COURSE NOTES: Understanding genetics for improving health outcomes

Course Code: CEUGH

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Course Description

The course is intended to provide the tools that holistic nutritionists can use to deliver personalized healthcare to their clients, catered to the genetic makeup of the client. Genetics plays a very robust role in nutrition, detoxification, weightloss and overall health and wellness. Furthermore, genetics can be used not only to improve the health of the clients but also to develop DNA based nutritional plans that can potentially prevent development of chronic diseases. Course participants will gain knowledge on how genetic information can be used to deliver nutritional plans, weightloss strategies, detoxification plans, hormonal balance plans along with nutritional plans to prevent development of chronic disease such as diabetes and cardiovascular diseases.

IHN has partnered with Anantlife Canada Inc., a leader in clinical grade genetic testing for healthcare providers all over the world, to offer a Certified Genetic Testing Provider Certificate upon successful completion of the course. Successful completion of the course implies that the candidates have received the education and training to not only understand genetic concepts pertaining to diet, nutrition, detoxification, fitness, hormonal health and metabolic disorders but have also been trained on interpretation of the genetic testing reports along with development of a DNA based health plan for better health outcomes.

SESSION 7:

SKIN GENETICS: HOW DO OUR GENES REGULATE AGING AND SKIN HEALTH? HOW CAN THIS INFORMATION BE UTILIZED TO IMPROVE SKIN HEALTH BY DEVELOPING DNA BASED PERSONALIZED PLANS?

Skin forms the largest organ in our bodies and genetics plays an essential role in determining its health and predispositions to various conditions. Genetics plays a role in determining the nutritional needs of your skin, predisposition of your skin to dryness, oxidative damage, glycation induced damage as well as wrinkles, skin elasticity and predisposition to varicose veins, stretch marks and cellulite, response of your skin to UV exposure including predisposition to pigmentation, sunspots and freckles, predisposition to inflammatory conditions including eczema, psoriasis and contact dermatitis. The readings herein are to provide an understanding of skin genetics and how providing DNA based skin health regimen can be utilized to maintain/improve skin health.

Clinical, Cosmetic and Investigational Dermatology

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ORIGINAL RESEARCH

Genetic polymorphisms and skin aging: the identification of population genotypic groups holds potential for personalized treatments

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Introduction: Skin changes are among the most visible signs of aging. Skin properties such as hydration, elasticity, and antioxidant capacity play a key role in the skin aging process. Skin aging is a complex process influenced by heritable and environmental factors. Recent studies on twins have revealed that up to 60% of the skin aging variation between individuals can be attributed to genetic factors, while the remaining 40% is due to non-genetic factors. Recent advances in genomics and bioinformatics approaches have led to the association of certain single nucleotide polymorphisms (SNPs) to skin properties. Our aim was to classify individuals based on an ensemble of multiple polymorphisms associated with certain properties of the skin for providing personalized skin care and anti-aging therapies.

Methods and results: We identified the key proteins and SNPs associated with certain properties of the skin that contribute to skin aging. We selected a set of 13 SNPs in gene coding for these proteins which are potentially associated with skin aging. Finally, we classified a sample of 120 female volunteers into ten clusters exhibiting different skin properties according to their genotypic signature.

Conclusion: This is the first study that describes the actual frequency of genetic polymorphisms and their distribution in clusters involved in skin aging in a Caucasian population. Individuals can be divided into genetic clusters defined by genotypic variables. These genotypic variables are linked with polymorphisms in one or more genes associated with certain properties of the skin that contribute to a person's perceived age. Therefore, by using this classification, it is possible to characterize human skin care and anti-aging needs on the basis of an individual's genetic signature, thus opening the door to personalized treatments addressed at specific populations. This is part of an ongoing effort towards personalized anti-aging therapies combining genetic signatures with environmental and life style evaluations.

Keywords: single nucleotide polymorphisms, genetic clustering, genotypic signature, treatment personalization, dermatology, cosmetics

Introduction

Skin aging

As developed countries grow older, scientists are trying to gain insight into the molecular and physiological events involved in the skin aging process. Skin changes are among the most visible signs of senescence, and have garnered more interest in a society that values youth and beauty.[1] In this scenario, the term "perceived age" refers to the age that someone is visually estimated to have in relation to their chronological age. This concept has implications that go beyond mere aesthetics: perceived age is a robust biomarker of aging that predicts survival in certain groups of patients[2] and correlates with important functional and molecular aging phenotypes.[3]

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Research on same-sex twins suggests that up to 60% of the variation in perceived age is influenced by genetics, while the remaining 40% can be explained by non-genetic factors.[3,4] Among the latter, smoking and sun exposure are the environmental factors of major importance for premature skin wrinkling or facial aging.[5] Furthermore, a direct effect of both smoking and light exposure on overall health status has been documented.[6]

Ethnicity and sex provide obvious evidence of genetic influence on skin aging. Wrinkling in Asians tends to occur later and with less severity than in Caucasians,[7] while another study revealed that dryness of the skin is higher in African-American and Caucasian women.[8] But the most explicit difference between ethnic groups is skin color, with more darkly pigmented subjects retaining younger skin properties.[9] On the other hand, physiological dissimilarities between men and women also highlight the role of genetics in skin aging. For example, male skin is more sensitive to environmental aggressors, particularly ultraviolet light exposure.[10]

Intrinsic and extrinsic factors converge in modifying certain biochemical properties that, in the end, are responsible for progressive skin decay. Significant changes driving this process are oxidative stress,[11] lack of elasticity, and reduced hydration.[12] Oxidative damage arises as a consequence of sun exposure and metabolic generation of free radicals and leads to loss of dermal cells and the extracellular matrix. Although elastic proteins in the matrix are long-lived, they also accumulate damage via crosslinking and other mechanisms. With the degeneration of glycolipids that maintain skin hydration, skin suffers severe loss of water.

