Research article

IL-38 gene expression: A new player in Graves' ophthalmopathy patients in Iraq

Nada Kadum Jawad[1], Ahmad Tarik Numan[2], Ahmed Ghaith Ahmed[3], Tahreer Hadi Saleh[4], Bahaa Abdullah Laftaah Al-Rubaii[5]

[1, 2] Department Pharmacy, Al-Mustafa University College, Baghdad, Iraq
[3] Department of Prosthodontics, College of Dentistry, Al-Farahidi University, Baghdad, Iraq
[4]Biology Department, College of Science, Mustansiriyah University, Baghdad, Iraq
[5]Biology Department, College of Science, University of Baghdad, Baghdad, Iraq

(Received: September 2022 Revised: January 2023 Accepted: January 2023)

Corresponding author: **Tahreer Hadi Saleh.** Email: dr.tahreer80@uomustansiriyah.edu.iq

ABSTRACT

Introduction and Aim: Thyroid eye illness is widely recognized as one of the most significant concerns confronting the medical profession today. The thyroid-related ophthalmopathy also known as Graves' ophthalmopathy is an autoimmune disorder that cannot be reversed and hence, research pertaining to the identification of novel markers that can explain both the prognosis and the recovery of the condition is needed. In this study, we aimed to investigate the *IL-38* gene expression levels among Graves' ophthalmopathy patients in Iraq.

Methodology: The TSH levels were measured with an enzyme-linked immunosorbent assay (ELISA). The level of *IL-38* gene expression in patient blood samples and normal healthy controls was measured qualitatively using RT-PCR.

Results: Patients who were diagnosed with Graves' ophthalmopathy were observed to have abnormally low levels of thyrotropin (TSH) in their serum. In these patients, the interleukin *IL-38* gene expression was observed to be significantly greater compared to healthy controls ($P<0.01$), the tendency of which continued even after one year of therapy with anti-thyroid drugs.

Conclusion: The findings of this study indicate that the IL-38 transcript is important in the autoimmune response. The identification of IL-38 expression levels could contribute in the early clinical diagnosis and treatment of thyroid eye disease

Keywords: Interleukin 38; ophthalmopathy disease; Graves' ophthalmopathy; cytokine gene expression.

INTRODUCTION

Graves' ophthalmopathy also known as Graves' orbitopathy, is an autoimmune disorder frequently associated with a hyperactive thyroid (1). Increased thyroid-stimulating antibody activity and cytokine release by cytotoxic T cells produce Graves' ophthalmopathy characterized by symptoms such as inflammation, fat accumulation, orbital connective tissue lengthening, and stronger eyelid muscles. The antibodies and cytokines in question also stimulate a variety of cells beyond just the thyroid, including preadipocytes and periorbital fibroblasts. As a result, fat deposits and an abundance of hydrophilic glycosaminoglycans which can bind water leading to proptosis, diplopia, periorbital edema, and congestion of the eyes. Untreated, it can cause irreparable muscular fibrosis and eye damage (2).

Over the past decade, thyroid test sensitivity and specificity have greatly altered thyroid eye problem treatment. Modern technologies use molecular assays to improve specificity and sensitivity (3). Interleukin 38 (IL-38), a member of the interleukin-1 family can downregulate the levels of numerous pro-inflammatory cytokines; for instance, IL-38 can inhibit the release of IL-17 by T helper cells (4). Patients with thyroid eye illness may experience a unique self-limiting inflammatory disease process, although the underlying mechanism is not well understood at this time (5). So, it is important to investigate both the mechanism and role played by IL-38 in ophthalmopathy patients. The *IL-38* gene is located between two antagonistic genes: the *IL-1Ra* gene (IL-1RN) and the *IL-36Ra* gene (IL-36RN) on chromosome 2q13-14 (6). The progression and result of inflammation depend on the equilibrium of anti- and pro-inflammatory substances. IL-38 is a cytokine with a broad anti-inflammatory effect. The anti-inflammatory effects of IL-38 in macrophages and fibroblasts have previously been demonstrated (7), and shown to reduce inflammation in arthritis. It was shown that patients with rheumatoid arthritis had an increased level of IL-38 gene expression, and it was also shown that IL-38 gene expression is a

negative regulator of inflammatory arthritis (7). Ophthalmopathy patients have not had their IL-38 function clearly defined till recently. As a result in this study we aimed to look into the expression levels of IL-38 in Graves' ophthalmopathy patients, which could probably be used as a biomarker to aid physicians in the early clinical diagnosis and treatment of thyroid eye disease.

