Transcriptomic Profiling Explains Racial Disparities in Pterygium Patients Treated With Doxycycline

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Purpose. To understand the differential responses to doxycycline between Caucasian and Hispanic patients observed in a previous clinical trial.

Methods. Primary cultures were established using pterygia excised from male Caucasian ($n = 3$) and Hispanic ($n = 6$) patients. The response of these cells to doxycycline was tested in a toxicity assay. In addition, a complete transcriptome was obtained from the nine samples, and the results were analyzed using false discovery rate statistics. Results were confirmed by quantitative RT (qRT)-PCR and Western blotting for a limited set of genes.

Results. Caucasian pterygium cells underwent apoptosis upon exposure to doxycycline, whereas Hispanic cells survived the treatment. Transcriptomic analysis showed profound differences between cells of both ethnicities, even before treatment, implicating important cellular pathways such as the mitochondrial oxidative phosphorylation chain, the proteasome, and the components of the extracellular matrix. Following exposure to doxycycline, there was a significant increase in proapoptotic proteins, regulators of the cell cycle, and components of the mitochondrial membrane in Caucasian cells but not in their Hispanic counterparts. There was a good correlation between data obtained by ultra-sequencing and those generated by qRT-PCR or Western blotting.

Conclusions. The lack of response to doxycycline observed in Hispanic pterygium patients in a previous clinical trial can be explained by the genetic protection afforded to the cells in this ethnic background against apoptosis and cell death. New therapeutic options must be devised for these patients.

Keywords: doxycycline, pterygium, racial disparities, transcriptomic analysis

Pterygium is a common eye disease characterized by abnormal growth of conjunctival and/or limbal cells that migrate toward the cornea, accompanied by an increase in fibrotic material and angiogenesis, and causing obvious vision distortions.[1–3] The pathogenesis of pterygium is not completely understood, but it seems that exposure to ultraviolet light, age, and sex play important roles[4–6]; moreover, human papilloma virus infection may be a cofactor in susceptible hosts.[7] Current treatment of the disease includes surgical removal of the lesion followed by conjunctival or limbal autografts in the presence of different adjuvants,[8] yet the recurrence rate remains high.[9] Experimental antiangiogenic therapies are being used but, so far, the results have been inconclusive.[10–12]

In a recent article, it was shown that doxycycline (Vibravenosa; Pfizer, Alcobendas, Spain), a common oral antibiotic, was able to dramatically reduce pterygium-like lesions in a mouse model through inhibition of matrix metalloproteinases and angiogenesis.[13] Further investigation using massive transcriptome sequencing revealed that doxycycline affects pterygium cells by regulating up to 332 genes involved in mitochondrial metabolism, endoplasmic reticulum stress response, and pathways related to integrins, extracellular matrix components, and growth factors.[14] A phase II clinical trial showed that oral doxycycline was able to reduce pterygium size in Caucasians but not in patients of Hispanic ethnicity.[15]

We decided to study the cause of this differential response to doxycycline by using whole-genome transcriptomic techniques to explore new pharmacological approaches to overcome the lack of response in Hispanic pterygium patients.

Materials and Methods

Patients and Primary Culture

Use of pterygium specimens was approved by our Institutional Review Board (Comité Ético de Investigación Clínica de La Rioja). All patients provided written informed consent, and specimens were handled in accordance with Declaration of Helsinki regulations. Nine male patients who underwent surgical removal of their primary pterygia were recruited. These included three Caucasian (76.7 ± 1.1 years of age) and six Hispanic (39.0 ± 8.2 years of age) patients. Excised pterygia were transferred to the laboratory for establishing primary cell cultures as described previously.[14] Briefly, each pterygium specimen was minced into small pieces and cultured for 3 days in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal calf serum, 200 mM L-glutamine, 0.5% DMSO, and 1% penicillin/streptomycin/amphotericin (all culture reagents are from Invitrogen, Carlsbad, CA, USA). Once the cells had migrated from the explants and attached to the dish, the medium was changed to keratinocyte-serum free medium

TABLE. TaqMan Probes (Invitrogen) Used for the Quantification of Gene Expression by qRT-PCR

Gene Family	Gene	TaqMan Catalog Number
ER stress	*PPIB*	Hs00168719
	HSPA5	Hs00946084
Cell cycle	*TP53*	Hs01034249
Oligotransferases	*SEC61A1*	Hs01037684
Cytokines	*VEGFA*	Hs00900055
	IL6	Hs00985639
Housekeeping gene	*GAPDH*	Hs99999905

Thermocycler parameters were as follows: 10 minutes of denaturation at 95°C, followed by 40 cycles at 95°C for 15 seconds and for 1 minute at 60°C. Values were interpolated into a standard curve to calculate absolute expression. These values were then divided by the expression level of GAPDH in the same samples to correct for potential pipetting errors.

with 5% fetal calf serum and 1% penicillin/streptomycin/amphotericin. These cells have been previously characterized as fibroblast-like cells due to their immunoreactivity to vimentin and to the expression of fibroblast genetic markers.[14] When cells reached 80% confluency, they were passed to fresh dishes using 0.25% trypsin. Medium was renewed every 2 to 3 days and replaced with fresh medium 24 hours before treatment of 50, 200, or 500 μg/mL doxycycline or the same volume of vehicle (PBS) for 24 hours. All experiments were performed with cells at passages 3 to 5.