Sequencing of the human genome and subsequent developments in genomics have provided new tools with which to investigate these factors at the molecular level, while bioinformatics allows an integrated analysis of the huge amount of data that are being generated.[13] At the same time, new techniques in genomic expression profiling are able to characterize individual genotypes, thereby opening the door to personalized dermatologic therapy.[14]

Single nucleotide polymorphisms (SNPs) occur when single bases in genes are changed or deleted, which may result in an amino acid change at a specific position and a change in phenotype. Numerous physiological states or diseases have been correlated with occurrence of SNPs associated with particular genes in the genome of a human who exhibits the physiological states or disease. For example, manganese superoxide dismutase (*SOD2V16A*) polymorphism has been shown to be associated with cancer risk,[15] and matrix metalloproteinases-2 (735C/T and 1306C/T) polymorphisms with the development of glaucoma.[16] SNPs have also been associated with skin features. Han et al identified novel SNPs associated with skin pigmentation and hair color.[17] Further supporting this, a recent genome-wide association study reported the role of *STXBP5L* gene variants in photoaging[18] or the association of mutations in genes coding for antioxidant response with the predisposition to higher oxidative stress, leading to accelerated deterioration.[19] But, we still lack information regarding the identification, frequency, and clustering of gene variants related to main functions of aging skin, such as antioxidative capacity, collagen metabolism and structure (elasticity), and natural hydration capacity in a healthy Caucasian population.

Taking advantage of the recent advances in genomics and bioinformatics, we describe herein how main skin functions can be classified according to genotypic signature and how pertinent personalized corrective treatments could be designed. With this aim, we classified a normal population into clusters according to the distribution of polymorphisms in genes associated with antioxidant capacity, hydration, and elasticity of the skin. As more precise methods providing personalized dermatological and anti-aging treatments are needed, skin therapies adapted to each one of these clusters could then be specifically designed. This work represents part of the technologies developed by the authors towards genetically personalized dermocosmetics treatments.

Methods and results

Characterization of skin aging at the gene and protein level

Aged skin is characterized, among other features, by dehydration, loss of elasticity, and increased oxidative damage.[11,12] These three main skin properties were selected because they are strongly related to well-known metabolic pathways, and because many other apparent skin properties, such as wrinkle extension and depth, can be attributed to one of these three (in this case, wrinkles can be attributed to loss of elasticity and collagen metabolism). Accordingly, through an extensive literature search, we identified the genes that encode proteins involved in skin hydration, skin elasticity, and antioxidant capacity of the skin. A total of 72 proteins were identified: the group of proteins related to skin elasticity is composed of 39 proteins, including matrix metalloproteases involved in collagen and elastin metabolism; the hydration group is composed of six proteins, mainly aquaporins and proteoglycans; and the group related to antioxidant capacity of the skin is composed of 27 proteins, including superoxide dismutase, catalase, and nicotinamide adenine dinucleotide phosphate-oxidase.

Selection of polymorphisms associated with skin aging

Among all the 72 proteins involved in skin aging, we further identified which proteins have associated SNPs in their genes that influence the corresponding protein product expression or functionality. A total of 13 SNPs in ten proteins were selected, NAD(P)H dehydrogenase [quinone] 1,[20] matrix metalloproteinase-1,[21] superoxide dismutase II,[22,23] nuclear factor erythroid 2-related factor 2,[24–26] glutathione peroxidase 1,[27] catalase,[28] matrix metalloproteinase-3,[29] interleukin-6,[30] matrix metalloproteinase-9,[31] and aquaporin-3.[32] The effect of the SNP on protein expression or activity was categorized as +1, which indicates that the SNP increases the protein expression or activity, or −1, which indicates that the SNP decreases protein expression or activity (Table 1).

Moreover, in order to study SNPs that are frequent in Caucasian populations, we checked the previously described population frequencies for these SNPs in NCBI database (http://www.ncbi.nlm.nih.gov/) in a Caucasian population. Genetic variants were selected as having a described population frequency higher than 10%.

Study population

A noninterventional, epidemiological, cross-sectional study was conducted in 120 female volunteers. The epidemiologic study was conducted at the Hospital of Nisa, Valencia, Spain. The study received the approval of the Autonomic Ethics Committee of Clinical Studies of Drugs and Medical Devices of the Valencian Community in Spain (CAEC).

