

TITLE: Can Total Antioxidant Capacity Be A Surrogate Marker In Chronic Obstructive Pulmonary Disease ?

ABSTRACT

Background: 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 antioxidant. Total antioxidant activity (TAC), represents the individual's overall antioxidant status and may give more relevant biological information compared to individual components.

Aims: Study correlation between TAC and bilirubin, albumin, urate & ceruloplasmin (CP) and explore the utility of serum TAC in diagnosis of COPD.

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 by using commercially available kits.

Results: CP were 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. Their 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: TAC is significantly decreased in COPD and definitely appears as a surrogate marker in the diagnosis of COPD but the estimation is to be done with extreme care.

28 Key Words: chronic obstructive pulmonary disease, antioxidants, total
29 antioxidant capacity, ceruloplasmin, albumin, urate.

30 **INTRODUCTION**

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

36 Lung with a large surface area is continuously exposed to endogenously or
37 exogenously oxidants [3, 4, 5] and is protected against oxidative challenge by
38 well-developed enzymatic and non-enzymatic antioxidant systems. Enzymatic
39 antioxidant include superoxide dismutase (SOD), ceruloplasmin, glutathione
40 peroxidase (GSH-Px), glutathione reductase (GSH-Rx), catalase etc [6, 7], while
41 non-enzymatic antioxidants include glutathione, ascorbate, urate, α -tocopherol,
42 bilirubin, albumin and lipoic acid. Increased pulmonary exposure to oxidative
43 source and/or reduced antioxidant defenses leads to an imbalance known as
44 oxidative stress [8]. Increased oxidative stress not only produces direct injurious
45 effects in the lungs, but also activates the molecular mechanisms that initiate
46 lung inflammation [9] and may have a role in many of the processes thought to
47 be involved in the complex pathological events that results in COPD.

48 Ceruloplasmin, the major serum inhibitor of lipid peroxidation [10] has
49 been documented as a main extracellular antioxidant in serum [11], inhibiting
50 ferrous ion stimulated lipid peroxidation [12], preventing lung injury and an
51 abnormality of ceruloplasmin oxidative inhibition could be involved in the
52 pathogenesis of COPD [13].

Urate has been found to be an excellent scavenger of singlet oxygen [14] and protects erythrocyte membrane from peroxidation [15]. Urate was effective in preventing lipid peroxidation at concentrations considerably below those normally found in plasma. Urate acts either by directly scavenging active oxygen species [16, 17] or by binding radical-generating transition metals [18]. Antioxidant activity of urate is an important aspect of its in vivo function [15, 19]. Albumin has been considered not only as an antioxidant, but as the major circulating antioxidant in plasma known to be exposed to continuous oxidative stress [4, 20, 21]. Ames et al [22, 23] demonstrated that antioxidant effect of bilirubin exceeds that of vitamin E toward lipid peroxidation and levels of serum bilirubin are high enough to account substantially towards total antioxidant capacity of serum [24, 25].

The sum of endogenous and exogenous antioxidants represents the total antioxidant activity (TAC). Measuring TAC can provide information on an individual's overall antioxidant status, which may include those antioxidants not yet recognized or not easily measured [26]. Cooperation of all the different antioxidants provides greater protection against attack by reactive oxygen or nitrogen radicals, than any single compound alone. Thus, the overall antioxidant capacity may give more relevant biological information compared to that obtained by the measurement of individual components.

To this end we estimated the levels of TAC, bilirubin, albumin, urate and ceruloplasmin in serum of COPD patients and compared their levels with that in normal subjects to assess the utility of serum TAC in COPD.

MATERIAL AND METHODS

Subject selection and Procedure

The study subjects comprises of two groups:

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

ii) Group II or COPD patients: Comprises of 86 patients of COPD diagnosed as per GOLD guidelines (GOLD report 2011). Subjects for this group were selected from the indoor and outdoor patients from Military Hospital- Cardio-Thoracic Centre, during Dec 2010 to Dec 2012.

Both the groups were further divided into equal number of 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.

