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Original research paper

Germline variants of the genes involved in NF-kB activation are associated with the risk of COPD and lung cancer development

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) and lung cancer (LC) are closely related diseases associated with smoking history and dysregulated immune response. However, not all smokers develop the disease, indicating that genetic susceptibility could be important. Therefore, the aim of this study was to search for the potential overlapping genetic biomarkers, with a focus on single nucleotide polymorphisms (SNPs) located in the regulatory regions of immune-related genes. Additionally, the aim was to see if an identified SNP has potentially an effect on proinflammatory cytokine concentration in the serum of COPD patients. We extracted summary data of variants in 1511 immune-related genes from COPD and LC genome-wide association studies (GWAS) from the UK Biobank. The LC data had 203 cases, patients diagnosed with LC, and 360 938 controls, while COPD data had 1 897 cases and 359 297 controls. Assuming 1 association/gene, SNPs with a p-value < 3.3×10^{-5} were considered statistically significantly associated with the disease. We identified seven SNPs located in different genes (*BAG6, BTNL2, TNF, HCP5, MICB, NCR3, ABCF1, TCF7L1*) to be associated with the COPD risk and two with the LC risk (*HLA-C, HLA-B*), with statistical significance. We also identified two SNPs located in the *IL2RA* gene associated with LC (rs2386841; $p = 1.86 \times 10^{-4}$) and COPD (rs11256442; $p = 9.79 \times 10^{-3}$) but with lower significance. Functional studies conducted on COPD patients showed that RNA expression of IL2RA, IFNγ and related proinflammatory cytokines in blood serum did not correlate with a specific genotype. Although results presented in this study do not fully support our hypothesis, it is worth to mention that the identified genes/SNPs that were associated with either COPD or LC risk, all were involved in the activation of the NF-κB transcription factor which is closely related to the regulation of the inflammatory response, a condition associated with both pathologies.

Keywords: GWAS, SNP, COPD, lung cancer, immune-related genes, NF-κB

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Chronic obstructive pulmonary disease (COPD) and lung cancer (LC) are complex diseases, caused as a result of complex interactions of genetics and environment. Both diseases are associated with exposure to tobacco smoke, airflow obstruction and impaired inflammatory response (1). Regardless of the smoking status, it has been shown that COPD increases the risk of development of LC up to 5 times. The data suggest that genetic background could be an important risk factor for developing both diseases (2). The airflow limitation is usually associated with an abnormal inflammatory response of the lungs to noxious particles or gases (3), characterized by activation of the cells of the innate and adaptive immune system (4). The regulation of the immune response in the respiratory tract is a complex process, involving a variety number of cells and molecules. Studies show that generally a small number of neutrophils in normal lung tissue is significantly raised in COPD lungs due to the release of the pro-inflammatory chemoattractant (LTB4, CXCL1, CXCL5, and CXCL8) (5, 6). The number of macrophages in COPD patients is also increased as a result of activated inflammation. It has been shown that macrophages in patients with COPD secrete increased amounts of inflammatory mediators (TNF-a, CXCL1, CXCL8, CCL2, LTB4 and reactive oxygen species (ROS)) (7) which are mostly regulated by the transcription factor nuclear factor kappa B (NF-kB) (8). It has also been shown that natural killer cells release inflammatory cytokines and chemokines (TNF-α and IL-8) that can induce pathological features of COPD (9).

It is not surprising that pathological conditions associated with inflammation, such as COPD, are induced by cytokines, which recruit immune cells like macrophages, T-lymphocytes, eosinophils and dendritic cells (10). It has been shown that CCR5 and CXCR3 cytokines and their ligands are responsible for the recruitment of the CD8+ T-lymphocytes in the lung parenchyma of COPD patients (11, 12). It is highly likely that their activation will cause the lung damage observed in COPD *via* their high levels of perforin and Fas ligand (13). In the context of LC, it is also associated with tissue damage and subsequent inflammatory reaction(14). Furthermore, genetic studies have shown an association between genetic alterations in DNA repair genes and susceptibility to LC (15). Since cigarette smoking may induce DNA damage, individuals with a reduced DNA damage repair capacity have a high level of carcinogen-DNA adducts in their tissues and have an increased risk of developing LC. Zhou *et al.* showed that patients with polymorphisms in XRCC1 (Arg399Gln) and ERCC2 (Asp312Asn and Lys751Gln), genes involved in DNA nucleotide excision repair, are associated with the development of LC (16).