MATERIALS AND METHODS

This study compared 30 Graves' disease patients to 20 healthy people. Female patients undergoing treatment at the Baghdad Governorate Private Hospital between 22, January and 22, March 2022 were included in the study. All patients and healthy controls were aged between 20 to 71 years. Prior to treatment, patients with ophthalmopathy underwent a battery of diagnostic procedures to determine the etiology of the disease, the size of the thyroid gland, and a clinical evaluation of nuclear thyroid by ultrasound.

Determination of serum thyroid stimulating hormone (TSH)

The serum TSH level was determined using an ELISA kit using the protocol describer earlier (8). The micro titer plate wells were designed to allow for the estimation of serum reference, control, and patient samples in duplicate. Control, standard, and specimen aliquots of 50 µl were pipetted into their corresponding wells, followed by additions of 50 µl of TSH-Antibody-Biotin solution and 50 µl of the active TSH-enzyme conjugate solution to each well. After gently mixing the contents for 25-30 seconds, the plate was covered and incubated at 25ºC for one hour at ambient temperature. The contents of each well was discarded and washed three times with 250- 300µl of washing buffer (1X), followed by addition of TMB substrate (100 µl) onto each well. After 15 mins, 50 µl of stop solution was added to each well, mixed carefully and the absorbance read at 450 nm using an ELISA reader.

Extraction and purification of RNA

RNA was extracted from each sample using the TRIzol™ Reagent kit (Promega, USA), as per the manufacturer's instructions. Briefly, 300µl of blood was added to 700 µl of Trizol™ Reagent in Eppendorf tubes, and the mixture lysed. For three phase's separation, 200µl of chloroform was added to the lysate in each tube, incubated for 2-3 minutes, followed by centrifugation at 12,000 rpm for 10 minutes. The RNA-containing aqueous phase was subsequently transferred to fresh Eppendorf tubes. For RNA precipitation, 500 µl of isopropanol was added to the aqueous phase, followed by incubation or 10 mins and centrifugation at 12,000 rpm for 10 mins. The supernatant was discarded and the total RNA precipitate, which appeared to be a gelatinous pellet of white color at the bottom of the tube, was collected. Then after adding 500 µl of 70% ethanol to each tube, a brief vortex was performed, and the tubes were centrifuged for 3 min. at a speed of 10000 rpm. The ethanol was aspirated and the pellet air-dried. The RNA solubility test was performed by rehydrating the pellet in 20 µl of nuclease-free water and storing it in the refrigerator overnight.

Estimation of RNA purity and concentration

RNA concentration was estimated using a quantiflour. For 1µl of RNA, 199µl of diluted QuantyFlour dye was mixed. After 5 minutes of incubation at 25ºC in a dark the RNA concentration was estimated.

Genes expression of *IL-38 and B-actin* using RT-PCR

One-step qRT-PCR SYBR Green assay was used to measure IL-38 and *β*-actin (9) gene expression. IL-38 and actin primers were obtained from Macrogene, Korea. The forward and reverse primer sequences for IL-38 and *β*-actin are given in Table1.

Stock solutions of the forward and reverse primers used in this study were prepared by dissolving the lyophilized primers in nuclease-free water to a concentration of 100 pM/µl; this stock solution was then diluted to a final concentration of 10 pM by dissolving 10 µl of primer in 90 µl of deionized water and stored at -23°C until use.

Quantitative Real-Time PCR (QRT-PCR)

The gene expression of *IL-38* and βactin genes was carried out using qRT-PCR. The qRT-PCR master mix consisted of the components listed out in Table (2). Appropriate volume of extracted RNA was added to master mix to get a 20µl final reaction volume. The qPCR reaction was run, using the cycling conditions given in Table 3 for the *IL-38* and *β* -actin genes. qRT-PCR was performed using a Real-time PCR machine that had been pre-configured with qPCR soft software before the experiment began (BioMolecular, Australia). By employing the threshold cycle (Ct) approach, the relative levels of gene expression as well as the fold changes that occurred were determined (10). There were two identical reactions that were carried out. PCR applied in different area (11-17).

The expression ratio was estimated without a calibrator sample $2^{-\Delta Ct}$ according to the following equation (18). Gene Expression Calculation (Relative quantification), The calibrator was chosen from the control samples. C_t values ≥ 38 were considered unreliable and neglected

ΔCt = Ct gene - Ct House Keeping gene ΔΔCt = ΔCt Treated - ΔCt Control,

Folding $=2^{-\Delta\Delta Ct}$ (Normalized expression ratio).

