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Original Scientific Paper

DIFFERENCES IN CLINICAL CHARACTERISTICS OF PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) IN OSIJEK AND ZADAR AND POSSIBLE ASSOCIATION WITH GLOBAL DNA METHYLATION AS AN EPIGENETIC BIOMARKER

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SUMMARY – Environmental factors play an important role in the onset and development of chronic obstructive pulmonary disease (COPD). Previous studies have shown that global DNA methylation could be a useful biomarker in COPD. We hypothesized that COPD patients from two different Croatian regions might have clinical characteristics, quality of life, and global DNA methylation, which could be used as a possible epigenetic marker. The study included 136 COPD patients with an average age of 70 years, of whom about 70% were men. Among them, 37% were active smokers, 58% ex-smokers, and 5% non-smokers. There were 69 patients in Osijek and 67 in Zadar. The control group consisted of 64 subjects who were not diagnosed with COPD or other respiratory diseases, 32 from each center. We analyzed risk factors, severity and type of symptoms, exacerbations, comorbidities, and numerous parameters of lung function, phenotypic characteristics, quality of life, and global DNA methylation. We found that COPD patients in Zadar had more pronounced emphysema (P<0.001), while COPD patients from Osijek had more severe symptoms of cough and expectoration (P<0.001 for both). Patients from Osijek had more cardiovascular comorbidities (P<0.001) and depression (P=0.01) than patients from Zadar. Analysis of global DNA methylation found that patients with COPD had lower values than the control group, but the difference was not statistically significant.

Keywords: *Epigenetics; Global DNA methylation; COPD; Quality of life; Lifestyle*

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a progressive disease with a poor prognosis and very heterogenus clinical presentation. COPD is the third leading cause of death worldwide (1). Both environmental and genetic factors play an important role in the development of COPD, but little is known about the specific epigenetic pathways involved in the development and course of this disease. Epigenetic

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mechanisms affect gene expression and subsequent protein expression, but do not alter the primary DNA sequence (2). DNA methylation is one of the main epigenetic mechanisms through which gene-environment interactions occur. Active methylation and demethylation are responsible for methylation in genome regions where the cytosine nucleotide is followed by the guanine nucleotide in a specific sequence (CpG islands) (3). DNA methylation sites can be inherited by DNA replication and catalyzed by DNA methyltransferases (DNMT). Hypermethylation of CpG islands in gene promoters usually leads to decreased gene expression, whereas hypomethylation leads to increased gene regulation (4). In recent years, the development of epigenetics and molecular biology have provided new potential opportunities to elucidate the pathogenesis and improve the diagnosis and treatment of COPD (5). Epigenetic changes may affect the expression of many genes in the lungs of patients with COPD, and enzymes that regulate these epigenetic changes may be activated by smoking, but the results of various studies have not always been consistent. In the study by Qiu et al (6), DNA methylation of six different methylation positions (DMPs) was associated with the smoking year, or pack year (PY), in active smokers, and DNA methylation of eight different methylating positions was related to smoking duration and time elapsed since smoking cessation in ex smokers. De Vries et al (7) found no association of DNA methylation with the presence of COPD or with smoking status. The fact that not all smokers suffer from COPD and that COPD is a highly heterogeneous disease could also be explained with epigenetic changes. Recent research hase shown that smoking is associated with only 45% of COPD patients, while other causes of the disease need to be further investigated (8). Atmospheric air pollution and exposure to the combustion of biofuels may contribute to the pathogenesis of COPD through aberrant DNA methylations (9). Protective effect of the Mediterranean diet on lung function in active smokers was shown by a Spanish study (10), and studies have also shown that smokers who eat less fruits and vegetables have a higher risk of developing COPD (11). Understanding the impact of environmental factors on the development of COPD through gene methylation could play a key role in preventing onset and progression of the COPD (12). As climate and lifestyle could have a different impact on COPD patients, we assumed that COPD patients from two geographically different regions, such as the cities Osijek and Zadar, might have different clinical features, disease severity, disease course, and quality of life. Global DNA methylation shows the overall degree of genome methylation. It is relatively simple, non-invasive, and easily used in clinical practice (13, 14). Based on the above, it is expected that the clinical characteristics of COPD and DNA methylation could differ between the group of patients treated in Osijek and the group treated in Zadar, and that global DNA methylation could be different in relation to individual characteristics of COPD patients.

