

## Article

# Biology of Drug-Induced Gingival Hyperplasia: In Vitro Study of the Effect of Nifedipine on Human Fibroblasts

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**Abstract:** Background: It has been proven that the antihypertensive agent nifedipine can cause gingival overgrowth as a side effect. The aim of this study was to analyze the effects of pharmacological treatment with nifedipine on human gingival fibroblasts activity, investigating the possible pathogenetic mechanisms that lead to the onset of gingival enlargement. Methods: The expression profile of 57 genes belonging to the “Extracellular Matrix and Adhesion Molecules” pathway, fibroblasts’ viability at different drug concentrations, and E-cadherin levels in treated fibroblasts were assessed using real-time Polymerase Chain Reaction, PrestoBlue™ cell viability test, and an enzyme-linked immunoassay (ELISA), respectively. Results: Metalloproteinase 24 and 8 (MMP24, MMP8) showed significant upregulation in treated cells with respect to the control group, and cell adhesion gene CDH1 (E-cadherin) levels were recorded as increased in treated fibroblasts using both real-time PCR and ELISA. Downregulation was observed for transmembrane receptors ITGA6 and ITGB4, the basement membrane constituent LAMA1 and LAMB1, and the extracellular matrix protease MMP11, MMP16, and MMP26. Conclusions: The obtained data suggested that the pathogenesis of nifedipine-induced gingival overgrowth is characterized by an excessive accumulation of collagen due to the inhibition of collagen intracellular and extracellular degradation pathways.

**Keywords:** drug-induced gingival overgrowth; nifedipine; human gingival fibroblasts; hypertension therapy side effects



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## 1. Introduction

The most frequent risk factor for myocardial infarction, congestive heart failure, and stroke is hypertension [1,2]. Nifedipine is used as an antihypertensive and antianginal agent, providing vascular smooth muscle relaxation and negative inotropic and chronotropic effects on. This drug is able to inhibit voltage-dependent L-type calcium channels in vascular smooth muscles and myocardial cells, preventing the entry of calcium ions. The reduction of intracellular calcium level causes a decrease of peripheral arterial vascular resistance and the dilatation of coronary arteries. These conditions result in systemic blood pressure reduction and in myocardial oxygen delivery increase [3–6]. The systematic review and meta-analysis by Ross et al. [7] analyzed the side effects of antihypertensive agents on 28,922 patients, reporting that calcium channel blockers are most associated with neurologic (17.6%), cardiovascular (11.9%), respiratory (7.8%), and dermatologic (6.8%) adverse events. The literature also proved gingival overgrowth (GO) to be a further side effect associated with the administration of calcium channel blockers,

including nifedipine. The cross-sectional study by Miranda et al. [8] recorded a significantly higher prevalence of gingival enlargement in 65 patients treated with nifedipine compared with the control group, and it also assessed, in line with other studies, that gingival lesions were more severe in the vestibular area of inferior and anterior teeth [9,10].

The onset of gingival overgrowth is not only associated with antihypertensive therapy, but also with anticonvulsant and immunosuppressant drugs' chronic administration: the prevalence of drug-induced gingival enlargement (DIGO) was demonstrated to be around 50–70%, 30%, and 25–30% in the case of phenytoin [11,12], nifedipine [8], and cyclosporine, respectively [12]. Clinical manifestation of GO is similar for all the cited drugs: it usually manifests itself during the first 3 months of the therapy, reaching its widest expression after 9–12 months [13]. The study of 2012 by Miranda et al. [14] described the manifestation of gingival lesions, evaluating two clinical indices, i.e., vertical and horizontal overgrowth: GO occurs at first in the area of interdental papilla, as localized nodular enlargement (horizontal growth), and it afterwards expands to the dental crown (vertical growth), interfering with mastication. The severity of drug-induced gingival enlargement (DIGO) seems to be influenced by plaque scores and gingival inflammation [15]. In particular, with reference to nifedipine, some studies demonstrated that the onset and the development of GO depends on high plaque index, administration of high drug doses, and genetic factors [16,17]. Moreover, Trackman et al. assessed that the characteristics of GO induced by nifedipine, phenytoin, and ciclosporin are different: gingival lesions associated with anticonvulsants are the most fibrotic, gingival tissue in patients treated with cyclosporin appears highly inflamed, while lesions related to nifedipine administration have intermediate characteristics [18].