Table 1: Primers used in this study (9)

Primer	Sequence (5`- 3`)	Tm (ºC)	Amplicon size
IL-38	F: AACTTGGCATTTGTGAATGGGAT	66	81bp
	R: CCATCGGCGGAGCCTCTTTT	60	
β-actin	F: TGTGATGGTGGGTATGGGTC	62	162 bp
	R: ACACGCAGCTCATTGTA	54	

Table 2: RT-PCR master mix for expression of *IL-38 and B- Action*

Master mix components	Volume (µl/sample)	Volume (96 samples)
qPCR Master Mix	5	480
RT mix	0.25	24
$MgCl_2$	0.25	24
Forward and reverse primers	0.5 pM each	48 each
Nuclease free water	2.5	240
RNA	1	
Total volume	10 µl	
Aliquot per single run	1 µl of Template	

Table 3: Real-Time PCR cycling conditions

Steps	Temp (°C)	Time (min: sec)	Cycle
RT. Enzyme activation	37	15:00	1
Initial Denaturation	95	10:00	
Denaturation	95	00:20	40
Annealing	63	00:20	
Extension	72	00:20	
Melting curve analysis	65-95	2:00	1

Statistical analysis

Data represented as means and standard deviations. The statistics analysis was performed using the Statistical Processing and Analysis System-SPSS (2019) software. The t-test was used to examine the statistical significance of differences between means and standard deviations for the study's parameters. The significance of differences was estimated at a p-value P≤0.05 and P ≤ 0.01.

RESULTS

A total of 30 patients diagnosed with Graves' Ophthalmopathy disease by clinical examination and 20 healthy controls were enrolled in this study. The serum levels of Thyroid stimulating hormone (TSH) measured in all of the participants is given in Table 4.

Table 4: Serum levels of thyroid-stimulating hormone in ophthalmopathy patients and healthy controls

Groups	TSH level (µl/dl) ± SD	P-value
Ophthalmopathy patients	$0.04^a \pm 0.37$	0.0029*
Healthy control	$2.21^b \pm 0.012$	0.0001*

Different letters in rows mean a significant difference (P≤0.01). SD: standard deviation**.**

As seen from Table, there was a significant decrease (P < 0.01) in serum levels of TSH in ophthalmopathy patients in comparison to the normal healthy control group.

IL-38 gene expression

In this study, using quantitative-reverse transcription-polymerase chain reaction (qRT-PCR), the amount of IL-38 mRNA transcripts that were present in the blood of case-control subjects (ophthalmopathy patients and healthy controls) were estimated. The amount of fluorescence that is measured at each cycle of PCR is directly connected to the amount of PCR products that are produced, as shown in Figures 1A and 2. Because only one peak appeared in the melting curve, it was clear that the primers for β-Actin and IL-38 were extremely

specific. This was shown by the fact that the curve had only one peak. To the best of our knowledge, primer dimer formation did not take place while the PCR was being performed (Figs. 3 and 4).

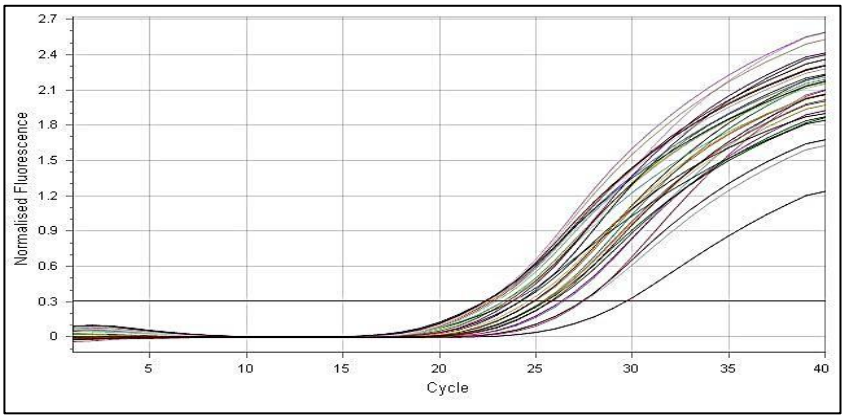

Fig. 1: Melting curve analysis of the RT-PCR (real-time polymerase chain reaction) for *β-actin* gene expression. A: Ct values pointed from 16 to 24. The photographs were taken directly from Mic-qPCR