Patients and methods

The study was first approved by the Ethics Committees of the Clinical Hospital Center Osijek and the Zadar General Hospital, and then by the Ethics Committee of the Faculty of Medicine, the University of Osijek (decision No. 2158-61-07-17-130, July 3, 2017). The study was performed from October 2017 to March 2019 and in that period all data were collected.

Patients

Based on the calculation of the mean effect in different numerical variants between the two independent groups, 136 patients with COPD, 69 patients treated in Clinical Hospital Center Osijek and 67 patients treated in General Hospital Zadar, were included in the study. There were 70% men and 30% women. According to smoking habits, there were 37% active smokers, 58% ex-smokers, and 5% non-smokers. The mean age of patients with COPD was 70 years (interquartile range 66-74). Patients in Osijek and patients in Zadar had similar demographic characteristics (Table 1). The age- and sex-matched control group was formed for the purposes of global DNA methylation analysis. It consisted of 64 subjects, 32 from each center, without diagnosis of COPD or other lung disease or respiratory symptoms and with normal spirometric parameters. The control group consisted of an equal number of men and women and an equal number of active/former smokers and non-smokers. The mean age of control subjects was 71 years (interquartile range

65-77). Each participant signed the informed consent form.

Patients with COPD were men and women over the age of 40 years with a previous diagnosis of COPD confirmed by spirometric findings of persistent airway obstruction according to Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) criteria (1). The only exclusion criterion was the exacerbation of COPD at the time of inclusion and conduction of study procedures. According to the GOLD (1), patients were classified by grade of obstruction (GOLD I-IV) and by grade of severity of symptoms and risk of exacerbation (GOLD A-D).

Method

Collection of detailed anamnestic data on smoking and other risk factors, symptoms, exacerbations, and comorbidities, and findings of pulmonary function (PFT), chest computed tomography (CT), cardiac ultrasound, and laboratory findings of eosinophils, total IgE, and global DNA methylation from peripheral blood were done in all patients. Relevant indicators of pulmonary function were obtained by spirometry, pulmonary diffusion, and arterial blood gas analysis (Table 2). Echocardiographic findings and chest CT scans were not older than 12 months before the enrollment and contained the data needed to assess the presence of pulmonary hypertension and the presence and extent of emphysema and bronchiectasis. They were interpreted by different experts in both centers, and there was no single method for evaluating these findings, nor were quantitative data used. If patients did not perform these tests or if the findings did not contain the required data, echocardiography and chest CT were performed subsequently.

Patients quality of life and symptoms were assessed by four validated questionnaires. Short form-36 questionnaire (SF-36) is the most commonly used validated generic questionnaire for a comprehensive assessment of health status in the domain of physical and emotional health (15, 16). It consists of 36 items grouped into eight conceptual categories. The St. Georges Respiratory Questionnaire (SGRQ) is a specific validated health quality assessment questionnaire for respiratory diseases (17). It contains 50 questions and is divided into 2 parts. The total score and results in 3 domains (symptoms, activity, and impact) are calculated. The score ranges from 0 to 100, with a higher score indicating a worse condition. The COPD Assessment test (CAT) is a questionnaire designed to assess the symptoms of COPD applicable in everyday clinical practice. It consists of 8 questions that give a possible sum of 0-40 and shows a good correlation with the more complicated SGRQ questionnaire (18). The modified Medical Research Council (mMRC) dyspnoea assessment scale was used for self-assess degree of shortness of breath on a scale of 0 to 4 in daily activities (19, 20). The Life Habits Form (LHF) was designed in collaboration with the Department of Sociology, University of Zadar for the purposes of this study to facilitate the collection of data on lifestyle and living conditions. LHF contains data on exposure to risk factors such as smoking, housing, heating, education, occupation, and eating habits. A part of the LHF is a guestionnaire that corresponds to a modified validated Mediterranean Diet Serving Score (MDSS) questionnaire (21).

