



## ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Chromatin textural parameters of blood neutrophils are associated with stress levels in patients with recurrent depressive disorder

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## SUMMARY

**Introduction/Objective** During the past 20 years, there have been numerous attempts to design and apply a simple, affordable blood analysis tool for diagnostic and prognostic purposes in psychiatry.

In this article we demonstrate that some mathematical parameters of chromatin organization and distribution in blood neutrophil granulocytes are related to stress levels in patients diagnosed with recurrent depressive disorder (RDD).

**Methods** The study was performed on 50 RDD participants who were asked to complete Depression, Anxiety and Stress Scales (DASS-21). Peripheral blood samples were obtained from all the participants, smeared on glass slides and stained using a modification of Giemsa method. A total of 500 representative chromatin structures (10 per patient) of neutrophil granulocytes were evaluated using textural analysis with the application of gray level co-occurrence matrix (GLCM) method. Parameters such as angular second moment (indicator of textural uniformity), inverse difference moment (textural homogeneity), and textural sum variance were calculated.

**Results** The results indicate that there is a statistically highly significant correlation ( $p < 0.01$ ) between certain chromatin GLCM parameters such as inverse difference moment, and DASS-21 stress score. There was also a significant difference ( $p < 0.05$ ) in some chromatin GLCM parameters in patients diagnosed with RDD with psychotic features, when compared to the ones without psychosis.

**Conclusion** These findings suggest that in the future, chromatin GLCM features might have a certain predictive value for some clinical features of recurrent depressive disorder.

**Keywords:** nucleus; structure; anxiety; depression

## INTRODUCTION

In recent years, in the rapidly growing field of neurosciences, there have been many attempts to develop an exact, objective, and affordable image analysis method that would be applied in clinical practice as a supplement to the conventional diagnostic protocols. Many new mathematical algorithms have been proposed, often with limited results and impact. One of the techniques that are today being frequently considered in neurology and biology studies includes the analysis of texture [1, 2]. Textural analysis can be used to quantify structural features such as homogeneity and uniformity [2, 3].

There are many ways to assess texture of a biophysical system. Some of the methods are based on higher mathematics and second order statistical calculations. One of these frequently used textural algorithms include the Gray level co-occurrence matrix (GLCM). The parameters of GLCM method may have certain value in medical image analysis due to their potential

ability to detect structural alterations in cells and tissues [4].

Textural parameters of tissue architecture were shown to be a potentially important addition to conventional cell biology and histology methods [5, 6]. For example, in a study published in 2009, Shamir and associates demonstrated that these features, when calculated on muscle tissue in an animal experimental model, can be a possible indicator of structural deterioration during physiological aging. In collagen morphology, textural indicators also exhibit a differentiating power to some extent [7]. Some textural parameters of spleen germinal center tissue might be associated with some physiological parameters such as humoral immune response to a foreign antigen [8].

Our recent studies have indicated that changes in chromatin structure in some cells can also be detected and evaluated using GLCM features. Our work on spleen lymphocytes in germinal centers indicated that some parameters are correlated with nuclear shape [9].

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Nuclear textural parameters of progenitor cells in spleen also exhibited statistically significant changes during aging [10]. Also, some of these determinants were proven to be very useful in discriminating two different lymphocyte populations in thymus [11]. Finally, in 2016, we indicated that textural features of blood lymphocyte chromatin may be important indicators of structural changes induced by oxidopamine [12].

