

Discovery of Effects of Retinol on Human Skin Aging in
Individuals of East Asian Descent

Protocol and Statistical Analysis Plan

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Anne Lynn S. Change, MD, Principal Investigator
Stanford University School of Medicine
Redwood City, California, 94063

Title of Proposal:

**Discovery of Rejuvenation Effects of Vitamin A (Retinol) Lotion
on Naturally Aged Skin in an East Asian Cohort**

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Principal Investigator:

**Anne Lynn S. Chang, MD
Stanford University School of Medicine
Assistant Professor, Department of Dermatology
450 Broadway Street [REDACTED]
Redwood City, CA 94063**

Introduction

Increasing evidence from multiple organ systems suggests that there are different rates of natural aging in humans. One of the best organs to study human aging is the skin, due to its amenability to clinical inspection and serial biopsy. Recently, genome-wide analysis in an Ashkenazi population has provided the evidence that skin can age at different rates and that youthful appearing skin is associated with different gene variants (Chang *et al.*, 2014). The first studies to demonstrate anti-aging effects of a topical agent were published in 1988 in which topical tretinoin was used to improve the appearance in photo-aged skin (Weiss *et al.*, 1988). Subsequently, topical retinol (vitamin A) lotion was shown in a placebo-controlled study to decrease fine wrinkling in photo-protected skin in older individuals residing in nursing homes (Kafi *et al.*, 2007).

Recent advances in deep sequencing technology have enabled the unbiased and detailed analysis of gene expression changes in the skin (Chang *et al.*, 2013). Furthermore, a type of deep sequencing technique called 3'-end sequencing has demonstrated the ability to identify changes in a class of transcripts called long non-coding RNAs (lnc RNAs) that are now understood to be a previously unrecognized way to regulate gene expression. Here, we propose to utilize this state-of-the-art technology to discover, in unbiased fashion, the rejuvenation effects of the most widely used class of topical anti-aging agents, retinoids. In this study, we will focus on topical retinol, as it is an over-the-counter agent that has minimal side effects.

Furthermore, studies on retinol to date in humans have occurred largely in white population even though individuals of Asian descent represent the majority of the world population. The potential effects of retinol on individuals of Asian descent are uncharacterized and represent an untapped potential to explore differences in aging across ethnic groups.

While prior studies have indicated an increase in glycosaminoglycans (GAGs) and collagen after topical retinol usage (Kafi *et al.*, 2007), the true breadth of anti-aging effects including the mechanisms by which these effects are controlled has not been explored. It is possible that topical retinol has additional effects on major pathways of skin aging, such as NFkB related genes (Chang *et al.*, 2013) as well as previously unknown aging pathways. This study aims to discover the molecular basis of rejuvenation effects of retinol so that new topical agents can be identified that might mimic aspects of the retinol rejuvenation effect. We aim to do this in a Han Chinese cohort, as this is the most populous ethnic group among the East Asian population. Given the similarity in haplotype of Han Chinese with Korean and Japanese populations, these latter groups will be included as well.

Objective:

To discover new pathways of rejuvenation using topical vitamin A application on naturally aged in East Asian women.

Specific Aims:

Specific Aim 1: To discover molecular pathways of skin rejuvenation by identification of gene expression profiles after topical vitamin A in East Asian women

Specific Aim 2: To identify changes in wrinkling and biometrics associated with skin rejuvenation after topical vitamin A application in East Asian women

Specific Aim 3: To identify type and severity of adverse events associated with topical retinol usage

Materials and Methods

Formulation of topical retinol per Kafi *et al.* by combining a 41% retinol solution in 55% polysorbate 20 in sufficient proportions with fragrance-free Norwegian Formula Neutrogena Body Moisturizer (Ortho-Neutrogena Co, Los Angeles, Calif) to yield a 0.4% retinol lotion. The vehicle was similarly prepared with 55% polysorbate 20 solution in Norwegian Formula Neutrogena Body Moisturizer. There were no discernable differences in color, odor, or consistency between the active lotion with retinol and the placebo lotion control. A stability study using high-performance liquid chromatography will be performed to demonstrate that more than 90% of 0.4% retinol remained in the moisturizer 3 months after preparation. Both 0.4% retinol lotion and its vehicle were placed in dark glass containers covered with foil to eliminate transmission of UV radiation and stored at 4°C.

After Stanford Human Subjects Panel approval and written informed consent, participants will be screened for eligibility as follows:

Inclusion Criteria

Able to provide written informed consent
Older group: Age between 50 and 75 years
Young group: Age less than or equal to 25 years
Female
Fine wrinkling score at baseline =7 or greater if in older group
All 4 grandparents of Han Chinese, Korean or Japanese descent
Willing and able to apply topical agents to inner arms
Uncontrolled medical problems including diabetes, active chemotherapy
Body mass index within normal or overweight range
No history of weight loss of >20 lbs. within past 5 years

Exclusion Criteria

Skin condition in the areas of skin biopsy that would obscure results of analysis
Topical creams or treatment to arms 2 weeks prior to study baseline visit, including sunscreens, topical moisturizers, sunless tanners
Individuals with known hypersensitivity to retinoid class of agents
Prior anti-aging treatments to arms including retinol, microdermabrasion within 2 weeks of baseline visit

Prior laser therapy or surgical procedure to arms
Prior radiation or other trauma (extensive burns or abrasions) to arm skin
Hormone-based therapy within 4 weeks of enrollment

Screening Visit (VISIT 1—older group only)

Only individuals fulfilling eligibility criteria will be enrolled.

