

TOTAL ANTIOXIDANT CAPACITY : CORRELATION WITH OTHER ANTIOXIDANTS AND CLINICAL UTILITY OF THEIR LEVELS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Aims: Oxidative stress is one of the major patho-physiologic hallmarks in the development of COPD and lung is protected against this by enzymatic and non-enzymatic antioxidants. Total antioxidant activity (TAC), may give more relevant biological information about the individual's overall antioxidant status compared to individual components. This study aims to evaluate correlation between TAC and other antioxidants bilirubin, albumin, urate & ceruloplasmin (CP) and explore the clinical utility of their levels in diagnosis of COPD.

Study design: Comparison study.

Place and Duration of Study: Cardio Thoracic Centre Pune and Department of Biochemistry, AFMC, Pune during Dec 2010 to Aug 2012.

Methodology: Study comprises of 86 normals as controls group and 86 confirmed COPD patients as COPD group. CP was estimated by patented kinetic method of Somani & Ambade, while all other analytes were estimated by using commercially available kits.

Results: CP was significantly higher in COPD patients (1392.8 ± 281.6 IU/L) as compared to controls (1006.2 ± 236.1 IU/L) while levels of albumin, urate and TAC were significantly lower in COPD patients. The levels in COPD and controls are albumin: 3.8 ± 0.5 & 4.3 ± 0.4 g/dL; urate: 4.4 ± 1.3 & 5 ± 1.3 mg/dL; TAC 27.7 ± 6.9 & 36 ± 8.2 mmolTE/L respectively. No appreciable correlation was noticed between any individual antioxidant and TAC.

Conclusion: CP and TAC showed statistically significant differences between controls and COPD patients and may have clinical utility in the management of COPD. However, the estimation of TAC is to be done with extreme care.

Keywords: chronic obstructive pulmonary disease, antioxidants, oxidative stress, ceruloplasmin, ferroxidase, albumin, urate.

1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is defined as a condition in which there is chronic obstruction to airflow due to chronic bronchitis and /or emphysema. It is the only chronic disease that has shown progressive upward trend in both mortality & morbidity and is expected to be the third leading cause of death by 2020 [1, 2].

Lung with a large surface area is continuously exposed to endogenous or exogenous oxidants [3, 4, 5] and is protected against oxidative challenge by well-developed enzymatic and non-enzymatic antioxidant systems. Enzymatic antioxidants include superoxide dismutase (SOD), ceruloplasmin, glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx), catalase etc [6, 7], while non-enzymatic antioxidants include glutathione (GSH), ascorbate, urate, α -tocopherol, bilirubin, albumin and lipoic acid. Increased pulmonary exposure to oxidative source and/or reduced antioxidant defenses leads to an imbalance known as oxidative stress [8]. Oxidative stress has important implications on several events

28 of lung physiology and for the pathogenesis of COPD. These include activation of the
29 molecular mechanisms that initiate lung inflammation [9], oxidative inactivation of
30 antiproteases and surfactants, mucus hypersecretion, membrane lipid peroxidation, alveolar
31 epithelial injury, remodeling of extracellular matrix, and apoptosis [10]. In the COPD patients
32 there also may be a reduction in the endogenous antioxidants as a result of reduction in a
33 Nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that regulates
34 several antioxidant genes. Suboptimal NRF2-regulated antioxidant defense in COPD patient
35 lungs accounts for the greater oxidant-antioxidant imbalance leading to COPD [11].

36 Ceruloplasmin, the major serum inhibitor of lipid peroxidation [12], has been documented as
37 a main extracellular antioxidant in serum [13], inhibiting ferrous ion stimulated lipid
38 peroxidation [14], preventing lung injury, and an abnormality of ceruloplasmin oxidative
39 inhibition could be involved in the pathogenesis of COPD [15]. The antioxidant action has
40 been proposed as a crucial function of ceruloplasmin. Among various substrates the highest
41 oxidizing activity has been found for Fe^{+2} and thus the alternative name of ferroxidase
42 (EC.1.16.3.1) has been proposed [16, 17]. Ceruloplasmin in this study was therefore
43 estimated by its ferroxidase activity, by virtue of which it plays an antioxidant role in COPD.

