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Cortisol in Schizophrenia: No Association with Tobacco Smoking, Clinical Symptoms or Antipsychotic Medication

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Abstract

Cigarette smoking is associated with higher cortisol levels in healthy subjects. In schizophrenia this relationship is not clear. There are divergent results on the association between cortisol with smoking, clinical symptoms and medication in schizophrenia. This study evaluated this association in 196 Caucasian inpatients with schizophrenia (51.30 ± 26.68 years old), subdivided into 123 smokers and 73 non-smokers. Basal salivary cortisol levels were measured twice, at 08.00 and 09.00 AM, 90-120 min after awakening. The effect of smoking on cortisol was evaluated according to current smoking status, the number of cigarettes/day and the nicotine addiction intensity. The influence of clinical symptoms and/or antipsychotic medication on cortisol was determined using the Positive and Negative Syndrome Scale (PANSS), and chlorpromazine equivalent doses.

Non-smokers were older, received lower doses of antipsychotics, had higher PANSS scores, and had longer duration of illness than smokers.

Salivary cortisol was similar in schizophrenic patients subdivided according to the smoking status, the number of cigarettes/day and nicotine addiction intensity. No significant correlation was found between salivary cortisol and PANSS scores, chlorpromazine equivalent doses, age of onset or the duration of illness.

The findings revealed no association between salivary cortisol and smoking, nicotine addiction intensity, or clinical symptoms. Our preliminary data showed no correlation between salivary cortisol and chlorpromazine equivalent doses and/or antipsychotic medication. Our findings suggest that smoking does not affect the cortisol response in schizophrenic patients as it has been shown in healthy individuals. Future studies should investigate a possible desensitization of the stress system to smoking.

Keywords: antipsychotic medication; Caucasians; chlorpromazine equivalent doses; cigarette smoking; nicotine addiction; salivary cortisol; schizophrenia; schizophrenia symptoms.

1. Introduction

Schizophrenia is a severe, heterogeneous chronic mental disorder with diverse clinical manifestations, influenced by various genetic risk factors and complex interplay between environmental risk factors such as stress exposure and gene–environment interactions. It affects about 1% of the population worldwide (Kahn et al., 2015). Patients with schizophrenia have dysregulated major system regulating the stress response, the hypothalamic–pituitary–adrenal (HPA) axis (Bradley and Dinan, 2010; Brenner et al., 2009; Girshkin et al., 2014; Girshkin et al., 2016; Walker et al., 2008), manifested in the form of both hyper- and hypofunction (Bradley and Dinan, 2010). Schizophrenic patients show altered response to stress compared to control subjects (Brenner et al., 2009; Girshkin et al., 2016). The HPA axis abnormalities in schizophrenia are indicated by elevated basal cortisol levels (Girshkin et al., 2014; Jakovljevic et al., 1998; Muck-Seler et al., 1999), non-suppression of cortisol after dexamethasone suppression test (DST) (Hori et al., 2012; Jakovljevic et al., 1998; Muck-Seler et al., 1999), and blunted cortisol awakening response (CAR) (Mondelli et al., 2010). Disrupted 24-h diurnal rhythm of cortisol secretion (Gallagher et al., 2007) and lower cortisol response to psychological stress (Brenner et al., 2009; Gispen-de Wied, 2000) were also reported. However, there are reports showing similar basal cortisol concentration between schizophrenia patients and controls (Bradley and Dinan, 2010). Some variations in the HPA axis are assumed (Murri et al., 2012) to be associated with severity of particular clinical symptoms evaluated by the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987). However, our previous study did not confirm these findings (Pivac et al., 1997). Cortisol was reported to be associated with positive

and depressive symptoms, excitement and disorganization in a small sample of first episode psychosis (Murri et al., 2012). Although schizophrenia is a neurodevelopmental disorder, the neural diathesis-stress model implicates the role of stress and HPA axis, and interaction with disturbed dopaminergic pathways, in the etiology of schizophrenia (Pruessner et al., 2016; Walker et al., 2008).

Smoking has been significantly associated with various neuropsychiatric disorders (Dome et al., 2010) and psychosocial stress (Slopen et al., 2013). The high incidence of tobacco smoking in schizophrenic patients (Sagud et al., 2009; Winterer, 2010) compared to the general population (de Leon and Diaz, 2005; Dome et al., 2010; Manzella et al., 2015) indicates their increased susceptibility to nicotine addiction.

In healthy subjects, elevated cortisol levels are associated with both passive and active tobacco smoking (Soldin et al., 2011). Cortisol levels were significantly higher in smokers than in non-smokers (Steptoe et al., 2004). These findings were confirmed in the large population cohorts of middle-aged (Badrick et al., 2007) and older (Direk et al., 2011) healthy subjects. Salivary cortisol levels were higher in current smokers than in non-smokers, but did not differ between former smokers and never-smokers (Badrick et al., 2007; Direk et al., 2011).

The primary hypothesis of this study was that smoking and/or intensity of nicotine addiction is significantly associated with salivary cortisol levels in patients with schizophrenia. As the effect of smoking on cortisol levels were evaluated in small number of patients with schizophrenia (Brenner et al., 2009; Hori et al., 2012; Iancu et al., 2007; Murri et al., 2012), we included a fairly large group of inpatients with schizophrenia. Given that smoking affects heart rate (Gillum, 1992) and heart rate is regulated by the autonomic nervous system, it was used in our study as an indicator of the cardiovascular system function. The second hypothesis of this study was that salivary cortisol levels are associated with various clinical symptoms of

schizophrenia (Girshkin et al., 2014; Murri et al., 2012) and/or with antipsychotic medication (Bradley and Dinan, 2010). Therefore, we assessed the possible associations between salivary cortisol, symptoms of schizophrenia, and different antipsychotic medication.

