ORIGINAL ARTICLE

The natural cytotoxic receptors (NKp30, NKp44 and NKp46) gene expression in Coeliac disease patients

Maryam Khodapanah, Nastaran Asri, Mohammad Rostami-Nejad, Mehrdad Hashemi, and Hamid Asadzadeh Aghdaei

ABSTRACT

Objectives: Defects in the expression of natural killer (NK) cells activating receptors (like NKp30, NKp44, and NKp46) impair the ability of NK cells to kill virus-infected cells and are associated with amplified local inflammation. This study aimed to evaluate the expression of these receptors in coeliac disease (CD) patients relative to controls.

Methods: We analysed the NKp30, NKp46 and NKp44 genes expression in intestinal biopsy specimens of 40 confirmed coeliac disease patients and 40 controls using the real-time PCR method.

Results: The NKp30 mRNA expression in coeliac disease patients was significantly lower than in controls (p=0.01) with a significant inverse correlation with the patient's weight loss symptom (p=0.0173). The NKp44 mRNA expression was extremely higher in coeliac disease patients than in controls (p=0.02). The mRNA expression of NKp46 did not differ between patients and controls (p=0.3). The expression levels of these receptors did not show any significant difference between Marsh II and Marsh III groups of patients and controls (p>0.05).

Conclusion: Further studies are needed to elucidate the predominant NKp30 isoform and possible SNP role in controlling its expression in coeliac disease which leads to designing innovative targeted therapy for coeliac disease patients.

Keywords: *Autoimmune Diseases, Biopsy, Coeliac disease (CD), Gene Expression*

NZ J Med Lab Sci 2023; 77(2): 66-70

INTRODUCTION

Coeliac disease (CD) is a chronic gastrointestinal autoimmune disorder that occurs in genetically predisposed individuals due to abnormal immune responses to gluten proteins (1, 2). The disease can present with gastrointestinal symptoms such as diarrhoea and bloating and extra-intestinal manifestations like weight loss, osteoporosis, fatigue, etc. (3-6). Marsh was the first who classified histopathological alterations of CD according to the Marsh grading (I to III) system (7). It has been reported that viral infections such as infections with adenovirus, enterovirus, hepatitis C virus, and etc. can be associated with an increased incidence of CD (8-10). Actually, mucosal inflammation in CD subjects is associated with high production of interferon (IFN)-α, a cytokine over-produced by virus-infected cells. Natural killer (NK) cells are innate immune system cells, which fight a wide variety of pathological challenges and infections. These cells have the ability to eliminate virally infected cells by cytocidal action or induction of apoptosis (11, 12). NK cells have two types of activating and inhibitory receptors. Natural cytotoxic receptors (NCRs), including NKp46, NKp44 and NKp30 are important NK cell-activating receptors that after engagement with their ligands deliver potent signals to NK cells. These signals lead to the lysis of harmful tumour-transformed or infected cells and regulation of the homoeostasis of immune responses (13-15). Defects in the expression of these activating receptors can cause local inflammation (12). Moreover, NK cells activation and increased cytotoxicity occur as a response to inflammatory conditions in CD patients' intestinal mucosa leading to epithelial cell death and villous atrophy (16-18).

The aim of this study was to evaluate the natural cytotoxic receptors (NKp30, NKp44 and NKp46) gene expression in coeliac disease patients compared to healthy subjects and evaluate their correlations with patient's reported clinical manifestations, which may lead to the design of further studies with the aim of finding novel therapeutic targets for this disorder.

MATERIALS AND METHODS

Sample Collection

Forty confirmed CD patients with a mean age of 41.78±12.8 who were referred to the coeliac disease department at the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences enrolled in this study Their disease was confirmed by serum IgA transglutaminase antibody (TGA) and gastroduodenal biopsy evaluation. Moreover, 40 normal subjects with a mean age of 36.55±10.07 with no symptoms or history of CD and other autoimmune diseases up to their first relative's degree, who attended the endoscopy unit because of digestive problems like dyspepsia or reflux but had normal upper endoscopy, served as controls. Subjects who had other autoimmune diseases, such as inflammatory bowel disease and type 1 diabetes, blood diseases, gastrointestinal infections, rheumatoid arthritis, and pregnant females were excluded from the study.