44 Urate has been found to be an excellent scavenger of singlet oxygen [18] and protects
45 erythrocyte membrane from peroxidation [19]. Urate was effective in preventing lipid
46 peroxidation at concentrations considerably below those normally found in plasma. Urate
47 acts either by directly scavenging active oxygen species [20, 21] or by binding radical-
48 generating transition metals [22]. Antioxidant activity of urate is an important aspect of its in-
49 vivo function [19, 23]. Albumin has been considered not only as an antioxidant, but as the
50 major circulating antioxidant in plasma known to be exposed to continuous oxidative stress
51 [4, 24, 25]. Ames et al [26, 27] demonstrated that antioxidant effect of bilirubin exceeds that
52 of vitamin E towards lipid peroxidation and levels of serum bilirubin are high enough to
53 account substantially towards total antioxidant capacity of serum [28, 29].

54 The sum of endogenous and exogenous antioxidants represents the total antioxidant activity
55 (TAC). Measuring TAC can provide information on an individual's overall antioxidant status,
56 which may include those antioxidants not yet recognized or not easily measured [30].
57 Cooperation of all the different antioxidants provides greater protection against attack by
58 reactive oxygen or nitrogen radicals, than any single compound alone. Thus, the overall
59 antioxidant capacity may give more relevant information on an individual's overall antioxidant
60 status compared to that obtained by the measurement of individual components.

61 To this end, we estimated the levels of TAC, bilirubin, albumin, urate and CP in serum of
62 COPD patients and compared the levels with that in normal subjects, studied correlation of
63 TAC with other antioxidant and their utility in diagnosis of COPD.

64 2. MATERIAL AND METHODS

65 2.1 Subject selection and Procedure

66
67 i) Group I or Controls: Comprises of 86 normal, age and sex matched individuals. Subjects
68 for this group were taken from normal healthy individuals coming to Department of
69 Biochemistry for routine check up and annual medical board and other volunteers.

70 ii) Group II or COPD patients: Comprises of 86 patients of COPD diagnosed as per GOLD
71 guidelines (GOLD report 2011). For this group subjects were selected from the patients
72 reporting with symptoms of COPD to respiratory OPD of Cardio-Thoracic Centre, Pune,
73 between Dec 2010 to Aug 2012.

Both the groups were further equally divided into smoker and nonsmoker group (43 smokers and 43 nonsmokers). The clinical assessment at the time of presentation, in terms of signs and symptoms and relevant investigation were recorded in a proforma. Informed consent was taken from the subjects before drawing their blood specimen.

2.2.1 Exclusion criteria for control and COPD patients:

Patients with a diagnosis of asthma, coronary artery disease (CAD), diffuse parenchymal lung disease, patients on long term oxygen therapy or unable to perform spirometry were excluded from the Group II. Similarly individuals with any respiratory or lung disease or past history of any lung/respiratory problem/disease were excluded from Group I.

All patients were administered a questionnaire to collect demographic data (name, age, sex, address) duration of symptoms, history of allergic symptoms (history of atopy, nasal allergies), episodic or progressive symptoms, chest examination (wheeze), radiological investigations (CXR, CT Scan), basis of initial diagnosis (ascertained from follow up book) and whether spirometry was performed for initial diagnosis. From the questionnaire an initial clinical impression of the diagnosis was made. Subjects were asked not to use short acting bronchodilators (e.g. beta-agonist salbutamol or anti cholinergic agent ipratropium bromide) within 04 hours of testing, long acting bronchodilators (salmeterol or formoterol) for 12 hours prior to testing and oral therapy with theophyllines or slow release B-agonists for 24 hrs prior to the test. All patients underwent spirometry for confirmation of the diagnosis of COPD. The presence of post bronchodilator FEV1/FVC <0.70 was used to confirm presence of persistent airflow limitation and thus of COPD [31].

Smoking index was used to quantify smoking exposure among the study subjects. Smoking index is defined as number of cigarettes or bidis smoked per day multiplied by the total duration of smoking in years. It is a better index to quantify smoking in Indian context as compared to pack years [32].