A peripheral blood sample for global DNA methylation was taken from all patients and stored at -20°C until laboratory analysis (22). DNA was isolated from leukocytes using the commercial set NucleoSpin Blood L (Macherey Nagel, Germany), followed by absolute colorimetric quantification of global DNA methylation with a commercial set Methylated DNA Quantification Kit (Colorimetic) (ab117128) Abcam (Cambridge, United Kingdom) according to the manufacturer's instructions. All laboratory analyses were carried out in the Laboratory for DNA Analysis of the Department of Medical Chemistry, Biochemistry and Laboratory Medicine, Faculty of Medicine Osijek, University J.J. Strossmayer in Osijek.

Statistical analysis

Category data were presented as absolute and relative frequencies. Differences in category variables were tested by the chi-square test and, if necessary, by Fisher's exact test. The normality of the distribution of numerical variables were tested by the Shapiro-Wilk test. Differences of normally distributed numerical variables between two independent groups were tested by Student's test, in case of deviation from normal distribution by Mann-Whitney U test, and in case of three or more independent groups by analysis of variance (ANOVA) or Kruskal-Wallis test (post hoc Conover). Bonferroni correction was used for all multiple

analysis. The correlations of normally distributed numerical variables were assessed by Pearson's correlation coefficient r, and in case of deviation from the normal distribution by Spearman's correlation coefficient ρ (rho). Regression analysis (bivariate and multivariate) will examine which factors influence higher values of global DNA (23, 24). To predict the possibility of occurrence of an event, an expectation maximisation (EM) cluster analysis is used (25). All P values were two-sided. The significance level was set at 0.05. The statistical programs MedCalc® Statistical Software version 20.010 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021) and SPSS Ver. 23.0 (Released 2015. IBM. Armonk, NY: IBM Corp.) were used.

Results

COPD patients from Osijek and Zadar did not differ significantly in age, sex, body mass index, age at diagnosis, symptoms, and lung function (Table 1). About 75% of patients in both groups were retired and no significant difference was found in terms of employment status between patients from Osijek and those from Zadar (Fisher's exact test, P=0.89). There were no differences in the exacerbation rates (chi-square test, P=0.52), hospitalization rates, or emergency visit rates during the last 12 months (chi-square test, P=0.30). No differences were found in the distribution of obstruction severity (GOLD I-IV, chi-square test, P=0.31) and symptom severity and risk of exacerbations (GOLD A-D, Fisher's exact test, P=0.89). There was also no statistically significant difference in any of the lung function indicators (Table 2). In terms of exposure to the risk factors, no significant difference in smoking habits was found between these two groups of COPD patients. In both groups of patients, the median PY was 45 (Mann-Whitney U test, P=0.85), and more than 90% of patients had more than 20 PY (Fisher's exact test, P>0.99). The median age of starting smoking was 17 years in patients in Osijek , and 19 years in patients from Zadar (Mann-Whitney U test P=0.16). In terms of exposure to biofuels during life, a total of 68 patients (51%) used woods for heating, significantly more in Zadar (61%) than in Osijek (41%) (chi-square test, P=0.02).

Chest CT scan showed that COPD patients in Zadar had more severe pulmonary emphysema than COPD patients in Osijek (chi-square test, P<0.001), while patients from Osijek had more pronounced symptoms of cough and expectoration (chi-square test, P<0.001 for both). Patients from Osijek had significantly more cardiovascular comorbidities (chi-square test, P<0.001), and depression (chi-square test, P=0.01) than patients from Zadar (Table 3).

