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Role of Plasma Level of Soluble Receptor for Advanced Glycation End-Products (S-RAGE) In COPD Patients-A Hospital-Based Case-Control Study

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ABSTRACT

Introduction

Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease, and a biomarker-based approach has the potential to improve the management of COPD. The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor expressed highly in the lungs and is a promising biomarker for COPD. Here, we measured the plasma levels of soluble forms of RAGE (S-RAGE) in COPD and healthy controls.

Methods

One hundred sixty-three subjects were enrolled, including 91 (55.8%) with stable COPD, and 72 (44.2%) were enrolled. Plasma S-RAGE was measured by enzyme-linked immunosorbent assay (ELISA)in a 96-well plate. Spirometry was done to confirm the diagnosis of COPD.

Results

The mean age of COPD and controls were 62.0±SD 10.46 and 49.88 ± SD 7.53 respectively. The mean S-RAGE level in the case and controls were 866.99 and 974.63 pg/ml respectively and was found to be statistically non-significant. Analysis using the data in various grades of COPD, the mean difference in S-RAGE levels was found to be statistically significant inmoderately severe COPD cases only (Mean Difference-372.2(157.9),(95%CI 60.3,680.1)(p=0.02) compared to controls.

Conclusion

This was a pilot study that failed to show a significant difference in mean S-RAGE levels between cases and controls. However, further study is required to elucidate the role of S-RAGE in COPD patients.

I. Introduction

The global strategy for prevention, diagnosis, and management of COPD 2022 report define COPD as a common, preventable, and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities[1]. COPD is usually caused by significant exposure to noxious particles or gases and is influenced by host factors such as abnormal lung development. Significant comorbidities may develop in patients with COPD with an impact on morbidity and mortality. Measure the group difference in the level of S-RAGE between patients with COPD and healthy controls and assess the association between the level of S-RAGE with the severity of COPD[2]. Inclusion criteria included stable patients with COPD and those who were willing to sign written consent. Exclusion criteria included **c**oexistent pulmonary disease (e.g., interstitial pulmonary fibrosis, bronchiectasis, or granulomatous lung disease), malignancy, heart diseases, diabetes, long-term oral corticosteroid therapy, renal impairment, and COPD with acute exacerbation.

All consecutive patients with COPD who met the inclusion criteria and agreed to provide written consent are included in the study[3,4]. The study was proposed with the objectives to measure the group difference in the level of S-RAGE between patients with COPD and healthy controls and assess the association between the level of S-RAGE with the severity of COPD[5].

II. Methodology

II.(A) Study Design

It was a hospital-based case-control study conducted by the Department of Pulmonary Medicine, Indira Gandhi Medical College, Shimla between 1/6/2018 to 06/06/2019. All consecutive patients with stable COPD who met the inclusion criteria and agreed to provide written consent are included in the study. Patients who met the inclusion criteria for chronic obstructive lung disease (GOLD) criteria for a clinical definition of COPD[6]. The diagnosis of COPD was subsequently confirmed by post-bronchodilator spirometry. Post-bronchodilator spirometry was performed 15 minutes after administration of four doses of salbutamol sulfate (100 µg). Pre-and post-bronchodilator spirometry would be performed according to American Thoracic Society/European Respiratory Society recommendations[7].The severity of COPD would be done based on the GOLD criteria. Stable COPD would be defined by the absence of the requirement of antibiotic and/or oral corticosteroid therapy in the preceding 6 weeks. Age and sex-matched healthy individuals who are attending the outpatient department were included as controls[8].All consecutive patients with stable COPD attending the Pulmonary Medicine outpatients and partners of COPD cases were enrolled in the study.Since there is no Indian study, we included 100 subjects in each group. All participants provided written informed consent. This study was approved by the Institutional Research Ethics Committee, IGMC, Shimla.

