



## Salivary Levels of Neurotoxic Versus Neuroprotective Indices Underpin the Aberrant Response to Psychological Stress in Schizophrenic Patients

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### ABSTRACT

**Background:** Schizophrenia is a chronic, recurrent disease that starts with a first psychotic episode and continues with periods of remission and acute psychosis with impaired interaction with stress. **Aims:** to allocate the gender differences in relation to distress in the salivary levels of the tryptophan (Trp), kynurenine (KYN) metabolites, cortisol and serotonin in patients with schizophrenia following psychological stress test. **Methods:** Eighty chronic schizophrenia patients and healthy controls, were subjected to a modified version of the Vienna test. Two saliva samples were gathered before and 10 minutes following stress test. Salivary concentration of cortisol, serotonin, Trp, L. KYN, Kynurenic acid (KYA), quinolinic acid (QUIN) and picolinic acid (PIC) were quantified by liquid chromatography triple quadrupole mass spectrometry. **Results:** Highly significant rise in the mean salivary post stress test cortisol concentration ( $P < 0.001$ ) and significant decrease in mean salivary PIC ( $P < 0.05$ ) of total schizophrenic patient group. Schizophrenic women showed decreased post stress salivary QUIN:KYA ratio ( $P < 0.001$ ), whereas men with schizophrenia exhibited increased QUIN:KYA ( $P < 0.001$ ) and KYN:Trp ( $P < 0.05$ ) ratios in comparison to their respective control values. Significant positive correlations of the pre-stress salivary PIC and QUIN ( $r = 0.344$ ,  $P = 0.05$ ) with highest sensitivity and specificities displayed by salivary cortisol and L.KYN values. **Conclusion:** Schizophrenic men showed aberrant response to psychological stress than women as reflected by highest neurotoxic QUIN: KYA index. This neurotoxic index is suggested as a marker for the therapeutic evaluation of the effect of antipsychotic drug on the patient-environment interaction.

**Keywords:** Schizophrenia, Post stress, Kynurenines, Mass spectrometry, Vienna test

### INTRODUCTION

Schizophrenia is a complex, multifactorial and polygenic mental disorder [1,2]. Twin and adoption studies point to the impact of genetic factors, among other contributing factors, in its etiology [3]. Most psychiatric disorders, including schizophrenia, develop in those who failed to take control of stress and do not achieve positive outcomes when faced with adversities and stressors. Resilience is modulated by neurotransmitters, neurotrophic factors, hypothalamic-pituitary-adrenal (HPA) axis, autonomic nervous system, oxidative stress, and metabolic markers). Serotonin is responsible for behaviors and somatic functions: cognition, including memory; perception and attention; sensory gating; mood; aggression; sexual drive; appetite; energy level; pain sensitivity; endocrine function; and sleep [4]. Schizophrenia is often described in terms of positive and negative symptoms [5].

Positive symptoms are described in persons who lost the touch with reality in certain ways [6]. Negative symptoms commonly include flat expressions or little emotion, poverty of speech, inability to experience pleasure, lack of desire to form relationships, and lack of motivation. Cognitive symptoms include trouble with prioritizing tasks, memory and organizing thoughts, anosognosia or (lack of insight) being unaware of having an illness [6].

Cognitive dysfunction in schizophrenia may be mediated by the metabolites of the kynurenine-nicotinic acids pathway,

such as QUIN and PIC (NMDA agonists) which stimulate the activity of the inducible nitric oxide synthase (iNOS). The nitric oxide derived from iNOS pathway mediates cellular toxicity and triggers a lipid peroxidation cascade, which contributes to myelin degeneration [7].

The HPA axis is central in mediating the human stress response. At times of stress, the anterior pituitary gland releases the adrenocorticotrophic hormone, which fosters the release of increasing amounts of circulating cortisol from the adrenals. After the acute stressor, mineralocorticoids are activated at the onset of the stressor and are involved in appraisal. glucocorticoids, on the other hand, terminate the acute stress response and aid in recovery, facilitating the return to homeostatic balance until called upon for the next stressor [8]. During chronic stress, adaptation normally occurs to the triggered cortisol release. Psychological stress can be defined as psychological tension or strain that is difficult to manage or endure [9,10]. Activation of the HPA axis represents the fundamental response to stress in human and other mammals, and dysfunction of this axis is associated with a range of physical and mental health disorders [11].

KYA is a metabolite of tryptophan produced from the breakdown of KYN by KYN aminotransferases (KAT), mainly within astrocytes. It is an endogenous neuroprotectant and an elevated level of KYA may affect memory and cognitive processes in animals [12]. QUIN, macrophage/microglia-derived excitotoxin that has functions such as neurotoxins, gliotoxin, and proinflammatory mediator that changes the integrity of blood brain barrier in variety of pathophysiological conditions [13]. PIC is an endogenous metabolite of L-Trp that has been reported to possess a wide range of neuroprotective, immunological, and anti-proliferative affects within the body [14]. Deficiency of serotonin (5-HT) that normally maintains mood balance, leads to depression. The observation of similarity between serotonin and hallucinogen drug like lysergic acid diethylamide led to hypothesis that schizophrenia may be associated with a dysfunction of serotonin system [3]. Moreover, some atypical antipsychotics, such as clozapine, improve symptoms of schizophrenia through modulating the 5-HT concentration [15]. So, in this study we determined the correlation of the salivary Trp and KYN metabolites in addition of serotonin and cortisol with the ability to tolerate induced stress in normal subjects and schizophrenic patients and to check for the gender difference in the levels of these salivary parameters in response to stress.

## MATERIALS AND METHODS

This is a case control cross sectional study conducted on total 80 subjects. Forty of them were patients with chronic schizophrenic who were recruited from Ibn-Rushud Teaching Hospital in Baghdad city during the period from January to August 2015.

The diagnosis of schizophrenia was carried out by senior Psychiatry at Ibn-Rushud hospital according to DSM-V criteria. Forty persons were enrolled in the study from the Baghdad populations who do not suffer from apparent psychiatric problems as volunteers with an age range of 18-45 years. Following patient interview, the symptom description and signs are rated according the brief psychiatric rating scale (BPRS) and the total item number from this rating guide is recorded for each patient [16,17]. The exclusion criteria for patients and controls encompass chronic diseases (diabetes mellitus, hypertension, or heart diseases, previous history of meningitis, multiple sclerosis, Alzheimer disease, Parkinson's disease, epilepsy), drug abusers, and alcoholism.

### Collection of Saliva

For every patient and normal was instructed to wash their mouth three times with tap water to clear food debris. A cotton roll was placed under the cheek to facilitate collection of unstimulated saliva by absorbing the saliva content of the mouth and also to avoid collection of the mucosa cells of the oral cavity. The cotton rolls were removed and placed in a plastic test tube. About 0.5-2 ml of saliva samples were collected and left in a cool box for subsequent processing. A second saliva sample was collected by the same procedure from all participants immediately at the end of stress test (after 10 minutes).

### Induction of Stress

After collection of the second saliva sample, each patient and control were interviewed and exposed to a list of questions that stress on the patient interest, educational level, and socioeconomic status of each subject. The interaction of each subject is carefully observed. After 10 minutes, the interview is stopped, and another saliva sample was collected. The questions are extrapolated from the computer program of the Vienna stress test panels because of unavailability

of specialized device used in this test. According to the patients and control response to stress induced questions, the patients and control subjects were further sub-grouped into Distress intolerant patient (n=11), Distress tolerant patient (n=29), Distress intolerant control (n=4), and Distress tolerant control (n=36).

### Saliva Processing

The cotton rolls containing plastic tubes were centrifuged at 3000 rpm for 10 minutes to facilitate elution of clear saliva. The samples were diluted with equal volume of ultra-pure deionized distilled water and then half volume of 6% perchloric acid was added to deproteinize as much as possible of saliva samples. The deproteinized samples were stored frozen at  $-20^{\circ}\text{C}$  for subsequent analyses quantitative analyses of salivary concentration of cortisol, serotonin, amino acid Trp and its metabolites (KYN, KYA, QUIN and PIC) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) Liquid chromatography triple quadrupole mass spectrometer model LCMS-8030 [18].

For chromatographic separation, a 3.5 Mm column ( $100 \times 3.0$  mm) with a guard column of same type was used. A gradient elution, at a flow rate of 0.2 ml/min, was performed with mobile phase A consisting of 0.1% acetic acid in HPLC water, and mobile phase B made of acetonitrile. The following optimized condition used: capillary voltage (0.3 kV); Cone gas flow-rate (50L/hr); desolvation gas flow rate (750 L/hr); Source temperature ( $120^{\circ}\text{C}$ ), desolvation temperature ( $200^{\circ}\text{C}$ ).