Toxicity Assay

Responses to doxycycline of primary cultures of pterygium cells from Hispanic or Caucasian patients were studied in 96-well plates as described previously.[16] Cells were seeded at a density of 25,000 cells/well and allowed to attach to the substrate for 4 hours. After that, 500 μg/mL doxycycline or the same volume of vehicle (PBS) was added to the wells, and cell numbers were estimated after 24 hours by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega, Madison, WI, USA). Results are presented as percentage of viability over untreated controls.

RNA Extraction and Transcriptomic Analyses

Total RNA was isolated from pterygium cell cultures using TRIzol (Invitrogen), purified using the RNeasy mini-kit (Qiagen, Valencia, CA, USA), and treated with DNase I (Qiagen) following the manufacturer's instructions. Complementary DNA (cDNA) library preparation and ultrasequencing were performed according to the manufacturer's protocols and using their reagents (Illumina, San Diego, CA, USA) as described previously.[14] Briefly, the integrity and quality of total RNA were assessed with an automated electrophoresis system (Experion; Bio-Rad, Hercules, CA, USA). Then, mRNA was isolated from 1 μg of total RNA using poly-T oligonucleotide-attached magnetic beads. This mRNA was fragmented into approximately 200 base pair (bp) pieces by using divalent cations under elevated temperature. Cleaved RNA fragments were reverse transcribed into first-strand cDNA using reverse transcriptase and random primers. Next, the second strand was synthesized using DNA polymerase I and RNase H. These double-stranded cDNA fragments were end-repaired by T4 DNA polymerase and Klenow DNA polymerase, phosphorylated by T4 polynucleotide kinase, and ligated to Illumina indexing adapters. These adapter-tagged libraries were amplified by using 15 cycles of PCR with DNA polymerase (Phusion; Finnzymes Reagents, Vantaa, Finland) and

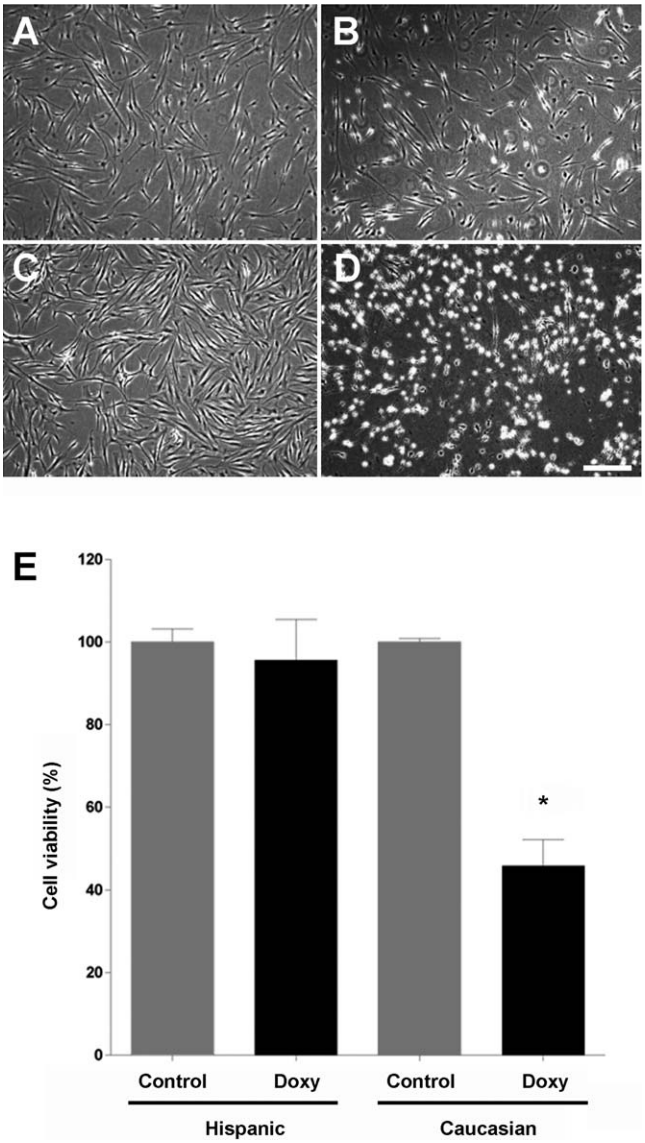

FIGURE 1. Phase-contrast views of pterygium cells obtained from Hispanic (**A**, **B**) or Caucasian (**C**, **D**) patients treated with PBS as a control (**A**, **C**) or with 500 μg/mL doxycycline (**B**, **D**) for 24 hours. (**D**) Dead cells can be distinguished by their round shape. (**E**) Quantification of the same experiment from the MTT assay. *Asterisk*: Statistically significant differences from PBS-treated controls ($P < 0.01$). *Scale Bar*: 100 μm.

validated and quantified by electrophoresis and quantitative PCR (qPCR). Pools of six indexed libraries were mixed (multiplexed) at equimolar ratios to yield a total oligonucleotide mixture concentration of 10 nM. Finally, the resulting libraries were sequenced with the Genome Analyzer IIx platform (Illumina) to generate 150-bp single reads. Six pooled indexed libraries were sequenced in each flow cell lane.

