NÖRO PSİKİYATRİ ARŞİVİ
ARCHIVES OF NEUROPSYCHIATRY

RESEARCH ARTICLE

Arch Neuropsychiatry 2023;60:28–36
https://doi.org/10.29399/npa.28127

Associations Between Blood Levels of NLRP3 Inflammasome Components and Obsessive Compulsive Disorder

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ABSTRACT

Introduction: Even though the effect of inflammation on pathogenesis of obsessive compulsive disorder (OCD) is known, information regarding the underlying mechanisms are yet to be revealed. The NLRP3 inflammasome complex is an important component of the innate immune system that initiates and mediates inflammatory response to a variety of stimuli. This study aims to inquire into a possible association between NLRP3 inflammasome complex and OCD.

Methods: This case-control study included 103 participants (51 cases with OCD and 52 healthy controls). All participants were evaluated with the Yale Brown Obsessive Compulsive Scale, Hamilton Depression Scale, and Hewitt Multidimensional Perfectionism Scale. RNA and proteins were extracted from peripheral blood mononuclear cells. Expression of NLRP3 inflammasome components were determined using quantitative real-time polymerase chain reaction (PCR) and Western blotting. Levels of Serum IL-1beta and IL-18 cytokine were determined by ELISA.

Results: NEK7 and CASP1 mRNA levels were significantly higher in OCD patients, compared to controls. Pro-caspase-1 protein levels were elevated, as well. Regression analysis showed that NEK7 mRNA and pro-caspase-1 protein levels can differentiate OCD and healthy control groups.

Conclusion: Our results provide insight into the molecular alterations that could explain the inflammation-OCD association.

Keyword: ASC, caspase-1, inflammasome, NEK7, NLRP3, obsessive-compulsive disorder

Cite this article as: Tetik M, Direk N, Önder Uzgan B, Aykac C, Ekinci B, Yaras T ve ark. Associations Between Blood Levels of NLRP3 Inflammasome Components and Obsessive Compulsive Disorder. Arch Neuropsychiatry 2023;60:28–36.

INTRODUCTION

Obsessive compulsive disorder (OCD) is characterized by repetitive, involuntary thoughts and behaviors that disrupts daily activities and functioning of affected person. Obsessive compulsive disorder is a symptomatically heterogeneous disorder with several factors involved in its etiology. One of these factors is inflammation, which has been studied in OCD continuously, yet results are inconclusive (1). Generally, peripheral levels of pro-inflammatory and anti-inflammatory cytokines were used as indicators of inflammation in OCD studies. However, using such indicators provide limited information about the role of inflammation on OCD etiology. Contrary to measuring cytokine levels, arising from the underlying inflammatory processes, observing the processes may provide insight regarding the etiology of the disease.

Highlights

- **Blood cells of OCD patients have increased expression of NLRP3 inflammasome.**
- **NEK7 mRNA and pro-Caspase-1 protein levels can distinguish between the two groups.**
- **Increased pro-Caspase-1 expression is independent of depression comorbidity.**

Inflammasomes are the sensors of various stress stimuli and mediators of inflammatory response. They are oligomeric structures composed of nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins. Nucleotide-binding oligomerization domain, leucine rich repeat (LRR) and pyrin domain (PYD) containing protein 3 (NLRP3) inflammasome is formed by the interaction of NLRP3, pro-caspase-1 and apoptosis-associated speck-like protein containing a CARD (PYCARD/ASC) protein. Upon stress stimuli, NLRP3 protein is activated

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Received: 06.02.2022, **Accepted:** 14.04.2022, **Available Online Date:** 26.02.2023

to initiate an oligomeric complex that is known as the 'inflammasome complex'. Active inflammasome complex cleaves pro-caspase-1 to yield active caspase-1 that processes cytokines such as IL-1β, IL-18 and IL-33. NLRP3 inflammasome has been shown to be related to neuropsychiatric conditions such as major depressive disorder (MDD), bipolar disorder, schizophrenia, Parkinson's disease and multiple sclerosis (2–7). It is postulated to precipitate and perpetuate inflammation in those diseases.

The Nima-Related Kinase 7 (NEK7), a known regulator of microtubule formation and mitosis, was recently identified as a regulator of NLRP3 inflammasome activity. Inflammasome-activating signals cause potassium efflux and therefore stimulate NEK7, which leads to NLRP3 oligomerization and speckle formation. NEK7 is required for activation of NLRP3 inflammasome and it is specific to NLRP3, as it has no function on the activation of other inflammasome complexes (8–10).

There are only few studies in which the association of inflammasomes and psychiatric disorders including psychosis, bipolar disorder and MDD have been tested. Yet, no study has explored such relationship between inflammasomes and OCD, so far. Considering the increasing evidence that suggests the involvement of neuroinflammation in OCD pathogenesis and the triggering role of NLRP3 inflammasome in pro-inflammatory states, we hypothesized that NLRP3 inflammasome might be associated with OCD. Therefore, we compared mRNA and protein levels of NLRP3 inflammasome components and NEK7 between OCD patients and healthy controls. We also investigated a possible association between NLRP3 inflammasome components and clinical features of OCD including severity, co-existing anxiety, depression, and perfectionism.