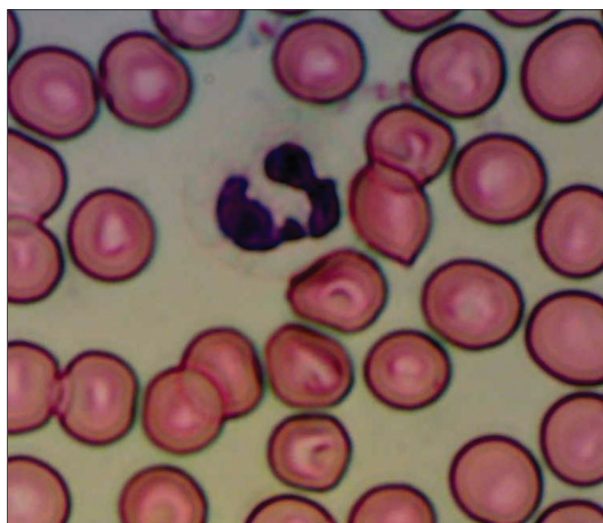
In this study we show that some chromatin GLCM parameters of peripheral blood neutrophil granulocytes are associated with self-reported stress levels in patients diagnosed with recurrent depressive disorder. To our knowledge, this is the first work to show that GLCM features calculated under these conditions correlate to a psychological parameter of stress. Also, this is the first study to apply GLCM analysis in peripheral blood cells on a sample of psychiatric patients with recurrent depression.

## METHODS

The study was performed on 50 patients (14 males, 36 females, average age  $53.9 \pm 6.4$  years) at Laza Lazarević University Clinic for Mental Disorders, Belgrade, Serbia. All the patients had been diagnosed with recurrent depressive disorder (F33 diagnosis code according to International classification of diseases ICD-10). Exclusion criteria were as follows: comorbid psychotic or other serious psychiatric disorder, substance abuse, serious non-psychiatric disorders that might have impacted the final results (i.e. immunological illnesses, blood cell disorders, endocrine diseases, etc.).

All the participants were asked to complete a questionnaire that included Depression, Anxiety and Stress Scales (DASS-21) [13]. This instrument is made up of 21 self-report items that need to be rated on a Likert scale (four point). The items reflect emotional symptoms and experiences over the last seven days. Each of the three scales (for depression, anxiety and stress, respectively) consists of three items. The depression scale evaluates symptoms such as anhedonia, loss of self-esteem, and hopelessness. The anxiety scale refers to the symptoms related to fear, panic, worry, and autonomic system dysfunction. The stress scale covers items on relaxation difficulties, agitation, tendency to overreact, etc.

A sample of peripheral blood was obtained from all the participants and smeared on glass slides. The smears were fixated in methanol and stained using Giemsa method (Figure 1). For the details regarding the Giemsa technique, the reader is referred to previously published works of other authors [14, 15]. This method has also been successfully applied for textural analysis of chromatin in spleen follicular cells in our recent research [16]. The study protocol regarding DASS-21 administration, blood smear preparation and staining was a part of a wider research for a PhD thesis which was approved by the ethics panel of the host institution. Informed signed consent for the participation in the PhD thesis research was obtained from all the patients. The research was conducted in accordance with



**Figure 1.** Giemsa-stained nucleus of a neutrophil granulocyte

■	□	■	■	0	3	2	1
■	■	□	□	2	2	3	3
□	■	■	□	3	1	1	3
□	■	□	■	3	0	3	1
■	■	□	■	2	2	3	0

**Figure 2.** An example of gray level designations (for a low-quality micrograph) used for later formation of gray level co-occurrence matrix

the Helsinki Declaration as revised in 1989 and conformed to the legal standards regarding research in medicine.

Nuclear structures of Giemsa-stained neutrophil granulocytes (10 cells per patient) were visualized using Pro-Micro Scan DEM 200 instrument (Oplenic Optronics, Hangzhou, CN) mounted on Olympus BX41 microscope (Olympus Corporation). Digital micrographs were created and saved in JPEG format. Dimensions of the micrographs were  $1600 \times 1200$  pixels (width 1600, height 1200), both horizontal and vertical resolutions were 96 dpi, and bit depth equaled 24. Regions of interest of neutrophil nuclei were created, after which textural GLCM analysis of regions of interest was performed.