Data Treatment and Statistical Analysis

Raw sequence data in FASTQ software (http://sourceforge.net/projects/maq, in the public domain) format were processed through a number of sequential steps[14] including adapter removal, mRNA alignment to DNA, gene expression quantification using the Tuxedo software suite (Oracle Corporation, Redwood Shores, CA, USA), and selection of differentially

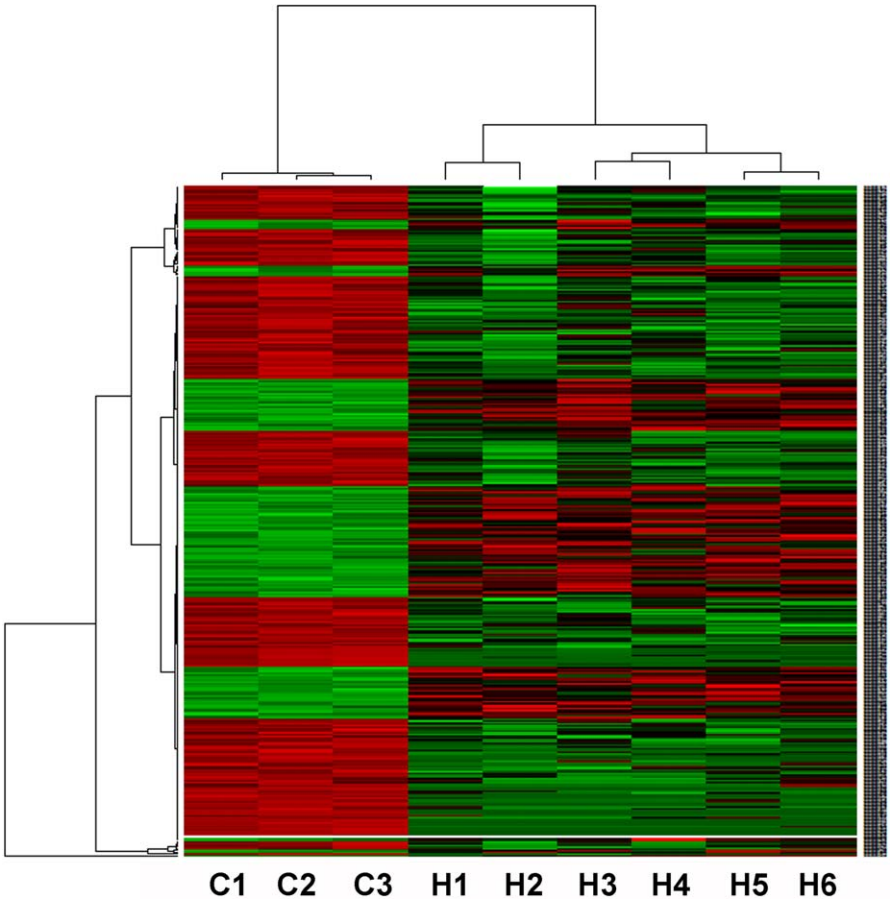

FIGURE 2. Hierarchical clustering (Manhattan plot) of the nine samples (*columns*) and the 3000 genes (*rows*) which showed larger differences between races. Notice that pterygium cells from Caucasian (C1–C3) or Hispanic (H1–H6) origin cluster together. *Red* indicates high expression levels, and *green/black* designate low expression levels.

expressed loci using the false discovery rate (FDR)-based significance analysis of microarrays (SAM) method. Resulting data were submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, in the public domain) under accession numbers GSE34736 and GSE58441.

The genes whose expression levels were most regulated by doxycycline for each racial group with a P value of <0.005 and an FDR lower than 0.2 were represented as heatmaps by using the Manhattan method for dissimilarity and the Ward method for hierarchical clustering, calculated in an R software environment (www.r-project.org, in the public domain). The Reactome FI cytoscape plugin (Reactome database; www.reactome.org, in the public domain) and the GeneMania cytoscape plugin (GeneMania; University of Toronto, Toronto, ON, Canada; www.genemania.org, in the public domain) were used to identify enriched pathways and networks regulated by doxycycline treatment in both racial backgrounds.[17]

qRT-PCR

Gene products whose expression levels were significantly different between the two were chosen for further confirmation. Complementary DNA was synthesized by reverse transcription of 1 μg of total RNA, using the SuperScript III first-strand synthesis kit (Invitrogen) in a total volume of 20 μL according to the manufacturer's instructions and was amplified by qRT-PCR by using TaqMan probes (Invitrogen; Table) with a model 7300 real time-PCR system (Applied Biosystems, Carlsbad, CA, USA), and their gene expression levels were calculated using absolute quantification by interpolation into a standard curve.[14] All values were divided by the expression value of the house keeping gene GAPDH to avoid potential pipetting errors.

Western Blotting

Some of the pterygium cells treated with doxycycline or PBS, as described above, were processed for protein analysis. Cells were homogenized in lysis buffer (PBS, pH 7.4, containing complete protease inhibitor cocktail [Roche, Madrid, Spain]) and centrifuged to eliminate solid debris. Total protein in the supernatants was calculated with the BCA kit (Pierce, Rockford, IL, USA). A 5× SDS sample buffer (Invitrogen) was added to all samples. Extracts were boiled for 10 minutes and run on standard 10% SDS polyacrylamide gels (Invitrogen). The antibodies tested in this study were as follows: mouse anti-CCAAT-enhancer-binding protein homologous protein (CHOP; Abcam, Cambridge, UK), 1:1000 dilution; rabbit anti-TIMP2 (Abcam), 1:1000 dilution; rabbit anti-TIMP3 (Abcam), 1:1000 dilution; and mouse anti-β-tubulin (Sigma-Aldrich Corp., St. Louis, MO, USA), 1:40,000 dilution. Peroxidase-conjugated goat anti-rabbit (1:6000 dilution) or donkey anti-mouse (1:30,000 dilution) antibodies were from Cell Signaling Technology (Danvers, MA, USA) and Jackson Immunoresearch Laboratories (West Grove, PA, USA), respectively. Peroxidase activity was detected with enhanced chemiluminescence (GE Healthcare,