This study was approved by the ethical committee of the Research Institute for Gastroenterology and Liver Diseases (RIGLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran (code: IR.SBMU.RIGLD.REC.1399.035, 18 July 2020) and written informed consent was obtained from patients.

RNA extraction and complementary DNA synthesis

Total RNA was extracted from fresh small intestinal biopsy samples using YTA RNA Extraction Kit (YEKTA TAJHIZ AZMA, Taiwan) according to the provided protocol. Isolated RNA quantity measurement was made on a NanoDrop photospectrometer and RNA with a 260 nm/280 nm ratio between 1.8 and 2 was used for further analysis. Complementary DNA (cDNA) synthesis was performed using the cDNA Synthesis Kit Biofact (2 step 2x RT- PCR Pre- mix Taq, Korea) according to the manufacturer's instructions.

Primer designing and quantitative real time PCR (RT-qPCR)

Specific primer sequences were designed by Gene runner software (V. 3.05). The gene-specific primer sequences are shown in Table 1. The specificity of the primers was checked by performing PCR experiments followed by electrophoresis on a 1.5% agarose gel. The qPCR was carried out using Ampliqon RealQ Plus 2x Master Mix Green on the thermocyclers Rotor Gene Q MDx (Qiagen, France). The relative expression level was calculated by the 2-ΔΔct method, in which the housekeeping gene β2M was used for normalisation. The cycling programme was 15 minutes at 95°C, 40 cycles of 20 seconds at 95°C and 60 seconds at 60°C.

Statistical analysis

Statistical analysis was performed using SPSS (V.21) and GraphPad, Prism (V.6). Comparisons between the groups were examined by t-TEST and ANOVA. The correlation between variables was assessed using the Spearman correlation test. The differences with P value < 0.05 were considered statistically significant.

RESULTS

Demographic and clinical characteristics

Forty confirmed CD patients including 24 (60%) females and 16 (40%) males were enrolled in this study: 3 with a Marsh I value, 16 with a Marsh II, and 21 with a Marsh III value. Moreover, 40 healthy subjects including 23 (57.5%) females and 17 (42.5%) males were considered as a control group. The mean age of cases and controls was 41.78±12.8 and 36.55±10.07, respectively. There was no significant difference between the age and sex of patients and controls (p>0.05).

Common gastrointestinal presentations of patients were bloating (55%) and diarrhoea (55%), and the predominant extra-intestinal symptoms were fatigue (67.5%) and anaemia (47.5%). (Figure 1.)

mRNA expression of NKp30, NKp44 and NKp46

We analysed the mRNA expression of NKp30, NKp44 and NKp46 in intestinal biopsy specimens of CD patients compared to healthy control subjects using β2M as a housekeeping gene. A significant decrease in NKp30 mRNA level was shown in the CD patients' intestinal tissue specimens (0.895 ± 0.74) compared with controls (1.506 ± 1.43), (p=0.0173), (Figure 2a). On the other hand, a significantly increased mRNA level of NKp44 was observed in the biopsy specimen of the patient group (1.908 ± 1.9) compared to the controls (1.242 ± 1.4), (p=0.02), (Figure 2b.). However, the mRNA expression of NKp46 did not show a significant difference between patients (1.016 ± 0.54) and controls (0.87 ± 0.65), (p=0.3), (Figure 2c.)

mRNA expression of NKp30, NKp44 and NKp46 in different Marsh classes biopsy specimens

Our findings showed that NKp30, NKp44 and NKp46 mRNA expression were not significantly different between Marsh II and Marsh III CD patients and control subjects (patients in Marsh I group were not analysed due to their small number) (p>0.05), (Figure 3.)

Correlations

Using Spearman correlation analysis, we found that there is a significant inverse correlation between NKp30 gene expression and weight loss (r = -0.403, p = 0.01). Also, the bivariate Spearman correlation showed no statistical correlation between NKp44 and NKp46 gene expression and symptoms in CD patients (Table 2).