Parameters of image texture were calculated using MaZda software, previously developed for COST B11 and B21 European projects by a researcher from the Institute of Electronics, Technical University of Lodz [17, 18, 19]. Textural parameters were calculated based on Gray level co-occurrence matrix (GLCM) where second order statistical analysis is performed on resolution unit pairs and their gray values. Before GLCM is constructed, each resolution unit is assigned a value based on its gray intensity. The example of such assignment on a simpler micrograph is shown on Figure 2. Our micrographs had much higher number of possible gray intensity values compared to the one in the figure.

Inverse difference moment (IDM) as a measure of textural homogeneity was calculated based on the following formula:

$$\text{IDM} = \sum_i \sum_j \frac{1}{1+(i-j)^2} p(i,j)$$

where 'i' and 'j' are the values of neighbor and reference pixels in GLCM. Details on GLCM creation and features can be found in various previous works [2, 20].

Apart from IDM, we also measured the texture angular second moment (ASM). This is an indirect parameter of textural uniformity and can be determined as:

$$\text{ASM} = \sum_i \sum_j \{p(i, j)\}^2$$

Sum variance (SVAR) of the co-occurrence matrix was calculated as:

$$\text{SVAR} = \sum_{i=2} \left[ i - \sum_{l=2} i p_{x \rightarrow y}(l) \right]^2$$

This parameter is related to local variations of the textural distribution in a micrograph.

All GLCM parameters were calculated on regions of interest (objects) directly from the micrograph which was previously converted to 8-bit gray scale format. No cropping or other modifications were performed.

As the addition to the textural measurements, we also calculated fractal dimension (FD) of the neutrophil chromatin structure. FD was determined on binarized nuclear images, using the FracLac plugin for ImageJ (A. Karperien, Version 2.5, Release 1e, Charles Sturt University, Australia) [21]. During the analysis, a special box-counting method is performed, the structure is covered by a number of boxes (N) on different scales ( $\epsilon$ ), after which FracLac forms a logarithmic graph (Figure 3) and calculates FD from the slope of the regression line:

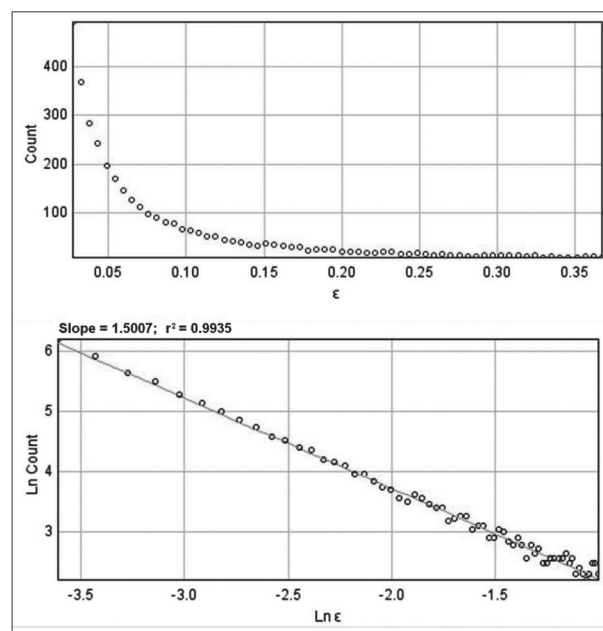
$$\text{FD} = \text{regression slope} [\ln(N)/\ln(\epsilon)]$$

FD was previously used on numerous occasions in cell biology in order to detect small structural alterations [16, 22]. Although not directly related to ASM or IDM, it may provide additional information on the nature and causes of their potential change.

## RESULTS

Mean score for the depression and anxiety scales of DASS-21 were  $10.9 \pm 5.3$  and  $9.2 \pm 4.4$ , respectively. The average value of stress score in DASS-21 was  $11.7 \pm 5.3$ . The mean value of IDM in neutrophil granulocytes was  $0.79 \pm 0.04$ . The average textural ASM of chromatin structure was  $0.041 \pm 0.021$ , and the mean value of nuclear FD was  $1.52 \pm 0.11$ . Mean SVAR of the GLCM equaled  $73.9 \pm 21.3$ .

