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Salivary Biomarker Alpha Defensins 1-4 In Bipolar Affective Disorder (Mania) And First Degree Relatives

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Declaration of interest- None

Conflict of interest-None

Highlights

- Significant reduction in levels of alpha defensins 1, 2, 3 and 4 in saliva, in course of 2 weeks with treatment of mania
- Significantly higher values of Human alpha defensins 1 and 2 in cases and first degree relatives (FDRs) when compared to controls, suggesting a possible association of elevated Alpha defensins and patho-physiology of mania

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Abstract

Saliva has been traditionally used to measure cortisol as a stress biomarker in neurology and neuropsychiatry in numerous studies. Recent studies demonstrated the whole saliva can be an alternative for detecting some relevant plasma clinical analytes. The goal of our study was to observe the levels of alpha defensins 1-4 levels in the saliva of patients with Bipolar disorder (BD), manic episode when they are symptomatic and after 2 weeks, in healthy controls and the first degree relatives (FDRs) of patients with BD .

Inpatients with the diagnosis were chosen after a complete physical examination. The samples taken were 33 Bipolar disorder (manic episode) patients and their respective FDRs along with 22 healthy controls. Our study found significant reduction in levels of alpha defensins 1, 2, 3 and 4 in saliva, in 2 weeks with treatment of mania. We also observed significantly higher values of Human alpha-defensins 1 and 2 in cases and FDRs when compared to controls, suggesting a possible association of Alpha defensins in pathophysiology of mania. Saliva's role as an alternate biological fluid in study of biomarkers in psychiatric disorders can also be explored further as suggested by the results .

Key words - Bipolar disorder; biomarkers; alpha-defensins; Saliva

1. Introduction

Recently, several studies showed the presence of significant alterations in the immune system of bipolar patients, like the elevated plasma levels of inflammatory markers, particularly Interleukin-1(IL-1) receptor antagonist (IL-1Ra) and soluble tumor necrosis factor receptor (sTNF-R1) were associated with general disease severity and psychotic features in bipolar (Hope et al.,2013). Elevated plasma levels of IL-1 receptor antagonist (IL-1Ra) and soluble tumor necrosis factor receptor (sTNF-R1) in bipolar were consistent with Dickerson et al. (2013) that showed a relationship between the elevated level of C- reactive protein and the severity of affective symptoms in patients with bipolar disorder in manic phase (Dickerson et al. 2013). These studies support a central role of immune activation in the core pathological mechanisms of severe mental illnesses. Some proteomic studies have also been carried out by de- Souza and colleagues (Martins de Souza et al., 2012) to identify specific markers of the disease. All the proteins and peptides which were significantly increased in the Schizophrenia (SZ) and the Bipolar disorder (BD) groups with respect to control are involved in the innate immunity. Defensins are small cationic peptides with antibacterial, antiviral, and immunomodulatory properties, part of the innate immunity (Lehrer et al, 2012).

Stimulated by these findings Iavarone et al. carried out a top-down proteomic analysis on the acidic soluble solution of the whole saliva of different individuals with psychiatric illness (schizophrenia, bipolar mood disorder) comparing them against a group of healthy controls further divided among smokers and non-smokers. This study confirmed increased levels of α- defensins 1-4 and evidenced increased levels of S100A12, Cystatin A and S- derivatives of cystatin B (Cabras T et al. 2012) in the whole saliva of individuals with psychiatric illness. It confirmed some of the results obtained by blood analysis, demonstrating the whole saliva can be a good alternative for detecting some relevant plasma clinical analytes (Iavarone et al.,2014). The collection is noninvasive, and saliva has many proteins (Scarano et al, 2010). Unlike blood, it does not require the depletion of abundant proteins and requires less processing before protein analysis.

Even though some of the salivary biomarkers are seen in Bipolar disorder, it is not conclusive whether these markers are present during symptomatic phase or remission phase. Therefore, we needed to assess whether it is a state marker or a trait marker to use it as a screening tool in the general population to prevent the risk factors.

The recent study by Ormerod et al in 2022, focused on composite immunity biomarkers scores associated with severe mental illness like Schizophrenia and bipolar disorder, where they observed that Human Neutrophil Protein (HNP) 1-3 (also called alpha-defensins) were associated with BD.

