Scientific Investigation and Clinical Summary: 
Age Intervention® Dark Circle Eye Defense

Aimed at Improving the Appearance of Dark Circles, Peri-Ocular Wrinkles and Skin Texture

INTRODUCTION
Dark under-eye circles are one of the most common cosmetic concerns today. The causes are varied but most commonly include vascular changes and leakage, discoloration from blood breakdown, iron deposition staining, inflammation, thinning skin and uneven texture. To address under-eye circles, a product ideally needs to address all of these conditions. Research has shown that the factors contributing to under-eye circles are addressable. Enzymes that break down blood are produced by fibroblasts and keratinocytes in the skin and are inducible. Inorganic iron deposits can be chelated to improve removal. Inflammation can be reduced by reducing bilirubin and iron. Skin texture can be restructured with matrikins and retinol. This paper explores whether these factors can be simultaneously addressed in a nightly application eye cream.

METHODS
Four in-vitro studies evaluated the efficacy of peptides, chelating agents and other significant ingredients. Two additional in-vivo studies – on 22 and 20 subjects, respectively – investigated the efficacy of products on the appearance of dark eye circles and wrinkles. The first examined a base formula applied 2X per day. The second examined the same base formula plus retinol and Microsponge® technology to improve product delivery and achieve once-a-day results.

RESULTS
Quantitative and qualitative measurements demonstrated significant improvement after 60 days of product usage. Results showed the subjects’ appearance improved dramatically based on the following metrics (measured %, dermatologist assessment %): Decreased under-eye discoloration (100%, 100%), wrinkle reduction (100%, 95%), increased smoothness (100%, 90%) and increased elasticity (100%, 80%). Subject satisfaction was high at 90%.

CONCLUSION
A single nightly application of Jan Marini Skin Research’s Age Intervention Dark Circle Eye Defense effectively reduces the appearance of dark under-eye circles and wrinkles and improves smoothness and elasticity.
A group of enzymes that helps break down blood is referred to as uderine diphosphate glucurononyltransferases, or UGTs. In specific, bilirubin is broken down by UGT1A1. While primarly produced in the liver, UGT1A1 is also expressed by cells in the skin including both keratinocytes and fibroblasts (2)(3).

Interestingly, the expression of these enzymes changes based on the necessary level of activity, meaning that expression of the enzyme is inducible, or can be modified (4) (2). This leads to the possibility of increasing the base expression of the enzyme, thus increasing the ability of the skin to quickly break down blood leakage.

Iron, accumulated in tissue as a result of blood breakdown, creates insoluble deposits of inorganic ferric hydroxides that are very slow to eliminate from tissue. In addition, bilirubin and inorganic iron create oxidative stress, inducing a pro-inflammatory situation leading to a self-perpetuating inflammatory cycle of vasodilatation and increased leakage (5).

Thin skin in the eye region exacerbates the problem and the appearance of dark circles. Skin around the eye is one of the thinnest areas on the face. Typical thickness ranges from as little as 1-2mm to as much as 3-4mm. Gravity and the loss of both collagen and elastin associated with aging further thins the skin around the eye, leading to increased wrinkles and uneven texture which increases the visibility of underlying vascular discoloration. Subsequently, discoloration is most often visible around the eye. Matrikin peptides Pal-GHK (Palmitoyl Oligopeptide) and Pal-GQPR (Palmitoyl Tetrapeptide-7) enhance cutaneous restructuring, (6) decreasing the fragility of the skin around the eyes while strengthening the dermal matrix supporting the microvasculature.

The product transport mechanism and texture on the skin can be improved using Microsponge® technology. This technology incorporates microscopic particles that can be infused with the active product. These microscopic particles are then deposited on the skin during application and continue delivering the product slowly over time to increase penetration, reduce irritancy, and provide longer lasting benefits than a typical nightly cream.

This paper summarizes research and testing from multiple studies (5) (7), including both in-vivo and in-vitro testing, to determine the efficacy of ingredients in Jan Marini Skin Research’s Age Intervention Dark Circle Eye Defense and the effect of the product in clinical use.

