

Article

Elovanoid 34 reduces proinflammatory cytokines and arrest the expression of genes that drive senescence, autophagy, unfolded protein response (UPR), matrix metalloproteinases (MMPs) and inflammation in human nasal cells exposed to house dust mite (HDM): Therapeutic Potential in Allergy

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Abstract: Elovanoids (ELV) are novel lipid mediators synthesized from very long-chain (VLC) omega-3 (n-3) polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA), by the elongase enzyme ELOVL4. ELV have been shown to play a fundamental role in maintaining brain and retina function, neuroprotection, maintaining the skin's permeability barrier, and sperm function, among other important cellular processes. Furthermore, the methyl ester or sodium salt of ELV-N32 and ELV-N34 have been found to be efficient in reduced infarct volumes, promoting cell survival and decreasing the alteration of the neurovascular unit in a model of ischemia due to occlusion of the middle cerebral artery. Their bioactivity includes enhancing of anti-apoptotic and pro-survival protein expression combined with the downregulation of pro-apoptotic proteins. House dust mite (HDM) is the most prevalent indoor allergen, and HDM exposure is frequently the cause of allergic respiratory disease, including allergic rhinitis and allergic asthma in HDM sensitized individuals. In our study, human nasal epithelial cells (HNEpC) were used as an in vitro model for allergy. The cells were challenged with HDM (mixture of *Dermatophagoides farinae* and *D. pteronyssinus* extract), with or without treatment with ELV-34. Phosphate-buffered saline (PBS) (vehicle) and ELV-34 (500 nM) alone were used as the control. Our findings show that ELV-34 promotes cell viability and reduces cytotoxicity upon HDM sensitization of HNEpC. ELV-34 treatment significantly reduces the levels of IL-6, IL-1 β , IL-8, VEGF, ICAM1, CXCL1 and CCL2, restored the IL-10 level and diminishes the induction of the transcription of inflammatory and senescence genes in HNEpC. Our data also shows that HDM triggered the expression of several key genes in human nasal epithelial cells, including autophagy, unfolded protein response (UPR), Matrix metalloproteinases (MMPs), senescence and inflammation markers. Together, our data reveal a novel pro-homeostatic and cell-protective lipid-signaling mechanism in nasal epithelial cells that envision novel potential therapeutic targets for the treatment of allergies.

Keywords: elovanoid; allergy; house dust mite, HNEpC.

1. Introduction

Elovanoids are a bioactive lipid mediators derived from VLC-PUFA, n-3, which are the biosynthetic products of elongase ELOVL4 newly-discovered that are necessary for

cell integrity and have shown very promising results in a cellular experimental allergy model in human nasal cells exposed to HDM. These novel lipid mediators have been shown to be able to downregulate senescence gene programming, SASP (senescence-associated secretory phenotype), and inflammaging (age-dependent low-grade chronic inflammation) which in turn, promotes and sustains cell structural integrity, viability, and function [10,14].

House dust mites (HDM) are the most important indoor allergen, approximately 1-2% of the global population suffers from house dust mite allergy. is the single most important domestic source for allergic diseases such as allergic rhinitis, asthma and atopic dermatitis. The most important house dust mites are *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (in drier areas) and *Euroglyphus maynei* (in humid areas). HDM allergen plays important role in allergic inflammation through innate immune system Toll-like receptors (TLRs), protease activated receptors (PARs) and DC-SIGN could be activated by different HDM major allergens, resulting in the upregulation of proinflammatory cytokines.

Because Elovonoids have been shown to play an important role in the control of mediators involved in inflammation, exerting a protective role of cellular integrity against inflammation[15], we proposed to determine whether this property could be useful to protect a human nasal epithelial cell line (HNEpC) exposed to HDM-induced deleterious action.

Some serum markers of inflammation, for example IL-6, commonly tend to be slightly higher in older adults than the usual baseline in younger adults, it has been proposed that this causes damage to normal tissues and contributes to degenerative diseases of aging or inflammaging phenomenon. The inflammaging is partly attributable to dysregulated immune system leading to the development of the SASP program consistent of the release of multiple cytokines and chemokines by accumulation of senescent nonimmune tissue cell with a proinflammatory effect. The dysregulation, not only in SASP program, but also in the processes of autophagy and mitophagy, have been associated with environmental allergen or pollutant [16].

2. Results

2.1 Elovonoids elicit potent cytoprotection against LPS, poly(I:C) and HDM extracts.

To test the efficacy of elovanoids on cell survival, human nasal epithelial cells HNEpC were incubated with LPS (30 µg/mL) or poly(I:C) (100 µg/mL) and 30 minutes later ELVN-32 and ELVN-34 (500nM) were added for 24h hours (Figure 1).

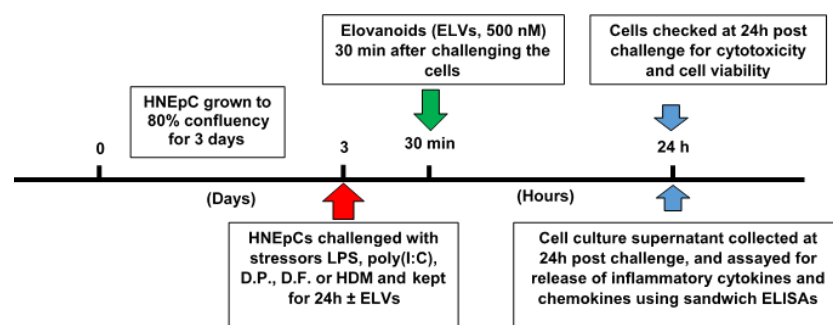


Figure 1. *In vitro* experimental design used in the study. Human nasal epithelial cells (HNEpC) challenged by allergen HDM were analyzed to determine the protective effect of Elovonoids.

LPS and poly(I:C) induced a decrease in cell viability and increase of LDH levels (Figure 2, red bars), indicating that HNEpC were subjected to cellular stress that compromises their viability. Our results indicate that both ELVN-32 and ELVN-34 successfully prevented cell death, restoring the cell viability (Presto Blue HS assay) (Figure 2, left panel)

and LDH levels (cytotoxicity assay, CyQuant LDH) (Figure 2, right panel) in all the conditions tested. Addition of ELVs increases cell viability and fosters protection to the HNEpC.