Saliva analysis has been proposed for clinical use because it is easier to collect than blood and can be collected by professionals without the requirement of formal medical training (Schulz et al, 2013) and is less anxiety-provoking than providing a blood sample and is less embarrassing than producing a urine specimen (Shetty et al, 2010). Unlike blood, it does not require depletion of abundant proteins and requires less processing before protein analysis.

Proteomics in psychiatric and neurology is a field still in its infancy. Consequently, there are currently few proteomic studies of saliva in CNS research; however, this may soon change as this biomaterial utility is recognized, along with recent advances in technology. Large scale studies validating salivary biomarkers are needed, as are eventual mechanistic explanations for the discovered biomarkers. The sensitivity and specificity of proteomic and other omic technologies are increasing at a rapid rate, ushering in an exciting climate of discovery.

2. Methods

This study was a hospital-based prospective study on inpatients diagnosed with first-episode mania or bipolar disorder, current episode mania, conducted for 2 weeks at a tertiary care referral psychiatric facility in India in August 2020- March 2021.

2.1. Sample

Saliva has been traditionally used to measure cortisol as a stress biomarker in neurology and neuropsychiatry in numerous studies. Recent studies demonstrated the whole saliva can be an alternative for detecting some relevant plasma clinical analytes. The goal of our study was to observe the levels of alpha defensins 1-4 levels in the saliva of patients with Bipolar disorder, manic episode when they are symptomatic and after 2 weeks, in healthy controls and the first degree relatives (FDRs) of patients with BD.

The sample consisted of 33 individuals chosen by Purposive sampling and who are diagnosed cases of Mania without psychotic symptoms, mania with psychotic symptoms and Bipolar Disorder, current episode mania without psychotic symptoms and Bipolar Disorder, current episode mania with psychotic symptoms according to Diagnostic Criteria for Research (DCR) of International Classification of Diseases-tenth edition (ICD-10, World health organization, 1993), 33 first degree relatives accompanying the patient , and 22 healthy individuals as controls were matched for age and sex before selecting for the study. The sample size initially calculated was 50 of each group but was reduced due to the COVID pandemic.

2.2. Procedure

The study was initiated after obtaining the permission from the Institutional Ethics Committee numbered IEC/CIP/2018-19/254. All the participants with a diagnosis of Bipolar affective disorder (manic episode) fulfilling the inclusion criteria were recruited after obtaining their or their guardian's written informed consent. (Fig 1)

Resting whole saliva was collected via Salivette® saliva collection kit. A synthetic sponge was placed for 5-10 mins at the base of the tongue, ensuring that the sample was collected at 30 min after consumption of food. The sample was then centrifuged at 10,00g for 15 mins, and the supernatant was stored at -50ºC within 40 minutes of collection. This process was carried out for group 1 at baseline and after 2 weeks; for group 2 and group 3 the process was followed once. Patients were started on any mood stabilizers (12 on Lithium Carbonate, 13 on Sodium Valproate and 8 on Carbamazepine), antipsychotics (15 on Olanzapine, 12 on Risperidone and 6 on other atypical antipsychotics) or benzodiazepines as decided by the treating team.

Group 1 was evaluated on Young Mania Rating Scale (YMRS) and Brief Psychiatric Rating Scale (BPRS) for the severity of the affective symptoms and psychosis at baseline. Patients belonging to the study groups were given mood stabilizer and antipsychotic as per requirement. They were assessed again on the same parameters after 2 weeks.

All the collected samples were sent to biochemistry lab at CIP and levels of alpha defensins 1-4, in saliva were measured using an Enzyme-linked immunosorbent assay (ELISA), the ELK Biotechnology Human DEFa 1, 1B (2), 3 & 4 Pre-Coated ELISA Kits solid phase immunoassay specially designed to measure Alpha defensins 1,2,3 & 4 with a 96-well strip plate that's precoated antibody specific for Alpha defensins 1,2,3 & 4 respectively and with the help of ELISA reader. The patients continued to receive mood stabiliser and antipsychotics depending upon the severity of illness.