The increase of the volume of gingival tissues represents the consequence of the connective tissue response: the alterations in cellular and extracellular matrix (ECM) metabolisms leads to an excessive storage of extracellular matrix proteins (collagens or amorphous ground substance) [15,19]. Calcium channel blockers can inhibit the intracellular calcium uptake, an event that leads to the stimulation of gingival fibroblasts, which are mainly involved in the synthesis and enzymatic degradation of ECM proteins. The pathogenetic hypothesis of DIGO is based on the presence of genetically predisposed gingival fibroblasts, which produce enormous quantity of collagen, consequently causing a disproportion in the collagen synthesis-degradation mechanism [13,20]. Some authors demonstrated otherwise that the storage of collagen in DIGO is the result of the inhibition of the collagen degradation process [21].

### *Objectives*

This study aimed to analyze the effects of pharmacological treatment with nifedipine on human gingival fibroblasts' activity, investigating the possible pathogenetic mechanisms that lead to the onset of gingival enlargement. The expression profile of 57 genes belonging to the "Extracellular Matrix and Adhesion Molecules" pathway, fibroblasts' viability at different drug concentrations, and E-cadherin levels in treated fibroblasts were assessed.

## **2. Materials and Methods**

### *2.1. Primary Human Fibroblast Cells' Culture*

Human gingival fibroblasts were purchased from ATCC<sup>®</sup> Cell Lines. Cells at the second passage were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Inc., St. Louis, MO, USA) supplemented with 10% fetal calf serum and antibiotics (penicillin 100 U/mL and streptomycin 100 micrograms/mL, Sigma Aldrich, Inc., St. Louis, MO, USA). Cells were replicated for subsequent experiments.

### *2.2. Cell Viability Test*

Human gingival fibroblasts were seeded into 96-well plates at a density of  $10^4$  cells per well containing 100  $\mu$ L of cell culture medium and incubated for 24 h to allow cell adherence.

A stock solution of nifedipine (Nifedipine, Meda Pharma Spa, Mylan, Italy) was prepared dissolving 1 mg of nifedipine powder in 1 mL of absolute ethanol.

The stock solution of Nifedipine (1 mg/mL) was dissolved in DMEM medium to prepare serial dilution at the concentrations of 5000, 2000, 1000, 500, and 100 ng/mL.

The vehicle absolute ethanol dissolved in the cell culture medium at the same concentration used to prepare the serial dilutions was used as a negative control.

After 24 h of incubation, cell viability was measured using PrestoBlue™ Reagent Protocol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, as previously described [22].

### 2.3. Cell Treatment

Once semi-confluence was reached (about 70% of confluence), cells were detached from flasks by trypsin-ethylenediaminetetraacetic acid (EDTA) and seeded at a density of  $1 \times 10^5$  cells/mL into 9 cm<sup>2</sup> (3 mL) wells. Cells were washed two times with phosphate-buffered saline (PBS) and incubated for 16 h at 37 °C with serum-free DMEM. After serum starvation, cells were treated for 24 h with 1000 ng/mL nifedipine solution prepared in DMEM supplemented with 2% FBS, antibiotics and amino acids. Absolute ethanol dissolved in the cell culture medium at the same concentration used to prepare the nifedipine solution treatment was used as a negative control.

Three biological replicates were performed.

Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. After the end of the treatment, cells were sub-confluent (about 90% of confluence) and RNA was extracted from the cells.

### 2.4. RNA Isolation, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells using the GenElute mammalian total RNA purification miniprep kit (Sigma-Aldrich) according to manufacturer's instructions. Pure RNA was quantified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

cDNA synthesis was performed starting from 500 ng of total RNA, using PrimeScript RT Master Mix (Takara Bio Inc.). The reaction was incubated at 37 °C for 15 min and inactivated by heating at 70 °C for 10 s.

cDNA was amplified by real-time quantitative PCR using the ViiA™ 7 System (Applied Biosystems, Foster City, CA, USA).

All PCR reactions were performed in a 20 µL volume. Each reaction contained 10 µL of 2x qPCR BIO SYGreen Mix Lo-ROX (Pcrbiosystems), 400 nM concentration of each primer, and cDNA.

Custom primers belonging to the "Extracellular Matrix and Adhesion Molecules" pathway were purchased from Sigma Aldrich. All experiments were performed including non-template controls to exclude reagents' contamination. PCR was performed including two analytical replicates.