Eligibility criteria for the study

Participants in the study were women aged between 41 and 49 years old, of any skin type, who regularly attended the dermatology clinic for dermo-aesthetic purposes, and who had a predominantly urban lifestyle. They had to have full

Table 1 List of selected polymorphisms in proteins associated with skin aging

SNP (rs number)	Protein name	UniProt code	Skin property	SNP variant	SNP effect		Reference list number
					Expression	Activity	
rs1800566	NAD(P)H dehydrogenase [quinone] 1	P15559	Skin antioxidant capacity	C:C		+1	20
				T:T		−1	
rs1799750	Matrix metalloproteinase-1	P03956	Skin elasticity	-:-	−1		21
				G:G	+1		
rs4880	Superoxide dismutase II	P04179	Skin antioxidant capacity	T:T	−1		22
				C:C	+1		
rs1141718	Superoxide dismutase II	P04179	Skin antioxidant capacity	T:T		+1	23
				C:C		−1	
rs35652124	Nuclear factor erythroid 2-related factor 2	Q16236	Skin antioxidant capacity	T:T	−1		24
				C:C	+1		
rs6706649	Nuclear factor erythroid 2-related factor 2	Q16236	Skin antioxidant capacity	C:C	+1		25
				T:T	−1		
rs6721961	Nuclear factor erythroid 2-related factor 2	Q16236	Skin antioxidant capacity	G:G	+1		26
				T:T	−1		
rs1050450	Glutathione peroxidase 1	P07203	Skin antioxidant capacity	C:C		−1	27
				T:T		+1	
rs1001179	Catalase	P04040	Skin antioxidant capacity	A:A	+1		28
				G:G	−1		
rs3025058	Matrix metalloproteinase-3	P08254	Skin elasticity	T:T	+1		29
				-:-	−1		
rs1800795	Interleukin-6	P05231	Skin elasticity	G:G	+1		30
				C:C	−1		
rs3918242	Matrix metalloproteinase-9	P14780	Skin elasticity	C:C	−1		31
				T:T	+1		
rs17553719	Aquaporin-3	Q92482	Skin hydration	A:A	+1		32
				G:G	−1		

Notes: Single nucleotide polymorphism (SNP) effect on protein expression means that the genetic variant leads to a modification in the quantity of protein that is expressed from the gene, and effect on activity means that the genetic variant leads to a modification of the functionality of the protein. UniProt are standard protein codes (www.uniprot.org). The effect of the SNP on protein expression or activity was categorized as +1, which indicates that the SNP increases the protein expression or activity, or −1, which indicates that the SNP decreases protein expression or activity.

capability to understand and sign the informed consent document with free will. Prospective participants were excluded if they were exposed to external factors that posed a serious risk for skin health or suffered from severe skin disorders.

Sample collection

Saliva samples were collected from study subjects by using DNA collection tubes (DNA Genotek Inc., Ottawa, ON, Canada). The samples were sent to Progenika Biopharma SA (Vizcaya, Spain) and to Centro Nacional de Genotipado (Santiago de Compostela, Spain) for the genotyping of selected SNPs by using Affymetrix (Santa Clara, CA, USA) and Sequenom, Inc. (San Diego, CA, USA) technologies.

In brief, DNA was extracted using ORAGENE (DNA Genotek Inc.) extraction reagent following the indications of the supplier. Quality and quantity DNA from saliva samples were assessed in a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer. Allele-specific probes were designed in order to discriminate different genotypes by means of fluorescence detection in real-time quantitative polymerase chain reaction for every SNP, except for rs3918242. The rs3918242 polymorphism was analyzed by polymerase chain reaction followed by restriction fragment length polymorphism analysis as described.[33]

Classification of individuals based on an ensemble of multiple genetic polymorphisms associated with certain properties of the skin

A total of 13 SNPs in genes associated with certain properties of the skin (phenotypes) were considered for the analysis (Table 1). The detailed population frequency of the analyzed SNPs is as described in Table 2.

Our next goal was to identify if the selected SNPs could define genetic groups or clusters in the study sample, and to see if these genetic groups could be linked to biochemical and metabolic skin properties. As it has been described, each SNP gives rise to two different genotypes with phenotype consequences, and we assigned a binary effect to each SNP. Thus, the variant contributing positively to the skin property was given a value of +1, whereas the variant not contributing positively to the skin property was given a value of −1.

The genetic variants were clustered using the following criteria: 1) obtaining a total number of clusters between two and 20; and 2) identifying genetic clusters that explain the differences in the effects of the SNP variants on the biochemical and metabolic properties of the skin.

The genetic clustering analysis was conducted by using the k-means technique for genetic cluster analysis as described elsewhere.[34]

The conducted analysis shows that the sample can be optimally classified into ten genetic clusters, each with its own genetic signature, as shown in Table 3. Each one of the SNPs exerts a specific and differential contribution on each of the ten genetic clusters identified. For example, SNP rs1800566 is very relevant for the definition of clusters 9 and 10 (contribution of 100% to both), whereas it is less relevant for the definition of clusters 4 and 5 (contribution of 16% and 29%, respectively). Each genetic cluster has its own combination of relevant SNPs. For example, cluster 1 is different from cluster 2, mainly because of the differential contribution of SNPs rs6721961 (46% versus 100%), rs1050450 (35% versus 76%), rs1001179 (18% versus 76%), rs1800795

Table 2 Single nucleotide polymorphisms and variant frequencies in the sample population

rs1800566		**rs1001179**		**rs35652124**		**rs3918242**	
C:C	62%	A:A	12%	C:C	8%	C:C	71%
T:C	31%	G:A	27%	T:C	39%	C:T	24%
T:T	8%	G:G	61%	T:T	53%	T:T	4%
rs1799750		**rs3025058**		**rs6706649**		**rs6721961**	
-:-	26%	-:-	29%	C:C	78%	G:G	72%
G:-	49%	T:-	44%	T:C	17%	T:G	27%
G:G	26%	T:T	27%	T:T	5%	T:T	1%
rs4880		**rs1800795**		**rs1050450**			
C:C	26%	C:C	9%	C:C	52%		
C:T	49%	C:G	52%	T:C	39%		
T:T	25%	G:G	39%	T:T	9%		
rs1141718		**rs17553719**					
T:T	100%	A:A	49%				
C:T	0%	G:A	42%				
C:C	0%	G:G	9%				