As already mentioned, proinflammatory cytokines (*e.g.* IL-1, IL-6, IL-8 and TNF-α) are key regulators of inflammation, participating in acute and chronic inflammation. During the inflammation, they amplify the inflammatory response and are often linked to disease severity (17). It has been shown that patients with severe COPD have higher IL-6 levels than patients with milder COPD (18). Furthermore, a study on inflammatory markers in COPD patients indicates that elevated IL-6 levels in serum, are predictive of increased mortality in COPD patients (19). Contrary to the role of proinflammatory cytokines in the development of COPD, their role in the development of cancer has been studied more. It is known that TNF-α, mediated by the activation of NF-κB, PKCα- and AP-1-dependent pathways can initiate tumor promotion and initiation by regulating the proliferation and survival of neoplastic cells (20). An important role of TNF-α in resistance to EGFR-targeted therapy in NSCLC models has also been shown, where in response to EGFR inhibitors an upregulation of TNF-α has been reported, regardless of the EGFR status (21).

Genome-wide association studies (GWAS) are a very useful tool for a better understanding of the genetic background of complex diseases (22). GWAS compares the frequency of common DNA variations between unrelated patients with a specific trait and healthy individuals (controls) giving genomic variants associated with the risk of getting the trait (23, 24). Several GWAS are done on COPD and LC that have pointed out single nucleotide polymorphisms (SNPs) mostly related to nicotine receptors, apoptosis and DNA repair (25), however, only a few GWAS studies have focused (26–28) on genes involved in the regulation of the immune response. Also, little is known about common genetic SNPs located in the regulatory region of candidate genes, which are able to quantitatively change the gene's expression and therefore affect susceptibility to the disease. Chen *et al.* discovered two SNPs (rs10499563 and rs1800796) in the promoter region of IL-6 associated with increased LC risk. These SNPs increase promotor activity which leads to increased expression of IL-6. Increased IL-6 expression can lead to subsequent chronic inflammation and it exhibits both promoting and suppressive roles in tumor development and other complex diseases (29).

Further on, in the context of the immune-related genes potentially associated with LC and COPD, there is no published GWAS. There are only a couple of GWAS that have overlapped GWAS results of COPD with that of LC. Those results show significant associations with SNPs in nicotinic acetylcholine receptors (25, 30). However, since the numerous findings suggest that dysregulated immune response could be the basis of both diseases, the aim of this case-control study was to explore the SNPs of immune-related genes, extracted from the UK Biobank summary statistics (31), and try to identify the common genetic nominator associated with COPD and LC.

EXPERIMENTAL

Study population

Summary statistics of GWAS for LC and early and later onset COPD were downloaded from the UK Biobank. The lung cancer data had 203 cases, consisting of patients diagnosed with LC (no further classification is known), and 360 938 healthy controls. COPD data had 1 897 cases, patients that were diagnosed with early or later onset of COPD, and 359 297 healthy controls.

For the purpose of DNA and RNA isolation and proinflammatory cytokine expression profiling, 213 COPD patients in the stable phase of the disease were enrolled in this study at the Clinical Department for Lung Diseases Jordanovac, University Hospital Centre Zagreb (Zagreb, Croatia). The study was approved by the Ethical Committee of the University Hospital Centre Zagreb. All participants provided written informed consent to participate in this study.

SNP selection

A list of 1511 immune-related genes with their location was downloaded from the Innate DB (https://www.innatedb.com/), including the ± 200 kb flanking region around the genes. All SNPs with minor allele frequency (MAF) > 0.05 and pairwise linkage disequilibrium $r^2 > 0.8$ were selected for the study. The associations were considered significant if

the *p*-value after Bonferroni correction was $< 3.3 \times 10^{-5}$, assuming 1 association/gene. Associated SNPs were analyzed with *in silico* tools. LocusZoom and UCSC Genome Browser were used for visualizing association profiles; HaploReg and Regulome DB were used for the evaluation of the regulatory nature and the possible functional effects of the SNPs (Supplementary Tables S1 and S2).