2. Materials and Methods

2.1. Participants

The study included 196 inpatients with schizophrenia (51.30 ± 26.68 years old), diagnosed using the Structured Clinical Interview for DSM-IV (SCID) (First et al., 1995) and recruited from the Psychiatric Hospital Vrapce, Zagreb, Croatia from January 2014 to December 2015. Schizophrenic patients were subdivided into 123 smokers (i.e. current smokers) and 73 non-smokers (i.e. never smokers and former smokers). All patients were Caucasians of Croatian origin. Besides the SCID, all patients were assessed for the presence or the severity of particular clinical symptoms of schizophrenia using the scores in the total PANSS and PANSS subscales. Inclusion criterion was a diagnosis of schizophrenia in subjects older than 18 years. All participants were screened for potential medical issues that may influence the HPA axis activity. Subjects were excluded if they had diabetes, significant alcohol or substance use, were pregnant, in lactation or breast-feeding, received Phenytoin, Ventolin, CRH, ACTH, dexamethasone, metyrapone or were treated for arthritis or multiple sclerosis. They were asked to restrain from physical activity before the protocol started. Besides nicotine dependence, no other co-morbid substance abuse or dependence was present.

All patients were treated with different antipsychotic medication: olanzapine (5-20 mg/day), clozapine (300-800 mg/day), risperidone (2-6 mg/day), fluphenazine (5-15 mg/day), haloperidol (4-15 mg/day), promazine (400-500 mg/day), alone or combined with benzodiazepines, i.e. diazepam (2-10 mg/day). Mean dose of antipsychotic medication, calculated into chlorpromazine

equivalent doses, was 309.5 ± 263.5 mg/day (range 50-1600 mg/day).

This study was conducted with the approval of the Ethics Committee of the Psychiatric Hospital Vrapce, Zagreb, Croatia, and in accordance with the ethical standards established by the 1975 Declaration of Helsinki. The procedures were discussed with all patients in detail. Patients were included in the study after they agreed to participate and provide written informed consent.

2.2. Assessment of smoking habits/nicotine exposure

Current smokers were asked to be overnight nicotine abstinent before the sample collection. Nicotine dependence was assessed by the medical charts, psychiatric interview and the Fagerstrom Test for Nicotine Dependence (FTND) (Heatherton et al., 1991). Patients were subdivided according to the FTND scores into those with mild (less than 5 scores), moderate (5-7 scores) and severe (more than 7 scores) addiction.

Patients who regularly smoke 10 or more cigarettes per day (N=123) were asked to smoke their own first cigarette with average nicotine amount (containing at least 1 mg of nicotine), in a "smoking" protocol, immediately after the first salivary cortisol sampling. Eight days later, a part of smokers (N=28) who were included in the previous "smoking" protocol, were asked to refrain from smoking their first cigarette, in another "nonsmoking" protocol, between the first and the second salivary cortisol sampling.

2.3. Symptom assessment

Based on previous factor analyses of PANSS (Citrome et al., 2011; Emsley et al., 2003; Murri et al., 2012), symptoms were assessed using the PANSS total and PANSS subscale scores.

2.4. Cortisol assessment

Since cortisol levels depend on the time of sampling and cortisol has its peak, i.e. CAR, 30-40 min after awakening (Girshkin et al., 2016; Stalder et al., 2016), morning salivary cortisol levels

(□mol/l) were determined in schizophrenic inpatients 90-120 min after awakening. Awakening time for all inpatients was between 06.00 – 06.15 AM. To avoid CAR, saliva sampling started at 08.00 AM after fasting overnight. Patients were asked to refrain from breakfast, coffee, tea, smoking, brushing their teeth, taking medication, or doing exercises before collecting salivary samples. Subjects were allowed to drink only plain water during the protocol, but not just before the salivary cortisol collections. Procedure started by measuring the patients' heart rate and taking the first saliva sample at 08.00 AM using Salimetrics oral swab storage tubes (Salimetrics Europe Ltd.) according to manufacturer's instructions (2 minutes under the tongue). Samples were immediately refrigerated at 4°C and later stored at -20°C. Second saliva sample (at about 09.00 AM) was scheduled 45-50 minutes after smoking, or 55-60 minutes after the first salivary sample for non-smokers. During this period smokers completed the FTND. Non-smokers rested between the first and the second saliva sampling.

Salivary cortisol levels were measured using Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics Europe Ltd.), according to the manufacturer's instructions. Detection was based on a reaction between cortisol conjugated horseradish peroxidase enzyme and tetramethyl-benzidine as a substrate. The optical density was read on a standard plate reader at 450 nm. All samples were run in duplicates. Samples from an individual patient (sampled at 08.00 and 09.00 AM) were analyzed within the same run. Coefficient of variation (CV) was 7.7%, and an inter-assay CV was 13.6%.

Salivary cortisol data were divided according to treatment with typical vs. atypical antipsychotics alone or in combination with benzodiazepines, or according to chlorpromazine equivalent doses.

2.5. Heart rate

Resting heart rate (beats/minute) was measured by a psychiatrist using the palpation

method at the wrist (radial artery), by counting the number of beats within a one minute period. This procedure was repeated two times: the first heart rate measurement was at 08.00 AM and the second measurement was 30 minutes after the first measurement (around 08.30 AM).