2.2 Subject collection and storage

Fasting venous blood samples, about 5 mL, were collected in a plain sterile gel vacutainer from all the subjects. Serum was separated by centrifuging the blood sample at 2000 rpm for 5 minutes. The supernatant was separated into micro-centrifuge vials/Eppendorf tubes. Estimation of CP, albumin, bilirubin, and urate was done immediately without any storage while remaining aliquots were stored frozen at -70°C in deep freezer until analysed for TAC.

2.3 Estimation of Ceruloplasmin

Ceruloplasmin (CP) was estimated by indigenous patented kinetic method of Somani and Ambade (Govt of India Patent No192356), [33] on fully automated analyzer XL 600 from Transasia Mannheim GmbH, Germany, using the reagents as below: Reagent-1: Chromogen (0.5 mmol/L) was made by dissolving 159.65 mg of norfloxacin in 1000 mL of acetate buffer (0.45 mol/L, pH 5.4) containing 0.2% Triton X-100. Reagent-2: Substrate (2.04 mmol/L) was made by sequentially dissolving, 320 mg of DTT and 800 mg of ferrous ammonium sulphate, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, in 1000 mL of deionised water (DIW). The analyser was set at following parameters: Assay Type: Rate A; Wavelength: Primary = 376nm, Secondary = 0nm; Assay Points 0, 0, 15, 18 cycles; Sample volume: 10µL; Reagent 1: 200µL; Reagent 2: 30µL; Factor 2010. This estimation is based on the principle that enzymatic oxidative property, that is, ferroxidase property oxidizes ferrous to ferric ion which then complexes with chromogen. The formation of this complex is measured kinetically at

118 376nm. The ferroxidase activity in serum was calculated from the factor and displayed
119 directly by the fully automated analyser in IU/L.

120 2.4 Estimation of albumin, bilirubin, urate

121 Albumin, bilirubin & urate were estimated using kits from Transasia on fully automated
122 analyzer XL 600 from Transasia Mannheim GmbH, Germany. Albumin was estimated by
123 BCG Dye Method, End Point Code No: 120223; Bilirubin by Diazo Method, End Point Code
124 No: 120244 and urate by Modified – Trinder Method, End Point Code No: 120216.

125 2.5 Estimation of Total Antioxidant Capacity

126 TAC was estimated using commercially available BioVision Total Antioxidant Capacity
127 (TAC) Assay Kit (Catalog No K274-100); BioVision Research Products, 980 Linda Vista
128 Avenue, CA 94043, USA. It is based on the principle that Cu^{++} ion is converted to Cu^+ by
129 antioxidants. The reduced Cu^+ ion is chelated with a colorimetric probe giving a broad
130 absorbance peak around 570nm, proportional to the TAC. As there is no specific substance
131 by the name 'antioxidant', the kit has been provided with Trolox which is the most widely
132 used standard for antioxidant and therefore TAC was measured as TEAC (trolox equivalent
133 antioxidant capacity) in nmol/ μL or mmol/L serum.