DNA isolation and SNP genotyping

In order to be able to stratify the COPD patients according to *IL2RA* genotypes, DNA from 213 COPD patients was extracted from blood cells by a standard salting-out protocol (32). DNA purity and concentration were confirmed using BioSpec-nano (Shimadzu, Japan). Genotyping was performed using TaqMan SNP Genotyping Assay (Applied Biosystems, USA) and Universal TaqMan Master Mix (Applied Biosystems). Analysis was performed on the 7300 Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocol.

RNA isolation and real-time quantitative PCR analysis

For the purpose of the gene expression analysis, total RNA from 53 previously genotyped COPD patients were isolated. Briefly, blood was centrifuged at 3,500 rpm for 10 min at 4 °C, and the RNA was extracted from buffy coat using the TRIzol (Thermo Fisher Scientific, USA)/chloroform method (33). DNA was removed using DNase I (NEB, USA) followed by the cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. For the assessment of *IL2RA* gene expression commercial TaqMan Gene Expression Assays were used, for *IL2RA* assay Hs00907777_m1 (Thermo Fisher Scientific) and for actin-β, endogenous control, assay Hs99999903_m1 (Thermo Fisher Scientific) was used. For determining the expression of *IFNγ* and endogenous control *GAPDH*, SYBR™ Green PCR Master Mix (Applied Biosystems) was used. Sequences of the specific primers are listed in Supplementary Table S3. Quantitative PCR was performed on the 7300 Real-Time PCR System (Applied Biosystems) using standard conditions and fold changes were calculated using the standard $2^{-\Delta\Delta Ct}$ method (34).

ELISA

Concentrations of different proinflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8 and TNFα) in the sera of 105 previously genotyped COPD patients were measured using a ProcartaPlex High Sensitivity Assay, with corresponding bead sets (Thermo Fisher Scientific), according to manufacturer's recommendation. The concentration of cytokines was determined by interpolation from a standard curve using the xPONENT software package (Luminex, USA).

Statistical analysis

For assessing the influence of the rs11256442 genotype on *IL2RA* and *IFNγ* expression and cytokine concentrations, a *t*-test was used in the co-dominant model using GraphPad Prism 8 (version 8.2.1; USA).

RESULTS AND DISCUSSION

SNPs associated with COPD and LC

For the purpose of this study, we downloaded the summary data from the UK Biobank and analyzed them for SNPs in 1511 immune-related genes with MAF > 0.05 and pairwise linkage disequilibrium $r^2 > 0.8$ that were associated with either COPD or LC risk. The analysis of the summary data from a GWAS on 1 897 patients diagnosed with COPD and 359 297 controls identified 7 independent SNPs in the vicinity of immune-related genes that were associated with the development of COPD at $p < 3.3 \times 10^{-5}$ (Fig. 1.). The most significantly associated SNPs were rs3132449 which is located in the 5′ region of *BAG6* gene ($p = 4.65 \times 10^{-9}$) and rs3129956 located in the intronic region of *BTNL2* gene ($p = 7.91 \times 10^{-8}$), rs1800628 located in 3′ region of *TNF* gene ($p = 1.21 \times 10^{-6}$), rs3132090 located in non-coding exon transcript ($p = 1.41 \times 10^{-6}$), rs3130614 located in the intronic region of *MICB* gene ($p = 2.40 \times 10^{-6}$), rs3130063 in the intronic region of *NCR3* gene ($p = 2.43 \times 10^{-6}$) and rs3132610 in the intronic region of *ABCF1* gene ($p = 1.83 \times 10^{-5}$). Other SNPs in functionally interesting genes, associated with COPD but with lower significance ($p < 0.05$), included rs11569805 in the intronic region of *TNRSF8*, rs1883832 in 5′-UTR of *CD40*, rs684014 2.8kb 5′ of *TNFRSF1B*, rs112786155 1.9kb 3′ of *TNFRSF9*, rs11256442 in the intronic region of *IL2RA* and rs11145626 in the intronic region of *GNAQ*. The list of all SNPs identified in the COPD/healthy cohort comparison, with a significance lower than 0.05, is listed in Supplementary Table S1, together with *in silico* analyses on the functional consequences of the SNPs.