### Statistical Analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS) version 20. The categorical data were presented as frequency and percentages. The continuous variables were presented as mean, and standard deviations. Fisher test, Chi-square, independent t-test, paired t-test, and Pearson's correlation test were used to assess the association between categorical variables. The specificity, sensitivity and area under curves were analyzed by the receiver operating characteristic (ROC) curve.  $P < 0.05$  was statistically worthwhile.

## RESULTS

The socio-demographic and clinical features of the studied schizophrenic patients and the controls are exemplified in Table 1 whereas, the means of the brief psychiatric rating scale (BPRS) are listed in Figure 1. Of the schizophrenia cases, 60% were not married as compared to the 40% married patients ( $P < 0.05$ ). On the contrary, 65.5% of the controls were married as compared to 34.5% un married controls (Figure 2).

**Table 1 Socio-demographic and clinical characteristics of the study groups**

Parameters		Patient N (%)	Control N (%)
Number		40	40
Age (years)		18-45	18-45
Gender	Men	45%	47.50%
	Women	55%	52.50%
Marital status:	Unmarried	40%	65.50%
	Married	60%	34.50%
Duration of disease (years)		$16.95 \pm 5.97$	-
Family history of disease		No	No
Smokers		65%	25%
Treatment		100%	-
History of ECT:	No	20%	-
	Yes	80%	-

ECT: Electroconvulsive Therapy

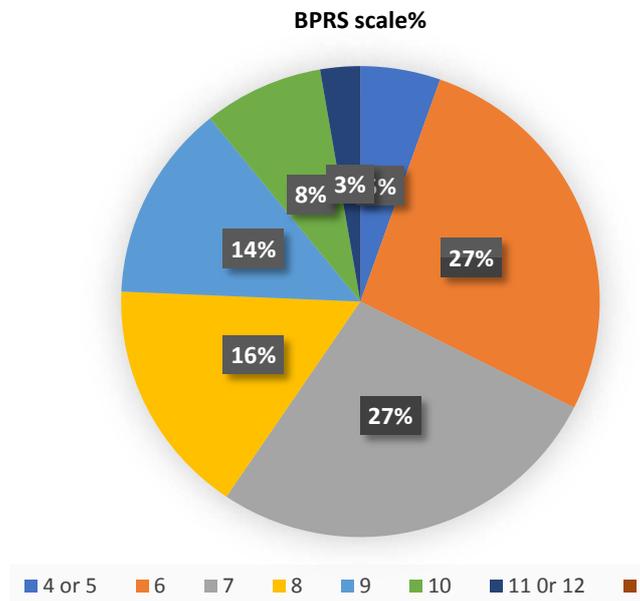


Figure 1 The brief psychiatric rating scale (BPRS) of schizophrenic patients

Table 2 Socio-demographic features among schizophrenic distress tolerant and intolerant cases and control groups

Variables	Control		Cases	
	Distress tolerant	Distress intolerant	Distress tolerant	Distress intolerant
Total No. (%)	36 (90.5%)	4 (10.5%)	29 (72.5%)	11 (27.5%)
Age (years) <sup>a</sup>	31.4 ± 5.9	32.6 ± 5.9	39.4 ± 5.7***	40.8 ± 4.9***
Gender <sup>b</sup>	Male	20 (55.6)	1 (25%)	15 (51.7)
	Female	16 (44.4)	3 (75%)	14 (48.3)
Marital status <sup>b</sup>	Unmarried	13 (36.1)	1 (25%)	19 (65.5)
	Married	23 (63.9)	3 (75)	10 (34.5)
Total	36 (100)	4 (10%)	29 (100)	11(27.5%)

a: Independent t-Test; B: Chi Square Test; SD: Standard Deviation

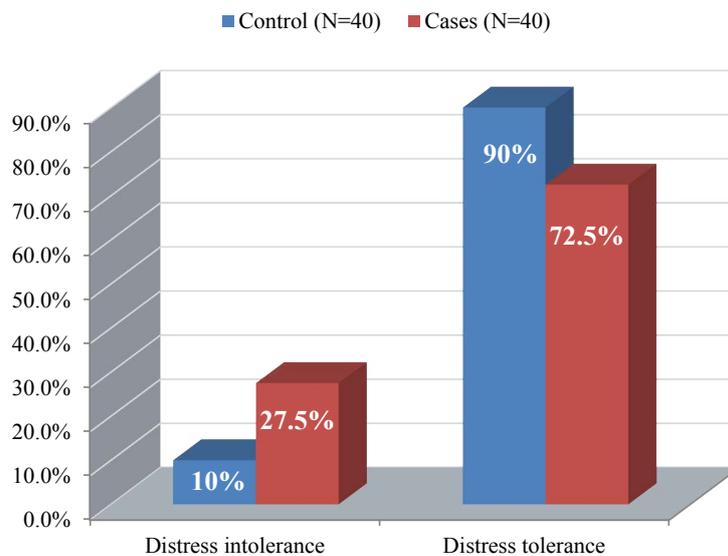
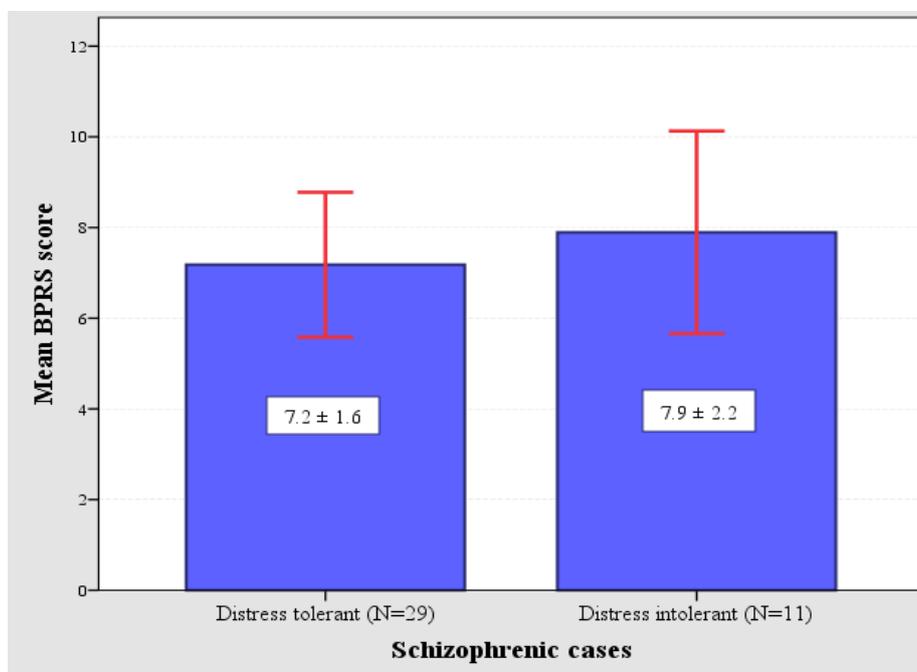


Figure 2 Histogram showing distress tolerance and intolerance of cases (patients with schizophrenia) and healthy controls (Fisher’s exact test, p<0.05)

Figure 3 depicts that the mean  $\pm$  SD of BPRS score was higher among distress intolerant cases of schizophrenia ( $7.9 \pm 2.2$ ) as compared to tolerant cases ( $7.2 \pm 1.6$ ). Nevertheless, this difference was insignificant as a matter of comparison ( $p=0.273$ ).



**Figure 3 Error Bar graph showing the mean  $\pm$  standard deviation of the BPRS scores among schizophrenic cases according to their tolerance to distress test**

The results of Tables 1 and 2, Figure 2 revealed no gender differences in the age of patients and control ( $P=758$ ). Of the controls, 90% were found to be distress tolerant and only 10% were distress intolerant whereas, 72.5% of schizophrenics were distress tolerant and 27.5% ( $P<0.05$ ) were distress intolerant. Distress intolerant patients with schizophrenia were significantly older than the control counterpart (mean  $40.8 \pm 4.9$  versus  $32.6 \pm 5.9$  years,  $P<0.001$ ). On the contrary, schizophrenic patients within the distress tolerant group were older in age than the distress tolerant control group ( $39.4 \pm 5.7$  years versus  $31.4 \pm 5.9$  years,  $p<0.001$ ). There were no significant differences in the gender ( $P=0.31$ ) or marital status ( $P=0.45$ ) in the schizophrenic cases with distress intolerance. Whereas, the percentages of married women in the distress intolerant control group (75%) were significantly higher ( $P<0.05$ ) than those of unmarried women (25%) in control distress intolerant group. Similar increase in percentages were recorded in distress tolerant unmarried schizophrenics as compared to married distress tolerant patients ( $P<0.05$ ).