The amplification profile was initiated by 10 min incubation at 95 °C, followed by two-step amplification of 15 s at 95 °C and 60 s at 60 °C for 40 cycles. As a final step, a melt curve dissociation analysis was performed [22–25].

### 2.5. Statistical Analysis

RPL13 is the reference gene used in this analysis and was selected by preliminary test among three housekeeping genes. The expression of RPL13 appeared more consistent with the amount of RNA input. Gene expression quantification was conducted with the delta/delta Ct calculation method [26] and RPL13 was used as a reference gene to normalize the gene expression levels. After normalization, quantification cycles of treated cells and control were compared by a paired sample *t* test. Mean expression levels of treated cells were calculated as fold changes relative to the expression of untreated cells with the delta/delta calculation method.

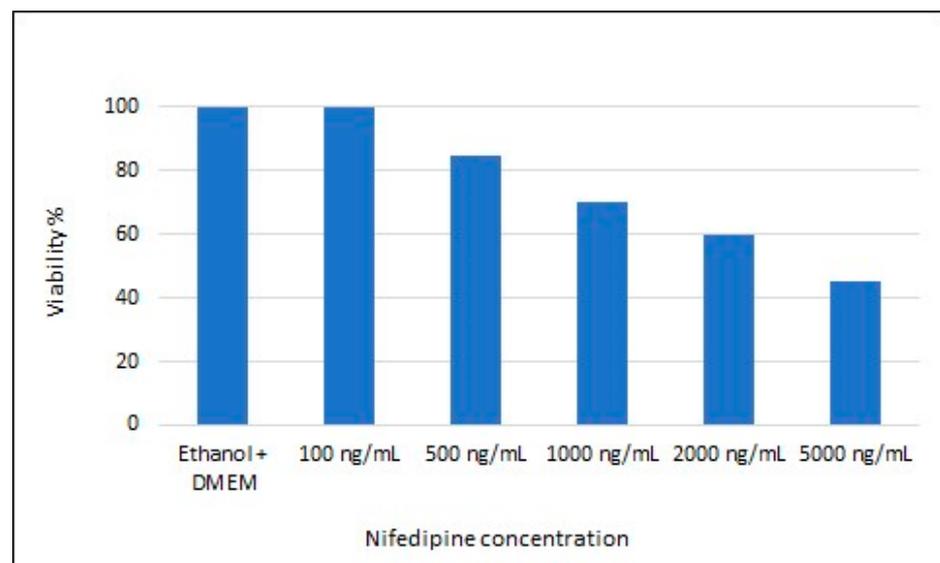
### 2.6. Detection of E-Cadherin Levels by Enzyme-Linked Immunosorbent Assay

E-cadherin levels were measured by sandwich enzyme-linked immunoassay (ELISA) after fibroblast treatment with nifedipine, by using a commercial kit, Human E-cadherin ELISA KIT (Bioassay Technology Laboratory, Shanghai, China), which uses monoclonal antibodies directed against distinct epitopes of human E-cadherin (E-Cad).

The plate has been pre-coated with human E-Cad antibody. Samples were added to these wells and binds to antibodies coated on the wells. Then biotinylated human E-Cad antibody was added and binds to E-Cad in the sample. Then, the secondary antibody streptavidin- horseradish peroxidase was added and binds to the biotinylated E-Cad antibody. After incubation, unbound streptavidin-HRP was washed away during the washing step. Substrate solution was then added, and color develops in proportion to the amount of human E-Cad. The reaction was terminated by addition of acidic stop solution and absorbance is measured at 450 nm by an automated microplate reader (Sunrise™, Tecan Trading AG, Switzerland). E-cadherin levels were expressed as ng E-Cad/ng of total protein.

### 3. Results

PrestoBlue™ cell viability test was conducted to determine the optimal concentration of nifedipine to be used for cell treatment that did not significantly affect cell viability. PrestoBlue™ data showed that nifedipine, regardless of treatment concentration, had no effect on cell viability compared to that of untreated cells (Figure 1). Considering the percentage of viability cells, the concentration of 1000 ng/ul was assumed as not lethal but sufficient to induce a significant response.



**Figure 1.** Nifedipine effect on cell viability.

Based on this test, the optimal concentration used for the treatment was 1000 ng/mL.