Table 1. Primers used in qPCR

Primer name	Primer sequence	Length
NKp46 (Forward)	GGATCTGAAAGCTGGTGTTGAG	22
NKp46 (Reverse)	TAGTTCTCCCACCCTCTGCAT	21
NKp44 (Forward)	AGGAGGCTTCAGCACTTGTG	20
NKp44 (Reverse)	GAGAGGTCCAAGCCATCGTC	20
NKp30 (Forward)	CAAGTGATGTGTGAGTCCCGT	21
NKp30 (Reverse)	AAGATGTCCCAGTTGGCGAA	20
β2M (Forward)	TGCTGTCTCCATGTTTGATGTATCT	21
β2M (Reverse)	TCTCTGCTCCCCACCTCTAAGT	21

Table 2. The correlation between clinical symptoms and NKp30, NKp44 and NKp46 gene expression in coeliac disease group.

Symptoms	NKp44 mRNA expression		NKp46 mRNA expression		NKp30 mRNA expression	
	P-value	Correlation coefficient	P-value	Correlation coefficient	P-value	Correlation coefficient
Diarrhoea	0.208	-0.209	0.480	-0.117	0.947	-0.011
Nausea and vomiting	0.941	-0.02	0.813	0.040	0.548	0.101
Bloating	0.419	0.135	0.688	-0.066	0.341	-0.155
Anaemia	0.800	0.043	0.056	0.312	0.249	-0.189
Weight loss	0.181	-0.222	0.083	-0.281	0.010*	-0.403
Aphthous	0.703	0.066	0.349	0.156	0.317	0.167
Infertility	0.539	0.106	0.160	0.232	0.280	0.180
Abortion	0.163	0.238	0.336	0.160	0.798	-0.043
Fatigue	0.422	-0.134	0.793	0.044	0.799	-0.042
Bone disease	0.071	0.300	0.060	0.351	0.523	-0.105

*significant correlation

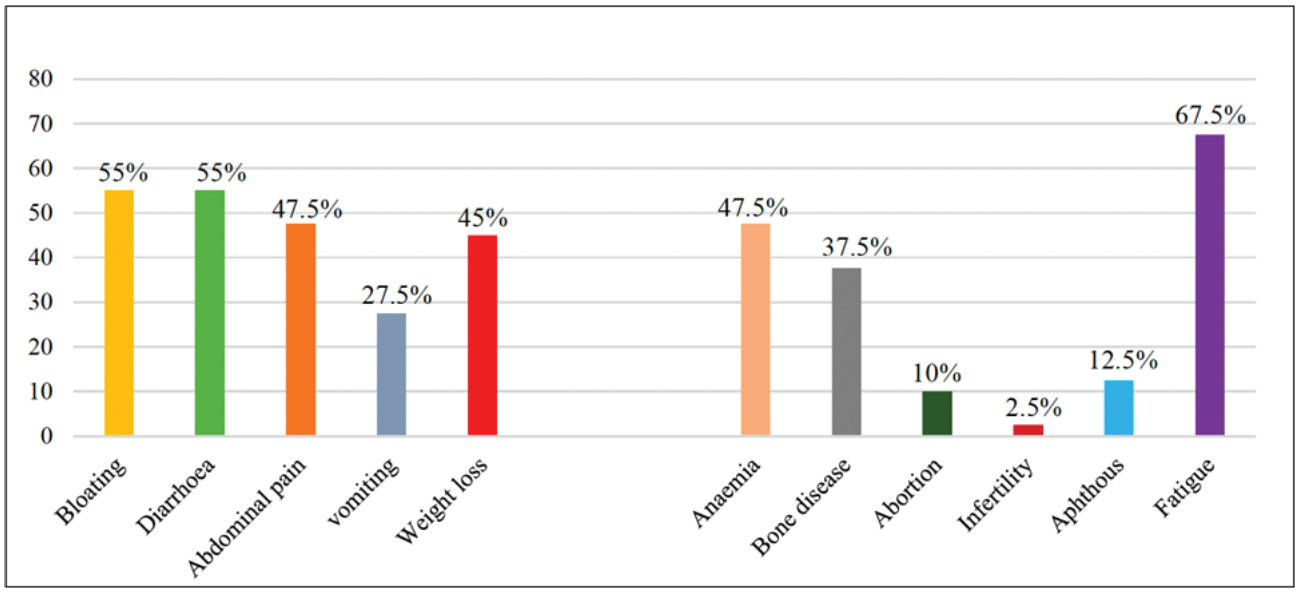

Figure 1. (a) Gastrointestinal and (b) extra-intestinal symptoms of patients.