**Table 3 Paired t test for the gender comparison of the salivary biochemical parameters at baseline and 10 minutes of stress in the schizophrenia patient group**

Stress test parameter <sup>a</sup>	Women Case (n=18)		Men Case (n=22)	
	Baseline	After 10 minutes	Baseline	After 10 minutes
Total Protein (%)	11.89 $\pm$ 1.05	11.83 $\pm$ 1.17	10.91 $\pm$ 1.12	11.45 $\pm$ 1.23
Cortisol (ng/dL)	178.89 $\pm$ 12.42	348.39 $\pm$ 29.42***	179.91 $\pm$ 22.06	357.41 $\pm$ 28.72***
Serotonin (ng/mL)	4.33 $\pm$ 2.56	4.56 $\pm$ 2.98	4.88 $\pm$ 2.41	5.61 $\pm$ 2.62
Trp (nmol/mL)	25.04 $\pm$ 19.70	28.79 $\pm$ 23.15	35.65 $\pm$ 28.52	29.73 $\pm$ 23.27¶
L-KYN (nmol/mL)	122.22 $\pm$ 123.49	142.22 $\pm$ 182.32	155.45 $\pm$ 136.41	115.45 $\pm$ 132.11
KYA (nmol/mL)	3.22 $\pm$ 0.79	4.78 $\pm$ 6.17	3.05 $\pm$ 1.46	2.68 $\pm$ 0.76*
QUIN (nmol/mL)	34.8 $\pm$ 19.24	30.22 $\pm$ 11.30	29.41 $\pm$ 12.34	30.45 $\pm$ 12.59
PICAcid (nmol/mL)	290.56 $\pm$ 46.12	276.67 $\pm$ 46.19**	295.00 $\pm$ 42.40	267.27 $\pm$ 58.63*
L-KYN:Trp	8.91 $\pm$ 11.99	11.09 $\pm$ 14.99	9.68 $\pm$ 11.13	10.01 $\pm$ 14.62

KYA:L-KYN	0.05 ± 0.03	0.05 ± 0.03	0.04 ± 0.06	0.04 ± 0.03
PIC Acid: L-KYN	4.42 ± 2.56	4.30 ± 2.95	3.42 ± 2.44	4.23 ± 2.48
QUIN:L-KYN	0.56 ± 0.49	0.4 ± 0.30	0.34 ± 0.33	0.47 ± 0.33†
QUIN: KYA	11.45 ± 6.94	9.71 ± 5.11	10.61 ± 5.11	11.97 ± 5.50
PIC Acid: KYA	96.04 ± 28.92	85.12 ± 34.39	110.66 ± 39.64	106.55 ± 31.49
PIC Acid: QUIN	10.19 ± 4.44	11.91 ± 9.65	12.11 ± 5.61	10.55 ± 4.74

a: Paired t-test (\*P<0.05, \*\*P<0.01; \*\*\*P<0.001); SD: standard deviation, Trp: Tryptophan; KYN: Kynurenine; KYA: Kynurenic acid; QUIN: Quinolinic acid; PIC: Picolinic acid

Using paired t-test, Figures 4A-4C revealed only significant increase in the post stress test mean salivary cortisol level (P<0.001) and significant decrease in the mean KYA:Trp ratio (P<0.01) in the control group as compared to their baseline values. Whereas in Figures 4D-4F, significant increases in the means of the salivary post stress test cortisol concentration (P=0.001) and PIC (P=0.025) were recorded in schizophrenic group as compared to the respective prestress test salivary mean values. All of the other studied parameters displayed non-significant changes (P>0.05). An independent t-test for the comparison of prestress schizophrenic mean values to those of prestress mean control values (Figure 5) revealed significant increases in the means of the salivary pre-stress test level of cortisol (P<0.01), PIC (P<0.05), PIC:KYA (P<0.001) and near significance rises in the means of PIC:QUIN ratios (P=0.063) in schizophrenic group. Moreover, there were significant decrease in the mean salivary Trp (P<0.05) and near significance decrease in the salivary KYA (P=0.063) as compared to those in the control group. Yet, non-significant changes were observed in the rest of the studied parameters (Figure 5A-5C). Comparison of the post stress test biomarkers, showed highly significantly increases in salivary mean levels of cortisol (P<0.001), KYN: Trp (P<0.01), and near significant increase in the mean QUIN:KYA ratio. Similarly, there were high significant decreases in the means of Trp concentration (P<0.001), PIC: KYA ratios (P<0.01), near significance reduction in the means of KYA (P=0.067) and KYA: KYN in the schizophrenic patient group as compared to their post stress levels of the control group (Figures 6D-6F).

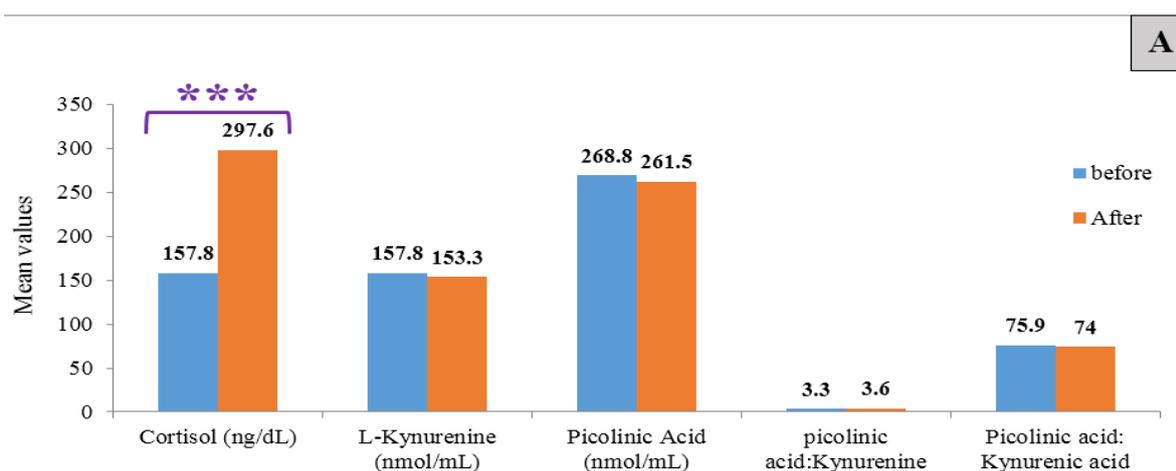


Figure 4A Paired t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*\*P<0.001, \*\*P<0.01, \*P<0.05; †Near significance)