Exclusion criteria for control and COPD patients:

Patients with a diagnosis of asthma, coronary artery disease, diffuse parenchymal lung disease, patients on Long Term Oxygen Therapy or unable to perform spirometry were excluded from the group II. Similarly individual with any respiratory or lung disease or past history of any lung/respiratory problem/disease were excluded from the control group (group I)

All patients were administered a questionnaire to know 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. All patients underwent spirometry for

confirmation of diagnosis. Presence of post bronchodilator $FEV_1/FVC < 0.70$ was used to confirm presence of persistent airflow limitation & thus COPD [27].

Smoking index, defined as no. of cigarettes or bidis smoked per day multiplied by the total duration of smoking in years was used to quantify smoking exposure among the study subjects. It is a better index to quantify smoking in Indian context as compared to pack years [28].

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 tube. 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

Estimation of ferroxidase activity

Ceruloplasmin (CP) was estimated by indigenous patented kinetic method of Somani and Ambade (Govt of India Patent No192356), [29] based on its ferroxidase activity on fully automated analyzer XL 600 from Transasia Mannheim GmbH, 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 fully automated analyser was set at following parameters: Assay Type: Rate A; Wavelength: Primary = 376nm, Secondary = 0nm; Assay Points 0, 0, 15, 18 cycles; Sample volume: 10 μL ; Reagent 1: 200 μL ; Reagent 2: 30 μL ;

Factor 2010. The CP in serum was calculated from the factor and displayed directly by the fully automated analyser in IU/L.

Estimation of albumin, bilirubin, urate and TAC

Albumin, bilirubin, urate was measured using commercially available kits on fully automated analyzer XL 600 from Transasia Mannheim GmbH . Albumin was estimated by BCG Dye Method, End Point Code No: 120223; Bilirubin by Diazo Method, End Point Code No: 120244 and urate by Modified – Trinder Method, End Point Code No: 120216. TAC was measured using commercially available BioVision Total Antioxidant Capacity (TAC) Assay Kit (Catalog No K274-100); BioVision Research Products, 980 Linda Vista Avenue, CA 94043, USA. It is based on the principle that Cu^{++} ion is converted to Cu^{+} by antioxidant. The reduced Cu^{+} ion is chelated with a colorimetric probe giving a broad absorbance peak around 570nm, proportional to the TAC in nmol/ μL or mmol/L serum.

Statistical analysis was carried out using SPSS 17.0 applying appropriate test.

RESULTS

The age & sex distribution of study groups is as per Table 1.

Serum levels of CP, albumin, bilirubin, urate and TAC were compared between the study groups using unpaired t-test and results are presented in Table 2.

The levels of these analytes were also compared between the smoker and nonsmoker group within the study groups (controls and COPD) and results are presented in Table 3, whereas inter-comparison (ANOVA) between all four groups (smoker controls, nonsmoker controls, smoker COPD & nonsmokers COPD) is presented in Table 4

Correlation between TAC and albumin, bilirubin, urate in controls and COPD are represented in Figure 1 and 2 respectively. The standard curve of Trolox at different times and variation in the absorbance of serum samples at different times is as per Figure 3 and 4 respectively.

DISCUSSION

Ceruloplasmin is a major extra cellular antioxidant in serum [11], and plays a role in preventing lung injury and an abnormality of its oxidative inhibition could be involved in the pathogenesis of COPD [13]. Urate acts as antioxidant at concentrations considerably below those normally found in plasma either by directly scavenging active oxygen species [16, 17] or by binding radical-generating transition metals [18]. Albumin is the major circulating antioxidant in plasma [4, 20, 21]. Antioxidant effect of bilirubin exceeds that of vitamin E toward lipid peroxidation [22, 23] and serum bilirubin levels are high enough to account for serum TAC substantially [24, 25]. Measuring TAC can provide information on an individual's overall antioxidant status, which may give more relevant biological information compared to that obtained by the measurement of individual components.

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).

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 that of Verrills et al [30] who found that a panel of four biomarkers ($\alpha(2)$ -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 [31]. No significant difference was found in albumin, urate and bilirubin between smoker and nonsmoker group either within controls or COPD (Table 3 and 4). This is in agreement with that of Lykkesfeldt et al [32]

The significantly lower serum levels of TAC in COPD patients as compared to controls is in agreement with that of Sahin et al [33] who reported lower antioxidant capacity in COPD.

