Tumor necrosis factor receptor 2 as a possible marker of COPD in smokers and ex-smokers

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Introduction: Oxidative stress and systemic inflammation are higher in smokers and patients with COPD; however, markers that may help differentiate between smokers and patients with COPD have not yet been identified. We hypothesized that tumor necrosis factor-alpha receptor (TNFR) and soluble form of the receptor for advanced glycation end products (sRAGE) can be indicators of COPD in asymptomatic patients.

Patients and methods: We evaluated 32 smokers (smoking history >10 pack-years), 32 patients with mild/moderate COPD (smokers and ex-smokers), and 32 never smokers. Concentrations of C-reactive protein (CRP), interleukin (IL)-6, TNFR1 and TNFR2, advanced glycation end products (AGEs), and the sRAGE were measured in serum.

Results: There were higher CRP and AGEs concentrations in smokers and in patients with COPD (P<0.001 and P=0.01, respectively) compared to controls, without statistical difference between smokers and patients with COPD. Concentrations of sRAGE, IL-6, and TNFR1 did not differ between study groups. TNFR2 was significantly higher in patients with COPD than in smokers (P=0.004) and controls (P=0.004), and the presence of COPD (P=0.02) and CRP (P=0.001) showed a positive association with TNFR2. Positive associations for smoking (P=0.04), CRP (P=0.03), and IL-6 (P=0.03) with AGEs were also found. The interaction variable (smoking x COPD) showed a positive association with IL-6.

Conclusion: Our data suggest that TNFR2 may be a possible marker of COPD in asymptomatic smokers and ex-smokers. Although smokers and patients with early COPD presented other increased systemic inflammation markers (eg, CRP) and oxidative stress (measured by AGEs), they did not differentiate smokers from COPD.

Keywords: smoking, chronic obstructive pulmonary disease, inflammation mediators, oxidative stress

Introduction
Oxidative stress and systemic inflammation are increased in smokers and patients with COPD, are responsible for disease progression, and seem to be associated with the severity of COPD.¹⁻³ However, whether specific inflammatory and oxidative stress blood markers are associated with systemic response to smoke or with initial stages of COPD are currently unknown.

Tobacco smoke can directly or indirectly induce the formation of advanced glycation end products (AGEs) via oxidative stress.⁴⁻⁵ AGEs are products of glycation and oxidation of proteins and lipids.⁶ Toxic glycation products are present in aqueous extracts of tobacco and can rapidly react with proteins to form AGEs or products such as methylglyoxal and glyoxal (AGE precursors), found in tobacco smoke.⁷ AGEs have been shown to be increased in smokers and patients with COPD.⁸⁻¹⁰
The AGES can bind to specific receptors such as the receptor for advanced glycation end products (RAGE) – a transmembrane receptor that belongs to the immunoglobulin family (mRAGE).\textsuperscript{11,12} This interaction and mRAGE itself activate inflammatory signaling pathways such as the nuclear factor kappa B (NFkB) and can trigger proinflammatory cytokine production, leading to inflammation in various organs.\textsuperscript{11–13} In contrast, soluble forms of the receptor (sRAGE), shed from the plasma membrane via proteolytic cleavage by matrix metalloproteinases, have been shown to be protective in preventing signaling through mRAGE.\textsuperscript{14} Another mechanism of NFkB activation is through tumor necrosis factor alpha (TNF-\(\alpha\)) receptors (TNFR); both TNFR1 and TNFR2 activation can lead to stimulation of nuclear factor NFkB in a variety of cells.\textsuperscript{15,16} Therefore, AGE, RAGE, and TNFR stimulate NFkB and can trigger the production of proinflammatory cytokines leading to inflammation in smokers and patients with COPD.\textsuperscript{11–15,17}

The role of sRAGE and TNFR in smokers and patients with COPD is unclear.\textsuperscript{18–22} Some studies have shown lower sRAGE values in patients with COPD than controls, a positive association between sRAGE and forced expiratory volume in 1 second (FEV\(_1\)), and a negative association between sRAGE concentrations and the presence of COPD – suggesting a protective role for sRAGE.\textsuperscript{18,19} The presence and role of TNFR1 and TNFR2 in smoking and COPD have also received little attention.\textsuperscript{21,22} A study by D’Hulst et al suggest that both receptors contribute to COPD pathogenesis, but TNFR2 seems to be more actively involved in the development of inflammation and airflow obstruction.\textsuperscript{22}