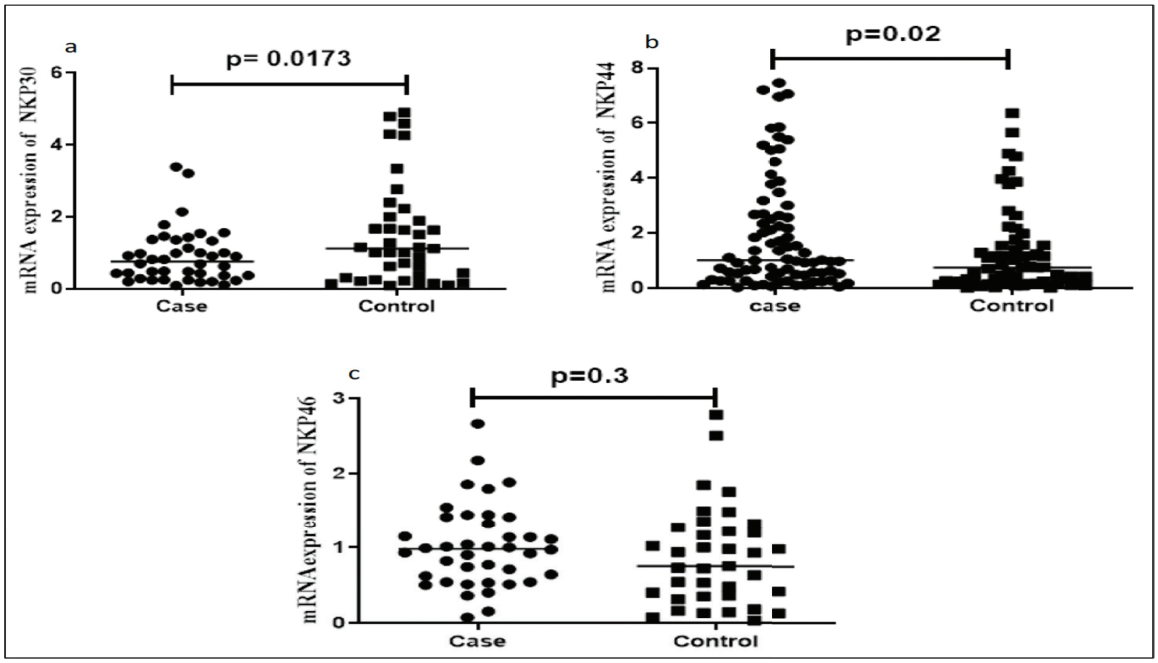

Figure 2. Comparison of NKp30, NKp44 and NKp46 gene expression in intestinal biopsy samples of CD patients and healthy controls.