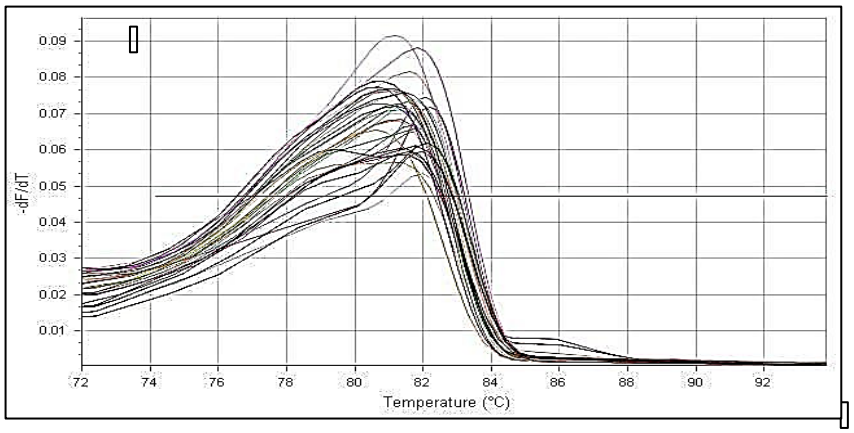

Fig. 2: β-actin gene melting curve

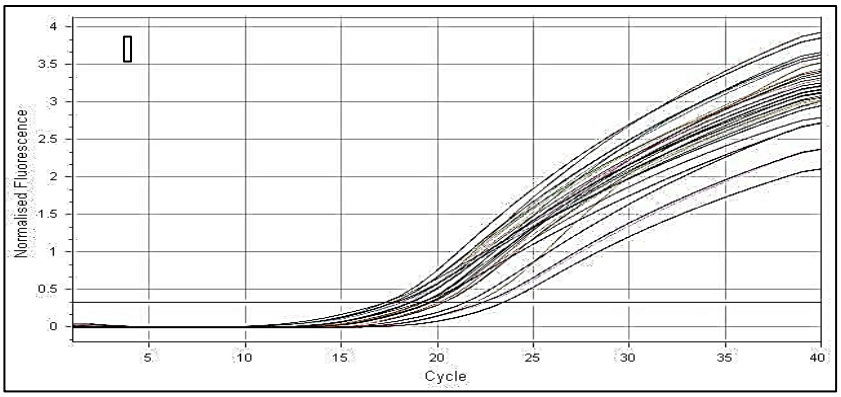

Fig. 3: Melting curve analysis of the RTPCR (real-time polymerase chain reaction) for *IL-38* gene expression. A: Ct values from 11 to 18. The photographs were taken directly from Mic-qPCR

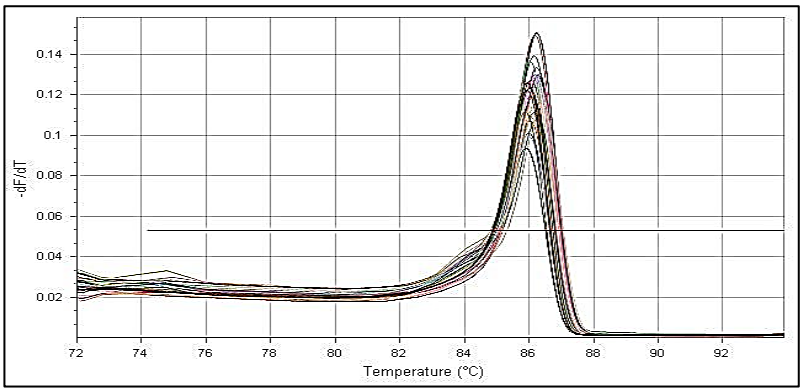

Fig. 4: Melting curve of *IL-38* gene

Table 5: Fold expression of *IL-38* gene in ophthalmopathy patients and healthy controls

IL-38 Fold expression (fold) ± SD		P-value
Ophthalmopathy patients	Healthy controls	
$0.69^{a} \pm 0.86$	$4.79^{b} \pm 0.11$	0.0031*

Different letters in raw indicate a significant difference *($P \leq 0.01$). SD: standard deviation.

This is the first study that we are aware of that evaluates the utility of monitoring the levels of *IL-38* gene expression in patients who have a severe condition caused by ophthalmopathy. The findings showed that the folding of the *IL-38* gene was significantly impaired in patients with ophthalmopathy as compared to healthy controls ($P \leq 0.01$; Table 5).