The results of the SGRQ questionnaire were worse in patients from Osijek than in patients from Zadar for the total score and the impact domain, but the differences were not statistically significant (Mann-Whitney U test, P=0.49 and P=0.60, respectively) (Table 3). However, the results of the SF-36 questionnaire showed that patients in Osijek had a statistically significantly worse result than patients in Zadar in the domain of physical health, for the conceptual category of physical functioning (Mann-Whitney U test, P=0.04), and for the conceptual category role limitations due to emotional problems in the domain of mental health, (Mann-Whitney U test, P=0.03), while in other conceptual categories and overall score, there were no statistically significant differences between patients in Osijek and Zadar (Table 4).

The EM (Expectation Maximisation) cluster analysis method found that subjects in the overall sample could be classified into two basic clusters according to global DNA methylation: 1. low-methylating DNA cluster with a larger number (n=170), and 2. high-methylating DNA cluster with a smaller number of participants (n=27) (Figure 1). Even though it was observed that control subjects and non-smokers had slightly higher values of global DNA methylation, but no statistically significant difference ware found between the group of COPD patients and the control group (Mann-Whitney U test, P=0.97), between smokers and non-smokers (Mann Whitney U test, P=0.41), and neither between men and women (Mann-Whitney U test, P=0.73). Besides, there was no statistically significant difference in global DNA methylation between COPD patients in Osijek and COPD patients in Zadar (Mann-Whitney U test, P=0.73).

It was noticed that control subjects in Zadar, and especially male control subjects, had higher values of global DNA methylation compared to other subjects. A statistically significant difference in global DNA

methylation was found only in the group of male subjects in Zadar between COPD patients (median 2,3, interquartile range 1.9–3.2) and control subjects (median 4.1, interquartile range 1.9–5.3) (Mann-Whitney U test, P=0.03). Female control subjects in Zadar had higher values of global DNA methylation (median 3.4, interquartile range 1.8–4.2) than COPD female patients in Zadar as well (median 2.4, interquartile range 2.0–2.9), but the difference was not statistically significant (Mann-Whitney U test, P=0.34). It was also found that non-smokers had statistically significantly higher values of global DNA methylation (median 3.8, interquartile range 2.3–4.9) than smokers (active and former) (median 2.4, interquartile range 1.9–3.4) (Mann-Whitney U test, P=0.01) only in participants in Zadar.

Global DNA methylation was statistically significantly lower in patients who had a cough symptom (median 2.3 interquartile range 1.9–2.9) compared to patients who did not have a cough symptom (median 2.6, interquartile range 2.1–3.5) (Mann-Whitney U test, P=0.04). Patients who had expectoration symptom also had lower values of global DNA methylation, but statistical significance was not achieved (Mann-Whitney U test, P=0.08). Global DNA methylation was statistically significantly higher in patients with COPD in Osijek, who had depression as comorbidity (median 2.8, interquartile range 2.4–3.2), compared to patients without depression (median 2.2), interquartile range 1.9–2.9) (Mann-Whitney U test, P=0.04).

Analysis of the distribution of low-methylating and high-methylating values of global DNA methylation, cluster 1 and cluster 2, within the examined subgroups showed that the highest share of high-methylating samples (cluster 2) was in the control group, both men and women, and in male COPD patients in Zadar (Figure 2).

The groups of subjects with global low-DNA methylation (1) and high-DNA methylation (2) were found to be statistically significantly different with regard to the presence of smoking (P=0.03), number of pack/year (P=0.002), the presence of cough (P=0.001) and the presence of expectoration (P=0.01) (Mann-Whitney U test).

Discussion

Demographic characteristics and COPD duration and severity did not differ significantly between the patients from Osijek and those from Zadar. Patients in Osijek and Zadar were similarly exposed to the main risk factors for COPD and did not differ significantly in smoking habits, occupational exposure, or eating habits. However, significanltly more COPD patients in Zadar used wood to heat their homes throughout their lifetimes in comparison with patients in Osijek. Biofuel combustion is associated with increased COPD prevalence in persons minimally exposed to tobacco smoke in low- and middle-income countries (26). Studies also confirm that this is an important risk factor for developing COPD in people who have never smoked (27). Data from the Croatian Bureau of Statistics show that about 50% of households in Croatia use different types of biofuels for heating, slightly more in the continental part of Croatia (55%) than in Dalmatia (49%) (28). It is possible that wood heating contributed to COPD in COPD patients in Zadar, but in general, the impact of this risk factor on Slavonians is probably greater than on Dalmatians due to colder winters and longer heating seasons in continental Croatia. Recent research warns about the harmful effects of heating indoor spaces with wood in highly developed countries (29).