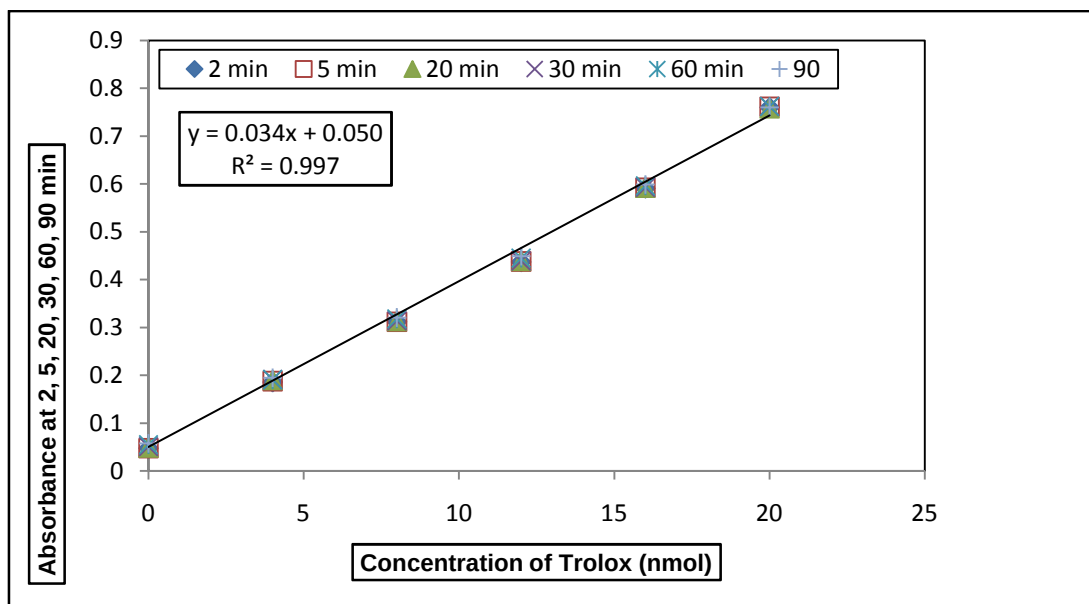
134 Kit has been provided with Cu^{++} Reagent, Assay Diluent and Protein Mask. Trolox Standard
135 provided in lyophilized form was dissolved in 20 μL of pure Dimethylsulfoxide (DMSO) and
136 made 1mL by DIW to get 1mM solution, which was used to prepare working standard by
137 adding 0, 4, 8, 12, 16, 20 μL to individual wells and adjusting the total volume to 100 μL with
138 DIW to get 0, 4, 8, 12, 16, 20nmol of working Trolox standard. Kit can be used to estimate
139 TAC or Antioxidant Capacity (AC) due to small molecule. If small molecule AC is desired,
140 serum samples are to be diluted 1:1 with protein mask. Therefore, it is not used in our study.
141 All well volumes were adjusted to 100 μL with DIW. Working coloring reagent (prepared by
142 diluting one part Cu^{++} reagent with 49 parts of Assay diluent) of 100 μL was added to all
143 standard & sample wells. Plate was incubated at room temperature for 1.5 hours followed
144 by reading the absorbance at 570nm using the plate reader.

145 After adding all reagents we measured absorbance at different times from 2 minutes to 90
146 minutes instead of direct 1.5 hours mentioned in the kit. We found that there was no change
147 in the absorbance of the standard (Figure 1), but the absorbance in the serum samples was
148 found increasing gradually (Figure 2). The absorbance of all the serum samples at any time
149 was within the standard curve (between 0.1 to 0.76 as shown in Figure 2). From the
150 standard curve which was very identical to that mentioned in the kit insert, regression
151 formula: $y = 0.034x + 0.050$, $R^2 = 0.997$ was calculated, from which concentration of TEAC
152 in samples was calculated. The concentration can also be calculated using standard formula
153 of colorimetry. In this study one of the sample has an absorbance of 0.151, the standard of
154 4nmol has an absorbance of 0.186, blank was 0.048 & therefore the concentration of TEAC
155 in this sample was 2.985nmol. But this value was for the 0.1 μL serum, as in each well 0.1 μL
156 serum was added & therefore TEAC of this sample was 29.85nmol/ μL or 29.85mmol/L. The
157 absorbance of the same sample after 90 minutes became 0.36 which was near to 8nmol
158 standard with an absorbance of 0.312. Thus same sample TEAC gets calculated as
159 9.45nmol/0.1 μL serum or 94.5 mmol/L. It is to be noted that 2 minutes incubation appears
160 more valid, as with time the increase in the absorbance starts deviating. The correlation
161 between absorbance at 2minutes and 5minutes ($R^2 = 0.997$) drops down systematically with
162 time ($R^2=0.84$ at 90 minutes), (Figure 2). Finally we decided to use exact 2 minutes
163 incubation time for accurate estimation of serum TAC levels in this study.