Most of the published studies reported the TAC in trolox equivalent (TE) per litre and mentioned levels as TEAC (trolox equivalent antioxidant capacity). The levels of TAC in healthy controls reported in various studies are 0.77 ± 0.09 mM/L [34], 1.69 ± 0.2 mmol/L [35], 675 ± 61 μmolTE/L [36], 12048 ± 2678 μmolTE/L [37], 1.0 ± 0.6 μmolTE/L [38], 1.31 mmol/L in healthy nonsmoker, 0.87 in healthy smokers [39]. Thus the values have been seen ranging from 1 μmolTE/L to 12048 μmolTE/L. This variation might be due to different methodologies used to estimate TAC [40].

In our study the values were much higher 36 ± 8.2 mmolTE/L. To understand the high values we looked into the methodology of the kit used to estimate TAC. Kit has been provided with Cu^{++} Reagent, Assay Diluent and

Protein Mask. Trolox Standard provided in lyophilized form was dissolved in 20µL of pure Dimethylsulfoxide (DMSO) and made 1mL by DIW to get 1mM solution, which was used to prepare working standard by adding 0, 4, 8, 12, 16, 20µL to individual wells and adjust the total volume to 100µL with DIW to get 0, 4, 8, 12, 16, 20nmol of working Trolox standard. Kit can be used to estimate TAC or AC due to small molecule. If small molecule AC is desired, samples should be diluted 1:1 with protein mask. For serum samples, 0.01-0.1µl serum without Protein Mask has been suggested. As we were interested in TAC only, serum volume 0.1µL without Protein Mask was used. All well volumes were adjusted to 100µL with DIW. Working coloring reagent (prepared by diluting one part Cu^{++} reagent with 49 parts of Assay diluent) of 100µL was added to all standard & sample wells. Plate was to be incubated at room temperature for 1.5 hours followed by reading the absorbance at 570nm using the plate reader. After adding all reagents we measured absorbance at different times from 2 min to 90 min instead of direct 1.5 hr mentioned in the kit. We found that there was no change in the absorbance of the standard (Figure 3), but the absorbance in the serum was found increasing gradually (Figure 4). The absorbance of all the samples at any time was within the standard curve (between 0.1 to 0.76 as shown in figure 4). From the standard curve which was very identical to that mention in the kit insert, regression formula $y = 0.034x + 0.050$, $R^2 = 0.997$ was calculated, from which concentration of TEAC in samples was calculated. The concentration can also be calculated using formula of colorimetry. In this study one of the sample has a absorbance of 0.151, the standard of 4nmol has absorbance of 0.186, blank was 0.048 & therefore the concentration of TEAC in this sample was 2.985nmol. But this value was for the 0.1µL serum, as in each well 0.1µL serum was added & therefore TEAC of this sample was 29.85nmol/µL or 29.85mmol/L.

233 The absorbance of the same sample after 90 min became 0.36 which was near to
 234 8nmol standard with absorbance of 0.312. Thus same sample TEAC gets
 235 calculated as 9.45nmol/0.1uL serum or 94.5 mmol/L. Thus using this kit if
 236 0.1uL of any serum gives absorbance within the absorbance range of standard
 237 curve will give minimum of 20mmol/L with just 5 min incubation. This
 238 increasing absorbance with time is definitely due to some antioxidants present in
 239 the serum. It cannot be due to any contamination in the DIW which was added in
 240 all the wells, because in two wells A1 and A2 of ELISA plate, 100μL of same DIW
 241 water was added as blank corresponding for 0nmol standard. Its absorbance was
 242 found almost constant till full 90min of incubation (0.048 at 2 min was found
 243 0.058 after 90 min). And more over all the standards were also diluted in the
 244 same DIW. Similar increase in the TEAC has been reported by Schofield &
 245 Braganza [41]. They reported that the measured TEAC of human plasma by Total
 246 Antioxidant Status kit (from Randox Labs, UK), increases with time. One healthy
 247 individual TEAC was found 1.06 mmol/L at 2 min which went up to 1.53
 248 mmol/L at 3 min. They also reported that dilution further increase the TEAC of
 249 same individual from 1.51 mmol/L to 1.80mmol/L. Due to such effect of dilution
 250 of serum on TEAC which is also mentioned in Randox kit instruction sheet, the
 251 dilution of serum sample is not recommended by Randox kit. In our kit
 252 (Biovision) 0.1μL serum was suggested and total volume was to be made 100μL
 253 leading to very high dilution and incubation time suggested was 90 min. Both
 254 these factors were found literally making the plasma a very efficient antioxidant
 255 and might be responsible for very high TEAC of controls and COPD in our study.
 256 The second reason could be that the Indian population is exposed to very
 257 pollution and as a response to that body might have generated an efficient
 258 antioxidant system. The increase in the TEAC as seen in this experiment in vitro