The gene expression profile of 57 genes belonging to the “Extracellular Matrix and Adhesion Molecules” pathway was analyzed using real-time PCR. Table 1 reports the list of genes and their fold change after treatment with nifedipine. Bold fonts indicate significant variation of gene expression level: fold change  $\geq 2$  and  $p$ -value  $\leq 0.05$  for upregulated genes, and fold change  $\leq 0.5$  and  $p$ -value  $\leq 0.05$  for significantly downregulated genes.

**Table 1.** Genes belonging to the “Extracellular Matrix and Adhesion Molecules” pathway analyzed using real-time Polymerase Chain Reaction. Fold changes represent the expression levels of genes after 24 h treatment with nifedipine, as compared with untreated cells ( $n = 3$ ). SD = standard deviation.

Gene	Fold Change	SD	Gene Function
CD44	0.64	0.08	Cell-Cell Adhesion
CDH1	<b>5.62</b>	2.06	Cell-Cell Adhesion
COL1A2	0.77	0.12	Collagens and Extracellular Matrix Structural constituent
COL2A1	0.57	0.15	Collagens and Extracellular Matrix Structural constituent
COL3A1	0.66	0.05	Collagens and Extracellular Matrix Structural constituent
COL4A1	0.85	0.14	Collagens and Extracellular Matrix Structural constituent
COL5A1	0.66	0.08	Collagens and Extracellular Matrix Structural constituent
COL6A1	0.90	0.17	Collagens and Extracellular Matrix Structural constituent
COL7A1	0.55	0.19	Collagens and Extracellular Matrix Structural constituent
COL8A1	0.92	0.09	Collagens and Extracellular Matrix Structural constituent
COL9A1	1.10	0.02	Collagens and Extracellular Matrix Structural constituent
COL10A1	1.54	0.06	Collagens and Extracellular Matrix Structural constituent
COL11A1	0.96	0.11	Collagens and Extracellular Matrix Structural constituent
CCTNA1	0.69	0.02	Cell Adhesion Molecule
CTNB	0.67	0.07	Cell Adhesion Molecule
CTNND2	0.96	0.30	Cell Adhesion Molecule
FN1	0.54	0.09	Cell Adhesion Molecule
HAS1	0.68	0.09	Transmembrane Receptor
ILF3	0.62	0.07	Transmembrane Receptor
ITGA1	0.95	0.10	Transmembrane Receptor
ITGA2	1.19	0.18	Transmembrane Receptor
ITGA3	0.88	0.15	Transmembrane Receptor
ITGA4	0.61	0.10	Transmembrane Receptor
ITGA5	0.70	0.03	Transmembrane Receptor
ITGA6	<b>0.41</b>	0.04	Transmembrane Receptor
ITGA7	1.48	0.31	Transmembrane Receptor
ITGA8	0.59	0.06	Transmembrane Receptor
ITGB1	0.87	0.09	Transmembrane Receptor
ITGB2	0.73	0.07	Transmembrane Receptor
ITGB4	<b>0.23</b>	0.03	Transmembrane Receptor
ITGB5	0.75	0.06	Transmembrane Receptor
LAMA1	<b>0.45</b>	0.04	Basement Membrane Constituent
LAMA2	1.06	0.19	Basement Membrane Constituent
LAMA3	0.64	0.08	Basement Membrane Constituent
LAMB1	<b>0.39</b>	0.02	Basement Membrane Constituent
LAMB2	0.75	0.10	Basement Membrane Constituent
LAMB3	0.88	0.11	Basement Membrane Constituent

Table 1. Cont.

Gene	Fold Change	SD	Gene Function
MMP2	0.73	0.10	Extracellular Matrix Protease
MMP3	0.90	0.08	Extracellular Matrix Protease
MMP7	1.03	0.42	Extracellular Matrix Protease
MMP8	<b>3.98</b>	1.47	Extracellular Matrix Protease
MMP9	1.04	0.35	Extracellular Matrix Protease
MMP10	1.24	0.05	Extracellular Matrix Protease
MMP11	<b>0.14</b>	0.05	Extracellular Matrix Protease
MMP12	1.20	0.13	Extracellular Matrix Protease
MMP13	1.14	0.19	Extracellular Matrix Protease
MMP14	0.96	0.07	Extracellular Matrix Protease
MMP15	1.90	0.08	Extracellular Matrix Protease
MMP16	<b>0.38</b>	0.04	Extracellular Matrix Protease
MMP24	<b>9.68</b>	1.05	Extracellular Matrix Protease
MMP26	<b>0.31</b>	0.01	Extracellular Matrix Protease
TGFB1	0.88	0.14	Transforming growth factor $\beta$ Signaling
TGFB2	1.20	0.12	Transforming growth factor $\beta$ Signaling
TGFB3	0.62	0.02	Transforming growth factor $\beta$ Signaling
TIMP1	0.76	0.11	Extracellular Matrix Protease Inhibitor
VCAN	0.75	0.03	Cell Adhesion Molecule
RPL13	1.00	0.00	Housekeeping gene