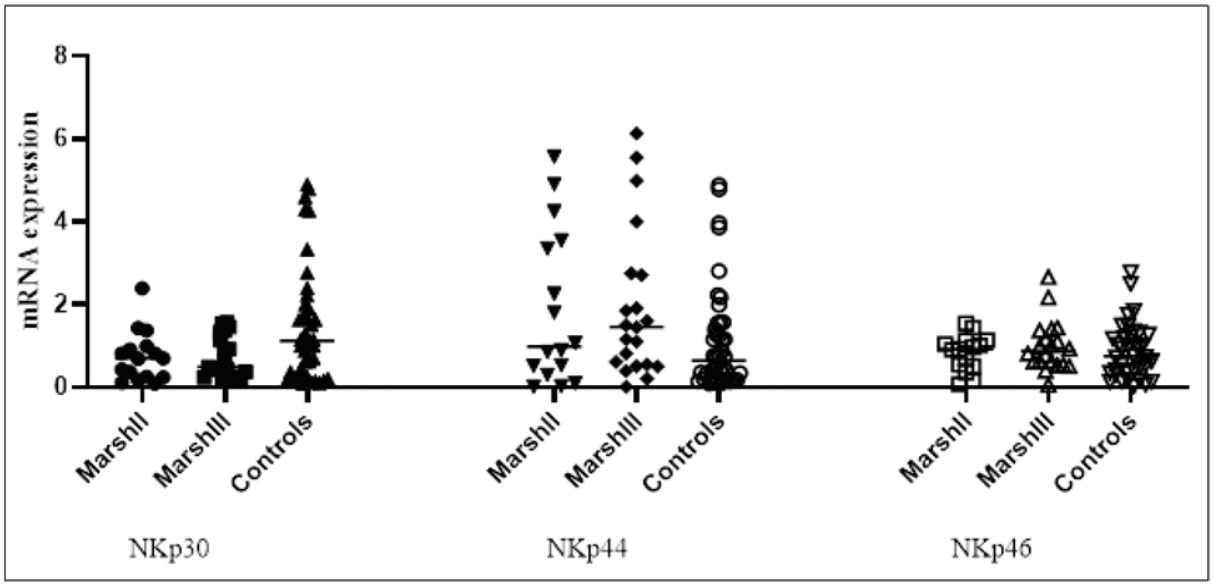

Figure 3. Comparison of NKp30, NKp44 and NKp46 gene expression in intestinal biopsy samples of CD patients with different Marsh classes and healthy controls.

DISCUSSION

Natural killer cells are among the important components of innate immunity and act as the first line of defence against pathological challenges and infections. These cells are also effective in controlling inflammatory and autoimmune disorders (16). It has been reported in various studies that, viral infections are among the environmental predisposing factors for coeliac disease and one of their immune system escaping strategies is the regulation of NK cells activating receptors including NCRs like NKp44, NKp46 and NKp30 (19). Moreover, NK cells activation and increased cytotoxicity may lead to epithelial cell death and villous atrophy in CD patients' intestinal mucosa (16-18).

In this report, we showed that NKp30 mRNA expression in CD patients' intestinal tissue specimens was significantly lower than in controls (p=0.0173) and it showed a significant inverse correlation with patients' weight loss symptom (p=0.01). Moreover, mean NKp44 mRNA levels were higher in our CD patients' group in comparison to controls (p=0.02). The mRNA expression of NKp46 did not differ between patients and controls (p=0.3). Receptor mRNA expression was not significantly different between Marsh II and Marsh III CD patients and controls (p>0.05).

Our study expands on such data reported by Marafini et al. study showing that the expression of NKp44 and NKp46 did not significantly differ between total intraepithelial cells extracted from CD patients and controls. They also reported that in active CD subjects, the percentage of NKp30+ NK cells was significantly higher than inactive CD patients and controls, while NKp30-expressing NKT cells fraction did not differ between coeliac patients and controls (12). Meresse and co-workers also observed up-regulation of NKp44, but not NKp46, in coeliac disease patients' NKG2C+ cells; however, its RNA expression level was apparently similar to the NKG2C− cells (20). Uhde et al. reported the lower NKp44 expression by intraepithelial innate lymphoid cells of active and refractory type 1 CD patients, which was restored in GFD-treated patients who showed evidence of mucosal healing (21). This apparent discrepancy showed that the expression of these markers can be different in various cell populations and specimens. The reason and the precise mechanisms underlying these changes have not been explored completely. It can be said that, since NKp44 is only expressed by NK cells that are activated (15), and as NK cells activation occurs as a response to an inflammatory condition in CD patient's intestinal mucosa leading to epithelial cell death and villous atrophy, increased in NKp44 mRNA expression in our CD patients intestinal mucosa may happen with the aim of intestinal destruction.