could be the one of the reason for advocating intake of sufficient amount of water daily for good health. This beneficial effect of water intake on the body can be explored further to prove this hypothesis.

It is to be noted that 2 min incubation appears more valid, as with time the increase in the absorbance starts deviating. The correlation between absorbances at 2min and 5min ($R^2 = 0.997$) drops down systematically with time ($R^2=0.84$ at 90 min), Figure 4. This deviation may be due to other substances also in serum might be behaving like antioxidants which is varying in different individuals. Such type of behaviour had been noticed in Jaffe's reaction used worldwide to estimate creatinine. Initial reaction was found to be due to creatinine and after few minutes other non-creatinine substances were also found to behave as creatinine increasing the absorbance giving erroneously high creatinine values. Based on this, the kit was later modified to fixed time assay where the absorbance was monitored from 20 to 80 seconds to get correct creatinine values.

However, our interest in the present study was to monitor alteration in the TAC in COPD patients and we found significant decrease in the TAC in COPD. As it is reported that the major contributions to TAC are provided by albumin and urate [42 - 44], so correlation between TAC and albumin, urate & bilirubin in controls and COPD were studied and found that none of the parameter to have any considerable correlation with TAC though albumin, urate were also significantly lower in COPD patients as compared to controls (Figure 3 & 4).

Thus the increased changes in the TAC could not be correlated to albumin, urate and bilirubin. This correlation was also studied in smoker and non-smoker groups of controls and COPD patients and found no appreciable correlation. Also no correlation was found between CP and TAC either in controls or COPD.

CONCLUSION: TAC which gets significantly decreased in COPD which is further reduced in smoker COPD definitely appears as a surrogate marker in the diagnosis of the COPD but the estimation is to be done with extreme care and the values are to be interpreted accordingly.

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 413 Table 1. The age and sex distribution of study groups
 414 Table 2. Comparison of levels of different analytes between controls and copd
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 416 Table 3. Comparison of levels of different analytes between controls and copd
 417 patients within smoker and nonsmokers group
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 419 Table 4. Inter comparision between all four groups (smoker controls, nonsmoker
 420 controls, smoker copd & nonsmoker copd)
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 423 Figure 1. Correlation between TAC and albumin, bilirubin & uric acid in Controls
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 425 Figure 2. Correlation between TAC and albumin, bilirubin & uric acid in COPD
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 427 Figure 3. Standard curve of Trolox at different times
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 429 Figure 4. Variation in the absorbance of serum samples at different times
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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)	92 (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

TABLE 2. Comparison of levels of different analytes between controls and copd

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

TABLE 3. Comparison of levels of different analytes between controls and copd patients within smoker and nonsmokers group

