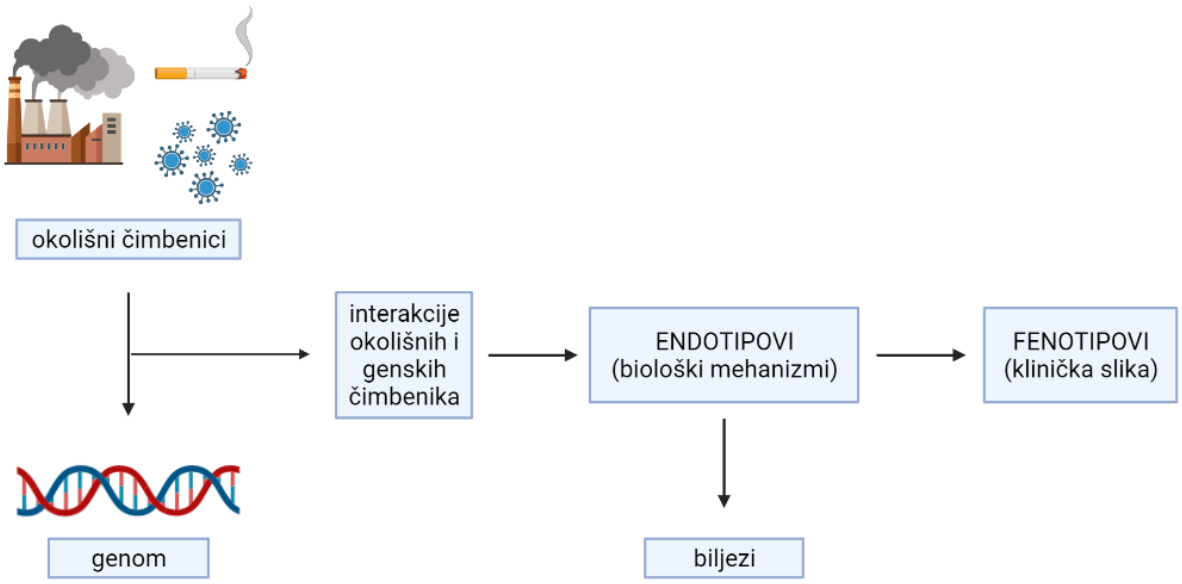
Trenutno dostupna i primjenjivana farmakoterapija za KOPB pomaže u smanjenju simptoma, broja egzacerbacija i njihove težine te povećava kvalitetu života pacijenata. Najčešće se radi o bronhodilatatorima koji se dijele na agoniste β2-adrenergijskih receptora koji utječu na glatko mišićje dišnih putova i poboljšavaju protok zraka pri izdahu što rezultira povećanjem vrijednosti FEV_1 i na muskarinske antagoniste koji utječu na motorički tonus bronha putem muskarinskih receptora. Dodatno, obje skupine bronhodilatatora dijele se na lijekove kratkog i na lijekove dugog djelovanja. Uz bronhodilatatore, koriste se inhalacijski ili sustavni kortikosteroidi. Odabir terapije ovisi o kategorizaciji pacijenata s KOPB-om prema GOLD smjernicama. Početna terapija za pacijente iz skupine A, prema ABCD klasifikaciji, su bronhodilatatori kratkog ili dugog djelovanja, ovisno o njihovom učinku na zaduhu. Početnu terapiju za pacijente iz skupine B čine dugodjelujući bronhodilatatori, dok se pacijentima iz skupine C preporučuje dugodjelujući muskarinski antagonist (LAMA, engl. *long-acting muscarinic antagonist*) pred dugodjelujućim beta agonistom (LABA, engl. *long-acting beta-agonist*) zbog prevencije egzacerbacija. Konačno, pacijentima iz skupine D daje se LAMA ili kombinacija LAMA-e i LABA-e, dok je kod pacijenata s uznapredovalim KOPB-om i učestalim egzacerbacijama potrebno uvesti kombinaciju s inhalacijskim kortikosteroidima (ICS, engl. *inhaled corticosteroids*) (40). Također, u pacijenata s čestim egzacerbacijama,

lošom plućnom funkcijom i kroničnim bronhitisom kod kojih bronhodilatatori ne postižu stabilizaciju stanja indicirana je primjena roflumilasta, inhibitora fosfodiesteraze 4 (1).

Na temelju dobro poznatih i opisanih mehanizama bolesti, tek za manji dio pacijenata postoji ciljana učinkovita terapija, a ona se odnosi na pacijente s nedostatkom A1AT-a. Daljnja istraživanja o endotipovima bolesti trebala bi otvoriti nove mogućnosti farmakoloških mjera i tako omogućiti bolju zdravstvenu skrb za veći dio pacijenata s KOPB-om.

1.1.6. Sustavna upala u KOPB-u

Heterogenost KOPB-a očituje se u tome što nisu sve komponente bolesti prisutne u svih pacijenata, a kompleksnost se dodatno povećava nizom dinamičkih interakcija promjena koje nisu linearne (41). S obzirom na navedeno, podjela na fenotipove, koji imaju slične kliničke karakteristike, olakšava procjenu prognoze bolesti i terapijski pristup. Fenotip se definira kao klinička manifestacija bolesti koja je rezultat interakcije genotipa i utjecaja okolišnih čimbenika (42) (Slika 3). Prve fenotipove KOPB-a opisao je Dornhorst 1950.-ih, a to su bili ružičasti puhaljko (engl. *pink puffer*) i modri naduvenko (engl. *blue bloater*) (43). Neki od današnjih osnovnih fenotipova KOPB-a su fenotip čestih egzacerbacija, fenotip emfizema, fenotip kroničnog bronhitisa, fenotip brzog gubitka plućne funkcije, sindrom preklapanja KOPB-a i astme i drugi (41, 44).

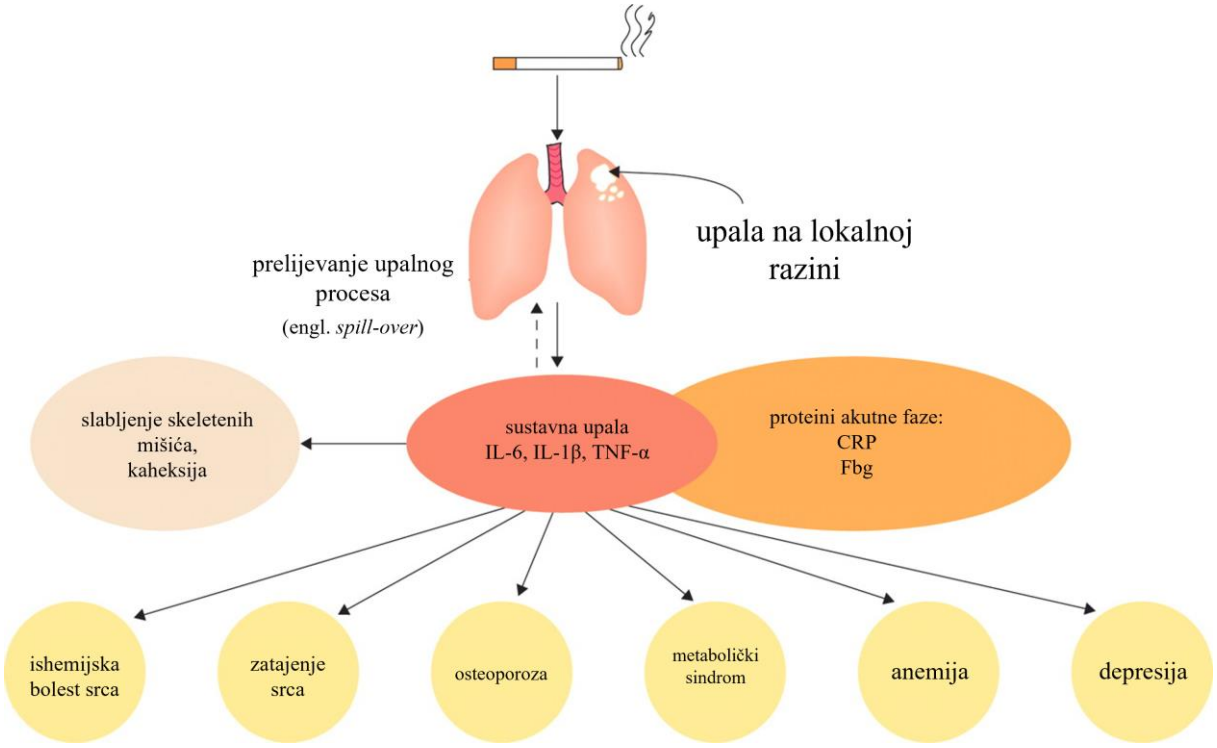


Slika 3. Povezanost između gena, okolišnih čimbenika, endotipova i fenotipova.

Napravljeno pomoću https://biorender.com/

Jedna od osnovnih zadaća kliničara i istraživača jest povezivanje fenotipova s mehanizmima bolesti koji se odvijaju u pozadini, odnosno endotipovima bolesti.

Iako je zasada jedini potvrđeni endotip KOPB-a, za kojeg postoji specifična terapija, endotip nedostatka A1AT-a, jedan od predloženih endotipova KOPB-a je endotip sustavne upale. Sustavna upala u KOPB-u dokazana je u podskupini pacijenata s KOPB-om, a u perifernoj cirkulaciji može se detektirati mjerenjem koncentracije općih upalnih biljega (45). Međutim, još uvijek nije poznato može li se tretirati farmakološki i koje bi farmakološke mete za to bile najpogodnije. Postoji nekoliko teorija kako dolazi do sustavne upale i kako je ona povezana s KOPB-om, no nijedna još uvijek nije potvrđena. Najčešća teorija pretpostavlja da se lokalni upalni proces u dišnom sustavu prelijeva (engl. *spill-over*) u perifernu cirkulaciju (Slika 4) (46, 47).



Slika 4. Sustavna upala u KOPB-u i njene posljedice.

KOPB – kronična opstrukcijska plućna bolest; IL-6 – interleukin-6; IL-1β – interleukin-1beta; TNF-α – čimbenik tumorske nekroze alfa; CRP – C-reaktivni protein; Fbg – fibrinogen.

Preuzeto iz: Barnes i Celli, 2009.

Druge teorije govore o postojanju i interakciji različitih čimbenika (npr. pušenje, starenje) koji uzrokuju i lokalnu i sustavnu upalu (48). Iako se u znanstvenoj zajednici mehanizmi nastanka sustavne upale još uvijek utvrđuju, poznato je da se posljedice trajne sustavne upale manifestiraju u obliku različitih komorbiditeta – poremećaja u krvožilnom sustavu, osteoporoze, metaboličkog sindroma, anksioznosti, depresije, disfunkcije mišića i mnogih drugih.

1.2. Biljezi sustavne upale u KOPB-u

Sustavna upala smatra se bitnom komponentom KOPB-a, a neki autori čak predlažu da je KOPB samo dio kroničnog sustavnog upalnog sindroma (49). U kompleksnoj mreži interakcija u sustavnoj upali sudjeluju aktivirane cirkulirajuće upalne stanice i niz molekula koje predstavljaju upalne biljege. Osim općih upalnih biljega, C-reaktivnog proteina (CRP), fibrinogena (Fbg) i ukupnog broja leukocita (lkc), značajnu ulogu u modulaciji imunosnog odgovora imaju i citokini.

1.2.1. Citokini

Citokini su izvanstanične signalne molekule čija je primarna funkcija nadzor imunosnih reakcija, a pritom posjeduju svojstva pleotropizma (jedan citokin ima više različitih funkcija) i redundancije (ista funkcija je posredovana s više citokina istovremeno). Najčešće se oslobađaju iz upalnih i imunosnih stanica u manjim skupinama u određenim obrascima.

Neutrofili potiču odvijanje upale tako što luče interleukin (IL)-8 i leukotrien (LT)B-4. IL-8 pripada skupini kemokina iz CXC porodice, što znači da jedna aminokiselina (X) odvaja dva N-terminalna cisteina (C), i luči se u ranom upalnom odgovoru, ali ostaje aktivan produljeni period. Glavna mu je uloga privlačenje i aktivacija upalnih stanica u akutnoj fazi (50). Neutrofili također sintetiziraju i otpuštaju proteolitičke enzime poput neutrofilne elastaze, proteinaze-3, katepsina-G i MMP-ova koji uništavaju plućni parenhim i doprinose kroničnoj sekreciji sluzi. Uz neutrofile, IL-8 luče monociti i makrofagi.

Aktivirani makrofagi također stvaraju i otpuštaju IL-6 i TNF-α, LTB-4 i ROS-ove koji privlače i aktiviraju ostale stanice imunosnog sustava, posebice neutrofile. IL-6 inducira sintezu proteina akutne faze (CRP, Fbg, serumski amiloid A), potiče upalnu signalizaciju te regulira metaboličke i regeneracijske mehanizme.

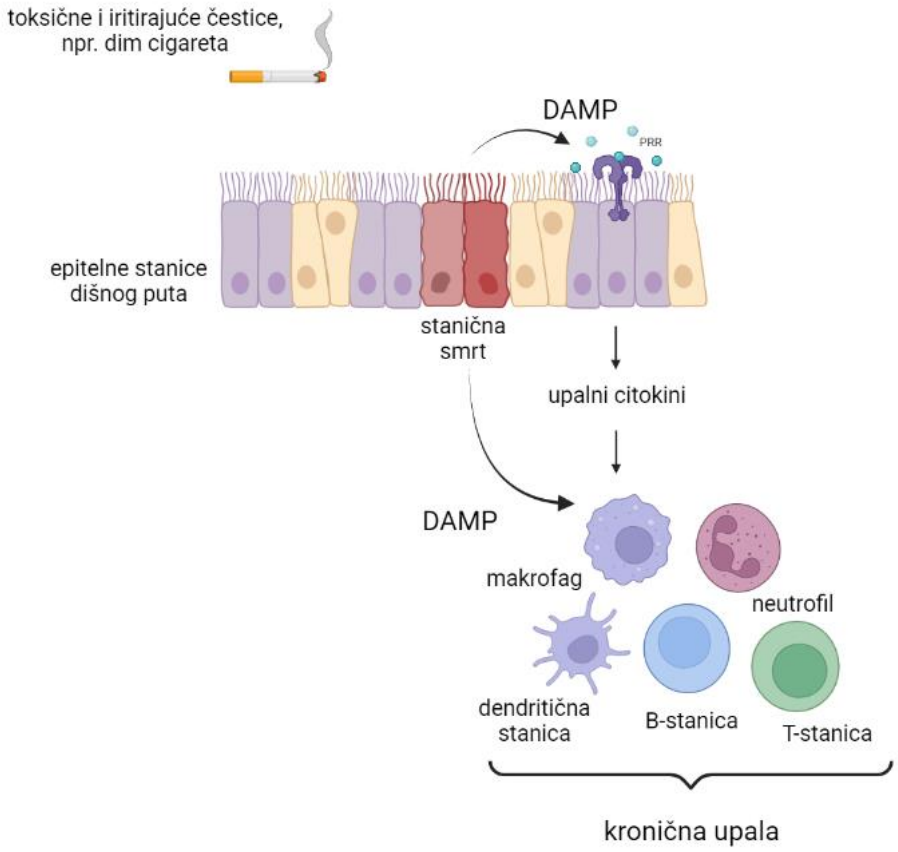
TNF-α je citokin koji aktivira transkripcijski čimbenik NF-κB te ima najšire djelovanje. Smatra se da stimulira sekreciju mucina 5AC (MUC5AC) iz bronhijalnih epitelnih stanica te pojačava ekspresiju adhezijskih molekula na upalnim, epitelnim i endotelnim stanicama dišnog sustava (51).

Osim IL-6, IL-8 i TNF-α, citokin značajan u upalnim odgovorima u KOPB-u je i IL-1, a pritom razlikujemo IL-1α i IL-1β koji su kodirani različitim genima, ali se vežu za isti receptor IL-1R. Nakon sinteze, pro-IL-1α uglavnom ostaje unutar stanice i biološki je aktivan. Uslijed različitih stimulansa prelazi iz citoplazme u jezgru i djeluje poput transkripcijskog čimbenika. Uz to, može se razgraditi pomoću proteaza do dodatnog aktivnog oblika, IL-1α. IL-1α otpušta se iz umirujućih stanica kao molekularni obarazac oštećenja (DAMP, engl. *damage-associated molecular pattern*) u izvanstanični prostor te potiče neutrofilnu upalu (52 – 54). Smatra se da tako potiče nastanak i odvijanje upalnog stanja. S druge strane, pro-IL-1β nije biološki aktivan te je potrebno djelovanje proteaza da bi nastao aktivan oblik IL-1β. IL-1β u većoj je mjeri istraživan u KOPB-u te predstavlja vrlo bitnu komponentu upalnog odgovora posredovanog aktivacijom NLRP3 inflamasoma, odnosno najbolje istraženog višeproteinskog kompleksa koji sadrži receptor sličan oligomerizacijskoj domeni koja veže nukleotide (NLR, engl. *nucleotide-binding oligomerization domain (NOD)-like receptor*) s N-terminalnom pirinskom domenom tipa 3 (NLRP3, engl. *NOD-like receptor pyrin 3*). Također, IL-1β u pojačanoj se mjeri otpušta iz makrofaga pušača i pacijenata s KOPB-om te se povezuje s leukocitozom (55, 56).

U okviru sustavne upale u KOPB-u, Agusti i suradnici uveli su termin inflamom (engl. *inflammome*) koji predstavlja obrazac interakcija u sustavnoj upali, koja može biti prolazna ili trajna, i obuhvaća šest biljega od kojih su tri citokina (CRP, Fbg, lkc, IL-6, IL-8 i TNF-α) (57). U najvećem longitudinalnom istraživanju o sustavnoj upali u stabilnom KOPB-u utvrđeno je da su znakovi trajne upale povezani s egzacerbacijama i većom stopom smrtnosti (57). Osim toga, pacijenti sa sličnim vrijednostima parametara plućne funkcije mogu imati različite ishode ovisno o prisutnosti i trajanju sustavne upale. Druga istraživanja također su dokazala povećane koncentracije CRP-a, Fbg-a, lkc te citokina IL-6, IL-8 i TNF-α u sustavnoj cirkulaciji pacijenata s KOPB-om (49, 57 – 59). Uz to, pacijenti s povećanom koncentracijom općih upalnih biljega pokazali su brži gubitak plućne funkcije i veći rizik od napredovanja u stadiju bolesti (57, 60, 61).

Mjerljivi biljezi koji su povezani s kompleksnim bolestima poput KOPB-a, a prisutni su u lako dostupnim uzorcima poput krvi, predstavljaju veliki značaj za dijagnostiku, terapijski pristup pacijentu, individualizaciju liječenja, praćenje progresije bolesti i odabir pacijenata za klinička ispitivanja. Stoga je od velike važnosti da se prepoznaju i utvrde različiti endotipovi bolesti sa svrhom boljeg razumijevanja heterogenosti KOPB-a. U sterilnoj upali u stabilnoj

fazi KOPB-a, osim opisanih općih upalnih biljega i citokina, na modulaciju imunosnog odgovora utječu i DAMP-ovi (Slika 5) (62).



Slika 5. Utjecaj DAMP-ova na nastanak kronične upale u KOPB-u.

KOPB – kronična opstrukcijska plućna bolest; DAMP – molekularni obrazac oštećenja; PRR – receptor za prepoznavanje obrazaca; IL-6 – interleukin-6; IL-8 – interleukin-8; TNF-α – čimbenik tumorske nekroze alfa.

Napravljeno pomoću https://biorender.com/

U sklopu ovog istraživanja ispitana je povezanost dviju DAMP molekula za koje se smatra da su povezane s KOPB-om – izvanstanični protein toplinskoga šoka 70 (eHsp70, engl. *extracellular heat shock protein 70*) i izvanstanični adenozin-trifosfat (ATP, engl. *extracellular adenosine triphosphate*).

1.2.2. Protein toplinskoga šoka 70

Proteini toplinskoga šoka čine skupinu proteina koji su prisutni u svim organizmima i evolucijski su očuvane molekule s primarnom zaštitnom ulogom u stanici, posebno kada je stanica izložena okolišnom stresu. Dijele se u pet velikih porodica ovisno o molekulskoj masi, homologiji aminokiselinskih slijedova i funkciji. Razlikujemo male Hsp-ove (npr. Hsp27), Hsp60, Hsp70, Hsp90 i Hsp110 (63), a svaka od skupina proteina ima svoje funkcije u organizmu. Geni proteina toplinskoga šoka (*HSP*) prvi put su otkiveni u žlijezdama slinovnicama voćne muhe *Drosophila melanogaster*, a sinteza Hsp proteina regulirana je okolišnim, fiziološkim i patofiziološkim procesima (64).

Hsp70 porodicu proteina čini skupina evolucijski visokoočuvanih proteina čija se molekulska masa nalazi unutar raspona od 68 do 75 kDa i čini ju 13 članova - HspA1A, HspA1B, HspA1L, HspA2, HspA5, HspA6, HspA7, HspA8, HspA9, HspA12A, HspA12B, HspA13, HspA14 (65). Razlikujemo konstitutivno eksprimirane oblike (Hsc70 ili Hsc73) te inducibilne oblike (iHsp70 ili Hsp72) Hsp70 proteina čija je sinteza regulirana na razini transkripcije (HspA1A, HspA1B, HspA5, HspA6 i HspA14). Svi članovi Hsp70 porodice građeni su od visokokonzerviranih N-terminalnih ATP-aznih domena i C-terminalnih domena gdje se odvija vezanje supstrata (66 – 68).

Hsp70 ima višestruke funkcije. Unutarstanična funkcija proteina Hsp70 kao molekulskog šaperona je potpomaganje pravilnog smatanja polipeptidnih lanaca, stabilizacija konformacije novosintetiziranih proteina prije daljnje obrade u citoplazmi ili procesa translokacije u endoplazmatski retikul i mitohondrije. Osim toga, Hsp70 sprječava agregaciju proteina i potiče popravak denaturiranih proteina te njihovu proteasomalnu razgradnju u slučaju velikog oštećenja nakon utjecaja stresnih čimbenika (69, 70). Unutarstanični Hsp70 ima zaštitnu staničnu ulogu i regulira imunosni odgovor (71, 72).

Na ekspresiju Hsp70 utječe prisutnost čimbenika stresa poput povišene temperature, oksidacijskog stresa, ultraljubičastog začenja, virusnih i bakterijskih infekcija i drugih. Razina ekspresije Hsp proteina regulirana je transkripcijskim čimbenicima toplinskoga šoka (HSF, engl. *heat shock factor*) od kojih razlikujemo 4 člana. Oni se nalaze u svom monomernom obliku kada nisu aktivirani (71). HSF-1 je primarni regulator transkripcije Hsp70 u sisavaca, a njegova se aktivacija sastoji od nekoliko koraka – translokacije u jezgru, trimerizacije i fosforilacije te vezanja na element toplinskoga šoka (HSE, engl. *heat shock element*) u

promotorskoj regiji *HSP70* gena. Aktivacija HSF-1 može biti negativno regulirana posttranslacijskim promjenama poput fosforilacije specifičnih serinskih ostataka.

Unutarstanični oblik Hsp70 svoje antiupalno djelovanje uglavnom vrši na način da potiskuje aktivaciju NF-κB signalnog puta. Osim toga, djeluje antiapoptozno, a smatra se i da je dio unutarstaničnih antioksidacijskih mehanizama (71).

Osim unutar stanice, Hsp70 može se nalaziti u izvanstaničnom prostoru u koji dolazi pasivnim i/ili aktivnim otpuštanjem iz stanica, poput mononuklearnih stanica iz periferne krvi, tijekom odgovora urođenog ili stečenog imunosnog sustava (36, 69). Ipak, najviše ga oslobađaju stanice prilikom oštećenja koje uzrokuje njihovu lizu (63). Hsp70, koji se otpušta iz nekroznih stanica, posjeduje jako imunostimulacijsko djelovanje (73). S obzirom da je eHsp70 povezan s aktivacijom imunosnog sustava, njegova povišena koncentracija povezuje se sa sustavnom upalom i oksidacijskim stresom (64). Hsp70 u izvanstaničnom prostoru djeluje kao DAMP molekula koja alarmira imunosni sustav te modulira lučenje upalnih citokina.

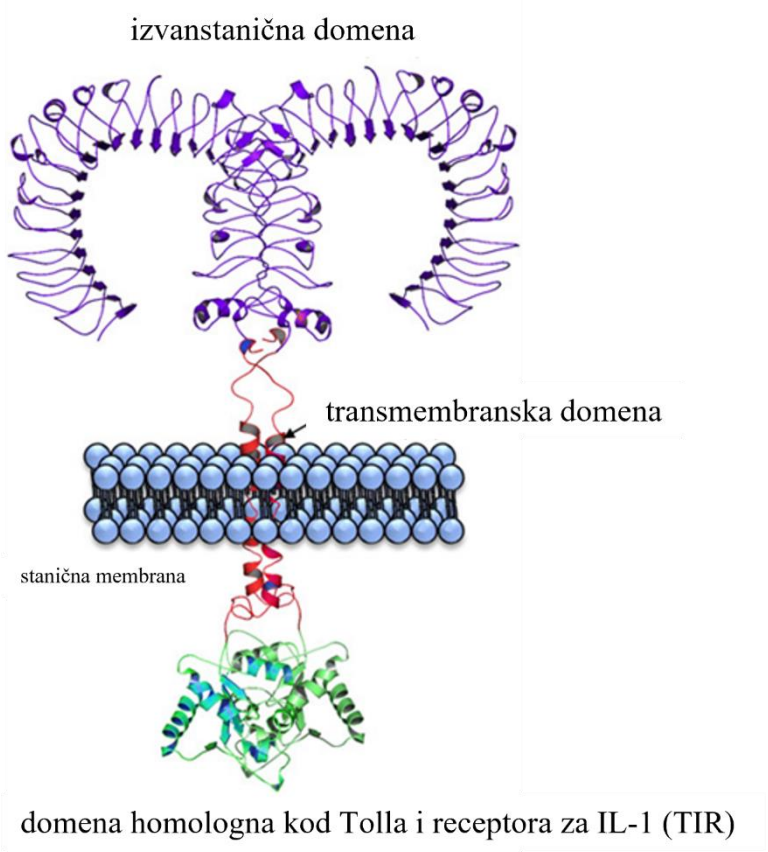
Konačan omjer unutarstaničnog i izvanstaničnog Hsp70 određuje smjer imunosnih reakcija što bi moglo predstavljati veliki značaj u bolestima koje u svojoj patogenezi imaju kroničnu upalu.

1.2.2.1. Receptori TLR2 i TLR4

U izvanstaničnom prostoru eHsp70 može se vezati na receptore koji su uključeni u imunosne odgovore. Jedan od receptora na koji se veže je receptor 1 sličan lektinu za oksidirani lipoprotein niske gustoće (LOX-1, engl. *lectin-like oxidized low-density lipoprotein receptor-1*) koji je pronađen na površini humanih dendritičnih stanica, a putem kojeg se aktivira imunosni odgovor posredovan CD8+ stanicama. Uz to, eHsp70 se veže i na lektine slične imunoglobulinu koji vežu sijalinsku kiselinu (Siglec, engl. *sialic acid-binding immunoglobulin-like lectin*) -5 i -14 koji se razlikuju u usmjeravanju imunosnog odgovora. LOX-1 te Siglec-5 i -14 primjeri su receptora „čistača“ (engl. *scavenger receptors*). Najviše istraženi receptori za eHsp70 su receptori slični Tollu (TLR, engl. *Toll-like receptor*) (65, 73).

TLR-ovi pripadaju skupini evolucijskih očuvanih receptora za prepoznavanje obrazaca (PRR, engl. *pattern recognition receptor*), koju još čine lektinski receptori tipa C (CLR, engl. *C-type lectin receptor*), receptori NLR, receptori krajnjih produkata uznapredovale glikacije (RAGE, engl. *receptor for advanced glycation end products*) i receptori slični genu 1 kojeg inducira retinoična kiselina (RLR, engl. *retinoic acid-inducible gene 1 (RIG-1)-like receptor*) (74). Aktiviraju se vezanjem molekularnih obrazaca povezanih s patogenima (PAMP, engl. *pathogen-associated molecular pattern*) i DAMP-ova kao što je eHsp70. Također, smatra se da neke od komponenata dima cigareta mogu izravno djelovati na TLR-ove. Aktivacijom TLR-ova pokreću se složeni signalni putovi koji su primarno odgovorni za aktivaciju i regulaciju imunosnog odgovora, odnosno za sazrijevanje stanica koje prezentiraju antigen (APC engl. *antigen presenting cell*).

TLR-ovi su transmembranski proteini, a TLR4 ponekad može biti internaliziran ili eksprimiran unutar stanice (75). U transmembranskom obliku imaju izvanstanično N-terminalno područje s ponavljajućim sljedovima bogatim leucinom (LRR, engl. *leucine-rich repeat*), transmembransko područje i citoplazmatsko područje odgovorno za prijenos signala, tzv. domenu homolognu kod Tolla i receptora za IL-1 (TIR, engl. *Toll/interleukin-1 receptor homology domain*) (Slika 6) (76).



Slika 6. Građa receptora TLR.

TLR – receptor sličan Tollu; TIR – domena homologna kod Tolla i receptora za interleukin-1.

Preuzeto iz: Anwar i sur., 2018.

TIR domena građena je od 5 centralnih β-nabranih ploča i okružena je sa 6 α-uzvojnica koje čine tri strukturne podjedinice nazvane Box1, Box2 i Box3 (77). TIR domena TLR-ova veže se za TIR domenu adaptacijskih molekula i tako pokreće unutarstaničnu signalnu kaskadu. Zajednički signalni put TLR-ova završava aktivacijom transkripcijskog čimbenika NF-κB i protein-kinaze aktivirane mitogenom (MAPK, engl. *mitogen-activated protein kinase*), a ključna adaptacijska molekula u tom procesu je čimbenik mijeloidne diferencijacije 88 (MyD88, engl. *myeloid differentiation factor 88*). Cilj aktivacije tih unutarstaničnih signalnih putova je umanjiti učinak štetnog izvora upalnog odgovora i popraviti oštećeno tkivo ukoliko ono postoji. Jaki upalni odgovor narušava homeostazu organizma i potiče upalno stanje

sintezom upalnih citokina poput IL-1β, IL-6 i TNF-α što doprinosi razvoju i progresiji bolesti (78, 79).

TLR2 i TLR4 glavni su receptori izvanstaničnog Hsp70. TLR2 prepoznaje i veže peptidoglikane, lipoproteine i lipoteikoičnu kiselinu Gram-pozitivnih bakterija te mikobakterijski lipoarabinomanan, neke vrste lipopolisaharida (LPS, engl. *lipopolysaccharide*) i lipoproteine pretežito porijeklom iz gljivica, npr. zimosan. TLR4 veže LPS iz vanjske membrane Gram-negativnih bakterija, oligosaharide hijaluronske kiseline, fibrinogen i proteine virusne ovojnice (80).

Povećana koncentracija eHsp70 u KOPB-u marker je aktivacije imunosnog sustava (36). Smatra se da je razlog povećanja koncentracije eHsp70 stalna prisutnost signala za upalni imunosni odgovor. Posljedično, u plućima dolazi do regrutacije neutrofila i monocita koji otpuštaju proteolitičke enzime i tako dolazi do uništavanja plućnog tkiva. Cigaretni dim i komponente zagađenog zraka mogu izravno aktivirati TLR-ove jer sadrže LPS ili neizravno poticanjem stanica na opuštanje DAMP-ova. Pritom valja naglasiti da aktivacija imunosnog sustava u KOPB-u nije ograničena samo na pluća, već se odvija na razini cijelog organizma (81).

1.2.3. Adenozin-trifosfat

Adenozin-trifosfat je nukleotid građen od dušične baze adenina, pentoznog šećera D-riboze i tri fosfatne skupine koje su vezane na 5'-OH skupinu riboznog prstena. Glavne uloge ATP-a unutar stanice su sudjelovanje u biosintezi i ionskom transportu, dok izvan stanice ulazi u interakciju s izvanstaničnim receptorima i enzimima. ATP se otpušta iz svih tipova živčanih završetaka, eritrocita, trombocita, skeletnih mišića, stanica u apoptozi, vaskularnih glatkih mišića, endotelnih i epitelnih stanica (82, 83) te uzrokuje različite stanične odgovore koji rezultiraju kontrakcijom mišića, vazodilatacijom krvnih žila, agregacijom trombocita, rastom stanica i dr. (84)

Međutim, u izvanstaničnom prostoru ATP se vrlo brzo razgrađuje djelovanjem biljega diferencijacije (CD, engl. *cluster of differentiation*) 39 i 73 do adenozin-difosfata, adenozin-monofosfata i adenozina. Produkti razgradnje vežu se za svoje receptore te dolazi do slabljenja upalnog imunosnog odgovora. Povećanim otpuštanjem i/ili nedostatnom razgradnjom ATP-a zbog smanjene ekspresije CD39 i CD73 narušava se ravnoteža otpuštanja i razgradnje ATP-a što je često uočeno u upalnim stanjima (82, 85).

Izvanstanični oblik ATP-a u krvi detektiran je 1969. godine (86), no dosad utvrđene koncentracije eATP-a su vrlo niske, od 20 do 100 nM (87). Smatra se da se ATP iz stanica može otpuštati aktivnim i pasivnim procesima.

Tkivna oštećenja različite etiologije (sepsa, ishemija) popraćena su povećanjem vaskularne permeabilnosti što dovodi do curenja tekućine u izvanstanični prostor nakon čega se sav sadržaj neselektivno ispušta u okoliš. ATP izlazi iz stanica niz koncentracijski gradijent uslijed mehaničkog oštećenja ili nekroze. Međutim, ATP se može otpuštati iz stanica i specifično reguliranom egzocitozom, mikrovezikularnim transportom te različitim tipovima kanala (koneksinski i paneksinski, kanali prolaznog potencijala) i prijenosnika (88, 89).

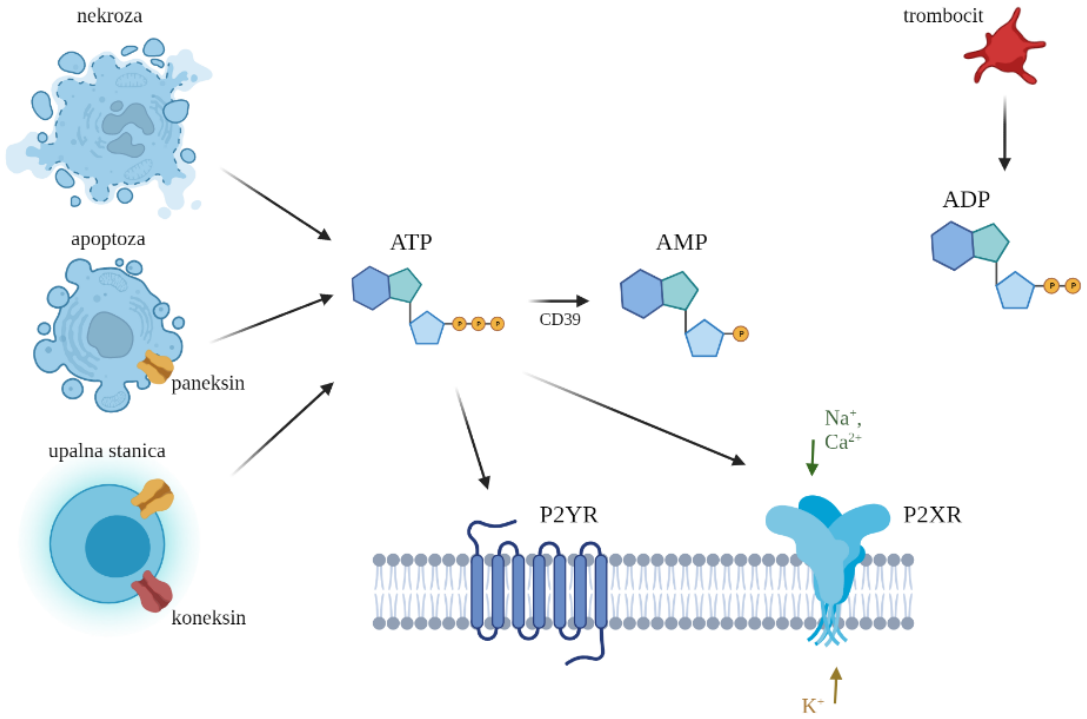
1.2.3.1. Purinergijski receptori P2X7R i P2Y2R

Slično kao i eHsp70, eATP predstavlja signal za opasnost koji okolne stanice primaju nakon što se eATP veže za transmembranske purinergijske receptore. Purinergijsko signaliziranje prvi puta je opisano 1970. godine kao oblik međustanične komunikacije (90). Veliki broj stanica na svojoj površini eksprimira P1 i P2 purinergijske receptore koji se međusobno, osim u građi, razlikuju u selektivnosti vezanja nukleotidnih liganada i afinitetu prema njima. Nukleotidi su vrlo dobri prijenosnici signala s obzirom na njihovu visoku

unutarstaničnu i nisku izvanstaničnu koncentraciju, topljivosti u vodi i brzu hidrolitičku razgradnju nukleotidazama (Slika 7) (88, 91).

Najznačajniji receptori za eATP su P2 receptori putem kojih eATP djeluje autokrino i parakrino. Selektivni su za dvo- i tro-fosfatne nukleotide, a razlikujemo dvije porodice receptora: P2X receptore koji su ionski kanali i P2Y receptore koji su povezani s G proteinom (86).

P2X porodica receptora obuhvaća 7 kationskih kanala moduliranih izvanstaničnim Ca^{2+}, Na^{+}, Mg^{2+} i H^{+} kationima od kojih je najviše istražen P2X7R. Aktivacija P2X receptora mobilizira unutarstanični Ca^{2+} i uzrokuje depolarizaciju stanične membrane. Vezanjem eATP-a na P2X7R smanjuje se unutarstanična koncentracija K^{+} te može doći do aktivacije NLRP3 inflamasoma, multimernog proteinskog kompleksa koji se sastoji od NLR receptora, adaptacijskog proteina nalik mrljicama povezanog s apoptozom koji sadrži domenu za aktivaciju i privlačenje kaspaza (ASC, engl. *apoptosis-associated speck like protein containing a caspase activation and recruitment domain*) i proteaze kaspaze-1 (92, 93). Aktivacija NLRP3 inflamasoma može dovesti do piroptoze (stanične smrti regulirane kaspazom-1 u kanonskom, odnosno kaspazama-4 i -5 u nekanonskom putu (94)) i do otpuštanja upalnih citokina IL-1β i IL-18. Porastom unutarstanične koncentracije Ca^{2+} dolazi do aktivacije kalcijevih signalnih kaskada, npr. p38 MAPK ili fosfolipaze A2. Kada je P2X7R dulje izložen visokim koncentracijama eATP-a (>10 sekundi), može stvarati velike makromolekulske pore kroz koje prolaze molekule < 900 Da (92, 95).