2.3. Statistical Analysis

Appropriate statistical measures were applied using Statistical Package for Social Sciences (SPSSver.25.0) in following steps. Socio-demographic variables were compared between the two groups using the Chi-square test. For the categorical variables, Chi-square test was applied to analyze differences between the groups and Fisher's exact test was computed wherever necessary. As the distribution was not normal as tested by the Shapiro-Wilk test, Nonparametric tests were applied. Kruskal-Wallis test was applied for comparison of biochemical markers between cases, FDRs and healthy controls. Wilcoxon Signed Ranks test was applied for comparison of biochemical markers of cases at baseline and 2 weeks. Spearman's correlation was used to see the correlation between various markers. Statistical Significance at $p<0.05$.

3. Results

The mean age of onset of the illness for mania in the present study was (24.27 ± 7.74) years. The demographic data of the samples of all 3 groups studied are presented in Fig. 2

Fig 3 shows the comparison overtime in biochemical variables Alpha-defensins 1 (ng/mL), 2 (pg/mL), 3(ng/mL) and 4(pg/mL), among cases. We observed there was a significant difference in values of α Defensins 1($p<0.001$), 2 ($p<0.001$), 3($p<0.001$) & 4($p<0.001$) overtime (2 weeks) in Group 1.

We also compared the levels of biomarkers in the saliva, that include alpha defensins 1, 2, 3 & 4, amongst cases, FDRs and healthy controls using Kruskal-Wallis test and Post-HOC Bonferroni correction. There was a significant difference in the levels of Alpha-defensins 1 & 2 between the control group and the cases and between controls and FDRs. There was no significant difference in levels of alpha-defensins 3 & 4 between all the three groups (Fig 4).

The correlation between clinical variables (YMRS & BPRS at 0 weeks) and biochemical variables (Alpha defensins 1, 2, 3 & 4 values taken at 0 weeks). There was significant positive correlation between the Alpha defensins 2 and BPRS at 0 week (rho=0.349; p= 0.04) and alpha defensins 4 and YMRS at 0 week (rho=0.463; p=0.007). Rest no significant correlation present in the above values. (Fig 5)

Furthermore, the correlation between clinical variables (YMRS and BPRS at 2 weeks) and biochemical variables (Alpha-defensins 1, 2, 3 & 4 values taken at 2 weeks) were calculated. There was significant positive correlation between alpha-defensins 2 and BPRS at 2 weeks (rho=0.372; p=0.033) and alpha-defensins 4 with YMRS (rho=0.544; p=0.001) and BPRS (rho=0.487; p=0.004) at 2 weeks.

4. Discussion and Conclusion

A longitudinal study was conducted on patients with bipolar disorders to determine the variation in the levels of alpha-defensins in saliva with the waning symptoms. It's an advantage over previous cross-sectional studies. As response to treatment is observed in 2 weeks, we reapplied the scales for an objective measurement of the symptoms and studied the biomarker levels in the saliva at the end of 2 weeks.

After going through a plethora of literature, we found very few studies dedicated to the topic of the Indian population using saliva as the biological fluid of interest in bipolar disorder.

The inference from the results suggests that with the improvement of symptoms, the levels of Alpha defensins 1, 2, 3 and 4 in the saliva of patients with mania decrease. When we compared the levels of Alpha defensins 1, 2, 3 and 4 in saliva of patients with BD with their FDRs and healthy controls, the results showed higher levels of alpha defensins 1 and 2 in patients with BD than in controls. Along with that, levels of only alpha-defensins

1 in saliva of FDRs were observed to be higher than that in controls. This is a novel finding of our study which is suggestive that alpha-defensins 1 could be an endophenotype related to the risk of bipolar disorder present in the saliva. But the study needs to be replicated when patients are euthymic to confirm this finding. The levels of alpha-defensins 3 and 4 did not show any significant difference when compared between the three groups. The current study also observed a positive relation between the decrease in the scores of YMRS and alpha defensins 4 when scored at baseline and 2 weeks and BPRS at 2 weeks with alpha defensins 4. Positive relations of decrease in BPRS scores with alpha-defensins 2 at baseline and at 2 weeks were observed. We can infer that as psychosis is decreasing in the patients there is decrease in alpha defensins 2 and 4 as the rho>0.3 but further studies are needed for concrete evidence.