METHODS

Study Population

The current study included 51 patients with OCD (aged 18 to 45 years) and 52 healthy (aged 18 to 45 years) controls who were recruited from the Department of Psychiatry, Dokuz Eylül University Hospital between February and November 2018. Healthy controls were age and sex-matched participants with no history of a chronic disease or psychiatric disorder. Participants with an inflammatory disorder such as rheumatoid arthritis, autoimmune disorders (i. e. Hashimoto's thyroiditis) and those on corticosteroid, statin, antihypertensive or non-steroid anti-inflammatory treatments were excluded from the current study. If a participant used an anti-inflammatory medication in the past five days, blood collection and psychiatric evaluations were postponed. Additionally, people with any sign of infection within one week prior to blood collection were not included in this study.

All protocols and methods were approved by the Dokuz Eylül University Clinical Studies Ethical Committee (Date: 11.05.2017, Decision no: 2017/08–06, Protocol number 378-SBKAEK). Oral and written informed consents were obtained from all participants.

Clinical Evaluation

Sociodemographic data were collected. The Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) Axis I Disorders was applied to confirm OCD diagnosis and to assess the presence of other psychiatric disorders. Yale-Brown Obsession and Compulsion Scale (Y-BOCS) was used to identify the severity of OCD (11,12). Y-BOCS consists of a symptom checklist of 74 questions under the classification headings for obsessions and compulsions. Hamilton Depression Rating Scale (HAM-D) was used to evaluate severity of depressive symptoms (13,14). To evaluate perfectionism levels, Hewitt's Multidimensional Perfectionism Scale (MPS) was used (15,16).

Molecular Analyses of Inflammatory Markers

Peripheral Blood Mononuclear Cell (PBMC) Isolation

For molecular studies, fasting peripheral venous blood samples (16.5 ml) were collected between 9:00 and 11:00 a.m. Peripheral blood mononuclear cells were isolated from fresh blood samples (6 ml) by adding 1:1 volume:volume Phosphate Buffered Saline (PBS) and overlaying on 3 ml lymphocyte separation medium (Lonza, Cat#BE17–829E), followed by centrifugation (Eppendorf 5810 R) at 2200 revolutions per minute (RPM) for 25 minutes. Isolated PBMCs were washed in PBS at 1500 RPM for 10 minutes. In total, 500 µl TRIzol (ThermoFisher) was added to pellets to preserve RNA integrity. Four aliquots/sample were stored at -80°C until RNA and protein isolation.

RNA Isolation and cDNA Synthesis

Aliquots in TRIzol were thawed on ice and 1:1 volume:volume EtOH (100%) was added. RNA extraction was performed using Direct-zol RNA Miniprep Plus kit (Zymo, Cat# R2072) according to the manufacturer's protocol and stored at -80°C until cDNA synthesis. RNA purity and quantity were assessed using Nanodrop 2100 c spectrophotometer (Thermo Scientific). $OD_{260/280}$ and $OD_{260/230}$ ratios were calculated and samples with 1.9–2.0 and >1.5, respectively were used for cDNA synthesis. One µg RNA was converted to cDNA using First Strand cDNA Synthesis kit (ProtoScript Cat# E6300).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

mRNA expression levels of NLRP3, ASC, CASP1 and NEK7 were determined using GoTaq qPCR Master Mix (Promega, Cat# A6001) on LightCycler480 (Roche). GAPDH was used as housekeeping gene for normalization and relative expression of each gene was calculated according to 2^{-DDCt} method. Primer pairs used for each gene are:

NEK7_forw 5′-TTTACACTCCTGACAGCG -3′,
NEK7_rev 5′-GCAACAGGAACTTTAGAACT -3′,
caspase-1_forw 5′-CTCAGGCTCAGAAGGGAATG-3′,
caspase-1_rev 5′-CGCTGTACCCCAGATTTTGT -3′,
NLRP3_forw 5′-GCAGCAAACTGGAAAGGAAG-3′,
NLRP3_rev 5′-CTTCTCTGATGAGGCCCAAG-3′, ASC_
forw 5′-AGTTTCACACCAGCCTGGAA-3′, ASC_rev
5′-TTTTCAAGCTGGCTTTTCGT-3′, GAPDH_forw
5′-ACCACAGTCCATGCCATCAC -3′, GAPDH_rev
5′-TCCACCCTGTTGCTGTA-3′.

Each reaction was performed in triplicates and melting-curve analysis was performed for each primer pair to ensure specificity.

Protein Extraction from PBMCs and Western Blotting

Chloroform was added on TRIzol-protected samples at 1:5 volume:volume ratio. Following the centrifugation and the EtOH wash, proteins were precipitated with 0.3 M guanidium hydrochloride. After washing, pellets were dissolved in 1% SDS solution. Protein concentration was measured using BCA Assay Kit (Thermo, Cat#: 23227). Equal amount of protein (30 µg) was mixed with SDS-sample buffer and boiled for 5 minutes to denature proteins. Proteins were separated on SDS-Polyacrylamide gel in Tris-Glycine Buffer for 2 hours and transferred to PVDF membrane at 250 mA for 2 hours. Membrane with transferred proteins was blocked with 5% fat-free milk in 1X TBS (Tris-buffered saline) solution. Optimal incubation times for each primary antibody was determined and followed by incubation with the appropriate horse-radish-peroxidase (HRP)-conjugated secondary antibody. Antibodies used for each protein are anti-CASP1 (Abcam Cat# ab1872), anti-ASC (AG, Cat# 25B-0006-C), anti-NEK7 (Abcam, Cat#ab13351), anti-NLRP3 (AG, Cat# 20B-OO14-C), anti-beta-actin (Abcam, Cat# ab6227). Finally, membranes were incubated with Luminata Forte Western HRP Substrate (Luminata, WBLUF0500) and

chemiluminescence was measured on imaging system (Vilber Lourmat, ECX-F20L). Image analysis was performed using the on-site software and protein levels were calculated with reference to beta-actin.