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2.6 Statistical analysis

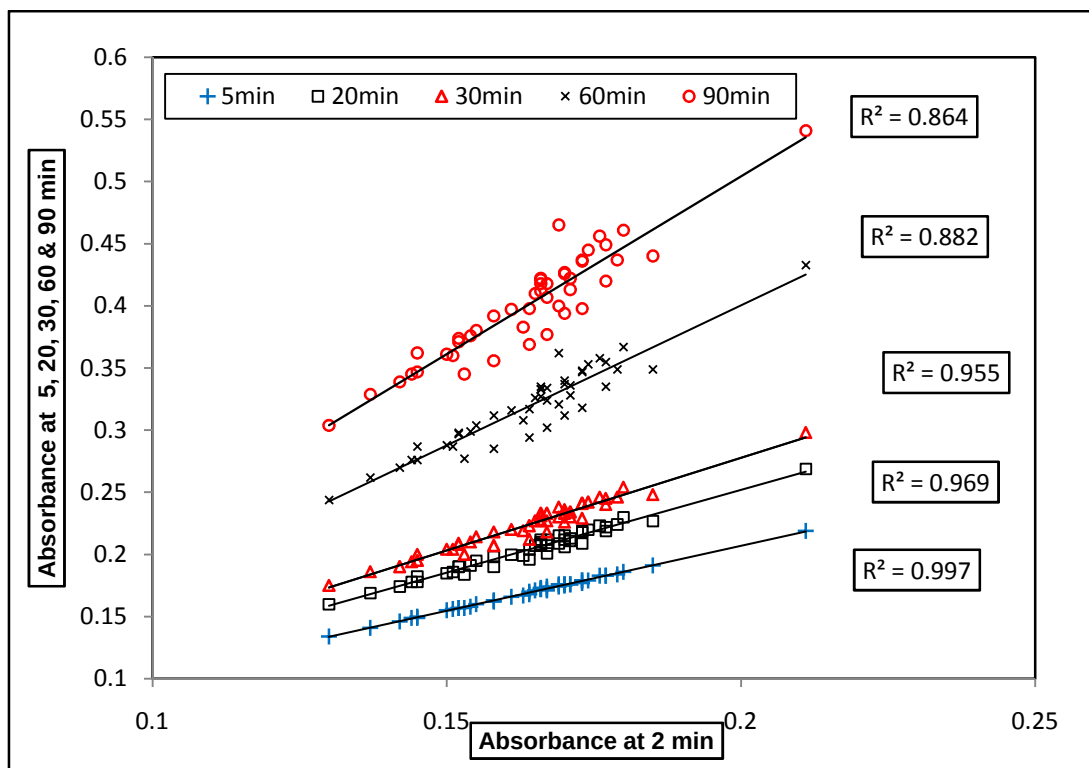
Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS version 17). Results have been expressed as mean $\pm$ SD. Mean differences were



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Figure 1. Standard curve of Trolox at different times

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Figure 2. Absorbance of serum samples at different times

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tested using unpaired two tailed 't' test. Correlation between the variables was calculated using Pearson's coefficient. ANOVA was applied on the four groups formed, based on disease and smoking status (nonsmoker controls, smoker controls, nonsmoker COPD and smoker COPD). Kolmogorov-Smirnov Test was applied to test normality for each group. ANOVA was applied to compare analyte levels between defined four groups. Post Hoc test (Bonferroni) was applied for multiple comparisons for the analytes where ANOVA was significant. Cut-off point for determining statistical significance levels was $P = .05$.

3. RESULTS AND DISCUSSION

In eighty six controls (Group I) male to female ratio was 64:22, Mean $\pm$ SD of age was 60.9 ± 11.78 years with range 27-90 years. In eighty six COPD patients (Group II) male to female ratio was 61:25; Mean $\pm$ SD of age 67.3 ± 8.36 years and range 45-97 yrs (Table 1). In 43 smoker controls and 43 smoker COPD, male to female ratio was 41:2.