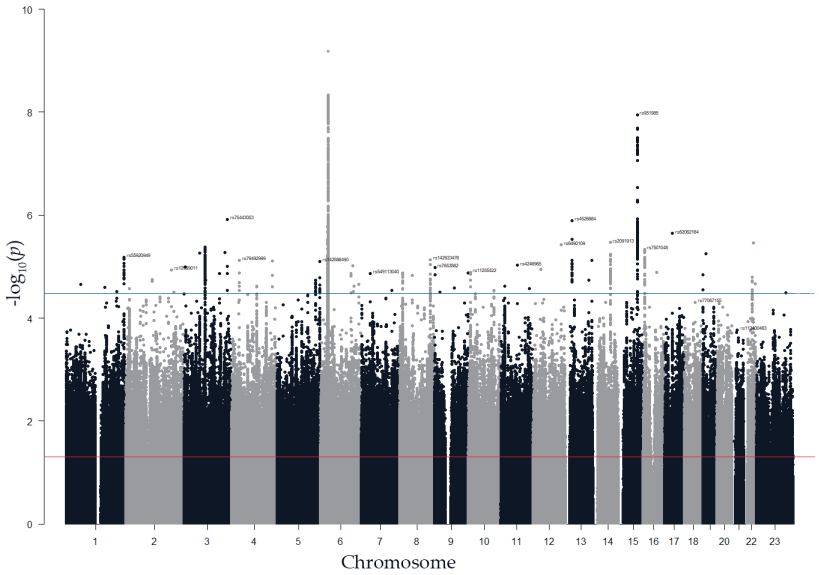

Fig. 1. Genome-wide signal intensity (Manhattan) plots showing the individual –log10 (p values) against the genomic position for COPD data, with the most associated SNP highlighted on each chromosome. The blue line indicates a suggestive level of significance ($p < 3.3 \times 10^{-5}$), whereas the red line indicates the significance level ($p < 0.05$).

Another UK Biobank GWAS SNP list, analyzed in this study, included 203 patients with LC and 360 938 controls in a case-control study (Fig. 2). Results of our analysis pointed to 2 SNPs in the vicinity of immune-related genes that were associated with the development of LC. SNPs associated with LC were rs1639113, located 15kb 3′ of the *HLA-C* gene ($p = 2.89 \times 10^{-6}$) and rs9265821, located 10kb 3′ of the *HLA-B* gene ($p = 4.13 \times 10^{-6}$). Other interesting SNPs, associated with LC, however with lower significance ($p < 0.05$) were observed for SNPs rs6806802 in *CCR9*, rs9766026 in *F13A1*, rs2386841 in *IL2RA*, rs2495366 in *TNRSF14*, rs1148471 in *TNRSF8*, rs1682802 in *GNA15* and rs1148038 in *IL31RA* genes. All SNPs, with a significance lower than 0.05, are listed in Supplementary Table S2.

Even though the results of our study did not pinpoint any common SNPs associated with both diseases, there was a common gene, *IL2RA*, with its two SNPs associated with LC (rs2386841) and COPD (rs11256442), although with a lower significance. Interleukin-2 (IL-2) and its receptor (IL-2R), consisting of 3 subunits (alpha, beta and gamma), play an important role in immune system homeostasis and tolerance and according to Haploreg, both SNPs affect chromatin enhancer marks, especially in T-cells.

Apart from this, we also identified rs3095276 in the intronic region and rs3095276 in the 3′ region of the ABC transporter ABCF1 to be associated with COPD. ABCF1 has been defined in the context of macrophage polarization and immune responses linked to interferon-β production and tolerance (35). The role of ABCF1 in human airway epithelial cells is to mediate CXCL10 production in response to dsDNA viral mimic challenge and it can interact with TLR signaling suggesting a multifactorial role for ABCF1 in innate immunity in human airway epithelial cells (36).