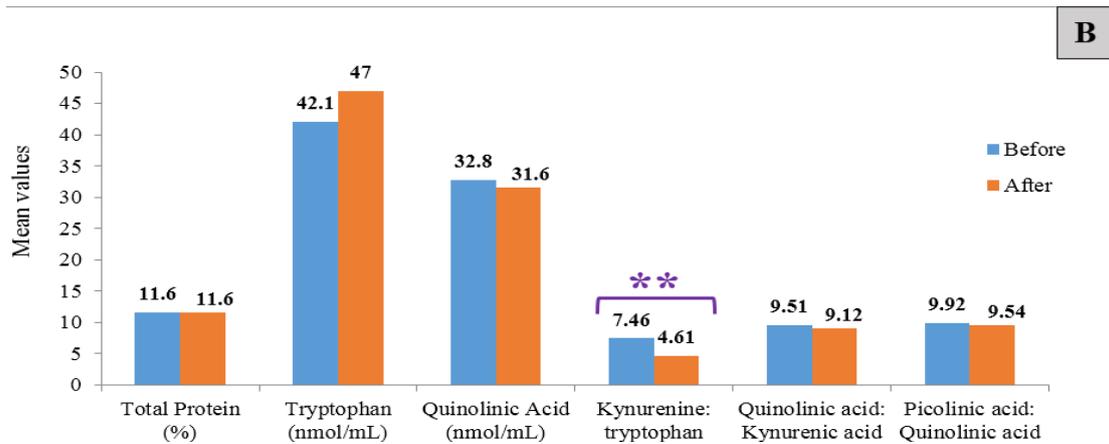


Figure 4B Paired t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

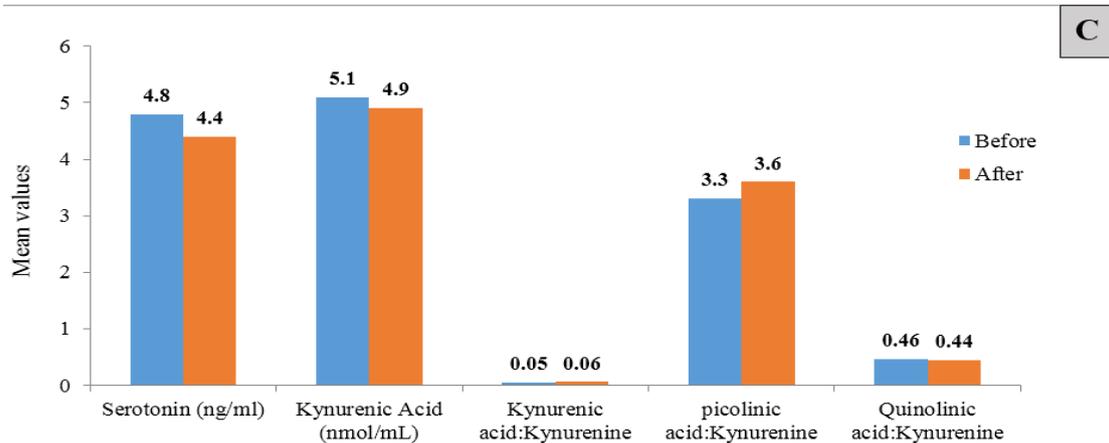


Figure 4C Paired t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

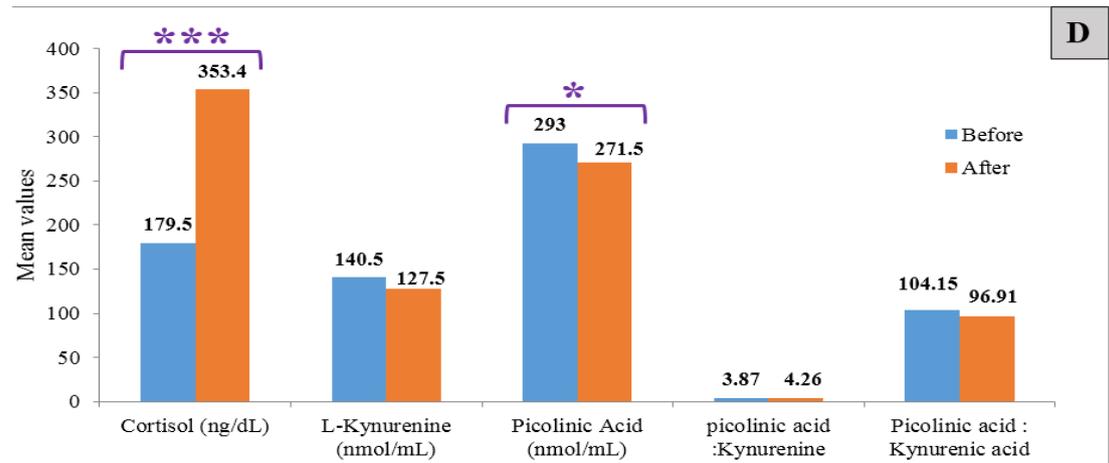


Figure 4D Paired t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

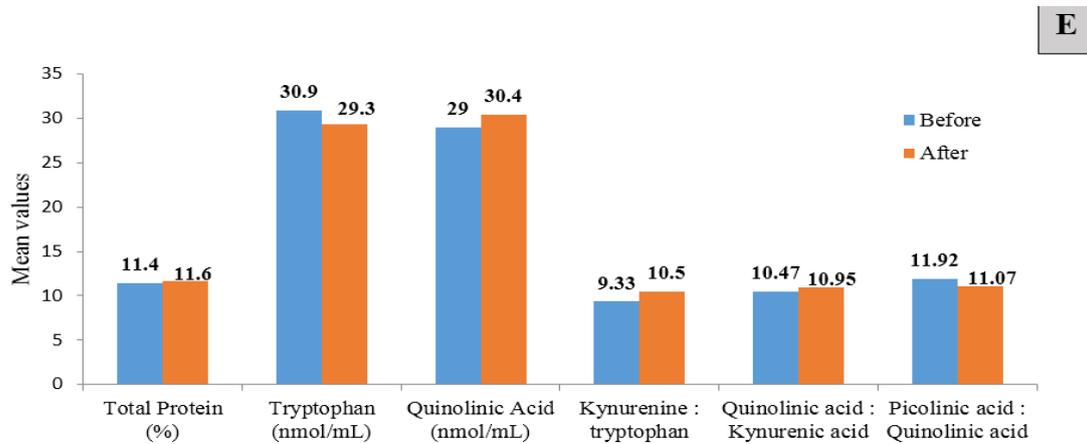


Figure 4E Paired t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

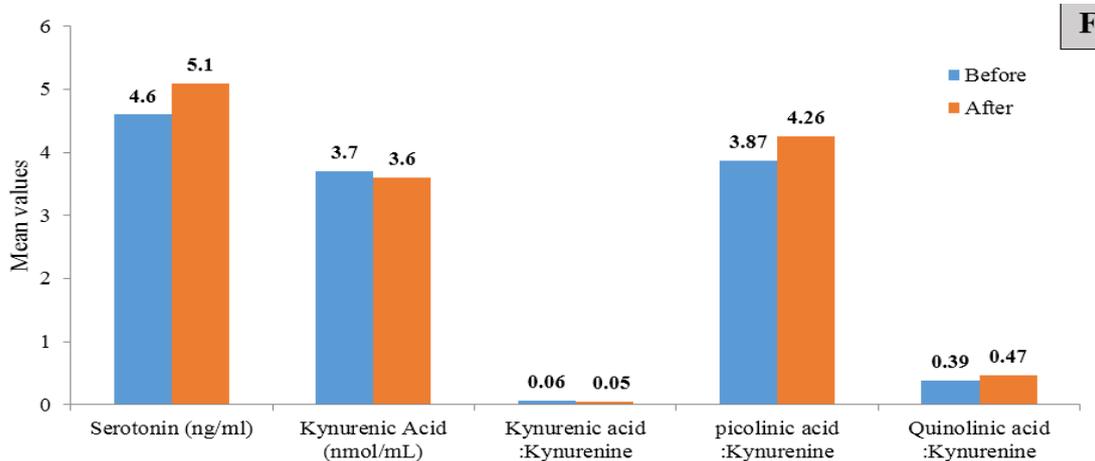


Figure 4F Paired t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

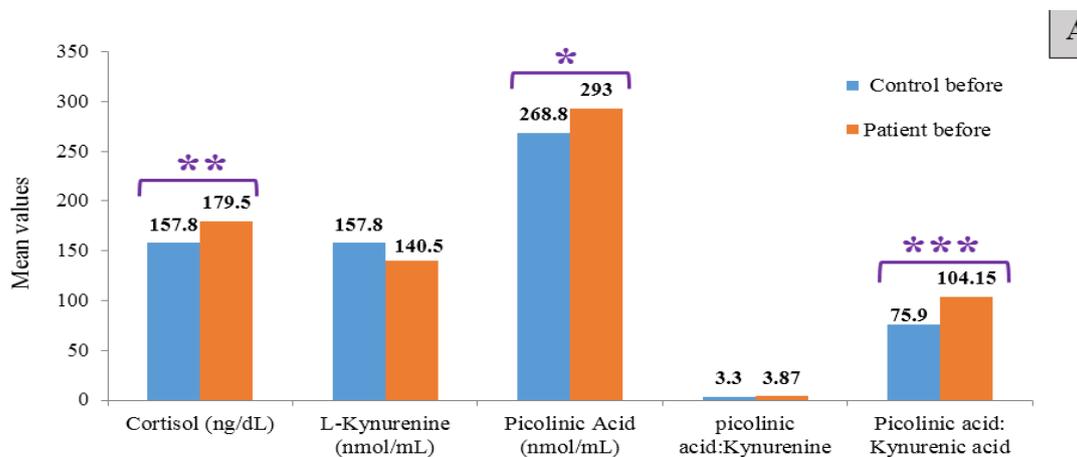


Figure 5A Independent t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

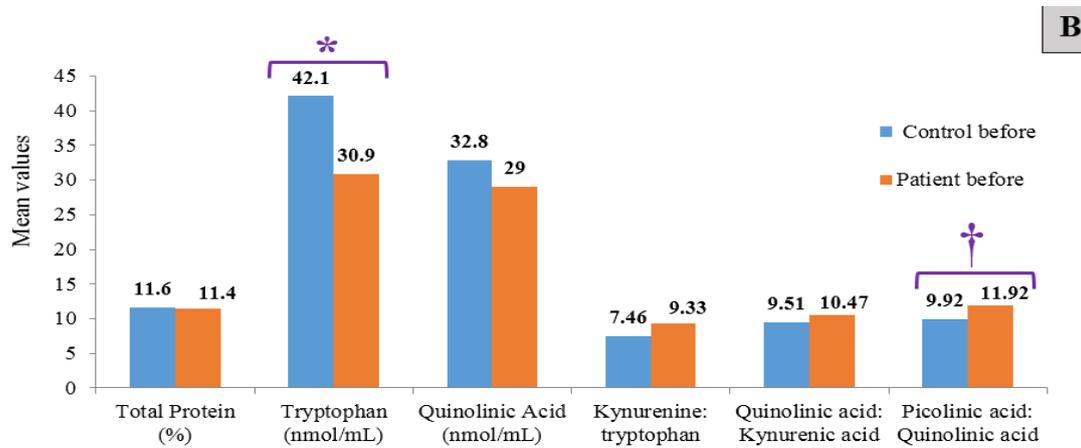


Figure 5B Independent t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

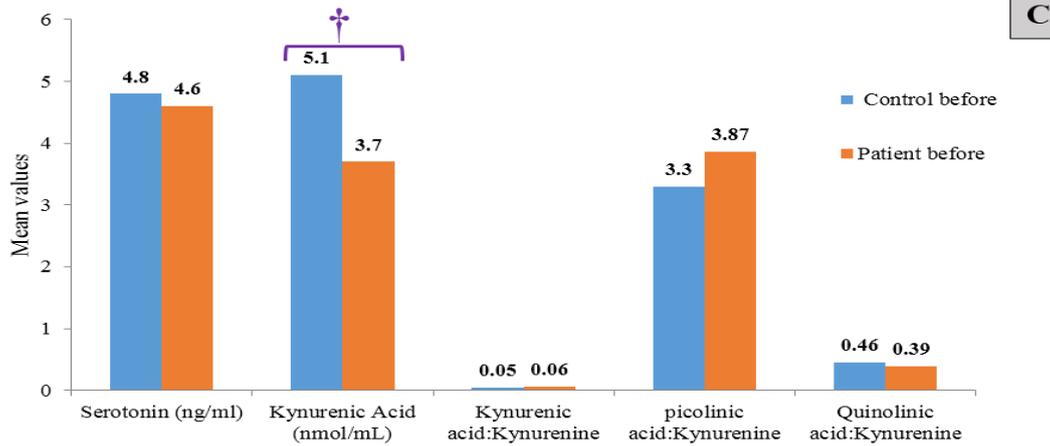


Figure 5C Independent t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

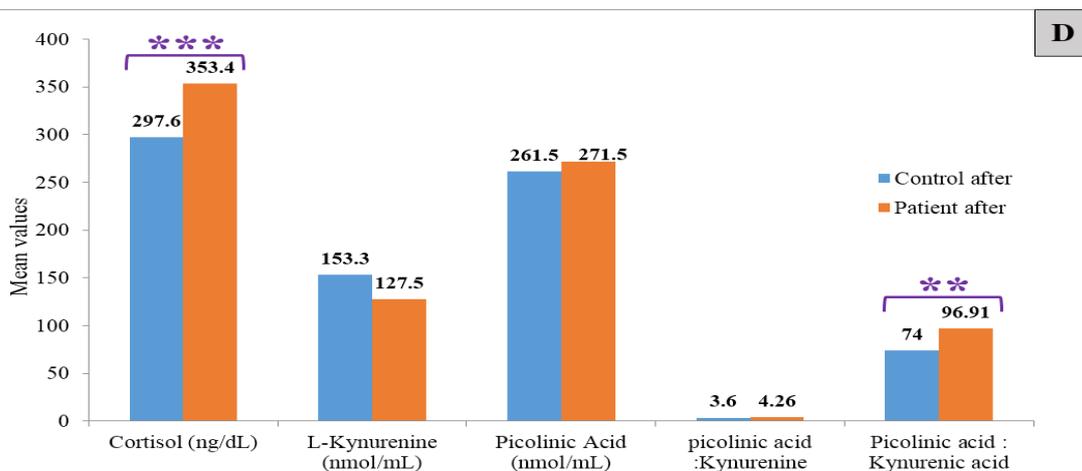


Figure 5D Independent t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*P<0.01, \*P<0.05; †Near significance)

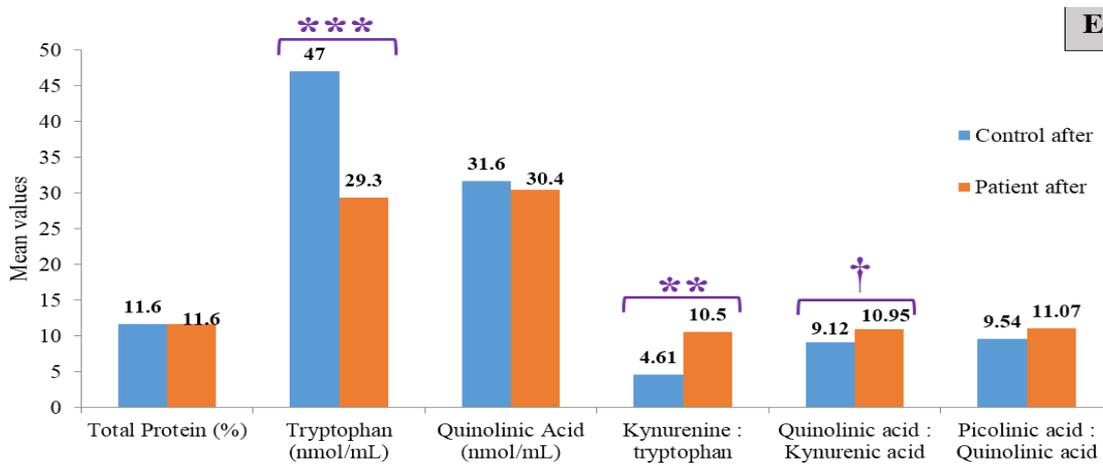


Figure 5E Independent t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*\*)P<0.001, \*\*P<0.01, \*P<0.05; †Near significance)

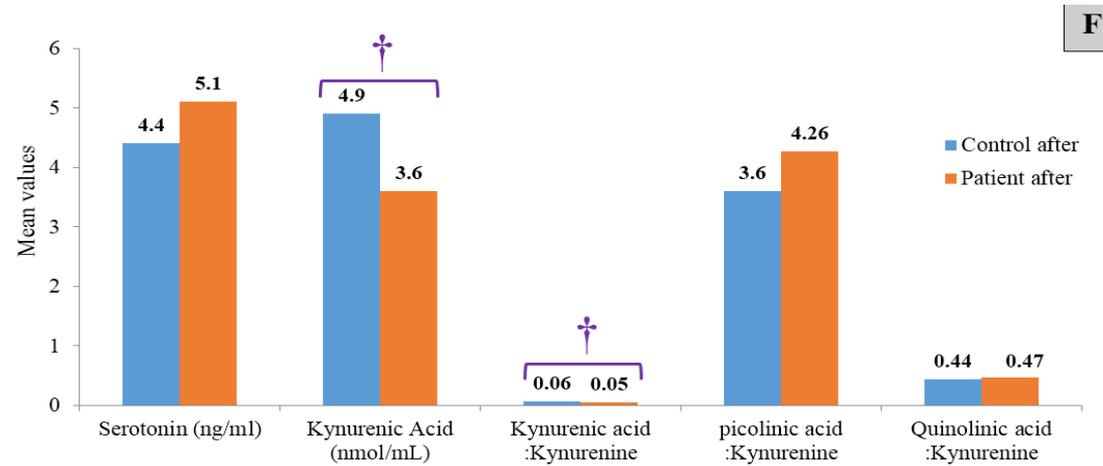