2.6. Data analyses

All results were evaluated with SigmaStat 3.5 (Jandel Scientific Corp., San Jose, California, USA). Normality of the distribution was assessed with the Kolmogorov–Smirnov test. Due to non-normal distribution of our data, all analyses were performed using non-parametric tests. Age, PANSS scores, age at onset, duration of illness, number of hospitalizations, total duration of hospitalizations and chlorpromazine equivalent doses are expressed as median (Q1-Q3); gender and employment as number (percentages); FTND scores as mean ± SD. Salivary cortisol levels (□mol/l), heart rate (bmp) and demographic data between smokers and nonsmokers were compared using Mann-Whitney test. Salivary cortisol levels in three different groups of subjects subdivided according to the number of cigarettes/day, or according to their physical addiction to nicotine, was evaluated using Kruskal Wallis ANOVA on ranks. Wilcoxon signed-rank test was used for the comparison of the dependent samples, such as the first and the second salivary cortisol concentration and heart rate measured in the same person. A multiple regression analysis was used to assess for the influence of gender and age on salivary cortisol levels and heart rate. All correlations, of salivary cortisol measured at 08.00 or 09.00 AM, absolute change in salivary cortisol and number of cigarettes/day, with 1) FTND scores; 2) clinical symptoms of schizophrenia (evaluated using the PANSS total, positive, negative, general psychopathology subscales and particular symptom dimensions: selected PANSS positive, selected PANSS negative, PANSS excited, PANSS disorganized, PANSS motor, PANSS depression, PANSS anxiety and PANSS cognition subscale scores); 3) chlorpromazine equivalent doses; 4) demographic variables, were examined using Spearman rank order

correlation.

G*Power 3 Software (Faul et al., 2007) was used for the *a priori* determination of sample size. For Mann-Whitney test (with $\alpha = 0.05$; power $(1 - \beta) = 0.800$; medium effect size ($\omega = 0.5$)) required sample size was 184. For Wilcoxon test (matched pairs) (with $\alpha = 0.05$; power $(1 - \beta) = 0.800$; medium effect size ($\omega = 0.5$)) required sample size was 47. For multiple linear regression, (with $\alpha = 0.05$; power $(1 - \beta) = 0.800$; medium effect size ($\omega = 0.15$), and 2 predictors) required sample size was 68. Hence the actual sample size of 196 had the needed statistical power (power $(1 - \beta) = 0.800$) and adequate sample size to detect significant differences among the groups.

3. Results

3.1. Demographic data in patients with schizophrenia

Demographic data, as well as PANSS and FTND scores, and chlorpromazine equivalent doses, are shown in Table 1. Non-smokers were significantly older, had significantly longer duration of illness and received significantly lower doses of antipsychotic medication (calculated as chlorpromazine equivalent doses) than smokers. There were no significant differences in the distribution of male or female subjects or employment between smokers and nonsmokers. Smokers and non-smokers did not differ significantly in the number of hospitalization and total duration of hospitalizations (Table 1).

3.2. Symptoms in smokers and non-smokers

The total PANSS scores and the PANSS general psychopathology scores were significantly higher in non-smokers compared to smokers (Table 1). The PANSS positive and negative scores were similar in smokers and non-smokers (Table 1).

3.3. Salivary cortisol in smokers and non-smokers with schizophrenia

Multiple linear regression analysis revealed no significant effect of gender or age on basal salivary cortisol levels: ($F_{2,195}$=0.33, p=0.717, $Radj^2$=0.000, β_{age}=-0.055, p_{age}=0.452, β_{gender}=0.030, p_{gender}=0.679 for the first cortisol sample and $F_{2,195}$=0.26, p=0.769, $Radj^2$=0.000, β_{gender}=-0.028, p_{gender}=0.700, β_{age}=0.048, p_{age}=0.509 for the second cortisol sample). Therefore, in all further analyses, cortisol data for men and women were merged. The first or the second cortisol measurement did not differ significantly between smokers and non-smokers (Figure 1). Both in smokers and in non-smokers, the second cortisol was significantly lower than the first cortisol sample (Figure 1).

To assess whether the number of cigarettes/day affects significantly salivary cortisol levels, smokers were subdivided into those smoking 10-20, 21-30 or more than 30 cigarettes per day. No significant differences (Kruskal Wallis ANOVA) were found between these groups in the first cortisol measurement (H=0.966, df=2, p=0.617). The second salivary cortisol levels were similar in smokers smoking 10-20, 21-30 or more than 30 cigarettes per day (H=3.115, df=2, p=0.211).

In order to evaluate the possible effect of the first cigarette of the day on salivary cortisol levels, the difference between the first and the second saliva cortisol sample (absolute change or Δ cortisol) was compared in smokers who smoked and smokers who refrained from smoking of their first cigarette that day (N=28). Similar change of cortisol was found between those who smoked the first cigarette and the same group 8 days later who refrained from smoking of their first cigarette (W=30.00, p=0.741, Wilcoxon test).