Table 3 Contribution of individual polymorphisms to the definition of each genetic cluster

Polymorphism	Genetic cluster									
	1	2	3	4	5	6	7	8	9	10
rs1800566	50%	75%	53%	16%	29%	45%	53%	78%	100%	100%
rs1799750	38%	33%	32%	32%	42%	24%	25%	28%	15%	100%
rs4880	15%	29%	79%	83%	19%	76%	100%	48%	21%	100%
rs1141718	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
rs35652124	47%	76%	75%	65%	75%	75%	75%	76%	100%	100%
rs6706649	20%	44%	100%	100%	31%	44%	66%	76%	50%	100%
rs6721961	46%	100%	51%	18%	31%	27%	32%	57%	100%	50%
rs1050450	35%	76%	42%	75%	40%	32%	41%	78%	76%	100%
rs1001179	18%	76%	44%	16%	41%	43%	52%	75%	27%	100%
rs3025058	77%	81%	27%	100%	100%	76%	45%	22%	81%	100%
rs1800795	73%	25%	75%	26%	76%	55%	24%	75%	81%	100%
rs17553719	50%	100%	100%	32%	27%	49%	26%	28%	44%	75%
rs3918242	77%	44%	100%	100%	85%	44%	100%	100%	100%	100%

Notes: Each genetic cluster is defined by a unique combination of polymorphisms and levels of contribution to the cluster definition. For example, single nucleotide polymorphism rs1800566 is very relevant for the definition of clusters 9 and 10 (contribution of 100%), whereas it is less relevant for the definition of clusters 4 and 5 (contribution of 16% and 29%, respectively).

(73% versus 25%), or rs17553719 (50% versus 100%). A given individual can then be classified into one of the ten genetic clusters according to his/her genetic signature. The frequency distribution of the 120 females of the study population in the ten genetic clusters is shown in Figure 1.

As shown in Table 1, each of the SNPs contributes to the expression and/or activity of certain proteins linked with skin properties, with an effect described as +1 (positive for skin property) or −1 (negative for skin property). Consequently, we were able to describe the contribution of the genetic profile of each cluster to each one of the three skin properties (antioxidant capacity, elasticity and collagen structure, and natural hydration ability). To conduct this calculation, we considered only polymorphisms with a contribution of over 75% to the group, and SNPs that exhibited variability between genotypic groups. For that reason, SNP rs1141718 was discarded for further analysis as it was predominant in all the study population. The values in Table 4 for each skin property and each cluster show the magnitude of the effect, by simple addition of the individual effect values of the relevant SNPs in the genetic group to that property. The plus sign indicates that the ensemble of the genotypic variants in the group has a positive effect on the skin property, and the minus sign indicates that it has a negative effect. The higher the value, the higher will be the effect on that skin property. It should be noted that certain SNP variants in different polymorphisms for the same property can cancel each other out. For example, if the variant C:C of rs1800566 (effect +1) and the variant T:T of rs4880 (effect −1) appear as relevant in the same cluster, the net effect value for antioxidant capacity is 0.

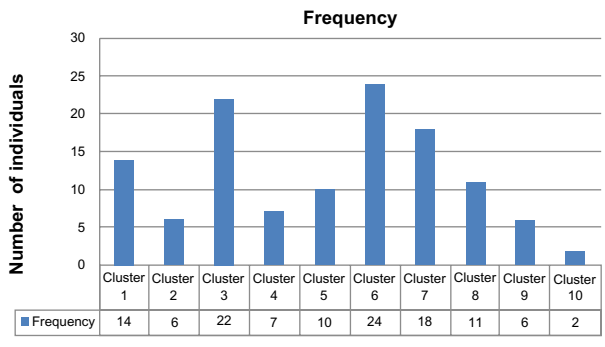

Figure 1 Distribution into ten genotypic groups of the 120 patients analyzed.

Based on the SNP contribution and particular effect to each one of the genetic groups, we can further describe the genetic clusters as follows (only relevant features are highlighted for each group):

Table 4 Overall contribution of the set of polymorphisms that define a genotypic group on skin properties involved in aging

Genetic cluster	Skin properties		
	Antioxidant capacity	Skin elasticity	Skin hydration
1	0	−2	0
2	−3	0	+1
3	0	0	+1
4	−1	+2	0
5	−1	0	0
6	−1	−1	0
7	−1	+1	0
8	−6	+1	0
9	+1	+1	0
10	+3	−3	−1

Notes: Only effects of single nucleotide polymorphisms with a contribution over 75% to the genetic cluster definition were considered. Positive or negative integers show the magnitude of the added effects of SNP variants on each skin property for each genetic cluster.