II.(B) GOLD Criteria

Patients met the Global initiative for chronic Obstructive Lung Disease (GOLD) criteria for a clinical definition of COPD. The diagnosis of COPD was subsequently confirmed by post-bronchodilator spirometry. Post-bronchodilator spirometry was performed 15 minutes after administration of four doses of salbutamol sulfate (100 µg). Pre-and post-bronchodilator spirometry would beperformed according to American Thoracic Society/European Respiratory Society recommendations. The severity of COPD would be done based on the GOLD criteria. Stable COPD would bedefined by the absence of the requirement of antibiotic and/or oral corticosteroid therapy in the preceding 6 weeks. Age and sex-matched healthy individuals who are attending the outpatient department were included as controls. All consecutive patients with stable COPD attending the Pulmonary Medicine outpatients and partners of COPD cases were enrolled for the study between 1/6/2018 to 06/06/2019. Patients with **c**oexistent pulmonary disease (e.g.,interstitial pulmonary fibrosis, bronchiectasis, or granulomatous lung disease), malignancy, heart diseases, diabetes, long-term oral corticosteroid therapy, renal impairment, and COPD with acute exacerbation were excluded. Since there is no Indian study, we included subjects in two groups. This study was approved by the Institutional Research Ethics Committee, IGMC, Shimla,and the local Research Advisory Committee of Multidisciplinary Research Unit, IGMC, Shimla.

II.(C) Data Collection

Approval of the Institutional Ethical Committee of Indira Gandhi Medical College, Shimla was taken. Patients were enrolled in the study after written informed consent. The demographic profiles, detailed medical history, and risk factors were recorded. Non-smoker was defined as individuals who had never regularly smoked or who had smoked one or more cigarettes/Bidis a day for less than one year. Smokers are individuals who reported regularly smoking one or more cigarettes/bidis a day for at least one year. Former smokers are individuals who reported smoking one or more cigarettes a day regularly in the past but who had not smoked during the last year. Dyspnea was graded by the modified medical research council (mMRC) scale[9].A general physical examination and examination of the system were done. Spirometry was done in all cases as perthe ATS/ERS standards. Depending on the post-bronchodilator FEV_1 value, the severity of COPD was classified as per GOLD recommendation i.e., stage I (FEV_1 %≥80),stage II (50%≤FEV_1 %<80), stage III (30≤FEV_1 %<30-49) and stage IV (FEV_1 %<30). Blood samples were also drawn from all the subjects for serum S-RAGE level estimation.

II.(D) Statistical analysis

Continuous variables were reported as mean ± SD or median and interquartile range depending on the distribution of the variables. Categorical variables were recorded as counts and percentages. The difference in the level of S-RAGE between cases and controls was analyzed using appropriate statistical tests. The association between the severity of COPD and the levels of S-RAGE was analyzed by logistic regression analysis. Multivariate logistic regression analysis was done to adjust the effects of confounding variables/covariates. A 2-tail test for significance would be taken at <0.5.

II.(E) Chemicals and Materials: HumanS-RAGE ELISA kit purchased from *Biovendor-Laboratornimedicinaa.s. (Karasek 1767/1) 62100 Brno Czeck Republic*.

Reagents Supplied in Kit

KIT COMPONENTS	STATE	QUANTITY
Antibody-coated microtiter strips	ready to use	96 wells
Biotin-labeled antibody conc.(100x)	concentrated	0.13ml
Streptavidin –HRP conjugate	Ready to use	13 ml
Master standard	Lyophilized	1 vial
Quality control HIGH	Lyophilized	2 vial
Quality control LOW	Lyophilized	2 vial
Dilution buffer	ready to use	20 ml
Biotin –Ab diluents	ready to use	13 ml
Wash solution conc. (10x)	concentrated	100ml
Substrate solution	Ready to use	13ml
Stop solution	Ready to use	13ml
Product data sheet +certificate of analysis	---------	1 pc.

Other materials used for performing the assay, deionized (distilled) water, a Test tube for dilution samples, Glassware (graduated cylinders and bottle) for wash solution (dilution buffer), precision pipettes to deliver 10-100ul with disposable tips, multichannel pipette to deliver 10ul with disposable tips, absorbent material (e.g. paper towels)for blotting the microtitrate plate after washing, vortex mixer, orbital microplate shaker capable of approximate 300rpm, microplate washer, microplate reader with 450nm filter,preferably with reference wavelength 630nm. Software package facilitating data generation and analysis MPM6.0 incorporation.