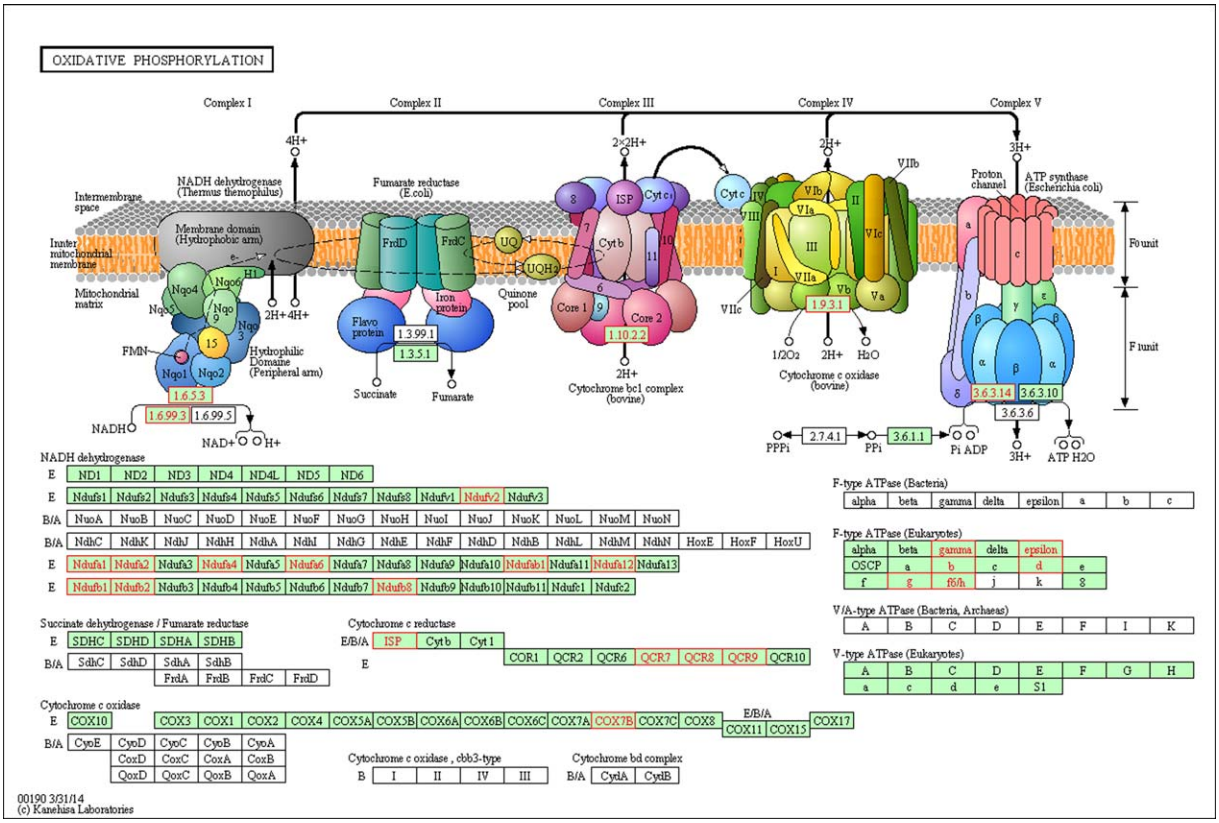

Figure 3. An inner mitochondrial membrane and the proteins involved in the process of oxidative phosphorylation. Proteins in *red* are expressed at higher levels in Caucasian than in Hispanic pterygium cells and include critical components of NADH dehydrogenase, cytochrome c reductase, cytochrome c oxidase, and even the F-type ATPase. Reprinted with permission from Kyoto Encyclopedia of Genes and Genomes (KEGG). Copyright 2013, KEGG, Kyoto, Japan.[38,39]