Slika 7. Otpuštanje ATP-a iz nekroznih, apoptoznih i upalnih stanica, njegov put hidrolitičke razgradnje izvan stanice i receptori na koje se veže.

ATP – adenozin-trifosfat; ADP – adenozin-difosfat; AMP – adenozin-monofosfat; CD – biljeg diferencijacije.

Napravljeno pomoću https://biorender.com/

S druge strane, P2Y porodica receptora obuhvaća 8 receptora povezanih s G proteinom koje mogu aktivirati ATP, uridin-trifosfat (UTP, engl. *uridine triphosphate*), uridin-difosfat (UDP, engl. *uridine diphosphate*) i/ili aktivirani šećeri vezani za UDP (96). P2Y2R također ima značajnu ulogu u oblikovanju imunosnog odgovora tako što privlači imunosne stanice, ponajviše makrofage i neutrofile, na mjesto ozljede koje fagocitiraju nekrozne i apoptozne stanice. Prilikom poremećaja u regulaciji P2Y2R, fagociti ne vrše svoju funkciju te dolazi do pojačane upale i oštećenja tkiva. P2Y2R najveći afinitet pokazuje prema vezanju ATP-a i UTP-a. Receptor je rasprostranjen na različitim stanicama poput plućnih stanica, stanica skeletnih mišića, slezene, srca, mozga, makrofaga, limfocita, neutrofila i drugih (97, 98).

Široka rasprostranjenost P2 purinergijskih receptora u stanicama dišnog sustava te sveprisutnost ATP-a ukazuju na mogućnost da je eATP važan medijator upalnog imunosnog odgovora te tako može biti povezan s patologijom različitih bolesti dišnog sustava, poput KOPB-a (82).

1.3. Genska komponenta KOPB-a

Iako su za nastanak i razvoj KOPB-a značajni okolišni čimbenici, ponajviše udisanje cigaretnog dima, vrlo bitnu ulogu u etiologiji ima i genetika.

SERPINA1 dosada je jedini gen za koji je utvrđena povezanost s većim rizikom od KOPB-a. *SERPINA1* kodira za proteinski produkt A1AT, koji je inhibitor leukocitne elastaze, enzima iz azurofilnih granula neutrofila. Nedostatak A1AT nasljedna je autosomna kodominantna bolest i najčešće je vezana uz Z alel koji vodi do promjena u kodirajućem slijedu i uzrokuje zamjenu aminokiselina zbog čega se stvaraju polimeri A1AT u hepatocitima (99 – 101). Zbog agregacije polimera, izlučivanje A1AT je smanjeno te je djelovanje proteolitičkih enzima izraženije što dovodi do pojave emfizema.

Iako dosada nije otkrivena nijedna druga genska komponenta koja je povezana s KOPB-om, genetička istraživanja o KOPB-u pridonose razumijevanju različitih fenotipova bolesti kao i razumijevanju patogeneze poligenskih kompleksnih bolesti. Pretpostavlja se da nacionalna pripadnost, spol, pušačke navike ispitanika, odnosno heterogenost ispitanika u istraživanjima kao i postojanje različitih fenotipova KOPB-a otežavaju detekciju gena koji su povezani s rizikom od razvoja KOPB-a (101, 102).

Populacijska istraživanja povezanosti između bolesti i promjene u lokusu u genomu najčešće ispituju polimorfizme jednog nukleotida (SNP, engl. *single nucleotide poylmorphism*). Genski polimorfizam definira se kao svaka strukturna promjena genomske DNA s pojavom više od jednog alela na istom lokusu, a koja se pojavljuje u više od 1 % sveukupne populacije. SNP uključuje izmjenu jednog nukleotida (A, T, G ili C) drugim tako uzrokujući promjenu u genskoj poruci zapisanoj u DNA. Pritom kompleksne bolesti poput KOPB-a nisu uzrokovane samo jednom genskom varijantom, već interakcijama između različitih genskih varijanti, interakcijama gena i proteina, odnosno gena i okolišnih čimbenika (1, 103). Složene interakcije utječu na razinu rizika od bolesti, klinički tijek i ishod bolesti. Smatra se da bi SNP-ovi iz ljudskog genoma mogli biti povezani s bolestima s obzirom na visoku učestalost (50 000 – 250 000 SNP-ova u cijelom genomu) i da mogu poslužiti kao biljezi radi utvrđivanja genske pozadine kompleksnih bolesti. Stoga su brojna istraživanja usmjerena na utvrđivanje povezanosti gena s rizikom od nastanka određenih bolesti te fenotipovima bolesti, utjecaja genskih varijanti na funkciju proteina i ostalo. Time se proširuje znanje o patogenezi bolesti na genskoj razini, otvara mogućnost razvoja novih terapijskih strategija te stvara potencijal za nove i/ili dodatne dijagnostičke biljege.

SNP-ovi se mogu nalaziti u kodirajućim i nekodirajućim područjima gena. Oni koji se nalaze u kodirajućim područjima razlikujemo prema tome utječu li na aminokiselinski slijed u proteinu (engl. *nonsynonymous SNP*) ili ne utječu (engl. *synonymous SNP*). Najčešće funkcionalne genske varijante imaju regulacijsku ulogu i smještene su u nekodirajućim dijelovima te mogu utjecati na transkripciju gena, stabilnost glasničke ribonukleinske kiseline (mRNA, engl. *messenger ribonucleic acid*), prekrajanje mRNA ili translaciju. Time mogu predstavljati predispoziciju za obolijevanje od bolesti.

Projekt ljudskog genoma (engl. *the Human Genome Project*) uvelike je pridonio identifikaciji gena u različitim populacijama, a time se stvorila velika baza podataka koja postaje temelj daljnjih istraživanja. Cjelogenomska asocijacijska istraživanja (GWAS, engl. *genome-wide association study*) postaju široko zastupljena zbog brzog tehnološkog napretka u genetici. Ona se temelje na dobivanju velike količine podataka bez postavljanja hipoteza te se njima pretražuje cijeli genom testiranjem i do nekoliko milijuna genskih varijanti čija je učestalost u pravilu > 5 % (104). Suprotno tome, analize gena kandidata (engl. *candidate gene analysis*) usmjeravaju se na utvrđivanje povezanosti točno definiranih varijacija gena s određenim ishodom, npr. bolesti na temelju poznatih uloga genskih produkata u bolesti.

Različita istraživanja identificirala su potencijalne gene kandidate za KOPB, a neki od kojih su *IL6R*, *MMP12*, *HHIP*, *AGER*, *FAM13A*, *CHRNA3/5*, *TGF-B2* (99, 100, 105 – 107). Publikacija o prvom GWAS istraživanju u kojem su se pretraživali geni povezani s KOPB-om objavljena je 2009. godine (108). Analizirano je 100 SNP-ova te su detektirane dvije varijante za alfa (α) 3 i 5 podjedinice nikotinskog acetilkolinergijskog receptora (CHRNA3/5, engl. *cholinergic receptor nicotinic alpha 3 and 5 subunits*) koje su bile povezane s plućnom funkcijom, ali i s rizikom od karcinoma pluća u prijašnjim istraživanjima. Međutim, zbog različitih pušačkih navika ispitanika, vrlo je složeno interpretirati rezultate s obzirom da postoje mnoga druga patološka stanja povezana s pušenjem. Osim CHRNA3/5, u manjoj skupini istog istraživanja primijećena je povezanost između proteina koji stupa u interakciju s hedgehog proteinima (HHIP, engl. *hedgehog interacting protein*) i plućne funkcije (108). Zhou i sur. kasnije su analizom interakcija između pojedinih regija gena i promotora utvrdili varijantu zbog koje dolazi do smanjene ekspresije gena *HHIP* (109). Istraživanja su dokazala da je gen *AGER*, koji kodira za receptor RAGE, povezan s rizikom od ubrzanog pada plućne funkcije i početkom razvoja KOPB-a (110, 111). Polimorfizam rs2070600 pokazao je snažnu povezanost s padom plućne funkcije i serumskom koncentracijom RAGE-a što znači da bi polimorfizam mogao imati funkcionalne posljedice povezane s KOPB-om (112).

Glikozilacijska promjena u RAGE-u koja se događa zbog polimorfizma u genu *AGER* mogla bi povećati aktivnost receptora RAGE i dovesti do povećanog otpuštanja upalnih citokina, odnosno oštećenja plućnog tkiva (113).

Osim u genu za RAGE, polimorfizmi u genima za TLR2 i TLR4 bili su povezani s padom FEV_1 (%) i povećanim brojem upalnih stanica u KOPB-u (114). KOPB je kompleksna poligenska bolest i još uvijek nije poznato koji su sve geni uključeni u imunosni odgovor, no s obzirom da postoji povezanost eHsp70 i njegovih receptora s KOPB-om, geni koji kodiraju za navedene proteine mogli bi imati ulogu u modulaciji imunosnog odgovora u KOPB-u. Poznato je da je ekspresija gena *HSP70*, *TLR2* i *TLR4* promijenjena u KOPB-u. Pouwels i sur. pretpostavili su da bi povećana ekspresija *TLR2* i *TLR4* iz neutrofila iz periferne krvi u pacijenata s KOPB-om mogla biti povezana s većom osjetljivosti neutrofila na DAMP-ove tijekom egzacerbacija (115). Stoga, polimorfizmi gena koji kodiraju za Hsp70, TLR2 i TLR4 mogli bi biti povezani s njihovom promijenjenom genskom ekspresijom, odnosno s aktivnošću receptora i upalnim odgovorom popraćenim visokim koncentracijama upalnih citokina i drugih upalnih biljega.

Funkcionalne varijante najčešće su regulacijske, a ne one koje izravno utječu na proteinsku strukturu. Osim toga, regulacija genske ekspresije postaje vrlo kompleksna s obzirom na tkivnu specifičnost pa postoji mogućnost da se utjecaj genske varijante na ekspresiju ne detektira u odabranoj vrsti uzorka u kojoj se provodi ispitivanje.

Nadalje, u poligenskim bolestima vrlo je teško odvojiti učinak pojedinog SNP-a. Smatra se da se pojedini polimorfizmi mogu nasljeđivati zajedno, odnosno da među njima postoji neravnoteža povezanosti (LD, engl. *linkage disequilibrium*). LD opisuje se veličinama r^2 i D' (Lewontin koeficijent). Obje veličine mogu poprimiti vrijednost od 0 do 1, a ukoliko je vrijednost blizu 1, znači da su polimorfizmi međusobno povezani, odnosno nasljeđuju se zajedno te čine blokove, tzv. haplotipove.

U sklopu ovog doktorskog rada za procjenu LD-a koristio se D' jer na r^2 iznimno utječe učestalost rjeđeg alela (MAF, engl. *minor allele frequency*) što može dovesti do pogrešnog zaključka o međusobnoj povezanosti polimorfizama ukoliko se analiziraju SNP-ovi s velikim razlikama u MAF-u (116). Kao kriterij za D' korištena je vrijednost 0,80 pa je za sve polimorfizme s $D' > 0,80$ smatrano da se nasljeđuju zajedno (117).

1.4. Obrazloženje teme

KOPB predstavlja značajni javnozdravstveni problem. Prema podacima Svjetske zdravstvene organizacije, KOPB je na trećem mjestu uzročnika smrti u svijetu, a procijenjena prevalencija za osobe starije od 40 godina je 13,1 %. KOPB je višekomponentna bolest s heterogenim kliničkim oblicima (fenotipovi) i patobiološkim mehanizmima (endotipovi). Smatra se da na pojavnost i razvoj KOPB-a utječe niz dinamičkih i kumulativnih promjena i interakcija između gena i okoliša. Ipak, utvrđeno je da KOPB nije isključivo plućna bolest, već je i sustavna bolest koja zahvaća niz drugih organa. Kronična upala smatra se središnjim patogenetskim procesom u KOPB-u te značajno utječe na tijek bolesti. Osim na lokalnoj razini u dišnom sustavu, u pojedinih pacijenata razvija se sustavni upalni odgovor. Stoga je **glavni cilj** ovog doktorskog rada ispitati promjene u plazmatskoj koncentraciji dviju molekula koje predstavljaju „signal za opasnost“ kada se nađu u izvanstaničnom prostoru, eHsp70 i eATP-a, te razjasniti patogenezu bolesti na genskoj razini putem određivanja relativne ekspresije gena *HSP70*, *TLR2*, *TLR4*, *P2X7R* i *P2Y2R* i polimorfizama u genima *HSP70*, *TLR2* i *TLR4*.

Uz glavni cilj, **specifični ciljevi istraživanja** su:

1. odrediti koncentracije eHsp70 u EDTA-plazmi pacijenata sa stabilnim oblikom KOPB-a i kontrolnim ispitanicima; u dosadašnjim istraživanjima nisu bile određivane koncentracije eHsp70 u perifernoj cirkulaciji u preporučenom tipu uzorka (EDTA-plazma) na dostatnom broju pacijenata;

2. odrediti koncentracije eATP-a u EDTA-plazmi pacijenata sa stabilnim oblikom KOPB-a i kontrolnim ispitanicima; ne postoje podaci o koncentracijama u perifernoj cirkulaciji pacijenata s KOPB-om;

3. ispitati promjene u koncentraciji općih upalnih biljega (lkc, CRP i Fbg) te upalnih citokina, (IL-1α, IL-1β, IL-8, IL-6 i TNF-α) u pacijenata sa stabilnim oblikom KOPB-a u odnosu na kontrolne ispitanike;

4. za sve parametre procijeniti predikcijski potencijal te u konačnici predložiti kombinirani model od više parametara s najvećim predikcijskim potencijalom za identifikaciju pacijenata oboljelih od KOPB-a.

5. utvrditi dolazi li do promjene u ekspresiji *HSP70, TLR2, TLR4, P2X7R* i *P2Y2R* te istražiti odabrane polimorfizme gena *HSP70, TLR2* i *TLR4.*

Glavna hipoteza istraživanja je da je sustavna upala izraženija u pacijenata s KOPB-om u stabilnoj fazi bolesti u usporedbi s kontrolnom skupinom te da su koncentracije DAMP molekula eHsp70 i eATP-a povećane u pacijenata sa stabilnim KOPB-om. Također, pretpostavljeno je da će se koncentracije eHsp70 i eATP-a mijenjati ovisno o stadiju bolesti na temelju razine plućne opstrukcije i/ili uznapredovalosti simptoma, ali i ovisno o pušačkom statusu budući da je pušenje glavni rizični čimbenik za razvoj KOPB-a. Konačno, pretpostavljeno je da bi pojedine varijante gena *HSP70, TLR2* i *TLR4* mogle biti povezane s rizikom od KOPB-a te se očekuje da će ekspresije gena *HSP70, TLR2, TLR4, P2X7R* i *P2Y2R* biti povećane u pacijenata s KOPB-om u usporedbi s kontrolnom skupinom.

2. POVEZANOST PLAZMATSKOG PROTEINA TOPLINSKOGA ŠOKA 70 SA STADIJEM BOLESTI, PUŠENJEM I PLUĆNOM FUNKCIJOM U PACIJENATA S KRONIČNOM OPSTRUKCIJSKOM PLUĆNOM BOLESTI

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Association of Plasma Heat Shock Protein 70 with Disease Severity, Smoking and Lung Function of Patients with Chronic Obstructive Pulmonary Disease

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Abstract: Extracellular heat shock protein 70 (eHsp70) might modulate immune responses in chronic obstructive pulmonary disease (COPD). The aim of the study was to explore eHsp70 concentration in stable COPD, its association with disease severity and smoking status as well as its diagnostic performance in COPD assessment. Plasma samples were collected from 137 COPD patients and 95 healthy individuals, and concentration of eHsp70 was assessed by commercially available enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Science, Farmingdale, NY, USA). COPD patients were subdivided regarding airflow obstruction severity and symptoms severity according to the Global Initiative for COPD (GOLD) guidelines. eHsp70 concentration increased in COPD patients when compared to controls and increased with the severity of airflow limitation as well as symptoms burden and exacerbation history. eHsp70 concentration did not differ among COPD patients based on smoking status, yet it increased in healthy smokers compared to healthy nonsmokers. In addition, eHsp70 negatively correlated with lung function parameters forced expiratory volume in one second (FEV_1) and FEV_1/ forced vital capacity (FVC), and positively with COPD multicomponent indices BODCAT (BMI, airflow obstruction, dyspnea, CAT score), BODEx (BMI, airflow obstruction, dyspnea, previous exacerbations), CODEx (Charlson's comorbidity index, airflow obstruction, dyspnea, previous exacerbations) and DOSE (dyspnea, airflow obstruction, smoking status, previous exacerbations) With great predictive value (OR = 7.63) obtained from univariate logistic regression, eHsp70 correctly classified 76% of cases. eHsp70 is associated with COPD prediction and disease severity and might have the potential for becoming an additional biomarker in COPD assessment.

Keywords: chronic obstructive pulmonary disease; extracellular heat shock protein 70; smoking; lung function; EDTA plasma

1. Introduction

Heat shock proteins (Hsps) are highly conserved and ubiquitously expressed proteins that normally act as molecular chaperones which help in the maintenance of protein homeostasis by assisting their folding processes [1,2]. Their involvement in proper protein folding, prevention of protein aggregation and apoptosis is of great importance for cellular function, especially when cells are exposed to stressful conditions [3–5]. The 72-kDa Hsp (Hsp70 in the following refers to this protein) is

located in the cytosol and nucleus, and its expression is induced as a part of the response to different stressors like heat, bacterial or viral infections [6,7]. However, apart from being an intracellular protein, Hsp70 can be released from cells passively following cellular lysis i.e., necrotic death, and/or actively through nonclassical exocytotic pathways [8–10]. When found in the extracellular milieu, Hsp70 becomes a damage-associated molecular pattern (DAMP) and represents a danger signal to the immune system [11].

eHsp70 acts mainly as proinflammatory and activates immune responses by engaging appropriate receptors (toll-like receptors (TLRs) 2 and 4, cluster of differentiation (CD) 14, CD40, CD91, lectin-like oxidized low-density lipoprotein-1 (LOX-1), receptor for advanced glycation end-products (RAGE)) [9,12,13]. On the other hand, eHsp70 might also modulate an adaptive immune response through binding to antigenic peptides and presenting them to antigen-presenting cells [14]. The sources of Hsp70 in peripheral circulation have not been fully elucidated. Still, various viable cells of both hematopoietic (e.g., peripheral blood mononuclear cells) and nonhematopoietic origin (e.g., epithelial cells) are being considered as potential candidates [8,15].

COPD is an inflammatory syndrome characterized by permanent airflow limitation. It is a multicomponent condition with both pulmonary and extrapulmonary effects [16]. Chronic respiratory inflammation involves activation and infiltration of macrophages and neutrophils and leads to abnormal immune responses, mucus hypersecretion, oxidant-antioxidant imbalance and apoptosis [17]. The role of Hsp70 in COPD pathogenesis is still unclear, despite the efforts of some researchers. It was found that Hsp70 is increased in sputum of COPD patients compared to both healthy smokers and nonsmokers [18]. In addition, increased expression of Hsp70 at both the mRNA and protein level was detected in lung tissue of COPD patients [17]. To the contrary, lower numbers of Hsp70 immunoreactive cells in bronchial tissue of COPD patients were detected when compared to healthy control subjects [19]. In our previous research, Hsp70 expression was significantly decreased in leukocytes of COPD patients, especially in COPD smokers, but also in healthy smokers in comparison to never-smoking individuals, and we suggested suppressed Hsp70 transcription or its increased release from cells as potential underlying mechanisms that could explain the observed phenomenon [20]. Later, we explored effects of extracellular Hsp70 by employing recombinant human Hsp70 protein (rhHsp70) on human monocytic and bronchial epithelial cellular models (primary cells and cell lines), and we confirmed that rhHsp70 alone, and in combination with cigarette smoke, stimulates TLR2 and/or TLR4 receptors, mitogen-activated protein kinase (MAPK) and/or nuclear factor kappa B (NF-κB) signaling pathways and proinflammatory cytokines release [21–23]. Positive associations between eHsp70 and cytokines, as well as other inflammatory markers in circulation, were reported [24,25], and we established the presence of systemic inflammation in our group of COPD patients [26]. Concentration of eHsp70 was assessed in peripheral circulation of COPD patients in only a few studies, but with inconsistent results in comparison to healthy subjects (increased or similar values) [4,16,27]. However, data about eHsp70's predictive value, and its association with disease severity, are lacking.

We hypothesized that the concentration of eHsp70 in plasma of patients with stable COPD is increased in comparison to healthy individuals, and that it increases with disease severity assessed by the level of airflow obstruction, as well as symptoms and history of exacerbations. In addition, we wanted to investigate the association between eHsp70 concentration and smoking status, as well as its associations with COPD multicomponent indices (BODCAT (BMI, airflow obstruction, dyspnea, CAT score), BODEx (BMI, airflow obstruction, dyspnea, previous exacerbations), CODEx (Charlson's comorbidity index, airflow obstruction, dyspnea, previous exacerbations) and DOSE (dyspnea, airflow obstruction, smoking status, previous exacerbations)) and lung function parameters. Finally, our aim was to evaluate diagnostic performances of eHsp70.

2. Materials and Methods

2.1. Participants

There were 137 patients at the stable phase of COPD and 95 healthy individuals matched by age and sex. COPD was diagnosed by a specialist pulmonologist at the Clinical Department for Lung Diseases Jordanovac, University Hospital Centre Zagreb (Zagreb, Croatia), in 2017 and 2018. Patients were in the stable phase of COPD without exacerbations during the last three months, without changes in their therapy regime and without infections in the lower respiratory tract. Health state of control subjects was established based on anamnestic data and normal spirometry test results. Both patients and healthy individuals had to be older than 40 years, could not have any lung disease (except COPD for COPD patients), could not have inflammatory diseases, manifest cardiovascular diseases, acute infections, diabetes with severe complications, severe liver diseases, severe kidney insufficiencies, malignant diseases, transplantations or other ongoing inflammations. All of them signed an informed consent for the scientific research they volunteered for and were introduced to the aims of the research. The research was performed in accordance with the Helsinki Declaration and was approved by the Ethics Committee of University Hospital Centre Zagreb (Approval Protocol Number: 02/21/JG) on 29 August 2014 and by the Ethics Committee for Experimentation of Faculty of Pharmacy and Biochemistry, University of Zagreb (Approval Protocol Number: 251-62-03-14-78) on 10 September 2014. In addition to the diagnosis criterion (forced expiratory volume in one second/forced vital capacity (FEV_1/FVC) < 0.70) by the Global Initiative for COPD (GOLD), there were classifications of disease severity based on airflow limitation assessed by FEV_1 measurements (GOLD 1–4 stages) as well as the history of symptoms and exacerbations assessed by the score from the COPD Assessment Test (CAT) (GOLD A–D groups) [28]. All participants reported data about smoking status, so groups of healthy nonsmokers (n = 48), healthy smokers (n = 47), COPD nonsmokers (n = 10), COPD former smokers (n = 90) and COPD smokers (n = 37) were formed. For calculation of multicomponent indices related to COPD assessment, the following data were collected for COPD patients: body mass index (BMI), score obtained from modified Medical Research Council (mMRC) Dyspnea Scale, number of previous exacerbations and Charlson's comorbidity index. Afterwards, BODCAT (BMI, airflow obstruction, dyspnea, CAT score), BODEx (BMI, airflow obstruction, dyspnea, previous exacerbations), CODEx (Charlson's comorbidity index, airflow obstruction, dyspnea, previous exacerbations) and DOSE (dyspnea, airflow obstruction, smoking status, previous exacerbations) were calculated [29].

2.2. Assessment of Lung Function

Airflow limitation was diagnosed by spirometry on a Master-Screen Pneumospirometer (Jaeger, Wurzburg, Germany), and airflow obstruction was confirmed if FEV_1/FVC was lower than 0.70 after three acceptable measurements. Furthermore, diffusing capacity for carbon monoxide (DLCO) was measured three times on a Master-Screen PFT Pro (Jaeger, Wurzburg, Germany), as described before [29].

2.3. Measurement of eHsp70

Blood samples were collected between 7 and 9 a.m. into tubes with anticoagulant ethylenediaminetetraacetic acid (EDTA) (Greiner Bio-One, GmbH, Kremsmünster, Austria) by venepuncture of a large antecubital vein after overnight fasting [26]. Plasma was separated after centrifugation at 1000× *g* for 15 min at 4 °C and stored immediately at 80 °C until eHsp70 determination. eHsp70 concentration was measured using the AMP'D HSP70 high sensitivity ELISA kit (Enzo Life Science, Farmingdale, NY, USA). All experiments were performed following the manufacturer's protocol and recommendations, including minimal 1:4 dilution of EDTA plasma samples with assay buffer for matrix interference removal. Concentration of eHsp70 was determined in a randomly chosen sample on all plates and was used for internal validation. Calculation of eHsp70 concentration in samples was performed by a four-parameter logistic curve fitting program within Origin software

(OriginLab Corporation, Northampton, MA, USA). The sensitivity or limit of detection of the assay was 0.007 ng/mL, as determined by the manufacturer.

2.4. Statistical Analysis

Data were tested for normality by the Kolmogorov-Smirnov test, and all data failed it. Therefore, a nonparametric Mann-Whitney test was performed for analysis between controls and COPD patients. When comparing more than two groups based on different classifications, Kruskal-Wallis one way analysis of variance on ranks with post hoc analysis was used. Categorical variables were tested by Chi-squared test. Spearman Rank Order was performed for testing the correlations between investigated parameters, while assessment of predictive value of eHsp70 was obtained by univariate logistic regression analysis. Statistical tests were run in MedCalc statistical software version 17.9.2. (Ostend, Belgium), and results were considered statistically significant if $p < 0.05$.

3. Results

3.1. Association of eHsp70 with COPD Severity

Patients with stable COPD were of similar age as control subjects, and gender distribution was also similar between patients and healthy individuals, while lung function was decreased in the COPD group as expected (Table 1). The concentration of eHsp70 was increased in the plasma of COPD patients (0.98 (0.63–1.29) ng/mL) in comparison to controls (0.37 (0.25–0.63) ng/mL) ($p < 0.001$). Moreover, it was associated with disease severity when COPD patients were subdivided regarding FEV_1-based airflow limitation (Figure 1A) as well as symptoms severity and history of exacerbations (Figure 1B). eHsp70 showed statistically significant differences regarding GOLD 2–4 stages in comparison to controls ($p < 0.001$) and throughout GOLD A–D groups in comparison to controls ($p < 0.001$). Increasing concentration of eHsp70 successfully distinguished each group of patients regarding both subdivisions.

Table 1. Basic characteristics and spirometry parameters of all participants. Age was shown as median with minimum and maximum, and gender as absolute number. All other data were presented as median with interquartile range. Data were tested by Chi-squared or Mann-Whitney test.

Parameter	Controls n = 95	COPD Patients n = 137	*p*-Value
age	64 (46–83)	65 (44–86)	0.073
gender male/female	49/46	86/51	0.118
FEV_1 (L)	2.60 (2.12–3.19)	1.08 (0.78–1.57)	<0.001
FEV_1 (% pred.)	93 (86–104)	39 (28–60)	<0.001
FVC (L)	3.35 (2.77–4.16)	2.28 (1.81–2.77)	<0.001
FEV_1/FVC (%)	81 (77–88)	48 (41–58)	<0.001

COPD–chronic obstructive pulmonary disease; FEV_1—forced expiratory volume in one second; FVC—forced vital capacity.

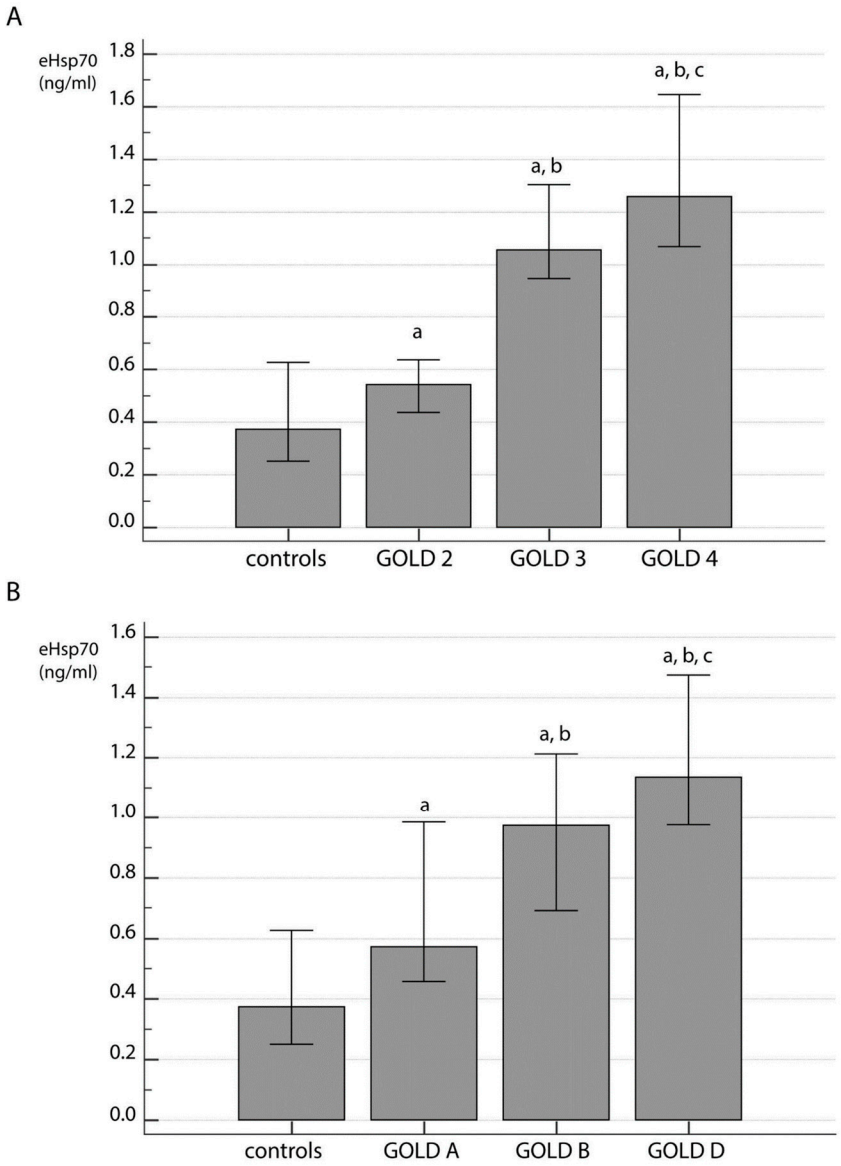

Figure 1. Concentration of eHsp70 in plasma of COPD patients regarding forced expiratory volume in one second (FEV_1)-based classification by Global Initiative for COPD (GOLD) (**A**) and ABCD classification based on symptoms severity and history of exacerbations (**B**). All data were presented as median with interquartile range. Statistical analysis was performed by Kruskal-Wallis one way analysis of variance on ranks. [a] statistically significant increase in eHsp70 concentration in comparison to controls; [b] statistically significant increase in eHsp70 concentration in comparison to GOLD 2 (**A**) or GOLD A (**B**); [c] statistically significant increase in eHsp70 concentration in comparison to GOLD 3 (**A**) or GOLD B (**B**).

3.2. Influence of Smoking Status on Plasma eHsp70 Concentrations

When all participants were compared based on self-reported smoking history, it was observed that there were significant differences in eHsp70 levels between controls and COPD patients ($p < 0.001$). More precisely, COPD patients had increased eHsp70 when compared to both healthy nonsmokers and healthy smokers, yet there was no difference between COPD patients according to their smoking status. However, healthy smokers had higher values of plasma eHsp70 concentrations in comparison to healthy nonsmokers (Figure 2). Additionally, when GOLD 2–4 stages (Figure 3A) and GOLD A–D groups (Figure 3B) were compared to healthy individuals who were grouped according to their smoking status, significant difference in eHsp70 concentration was observed ($p < 0.001$). Healthy smokers had

similar levels of eHsp70 in plasma as COPD patients at GOLD 2 stage and those in the GOLD A group, while patients at more advanced disease stages showed increased levels of plasma Hsp70 (Figure 3).

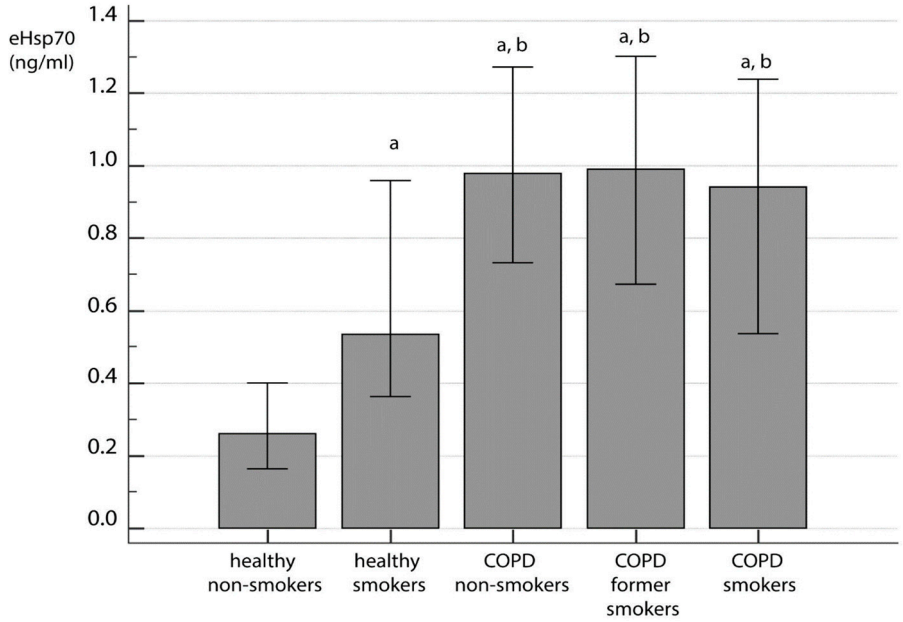

Figure 2. eHsp70 in healthy individuals and COPD patients regarding smoking status. All data were presented as median with interquartile range. Statistical analysis was performed by Kruskal-Wallis one-way analysis of variance on ranks. [a] statistically significant increase in eHsp70 concentration in comparison to healthy nonsmokers; [b] statistically significant increase in eHsp70 concentration in comparison to healthy smokers.

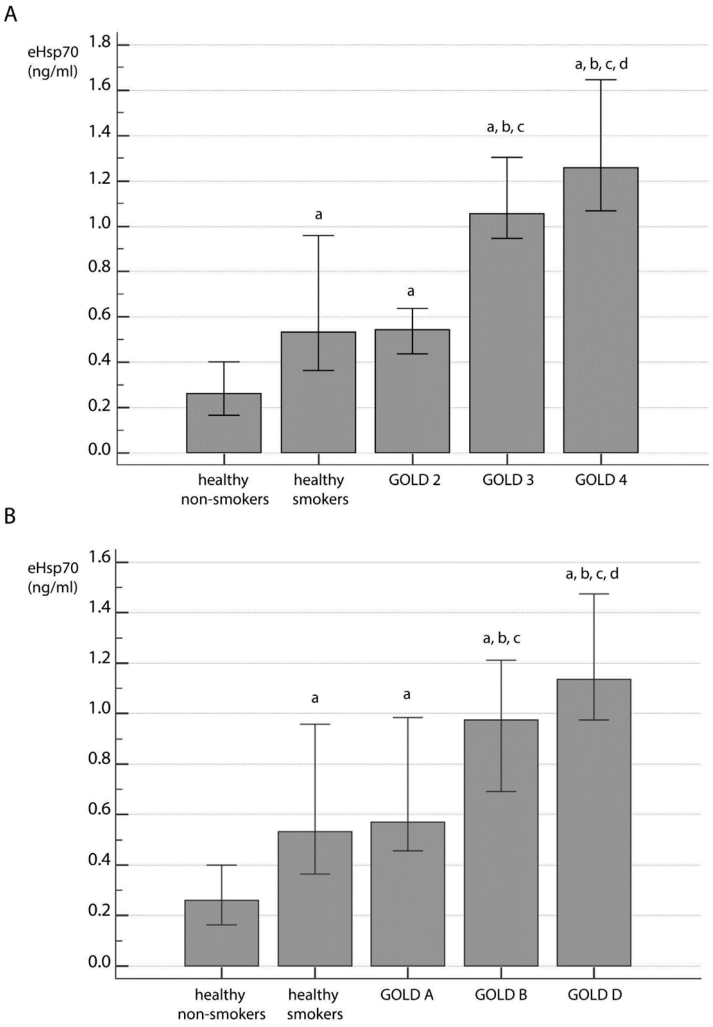

Figure 3. eHsp70 concentration in COPD patients at different stages of FEV_1-based airflow limitation (**A**) and based on symptoms severity (**B**) compared to healthy subjects regarding their smoking status. All data were presented as median with interquartile range. Statistical analysis between five groups of participants was performed by Kruskal-Wallis one-way analysis of variance on ranks. Significant difference in eHsp70 concentration was observed throughout GOLD 2–4 stages and GOLD A–D groups when healthy individuals were subdivided based on their smoking status ($p < 0.001$). Afterwards, post hoc analysis was performed. [a] statistically significant increase in eHsp70 concentration in comparison to healthy nonsmokers; [b] statistically significant increase in eHsp70 concentration in comparison to healthy smokers; [c] statistically significant increase in eHsp70 concentration in comparison to GOLD 2 (**A**) or GOLD A (**B**); [d] statistically significant increase in eHsp70 concentration in comparison to GOLD 3 (**A**) or GOLD B (**B**).

3.3. Associations of Lung Function Parameters and COPD Multicomponent Indices With eHsp70

We found no association between eHsp70 levels and age or gender in either COPD patients or controls. However, eHsp70 showed moderate to good positive correlation with COPD multicomponent indices BODCAT, BODEx (Figure 4A), CODEx and DOSE, as well as moderate to good negative correlation with lung function parameters FEV_1 (L), FEV_1 (% pred.) (Figure 4B) and FEV_1/FVC. DLCO and eHsp70 showed to be poorly negatively correlated ($p < 0.001$ for all correlations) (Table 2).