Enzyme-linked Immunosorbent Assay (ELISA) Analysis

ELISA analysis was performed on serum samples which have been isolated from blood and collected into yellow-cap gel-bottom tubes. Following a 30-minute incubation at room temperature, samples were centrifuged at 4000 rpm (2850 g) for 5 minutes and 500 µl aliquots were stored at -80°C until measurement. ELISA was performed according to the manufacturer's instructions for IL-1beta (Thermo Scientific, Invitrogen, Cat# BMS224–2) and IL-18 (Thermo Scientific, Invitrogen, Cat# BMS267NST). Fifty µl serum samples from each participant were used and absorbance at 450 nm – 620 nm was measured (Varioskan, Thermo). In order to determine IL-1beta and IL-18 concentration of each sample standart curves were employed.

Statistical Analysis

We tested the distribution of the continuous variables using histograms with normality curves. Variables with non-normal distribution were transformed using natural logarithm (LN). We excluded outliers (n=3) when analyzing inflammatory parameters that were assessed with Western blotting. Group comparisons were done with a chi-square test for categorical variables and with a t-test for continuous variables.

The associations of inflammasome components, NEK7, interleukin levels with OCD status were evaluated by means of logistic regression analyses. In the first model, analysis was performed according to age, gender and education level. In the second model, total Hewitt's MPS score was also included into the model. Within the context of the analyses, LN-transformed values of inflammatory parameters were used and results were given as back-transformed values.

In the OCD group, we tested possible predictors of the inflammasome components, NEK7, interleukin levels using a linear regression analyses. In these analyses, age, gender, education level, Y-BOCS score, total duration since OCD diagnosis, presence of OCD medication, Hewitt's MPS total score and HAM-D total scores were used as predictors. Again, back-transformed values were presented.

IBM Statistical Package for the Social Sciences (SPSS) version 22.0 was used for statistical analyses and type I error threshold of 0.05 was used for statistical significance.

RESULTS

In total, 51 participants with OCD and 52 healthy controls were enrolled in the current study. The mean age of OCD patients and healthy controls were respectively 30.41±8.90 and 30.56±7.89 years, (p>0.05). In total, 60.8% (n=31) of the OCD group and 59.6% (n=31) of the healthy controls were female (p>0.05). Obsessive compulsive disorder cases had significantly lower education level than healthy controls (p=0.04). Number of employed participants were significantly lower in OCD group than in healthy controls (p<0.001). Detailed characteristics of study population are presented in Table 1.

In total, 45.1% (n=23) of OCD patients were on antidepressant treatment. Average Y-BOCS score was 25.1±6.5 and average HAM-D score was 14.8±8.3 in OCD group. Hewitt's MPS scores were higher in OCD patients compared to healthy controls. However, the difference was not statistically significant (184.5±48.1 vs. 167.1±41.2, p=0.05) (Table 1).

NEK7 mRNA (p=0.001), CASP1 mRNA (p=0.006) and pro-caspase-1 protein levels (p=0.002) were higher in OCD patients compared to healthy controls, while the other components were not significant between the two groups at mRNA or protein levels (Figure 1, Table 1).

A series of logistic regression analyses were performed to test the association of mRNA and protein levels of NLRP3, caspase-1, ASC, NEK7, IL-1beta and IL-18 with OCD status. Final models in which analyses were adjusted for age, sex, education level, Hewitt's MPS scores indicated that NEK7 mRNA and pro-caspase-1 protein levels predicted OCD status (Table 2). No statistically significant association was present between NLRP3, ASC mRNA/protein levels with OCD.

Linear regression analysis was conducted to examine possible predictors of inflammasome components, NEK7, and interleukin levels in OCD group. There was no significant predictor of mRNA levels in OCD group. However, protein levels of NLRP3 and ASC were predicted by antidepressant treatment in OCD patients (Supplementary Table S1-S2, Figure 2). In the same model, age and Y-BOCS score were negatively associated with ASC protein levels in OCD patients. There was no significant predictor of NEK7, pro-caspase-1, IL-1b or IL-18 levels in the OCD group.

Furthermore possible associations between NLRP3 inflammasome components, NEK7 and interleukin levels and severity of depression in OCD patients was investigated. Patients with OCD were grouped according to the commonly used cut-off score for HAM-D scores (i. e. <18 and ≥18). mRNA or protein levels of NLRP3, ASC, or caspase-1 were not related to depression status in OCD group. On the other hand, NEK7 protein levels were significantly different between the two groups (HAM-D<18 vs. HAM-D≥18, 6.156±19.460 vs. 0.726±0.760, mean ± SD respectively, Kruskal-Wallis p=0.03), whereas no such association was observed for NEK7 mRNA levels. There was no association of IL-1b or IL-18 levels with depression status in the OCD group (Supplementary Table S3).