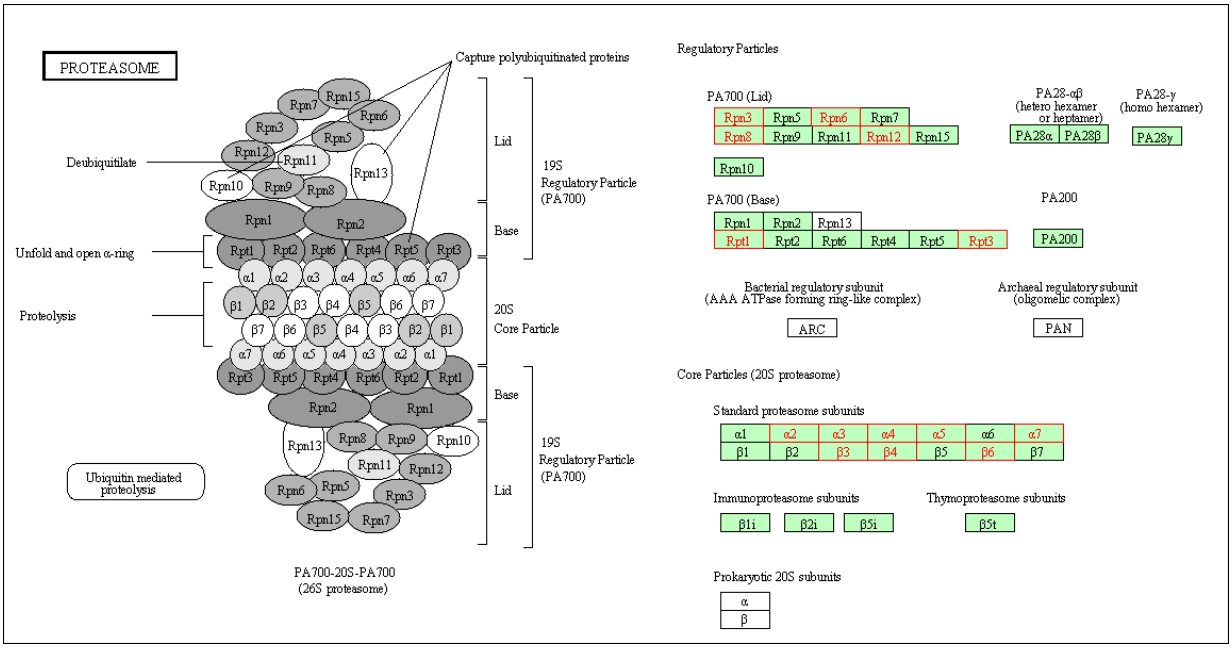

Figure 4. The proteasome and the proteins involved in the process of ubiquitin-mediated proteolysis. Proteins in *red* are expressed at higher levels in Caucasian than in Hispanic pterygium cells and include important components of the lid, base, and core of the proteasome complex. Reprinted with permission from Kyoto Encyclopedia of Genes and Genomes (KEGG). Copyright 2013, KEGG, Kyoto, Japan.[38,39]

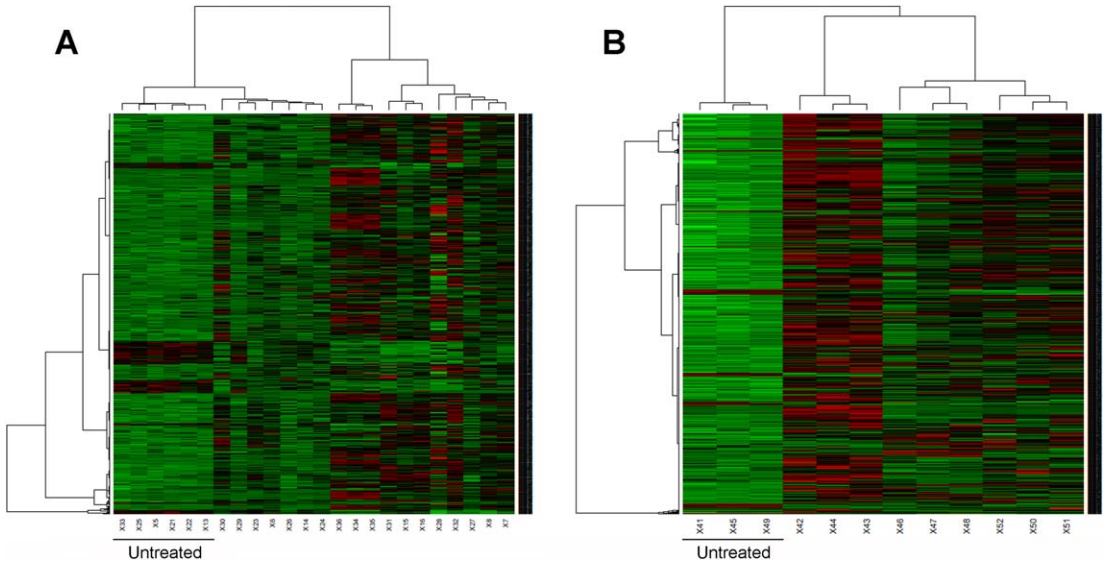

FIGURE 5. Hierarchical clustering (Manhattan plot) of the Hispanic (**A**) and Caucasian (**B**) pterygium cells treated with and without doxycycline. With an FDR = 0.2 cut-off there are 839 differentially expressed genes in Hispanics and 785 genes in Caucasians. *Red* indicates high expression levels, and *green/black* designate low expression levels.

Buckinghamshire, UK) and subsequently exposed on x-ray films (GE Healthcare). All experiments were repeated at least three times.

RESULTS

Pterygium Cell Sensitivity to Doxycycline in Primary Cultures

Primary cultures were successfully established from surgical specimens of pterygia obtained from three Caucasian and six Hispanic patients. When these cells were treated with doxycycline, we observed a behavior that confirmed previous descriptions in a clinical trial,[15] with cells of Hispanic origin not responding to doxycycline (Figs. 1A, 1B, 1E), whereas cells of Caucasian origin suffered apoptosis and cell demise (Figs. 1C–E).

Hispanic and Caucasian Pterygium Cells Express Different Genes Even Before Treatment

The complete transcriptomes of the nine pterygium specimens were analyzed, and important differences in gene expression were observed between cells of the two races. At an FDR cutoff of 0.2, 5342 genes were found to be differentially expressed between the races. A heatmap of the 3000 most differentially expressed genes (Fig. 2) shows a clear genetic signature differentiating Caucasian from Hispanic samples. To investigate which cellular pathways were differentially expressed, ultrasequencing results were analyzed with specialized software (Genemania; Reactome) and shown with Kyoto Encyclopedia of Genes and Genomes (KEGG). The three pathways best represented among the genes that showed the largest variations between races were (1) those genes expressing mitochondrial proteins involved in the oxidative phosphorylation cascade (Fig. 3); (2) those regulating the proteasome (Fig. 4); and (3) genes related to extracellular matrix (collagen, laminin, and tenascin). In addition, many ribosome-encoding genes and some cytoskeleton-related genes were also included. TIMP2 and TIMP3 were more highly expressed in Hispanic than in Caucasian cells.