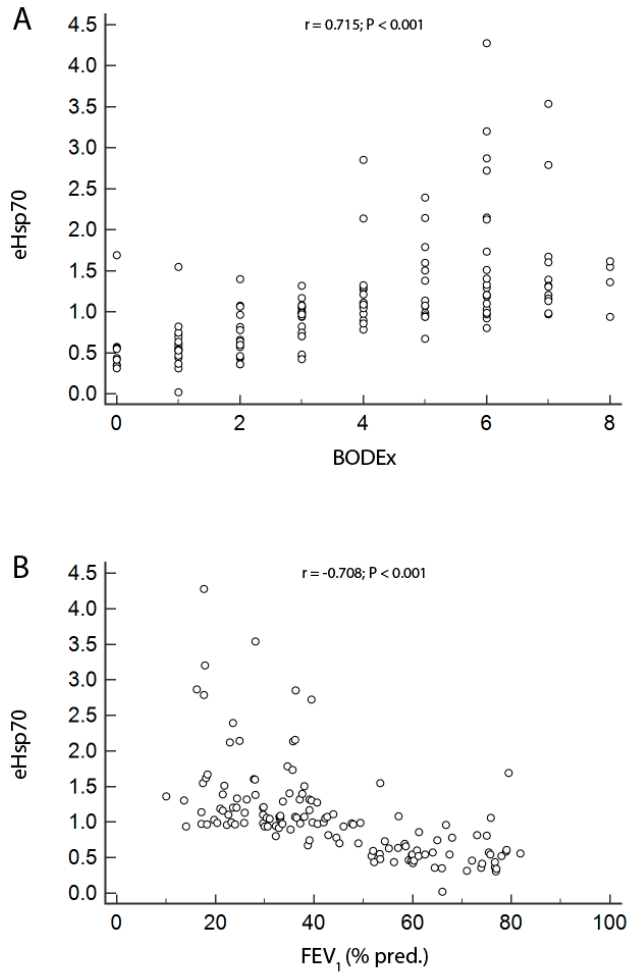

Figure 4. Association of eHsp70 with BODEx (**A**) and FEV_1 (% pred.) (**B**). r-Spearman's correlation coefficient; BODEx—BMI, airflow obstruction, dyspnea, previous exacerbations; FEV_1—forced expiratory volume in one second.

Table 2. Spearman Rank Order analysis was performed between eHsp70 and COPD multicomponent indices as well as lung function parameters.

Parameter	Spearman's Correlation Coefficient, r	*p*-Value
BODCAT	0.712	<0.001
BODEx	0.715	<0.001
CODEx	0.710	<0.001
DOSE	0.672	<0.001
FEV_1 (L)	−0.658	<0.001
FEV_1 (% pred.)	−0.708	<0.001
FEV_1/FVC	−0.644	<0.001
DLCO	−0.479	<0.001

BODCAT—BMI, airflow obstruction, dyspnea, score from COPD assessment test (CAT); BODEx—BMI, airflow obstruction, dyspnea, previous exacerbations; CODEx—comorbidities (Charlson's index), airflow obstruction, dyspnea, previous exacerbations; DOSE—dyspnea, airflow obstruction, smoking status, previous exacerbations; FEV_1—forced expiratory volume in one second; FVC—forced vital capacity; DLCO—diffusing capacity for carbon monoxide. Previous exacerbations are defined as the number of exacerbations in the previous year.

3.4. Predictive Performance of eHsp70

Univariate logistic regression analysis with a defined cut-off value of 0.5 showed that eHsp70 had great predictive value with its odds ratio (OR) of 7.63, 95% confidence interval (CI) = 3.68–15.82 and there were 76% cases correctly classified ($p < 0.001$).

4. Discussion

COPD is a highly prevalent yet underdiagnosed disease, with increasing morbidity and mortality rates. Complex underlying mechanisms are reflected by diverse clinical presentation and are making this disease challenging for specific diagnosis and therapy. Due to COPD heterogeneity, to personalize the treatment, particular endotype and phenotype should be recognized for each patient.

It is now recognized that inflammation in COPD is not present only at the local level i.e., in lungs and airways, but also at the whole-body level, with persistent systemic inflammation being demonstrated in some patients [30]. A blood sample may be obtained in a relatively noninvasive way which makes it easily accessible. Therefore, searching for a good peripheral blood diagnostic, prognostic, predictive biomarker and/or biomarker of disease severity in any disease is recommendable, especially in complex and heterogeneous diseases.

The potential pathogenetic role of eHsp70 is still quite obscure. However, it seems to be associated with an eHsp70 immunomodulatory function. Immune cells can recognize eHsp70, which initiates signal transduction and results in the release of cytokines. Moreover, crosstalk with TLRs activates proinflammatory signals which result in promoting and prolonging chronic inflammation [31]. Also, inflammation-related outcomes of eHsp70 might be executed by NLRP3 inflammasome activation [32]. Besides COPD, eHsp70 seems to be implicated in other respiratory diseases, such as asthma, with a similar pathogenetic background. It was reported that plasma eHsp70 was increased in asthmatic patients compared to healthy individuals [3], as well as in pregnant asthmatics, in comparison to healthy pregnant women [33]. Some studies also detected elevated concentrations of Hsp70 in the sera of lung cancer patients [34,35].

In this study, we assessed the concentration of Hsp70 in the peripheral blood of patients with stable COPD, and association with disease characteristics defined by spirometry and clinical presentation. eHsp70 was significantly elevated overall in COPD patients compared to healthy subjects. This increase was related to the degree of airflow limitation as well as symptoms burden and history of exacerbation. eHsp70 concentrations were the highest in GOLD 4 stage and GOLD D group. It is also important to emphasize that eHsp70 was elevated even in GOLD A and GOLD 2 (which, in clinical practice, is often the lowest GOLD stage) compared to the overall control group. To the best of our knowledge, this is the first study that has assessed eHsp70 concentrations in COPD patients subdivided by the GOLD ABCD classification, and the first to show differences in eHsp70 levels according to GOLD stages.

Dong et al. reported that the expression of intracellular Hsp70 was closely related with COPD severity, and was higher in GOLD 2, 3 and 4 stages compared to the GOLD 1 stage [17]. However, the association of extracellular Hsp70 with disease severity has not yet been shown for COPD, but demonstrated for some other diseases, namely asthma, chronic heart failure and rheumatoid arthritis [3,36,37].

In the present study, when participants of the control group were subdivided according to their smoking status, patients belonging to the GOLD 2 and GOLD A subgroups had higher eHsp70 levels than never-smoking individuals, but similar eHsp70 levels as smokers with normal lung function. Therefore, it could be suggested that so-called healthy smokers might be more susceptible to altered inflammatory responses provoked by eHsp70 being a danger signal to the immune system, and some of them might even develop COPD in the future. This assumption should be tested in future studies. In addition, as only 20% of smokers develop COPD, a specific individual genetic makeup seems to be a determined for the disease.

By searching the literature, we found only three studies that assessed Hsp70 concentration in the blood of COPD patients [4,16,27]. However, in addition to being performed on significantly lower

number of participants compared to our study, there are some concerns regarding their sample and ELISA kit selection. Reported concentrations of eHsp70 are very dependent on the matrix in which it is measured. Whitham and Fortes demonstrated that eHsp70 concentrations were the highest in EDTA plasma. However, values in heparinized plasma were somewhat lower, while the lowest eHsp70 levels were measured in serum, and they hypothesized that this was due to the binding of eHsp70 to the aggregated clotting proteins in serum. Therefore, they recommended EDTA plasma as a sample of choice in future investigations [8]. This is important for studies with healthy participants at rest as their eHsp70 values tend to be low, as well as for the studies with elderly subjects since their eHsp70 concentrations are significantly lower than in young individuals [25].

As already mentioned, eHsp70 in COPD patients was assessed in serum [4,27] or heparinized plasma [16]. However, although Ünver et al. used serum samples, the measurement of eHsp70 was performed by an adopted ELISA kit that was not entirely appropriate for blood as a matrix, and this could be the reason for the extremely high eHsp70 values obtained in the study [4]. Hacker et al. also used an adopted ELISA kit that was specific for intracellular Hsp70 determination in cell lysates [27]. Limitations of these assays were caused by the lack of optimization for biological fluids such as blood. On the other hand, Cui et al. selected a proper ELISA kit which was validated for serum and EDTA plasma, but they chose heparinized plasma as the sample for eHsp70 measurement, which might be the reason for obtaining higher eHsp70 values [16]. In the present study, we used EDTA plasma and an ELISA kit that was more sensitive and could detect lower eHsp70 concentrations compared to other ELISA kits also validated for EDTA plasma and serum [38]. With this choice of sample and kit we were able to detect eHsp70 in each study participant (controls and patients).

In this study, we obtained positive associations between eHsp70 and multicomponent COPD indices (BODCAT, BODEx, CODEx, DOSE) that reflect airflow obstruction, smoking status, symptoms and history of exacerbations, which are all important in the assessment of the patients' overall condition. We also obtained significant negative associations between eHsp70 and lung function parameters, and this was only reported for the expression of intracellular Hsp70 in COPD patients with good correlation for FEV_1/FVC and poor correlation for FEV_1 (% pred.) [17]. Finally, eHsp70 was shown to have a good predictive characteristic with its OR of 7.63 (95% CI = 3.68–15.82).

Although we presented some novel and interesting results, our study had some limitations. It did not include COPD patients from the GOLD C group or the GOLD 1 stage. However, in clinical practice COPD patients belonging to the GOLD 1 group rarely contact their physician due to very mild symptoms, and the GOLD C category of patients is also very rare as they do not have many symptoms and are not usually frequent exacerbators. A larger number of participants should be recruited in further studies, and a longitudinal study design should be considered.

5. Conclusions

This study demonstrated that eHsp70 concentrations were increased in EDTA plasma of COPD patients in the stable phase of the disease when compared to healthy subjects, and its levels were associated with airflow limitation as well as symptoms burden and history of exacerbations. Smokers with normal lung function had significantly higher eHsp70 values than healthy never-smokers, and chronically elevated eHsp70 might contribute to the development of some pathologies in the future, including COPD, in some genetically or otherwise susceptible healthy smokers. We suggest that eHsp70 has a potential to become a new biomarker in COPD assessment, and its evaluation in healthy smokers might also merit further investigation.

Author Contributions: I.H. and L.R. wrote the main manuscript text, and all authors contributed to the design of the work. A.H.-T., M.G.R. and I.H. performed the experiments. A.V.D. and S.P.-G. were responsible for collecting the samples, performing spirometry and DLCO analysis, and collecting data about participants. L.R. performed statistical analysis and interpreted it with I.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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3. IZVANSTANIČNI ADENOZIN-TRIFOSFAT POVEZAN JE SA STUPNJEM PLUĆNE OPSTRUKCIJE I PROGRESIJOM SIMPTOMA U PACIJENATA S KRONIČNOM OPSTRUKCIJSKOM PLUĆNOM BOLESTI

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Extracellular adenosine triphosphate is associated with airflow limitation severity and symptoms burden in patients with chronic obstructive pulmonary disease

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Extracellular adenosine triphosphate (eATP)-driven inflammation was observed in chronic obstructive pulmonary disease (COPD) but was not investigated in patients' blood. Therefore, this study aimed to investigate eATP concentration in plasma of COPD patients and its association with disease severity and smoking. Study included 137 patients with stable COPD and 95 control subjects. eATP concentration was determined in EDTA plasma by luminometric method, and mRNA expression of eATP receptors P2X7R and P2Y2R was analysed by quantitative polymerase chain reaction (qPCR). eATP concentration was increased in COPD patients compared to controls ($P < 0.001$). Moreover, it was increasing with disease severity (GOLD 2–4) as well as symptoms burden and exacerbations history (GOLD A–D) ($P < 0.05$). eATP in healthy smokers differed from healthy non-smokers ($P < 0.05$) but was similar to GOLD 2 and GOLD A patients. eATP showed great diagnostic performances (OR = 12.98, $P < 0.001$) and correctly classified 79% of study participants. It demonstrated association with FEV_1 and multicomponent indices (ADO, BODEx, BODCAT, CODEx, DOSE). Regarding gene expression, P2Y2R was increased in the blood of COPD patients. Plasma eATP could become a diagnostic and/or prognostic biomarker in COPD, as it seems to be associated with patients' condition, quality of life and disease progression.

Adenosine triphosphate (ATP) might be an important molecule in the pathogenesis of airway diseases due to its secretion by activated macrophages, neutrophils, epithelial cells, endothelial cells and platelets[1,2]. Once released in extracellular space, ATP contributes to the exacerbation of inflammation, bronchoconstriction and cough. Moreover, it modulates airway hypersensitivity and immune cells function[3]. Therefore, if there is an accumulation of extracellular ATP (eATP) in the airways, patients are prone to pro-inflammatory responses[4]. ATP is present in extracellular matrix in lower concentrations when inflammation is induced as a part of normal physiological response. However, eATP can be found in higher concentrations when cell death or cell activation are stimulated[2,5,6].

Most airway diseases are associated with ATP accumulation in bronchoalveolar lavage (BAL), sputum or exhaled breath condensate (EBC). In general, BAL samples reflect only the peripheral airways, sputum represents situation in the central airways, while the origins and the mechanism of airway secretions in EBC are not fully understood[7]. Chronic obstructive respiratory disease (COPD) is a heterogeneous and complex disease characterized by airway obstruction and inflammation[8]. Increased eATP concentrations were determined in the airways

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of patients with COPD when measured in BAL, and the highest concentration was observed in COPD patients with more advanced disease[9].

Although inflammation in lungs can be caused by different harmful particles, cigarette smoke is considered the most common risk factor for development of COPD[10]. The effect of cigarette smoke on ATP release was investigated in a mouse model of smoke-induced acute lung inflammation and emphysema, and an increase in eATP was found in their BAL[11]. The effect of smoking was investigated in human samples, too. Neutrophils were isolated from blood of healthy non-smoking donors and stimulated with cigarette smoke. Afterwards, concentration of eATP was measured and it was increased in comparison to non-stimulated neutrophils[12]. It was also shown that higher eATP concentrations were present in BAL of COPD patients compared to healthy subjects, even after smoking cessation[9,13]. However, when ATP was measured in EBC, no differences between healthy non-smokers, healthy smokers and COPD patients were found[7]. Nevertheless, to the best of our knowledge, eATP concentrations in COPD patients' blood were not determined so far.

ATP is a ligand for some of the purinergic receptors (P2Rs) that are widely expressed in the lungs – trans-cell membrane cationic channels (P2XRs) and trans-membrane domain G protein-coupled receptors (P2YRs). When ATP binds to the P2Rs, macrophages and neutrophils secrete pro-inflammatory molecules and mediators of tissue degradation, all of which contribute to the chronic inflammation in COPD[13]. There are seven P2XRs ($P2X_{1-7}R$) and eight P2YRs ($P2Y_{1/2/4/6/11/12/13/14}R$). Common receptors investigated from those two families are P2X7R and P2Y2R[14,15]. P2X7R receptors are predominantly intracellular and they locate to the plasma membrane upon differentiation of monocytes to macrophages[16]. Once bound to P2X7R, eATP induces NLRP3 inflammasome activation that leads to the maturation and release of IL-1β[17]. Macrophages from BAL and blood neutrophils of COPD patients showed higher expression of the P2X7R[11]. While P2X7R is activated only by ATP, P2Y2R can be activated by ATP and UTP[3,18]. Nevertheless, it is considered that activated P2Y2Rs have a role in directing neutrophil chemotaxis and amplifying chemotaxis signals, so eATP can be an autocrine and paracrine messenger[5,15,19].

This study aims to investigate for the first time eATP in peripheral blood of COPD patients. Pattern of eATP fluctuation associated with smoking, severity of airflow limitation (Global Initiative for Chronic Obstructive Lung Disease (GOLD) 1–4 classification assessment) as well as symptoms and history of exacerbations (GOLD A–D classification assessment) was also determined, as none of this was explored so far. Finally, mRNA expression level of P2X7R and P2Y2R, two common eATP receptors that could have a role in COPD pathogenesis associated with eATP, was investigated. We hypothesized that eATP would be elevated in peripheral blood of COPD patients when compared to age- and sex-matched control subjects, and related to smoking status as well as severity of disease.

Methods

Participants of the study. There were 232 participants in this retrospective study. 95 were in control group and 137 were COPD patients in stable phase of the disease. They were recruited at the Clinical Department for Lung Diseases Jordanovac, University Hospital Centre Zagreb (Zagreb, Croatia), signed an informed consent for scientific research and agreed to volunteer. Ethical Committee of University Hospital Centre Zagreb and Ethical Committee for Experimentation of Faculty of Pharmacy and Biochemistry, University of Zagreb (Zagreb, Croatia), approved the study (Approval Protocol Numbers: 02/21/JG and 251-62-03-14-78, respectively).

COPD was confirmed by pulmonologists according to the ratio of spirometry parameters forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) measured in litres ($FEV_1/FVC < 0.70$), as described by the GOLD guidelines[8]. Stable phase of the disease was defined as no exacerbations in the last three months, no changes in medications for respiratory system and no symptoms of infection in lower respiratory tract. Exclusion criteria for COPD patients were as follows: age under 40, lung diseases other than COPD, inflammatory systemic diseases, acute infections, diabetes with severe complications, severe liver diseases, severe kidney insufficiency, malignant diseases, transplantations, and other specific or non-specific acute inflammations.

Health state of control subjects was established based on anamnestic data and spirometry test results. Control individuals had to meet the same inclusion and exclusion criteria as COPD patients, except for the findings of post-bronchodilator spirometry test results (that were normal for control subjects). They were age- and sex-matched to their COPD counterparts, living in the same area of Croatia as COPD patients.

Both control and COPD groups of patients were subdivided according to the smoking status, so there were non-smokers (n = 48) and smokers (n = 47) in control group, and non-smokers (n = 10), former smokers (n = 90) and smokers (n = 37) in COPD group of patients. Moreover, COPD patients were classified into different GOLD stages based on FEV_1, as recommended by GOLD: GOLD 1 ($FEV_1 \geq 80\%$) (n = 0), GOLD 2 ($50\% \leq FEV_1 < 80\%$) (n = 47), GOLD 3 ($30\% \leq FEV_1 < 50\%$) (n = 50) and GOLD 4 ($FEV_1 < 30\%$) (n = 40) stage of the disease. Nevertheless, besides airflow limitation severity, COPD patients were distinguished in the groups according to the symptoms and history of exacerbations (ABCD assessment): GOLD A (n = 27), GOLD B (n = 70), GOLD C (n = 0) and GOLD D (n = 40). COPD patients completed both Modified Medical Research Council (mMRC) Dyspnoea Scale and COPD Assessment Test (CAT) questionnaires. Body mass index (BMI), number of exacerbations during previous year and Charlson comorbidity index were additionally matched to every COPD patient. Afterwards, some multicomponent indices already established for evaluation of patient's condition were calculated: ADO, BODCAT, BODEx, CODEx and DOSE[20–23]. ADO is composed of age, dyspnoea, and airflow obstruction; BODCAT is composed of BMI, airflow obstruction, dyspnoea, and CAT score; BODEx is composed of BMI, airflow obstruction, dyspnoea, and previous exacerbations; CODEx is composed of comorbidities (Charlson index), airflow obstruction, dyspnoea, and previous exacerbations; DOSE is composed of dyspnoea, airflow obstruction, smoking status, and previous exacerbations. Previous exacerbations were defined as a number of exacerbations in the previous year.

Spirometry. Spirometry is a common method in diagnosing the airflow limitation. The spirometry was performed in outpatient clinic by trained technicians on each visit for COPD patients and once for control subjects. The spirometry was done on a MasterLab (Jaeger, Würzburg, Germany), according to the recommendations of the European Respiratory Society and American Thoracic Society. Spirometry was repeated at least three times, sometimes eight times, until two reproducible efforts were obtained. The exhalation effort had to be at least 6 seconds, or until the end-expiratory plateau was reached. The two largest FVC and FEV_1 values had to show less than 5% variability, according to the standardized procedure[24]. Predicted values were the most commonly used European Community of Coal and Steel (ECCS) values[25]. The lung function parameters measured were FEV_1, FVC and FEV_1/FVC. A bronchodilator test with 400 µg of salbutamol was taken for each individual. Spirometry measurements were performed 15–30 minutes after salbutamol application. The same rules and standards were applied in prebronhodilator spirometry, and the best values for FEV_1 and FVC were chosen.

Diffusion capacity for carbon monoxide (DLCO) measurement. Pulmonary diffusing capacity was measured by single-breath method with carbon monoxide, on MasterScreen PFT Pro (Jaeger, Würzburg, Germany), according to the guidelines[26]. Helium dilution was used to determine alveolar volume (VA) and calculate the transfer coefficient for carbon monoxide (KCO) - DLCO/VA, as a unit diffusion indicator. Measurement results were correlated with the subjects' haemoglobin values. Each subject made three measurements. Predictive values were estimated according to Cotes[27].

Blood sampling and preparation. Blood samples were collected from 7 a.m. to 9 a.m. after overnight fasting by venepuncture of a large antecubital vein into two tubes (6 mL) with K_3-ethylenediaminetetraacetic acid (K_3EDTA) anticoagulant (Greiner Bio-One, GmbH, Kremsmünster, Austria), and were mixed by an inversion for 8 times. For venepuncture, order of blood sampling and mixing, the guidelines were followed according to the national recommendations for venous blood sampling[28]. Samples were centrifuged immediately after blood collection for the two times as follows - firstly, 10 minutes at 3500 rpm at +4 °C and secondly, 15 minutes at 4000 rpm at +4 °C, as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines[29]. After first centrifugation, buffy coat layers were removed into two 2 mL tubes with 1 mL of TRI Reagent Solution (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and stored at −80 °C for isolation of RNA. After second centrifugation, remaining EDTA plasma was prepared for eATP determination. Those EDTA plasma samples were immediately mixed (2:1) with ATPlite Mammalian Cell Lysis Solution from the ATPlite kit for measurement of ATP (Perkin Elmer, Waltham, Massachusetts, USA), briefly mixed on vortex, and stored at −80 °C until analysis. ATPlite Mammalian Cell Lysis Solution was added to inactivate endogenous ATPases and to stabilize eATP, as suggested by manufacturer.

Relative mRNA expression. Total RNA was isolated from buffy coats stored with TRI Reagent Solution by conventional method[30]. Concentration of RNA was measured by Nanodrop 8000 (Thermo Fischer Scientific, Wilmington, USA) and its quality was assessed by A_{260}/A_{280} ratio with criteria $A_{260}/A_{280} = 1.9–2.1$. cDNA was prepared with random primers and RevertAid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA) by GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA), with PCR conditions being 5 min at 25 °C, 60 min at 42 °C and 5 min at 70 °C. Quantitative polymerase chain reaction (qPCR) was performed with Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, USA), and unlabelled PCR primers and Taqman probe from Taqman Gene Expression Assays (Applied Biosystems, Foster City, USA). PCR conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 60 °C for 1 minute, using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA). For the detection of mRNA expression, specific primers to P2X7R (Hs00175721_m1; Applied Biosystems, Foster City, USA) and P2Y2R (Hs04176264_s1; Applied Biosystems, Foster City, USA) were used. Beta-2-microglobulin (B2M) (Hs99999907_m1; Applied Biosystems, Foster City, USA) and peptidylprolyl isomerase (PPIA) (Hs99999904_m1; Applied Biosystems, Foster City, USA) were used as reference genes and were performed during each run for each sample for the normalization between the samples. Results of gene expression in controls and COPD patients were compared to the results of a randomly selected healthy control's sample that was included in every plate as internal control. Data were expressed as a fold change of mRNA after following calculation $\Delta\Delta Ct$ = mean ΔCt value (target samples) – mean ΔCt value (control samples), where fold change value corresponds to the $2^{-\Delta\Delta Ct}$[31].

Measurement of ATP. ATP levels were measured using ATPlite assay (Perkin Elmer, Waltham, Massachusetts, USA), according to the instructions of the manufacturer (ATPlite Luminescence ATP Detection Assay System, Rev. F – April 2015), with some modifications due to the human plasma used as a sample.

Dilutions of ATP standards (0.016–2 µM) and prepared plasma samples (1:10) were done with water. The luminescence in white 96-well plates was measured by the multilabel plate reader Victor 3 (Perkin Elmer, Waltham, Massachusetts, USA). Afterwards, four parameter logistic curve fit was used for calculating ATP concentrations by OriginPro 9 software program (OriginLab Corporation, Northampton, Massachusetts, USA). We determined intra-assay and inter-assay variations for the measurement of ATP in human EDTA plasma. Intra-assay variation analysis was run with a randomly selected healthy control's sample at one plate, and coefficient of variation (CV) for plasma ATP concentration was 7.23% (n = 20). Inter-assay variation analysis was performed with the same randomly chosen healthy control's sample, which was included as internal control at every plate run with participants' samples. Inter-assay CV was 10.38% (n = 14).

Statistics. Data were tested for normality by Kolmogorov-Smirnov test. As all data failed a normality test, a nonparametric Mann-Whitney test was used for the analysis of the difference between controls and COPD. Kruskal-Wallis test followed by a post-hoc analysis was used in a case of comparison of more than two groups. Chi-squared test was used for comparison of categorical variable (sex). For determination of association between

parameter	controls n = 95	COPD patients n = 137	P-value
age/years	64 (46–83)	65 (44–86)	0.073
sex			
males	49	86	0.118
females	46	51	
FEV_1*/L	2.60 (2.12–3.19)	1.08 (0.78–1.57)	<0.001
FEV_1/%	93.30 (86.38–104.20)	39.00 (28.08–59.73)	<0.001
FVC¤/L	3.35 (2.77–4.16)	2.28 (1.81–2.77)	<0.001
FEV_1/FVC	0.81 (0.77–0.88)	0.48 (0.41–0.58)	<0.001

Table 1. Basic characteristics and lung function parameters of participants included in the study. Age is presented as median with minimum – maximum, sex is presented as absolute number, while all other parameters are presented as median with interquartile range. Chi-squared test was used for comparison of males and females, while all other parameters were tested by Mann-Whitney test. Data were considered significant if $P < 0.05$. *FEV_1 – forced expiratory volume in 1 second; ¤FVC – forced vital capacity.

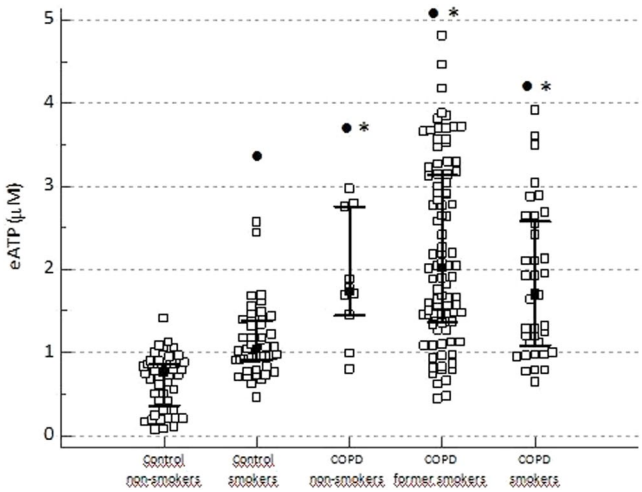

Figure 1. Influence of smoking history on eATP concentration determined in EDTA plasma of control non-smokers, control smokers, COPD non-smokers, COPD former smokers and COPD smokers. Data are shown as median with interquartile range for all the groups. Kruskal-Wallis test showed there was a significant difference between the groups ($P < 0.001$), and post-hoc analysis was performed. No significant difference was found between COPD patients subdivided according to their smoking status. •Statistically significant difference in comparison to control non-smokers; *statistically significant difference in comparison to control smokers.

the parameters, Spearman Rank Order was performed[32]. Univariate logistic regression analysis was also performed, and odds ratio (OR) with 95% confidence interval (95% CI) values were obtained. Results were considered statistically significant if $P < 0.05$. MedCalc statistical software version 17. 9. 2. (MedCalc Software, Ostend, Belgium) was used in the study.

Results

Basic characteristics and lung function parameters of participants are shown in Table 1. There was no difference in age ($P = 0.073$) or sex ($P = 0.118$) between controls and COPD patients. However, spirometric variables were significantly lower in patients with COPD compared to healthy individuals, as expected ($P < 0.001$).

Influence of smoking history on eATP concentration. We found that eATP was significantly increased in plasma of patients with COPD compared to control subjects [1.879 (1.262–2.888) μM *vs*. 0.875 (0.701–1.074) μM, respectively; $P < 0.001$]. When participants were subdivided according to their smoking history, eATP concentration was found to be elevated in healthy smokers in comparison to healthy non-smokers ($P < 0.05$). Moreover, eATP was increased in total cohort of COPD patients in comparison to both control smokers and non-smokers ($P < 0.001$). However, smoking history did not influence eATP level in patients with COPD, as no significant difference between COPD patients subdivided according to their smoking status was observed when they were compared to each other (Fig. 1).

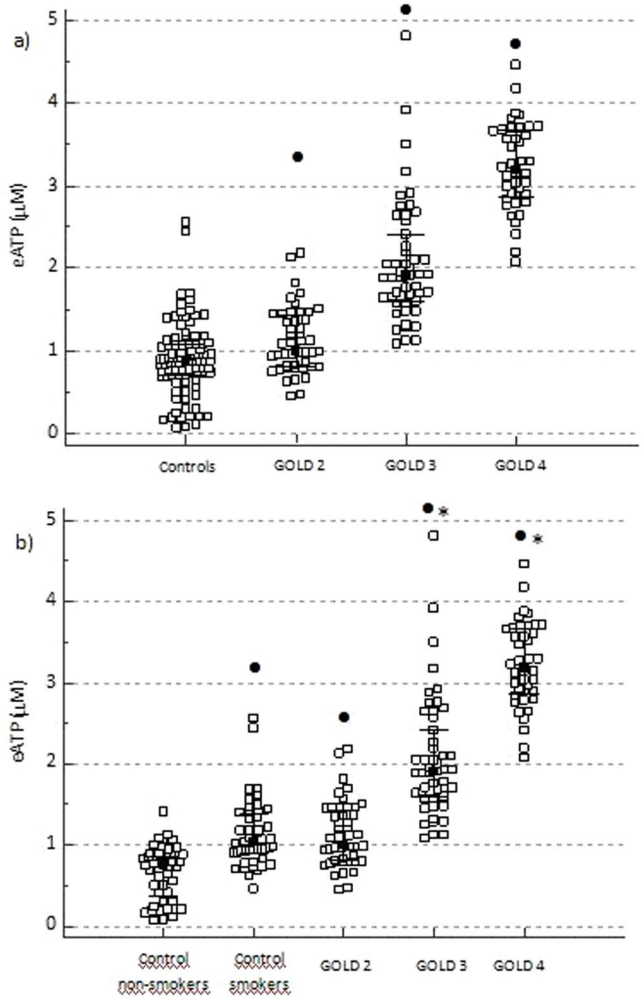

Figure 2. Influence of airflow limitation severity (GOLD 1–4 classification assessment) on eATP concentration. (**a**) eATP concentration was measured in EDTA plasma of control subjects and COPD patients subdivided by severity of airflow limitation into GOLD 2, GOLD 3 and GOLD 4 groups. (**b**) COPD patients were subdivided into GOLD 2–4 stages and compared to healthy individuals based on their smoking status (healthy non-smokers and healthy smokers). Data are shown as median with interquartile range for all the groups. Kruskal-Wallis test showed there was a significant difference between the groups ($P < 0.001$ for both **a,b**), and post-hoc analysis was performed. In COPD patients, a statistically significant increase in GOLD 3 in comparison to GOLD 2, and in GOLD 4 in comparison to GOLD 2 and GOLD 3 stages was also found. •Statistically significant difference in comparison to total controls (**a**) or control non-smokers (**b**); *statistically significant difference in comparison to control smokers (**b**).

GOLD classifications and eATP concentration. Next, we wanted to explore influence of airflow limitation severity as well as symptoms and exacerbations on eATP levels in peripheral blood. When patients were classified according to the airflow limitation severity characterized by FEV_1 (GOLD 2, 3 and 4 stages; no patients fulfilled conditions for GOLD 1 group in our study), they all had significantly elevated plasma levels of eATP compared to controls. More importantly, concentration of eATP was significantly increasing with the severity of airflow limitation (Fig. 2a). When control subjects were subdivided due to their smoking history, patients with GOLD 3 and GOLD 4 stages had increased eATP concentration in comparison to both control non-smokers and control smokers ($P < 0.05$); however, difference was not observed between healthy smokers and patients in GOLD 2 stage (Fig. 2b).

COPD patients, when subdivided according to the symptoms burden and exacerbations history (GOLD A, B and D groups; no patients fulfilled conditions for GOLD C group in our study), had increased eATP concentration in comparison to the controls. Moreover, those three GOLD groups were significantly different from each other, and eATP was increasing with the severity of the symptoms ($P < 0.05$) (Fig. 3a). Also, eATP was increased in GOLD B and GOLD D patients' groups when compared to both control non-smokers and control smokers ($P < 0.05$), while the levels of eATP did not differ between patients in GOLD A group and healthy smokers (Fig. 3b).

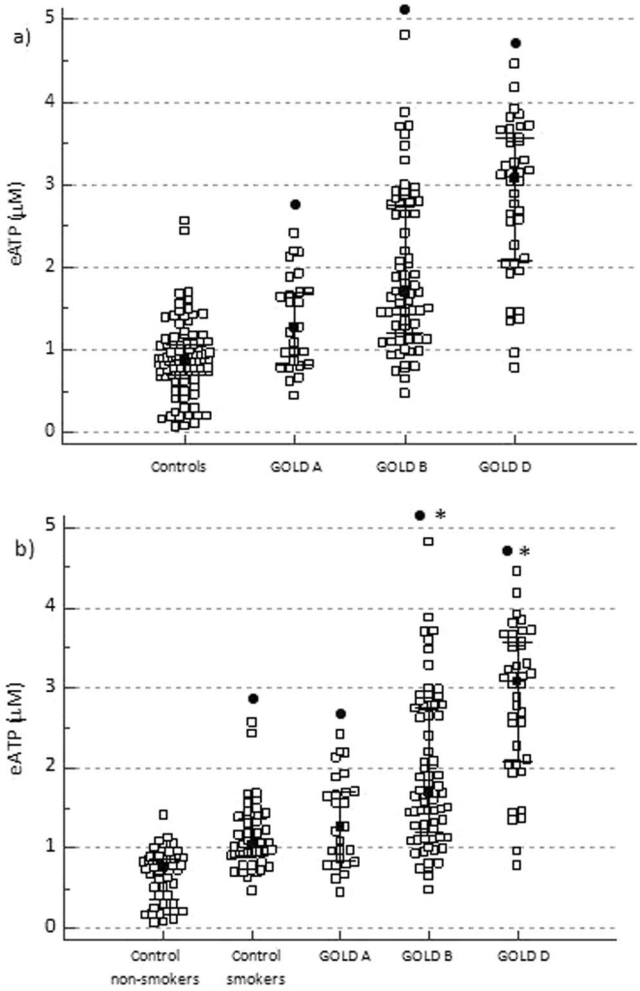

Figure 3. Influence of symptoms and history of exacerbations (GOLD A–D classification assessment) on eATP concentration. (**a**) eATP concentration was determined in EDTA plasma of control individuals and patients with COPD subdivided into GOLD A, GOLD B and GOLD D groups. (**b**) COPD patients were subdivided into GOLD A - D groups and compared to healthy subjects according to their smoking status (healthy non-smokers and healthy smokers). Data are shown as median with interquartile range for all the groups. Kruskal-Wallis test showed there was a significant difference between the groups ($P < 0.001$ for both **a,b**), and post-hoc analysis was performed. In COPD patients, a statistically significant increase in GOLD B in comparison to GOLD A, and in GOLD D in comparison to GOLD A and GOLD B groups was also found. •Statistically significant difference in comparison to total controls (**a**) or control non-smokers (**b**); *statistically significant difference in comparison to control smokers (**b**).

Diagnostic value of eATP and correlation analysis. In order to evaluate eATP diagnostic performances, univariate logistic regression analysis was performed and OR of even 12.98 (95% CI = 6.10–27.62, $P < 0.001$) was obtained for eATP in EDTA plasma. In addition, 79% of cases were correctly classified by eATP measurement.

We also performed nonparametric measurement of rank correlation in COPD patients, and Spearman's rank correlation coefficient (rho) and P-values were obtained (Table 2).

Regarding basic spirometry parameters, eATP showed a very good to excellent negative correlation with FEV_1 when expressed in litres and in percentage (%) of predicted values, and a moderate to good negative correlation with FEV_1/FVC. DLCO, used for the assessment of the diffusion properties of the alveolar capillary membrane, negatively correlated moderately to well with concentration of eATP. When multicomponent indices ADO, BODCAT, BODEx, CODEx and DOSE that reflect patients' condition (airflow limitation, dyspnoea, exacerbations, BMI, smoking, comorbidities and/or age) were correlated with eATP, they also showed a very good to excellent positive association, except ADO that demonstrated a moderate to good positive correlation with eATP concentration (Table 2).

mRNA expression of eATP receptors. Finally, we explored mRNA expression of the two eATP receptors in the blood (buffy coat containing leukocytes and platelets) of participants from the study. The expression levels

parameter	Spearman's correlation coefficient, rho	P-value
ADO*	0.611	<0.001
BODCAT[¤]	0.785	<0.001
BODEx[•]	0.808	<0.001
CODEx[▪]	0.819	<0.001
DOSE[°]	0.765	<0.001
DLCO[◊]	−0.611	<0.001
FEV_1[Δ] (L)	−0.764	<0.001
FEV_1 (%)	−0.826	<0.001
FEV_1/FVC[§]	−0.661	<0.001

Table 2. Association between eATP and multicomponent indices as well as lung function parameters in COPD patients. Data were analysed by Spearman Rank Correlation. Results are described with Spearman's correlation coefficient (rho) and P-value. *ADO – age, dyspnoea, airflow obstruction; [¤]BODCAT – BMI, airflow obstruction, dyspnoea, score from CAT; [•]BODEx – BMI, airflow obstruction, dyspnoea, previous exacerbations; [▪]CODEx – comorbidities (Charlson index), airflow obstruction, dyspnoea, previous exacerbations; [°]DOSE – dyspnoea, airflow obstruction, smoking status, previous exacerbations; [◊]DLCO – diffusion capacity for carbon monoxide; [Δ]FEV_1 – forced expiratory volume in 1 second; [§]FVC – forced vital capacity. Previous exacerbations are defined as a number of exacerbations in the previous year.