Because a higher concentration of sRAGE may protect smokers from the evolution of COPD, and a higher concentration of TNFR2 seems to be associated with the development of airway obstruction, we hypothesized that serum concentrations of these mediators can be indicators of COPD in asymptomatic smokers. Therefore, our aim was to evaluate serum markers of oxidative stress (AGEs and sRAGE) and systemic inflammatory [TNFR1, TNFR2, interleukin (IL)-6, and C-reactive protein (CRP)] mediators to verify whether they could distinguish smokers from patients with COPD. To our knowledge, this is the first study evaluating the role of TNF receptors as a marker of COPD in smokers and ex-smokers.

**Patients and methods**

From March 2013 to November 2014, 157 individuals (never smokers, smokers, and patients with mild/moderate COPD) from the Pulmonology Outpatient and Smoking Cessation units at Botucatu Medical School were evaluated, and 96 patients were included in a cross-sectional study. The other 61 patients were excluded according to exclusion criteria (Figure 1). Never smokers, active smokers (smoking history \(>10\) pack-years), or patients with mild/moderate COPD (smoker or ex-smoker, post-bronchodilator FEV\(_1\)/forced vital capacity (FVC) \(<0.70\) and FEV\(_1\) \(>50\%\))\textsuperscript{23} were consecutively selected. Exclusion criteria included primary diagnosis of other respiratory diseases such as asthma, restrictive disorders (tubercular sequelae, interstitial fibrosis), sleep apnea/hypopnea syndrome, or lung cancer. In addition, a primary diagnosis of unstable angina, congestive heart failure (New York Heart Association classes III or IV), or other chronic diseases, such as uncontrolled diabetes mellitus, kidney or liver failure, and cancer, were also grounds for exclusion. Patients with mild/moderate obstruction reported they were asymptomatic and not on maintenance medications and did not report exacerbation in the previous year. After initial screening, those included were evaluated over 3 days in the same week.

Sample size was calculated for a multiple linear regression effect size of 0.15, with an estimated multiple correlation squared of 0.50 and the addition of five predictors in the model (G Power 3.1.3).

Participants were made aware of the proposed study procedures and provided written informed consent for study participation. All procedures were approved by the Botucatu Medical School University Hospital Research Ethics Committee (IRB approval number 4415-2012).

**Pulmonary function, pulse oximetry, smoking status, and dyspnea score**

Pre and post-bronchodilator spirometry were performed using a KOKO spirometer (Ferrari KOKO Louisville, CO 80027, USA) according to criteria set by the American Thoracic
Society (ATS). 24 FEV1 values were expressed in liters and as percentages of FVC and reference values. 25 Pulse oximetry (SpO2) was assessed using an Onyx oximeter (Model 9500 Oximeter; Nonin Medical Inc., Minneapolis, MN, USA) while the patients were breathing room air. Smoking history and current smoking status were investigated and complemented by assessing nicotine dependence intensity (Fargestrom Test). 26 Confirmation of smoking status was performed by measuring carbon monoxide (CO) in exhaled air by a standardized technique (Micro CO Meter, 6Cardinal Health, England, UK). A value of exhaled CO > 6.0 ppm was considered to indicate active smoking. 27, 28 Dyspnea was evaluated by the Borg Dyspnea Scale Score. 29

**Nutritional assessment**

Body weight and height were measured by the Filizola® scale, and the body mass index ([BMI] = weight [kg]/height [m2]) was calculated. Body composition was evaluated by bioelectrical impedance (BIA 101; RJL Systems, Detroit, MI, USA) according to the European Society for Parenteral and Enteral Nutrition guidelines. 30 Fat-free mass (FFM, kg) was calculated using a group-specific regression equation developed by Kyle et al. 30 The FFM index (FFMI = FFM/height2) was also calculated. Lean body mass depletion was defined as an FFMI < 15 kg/m2 for women and < 16 kg/m2 for men. 31

**Blood sampling and analysis of oxidative stress and systemic inflammation: AGEs, sRAGE, IL-6, CRP, and TNFR1 and TNFR2**