Defensins are diverse members of a large family of antimicrobial peptides, contributing to the antimicrobial action of granulocytes, mucosal host defense in the small intestine and epithelial host defense in the skin and elsewhere (Ganz et al,1985). In the immune system, effector cells are the relatively short-lived activated cells that defend the body in an immune response. The production of effector cells in response to first-time exposure to an antigen is called the primary immune response. Recent data suggests that defensins may play a key role in the recruitment and activation of the appropriate adaptive effector response (Krutzik et al, 2001). Initially human alpha defensin peptides were isolated from the neutrophils and are thus called human neutrophil peptides (Ganz et al, 1985).

Defensin Alpha gene (DEFA) DEFA1 and DEFA3 genes show extensive copy number polymorphism, and the number of gene copy per diploid genome varies between 4 and 14 with a mean value of 10. On the contrary, the DEFA4 gene, responsible for the expressions of α-defensin 4, is not duplicated and only two copies exist in a diploid genome. Therefore, the relative amount of α-defensin 4 is noticeably lower,

and its structure is somewhat different from that of α-defensins 1–3 (Lehrer et al, 2012). Three of these (Alpha defensins -1, Alpha defensins -2, and Alpha defensins -3) are highly homologous (Selsted et al, 1984) constitute between 30% and 50% of the total protein in azurophil granules of human neutrophils (Rice et al, 1987). The fourth, Alpha defensins -4, has a distinctly different primary structure (Singh et al, 1988) and accounts for only 1% to 2% of the neutrophil's total defensins (Gabay et al, 1989) The minor variations within a family of defensins significantly affect their specificity and microbicidal potency. Alpha-Defensins are produced and stored as pre-propeptides in matured Polymorphic neutrophils (PMNs) and Paneth cells (Diamond et al,1996). The activity of a few alpha-defensins as specific antagonists of adrenocorticotropin has led to their classification as the corticostatin/defensin family. Human alpha-defensins are chemotactic to monocytes, dendritic and T-cells, suggesting their importance in shaping the adaptive immune response (Tang et al, 1999)

Alpha defensins 1-3 have been reported to increase the production of tumor necrosis factor (TNF) and IL-1, while decreasing the production of IL-10 by monocytes.Increased levels of proinflammatory factors (e.g., IL-1, TNF, histamine and prostaglandin D2) and suppressed levels of IL-10 at the site of microbial infection are likely to amplify local inflammatory responses. The capacity of defensins to enhance phagocytosis, promote neutrophil recruitment, enhance the production of proinflammatory cytokines, suppress anti-inflammatory mediators and regulate complement activation argues that defensins upregulate innate host inflammatory defenses against microbial invasion (Raj et al, 2002).

Defensins are found to induce proteoglycan-dependent catabolism of low-density lipoprotein (LDL) by vascular cells, leading to a new class of inflammatory apolipoprotein (Risso, 2000). Defensins increase proliferation of epithelial cells, suggesting their involvement in wound healing (Sterk et al, 1999). Defensins also have regulatory functions in addition to their role in integrating the innate and adaptive immune responses (Raj et al, 2002)

In 2014 Iavarone et al carried out a top-down proteomic analysis, using Liquid chromatography–mass spectrometry, on the acidic soluble solution of the whole saliva of different individuals with psychiatric illness (schizophrenia, bipolar mood disorder) comparing them against a group of healthy controls further divided among smokers and non-smokers. In this study, the levels of α-defensins 1–3 was more than α-defensin 4 in schizophrenia and bipolar patients, suggesting that their increased concentration could not only be linked to an increased neutrophil activity but could rather be derived also from the contribution of other cells devoted to the adaptive immunity. The mean levels of α-defensins 1–3 in whole saliva of schizophrenic and bipolar patients were higher with respect to controls, while the increase of α-defensin 4 was not computable, because this defensin was frequently undetectable in the healthy subject.

Composite scores of S100B, furin, Human Neutrophil Protein (HNP) 1-3 (also called alpha-defensins) and BD-1 were associated with BD, a finding that requires further investigation given the lack of studies of blood brain barrier markers in BD. Although alpha-defensins are known to induce immune responses, their anti-inflammatory activity has also been documented. This could explain their finding that furin and the alpha-defensins HNP1-3 all loaded negatively together with S100B, in BD patients (Ormerod et al, 2022).