Doxycycline Treatment Alters Different Pathways in Hispanic and Caucasian Cells

All pterygium cells were exposed to three concentrations of doxycycline for 24 hours, and their transcriptomes were compared to untreated cells. Heatmaps of differentially expressed genes in treated or untreated Hispanic (Fig. 5A) and Caucasian (Fig. 5B) cells showed a large number of genes whose expression was modulated by doxycycline (839 in Hispanics and 785 in Caucasians). When these genes were organized into pathways, there were some pathways which were regulated in the same way in both racial backgrounds. These included vesicle targeting, protein folding, endoplasmic reticulum stress, and some mitochondrial components such as cytochrome c oxidase subunits. Other pathways were statistically significantly activated by doxycycline in Caucasians but not in Hispanics, including regulatory proteins of the execution phase of apoptosis (BCL2L1, CHOP, FADD, FXN, NKX3-1, HTRA2, TRAF2, ZNF205, ZNF622, and others), cell cycle arrest (CDK9, CDK10, CDK20, HRAS, MYC, NKX3-1, PSMA4, PSMA5, PSMD8, PSME2, RRP8, GAK, CCNB1IP1, and others), and proteins of the mitochondrial membrane (AK2, BNIP3, CCDC90A, CHCHD3, CHCHD4, COX18, DHODH, FLCN, FXC1, HSPA9, LONP1, MRPL18, NDUFS8, TIMM44, TMEM70, and others). On the other hand, there were some pathways that were statistically significantly upregulated in Hispanics but not in Caucasians. This happened with genes involved in tRNA synthesis (CARS, GARS, KARS, SARS, WARS, YARS, and others) and some genes involved in protein N-linked glycosylation (ALG2, ALG3, ALG5, ALG12, EDEM1, GANAB, MPI, OSTC, PMM2, ST6GALNAC4, SYVN1, and others).

Confirmation of Differential Expression by qRT-PCR and Western Blotting

To confirm some of the results obtained by ultrasequencing, qRT-PCR was performed with specific probes, providing very parallel results (Fig. 6). In fact, a correlation between qRT-PCR and ultrasequencing values rendered a Pearson's coefficient $r^2 = 0.87$. Some relevant proteins such as CHOP, TIMP2, and TIMP3 were also studied in parallel by ultrasequencing and