Significant differences in the characteristics of the disease, quality of life, and comorbidities were found between COPD patients in Osijek and Zadar. Health-related quality of life (HRQoL) of COPD patients in Osijek was worse than of those in Zadar. COPD patients in Osijek had significantly more cardiovascular comorbidities and depression, which certainly affected their quality of life. Patients in Zadar were found to have more pronounced emphysema, while patients in Osijek had more pronounced coughing and expectoration, i.e., symptoms of chronic bronchitis. These physical characteristics of patients with COPD have been recognized for a long time and are traditionally considered the main phenotypic differences of COPD patients – the phenotype of the so-called "pink puffer" with dominant emphysema and the phenotype of the so-called "blue bloater" with dominant chronic bronchitis (30). Epigenetic mechanisms involved in hypersecretion (31) and development of emphysema

(32) are mostly known, and the main environmental factors influencing these characteristics of the disease are common. However, climates in Slavonia and Dalmatia are quite different and could have a significant impact on the diversity of COPD manifestations, the quality of life, and the presence of comorbidities in COPD patients. Much attention has been paid to the possible protective immunomodulatory role of vitamin D as an epigenetic regulator of genes associated with lung disease (33), especially the protective role in the development of COPD and the prevention of moderate and severe exacerbations of COPD (34). Reduced sun exposure and vitamin D deficiency can increase susceptibility to respiratory tract infections and the tendency to cough (35), which was the main feature of COPD patients in Osijek. There is also a strong link between vitamin D deficiency and the risk of various mental health disorders (36, 37). Climate influence is one of the main risk factors for depression, which was significantly more present in patients in Osijek than in Zadar. Cardiovascular comorbidities are the most important comorbidities in COPD patients, associated with COPD mortality (38). The results of some studies suggest that COPD patients with higher body mass index and chronic bronchitis may be more susceptible to cardiovascular comorbidities (39), while patients with emphysema may be less susceptible (40). Our results, that COPD patients in Osijek predominantly have chronic bronchitis and significantly more cardiovascular comorbidities, confirm the thesis that less sun exposure disrupts the anti-inflammatory and other protective properties of vitamin D in the respiratory (33, 34) and cardiovascular system (41).

In our study sample, there were all degrees of COPD severity, but global DNA methylation was not associated with either degrees of obstruction or severity of symptoms. In the study by He et al (42), lower levels of DNA methylation were associated with the presence of COPD Qui et al (43) found an association between DNA methylation and more severe forms of COPD. Zinellu et al (44, 45) also found that global DNA methylation was lower in patients with mild to moderately stable COPD. The authors assumed that global DNA methylation was associated with disease severity and oxidative stress, but severe COPD patients were not included in their study, and the link between DNA methylation and oxidative stress has not been confirmed.

In our study, global DNA methylaton was lower in patients than in control subjects, but a significant difference was found only between a subgroup of male COPD patients and male controls in Zadar. In many malignant diseases, and other diseases as well, in which the association between DNA methylation disorders of certain genes and disease has been confirmed, global DNA methylation does not reflect the methylation of specific CpG sites of individual genes, but may indicate disease activity or treatment effect (46, 47). In many haematological and other malignant (48) and cardiovascular diseases (49), patients had lower global DNA methylation values than control subjects. On the other hand, global DNA methylation in COPD patients with depression in Osijek was significantly higher, which is in line with previous research by Reszko et al (50) who found that women with depression had higher global DNA methylation than the control group. It can be assumed that the value of global DNA methylation in COPD patients was influenced by a number of comorbidities, especially cardiovascular, which are known to interact with COPD (51). Lower values of global DNA methylation that reach statistically significant differences only in certain groups of COPD patients can be explained by a relatively small sample size due to the high heterogeneity of the disease. The association of DNA methylation with diseases, but also the characteristics of patients, indicates a complex involvement of DNA methylation in the pathogenesis of different diseases.