Several studies including the study done by Bai YM et al in 2014 have documented increased levels of circulating pro-inflammatory cytokines in different phases of bipolar disorder. In repeated mood episodes of bipolar disorder, these cells get activated chronically affecting homeostatic balance. This leads to a constant inflammatory environment which causes damage to the neurons. (Watkins CC et al 2014).

High levels of IL-6 and TNF-alpha have been reported during mania with IL-6 levels returning to baseline after treatment with mood stabilizers while TNF-alpha continued to remain high in the study conducted by Kim YK et al in 2007. This showed the pro-inflammatory state resolves in euthymia, and that lithium influenced the cytokine profile. In recent studies, lithium was shown to have neuroprotective activity in preclinical models.

Valproate (VPA) has shown anti-inflammatory properties. (Zhang et al, 2012) The administration of VPA induced the activation of the extracellular signal-regulated kinase pathway downregulating proinflammatory cytokines such as inducible nitric oxide synthase and cyclooxygenase-2 (Frey et al, 2006). Antipsychotic drugs (APD) have also shown anti-inflammatory action by attenuating cell-mediated immune activation as well as oxidative and nitrosative stress (Cox et al, 2015; Debnath&Venkatasubramanian 2013). Hence, we may conclude that mood stabilizers might be attenuating the proposed over-activated inflammatory process of neutrophil-origin alpha-defensins in psychotic states. It resembles our study with a decrease of Alpha defensins 1, 2, 3 and 4 in the saliva of patients with BPAD and a decrease in the YMRS and BPRS scores. In BPAD patients, there is decreased need for sleep. Studies have shown that chronic sleep loss is a cause of low-grade inflammation in the brain. Hence, the use of benzodiazepines in assisting patients with BPAD to improve their sleep duration (Eugene et al, 2015), might also be a factor in the decrease of Alpha defensins 1, 2, 3 and 4 in the 2-week duration as seen in other inflammatory markers.

In conclusion, significant reduction in levels of alpha-defensins 1, 2, 3 and 4 in saliva, over 2 weeks with treatment of mania. Furthermore, significantly higher values of Human alpha defensins 1 in cases and first-degree relatives (FDRs) when compared to controls, suggesting a possible association of elevated Alpha defensins and patho-physiology of mania.

5. Limitations

Our sample size was modest which is liable to a type I error, which also makes it difficult to generalize the result. This study examined a specific segment of the population drawn out of a purposive method of sampling. Therefore, the conclusions derived from the study might not hold for all patients with mania. This study needs to be replicated in other populations. Treatment was not controlled, including varying choices and

dosages of medications used. The effect of drugs on the biomarkers could be varied and hence could confound the results. The interval between saliva sample collection from patients (2 weeks) is relatively short which could affect the levels of the biochemical parameters due to this we could not establish a possible role of Alpha defensins 1,2 3 and 4 as trait markers as the patients were not euthymic. As levels of alpha-defensins 1,2 3 and 4 are found raised in other inflammatory, neoplasms, and infections, they have low sensitivity and specificity for bipolar disorder.

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Fig 1

Inclusion Criteria for Patients (G1)

1. Diagnosis of Bipolar Affective Disorder current episode Mania using Diagnostic Criteria for Research (DCR) of International Classification of Disease-10th edition (ICD-10, World Health Organization,1992)
2. YMRS > 20
3. Age between 18- 60 years of male or female sex.
4. Patients giving written informed consent.

Exclusion Criteria for Patients (G1)

1. Presence of co-morbid neurological or other psychiatric disorder(s).
2. Patient with co morbid substance dependence, except nicotine and caffeine.
3. Not willing to give written informed consent
4. Patients with any neurological or physical disorder.
5. Patients suffering from Xerostomia

Inclusion Criteria for First degree relatives (G2)

1. First degree relative not having the manifested disorder
2. General Health Questionnaire (GHQ) (12) less than or equal to 3
3. Giving written informed consent.
4. Order of selection – Sibling > Parents > Offspring

Exclusion Criteria forFirst degree relatives (G2)

1. General health questionnaire (GHQ) score more than 3
2. Diagnosed with major psychiatric illness
3. Diagnosed with medical disorder.
4. Not willing to give written informed consent.

Inclusion Criteria for Controls (G3)

1. Persons not having any major physical/mental morbidity.
2. General Health Questionnaire (GHQ) (12) less than or equal to 3
3. Giving written informed consent.