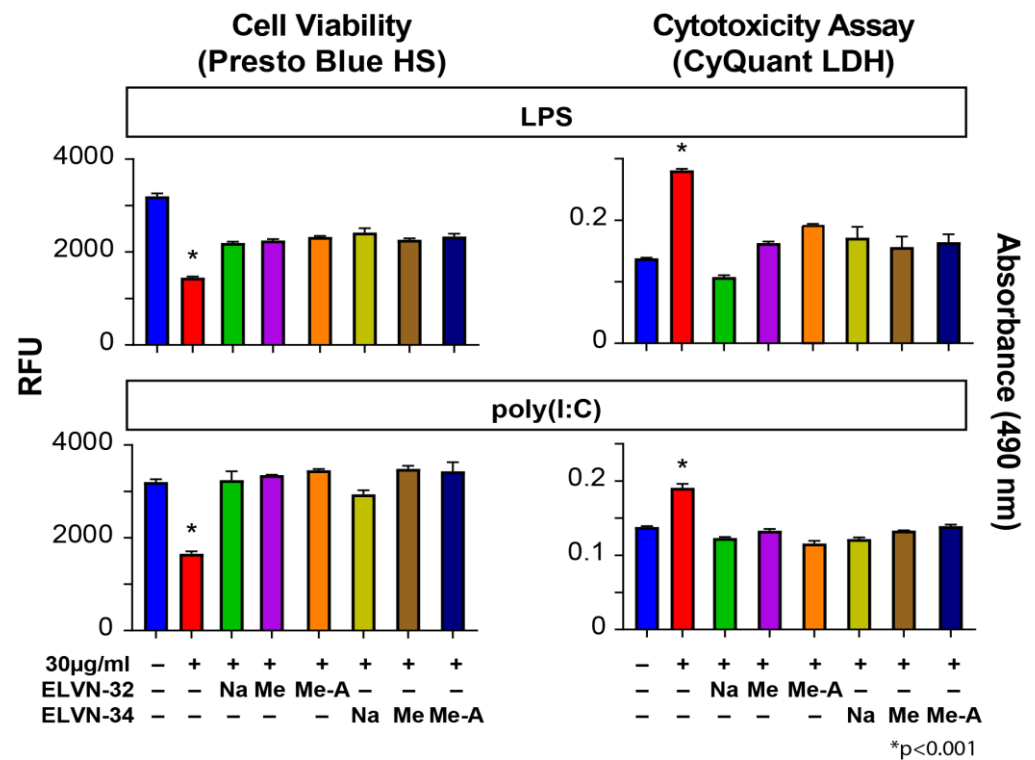


Figure 2. ELVN-32 and ELVN-34 protect HNEpC from cytotoxicity induced by LPS or poly(I:C). Cytotoxicity assay (CyQuant LDH, right panels) shows that ELVN-32 and ELVN-34 elicit potent HNEpC cytoprotection when are exposed to the stressors LPS (red bars, upper panel) and poly(I:C) (red bars, bottom panel). HNEpC treated with ELVN-32 and ELVN-34 (500nM) were protected from the cell death (Presto Blue HS, left panels) induced by LPS (30 µg/mL) or poly(I:C) (100 µg/mL). The results showed the averages of three independent experiments. *p<0.001

Asthma is caused by exposure to allergens such as dust mites, pet dander, pollen, or mold. Of these allergens, house dust mite (HDM) is the most important indoor allergen for humans, although two other mite species, *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) and *Dermatophagoides farinae* (*D. farinae*), have been also shown to play a relevant role in the development of asthma in human patients.

HDM extracts (*D. farinae*, *D. pteronyssinus* and HDM) induced a decrease in cell viability (Figure 3, red bars at left panel) and increase of LDH levels (Figure 3, red bars at right panel), indicating that HNEpC were subjected to cellular stress that compromises their viability.

Cytotoxicity assay (LDH) shows that the stressor HDM extracts (*D. farinae*, *D. pteronyssinus* and HDM) increase formation of red formazan indicating cytotoxicity, which is reduced by ELVs (Figure 3, right panel). Cell viability assay using PrestoBlue HS reagent also shows more resorufin production to cells challenged with the stressor HDM extracts (*D. farinae*, *D. pteronyssinus* and HDM) (Figure 3, left panel).