It has been reported that NKp30 expression, which is specifically expressed on NK cells, plays an important role in the prognosis of infectious diseases. In fact, NKp30 has three isoforms with contrasting effects including immunostimulatory isoforms (NKp30a, NKp30b) and one immunosuppressive isoform (NKp30c) and could mediate both dendritic cells (DC) killing and maturation (22). These limited and contradictory data about its expression in different specimens of coeliac disease patients can help in designing an innovative study examining what kind of NKp30 isoforms has predominant expression in CD subjects and if a single-nucleotide polymorphism (SNP) at the gene encoding NKp30 has a strong role in controlling this phenomenon. Finding the NKp30 expression profile may guide in designing innovative targeted therapy for CD patients. Most importantly, the phenotype of NK cells has been proven to be affected by hormones and cytokines secreted by adipose tissue. In our study, NKp30 expression showed a significant inverse correlation with weight loss, which is one of the main symptoms of CD patients (23). Researchers in previous studies reported its decreased expression in normal obese subjects and linked it to these people's higher susceptibility to infections and increased cancer risk. In fact, researchers believe that NK cell functionality is disturbed in obesity and altered NK cell activity could be reactivated following weight loss (24, 25). Therefore, it can be assumed that weight loss in CD patients may happen with the goal of NK cells reactivation and it doubles the importance of studying NKp30 in patients with coeliac disease.

Enterovirus (EV) is among the most important viruses involved in the pathogenesis of coeliac disease and has a role in the activation/regulation of NKp44 (26-28), thus preventing or treating EV infection in CD patients is of great importance.

One of our study limitations was that NK cells were not isolated from biopsy samples and further studies on isolated NK cells are needed to confirm/ reject the result of our study.

CONCLUSION

In summary, we identified altered expression profiles of natural cytotoxicity receptors NKp30 and NKp44 in CD patients, which in some cases were consistent with previous studies and in some cases were not. It remains to be determined whether the increase in NKp44 in CD patients' intestinal mucosa is stimulated with the aim of intestinal destruction and further studies are awaited to elucidate the predominant NKp30 isoform and possible SNP role in controlling its expression that leads to designing innovative targeted therapy for CD patients.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Gastroenterology and Liver Diseases Research Center of Shahid Beheshti University of Medical Sciences for their support and contribution to this study.

AUTHOR INFORMATION

Maryam Khodapanah, MSc, Researcher[1]
Nastaran Asri, PhD, Researcher[2]
Mohammad Rostami-Nejad, PhD, Associate Professor of Clinical Immunology[2]
Mehrdad Hashemi, PhD, Associate Professor of Molecular Genetics[1,3]
Hamid Asadzadeh Aghdaei, MD, PhD, Associate Professor[4]

[1]Department of Genetics, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
[2] Celiac Disease and Gluten Related Disorders Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Farhikhtegan Medical Convergence Science Research Center, Farhikhtegan Hospital, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
[4]Basic and Molecular Epidemiology of Gastrointestinal Disorders Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Corresponding Author: Dr. Mohammad Rostami-Nejad
Email: m.rostamii@gmail.com

REFERENCES

1. Taraghikhah N, Ashtari S, Asri N, et al. An updated overview of spectrum of gluten-related disorders: clinical and diagnostic aspects. *BMC Gastroenterol* 2020; 20(1)258: 1-12.
2. Asri N, Rostami-Nejad M, Anderson RP, Rostami K. The Gluten Gene: Unlocking the Understanding of Gluten Sensitivity and Intolerance. *Appl Clin Genet* 2021; 14: 37-50.
3. Sharma N, Bhatia S, Chunduri V, et al. Pathogenesis of celiac disease and other gluten related disorders in wheat and strategies for mitigating them. *Front Nutr* 2020; 7:6
4. Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol* 2002; 2(9): 647-55.