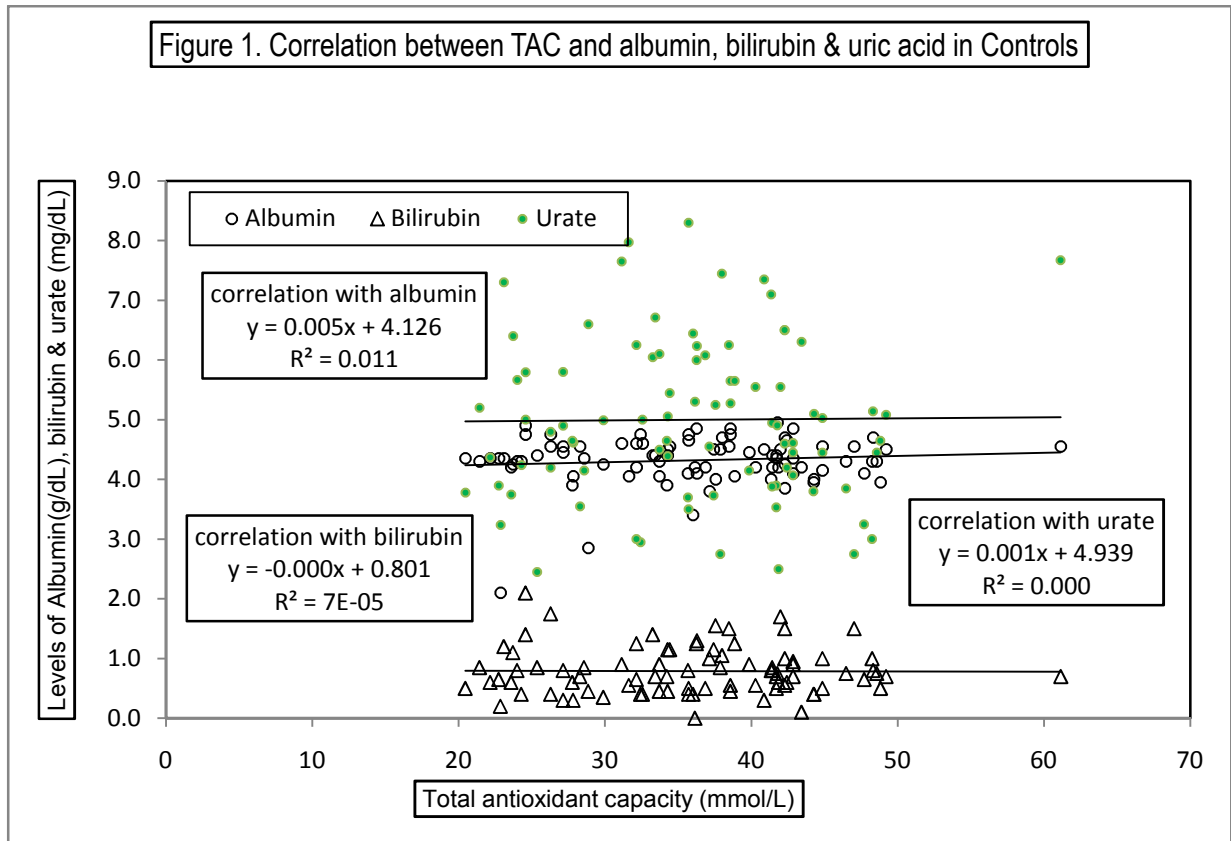
Bio marker	Controls (n=86)		COPD patients (n=86)	
	Non Smokers controls (n=43)		Non Smokers COPD (n=43)	
	Mean	SD	Mean	SD
Total Antioxidant capacity (mmolTE/L)	38.1	8.1	31.8	5.1
Albumin (g/dL)	4.3	0.2	3.8	0.5
Total Bilirubin (mg/dL)	0.8	0.4	0.8	0.5
Uric acid (mg/dL)	4.9	1.4	4.6	1.2
Ceruloplasmin (IU/L)	1033.2	262.0	1435.3	264.9
	Smokers controls (n=43)		Smokers COPD (n=43)	
	Mean	SD	Mean	SD
Total Antioxidant capacity (mmolTE/L)	33.9	7.8	23.6	5.9
Albumin (g/dL)	4.3	0.5	3.8	0.5
Total Bilirubin (mg/dL)	0.8	0.4	0.8	0.3
Uric acid (mg/dL)	5.1	1.3	4.3	1.4
Ceruloplasmin (IU/L)	979.2	206.6	1350.3	294.2