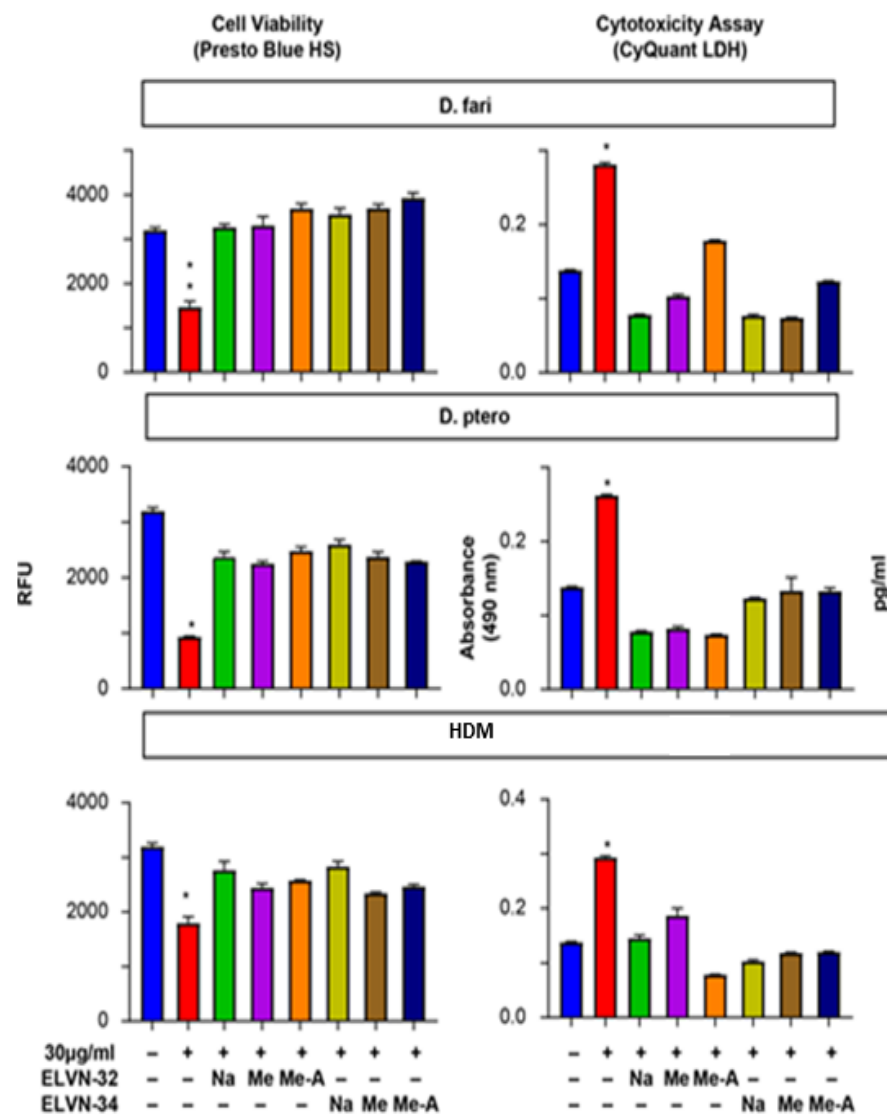


Figure 3. ELVN-32 and ELVN-34 protect HNEpC from cytotoxicity induced by HDM extracts. Cytotoxicity assay (CyQuant LDH, right panels) shows that ELVN-32 and ELVN-34 elicit potent HNEpC cytoprotection when are exposed to the stressors HDM extracts (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* and HDM). HNEpC treated with ELVN-32 and ELVN-34 (500nM) were protected from the cell death (Presto Blue HS, left panels) induced by HDM extracts. The results showed the averages of three independent experiments. *p<0.001

Our results indicate that both ELVN-32 and ELVN-34 successfully prevented cell death, restoring the cell viability (Presto Blue HS assay) (Figure 3, left panel) and LDH levels (cytotoxicity assay, CyQuant LDH) (Figure 3, right panel) after the challenge with different HDM extracts. In summary, addition of ELVs increases cell viability as well as HNEpC protection.

2.2 Elovonoids downregulate the expression of pro-inflammatory cytokines and chemokines induced by LPS, poly(I:C) and HDM extracts in human epithelial nasal cells.

HNEpC were exposed 30 minutes to LPS (30 $\mu\text{g/mL}$) or poly(I:C) (100 $\mu\text{g/mL}$) and their supernatants were collected in order to determine the expression of pro-inflammatory cytokines and chemokines IL-6, IL-1 β , IL-8/CXCL8, CCL2/MCP-1, CXCL1/KC/GRO, VEGF, ICAM1(CD54). Our results demonstrate that the challenge of HNEpC with LPS or poly(I:C) induce the expression of pro-inflammatory cytokines and chemokines IL-6, IL-1 β , IL-8/CXCL8, CCL2/MCP-1, CXCL1/KC/GRO, VEGF, ICAM1(CD54) (Figure 4, red bars). The treatment of human nasal epithelial cells with ELVN-32 (500nM) or ELVN-34 (500nM) reduce the expression of pro-inflammatory cytokines and chemokines (Figure 4). However, LPS downregulates the expression IL-10 in HNEpC that is restored to normal levels after treatment with ELVN-32 or ELVN-34 (Figure 5, panel IL-10). These results show that treatment with elovanoids protects human nasal epithelial cells from the expression of pro-inflammatory cytokines under the stressors LPS and poly(I:C), favoring the expression of anti-inflammatory cytokines such as IL-10.

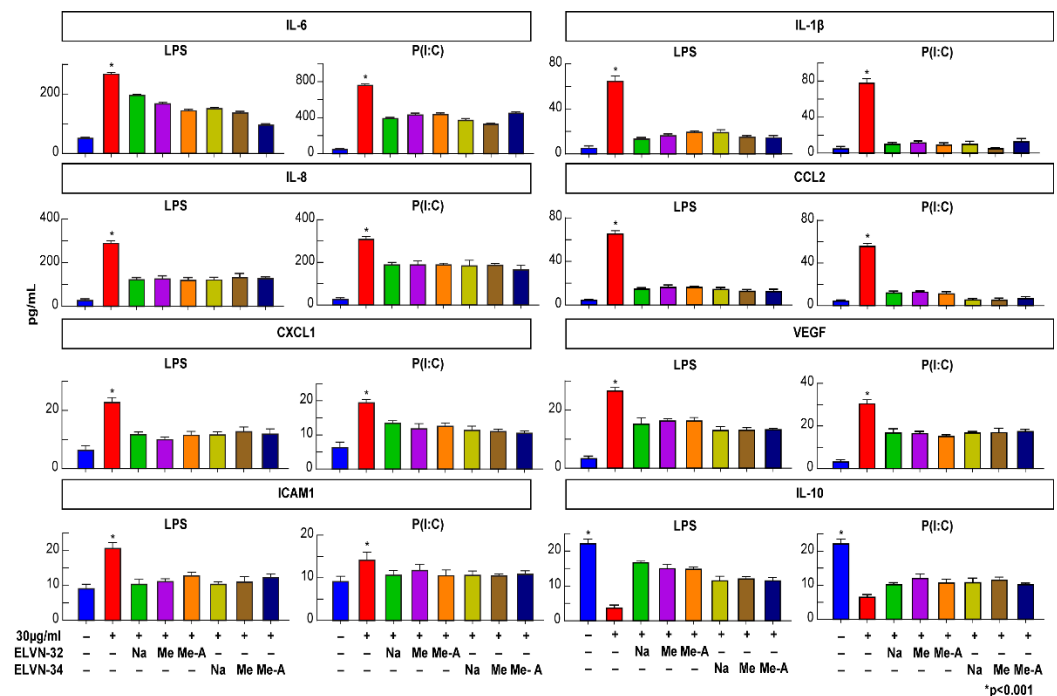


Figure 4. ELVN-32 and ELVN-34 reduce the expression of pro-inflammatory cytokines and chemokines in HNEpC treated with LPS or poly(I:C). HNEpC challenged with LPS (30 $\mu\text{g/mL}$) or poly(I:C) (100 $\mu\text{g/mL}$) display enhanced production of pro-inflammatory cytokines and chemokines IL-6, IL-1 β , IL-8/CXCL8, CCL2/MCP-1, CXCL1/KC/GRO, VEGF, ICAM1(CD54) compared to non-treated cells. This increase in the production of pro-inflammatory cytokines and chemokines is abrogated by the addition of ELVN-32 or ELVN-34 (500 nM) 30 minutes post-challenge. LPS induces a reduction of IL-10 expression in HNEpC that is restored to normal levels after treatment with ELVN-32 or ELVN-34. The results showed the averages of three independent experiments. *p<0.001

Similar results were observed when HNEpC were challenged with HDM stressors (*D. farinae*, *D. pteronyssinus* and HDM) (30 $\mu\text{g/mL}$) (Figure 5, red bars). The treatment of human nasal epithelial cells with ELVN-32 (500nM) or ELVN-34 (500nM) reduce the expression of pro-inflammatory cytokines and chemokines induced by HDM stressors (Figure 5). Again, HDM stressors showed a significant downregulation of the IL-10 expression in HNEpC that is restored to normal levels after treatment with ELVN-32 or ELVN-34 (Figure 5, panel IL-10).

