DEVELOPMENT AND CLINICAL ASSESSMENT OF A COMPREHENSIVE PRODUCT FOR PIGMENTATION CONTROL IN MULTIPLE ETHNIC POPULATIONS

Elizabeth T. Makino BS CCRA MBA, Kuniko Kadoya PhD, Monya L. Sigler PhD, Peter D. Hino MD FAAD, and Rahul C. Mehta PhD
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Elizabeth T. Makino BS CCRA MBA, a Kuniko Kadoya PhD, a Monya L. Sigler PhD, b Peter D. Hino MD FAAD, b and Rahul C. Mehta PhD a

aSkinMedica, Inc., An Allergan Company, Irvine, CA
bThomas J. Stephens & Associates, Inc., Richardson, TX

ABSTRACT

Background: Pigmentary changes in people of different ethnic origins are controlled by slight variations in key biological pathways leading to different outcomes from the same treatment. It is important to develop and test products for desired outcomes in varying ethnic populations.

Objectives: To develop a comprehensive product (LYT2) that affects all major biological pathways controlling pigmentation and test for clinical efficacy and safety in different ethnic populations.

Methods: A thorough analysis of biological pathways was used to identify ingredient combinations for LYT2 that provided optimal melanin reduction in a 3-D skin model. Expression of four key genes for melanogenesis, TYR, TYRP-1, DCT, and MITF was analyzed by qPCR. Clinical study was conducted to compare the efficacy and tolerability of LYT2 against 4% hydroquinone (HQ).

Results: Average melanin suppression by LYT2 in 7 independent experiments was 45%. All four key genes show significant down-regulation of expression. LYT2 provided statistically significant reductions in mean overall hyperpigmentation grades as early as week 2 compared to baseline, with continued significant improvements through week 12 in all ethnic groups tested.

Conclusion: We have successfully combined management of 6 categories of pathways related to melanogenesis: melanocyte activation, melanosome development, melanin production, melanin distribution, keratinocyte turnover, and barrier function to create a comprehensive HQ-free product. The outcome clearly shows greater pigmentation control with LYT2 compared to other HQ-free products in skin tissue models and earlier control in clinical studies compared to 4% HQ. Clinical study shows pigmentation control benefits of LYT2 in people of Caucasian, Hispanic, and African ethnic origins.


INTRODUCTION

Skin conditions related to hyperpigmentation continue to contribute to psychosocial anxiety that leads to poor quality-of-life measures.1,2 Pigmentary changes are the first sign of aging in a majority of population, and they are self-described by patients using various expressions including dark spots, blotches, scars, dirty skin, stains etc. The desired outcome of pigment correction in these patients is to obtain skin with homogeneous pigmentation. In some cultures, dark skin represents a lower socioeconomic status, encouraging people to seek skin bleaching options for otherwise normal healthy skin. Regardless of the reasons, pigment correction remains one of the most sought after goals of aesthetic treatments worldwide.

Melanin, in various forms and distribution pattern, is the primary pigment responsible for constitutive human skin color.3 It is synthesized by melanocytes at the base of the epidermis and transported to keratinocytes via dendrites using a series of complex steps. One of the primary functions of epidermal melanin is to provide protection against the constant onslaught of solar radiation. More than 375 different genes involved in pigmentation processes have been identified to date, and research is underway to further understand their role.4

In order to create a product to address the multitude of skin hyperpigmentation issues, it is important to understand the chemistry, biology, and genetics of melanin production and distribution in all ethnicities, which would allow rational combination of ingredients for correcting a wide range of pigimentary conditions.