Table 2 reports only the significantly deregulated genes after nifedipine treatment.

**Table 2.** Significant gene expression levels after 24 h treatment with nifedipine, as compared with untreated cells ( $n = 3$ ).

Gene	Fold Change	SD	Gene Function
CDH1	5.62	2.06	Cell-Cell Adhesion
ITGA6	0.41	0.04	Transmembrane Receptor
ITGB4	0.23	0.03	Transmembrane Receptor
LAMA1	0.45	0.04	Basement Membrane Constituent
LAMB1	0.39	0.02	Basement Membrane Constituent
MMP8	3.98	1.47	Extracellular Matrix Protease
MMP11	0.14	0.05	Extracellular Matrix Protease
MMP16	0.38	0.04	Extracellular Matrix Protease
MMP24	9.68	1.05	Extracellular Matrix Protease
MMP26	0.31	0.01	Extracellular Matrix Protease

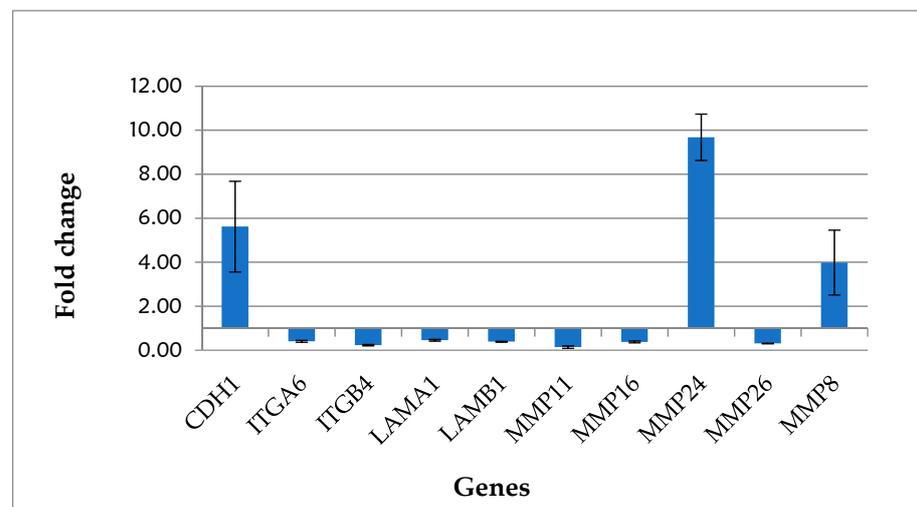
Genes significantly upregulated in treated cells with respect to the control were CDH1 (E-cadherin), belonging to cell adhesion genes, and MMP24 and MMP8, involved in extracellular matrix deposition.

The CDH1 (E-cadherin) levels measured by enzyme linked immunoassay (ELISA) after nifedipine treatment showed an increase of CDH1 levels (5.34-fold  $\pm$  0.19) in treated

fibroblasts vs. untreated control, confirming the gene expression results obtained by real-time PCR.

Among the significant downregulated genes induced by the treatment, there were transmembrane receptor ITGA6 and ITGB4, the basement membrane constituent LAMA1 and LAMB1, and the extracellular matrix protease MMP11, MMP16, and MMP26.

Figure 2 shows the gene expression profile of the significant deregulated genes belonging to the “Extracellular Matrix and Adhesion Molecules” pathway.