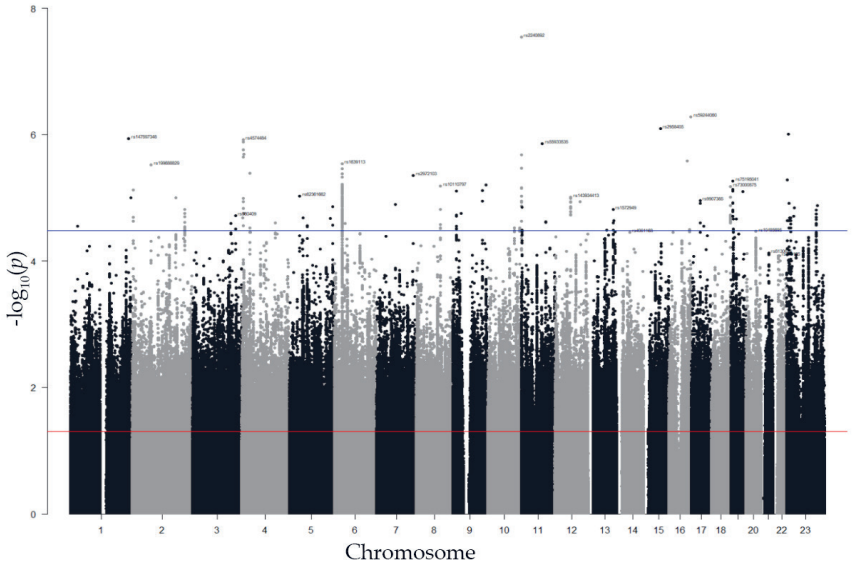

Fig. 2. Genome-wide signal intensity (Manhattan) plots showing the individual –log10 (p values) against the genomic position for LC data, with the most associated SNP highlighted on each chromosome. The blue line indicates a suggestive level of significance ($p < 3.3 \times 10^{-5}$), whereas the red line indicates the significance level ($p < 0.05$).

Interestingly, we also identified several variants in the vicinity of genes involved in the NF-κB activation; two SNPs in the vicinity of G protein family genes, GNAQ (rs11145626) associated with COPD and GNA15 (rs1682802) associated with LC. Those genes are involved in GPCR (G protein-coupled receptor) pathway. For example, during an inflammatory response activated Gα subunit, which is part of a G protein, can activate protein kinase C (PKC), in lymphocytes and macrophages, which regulates NF-κB activity through multiple intracellular mechanisms (37, 38).

We also identified rs1800628 in the vicinity of tumor necrosis factor (TNF) associated with COPD. TNF is a cytokine, that acts as a mediator of immune and inflammatory responses. It is involved in the promotion of inflammatory responses and plays a critical role in the pathogenesis of inflammatory, autoimmune, and malignant diseases (39). Some studies have shown that polymorphisms in the promoter region of TNF are associated with an increased risk of COPD and LC. For example, TNF-α-308 polymorphism has been shown to be associated with COPD (40) and both TNF-α-308 and TNF-α-238 polymorphisms with LC (41). Another gene associated with COPD and LC is TNFRSF8, however with lower significance. The SNP associated with COPD was rs11569805, while rs1148471 was associated with LC. TNFRSF8 is tumor necrosis factor, also known as CD30. Its stimulation, mediated through tumor necrosis factor receptor-associated proteins (TRAFs), activates the nuclear factor-kappa B (NF-κB) pathway. In addition to this, CD30 can also activate mitogen-activated protein kinase (MAPK) pathways (42). Since inflammation, a common symptom in both COPD and LC, is mainly regulated through the NF-κB activation (43, 44), altered expression of TNFRSF8 could be a potential link between these two diseases. Our results also showed that SNPs in the vicinity of other genes belonging to the TNF-receptor superfamily were associated with the observed diseases. We found that rs2495366 (TNFRSF14) was associated with LC, and rs1883832 (CD40, TNFRSF5), rs684014 (TNFRSF1B) and rs112786155 (TNFRSF9) were associated with COPD. Just like TNFRSF8, TNF receptors are involved in apoptosis and inflammation, but can also activate other pathways, like cell proliferation, differentiation and survival, all regulated with NF-kB activation (42, 45–47). Also, it is important to mention that rs8010152 SNP in the 5′ region of TRAF3 was associated with COPD. TRAF3 is a part of the TRAF family of proteins and it binds to TNFRSF5 as the part of the TNFRSF5 signaling pathway (48, 49).