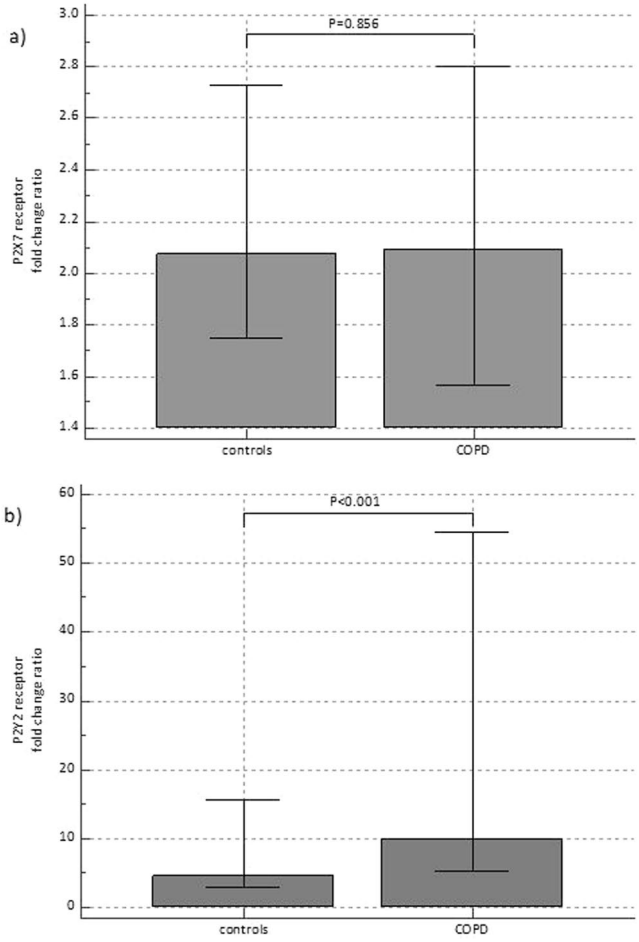

Figure 4. mRNA expression of eATP receptors P2X7R (**a**) and P2Y2R (**b**) in healthy subjects and patients with COPD. Results of gene expression are shown as fold change ratio with its median and interquartile range. Mann-Whitney test was used.

of P2X7R were similar in control and COPD patients (P = 0.856) (Fig. 4a), while P2Y2R showed twice as high expression in COPD patients in comparison to healthy individuals (P < 0.001) (Fig. 4b). The levels of P2Y2R mRNA expression did not differ between COPD non-smokers and healthy smokers (data not shown).

Discussion

In this study, concentration of eATP was measured in the blood of COPD patients for the first time, and it was significantly elevated when compared to age- and sex-matched control subjects. Interestingly, eATP concentration differed between healthy non-smokers and smokers, and in COPD patients was associated with both airflow limitation severity (GOLD 1–4 stages) as well as with symptoms burden and history of exacerbations (assessed by GOLD A–D classification).

Different studies demonstrated an increase in eATP in media of cultured human bronchial epithelial cells[14], BAL fluid of mice[12,33], BAL fluid of COPD patients[9] and EBC of COPD patients[7], but no one measured eATP concentration in COPD patients' blood samples – either plasma or serum. Serum is not an appropriate sample for quantification of eATP due to a potential interference of intracellular ATP from erythrocytes and platelets. Moreover, blood cell-derived ectonucleotidases in serum can affect eATP concentration[9]. Therefore, EDTA plasma was chosen as a representative sample of peripheral processes in COPD patients.

ATP could be released from different cells due to the cell injury or cell death. However, it can also be secreted through pannexin and connexin channels from intact activated cells (neutrophils, macrophages, platelets, epithelial and endothelial cells) as a result of nonlytic processes[2,14,15]. eATP acts as a damage-associated molecular pattern (DAMP)[34,35], and we suggest that the levels of eATP determined in the blood of COPD patients might reflect multicomponent pathophysiological background of COPD.

Cigarette smoking is the most common underlying mechanism of COPD development, and COPD patients are mostly current or former smokers, which was confirmed by our study. Previously, an increase in eATP was observed in BAL of a mouse model of smoke-induced acute lung inflammation and emphysema[12,33] as well as in BAL of COPD patients, even after smoking cessation[9,13]. In this study, control smokers had higher eATP concentrations in plasma from control non-smokers, although the levels were lower than in COPD patients, which might suggest some initial tissue changes (*e.g.* cellular destruction) and/or amplification of inflammatory responses in so-called healthy smokers. However, eATP concentrations in COPD patients were not associated with smoking history. Therefore, factors other than smoking seem to be primarily responsible for inducing eATP release that could aggravate ongoing inflammation in patients with COPD, making in such way a vicious circle.

It was reported that severity of airflow limitation and hypoxia are often associated with COPD exacerbations and could lead to ATP release from cells of the respiratory system[1]. In this study, eATP concentration in EDTA plasma was increasing with the disease severity based on airflow limitation, and the highest concentration was observed in GOLD 4 stage. Interestingly, there was no difference between control smokers and patients in GOLD 2 stage, while eATP in patients in GOLD 3 and GOLD 4 stages was increased in comparison to both control groups (smokers and non-smokers). Similar results were observed when symptoms and exacerbation history were assessed (GOLD A–D groups of patients), showing no difference between eATP levels in healthy smokers and patients in GOLD A group. This could become yet another reason for pro-active non-smoking campaign, as seems plausible that less severe COPD patients have similar pathophysiologic background with smokers who did not develop COPD. Naturally, pathogenesis gets more complex with the severity of the disease. Moreover, COPD patients from our study had lower value of pO_2 (mmHg) = 70 (68–71) and normal value of sO_2 (%) = 96 (95–98). Although we could not say with certainty if the COPD patients from our research were or were not hypoxic, eATP showed an association with oxygenation status. Indeed, eATP showed poor negative correlations with both parameters used for the assessment of oxygenation status ($rho = -0.486$, $p < 0.001$ and $rho = -0.471$, $p < 0.001$ for pO_2 and sO_2, respectively). Therefore, increased levels of eATP in plasma of COPD patients could be, at least partly, due to their poor oxygenation status.

Increased amount of eATP in the lungs of COPD patients leads to the activation of purinergic receptors that are widely expressed across all cells in the lungs[3]. It was shown that when activated by eATP, P2Y2Rs lead to further ATP release by pannexin dependant mechanism, which then activates P2X7Rs. Although it was only investigated in viral, bacterial and protozoa infections, suggested cooperation between purinergic receptors driven by ATP could be observed in other conditions when immune response is needed due to inflammatory processes[36,37]. Careta *et al.* demonstrated that expression of P2X7R and P2Y2R were down-regulated in the lungs of non-obstructed smokers and COPD patients, but their study included patients in an early stage of COPD and a very few of them. Moreover, their study population had both lung cancer and COPD, so there might had been a bias regarding the observations associated with COPD[13]. Increased P2X7R expression in the lungs and BAL cells after LPS stimulation was reported[38]. In addition, P2X7R was up-regulated in BAL macrophages and blood neutrophils from patients with COPD[9]. On the other hand, P2Y2R showed an important role as a mediator in the recruitment of macrophages and neutrophils at the site of inflammation where damaged cells are being phagocytosed by macrophages and neutrophils. P2Y2Rs could induce hyperinflammation and tissue damage by promoting a chronic state of the disease by increasing activity and migration capacity of neutrophils[39]. Moreover, eATP acts like an autocrine messenger through activation of P2Y2Rs and amplifies chemotaxis signals[15]. The P2X7R is ATP-selective receptor, but it is considered to have a low affinity for ATP[40]. If the translocation of P2X7R to the cell membrane is regulated by eATP concentration at the transcriptional and/or post-transcriptional levels, it is possible that observed concentrations of eATP in patients with COPD were not high enough to activate P2X7Rs, since in our study mRNA expression of this receptor was not different in comparison to healthy subjects. On the other hand, mRNA expression level of P2Y2R was twice as high in patients with COPD in comparison to control individuals. This could reflect on higher activity of neutrophils, and we detected a significantly increased number of neutrophils in COPD patients (data not shown). What is also interesting is that the levels of P2Y2R mRNA expression did not differ between COPD non-smokers and healthy smokers, which confirms once again significant disturbances within healthy smokers' organisms.

In our study, eATP with an OR of 12.98 showed a great predicting power, and correctly classified 79% of cases. In addition, eATP demonstrated a very good to excellent positive correlation with multicomponent indices BODCAT, BODEx, CODEx and DOSE that indirectly reflects an association with most of the major

COPD-related factors, such as airflow limitation, dyspnoea, exacerbations, BMI, smoking, comorbidities and/or age. On the other hand, we also showed a very good to excellent negative correlation with FEV_1, meaning that the severity of the disease was followed by an increase in eATP concentration, and it might be that eATP-driven inflammation is a part of the disease progression. There are several methods for the assessment of pulmonary function. Besides spirometry, in the present study DLCO was determined in COPD patients. As expected, it was decreased due to emphysema present in COPD and was decreasing with more advanced severity stages of disease (data not shown). The loss of alveoli in emphysema is resulting in smaller surface area available for diffusion of respiratory gases, so carbon monoxide (CO) transfer from the alveolar gas to the haemoglobin of the erythrocytes in the pulmonary circulation is also diminished[41]. Based on the results from this study, it could be suggested that eATP concentration was a reflection of decreased lung function, since there was a negative correlation between eATP and DLCO as well as FEV_1 in COPD patients.

Limitation of this study is a lack of participants in GOLD 1 stage and GOLD C group of COPD patients. It would be interesting to investigate eATP at the beginning of the disease development (GOLD 1 stage), but, unfortunately, this group of COPD patients rarely contacts physicians in our outpatient clinic due to very mild symptoms. In addition, GOLD C category of patients is also very rare, as patients that do not have many symptoms usually are not frequent exacerbators. Also, the sample size was small for group of COPD non-smokers. As we found that P2Y2R expression is similar between this subgroup of patients and healthy smokers, it would be interesting to confirm that the observed effect was not just a bias due to an inadequate number of patients. Therefore, a prospective study with more participants is suggested.

In conclusion, eATP concentration in EDTA plasma of COPD patients in stable phase showed great diagnostic performances and was associated to the disease progression described by airflow limitation severity as well as symptoms and exacerbation history. Moreover, it might be that smoking is a part of eATP-driven systemic inflammation, especially in healthy smokers, who had increased levels of eATP when compared to control non-smokers, their P2Y2R expression levels were similar to COPD non-smokers, and their eATP concentration was similar to COPD patients in GOLD 2 stage as well as in GOLD A group. Blood is an easily and non-invasively obtained sample, and plasma eATP could become a diagnostic and/or prognostic biomarker in COPD, as it seems to be associated with patients' condition, quality of life and disease progression.

Data availability

All data generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

I.H. and L.R. wrote the main manuscript text and prepared figures and tables. All authors contributed to the design of the work, while I.H., A.H.T., A.S.B. and M.G.R. performed the experiments. A.V.D. and S.P.G. were responsible for collecting the samples, performing spirometry and DLCO analysis, and collecting data about participants. L.R. performed statistical analysis and interpreted it with I.H. All authors reviewed the manuscript and approved the submitted version (and any substantially modified version). In addition, all authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

Competing interests

The authors declare no competing interests.

Additional information

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4. KOMBINACIJA BILJEGA SUSTAVNE UPALE U PROCJENI KRONIČNE OPSTRUKCIJSKE PLUĆNE BOLESTI: DIJAGNOSTIČKA VRIJEDNOST I IDENTIFIKACIJA MREŽNE POVEZANOSTI I KLASTERA

diagnostics

MDPI

Article

Combination of Systemic Inflammatory Biomarkers in Assessment of Chronic Obstructive Pulmonary Disease: Diagnostic Performance and Identification of Networks and Clusters

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Abstract: Interleukin (IL)-1α, IL-1β, IL-6, IL-8 and tumor necrosis factor (TNF)α contribute to inflammation in chronic obstructive pulmonary disease (COPD). We wanted to investigate their interrelations and association with disease severity, as well as to combine them with other inflammation-associated biomarkers and evaluate their predictive value and potential in identifying various patterns of systemic inflammation. One hundred and nine patients with stable COPD and 95 age- and sex-matched controls were enrolled in the study. Cytokines' concentrations were determined in plasma samples by antibody-based multiplex immunosorbent assay kits. Investigated cytokines were increased in COPD patients but were not associated with disease or symptoms severity. IL-1β, IL-6 and TNFα showed the best discriminative values regarding ongoing inflammation in COPD. Inflammatory patterns were observed in COPD patients when cytokines, C-reactive protein (CRP), fibrinogen (Fbg), extracellular adenosine triphosphate (eATP), extracellular heat shock protein 70 (eHsp70) and clinical data were included in cluster analysis. IL-1β, eATP and eHsp70 combined correctly classified 91% of cases. Therefore, due to the heterogeneity of COPD, its assessment could be improved by combination of biomarkers. Models including IL-1β, eATP and eHsp70 might identify COPD patients, while IL-1β, IL-6 and TNFα combined with CRP, Fbg, eATP and eHsp70 might be informative regarding various COPD clinical subgroups.

Keywords: chronic obstructive pulmonary disease; cytokines; systemic inflammation; clusters; adenosine triphosphate; heat shock protein 70

1. Introduction

Cytokines are small proteins (5–30 kDa) with a short half-life and they are usually circulating in body fluids in picomolar concentrations. While being produced by a variety of cells, their main role is regulation of the immune system, so they or their receptors are often being recognized as targets for potential therapeutic interventions in many different diseases [1]. Cytokines are not disease-specific biomarkers, yet they are considered to be surrogate biomarkers for inflammation

in chronic obstructive pulmonary disease (COPD), as it seems they have an important role in COPD-associated inflammatory responses [2]. COPD is a complex, heterogeneous disease at the genetic (e.g., alpha-1 antitrypsin deficiency), cellular and molecular levels, and its manifestations are both pulmonary and extrapulmonary [3]. Currently, it is the fourth leading cause of death in the world, and it represents an important public health challenge as its global prevalence of 11.7% is expected to rise for many years to come [4]. Although COPD is characterized by respiratory symptoms and airflow limitation, systemic inflammation may be developed in some patients and it contributes to the progression of the disease and development of comorbidities that might have an impact on morbidity and mortality [5–9]. Various clinical studies reported elevated levels of inflammatory cytokines in respiratory tract and/or peripheral blood of COPD patients in comparison to healthy controls [2,7,10–12]. Our study focused on several cytokines as it follows: interleukin (IL)-1α, IL-1β, IL-6, IL-8 and tumor necrosis factor (TNF)α.

IL-1 is mainly produced by the airway epithelium and macrophages, and it is released along with IL-6, IL-8 and TNFα. It causes neutrophilia, macrophage activation and responses by T cells [13]. Both pro-IL-1α and its mature IL-1α form are biologically active [14]. Contrary to this, pro-IL-1β has to be cleaved to be biologically active, mostly by caspase-1 through nucleotide-binding domain (NOD)-like receptor protein (NLRP)3 inflammasome activation [15]. IL-6 is a pro-inflammatory cytokine synthesized by the airway epithelium, macrophages, and other cells at the site of inflammation in response to environmental stressful stimuli such as smoking, and it is participating in the activation, proliferation and differentiation of T cells [6,16,17]. IL-8 is a multifunctional chemokine involved in inflammatory processes including neutrophil infiltration and chemotaxis [16,18]. It is secreted from macrophages, T cells, airway epithelium and neutrophils [19]. TNFα is produced by T cells, mast cells, and cells of airway epithelium. Its main functions are control of cellular migration and stimulation of secretion of other cytokines [20].

There are inconsistent observations regarding the association of cytokines with COPD severity, prognostic value and cytokine-targeted therapeutic approach. In addition, due to the disease complexity and different underlying mechanisms, clinical manifestations of COPD are presented differently. Therefore, instead of one, a group of biomarkers might better represent a specific COPD phenotype. In line with this, it was shown that persistent systemic inflammation is present in some of COPD patients and accompanied with an increase in CRP, fibrinogen (Fbg), white blood cells (WBC) and inflammatory cytokines [9]. Agusti et al. investigated six inflammatory parameters (CRP, Fbg, WBC, IL-6, IL-8 and TNFα) which form "inflammome" and showed that 70% of COPD patients had some of the components of systemic inflammation. Among them, in 16% of COPD patients inflammation was persistent, and associated with mortality and exacerbations [21]. From our previous studies, we observed that our COPD cohort might also show characteristics of systemic inflammation because of increased concentrations of CRP and Fbg [22] that are being common inflammatory parameters as well as extracellular adenosine triphosphate (eATP) [23] and extracellular heat shock protein 70 (eHsp70) [24] which act like damage-associated molecular patterns (DAMPs). In addition, a previous investigation showed that there were significant associations between the aforementioned parameters with lung function and disease severity, as well as symptoms severity and history of exacerbations, and different multicomponent clinical parameters used for the assessment of dyspnoea, exacerbations and lung impairment. These parameters have not been studied together before, and we wanted to evaluate their combined performances. First, our aim was to determine concentrations of cytokines IL-1α, IL-1β, IL-6, IL-8 and TNFα in COPD patients in comparison to healthy subjects and to investigate their association with disease and symptoms severity. As cytokines exhibit pleiotropy and redundancy, we also wanted to assess relations between them in healthy non-smokers, healthy smokers and COPD patients. We hypothesized that the combination of common inflammatory biomarkers (CRP and Fbg), DAMPs (eATP and eHsp70) and cytokines might ameliorate the understanding of relations between different inflammatory parameters and help to identify some potential COPD subgroups regarding

systemic inflammation. Finally, we wanted to suggest a model of combined parameters for recognizing COPD patients based on predictive value.

2. Materials and Methods

2.1. Participants

The current cross-sectional case-control study included 109 patients with stable COPD and 95 healthy individuals. For the additional analyses that involved eATP, one COPD patient was excluded because the plasma sample for the determination of eATP could not be obtained. For the determination of eATP and eHsp70, all individuals from the study (137 COPD patients and 95 controls) were recruited during 2017 and 2018 at the Clinical Department for Lung Diseases Jordanovac, University Hospital Centre Zagreb (Zagreb, Croatia) according to the predefined inclusion and exclusion criteria, while additional recruitment for the investigation of common inflammatory biomarkers and cytokines was performed during 2019 (109 COPD patients and 95 controls). During the second recruitment, not all participants were suitable to be included in the study because some of them died (n = 10), while others did not match inclusion criteria (lung transplantation, n = 5; acute exacerbations, n = 4) or could not be reached (n = 9). All participants agreed to take a part in the study as volunteers and confirmed it by signing an informed consent. The study was approved by the Ethics Committee of University of Hospital Centre Zagreb and Ethics Committee for Experimentation of Faculty of Pharmacy and Biochemistry, University of Zagreb (Zagreb, Croatia) (Approval Protocol Numbers: 02/21/JG on 29 August 2014 and 251-62-03-14-78 on 10 September 2014, respectively). Pulmonology specialists confirmed diagnosis of COPD after symptoms evaluation and spirometry measurements according to the guidelines by the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) [4]. All patients were in the stable phase of the disease with no exacerbations in the last three months since the recruitment, no changes in therapy and no symptoms of infection in lower respiratory tract. On the other hand, healthy individuals were included in the study based on anamnestic data and spirometry results that were among normal values. They were age- and gender-matched to the COPD patients. Exclusion criteria were same for all participants and they included as follows: age under 40, lung diseases other than COPD (except COPD for COPD patients), systemic inflammatory diseases, acute infections, diabetes with severe complications, severe liver diseases, severe kidney insufficiency, malignant diseases, transplantations, and other specific or non-specific acute inflammations. In addition, smoking data was obtained from all participants. COPD patients were divided in GOLD 2-4 stages according to the level of airflow limitation, as suggested by GOLD guidelines [4]. Besides forced expiratory volume in one second (FEV_1)-based disease severity, COPD patients were divided in GOLD A-D groups based on the assessment of symptoms severity and history of exacerbations. Evaluation of the symptoms and health-related quality of life was assessed by COPD Assessment Test (CAT), modified Medical Research Council (mMRC) Dyspnoea Scale as well as St George Respiratory Questionnaire for COPD patients (SGRQ-C). Additionally, data about previous exacerbations were obtained from the COPD patients. Finally, the Charlson comorbidity index was matched to every COPD patient, so that the multicomponent parameter CODEx could be established. CODEx stands for comorbidities (Charlson index), airflow obstruction, dyspnoea, and previous exacerbations [25].

2.2. Evaluation of Lung Function

Diagnosis of airflow limitation was established by spirometry when FEV_1 and forced vital capacity (FVC) ratio was <0.70. Measurements were performed by trained technicians at the Clinical Department for Lung Diseases Jordanovac, University Hospital Centre Zagreb. Moreover, the pulmonary diffusion capacity for carbon monoxide (DLCO) was measured for the assessment of lung function in COPD patients. Both procedures were performed as already described in detail in [23].

2.3. Blood Sampling and Cytokine Determination

Peripheral venous blood was collected from 7 a.m. to 9 a.m. by venepuncture of a large antecubital vein after overnight fasting. Tubes with K_3-ethylenediaminetetraacetic acid (K_3 EDTA) anticoagulant (Greiner Bio-One, Kremsmünster, Austria) were used for the blood collection. Afterwards, tubes were mixed by an inversion 8×, and centrifuged immediately, as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines [26]. Obtained EDTA plasma samples were stored at −80 °C until the analysis. Concentration of IL-1α in plasma was determined by Platinum Procarta Plex Kit (Thermo Fischer Scientific, Waltham, MA, USA), while levels of IL-1β, IL-6, IL-8 and TNFα were determined by Procarta Plex High Sensitivity Luminex kit (Thermo Fischer Scientific), according to manufacturer's recommendations. Antibody-coated magnetic beads were transferred to wells of a 96-well plate and washed. Afterwards, 25 µL of assay buffer was added to wells followed by addition of 25 µL of samples or standards. For IL-1α, determination plate was incubated for 120 min at room temperature (RT) with shaking, while for determination of the other cytokines, plates were incubated for 30 min at RT followed by an overnight incubation at 4 °C. At the end of incubation, plates were washed and 25 µL of detection antibodies were added to wells. Plates were then incubated for 30 min at RT, with shaking. After the washing step, 50 µL of streptavidin-phycoerythrin conjugate was added to wells and plates were incubated for 30 min at RT with shaking. At the end of incubation, plates for IL-1α determination were washed, 120 µL of reading buffer was added to wells and samples were analyzed by use of Luminex 200 instrument (Luminex Corporation, Austin, TX, USA). On the other hand, for IL-1β, IL-6, IL-8 and TNFα determination, 50 µL of amplification reagent 1 was added to wells. After 30 min incubation, 50 µL of amplification reagent 2 was added to wells and incubation continued for additional 30 min. Finally, plates were washed, beads were resuspended in 120 µL of reading buffer, and samples were analyzed by a Luminex 200 instrument. Cytokines concentrations were determined by interpolation from a standard curve using the xPONENT software package (Luminex Corporation).

2.4. Statistics

Normality of all data was tested by Kolmogorov–Smirnov test, and since all data failed a normality test, results were shown as median with interquartile range (IQR). Only age was shown as median with minimum and maximum, while gender was shown in absolute numbers. Non-parametric Mann–Whitney test and Kruskal–Wallis test were used for the analyses of differences between the groups of interest. Gender was tested by Chi-squared test. Univariate and multivariate logistic regression analyses were used to investigate COPD-inflammation contributing factors, and odds ratio (OR) with 95% confidence interval (CI) were obtained. Variables were added in the binary logistic regression analysis as continuous variables. Described analysis were performed in MedCalc statistical software version 17.9.2. (MedCalc Software, Ostend, Belgium).

Network analysis was used for the assessment of relations between investigated parameters. Values of the 95th percentile of each parameter in healthy non-smokers were considered as the criteria for the evaluation of the patterns between the parameters in healthy non-smokers, healthy smokers and COPD patients. Differences in the number of patients with abnormal levels between the groups were analyzed using Fisher's exact tests. Prior hierarchical clustering, variables were transformed to standard normal distribution by inverse transformation of ranks to normality (R package "Gen ABEL") [27]. Distance between the subjects was calculated using Euclidian method, and group of subjects were merged by complete linkage method. Optimal number of clusters was determined combining 30 indices apply NbClust function (R package "NbClust") [28]. Variables used for clustering and reference variables presented next to cluster were compared using a Kruskal–Wallis test or Fisher's exact test, depending on the data type. Network and clustering analyses were performed in R programming software (R Core Team) [29]. For all analyses, the false discovery rate was controlled using the Benjamini–Hohcberg method at significance level of 0.05.

3. Results

3.1. Basic Characteristics and Cytokines' Concentrations of All Participants

One hundred and nine patients with stable COPD were compared to age- and gender-matched healthy subjects (total healthy participants and only healthy non-smokers). COPD patients showed to have declined lung function in comparison to controls assessed by spirometry parameters. All investigated cytokines were elevated in peripheral circulation of COPD patients when compared to healthy individuals (Table 1). As smoking data were obtained from all participants, it was shown that only TNFα was increased in healthy smokers in comparison to healthy non-smokers. In addition, concentrations of TNFα and IL-6 were increased in both COPD former smokers and COPD smokers when they were compared to healthy controls regarding their smoking status as well as to COPD non-smokers. On the other hand, IL-1α was increased only in COPD smokers in comparison to both non-smoking controls and smoking controls, while IL-1β showed to be increased in each of COPD groups regarding smoking status when compared to healthy non-smokers and healthy smokers (see Supplementary Table S1).

Table 1. Demographic characteristics, spirometry parameters and cytokines' concentrations in participants from the study.

	Total Healthy Subjects n **= 95**	**Healthy Non-Smokers** n **= 48**	**COPD Patients** n **= 109**	p_1	p_2
age	64 (46–83)	65 (52–83)	65 (45–87)	0.069	0.600
gender male female	 49 46	 23 25	 69 40	0.121	0.104
FEV_1 (L)	2.60 (2.12–3.19)	2.82 (2.28–3.19)	1.08 (0.69–1.60)	**<0.001**	**<0.001**
FEV_1 (% pred.)	93.3 (86.4–104.2)	101.1 (90.6–110.4)	40.8 (27.9–61.7)	**<0.001**	**<0.001**
FVC (L)	3.35 (2.77–4.16)	3.58 (2.76–4.18)	2.28 (1.74–2.77)	**<0.001**	**<0.001**
FEV_1/FVC (%)	80.6 (76.8–87.6)	83.0 (78.1–91.8)	51.3 (40.7–58.7)	**<0.001**	**<0.001**
IL-1α (pg/mL)	0.30 (0.30–0.97)	0.31 (0.31–0.71)	0.43 (0.30–2.13)	**0.003**	**0.007**
IL-1β (pg/mL)	0.10 (0.10–0.61)	0.10 (0.10–0.17)	6.90 (0.61–23.91)	**<0.001**	**<0.001**
IL-6 (pg/mL)	4.85 (3.45–7.09)	4.41 (3.29–6.17)	32.17 (10.64–64.30)	**<0.001**	**<0.001**
IL-8 (pg/mL)	6.36 (4.07–11.17)	6.22 (4.34–11.33)	8.73 (3.56–17.76)	**0.040**	**0.049**
TNFα (pg/mL)	0.40 (0.35–1.36)	0.35 (0.35–0.53)	8.24 (0.35–19.23)	**<0.001**	**<0.001**

Age was shown as median with minimum and maximum, while gender was presented as an absolute number. Results of spirometry and cytokines' measurements were shown as median with interquartile range (IQR). Comparison of males and females was performed by Chi-squared test, while all other parameters were tested by Mann–Whitney Rank Sum test. Data were considered significant if $p < 0.05$. FEV_1–forced expiratory volume in one second; FVC–forced vital capacity; IL-1α–interleukin-1alpha; IL-1β–interleukin-1beta; IL-6–interleukin-6; IL-8–interleukin-8; TNFα–tumor necrosis factor alpha. alpha; p_1–statistical significance of differences between total healthy subjects and chronic obstructive pulmonary disease (COPD) patients; p_2–statistical significance of differences between healthy non-smokers and COPD patients. All p-values that are <0.05 are in bold.

3.2. Association of Cytokines' Concentrations with the Severity of Airflow Limitation and Symptoms Severity

All the cytokines were investigated regarding the severity of COPD based on GOLD guidelines (Table 2). None of the cytokines was associated with the severity of airflow obstruction or the symptoms severity and history of exacerbations, since the concentrations were only elevated in each of GOLD 2-4 stages and GOLD A-D groups when being compared to healthy subjects but did not differ between GOLD 2-4 or GOLD A-D. IL-8 was the only cytokine whose level did not show significant difference between healthy subjects and COPD patients with moderate COPD in GOLD 2 stage, and there was no change of IL-8 concentration in either of GOLD A-D groups. As well as in combined ABCD assessment, we have compared cytokines' concentrations in COPD frequent exacerbators and non-frequent exacerbators, but no statistically significant difference was found (data not shown). In addition, cytokines' concentrations were similar in men and women (in both healthy and COPD groups). Regarding comorbidities (cardiovascular diseases or metabolic diseases), we also found no statistically significant difference in circulating cytokines' levels.

Table 2. Concentration of cytokines in healthy participants and COPD patients regarding the severity of airflow obstruction assessed by FEV_1 (GOLD 2-4 stages) and the severity of symptoms and exacerbation history (GOLD A-D groups).

	IL-1α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	TNFα (pg/mL)
controls *n* = 95	0.30 (0.30–0.97)	0.10 (0.10–0.61)	4.85 (3.45–7.09)	6.36 (4.07–11.17)	0.40 (0.35–1.36)
GOLD 2 *n* = 39	0.40 (0.30–2.37) [1]	8.77 (0.70–20.40) [1]	30.14 (10.54–58.01) [1]	6.98 (3.27–15.25)	11.04 (0.39–19.37) [1]
GOLD 3 *n* = 36	0.63 (0.30–2.04) [1]	7.57 (0.75–22.63) [1]	34.75 (8.25–56.75) [1]	8.77 (3.50–23.59) [1]	7.40 (0.77–14.08) [1]
GOLD 4 *n* = 34	0.48 (0.30–1.60) [1]	5.54 (0.56–42.23) [1]	27.23 (12.51–106.87) [1]	9.74 (4.56–22.89) [1]	6.63 (0.35–31.37) [1]
p_1	**0.031**	**<0.001**	**<0.001**	**0.041**	**<0.001**
GOLD A *n* = 14	2.04 (0.30–3.09) [1]	8.72 (3.55–20.63) [1]	33.33 (11.95–56.65) [1]	6.07 (3.55–14.00)	12.31 (3.34–18.65) [1]
GOLD B *n* = 63	0.40 (0.30–1.84) [1]	8.27 (0.56–25.78) [1]	33.36 (10.85–71.16) [1]	9.40 (3.64–19.89)	8.60 (0.35–19.46) [1]
GOLD D *n* = 32	0.48 (0.30–2.56) [1]	4.25 (0.53–21.72) [1]	24.07 (8.25–52.93) [1]	8.20 (3.37–18.60)	4.29 (0.35–17.28) [1]
p_2	**0.018**	**<0.001**	**<0.001**	**0.398**	**<0.001**

Data were presented as median with IQR after performing Kruskal–Wallis one-way analysis of variance test. Data were considered significant if $p < 0.05$. Afterwards, post-hoc analysis was performed. GOLD–Global Initiative for chronic obstructive pulmonary disease; IL-1α—interleukin-1alpha; IL-1β—interleukin-1beta; IL-6–interleukin-6; IL-8—interleukin-8; TNFα—tumor necrosis factor alpha; p_1—statistical significance of differences between controls, GOLD 2, GOLD 3 and GOLD 4; p_2—statistical significance of differences between controls, GOLD A, GOLD B and GOLD D. [1] statistically significant in comparison to controls. All *p*-values that are <0.05 are in bold.

3.3. Cytokines' Interrelations

Network analysis was performed for the assessment of relations between investigated cytokines. Every cytokine was presented by an individual node, and its size was proportional to the percentage of defined abnormal values, as described (see Supplementary Table S2). Links between the nodes were present when at least 1% of the participants shared abnormal values for linked parameters. Moreover, the width of the link presented the percentage of the participants sharing abnormal values (Figure 1). There were no significant cytokine-based interrelations in healthy non-smokers as the nodes were small and the links between them were rare. Interestingly, healthy smokers showed to have larger nodes of IL-1β ($p < 0.05$) and TNFα ($p < 0.01$), and there were more linking nodes in comparison to healthy non-smokers. The cytokine network is even more developed in COPD patients with increased nodes of IL-1β, IL-6 and TNFα ($p < 0.001$ in comparison to both healthy non-smokers and healthy smokers for all three parameters) as well as IL-8 ($p < 0.05$ in comparison to healthy non-smokers).

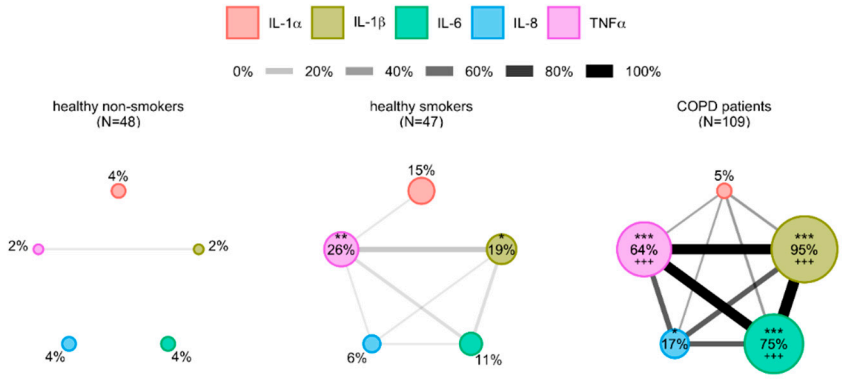

Figure 1. Network layout of cytokines determined in healthy non-smokers, healthy smokers and COPD patients. Cytokines are shown as different nodes of the network whose size is in proportion with the prevalence of their abnormal values defined by 95th percentile of healthy non-smokers. Two nodes are linked when more than 1% of participants in the network share abnormal values of these two parameters, and width of a line is proportional to that proportion. IL-1α–interleukin-1alpha; IL-1β–interleukin-1beta; IL-6–interleukin-6; IL-8–interleukin-8; TNFα–tumor necrosis factor alpha. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to healthy non-smokers, +++ $p < 0.001$ in comparison to healthy smokers.

3.4. The Potential of Cytokines in Identifying COPD Patients

To determine the potential of cytokines regarding identifying COPD patients, univariate logistic regression analysis was performed for cytokines whose concentrations were determined in plasma of all participants from the study. IL-1α had no statistically significant predictive performances, while IL-8 showed to have the lowest OR as well as number of correctly classified cases. ORs of IL-1β, IL-6 and TNFα were 5.53, 1.14 and 1.27 ($p < 0.001$ for all) (Table 3).

Table 3. Univariate logistic regression analysis of all cytokines investigated.

	OR	*p*	95% CI	Cases Correctly Classified (%)
IL-1α	1.00	0.536	0.99–1.01	53
IL-1β	5.53	**<0.001**	2.05–14.90	84
IL-6	1.14	**<0.001**	1.08–1.19	80
IL-8	1.03	**0.010**	1.01–1.05	56
TNFα	1.27	**<0.001**	1.16–1.40	74

OR—odds ratio; CI—confidence interval; IL-1α—interleukin-1alpha; IL-1β—interleukin-1beta; IL-6—interleukin-6; IL-8—interleukin-8; TNFα—tumor necrosis factor alpha. All *p*-values that are <0.05 are in bold.

3.5. Analysis of Relations between Inflammation-Driven Parameters in COPD Patients and Identification of COPD Clusters Regarding Systemic Inflammation

Based on the differences obtained in cytokines' concentrations between healthy participants and COPD patients, cytokine network analysis and evaluation of predicting potential of investigated cytokines, IL-1β, IL-6 and TNFα showed statistically the most significant results. As systemic inflammation in COPD goes beyond increased production of cytokines, we wanted to broaden our view regarding complexity and networking of inflammatory parameters in blood of patients. Therefore, based on our previous research, common inflammatory parameters CRP and Fbg as well as DAMPs eATP and eHsp70 were included together with cytokines IL-1β, IL-6 and TNFα in further analysis. Now, we wanted to assess the relations between all those parameters, so additional network analysis was performed (Figure 2). Potential relations between them were also investigated in three groups of participants (healthy non-smokers, healthy smokers and COPD patients) with reference values of the 95th percentile of each parameter in healthy non-smokers as well (see Supplementary

Table S2). Nodes were small in their size in healthy non-smokers, and only two links were present–one between CRP and IL-6 and the other between IL-1β and TNFα. Nodes with IL-1β ($p < 0.01$), TNFα ($p < 0.01$), eATP ($p < 0.001$) and eHsp70 ($p < 0.001$) were larger in healthy smokers in comparison to healthy non-smokers, and there were more links between all the parameters. Similar to cytokine network analysis, the most developed network could be seen in COPD patients where nodes of IL-1β, IL-6, TNFα, CRP, Fbg, eATP and eHsp70 were significantly larger in comparison to both healthy non-smokers ($p < 0.001$ for all except for CRP whose p value is <0.01) and healthy smokers ($p < 0.001$ for all except for CRP whose p value is <0.05, and Fbg whose p value is <0.01).

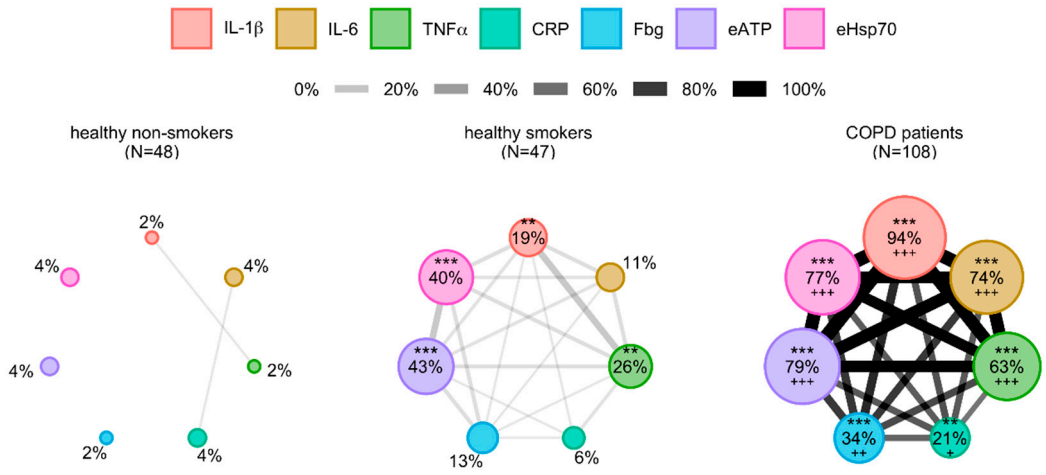

Figure 2. Network layout of cytokines combined with common inflammatory parameters and DAMPs in healthy non-smokers, healthy smokers and COPD patients. Parameters are shown as different nodes of the network whose size is in proportion with the prevalence of their abnormal values defined by 95th percentile of healthy non-smokers. Two nodes are linked when more than 1% of participants in the network share abnormal values of these two parameters, and width of a line is proportional to that proportion. DAMP–damage-associated molecular pattern; IL-1β—interleukin-1beta; IL-6—interleukin-6; TNFα—tumor necrosis factor alpha; CRP—C-reactive protein; Fbg—fibrinogen; eATP—extracellular adenosine-triphosphate; eHsp70—extracellular heat shock protein 70. ** $p < 0.01$, *** $p < 0.001$ in comparison to healthy non-smokers; +++ $p < 0.001$ in comparison to healthy smokers.