To assess (with Kruskal Wallis ANOVA) the possible influence of nicotine dependence on second salivary cortisol sample, smokers were subdivided according to the FTND scores. Their salivary cortisol levels were similar between 56 patients with mild (9.9 (6.1-16.4) □mol/l), 41 patients with moderate (10.2 (7.8-14.9) □mol/l) and 26 patients with severe (10.8 (7.7-16.5)

□mol/l) physical addiction to nicotine (H=0.181; df=2; p=0.913). The absolute change of cortisol between the first and the second salivary sample did not differ significantly when smokers were subdivided into those with mild (4.5 (1.3-8.1) □mol/l), moderate (5.2 (3.0-8.0) □mol/l) and severe (3.6 (1.8-6.0)□□mol/l) physical addiction to nicotine (H=2.379; df=2; p=0.304). Spearman rank order correlation revealed a lack of significant correlation between a change in salivary cortisol levels and number of smoked cigarettes (r=0.02; p=0.769). No significant correlation was detected between a change in salivary cortisol levels and total FTND scores (r=0.07; p=0.420).

3.4. Correlation between salivary cortisol and PANSS scores in smokers and non-smokers

To examine the association between cortisol and the PANSS scores, Spearman rank order correlation was used in patients subdivided into smokers and non-smokers. No significant correlations (p>0.05) were found between salivary cortisol levels and positive, negative, general psychopathology and total PANSS scores in both smokers and non-smokers with schizophrenia (data available on request).

3.5. Correlation between salivary cortisol and PANSS total and selected PANSS subscale scores in all patients with schizophrenia

Spearman rank order correlation revealed a lack of significant correlation between the PANSS total or the selected PANSS subscale scores and the first or the second salivary cortisol levels, or absolute change (Δ) in salivary cortisol levels in schizophrenic patients (Table 2). No significant (p>0.05) correlations were found between the cortisol levels (the first and the second salivary cortisol sample and a change of salivary cortisol) with the age of onset, duration of illness, number of hospitalizations or total duration of hospitalizations in all patients with schizophrenia (data available on request).

3.6. The association between salivary cortisol concentration and antipsychotic medication and/or chlorpromazine equivalent doses in patients with schizophrenia

Salivary cortisol in patients treated with different antipsychotics alone or in combination with benzodiazepines is shown in Table 3 and Supplementary Table 1. The first or the second salivary cortisol sample, or a change (Δ) of cortisol did not differ significantly between patients treated with different medication (Table 3, Supplementary Table 1). Spearman coefficient of correlation revealed that chlorpromazine equivalent doses were not significantly correlated with the first and the second salivary cortisol levels, or a change (Δ) of cortisol in patients with schizophrenia (Table 3). When patients were divided into smokers and non-smokers, no significant correlation was detected between chlorpromazine equivalent doses and the first and the second salivary cortisol levels, or a change (Δ) of cortisol (Supplementary Table 2).

3.7. Heart rate in smokers and non-smokers with schizophrenia

Multiple linear regression analysis revealed no significant effect of age or gender on the first heart rate ($F_{2,195}$=0.37, p=0.690, $Radj^2$=0.000, β_{age}=-0.012, p_{age}=0.871, β_{gender}=0.062, p_{gender}=0.390). A significant effect of age and a lack of effect of gender was found on the second heart rate ($F_{2,194}$=4.21, p=0.016, $Radj^2$=0.032, β_{gender}=0.047, p_{gender}=0.511, β_{age}=-0.206, p_{age}=0.004). Since gender did not significantly affect heart rate, all further analyses for heart rate were performed for men and women together. The first heart rate was similar in smokers and non-smokers (Figure 2). The second heart rate was significantly higher in smokers compared to non-smokers. When the first and the second heart rate were compared in the same person, a significantly higher second heart rate was found in smokers after they smoked their first cigarette of the day compared to their first measurement. In non-smokers, the second heart rate was significantly lower than their first heart rate, and significantly lower compared to the second heart rate in smokers (Figure 2).

4. Discussion

The results of the present study showed that 1) patients with schizophrenia who were non-smokers were older, had longer duration of schizophrenia, had more severe symptoms and were treated with lower doses of antipsychotics than smokers; 2) salivary cortisol was similar between smokers and non-smokers; and was not associated with smoking the first cigarette of that day, the number of cigarettes/day, or the intensity of physical addiction to nicotine; 3) salivary cortisol was not correlated with clinical symptoms of schizophrenia; 5) salivary cortisol was not associated with chlorpromazine equivalent doses or antipsychotic medication.

In line with the previous findings (Dickerson et al., 2013; Salokangas et al., 2006) the prevalence of tobacco smoking was 64% in this study, and patients smoked around 20 cigarettes/day. This number might be under-estimated because the inpatients are restricted to smoke due to the hospital's regulations. Non-smokers were older than smokers, had significantly higher PANSS total and PANSS general psychopathology scores, longer duration of the illness and were treated with lower doses of antipsychotics. These more pronounced symptoms in non-smokers are presumably due to the longer duration of schizophrenia and treatment with the lower doses of antipsychotics compared to smokers. More severe symptoms in non-smokers agree with higher negative symptoms and total PANSS scores in Chinese light smokers with schizophrenia compared to heavy smokers (Zhang et al., 2014). These results disagree with other data (Aguilar et al., 2005; Iancu et al., 2007; Salokangas et al., 2006). The differences between studies might be explained by the small number of patients (Iancu et al., 2007), the use of different clinical scales (Salokangas et al., 2006), or the subdivision of schizophrenic patients into heavy, mild or light smokers (Aguilar et al., 2005; Zhang et al., 2014) compared to smokers and non-smokers in the present study. More frequent smoking in schizophrenic patients than in healthy subjects and

more severe symptoms in non-smokers might be explained by a form of self-medication, where patients smoke since they try to alleviate cognitive and negative symptoms and reduce side effects of antipsychotics (Sagud et al., 2008; Winterer, 2010). However, this hypothesis was recently criticized, since some predictors for self-medication associated with smoking were not confirmed (Manzella et al., 2015). Both regular (Salokangas et al., 2006) and heavy (Zhang et al., 2014) smoking are general health risks for schizophrenia, and additional health risks for cancer, cardiovascular disease and increased mortality. Hence clinicians should focus on the interventions for cessation, or at least a reduction of the number of cigarettes smoked per day, as integral part of schizophrenia treatment.