5. Rostami-Nejad M, Rostami K, Emami MH, et al. Epidemiology of celiac disease in Iran: a review. *Middle East J Dig Dis* 2011; 3(1): 5-12.
6. Rostami-Nejad M, Razzaghi Z, Esmaeili S, et al. Immunological reactions by T cell and regulation of crucial genes in treated celiac disease patients. *Gastroenterol Hepatol Bed Bench* 2020; 13(2): 155-160.
7. Kneepkens F, von Blomberg B. Clinical practice Coeliac disease. Eur J Pediatr 2012; 171(7): 1011-1021.
8. Plot L, Amital H. Infectious associations of Celiac disease. *Autoimmun Rev* 2009; 8(4): 316-319.
9. Stene LC, Honeyman MC, Hoffenberg EJ, et al. Rotavirus infection frequency and risk of celiac disease autoimmunity in early childhood: a longitudinal study. *Am J Gastroenterol* 2006; 101(10): 2333–2340.
10. Brown JJ, Jabri B, Dermody TS. A viral trigger for celiac disease. *PLoS Pathog* 2018; 14(9): e1007181.
11. Schmidt KN, Leung B, Kwong M, et al. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J Immunol* 2004; 172(1): 138-43.
12. Marafini I, Monteleone I, Di Fusco D, et al. Celiac disease-related inflammation is marked by reduction of Nkp44/Nkp46-double positive natural killer cells. *PloS One* 2016; 11(5): e0155103.
13. Hudspeth K, Silva-Santos B, Mavilio D. Natural cytotoxicity receptors: broader expression patterns and functions in innate and adaptive immune cells. *Front Immunol* 2013; 4: 69.
14. Ma Y, Li X, Kuang E. Viral evasion of natural killer cell activation. **Viruses** 2016; 8(4): 95.
15. Barrow AD, Martin CJ, Colonna M. The Natural Cytotoxicity Receptors in Health and Disease. *Front Immunol* 2019; 10: 909
16. Aghamohamadi E, Kokhaei P, Rostami-Nejad M, et al. Serum Level and Gene Expression of Interleukin-15 Do Not Correlate with Villous Atrophy in Celiac Disease Patients. *Genet Test Mol Biomarkers* 2020; 24(8): 502-507.
17. Abadie V, Jabri B. IL-15: a central regulator of celiac disease immunopathology. *Immunol Rev* 2014; 260(1): 221-234.
18. Mention JJ, Ben Ahmed M, Bègue B, et al. Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. *Gastroenterology* 2003; 125(3): 730-745.
19. Marafini I, Imeneo M, Monteleone G. The Role of Natural Killer Receptors in Celiac Disease. *Immunome Res* 2017; 13(1).
20. Meresse B, Curran SA, Ciszewski C, et al. Reprogramming of CTLs into natural killer-like cells in celiac disease. *J Exp Med* 2006; 203(5): 1343-1355.
21. Uhde M, Yu X, Bunin A, et al. Phenotypic shift of small intestinal intraepithelial type 1 innate lymphoid cells in celiac disease is associated with enhanced cytotoxic potential. *Clin Exp Immunol* 2020; 200(2): 163-175.
22. Delahaye NF, Rusakiewicz S, Martins I, et al. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nat Med* 2011; 17(6): 700-707.
23. Caio G, Volta U, Sapone A, et al. Celiac disease: a comprehensive current review. *BMC Med* 2019; 17(1): 1-20.
24. Bähr I, Goritz V, Doberstein H, et al. Diet-Induced Obesity is associated with an impaired NK cell function and an increased colon cancer incidence. *J Nutr Metab* 2017; 2017: e4297025.
25. Bähr I, Spielmann J, Quandt D, Kielstein H. Obesity-associated alterations of natural killer cells and immunosurveillance of cancer. *Front Immunol* 2020; 11: (245)
26. Sharquie IK, Abdullah S, Bayati A. The role of human cytomegalovirus (HCMV) in patients with active coeliac disease (CD). *East J Med* 2021; 26(2): 204-209.
27. Oikarinen M, Puustinen L, Lehtonen J, et al. Enterovirus infections are associated with the development of celiac disease in a birth cohort study. *Front Immunol* 2021; 11: e604529.
28. Pazina T, Shemesh A, Brusilovsky M, et al. Regulation of the functions of natural cytotoxicity receptors by interactions with diverse ligands and alterations in splice variant expression. *Front Immunol* 2017; 8: 00369.

Copyright: © 2023 The author(s). This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.