As part of the screening process, clinical histories, physical examinations including vital signs, and photography of sun exposed and non sun-exposed arm will be performed. Cutometry, Raman spectroscopy (Biophotonic scanning) and transepidermal water loss will be measured.

Enrollment Visit (VISIT 2—subset of 30 participants from the older group above will be invited for this visit)

Upon enrollment into the study, patients will be instructed on proper usage topical retinol versus vehicle placebo. The placebo versus treatment arm will be clearly labeled on the subject's diary to minimize subject error. Participants will apply treatment to one arm and placebo to the other arm based on computer generated randomized assignment of left or right for the retinol. Participants are blinded to the treatment. Participants will be given a diary to record adherence to study procedures. Participants will be instructed to discontinue study if there is any itching or burning sensation that is moderate or severe and to contact study personnel immediately should this occur.

Week 12 visit (VISIT 3—the 30 participants from VISIT 2 (older group) only)

Adverse events and concomitant medication changes will be assessed and recorded. Study diary will be reviewed for adherence to study regimen. Photographs, cutometry and TEWL measurements will be taken. Two 4 mm skin biopsies will be taken at this time, one from the side with the retinol exposure and one from the side without retinol exposure after injection of a small amount of lidocaine anesthetic. Skin biopsies will be bisected and placed in either formalin for hematoxylin and eosin staining and mucin staining (for GAG), or placed into RNA later for subsequent gene expression analysis by 3'-end sequencing as described in Chang *et al.*, 2013. Tissue will also be sent for microRNA analysis.

VISIT 1—age<25 years group only

As part of the screening process, clinical histories, physical examinations including vital signs, and photography of face, sun exposed and non sun-exposed arm skin will be performed. Cutometry, Raman spectroscopy (non invasive Biophotonic scanning) and transepidermal water loss will be measured. Photographs, cutometry and TEWL measurements will be taken. A 4 mm skin biopsies will be taken from the inner arm skin after injection of a small amount of lidocaine anesthetic. Skin biopsies will be bisected and placed in either formalin for hematoxylin and eosin staining and mucin staining (for GAG), or placed into RNA later for subsequent gene expression analysis by 3'-end sequencing as described in Chang *et al.*, 2013. Tissue will also be sent for microRNA analysis.

Statistical Considerations

This study is powered using data from Kafi *et al.* for the primary clinical endpoint of fine

wrinkling score as measured by semi-quantitative scale of 0 to 9. A sample size of 36 provides a power level of 0.80 in detecting a difference of 1.00 unit on mean value before and after treatment for the fine wrinkling scale, with a type I error rate of 0.05 for a 2-tailed hypothesis, assuming a standard deviation of differences to be 1.8. Assuming a dropout rate of 10%, the sample size needed is 28 subjects.

Anticipated Results

Results of photographs will be analyzed in blinded fashion by a board certified dermatologist for skin aging parameters including fine wrinkling (tertiary endpoint), dyspigmentation, and surface texture with results graded on a 9 point Likert scale per Kafi *et al.* We anticipate that there will be statistically significant differences in mean score of these parameters between pre- and post- treatment groups for both vitamin A and placebo.

Results of histology will be evaluated in blinded fashion by a dermatopathologist. Parameters of skin aging will include measurement of epidermal thickness, and density of collagen and elastin. Results from the pre- and post- treatments groups for topical vitamin A versus placebo will be compared.

Results of the cutometry and TEWL measurements will be grouped and mean and standard deviations for the older patients before and after topical vitamin A treatment will be calculated and compared to placebo. We anticipate there will be statistically significant differences in the mean scores in cutometry and TEWL measurements between treated and placebo groups.

Whole transcriptome libraries will be created from the samples stored in RNA later and sent for 3'-end sequencing at Stanford Functional Genomics Core Facility after de-identification. We have utilized this Core facility for other projects in the past with high quality results. Results will be analyzed by our bioinformatician using the methods outlined in Chang ALS *et al.*, 2013. Specifically, comparisons will be made in skin from older participants between the pre and post treatment compared to placebo. All results of interest from 3'-end sequencing will be confirmed by RT-qPCR.

We anticipate that the adverse event rate (deemed related to study agent by investigator) for participants is less than 10% and may include mild (grade 1) itching or burning.

Significance

This study will be the first broad based, unbiased investigation into the molecular basis of the anti-aging effects of topical vitamin A. This study is a high quality, well-controlled study using state-of-the art technology of 3-Seq and microRNA analysis. Our research group has established the workflow from previous larger studies in skin aging (unpublished data) and can now leverage this experience for maximal efficiency and positive results. Specifically, we would be able to compare our results to inner arm skin from females younger than 30 years whose gene expression profiles are already contained in our database.

This study will provide novel candidate gene pathways as well as confirm suspected/known genes important for skin rejuvenation in humans. This information will aid in discovery of new topical agents that might have rejuvenative capacity due to their ability to mimic aspects of the vitamin A gene expression changes.

References

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