Although our study showed some differences in the characteristics of COPD between patients from Osijek and Zadar, two different environments in which they were exposed to different environmental influences, we did not find a clear association with global DNA methylation as an epigenetic factor. Given the outstanding results observed in individual patient subgroups, a much larger sample size should be included in future studies to address the questions regarding this association. Better and more accurate results are likely to be obtained by measuring DNA methylation in sputum and/or exhaled air samples (52), but these methods are currently too complex, expensive, and unsuitable for clinical research.

Conclusion

Global DNA methylation is influenced by various factors, including host characteristics and lifestyles, all of which need to be carefully considered in the research aimed at establishing the association between global DNA methylation and health outcomes (53). Extreme complexity, frequent inconsistency, and reverse causality create confusion and pose problems in interpreting the results of epigenetic DNA methylation studies. Global DNA methylation is not a suitable and reliable biomarker in COPD, but the results of previous studies of global DNA methylation in COPD and the results of our study suggest that some subsets of data may still be important as epigenetic markers. Recent genome-wide research suggests that the classical model of interpretation of molecular mechanisms of abnormal DNA methylation and their possible therapeutic potential needs to be re-examined (54). Further research on global DNA methylation should contribute to the development of more accurate biomarkers for the detection, diagnosis, prediction of response to therapy, and prognosis of not only malignant, but also other diseases including COPD (55).

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Sažetak

RAZLIKE U KLINIČKIM KARAKTERISTIKAMA BOLESNIKA S KRONIČNOM OPSTRUKTIVNOM PLUĆNOM BOLEŠĆU (KOPB) U OSIJEKU I ZADRU I MOGUĆA POVEZANOST S GLOBALNOM DNA-METILACIJOM KAO EPIGENETSKIM BIOMARKEROM

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Poznato je da u nastanku i razvoju kronične opstruktivne plućne bolesti (KOPB) važnu ulogu imaju okolišni čimbenici. Dosadašnja istraživanja pokazala su da bi globalna DNA metilacija mogla biti korisna kao biomarker u KOPB-u. Pretpostavili smo da bi se bolesnici s KOPB-im iz dvije različite hrvatske regije mogli razlikovati u kliničkim karakteristikama, kvaliteti života i globalnoj DNA metilaciji kao mogućem epigenetskom markeru. Ispitivanje je provedeno na uzorku od 136 bolesnika s KOPB-om prosječne dobi od 70 godina (70% muškarci). Među njima je bilo 37% aktivnih pušača, 58% bivših pušača i 5% nepušača. U Osijeku je bilo 69 bolesnika, a u Zadru 67 bolesnika. Kontrolnu skupinu činila su 64 ispitanika koji nisu bolovali od KOPB-a i drugih respiratornih bolesti, po 32 iz svakog centra. U bolesnika s KOPB-om analiziran je veliki broj podataka uključujući čimbenike rizika, težinu i tip simptoma, egzacerbacije, komorbiditete, kao i brojne pokazatelje plućne funkcije, fenotipske karakteristike, kvalitetu života i globalnu DNA metilaciju. Nađeno je da su bolesnici s KOPB-om u Zadru imali jače izražen emfizem ($P < 0,001$), dok su bolesnici u Osijeku imali jače izražene simptome kašlja i iskašljavanja ($P < 0,001$). Bolesnici u Osijeku u odnosu na bolesnike u Zadru imali su više kardiovaskularnih komorbiditeta ($P < 0,001$) i depresije ($P = 0,01$). Analizom globalne DNA metilacije nađeno je da su bolesnici s KOPB-om imali niže vrijednosti od kontrolne skupine, ali razlika nije bila statistički značajna.

Ključne riječi: *Epigenetika; Globalna DNA metilacija; KOPB; Kvaliteta života; Način života*