**METHODS**

**In-Vitro Test 1 – Iron Chelation Efficacy**

N-Hydroxysuccinimide (NHS) was tested for its efficacy as an iron chelating agent. Testing was performed using Ferrospectral (MERCK), which turns a violet color in the presence of unbound iron. N-Hydroxysuccinimide was tested vs. a non-binding control, Gluc, and a known binding control Desferrioxamine.

**In-Vitro Test 2 – Iron Chelation Concentration**

The nominal concentration of NHS to eliminate iron from blood was determined using water samples with an iron concentration equivalent to the iron content of 100mL of blood. Increasing concentrations of the chelating agent were evaluated using Ferrospectral and compared to a water sample with an equivalent iron concentration and no Ferrospectral. The nominal concentration was determined as the minimum percentage necessary to fully bind the iron.

**In-Vitro Test 3 – Chrysin induction of UGT**

UGT expression was determined indirectly by measuring mRNA induction using the quantitative RT-PCR test. HepG2 cells were used for the test due to their ability to express all forms of UGT, including UGT1A1. Cells were cultured in a Williams medium in the presence, or absence, of Chrysin for three days. The cell medium was replaced every 24 hours. Cells were lysed at the end of the three day period, mRNA was extracted and RT-PCR testing was conducted.

An internal amplification control, using a gene considered stable and not involved in the action of the test substance, was systematically run for each sample. In this case, the gene GAPDH was used. The amplicons (amplification products resulting from RT-PCR) were quantified directly, as they were detected, on the RT-PCR system before being subjected to electrophoresis to illustrate the results obtained.

A pair of specific probes was selected for the UGT1A1 gene, which codes for synthesis of the bilirubin glucuroconjugation enzyme (8).

**In-Vitro Test 4 – Reduction in Inflammation**

Inflammation reduction is expected due to the effects of iron chelating and increased expression of UGT1A1 to assist in the breakdown of bilirubin. Prostaglandins, which are conventionally considered mediators of inflammatory stress, especially following UV exposure, were used to determine the probable reduction in inflammation.

Human keratinocytes and fibroblasts were cultured in an appropriate medium in the presence of various concentrations of peptides and chelating agents for 24 hours. The cells were then transferred to a product-free medium and exposed to a pro-inflammatory dose of UVB radiation at a dosage of 35mJ/cm2 for fibroblasts and 30mJ/cm2 for keratinocytes.

Following irradiation, the cells were post-incubated for 24 hours in either medium alone or in medium containing various concentrations of product. Results were compared against a known positive control medium, aspirin. PGE2 release into the culture medium after 24 hours was determined using an ELISA method.

**In-Vivo Test 1 – Placebo Control Trial**

A placebo controlled test using a cream based solution with and without a 2% blend of Chrysin, NHS, Palmitoyl...
Oligopeptide, and Palmitoyl Tetrapeptide-7 was conducted on 22 female subjects with a mean age of 32.7 +/- years.

A standardized digital photographic system with a computerized positioning template was used for before and after images. This enabled faithful repositioning and superimposition of the ring zones for photographic assessment. Image processing software captured Red (R) Green (G) and Blue (B) color parameters and converted them to L, a* and b* for analysis. Photographs were taken twice with a two day interval, and at the beginning and end of the test. Results were assessed using the mean scores from both images.

Volunteers applied a different product to each half of the face. The placebo cream was applied to the left side of the face and the blend of Chrysin, NHS, Palmitoyl Oligopeptide, and Palmitoyl Tetrapeptide-7, was applied to the right side. Applications were conducted twice daily for 56 days. Each subject acted as her own control. Voluntary UV or sun exposure sessions were prohibited for the duration of the study. The volunteers were asked not to drink/party the evening prior to each photography session. All photographs were taken without makeup.

In-Vivo Test 2 – Jan Marini Skin Research’s Age Intervention Dark Circle Eye Defense
In-Vivo Test 2 was conducted using the final formulation of Jan Marini Skin Research’s Age Intervention Dark Circle Eye Defense with the peptides tested in the prior tests plus retinol and Microsponge® delivery technology. Twenty female subjects applied product under both eyes once per day prior to bed for 60 continuous days. Subjects were evaluated every 15 days for the duration of the study (day 0, 15, 30, 45 and 60). Subject inclusion criteria included: peri-orbital wrinkles, under-eye circles, no eye product use for two months prior to enrollment and absence of any skin disease.