Melanocyte Activation

Keratinocytes, melanocytes and fibroblasts secrete a number of cytokines in response to environmental damage and stress. α-Melanocyte stimulating hormone (αMSH)/melanocortin 1 receptor (MC1R); endothelin-1 (ET-1); Endothelin B receptor (ET-B); and stem cell factor (SCF)/c-KIT receptor, have been shown to substantially contribute to melanogenesis. Overexpression of these key markers is consistently observed in hyperpigmented skin compared to even-toned skin.5 Proopiomelanocortin (POMC), is a precursor of αMSH6 which binds to MC1R causing up-regulation of microphthalmia transcription factor (MITF). MITF functions
as a master transcription factor upregulating expression of pigmentation-related genes tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and Dopachrome tautomerase (DCT/TYRP2). MC1R also plays a critical role in pigment-independent UV protection by stimulation of antioxidant defense and DNA repair. ET-1 mediated activation of ET-B receptor affects melanosome formation and maturation and MITF activation. Furthermore, environmental damage can result in local inflammation releasing inflammatory cytokines which modulate MITF activity via pathways such as prostaglandins, TNFα, and defensins. Activation of plasmin (PLG) stimulates melanogenesis via mechanisms preferentially affecting women.

Melanocytes are generated from melanocyte stem cells (McsCs) in the hair follicles. Wingless-related integration site (Wnt) proteins secreted by fibroblasts modulate melanocyte proliferation via Wnt/β-catenin signaling pathway by activating McScs. Dickkopf 1 (DKK1) is an inhibitor of Wnt signaling and reduces Wnt-mediated MITF activation leading to reduced melanogenesis. SCF via c-KIT signaling also promotes McScs into epidermal melanocytes. Protease-activated receptor-2 (PAR-2) plays an important role in inducing SCF secretion from keratinocytes.

Melanosomes Development
Melanosomes are subcellular lysosome-derived organelles where melanin is synthesized, stored, and transported. They impart color to skin and provide photoprotection. There are four stages in the melanosome development process. Stage I pre-melanosomes are non-pigmented vesicles derived from the endosomal system. Pre-melanosomal protein (Pmel-17) and melanoma antigen recognized by T cells 1 (melan-A/MART1) are critical structural proteins present at this stage. Stage II melanosomes are characterized by intraluminal fibrillar amyloid sheets formed by Pmel17. Delivery of melanogenic enzymes TYR, TYRP1, and DCT/TYRP2 initiate melanogenesis and maturation to stage III melanosomes. Distribution of these key melanogenic enzymes to the correct melanosomes is controlled by adaptor complex proteins (AP1, AP3) and biogenesis of lysosome-related organelles complex (BLOC1, BLOC2). Melanin deposition on the fibers of stage III melanosomes continues until all internal structures are masked in stage IV melanosomes.

Melanin Synthesis
Dark and light eumelansins and reddish-brown pheomelansins are the primary determinants of visible pigmentation. The enzyme TYR catalyzes the initial rate-limited step of converting tyrosine into Dopachrome, the precursor of melanins. Incorporation of cysteine forms cysteinylDOPA which forms pheomelansins. Dopachrome, which forms either light brown DHI or black insoluble DHI melanin. Two key properties of melanin are absorption of UV and visible light and providing antioxidant properties due to the presence of unpaired electrons. DHI melanin is a stronger absorber of light whereas DHICA melanin is a stronger antioxidant. The ratio of pheomelansins, DHI eumelanin and DHICA eumelanin may be critical in providing the full spectrum of visible skin color and antioxidant protection.

Melanosome Transfer
Melanin synthesized in melanocytes and stored in melanosomes is transferred to keratinocytes. Each melanocyte uses dendrites to contact about 36 keratinocytes in the basal and supra-basal layers to transfer melanin. This epidermal melanin unit contains keratinocytes that are actively dividing and regenerating. Pigmented melanosomes translocate to keratinocyte dendrites via motor proteins such as Rab27a by transfer to keratinocytes via multiple proposed mechanisms. Two of the most supported mechanisms of transfer include shedding individual pigmented melanosome cores or pigment-ed melanosome-rich globules into extracellular space followed by phagocytosis by keratinocytes. The role of PAR-2 in the phagocytosis process is well established. DKK1 suppresses PAR-2 activity thereby reducing melanosome transfer. The final step is to distribute pigmented melanosomes within keratinocytes. Dark skin contains large individual melanosomes with dark eumelanin distributed throughout the cytosol. Light skin contains smaller melanosomes in clusters with light melanin predominantly accumulating as a cap over the nucleus.

