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The role of interleukin genes in the course of depression

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Abstract: Background: Research studies conducted in recent years have confirmed that in the absence of medical illnesses, depressive disorders are associated with upregulation of many inflammatory cytokines such as tumor necrosis factor-aplha (TNF-α), interleukin-1 and 6 (IL-1,IL-6). The main objective of the study was to examine whether recurrent depressive disorders (rDD) are accompanied by more profound inflammatory disturbances than the first episode of depression (ED-I). The analysis included the expression of mRNA and protein levels of three interleukins namely. IL-1, IL-6 and IL-10.

Methods: The study was carried out in a cohort of 130 patients: ED-I group – 44 patients, rDD group – 86 patients respectively.

Results: Our results suggest that there was no significant statistical difference between the analyzed groups as regards the intensity of the depressive disorders. Furthermore, No differences in the expression of IL-1, IL-6 and IL-10 genes on the level of both mRNA and protein were observed among the groups. Additionally, there was no significant interrelation been documented between the number of depression episodes experienced v/s the expression of selected genes.

Conclusions: There is no significant difference in IL-1, IL-6 and IL-10 expression between patients with recurrent depressive disorders and those suffering from the first episode of depression. 2. There seems to be no difference in acute first episode depression vs. acute episode of depression in patients with a recurrent disorder. Further larger trials are needed.

Keywords: First episode, recurrent depressive disorder, IL-1, IL-6, IL-10

1 Introduction

Cytokines are small pleiotropic proteins discovered in the context of cellular activation and cell-to-cell communication in the immune system. They can be viewed as either 'pro-inflammatory' or 'anti-inflammatory', depending on their aggregate effects on target cells [1]. The expression and activity of cytokines are induced in response to an infection, traumatic brain injury, ischemic or hemorrhagic stroke, or various other neurodegenerative diseases; however, pro-inflammatory cytokines (interleukin-1 (IL-1), IL-18, and tumor necrosis factor alpha (TNF-α)) are expressed normally in the brain and also play an active role in cellular events that induce structural changes at the synaptic level [1]. Research studies conducted in recent years confirm that in the absence of medical illnesses, depressive disorders are associated with increased levels of many inflammatory cytokines (e.g. TNF-α, IL-1, IL-6) [2]. The "cytokine hypothesis of depression" [3] is based on an assumption that inflammatory mediators play a vital role in the pathophysiology of depressive disorders.

There are various underwritten pathways through which cytokines may influence the pathophysiology of depression. The most important include cytokine-induced changes in the metabolism of monoamines, such as dopamine, noradrenalin and serotonin, specifically in midbrain nuclei with widespread projections. In addition, cytokines induce cortisol hypersecretion, directly by stimulating the hypothalamic-pituitary-adrenal axis (HPA axis) and indirectly by modifying the sensitivity of the glucocorticoid receptor [4]. Chronic hyperactivity of the HPA axis instigates hypercortisolism, which in turn affects the

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nervous, endocrine, and immune systems. The HPA axis function is upregulated by pro-inflammatory cytokines through the brain receptors for soluble molecules which are expressed mostly in the hypothalamus [5].

A particular role in the etiology of rDD is assigned to two pro-inflammatory interleukins (IL-1 and IL-6) and to one anti-inflammatory interleukin, i.e. IL-10 [6, 7]. Elevated levels of IL-1 and IL-6 have served as consistent biomarkers of depression, while IL-10 found to be persuading depressive behavior through its ability to counterbalance pro-inflammatory cytokine expression [7, 8].

The main objective of the study was to examine whether rDD are accompanied by more profound inflammatory disturbances than the first episode of depression (ED-I). The analysis included the expression of mRNA and protein levels of three interleukins, i.e.: IL-1, IL-6 and IL-10. We have formulated a working hypothesis, according to which successive episodes of rDD are linked with increased dysregulation of pro- and anti-inflammatory cytokines production.

2 Material and methods

2.1 Subjects

The study was carried out in a cohort of 130 patients: ED-I group – 44 patients, rDD group – 86 patients. All patients were hospitalized at the Department of Adult Psychiatry of the Medical University of Lodz, Poland. The selection of the individuals for the study group was performed randomly without replacement sampling.