In addition, hierarchical cluster analysis was conducted with seven variables and clinical data obtained from COPD patients (Figure 3, see Supplementary Table S3). FEV_1 (% predicted) was included for the evaluation of airflow limitation-based severity. However, FEV_1 is insufficient for the disease severity assessment, and there was a need for additional assessment by DLCO (as a measure of the diffusion properties of the alveolar capillary membrane), number of exacerbations, mMRC (for dyspnoea severity), CAT and SGRQ-C (for more comprehensive assessment of symptoms and quality of life related to health status) and multicomponent index CODEx that incorporates several variables with great emphasis on comorbidities. Division of COPD patients based on FEV_1 was defined by GOLD guidelines [4], and there were four groups of COPD patients according to the severity of airflow limitation, as already described. Criteria for the diffusion limitation severity assessed by DLCO was defined by literature as well [30]. According to Fragoso et al., in our cluster analysis COPD patient that experienced two or more exacerbations or at least one exacerbation which led to hospitalization were considered to have a phenotype of exacerbator, while those that had less than two exacerbations during the previous year without hospitalization were considered to be non-exacerbators [31]. The division criteria for other parameters (mMRC, CAT, SGRQ-C, CODEx) were established by median values in COPD patients from the study.

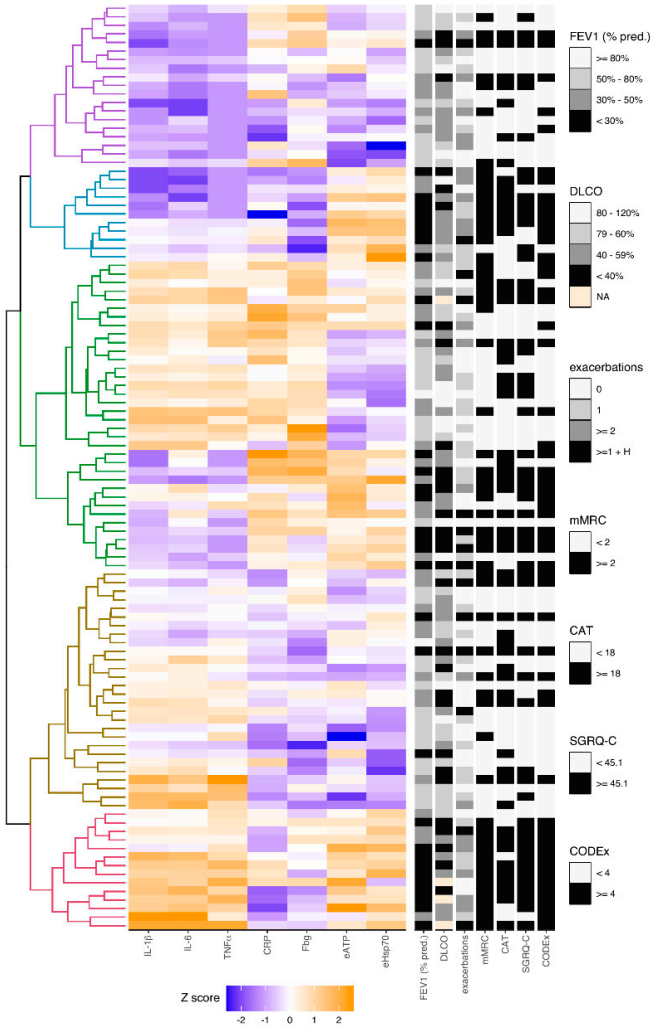

Figure 3. The heatmap of clusters of COPD patients regarding concentrations of cytokines (IL-1β, IL-6, TNFα), common inflammatory parameters (CRP, Fbg) and DAMPs (eATP, eHsp70). Hierarchical clustering analysis was used to characterize different phenotypes in COPD patients based on concentrations of cytokines (IL-1β, IL-6, TNFα), common inflammatory parameters (CRP, Fbg) and DAMPs (eATP, eHsp70). All concentrations were rank-based inverse normal transformed, and Euclidian correlation test with complete linkage was used for the clustering analysis. The heatmap shows colored squares, which present values of each parameter in each COPD patient. Five clusters of COPD patients were identified (left), and their clinical data (right) was included in the analysis, too. DAMP–damage-associated molecular pattern; IL-1β—interleukin-1beta; IL-6—interleukin-6; TNFα—tumor necrosis factor alpha; CRP—C-reactive protein; Fbg—fibrinogen; eATP—extracellular adenosine-triphosphate; eHsp70—extracellular heat shock protein 70; FEV_1 (% pred.)—forced expiratory volume in one second (% predicted); DLCO—diffusing capacity for carbon monoxide; exacerbations–number of exacerbations reported in previous year; mMRC—modified Medical Research Council; CAT–COPD Assessment Test; SGRQ-C—St George's Respiratory Questionnaire for COPD patients; CODEx—comorbidities, obstruction, dyspnoea, previous exacerbations; NA—not applicable; H–hospitalization.

Patients from cluster group 1 showed to have decreased concentrations of all three cytokines as well as mostly lower concentrations of eATP and eHsp70, while levels of CRP and Fbg seemed to be heterogeneous. Based on clinical data, those were predominantly the patients with mild clinical phenotype. COPD patients from cluster group 2 shared decreased values of all cytokines and they also mostly had lower levels of common inflammatory parameters, yet they had increased levels of DAMPs.

All the patients from cluster group 2 were in GOLD 3 or GOLD 4 stages of the disease and were above the median of mMRC score. Most of them had a phenotype of exacerbator. Predominantly, patients in cluster group 2 had higher scores of CAT, SGRQ-C and CODEx. Cluster groups 3 and 4 showed to have various changes of all parameters included in the analysis, and there was no unambiguous clinical phenotype regarding observed changes. However, cluster group 4 showed to recruit more patients with milder clinical phenotype. Patients in cluster group 5 showed mostly lower values of CRP and Fbg and increased levels of all cytokines, eATP and eHsp70. All of them were in GOLD 3 and GOLD 4 stages of the disease, had at least one exacerbation in the previous year as well as lower DLCO (a few patients could not perform this analysis due to their severe symptoms; therefore, not applicable (NA) was designated for them on the heatmap). Almost whole group had great impact of dyspnoea on everyday life assessed by mMRC and great impact of comorbidities assessed by CODEx score, while most of the patients from the group had more severe symptoms that affect their quality of life.

3.6. Model Combined of IL-1β, eATP and eHsp70 as the Best Combination for Identifying COPD Patients

Finally, evaluation of predictive performances of additional parameters was performed by univariate logistic regression analysis, and it was shown that all parameters had potential in identifying COPD patients. CRP showed to have OR of 1.24 (95% CI = 1.09–1.41, $p = 0.001$), OR of Fbg was 2.55 (95% CI = 1.65–3.95, $p < 0.001$), while eATP showed to have OR of 20.16 (95% CI = 8.40–48.38, $p < 0.001$), and eHsp70 OR of 5.20 (95% CI = 3.02–8.96, $p < 0.001$). Based on all statistically significant results from univariate logistic regression analysis (cytokines in Table 3 as well as CRP, Fbg, eATP and eHsp70), multivariate logistic regression analysis suggested a model composed of IL-1β (OR = 3.58, 95% CI = 1.71–7.47), eATP (OR = 6.08, 95% CI = 1.79–20.64) and eHsp70 (OR = 3.10, 95% CI = 1.59–6.03). This model showed the greatest predictive performance in comparison to other models and successfully classified 91% of cases, while area under the curve (AUC) was 0.966 (95% CI = 0.931–0.987, $p < 0.001$) which was the highest one when compared to other AUCs of all investigated models.

4. Discussion

Our study showed that all investigated cytokines (IL-1α, IL-1β, IL-6, IL-8 and TNFα) were increased in plasma of COPD patients, yet there was no association with airflow obstruction and symptoms severity. Inflammation was more developed in healthy smokers when compared to healthy non-smokers, and even more in COPD patients which was assessed by two network analyses–one including only cytokines, and the other conducting IL-1β, IL-6 and TNFα as well as additional inflammation-associated parameters CRP, Fbg, eATP and eHsp70. Moreover, when all parameters and clinical data were included in cluster analysis, different COPD clusters were observed. Finally, our study suggested combination of IL-1β, eATP and eHsp70 as the best model in identifying COPD patients with 91% correctly classified cases.

COPD is characterized by persistent inflammation predominantly localized to the peripheral airways and lung parenchyma, but it is also recognized that systemic inflammation might have an important role in development and progression of the disease and its comorbidities. Underlying mechanism of systemic inflammation in COPD include oxidative stress and altered circulating levels of inflammatory mediators [6,7]. It was shown that IL-1α and IL-1β were increased in lung samples and sputum of COPD patients. Additionally, it has been established that IL-1β was increased in peripheral circulation, while no study, to the best of our knowledge, investigated levels of blood IL-1α in patients with COPD [32,33]. IL-6 was increased in blood samples as well as in samples obtained from respiratory system of COPD patients when compared to controls [2,12]. In addition, IL-8 was increased in plasma [7] and sputum [8] of COPD patients as well as TNFα [5,7]. Increased cytokine levels in patients with COPD from the current study in comparison to controls might be more related to the systemic inflammation present in stable COPD than to pulmonary function impairment. Cytokines are the markers of low-grade inflammation, which was significantly developed in COPD patients. This was observed from network analysis of all cytokines, and IL-1β, IL-6 and TNFα were the best

discriminators of COPD patients with cytokine levels being >95th percentile of the group of healthy non-smokers. However, one should have in mind that those cytokines are not specific to COPD and that systemic inflammation develops later in the disease course. Therefore, association of cytokines with disease severity parameters and/or outcomes would be preferable. It was shown that IL-1β from peripheral circulation positively correlated with CRP and negatively with FEV_1 [33]. Additionally, IL-1β is a dominant part of systemic pro-inflammatory response in COPD, and its high levels in sputum were associated with impaired lung function [11,34]. Negative correlation was also observed between IL-6 and FEV_1 [35]. However, IL-6 was not associated with decline in lung function in COPD patients from the ECLIPSE cohort [11]. Cytokines IL-1β, IL-6 and TNFα showed to be associated with the severity of COPD [10,16,36,37]. Our study did not show an increase in cytokine concentration regarding the severity of airflow obstruction or symptoms severity and history of exacerbations. Association of cytokines with disease severity was not successfully replicated either in some other studies, which might indicate heterogeneity within patient populations [6,17,38]. Kleniewska et al. showed that COPD patients had increased IL-1β, IL-6 and TNFα in induced sputum, but there was no difference in their concentration in serum in comparison to healthy subjects. However, CRP and Fbg showed to be increased in serum of COPD patients from the same study [39]. Cigarette smoking is one of the main environmental contributors to the development and progression of COPD. Still, our results indicate that the elevation of plasma cytokine levels was a consequence of COPD rather than smoking status. Similar observation was present in the study by Selvarajah et al. [40]. Besides cytokines, other parameters significantly contribute to the inflammatory processes as well, so they should be also explored as potential diagnostic and/or therapeutic targets. Additionally, it is considered that spirometry, symptoms assessment and data regarding exacerbations are not sufficient to reflect entirely the heterogeneity of COPD [31], and similar levels of airflow obstruction might result with different outcomes depending on the presence or absence of persistent systemic inflammation [21]. Therefore, investigation of biomarkers is important because of the possible distinction of COPD patients based on various patterns in alteration of investigated biomarkers. In our previous publications that included the same subjects as the current study, common inflammatory parameters CRP and Fbg were increased in COPD patients, and their predictive potential was observed as well [22]. Additionally, in the same patients we also assessed eATP and eHsp70 and showed that both of those DAMPs were associated with smoking status, airflow obstruction severity as well as symptoms severity and history of exacerbations [23,24]. Moreover, their great predictive values and association with multicomponent clinical parameters used for COPD assessment indicate there might be eATP- and eHsp70-driven inflammation as a part of disease progression. When all aforementioned parameters were combined with IL-1β, IL-6 and TNFα in network analysis, it was shown that not all patients with stable COPD have increased systemic inflammatory parameters, and even already well-known inflammatory parameters like CRP and Fbg were increased in only 21% and 34% of COPD patients, respectively, in comparison to the 95th percentile of healthy non-smokers. This suggest that other parameters (IL-1β, IL-6, TNFα, eATP, eHsp70) might have more important role in ongoing inflammation. In addition, increasing compactness of connected lines indicates there is a significant progression of inflammation in COPD patients. Still, systemic inflammation does not have to be persistent. It was demonstrated after three years follow-up that if duration of systemic inflammation was at least one year, it could lead to worse COPD outcomes (all-cause mortality and/or exacerbation frequency) [21]. Therefore, our suggestion for the future studies is to include a measurement of the same parameters after prolonged period with the aim to assess the persistence of systemic inflammation. Furthermore, different phenotypes of COPD might be identified with the purpose of better prognosis, diagnosis, and targeted treatment of COPD. Some of advanced statistical techniques may prove to be useful in identifying candidate phenotypes. Cluster analysis encompasses different algorithms for grouping objects without a priori hypothesis. By applying cluster data analysis, we studied concentrations of various parameters and clinical data obtained from our patients with stable COPD. Therefore, the goal was to classify overall data into relatively homogeneous cluster groups. There were previous studies with cluster analyses of

various cytokines in COPD. Cluster group of COPD patients with lower cytokines values regarding statin therapy was suggested as an important one by Marević et al. [41]. Additionally, comorbidity clusters of COPD patients were associated with systemic inflammation [42], while other studies suggested several COPD subtypes after applying cluster analyses that explored clinical variables and outcomes [43–45]. Our cluster analysis joined IL-1β, IL-6 and TNFα with CRP, Fbg, eATP and eHsp70 since they were recognized as potential parameters in identifying various subgroups among COPD patients with systemic inflammation. In addition, we included clinical data as referent variables. There were several observations from cluster analysis that are worth mentioning. Cluster groups 2 and 5 showed the worst status regarding all clinical variables in the study. Cluster group 2 had increased both eATP and eHsp70, while other investigated parameters were decreased. On the other hand, cluster group 5 encompassed patients with increased cytokines as well as eATP and eHsp70, while they had mostly intermediate or decreased levels of CRP and Fbg. Patients from both cluster groups were the ones with lowest FEV_1 that was accompanied with lower health-related quality of life and possible significant impact of comorbidities assessed by CODEx. Considering that an increase in eATP and eHsp70 was accompanied by more severe clinical features, it could be suggested to include them in the assessment of COPD. Cluster groups 3 and 4 comprised most of the patients and it seems they represent the heterogeneity of COPD. All clinical variables significantly differed among these COPD patients. The heatmap shows there might be additional subgroups within groups 3 and 4 that were not separated by the cluster analysis. Finally, COPD patients from cluster group 1 had lower levels of all cytokines and mostly also of eATP and eHsp70, while CRP and Fbg differed. Predominantly, they showed to have mild to moderate clinical phenotype when the assessment of airflow limitation, exacerbations, symptoms severity and comorbidities were considered. Results from cluster analysis suggest there might be several patterns of inflammation in COPD patients, and similar was observed in the study of Rennard et al. [45]. It would be interesting to evaluate observed patterns in investigated parameters regarding commonly present comorbidities and potential effect of therapy. Cluster analysis might be a part of targeted approach towards future study designs, so the questions of interest could be directed to the specific COPD subgroups. Finally, when all statistically significant predictors from this study were included in multivariate logistic regression analysis, a combination of IL-1β, eATP and eHsp70 showed to have great performances in identifying COPD patients. The suggested model successfully classified 91% of all cases. Therefore, combined three-parameter model might have a great value in recognizing COPD patients, while patterns in concentrations of cytokines, CRP, Fbg, eATP and eHsp70 might be useful in identifying different COPD subgroups.

Several shortcomings are present in the current study. There were no patients in GOLD 1 stage or GOLD C group since COPD patients in GOLD 1 stage of the disease mostly do not contact their physician because of the very mild symptoms, while patients in GOLD C group usually do not manifest many symptoms and are not frequent exacerbators. However, larger number of participants in general should be considered in the further studies and a longitudinal study should be preferred over a cross-sectional case-control study.

5. Conclusions

Cytokines are one of the contributors in inflammatory processes present in COPD patients. However, by itself they are insufficient for the assessment of COPD, so additional biomarkers should be also evaluated. Models that include IL-1β, eATP and eHsp70 might prove to be useful in recognizing COPD patients because of its great predictive value, while combinations of IL-1β, IL-6 and TNFα with CRP, Fbg, eATP and eHsp70 might have a potential in differentiating COPD patients regarding clinical subgroups, with eATP and eHsp70 being particularly useful in identifying patients with severe COPD.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4418/10/12/1029/s1, Table S1: Levels of cytokines in all participants regarding their smoking status, Table S2: Values of 95th percentile of the parameters determined in healthy non-smokers that are used in network analyses, Table S3: Clinical characteristics and concentrations of cytokines (IL-1β, IL-6, TNFα), common inflammatory parameters (CRP, Fbg) and DAMPs (eATP, eHsp70) in COPD patients according to the five clusters after unsupervised hierarchical clustering analysis.

Author Contributions: I.H. and L.R. wrote the main manuscript text and prepared figures and tables; L.R. and D.K. performed statistical analysis and interpreted it with I.H.; all authors contributed to the design of the work, while D.B. and M.B. performed the experiments; A.V.D. was responsible for collecting the samples, performing spirometry and DLCO analysis, and collecting data about participants; all authors reviewed the manuscript and approved the submitted version (and any substantially modified version). In addition, all authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature. All authors have read and agreed to the published version of the manuscript.

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Supplementary material

Table 1. Levels of cytokines in all participants regarding their smoking status.

	healthy non-smokers N = 48	healthy smokers N = 47	COPD non-smokers N = 5	COPD former smokers N = 75	COPD smokers N = 29	P
IL-1α (pg/ml)	0.30 (0.30 - 0.71)	0.30 (0.30 - 1.78)	0.30 (0.30 - 1.43)	0.40 (0.30 - 1.84)	1.57 (0.30 - 2.66) [1,2]	**0.010**
IL-1β (pg/ml)	0.10 (0.10 - 0.16)	0.10 (0.10 - 2.76)	0.62 (0.28 - 2.24) [1,2]	8.24 (0.56 - 26.23) [1,2]	9.65 (1.52 - 17.54) [1,2]	**<0.001**
IL-6 (pg/ml)	4.41 (3.29 - 6.17)	5.37 (3.79 - 8.01)	10.40 (2.88 - 15.51)	36.05 (10.75 - 72.66) [1,2,3]	33.05 (11.09 - 52.03) [1,2,3]	**<0.001**
IL-8 (pg/ml)	6.22 (4.34 - 11.33)	6.36 (3.99 - 11.17)	3.25 (2.32 - 6.92)	8.20 (3.61 - 20.22)	9.65 (4.39 - 17.54)	0.172
TNFα (pg/ml)	0.35 (0.35 - 0.53)	0.70 (0.35 - 3.04) [1]	0.35 (0.35 - 0.71)	8.18 (0.46 - 23.32) [1,2,3]	11.08 (1.13 - 17.55) [1,2,3]	**<0.001**

Data were tested by Kruskal-Wallis one-way analysis of variance and presented as median with IQR. Results were statistically significant if P<0.05. Afterwards, post-hoc analysis was performed.

IL-1α – interleukin-1alpha; IL-1β – interleukin-1beta; IL-6 – interleukin-6; IL-8 – interleukin-8; TNFα – tumour necrosis factor alpha.

[1] statistically significant in comparison to healthy non-smokers;

[2] statistically significant in comparison to healthy smokers;

[3] statistically significant in comparison to COPD non-smokers.

Table 2. Values of 95th percentile of the parameters determined in healthy non-smokers that are used in network analyses.

network analysis of all cytokines	
parameter	95th percentile value
IL-1α (pg/ml)	6.18
IL-1β (pg/ml)	0.10
IL-6 (pg/ml)	10.48
IL-8 (pg/ml)	26.14
TNFα (pg/ml)	2.65
network analysis of selected parameters	
parameter	95th percentile value
IL-1β (pg/ml)	0.10
IL-6 (pg/ml)	10.48
TNFα (pg/ml)	2.65
CRP (mg/l)	5.15
Fbg (g/l)	4.2
eATP (μmol/l)	1.10
eHsp70 (ng/ml)	0.74

Table S3. Clinical characteristics and concentrations of cytokines (IL-1β, IL-6, TNFα), common inflammatory parameters (CRP, Fbg) and DAMPs (eATP, eHsp70) in COPD patients according to the five clusters after unsupervised hierarchical clustering analysis.

parameter	CLUSTER 1 N=19	CLUSTER 2 N=11	CLUSTER 3 N=36	CLUSTER 4 N=28	CLUSTER 5 N=14	P	after adjusted P-value *
FEV_1 (% pred.)	61.8 (40.4 – 61.8)	26.0 (20.8 – 30.0)	43.8 (29.4 – 56.9)	54.5 (34.4 – 64.0)	27.3 (23.1 – 30.6)	**<0.001**	1 vs. 2 1 vs. 3 1 vs. 5 2 vs. 3 2 vs. 4 3 vs. 5 4 vs. 5
DLCO	55.7 (45.1 -74.4)	44.6 (37.5 – 46.2)	50.1 (36.3 – 74.2)	56.7 (36.2 – 70.0)	40.7 (34.8 – 44.6)	0.319	NS
exacerbations (N) 0 1 ≥2 ≥1 + H	 8 5 5 1	 1 4 5 1	 13 14 3 6	 13 9 2 4	 3 4 5 2	0.130	NS
mMRC	1 (1 – 2)	3 (2 – 3)	1 (1 - 2)	1 (1 – 2)	2 (2 - 3)	**<0.001**	1 vs. 2 1 vs. 5 2 vs. 3 2 vs. 4 3 vs. 5 4 vs. 5
CAT	15 (11 - 20)	20 (16 – 27)	17 (13 – 24)	17 (8 – 21)	22 (14 – 26)	**0.016**	NS
SGRQ-C	32.5 (24.1 – 49.8)	59.5 (45.5 – 79.4)	47.3 (31.3 – 62.5)	35.2 (20.5 – 47.1)	64.2 (54.7 – 69.0)	**<0.001**	1 vs. 2 1 vs. 5 2 vs. 4 3 vs. 4 4 vs. 5

CODEx	1 (1 – 3)	6 (5 – 6)	4 (2 – 5)	2 (1 – 4)	5 (4 - 6)	**<0.001**	1 vs. 2 1 vs. 5 2 vs. 3 2 vs. 4 3 vs. 5 4 vs. 5
IL-1β (pg/ml)	0.38 (0.24 – 0.65)	0.21 (0.12 – 3.82)	13.22 (1.08 – 25.90)	8.81 (3.90 – 20.25)	47.55 (12.98 – 56.40)	**<0.001**	1 vs. 3 1 vs. 4 1 vs. 5 2 vs. 3 2 vs. 4 2 vs. 5 3 vs. 5 4 vs. 5
IL- 6 (pg/ml)	3.84 (1.87 – 9.26)	10.72 (1.56 – 14.73)	39.91 (29.98 – 72.15)	37.67 (19.08 – 63.71)	118.73 (40.21 – 189.82)	**<0.001**	1 vs. 3 1 vs. 4 1 vs. 5 2 vs. 3 2 vs. 4 2 vs. 5 3 vs. 5 4 vs. 5
TNFα (pg/ml)	0.35 (0.35 – 0.35)	0.35 (0.35 – 4.22)	12.31 (0.77 – 20.00)	9.22 (5.77 – 18.12)	27.56 (18.48 – 56.77)	**<0.001**	1 vs. 3 1 vs. 4 1 vs. 5 2 vs. 3 2 vs. 4 2 vs. 5 3 vs. 5 4 vs. 5
CRP (mg/l)	2.21 (1.09 – 4.53)	1.75 (0.98 – 2.96)	5.34 (3.82 – 8.35)	1.24 (0.89 – 1.77)	1.21 (0.77 – 3.21)	**<0.001**	1 vs. 3 2 vs. 3 3 vs. 4

							3 vs. 5
Fbg (g/l)	3.8 (3.6 – 4.5)	3.0 (2.5 – 3.3)	4.5 (4.2– 5.1)	3.5 (3.1 – 3.7)	3.7 (3.5 – 3.9)	**<0.001**	1 vs. 2 1 vs. 3 1 vs. 4 2 vs. 3 2 vs. 4 2 vs. 5 3 vs. 4 3 vs. 5
eATP (μmol/l)	1.19 (0.93 – 1.52)	1.91 (1.84 – 2.23)	1.63 (1.24 – 2.04)	1.38 (1.12 – 1.52)	1.94 (1.92 – 2.33)	**<0.001**	1 vs. 2 1 vs. 3 1 vs. 5 2 vs. 4 3 vs. 4 3 vs. 5 4 vs. 5
eHsp70 (ng/ml)	0.99 (0.58 – 1.49)	3.41 (2.94 – 6.84)	2.06 (1.09 – 2.96)	0.82 (0.51 – 1.54)	3.45 (3.15 – 5.10)	**<0.001**	1 vs. 2 1 vs. 3 1 vs. 5 2 vs. 3 2 vs. 4 3 vs. 4 3 vs. 5 4 vs. 5

DAMP – damage-associated molecular pattern; FEV_1– forced expiratory volume in one second; DLCO – diffusing capacity for carbon monoxide; exacerbations – number of exacerbations reported in previous year; mMRC – modified Medical Research Council; CAT – COPD Assessment Test; SGRQ-C – St George's Respiratory Questionnaire for COPD patients; CODEx – comorbidities, obstruction, dyspnoea, previous exacerbations; IL-1β – interleukin-1beta; IL-6 – interleukin-6; TNFα – tumour necrosis factor alpha; CRP – C-reactive protein; Fbg – fibrinogen; eATP – extracellular adenosine-triphosphate; eHsp70 – extracellular heat shock protein 70; H – hospitalization; NS – not significant after the adjustment of P-value.

* significant differences between the groups after the adjustment of P-value by Benjamini-Hochberg method.

5. POVEĆANA EKSPRESIJA GENA *HSP70* I *TLR2* I POVEZANOST POLIMORFIZMA JEDNOG NUKLEOTIDA rs6457452 U GENU *HSP70* S RIZIKOM OD KRONIČNE OPSTRUKCIJSKE PLUĆNE BOLESTI U HRVATSKOJ POPULACIJI

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Article

Increased *HSP70* and *TLR2* Gene Expression and Association of *HSP70* rs6457452 Single Nucleotide Polymorphism with the Risk of Chronic Obstructive Pulmonary Disease in the Croatian Population

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Abstract: Heat shock protein 70 (Hsp70) engages Toll-like receptors (TLR) 2 and 4 when found in the extracellular compartment and contributes to inflammation in chronic obstructive pulmonary disease (COPD). Since there is growing evidence for the genetic risk factors for COPD, the gene expression of *HSP70*, *TLR2* and *TLR4* was determined, as well as the association between *HSP70*, *TLR2* and *TLR4* single nucleotide polymorphisms, (SNPs) and COPD. The gene expression was assessed in peripheral blood cells of 137 COPD patients and 95 controls by a quantitative polymerase chain reaction (qPCR), while a total of nine SNPs were genotyped by TaqMan allelic discrimination real-time PCR. *HSP70* and *TLR2* gene expression was increased in COPD patients compared to the controls, regardless of the disease severity and smoking status of participants. The rs6457452 SNP of *HSP70* was associated with COPD, indicating the protective role of the T allele (OR = 0.46, 95% CI = 0.24–0.89, $p = 0.022$). Furthermore, COPD C/T heterozygotes showed a decreased *HSP70* mRNA level compared to COPD C/C homozygotes. In conclusion, *HSP70* and *TLR2* may have a role in the pathogenesis of COPD, and the *HSP70* rs6457452 variant might influence the genetic susceptibility to COPD in the Croatian population.

Keywords: chronic obstructive pulmonary disease; heat shock protein 70; toll-like receptor 2; toll-like receptor 4; single nucleotide polymorphism

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is a multicomponent heterogeneous inflammatory disease characterized by persistent respiratory symptoms and airflow limitation [1]. It is known that the development of COPD is influenced by several environmental and genetic factors. Tobacco exposure has been recognized as the main environmental

risk factor for COPD [2], yet only 10–20% of smokers develop airway obstruction [3]. COPD has both pulmonary and extrapulmonary manifestations, and systemic chronic inflammation has been identified as a potential COPD endotype [4]. It is considered that the persistent low-grade inflammation in stable COPD might be caused by increased levels of many inflammatory mediators [5], reactive oxygen and nitrogen species [6], and pathogen-associated molecular patterns or damage-associated molecular patterns (DAMPs) such as extracellular heat shock protein 70 (eHsp70) [7–9]. eHsp70 is thought to activate proinflammatory responses by binding to Toll-like receptors (TLRs) 2 and 4, leading to increased cytokines concentrations [10].

Hsp70 is predominantly an intracellular protein involved in the maintenance of protein homeostasis, especially in the presence of stressors such as heat, bacterial, and viral infections [11]. However, the role of Hsp70 in COPD pathogenesis remains to be elucidated. Increased gene expression of *HSP70* and its protein concentration was observed in lung tissues of COPD patients, and *HSP70* mRNA levels were negatively correlated with lung function. Moreover, *HSP70* expression in lung tissues was associated with airway obstruction severity and smoking status [12]. In the study by Ambrocio-Ortiz et al., expression of *HSP70* was examined in sputum, and they found a decrease in the COPD smoking group compared to controls who were mostly smokers [13].

Considering TLR2 and TLR4, they are expressed by a variety of cells involved in innate and adaptive immune responses. Cigarette smoke and Hsps can affect TLR2 and TLR4 protein concentration and the mRNA level in the respiratory tract. Our previous study showed that cigarette smoke or eHsp70 can inhibit the expression of *TLR2* and *TLR4* in bronchial epithelial cells in COPD [14]. Moreover, *TLR2* and *TLR4* expression was decreased in the lung parenchyma of COPD patients with severe disease, while the gene expression of both receptors was increased in the airway epithelium of COPD patients in mild and moderate stages [15]. However, when *TLR2* and *TLR4* gene expression was determined in alveolar macrophages from the bronchoalveolar lavage of COPD smokers, there was no difference in the mRNA levels compared to both the control of non-smokers and control of smokers [16].

In general, the gene expression could differ in various types of samples used in the analysis. Sputum reflects the central airways, while bronchoalveolar lavage represents the peripheral airways [17]. On the other hand, blood is an easily available sample that is representative of systemic compartment, which is also important in COPD. To the best of our knowledge, this is the first study in which blood samples were used to assess the basal *HSP70*, *TLR2* and *TLR4* gene expression in stable COPD without any further in vitro treatments or stimulations.

COPD is a complex disease with a genetic background. Single nucleotide polymorphisms (SNPs) may be associated with changes in gene expression, affect gene product and thus contribute to susceptibility to disease. *HSP70* polymorphisms have been found to be related to various diseases such as pulmonary fibrosis [18,19], atherosclerosis [20], coronary heart disease [21] and COPD [13]. Furthermore, the *HSP70* rs1061581 variant was associated with COPD in the Croatian population [22]. Genetic polymorphisms in *TLR2* and *TLR4* genes were also significantly related to altered risk of COPD [23–25] as well as to reduced lung function and an increased number of inflammatory cells in the sputum of COPD patients [24].

The aim of this study was to investigate the gene expression of *HSP70*, *TLR2* and *TLR4* in the peripheral blood cells of COPD patients and to explore whether there is an association between the gene expression and disease severity, and smoking. Genotype and allele distribution of selected *HSP70*, *TLR2* and *TLR4* polymorphisms were determined in healthy subjects and COPD patients, and a potential association between SNPs and COPD was assessed. Finally, we explored the relation between *HSP70*, *TLR2* and *TLR4* mRNA levels, and investigated polymorphisms.

2. Materials and Methods

2.1. Study Population

The retrospective study comprised of 137 COPD patients in the stable phase of the disease, and 95 controls. Stable COPD was defined as no exacerbations during at least three physician visits in the previous 3 months, with no changes in respiratory therapy and no symptoms of a lower respiratory tract infection. All participants were recruited at the Clinical Department for Lung Diseases Jordanovac, University Hospital Centre Zagreb (Zagreb, Croatia) after they agreed to participate voluntarily in the study and signed an informed consent form. The study was approved by the Ethical Committee of the University Hospital Centre Zagreb and the Ethical Committee for Experimentation of the University of Zagreb Faculty of Pharmacy and Biochemistry (Zagreb, Croatia) (approval protocol numbers: 02/21/JG on 29 August 2014 and 251-62-03-14-78 on 10 September 2014, respectively).

The diagnosis of COPD was made according to the guidelines [1], after measuring the spirometry parameters—forced expiratory volume in the first second (FEV_1) and forced vital capacity (FVC). If FEV_1/FVC was under the value of 0.70, airway obstruction was confirmed. COPD patients were classified into GOLD stages based on FEV_1 as follows: GOLD 1 ($FEV_1 \geq 80\%$) ($n = 0$), GOLD 2 ($50\% \leq FEV_1 < 80\%$) ($n = 47$), GOLD 3 ($30\% \leq FEV_1 < 50\%$) ($n = 50$) and GOLD 4 ($FEV_1 < 30\%$) ($n = 40$). In addition to airflow limitation severity, data on symptoms severity and history of exacerbations were used to differentiate COPD patients into GOLD groups based on COPD Assessment Test (CAT). There were GOLD A ($n = 27$), GOLD B ($n = 70$), GOLD C ($n = 0$) and GOLD D ($n = 40$) groups.

The control subjects were age- and sex-matched with the COPD subjects, and their medical history data and spirometry results were used to determine their health status. Subjects in both groups were older than 40 years, had no lung diseases (except COPD in COPD patients), inflammatory systemic diseases, acute infections, diabetes with severe complications, severe liver disease, severe renal insufficiency, malignant diseases, transplantations, or other specific or non-specific acute inflammation. In addition, all participants provided information on smoking status, so that there were 48 healthy non-smokers, 47 healthy smokers, 10 COPD non-smokers, 90 COPD former smokers, and 37 COPD smokers.

2.2. RNA Isolation and Gene Expression Analysis

Blood samples were collected in two tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant (Greiner Bio-One, GmbH, Kremsmünster, Austria). One tube was used for DNA isolation, while the other was used for RNA extraction. After centrifugation at 3500 rpm for 10 min at +4 °C, RNA was extracted from buffy coat using the TRIzol/chloroform method [26], followed by the cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific, Waltham, MA, USA) with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) [27]. Commercial TaqMan Gene Expression Assays were used for the assessment of gene expression (Hs00359163_s1 for *HSP70*, Hs02621280_s1 for *TLR2*, Hs00152939_m1 for *TLR4*; Applied Biosystems, Foster City, CA, USA) as well as Taqman Universal Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's guidelines. Beta-2-microglobulin (*B2M*) and peptidylprolyl isomerase (*PPIA*) ((Hs99999907_m1 for *B2M*, Hs99999904_m1for *PPIA*; Applied Biosystems, Foster City, CA, USA) were used as the endogenous controls for data normalization. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of target genes in controls and COPD patients [28]. One randomly chosen control sample was included in each plate as a calibrator.

2.3. Determination of eHsp70 Concentration

Measurement of eHsp70 was determined in the same cohort in our previous study (8) in EDTA plasma using the commercially available enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Science, Farmingdale, NY, USA).

2.4. DNA Isolation and SNP Genotyping

DNA was extracted from blood cells by a standard salting-out procedure [29]. SNPs in the *HSP70*, *TLR2* and *TLR4* genes were selected based on a literature search of studies associated with COPD, smoking or other respiratory diseases, and when the minor allele frequency was (MAF) ≥0.05 in the European population, according to the 1000 Genomes Project. A total of nine polymorphisms shown in Table 1 were selected for genotyping by the Taqman allelic discrimination real-time PCR. Reaction was carried out in a total volume of 10 µL with the corresponding SNP Genotyping Assays (Applied Biosystems, Waltham MA, USA), TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA), and an adjusted volume of DNA templates, according to the manufacturer's instructions. Analysis was performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a default PCR amplification protocol. The successful call rate was greater than 96%, while 10% of samples were genotyped additionally to assess the reproducibility of the genotyping protocol. Data were interpreted using the Sequence Detection Software (SDS v. 1.4., Applied Biosystems, Waltham, MA, USA).

Table 1. Basic information about investigated polymorphisms in *HSP70*, *TLR2* and *TLR4* genes.

Gene Name	Gene ID	SNP ID	Assay ID	Functional Relevance	Nucleotide Substitution	Chromosome Position	MAF
HSPA1A	3303	rs1008438	ANCFDUH *	2 KB upstream variant	A > C	chr6:31815431	0.4036 (C)
HSPA1A	3303	rs1043618	C__11917510_10	5′UTR variant	G > C	chr6:31815730	0.3926 (C)
HSPA1B	3304	rs6457452	C___3052604_10	5′UTR variant	C > T	chr6:31827773	0.0775 (T)
TLR2	7097	rs1898830	C__11853988_10	intron variant	A > G	chr4:153687301	0.3250 (G)
TLR2	7097	rs3804099	C__22274563_10	synonymous variant	T > C	chr4:153703504	0.4354 (C)
TLR2	7097	rs13150331	C__11853987_10	intron variant	A > G	chr4:153678470	0.4056 (G)
TLR4	7099	rs2737190	C___2704047_10	upstream variant	G > A	chr9:117701903	0.3290 (G)
TLR4	7099	rs10759932	C__31783996_10	2 KB upstream variant	T > C	chr9:117702866	0.1531 (C)
TLR4	7099	rs7846989	C_189478937_10	3′UTR variant	T > C	chr9:117720662	0.1004 (C)

HSP—heat shock protein; TLR—Toll like receptor; SNP—single nucleotide polymorphism; MAF—minor allele frequency (1000 Genomes, European population); UTR—untranslated. * Custom Taqman$^{(R)}$ SNP Genotyping Assay.