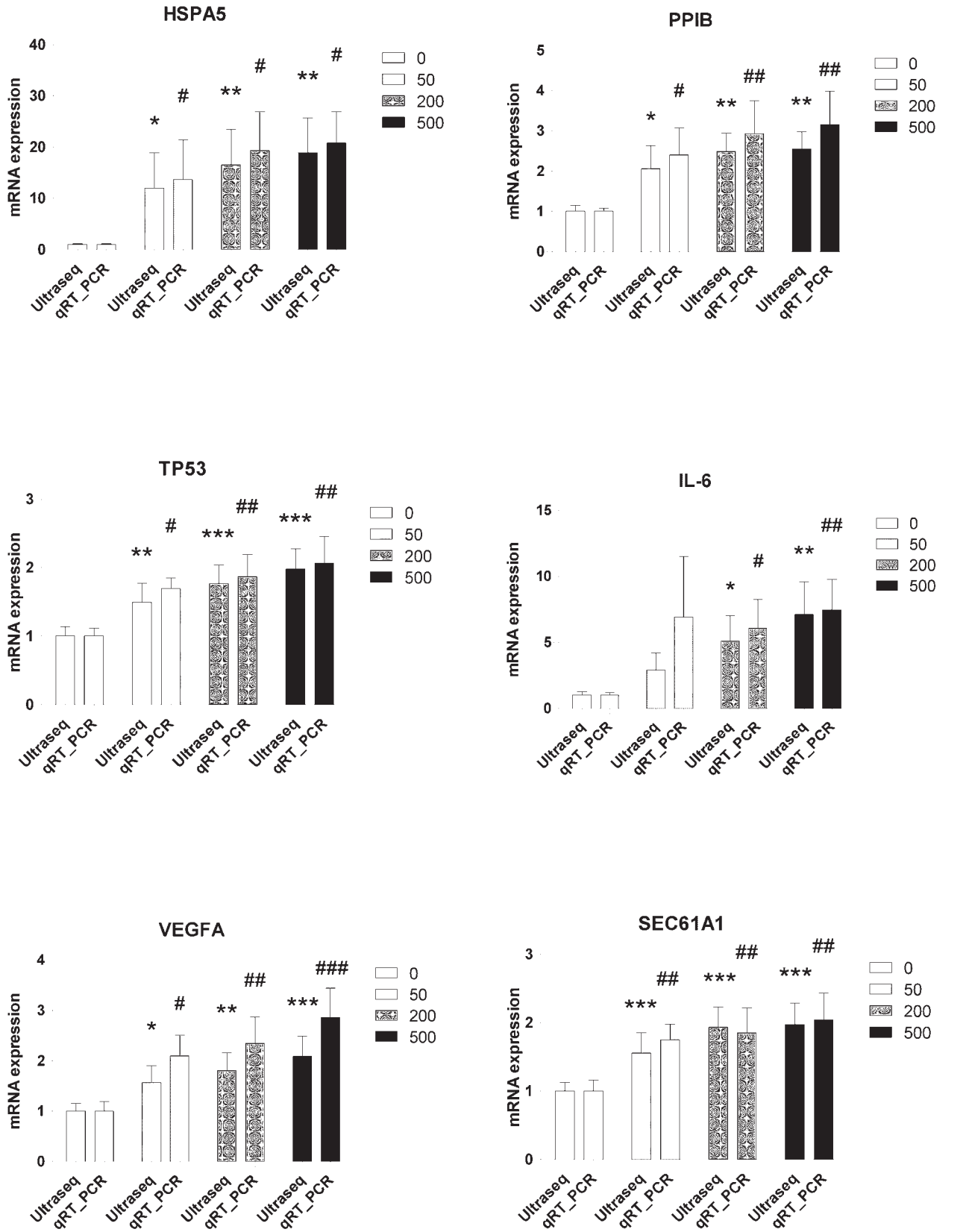

FIGURE 6. Gene expression quantification by ultrasequencing and qRT-PCR for six sample genes whose expression was regulated by the treatment (0, 50, 200, and 500 μg/mL doxycycline). *Bars*: means ± SEM of the nine samples used in the study. *Symbols*: statistically significant differences from PBS-treated controls (* or #: $P < 0.05$; ** or ##: $P < 0.01$; *** or ###: $P < 0.001$).

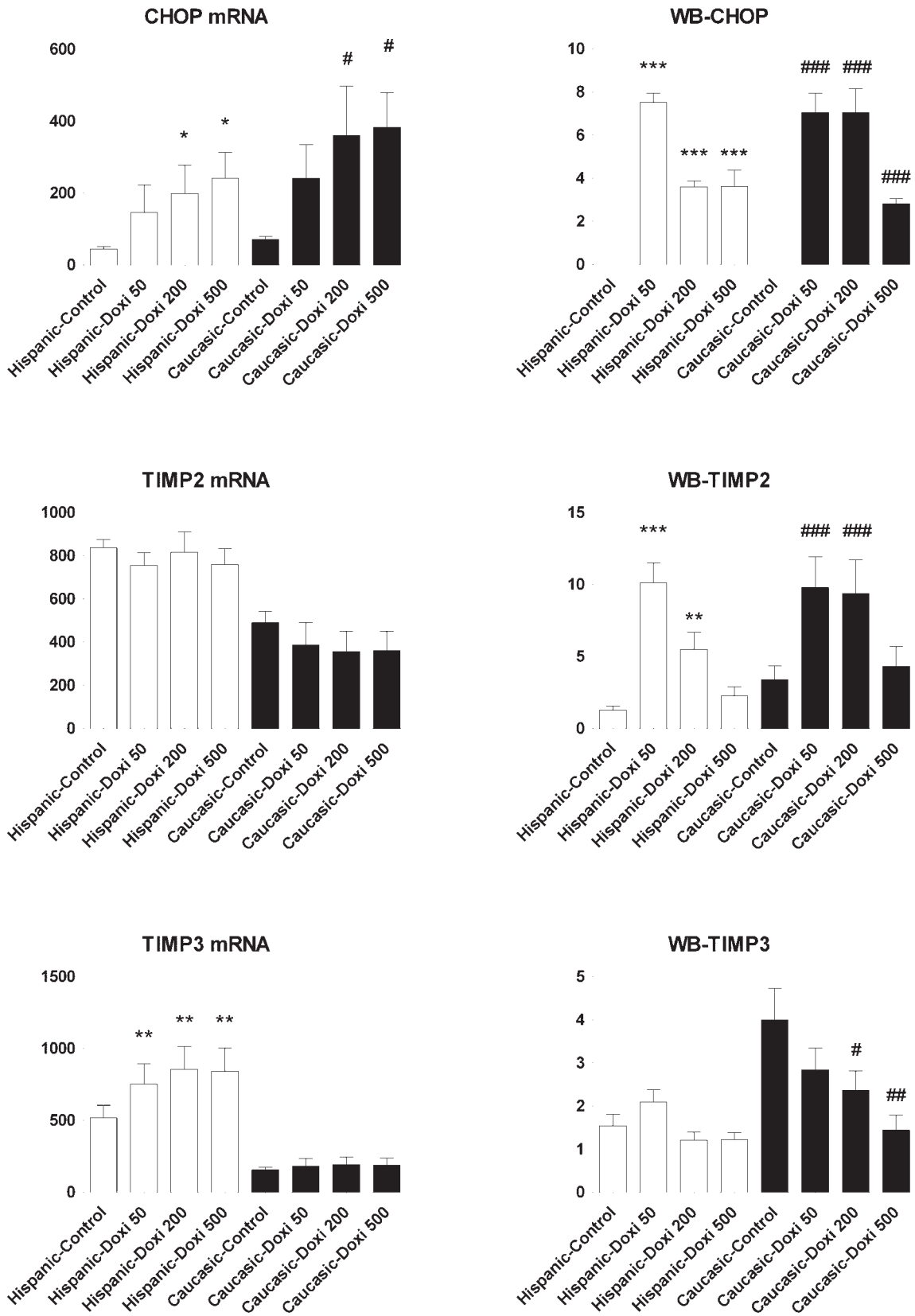

FIGURE 7. Comparison of ultrasequencing (mRNA) values (*left*) and Western blotting (WB [*right*]) for CHOP, TIMP2, and TIMP3. CHOP mRNA shows a dose-dependent increase due to doxycycline treatment, which is higher in Caucasian cells than in Hispanic counterparts. This also can be appreciated in the Western blot. TIMP2 and TIMP3 mRNA expression levels are higher in Hispanic cells, but the protein levels are higher in Caucasian cells. Tubulin was used as a housekeeping protein. *Bars*: mean ± SEM of the six to eight samples used in the study. *Symbols*: statistically significant differences from PBS-treated controls (* or #: $P < 0.05$; ** or ##: $P < 0.01$; *** or ###: $P < 0.001$).