- Cluster 1: genetic susceptibility to loss of elasticity of the skin due to polymorphisms in genes that encode matrix metalloproteinase-3 and matrix metalloproteinase-9.
- Cluster 2: genetic susceptibility to oxidative damage mainly due to polymorphisms in genes that encode NAD(P)H dehydrogenase [quinone] 1, nuclear factor erythroid 2-related factor 2, glutathione peroxidase 1, and catalase, together with a genetic capability to maintain the appropriate hydration levels of the skin due to polymorphism in genes for aquaporin-3.
- Cluster 3: genetic capability to maintain the appropriate hydration levels of the skin due to polymorphism in genes for aquaporin-3.
- Cluster 4: genetic susceptibility to oxidative stress damage of the skin due to polymorphism in genes that encode glutathione peroxidase 1 protein, together with a genetic capability for appropriate elasticity of the skin due to polymorphism in genes for matrix metalloproteinase-9 and matrix metalloproteinase-3.
- Cluster 5: genetic susceptibility to oxidative stress damage of the skin due to polymorphism in genes for nuclear factor erythroid 2-related factor 2.
- Cluster 6: genetic susceptibility to loss of elasticity of the skin due to polymorphisms in genes that encode matrix metalloproteinase-3.
- Cluster 7: genetic capability for appropriate skin elasticity and antioxidant capacity due to polymorphisms in genes that encode matrix metalloproteinase-9, superoxide dismutase II, and nuclear factor erythroid 2-related factor 2.
- Cluster 8: genetic susceptibility to oxidative stress damage of the skin due to polymorphism in genes that encode nuclear factor erythroid 2-related factor 2, NAD(P)H dehydrogenase [quinone] 1 protein, glutathione peroxidase 1, and catalase.
- Cluster 9: genetic capability for good skin elasticity due to polymorphisms in genes for matrix metalloproteinase-9.
- Cluster 10: genetic capability against oxidative damage due to polymorphisms in genes that encode nuclear factor erythroid 2-related factor 2, superoxide dismutase II, and catalase, together with a genetic susceptibility for loss of elasticity and hydration of the skin due to polymorphisms in genes that encode matrix metalloproteinase-9, interleukin-6, matrix metalloproteinase-1, and aquaporin-3.

Discussion

This is the first study that describes the actual frequency of genetic polymorphisms involved in skin aging in a Caucasian population. We identified 13 SNPs in genes coding for proteins that play a role in skin properties associated with aging, namely, oxidative stress, elasticity, and hydration. The genetic polymorphism frequency and the SNP clustering analysis in the study sample allowed us to classify the 120 female volunteers into ten genotypic groups or clusters. Taking into account the different protein functionalities from each one of the genetic variants as described in the literature (Table 1), a global effect was assigned to each cluster on the basis of the collective contribution of the set of polymorphisms to the biochemical and metabolic properties of the skin.

This clustering analysis suggests that different skin care needs depend on the naturally occurring single genetic variants present in each one of the genetic clusters. For example, cluster 8 shows the worst combination of antioxidant genes, having the weakest versions of NAD(P)H dehydrogenase [quinone] 1 protein, glutathione peroxidase 1, and catalase, which are enzymes linked with the antioxidant pathways, whereas cluster 10 shows a good genetic capability against oxidative damage due to good versions of genes for nuclear factor erythroid 2-related factor 2, superoxide dismutase II, and catalase. Thus, cosmetic products or therapies for individuals from cluster 8 should be more concentrated in antioxidative ingredients than the cosmetics aimed at those in cluster 10 – not only because cluster 8 seems to present a reduced natural antioxidative potential, which is why they would need an antioxidative supplement, but because the skin of individuals in cluster 10, which has a good natural antioxidative capacity, could be damaged by an excess of antioxidative ingredients (the genetic natural plus using cosmetics with even more antioxidative capacity). It is clear, then, that each one of the different genetic clusters shows a unique combination of needs for each one of the three main studied features. We suggest that it is not only necessary to supplement the lack or low activity of certain skin functions, but also that the excess of active ingredients in cosmetics or skin therapies can even be harmful for those individuals whose genetic background already provides strong natural capabilities.

In consequence, we suggest that genomic analysis can contribute to the characterization of human skin care and anti-aging needs, by conducting a simple genetic test and assigning an individual to one of the ten genetic groups.

Study limitations

This study presents the following limitation: only genetic variants with sufficiently described effects on skin properties were included for analysis. Other well-known variants related

to skin aging but which do not have a strong genetic background or expected variability in a normal population have not been considered; for example, synthesis of elastic fibers or hyaluronan, or other skin matrix or cellular components known to be linked with skin aging processes. Once well-established genetic and phenotypic information about these processes are available, they could be included in further analyses.

This study nevertheless, for the first time, sets the ground for the description of the actual prevalence of a number of the main relevant genetic variants in a normal Caucasian population. We suggest that, in order to complete the skin aging evaluation and to apply corrective measures when needed, it is necessary to combine genetic analysis with direct evaluation of skin needs.

Conclusion

The use of genetic signature for the identification of skin care individual requirements opens the door to personalized treatments for specific populations. With genotypic services becoming increasingly affordable, this perspective could be a reality in the not-so-distant future. Suggestions for further research include the discovery of new proteins associated with skin aging, additional polymorphisms that modify their activity or expression, and epigenetic modifications of DNA affecting gene regulation. One can even envisage having the complete DNA sequence of an individual available as an aid to designing a personalized skin care and anti-aging treatment. Authors are currently developing new avenues for personalized dermo-aesthetics by exploring new genetic signatures combined with enviromental and lifestyle factors.