Fasting peripheral blood was collected (08.00 hours) and serum stored at −80°C until analysis. IL-6, TNFR1, TNFR2, AGEs, and sRAGE (lower detection limit of 0.70, 0.16, 0.2, 0.5, and 1.25 pg/mL, respectively) were measured in duplicate by high-sensitivity commercial kits using enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s instructions (BioSource International Inc., CA, USA). AGEs represent a class of covalently modified proteins generated by oxidative and non-oxidative pathways, involving sugars or their degradation products. Further, AGEs exert their damaging and pro-inflammatory effects by activating signaling cascades via specific receptors named RAGE. 32 Soluble RAGE (sRAGE), a free form of RAGE, acts as a suppressor of RAGE activation and signaling, because it plays its role as a decoy for RAGE ligands in order to prevent signal transduction and to avoid the effects of the RAGE-binding interaction. Readings were obtained in a Spectra Max 190 microplate spectrophotometer (Molecular Devices®, Sunnyvale, CA, USA). CRP was measured in duplicate by high-sensitivity particle-enhanced immunonephelometry (Cardio-Phase, Dade Behring Marburg GmbH, Marburg, USA), with a lower detection limit of 0.007 mg/L. All measurements were performed after the final evaluation using kits with the same lot number to avoid measurement bias.

**Statistical analysis**

Descriptive statistics were used to describe the features of all participants. Mean ± SD or medians and interquartile range (25%–75%) were used depending on data distribution. Categorical variables were expressed as percentages. Chi-square was used to compare the values of categorical variables.

Multiple linear regression analysis was used to assess associations between COPD (absence = 0, presence = 1), smoking status (absence = 0, presence = 1), interaction variable (smoking × COPD), exercise capacity, health status, body composition, systemic inflammatory status (CRP, IL-6, TNFR1, and TNFR2), and oxidative stress (AGEs and sRAGE) in the three study groups. Setting variables in the model were: sex and age. Collinearity was prevented by deleting a variable showing correlation. The level of significance was set at 5%. All analyses were performing using IBM SPSS Statistics 22 and Sigma Plot 11.0.

**Results**

Table 1 shows the general characteristics of the 96 subjects enrolled in the study and the comparison between never smokers, smokers, and patients with mild/moderate COPD. Patients with COPD presented reduced lean body mass index; however, the proportion of depleted and non-depleted subjects did not differ between groups. In addition, 44% of patients with COPD were active smokers. Smoking history was higher in the COPD group compared to smokers and never smokers. Spirometry variables were lower in patients with COPD versus smokers as well as in smokers versus never smokers.

Figure 2 shows AGEs concentrations in the three groups. Smokers (P<0.001) and patients with COPD (P<0.001) showed higher values than never smokers. There was no statistically significant difference between smokers and patients with COPD. sRAGE concentrations did not differ between groups (P=0.92).
Systemic inflammatory status assessed by TNFR1 concentrations did not differ between groups [never smokers: 119.2 (104.3–139.5) pg/mL; smokers: 122.8 (103.7–136.7) pg/mL; COPD I/II: 134.0 (115.7–210.3) pg/mL, \( P = 0.07 \)]; however, patients with COPD showed higher TNFR2 values than smokers and never smokers (Figure 3).

Smokers and patients with COPD presented higher CRP values than never smokers. However, there was no statistically significant difference between smokers and patients with COPD (Figure 4). Concentrations of IL-6 did not differ between groups [never smokers: 1.2 (0.9–3.4) pg/mL; smokers: 1.9 (1.5–3.6) pg/mL; COPD I/II: 2.8 (1.3–4.7) pg/mL, \( P = 0.07 \)].

In the analysis of predictors of oxidative stress and systemic inflammation, smoking presented a positive association with AGEs and CRP, whereas the presence of COPD and FEV\(_1\) % predicted values were associated with TNFR2; moreover, there was an association between CRP and AGEs, TNFR1 and -2, and also between AGEs and IL-6 (Table 2). We included the interaction variable (smoking \(\times\) COPD) in all models (data not shown); however, the interaction variable showed association only with IL-6 (Table 2).