Figure 5F Independent t-test for the comparison of the studied salivary parameters before and after 10 minutes of stress test in the schizophrenic patients (n=40) and control (n=40) groups (\*\*\*)P<0.001, \*\*P<0.01, \*P<0.05; †Near significance)

The gender influence on the levels of the studied parameters in control and schizophrenia groups is shown in Table 3. Following 10 minutes of stress in control women subgroup, there were significant increases in the means of salivary cortisol (P<0.001), the ratio of KYA:KYN (P<0.05), PIC:KYN (P<0.05) and significant decrease in the mean salivary KYN:Trp ratio after stress in the control women as compared to their prestress salivary mean values (P<0.05). In the control men, there were only significant rise in the mean salivary cortisol after 10 minutes of stress (P<0.001) and a near significant decrease in the salivary PIC concentration (P=0.085) as compared to their baseline values.

In Table 4, women with schizophrenia displayed significant increase in the salivary values of cortisol (P<0.000) with only significant decrease in the mean PIC level (P<0.05) following 10 minutes of stress as compared to their baseline values. Schizophrenic men have significant elevation in the post stress salivary cortisol (P<0.001), near significant rise in QUIN: KYN (P=0.065), significant reduction in KYA (P<0.05), PIC (P<0.05) and a near significant decrease in Trp (P=0.08) in comparison to their corresponding mean salivary baseline values.