Table 4. Inter comparison between all four groups (smoker controls, nonsmoker controls, smoker COPD & nonsmoker COPD) (ANOVA)

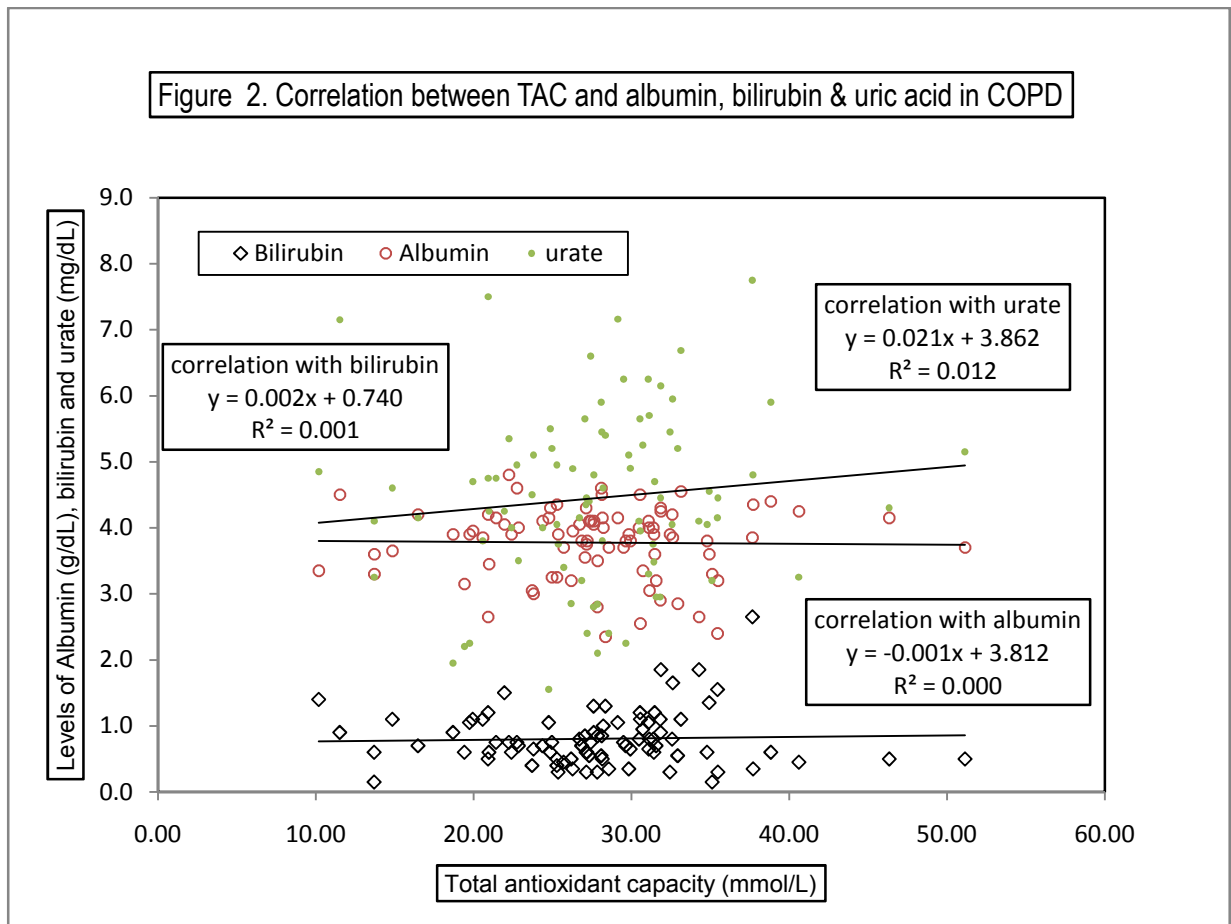
Dependent Variable	Group	Group	Mean Difference between groups	Std. Error	Sig.
Total Antioxidant Capacity	Non-smoker Controls	Smoker Control	4.17*	1.48	.032
		Non-smoker COPD	6.22*	1.48	.000
		Smoker COPD	14.46*	1.48	.000
	Smoker Control	Non-smoker COPD	2.04	1.48	1.000
		Smoker COPD	10.28*	1.48	.000
	Non-smoker COPD	Smoker COPD	8.23*	1.48	.000
Albumin	Non-smoker Controls	Smoker Control	-0.051	0.10	1.000
		Non-smoker COPD	0.51*	0.10	.000
		Smoker COPD	0.52*	0.10	.000
	Smoker Control	Non-smoker COPD	0.565*	0.10	.000
		Smoker COPD	0.576*	0.10	.000
	Non-smoker COPD	Smoker COPD	0.009	0.10	1.000
Total Bilirubin	Non-smoker Controls	Smoker Control	-0.006	0.09	1.000
		Non-smoker COPD	-0.051	0.09	1.000
		Smoker COPD	0.012	0.09	1.000
	Smoker Control	Non-smoker COPD	-0.044	0.09	1.000
		Smoker COPD	0.018	0.09	1.000
	Non-smoker COPD	Smoker COPD	0.062	0.09	1.000
Urate	Non-smoker Controls	Smoker Control	-0.228	0.28	1.000
		Non-smoker COPD	0.305	0.28	1.000
		Smoker COPD	0.576	0.28	.276
	Smoker Control	Non-smoker COPD	0.531	0.28	.385
		Smoker COPD	0.802*	0.28	.033
	Non-smoker COPD	Smoker COPD	0.271	0.28	1.000
Ceruloplasmin	Non-smoker Controls	Smoker Control	53.93	55.83	1.000
		Non-smoker COPD	-402.15*	55.83	.000
		Smoker COPD	-317.12*	55.83	.000
	Smoker Control	Non-smoker COPD	-456.08*	55.83	.000
		Smoker COPD	-371.06*	55.83	.000
	Non-smoker COPD	Smoker COPD	85.025	55.83	.778