DISCUSSION

In adults, the most common ophthalmopathy disease is the Grave's disease in which the excess amount of thyroid stimulating hormone is secreted by an overactive thyroid. Many studies have shown that females are more likely than males to develop ophthalmopathy due to the estrogen hormone, due its key role in the physiologic role in female development, reproduction, and regulation of tissues and organ systems that support reproduction. Estrogen is also affected by the development of thyroid disorders (18). According to Zaman and his colleagues, Grave's disease had a higher prevalence of increased TSH levels (20.55%) in females than in males (19). It has been demonstrated that Grave's disease is an autoimmune illness that is brought on by a convoluted interaction of hereditary and environmental variables (20). According to Smith and Hegedüs (21), women have a 3% lifetime risk of Grave's disease, while men have a 0.5% risk.

Research conducted on IL-38 transcripts has shown that they play an important role in avoiding the advancement of autoimmune Graves' disease. This is accomplished by reducing the activation of self-reactive T cells and delaying the beginning of illness. This disorder manifests itself in a patient whenever their immune system begins to attack their own body (22). These findings provide credence to the findings by (23), who found that the blood IL-38 concentrations of individuals with Graves' disease and Hashimoto's illness were significantly different from those of healthy controls. Recently discovered IL-38 is a member of the IL-1 family that inhibits inflammatory signaling and, by extension, the generation of inflammatory mediators. Because it binds to important receptors like the IL-1 receptor 1, the IL-36 receptor, and the IL-1 receptor 10, IL-38 is able to make its anti-inflammatory effects last for a longer period of time (23, 24). Recent research has shown conclusive evidence that IL-38 plays a role in the development of autoimmune diseases. Recent studies on IL-38 have mostly concentrated their attention on the eye illness known as ophthalmopathy (25). Thus the elevated levels of IL-18 observed among Graves' ophthalmopathy patients in this study assumes significance, and could be used as a biomarker in detecting the development of this thyroid eye illness in females with decreased TSH levels**.**

CONCLUSION

The expression of the IL-38 gene is an excellent diagnostic test that can be used to determine the severity of Graves' disease of the eye. The prognosis of autoimmune thyroid eye disease will help with therapeutic applications, and the titer will determine the severity of the condition.

ACKNOWLEDGMENT

The authors acknowledge all patients and healthy volunteers who participated in this research work.

REFERENCES

1. Diana, T., Olivo, P.D., Kahaly, G.J. Thyrotropin receptor blocking antibodies. Hormone and metabolic research, 2018; 50(12):853-862.