These results indicate that treatment with elovanoids protects human nasal epithelial cells from the expression of pro-inflammatory cytokines, in favor of anti-inflammatory cytokines, suggesting a possible beneficial therapeutic effect for the treatment of allergies.

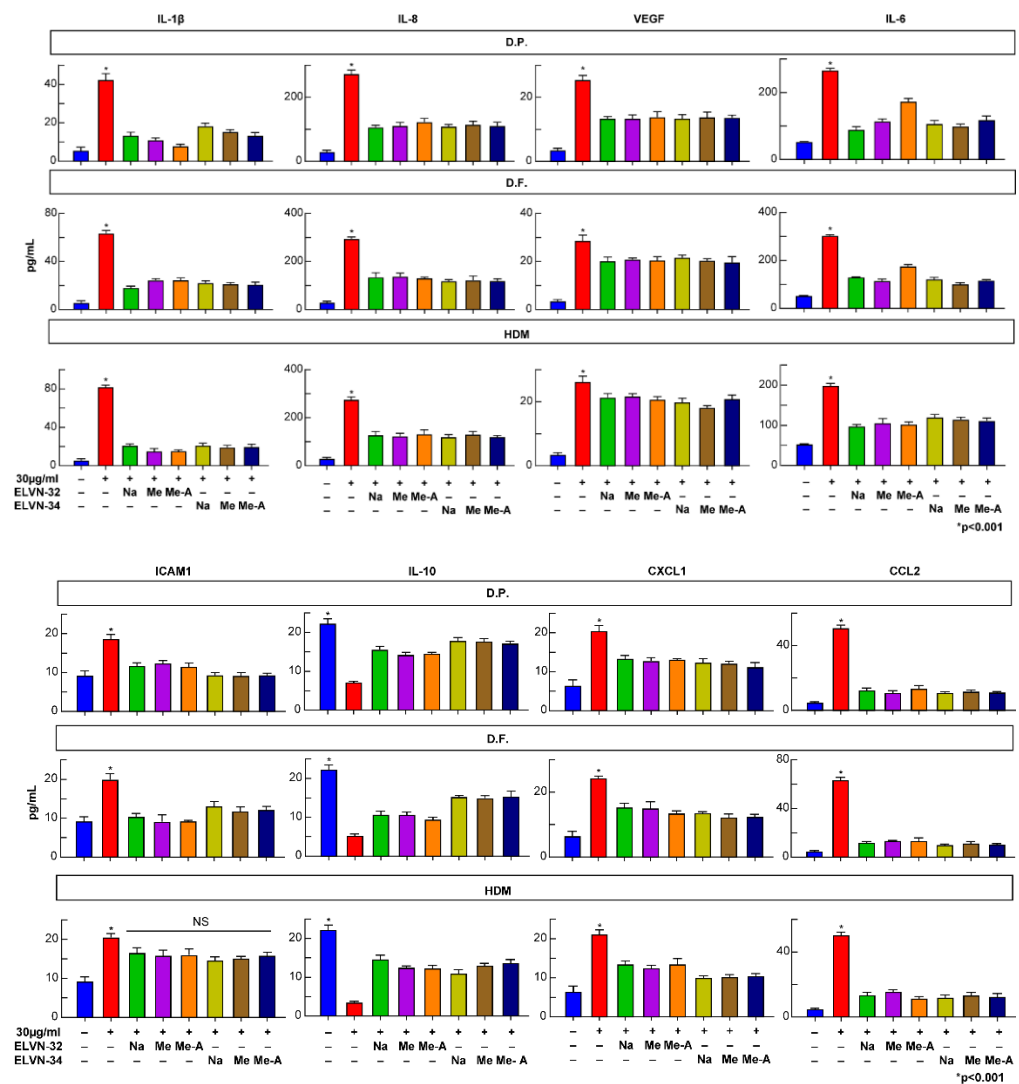


Figure 5. ELVN-32 and ELVN-34 reduce the expression of pro-inflammatory cytokines and chemokines in HNEpC exposed to HDM extracts. A remarkable decrease was observed in the release of anti-inflammatory cytokine IL-10 in HNEpC challenged with the HDM stressors (*D. farinae*, *D. pteronyssinus* and HDM) (30 μ g/mL). This decreased production of anti-inflammatory cytokine are reversed by the addition of ELVN-32 or ELVN-34 (500 nM), 30 minutes post challenge with the stressor. The results showed the averages of three independent experiments. *p<0.001

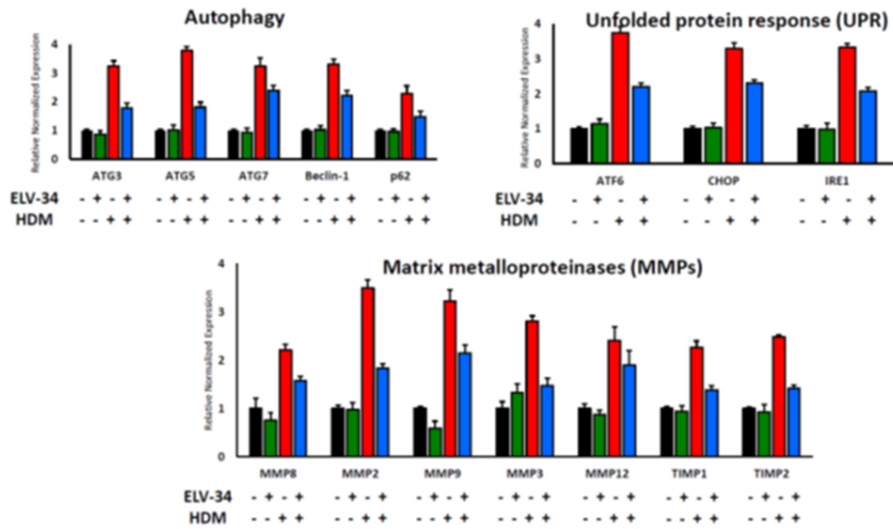
2.3 Elovonoids regulate the expression of autophagy, UPR, MMPs, senescence and inflammation markers in human epithelial nasal cells.