There was a statistically highly significant ( $p < 0.01$ ) correlation between neutrophil chromatin IDM and the score of the stress scale within DASS-21 (Figure 4). The correlation was negative, meaning that as the IDM increased, the stress level decreased, and vice versa. In Figure 4, plotted values of chromatin IDM and stress levels are shown. It was concluded that the age of the patients was not a contributing factor to this correlation. The relationship between stress score and IDM suggests that chromatin



**Figure 3.** As an addition to this study, fractal analysis was performed using the box-counting method; in this method, the structure is covered by a number of boxes (N) on different scales ( $\epsilon$ ), after which the software forms a logarithmic graph and calculates fractal dimension from the slope of the regression line

IDM of blood neutrophils is a potentially good indicator of psychological distress in patients diagnosed with recurrent depressive disorder. No such correlation ( $p > 0.05$ ) was observed between the values of chromatin IDM and the result of DASS-21 depression scale. Neither chromatin FD nor the values of chromatin ASM and SVAR in blood neutrophils were related to the scores of the DASS-21 scales. There was a much weaker negative relationship between IDM and DASS-21 anxiety score ( $p < 0.05$ , Figure 5).

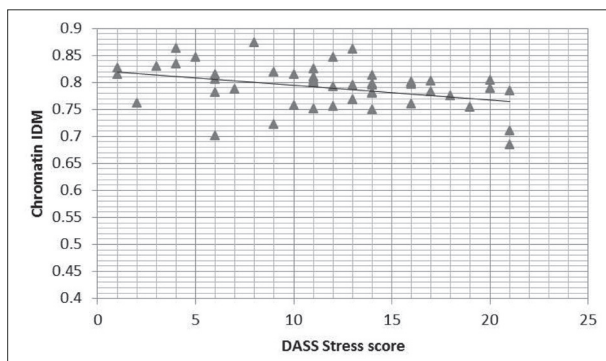
Patients with psychotic features ( $n = 10$ ) had significantly higher ( $p < 0.01$ ) values of chromatin SVAR, compared to the patients without psychosis ( $n = 40$ ). The values of SVAR equaled  $92.7 \pm 17.9$  and  $69.2 \pm 19.5$ , respectively. ASM, IDM, and FD did not significantly differ between these two subgroups.

## DISCUSSION

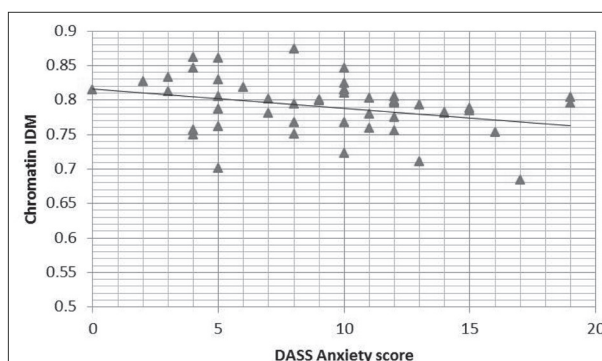
In this study in patients diagnosed with recurrent depressive disorder, we investigated the potential relationship between mathematical parameters of chromatin organization in peripheral blood neutrophils, and determinants of depression, anxiety, and stress. The main finding is the detected correlation between chromatin textural IDM and self-reported stress levels using the DASS scale. Other mathematical parameters were not significantly related to the scores of DASS subscales. These results imply that GLCM inversed difference moment of chromatin is potentially a valuable indicator of stress, and that it may in the future be used as an integral part of a biosensing system in psychology, psychiatry, and related disciplines.