2.5. Statistics

Data were analyzed using MedCalc statistical software version 17.9.2. (MedCalc Software, Ostend, Belgium). All data failed a normality test performed with the Kolmogorov-Smirnov test, so a nonparametric Mann-Whitney test was used to assess the differences in age, spirometry parameters and the relative expression of target genes between the controls and COPD subjects. The Kruskal-Wallis test was used when more than two groups were compared. χ^2 test was used to compare the categorical variable (sex), while the χ^2 and Fisher's exact test were used to test the relation between genotype and allele distribution. Logistic regression was performed for an association analysis between genotype and allele distribution, and COPD risk by calculating the odds ratio (OR) and the corresponding 95% confidence interval (CI).

The SNPStats software (https://www.snpstats.net/start.htm, accessed on 15 October 2020) was used for the assessment of Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD), and haplotype analysis. A strong LD was considered when D′ > 0.80 [30].

The p value of <0.05 was considered statistically significant in all analyses.

3. Results

3.1. Basic Population Characteristics

The general information and spirometry results of the enrolled participants are shown in Table 2. COPD patients had decreased values of spirometry parameters in comparison to the controls. There was no difference in age or sex distribution between the two groups.

Table 2. Basic characteristics and spirometry parameters of healthy controls and COPD patients included in the study.

Parameter	Controls (n = 95)	COPD Patients (n = 137)	p
sex male female	 49 46	 86 51	0.118
age	64 (46–83)	65 (44–86)	0.073
FEV_1 (L)	2.60 (2.12–3.19)	1.08 (0.78–1.57)	<0.001
FEV_1 (% predicted)	93 (86–104)	39 (28–60)	<0.001
FVC (L)	3.35 (2.77–4.16)	2.28 (1.81–2.77)	<0.001
FEV_1/FVC (%)	81 (77–88)	48 (41–58)	<0.001

COPD—chronic obstructive pulmonary disease; FEV_1—forced expiratory volume in the first second; FVC—forced vital capacity.

Age is shown as a median (minimum and maximum), while sex is shown as an absolute number. Other results are presented as a median (interquartile range). Data were analyzed by the χ^2 or Mann-Whitney test.

3.2. HSP70, TLR2 and TLR4 Gene Expression in Controls and COPD Patients

The gene expression of *HSP70*, *TLR2* and *TLR4* was determined in peripheral blood cells, and it was found that the mRNA level was increased for *HSP70* and *TLR2* in patients with COPD, when compared to healthy subjects (Table 3).

Table 3. Relative expression of *HSP70*, *TLR2* and *TLR4* in controls and COPD patients.

	Controls	COPD Patients	p
HSP70	1.01 (0.74–2.07)	1.73 (1.11–7.46)	<0.001
TLR2	1.03 (0.69–2.65)	1.93 (0.95–6.10)	0.002
TLR4	0.72 (0.56–0.94)	0.77 (0.56–1.07)	0.323

HSP70—heat shock protein 70; TLR—Toll like receptor; COPD—chronic obstructive pulmonary disease. Data were analyzed by Mann-Whitney test and shown as a median (interquartile range).

Regarding the severity of airway obstruction as well as symptoms burden and the history of exacerbations (assessed by CAT), the gene expression of *HSP70* and *TLR2* was up-regulated in GOLD 2, GOLD 3 and GOLD 4 stages as well as in GOLD A, GOLD B and GOLD D groups, respectively, in comparison to the control group (Table 4). However, no significant differences between GOLD stages or groups were found.

HSP70 gene expression showed to be increased in COPD non-smokers, COPD former smokers and COPD smokers in comparison to healthy non-smokers (Table 5). On the other hand, there was no difference between COPD non-smokers and healthy smokers, while the *HSP70* mRNA level was increased in COPD former and current smokers compared to healthy smokers. However, *HSP70* expression amongst COPD patients was independent of their smoking history. *TLR2* expression was increased in former COPD smokers and COPD smokers in comparison to healthy non-smokers, and only in COPD smokers when compared to healthy smokers. On the other hand, gene expression of *TLR4* did not differ between controls and COPD patients with different smoking history.

Table 4. Relative expression of *HSP70*, *TLR2* and *TLR4* in controls and COPD patients according to the severity of airflow obstruction and symptoms severity.

	Controls (n = 95)	GOLD 2 (n = 47)	GOLD 3 (n = 50)	GOLD 4 (n = 40)	p [1]	GOLD A (n = 27)	GOLD B (n = 70)	GOLD D (n = 40)	p [2]
HSP70	1.01 (0.74–2.07)	1.67 (1.00–10.21)	1.92 (1.22–12.09)	1.68 (1.06–5.50)	<0.001	1.72 (1.12–11.61)	1.67 (1.09–6.04)	2.79 (1.17–7.46)	<0.001
TLR2	1.03 (0.69–2.65)	1.83 (1.00–3.67)	1.96 (1.02–10.64)	1.83 (0.90–6.92)	0.019	1.89 (1.18–5.26)	1.63 (0.82–3.81)	2.11 (1.00–8.51)	0.009
TLR4	0.72 (0.56–0.94)	0.78 (0.61–1.07)	0.80 (0.56–1.09)	0.71 (0.56–0.99)	0.690	0.76 (0.59–1.06)	0.76 (0.56–1.09)	0.80 (0.56–0.98)	0.797

HSP70—heat shock protein 70; TLR—Toll like receptor; COPD—chronic obstructive pulmonary disease; GOLD—Global Initiative for Chronic Obstructive Pulmonary Disease. Results are shown as a median (interquartile range). p [1]—comparison between controls and GOLD 2–4 by Kruskal-Wallis test; p [2]—comparison between controls and GOLD A-D by Kruskal-Wallis test.

Table 5. Relative expression of *HSP70*, *TLR2* and *TLR4* in controls and COPD patients based on smoking status.

	Healthy Non-Smokers (n = 48)	Healthy Smokers (n = 47)	COPD Non-Smokers (n = 10)	COPD Former Smokers (n = 90)	COPD Smokers (n = 37)	p
HSP70	0.93 (0.64–1.78)	1.09 (0.90–2.07)	1.87 [a] (1.49–2.11)	1.72 [a,b] (1.05–6.53)	2.14 [a, b] (1.21–12.93)	<0.001
TLR2	0.98 (0.70–3.08)	1.18 (0.65–2.65)	2.22 (1.17–3.94)	1.81 [a] (0.86–6.34)	1.98 [a,b] (1.32–12.59)	0.022
TLR4	0.65 (0.54–0.93)	0.78 (0.63–0.96)	0.78 (0.61–1.07)	0.80 (0.56–1.09)	0.71 (0.56–0.99)	0.482

HSP70—heat shock protein 70; TLR—Toll like receptor; COPD—chronic obstructive pulmonary disease. Data were tested by Kruskal-Wallis test and shown as median (interquartile range). [a] statistically significant difference in comparison to healthy non-smokers; [b] statistically significant difference in comparison to healthy smokers.

3.3. Association Analysis between HSP70, TLR2 and TLR4 SNPs and COPD

A total of nine SNPs of the *HSP70*, *TLR2* and *TLR4* genes were examined, and all SNPs had a call rate >96%. The genotype frequency distributions were consistent with HWE ($p > 0.05$), except for the *TLR2* rs1898830 SNP, so this variant was not included in further statistical analysis. Genotype and allele distribution of the studied polymorphisms was determined, and an association analysis was performed. It was found that the *HSP70* rs6457452 variant was associated with COPD risk when C/T and T/T carriers (OR = 0.48, 95% CI = 0.24–0.97, p = 0.040) were compared to C/C carriers (Table 6). In addition, there was a significant difference in allele distribution, and the T allele showed OR of 0.46 (95% CI = 0.24–0.89, p = 0.022) in comparison to the reference C allele. However, no significant relation between *TLR2* and *TLR4* polymorphisms and the risk of COPD was found (Table 6).

The associations between gene expression and polymorphisms of each gene were then examined. *HSP70* expression was increased in COPD C/C carriers of the rs6457452 polymorphism compared to COPD C/T carriers as well as in comparison to control C/C carriers (Table 7). On the other hand, relative gene expression of *HSP70* did not differ in the carriers of the C/T genotype when controls and COPD patients were compared. Reference homozygotes and heterozygotes of rs1008438 and rs1043618 who were COPD patients showed increased *HSP70* gene expression, while there was no difference in *HSP70* expression between control subjects and the COPD patient group within the variant homozygotes. The same was observed regarding the *TLR2* rs3804099 polymorphism, while the COPD carriers of the G/G genotype of the rs13150331 showed increased *TLR2* expression in comparison to healthy participants with the same genotype (Table S1). Finally, since *TLR4* expression did not differ between controls and COPD patients, there were no significant

observations when *TLR4* expression was analyzed with respect to the three *TLR4* SNPs in all participants (Table S2).

Table 6. Genotype and allele distribution of *HSP70*, *TLR2* and *TLR4* polymorphisms in controls and COPD patients, and association analysis between investigated polymorphisms and COPD risk.

HSP70			**Controls n (%)**	**COPDn (%)**	p [1]	**OR (95% CI)**	p [2]
rs1008438	genotype	A/A	33 (35)	60 (44)	0.192	1.00	
		A/C	50 (54)	57 (41)		0.61 (0.34–1.08)	0.089
		C/C	10 (11)	20 (15)		0.97 (0.41–2.27)	0.943
	allele	A	116 (62)	177 (65)	0.697	1.00	0.625
		C	70 (38)	97 (35)		0.91 (0.62–1.34)	
rs1043618	genotype	G/G	33 (36)	61 (45)	0.315	1.00	
		C/G	46 (51)	55 (40)		0.65 (0.36–1.15)	0.139
		C/C	12 (13)	20 (15)		0.90 (0.39–2.07)	0.807
	allele	G	112 (62)	177 (65)	0.504	1.00	
		C	70 (38)	95 (35)		0.86 (0.58–1.27)	0.443
rs6457452	genotype	C/C	71 (77)	120 (88)	0.046	1.00	
		C/T	19 (21)	17 (12)		0.48 (0.24–0.97)	0.040
		T/T	2 (2)	0 (0)			
	allele	C	161 (88)	257 (94)	0.030	1.00	
		T	23 (12)	17 (6)		0.46 (0.24–0.89)	0.022
TLR2							
rs3804099	genotype	T/T	29 (32)	45 (33)	0.668	1.00	
		C/T	44 (48)	59 (43)		0.86 (0.47–1.59)	0.638
		C/C	18 (20)	33 (24)		1.18 (0.56–2.48)	0.659
	allele	T	102 (56)	149 (54)	0.800	1.00	0.726
		C	80 (44)	125 (46)		1.07 (0.73–1.56)	
rs13150331	genotype	A/A	27 (29)	48 (36)	0.547	1.00	
		A/G	48 (52)	60 (44)		0.73 (0.39–1.34)	0.310
		G/G	17 (19)	27 (20)		0.95 (0.44–2.06)	0.903
	allele	A	102 (55)	156 (58)	0.690	1.00	
		G	82 (45)	114 (42)		0.91 (0.62–1.33)	0.621
TLR4							
rs2737190	genotype	G/G	14 (15)	19 (14)	0.600	1.00	
		A/G	35 (38)	59 (45)		1.24 (0.55–2.78)	0.599
		A/A	43 (47)	54 (41)		0.92 (0.42–2.06)	0.849
	allele	G	63 (34)	97 (37)	0.657	1.00	
		A	121 (66)	167 (63)		0.90 (0.60–1.33)	0.586
rs10759932	genotype	T/T	65 (72)	95 (70)	0.881	1.00	
		C/T	21 (23)	40 (30)		1.09 (0.61–1.98)	0.764
		C/C	4 (5)	0 (0)			
	allele	T	151 (84)	230 (85)	0.810	1.00	
		C	29 (16)	40 (15)		0.91 (0.54–1.52)	0.709
rs7846989	genotype	T/T	71 (77)	111 (83)	0.301	1.00	
		C/T	20 (22)	22 (17)		0.67 (0.34–1.31)	0.241
		C/C	1 (1)	0 (0)			
	allele	T	162 (88)	244 (92)	0.257	1.00	
		C	22 (12)	22 (8)		0.66 (0.36–1.24)	0.198

HSP70—heat shock protein 70; TLR2—Toll like receptor 2; TLR4—Toll-like receptor 4; OR—odds ratio; COPD—chronic obstructive pulmonary disease; CI—confidence interval. Allele and genotype distributions are presented as absolute numbers with corresponding percentages. p [1]—p-value were analyzed by χ^2 test or Fisher's exact test where appropriate; p [2]—p-value were analyzed by logistic regression.

Table 7. Association of *HSP70* polymorphisms with *HSP70* expression.

SNP	Genotype	*HSP70* Expression		p [1]
		Controls	COPD	
rs1008438	A/A	0.90 (0.72–1.35)	2.02 (1.10–6.84)	<0.001
	A/C	1.09 (0.76–3.03)	1.72 (1.17–12.59)	0.005
	C/C	1.01 (0.71–1.38)	1.38 (1.07–2.78)	0.099
	P[2]	0.360	0.147	
rs1043618	G/G	0.90 (0.71–1.25)	2.02 (1.18–6.84)	<0.001
	C/G	1.09 (0.79–3.92)	1.72 (1.16–12.76)	0.008
	C/C	1.13 (0.78–1.67)	1.38 (1.07–2.78)	0.276
	P[2]	0.178	0.127	
rs6457452	C/C	0.98 (0.73–1.87)	1.83 (1.16–10.17)	<0.001
	C/T	1.31 (0.93–1.97)	1.27 (0.86–3.79)	0.921
	T/T	3.26 (1.01–5.50)	-	-
	P[2]	0.336	0.047	

SNP—single nucleotide polymorphism; HSP70—heat shock protein 70; COPD—chronic obstructive pulmonary disease. Results are shown as median (interquartile range). p [1]—difference in *HSP70* expression between control group and patients with COPD within the same genotype of *HSP70* polymorphisms, checked by Kruskal-Wallis test. p [2]—difference in *HSP70* expression between genotypes of *HSP70* polymorphisms in control group and in patients with COPD, checked by Kruskal-Wallis test.

Our previous research demonstrated that COPD patients had increased concentrations of plasma eHsp70 [8], and TLR2 and TLR4 are its main receptors [10]. Therefore, we evaluated the association between *HSP70*, *TLR2* and *TLR4* polymorphisms and eHsp70 concentrations (Table S3). COPD patients had increased eHsp70 concentrations compared to controls within the same genotype, while there was no difference in eHsp70 concentrations either in controls or COPD patients when heterozygotes and variant homozygotes were compared to reference homozygotes in terms of each polymorphism.

3.4. Haplotype Analysis

All three investigated *HSP70* SNPs were in strong LD, meaning that they are expected to be inherited together and therefore were included in the haplotype analysis. Table 8 shows that the $C_{rs1008438}C_{rs1043618}T_{rs6457452}$ haplotype was associated with COPD risk with OR of 0.47 (95% CI = 0.23–0.97, $p = 0.043$) in comparison to the reference haplotype $A_{rs1008438}G_{rs1043618}C_{rs6457452}$. Two *TLR2* polymorphisms were included in LD analysis. However, the D' value between rs3804099 and rs13150331 was 0.65, so no haplotype analysis was performed due to the applied criterion of D' > 0.80. Of the three *TLR4* SNPs examined, rs2737190 and rs10759932 as well as rs2737190 and rs7846989 were found to be strongly inherited. However, none of the common haplotypes with a frequency >0.01 was associated with COPD risk (Table S4).

Table 8. Association analysis of *HSP70* haplotypes with COPD risk.

rs1008438	rs1043618	rs6457452	Frequency		OR (95% CI)	p
			Controls	COPD		
A	G	C	0.570	0.646	1.00	-
C	C	C	0.230	0.285	1.03 (0.66–1.60)	0.910
C	C	T	0.107	0.062	0.47 (0.23–0.97)	0.043
C	G	C	0.039	0.007	0.19 (0.04–1.00)	0.052
A	C	C	0.035	0	NA	1

HSP70—heat shock protein 70; COPD—chronic obstructive pulmonary disease; OR—odds ratio; CI—confidence interval; NA—not applicable. Rare haplotypes ($A_{rs1008438}C_{rs1043618}T_{rs6457452}$, $C_{rs1008438}G_{rs1043618}T_{rs6457452}$ and $A_{rs1008438}G_{rs1043618}T_{rs6457452}$) with total frequency <0.01 were not included in the association analysis.

4. Discussion

This study showed that the gene expression of *HSP70* and *TLR2* was increased in COPD patients compared to controls. This increase was independent of COPD severity assessed by both airway obstruction as well as symptoms burden and exacerbation history, and was also independent of the subjects' smoking status. Regarding SNPs analysis, the T allele of *HSP70* rs6457452 polymorphism as well as *HSP70* haplotype consisting of the rs6457452 T allele had a protective role in COPD. Finally, decreased *HSP70* gene expression was observed in COPD C/T carriers of the rs6457452 polymorphism compared to COPD C/C homozygotes.

Dong et al. demonstrated increased *HSP70* gene expression in the peripheral lung tissues of COPD patients, and the *HSP70* expression negatively correlated with lung function which was assessed by FEV_1. Moreover, it was observed that expression of Hsp70 protein and its mRNA level was up-regulated by cigarette smoke in vitro in 16HBE human bronchial epithelial cells [12]. In our study, gene expression of *HSP70* was increased in peripheral blood cells of COPD patients compared to the controls. We found that HSP70 gene expression amongst COPD patients was independent of their smoking history, and we suggest that observed increase of its level compared to controls was associated with disease itself and not with smoking. However, those conclusions should be taken with caution due to the small number of COPD non-smokers (n = 10). In the study by Rumora et al., Hsp70 protein expression was explored in relation to smoking status of both control and COPD subjects. Decreased Hsp70 expression in COPD individuals, especially in COPD smokers, was explained by its potentially increased release from peripheral blood leukocytes [31]. Indeed, we have previously reported increased plasma concentration of eHsp70 in COPD patients compared to healthy individuals [8].

The main eHsp70 receptors TLR2 and TLR4 are activated mostly by pathogen components but can also be activated by proteins released from dying cells, including Hsps that act like DAMPs. Activation of the TLR signaling pathway leads to the production of proinflammatory cytokines [32]. Overall, available data on *TLR2* and *TLR4* expression are conflicting, and results seem to be by cell type i.e., tissue specific. High gene expression of *TLR2* and *TLR4* was found in peripheral blood mononuclear cells of obese patients suggesting that up-regulated expression of *TLR*s is associated with persistent low-grade inflammation [33]. Both *TLR2* and *TLR4* mRNA levels were increased in circulating neutrophils of COPD patients, and they were also increased in COPD smokers compared to non-smoking controls [34]. Our previous in vitro studies demonstrated that eHsp70 increased only *TLR2* but not *TLR4* expression in human monocyte-derived macrophages [35]. Similarly, in the current study, *TLR2* gene expression was increased in peripheral blood cells of COPD patients, but *TLR4* expression in COPD subjects did not differ from the control group. This increase might be associated with increased levels of eHsp70. However, TLR2 is a receptor of many other ligands apart from eHsp70, and further investigation is needed to explore a possible association between TLR2 and eHsp70. *TLR2* showed similar pattern of behavior as *HSP70* in terms of association with disease severity but not entirely with smoking. *TLR2* expression increased only in former COPD smokers and COPD smokers compared with non-smoking controls, and in COPD smokers in comparison to smoking controls. It could be suggested that in stable COPD, especially in COPD smokers, there is a persistent low-grade inflammation that contributes to the increase of *HSP70* and *TLR2* mRNA levels. In addition, there may be differential transcription of the *HSP70* and *TLR2* genes in COPD patients, which could finally lead to differential gene expression.

A growing number of large-scale genome-wide association studies, e.g., COPD Gene and International COPD Genetics Consortium, have been trying to identify genetic variants associated with the risk of COPD that might help the diagnosis or become therapeutic targets. However, the heritability of complex diseases, such as COPD, remained unsolved. In order to explore the risk of developing COPD in the Croatian population, a total of nine SNPs of *HSP70*, *TLR2* and *TLR4* were genotyped. Only the relatively rare (MAF = 0.08) *HSP70* rs6457452 SNP, located in the 5′UTR region, showed an association with the risk of

COPD. SNPs localized in regulatory part of a gene such as the 5′UTR region could affect the mRNA stability and expression, contributing to a disease susceptibility [36]. It was reported that rs6457452 was associated with increased risk of COPD in smokers, but the rs6457452 did not follow HWE in that study [13]. However, rs6457453 met the HWE criterion in our study and demonstrated a lower risk of COPD in the C/T and T/T carriers compared to C/C carriers, and the T allele showed protective effect. In the study by Guo et al. the T allele of rs6457452 was associated with an increased risk of lung cancer in two independent lung case-control studies within the Chinese population, and they demonstrated that the T allele was associated with lower Hsp70 protein expression in both normal bronchial epithelial cells and malignant cancer cells [37]. In addition, the C allele of rs6457452 was associated with decreased *HSP70* expression in sepsis. The same authors also estimated that the peripheral blood mononuclear cells of individuals with the haplotype consisting of the C allele of rs6457452 were producing less Hsp70 [38]. Haplotype analysis of *HSP70* from this study suggested an association of the haplotype containing the T allele of rs6457452 variant with the protective effect in COPD. When dealing with complex diseases, haplotype analysis is a useful and inexpensive method. Therefore, larger studies with a more detailed, statistically sophisticated assessment needs to be performed to explore further the effect of Hsp70 on the risk of COPD.

Although other polymorphisms studied did not show significant association with COPD risk in our cohort of participants, there were few studies that demonstrated their significant relations. The *HSP70* rs1008438 polymorphism was associated with COPD in all COPD subjects and in the smoking subgroup, and smoking parameter pack-years was higher in the COPD C/C carriers in the study by Korytina et al. [3]. In addition, it was reported that the A allele of rs1008438 was associated with increased COPD risk in smoking participants and those who were exposed to biomass burning smoke, but rs1008438 was not related to mRNA or protein levels of Hsp70 [13]. The C/C carriers of the *HSP70* rs1043618 were associated with an increased risk of lung cancer [39], and with higher risk of coronary heart disease, while reduced expression of Hsp70 protein was affected by the polymorphism [21]. *TLR2* and *TLR4* polymorphisms were associated with various conditions such as asthma [40], pulmonary tuberculosis [32,41], Helicobacter pylori infection [42], as well as alterations in levels of high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides [36]. They were also associated with susceptibility to COPD [25], the level and decline of lung function, an increased number of inflammatory cells in induced sputum of COPD patients [24], and an increased risk of COPD in smokers [43] or an earlier stage of COPD [44]. However, the ethnic context of polymorphisms as well as sample size should be considered regarding the results of the genetic association studies.

In this study, we found no association between plasma eHsp70 concentrations in COPD patients and selected *HSP70*, *TLR2* and *TLR4* polymorphisms. However, *HSP70* gene expression was affected by the *HSP70* rs6457452 SNP, and COPD patients with C/T genotype had reduced gene expression of *HSP70* in comparison to those with the C/C genotype. Furthermore, there was no difference in *HSP70* expression between the controls and COPD subjects within the same C/T genotype, suggesting that the T allele of the rs6457452 polymorphism may have an impact on gene transcription. However, this remains to be confirmed with a larger sample size that would include the COPD patients with T/T genotype, and the role of rs6457452 in COPD should be explored in a functional genomic study.

The main limitation of the current study is a small sample size, especially our results regarding the effect of smoking on *HSP70* and *TLR2* expression, and the association of *HSP70* rs6457452 with COPD and *HSP70* expression should be taken with caution. Another limitation of our study was the lack of GOLD 1 and GOLD C patients. However, COPD patients in GOLD 1 stage rarely contact physicians due to very mild symptoms, while GOLD C group of patients is also very rare since patients do not have many symptoms and are not frequent exacerbators.

5. Conclusions

To conclude, *HSP70* and *TLR2* gene expression may have a significant role in the pathogenesis of COPD. The current study is the first to demonstrate that *HSP70* rs6457452 polymorphism may affect *HSP70* gene expression and may be associated with the risk of COPD in the Croatian population. Thus, identification of specific molecular markers in peripheral circulation of COPD patients could be useful for achieving a better understanding of the systemic component of the disease.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/diagnostics11081412/s1, Table S1: Association of *TLR2* polymorphisms with *TLR2* expression; Table S2: Association of *TLR4* polymorphisms with *TLR4* expression; Table S3: Association of HSP70, *TLR2* and *TLR4* polymorphisms with plasma Hsp70 concentrations; Table S4: Association analysis of *TLR4* haplotypes with COPD risk.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Supplementary material

Table S1. Association of *TLR2* polymorphisms with *TLR2* expression.

SNP	genotype	*TLR2* expression		P*
		controls	COPD	
rs3804099	T/T	1.02 (0.64 – 2.25)	2.07 (1.01 – 5.56)	**0.027**
	C/T	0.99 (0.71 – 2.01)	1.83 (0.93 – 10.17)	**0.026**
	C/C	1.31 (0.80 – 7.24)	1.97 (0.91 – 5.14)	0.657
	P**	0.492	0.827	
rs13150331	A/A	1.15 (0.74 – 1.70)	1.98 (0.75 – 3.67)	0.169
	A/G	1.16 (0.69 – 7.24)	1.93 (0.94 – 7.73)	0.132
	G/G	1.00 (0.66 – 1.26)	1.78 (1.13 – 9.19)	**0.007**
	P**	0.410	0.754	

SNP – single nucleotide polymorphism; TLR2 – Toll like receptor 2; COPD – chronic obstructive pulmonary disease.

Results are shown as a median (interquartile range).

P* - difference in *TLR2* expression between control group and patients with COPD within the same genotype of *TLR2* polymorphisms, checked by Kruskal-Wallis test.

P** - difference in *TLR2* expression between genotypes of *TLR2* polymorphisms in control group and in patients with COPD, checked by Kruskal-Wallis test.

Table S2. Association of *TLR4* polymorphisms with *TLR4* expression.

SNP	genotype	*TLR4* expression		P*
		controls	COPD	
rs2737190	G/G	0.84 (0.64 – 0.95)	0.87 (0.60 – 1.26)	0.687
	A/G	0.72 (0.59 – 0.89)	0.75 (0.56 – 1.09)	0.745
	A/A	0.65 (0.55 – 0.94)	0.78 (0.55 – 0.96)	0.371
	P**	0.347	0.470	
rs10759932	T/T	0.68 (0.55 – 0.94)	0.79 (0.56 – 1.03)	0.288
	T/C	0.69 (0.54 – 0.86)	0.71 (0.59 – 1.11)	0.333
	C/C	0.90 (0.86 – 1.43)	-	-
	P**	0.200	0.755	
rs7846989	T/T	0.72 (0.55 – 0.94)	0.76 (0.56 – 1.08)	0.350
	C/T	0.69 (0.63 – 0.85)	0.77 (0.61 – 0.98)	0.583
	C/C	0.99 (0.99 – 0.99)	-	-
	P**	0.541	0.693	

SNP – single nucleotide polymorphism; TLR4 – Toll like receptor 4; COPD – chronic obstructive pulmonary disease.

Results are shown as a median (interquartile range).

P* - difference in *TLR4* expression between control group and patients with COPD within the same genotype of *TLR4* polymorphisms, checked by Kruskal-Wallis test.

P** - difference in *TLR4* expression between genotypes of *TLR4* polymorphisms in control group and in patients with COPD, checked by Kruskal-Wallis test.

Table S3. Association of *HSP70*, *TLR2* and *TLR4* polymorphisms with plasma Hsp70 concentrations.

gene	SNP	genotype	eHsp70 (ng/ml)		P*
			controls	COPD	
HSP70	rs1008438	A/A	0.35 (0.24 – 0.48)	0.99 (0.59 – 1.20)	**<0.001**
		A/C	0.40 (0.25 – 0.72)	0.99 (0.70 – 1.31)	**<0.001**
		C/C	0.41 (0.25 – 0.82)	0.93 (0.58 – 1.43)	**0.019**
		P**	0.316	0.841	
	rs1043618	G/G	0.38 (0.26 – 0.62)	0.97 (0.57 – 1.20)	**<0.001**
		C/G	0.39 (0.25 – 0.74)	0.99 (0.74 – 1.31)	**<0.001**
		C/C	0.37 (0.26 – 0.63)	0.93 (0.58 – 1.43)	**0.004**
		P**	0.920	0.605	
	rs6457452	C/C	0.37 (0.25 – 0.60)	0.99 (0.62 – 1.28)	**<0.001**
		C/T	0.37 (0.21 – 0.70)	0.98 (0.66 – 1.31)	**<0.001**
		T/T	0.79 (0.37 – 1.22)	-	-
		P**	0.563	0.901	
TLR2	rs3804099	T/T	0.37 (0.27 – 0.56)	0.94 (0.57 – 1.24)	**<0.001**
		C/T	0.37 (0.25 – 0.67)	1.06 (0.80 – 1.26)	**<0.001**
		C/C	0.34 (21 – 0.74)	0.94 (0.65 – 1.32)	**0.001**
		P**	0.831	0.315	
	rs13150331	A/A	0.38 (0.23 – 0.70)	0.98 (0.57 – 1.30)	**<0.001**
		A/G	0.37 (0.27 – 0.88)	0.99 (0.63 – 1.30)	**<0.001**
		G/G	0.31 (0.17 – 0.41)	0.98 (0.71 – 1.18)	**<0.001**
		P**	0.252	0.972	

TLR4	rs2737190	G/G	0.41 (0.26 – 0.50)	0.98 (0.73 – 1.28)	**<0.001**
		A/G	0.47 (0.26 – 0.75)	1.03 (0.59 – 1.29)	**<0.001**
		A/A	0.35 (0.22 – 0.48)	0.96 (0.60 – 1.20)	**<0.001**
		P**	0.467	0.616	
	rs10759932	T/T	0.37 (0.27 – 0.64)	0.98 (0.60 – 1.33)	**<0.001**
		T/C	0.42 (0.16 – 0.76)	0.98 (0.78 – 1.19)	**<0.001**
		C/C	0.31 (0.25 – 0.42)	-	-
		P**	0.784	0.931	
	rs7846989	T/T	0.37 (0.23 – 0.64)	0.98 (0.60 – 1.29)	**<0.001**
		C/T	0.40 (0.28 – 0.62)	1.03 (0.75 – 2.14)	**<0.001**
		C/C	0.25 (0.25 – 0.25)	-	-
		P**	0.635	0.257	

HSP70 – heat shock protein 70; TLR – Toll like receptor; SNP – single nucleotide polymorphism; COPD – chronic obstructive pulmonary disease; eHsp70 – extracellular Hsp70.

Results are shown as a median (interquartile range).

P* - difference in eHsp70 concentration between control group and patients with COPD within the same genotype of *HSP70, TLR2* or *TLR4* polymorphisms, checked by Kruskal-Wallis test.

P** - difference in eHsp70 concentration between genotypes of *HSP70, TLR2* or *TLR4* polymorphisms in control group and in patients with COPD, checked by Kruskal-Wallis test.

Table S4. Association analysis of *TLR4* haplotypes with COPD risk.

rs2737190	rs10759932	rs7846989	frequency		OR (95% CI)	P
			controls	COPD		
A	T	T	0.641	0.615	1.00	-
G	T	T	0.121	0.179	1.40 (0.83 – 2.35)	0.210
G	C	T	0.111	0.113	1.09 (0.57 – 2.09)	0.790
G	T	C	0.062	0.057	0.89 (0.38 – 2.10)	0.800
G	C	C	0.051	0.019	0.39 (0.12 – 1.30)	0.130

TLR4 – Toll like receptor 4; COPD – chronic obstructive pulmonary disease; OR – odds ratio; CI – confidence interval.

Rare haplotypes ($A_{rs2737190}C_{rs10759932}T_{rs7846989}$, $A_{rs2737190}T_{rs10759932}C_{rs7846989}$ and $A_{rs2737190}C_{rs10759932}C_{rs7846989}$) with total frequency <0.01 were not included in the association analysis.

6. RASPRAVA

KOPB je višekomponentna bolest koja ne zahvaća samo dišni sustav, već se može manifestirati na razini cijelog organizma zahvaćajući tako i druge organske sustave što je vidljivo u obliku razvoja različitih komorbiditeta.

Povećanje smrtnosti od KOPB-a u stalnom je porastu u svjetskoj populaciji i predstavlja značajni javnozdravstveni problem koji podrazumijeva velike troškove liječenja. Oko 3 milijuna ljudi godišnje umire od KOPB-a i njegovih komplikacija, a procjenjuje se da će do 2060. godine taj broj porasti do 5,4 milijuna (1). Smatra se da još uvijek nije dovoljno prepoznata važnost KOPB-a te je potrebno podizanje svijesti populacije o ovoj bolesti. Ova disertacija svojim znanstvenim doprinosima također nastoji pridonijeti što boljem razumijevanju KOPB-a i biokemijskih promjena prisutnih u perifernoj cirkulaciji, ali i saznanjima o bolesti na razini gena.

Mnogi slučajevi KOPB-a dijagnosticiraju se u kasnijim fazama bolesti jer se simptomi poput kašlja, umora pri fizičkim naporima i otežanog disanja zanemaruju, posebno ukoliko su oboljele osobe pušači. Upalni odgovor, koji je sastavni dio patogeneze bolesti, pojačan je ako su osobe izložene duhanskom dimu što se smatra jednim od glavnih čimbenika rizika. Osim na lokalnoj razini, upalni odgovor može biti prisutan i na sustavnoj razini i time pridonijeti općem pogoršanju cjelokupnog zdravlja pojedinca.

Zbog upalnih procesa oštećene ili umiruće stanice otpuštaju DAMP-ove koji mogu alarmirati imunosni sustav nakon vezanja na PRR-ove i aktivacije specifičnih unutarstaničnih signalnih putova (118). DAMP-ovi koji djeluju kao signal za opasnost mogu biti protein visoke pokretljivosti iz skupine 1 (HMGB1, engl. *high mobility group box 1*), Hsp-ovi, mokraćna kiselina, IL-1α, ATP, S100 proteini i mnogi drugi, a neki od njih nalaze se u povećanim koncentracijama u iskašljaju i serumu pacijenata s KOPB-om (118 – 120).

Dvije vrlo značajne DAMP molekule koje su istraživane u sklopu ove disertacije su eHsp70 i eATP.

Hsp70 obitelj proteina unutar stanice ima zaštitnu ulogu molekulskog šaperona (64). S druge strane, izvanstanični Hsp70 je signalna molekula koja vezanjem na svoje receptore, poput TLR2 i TLR4, potiče kaskadu unutarstaničnih događanja što dovodi do povećane sinteze upalnih citokina i specifičnog odgovora imunosnog sustava (36, 121).

Osim eHsp70, eATP također je signalna molekula koja može potaknuti upalni proces u KOPB-u. U fiziološkim uvjetima eATP se održava u niskim koncentracijama finom

regulacijom i dinamičkom ravnotežom između njegovoga otpuštanja i razgradnje. Narušavanjem te ravnoteže dolazi do povećanja koncentracije eATP-a u upalnim stanjima, no još uvijek nije razjašnjeno je li to posljedica povećanog otpuštanja ATP-a, smanjene razgradnje ili istodobnog odvijanja oba procesa (122).

Ovo istraživanje pokazalo je da pacijenti sa stabilnim oblikom KOPB-a imaju povećanu koncentraciju DAMP-ova eHsp70 i eATP-a u uzorcima plazme s etilendiamintetraoctenom kiselinom (EDTA, engl. *ethylenediaminetetraacetic acid*). Po prvi je puta pokazano da je koncentracija plazmatskog eHsp70 i eATP-a povezana sa stadijem KOPB-a procijenjenog na temelju razine plućne opstrukcije te s težinom simptoma i povijesti egzacerbacija na temelju ABCD klasifikacije. Na temelju ovih rezultata, može se pretpostaviti da stanice iz periferne cirkulacije u pacijenata s KOPB-om u povećanoj količini otpuštaju Hsp70 i ATP pasivnim i/ili aktivnim procesima. Ukoliko se eHsp70 i eATP promatraju kao biljezi aktivacije imunosnog sustava, može se zaključiti da se imunosni odgovor pojačava s opadanjem plućne funkcije procijenjene prema FEV_1 (%) i uznapredovalosti simptoma. Također, ABCD klasifikacija vrlo je bitna u primjeni farmakoloških mjera prema GOLD smjernicama što znači da bi se eHsp70 i eATP mogli koristiti za praćenje učinkovitosti terapije i/ili kao potencijalne buduće farmakološke mete ukoliko se budućim istraživanjima dokažu mehanizmi povećanja koncentracije eHsp70 i eATP-a u KOPB-u. Osim toga, oba parametra ukazala su na značajnu predikcijsku vrijednost na temelju statističke analize logističkom regresijom. Na temelju dobivenih vrijednosti omjera izgleda (OR, engl. *odds ratio*), pojedinci s povećanom koncentracijom eHsp70 u perifernoj cirkulaciji imaju više od 7 puta (OR = 7,63) veću vjerojatnost oboljevanja od KOPB-a, dok je kod pojedinaca s povećanom koncentracijom eATP-a vjerojatnost od oboljevanja od KOPB-a gotovo 13 puta (OR = 12,98).

Hsp70 u izvanstaničnom prostoru može potjecati iz nekroznih plućnih stanica ili mononuklearnih stanica iz periferne krvi. Prvo istraživanje o eHsp70 u pacijenata s KOPB-om utvrdilo je povećane koncentracije eHsp70 u serumu, ali promjene u koncentraciji nisu bile povezane s razinom plućne opstrukcije (81). U kasnijem istraživanju Ünver i sur., serumska koncentracija Hsp70 nije bila statistički značajno drugačija u pacijenata s KOPB-om u usporedbi s kontrolnom skupinom, no potencijalno objašnjenje autori su pronašli u malom broju ispitanika. Također, ista istraživačka skupina nije utvrdila postojanje razlike u koncentraciji IL-6 između pacijenata s KOPB-om i kontrolnih ispitanika, dok je CRP bio značajno povišen u skupini pacijenata s KOPB-om (123). Nadalje, u istraživanju koje je uključivalo radnike u rudnicima određivale su se koncentracije Hsp70 i Hsp27 u

hepariniziranoj plazmi i limfocitima iz periferne cirkulacije. Plazmatska koncentracija Hsp70 bila je povećana u pacijenata s KOPB-om u usporedbi s kontrolnom skupinom. Također, povećanje koncentrancije eHsp70 uočeno je u rudara s KOPB-om i pneumonikozom u usporedbi s kontrolama i u usporedbi s rudarima s KOPB-om bez pneumonikoze. Ipak, koncentracija Hsp27 bila je snižena u osoba s KOPB-om. Konačno, utvrđeno je da visoka koncentracija Hsp70 u rudara povećava rizik od KOPB-a te bi Hsp70 tako mogao poslužiti kao biljeg za praćenje osoba koje su u rudnicima izložene prašini i česticama koje predstavljaju rizični čimbenik za oboljevanje od KOPB-a (124).