Comparing the results of our case-control studies we can see that there are SNPs in the vicinity of interleukin receptor genes and genes of their ligands that are associated with COPD, like IL1RL1 (rs13019081), IL18R1 (rs2241116) or IL18RAP (rs2293225), or LC, like IL31RA (rs1148038). Interestingly, IL1RL1, IL18R1, IL18RAP and IL31RA are all involved in different interleukin pathways and are associated with NF-κB activation, an inducible transcription factor that regulates expression of genes involved in different processes of the immune and inflammatory responses (50). We found this very interesting, because it is well-accepted that sustained activation of the NF-κB pathway links COPD and LC by generating a pro-tumorigenic inflammatory environment by maintaining immunosuppressive conditions (51).

Functional analysis

Because our *in silico* analysis presented in Supplementary Table S1 showed that rs11256442 can modulate chromatin state by either enhancing or repressing expression of the *IL2RA* gene in specific tissues and cell lines, we wanted to investigate if rs11256442 has

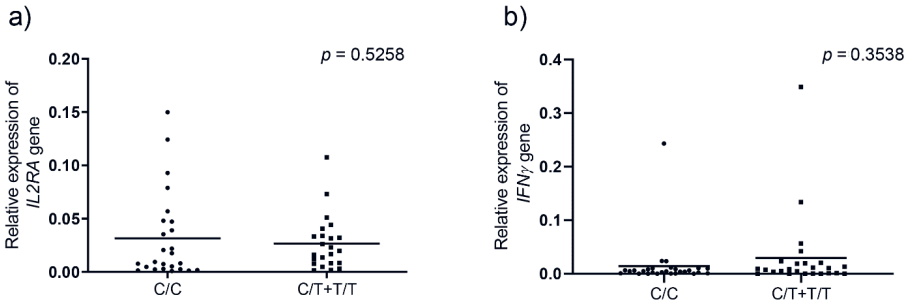

Fig. 3. Expression analysis of *IL2RA* and *IFNγ* in COPD patients in relation to the rs11256442 genotype. Real-time PCR analysis was performed on cDNA samples from COPD patients (N(C/C) = 28, N(C/T) = 20 and N((T/T) = 5).

any impact on the expression of *IL2RA* in COPD patients. In addition, it is known that IL2RA signaling could lead to the expansion of IFNγ^+ T cells, therefore we also wanted to study if the rs11256442 has any impact on *IFNγ* expression in peripheral mononuclear

Table I. The clinical and demographic characteristics in the cohort of COPD patients and the frequency of the comorbidities

Characteristic	No. of COPD patients
Age, year, median (SD[a], minimum and maximum)	63 (10.7, 33–87)
GENDER	
male, *n* (%)	141 (66.2)
female, *n* (%)	70 (32.9)
missing information, *n* (%)	2 (0.9)
SMOKING STATUS	
smoker, *n* (%)	51 (23.9)
ex-smoker, *n* (%)	133 (62.5)
non-smokers, *n* (%)	13 (6.1)
missing information, *n* (%)	16 (7.5)
FEV1*, %, mean (SD*)	46.03 (20.7)
FEV1*	
1 (80–120 %), *n*	7
2 (50–80 %), *n*	70
3 (30–50 %), *n*	63
4 (0–30 %), *n*	51
missing information, *n*	22

[a] SD – standard deviation, FEV1 – forced expiratory volume in one second

Table II. Concentration (γ) of serum IL-1α, IL-1β, IL-6, IL-8 and TNFα in COPD patients with specific rs11256442 genotype. The concentration of plasma cytokines of total of 105 COPD patients was determined, which included 44 samples of the C/C rs11256442 genotype, 51 with the C/T genotype and 10 with the T/T genotype