Changes in the under-eye color were assessed clinically by a dermatologist (principal investigator) and measured by determining the change in red (a*) and blue (b*) color components in a digital image. Images were taken using a KODAK EASY SHARE DX6490 camera (© Eastman Kodak Company, 2003 – Kodak and EasyShare are trademarks of Eastman Kodak Company) using an image resolution of 2304 x 1728 pixels. Red (-a*) and blue (-b*) components of the skin were measured using dedicated image analysis software.

Skin quality and texture were also evaluated qualitatively and quantitatively. Physician assessment measured skin smoothness, elasticity (compactness) and wrinkles.

Quantitative measurements evaluated skin elasticity, skin profilometry, skin micro-wrinkles, skin smoothness, and wrinkle volume. Changes in skin elasticity were measured using a cutometer (CUTOMETER® MPA 580).

Results of the study indicated that the blend of peptides, Chrysin, NHS, Palmitoyl Oligopeptide, and Palmitoyl Tetrapeptide-7, had significant effects on the appearance of dark circles. The increase in Bilirubin Enzyme UGT1A1 was statistically significant at 247% and 600%, respectively (Figure 2).

In-Vitro Test 3 – Chrysin induction of UGT
Chrysin is a peptide intended to induce an increase in expression of UGT1A1 to assist in the breakdown of bilirubin. Expression of UGT1A1 was determined after a 3 day incubation period and expression of mRNA for UGT1A1 was measured and quantified vs. a reference. The increase in expressions for both 2% and 3% concentrations of a blend with Chrysin were statistically significant at 247% and 600%, respectively (Figure 2).
**In-Vitro Test 4 – Reduction in Inflammation**

Anti-inflammatory effects were tested by measuring prostaglandin PGE2 inhibition from keratinocytes and fibroblasts. Inhibition was significant at the lowest concentration of 0.5% in both keratinocytes and fibroblasts (Figure 3).

<table>
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<th>% Inhibition</th>
<th>0.5%</th>
<th>1.0%</th>
<th>2.0%</th>
<th>3.0%</th>
<th>Aspirin</th>
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<td>Placebo</td>
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With 1% concentration the inhibition in keratinocytes reached ~85% and did not change with increased concentrations. Expression of fibroblasts continued increasing slightly with increased dosage, becoming nearly comparable to that of aspirin by 3%.

**In-Vivo Test 1 – Placebo Control Trial**

Reduction in both red and blue discoloration was significant with a response rate of 73% and a significant difference between the treatment and placebo sides. The mean reduction across all subjects was 12.5% for red and 10.2% for blue on the treatment side while the mean reductions on the placebo side were only 2.8% and 2.6%, respectively.

**In-Vivo Test 2 – Jan Marini Skin Research’s Age Intervention Dark Circle Eye Defense**

Subjects applied Jan Marini Skin Research’s Age Intervention Dark Circle Eye Defense to both eyes each night for 60 consecutive days. and were monitored every 15 days.

**Reduction in the Appearance of Under-eye Circles**

Investigator assessment showed an improvement in the appearance of under eye circles in 100% of subjects by day 45 with a mean score of 1.4 post vs. 2.6 pre (1 = normal skin, 2 = slightly dark rings, 3 = dark rings).

**Figure 3**

Inflammatory Inhibition

**Figure 4**

Improvement in Under-Eye Color

**Figure 5**

Mean Reduction in Red and Blue Discoloration

Reduction in red/blue under-eye circles are determined by measuring changes in a* and -b*. There was a significant difference between treated and placebo eyes (Figure 4). Sixteen of twenty-two subjects (73%) observed a reduction in red discoloration and 14 subjects (64%) observed a decrease in blue discoloration. Nonresponding subjects did not experience increased discoloration. There were zero subjects with an increase of more than 10% in either red or blue discoloration. Within responding subjects, the mean reduction was ~19% for both red and blue discoloration.
Quantitative assessment showed statistically significant improvement \((p<0.001)\) in discoloration by day 15. By day 60 average improvement in red discoloration \((a^*)\) was 12.1% with an individual subject range of 3.9 to 20.1%. Improvement in blue discoloration \((b^*)\) at day 60 was 10.2% with an individual subject range of 1.8% to 24.2% (Figure 5).