Table 4 Paired t test for the gender comparison of the salivary biochemical parameters at baseline and 10 minutes of stress in the intolerant patient and control groups

Stress test parameter <sup>a</sup>	Intolerant patients (N=11)		Intolerant control (N=4)	
	Baseline	After 10 minutes	Baseline	After 10 minutes
Total protein (%)	11.5 ± 1.19	11.8 ± 1.16	11.6 ± 1.84	11.5 ± 1.66

Cortisol (ng/dL)	181.5 ± 18.48	339.3 ± 21.25***	157.6 ± 41.04	284.0 ± 57.23***
Serotonin (ng/ml)	4.47 ± 2.44	5.34 ± 2.80¶	4.7 ± 2.45	4.4 ± 2.41
Trp (nmol/mL)	28.29 ± 22.89	30.26 ± 23.99	42.2 ± 22.36	47.1 ± 24.15
L-KYN (nmol/mL)	113.4 ± 109.5	107.9 ± 156.3	153.9 ± 84.83	154.7 ± 91.61
KYA (nmol/mL)	3.96 ± 5.09	4.03 ± 4.98	4.7 ± 3.19	4.8 ± 2.98
QUIN (nmol/mL)	28.97 ± 12.67	28.24 ± 11.65	32.0 ± 11.38	31.9 ± 10.12
PIC Acid (nmol/mL)	297.0 ± 45.33	269.0 ± 8.05*	270.0 ± 52.65	266.0 ± 47.38

a Paired t-test: \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; SD: Standard Deviation; Trp: Tryptophan; KYN: Kynurenine; KYA: Kynurenic acid; QUIN: Quinolinic acid; PIC: Picolinic acid; ¶Near significance

In the distress intolerant schizophrenic cases and control subjects (Table 5), the paired t-test revealed highly significant increases in the post stress salivary mean cortisol concentration in both patients and controls (P<0.001) as compared to their prestress mean values. Furthermore, there was a highly significant reduction in the mean post stress salivary PIC (P=0.05) and a near significance increase in the post stress salivary serotonin concentration as compared to the baseline values (P=0.086) of the distress intolerant patient subgroup. Whereas, in the distress tolerant cases, there were statistically highly significant increase in the mean post stress salivary cortisol (P<0.001), a near significant increase in the mean salivary QUIN (P=0.081), near significant decrease in the salivary mean KYA (P=0.07) of the schizophrenic group as compared to their respective baseline mean values of the distress tolerant schizophrenics (Table 6). In the distress tolerant control group there were significant increases in the means of post stress salivary cortisol (P<0.001), Trp level (P<0.05) and significant reduction in the mean salivary QUIN (P=0.054) as compared to their respective baseline values of the distress tolerant control group. No statistical significant changes were reported in the other parameters.

**Table 5 Paired t test for the comparison of the salivary biochemical parameters at baseline and 10 minutes of stress in the tolerant patient and control groups**

Stress test parameter <sup>a</sup>	Tolerant patients (N=29)		Tolerant control (N=36)	
	Baseline	After 10 minutes	Baseline	After 10 minutes
(%)Total Protein	9.3 ± 4.64	10.2 ± 3.54	11.0 ± 1.9	12.0 ± 1.58
Cortisol (ng/dL)	136.5 ± 69.67	341.7 ± 126.66***	159.5 ± 37.03	419.8 ± 5.31***
Serotonin (ng/ml)	4.3 ± 2.65	4.6 ± 2.52	5.4 ± 2.4	4.3 ± 2.69
Trp (nmol/mL)	29.3 ± 25.94	26.5 ± 21.49	41.4 ± 22.86	46.0 ± 2.69*
L-KYN (nmol/mL)	191.0 ± 141.34	154 ± 96.29	192.2 ± 95.23	140.01 ± 97.21
KYA (nmol/mL)	2.4 ± 1.29	2.4 ± 0.92¶	8.8 ± 0.83	6.3 ± 2.86
QUIN (nmol/mL)	26.9 ± 14.94	33.9 ± 12.7¶	39.5 ± 58.04	28.5 ± 6.02¶
PIC Acid (nmol/mL)	225.7 ± 117.22	247.1 ± 83.85	257.5 ± 58.04	217.5 ± 68.33

a Paired t-test: \*P<0.05, \*\*P<0.01, \*\*\*P<0.001; SD: Standard Deviation; Trp: Tryptophan; KYN: Kynurenine; KYA: Kynurenic acid; QUIN: Quinolinic acid; PIC: Picolinic; ¶Near significance

Figure 6A reveals the results of ROC curve of salivary cortisol concentration in patients with schizophrenia before and after stress test. At cut off value of 165.5 ng/dL, the sensitivity specificity and area under curve (AUC) at baseline were 72.5%, 60%, and 0.657, respectively whereas in post stress analyses, they were 70%, 72.5%, 0.752, in an ordered manner (Table 6). The pre-stress salivary KYA concentration in schizophrenic patients displayed a low sensitivity (45%) and 75% specificity at a cut off value of 3.5 nmole/ml (Figure 6B). The post stress salivary KYA sensitivity and specificity were 60% and 65%, in an order, at the same cut off value and AUC of 0.672 (Figure 6C). Significant positive correlation of the pre-stress salivary PIC with QUIN (r=0.344, P=0.029; Figure 7A) and between the salivary post stress KYN and KYA concentration (r=0.674, P=0.000) were recorded in schizophrenic patient group (Figure 7B).

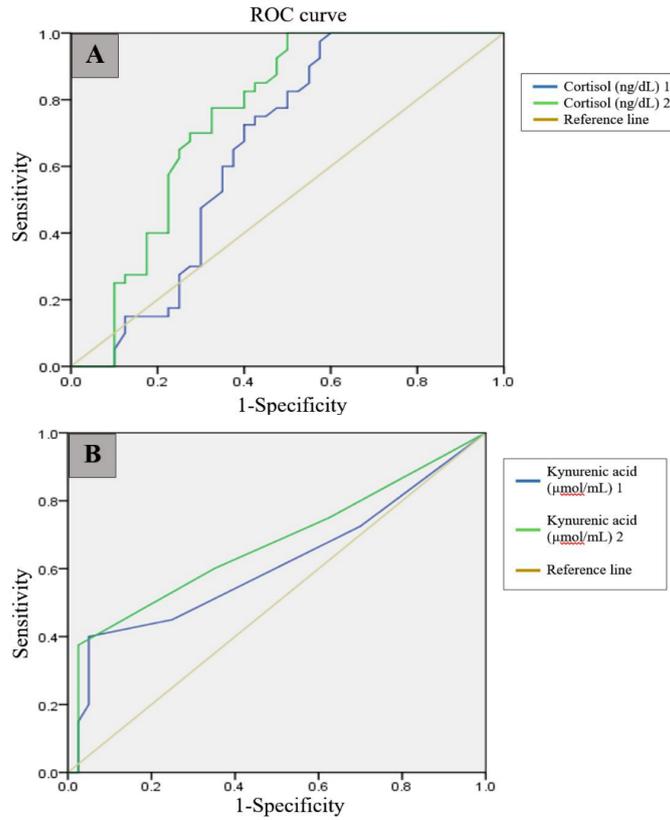


Figure 6 The receiver operating characteristic (ROC) curve of (A) the salivary cortisol (B) Kynurenic acid. 1: before, 2: after stress test

Table 6 Statistics of the salivary cortisol Kynurenic acid before and after stress test

Parameter	Cut-off value	Sensitivity	Specificity	Area under curve
Salivary cortisol 1	165.5	72.50%	60%	0.657
Salivary cortisol 2	339.5	70%	72.50%	0.752
Kynurenic Acid 1	3.5	45%	75%	0.613
Kynurenic Acid 2	3.5	60%	65%	0.672

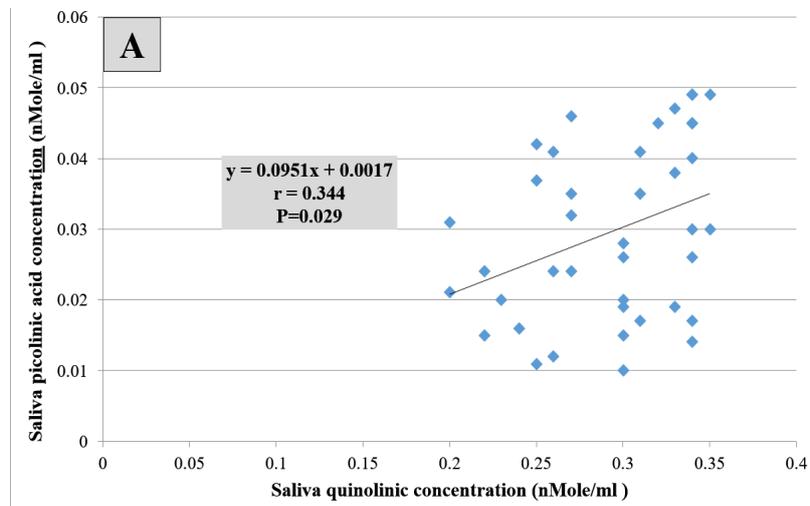


Figure 7A Correlation between patient saliva concentration of prestress picolinic acid and quinolinic acid