### Table 1 General characteristics of individuals in the study

| Variables                      | Never smokers \((n = 32)\) | Smokers \((n = 32)\) | COPD I/II \((n = 32)\) | \(P\)-value |
|--------------------------------|---------------------------|---------------------|-------------------------|-------------|
| Age (years)                    | 49.5 (46.0–58.5)\(^a\)    | 53.0 (51.0–55.0)\(^a,b\) | 64.5 (58.0–74.5)\(^a\) | \(<0.001\) |
| Sex (M/F)                      | 9/23                      | 7/25                | 12/20                   | 0.75        |
| BMI (kg/m\(^2\))              | 26.8±4.5                  | 25.8±3.6            | 24.4±4.2                | 0.07        |
| FFMI (kg/m\(^2\))             | 23.3 (20.3–29.8)\(^a\)    | 19.8 (17.4–24.3)\(^a,b\) | 18.4 (16.7–22.5)\(^a\) | 0.008      |
| Current smokers (n/%)          | 0/0                       | 32/100              | 1/44                    | \(<0.001\) |
| Smoking history (pack-years)   | 0\(^a\)                   | 38.0 (21.5–50.7)\(^b\) | 55.5 (39.5–77.5)\(^c\) | \(<0.001\) |
| CO (ppm)                       | 0\(^a\)                   | 10.0 (8.2–15.7)\(^b\) | 0.0 (0–12.0)\(^c\)      | \(<0.001\) |
| FVC (L)                        | 102.0 (93.0–110.0)         | 97.0 (90.0–103.0)    | 93 (79.5–103.0)         | 0.06        |
| FEV\(_1\) (L)                  | 2.9±0.4\(^a\)             | 2.3±0.5\(^b\)       | 1.8±0.5\(^c\)           | \(<0.001\) |
| FEV\(_1\) (% predicted)       | 100.8±12.9\(^a\)          | 91.1±14.5\(^b\)     | 77.4±17.0\(^c\)         | \(<0.001\) |
| FEV\(_1\)/FVC                 | 0.81 (0.79–0.85)\(^a\)    | 0.78 (0.73–0.83)\(^b\) | 0.63 (0.59–0.67)\(^c\) | \(<0.001\) |
| \(\text{SpO}_2\) (%)          | 96.0 (95.0–97.5)\(^a\)    | 96.0 (95.0–97.0)     | 95.0 (93.0–97.0)         | 0.16        |
| Borg Dyspnoea Scale score      | 0 (0–0)                   | 0 (0–0)             | 0 (0–0)                 | 1.00        |

**Notes:** Values expressed as mean ± standard deviation or median (quartile 1 – quartile 3); different letters and bold values indicate statistically significant difference. \(P\leq0.05\), \(\chi^2\), ANOVA and Tukey or Kruskal–Wallis and Dunn’s tests.

**Abbreviations:** COPD I/II, mild/moderate; GOL, Global Initiative for Chronic Obstructive Lung Disease; kg, kilogram; m, meter; BMI, body mass index; FFMI, fat-free mass index; CO, carbon monoxide; FEV\(_1\), forced expiratory volume in 1 second; FVC, forced volume capacity; \(\text{SpO}_2\), pulse oximetry; M, male; F, female; ANOVA, analysis of variance.

**Figure 2** AGEs concentrations in never smokers, smokers, and patients with mild/moderate COPD; \(P<0.05\) (Kruskal–Wallis and Dunn’s tests).

**Abbreviations:** AGEs, advanced glycation end products; NS, no significance.

**Figure 3** TNFR2 concentrations in never smokers, smokers, and patients with mild/moderate COPD; \(P<0.05\) (Kruskal–Wallis and Dunn’s tests).

**Abbreviations:** NS, no significance; TNFR2, tumor necrosis factor alpha receptor 2.
Discussion

The main result of this study indicates that TNFR2 may be a possible marker of COPD in asymptomatic smokers and ex-smokers because its concentration is different between smokers and asymptomatic patients with COPD. Elevated oxidative stress measured by AGEs, and systemic inflammation measured by CRP and IL-6, did not differ between patients with COPD and smokers. sRAGEs that may protect smokers from COPD did not differ between study groups.