**Skin Elasticity**

Improvement in skin elasticity was statistically significant by day 15 \((p<0.001)\) and improved over the course of the study. 100% of subjects’ measured elasticity improved, ranging from 0.3% to 30.6%, with only 3 subjects experiencing less than a 5% improvement and a mean improvement across all subjects of 15% (Figure 6).

Investigator assessment of skin elasticity showed improvement in 80% of subjects with an elasticity (compactness) improvement of 22%, improving from a mean score of 2.2 to 3.1 on a 4 point scale.

**Skin Profilometry**

Skin smoothness, wrinkles and wrinkle volume were assessed both quantitatively and qualitatively. Investigator assessment showed improvement in skin smoothness and in the appearance of wrinkles in 90% and 95% percent of all subjects, respectively. On average, significant improvement was observed between 30-45 days. Figure 7 shows before and 60-day post profilometry images.

Quantitative analysis showed a 100% response rate with statistically significant improvement \((p<0.001)\) in all categories (skin smoothness, wrinkles and wrinkle volume) beginning at the first visit (Figure 8).

Negative values for wrinkles and volume indicate product efficacy as they show a reduction in the number and volume of wrinkles (Figure 9).

The mean improvement and individual subject ranges were as follows: smoothness 23.2% (1.2% to 45.5%), wrinkles
-12.7% (-1.3% to 26.2%), and wrinkle volume -21% (-3.8% to 41.6%) at 60 days.

**Subject Self Assessment**

Subjects were asked to complete a series of questions at the completion of the study. Subjects were highly satisfied with the product. The favorable response was 90% for three separate questions:

1) Do you wish to continue using the product,
2) Would you purchase the product, and
3) Would you refer the product to a friend.

Cosmetically, the product was easy to apply to the skin (70% very easy, 30% sufficiently easy, 0% moderately easy or difficult) and pleasant to use (75% very pleasant, 25% pleasant, 0% minimally or not pleasant). There were no complications and no subjects (0%) experienced any dryness, redness, itchiness, stinging or desquamation.

**DISCUSSION**

The proprietary combination of Chrysin, peptides, retinol and iron chelating agents in Jan Marini Skin Research’s Age Intervention Dark Circle Eye Defense substantially decreases the appearance of dark under-eye circles.

In-vitro, its components are shown to increase the expression of UGT1A1 in keratinocytes and fibroblasts to assist in the removal of bilirubin and to chelate iron to facilitate iron removal, which reduces sources of discoloration under the eyes. The anti-inflammatory effects of the product, coupled with the reduction of pro-inflammatory factors bilirubin and iron, further reduce the appearance of dark circles.

Finally, retinol and matrikins promote cellular turnover and increase collagen production to thicken the skin in the under-eye area. This reduces the appearance of under-eye circles and provides a more effective support structure for the microvasculature, preventing further damage and blood leakage.

In-vivo testing showed a significant improvement in the appearance of dark circles in 100% of subjects (measured both by physician assessment and quantitative analysis). Changes were also visible in split face comparisons vs. a placebo control.

Additionally, subjects experienced a significant reduction in the appearance of lines and wrinkles with 100% of subjects observing improvement by quantitative measurement and 75% observing improvement by the investigating dermatologist’s assessment.

Subject satisfaction was extremely high with 90% of subjects responding that they:

1) Wished to continue using the product,
2) Would purchase the product, and
3) Would refer the product to a friend.

**CONCLUSION**

When used nightly, Jan Marini Skin Research’s product Age Intervention Dark Circle Eye Defense, which has a proprietary blend of Chrysin, peptides, retinol and N-hydroxysuccinimide, effectively reduces the appearance of dark circles around the eyes and significantly improves the appearance of lines and wrinkles.

Its efficacy – combined with a 0% rate of irritation or complications and extremely high subject satisfaction – makes it a safe and effective product to reduce the appearance of dark circles and to improve the appearance of lax, inelastic wrinkled skin around the eyes.
PHOTOGRAPHIC EVALUATIONS

BASELINE

AFTER 60 DAYS

BASELINE

AFTER 60 DAYS

BASELINE

AFTER 60 DAYS

BASELINE

AFTER 60 DAYS

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