DISCUSSION

To our knowledge, this is the first study exploring and providing evidence for an association between NLRP3 inflammasome pathway and OCD (17). CASP1 mRNA and pro-caspase-1 protein levels, and NEK7 mRNA levels were higher in OCD patients compared to healthy controls. Also, NEK7 mRNA and pro-caspase-1 protein levels were related to OCD status regardless of confounders.

As is true for all complex human phenotypes, measurement of inflammation-associated molecules in humans is affected by a multitude of factors.. Despite this difficulty, our observation of two different components of NLRP3 inflammasome being significantly associated with OCD in a relatively small sample is remarkable: NEK7 at mRNA level and pro-caspase-1 at protein level. While the other components of the pathway failed to show any such association, there was a common trend towards upregulation that did not reach statistical significance. Severity of disorder is an important aspect that should be considered when testing potential association of inflammation and OCD. Although there is no study investigating the impact of OCD severity on the level of inflammation, there are several studies showing positive correlations between inflammatory molecules and symptom severity in different phenotypes (18). In the current study, the mean Y-BOCS score is 25.1 for OCD group, which is thought to be moderate. Investigating these relations in a cohort with more severe cases could have led to more pronounced differences.

Although we did not observe any significant differences in NLRP3 and ASC mRNA/protein expression levels between OCD and healthy controls, it should be kept in mind that regulation of biological processes and pathways are complex. Upregulation/activation or downregulation/inhibition of a limited number of components is usually the case in several diseases and phenotypes. Indeed, in a study that compared the mRNA and protein levels of NLRP3, NEK7, caspase-1, and ASC in PBMCs, and IL1–1beta/IL18 in serum of patients with systemic lupus erythematosus

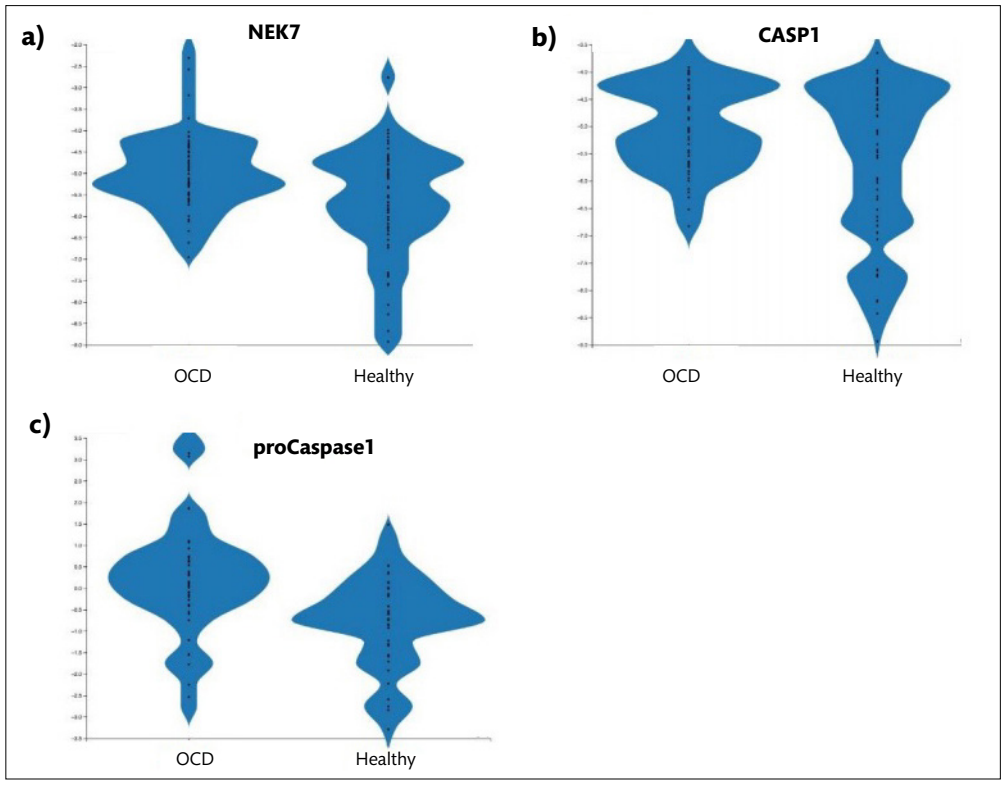

Figure 1. Comparison of NEK7 and caspase-1 levels between OCD patients and healthy controls. Violin plots showing distribution of mRNA (a, b) and protein (c) levels. Expression levels were LN-transformed and the y-axis shows relative expression compared to GAPDH mRNA (a, b) or beta-actin protein (c).