Keratinocyte Differentiation and Desquamation
As pigmented keratinocytes undergo terminal differentiation, their melanosomes degrade. Melanosome structure is not detected in cornocytes, except rarely in very dark skin. Autophagy-related

**TABLE 1.**

| Mediators of Melanogenesis During Different Stages, (-) Indicates an Inhibitory Effect on Melanogenesis, All Others Produce an Increase in Pigmentation |
|-----------------------------|-----------------------------|
| **Melanocyte Activation** | **Melanosome Development** |
| POMC                        | Pmel-17                     |
| αMSH, ASP (-)               | MART1/melan-A               |
| MC1R                        | AP1, AP3                    |
| MITF                        | BLOC1, BLOC2                |
| ET-1                        | Melanin Synthesis           |
| SCF                         | TYR                         |
| Wnt                         | DCT/TYRP2                   |
| DKK1 (-)                    | Rab27a                      |
| PAR-2                       | PAR-2                       |
| PGE2                        | DKK1 (-)                    |
| β-Defensin 3 (-)            |                             |

protein 7 (ATG7) appears to be a key protein inducing autophagy and degradation of melanosomes. A more common approach to remove existing melanin is desquamation which stimulates keratinocyte turnover removing pigmented keratinocytes. Enzymes transglutaminase (TGM) and kallikreins (KLK) stimulate epidermal turn-over and barrier repair. Cellular markers involucrin (INV) and filaggrin (FLG) are upregulated during barrier repair.

Development of a Product to Control Pigmentation Regardless of its Origin

Table 1 lists some of the important pathways contributing to melanogenesis based on the literature review presented here. Combination of ingredients (Table 2) that can modulate many of the key pathways were used to create a comprehensive product to address multiple targets. Pigment reducing activity of products were tested in a 3-dimensional tissue culture model before conducting a controlled split-face clinical study comparing efficacy of the test product to 4% hydroquinone in patients of different ethnic origin and different pigmentation type.

### MATERIALS AND METHODS

**Tissue Culture**

The in vitro study was conducted using epidermal equivalent MelanoDerm® MEL-300-B from MatTek Corp. (Ashland, MA, USA),

<table>
<thead>
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<th>TABLE 2.</th>
<th>Key Ingredients in LYT2 and Other Commercial Products With Potential Biological Pathways Affected by Them</th>
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<td>Melanocyte Activation</td>
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<tr>
<td>LYT2</td>
<td>PLG, PGE2</td>
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<tr>
<td>Tranexamic Acid</td>
<td>POMC</td>
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<tr>
<td>Tetrapeptide-30</td>
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<td>Vitis Vinifera SCE</td>
<td>Wnt, KKK1, ET-1</td>
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<td>Hydroxyacetophenone</td>
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<tr>
<td>Phenylethyl Resorcinol</td>
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<td>PER</td>
<td>Phenylethyl Resorcinol</td>
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<tr>
<td>Undecylenoyl Phenylalanine</td>
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<td>Aminopropyl Asc Phosphate</td>
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<td>Leucine</td>
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<tr>
<td>HAK</td>
<td>PLG, PGE2</td>
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<td>4-Methox K Salicylate</td>
<td>TYR</td>
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<td>LUC</td>
<td>Prunus yedoensis</td>
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<td>2-O-Ethyl Ascorbic Acid</td>
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<tr>
<td>APC</td>
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<td>Ellagic Acid</td>
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<td>Salicylic Acid</td>
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which consists of normal human-derived epidermal keratinocytes with melanocytes from Black donor. Tissues were cultured with maintenance medium, MEL-NMM-113 (MatTek Corp) containing KGF, bFGF and α-MSH, for 14 days. Medium was changed every other day and at the same time 2 µL of testing formulas, 25µL of 2% kojic acid (positive control) or water (negative control) were applied on the tissues after washing with Dulbecco PBS.