Exclusion Criteria for Controls (G3)

1. General health questionnaire (GHQ) score more than 3
2. Any medical or psychiatric disorder.
3. Not willing to give written informed consent

TOOLS

1. Informed Consent and Patient's Instruction Form
2. Socio-demographic and Clinical Data Sheet
3. Youngs Mania Rating Scale (YMRS) (Young, 1978)
4. Brief Psychiatric Rating Scale (John Overall, 1962)
5. General Health Questionnaire 12 (GHQ12) (Goldberg, D. P.et al.,1979)
6. Enzyme linked immunosorbent assay(ELISA)
7. Saliva collection device

Fig 2 Demographics of the sample

	Group			Total
	study group (33)	FDR (33)	Control (22)	
Gender				
Male	21 (63.6%)	27 (81.8%)	10 (45.5%)	58 (65.9%)
Female	12 (36.4%)	61 (8.2%)	12 (54.5%)	30 (34.1%)
Religion				
Hindu	29 (87.9%)	29 (87.9%)	17 (77.3%)	75 (85.2%)
Others	4 (12.1%)	4(12.1%)	5(22.7%)	13(14.8%)
Occupation				
Employed	15 (45.5%)	23 (69.7%)	16 (72.7%)	54 (61.4%)
Unemployed	18 (54.5%)	10 (30.3 %)	6 (27.3%)	34 (38.6%)
Marital status				
Single	14 (42.4%)	10 (30.3%)	11 (50.0%)	35 (39.8%)
Married	19 (57.6%)	23 (69.7%)	11 (50.0%)	53 (60.2%)
Residence				
Urban	7 (21.2%)	7 (21.2%)	4 (18.2%)	18 (20.5%)
Rural	26 (78.8%)	26 (78.8%)	18 (81.8%)	70 (79.5%)
Literacy				
Literate	31 (93.9%)	24 (72.7%)	14 (63.6%)	69 (78.4%)
Illiterate	2 (6.1%)	9 (27.3%)	8 (36.4%)	19 (21.6%)

Fig 3: Comparison of changes over time in biochemical variables among cases

Variables		Mean rank	Z	Sig(p)
α Defensin 1	0 week	38.82	5.012	**0.000****
	2 week	17.00		
α Defensin 2	0 week	40.21	4.851	**0.000****
	2 week	17.00		
α Defensin 3	0 week	37.85	4.959	**0.000****
	2 week	17.00		
α Defensin 4	0 week	38.12	4.994	**0.000****
	2 week	17.00		

*p=0.05; **p<0.001

Fig 4: Comparison of biomarkers across three groups at 0 week of cases

Variables	Cases (Mean Rank) (A)	FDRs (Mean Rank) (B)	Controls (Mean Rank) (C)	df	Kruskal-Wallis H	Sig(p)	Post-HOC
α Defensin 1	60.73	48.39	14.32	2	44.788	0.000**	A>C B>C
α Defensin 2	59.18	41.82	26.50	2	22.184	0.000**	A>C
α Defensin 3	49.44	38.23	46.50	2	3.358	0.187	-
α Defensin 4	49.67	36.61	48.59	2	5.065	0.079	-

Fig 5

Correlation between clinical and biochemical variables (at 0 week) of cases.

		α Defensins 1 (0 wk)	α Defensins 2 (0 wk)	α Defensins 3 (0 wk)	α Defensins 4 (0 wk)
YMRS 0 week	ρ	-0.072	0.194	0.278	**0.463****
	p	0.692	0.278	0.118	**0.007****
BPRS 0 week	ρ	0.058	**0.359***	0.184	0.310
	p	0.750	**0.040***	0.306	0.079

*p=0.05; **p=0.01

Correlation between clinical and biochemical variables of cases at 2 week

		α Defensins 1 (2 wk)	α Defensins 2 (2 wk)	α Defensins 3 (2 wk)	α Defensins 4 (2 wk)
YMRS 2 week	ρ	0.151	0.305	0.318	**0.544*****
	p	0.402	0.084	0.071	**0.001*****
BPRS 2 week	ρ	0.203	**0.372***	0.209	**0.487****
	p	0.257	**0.033***	0.243	**0.004****

*p=0.05; **p=0.01; ***p=0.001