II.(F) Principle (S-RAGE ELISA)

In the standards, quality controls and samples are incubated in microplate wells pre-coated with polyclonal antihuman serum S-RAGE antibody. After120 minutes of incubation and washing, a biotin-labelled polyclonal anti-humanS-RAGE antibody is added and incubated with capture S-RAGE for 60 minutes. After another washing, streptavidin–HRP conjugate is added. After 30 minutes of incubation and the last washing step, the remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by the addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of S-RAGE. A standard is constructed by plotting absorbance value versus S-RAGE concentrations of standards, and concentrations of unknown samples are determined using the standard curve.

II.(G) Preparations of Reagents

All reagents were brought to room temperature before use, prepared only the appropriate quantity of reagents for the test, and none of the components after the expiration date was used. Prepared the working solution of labelled antibody solution by adding 1 part antibody concentrate (100x) with 99 parts biotin–Ab diluents (10ul of biotin-labelled antibody concentrate (100x) +990 ul of biotin-Ab diluents for 1 strip (8 wells). Dilute wash solution concentrate (10x) tenfold distilled water to prepare a 1x working solution.

II.(H) Preparation of Samples

Samples were collected from human S-RAGE in serum and plasma (EDTA).Diluted sample 3x with dilution buffer just before the assay, e.g., 50ul of sample +100ul of dilution buffer for singlets, or preferably 100ul of sample +200ul of dilution buffer for duplicates, mix well (not to foam) by vertexing.

II.(I) Assay Procedure

Pipetted out 100ul of standards, quality controls,dilution buffer (=blank), and diluted samples, preferably in duplicates, into the appropriate wells.Incubate the plate at room temperature (25℃)for 2 hours shaking at 300rpm on an orbital microplate shaker.Washed the well 5 times with the

wash solution (0.35ml per well). After the final wash inverts and taps the plate strongly against a paper towel. Added 100ul of biotin-labelled antibody solution to each well.Incubate the plate at room temperature (i.e., 25℃) for 1 hour, shaking at 300rpm on an orbital microplate shaker.Wash the well 5 times with wash solution (0.35ml per well). After the final wash,invert and tap the plate strongly against a paper towel. Add 100ul of streptavidin–HRP conjugate to each well. Incubate the plate at room temperature (i.e., 25℃) for 30 minutes shaking at 300rpm on an orbital microplate shaker. Wash the well 5 times with wash solution (0.35ml per well). After the final wash, invert and tap the plate strongly against a paper towel. Add 100ul of substrate solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covered the plate with e.g., aluminum foil is recommended. Incubate the plate for 10 minutes at room temperature. The incubation time may be extended (up to 20 minutes) if the reaction temperature is below 20℃. Do not shake the plate during the incubation. Stopped color development by adding 100ul of stop solution. Determined the absorbance of each well using a microplate reader set at 450nm preferably with the reference wavelength set at 630nm (acceptable range:550-650nm) subtract readings at 630nm (550-650nm) from the readings at 450nm. The absorbances recorded read within 5 minutes of the last step.

III. Results

A total of 161 subjects were included, out of which, 89 were grouped as cases and 72 were grouped as controls. The mean age of the case was 62.00 ± SD10.455 and the mean age of controls was 49.88 ± SD7.526. The mean RAGE in cases (n=89) was 896.78 (416.13) and in controls (N=71) was 783.07 (367.99). The serum RAGE levels were lower in controls with a mean difference of 113.70(95% CI -238.01, 10.60) with t (-1.81) df =158, p= 0.07, but this was found to be statistically no significant. However, when mean RAGE levels were compared between controls and four grades of increasing severity of COPD cases, the ANOVA test F (3.09, p-value =0.02) was found to be statistically significant. On post-hoe comparisons using LSD, the difference was found to be statistically significant only between controls and grade 3 severity of COPD with a mean difference of - 270.19(95% CI -429.43, -110.94p-value = 0.001.