	C/C		C/T + T/T		
γ (pg mL^{-1})	Mean	SD[a]	Mean	SD[a]	*p*-value
IL-1α	3.22	11.49	1.33	2.05	0.29
IL-1β	20.69	33.13	15.17	22.47	0.34
IL-6	52.34	60.31	48.63	61.44	0.76
IL-8	19.22	33.02	17.92	24.69	0.83
TNFα	14.55	18.02	13.48	18.54	0.77

[a] SD – standard deviation

cells. Functional analyses were focused only on COPD patients due to the fact that for them we had the necessary biological material. Demographic and clinical characteristics of the tested cohort are presented in Table I. The real-time quantitative PCR (RT-qPCR) was performed on total RNA extracted from peripheral blood cells of the COPD patients, previously genotyped for rs11256442. The expression levels of tested genes were stratified according to the rs11256442 genotype and compared between the groups. The results of those analyses are shown in Fig. 3. Results of the expression analyses indicated that the tested regulatory polymorphism does not affect the expression levels of *IL2RA* and *IFNγ* genes in the group of COPD patients.

Further on, we wanted to test if the rs11256442 genotype could be associated with the serum concentration of the proinflammatory cytokines, well-known mediators of inflammation in COPD. Here we tested if the rs11256442 genotype can affect the serum concentration of IL-1α, IL-1β, IL-6, IL-8 and TNFα. For the purpose of this analysis, a total of 105 COPD patients with known rs11256442 genotype were available. Our results, presented in Table II, show that the rs11256442 SNP does not affect the serum levels of the tested cytokines.

As already mentioned, IL-2 is a pleiotropic cytokine with many different functions. The binding of IL-2 to IL-2R activates the signal *via* 3 different pathways: activation of the mammalian target of rapamycin (mTOR), phosphorylation and dimerization of STAT5, and activation of MAPK (52). It has been shown that the IL-2 signal, usually mediated through STAT5 in many immune cells, activates IFNγ production (53, 54). Interestingly, overexpression of IL2RA mRNA has been found in LC in comparison to normal lung tissue (55), while Jia *et al.* also found several SNPs (rs12569923, rs791588, and rs12722498) in the IL2RA gene that are associated with LC in a Chinese population (56). Higher IL2RA expression has also been associated with COPD (57) and increased IFNγ production (58). Also, increased soluble IL2RA levels are correlated with the risk of adverse outcomes in COPD patients with acute exacerbation (59). Our results show that there is no impact of rs11256442 neither on IL2RA expression nor on IFNγ expression in our tested COPD group. Also, we were not able to detect any differences in the proinflammatory peripheral cytokine concentration. Our hypothesis of a common genetic nominator for COPD and LC was

only partially confirmed by showing that IL2RA could be recognized as a potentially important regulator of inflammation. However, this was not confirmed in the *ex vivo* performed functional study, conducted on a relatively small number of patients. Finally, it is important to say that our genomic study also suffers from certain limitations. The UK Biobank data sets contain a large number of controls when compared to cases for both of our traits of interest. This low ratio between cases and controls could lead to false positive findings and, more importantly, false negative findings. Also, our COPD cohort is small and genetically different from the one from UK Biobank which could explain different results. Further investigations are needed to see how rs11256442 affects the development of COPD.

In conclusion, we overlapped the results of two GWAS studies on COPD and LC with a focus on immune-related genes. We found that there are several significantly associated SNPs in both diseases related to immune pathways and which all in the end participate in the NF-κB regulation.

Supplementary material is available upon request.

Conflict of interest. – The authors declare no competing interests.

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Authors contributions. – Conceptualization, A.F. and J.K.; methodology, J.B. and Y.N.; data analysis, J.B., Y.N. and L.R.; data extraction, S.C.; recruitment of patients and clinical data collection, L.Ć., A.V.D, M.J. and M.S., writing, J.B. and Y.N; review, L.Ć., A.V.D, M.J. and M.S.; editing, A.F. and J.K. All authors have read and agreed to the published version of the manuscript.

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