Table 1. Characteristics of the study population

Characteristics	OCD n=51	Control n=52	Inferential statistics	
			χ^2 *(df)*/*t (df)*	*p*
Age, years, mean (SD)	30.41 (8.90)	30.56 (7.89)	28.32 (27)	0.39
Sex, n (%)			0.020 (1)	0.90
Women	31 (60.80)	31 (59.60)		
Men	20 (39.20)	21 (40.40)		
Education, years, mean (SD)	12.65 (3.41)	16.31 (4.15)	29.88 (18)	0.04
Marital Status, n (%)			0.007 (1)	0.94
Married	21 (41.18)	21 (40.38)		
Single	30 (58.82)	31 (59.62)		
Employment status, n (%)			14.76 (1)	<0.001
Employed	17 (33.30)	37 (71.20)		
Unemployed	34 (66.70)	15 (28.80)		
BMI, kg/m^2, mean (SD)	25.76 (4.29)	23.36 (3.77)	94.33 (93)	0.44
Smoking status, n (%)			0.81 (1)	0.37
Smoker	20 (39.20)	16 (30.80)		
Non-smoker	31 (60.80)	36 (69.20)		
Hewitt's MPS, mean (SD)	184.50 (48.10)	167.10 (41.20)	1.98 (101)	0.05
Y-BOCS, mean (SD)	25.10 (6.50)	NA	NA	NA
HAM-D, mean (SD)	14.80 (8.30)	NA	NA	NA
NEK7 mRNA	0.11 (0.017)	0.006 (0.009)	-3.34 (90.98)	0.001
CASP1 mRNA	0.008 (0.006)	0.007 (0.006)	-2.82 (78.70)	0.006
ASC mRNA	0.002 (0.003)	0.002 (0.003)	0.31 (100)	0.760
NLRP3 mRNA	0.004 (0.008)	0.003 (0.006)	-0.76 (100)	0.450
NEK7 protein	1.33 (2.20)	0.82 (0.76)	-0.31 (64.39)	0.760
Pro-caspase-1 protein	1.43 (1.41)	0.71 (0.74)	-3.15 (76)	0.002
ASC protein	2.45 (6.03)	0.73 (0.52)	-1.49 (76)	0.140
NLRP3 protein	1.04 (1.04)	0.70 (0.54)	-1.83 (76)	0.071
IL-1β	4.60 (2.31)	4.50 (2.04)	-0.04 (101)	0.965
IL-18	40.95 (39.77)	46.16 (47.71)	0.95 (101)	0.343

ASC: apoptosis-associated speck-like protein containing a CARD; BMI: Body Mass Index; CASP1:caspase-1; HAM-D: Hamilton Depression Rating Scale; Hewitt's MPS: Hewitt's Multidimensional Perfectionism Scale score; IL-1β: interleukin 1 beta; IL-18: interleukin 18; mRNA: Messenger RNA; NA: Not applicable; NEK7: Nima-related kinase 7; NLRP3: NLR family pyrin domain containing 3; OCD: Obsessive Compulsive Disorder; SD: Standard deviation; Y-BOCS: Yale-Brown Obsession and Compulsion Scale score.

Table 2. Associations of NLRP3 inflammasome components, NEK7 with OCD status

	OR	95% CI	p
NEK7 mRNA			
Model 1	1.89	0.348–0.970	0.003
Model 2	1.63	0.387–0.970	0.036
CASP1 mRNA			
Model 1	0.61	0.418–0.884	0.009
Model 2	0.70	0.463–1.061	0.093
ASC mRNA			
Model 1	1.06	0.746–1.498	0.755
Model 2	1.16	1.162–1.739	0.467
NLRP3 mRNA			
Model 1	1.13	0.822–1.560	0.446
Model 2	1.16	0.805–1.674	0.424
NEK7 protein			
Model 1	1.07	0.707–1.614	0.754
Model 2	0.98	0.625–1.540	0.934
Pro-caspase-1 protein			
Model 1	2.10	1.252–3.533	0.005
Model 2	2.12	1.190–3.791	0.011
ASC protein			
Model 1	1.35	0.900–2.034	0.147
Model 2	1.25	0.811–1.923	0.312
NLRP3 protein			
Model 1	1.50	0.955–2.341	0.079
Model 2	1.50	0.925–2.428	0.101

ASC: apoptosis-associated speck-like protein containing a CARD; CASP1: caspase-1; CI: confidence interval; IL-1β: interleukin 1 beta; IL-18: interleukin 18; mRNA: Messenger RNA; NEK7: Nima-related kinase 7; NLRP3: NLR family pyrin domain containing 3; OCD: Obsessive Compulsive Disorder; OR: odds ratio.
Model 1 was adjusted for age, sex, duration of education.
Model 2 was adjusted for Hewitt's Multidimensional Perfectionism Scale scores in addition to variables in model 1.