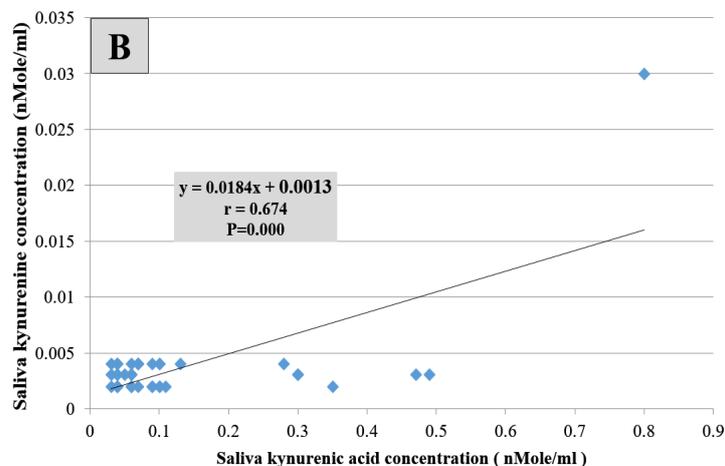


Figure 7B Correlation between patient saliva concentration of post stress kynurenine and kynurenic acid

## DISCUSSION

This study revealed that patients with schizophrenia were older in age than controls. The men to women ratio was about to 1.2:1, which is comparable to the ratio of 1.4:1 reported by McGrath, et al. [19].

According to patient response to the stress induced questionnaire using Vienna test, one tenth of the controls and more than one fourths of the studied schizophrenic cases didn't tolerate the stress test applied on them, this mean that intolerance to distress is significantly associated with diseased group. In harmony with this observation, Chiappelli, et al. [18] reported that schizophrenics displayed higher rate of distress intolerance as compared with the studied healthy controls. The BPRS clinical score was insignificantly higher among distress intolerant cases of schizophrenia as compared to tolerant cases. These findings suggest that the 24-item BPRS could be a useful metrics to symptom severity in patients with schizophrenia presented at outpatient's clinics [17]. Distress tolerance (DT) refers to the degree to which an individual can tolerate negative or aversive psychological states [20]. Individuals with weak DT may find negative emotions unbearable, believe they are incapable of coping with distress, try to avoid negative emotions or rid themselves of those emotions as quickly as possible, and find that negative emotions absorb their attention to the extent that their functioning is impaired [20,21]. DT is related to symptoms severity in multiple disorders, such as obsessive-compulsive disorder, anxiety disorders and depression [22-24].

We observed highly significant increases in salivary cortisol level in both patient and control group, but the rise is more significant in schizophrenic patients at post stress period as compared to prestress salivary mean values. Some investigators reported that following an acute psychosocial stressor the activity of the hypothalamic-pituitary-adrenal axis (HPA axis) is expected to increase, leading to high cortisol levels, increases in heart rate and blood pressure [25,26]. In schizophrenic patients this dysregulation in HPA is manifested in the form of both hyper- and hypofunction [27,28]. On the contrary, Gispens-de Wied [29] found that schizophrenia patients have similar baseline cortisol levels to controls.

The Kynurenine pathway (KP) of Trp metabolism has been shown recently to regulate formation of both neuroprotective (e.g. KYA and PIC and the essential cofactor, NAD<sup>+</sup>), and neurotoxic metabolites such as QUIN, 3-hydroxy KYN [30]. Researchers have indicated associations between levels of cortisol and cytokines, and psychological measures, such as subjective stress [31,32].

There was significant decrease in the mean salivary baseline Trp and near significance decrease in the salivary Trp degradation product, KYA, with highly significant increase in the mean salivary PIC, ratio of PIC: KYA and near significance rises in the mean PIC: QUIN ratio in schizophrenic patients as compared to those of control. These findings are probably due to significant decrease in TDO activity with age progression in the brain, liver, and kidney [33]. Additionally, they observed significant increase in brain IDO activity with age, which is consistent with finding of age-dependent increase in the brain KYN levels. This explains that the elevation in the KYN level to permit its availability to result in the increased level of KYA, PIC, and QUIN observed in this study [34]. The low Trp values

is attributed to its metabolic consumption through two major pathways, the KP and the methoxyindole pathway. The methoxy indole pathway generates 5-HT, which is a further substrate for melatonin biosynthesis [35]. This pathway accounts for approximately 5% of Trp metabolism. The other 95% of Trp is catabolized through the KP.

The low salivary KYA recorded in this study was confirmed by Birner, et al. [36] who revealed a decrease in the levels of KYA ( $p < 0.05$ ) in BD as compared to healthy control. Wurfel, et al. [37] also observed a 20% decrease in the peripheral concentration of KYA in subjects with psychosis. In contrast, BD patients with psychosis have been reported to display elevations in KYA in the CSF and decreases in KMO gene expression at post-mortem study that could theoretically increase the KYA concentrations in the brain [38,39]. Conceivably, the increase in KYA in these patients, who are currently on antipsychotic medications, may be the reflective of recovery from illness rather than being a pathogenic marker, per se. Myint and Kim [40] suggested an imbalance in the levels of neurotoxic and neuroprotective metabolites rather than the reduction in Trp that is central to the pathogenesis of depressive illness. The increase in the PIC and PIC: KYA and PIC: QUIN ratios pinpoint to the increased balance toward the production of neuroprotective antioxidant Trp metabolites and favors the supervening of the KAT to kynureninase Trp metabolic pathways in these schizophrenic patients.

Following stress test, there were highly significant increase in the salivary concentration of cortisol, KYN: Trp and PIC: KYA ratios which were associated with decrease in salivary Trp concentration and near significant rise in the mean QUIN: KYA ratio in total schizophrenic patient group. This further increase in salivary cortisol level was affirmed by Brenner, et al. [26] who proposed that patients can be discriminated from controls with a smaller change in cortisol between baseline and 15 min post-TSST, controlling for body mass index and severity of positive symptoms with a trend for lower overall cortisol secretion in patients. On the contrary, Wurfel, et al. [37] reported reduction in the mean KYN: Trp ratio in schizoaffective disorder, MDD and BD groups compared with healthy controls. The increase in KYN/Trp ratio was mostly because of a significant low Trp levels, associated with non-significant increase in KYN levels. Which is likely because of elevated catabolism of KYN to downstream metabolites [41]. Similar increase in the mean Trp breakdown index (KYN: Trp) was reported by Barry, et al. [42] in the depressed patient group compared with controls. This indicates a decreased conversion of Trp into Kynurenine and is in agreement with the lower expression of the IDO1, IDO2 and TDO2 genes [43]. In this study near significance reduction in the mean salivary KYA, KYA: KYN were recorded in schizophrenic cases as compared to those of the control group. The increased ratio KYN/KYA, points out an imbalance between KYN and KYA, or between QUIN and KYA. Some studies have demonstrated the neurotoxicity of KYN and its metabolite QUIN by stimulating NMDA receptor [44,45]. However, KYA, another metabolite of KYN, is known as an antagonist of the NMDA receptor and can reduce NMDA overstimulation to protect neurons [46]. Thus, the neurotoxic metabolites could overstimulate hippocampal NMDA receptor to signalize intracellular calcium overload and neuronal injury [47].

The significant decrease in mean salivary post stress PIC concentration as compared to baseline mean values were in line with those of Sublette, et al. [48] who reported similar decrease in PIC and a low PIC/QUIN ratio in both CSF ( $P < 0.001$ ) and blood ( $P = 0.001$  and  $P < 0.01$ , respectively) of suicide attempters. These findings opened the possibility of assessing PIC, or the ratio of PIC/QUIN in the blood as a potential risk marker of suicidal behavior as deficient amino- $\beta$ -carboxymuconate-semialdehyde-decarboxylase (ACMSD) enzyme activity underlies suicidal behavior. The enzyme ACMSD diminishes the QUIN formation by competitive production of the neuroprotective metabolite PIC. Therefore, reduced ACMSD activity can lead to high QUIN [49].

Schizophrenic women have additional increase in the mean salivary PIC: KYA, and KYA: KYN ratios, near significant increase in serotonin in addition to significant decreases in the means of KYN and QUIN:KYA ratios as compared to those of control women. There was no gender difference in the means on salivary cortisol levels. On the other side, Rajewska and Rybakowski [50] recorded HPA axis dysregulation in depression due to increased baseline plasma cortisol levels in female depressed patients with unchanged baseline plasma ACTH compared with healthy controls.