Table 1. The age and sex distribution of study groups

Parameter	Controls (n=86)			COPD patients (n=86)		
	Males	Females	Total	Males	Females	Total
Number (%)	64 (74.4)	22 (25.6)	86 (100)	61 (71)	25 (29)	86 (100)
Mean (yrs)	60.8	61.2	60.89	67.3	64	67.32
SD (yrs)	13.2	5.7	11.78	8.3	7.6	8.36
Range (yrs)	27-90	50-77	27-90	45-97	45-76	45-97

Serum levels of CP were significantly higher in COPD patients as compared to controls. Mean $\pm$ SD of CP in COPD versus controls respectively are: 1392.8 ± 281.6 IU/L versus 1006.2 ± 236.1 IU/L; (Table 2). This is in agreement with the study by Verrills et al [34], who found that a panel of four biomarkers (α (2)-macroglobulin, haptoglobin, ceruloplasmin, and hemopexin) was able to discriminate with statistical significance between the clinical groups of patients with asthma, patients with COPD, and control subjects and reported increased ceruloplasmin in COPD patients as compared to controls. Serum levels of albumin, urate and TAC were significantly lower in COPD patients as compared to controls. Levels of albumin, urate and TAC in COPD versus controls respectively are albumin: 3.8 ± 0.5 g/dL versus 4.3 ± 0.4 g/dL; urate: 4.4 ± 1.3 mg/dL versus 5 ± 1.3 mg/dL; TAC 27.7 ± 6.9 mmolTE/L versus 36 ± 8.2 mmolTE/L (Table 2). The fall in albumin in COPD in our study is similar to that reported by Moison et al [35]. Kolmogorov-Smirnov test for testing the normality of each analyte in each of the four groups namely nonsmoker controls, smoker controls, smoker COPD & nonsmoker COPD was applied and was found not significant for all the analyte ($P > 0.05$) (Table 3). ANOVA was accordingly applied to all the analytes. Except total bilirubin ($P = 0.9$), all the other four analytes were found to have significant differences between all four groups (Table 4). Post Hoc Test – Bonferroni was applied to these analytes for multiple comparisons between four groups. No significant difference was found in albumin and urate between smoker and nonsmoker group either within controls or COPD [Table 5]. This is in agreement with that of Lykkesfeldt et al [36]. CP levels were found significantly different between all the groups except nonsmoker & smoker control and

212 nonsmoker & smoker COPD, while TAC was found significantly different between all groups,
 213 except between smoker control and nonsmoker COPD (Table 5). The significantly lower
 214

215 **Table 2. Comparison of levels of different analytes between controls and COPD**
 216

Analyte	Controls (n=86)		COPD patients (n=86)		significance p Value
	Mean	SD	Mean	SD	
Total Antioxidant capacity (mmolTE/L)	36.0	8.2	27.7	6.9	<0.001
Albumin (g/dL)	4.3	0.4	3.8	0.5	<0.001
Total Bilirubin (mg/dL)	0.8	0.4	0.8	0.4	0.899
Uric acid (mg/dL)	5.0	1.3	4.4	1.3	0.031
Ceruloplasmin (IU/L)	1006.2	236.1	1392.8	281.6	<0.001

217 **Table 3. Descriptive statistics showing One-Sample Kolmogorov-Smirnov Test**
 218
 219