Razlike u korištenim uzorcima (serum, EDTA-plazma, heparinizirana plazma) i korištenim metodama iz navedenih istraživanja otežavaju intepretraciju i usporedbu dobivenih rezultata i potrebno ih je uzeti u obzir. U ovom istraživanju koncentracije eHsp70 određene su u uzorcima EDTA-plazme prema preporukama Whitham i sur. (125) koristeći komercijalni enzimski povezan imunosorpcijski test (ELISA, engl. *enzyme-linked immunosorbent assay*) (126) predviđen i validiran upravo za takvu vrstu uzorka.

Osim u KOPB-u, povećana izvanstanična koncentracija Hsp70 uočena je u pacijenata s drugim bolestima dišnog sustava, poput astme (119) i karcinoma pluća (121, 127).

eATP je dosada određivan u humanim uzorcima porijeklom samo iz dišnog sustava, a ovo istraživanje prvo je koje donosi rezultate o koncentraciji eATP-a u perifernoj cirkulaciji pacijenata s KOPB-om. Za analizu eATP-a odabran je uzorak EDTA-plazme, a ne seruma, kako bi se što više umanjile interferencije zbog otpuštanja ATP-a iz krvnih stanica. EDTA-plazmi odmah je dodan specifičan pufer iz komercijalno dostupnog testa korištenog za određivanje koncentracije eATP-a kako bi se inhibirale ATP-aze. Budući da se ATP brzo hidrolitički razgrađuje, na taj su način osigurani optimalni uvjeti za mjerenje koncentracije eATP-a kako bi se što reprezentativnije prikazala slika stanja u trenutku uzorkovanja, odnosno uključivanja ispitanika u istraživanje.

Dokazano je da eATP djeluje kao upalni medijator u KOPB-u (128). Povišene koncentracije izmjerene su u bronhoalveolarnom ispirku (BAL, engl. *bronchoalveolar lavage*) u *in vivo* modelu emfizema izazvanog cigaretnim dimom (85) te u BAL-u pacijenata s KOPB-om, no dosada nije bilo dokaza o istome u krvi (122).

Pretpostavljeni mehanizam djelovanja eATP-a temelji se na otpuštanju ATP-a kroz paneksinske i koneksinske kanale te vezikularnom egzocitozom iz stanica i vezanju na receptor P2X7R putem kojeg utječe na aktivaciju NLRP3 inflamasoma i sazrijevanje IL-1β

(129–131). Aktivacijom NLRP3 inflamasoma, eATP može doprinijeti piroptozi, ali i drugim oblicima stanične smrti poput apoptoze, nekroptoze i feroptoze (132).

Povezanost eHsp70 i eATP-a s plućnom funkcijom u ovom istraživanju dodatno je utvrđena korelacijskom analizom gdje su eHsp70 i eATP pokazali značajnu negativnu povezanost s FEV_1 (%), FEV_1 (L) i FEV_1/FVC. Negativna korelacija ATP-a i FEV_1 (%) zabilježena je jedino u BAL-u pacijenata s KOPB-om u istraživanju Lommatzsch i sur. (122).

Klasifikacija stupnjeva plućne funkcije procjenjuje se na temelju spirometrijskih parametara, no poznato je da su oni slabo povezani s klinički značajnim varijablama koje procjenjuju sveukupno zdravstveno stanje pacijenta. Osim toga, spirometrijska su mjerenja vrlo često neugodna za pacijente u naprednijim stadijima bolesti kada je opstrukcija dišnih putova veća pa je vrlo važno istraživati i identificirati pomoćne biljege koji bi doprinijeli u procjeni vrlo heterogenih kliničkih slika KOPB-a i omogućili praćenje bolesti. Na temelju toga, dodatnu vrijednost ovome istraživanju donose rezultati povezanosti eHsp70 i eATP-a s višekomponentnim kliničkim indeksima (ADO, BODCAT, BODEx, CODEx, DOSE) koji omogućavaju sveobuhvatniji pristup pacijenatima s KOPB-om, a koji uključuju razinu plućne opstrukcije, pušački status, simptome, povijest egzacerbacija, dob, stupanj uhranjenosti i komorbiditete. Indeksi nastali kombinacijom različitih parametara bolji su u procjeni prognoze, procjeni rizika od egzacerbacija i komorbiditeta (21) te mogu poslužiti kao korisni dodatni parametri u kliničkoj praksi. Dosada su se ADO i DOSE pokazali kao značajni prediktori budućih egzacerbacija u pacijenata s KOPB-om (22), dok je za BODEx, CODEx i ADO utvrđen bolji prognostički kapacitet u odnosu na pojedinačne parametre od kojih su sastavljeni (20). Povezanost eHsp70 i eATP-a s višekomponentnim kliničkim indeksima ukazuje na mogućnost uključivanja DAMP-ova u daljnja istraživanja kojima bi se istražila mogućnost kliničke primjene dva parametra za što bolju kliničku procjenu pacijenata s KOPB-om kao dodatni dijagnostički i/ili prognostički biljezi uz spirometriju.

Nekroza je česti uzrok otpuštanja eHsp70 i eATP-a u izvanstanični prostor, a cigaretni dim potiče nekrozno propadanje stanica. Poznato je da pušenje svojim posrednim mehanizmima doprinosi jačanju sustavne upale, a k tome je rizični čimbenik kod šest vodećih uzročnika smrti na svijetu, poput bolesti dišnog i krvožilnog sustava te različitih karcinoma. Mnogi pušači ne prepoznaju simptome KOPB-a zbog čega se bolest ne dijagnosticira pravovremeno. Reaktivni oksidansi iz cigaretnog dima potiču upalni odgovor u dišnom sustavu (133) što dovodi do povećanog broja upalnih stanica i povećane sinteze upalnih citokina, poput TNF-α,

IL-1, IL-6, IL-8 i čimbenika koji stimulira rast granulocitno-makrofagnih kolonija (GM-CSF, engl. *granulocyte-macrophage colony-stimulating factor*) (10). Iako prestanak pušenja u pacijenata s KOPB-om doprinosi sporijoj progresiji bolesti i smanjenju rizika od razvitka komorbiditeta (134, 135), posebno u ranijoj fazi bolesti, uočeno je da se upalni procesi u dišnom sustavu nastavljaju bez obzira na prestanak pušenja (136, 137). To se djelomično može objasniti promjenama u ekspresiji gena uključenih u upalni odgovor ili promjenama na epigenskoj razini. Također, pretpostavljeno je da bi potencijalni autoimuni odgovor koji se može razviti u KOPB-u također mogao imati bitnu ulogu u kroničnoj upali kao i zaostale čestice unijete u sustav prilikom pušenja ili kod bakterijskih infekcija (45, 138). Osim u dišnom sustavu, izlaganje cigaretnom dimu posredno rezultira upalnim procesom i na sustavnoj razini (139, 140).

Rezultati ove disertacije pokazuju da koncentracije eHsp70 i eATP-a rastu u pacijenata s KOPB-om u usporedbi s kontrolnim nepušačima i kontrolnim pušačima, no među pacijentima s KOPB-om nije bilo razlika pa su tako pacijenti koji su nepušači, bivši pušači ili aktivni pušači pokazivali slične koncentracije eHsp70 i eATP-a. Ipak, koncentracije eHsp70 i eATP-a bile su više u kontrolnih pušača u usporedbi s kontrolnim nepušačima što može ukazivati na početak alarmiranja organizma na opasnost oslobađanjem DAMP-ova iz stanica. Dodatno, kada su pacijenti s KOPB-om podijeljeni u GOLD 2 - 4 stadije i GOLD A - D skupine i uspoređeni s kontrolnim skupinama na temelju koncentracija eHsp70 i eATP-a, uočeno je da se jedino GOLD 2 stadij i GOLD A skupina ne razlikuju od kontrolnih pušača ukazujući da je izloženost dimu cigareta značajan stimulans za oslobađanje Hsp70 i ATP-a u izvanstanični prostor u perifernoj cirkulaciji. Prema tome, pretpostavljeno je da u pušača koji nisu razvili KOPB postoji upalni proces niske razine koji se može povezati s povećanom koncentracijom eHsp70 i eATP-a, a koji ima zajedničke značajke s upalnim procesom i povećanom koncentracijom eHsp70 i eATP-a u ranijim i blažim oblicima KOPB-a. Ipak, bilo bi korisno provesti istraživanje u uzorcima pacijenata u najblažem GOLD 1 stadiju bolesti koji vrlo često nije prepoznat i dijagnosticiran u toj fazi kako bi se utvrdilo postoje li razlike u koncentraciji eHsp70 i eATP-a već u ovom stadiju bolesti u odnosu na zdrave ispitanike.

Upalni učinci cigaretnog dima dokazani su povećanom sintezom citokina, posebice IL-8, na različitim staničnim linijama (141 – 143). Također, u prijašnjim istraživanjima, humana monocitna stanična linija THP-1 i monociti izolirani iz periferne cirkulacije zdravih ispitanika korišteni su kao stanični modeli sustavne komponente bolesti te je uočeno povećanje koncentracije IL-8 nakon tretiranja stanica rekombinantnim humanim (rh) Hsp70 koji se

koristi u *in vitro* istraživanjima sa svrhom ispitivanja djelovanja eHsp70. Na istim staničnim modelima ispitan je istovremeni utjecaj rhHsp70 i cigaretnog dima te je samo kod monocita uočen antagonistički tip interakcija na lučenje citokina, dok kod THP-1 stanične linije nije bilo značajnih razlika u odnosu na individualan učinak cigaretnog dima (144). Uspoređujući s istim tretiranjima primijenjenim na staničnim modelima plućne komponente bolesti (npr. humana bronhijalna epitelna stanična linija, NCI-H292) (142), zaključuje se da utjecaj cigaretnog dima zasebno ili u kombinaciji s rhHsp70 varira ovisno o tipu uzorka, odnosno vrsti tretiranih stanica.

Rumora i sur. primijetili su smanjenje unutarstanične ekspresije Hsp70 u perifernim leukocitima kod bivših pušača s KOPB-om i zdravih pušača, a najveće smanjenje uočeno je kod pušača s KOPB-om. Uzrok unutarstanične smanjene ekspresije mogla bi biti ili smanjena transkripcijska aktivnost i vezanje HSF-a na HSE (npr. zbog vezanja kinaze regulirane izvanstaničnim signalom (ERK, engl. *extracellular signal-regulated kinase*) i kinaze koja fosforilira N-terminalni dio transkripcijskog čimbenika c-Jun-a (JNK, engl. *c-Jun N-terminal kinase*) te fosforilacije HSF-1 ili povećano otpuštanje iz unutarstaničnog u izvanstanični prostor (145).

Povišenje koncetracije eATP-a u BAL-u zabilježeno je u pušača bez plućne opstrukcije i pušača s KOPB-om kad su uspoređeni sa zdravim nepušačima. Isto istraživanje bilo je prvo koje je pokazalo da se koncentracija eATP-a povećava u dišnim putovima nakon akutnog i kroničnog izlaganja cigaretnom dimu te je uočeno da visoke koncentracije eATP-a ostaju prisutne u pacijenata s KOPB-om čak i nakon prestanka pušenja (122). Dodatno, u plućnim uzorcima pušača s KOPB-om određeno je značajno smanjenje ekspresije *CD39* u usporedbi s pušačima bez opstrukcije i zdravim nepušačima te je smanjenje ekspresije koreliralo s razinom sustavne upale procijenjene na temelju vrijednosti CRP-a. Osim toga, utvrđena je niža proteinska ekspresija CD39 i ATP-azna aktivnost u plućima pacijenata s KOPB-om u usporedbi s pušačima bez opstrukcije dišnih putova i nepušačima. Time se dokazalo da smanjena ekspresija razgradnih enzima može biti jedan od uzroka povećanja koncentracije eATP-a što u konačnici dovodi do upale i plućnog emfizema (146). *In vitro* eksperimenti provedeni na stanicama bronhijalnog epitela (NCI-H292, 16HBE, NHBE) i monocitnim stanicama (MDM, THP-1) pokazali su da uslijed tretiranja stanica ekstraktom cigaretnog dima dolazi do povećanja koncentracija eATP-a u usporedbi s netretiranim stanicama (147). Također, u mišjem modelu akutne upale pluća i emfizema izazvanog cigaretnim dimom uočen je porast koncentracije eATP-a u BAL-u i pretpostavljena je signalizacija putem

P2Y2R (148), pri čemu aktivacija purinergijskih receptora vodi do otpuštanja IL-8 i elastaze iz neutrofila (130). Konačno, osim cigaretnog dima, ishemija i ograničen protok zraka mogu doprinijeti pojačanom oslobađanju ATP-a iz stanica (149).

Povišene koncentracije IL-1α, IL-1β, IL-6, IL-8 i TNF-α uočene su u pacijenata s KOPB-om iz ovog istraživanja u usporedbi s koncentracijama iz uzoraka kontrolne skupine ispitanika. Nijedan citokin nije pokazao povezanost s razinom plućne opstrukcije ni sa simptomima među pacijentima s KOPB-om kada su oni bili podijeljeni na GOLD 2 – 4 stadije i GOLD A – D skupine, ali su postojale značajne razlike između svakog GOLD stadija i svake GOLD skupine u usporedbi s kontrolnom skupinom za sve citokine osim za IL-8. Koncentracije različitih citokina mjerene su u uzorcima porijeklom iz dišnog sustava ili iz periferne krvi u pacijenata s KOPB-om (51, 55, 150 – 152) te se citokini smatraju bitnim biljezima upale u KOPB-u. Budući da nijedan citokin nije specifičan ni za jednu bolest, veliki naglasak stavlja se na obrasce u promjenama koncentracija nekoliko citokina koji bi jasnije mogli biti povezani s određenim patologijama u usporedbi s individualnim promjenama pojedinih citokina. Mrežnom analizom citokina IL-1α, IL-1β, IL-6, IL-8 i TNF-α u kontrolnih nepušača, kontrolnih pušača i pacijenata s KOPB-om ispitalo se postoji li povezanost i koji je stupanj povezanosti između citokina unutar i između svake skupine. Najznačajniju povezanost pokazuju IL1β, IL-6 i TNF-α te se ona povećava u pacijenata s KOPB-om u usporedbi s kontrolnim nepušačima i kontrolnim pušačima. Ta tri citokina pokazala su i najveći dijagnostički potencijal na temelju univarijatne logističke regresije. Zbog toga je napravljena mrežna analiza IL-1β, IL-6 i TNF-α u kombinaciji s CRP-om, Fbg-om, eHsp70 i eATP-om. Vidljive razlike uočene su već u kontrolnih pušača u usporedbi s kontrolnim nepušačima ukazujući na značajne upalne procese koji se odvijaju zbog izlaganja cigaretnom dimu. Ipak, najveći rast povezanosti između svih paramatera događa se u pacijenata s KOPB-om, posebno između DAMP-ova eHsp70 i eATP-a te citokina IL-1β, IL-6 i TNF-α u usporedbi s obje kontrolne skupine. Može se pretpostaviti da u pacijenata u stabilnoj fazi KOPB-a postoji značajan obrazac otpuštanja citokina IL-1β, IL-6 i TNF-α te dviju DAMP molekula, eHsp70 i eATP-a. Zaista, koncentracije IL-6 i TNF-α značajno su povišene u serumu pacijenata sa stabilnim KOPB-om, ali i s egzacerbacijama (57, 59, 151). Ipak, istraživanja su zabilježila različite rezultate o povezanosti koncentracije citokina u perifernoj cirkulaciji pacijenata s KOPB-om ovisno o razini plućne opstrukcije (151, 153) te su potrebna dodatna istraživanja koja bi razjasnila postoji li doista ta povezanost.

Nadalje, smatra se da IL-6 snažno potiče sintezu CRP-a u jetri, a povišene koncentracije IL-6 u KOPB-u povezane su s povišenim koncentracijama CRP-a (154). Ta je povezanost vidljiva u mrežnoj analizi već u kontrolnih nepušača, ali izostaje u kontrolnih pušača te se ponovno pojavljuje u pacijenata s KOPB-om. S obzirom da porast IL-6 i CRP-a nije bio statistički značajan u kontrolnih pušača uspoređujući s kontrolnim nepušačima, moguće je da je izostanak povezanosti ta dva parametra djelomično posljedica nedovoljnog broja ispitanika u podskupinama kada se kontrolna skupina podijeli na temelju pušačkog statusa.

Zbog vrlo čestih heterogenosti u rezultatima između različitih istraživanja, napravljena je hijerarhijska klasterska analiza kojom se ispitanici statističkim metodama nastoje objediniti u relativno homogene skupine sa sličnim zajedničkim karakteristikama u usporedbi s drugim podskupinama. Na taj se način može uključiti veći set potencijalnih biljega kojima bi se mogli razjasniti različiti fenotipovi iste bolesti. Klasteri su diferencirani na temelju 7 parametara koji su bili uključeni u mrežnu analizu i pokazali su značajnu međusobnu povezanost u usporedbi s kontrolnim nepušačima i kontrolnim pušačima. Iako je statističkom analizom identificirano 5 klasterskih skupina (155), najzanimljivije su promjene uočene kod skupina pacijenata s najvećim stupnjem plućne opstrukcije te s vrijednostima mMRC-a, CAT-a, SGRQ-C-a i CODEx-a iznad medijana. Tako se izdvajaju klaster 2 (plave boje), (donja) podskupina klastera 3 (zelene boje) i klaster 5 (crvene boje) koji većinski objedinjuju pacijente s klinički težom slikom na temelju odabranih parametara. Međutim, obrasci parametara IL-1β, IL-6 i TNF-α, CRP-a, Fbg-a, eATP-a i eHsp70 razlikuju se u tim klasterima. U klasteru 2 nalaze se pacijenti s najvećim koncentracijama DAMP-ova, eHsp70 i eATP-a, dok ostali parametri naginju prema nižim vrijednostima. Nadalje, donja podskupina klastera 3 objedinjuje pacijente s većim vrijednostima eHsp70 i eATP-a te CRP-a i Fbg-a, ali ne i citokina, dok klaster 5 objedinjuje pacijente s većim vrijednostima eHsp70 i eATP-a te citokina, ali ne i CRP-a i Fbg-a. Čini se da je svim pacijentima s težom kliničkom slikom zajednički porast koncentracije DAMP-ova u perifernoj cirkulaciji, dok su promjene u općim upalnim biljezima i citokinima heterogene te se na temelju njih mogu razlikovati dodatne tri podskupine pacijenata s KOPB-om s težom kliničkom slikom. Kompleksnost KOPB-a očituje se u raznolikosti patogenetskih mehanizama iako se radi o pacijentima koji su pokazivali vrlo slične značajke procijenjene na temelju često korištenih kliničkih parametara. Stoga je vrlo važno prepoznati i opisati endotipove bolesti te uočiti postoji li povezanost s kliničkim slikama (fenotipovima) kako bi se pristup pacijentima individualizirao što je više moguće. Nekoliko klasterskih analiza pokušalo je grupirati pacijente s KOPB-om na temelju

zajedničkih značajki (156 – 160), no uočeno je da je jedan od značajnih nedostataka ovih istraživanja uključivanje pacijenata iz samo jednog centra. Osim toga, potrebna su validacijska istraživanja koja bi potvrdila uočene promjene. Ipak, klasterska ispitivanja mogu poslužiti kao vrijedna preliminarna ispitivanja.

Konačno, zbog heterogenosti i kompleksnosti KOPB-a, smatra se da kombinacija parametara ima veći dijagnostički potencijal u kliničkoj praksi od pojedinačnih parametara za koje je utvrđena povezanost s KOPB-om. U ovom istraživanju utvrđeno je da model sastavljen od eHsp70, eATP-a i IL-1β ima najbolju predikcijsku vrijednost na temelju dobivenih rezultata omjera izgleda te bi takav model mogao poslužiti kao dodatni biljeg u prepoznavanju pacijenata s KOPB-om.

S obzirom na očekivano povećanje koncentracije eHsp70 i eATP-a u KOPB-u, ovo istraživanje uključilo je određivanje relativne ekspresije gena za Hsp70 i njegova dva receptora TLR2 i TLR4 te dva receptora za eATP, P2X7R i P2Y2R. Postavljena je hipoteza da će se ekspresije svih ispitivanih gena povećati u pacijenata s KOPB-om. Ipak, podaci iz literature pokazuju da promjene u ekspresiji nisu jednoznačne i uvelike ovise o metodama određivanja, vrstama uzoraka koji se koriste u istraživanjima i populaciji ispitanika.

Razina ekspresije gena *HSP70* i *TLR2* bila je gotovo duplo veća u pacijenata s KOPB-om u odnosu na kontrolnu skupinu, dok ekspresija *TLR4* nije ukazivala ni na kakve razlike između dvije uspoređivane skupine ispitanika.

Budući da je u skupini pacijenata s KOPB-om primijećena prisutnost sustavne upale na temelju povećanih vrijednosti lkc, Fbg-a i CRP-a te upalnih citokina, očekivana je povećana ekspresija gena koji kodira za Hsp70. Trajna kronična upala stvara stresni okoliš za stanice koje na to odgovaraju povećanom genskom ekspresijom i sintezom Hsp70 kako bi se održala proteinska stabilnost u stanici. Zbog toga, povećana koncentracija eHsp70 mogla bi biti posljedica pojačane ekspresije *HSP70* i sinteze Hsp70 koji se otpušta u izvanstanični prostor. Ipak, vrlo je bitno koji je izvor eHsp70 s obzirom da kod nekih stanica ne mora postojati opisani utjecaj na ekspresiju *HSP70*. Naime, takve stanice mogu doprinijeti povećanoj koncentraciji eHsp70 svojim povećanim brojem u perifernoj cirkulaciji i otpuštanjem unutarstaničnog Hsp70 aktivnim i pasivnim mehanizmima zbog čega dolazi do pada unutarstanične koncentracije. Osim toga, bilo bi korisno kada bi buduća istraživanja proučavala otpušta li se Hsp70 samostalno ili u određenim obrascima s drugim proteinima toplinskoga šoka.

Dong i sur. dokazali su pojačanu ekspresiju gena *HSP70* u plućnom tkivu u KOPB-u, a razina mRNA korelirala je s plućnim parametrima. Pritom je uočena umjerena korelacija razine mRNA za Hsp70 s FEV_1/FVC, a slaba s FEV_1 (%) (36). Autori su zaključili da je ekspresija *HSP70* povezana sa stadijem bolesti, no taj podatak treba intepretirati s oprezom uzimajući u obzir slabu negativnu korelaciju između razine mRNA za Hsp70 i FEV_1 (%) ($r = -0,29$, $P < 0,05$). Dosada nije bilo podataka o ekspresiji *HSP70* u perifernoj cirkulaciji pacijenata s KOPB-om ovisno o stadiju bolesti i uznapredovalosti simptoma te je ovo istraživanje prvo koje iznosi da nema povezanosti između navedenih parametara u perifernoj cirkulaciji.

Također, u ovom istraživanju pokazano je da nema povezanosti između razine mRNA za TLR2 sa stadijem bolesti ni s uznapredovalosti simptoma prema ABCD klasifikaciji.

Istraživanje Zhang i sur. povezalo je ekspresiju *TLR2* s progresijom bolesti koristeći krvne uzorke, no pacijenti s KOPB-om u tom su istraživanju bili podijeljeni samo u dvije skupine prema stadiju bolesti procijenjenom na temelju razine plućne opstrukcije i to tako što su jednu skupinu činili svi pacijenti s GOLD 1 i GOLD 2 stadijem, a drugu skupinu pacijenti s GOLD 3 i GOLD 4 stadijem. Isto istraživanje utvrdilo je da je ekspresija gena za TLR2 značajno povezana s upalnim odgovorom (161). Nadalje, pacijenti s naprednijim stadijem KOPB-a iz istraživanja skupine Simpson i sur. imali su veću ekspresiju gena *TLR2* i veću koncentraciju TLR2 u iskašljaju u usporedbi s pacijentima s blažim oblikom bolesti (162). Međutim, usporedbe različitih stadija bolesti rađene su na različitim vrstama uzoraka što predstavlja značajnu ograničenost u interpretaciji rezultata.

Povećanje ekspresije *TLR4* povezano je s pojačanim upalnim odgovorom u KOPB-u, no utvrđena je i potencijalna zaštitna uloga u obrani od oksidacijskog stresa. Na temelju povezanosti povećane ekspresije *TLR4* s povećanom vrijednosti FEV_1/FVC i smanjenim emfizemom, predloženo je da bi smanjena ekspresija *TLR4* mogla poslužiti kao predikcijski biljeg emfizema u pušača (163).

Iako su provedena brojna istraživanja o ekspresiji gena *TLR2* i *TLR4*, rezultati se razlikuju ovisno o vrsti uzorka koji je analiziran i koja komponenta bolesti je proučavana.

U uzorcima iskašljaja pacijenata s KOPB-om pokazano je da je ekspresija *HSP70* smanjena u pušača s KOPB-om u usporedbi s pušačima bez opstrukcije, a ekspresija je bila povećana u pacijenata s KOPB-om izloženim dimu biomase u usporedbi s ispitanicima koji su također izloženi dimu biomase, ali nemaju prisutnu plućnu opstrukciju (164). Ovakvi rezultati u pušača s KOPB-om mogu biti posljedica farmakoloških mjera koje smanjuju sustavnu upalu,

dok kod pušača bez opstrukcije postoji neregulirana upala, ali i viša razina oksidacijskog stresa koja može potaknuti veću ekspresiju *HSP70*. Uz to, prijašnja istraživanja pokazala su smanjenu proteinsku ekspresiju Hsp70 u pacijenata s KOPB-om, posebice pušača s KOPB-om, što može biti posljedica pojačanog otpuštanja u izvanstanični prostor (145). Daljnja istraživanja svakako bi trebala biti usmjerena na određivanje svih izvora Hsp70 u izvanstaničnom prostoru.

U ovom istraživanju, uočeno je da se nepušači s KOPB-om ne razlikuju od kontrolnih pušača, dok je ekspresija *HSP70* duplo veća u nepušača s KOPB-om u usporedbi s kontrolnim nepušačima. Razlike u ekspresiji *HSP70* između tri podskupine pacijenata s KOPB-om prema pušenju (nepušači, bivši pušači i pušači) nije bilo. Rezultati ekspresije gena *HSP70* djelomično se podudaraju s rezultatima koncentracije eHsp70. Razina transkripcije i koncentracija ciljnog proteina ne moraju biti u korelaciji te razina genske ekspresije ne predstavlja pouzdanu procjenu proteinske koncentracije (165). Kada se uspoređuju rezultati ekspresije gena *HSP70* i koncentracije eHsp70, bitne razlike koje se ne očituju u ekspresiji *HSP70* su one između kontrolnih nepušača i pušača te između nepušača s KOPB-om i kontrolnih pušača, dok između tih podskupina postoje razlike u koncentraciji eHsp70. Može se pretpostaviti da cigaretni dim kod pojedinaca bez plućne opstrukcije i simptoma KOPB-a nema toliki utjecaj na gensku ekspresiju, već je eHsp70 povišen zbog mogućih posrednih utjecaja cigaretnog dima pa se Hsp70 pojačano oslobađa iz stanica.

Imunomodulacijsko djelovanje eHsp70 ovisi o vrsti receptora na koji se veže. Glavni receptori na koje se veže eHsp70 su TLR2 i TLR4. Povećana genska ekspresija za TLR2 i TLR4 utvrđena je u humanim bronhijalnim epitelnim stanicama NCI-H292 nakon tretiranja s rhHsp70 i 15 %-tnom koncentracijom ekstrakta cigaretnog dima, dok su isti agensi smanjili ekspresiju receptora u primarnim epitelnim plućnim stanicama izoliranim od pacijenata s KOPB-om. Također, nakon tretiranja cigaretnim dimom razine mRNA za oba receptora bile su povišene u humanim monocitnim THP-1 stanicama, dok je razina mRNA za TLR2 u monocitima izoliranima iz periferne krvi zdravih osoba bila smanjena (142, 166). Istraživanje skupine Haw i sur. ukazalo je na povećanu ekspresiju gena *TLR2*, dok nije bilo promjena u ekspresiji *TLR4* u plućnom tkivu miševa koji su bili izloženi cigaretnom dimu. Također je utvrđeno da mRNA razine za TLR2 i TLR4 nisu bile promijenjene u plućnim makrofagima miševa izloženima cigaretnom dimu. Stoga je potrebno utvrditi doprinose li plućni makrofagi upalnim procesima samo svojim povećanim brojem bez promjene u ekspresiji gena koji kodiraju za TLR-ove. S druge strane, u epitelnim stanicama dišnih putova vjerojatnije je da se

mijenja genska ekspresija receptora (75). U uzorcima pacijenata s KOPB-om utvrđeno je da mRNA razine za TLR2 i TLR4 ovise o vrsti uzorka iz dišnog sustava te da postoje razlike u genskim ekspresijama receptora između uzoraka iz dišnih putova i uzoraka plućnog parenhima. Nadalje, genske ekspresije oba receptora povećane su u plućnom parenhimu u ranijim stadijima bolesti i kod kroničnog izlaganja cigaretnom dimu, a smanjene u naprednijim stadijima KOPB-a. Moguće objašnjenje pada ekspresije u naprednijim stadijima KOPB-a leži u povećanom razaranju stanica tijekom progresije bolesti pa se zbog toga i smanjuje razina mRNA (75).

Od dva receptora za eATP čija se ekspresija na razini gena određivala u uzorcima periferne krvi, samo je mRNA razina za P2Y2R pokazala povećanje u pacijenata s KOPB-om u usporedbi sa zdravima, dok se ekspresija gena *P2X7R* statistički nije razlikovala između pacijenata s KOPB-om i kontrolnih ispitanika. S obzirom na vrstu uzorka odabranu za ispitavanje genske ekspresije u ovom istraživanju, pojačana ekspresija *P2Y2R* bi mogla ukazivati na prisutnost čimbenika koji znatno mijenjaju ekspresiju *P2Y2R* u sustavnoj upali u KOPB-u.

Nakon aktivacije P2Y2R, unutarstanični C-terminalni dio receptora, koji je povezan s G-proteinom, potiče kaskadu koja dovodi do različitih fizioloških odgovora. Aktivacija receptora potiče izlučivanje vode i otpuštanje mucina u dišne putove (167), posebno tijekom egzacerbacija KOPB-a uzrokovanim virusom (168). ATP djeluje kemotaksično putem P2Y2R te na taj način privlači neutrofile i makrofage na mjesto upale (167, 169). Signal aktiviran vezanjem ATP-a na P2Y2R mogao bi imati ulogu i u mehanizmima unakrsnog razgovora (engl. *cross-talk*) u neutrofilima što bi moglo imati značajni učinak u pojačanju upalnog odgovora (170). Dokazano je da u cirkulirajućim neutrofilima pacijenata s KOPB-om dolazi do pojačane genske ekspresije purinergijskih receptora, posebno *P2Y2R*, te je ta promjena primijećena u skladu s povećanjem koncentracije eATP-a u BAL-u (122). Također, povećana ekspresija *P2Y2R* utječe na migraciju upalnih stanica i pojačano otpuštanje neutrofilne elastaze (169). Osim u KOPB-u, primijećeno je da P2Y2R ima bitnu ulogu u patofiziologiji nekih drugih bolesti dišnog sustava poput astme tako što je povezan s migracijom dendritičnih stanica i eozinofila (169) te u idiopatskoj plućnoj fibrozi gdje, osim na regrutaciju neutrofila, utječe na migraciju i proliferaciju fibroblasta u plućima te na otpuštanje citokina (171).

P2X7R, osim na površini stanice, može biti internaliziran unutar stanice. Čini se da u ispitivanoj skupini pacijenata s KOPB-om u ovom istraživanju ne dolazi do promjene u

ekspresiji gena *P2X7R* u usporedbi sa zdravom skupinom. Prijašnja istraživanja pokazala su da do aktivacije P2X7R dolazi u makrofagima u BAL-u pacijenata s KOPB-om te da ATP preko P2X7R regulira sekreciju MMP-9 i tkivnog inhibitora metaloproteinaza matriksa 1 (TIMP-1, engl. *tissue inhibitor of metalloproteinase-1*) (122). Izlaganje cigaretnom dimu povezano je s pojačanom aktivacijom P2X7R u plućnim makrofagima i krvnim neutrofilima koja može dovesti do pojačane regrutacije neutrofila. Također, eATP, čije otpuštanje može biti potaknuto cigaretnim dimom i stvaranjem ROS-ova, vezanjem za P2X7R potiče aktivaciju NLRP3 inflamasoma i otpuštanje IL-1β (130, 172, 173). S obzirom da u ovom istraživanju nije došlo do statističke značajne razlike u ekspresiji *P2X7R*, može se zaključiti da u perifernoj cirkulaciji pacijenata s KOPB-om nema značajnog učinka čimbenika koji bi utjecali na promjenu ekspresije *P2X7R*. P2X7R ne pokazuje visoki afinitet prema ATP-u iako je njegov selektivni receptor te aktivacija receptora ovisi i o izoformama receptora i o koncentraciji dvovalentnih kationa (92, 174). Stoga, iako je u ovom istraživanju uočena povišena koncentracija IL-1β u perifernoj cirkulaciji pacijenata s KOPB-om, čini se da ona nije u potpunosti i isključivo povezana s aktivacijom P2X7R zbog potencijalno preniske koncentracije eATP-a.

Razvojem tehnologije, veliki pomaci napravljeni su u istraživanjima povezanosti između genske podloge i KOPB-a. Poznato je da genski čimbenici doprinose riziku od KOPB-a, a genske varijante povezane s plućnom funkcijom u KOPB-u omogućavaju bolji uvid u etiologiju bolesti i mogu pomoći u kreiranju novih farmakoterapijskih rješenja. Najveća GWAS istraživanja identificirala su značajne varijante povezane s plućnom funkcijom pacijenata pa su tako naglašene važnosti gena i unutarstaničnih signalnih putova za razvoj terapije KOPB-a (npr. muskarinski acetilkolinergijski receptor 3 (*CHRM3*, engl. *muscarinic cholinergic receptor 3*, *MAPK*, receptor D za interleukin 17 (*IL17RD*)), ali i gena koji kodiraju komponente izvanstaničnog matriksa, produkte bitne u međustaničnoj komunikaciji, procesu adhezije stanice i ostalome (100, 175, 176). Dodatni podaci, poput genske ekspresije, regulacije gena, kromatinske interakcije, koregulacije genske ekspresije i slično, omogućavaju karakterizaciju funkcionalnih varijanti što pridonosi boljoj klasifikaciji bolesti i terapijskom pristupu.

Cilj ovog rada bio je ispitati postoji li povezanost između KOPB-a i polimorfnih biljega koji se nalaze unutar gena koji kodiraju za Hsp70 te njegove receptore TLR2 i TLR4, a koji utječu na upalni proces i konačni imunosni odgovor stanica na podražaje.

U uzorku hrvatske populacije oboljelih od KOPB-a dosada je samo polimorfizam rs1061581 u genu *HSP70* bio značajno povezan s KOPB-om. Opažena je povezanost G alela i G/G genotipa spomenutog polimorfizma s rizikom od KOPB-a (177).

Pretraživanjem dostupnih literaturnih podataka odabrano je ukupno devet polimorfizama unutar tri gena (*HSP70*, *TLR2* i *TLR4*) za koje je u ovom doktorskom radu ispitivano postoji li povezanost s rizikom od KOPB-a u odabranoj hrvatskoj populaciji pacijenata sa stabilnim oblikom KOPB-a. Budući da jedan polimorfizam (rs1898830) u genu *TLR2* nije zadovoljio uvjete Hardy-Weinbergove ravnoteže (HWE, engl. *Hardy-Weinberg equilibirum*), u daljnjem razmatranju i interpretaciji rezultata proučavano je preostalih osam polimorfizama od kojih je statistički značajnu povezanost s KOPB-om pokazao polimorfizam gena *HSP70* rs6457452. T alel pokazao je zaštitnu ulogu u usporedbi s C alelom te su nositelji C/T i T/T genotipova zajedno, na temelju analize logističkom regresijom, imali manji rizik od razvitka KOPB-a u odnosu na nositelje C/C genotipa. Svi ostali analizirani polimorfizmi nisu pokazali statistički značajnu povezanost s rizikom od KOPB-a.

Polimorfizam rs6457452 gena koji kodira za Hsp70 smješten je u 5'-netranslatiranom području (UTR, engl. *untranslated*) gena *HSPA1B* koje je zaduženo za regulaciju translacije, vezanje proteina koji reguliraju ulazak mRNA u ribosome i gensku ekspresiju (178). Pomoću dostupnog softvera HaploReg v4.1 (179) uočeno je da se na varijantno mjesto vežu 34 proteina, jedan od kojih je i NF-κB, a koji je od posebnog interesa s obzirom na povezanost s patogenezom KOPB-a. Dosada je objavljeno samo jedno istraživanje koje je uključivalo polimorfizam rs6457452 u pacijenata s KOPB-om te su nositelji C/T i T/T genotipova nosili veći rizik od KOPB-a što je suprotno rezultatima iz ovog rada. Međutim, to istraživanje bilo je usmjereno na pacijente koji su pušači ili koji su izloženi izgaranju biomase. Osim toga, u istom istraživanju, rs6457452 nije zadovoljio kriterij HWE pa bi se rezultati trebali interpretirati vrlo oprezno (164). Osim u KOPB-u, rs6457452 istraživan je u pacijenata s karcinomom pluća u kineskoj populaciji (180), s paranoidnom shizofrenijom u poljskoj populaciji (181), s alopecijom areata u korejskoj populaciji (182) i sa sepsom kod odraslih i djece u grčkom pilot istraživanju (183). Guo i sur. utvrdili su povećani rizik od karcinoma pluća kod nositelja T alela te je uočeno da T alel polimorfizma rs6457452 gena *HSP70* vodi do smanjene transkripcijske aktivnosti u normalnim bronhijalnim epitelnim i malignim stanicama (180), dok su Seok i sur. detektirali zaštitnu ulogu T alela u bolesti alopecija areata (182). Suprotni rezultati istraživanja mogu se objasniti populacijskim skupinama različitih etniciteta uključenih u istraživanja kao i time što je polimorfizam rs6457452 relativno rijetki

polimorfizam (MAF (T) = 12 % za svjetsku populaciju, MAF (T) = 8 % za europsku populaciju prema projektu 1000 Genoma) što utječe na snagu istraživanja i potreban broj sudionika.

Tek opsežna funkcionalna istraživanja i integrirani pristup koji kombinira podatke o genskim varijantama s transkriptomikom, epigenetikom i proteomikom mogu dati bolji uvid u cjelovitu gensku podlogu kompleksne bolesti poput KOPB-a.