**Melanin Quantification**

At the end of the study, three tissues from each treatment were washed, freeze-thawed, and submerged in 1% sodium-bicarbonate and placed into microcentrifuge tubes with SolvableTM (Tissue and Gel Solubilizer 0.5 M, Packard BioScience Co.) and incubated overnight at 95°C. Next day, they were centrifuged and supernatant was measured with 490 nm wavelength to quantify melanin. Total protein was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and used to normalize melanin concentrations. Results were calculated as percentage of negative control.

**Quantitative Real-Time PCR**

Six tissues from each treatment were collected for qPCR (Quantitative real-time PCR) analysis. mRNA was extracted using RNaqueous Total RNA Isolation Kit (Thermo Fisher Scientific). qPCR analysis was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). The expression level of TYR, TYRP1, DCT, and MITF were analyzed using TaqMan Gene Expression Assays (Thermo Fisher Scientific).

**Histology**

Tissues were fixed overnight with 10% NBF and embedded into paraffin blocks. Samples were sectioned and stained with Fontana Masson (American Mastertech Scientific). Images were captured using digital image scanner Nanozoomer (Hamamatsu). Three samples from each treatment were collected for histological analysis.

**Clinical Study Design**

A randomized, double-blind comparison study was conducted to assess the efficacy and tolerability of a comprehensive HQ-free and retinol-free test product (LYT2) and another retinol-containing prototype (Serum 2) against the positive control 4% hydroquinone cream (4% HQ; Bi-Coastal Pharma International, LLC). Criteria for study participation included female subjects aged 30-65 years, presenting moderate to severe facial hyperpigmentation (as determined by a grade of 4-9 on the overall hyperpigmentation scale) on both their left and right facial sides. Subjects were not allowed to apply any other topical products, nor oral products known to affect the appearance of hyperpigmentation or skin aging throughout the duration of the study.

Institutional Review Board approval (IntegReview IRB, Austin, Texas) was obtained prior to conduct of any study procedures. The conduct of the study followed all applicable guidelines for the protection of human subjects for research as per 21 CFR 50, in accordance with accepted standards for Good Clinical Practices.
FIGURE 4. Down-regulation of TYR, TYRP1, DCT and MITF gene expression was observed with LYT2 compared to negative control.

(GCP) and International Conference on Harmonization (ICH). All subjects provided informed consent prior to study participation.

All subjects were randomized to apply LYT2 on their left or right facial side. Of these subjects, half were randomized to apply 4% hydroquinone (4% HQ) on their other facial side, and the remaining half applied Serum 2 on their other facial side. Subjects were also provided with a basic skin care regimen (cleanser, oil-free moisturizer, and SPF 30 sunscreen) during the study. Study visits occurred at baseline, weeks 2, 4, 8, and 12.

Clinical efficacy was assessed at all study visits by the investigator who was blinded to the treatment randomization of the subject. The investigator evaluated the left and right facial sides using the following grading scales:

**Overall Hyperpigmentation:**
None (Score of 0): Best possible condition; even skin color, no hyperpigmentation.

Mild (Score of 1, 2, or 3): Few to several brown spots with increased pigmentation; they are small in size and slightly darker than surrounding skin.

Moderate (Score of 4, 5, or 6): Many brown spots with increased pigmentation; they are medium in size and much darker than surrounding skin.

Severe (Score of 7, 8, or 9): Many large brown spots with increased pigmentation; they are large in size and markedly darker than surrounding skin.

Global Improvement:

- 0=No change or worsening
- 1=Mild improvement (~25% overall improvement)
- 2=Moderate improvement (~50% overall improvement)
- 3=Marked improvement (~75% overall improvement)
- 4=Complete clearing/Dramatic improvement (~95%+ overall improvement)

The investigator assessed tolerability at all visits (erythema, dryness/scaling) on a four-point scale where 0=none, 1=mild, 2=moderate and 3=severe. Subjects were also asked to assess tolerability (burning/stinging, itching, tingling) on the same four-point scale for the left and right facial sides. Adverse events were captured during the study.