Recurrent depressive disorder is one of the most common mental disorders seen in contemporary psychiatry





**Figure 4.** There was a statistically highly significant ( $p < 0.01$ ) positive correlation between chromatin inverse difference moment (IDM) and Depression, Anxiety and Stress Scales (DASS-21) stress scores



**Figure 5.** Plotted values of chromatin inverse difference moment (IDM) and Depression, Anxiety and Stress Scales (DASS-21) anxiety score; weak but significant correlation ( $p < 0.05$ ) was detected

practice. The exact cause of depression has long been debated in medical research and today there are numerous theories trying to explain its pathogenesis and associated molecular mechanisms. One of such theories focuses on the possible impact of immune system and inflammation in the development of unipolar depression [23, 24]. Various cytokines and other immune-related mediators have been mentioned as potential contributors to depressive mood. Function of both neutrophils and lymphocytes may be changed during this disorder; however, the exact nature of these changes remains unclear.

Depression is closely related to stress, and the major finding of our study implies that the “stress component” of RDD is related to changes in neutrophil chromatin morphology. This may be due to several reasons. First, it is possible that cortisol, a well-known stress hormone, associated with “fight or flight” responses, may be involved in the manifestation of these chromatin changes. Depressed patients often have increased levels of cortisol, and this hormone when chronically increased might be a significant factor in depression development [25]. On the other hand, glucocorticoids exhibit significant genomic effects in neutrophil granulocytes [26]. Glucocorticoids may cause significant changes in expression of numerous genes, such as the ones for inflammatory cytokines, glutamine synthetase and other proteins. The magnitude of these changes may be similar to the ones seen in other leucocyte populations [26].

Second, it is possible that epinephrine and norepinephrine also significantly influence the chromatin organization of blood neutrophils. Kim et al. [27] showed that chronic catecholamine stress induced by prolonged delivery of epinephrine may significantly change the level of neutrophil trafficking. In some cell populations, epinephrine may substantially influence inflammation-related gene expression [28]. Changes in gene expression, if of sufficient magnitude, may alter the patterns associated with higher levels of DNA/chromatin organization, which can manifest itself as change in texture during conventional microscopy.

Finally, as correlation is not necessarily the proof of causality, we could speculate that the patients with specific patterns of neutrophil chromatin may have different self-perceived stress levels due to some specific properties of

neutrophil function. Neutrophils are secretory active cells which produce a variety of chemical mediators. Some of those mediators (i.e. specific interleukins) might be responsible for the subjective feeling of distress. It can be assumed that neutrophils that are more (or less) secretory active will differ from others in terms of their chromatin organization. This may be related to altered euchromatin/heterochromatin ratio, different chromatin distribution within the nucleus, different interaction between chromatin and nuclear envelope. All these factors might be associated with changes in the values of overall chromatin homogeneity.

According to our opinion, the major contribution of our present study is not the investigation of a specific physiological mechanism of stress and depression, but the fact that the GLCM IDM as a mathematical parameter has some prognostic value in assessing the stress levels in depressed patients. To our knowledge, to this date no parameter of blood neutrophils exists that would be able to serve as an indicator of stress in RDD. In fact, this is probably one of the first works to demonstrate the potential clinical value of neutrophil chromatin mathematical analysis, not only in RDD, but in psychiatry in general. In the future, it would be interesting to see if chromatin IDM is capable of predicting the outcomes of RDD therapy, or to correlate it with other biological tests used in contemporary psychiatry research.

Also, the potential value of our results reflects in the fact that GLCM analysis of blood neutrophils is an exact, objective, and relatively affordable method which does not require significant time and financial resources. Conventional histology and pathology analysis often rely on the subjective opinion of the professional on the appearance of cell and subcellular components. For example, to a pathologist, a nucleus may “appear” more or less disorderly in its structure and texture, but using the conventional means, so far it hasn’t been possible to assign quantification to this evaluation. Mathematical textural analysis overcomes this issue and applies a precise and objective estimate of structural features which can be performed by almost any medical or biological professional. This opens up numerous future possibilities regarding a design of modern easy-to-use biosensors in psychophysiology and psychiatry.