Similarly to previous results (Girshkin et al., 2016; Hori et al., 2012; Murri et al., 2012), and a meta-analysis with 2613 schizophrenic patients (Girshkin et al., 2014), in this study salivary cortisol levels were not influenced by age or gender in schizophrenic patients. Salivary cortisol levels (sampled either at 08.00 or at 09.00 AM) and a change in salivary cortisol were not associated with current smoking; the first cigarette smoked that day, the number of cigarettes/day and the intensity of physical addiction to nicotine in the present study. These findings might be related to the disturbed dopamine metabolism in schizophrenia (Sagud et al., 2009; Walker et al., 2008; Winterer, 2010). Our data agree with the previous results in patients with schizophrenia (Hori et al., 2012; Iancu et al., 2007) or the first episode of psychosis (Mondelli et al., 2010). The lack of cortisol response to smoking might be explained with desensitization to smoking, possibly due to the dysregulated dopamine metabolism and/or medication use. Namely, cigarette smoking usually increases dopamine concentration in mesolimbic system; however schizophrenia is characterized by dopaminergic hyperfunction in these regions. Therefore the effects of smoking on limbic regions including the HPA axis could be alleviated in schizophrenic patients, resulting in a lack of cortisol response (Sagud et al., 2008; Walker et al., 2008; Winterer,

2010). Our results reveal that smokers and non-smokers with schizophrenia have similar salivary cortisol levels, while healthy control smokers have significantly higher salivary cortisol levels compared to non-smokers (Badrick et al., 2007; Direk et al., 2011). These findings collectively suggest that salivary cortisol is differently affected by smoking in patients with schizophrenia, presumably due to the clinically relevant HPA axis dysfunction in schizophrenic patients (Bradley and Dinan, 2010), compared to cohorts of general population (Badrick et al., 2007; Direk et al., 2011). The possible influence of psychological stress was excluded since the identical protocols were used for all patients, suggesting exposure to similar stress levels. A clear effect of the diurnal rhythm was confirmed (Hempel et al., 2010) since the second salivary cortisol levels were significantly lower in both smokers and non-smokers compared to the first measurement at 08:00 AM.

No correlation was observed between cortisol levels and severity of symptoms in the first episode of psychosis patients (Mondelli et al., 2010), or the Brief Psychiatric Rating Scale total and subscale scores (Markianos et al., 1999), or positive and negative symptoms of schizophrenia (Pivac et al., 1997). In addition, no significant association between plasma/salivary cortisol and total PANSS scores (Girshkin et al., 2016; Hori et al., 2012; Strous et al., 2009), depressive symptoms (Munro et al., 1984), positive and negative symptoms (Girshkin et al., 2016; Hori et al., 2012; Markianos et al., 1999), and general psychopathology symptoms (Hori et al., 2012; Markianos et al., 1999) was reported in schizophrenia. Our results confirm these data since no significant correlation was detected between the total and the PANSS subscale scores and salivary cortisol levels determined either at 08.00 AM or at 09.00 AM or a change of cortisol. However, some studies that included rather small number (10-91) of subjects and may be underpowered detected positive or negative correlations between plasma or salivary cortisol and clinical symptoms of psychosis (Bradley and Dinan, 2010; Murri et al., 2012). These

discrepancies might be explained by the differences in diagnoses, rating scales, instruments used for assessing clinical symptoms, effect sizes, ethnicities, illness characteristics, illness stage and assay methods, plasma vs. salivary cortisol, timing of the cortisol measurement and circadian variability of cortisol (Girshkin et al., 2014; Murri et al., 2012).

In contrast to previous reports (Hatzimanolis et al., 1998; Jakovljevic et al., 2007; Markianos et al., 1999; Popovic et al., 2007; Tanaka et al., 2008; Zhang and Zhou, 2005), the present results revealed that antipsychotic medication (either neuroleptics or atypical antipsychotics), given as a monotherapy or in combination with benzodiazepines, did not affect salivary cortisol levels in patients. In addition, the first or the second cortisol measurements or a change in cortisol were not significantly associated with chlorpromazine equivalent doses. Smoking also affects degradation of most antipsychotics by increasing the activity of the cytochrome P450 isoforms and reducing plasma levels of various antipsychotics (Sagud et al., 2008; Winterer, 2010). Therefore, the finding of the similar cortisol values in smokers and nonsmokers, and a lack of association between salivary cortisol and chlorpromazine equivalent doses or antipsychotic medication, might be explained with the inhibitory effect of smoking on antipsychotic doses, resulting in reduced antipsychotic plasma levels in smokers and consequently similar cortisol values as in nonsmokers. The differences across studies may be explained by differences in plasma vs. salivary cortisol measurement or in small number (17-48) of patients in previous studies (Hatzimanolis et al., 1998; Jakovljevic et al., 2007; Markianos et al., 1999; Popovic et al., 2007; Tanaka et al., 2008; Zhang and Zhou, 2005). The discrepancies might also be due to differences in the schizophrenia severity, socio-cultural and economic factors, or biological heterogeneity (Zhang et al., 2014). In our study patients had similar severity of symptoms (total PANSS score range from 61-86), and were ethnically homogeneous and therefore severity of the illness and biological heterogeneity might be neglected. However, economic or socio-cultural

factors could contribute to these differences. Significant associations between cortisol and clinical symptoms of schizophrenia might be lost after clinical improvement and successful treatment (Bradley and Dinan, 2010). Since our patients received long-term antipsychotic treatment, and their median total PANSS scores (69-79) revealed that they were clinically improved, this might explain the lack of association between cortisol, clinical symptoms of schizophrenia and antipsychotic medication and/or chlorpromazine equivalent doses.