Patients with COPD showed increased TNFR2 concentrations in comparison to smokers and never smokers, and a positive association between COPD presence and TNFR2 was found. No previous study with a similar sample and design was identified in the literature. However, rats with TNFR1 and TNFR2 deficiency,22 after 6 months of exposure to tobacco, showed increased concentrations of both receptors; however, the relative increase in TNFR2 was higher than that of TNFR1. Vernooij et al showed higher TNFR2 concentrations in moderate COPD (FEV1% predicted =55±14) compared to healthy smokers (FEV1% predicted =98±16); however, they did not show any correlation between the degree of airflow limitation and TNFR2 concentration.33 The actual involvement of TNF receptors in the pathogenesis of COPD remains unknown and the mechanism that defines the dominant effect is still unclear. D’Hulst et al suggest that both TNFRs contribute to the pathogenesis of COPD, but that TNFR2 is the most active receptor in the development of inflammation and emphysema.22

Although most of the biological effects induced by TNF-α have been attributed to TNFR1, TNFR2 has been reported to stimulate TNF-α-induced T-cell proliferation.34 It appears that TNFR2 induces activation of NFκB signaling, differentially, under specific inflammatory conditions with assistance from some co-factors.15 According to D’Hulst et al, observations of reduced T-lymphocyte activation in the lungs of mice lacking TNFR2 and high lymphocyte numbers in bronchoalveolar lavage fluid of TNFR1 knockout (KO) mice exclusively expressing TNFR2 then further underline the importance of the R2 receptor in T-cell proliferation.32 NFκB activation can also be induced by TNFR2 that can be activated by TNFα to further activate NFκB.15 Therefore, the present data confirm the importance of this marker in COPD pathogenesis and that, although both TNFRs are involved in long-term cigarette smoke-induced pulmonary inflammation and emphysema, the contribution of TNFR2 is most prominent.

The increase of AGEs in patients with COPD is consistent with results from previous studies,9,10 and we did not
find differences in sRAGE concentration between groups. The Tesra study, evaluating only patients with COPD, did not identify differences in sRAGE concentrations between COPD II and III; however, a negative association was identified between sRAGE and emphysema after adjustment for spirometry and demographic variables. According to the data from the Tesra study, a lower sRAGE concentration may be associated with emphysema, regardless of disease stage.\textsuperscript{35} We can speculate that an sRAGE deficiency is involved in the pathogenesis of emphysema and could possibly provide targets for new treatments.\textsuperscript{36} However, Smith et al showed that circulating sRAGE is lower in severe and very severe COPD [FEV\textsubscript{1\% predicted} =37 (23–49)] and their concentrations present a correlation to the degree of airflow limitation.\textsuperscript{19} Patients with mild/moderate COPD, evaluated in the present study, may have associated lower intensity emphysema and inflammation, and our data suggest that sRAGE does not differentiate smokers from patients with COPD. However, large-scale studies are needed to determine whether sRAGE is a marker of COPD or is involved in the pathogenesis of emphysema.

Smokers and patients with COPD showed higher CRP concentrations than never smokers. Our data are consistent with recent studies.\textsuperscript{21,37–39} Although IL-6 concentrations did not differ between groups, we identify a positive association between AGEs and IL-6/CRP, which is in agreement with studies showing the effects of AGEs on cytokine production and suggests a dose-dependent association between AGEs and concentrations of IL-6 and CRP.\textsuperscript{40,41} We found a positive association between the interaction “smoking and COPD” with IL-6 concentrations. This result has not been previously described, although a higher systemic concentration of IL-6 in smokers and COPD is well established.\textsuperscript{42–44} Our study has some potential limitations. A recent study\textsuperscript{44} showed that symptoms are more prevalent in current or ex-smokers with preserved pulmonary function than in healthy controls who had never smoked. The authors showed respiratory symptoms in 50\% of current or ex-smokers with preserved pulmonary function evaluated by the COPD Assessment Test (CAT).\textsuperscript{45} We did not measure respiratory symptoms using the CAT; however, we evaluated dyspnea by the Borg Dyspnea Scale score, and no difference was found between groups. In addition, our conclusion is based on a cross-sectional study and indicates the need for long-term follow-up studies of inflammatory mediators in smokers to confirm the present findings.

**Conclusion**

Smokers and patients with early COPD present with increased systemic inflammation, measured by TNFR2 and CRP, and oxidative stress, measured by AGEs. TNFR2 was the only indicator of inflammation that differed between smokers and patients with COPD. The increase in TNFR2 suggests greater inflammation intensity in individuals with COPD. Therefore, we suggest that TNFR2 is more sensitive in identifying increased inflammation in the early stages of COPD, and could be a possible marker of COPD in asymptomatic smokers and ex-smokers.

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**Disclosure**

The authors report no conflicts of interests in this work.

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