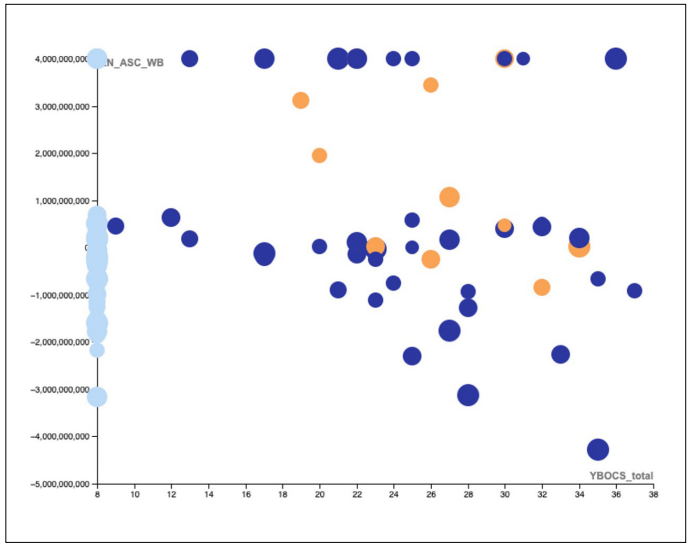

Figure 2. ASC protein levels are negatively associated with medication use and Y-BOCS score. Bubble plot shows ASC protein levels are higher in OCD patients who do not use medication (yellow bubbles) compared to OCD patients who use at least one psychiatric medicine (blue). The plot also shows that patients with lower Y-BOCS scores tend to have higher ASC protein levels. Bubble size indicates patient age. Light-blue bubbles indicate healthy controls (ASC: apoptosis-associated speck-like protein containing a CARD; OCD: Obsessive compulsive dioserder; Y-BOCS: Yale-Brown Obsession and Compulsion Scale score).

(SLE) to healthy controls, levels of NLRP3, NEK7, and ASC were found to be lower, whereas caspase-1, IL-1beta, and IL18 were higher (19).

It is possible that increased NEK7 mRNA levels in OCD patients reflect altered epigenetic states that lead to increased NEK7 transcription and/or mRNA stability. NEK7 transcription was recently shown to be regulated by NF-KB, in addition to the well-known targets of the 'priming' step, namely IL-1b and IL-18 (20). As an upstream regulator, NEK7 was shown to be critical for transmission of activation signals to NLRP3 inflammasome and it could be speculated that its levels determine the sensitivity or strength of inflammasome activation. In other words, higher NEK7 levels may make cells more sensitive to even low levels of inflammasome-activating signals. Further studies where cells isolated from OCD patients and exposed to various stimuli (e.g. LPS) would help to test this hypothesis.

A limitation of our study is the high rate of psychiatric medicine use among our OCD patients (n=40/51, 88%), which may skew the results. Accordingly, fluoxetine use was shown to suppress activation of NLRP3 activation (21). In another study, nine different antidepressants were shown to reduce IL-1β and IL-18 levels in patients with depression, both *in vivo* and *in vitro* (22). Moreover, the authors suggested use of IL-1β and IL-18, as well as NLRP3 inflammasome levels, as indicators of response to anti-depressants in depressive patients. However, no such association is currently known between antidepressant use and

the levels of NEK7. It is possible that our observation of upregulation of some components of NLRP3 inflammasome in OCD may be at least partially explained by suppression of the other components by long-term antidepressant use. Indeed, regression analysis revealed a negative relation between ASC and NLRP3 protein levels and antidepressant use among OCD patients. Intriguingly, a much weaker but significant negative association between ASC protein levels and Y-BOCS score was also observed. This observation could possibly explain normal levels of IL-1beta/IL-18 levels, as reduced ASC expression with increased disease severity would oppose the effects of increased caspase-1 and NEK7 in OCD patients. We speculate that such seemingly contradictory changes could be extended to the other inflammatory processes and could be the underlying reason for inconsistent changes in inflammatory markers in OCD patients (23). Therefore, the relation between NLRP3 inflammasome and OCD seems complex and further studies are required to develop a more mechanistic understanding.

Even though a substantial number of participants with OCD were on antidepressant medication, mRNA levels of NEK7 and protein levels of pro-caspase-1 were significantly higher in patients, and tended to be even higher in patients who do not use psychiatric medication (Supplementary Figure 1). It should be noted that caspase-1 is also part of different inflammasome complexes; however, NEK7 is thought to be a specific regulator of NLRP3 inflammasome. Thus, simultaneous increase of NEK7 and pro-caspase-1 in the OCD group points to the NLRP3 inflammasome as the culprit.

Another confounding factor may be the inclusion of patients with depression diagnosis, as NLRP3 activation has been linked to major depressive disorder (MDD) (4). However, currently there is no evidence on such an association between NEK7 and MDD. Therefore, increased NEK7 expression in OCD patients cannot simply be explained by MDD comorbidity. Similarly, we assessed possible interference of depression on increased pro-caspase-1 levels by comparing patients with HAM-D score<18 to those with HAM-D score≥18, which is close to syndromal level. Lack of any difference between the two groups suggests that increased pro-caspase-1 levels in OCD patients is independent of depression comorbidity. We also assessed possible interference of depression with NEK7 levels and observed a negative association between HAM-D severity scores and NEK7 protein levels (Supplementary Table 3). Therefore, increased NEK7 mRNA levels in OCD patients is irrelevant with the depression status. Role of inflammation is much less clear in the pathogenesis of OCD compared to other neuropsychiatric diseases (e.g. MDD) and has largely been limited to measurement of cytokines in blood or isolated macrophages. By providing evidence that suggests possible involvement of NLRP3 pathway alterations in OCD pathogenesis, our findings may lead to new avenues of research into OCD-inflammation association. At the very least, addition of inflammasome components to the list of frequently measured inflammation-associated molecules can be expected. Particularly NEK7 and caspase-1 may provide better understanding of the pathogenesis of OCD and further help researchers improve the existing treatment approaches. Another interesting finding is that ASC protein levels could be used to determine severity of OCD patients, as its correlated with Y-BOCS score non-aligned with medication use. Studies on larger samples will test our findings and will ideally include drug-naive and depression-free patients. Mechanistic studies will also help establish the causal relationship between NLRP3 inflammasome and OCD.