* The mean difference is significant at the 0.05 level.

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Figure 3. Standard curve of Trolox at different times

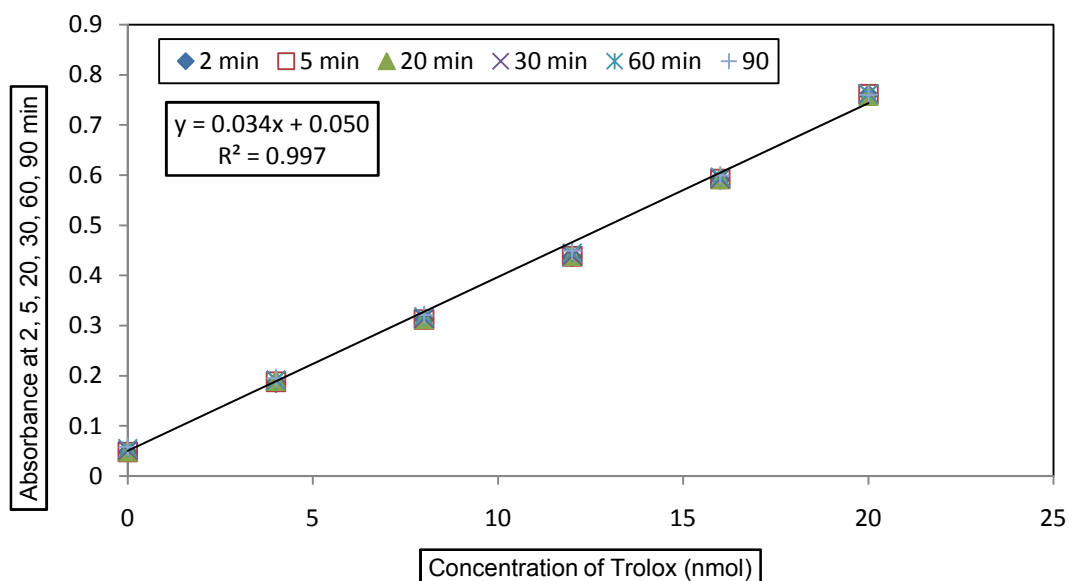


Figure 4. Variation in the absorbance of serum samples at different times