As expected (Gillum, 1992), in our study heart rate was higher in smokers compared to non-smokers. Smokers and non-smokers had similar first heart rate; while the second heart rate, measured after the first cigarette smoked that day in smokers, was higher than the first heart rate in smokers, and higher than the second heart rate in non-smokers. Since all non-smokers were resting between the first and the second heart rate monitoring, their second heart rate was lower than the first measurement due to the rest period. In line with the lack of association between increased heart rate and elevated salivary cortisol in our study, in patients with chronic schizophrenia, heart rate and salivary cortisol did not follow the same pattern of changes following stress response (Brenner et al., 2009).

Limitations of the study: 1) The possible influence of menstrual cycle, use of oral contraceptives and hormone replacement therapy (Kudielka et al., 2009) might affect cortisol. However our previous study revealed that menstrual status (pre- vs. post-menopausal) did not significantly affect plasma cortisol levels (Muck Seler et al., 2004); 2) The influence of genetic factors, early stress, social support, social hierarchy and psychological interventions, could also affect salivary cortisol and was not determined (Kudielka et al., 2009); 3) Due to the very strict protocol, it was not possible to compare the effect of smoking on cortisol between patients with schizophrenia and mentally healthy subjects, and/or patients in the first episode or acute phases of schizophrenia; 4) since this was a naturalistic study, patients were treated for a long time with

different types of antipsychotic medication. Therefore, the data showing a lack of association between salivary cortisol and antipsychotic medication and/or chlorpromazine equivalent doses are preliminary.

The strengths of the study were: 1) this study included the largest sample of inpatients with schizophrenia, a homogenous population of the same ethnic origin, recruited from the same center, allowing adequate statistical power and sample size, calculated "a priori" as suggested (Kudielka et al., 2009); 2) basal salivary cortisol was evaluated in a hospital setting in a closely followed protocol (Stalder et al., 2016); 3) smoking behavior was evaluated in details. Therefore, these data might be used for the future meta-analyses.

5. Conclusions

The results of the present study revealed that salivary cortisol was not influenced by smoking, nicotine addiction intensity or clinical symptoms of schizophrenia. Our preliminary data showed no correlation between salivary cortisol and chlorpromazine equivalent doses. The data suggest that patients with schizophrenia differ from the general population in the HPA axis' responses to smoking and nicotine addiction. The differences might be explained by the HPA axis dysfunction in patients with schizophrenia (Bradley and Dinan, 2010), and a disrupted cortisol stress reactivity (Girshkin et al., 2016), presumably related to frequent hospitalizations, long duration of illness and long term antipsychotic treatment, possible lack of social support or particular genetic factors (Kudielka et al., 2009).

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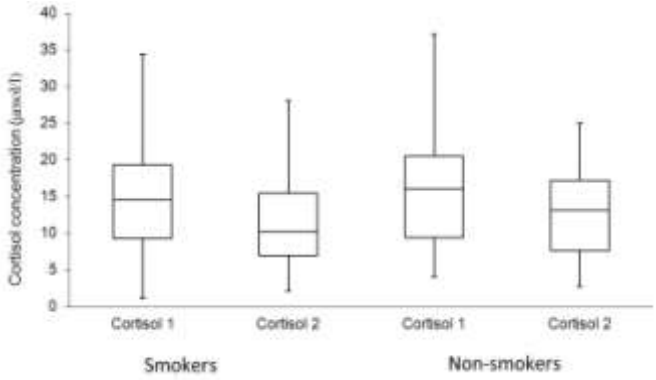

Figure 1. The first (Cortisol 1) and the second (Cortisol 2) salivary cortisol sample in schizophrenic smokers and non-smokers. *(W=-4583.00; p<0.001, Wilcoxon test) vs. the first cortisol sample in smokers; **(W=-1669.00; p<0.001, Wilcoxon test) between smokers and nonsmokers in the first cortisol sample; (U=4067.00; p=0.272, Mann Whitney test) between smokers and nonsmokers in the first cortisol sample; (U=3951.00; p=0.161; Mann Whitney test) between smokers and nonsmokers in the second cortisol sample.