Acknowledgements: *The authors would like to thank Prof. Dr. Sermin Genc (Dokuz Eylül University) for her valuable suggestions regarding study design and reagents that she provided. The authors would like to thank participants for their contribution.*

Ethics Committee Approval: All protocols and methods were approved by the Dokuz Eylül University Clinical Studies Ethical Committee (Date: 11.05.2017, Decision no: 2017/08–06, Protocol number 378-SBKAEK).

Informed Consent: Oral and written informed consents were obtained from all participants.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept- MT, TA, YO, ND; Design- MT, TA, YO, ND; Supervision- MT, TA, YO, ND, TY; Resource- TA, YO, MT; Materials- MT, TA, YO, BÖU, CA, CE, TY, AK, BE; Data Collection and/or Processing- MT, YO, BÖU, CA, CE, TY, AK, BE; Analysis and/or Interpretation- MT, YO, TA, ND, TY, BE, AK, FÖ; Literature Search- MT, TA, YO, ND, FÖ; Writing- MT, YO, TA, ND, FÖ, TY, BE, AK, BÖU, CA, CE; Critical Reviews- MT, TA, YO, ND, FÖ.

Conflict of interest: The authors declared that there is no conflict of interest.

Financial Disclosure: The project was supported by TUBITAK (Project No: 217S128, TA, YO).

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SUPPLEMENTARY TABLES and FIGURES

Table S1. Linear regression analysis of NLRP3 complex mRNA levels using possible predictor variables in OCD group

	NEK7 mRNA			Caspase-1 mRNA			ASC mRNA			NLRP3 mRNA			IL-1β			IL-18		
	Beta	SE	p	Beta	SE	p	Beta	SE	p	Beta	SE	p	Beta	SE	p	Beta	SE	p
Age	-0.009	0.019	0.651	0.019	0.016	0.265	-0.014	0.022	0.508	0.014	0.028	0.610	0.001	0.010	0.922	0.008	0.011	0.452
Gender	-0.403	0.297	0.182	0.100	0.256	0.697	-0.633	0.338	0.068	-0.211	0.432	0.627	-0.057	0.152	0.707	-0.135	0.166	0.420
Level of education	-0.065	0.044	0.150	0.002	0.038	0.964	-0.061	0.050	0.232	-0.041	0.064	0.529	0.004	0.022	0.872	-0.045	0.024	0.071
Y-BOCS score	0.006	0.022	0.805	-0.006	0.019	0.749	-0.008	0.025	0.744	0.008	0.032	0.804	0.016	0.012	0.176	-0.007	0.013	0.606
Duration of illness	-0.029	0.028	0.299	-0.013	0.024	0.601	-0.025	0.032	0.428	-0.048	0.040	0.244	-0.008	0.014	0.576	-0.017	0.016	0.275
Medication use	-0.336	0.330	0.315	-0.474	0.285	0.104	0.326	0.375	0.389	0.541	0.479	0.265	0.040	0.164	0.808	0.001	0.179	0.994
Hewitt's MPS score	-0.003	0.003	0.344	-0.002	0.003	0.497	0.000	0.004	0.933	0.005	0.005	0.287	-6.995E-5	0.002	0.967	1.222E-6	0.002	0.999
HAM-D score	0.023	0.021	0.280	0.021	0.018	0.262	0.027	0.024	0.274	-0.005	0.031	0.864	-0.003	0.011	0.807	0.002	0.011	0.834

ASC: apoptosis-associated speck-like protein containing a CARD; CASP1: caspase-1; HAM-D: Hamilton Depression Rating Scale; Hewitt's MPS: Hewitt's Multidimensional Perfectionism Scale score; IL-1β: interleukin 1 beta; IL-18: interleukin 18; mRNA: Messenger RNA; NEK7: Nima-related kinase 7; NLRP3: NLR family pyrin domain containing 3; OCD: Obsessive Compulsive Disorder; SE: Standard error; Y-BOCS: Yale-Brown Obsession and Compulsion Scale score;

Table S2. Linear regression analysis of NLRP3 complex inflammasome protein levels using possible predictor variables in OCD group