Standardized digital photographs of the left and right facial sides were taken at all visits using the VISA/A-CR photo system (Canfield Imaging Systems, Fairfield, New Jersey) with a Canon Mark II 5D digital SLR camera (Canon Inc., Tokyo, Japan). Prior to photography, subjects cleansed their face and equilibrated in the clinic for at least fifteen minutes. The RGB digital images were then analyzed for L* (brightness) in a target location on the left and right facial sides.

Subjects completed a self-assessment questionnaire at all follow-up visits (weeks 2, 4, 8, and 12) regarding self-perceived efficacy for each facial side and product attributes.

**RESULTS**

**Melanin Suppression in Human Tissue Model**

Average melanin suppression by LYT2 in 7 independent experiments was 45.0±7.66%. (Figure 1, Negative control = 0%). This reduction in melanin by 2 μL of LYT2 was comparable to that of 25 μL of positive control (53.14±7.8% for 2% kojic acid, a TYR inhibitor), demonstrating the superior ability of the product designed for comprehensive melanin control vs product containing TYR inhibitor alone. Histological observation showed great reduction of melanin accumulation in melanocytes and keratinocytes with LYT2 compared to negative control. No cytotoxicity was observed with LYT2 indicating that melanin reduction is in fact due to suppression of melanocyte activity and decreased dendrite formation and not due to melanocytolysis (Figure 2). It is important to recall that hydroquinone (HQ), one of the most commonly used prescription treatments for hyperpigmentation, decreases melanin partly due to its melanocytolytic action.29

Melanin suppression study was repeated to compare activity of LYT2 to other HQ-free and retinol-free commercial products. The results (Figure 3) show that comprehensive approach of LYT2, outlined in Tables 1 and 2, leads to greater melanin reduction than partial suppression by products designed to manipulate a few, but not all, critical pigmentation pathways.
Suppression of Key Genes in Melanogenesis Pathways
Expression of four key genes for melanogenesis, TYR, TYRP-1, DCT, and MITF was analyzed by qPCR (Figure 4). 2 μL LYT2 resulted in reduced expression of MITF by 54%, as compared to 64% with 25 μL positive control. Expression of TYR, TYRP-1, and DCT also showed significant down-regulation, strongly indicating that multiple pathways of melanogenesis are affected by LYT2. Taken together, these in vitro results demonstrate that LYT2 regulates multiple genes in melanogenesis.

Determination of Clinical Efficacy and Safety of LYT2
Forty-five female subjects aged thirty-one to sixty-five years old (mean age of 50 years) with Fitzpatrick Skin Types III-V were enrolled in the twelve week clinical study. Subjects identified

FIGURE 6. Improvement in appearance of PIH in female subject, age 40, Fitzpatrick Skin Type V in standard lighting. (A) Baseline, (B) 2 weeks, (C) 8 weeks.
FIGURE 7. Improvement in intensity and appearance of dark patches in female subject, age 54, Fitzpatrick Skin Type IV in standard lighting. (A) Baseline, (B) 12 weeks.

as African American (44.4%), Hispanic (26.7%), Asian (6.7%), or Caucasian (22.2%) ethnicities. Forty-three subjects completed the study (LYT2=43; Serum 2=23; 4% HQ=20): one subject discontinued due to treatment-related adverse event and the other subject was lost to follow-up. The results presented herein will focus on the comparison between LYT2 and 4% hydroquinone.