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Western blotting (Fig. 7). Interestingly, TIMP2 and TIMP3 mRNAs were lower in Caucasian cells (Figs. 7B, 7C), but the proteins behaved in the opposite way (Fig. 7E, 7F).

Discussion

According to the out-of-Africa theory of human migration, Caucasian and Hispanic populations are almost as distant from each other as is possible, both geographically and genetically.[18] Obviously, following the colonization of America by Europeans in the 15th century, a certain degree of genetic admixture has occurred,[19] yet there is a high level of genetic variation between these two populations. It has been known for quite some time that pterygium has a high prevalence among Hispanics and Latinos[20,21] and that these patients present with a high recurrence rate,[9,22] but many authors have attributed these characteristics to higher exposure to ultraviolet (UV) light and a larger dedication to outside activities.[4,5] In view of our results, we propose that most of these differences may have a genetic origin, although we cannot preclude environmental influences.

One of the shortcuts of the present report is the wide age differences between the two racial groups, with Caucasian patients being much older than the Hispanic ones. Nevertheless, this is a faithful representation of the clinical reality found in ophthalmologic services across Spain. Hispanic patients requiring pterygium excision in this context are always younger. Until now, we blamed a longer exposure to UV light during their childhood/youth in their countries of origin, although now we know that deep genetic differences may be at the root of this observation. In contrast, Caucasian patients (Spain's nationals in this case) tend to develop pterygia at a more advanced age and with a much lower incidence; thus, it was impossible to obtain age-matched pterygia for this racial group.

In our present study, we have shown that Caucasian pterygium cells in culture respond to doxycycline treatment by becoming apoptotic, whereas Hispanic cells do not. This confirms our previous observations in a clinical trial,[15] but at that time, we did not know whether the observed disparities were due to different rates of doxycycline transport from the blood to the cornea or to other pharmacodynamic factors. Now we can affirm that pterygium cells are intrinsically different between these two populations and that the lack of response in the Hispanics cells are due to genetic reasons.

When the genetic backgrounds were studied in untreated cells, we found a great number of genes (5342 genes) with significantly different expressions between the races. These included genes with very important roles in maintaining cell homeostasis, such as those involved in the oxidative phosphorylation chain, the proteasome, or the extracellular matrix. Of course, these three pathways are paramount in triggering either apoptosis or cell survival.[23,24] Two of the genes that were differentially expressed were TIMP2 and TIMP3. Although we did not find a metalloproteinase (MMP) gene altered when comparing races, TIMP mRNAs were higher in Hispanic cells, although when investigated by Western blotting, TIMP proteins were higher in Caucasian cells.

The importance of MMPs and TIMPs in pterygium pathogenesis has been known for some time, especially as they relate to invasion and migration of the advancing cells.[25–27]

The analysis of gene expression after doxycycline treatment was very informative. First, we found some pathways that were activated in both races and which coincided with previous studies.[14] However, more interesting were those pathways that became activated in one race or the other. One of these included the genes involved in tRNA synthesis, which is upregulated in Hispanic cells but not in Caucasian ones. The mechanism of action by which doxycycline acts on prokaryotic cells includes targeting bacterial aminoacyl-tRNA synthetase.[28] Accepting a similar mechanism in eukaryotes, Hispanic pterygium cells would be more protected against the drug because they produce more active enzymes.

Apoptosis regulation is one of the major mechanisms by which doxycycline reduces pterygium growth.[14] In this study, we found many regulatory proteins of the execution phase of apoptosis that are upregulated by doxycycline in Caucasian cells but not in Hispanic ones. For instance, ZNF205 (also known as Rhit) is related with the generation of intracellular oxidative stress through the production of H_2O_2.[29] This molecule is upregulated in Caucasians following application of doxycycline, but its expression does not change in Hispanics. Therefore, Caucasian cells would be more sensitive to oxidative stress and initiation of the mitochondrial apoptotic cascade. The proteasome is also related to apoptosis and oxidative stress.[30] As happens with so many other genes, critical components of the proteasome are upregulated upon doxycycline treatment in Caucasians but not in Hispanics, making the former more susceptible to apoptotic cell death.

The cell cycle is another obvious pathway whose regulation may result in differential cell survival. Here we saw that several cyclin-dependent kinases (CDK9, CDK10, CDK20) and proto-oncogenes (MYC, HRAS) were upregulated in doxycycline-treated Caucasian cells but not in their Hispanic counterparts.

Other genes overexpressed in Caucasian but not in Hispanic cells included MT2A, a molecule that inhibits cell growth through apoptosis, G2/M arrest, and negative regulation of NFκB,[31] and many components of the chemokine family such as CXCCL1, CXCL1, CXCL2, CXCL3, and CCL5. Chemokines have been described previously as molecules that are upregulated in pterygium compared with normal conjunctiva[24] and can act as chemoattractants for neutrophils, monocytes, and lymphocytes, thus fighting the excessive growth of pterygium cells.[32]

This is not the first time that differential pharmacological responses have been described depending on the patient's race. Some examples include pharmacokinetic changes,[33] varying tolerance to the drug,[34] or different side effects.[35] Nevertheless, this is the first time this has been reported for pterygium.

Other studies have also found racial disparities as an explanation for the prevalence of pterygium.[36,37] These differences have often been explained by variations in lifestyle, but given our results, those studies should be revisited, looking for potential genetic diversity. The present study also underscores the importance of including patients from different ethnic backgrounds in clinical trials, as a drug can be active in some people but not in others.

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