Bio marker						
Nonsmoker controls(n=43)						
	Mean	SD	SE	95% CI for mean	Kolmogorov-Smirnov Z	Kolmogorov-Smirnov Sig (2 tailed)
Total Antioxidant capacity (mmolTE/L)	38.1	8.1	1.24	35.6-40.6	0.65	0.79
Albumin (g/dL)	4.3	0.2	0.04	4.2- 4.4	0.75	0.63
Total Bilirubin (mg/dL)	0.8	0.4	0.06	0.7-0.9	0.89	0.41
Uric acid (mg/dL)	4.9	1.4	0.21	4.5-5.3	0.68	0.74
Ceruloplasmin (IU/L)	979.2	206.6	31.51	915.6-1042.8	0.54	0.93
Smoker controls (n=43)						
Total Antioxidant capacity (mmolTE/L)	33.9	7.8	1.20	31.5-36.5	0.62	0.84
Albumin (g/dL)	4.3	0.5	0.08	4.2-4.5	1.23	0.1
Total Bilirubin (mg/dL)	0.8	0.4	0.06	0.7-0.9	0.82	0.51
Uric acid (mg/dL)	5.1	1.3	0.20	4.7-5.5	0.4	1.0
Ceruloplasmin (IU/L)	1033.2	262.0	39.96	952.5-1113.8	0.60	0.86
Nonsmoker COPD (n=43)						
Total Antioxidant capacity (mmolTE/L)	31.8	5.1	0.78	30.3-33.4	1.1	0.18
Albumin (g/dL)	3.8	0.5	0.08	3.6-3.9	0.91	0.38
Total Bilirubin (mg/dL)	0.8	0.5	0.08	0.7-1.0	0.95	0.32
Uric acid (mg/dL)	4.6	1.2	0.19	4.2-5.0	0.66	0.77
Ceruloplasmin (IU/L)	1350.3	294.2	44.87	1259.7-1440.8	1350.3	294.2
Smoker COPD (n=43)						
Total Antioxidant capacity (mmolTE/L)	23.6	5.9	0.90	21.8-25.4	0.49	0.97
Albumin (g/dL)	3.8	0.5	0.08	3.6-3.9	1.0	0.27
Total Bilirubin (mg/dL)	0.8	0.3	0.05	0.7-0.9	0.87	0.44
Uric acid (mg/dL)	4.3	1.4	0.21	3.7-4.7	0.5	0.96
Ceruloplasmin (IU/L)	1435.3	264.9	40.39	1353.8-1516.8	0.73	0.67

220

221 **Table 4. ANOVA test for analytes**

222

Dependent Variable	Group	Sum of Squares	df	Mean Square	F	Sig.
Total Antioxidant Capacity	Between Groups	4765.8	3	1588.59	33.68	0.00
	Within Groups	7925.2	168	47.17		
Albumin	Between Groups	12.8	3	4.26	18.52	0.00
	Within Groups	38.7	168	0.23		
Total Bilirubin	Between Groups	0.1	3	0.03	0.20	0.90
	Within Groups	27.9	168	0.17		
Urate	Between Groups	15.8	3	5.26	3.02	0.03
	Within Groups	292.7	168	1.74		
Ceruloplasmin	Between Groups	6644983.6	3	2214994.53	33.05	0.00
	Within Groups	11259730.8	168	67022.21		

223 **Table 5. Post Hoc Test – Bonferroni: Multiple comparisons between all four groups**
 224 (smoker controls, nonsmoker controls, smoker COPD & nonsmoker COPD)
 225

Dependent Variable	Group (x)	Group (y)	Mean Difference between groups (x-y)
Total Antioxidant Capacity	Nonsmoker Controls	Smoker Controls	4.17*
		Nonsmoker COPD	6.22*
		Smoker COPD	14.46*
	Smoker Controls	Nonsmoker COPD	2.04
		Smoker COPD	10.28*
		Nonsmoker COPD	8.23*
Albumin	Nonsmoker COPD	Smoker COPD	8.23*
	Nonsmoker Controls	Smoker Controls	-0.051
		Nonsmoker COPD	0.51*
		Smoker COPD	0.52*
	Smoker Controls	Nonsmoker COPD	0.565*
		Smoker COPD	0.576*
Urate	Nonsmoker COPD	Smoker COPD	0.009
	Nonsmoker Controls	Smoker Controls	-0.228
		Nonsmoker COPD	0.305
	Smoker Controls	Smoker COPD	0.576
		Nonsmoker COPD	0.531
		Smoker COPD	0.802*
Ceruloplasmin	Nonsmoker COPD	Smoker COPD	0.271
	Nonsmoker Controls	Smoker Controls	-53.93
		Nonsmoker COPD	-371.06*
	Smoker Controls	Smoker COPD	-456.08*
		Nonsmoker COPD	-317.12*
		Smoker COPD	-402.15*
	Nonsmoker COPD	Smoker COPD	-85.025

226 * The mean difference is significant at the 0.05 level.

227 serum levels of TAC in COPD patients as compared to controls is in agreement with that of
228 Sahin et al [37] who reported lower antioxidant capacity in COPD. The lower levels of TAC
229 may be due to decreased synthesis of antioxidants or increased consumption of antioxidants
230 by oxidants or may be by oxidative modifying the antioxidant property of the antioxidant by
231 oxidative stress [38]. In nonsmokers, exposure to smoke from biomass fuel has been
232 suggested as cause for the COPD [39].