Kako bi se provjerilo postoji li povezanost rs6457452 s razinom ekspresije gena *HSP70*, provedena je statistička analiza s tim ciljem. Budući da u ovom istraživanju nisu detektirani T/T nositelji s KOPB-om, uspoređeni su samo C/C i C/T pacijenti s KOPB-om te je utvrđeno smanjenje ekspresije *HSP70* u C/T nositelja u usporedbi s nositeljima C/C genotipa. Također, ako se uspoređuju zdravi s oboljelima od KOPB-a unutar iste genotipske podskupine, vidljivo je da kod C/T nositelja nema razlike između zdravih i bolesnih, dok je ekspresija *HSP70* veća u pacijenata s KOPB-om u odnosu na zdrave s C/C genotipom. Dakle, može se uočiti smanjenje ekspresije *HSP70* u perifernom krvnom uzorku u KOPB-u kod nositelja T alela. Ipak, vrlo je bitno napomenuti da je rezultate potrebno oprezno interpretirati s obzirom da nisu detektirani nositelji T/T genotipa koji su oboljeli od KOPB-a. Osim toga, potrebno je uzeti u obzir da se genska ekspresija uvelike može razlikovati ovisno o tipu uzorka i o heterogenosti unutar populacije istih stanica. Kako bi se genska ekspresija povezala s određenom genskom varijantom, potrebno je provesti funkcionalne testove.

Također, provedena je statistička analiza kojom se ispitalo postoji li povezanost pojedinih polimorfizama s koncentracijama eHsp70 u plazmi. Utvrđeno je povećanje koncentracije eHsp70 u genotipskoj podskupini pacijenata s KOPB-om svakog ispitivanog polimorfizma u usporedbi sa zdravim ispitanicima istog genotipa. Razlika između različitih genotipova unutar zdrave populacije i populacije s KOPB-om nije bilo, čak ni kod polimorfizma rs6457452 kod kojeg je uočena promjena ekspresije gena *HSP70* u pacijenata s KOPB-om s obzirom na različite genotipove. Dakle, uočeno povećanje koncentracije eHsp70 u pacijenata sa stabilnim oblikom KOPB-a, koje je također povezano sa stadijima bolesti prema GOLD klasifikaciji, nije povezano s ispitivanim polimorfizmima u genima *HSP70, TLR2* i *TLR4*.

Dodatna provedena analiza koja je uključivala podatke o polimorfizmima bila je analiza povezanosti između haplotipova i rizika od KOPB-a. Haplotipovi predstavljaju kombinacije prostorno bliskih alela unutar homolognih kromosoma. Na taj se način dobiva podatak o neizravnoj povezanosti s ispitivanom bolesti kada pojedini polimorfizam nije izravno povezan

s ispitivanim ishodom jer se smatra da su za neke regije upravo haplotipovi funkcionalne jedinice koji pokazuju veću povezanost s bolesti (184). Prema postavljenom kriteriju (D' > 0,80), ispitivani polimorfizmi gena *TLR2* nisu pokazali LD, dok polimorfizmi gena *TLR4* jesu, ali nijedan haplotip nije bio povezan s rizikom od KOPB-a. Suprotno tome, od analiziranih haplotipova gena *HSP70*, $C_{rs1008438}C_{rs1043618}T_{rs6457452}$ statistički značajno je ukazivao na zaštitnu ulogu u KOPB-u.

Polimorfizmi rs3804099 i rs13150331 gena *TLR2*, polimorfizmi rs2737190, rs10759932 i rs7846989 gena *TLR4* te polimorfizmi rs1008438 i rs1043618 gena *HSP70* nisu bili povezani s rizikom od KOPB-a u provedenom istraživanju. Ipak, druga istraživanja koja su ispitivala iste polimorfizme u KOPB-u uspjela su dokazati njihovu povezanost s bolesti u odabranim populacijskim skupinama.

U istraživanju Korytina i sur. polimorfizam rs1008438 gena *HSP70* povezan je s KOPB-om u sveukupnoj populacijskoj skupini KOPB-a iz istraživanja kao i s podskupinom pušača. Također, indeks pušenja (pack-years) bio je značajno veći kod nositelja C/C genotipa (185). Nadalje, isti polimorfizam pokazao je povezanost s rizikom od KOPB-a u populaciji pušača i osoba izloženih izgaranju biomasa, ali se razina mRNA i koncentracija proteina nisu razlikovale prema genotipu (164). U spomenutom istraživanju ispitivana je i povezanost polimorfizma rs1043618 u genu *HSP70* s KOPB-om, no statistički značajni rezultati dobiveni su samo u skupini pojedinaca izloženima izgaranju biomase (164). Od drugih bolesti koje pogađaju dišni sustav, polimorfizam rs1043618 pokazao je povezanost s idipatskom plućnom fibrozom (186) i karcinomom pluća (187).

Polimorfizmi gena koji kodiraju za receptore za eHsp70, TLR2 i TLR4, puno su češće ispitivani nego *HSP70*, no njihova točna uloga u patogenezi KOPB-a još uvijek nije razjašnjena. Budulac i sur. uspjeli su dokazati povezanost pojedinih polimorfizama gena *TLR2* i *TLR4* sa stadijem i progresijom KOPB-a u prvom longitudinalnom istraživanju o SNP-ovima gena *TLR2* i *TLR4* u KOPB-u. Polimorfizam rs1898830 iz intronske regije gena *TLR2* pokazao je povezanost sa smanjenom plućnom funkcijom i njenim ubrzanim padom tijekom vremena te povećanim brojem upalnih stanica u iskašljaju. Smatra se da je ovaj polimorfizam povezan sa staničnom aktivnosti leukocita iz periferne cirkulacije posredovanom putem TLR2 receptora nakon stimulacije bakterijskim lipoproteinom, a koja se odnosi na povećanu sintezu IL-10, IL-8 i TNF-α (188). Drugi ispitivani polimorfizam rs13150331 gena *TLR2* u snažnoj je povezanosti (visoki LD) s rs1898830 (114).

Također, u drugim bolestima dišnog sustava, rs1898830 u genu *TLR2* povezan je sa smanjenim rizikom od tuberkuloze u kineskoj Han populaciji te je isto potvrđeno u populaciji Tibetanaca (189), dok u ranijem istraživanju provedenom u kineskoj populaciji isti polimorfizam nije zadovoljio kriterij HWE (190). Zhao i sur. u meta-analizi utvrdili su povezanost polimorfizma rs3804099 u genu *TLR2* s rizikom od astme (191). U istraživanju Budulac i sur. varijantni homozigoti istog polimorfizma imali su manji pad FEV_1 u usporedbi s divljim tipom te je polimorfizam pokazao povezanost s padom neutrofila i makrofaga u iskašljaju tijekom vremenskog praćenja pacijenata s KOPB-om. Dva polimorfizma (rs10759932 i rs2737190) gena *TLR4* značajno su bili povezani s rizikom od KOPB-a i plućne tuberkuloze u istraživanjima koja su uključivala kinesku Han populaciju (192, 193) te su povezani s upalom u plućima i dišnim putovima (114). Također, varijantni homozigoti polimorfizma rs7846989 u genu za TLR4 pokazivali su ubrzani pad FEV_1 u usporedbi s divljim tipom (114).

Danas se smatra da su varijante u nekodirajućim sljedovima DNA od velikog značaja te se njihovi pretpostavljeni molekularni mehanizmi i utjecaji, poput utjecaja na transkripciju gena ili stabilnost mRNA, moraju potvrditi funkcijskim testovima. Tek tako se može objasniti pravi utjecaj genotipova i alela. Najveći izazov istraživanjima koja se bave genskom pozadinom kompleksnih bolesti jest u kompleksnosti potvrde rezultata zbog velikog utjecaja etniciteta, heterogenosti unutar iste populacije i niza okolišnih čimbenika na statistički značajnu povezanost između ispitivanih varijanti i bolesti. Također, u poligenskim bolestima, informacija o haplotipu može dati bolji uvid u patogenezu bolesti od informacije o genotipu jer neki polimorfizmi međusobnom interakcijom mogu imati funkcionalnu ulogu, ali se ne može pouzdano utvrditi koji je polimorfizam funkcionalan unutar haplotipa.

Dosadašnje spoznaje iz znanstvenih istraživanja o KOPB-u pretežito su vezane uz promjene i mehanizme prisutne u dišnom sustavu, dok je veliki znanstveni doprinos ovog istraživanja upravo u dokazivanju povezanosti između KOPB-a i DAMP-ova eHsp70 i eATP-a iz uzoraka periferne cirkulacije. Štoviše, eHsp70 i eATP pokazali su značajan predikcijski potencijal individualno i u kombiniranom modelu s IL-1β što bi moglo unaprijediti buduće dijagnostičke i/ili prognostičke smjernice. Predloženo je da se eHsp70 i eATP mogu razmatrati kao biljezi sustavne upale u KOPB-u te kao biljezi razine plućne opstrukcije i uznapredovalosti simptoma što bi ponajviše moglo doprinijeti razvoju i praćenju budućih terapijskih pristupa. Konačno, znanstveni doprinos ove disertacije je i u dodatnim saznanjima o promjenama u KOPB-u na molekularnoj razini u obliku relativne genske ekspresije i analize polimorfizama

koja pružaju bolje razumijevanje mehanizama bolesti i usmjeravaju na buduća potrebna istraživanja.

7. ZAKLJUČCI

- Koncentracije eHsp70 i eATP-a u perifernoj cirkulaciji pacijenata s KOPB-om bile su veće nego u kontrolnoj skupini te su povezane sa stupnjem plućne opstrukcije na temelju FEV_1 (%) i s težinom simptoma na temelju CAT upitnika. Oštećenje stanica i upalni procesi u porastu su s progresijom bolesti.

- Koncentracije eHsp70 i eATP-a veće su u skupini zdravih pušača nego u zdravih nepušača što ukazuje na pojavu upalnih procesa prilikom izlaganja cigaretnom dimu, dok razlika u koncentraciji nije bilo u pacijenata s KOPB-om kad su se uspoređivale skupine nepušača, bivših pušača i trenutnih pušača.
- Koncentracije svih određivanih citokina, IL-1α, IL-1β, IL-6, IL-8 i TNF-α, povišene su u pacijenata s KOPB-om u odnosu na zdrave ispitanike, no nije utvrđena povezanost sa stupnjem plućne opstrukcije i težinom simptoma.
- Mrežnom analizom utvrđena je značajna povezanost IL-1β, IL-6, TNF-α, CRP-a, Fbg-a, eHsp70 i eATP-a u pacijenata s KOPB-om.
- Hijerarhijskom klasterskom analizom dokazana je heterogenost bolesti te je na temelju zajedničkih promjena u koncentracijama IL-1β, IL-6, TNF-α, CRP-a, Fbg-a, eHsp70 i eATP-a identificirano 5 klastera ispitanika.
- Relativna ekspresija gena *HSP70, TLR2* i *P2Y2R* povećana je u pacijenata s KOPB-om u usporedbi s kontrolnom skupinom, dok do promjene u ekspresiji nije došlo u genima *TLR4* i *P2X7R.*
- Analiza polimorfizama ukazala je da postoji povezanost između rizika od KOPB-a i SNP-a rs6457452 u promotorskoj regiji gena *HSP70,* odnosno T alel pokazao je zaštitni potencijal u odnosu na C alel polimorfizma.
- Skup biljega IL-1β, eHsp70 i eATP-a mogao bi se koristiti za dijagnozu KOPB-a.

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9. PRILOZI

Prilog 1: Popis kratica

A1AT	alfa-1 antitripsin (engl. *alpha-1 antitrypsin*)
ADO	dob, razina zaduhe i razina opstrukcije (engl. *age, dyspnoea, airflow obstruction*)
APC	stanica koja prezentira antigen (engl. *antigen presenting cell*)
ASC	adaptacijski protein nalik mrljicama povezan s apoptozom koji sadrži domenu za aktivaciju i privlačenje kaspaza (engl. *apoptosis-associated speck like protein containing a caspase activation and recruitment domain)*
ATP	adenozin-trifosfat (engl. *adenosine triphosphate*)
B2M	beta-2-mikroglobulin (engl. *beta-2-microglobulin*)
BAL	bronhoalveolarni ispirak (engl. *bronchoalveolar lavage*)
BMI	indeks tjelesne mase (engl. *body mass index*)
BODCAT	procjena uhranjenosti, razina opstrukcije, razina zaduhe, težina simptoma (engl. *BMI, airflow obstruction, dyspnoea, COPD Assessment Test score*)
BODE	procjena uhranjenosti, razina opstrukcije, razina zaduhe, fizička sposobnost (engl. *BMI, airflow obstruction, dyspnoea, exercise capacity*)
BODEx	procjena uhranjenosti, razina opstrukcije, razina zaduhe i egzacerbacije tijekom prethodnih godinu dana (engl. *body mass index, airflow obstruction, dyspnoea, previous exacerbations*)
CAT	upitnik za procjenu simptoma KOPB-a (engl. *COPD Assessment Test*)
CD	biljeg diferencijacije (engl. *cluster of differentiation*)
CHRNA3/5	alfa (α) 3 i 5 podjedinice nikotinskog acetilkolinergijskog receptora (engl. *cholinergic receptor nicotinic alpha 3 and 5 subunits*)
CHRM3	muskarinski acetilkolinergijski receptor 3 (engl. *muscarinic cholinergic receptor 3*)
CI	interval pouzdanosti (engl. *confidence interval*)
CLR	lektinski receptori tipa C (engl. *C-type lectin receptor*)

CLSI	Institut za standarde u kliničkom laboratoriju (engl. *Clinical Laboratory Standards Institute*)
CODEx	komorbiditeti, razina opstrukcije, razina zaduhe, egzacerbacije tijekom prethodnih godinu dana (engl. *Charlson's comorbidity index, airflow obstruction, dyspnoea, previous exacerbations*)
CRP	C-reaktivni protein (engl. *C-reactive protein*)
DAMP	molekularni obrazac oštećenja (engl. *damage-associated molecular pattern*)
DLCO	difuzijski kapacitet pluća (engl. *diffusion capacity for carbon monoxide*)
DNA	deoksiribonukleinska kiselina (engl. *deoxyribonucleic acid*)
DOSE	razina zaduhe, razina opstrukcije, status pušenja i egzacerbacije tijekom prethodnih godinu dana (engl. *dyspnoea, airflow obstruction, smoking status, previous exacerbation*)
eATP	izvanstanični adenozin-trifosfat (engl. *extracellular adenosine triphosphate*)
EBC	kondenzat izdahnutog daha (engl. *exhaled breath condensate*)
EDTA	etilendiamintetraoctena kiselina (engl. *ethylenediaminetetraacetic acid*)
eHsp70	izvanstanični protein toplinskoga šoka 70 (engl. *extracellular heat shock protein 70*)
ELISA	enzimski povezan imunosorpcijski test (engl. *enzyme-linked immunosorbent assay*)
ERK	kinaza regulirana izvanstaničnim signalom (engl. *extracellular signal-regulated kinase*)
Fbg	fibrinogen
FEV_1	forsirani izdisajni volumen u prvoj sekundi (engl. *forced expiratory volume in the first second*)
FVC	forsirani vitalni kapacitet (engl. *forced vital capacity*)
GM-CSF	čimbenik koji stimulira rast granulocitno-makrofagnih kolonija (engl. *granulocyte-macrophage colony-stimulating factor*)

GOLD	Globalna inicijativa za kroničnu opstrukcijsku plućnu bolest (engl. *Global Initiative for Chronic Obstructive Lung Disease*)
GWAS	cjelogenomska asocijacijska istraživanja (engl. *Genome-wide association study*)
HHIP	protein koji stupa u interakciju s hedgehog proteinima (engl. *hedgehog interacting protein*)
HMGB1	protein visoke pokretljivosti iz skupine 1 (engl. *high mobility group box 1*)
HSE	element toplinskoga šoka (engl. *heat shock element*)
HSF	transkripcijski čimbenik toplinskoga šoka (engl. *heat shock factor*)
Hsp	protein toplinskoga šoka (engl. *heat shock protein*)
HWE	Hardy-Weinbergova ravnoteža (engl. *Hardy-Weinberg equilibrium*)
ICS	inhalacijski kortikosteroidi (engl. *inhaled corticosteroids*)
iHsp70	inducibilni oblik proteina toplinskoga šoka 70
IL	interleukin
IL17RD	receptor D za interleukin-17
IQR	interkvartilni raspon (engl. *interquartile range*)
JNK	kinaza koja fosforilira N-terminalni dio transkripcijskog čimbenika c-Jun-a (engl. *C-Jun N-terminal kinase*)
KOPB	kronična opstrukcijska plućna bolest
LABA	dugodjelujući beta-agonist (engl. *long-acting beta-agonist*)
LAMA	dugodjelujući muskarinski antagonisti (engl. *long-acting muscarinic antagonist*)
LD	neravnoteža povezanosti (engl. *linkage disequilibrium*)
lkc	ukupan broj leukocita
LOX-1	receptor 1 sličan lektinu za oksidirani lipoprotein niske gustoće (engl. *lectin-like oxidized low-density lipoprotein receptor-1*)

LTB-4	leukotrien B-4
LPS	lipopolisaharid (engl. *lipopolysaccharide*)
LRR	ponavljajući sljedovi bogati leucinom (engl. *leucine-rich repeat*)
MAF	učestalost rjeđeg alela (engl. *minor allele frequency*)
MAPK	protein-kinaza aktivirana mitogenom (engl. *mitogen-activated protein kinase*)
MMP	metaloproteinaza matriksa (engl. *matrix metalloproteinase*)
mMRC	modificirani upitnik Vijeća za medicinska istraživanja (engl. *Modified Medical Research Council*)
mRNA	glasnička ribonukleinska kiselina (engl. *messenger ribonucleic acid*)
MyD88	čimbenik 88 mijeloidne diferencijacije 88 (engl. *myeloid differentiation factor 88*)
NF-κB	jezgrin čimbenik kappa B (engl. *nuclear factor kappa B*)
NLR	receptor sličan oligomerizacijskoj domeni koja veže nukleotid (engl. *nucleotide-binding oligomerization domain-like receptor*)
NLRP3	višeproteinski kompleks koji sadrži receptor sličan oligomerizacijskoj domeni koja veže nukleotide s N-terminalnom pirinskom domenom tipa 3 (engl. *nucleotide-binding oligomerization domain-like receptor pyrin 3*).
NOD	oligomerizacijska domena koja veže nukleotide (engl. *nucleotide-binding oligomerization domain*)
OR	omjer izgleda (engl. *odds ratio*)
PAMP	molekularni obrazac povezan s patogenima (engl. *pathogen-associated molecular pattern*)
PCR	lančana reakcija polimerazom (engl. *polymerase chain reaction*)
PPIA	peptidilprolil izomeraza A (engl. *peptidylprolyl isomerase A*)
PRR	receptor za prepoznavanje obrazaca (engl. *pattern recognition receptor*)
qPCR	kvantitativna lančana reakcija polimerazom (engl. *quantitative polymerase chain reaction*)

RAGE	receptor krajnjih produkata uznapredovale glikacije (engl. *receptor for advanced glycation end products*)
rh	rekombinantni humani
RLR	receptor sličan genu 1 kojeg inducira retinoična kiselina (engl. *retinoic acid-inducible gene 1 (RIG-1)-like receptor*)
ROS	reaktivni kisikovi spojevi (engl. *reactive oxygen species*)
SGRQ-C	upitnik za procjenu zdravstvenog stanja pacijenta s KOPB-om (engl. *St. George's Respiratory Questionnaire*)
Siglec	lektini slični imunoglobulinu koji vežu sijalinsku kiselinu (engl. *sialic acid-binding immunoglobulin-like lectin*)
SNP	polimorfizam jednog nukleotida (engl. *single nucleotide polymorphism*)
TIMP-1	tkivni inhibitor metaloproteinaza matriksa 1 (engl. *tissue inhibitor of metalloproteinase-1*)
TIR	domena homologna kod Tolla i receptora za IL-1 (engl. *Toll/interleukin-1 receptor homology domain*)
TLR	receptor sličan Tollu (engl. *Toll-like receptor*)
TNF-α	čimbenik tumorske nekroze alfa (engl. *tumour necrosis factor alpha*)
UDP	uridin-difosfat (engl. *uridine diphosphate*)
UTP	uridin-trifosfat (engl. *uridine triphosphate*)
UTR	netranslatirano područje (engl. *untranslated region*)
WHO	Svjetska zdravstvena organizacija (engl. *World Health Organization*)

Prilog 2: CAT upitnik

Vaše ime i prezime:	Današnji datum:

COPD Assessment Test

Kako je Vaša KOPB? Ispunite Test za procjenu KOPB™ (CAT)

Ovaj će upitnik pomoći Vama i Vašemu zdravstvenom djelatniku u mjerenju utjecaja KOPB (kronične opstruktivne plućne bolesti) na Vaše tjelesno i mentalno zdravlje te svakodnevno funkcioniranje. Vaši će odgovori, kao i ukupan rezultat na testu, koristiti Vama i Vašemu zdravstvenom djelatniku kao pomoć u poboljšanju zbrinjavanja Vaše KOPB i dobivanju najveće moguće koristi od liječenja.

Za svaku tvrdnju navedenu ispod, označite (znakom X) kućicu koja Vas **TRENUTNO** najbolje opisuje. Pazite da za svako pitanje odaberete samo jedan odgovor.

Primjer: Jako sam sretan/sretna 0 X 1 2 3 4 5 Jako sam tužan/tužna

			REZULTAT
Nikada ne kašljem	0 1 2 3 4 5	**Stalno kašljem**	
Uopće nemam sekreta (sluzi) u plućima	0 1 2 3 4 5	**Moja su pluća u cijelosti puna sekreta (sluzi)**	
Uopće ne osjećam stezanje u prsnom košu	0 1 2 3 4 5	**Osjećam jako stezanje u prsnom košu**	
Kada se penjem uzbrdo ili po stepenicama ne nedostaje mi zraka	0 1 2 3 4 5	**Kada se penjem uzbrdo ili po stepenicama jako mi nedostaje zraka**	
Nisam ograničen/ograničena u obavljanju bilo kojih kućanskih aktivnosti	0 1 2 3 4 5	**Vrlo sam ograničen/ ograničena u obavljanju kućanskih aktivnosti**	
S povjerenjem izlazim iz kuće bez obzira na svoju plućnu bolest	0 1 2 3 4 5	**Uopće nemam povjerenja kad izlazim iz kuće zbog svoje plućne bolesti**	
Čvrsto spavam	0 1 2 3 4 5	**Ne spavam čvrsto zbog svoje plućne bolesti**	
Imam puno energije	0 1 2 3 4 5	**Uopće nemam energije**	
		UKUPAN REZULTAT	

Last Updated: February 24, 2012

Prilog 3: mMRC skala zaduhe

mMRC skala zaduhe		
Molimo označiti samo jedan kvadratić.		
stupanj 0	nema tegoba	☐
stupanj 1	tegobe pri penjanju uz brežuljak ili do 4. kata	☐
stupanj 2	zamaranje pri hodu po ravnom s vršnjakom	☐
stupanj 3	ne može prehodati 100 m bez stajanja	☐
stupanj 4	toliko dispnoičan da ne izlazi iz kuće	☐

Prilog 4: SGRQ-C upitnik

UPITNIK O DISANJU ST. GEORGE ZA PACIJENTE S KOBP (SGRQ-C)

Ovaj je upitnik osmišljen kako bi nam pomogao da naučimo više o tome u kojoj mjeri vam disanje uzrokuje poteškoće i kako utječe na vaš život. Koristimo ga kako bismo saznali koji vam aspekti bolesti uzrokuju najviše problema za razliku od toga što liječnici i sestre misle koji su vaši problemi.

Pročitajte pažljivo upute i pitajte ukoliko nešto ne razumijete. Nemojte provesti previše vremena u odlučivanju vaših odgovora.

Prije popunjavanja ostatka upitnika:

Odaberite jedan kvadratić kako biste opisali svoje trenutno zdravstveno stanje:

Vrlo dobro	Dobro	Osrednje	Loše	Vrlo loše
☐	☐	☐	☐	☐

1. DIO

Pitanja o tome koliko poteškoća imate s prsnim košem.

Odaberite **JEDAN** kvadratić za svako pitanje:

1. pitanje. Kašljem:

- većinu dana u tjednu ..... ☐ a
- nekoliko dana u tjednu..... ☐ b
- samo s infekcijama prsnog koša ..... ☐ c
- nimalo ..... ☐ d

2. pitanje. Izbacujem sluz (ispljuvak):

- većinu dana u tjednu ..... ☐ a
- nekoliko dana u tjednu..... ☐ b
- samo s infekcijama prsnog koša ..... ☐ c
- nimalo ..... ☐ d

3. pitanje. Imam kratkoću daha:

- većinu dana u tjednu ..... ☐ a
- nekoliko dana u tjednu..... ☐ b
- nimalo ..... ☐ c

nastavak...

Odaberite **JEDAN** kvadratić za svako pitanje:

4. pitanje. Imam epizode pištanja u prsima:

- većinu dana u tjednu ☐ a
- nekoliko dana u tjednu ☐ b
- nekoliko dana na mjesec ☐ c
- samo s infekcijama prsnog koša ☐ d
- nimalo ☐ e

5. pitanje. Koliko ste epizoda problema s prsnim košem imali tijekom protekle godine?

- 3 ili više epizoda ☐ a
- 1 ili 2 epizode ☐ b
- bez epizoda ☐ c

6. pitanje. Koliko često imate dobre dane (malo problema s prsnim košem)?

- nema dobrih dana ☐ a
- nekoliko dobrih dana ☐ b
- većina dana je dobra ☐ c
- svaki dan je dobar ☐ d

7. pitanje. Ako vam pišti u prsima, je li to gore ujutro?

- ne ☐
- da ☐

nastavak...

2. DIO

8. ***Kako biste opisali stanje vašeg prsnog koša?***

Odaberite ***JEDAN***:

Uzrokuje mi puno problema ili je najvažniji problem koji imam ..... ☐ a

Uzrokuje mi nekoliko problema..... ☐ b

Ne uzrokuje mi nikakav problem..... ☐ c

9. ***Pitanja o aktivnostima kod kojih vam se obično javlja nedostatak daha.***

Za svaku izjavu izaberite ***kvadratić*** koji se odnosi na vas **ovih dana**:

	Točno	**Netočno**
Pri pranju ili odijevanju.....	☐	☐ a
Hodanje po kući	☐	☐ b
Hodanje vani na ravnom	☐	☐ c
Penjanje uz slijed stepenica.....	☐	☐ d
Hodanje uzbrdo.....	☐	☐ e

nastavak...

2. DIO

10. ***Još nekoliko pitanja o vašem kašlju i gubitku daha.***

Za svaku izjavu izaberite ***kvadratić*** koji se odnosi na vas **ovih dana**:

	Točno	**Netočno**
Moj kašalj je bolan	☐	☐ a
Kašalj me umara	☐	☐ b
Ostajem bez daha dok govorim	☐	☐ c
Ostajem bez daha kad se sagnem	☐	☐ d
Moj kašalj ili disanje ometaju moje spavanje	☐	☐ e
Lako se iscrpim	☐	☐ f

11. ***Pitanja o mogućim drugim učincima na vas zbog vašeg problema s prsnim košem.***

Za svaku izjavu odaberite ***kvadratić*** koji se odnosi na vas **ovih dana**:

	Točno	**Netočno**
Moj kašalj ili disanje sramote me u javnosti	☐	☐ a
Moj problem s prsnim košem je smetnja za moju obitelj, prijatelje ili susjede	☐	☐ b
Uplašim se ili uspaničim kad ne mogu doći do daha	☐	☐ c
Osjećam da nemam nadzor nad problemom sa svojim prsima.....	☐	☐ d
Postao/la sam slab/a ili sam invalid zbog mojeg prsnog koša.....	☐	☐ e
Vježbanje nije sigurno za mene	☐	☐ f
Sve mi se čini previše napornim	☐	☐ g

nastavak...

2. DIO

12. ***Ovo su pitanja o tome na koji način vaše disanje utječe na vaše aktivnosti.***

Za svaku izjavu odaberite ***kvadratić*** koji se odnosi na vas **zbog vašeg disanja**:

	Točno	**Netočno**
Treba mi puno vremena da se operem ili odjenem	☐	☐ a
Ne mogu se okupati ili istuširati ili mi treba puno vremena	☐	☐ b
Hodam sporije od ostalih ili uzimam stanku za odmor	☐	☐ c
Poslovi kao što su kućanski oduzimaju mi puno vremena ili moram uzeti stanku za odmor	☐	☐ d
Ako se uspnem jednim slijedom stepenica, moram zastati ili usporiti	☐	☐ e
Ukoliko se žurim ili hodam brzo, moram se zaustaviti ili usporiti	☐	☐ f
Moje disanje otežava mi obavljanje stvari poput hodanja uzbrdo, nošenje stvari po stepenicama, laganih vrtlarskih poslova poput plijevljenja, plesanja, boćanja ili golfa	☐	☐ g
Moje disanje otežava mi obavljanje radnji poput nošenja teškog tereta, kopanja u vrtu ili razgrtanja snijega, trčanja ili hodanja brzinom od 8 km/sat, igranja tenisa ili plivanja	☐	☐ h

nastavak....

2. DIO

13. ***Htjeli bismo znati kako vaši problemi s prsnim košem <u>obično</u> utječu na vaš svakodnevni život.***

Za svaku izjavu odaberite ***kvadratić*** koji se odnosi na vas **zbog vašeg disanja**:

	Točno	**Netočno**	
Ne mogu se baviti sportom ili igrama.....	☐	☐	a
Ne mogu izaći zabavljati se ili na rekreaciju	☐	☐	b
Ne mogu izaći van kuće u kupovinu	☐	☐	c
Ne mogu obavljati kućanske poslove	☐	☐	d
Ne mogu se odmaknuti daleko od svog kreveta ili stolice	☐	☐	e

14. ***Kako na vas utječu problemi s prsnim košem?***

Odaberite ***JEDAN***.

Ne sprječava me u obavljanju nijedne željene aktivnosti	☐ a
Sprječava me u obavljanju jedne ili dvije željene aktivnosti.....	☐ b
Sprječava me u obavljanju većine željenih aktivnosti	☐ c
Sprječava me u obavljanju svih željenih aktivnosti	☐ d

Zahvaljujemo vam na popunjavanju ovog upitnika.

Molimo vas da prije nego završite provjerite jeste li odgovorili na sva pitanja.

10. ŽIVOTOPIS

Iva Hlapčić rođena je 24. ožujka 1994. godine u Čakovcu gdje je završila Gimnaziju Josipa Slavenskog, prirodoslovno-matematički smjer. 2012. godine započinje studij medicinske biokemije na Farmaceutsko-biokemijskome fakultetu u Zagrebu. Nakon završenog stručnog osposobljavanja u Kliničkom bolničkom centru Sestre milosrdnice u Zagrebu, diplomirala je 2018. godine.

U sklopu „Projekta razvoja karijera mladih istraživača – izobrazba novih doktora znanosti“ Hrvatske zaklade za znanost (HRZZ) koji je financirala Europska unija iz Europskog socijalnog fonda, 2018. godine zapošljava se na znanstvenom istraživačkom projektu HRZZ-a „Uloga stresnog proteina Hsp70 u imunosno-upalnom odgovoru kod kronične opstrukcijske plućne bolesti“ (IP-2014-09-1247) čija voditeljica je prof. dr. sc. Lada Rumora. Od 2020. godine postaje suradnica na znanstvenom istraživačkom projektu HRZZ-a pod vodstvom prof. dr. sc. Karmele Barišić naziva „Gensko, proteinsko i RNA profiliranje kolorektalnog karcinoma primjenom tekuće biopsije“ (IP-2019-04-4624). Svoj znanstveni rad obavlja na Zavodu za medicinsku biokemiju i hematologiju Farmaceutsko-biokemijskog fakulteta Sveučilišta u Zagrebu. Na Zavodu sudjeluje i u nastavi kao suradnica na kolegijima Biokemija, Opća klinička biokemija, Molekularna dijagnostika te Biološke membrane i stanična signalizacija. Akademske godine 2018./2019. upisuje poslijediplomski doktorski studij Farmaceutsko-biokemijske znanosti. Na temelju svoje znanstvene aktivnosti, tijekom doktorskog studija postaje stipendistica European Respiratory Society-a i dobiva Godišnju nagradu za mladog znanstvenika za 2020. godinu od Hrvatskog društva za medicinsku biokemiju i laboratorijsku medicinu. Autorica je 13 znanstvenih radova i 13 kongresnih priopćenja.

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TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu **Doktorski rad**
Farmaceutsko-biokemijski fakultet

POVEZANOST IZVANSTANIČNIH MOLEKULA PROTEINA TOPLINSKOGA ŠOKA 70 I ADENOZIN-TRIFOSFATA SA SUSTAVNIM UPALNIM ODGOVOROM U PACIJENATA S KRONIČNOM OPSTRUKCIJSKOM PLUĆNOM BOLESTI

Iva Hlapčić

Kronična opstrukcijska plućna bolest (KOPB) kompleksna je i heterogena bolest s kroničnom upalom. Sustavni upalni odgovor vidljiv je u povećanju koncentracije općih upalnih biljega poput C-reaktivnog proteina (CRP), fibrinogena (Fbg), ukupnog broja leukocita (lkc) i upalnih citokina. Uslijed upalnih reakcija i oštećenja stanica u KOPB-u, povećava se koncentracija izvanstaničnog proteina toplinskoga šoka 70 (eHsp70) i izvanstaničnog adenozin-trifosfata (eATP). U ovom istraživanju određene su plazmatske koncentracije eHsp70 i eATP-a u 137 pacijenata sa stabilnim KOPB-om i uspoređene s koncentracijama u 95 kontrolnih ispitanika. Ispitano je postoji li povezanost ovih parametara sa stupnjem plućne opstrukcije, simptomima i povijesti egzacerbacija te pušačkim statusom. Određena je relativna razina ekspresije gena *HSP70*, *TLR2* i *TLR4* (dva receptora za eHsp70) te *P2X7R* i *P2Y2R* (dva receptora za eATP) te genotipizacija polimorfizama u genima *HSP70, TLR2* i *TLR4*. eHsp70 i eATP povezani su s KOPB-om, sa stupnjom plućne opstrukcije i progresijom simptoma. Također, koncentracije eHsp70 i eATP-a veće su u skupini zdravih pušača nego u zdravih nepušača što ukazuje na pojavu upalnih procesa prilikom izlaganja cigaretnom dimu prije pojave bolesti. Značajna povezanost uočena je između IL-1β, IL-6, TNF-α, CRP-a, Fbg-a, eHsp70 i eATP-a u pacijenata s KOPB-om, a hijerarhijskom klasterskom analizom potvrđena je heterogenost i kompleksnost bolesti. Model sastavljen od IL-1β, eHsp70 i eATP-a pokazao je veliku predikcijsku vrijednost. Značajno povećanje relativne genske ekspresije u pacijenata s KOPB-om u odnosu na zdrave ispitanike opaženo je za *HSP70*, *TLR2* i *P2Y2R* gene, dok je analiza polimorfizama ukazala da postoji povezanost između rizika od KOPB-a i polimorfizma rs6457452 u promotorskom dijelu gena *HSP70*. eHsp70 i eATP biljezi su sustavnog upalnog odgovora u KOPB-u i dio su patogeneze KOPB-a, a u kombinaciji s IL-1β čine model s velikim predikcijskim potencijalom. Dodatni doprinos istraživanja postignut je analizom genske ekspresije i polimorfizama koje pružaju bolje razumijevanje mehanizama i patogeneze KOPB-a.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskoga fakulteta.

Rad sadrži: 172 stranice, 18 slika, 23 tablice, 193 literaturnih navoda. Izvornik je na hrvatskom jeziku.

Ključne riječi: kronična opstrukcijska plućna bolest; sustavna upala; izvanstanični protein toplinskoga šoka 70; izvanstanični adenozin-trifosfat; genska ekspresija; polimorfizam jednog nukleotida

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Datum prihvaćanja: 18. svibnja 2022.

BASIC DOCUMENTATION CARD

University of Zagreb **Doctoral thesis**
Faculty of Pharmacy and Biochemistry

ASSOCIATION OF EXTRACELLULAR MOLECULES HEAT SHOCK PROTEIN 70 AND ADENOSINE TRIPHOSPHATE WITH SYSTEMIC INFLAMMATORY RESPONSE IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Iva Hlapčić

Chronic obstructive pulmonary disease (COPD) is a complex and heterogenous disease with chronic inflammation. Systemic inflammatory response is followed by increased concentration of common inflammatory biomarkers as C-reactive protein (CRP), total count of leukocytes (lkc), fibrinogen (Fbg) and inflammatory cytokines. There is an increase in extracellular heat shock protein 70 (eHsp70) and extracellular adenosine triphosphate (eATP) due to inflammatory response and cell damage in COPD. In this thesis, eHsp70 and eATP were determined in 137 patients with stable COPD and compared with the concentrations in 95 controls. It was assessed if there is the association between eHsp70 and eATP and the severity of the airflow limitation, symptoms burden and history of excerbations as well as smoking status. The relative gene expression *HSP70*, *TLR2* and *TLR4* (eHsp70 receptors) as well as *P2X7R* and *P2Y2R* (eATP receptors) was determined. Moreover, selected *HSP70*, *TLR2* i *TLR4* polymorphisms were genotyped. There was an association of eHsp70 and eATP with COPD compared to healthy subjects, airflow limitation and symptoms severity. eHsp70 and eATP were increased in control smokers compared to non-smoking controls which indicates there is an inflammatory response due to the expose of cigarette smoke even before the disease occurs. Significant association was observed between IL-1β, IL-6, TNF-α, CRP, Fbg, eHsp70 and eATP in COPD patients, while hierarchical cluster analysis confirmed heterogeneity and complexity of COPD. Based on logistic regression analysis, combination of IL-1β, eHsp70 and eATP-a showed significant predictive potential in identifying COPD subjects. There was a significant increase in the relative gene expression of *HSP70*, *TLR2* and *P2Y2R* genes in COPD patients, while the analysis of single nucleotide polymorphisms showed an association of rs6457452 in the promoter region of *HSP70* with the risk of COPD. In conclusion, eHsp70 and eATP are biomarkers of systemic inflammatory response in COPD and it seems they might be useful as prognostic biomarkers, especially in a combination with IL-1β. This thesis contributed with the data at genetic level which could be useful for better understanding of the COPD pathogenesis.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 172 pages, 18 figures, 23 tables and 193 references. Original is in Croatian language.

Keywords: chronic obstructive pulmonary disease; systemic inflammation; extracellular heat shock protein 70; extracellular adenosine triphosphate; gene expression; single nucleotide polymorphism.

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