The patients from the rDD group, before being qualified to take part in the study, were subject to an antidepressant therapy with drugs from the Selective Serotonin Reuptake Inhibitors (SSRI): 54 people were administered fluoxetine (initial dosage: 20 mg/day, maximum dosage: 60 mg/day), 8 people were taking sertraline (initial dosage: 50 mg/day, maximum dosage: 200 mg/day), 12 people were administered citalopram (initial dosage: 20 mg/day, maximum dosage: 40 mg/day), and 12 people were taking paroxetine (initial dosage: 20 mg/day, maximum dosage: 60 mg/day).

The patients were selected for the study based on the inclusion criteria for ED and rDD outlined in ICD-10 (F32.0-7.32.2, F33.0-F33.8) [9]. The presence of axis I and II disorders, other than depressive episodesand the diagnosis of somatic diseases and injuries of the central nervous system (CNS) were regarded as exclusion criteria. Other exclusion criteria included: inflammatory or autoimmune disorders and unwillingness to give informed consent. For all the subjects, a case history was obtained prior to participation using the standardized Composite International Diagnostic Interview (CIDI) [10].

All the subjects were free from medical illnesses, including infections and inflammatory or allergic reactions. None of the depressed patients was treated with drugs known to influence lipid metabolism, immune response or endocrine function. The participants wereclassified as non-alcoholics and light or non-smokers, and none had ever taken psychotropic drugs.

2.2 Methods

2.2.1 Severity of depression

Depression severity was assessed using the 17-item Hamilton Depression Rating Scale (HDRS). A description of HDRS was presented elsewhere [11]. All the subjects were examined during the course of their hospitalization. The study group included subjects hospitalized for the first time due to a depressive episode and depression treatment-naïve, as well as those treated for many years earlier and with multiple hospitalization episodes in the past; the latter were admitted for various degrees of health deterioration. The number of depression episodes and disease duration were recorded for each patient.

HDRS was administered at the point of admission during the symptomatic phase, which would generally be either before or shortly after a modification of the previous antidepressant drug regimen. Reassessment of the mental condition was conducted 8 weeks after the pharmacological treatment, also with the use of the HDRS scale. Patient examinations were conducted by the same person in each case. Blood samples were obtained from the patients and were further allocated in 5 ml test tubes containing EDTA, which were later centrifuged at 1000 xg for 10 minutes at 4°C to isolate peripheral blood lymphocytes. The acquired lymphocytes and serum were stored at -70°C until analyzed.

2.3 Evaluation of selected genes expression at the level of protein

2.3.1 Determining protein concentration

To assess Total protein concentration in blood plasma we used Micro BCA™ Protein Assay Kit (ThermoSCIENTIFIC)

based on the manufacturer's recommendations. 150 µl of the reaction mixture was added to pits containing 150 µl of serum, diluted 10 times in 10 mM of phosphate buffered saline, pH 7.4, and incubated for2 hours, at 37°C. In order to measure protein concentration, an analytical curve for serum albumin was determined. Both the examined samples and the reference samples were positioned parallel in three repetitions. Eventually, Sample absorbance was measured using Multiskan Ascent Microplate Photometer (Thermo Labsystems) at λ = 570 nm and total protein concentration was calculated from the standard curve equation.