To search for the mechanism involved in ELVs protection against HDM-mediated damage, UPR, MMPs, senescence and inflammation markers were analyzed by quantitative PCR on HNEpC challenged with HDM stressors (*D. farinae*, *D. pteronyssinus* and HDM) (30 μ g/mL). We selected genes involved in autophagy (ATG3, ATG5, ATG7, BECLIN-1 and P62), unfolded protein response (UPR) (ATF6, CHOP and IRE1) and matrix metalloproteinases (MMPs) genes (MMP8, MMP2, MMP9, MMP3, MMP12, TIMP12 and TIMP2) (Figure 6A), as well as, the expression of senescence (P21, P16, P27 and P53) and inflammation genes (IL-1 α , IL-6 and IL-1 β) (Figure 6B). All the markers analyzed showed high expression in HNEpC after HDM treatment, with the exception of p53 (Figure 6, red bars). The treatment of human nasal epithelial cells with ELVN-32 (500nM) or ELVN-34 (500nM)

significantly reduce the expression of all genes mentioned above after challenge with HDM stressors (Figure 6, blue bars).

These results clearly indicate that elovanoids have a protective effect on the survival, integrity, senescence and inflammation, as well as, autophagy process at the cellular level.

A.



B.

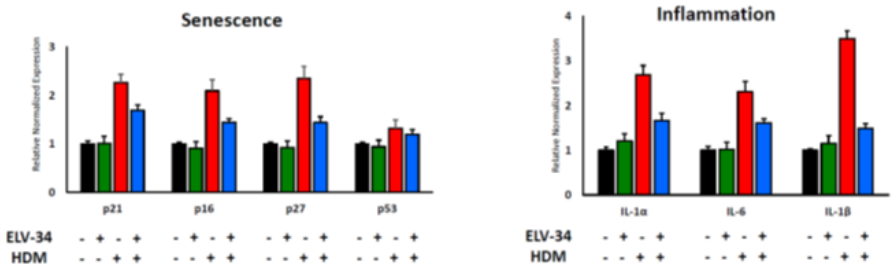


Figure 6. HDM triggered multiple signaling in human nasal epithelial cells. The challenge of HNEpC with HDM stressors (*D. farinae*, *D. pteronyssinus* and HDM) (30 μ g/mL) induce the expression of autophagy (ATG3, ATG5, ATG7, BECLIN-1 and P62), unfolded protein response (UPR) (ATF6, CHOP and IRE1) and matrix metalloproteinases (MMPs) genes (MMP8, MMP2, MMP9, MMP3, MMP12, TIMP12 and TIMP2) (panel A). HDM stressors also induce the expression of senescence (P21, P16, P27 and P53) and inflammation genes (IL-1 α , IL-6 and IL-1 β) on HNEpC (panel B). The treatment with ELVN-34 (500 nM) reduce the expression of autophagy, UPR, MMPs, senescence (except P53) and inflammation genes induced by HDM extracts. The results showed the averages of three independent experiments. * $p < 0.001$

2.4 Elovonoids protect human nasal epithelial cells from senescence.

Finally, our study also evaluated the Senescence-Associated Secretory Phenotype (SASP response) in human nasal epithelial cells (HNEpC), finding that treatment with elovanoids protected these cells from this phenomenon. HDM extract (30 μ g/mL) induced an increased number of beta-galactosidase positive cells (Figure 7, red bar) indicating that cellular senescence has been activated as a consequence of the stressor used. ELVN-34 (500 nM) elicit a robust protection of HNEpC exposed to HDM extract (Figure 7, green

bar), suggesting that this novel compound could protect HNEpC from premature senescence induced by the exposure to allergens.

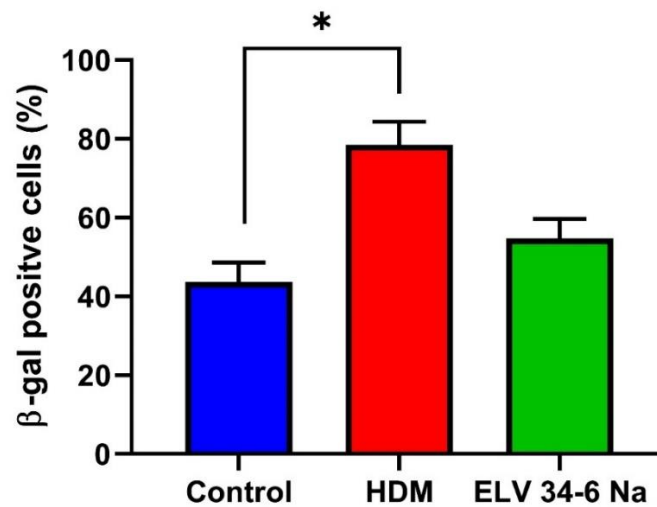


Figure 7. ELVN-34 protect HNEpC from the senescence-associated secretory phenotype (SASP response). Quantitative assessment of human nasal cells increased the SASP response (measured by the spider β -galactosidase staining) upon HDM exposure (30 μ g/mL). ELV 34:6 (500nM) treatment reduces the number beta-gal positive HNEpC induced by HDM. The results showed the averages of three independent experiments. * $p < 0.001$

3. Discussion

In the present study we use a model of human nasal epithelial cells (HNEpC) to study the release of inflammatory cytokines and the expression of adhesion molecules when interacting with already known inflammatory inducers, LPS and Poly (I: C), as well as when reproducing the in vivo conditions of HDM allergen-stimulated allergic rhinitis. Both cytotoxicity (damage to the plasma cell membrane) and cell viability (metabolic activity of cells) have been altered with exposure to these known inflammatory inducers of innate immunity LPS [17] and Poly (I: C) [18], as well as against house dust mites *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* or a 50% mixture of both (HDM).