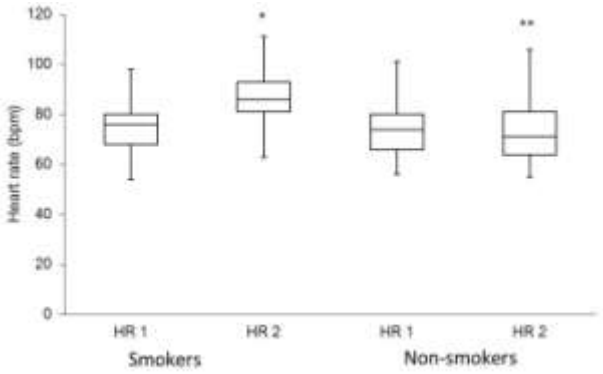

Figure 2. The first (HR1) and the second (HR2) heart rate in schizophrenic smokers and non-smokers. *(W=9641.00, p<0.001, Wilcoxon test) vs. the first heart rate sample in smokers; **(W=-1343.00, p=0.003, Wilcoxon test) vs. the first heart rate in non-smokers; $^{\#}$(U=7227.00; p<0.001, Mann-Whitney test) vs. the second heart rate in smokers; (U=701.50, p=0.754, Mann-Whitney test) for the first heart rate between smokers and non-smokers.

Table 1. Demographic data, PANSS, FTND and VAS scores for patients with schizophrenia subdivided according to their smoking status

		Smokers	Non-smokers	Statistical analysis
Smoking status N (%)		123 (62.8)	73 (37.2)	
Age (years)		49.0 (39.0-54.0)	56.0* (45.0-62.0)	U=2793.5; p=0.001; MW test
Gender	Females	76 (38.8)	53 (27.0)	χ^2=1.925; df=1; p=0.165; χ^2 test
	Males	47 (24.0)	20 (10.2)	
Employment	Yes	15 (7.6)	7 (3.6)	χ^2=0.105; df=1; p=0.745; χ^2 test
	No	108 (55.1)	66 (33.7)	
Number of cigarettes		22.6±4.9	0	
Age at onset (years)		30.0 (25.0-36.0)	32.0 (30.0-36.0)	U=3973.0; p=0.207; MW test
Duration of illness (years)		15.5 (7.0-24.0)	20.0* (11.5-29.0)	U=3298.0; p=0.002; MW test
Number of hospitalizations		4.0 (2.0-5.0)	4.0 (3.0-5.0)	U=4047.0; p=0.281; MW test
Total duration of hospitalizations (months)		6.0 (3.0-9.0)	6.0 (4.0-12.0)	U=3878.0; p=0.130; MW test
Chlorpromazine equivalent doses		200 (200-400)	200* (100-300)	U=3011.0; p=0.0001; MW test
PANSS total scores		69.0 (61.3-79.8)	79.0 (70.0-86.3)*	U=2950.0; p=0.001; MW test
PANSS positive scores		16.0 (13.0-18.8)	17.0 (14.0-19.0)	U=3811.0; p=0.076; MW test
PANSS negative scores		20.0 (17.0-22.0)	21.0 (18.0-24.0)	U=3750.5; p=0.053; MW test

PANSS general scores	34.0 (29.0-41.0)	41.0 (37.0-46.0)*	U=2677.0; p=0.001; MW test
FTND total scores	4.8±2.2	0	

PANSS: Positive and Negative Syndrome Scale; FTND: Fagerstrom Test for Nicotine Dependence; age, PANSS scores, age at onset, duration of illness, number of hospitalizations, total duration of hospitalizations and chlorpromazine equivalent doses are expressed as median (Q1-Q3); gender and employment as number (percentages); FTND scores as mean ± SD; MW: Mann Whitney test; χ^2 test=chi square test. *= significant

Table 2. The lack of correlation between the PANSS total or the selected PANSS subscale scores and the first (salivary cortisol 1), the second (salivary cortisol 2) salivary cortisol concentration, or the absolute difference between the first and the second salivary cortisol concentration (Δ cortisol) in 196 patients with schizophrenia

PANSS scale and subscales (scores)	**Cortisol 1 (□mol/l)**	**Cortisol 2 □□mol/l)**	**Δ cortisol □□mol/l)**
PANSS total			
Correlation Coefficient	-0.05	-0.01	-0.05
p Value	0.523	0.875	0.483
PANSS Positive total			
Correlation Coefficient	0.06	0.04	0.02
p Value	0.378	0.537	0.813
PANSS Negative total			
Correlation Coefficient	-0.08	-0.02	-0.02
p Value	0.269	0.748	0.818
PANSS General psychopathology total			
Correlation Coefficient	-0.07	-0.03	-0.08
p Value	0.325	0.641	0.245
PANSS delusions (P1)			
Correlation Coefficient	0.01	-0.12	0.02
p Value	0.898	0.138	0.759
PANSS hallucinatory behavior (P3)			
Correlation Coefficient	0.01	0.02	0.00
p Value	0.918	0.823	1.000
Selected PANSS positive (P1+P3+P5+P6+G9)			
Correlation Coefficient	0.01	-0.05	-0.05
p Value	0.862	0.445	0.496

Selected PANSS negative (N4+N2+N3+G16+N6+N1+G13)			
Correlation Coefficient	-0.08	-0.03	0.00
p Value	0.289	0.713	0.996
Selected PANSS positive (P1+G9+P3+P6+P5+G12+G15)			
Correlation Coefficient	-0.02	-0.05	-0.08
p Value	0.800	0.457	0.262
PANSS disorganized (G10+G11+N5+P2+N7)			
Correlation Coefficient	-0.04	0.01	-0.04
p Value	0.565	0.913	0.562
PANSS excited (P7+G14+P4+G8)			
Correlation Coefficient	-0.01	0.06	-0.03
p Value	0.866	0.377	0.683
PANSS motor (G5+G7)			
Correlation Coefficient	-0.10	-0.03	-0.09
p Value	0.150	0.639	0.188
PANSS depression (G6+G3)			
Correlation Coefficient	0.04	-0.00	-0.00
p Value	0.587	0.983	0.951
PANSS anxiety (G1+G2+G4)			
Correlation Coefficient	-0.04	-0.03	0.00
p Value	0.553	0.695	0.986
PANSS cognition (P2+N5+N7+G5+G10+G11+G12+G13+G1)			
Correlation Coefficient	-0.06	-0.01	-0.06
p Value	0.400	0.934	0.429