2.3.2 Enzyme-linked immunosorbent assay (ELISA)

The concentration of proteins IL-1, IL-6 and IL-10 in the patients' serum was determined using specific Elisa kits (R&D Systems, Inc, Minneapolis, MN, USA) for each cytokine respectively. β-actin was utilized for endogenous control of protein concentration in the samples and determined with the help of Human Actin Beta (ACTb) ELISA Kit (BMASSAY) based on the manufacturer's recommendations. Furthermore, 100 µl of serum ($\rho_{protein}$ = 0.5 mg/ml) was added to pits coated with antibodies specific for the analyzed proteins and then incubated (1.5 hours, 37°C). Following incubation contents were removed and the pits were rinsed three times in 10 mM of phosphate buffered saline and re-incubated (1 hour, 37°C) with 100 µl of biotinylated antibodies specific for the analyzed proteins. Then, the content was removed and the pits were rinsed three times in 10 mM of phosphate buffered saline and incubated (30 minutes, 37°C) with 100 µl of ABC working solution. The content was again removed and the pits were rinsed five times in 10 mM of phosphate buffered saline and incubated (10 minutes, 37°C) with 90 µl of TMB substrate. After adding 100 µl of TMB Stop Solution, the absorbance of the samples was measured using Multiskan Ascent Microplate Photometer (Thermo Labsystems) at λ = 450 nm. In order to determine protein concentration, analytical curves for the proteins were created.

2.4 Evaluation of selected genes expression at the level of mRNA

2.4.1 Total RNA isolation

Total RNA isolation from the patients' blood was performed using InviTrap Spin Universal RNA Kit (Stratec molecular, Berlin, Germany) based on the manufacturer's recommendations. Likewise, 300 µl of blood in a test tube was incubated with 300 µl of Lysis/Binding Buffer. Then, 300 µl of Acid Phenol: Chloroform mixture was added to the cellular lysate and, after mixing, the sample was centrifuged (5 minutes, 10000 xg) to separate the aqueous phase from the organic phase. The upper fraction (aqueous) was taken to a fresh test tube, which contained 375 µl of 96% ethanol and, after mixing, the entire content was poured into a test tube with a column and a filter. After centrifugation (15 seconds, 10000 xg), the column with the filter was put into fresh test tubes and rinsed in 700 µl of RNA Wash Solution 1, and then centrifuged (10 seconds, 10000 xg). The column with the filter was rinsed twice in 500 µl of wash solution 2/3 and centrifuged (1 minute, 10000 xg). The column with the filter was placed in a fresh test tube and isolated RNA was subject to elution in 30 µl of water free from nucleases (temperature of 95°C) by means of centrifugation (30 seconds, 10000 xg). Absorbance was measured using a spectrophotometer (Picodrop) at λ = 260 nm in order to determine total RNA concentration. Isolated RNA was stored at a temperature of -80°C for further use.

2.4.2 Quality analysis of isolated RNA

The quality of total RNA was checked with Agilent RNA 6000 Nano Kit (Agilent Technologies) in accordance with the manufacturer's recommendations. 1 µl of RNA 6000 Nano dye was added to a test tube containing 65 µl of Agilent RNA 6000 Nano gel matrix and then centrifuged (10 minutes, 13000 xg). The gel-fluorescent dye mixture was applied on the surface of a Nano chip placed in a workstation. Then, 5 µl of RNA Nano marker was added to selected pits. Isolated samples of RNA and RNA size marker were subject to denaturation (2 minutes, 70°C), and then 1 µl of the sample was pipetted into selected pits of the Nano chip and mixed (1 minute, 2400 rpm). The quality of the isolated RNA was checked using 2100 bio-analyzer (Agilent Technologies). The degradation level of total RNA was determined with the use of an electrophoretogram and RIN values were recorded. Only the samples with RIN value > 7 were subject to further analysis.

2.4.3 RT-PCR reverse transcription

An RT reaction was carried out using TaqMan® RNA Reverse Transcription Kit (Applied Biosystems) based on the manufacturer's recommendations and using specific starters and Hs 00174092_m1, Hs 00985639_m1, Hs

00961622_m1 probes, respectively for IL-1, IL-6 and IL-10, delivered by Applied Biosystems. The samples were incubated (30 minutes in 16°C and 30 minutes in 42°C) in a thermocycler (Biometra). RT was inactivated (5 minutes, 85°C) and the obtained cDNA which was further stored at a temperature of -20°C.

2.4.4 Real-Time PCR reaction – scanning miRNA panel

A Real-Time PCR reaction was conducted using TaqMan® Universal PCR Master Mix, No UNG (Applied Biosystems) according to the protocol provided by the manufacturer. The reaction mixture ratio was presented using a table. To calculate relative expression of miRNA genes, the Ct comparative method was used [12]. The level of IL-1, IL-6 and IL-10 gene expression in particular tissues was normalized in relation to the RPL13A reference gene.