The high sensitization capacity of HDM is known by inducing the proliferation of Th0 to Th2 lymphocytes, limiting the differentiation to Th1, and it has been attributed to the ability of group 1 allergens (Der p1), for example, to cleave IL-2R [19], activate dendritic cells for Th2 polarization, suppress IL-12 by CD40 cleavage, reduce INF- γ release and increase IL-4 production [20], thus favoring an allergic microenvironment by acting on adaptive immunity. But there is growing evidence that these group 1 allergens are mainly responsible for activating the innate immune response, cleaving the binding proteins of epithelial cells, activating protease-sensitive receptors (PAR) and TLR4, activating NOD-like receptors. (NLR) or interacting with ILC [21]. Wong *et al.* [22] studied the effects of HDM *Dermatophagoides pteronyssinus* [1] on the activation of eosinophils and bronchial epithelium cells; observed an increase in the release of inflammatory cytokines (NF-KB, AP-1, MAPK, IL-1, IL-6, TNF-alpha) [23] and in the expression of adhesion molecules, such as ICAM-1, in the co-culture systems. In our cellular model of human nasal epithelial cells, we have confirmed the elevation of the levels of pro-inflammatory cytokines of epithelial origin and alarmines such as IL-6, IL-8, IL-1 β , as well as the pro-inflammatory chemokine CCL2 associated with Th2 response and monocyte chemotactic, as well as the pro-inflammatory chemokine CXCL1 potent chemotactic for neutrophils.

Bronchial epithelial cells act as immunoregulators in allergic respiratory diseases through the expression of adhesion molecules on the cell surface, such as ICAM [24,25]. We have also confirmed the HDM-induced expression of VEGF (vascular endothelial growth factor) and ICAM1 (intercellular adhesion molecule of endothelial and immune cells). Although the increase in angiogenesis is a characteristic feature of the remodeling of the asthmatic airways as a consequence of inflammation, its activation seems to be confirmed from the beginning of exposure to the allergen, associated with an expression of ICAM-1 molecules that will favor the adhesion of inflammatory effector cells.

IL-10 is expressed in the nasal mucosa of allergic patients to HDM both in the epithelium and in the blood vessel endothelial cells, playing a role in the pathogenesis of allergic rhinitis as an immunosuppressive cytokine and revealed by Muller *et al.* [26] after nasal challenges with allergens and subsequent biopsies. Similarly, in our experimental model, we have observed a marked decrease in the production of IL-10 (an anti-inflammatory cytokine) after induction with LPS, Poly (I: C) and house dust mites.

On the other hand, autophagy has been shown to play an essential role in the lung inflammatory response induced by infection and stress. According to Racanelli *et al* [27], autophagy seems to play an important role in the pathogenesis of asthma. High levels of autophagy have been seen in sputum granulocytes, peripheral blood cells, and eosinophils from patients with asthma, although little is known about their role in allergic rhinitis. Also, genetic mutations in autophagy genes have been associated with asthma; for example, polymorphisms in ATG5 correlate with airway remodeling and loss of lung function in asthma [28]. Environmental pollutants and allergens can induce autophagy, and autophagy has been suggested to be essential for inhibiting spontaneous lung inflammation and for secretion of airway mucus by goblet cells. Mice with autophagy deficiency (Atg5^{-/-} and Atg7^{-/-}) develop spontaneous sterile lung inflammation [29]. Cell culture experimental model systems suggest that, in the lung epithelium, activation of autophagy may be deleterious inducing disease progression and promote the Th2 epithelial response in asthma. Our data also show that HDM triggered the expression of several key genes in the nasal epithelium, including autophagy, unfolded protein response (UPR), matrix metalloproteinases (MMP), senescence, and inflammation. HDM activates the autophagy genes: ATG3, ATG5, ATG7, BECLIN-1 and P62. These data support other previous studies in other respiratory diseases, as well as in chronic obstructive pulmonary disease (COPD) [27,30].

Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) pathway may play a fundamental role in the regulation of epithelium-dendritic cell interaction, which is crucial for the initiation and amplification of the allergic response [21]. The accumulation of misfolded proteins in the ER can be signaled through IRE1 α (transmembrane receptor that includes the enzyme 1 α that requires inositol), ATF6 (activation of transcription factor 6) and PERK (double-stranded RNA-dependent protein kinase PKR - ER kinase). If the first two signaling pathways fail to restore ER stress initiated by the GRP78 chaperone, homologous protein C / EBP (CHOP) will mediate apoptosis using PERK signaling. In our cell model, treatment with HDM also increases the expression of UPR, including the ATF6, CHOP and IRE1 genes, which are critical in allergic reactions and in asthmatic patients [21,31].

It has been described that cellular senescence is characterized by irreversible cell cycle arrest, leading to an increase in pro-inflammatory mediators, less capacity for tissue regeneration or repair, greater accumulation of senescent cells [32], being an important risk factor in the lung for the development of the asthma [33]. Among the studies that relate cell senescence and asthma, it is worth mentioning the research performed by Wu *et al.* [34] who observed cellular senescence induced by TSLP (*Thymic Stromal Lymphopoietin*) with elevated levels of P21 and P16 in human epithelial cells, being essential for the remodeling of the airways in vitro; as well as the study with a plasminogen activator inhibitor (PAI-1), mediator of cell senescence and fibrosis, which could activate p53 and mediate the senescence of type II alveolar cells (ATII) induced by bleomycin and doxorubicin.

bicin [35]. Therefore, senescence is triggered by factors such as aging, DNA damage, oxidative stress, mitochondrial dysfunction [16]. In order to study the activation of the cell senescence pathways we can use different markers: p53-ARF pathway (crucial mediator which plays a role in the cell responses to DNA damage) and the p16-pRb pathway (induced by a variety of stressful *stimuli*). In our results, the inflammatory genes IL-1a, IL-1b, and IL-6 are also activated, and finally, HDM also triggered senescence genes, including Cdkn1a (p21), Cdkn2a (p16), and Cdkn1b (p27) but not p53.

Elovanoids have been recently studied in an experimental model of retinal cells showing protection against neurodegenerative diseases such as age-related macular degeneration (AMD) and Alzheimer's, promoting repair, remodeling and regeneration [12]. In this study, ELV-34 was shown to play a relevant role in promoting cell viability and decreasing cytotoxicity after HNEpC sensitization to HDM. Furthermore, our results indicate that the levels of IL-6, IL-1 β , IL-8, VEGF, ICAM1, CXCL1, and CCL2 that had increased with HDM sensitization, were significantly reduced with ELV-34 treatment. Likewise, the level of IL-10 was restored by treatment with ELV34. Therefore, ELV-34 manages to decrease the production of pro-inflammatory cytokines, increasing the production of anti-inflammatory cytokines and decreasing the expression of adhesion molecules and vascular endothelial growth factor after exposure to inflammatory inducers and the HDM allergen.