At baseline, subjects’ facial sides randomized to the LYT2 and 4% HQ treatment groups presented with mean overall hyperpigmentation grades of 5.47 and 5.10, respectively. LYT2 provided statistically significant reductions in mean overall hyperpigmentation grades as early as week 2 compared to baseline, with continued significant improvements through week 12 (all P<0.001; Wilcoxon signed-rank test). The 4% HQ-treated group did not show significant reductions until week 4 compared to baseline, with continued significant reductions through week 12 (all P<0.001; Wilcoxon signed-rank test). Figure 5A shows mean percentage of baseline hyperpigmentation for LYT2 and 4% HQ treated subjects at all visits. Comparing treatment groups, LYT2 approached significantly greater reductions compared to 4% HQ at week 8 (P<0.07; mixed model on ranks). Figure 5B-D shows the clinical efficacy of LYT2 compared to HQ in sub-groups based on subject ethnicity; African American, Hispanic, and Caucasian sub-groups are presented. Both treatments were well-tolerated with mean scores for all tolerability parameters remaining below mild at all study visits.

LYT2 and 4% HQ were highly-rated by subjects on self-perceived efficacy in the following parameters (% of subjects agree strongly or agree): “decreased the appearance of uneven skin tone discolorations” (LYT2=84%; 4% HQ=75%), “improved the evenness of my skin tone” (LYT2=91%; 4% HQ=90%), and “helped to fade brown spots on my skin” (LYT2=84%; 4% HQ=80%). Mean global improvement scores at the end of treatment were greater for LYT2 treated group as compared to the 4% HQ treated group (1.76 vs. 1.50, respectively). Objective
image analysis for L* (brightness) showed consistent significant increases in L* at all follow-up visits (weeks 2, 4, 8, and 12) for those subjects treated with LYT2 (all P<0.02; paired t-test).

Figure 6 and Figure 7 show representative standardized digital photographs of African American and Hispanic subjects, treated with twice-daily use of LYT2.

**DISCUSSION**

Changes in skin pigmentation can be elicited within days (UV exposure), weeks (Post-Inflammatory Hyperpigmentation), months (Long Lasting Pigmentation) or years (sun/age spots).\(^4\) Differences in chemical structure, rate of synthesis, concentration and distribution of melanin as well as changes in melanocyte function and skin architecture are all responsible for the variety of hyperpigmentary skin conditions. In order to develop a comprehensive product that works on multiple types of pigmentary conditions in people of various ethnic origins, we must utilize the knowledge of complex biology of melanogenesis. TYR inhibition is one of the key intervention points of any successful pigmentation product; however, it alone is not sufficient to provide maximal pigmentation control. We have successfully combined management of 6 categories of pathways related to melanogenesis: melanocyte activation, melanosome development, melanin production, melanin distribution, keratinocyte turnover, and barrier function to create a comprehensive HQ-free product. The outcome clearly shows greater pigmentation control compared to other HQ-free products in skin tissue models and earlier control in clinical studies compared to 4% HQ.

Limited studies have been conducted comparing efficacy to HQ in people of various ethnic origins and Fitzpatrick Skin Types. The majority of studies are conducted in Caucasian and/or Fitzpatrick Skin Types I-III. Since a growing body of research has shown innate differences in the biochemical processes of melanogenesis between people of different ethnic origins, this study enrolled subjects with broader Fitzpatrick Skin Types which allowed us to conduct sub-group analysis of data in people of different ethnic origins. The Fitzpatrick Skin Type designation categorizes a patient's skin based on their response to sun exposure; those in the higher Fitzpatrick Skin Type range have a stronger ability to protect against sunlight (ie, rarely burns), due to the type and distribution of melanin present in their skin. However, these differences also make treatment of hyperpigmentation disorders a greater challenge in these populations. This clinical study shows benefits of LYT2 in people of Caucasian, Hispanic and African ethnic origins. Additional studies are underway in light and dark Asian origin populations and in subjects with hyperpigmentation caused by different conditions (PH, melasma, etc.) to complete the assessment of the benefits of a product designed for comprehensive pigmentation control.

**REFERENCES**


**DISCLOSURES**

Drs. Sigler, Hino are investigators for Allergan plc. Drs. Mehta, Kadoya and Ms. Makino are employees of Allergan plc, Irvine, CA.


**AUTHOR CORRESPONDENCE**

Rahul C. Mehta PhD

E-mail: rahul.mehta@allergan.com