Each target probe was amplified in a separate 96-well plate. All the samples were incubated at 50°C for 2 minutes and at 95°C for 10 minutes, and then cycled at 95°C for 30 seconds, at 60°C for 30 seconds and at 72°C for 1 minute; 40 such cycles were performed in total. Fluorescence emission data were captured and mRNA levels were quantified using the critical threshold (C_t) value. Analyses were performed using ABI Prism 7000 (SDS Software). Controls without RT and with no template cDNA were performed with each assay. Relative gene expression levels were obtained using the $\Delta\Delta C_t$ standard $2^{-\Delta\Delta ct}$ calculations and expressed as a fold change of the control sample [12, 13]. Amplification specific transcripts were further confirmed by obtaining melting curve profiles.

3 Statistical analysis

A statistical analysis of the collected material included calculation of both descriptive and inferential statistics. A two-tailed critical region was employed in the statistical hypothesis testing.

Qualitative characteristics of the study and control groups were expressed as frequencies shown as percentages. To characterize the average values for quantitative features, the arithmetical mean (M) and median (Me) were calculated. The measures of statistical dispersion included the range of values between the minimum and the maximum, and the standard deviation (SD).

Distributions were analyzed using the Shapiro-Wilk test. To compare non-parametric variables in the test groups, the following tests were used: the Pearson $\chi 2$ for qualitative variables, the Wilcoxon signed-rank test for two related groups for quantitative variables, and the Mann-Whitney U test for two independent groups to determine the coincidence of distributions. To evaluate the relations between the analyzed variables, Spearman's R rank order correlation coefficients were estimated. For all the analyses, statistical significance was set at the level of $p < 0.05$ [14]. All the data analyses were performed using STATISTICA PL, version 10.

4 Ethics

The patients were all native Poles, inhabitants of central Poland and unrelated. Selection of the individuals to the test group was random, without replacement sampling. The study group was randomly chosen from the patients treated at the Babinski Memorial Hospital in Lodz, Poland. The HS group was selected from among the staff of the same hospital.

Before deciding to participate in the study, the subjects were informed of its purpose, assured that the participation was voluntary, and guaranteed that personal data and the results of the tests would be kept confidential. Written informed consent was obtained from each subject according to the study protocol that had been approved by the Bioethical Committee of the Medical University of Lodz (No. RNN/728/12/KB).

5 Results

Average age of all the examined patients ($N = 130$) was: $M = 48.11$ years, $SD = 10.01$ (minimum age – 20 years, maximum – 67 years). In the ED-I group, average age was: $M = 45.13$, $SD = 11.01$, and in the rDD group: $M = 49.63$, $SD = 10.19$. The characteristics of the examined group in terms of sex and education are presented in table 1.

No significant statistical differences were observed between the examined groups in terms of sex ($\chi 2 = 2.01$, $p = 0.16$) and education ($\chi 2 = 1.46$, $p = 0.69$), only in terms of age ($Z = -2.01$, $p = 0.04$).

Significant statistical differences were observed between the severity of depression as measured using the HDRS scale in the ED-I and rDD groups on the day of admission to the study and after obtaining a response to the applied pharmacological treatment ($Z = 5.64$, $p < 0.001$ and $Z = 8.01$, $p < 0.001$, respectively). Our results indicate

Table 1: The comparison of the study groups in terms of sex and education.

Sex	**ED-I (n = 44)**	**rDD (n = 86)**	**p**	**Total (N = 130)**
	N (%)	**N (%)**		**N (%)**
Female	23 (52.27)	56 (65.12)	0.41	79 (60.77)
Male	21 (47.73)	30 (34.88)	0.52	51 (39.23)
Education	**ED-I (N = 42)**	**rDD (N = 89)**	**p**	**Total**
	N (%)	**N (%)**		**N (%)**
Primary	1 (2.27)	6 (6.97)	0.59	7 (5.38)
Vocational	11 (25.01)	18 (20.93)	0.52	29 (22.31)
Secondary	22 (50.01)	44 (51.16)	0.56	66 (50.76)
High	10 (22.73)	18 (20.93)	0.47	28 (21.54)

ED-I – first episode of depression, rDD – recurrent depressive disorders, n – number of samples, % – percentage, * – p statistically significant.

an improvement in the effect of the incorporated treatment in both the examined groups.