Our study had certain limitations that need to be pointed out. First, we opted for Giemsa staining method, which is today commonly used in cytogenetics. This method targets the phosphate groups of DNA, and especially the areas of genome rich with adenine–thymine segments. There are however numerous other staining techniques that are able to adequately assess the structure of a nucleus. In the future, we recommend additional experiments to be performed using DNA-specific Feulgen method, as used in our previous study on adrenal gland tissue [29]. In terms of some aspects of interaction with the DNA molecule, this method might be superior to Giemsa, and it would certainly be interesting to see results on textural analysis after administration of this stain. Also, our study measured the levels of stress in depressed patients using DASS-21 psychiatric scale, which is today frequently used to quantify subjective distress in both patients and healthy subjects; there are, however, numerous other means to evaluate stress in clinical conditions. Future studies would also need to include various different physiological and psychiatric methods for stress assessment and try to test if the mathematical analysis is still a good indicator of stress under the new conditions.

## CONCLUSION

Our study indicates that in patients diagnosed with recurrent depressive disorder, some textural features of blood neutrophil granulocytes are associated with the levels of subjective distress. To our knowledge, this is the first study

to perform textural analysis of blood leucocyte chromatin in this experimental setting. The results suggest potential applicability of mathematical analysis of blood cell chromatin in future psychiatry and psychophysiology research.

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## Note on previous publications

This paper has been presented in a form of abstract at the Second National Congress of Hospital Psychiatry with International Participation, Belgrade, Serbia, October 10–12, 2018.

The research protocol regarding the administration of the DASS-21 and obtaining/staining the patients' blood was done as a part of PhD thesis work of the co-author Draga Dimitrijević.

**Conflict of interest:** None declared.

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## Текстурални параметри хроматина неутрофилних гранулоцита крви су асоцирани са степеном стреса код болесника са рекурентним депресивним поремећајем

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### САЖЕТАК

**Увод/Циљ** Током протеклих 20 година постојали су бројни покушаји дизајнирања и примене једноставног, приступачног метода за анализу крви за дијагностичке и прогностичке потребе у психијатрији.

У овом раду показујемо да су неки математички параметри организовања и дистрибуције хроматина у неутрофилним гранулоцитима крви повезани са нивоима стреса код болесника са дијагнозом рекурентног депресивног поремећаја.

**Метод** Студија је спроведена на 50 болесника са дијагнозом рекурентног депресивног поремећаја, од којих је затражено да попуне упитник за депресију, анксиозност и стрес (DASS-21). Узорци периферне крви добијени су од свих учесника, направљени су размази на предметним стаклима и обојени коришћењем модификације методе по Гимзи. Укупно 500 репрезентативних хроматинских структура (10 по болеснику) неутрофила анализирано је коришћењем тек-

стуралне анализе, односно математичког алгоритма *GLCM* (*gray level co-occurrence matrix*). Израчунати су параметри попут аугуларног другог момента (индикатор текстуралне униформности), инверзног момента разлике (текстурална хомогеност) и текстуралне суме варијансе.

**Резултати** Резултати показују да постоји статистички значајна корелација ( $p < 0,01$ ) између одређених *GLCM* параметара хроматина, као што је инверзни моменат разлике, и *DASS-21* скор за стрес. Постојала је и значајна разлика ( $p < 0,05$ ) у неким *GLCM* параметрима хроматина код болесника са дијагностификованим депресивним поремећајем са психотичким карактеристикама у поређењу са онима без психозе.

**Закључак** Ови налази указују на то да текстурална анализа хроматина крвних ћелија у будућности може имати одређену предиктивну вредност за неке клиничке особине рекурентног депресивног поремећаја.

**Кључне речи:** једно; структура; анксиозност; депресија