2. Li, Z., Cestari, D. M., Fortin, E. Thyroid eye disease: what is new to know? Current opinion in ophthalmology, 2018; 29(6):528-534.
3. Joshi, S.R. Laboratory evaluation of thyroid function. J Assoc Physicians India, 2011; 59 (Suppl):14-20.
4. Abdulahad, W.H., Boots, A.M.H., Kallenberg, C.G.M. FoxP3+ CD4+ T cells in systemic autoimmune diseases: The delicate balance between true regulatory T cells and vector Th-17 cells. Rheumatology 2011;50(4):646–656.
5. Boutet, M.A., Bart, G., Penhoat, M., Amiaud, J., Brulin, B., Charrier, C., *et al*. Distinct expression of interleukin (IL)-36alpha, beta and gamma, their antagonist IL-36Ra and IL-38 in psoriasis, rheumatoid arthritis and Crohn's disease. Clin. Exp. Immunol. 2016; 184(2):159–173.
6. Nicklin, M.J., Barton, J.L., Nguyen, M., Fitzgerald, M.G., Du, G.W., Kornman, K. A sequence-based map of the nine genes of the human interleukin-1 Cluster. Genome., 2000; 79(5):718–725.
7. Ummarino, D. IL-38 promotes anti-inflammatory effects. Nature Reviews Rheumatology, 2017; 13(5):260-260.
8. Wild, D. The immunoassay handbook: theory and applications of ligand binding, ELISA and related techniques. Newnes., Stockton, ed. 2013, 339.
9. Jasper, M.J., Tremellen, K.P., Robertson, S.A. Primary unexplained infertility is associated with reduced expression of the T-regulatory cell transcription factor Foxp3 in endometrial tissue. MHR: Basic science of reproductive medicine, 2006; 12(5): 301-308.
10. Khalaf, Z.A., Jasim, H.M.,Mahdi, A.A. FOXP3 and IL-10 overexpression: A novel diagnostic biomarker in Iraqi patients having hyperthyroidism treated with radioactive iodine. Gene Reports, 2021; 25, 101384.
11. Rasin, K.H., Algabar, F.A.A., Al-Obaidi, M., Krayfa, A.H. Evaluation of medical waste management during the COVID -19 in Diyala government. Environmental Engineering and Management Journal, 2022; 21(12):1931–1944.
12. Kadhim AL-Imam, M.J., AL-Rubaii, B.A. The influence of some amino acids, vitamins and anti-inflammatory drugs on activity of chondroitinase produced by *Proteus vulgaris* caused urinary tract infection. Iraqi J Sci., 2016; 57 (4A):2412-2421.
13. Salih, H.S., Al-Shammari, A.M., Laftaah, B.A. Intratumoral co-administration of oncolytic newcastle disease virus and bacterial hyaluronidase enhances virus potency in tumor models. J Glob Pharma Technol. 2018; 10(10):303-310.
14. Ahmed, A.W., Ahmed, R.N., Naif, M.M., Yahya, M.T., Maulood, K.S., Alchalabi, G.B., *et al.* Chest CT findings and experience in 100 COVID19 patients in Mosul city, Iraq. Biomedicine. 2021; 41(4):793-798.
15. Al-Rubii, B.A.L. Cloning *Las*B gene of *Pseudomonas aeruginosa* eiastase 10104-2aI in *E. coli* BL21 and *E.coli* DH5α and investigated their effect on the stripping of Vero cells. Pakistan J. Biotechnol. 2017; 14(4):697-705.
16. Abdulrazaq, R.A., Mahmood, W.S., Alwan, B., Saleh, T.H., Hashim, S.T., Al-Rubaii, B.A.L. Biological study of protease produced by clinical isolates of *Staphylococcus aureus*. Res J Pharm Technol.2022; 15(12):5415-5420.
17. Abdul-Gani, M.N., Laftaah, B.A. Purification and characterization of chondroitinase ABC from *Proteus vulgaris*, an Iraqi clinically isolate. Curr Sci. 2017:2134-2140.
18. Jeng, J., Watson, S. Combinations of physiologic estrogens with xenoestrogens alter ERK phosphorylation profiles in rat pituitary cells. Environ Health Perspect; 2011; 119 (1): 104-112.
19. Zaman, B., Rasool, S. O., Sabri, S. M., Raouf, G. A., Balatay, A. A., Abdulhamid, M. A., *et al.* Prevalence of thyroid dysfunctions in a large, unselected population in Duhok city, Iraqi Kurdistan: A cross-sectional study. Journal of Biological Research - Bollettino Della Società Italiana Di Biologia Sperimentale, 2021; 94(2):10067
20. Brix, T.H., Kyvik, K.O., Christensen, K., Hegedüs, L. Evidence for a major role of heredity in Graves' disease: a population-based study of two Danish twin cohorts. J Clin Endocrinol Metab. 2001; 86(2):930-934.
21. Smith, T.J., Hegedüs L. Graves' disease. N Engl J Med; 2016; 375(16): 1552-1565.
22. Rahman, S., Kumar, V., Kumar, A., Abdullah, T.S., Rather, I.A.,Jan, A.T Molecular perspective of nanoparticle mediated therapeutic targeting in breast cancer: an odyssey of endoplasmic reticulum unfolded protein response (UPRER) and beyond. Biomedicines, 2021; 9(6):635.
23. Lin, H., Ho, A.S., Haley-Vicente, D., Zhang, J., Bernal-Fussell, J., Pace, A.M., Hansen, D., *et al.* Cloning and Characterization of IL-1HY2, a Novel Interleukin-1 Family Member. J. Boil. Chem., 2001; 276 (23):20597-20602.
24. Mora, J., Schlemmer, A., Wittig, I., Richter, F., Putyrski, M., Frank, A.C., *et al.* Interleukin-38 is released from apoptotic cells to limit inflammatory macrophage responses. J Mol Cell Biol. 2016; 8(5):426-438.
25. Tsang, M.S.M., Sun, X., Wong, C.K. The role of new IL-1 family members (IL-36 and IL-38) in atopic dermatitis, allergic asthma, and allergic rhinitis. Curr Allergy Asthma Rep. 2020; 20(40):1-8.