Disclosure

The authors report no conflicts of interest in this work.

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Research Article Open Access

Genetic Customization of Anti-aging Treatments

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Abstract

Skin aging is a multifactorial process that involves both intrinsic factors of genetic and hormonal origin and extrinsic factors of environmental and nutritional nature.

The purpose of this open study on a case series of volunteers is to evaluate the impact of genetic customization of common anti-aging dermocosmetic treatments. We report how the treatment may be customized by acting selectively on the metabolic impairments identified by the analysis of specific DNA variants. The customized cosmetic method shows a significantly higher efficacy compared to non-specific cosmetic treatments such as radiofrequency, suggesting that the combination genetic signature may provide a useful tool for personalized and more effective anti-aging therapies.

Keywords: Genetic risk score; Aging skin; Stratum corneum; SNPs; Skin elasticity; Hydration; Skin texture

Introduction

Aging is caused by the accumulation of cell damages and nonrepaired cells, which are an uncommon process between all species. Some types of damages are unavoidable such as ultraviolet (UV) radiation, free radicals, and genetic effects, and others involve environmental and behavioural influences.

There are two distinct types of skin aging: chronoaging and photoaging. Chronoaging, the natural aging process, is a continuous process that normally begins in our mid-20s with reducing collagen and production, and that enables skin to conserve its original status: it causes cell hypo activity, i.e., a continuous and progressive slowing of the cell repair and renewal processes, resulting in a decrease in cell efficiency.

Photo aging instead is caused by sun exposure and is characterized by the activation of oxidative stress phenomena and therefore, by cell hyperactivity, whose main outcome is damage to nucleic acids, proteins, and lipids.

Chronoaging and photoaging act synergistically in the generation of the typical signs of skin aging.

The structural alterations responsible for the visible signs of skin aging mainly affect the surface layers of the skin: the increase in keratinocyte terminal differentiation causes a progressive thickening of the stratum corneum due to an accumulation of dead cells at the surface level, forming a compact matrix which alters the hydration functions of the skin and gives it a dry and wrinkled appearance [1]. The lower production of collagen and elastin is responsible for the thinning of the dermis, whose degeneration leads to a reduction in skin elasticity and firmness [2]. Frequent sun exposure can cause photoaging that includes noticeable changes to the skin such as freckles, age spots, telangiectasia, rough and leathery skin, loose skin, actinic keratoses, and eventually skin cancer. Furthermore, repetitive facial exercise and movements actually lead to fine lines and wrinkles; photo-induced genetic damage is, in fact, responsible for the increased expression of inflammatory cytokines, involved in oxidative stress phenomena and in the generation of accelerated aging phenotypes and skin cell senescence phenotypes [1].

In response to genetic and environmental factors, aging skin can be defined as a chronic degenerative disease in which the combination of intrinsic and extrinsic factors play an important role in modifying regenerative, structural, and defensive capability of the epidermis. The importance of genetic variability on the development of complex diseases is well known. In recent years, research focused the role of genes and their variants in the onset of specific diseases.

Modifications to a coding gene may result in the production of proteins with a different functionality, characterized by primary and tertiary structures, different from those expected and potentially responsible for individual predisposition to certain diseases. Single-Nucleotide Polymorphisms (SNPs) are the most common genetic modifications.

In the context of chronoaging, modifications to the genes that encode for type 1 collagen (*COL1A1*) and elastin (*ELN*) are among the most studied individual variability factors. Type 1 collagen is the main structural component of the extracellular matrix of the dermis and its decline in quality and quantity is directly involved in tissue relaxation phenomena typical of senescence. Numerous studies have shown that common polymorphisms of the *COL1A1* gene may change the expression of the above-mentioned protein, consequently altering its production and turnover [3]. Elastin is a structural protein of the connective tissue and is the main component of the elastic fibers that make up the dermis. There are polymorphisms associated to the *ELN* gene that code for proteins with altered mechanical properties, which

are, therefore, responsible for an increased risk of impairment of skin elasticity [4].

In the context of photoaging, various genetic variability factors can take part in degenerative metabolic processes.

Metalloprotease 3 (also known as MMP3) is a protease involved in the degradation of the constituent components of the extracellular matrix of the dermis and in the tissue remodeling process that is commonly activated during inflammatory phenomena. After sunlight exposure, inflammation, or skin oxidative stress, MMP3 is activated and takes part in the degradation process of collagen fibers and elastic fibers that comprise the dermis; enzymatic activity of MMP3 can be modulated by genetic polymorphisms as shown by the literature [5].

Free radicals (ROS=Reactive Oxygen Species) are highly reactive substances derived from molecular oxygen that can damage the DNA and cell structures, thus altering the metabolic processes. Improper diet, stress, and exposure to cigarette smoke and pollutants are just some of the factors involved in increasing the cellular production of reactive oxygen species; our body is physiologically equipped with protective systems against ROS, in which the enzymes superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPX1) and catalase (CAT) play a key role in the transformation of radical species into inert species, which can be easily removed.

It is well known that the variability due to the presence of polymorphisms of the coding genes for these proteins modulates enzymatic activity, thereby causing a different individual susceptibility to oxidative stress [6]. Similarly, allelic variants of the genes that code for the cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) can determine a different susceptibility to inflammatory stimuli [7-9].