**Figure 2.** Gene expression profile of fibroblasts treated with nifedipine.

#### 4. Discussion

Gingival enlargement occurs as an adverse reaction in patients treated with calcium channel blocker agents (CCBs). It has been reported that DIGO is the most frequent periodontal side effect of CCBs, leading to mastication and speech problems, altering aesthetics, complicating oral hygiene maneuvers, and increasing the risk of bacterial infection [27]. CCBs take action in calcium metabolism: these drugs can decrease cellular  $Ca^{2+}$  influx and consequently reduce the uptake of folic acid, leading to a decreased synthesis of active collagenase, implicated in collagen degradation. Collagen degradation inhibition results in collagen accumulation [28,29]. In our study, fibroblasts were treated with nifedipine solution at a concentration of 1000 ng/mL for 24 h, and the obtained data showed a significant downregulation of genes coding for metalloproteinase (MMP) 11, 16, and 26. MMPs are enzymes involved in the connective tissue catabolic turnover, whose activation inhibition leads to connective tissue accumulation [30,31]. These results confirm that the increase of connective tissue collagen fibers induced by CCBs derives from the inhibition of collagen extracellular degradation pathways rather than from the increase in its production [21]. Downregulation was also observed for integrins ITGB4 and ITGA6, transmembrane cell-matrix adhesion receptors in fibroblasts that are responsible for collagen phagocytosis (collagen intracellular degradation pathway). The inhibition of MMPs expression occurs not only in nifedipine-induced GO, but it is also observable in gingival enlargement associated with immunosuppressants administration. The research of Lauritano et al. [32] recorded a downregulation of MMP8, MMP11, MMP15, MMP16, and MMP24 in gingival fibroblasts treated with cyclosporine and mycophenolate mophetil solutions. Many other authors demonstrated that anticonvulsant and immunosuppressant drugs are able to reduce the expression of  $\alpha$  and  $\beta$  integrins or to interfere with collagen adhesion, thanks to pro-inflammatory cytokines action and, consequently, altering collagen phagocytosis mechanisms [33–35]. Kataoka et al. demonstrated, in particular, that the lack of collagen degradation, in a sample of 20-day-old rats treated with nifedipine, was associated with the storage of Type I collagen [36]. According to Kim et al. [37], nifedipine-induced gingival enlargement is characterized by an increase in Transforming

growth factor  $\beta$  signaling, known as a mediator of fibrotic processes and able to regulate extracellular matrix remodeling and inflammatory response [36,38]. The authors of this study demonstrated that TGF- $\beta$ , in nifedipine-induced GO, causes an upregulation of gingival fibroblasts periostin, a matricellular protein involved in functional and structural regulation of connective tissue [39]. TGF- $\beta$  also stimulates fibroblastic population and ECM deposit of fibronectin and glycosaminoglycans [40]. According to the literature, interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 have been demonstrated to play a pathogenic role in drug-induced gingival fibrosis [12,41]. The authors of our study also assessed CDH1 (E-cadherin) level by performing an enzyme-linked immunoassay (ELISA): an increase of its levels was recorded in treated fibroblasts vs. untreated control, confirming the gene expression results obtained by real-time PCR. Cadherins 1 are calcium-dependent cell adhesion proteins and are involved in mechanisms regulating cell–cell adhesion, mobility, and proliferation of epithelial cells [42]. This data is in contrast with those provided by Kantarci et al. [43], who studied the effect of phenytoin, cyclosporine, and nifedipine administration on gingival tissues' basement membranes. Kantarci et al. [43], according to other authors [44], demonstrated that DIGO shows signs of the “epithelial to mesenchymal transition” (EMT) process, in which epithelial cells differentiate into fibrogenic fibroblast-like cells, losing epithelial cell–cell or cell–ECM contacts and migrating into connective tissue. This fibrotic enlargement is supported by TGF- $\beta$ 1, that stimulates collagen accumulation and deposition by fibroblasts, by increasing the expression of connective tissue growth factor (CTGF) and that leads to the EMT phenomenon.

## 5. Conclusions

Gingival overgrowth has been proven to be an adverse reaction in patients taking the CCB agent nifedipine. Enzyme-linked immunoassay (ELISA) and the expression profile analysis of 57 genes belonging to the “Extracellular Matrix and Adhesion Molecules” pathway in treated fibroblasts highlighted increased CDH1 levels and a downregulation of MMP11, MMP16, and MMP26, and of integrins ITGB4 and ITGA6, respectively. This data suggested that the pathogenesis of nifedipine-induced GO is characterized by an excessive accumulation of collagen due to the inhibition of collagen intracellular and extracellular degradation pathways.

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