Liu *et al.* [36], reviewing the role of autophagy in allergic inflammation, observed that inhibition of autophagy in a murine OVA asthma model reduces airway response, eosinophilia, and inflammation. Interestingly, treatment with ELV-34 decreases the induction of transcription of these genes. Furthermore, β -galactosidase staining, which is a marker of the senescence-associated secretory phenotype (SASP), was also performed on HDM-challenged nasal epithelial cells with or without ELV-34 [37]. Treatment with ELV-34 showed a marked decrease in β -galactosidase compared to cells treated with HDM alone.

In summary, our results shows that ELV-34 promotes cell viability and sequesters cytotoxicity upon HDM sensitization of HNEpC. Also, the levels of IL-6, IL-1 β , IL-8, VEGF, ICAM1, CXCL1 and CCL2 increased upon HDM sensitization, which was reduced significantly upon ELV-34 treatment. IL-10 level was reduced upon HDM sensitization, which was restored by ELV34 treatment. Our data also shows that HDM triggered the expression of several key genes in nasal epithelium, including autophagy, unfolded protein response (UPR), Matrix metalloproteinases (MMPs), senescence and inflammation [16]. HDM activates the autophagy genes: ATG3, ATG5, ATG7, BECLIN-1 and P62. This data supports other previous studies in other respiratory diseases as well as in chronic obstructive pulmonary disease (COPD) [27,30]. HDM treatment also increase the expression of UPR, including Atf6, Chop and Ire1 genes, which are critical in allergic reactions and asthmatic patient [21,31,38].

In addition, the inflammatory genes, IL-1 α , IL-1 β and IL-6, are activated as well and finally, the HDM also triggered the senescence genes, including Cdkn1a (p21), Cdkn2a (p16) and Cdkn1b (p27) but not p53. Interestingly, ELV-34 treatment diminishes the induction of the transcription of these genes. Moreover, β -galactosidase staining which is a marker of senescence associated secretory phenotype (SASP) was also performed in the nasal epithelial cells challenged with HDM with or without ELV-34 [37]. ELV-34 treatment showed marked decrease in β -galactosidase when compared with cells treated with HDM alone.

Although more studies are needed in this regard, our results show that ELV-34 is capable of inhibiting cytokines derived from epithelial cells, reducing the activation signal of the innate immune system by HDM, necessary to maintain adaptive immunity, restore the epithelial mucosal barrier and reduce the transcription of senescence and autophagy genes, being able to constitute a therapeutic option in the prevention and treatment of allergy to HDM.

Allergens are associated with the SASP program, the inflammaging phenomenon and senescent cells as a consequence of immuno-inflammatory dysfunction that leads to

increases in oxidative stress, mitochondrial dysfunction and the secretion of cytokines that contribute to cellular damage and chronic inflammation.

Elovanoids may represent a new therapeutic as shown by the results provided in relation to their activity on SASP, inflammaging, matrix remodeling, autophagia and accumulation of senescent cells, restoring immune-inflammatory dysfunction and regulating homeostasis in the prevention and progression of allergic pathologies.

4. Materials and Methods

4.1 Experimental design

The experimental design (Figure 1) with primary human nasal epithelial cells (HNEpC) allowed to assess the prohomeostatic protective signaling of ELV32 and ELV34 in relation to cytotoxicity, cell viability, cytokine production and expression of genes involved in inflammatory and senescence processes. HNEpC were challenged with different stressors (LPS, poly(I:C) or house dust mite extract and, 30 minutes later, the cells were treated with ELV32 or ELV34 (500nM). Cells were analyzed for cytotoxicity and cell viability and also cell supernatants were collected at 24 hours in order to assay the expression of pro-inflammatory cytokines and chemokines.

4.2 Cell line

Cryopreserved human nasal epithelial cells HNEpC were purchased from PromoCell GmbH, Heidelberg, Germany. (Catalog# C-1260, Lot# 436Z028). These cells are primary nasal epithelial cells obtained from the nasal mucosa of a 50 year old Caucasian male. HNEpC were grown to 80% confluence in Promocell's Airway epithelial cell growth medium (Catalog# C-21060) which was supplemented with airway epithelial cell growth medium supplement pack (Catalog# C-39160) and Penicillin/Streptomycin.

4.3 Cell inducers

HNEpC were challenged using several stressors: House Dust Mite extract from *Dermatophagoides pteronyssinus* (D.P.) (Catalog# 3033) lyophilized extract were obtained from Chondrex, Inc. and used at 30 µg/mL to challenge HNEpC. House Dust Mite extract from *Dermatophagoides farinae* (D.F.) (Catalog# 3040) lyophilized extract were obtained from Chondrex, Inc. D.F. and used at 30 µg/mL to challenge HNEpC. House Dust Mite extract – HDM a mixture of both (D.P.) & (D.F.) was used at (15 µg/mL + 15 µg/mL) to challenge HNEpC.

Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich Catalog# L4391). LPS is the principal component of Gram-negative bacteria that activates the innate immune system through its recognition by Toll-like receptor 4 (TLR4). This leads to a signaling cascade that ultimately results in the activation of NF-κB and the production of proinflammatory cytokines. LPS we used for our experiments is a preparation of smooth (S)-form LPS purified from the Gram-negative E. coli 0111:B4 that was used at 30 µg/mL to challenge HNEpC.

Polyinosinic-polycytidylic acid (poly(I:C) or poly(rI):poly(rC)) is a synthetic analog of double-stranded viral RNA (dsRNA), a molecular pattern associated with viral infection such as loss of epithelial integrity, increased production of mucus and inflammatory cytokines. Poly(I:C), a TLR3 agonist activates the antiviral pattern recognition receptors TLR3, RIG-I/MDA5 and PKR, thereby inducing signaling via multiple inflammatory pathways, including NF-κB and IRF. High Molecular Weight Poly(I:C) comprises long strands of inosine poly(I) homopolymer annealed to strands of cytidine poly(C) homopolymer.

The average size of Poly(I:C) HMW is from 1.5 kb to 8 kb. Poly(I:C) (Catalog# P1530) were obtained from Sigma-Aldrich and used at 100 µg/mL to challenge HNEpC.

4.4 Cell survival

Cell viability was analyzed using PrestoBlue HS (ThermoFisher Scientific) reagent (Catalog# C50201, Invitrogen). The PrestoBlue HS Cell Viability Reagent is a complete add and read, nontoxic reagent that does not require cell lysis. The highly purified resazurin that is used for PrestoBlue HS results in a reagent with a >50% decrease in background fluorescence and a >100% increase in the signal to background ratio.