The growing understanding of the genetic basis of many common multifactorial diseases has opened the way to personalized medicine, which means the creation of preventive and personalized therapeutic actions based on genetics. Numerous studies have introduced analytical methods able to assess the contribution of multiple variants in the development of complex diseases, significantly increasing the predictive value of the test [10]. Using this approach we have demonstrated how it is possible to correlate a genetic index (GRS-Genetic Risk Score) that takes into account the contribution of individual SNPs involved in the metabolic processes of the skin (collagen turnover, elastin structure, and susceptibility to inflammation and oxidative stress) and that can be used for the formulation of personalized beauty treatments.

The purpose of this work is to evaluate the possibility of genetic customization of an anti-aging radiofrequency treatment, combined with the simultaneous administration of phytotherapeutic active ingredients acting selectively on the metabolic impairments identified by the analysis of specific DNA variants.

Materials and Methods

Individual genetic susceptibility to skin aging

The GRS index (Genetic Risk Score) is constructed by means of sampling and genotyping the patient's DNA, i.e., characterization of the genetic constitution of an individual by identifying specific polymorphisms of *COL1A1* genes, *ELN* involved in the chronoaging process, and specific polymorphisms of the genes CAT, GPX, MnSOD2, IL-1β, TNF-α, and MMP3 involved in the photoaging process.

The selected SNPs were *COL1A1* rs1800012, involved in the type I collagen turnover [3], MMP3 rs3025058, that influences the breakdown of extracellular matrix and tissue remodelling [5]; *ELN* rs2071307, that affects assembly and mechanical properties of the elastic matrix4; CAT rs1001179, GPX rs1050450 and MnSOD2 rs1799725, that influence individual antioxidant capacity [6]; IL-1β rs1143634 and TNF-α rs1800629 that modulates anti-inflammatory response [7-9].

Each polymorphism analyzed is assigned an arbitrary numerical value that quantifies the impact of the previously mentioned genetic variation on individual susceptibility to aging, based on the information available in the literature cited above. This arbitrary numerical value is equal to 1 if the detected genotype contains two alleles considered to be unfavorable, and therefore is associated with increased susceptibility to skin aging; it is equal to 0 if only one unfavorable allele was detected; and it is equal to -1 if no unfavorable alleles were detected. The correlation between the assigned numerical value and the genotype and is exemplified in Table 1.

For each patient the GRS is calculated by means of an additive model, by adding the scores obtained for each of the single-nucleotide polymorphisms identified in the patient's genome and listed in Table 1, according to the "single SNP based test" model described in the work of Ballard and colleagues [11]. The genetic risk index can ideally assume any value between -8 and 8, in which GRS=-8 indicate the lowest genetic predisposition to skin aging, while GRS=8 indicate the highest susceptibility. Once the patient's GRS has been calculated, individual sensitivity to chronoaging and photoaging is determined by comparing each patient's GRS (Table 2).

Table 2 was created by combining the calculation of GRS with the frequencies of the polymorphisms under examination in the population of European origin, as can be derived from published data collected in the HapMap database [12] and in searchable databases on the website of the National Centre for Biotechnology Information [13].

Area	Sensitivity	GRS Values	Frequency
Chronoaging	Low	0 ≤ GRS ≤ -2	36.72%
	Intermediate	1	40.86%
	High	2	22.42%
Photoaging	Low	-1 ≤ GRS ≤ -6	41.70%
	Intermediate	0	26.10%
	High	1 ≤ GRS ≤ 6	32.20%

Table 2: Individual sensitivity to chronoaging and photoaging is determined by comparing each patient's GRS.

The distribution of the GRS, in relation to the genotype frequencies of the polymorphisms analyzed, makes it possible to classify the patients' genotypes into three arbitrary categories of sensitivity to chronoaging and photoaging. For each GRS there exists, therefore, a combination of two cosmetic compositions, suitable respectively to chronoaging and photoaging, standardizing the choice of products to the categories of low, intermediate, and high sensitivity.

Choice of active ingredients for the personalized treatment

The treatment include compositions prepared in the form of inert conductive gels, enriched with specific active ingredients and applied to the patient by means of a radio frequency device, which facilitates the deep absorption of the active ingredients.

The cosmetic compositions are divided into six formulations: three developed for the prevention and personalized treatment of the effects connected with Chronoaging (Table 3) and three containing specific active ingredients for treating the effects of photoaging (Table 4).

Sensitivity	Composition
Low	Aqua [Water], Propylene glycol, Saccharide isomerate, Ammonium acryloyldimethyltaurate/VP copolymer, Sodium gluconate, Benzyl alcohol, Coceth-7, PPG-1-PEG-9 lauryl glycol ether, Dehydroacetic acid, Parfum Fragrance], Hydrolyzed soy protein, PEG-40 hydrogenated castor oil.
Intermediate	Aqua [Water], Propylene glycol, Glycerin, Ammonium acryloyldimethyltaurate/VP copolymer, Saccharide isomerate, Sodium gluconate, Benzyl alcohol, Palmitoyl tripeptide-5, Coceth-7, PPG-1-PEG-9 lauryl glycol ether, Dehydroacetic acid, Parfum [Fragrance], PEG-40 hydrogenated castor oil.
High	Aqua [Water], Propylene glycol, Glycerin, Ammonium acryloyldimethyltaurate/VP copolymer, Saccharide isomerate, Sodium gluconate, Benzyl alcohol, Fagus sylvatica bud extract, Palmitoyl tripeptide-5, Coceth-7, PPG-1-PEG-9 lauryl glycol ether, Dehydroacetic acid, Parfum [Fragrance], PEG-40 hydrogenated castor oil, Lecithin, Tocopherol, Ascorbyl palmitate, Citric acid.