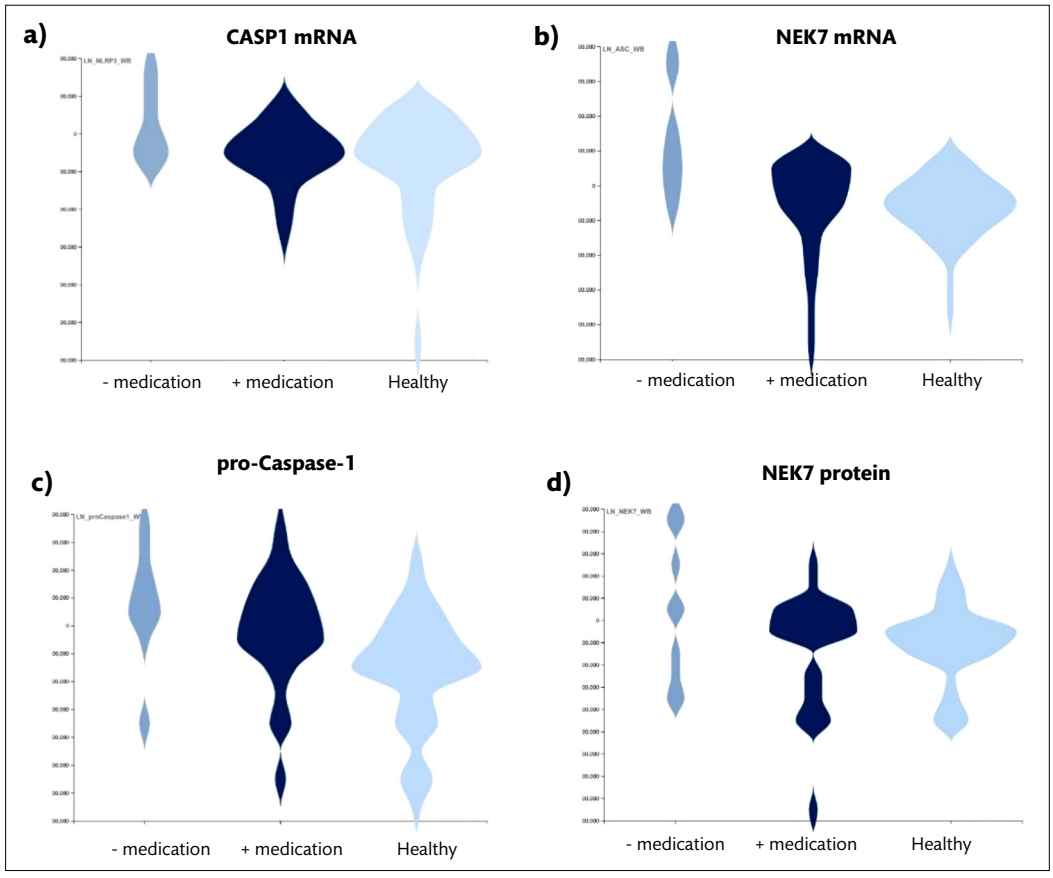
	NEK7 protein			Pro-caspase-1 protein			ASC protein			NLRP3 protein		
	Beta	SE	p	Beta	SE	p	Beta	SE	p	Beta	SE	p
Age	-0.065	0.032	0.051	-0.025	0.027	0.361	-0.073	0.028	0.015	-0.001	0.026	0.982
Gender	0.046	0.473	0.924	0.302	0.400	0.456	-0.222	0.419	0.599	0.078	0.379	0.839
Level of education	-0.015	0.067	0.819	0.022	0.057	0.698	-0.013	0.059	0.829	-0.006	0.054	0.910
Y-BOCS score	-0.036	0.033	0.278	0.013	0.028	0.650	-0.082	0.029	0.008	0.025	0.026	0.340
Duration of illness	0.093	0.048	0.059	0.040	0.040	0.329	0.058	0.042	0.182	0.019	0.038	0.617
Medication use	-0.859	0.474	0.080	-0.464	0.402	0.257	-1.731	0.420	0.000	-0.848	0.380	0.033
Hewitt's MPS score	0.006	0.005	0.227	-0.002	0.004	0.568	0.008	0.004	0.074	0.002	0.004	0.622
HAM-D score	-0.031	0.031	0.327	0.010	0.026	0.701	-0.004	0.027	0.884	-0.010	0.025	0.696

ASC: apoptosis-associated speck-like protein containing a CARD; HAM-D: Hamilton Depression Rating Scale; Hewitt's MPS: Hewitt's Multidimensional Perfectionism Scale score; IL-1β: interleukin 1 beta; IL-18: interleukin 18; NEK7: Nima-related kinase 7; NLRP3: NLR family pyrin domain containing 3; OCD: Obsessive Compulsive Disorder; SE: Standard error; Y-BOCS: Yale-Brown Obsession and Compulsion Scale score.

Table S3. Comparison of molecular markers between OCD patient with HAM-D <18 and HAM-D ≥18

Characteristics	HAM-D <18 Mean (SD)	HAM-D ≥18 Mean (SD)	p
NEK7 mRNA	0.010 (0.015)	0.012 (0.021)	0.201
CASP1 mRNA	0.007 (0.005)	0.010 (0.006)	0.171
ASC mRNA	0.002 (0.002)	0.002 (0.002)	0.156
NLRP3 mRNA	0.004 (0.008)	0.004 (0.008)	0.803
NEK7 protein	6.16 (19.46)	0.73 (0.760)	0.034
Pro-caspase-1 protein	3.13 (5.83)	1.39 (1.46)	0.422
ASC protein	6.78 (16.82)	0.99 (0.69)	0.483
NLRP3 protein	6.70 (15.71)	0.84 (0.52)	0.365
IL-1β	4.69 (2.40)	4.07 (1.69)	0.977
IL-18	39.98 (40.10)	38.96 (38.04)	0.650

ASC: apoptosis-associated speck-like protein containing a CARD; CASP1: caspase-1; HAM-D: Hamilton Depression Rating Scale; IL-1β: interleukin 1beta; IL-18: interleukin 18; mRNA: Messenger RNA; NEK7: Nima-related kinase 7; NLRP3: NLR family pyrin domain containing 3; OCD: Obsessive Compulsive Disorder; SE: Standard error



Supplementary Figure 1. Obsessive compulsive disorder patients who do not use medication (-medication) tend to have higher levels of NEK7 and caspase-1, both as mRNA and protein.