No significant statistical differences were found between the analyzed groups as regards the severity of depressive disorders (Table 2). Neither was such differences observed on the day of admission of the patients to the study nor after receiving a response to the implemented pharmacological treatment. In both groups, the average level of the symptoms of depressive disorders on the first day of the study corresponded to a severe level of depressive disorders according to the HDRS scale and remission of depressive disorders based on the HDRS scale after 8 weeks of the pharmacological therapy.

Table 3 presents average values, standard deviation, minimum and maximum values of expression at the mRNA level and the protein level for the analyzed inflammation enzymes: IL-1, IL-6 and IL-10 in the study group ($N = 130$).

Table 3 also presents a comparison of the analyzed genes expression at the mRNA level and the protein level in the ED-I and rDD group.

In the case of all the variables included in the analysis, no significant statistical differences were found between the ED-I and rDD group. No differences in the expression of IL-1, IL-6 and IL-10 genes at the level of both mRNA and protein were observed between the patients with the first episode of depression and the ones diagnosed with rDD.

An average number of depression episodes were 6.85 among the patients suffering from rDD (table 2). No significant interrelation was noticed between the number of depression episodes experienced and the expression of selected genes at the mRNA level and protein level.

There was no significant relationship in the examined group between the severity of depressive disorders measured before and after the pharmacotherapy and the expression at the mRNA level and protein level for the analyzed genes.

Table 2: The severity of depressive disorders among ED-I group and rDD group.

Variable	**ED-I (n = 44)**	**rDD (n = 86)**	**Mann-Whitney U test**	
	M (SD)	**M (SD)**	**Z**	**p**
HDRS-I	22.71 (7.42)	23.47 (6.86)	-0.25	0.81
HDRS-II	6.28 (4.48)	6.67 (4.36)	-0.44	0.66
Number of depression episodes	-	6.85 (4.81)	-	-

ED-I – first episode of depression, rDD-recurrent depressive disorders, HDRS-I – Hamilton Depression Rating Scale at the onset of therapy, HDRS-II – Hamilton Depression Rating Scale after pharmacological treatment, M – mean, SD – standard deviation, * – p statistically significant.

Table 3: Comparison of expression of the analyzed genes at the mRNA level and protein level in the affected from the ED-I and rDD group.

Variable	N = 130 ED-I + rDD			ED-I	rDD	Mann-Whitney U test	
	M (SD)	Min.	Max.	M (SD)	M (SD)	Z	p
IL-1 mRNA (2-ΔΔct)	0.67 (0.09)	0.43	0.83	0.66 (0.11)	0.68 (0.09)	-0.91	0.361
IL-1 protein (pg/ml)	11.19 (1.6)	7.11	13.81	10.99 (1.75)	11.29 (1.51)	-0.91	0.364
IL-6 mRNA (2-ΔΔct)	0.33 (0.05)	0.18	0.41	0.33 (0.05)	0.33 (0.05)	0.52	0.602
IL-6 protein (ng/ml)	5.46 (0.86)	3.01	6.91	5.53 (0.91)	5.43 (0.85)	0.72	0.474
IL-10 mRNA (2-ΔΔct)	0.37 (0.06)	0.25	0.53	0.37 (0.06)	0.37 (0.06)	0.13	0.898
IL-10 protein (ng/ml)	6.22 (0.94)	4.21	8.81	6.22 (0.94)	6.21 (0.95)	-0.03	0.978

IL-1 – interleukin 1, IL-6 – interleukin 6, IL-10 – interleukin 10, M – mean, SD – standard deviation,
* – p statistically significant.

6 Discussion

The working hypothesis presented in the introduction was not confirmed. The obtained results indicate no differences in the expression of cytokines (IL-1, IL-6, IL-10) between the patients with the first episode of depression and rDD. Therefore, the results may indicate actively progressing immune-inflammatory and oxidative stress underwritten in the patients with depression, although the magnitude of the biomarkers measured does not depend on the stage of the disease.