On entering live cells, the cellular reducing environment reduces resazurin to resorufin a compound that is red and highly fluorescent. Viable cells continuously convert resazurin to resorufin increasing the overall fluorescence and color of the media surrounding the cells. Also, the conversion of resazurin to resorufin results in a pronounced color change, therefore cell viability can be detected using absorbance-based plate readers. Fluorescence is read using a fluorescence excitation wavelength of 560 nm (excitation range is 540–570 nm) and an emission of 590 nm (emission range is 580–610–nm).

4.5 Cytotoxicity analysis

Cytotoxicity was measured using CyQuant LDH assay kit (Catalog# C20301, ThermoFisher Scientific) what evaluates damage to the plasma membrane releases LDH into the surrounding cell culture media. The extracellular LDH in the media can be quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD⁺ reduction to NADH. Oxidation of NADH by diaphorase leads to the reduction of a tetrazolium salt (INT) to a red formazan product that can be measured spectrophotometrically at 490 nm. The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity.

4.6 Pro-inflammatory cytokine profile analysis

In addition, the expression of the following cytokines were analyzed in the supernatant of HNEpC exposed to different allergic inducers: IL-6 (Catalog# 6802, Chondrex), IL-1β (Catalog# 6805, Chondrex), IL-8/CXCL8 (Quantikine ELISA Kit, Catalog# D8000C, R&D Systems), VEGF (Catalog# 6810, Chondrex), ICAM1(CD54) (ELISA Kit, Catalog# ab100640, Abcam), CXCL1/KC/GRO (Catalog# 6825, Chondrex) and CCL2/MCP-1 (Catalog# 6821, Chondrex), and IL-10 (Catalog# 6806, Chondrex).

4.7 Cellular Senescence Assay (β-galactosidase expression)

Cellular senescence was measured in HNEpC by analyzing the expression of beta-galactosidase using a beta-gal staining kit (Spider β-gal Kit, Catalog#SG04, Dojindo). SPiDER-βGal allows to detect senescence-associated (SA)-β-gal with high sensitivity being a reagent which possesses a high cell-permeability and a high retentivity inside cells. SA-β-gal can be detected specifically not only in living cells but also fixed cells by using a reagent (Bafilomycin A1) to inhibit endogenous β-galactosidase activity.

4.8 Gene expression

Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen). Quantitative PCR was performed in a CFX-384 Real-Time PCR system (Bio-Rad). The expression of target genes was normalized to the geometric mean of housekeeping genes and relative expression was calculated by the comparative threshold cycle method ($\Delta\Delta CT$). Table 1 shows a list of the primers used in the study.

Table 1. List of primers

Protein	Gene Name	Forward Sequence	Reverse Sequence
ATG3	<i>Atg3</i>	GATGGCGGATGGGTAGATACA	TCTTCACATAGTGCTGAGCAATC
ATG5	<i>Atg5</i>	AGAAGCTGTTTCGTCCTGTGG	AGGTGTTTCCAACATTGGCTC
ATG7	<i>Atg7</i>	ATGATCCCTGTAACTTAGCCCA	CACGGAAGCAAACAACCTCAAC
Beclin-1	<i>Becn1</i>	GGTGTCTCTCGCAGATTCATC	TCAGTCTTCGGCTGAGGTTCT
p62	<i>Nup62</i>	CCTTCCAACCAGCCACAAGTAC	CGAAGCAAGAGTCGCTGTTCCA
ATF6	<i>Atf6</i>	GACTCACCCATCCGAGTTGTG	CTCCCAGTCTTCATCTGGTCC
CHOP	<i>Aff4</i>	CTGGAAGCCTGGTATGAGGAT	CAGGGTCAAGAGTAGTGAAGGT
IRE1	<i>Ern1</i>	CCGAACGTGATCCGCTACTTCT	CGAAAGTCCTTCTGCTCCACA
MMP8	<i>Mmp8</i>	AATCCTTGCCCATGCCTTTCAACC	CCAAATTCATGAGCAGCCACGAGA
MMP2	<i>Mmp2</i>	CCTTAAAGTATGGAGCGACGTCA	AGCGTTCCCATACTTTACGCG
MMP9	<i>Mmp9</i>	ACGACATAGACGGCATCCAGTATC	AGGTATAGTGGGACACATAGTGGG
MMP3	<i>Mmp3</i>	ACCAACCTATTCCTGGTTGCTGCT	ATGGAAACGGGACAAGTCTGTGGA
MMP12	<i>Mmp12</i>	TAGAAGCAACTGGGCAACTGGACA	ACCGCTTCATCCATCTTGACCTCT
TIMP1	<i>Tipm1</i>	GGAGAGTGTCTGCGGATACTTC	GCAGGTAGTGATGTGCAAGAGTC
TIMP2	<i>Tipm2</i>	ACCCTCTGTGACTTCATCGTGC	GGAGATGTAGCACGGGATCATG
p21 ^{CIP}	<i>CDKN1A</i>	TGTCCGTCAGAACCCATGC	AAAGTCGAAGTTCCATCGCTC
p16 ^{INK4a}	<i>CDKN2A</i>	GGGGGCACCAGAGGCAGT	GGTTGTGGCGGGGGCAGTT
p27 ^{KIP}	<i>CDKN1B</i>	TAATTGGGGCTCCGGCTAACT	TGCAGGTCGCTTCCTTATTCC
p53	<i>TRP53</i>	CCCCTCCTGGCCCCTGTCATCTTC	GCAGCGCCTCACAACCTCCGTCAT
IL-1α	<i>IL-1α</i>	TGTATGTGACTGCCCAAGATGAAG	AGAGGAGGTTGGTCTCACTACC
IL-6	<i>IL-6</i>	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-1β	<i>IL-1β</i>	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCTGAAGAA
ACTB	<i>ActB</i>	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG
GAPDH	<i>GAPDH</i>	TGGACCTGACCTGCCGTCTA	CCCTGTTGCTGTAGCCAAATTC

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Conflicts of Interest: Ricardo Palacios (CEO) and David Rodríguez (Research Department Director) work at Diater Laboratorio de Diagnóstica y Aplicaciones Terapéuticas S.A and declare that they had no involved in the final decision to publish the results neither in the orientation of the results obtained in the present study. The other authors declare no conflict of interest.

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