The increase in KYA: KYN ratio or the decrease in the QUIN: KYA reported herein is due to the increase in KYA or a relative decrease in KYN and QUIN in these patients. KYA and QUIN have neuroactive properties and exert multiple effects in the central nervous system. KYA is an antagonist of the glutamatergic NMDA receptor and the cholinergic nicotinic  $\alpha 7$ -receptor and as such an endogenous protector against excitotoxicity [51]. QUIN is an agonist of the NMDA receptor and may cause excitotoxicity [52]. Reduced plasma levels of KYA have been reported in depression [53] and in patients with BD [54].

Although, schizophrenic patients within the distress tolerant group were older in age than their respective in the control group, we found some elevation in the post stress salivary serotonin in patients as compared to those of the controls. The cause of this elevation partly explained by effect of some medication used by these patients and it opens fields for the future extensive studied. Persons with low level of serotonin are more prone to depression, suicide, and violence. The rate of the brain serotonin biosynthesis in women is 50% of that in men, which may explain why the women have a higher probability of distressing depressive disorders [55]. Similarly, the diminished serotonin biosynthesis and high rate of depressive disorders can also be encountered in aged human beings [30]. Serotonin is noticeable in human saliva [56-58] and possibly that salivary serotonin is derived from the blood, as it has not yet been confirmed that the serotonergic system directly projects from the brain to salivary glands [59].

Ballester, et al. [56] declared that, although these data obtained from measurement of blood (peripheral) biochemical markers, yet, they indicated that depression and schizophrenia are characterized by defective 5-HT transmission and disturbed HPA axis activity. Paired t test revealed that both men and women patients exposed to stress exhibited consistent increase in post stress salivary cortisol and decrease in PIC, with near significant increase in the mean QUIN: KYN ratio, significant decrease in the means of post stress mean KYA, with near significant reduction in the mean post stress salivary Trp level as compared to their baseline values.

The QUIN, a neuroactive metabolite of the KYN pathway (QUIN is an agonist of NMDA receptor), is normally exists in human brain and CSF. Kegel, et al. [57] reported no difference in the CSF QUIN concentration between patients with schizophrenia and controls with lower QUIN/KYA ratio (neurotoxic index) in schizophrenia than in controls. They suggested an over-activated and imbalanced Kynurenine pathway in people with schizophrenia, favoring the production of KYA over QUIN.

Yet, control women showed significant increase in the KYA: KYN and significant decrease in the mean salivary KYN: Trp ratio after stress as compared to the baseline values. In the control men, there were only a near significant decrease in the mean post stress salivary PIC acid concentration as compared to their baseline values. The higher QUIN/KYA ratio in controls is in line with other studies and support the hypothesis that KYA is elevated in patients with schizophrenia (as reflected by finding herein of lower ratio of QUIN/KYA) is likely because of a lower input of KYN into the QUIN branch of the pathway. In consistent to these findings, Chiappelli, et al. [18] recorded a rise in the mean salivary KYA levels between baseline and 20 minutes following the stress task in both patients and controls. Patients who were unable to tolerate the stressful tasks and quit early showed significantly higher levels of KYA than patients who tolerated the psychological stressor or healthy controls. In this research study, stress test cannot be extended to 20-30 minutes as in the preliminary proposal because we observed that majority of distress intolerant patients could not tolerate and quit the test in 10 minutes period.

Under conditions of inflammation, the production of QUIN predominates over KYA, possibly because of the upregulation of KYN mono-oxygenase, KMO [60,61]. KYA may contribute to cellular signaling mechanisms by binding to GPR 35. KYA affects cyclic adenosine monophosphate production and subsequent  $Ca^{2+}$  flux in astrocytes. The signaling properties of KYA at GPR 35 may contribute to its regulatory role of neurotransmission [62]. This blunted release in salivary cortisol is similar to those reported by Marcellis, et al. [63] who elucidated that when experiencing chronic psychosocial stress, the individual's HPA axis alters or adapts its reactions to subsequent psychosocial stress. Consequently, the abnormal or depressed cortisol reaction could be explained as a symptom-related effect reflecting desensitization of the HPA system by reiterated "environmental" stressors. However, other researchers suggested that the HPA axis itself is intact as it functions normally under influence of more physical stressors, but only shows impairment when faced with a more psychosocial event [64]. This HPA axis responsible for the cortisol secretion has a slower reaction time that takes 10-15 min to be reflected in saliva [25].

The increase in post stress KYN and QUIN in these patients indicates the activation of the KAT and IDO enzymatic pathways as the important Trp degradation pathways. In such patients the post stress decrease in the salivary KYA is due to its consumption in response to the increased level of the neuroactive QUIN. With the capacity to stimulate excitatory glutamatergic signaling, extra-physiological concentrations of QUIN can result in neurotoxicity or neuronal death, tissue lesions, and seizures [65,66]. Additionally, QUIN neurotoxicity is also attributed to the generation of reactive oxygen species and lipid peroxidation [67]. Due to its potent neurotoxic properties, any elevation or accumulation of QUIN can have detrimental effects on not only the local cellular environment but also can impact

behavior as well [68]. Altogether, this clue strongly suggested that KYN can be considered as an antioxidant, which can endogenous donate an electron and protect macromolecules against oxidative modifications. These properties can be independent of the KYA formation [34]. Moreover, PIC also has been shown to defend the cholinergic neurons and the nicotinamide adenine dinucleotide diaphorase of neurons of rat striatum by its copper and iron chelator properties against QUIN-induced neurotoxicity [69]. The QUIN can act as a substrate for synthesis of NAD<sup>+</sup> at concentrations of less than 50 nM but can also be a cytotoxic agent at sub physiological concentrations (>150 nM) through the NMDA over activation, NOS induction, and nitric oxide; all together increase the free-radical damage in neurons and astrocytes [70,71].

The changes in the mean concentration of baseline values salivary cortisol, PIC, Trp and KYN in distress intolerant cases were more pronounced after stress in distress intolerant cases in comparison to their mean values of the control group. In consistent to these findings, Clark, et al. [43] reported no significant differences in the adjusted means of control and depressed individuals for either Trp or KYN. Yet, they reported significant low KYN:Trp ratio and QUIN levels as compared to control means. The lower serum Trp, and less so KYN:Trp, were linked with loss of cognitive function, and there was no such correlation with the levels of KYN [71].

Paired t-test displayed a near significance increase in the post stress salivary serotonin concentration, which probably due to, apart from small number of studied patients, the effect of the antipsychotic drugs used by these patients. On the other hand, Veen, et al. [72] reported that mood changes are related to alteration in monoaminergic neurotransmitters, for example, decreased serotonin. Neuropsychiatric consequence of Trp and serotonin paucity could represent the involvement of serotonergic neurotransmission in cognitive function, memory and learning capabilities. Functions of 5-HT<sub>2A</sub> are of greater relevance in mental disorders [73]. A study of Tan, et al. in 2007, revealed a direct association between the rate of recovery from depressive symptoms, and the elevation in circadian amplitude of salivary serotonin secretion in depressed patients after fluoxetine treatment [74]. This suggests that salivary serotonin may help in the assessment of serotonergic functions [58].

Drugs that cause blockade of KAT II brings about a decrease in brain KYA but can be related to cognition-enhancing effects [75,76]. The concomitant vitamin B6 deficiency may block the KP, resulting in a toxic build-up of certain KYN downstream metabolites. The deficiency of B vitamins, is not necessarily due to insufficient dietary intake, rather by the increase in demand and oxidation [77,78] as availability of pyridoxal 5-phosphate influences the activities of kynureninase and KATs. Also, for the decarboxylation of 5-HTP by AADC in serotonin synthesis. Not surprisingly, low Trp, vitamins B6 and E play role in the pathogenesis of depression [79,80]. So, achieving adequate B6 intake may help ensure optimal KP function [81]. Rios-Avila, et al. [82] found that moderate deficiency yielded increased 3-hydroxy KYN and a decrease in KYA and anthranilic acid. More severe deficiency also yielded an increase in KYN and xanthurenic acid and more pronounced effects on the other metabolites [62,83].

## CONCLUSION

Men with schizophrenia showed aberrant response to psychosocial stress than diseased women, as reflected by increased QUIN and KYA: QUIN ratio. The modified version of Vienna test triggers an acute psychosocial stress and increases the activity of the hypothalamic-pituitary-adrenal axis as reflected by an augmented rise in cortisol levels although, heart rate and blood pressure were not monitored. The ratios of neuroprotective to neurotoxic metabolites or neurotoxic to neuroprotective metabolites rather than the absolute values could delineate the changes in the neurobiology of schizophrenia.

## DECLARATIONS

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**Conflict of Interest**

Author declared that there are no conflicts of interest.

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