There are no such studies which would compare the biomarkers used in this study in both depression groups and, therefore, we cannot compare our results with other researchers' reports. Many research studies conducted earlier demonstrated that the level of pro-inflammatory interleukins was elevated and the level of anti-inflammatory interleukins reduced in the people with symptoms of depression as compared to healthy subjects [15, 16]. Moreover, Dahl et al. [17] indicated that plasma levels of various cytokines were increased during ongoing depression and reduced to normal levels after recovery.

It seems that the last of the quoted conclusions remains in conflict with the result of the studies conducted by us. However, in successive works one may find hypotheses in favor of the results obtained in our research work. Milaneschi et al. [18] stated that higher plasma levels of inflammatory markers (IL-1 and IL-6) in elder people predict the development of depressive symptoms during a 6-year follow-up. Such data would indicate increased pro-inflammatory readiness in the patients with rDD, even before the occurrence of the first symptoms of the disease. Moreover, in their research studies, Dannehl et al. [15] investigated whether changes in IL-6 and TNF-α over 4 weeks could be predicted by measuring cognitive-affective and somatic symptoms in patients with major depression. They found that higher somatoform symptoms during the last 2 years significantly predict an increase in TNF-α in women with major depression, but not in men. Exploratory analyses indicated high stability of TNF-alpha over 4 weeks in patients with major depression and moderate stability in healthy controls. IL-6 was less stable in both groups.

On the other hand, Baune et al. [19] examined the association between cytokines (IL-1β, sIL-4R, IL-6, IL-8, IL-10, IL-12, TNF-α) and subcortical white matter lesions, cortical atrophy and lacunar infarctions of an ageing brain. The link between an atrophy and the chemokine-cytokine factor (containing sIL-4R, IL-6, IL-8) remained significant after adjustment for age, sex, education, depressive symptoms, diabetes mellitus, cardiovascular diseases (stroke, TIA, myocardial infarction, myocardial insufficiency, arrhythmic heart), hypertension, body-mass index, smoking status and aggregation inhibitors as opposed to single cytokines. An atrophy of the parietal, temporal and occipital lobes was associated with the same cytokine-chemokine factor for the entire sample or restricted to those without a history of stroke/transient ischemic attacks. Therefore, a constant level of pro-inflammatory and anti-inflammatory cytokines may be a common denominator of neurodegenerative changes observed in the course of dementia and neurodegenerative processes

diagnosed in rDD [20]. However, in the case of subjects suffering from rDD, such changes are not an indication of permanent cognitive dysfunctions (evaluated using available psychological methods) that would preserve in periods of remission [20].

Latest research studies emphasize the beneficial effect of antidepressant treatment on the secretion of cytokines [21, 22, 23, and 24]; however, the results presented herein support the hypothesis that highlights the necessity of accepting dysregulation in the production of cytokines as a consistent biomarker of rDD. IL-1 and IL-6 are considered some of the most consistent biomarkers of depression [25], which is in conformity with the results obtained by us. Their increased level is also linked with treatment resistance [26]. Similarly, anti-inflammatory IL-10 fluctuations are associated with depressive symptoms in humans and are said to influence depressive behavior [27, 28].

In summary, it is possible to confirm the necessity for further research studies as regards the presented issues and mutual interrelations between inflammatory processes and emotional and cognitive signs of depression.

7 Conclusions

There is no significant difference in IL-1, IL-6 and IL-10 expression between patients with recurrent depressive disorders and those suffering from the first episode of depression. There seems to be no difference in acute first episode vs. acute episode of depression in patients with a recurrent disorder. Further larger trials are needed to evaluate.

8 Limitations

Although our study has strong points, some limitations have to be noted.

The results should be interpreted with caution given the relatively small sample size, which indicates a necessity of carrying out analyses taking statistical power into account during further studies.

Additionally, it is important to consider the possibility of making a type I error associated with the zero hypothesis (H_0).

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