Table1: Mean S-RAGE level in case and controls:

Group Statistics					
Patient status		N	Mean	Std. Deviation	Std. Error Mean
RAGE	Case	89	896.77	416.13	44.11021
	Controls	71	783.07	367.98	43.67190

Table2.(a) Mean S-RAGE level in cases and grades of COPD

Subject/grades of COPD	Mean	N	Std. Deviation
Controls	779.87	73	365.74
Mild	725.22	5	235.97
Moderate	802.14	27	385.57
Severe	1050.06	34	450.38
Very severe	833.09	21	384.21
Total	846.32	160	398.33

Table 2.(b) Mean difference & 95% CI

CONTROL	CASES	Mean Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
0	Mild	54.65	.76	-299.89	409.20
	Moderate	-22.26	.80	-195.01	150.49
	Severe	**-270.19***	**.00**	**-429.43**	**-110.94**
	Very severe	-53.21	.58	-243.13	136.70

Table 3. Independent Sample Test

Criteria		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Age	Equal variances assumed	7.57	.007	8.30	159	.000	12.237	1.47	9.32	15.14
	Equal variances not assumed			8.59	156.83	.000	12.237	1.42	9.42	15.05
Gender	Equal variances assumed	2.18	.14	.74	159	.457	.050	.07	-.08	.18
	Equal variances not assumed			.74	146.91	.461	.050	.07	-.08	.18
Smoking duration	Equal variances assumed	.002	.96	21.22	159	.000	33.06	1.56	29.98	36.14
	Equal variances not assumed			21.18	151.03	.000	33.06	1.56	29.981	36.14
Smoking status	Equal variances assumed	.27	.60	11.44	159	.000	1.09	.096	.905	1.28
	Equal variances not assumed			11.11	128.93	.000	1.09	.098	.89	1.28
POSTFEV1	Equal variances assumed	1.03	.31	.008	86	.994	.0026	.31	-.62	.63
	Equal variances not assumed			.005	1.02	.997	.0026	.54	-6.64	6.64
POSTBESTFEV1	Equal variances assumed	.49	.48	.940	86	.350	12.25	13.02	-13.64	38.15
	Equal variances not assumed			.617	1.02	.646	12.25	19.84	-228.76	253.2
POSTBESTFVC	Equal variances assumed	.10	.74	-.243	86	.809	-.67	2.79	-6.23	4.88
	Equal variances not assumed			-1.25	6.57	.252	-.67	.54	-1.97	.61
POSTBESTFVC	Equal variances assumed	1.04	.31	-.12	86	.906	-1.88	15.96	-33.61	29.84
	Equal variances not assumed			-.23	1.20	.849	-1.88	8.07	-70.97	67.19
EDUCATION STATUS	Equal variances assumed	.29	.58	-7.49	159	.000	-1.35	.180	-1.70	-.99
	Equal variances not assumed			-7.47	150.32	.000	-1.34	.181	-1.70	-.99

Table-4.Linear Regression

(Tests Between-Subjects Effects: Dependent Variable: S-RAGE)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2192360.925[a]	5	438472.185	1.161	.331
Intercept	758282.715	1	758282.715	2.007	.159
Age	286950.705	1	286950.705	.760	.385
Gender	37433.156	1	37433.156	.099	.753
Case/Controls	709637.563	1	709637.563	1.878	.173
COPD severity grading	200488.787	1	200488.787	.531	.467
Smoking duration	248217.323	1	248217.323	.657	.419
Error	58559767.392	155	377804.951		
Total	186411817.661	161			
Corrected Total	60752128.317	160			
a. R Squared = .036 (Adjusted R Squared = .005)					

IV. Discussion

The sample size was not calculated. An adequate number of the samples could not be collected due to the lesser number of volunteer participants as controls in this study. A recent review on the potential role of S-RAGE as a biomarker for COPD described several limitations of S-RAGE [10,11] including the lack of a clinically validated assay, leading to a rather wide disparity in absolute values found between studies. The current study used a fully validated S-RAGE assay following FDA guidelines[12] . We agree that this should be taken into account in larger studies than the current one. Another limitation is the scarce available information on the therapeutic modulation of RAGE signaling in human disease.

V. Conclusion

We did not observe any significant differences in the mean S-RAGE level in this study. It was a pilot study with a limited number of subjects. Moreover, the long storage of samples of more than 3 months before performing the test might have an impact on the final value. Therefore, we could not comment on the relevance of this study.

VI. Future perspectives

However, in the future we will plan a better-designed study to elucidate the role of S-RAGE in COPD patients.

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