Correlation between the PANSS scores and salivary cortisol was evaluated using the Spearman rank order correlation; PANSS: Positive and Negative Syndrome Scale

Table 3. The first, the second and a change (Δ) of salivary cortisol concentration in schizophrenic patients divided according to their pharmacotherapy

		N	Cortisol 1 (□mol/l)	Cortisol 2 (□mol/l)	Δ cortisol (□mol/l)
Antipsychotics	Atypical	171	15.9 (9.3-19.7)	11.2 (7.0-17.0)	4.9 (2.1-7.7)
	Typical	25	14.0 (9.3-16.3)	9.5 (7.4-14.1)	5.0 (1.0-7.6)
	MW test		U=2447.00 p=0.243	U=2475.00 p=0.203	U=2249.00 p=0.675
Antipsychotics	+benzodiazepines	10	14.5 (5.7-18.7)	9.7 (4.5-19.3)	2.5 (0.9-7.6)
	- benzodiazepines	186	15.2 (9.4-19.6)	10.9 (7.2-16.1)	4.9 (2.1-7.7)
	MW test		U=1087.00 p=0.370	U=999.00 p=0.695	U=1112.00 p=0.299
Chlorpromazine equivalent doses (mg/day)	Spearman Correlation Coefficient	196	r=0.087; p=0.225	r=0.056; p=0.431	r=-0.004; p=0.578

MW test: Mann Whitney test; Salivary cortisol concentration is expressed as median (Q1-Q3), N=number of patients.

Supplementary Table 1. The first (salivary cortisol 1), the second (salivary cortisol 2) salivary cortisol concentration, or the absolute difference between the first and the second salivary cortisol concentration (Δ cortisol) in schizophrenic patients divided according to monotherapy with antipsychotic medication

Drug		**N**	**Cortisol 1 (□mol/l)**	**Cortisol 2 (□mol/l)**	**Δ cortisol (□mol/l)**
Olanzapine	Yes	116	14.2 (8.5-19.5)	10.9 (6.2-16.1)	4.7 (2.1-7.9)
	No	80	15.8 (10.7-19.6)	10.9 (7.8-16.2)	4.9 (1.7-7.5)
	MW test		U=4280.00 p=0.357	U=4375.00 p=0.498	U=4833.00 p=0.622
Risperidone	Yes	57	16.2 (11.2-20.1)	13.1 (7.8-17.6)	4.9 (1.8-7.6)
	No	139	14.1 (9.0-19.4)	10.2 (6.7-15.4)	4.7 (2.0-7.7)
	MW test		U=3386.00 p=0.111	U=3439.00 p=0.148	U=3374.00 p=0.823
Clozapine	Yes	9	16.2 (13.8-20.5)	13.3 (11.3-17.6)	3.5 (2.4-5.1)
	No	187	14.7 (9.3-19.5)	10.8 (6.8-16.1)	4.9 (2.0-7.7)
	MW test		U=726.00 p=0.489	U=576.00 p=0.111	U=999.00 p=0.345
Fluphenazine	Yes	17	14.0 (10.4-16.1)	9.5 (7.1-12.7)	3.8 (1.0-6.7)
	No	179	15.8 (9.3-19.7)	11.1 (7.2-16.5)	4.9 (2.1-7.8)
	MW test		U=1783.00 p=0.243	U=1796.00 p=0.220	U=1783.00 p=0.243
Haloperidol	Yes	6	13.9 (9.1-20.3)	9.3 (8.8-14.1)	6.3 (0.6-10.5)

	No	190	15.2 (9.4-19.5)	10.9 (7.2-16.2)	4.8 (2.0-7.6)
	MW test		U=589.00, p=0.892	U=648.00 p=0.571	U=530.00 p=0.773
Promazine	Yes	2	13.0 (9.4-16.5)	12.5 (7.9-17.1)	8.2 (7.7-8.6)
	No	194	14.9 (9.3-19.6)	10.9 (7.2-16.1)	4.8 (2.0-7.6)
	MW test		U=223.00 p=0.721	U=179.00 p=0.856	U=84.00 p=0.170

Salivary cortisol concentration is expressed as median (Q1-Q3), N=number of patients, data analyzed by Mann-Whitney test.

Supplementary Table 2: Lack of correlation between chlorpromazine equivalent doses and the first (salivary cortisol 1), the second (salivary cortisol 2) salivary cortisol, or the absolute difference (Δ cortisol) between the first and the second salivary cortisol levels in 123 smokers and 73 non-smokers with schizophrenia

Chlorpromazine equivalent doses (mg/day)	**Cortisol 1 (□mol/l)**	**Cortisol 2 (□mol/l)**	**Δ cortisol (□mol/l)**
Smokers			
Correlation Coefficient	r=0.088	r=0.127	r=-0.025
p Value	0.329	0.160	0.786
Non-smokers			
Correlation Coefficient	r=0.196	r=0.068	r=-0.023
p Value	0.096	0.568	0.846

Correlation between cortisol chlorpromazine equivalent doses and salivary cortisol was evaluated using the Spearman Correlation Coefficient.