233 Most of the published studies reported the TAC in trolox equivalent (TE) per liter and
234 mentioned levels as TEAC (trolox equivalent antioxidant capacity). The levels of TAC in
235 healthy controls reported in various studies are $0.77 \pm 0.09\text{mM/L}$ [40], $1.69 \pm 0.2\text{mmol/L}$ [41],
236 $675 \pm 61\mu\text{molTE/L}$ [42], $12048 \pm 2678\mu\text{molTE/L}$ [43], $1.0 \pm 0.6\mu\text{molTE/L}$ [44], 1.31mmol/L in
237 healthy nonsmoker, 0.87 in healthy smokers [45]. Thus the values from the published
238 studies have been seen ranging from $1\mu\text{molTE/L}$ to $12048\mu\text{molTE/L}$. This variation might be
239 due to different methodologies used to estimate TAC [46]. In our study the values were $36 \pm$
240 8.2 mmolTE/L . We looked into the methodology of the kit used (TAC Assay Kit, BioVision,
241 USA) and first standardized the incubation time to exact 2 minutes so as to minimize the
242 errors and variation due to differences in the incubation time.

243 As it is reported that, the contributions to TAC are provided by albumin and urate [47 - 49],
244 so correlation studies between TAC and albumin, urate & bilirubin were conducted, and
245 found that none of the parameters have any considerable correlation with TAC in controls (r
246 $= 0.11, 0.01$ and 0.008 respectively) or in COPD ($r = 0.017, 0.112$ and 0.037 respectively),
247 though albumin and urate were also significantly lower in COPD patients as compared to
248 controls.

249 Thus the changes in the levels of TAC could not be correlated with proportionate changes in
250 albumin, urate and bilirubin. This correlation was also studied in smoker and nonsmoker
251 within controls and COPD, and no noticeable correlation was found. Also, no correlation was
252 found between CP and TAC either in controls or COPD. This suggests that TAC is not
253 limited to albumin, urate, bilirubin, CP but due to any other enzymatic or nonenzymatic
254 antioxidants. There are studies where TAC has been reported to correlate positively with
255 vitamin E levels ($P=0.05$), GSH ($P=0.02$) but not with vitamin C [40]. The depletion of plasma
256 GSH has been suggested as a key point in depletion of other antioxidants [50].

257
258 Clinical trials directed toward enhancing lung antioxidants using direct antioxidant molecules,
259 such as vitamin E, showed almost no effect on FEV_1 indicating a single antioxidant molecule
260 not efficacious in affording protection against the oxidative stress in COPD and accordingly
261 combination of antioxidants were suggested effective in the treatment of COPD [10].
262 Strategy based on targeting NRF2, which up-regulates a wide range of antioxidants genes,
263 was suggested as a more efficient COPD therapy [11]. These experiments also indirectly
264 favour TAC measurement over an individual antioxidant in the COPD diagnosis.

265 266 4. CONCLUSION

267
268 CP was significantly increased while TAC gets significantly decreased in COPD. CP and
269 TAC showed statistically significant differences between controls and COPD patients and
270 may have clinical utility in the management of COPD. However the estimation of TAC is to
271 be done with extreme care. Study with enough patients in each COPD GOLD group may be
272 needed to specifically link the clinical utility of these antioxidant measurements.

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281
282 **COMPETING INTERESTS**

283
284 Declared that no competing interests exist.
285

286 **AUTHORS' CONTRIBUTIONS**

287
288 Author ANS: Supervised the study; VA: Concept, designed, conducted the study, review of
289 literature and wrote the manuscript.

290
291 **CONSENT**

292
293 Declare that written informed consent was obtained from the patient for sample.

294 **ETHICAL APPROVAL**

295
296 All authors hereby declare that the study was approved by the appropriate ethics committee
297 and have therefore been performed in accordance with the ethical standards laid down in the
298 1964 Declaration of Helsinki. Obtained necessary Institutional ethical approval.

299
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