Table 3: Formulae used according to the genetic predisposition of sensitivity to chronoaging (respectively: low, intermediate, high).

For skin that does not show an impairment in the production of collagen and elastin, the cosmetic composition will be dedicated to increasing the hydration and nourishment of the skin to allow good cell functioning.

In the opposite case, if the genetic test detects a potential impairment in the expression of the proteins that maintain dermal tone, the composition of the gel used for the prevention and treatment of Chronoaging is targeted at stimulating the metabolism of fibroblasts, the cells responsible for collagen and elastin synthesis.

The extract of *Fagus sylvatica* (Beech tree bud) contains a high amount of phytostimulines, which are molecules that are known for their important effect of metabolic activation and have been shown *in vitro* to significantly stimulate the protein synthesis of keratinocyte cultures. *In vivo* studies have shown that *Fagus sylvatica* extract increases the smoothness of the skin by reducing the depth of wrinkles and improving skin hydration [14].

The sugar moisturizing factor is a carbohydrate complex similar to that contained in the skin, which acts by binding to the lysine amino acid residues exposed by the keratins, attracting water [15] and providing deep and lasting hydration, contributing to maintaining the skin barrier's functionality.

Palmitoyl tripeptide-3 is a synthetic peptide, able to penetrate the skin and increase the fibroblasts' production of collagen [16], my mimicking the action of thrombospondin-1, a multifunctional protein that activates the transforming growth factor beta (TGF-β) [17].

It has been shown that the peptides or proteins naturally extracted from soybeans may inhibit the action of the proteinases of the extracellular matrix, helping to maintain the integrity of the skin structure. The use of hydrolyzed soy protein increases the tropism of the fibroblasts [18], thereby promoting the synthesis of collagen and glycosaminoglycans [19]. Moreover, the hydrolyzed soy protein extract contains antioxidant peptides [20] with protective action towards the peroxidation of linoleic acid, neutralizing the effects of the peroxynitrite and oxygen free radicals [21].

The cosmetic method for the prevention and treatment of photoaging employs cosmetic compositions that contain active ingredients capable of combating and preventing the signs of photoinduced skin aging, formulated according to the genetic predisposition of sensitivity to dermatoheliosis (respectively: low, intermediate, high).

These compositions, which are shown in Table 4, act to preventively protect the skin from photoinduced damage, while maintaining over time an effective moisturizing action, preventing damage from free radicals on the cell membranes and DNA, reducing damage from solar radiation, and improving the sensation of well-being of the skin, neutralizing the sensory manifestations of inflammation.

Sensitivity	Composition
Low	Aqua [Water], Propylene glycol, Mentha piperita extract [Mentha piperita (Peppermint) extract], Ammonium acryloyldimethyltaurate/VP copolymer, Saccharide isomerate, Sodium gluconate, Benzyl alcohol, Pyrus malus extract [Pyrus malus (Apple) fruit extract], Coceth-7, PPG-1-PEG-9 lauryl glycol ether, Dehydroacetic acid, Parfum [Fragrance], PEG-40 hydrogenated castor oil, Lecithin, Tocopherol, Ascorbyl palmitate, Citric acid.
Intermediate	Aqua [Water], Propylene glycol, Mentha piperita extract [Mentha piperita (Peppermint) extract], Hydrolyzed grape fruit, Ammonium cryloyldimethyltaurate/VP copolymer, Sodium gluconate, Benzyl alcohol, Coceth-7, PPG-1-PEG-9 lauryl glycol ether, Dehydroacetic acid, Parfum [Fragrance], PEG-40 hydrogenated castor oil, Lecithin, Tocopherol, Ascorbyl palmitate, Citric acid.
High	Aqua [Water], Propylene glycol, Mentha piperita extract [Mentha piperita (Peppermint) extract], Hydrolyzed grape fruit, Ammonium acryloyldimethyltaurate/VP copolymer, Sodium gluconate, Benzyl alcohol, Coceth-7, PPG-1-PEG-9 lauryl glycol ether, PEG-40

	hydrogenated castor oil, Dehydroacetic acid, Oleyl alcohol, Parfum [Fragrance], Zanthoxylum bungeanum fruit extract, Lecithin, Tocopherol, Ascorbyl palmitate, Citric acid.

Table 4: Formulae used according to the genetic predisposition of sensitivity to photoaging (respectively: low, intermediate, high).

Peppermint is a perennial herbaceous, stoloniferous, and highly aromatic plant belonging to the Labiatae family (Lamiaceae) and to the genus *Mentha*. *In vitro* studies have demonstrated that peppermint possesses significant antimicrobial, antiviral, and antioxidant action (especially from eriocitrin) as well as anti-allergic action, and that some of the flavonoid glycosides it contains, such as luteolin-7-O-rutinoside, have a powerful effect on the release of histamine triggered by antigen/antibody reactions. Moreover, menthol can significantly suppress the production of inflammatory mediators such as leukotrienes (LT) B4, prostaglandin (PG) E2, and interleukin (IL)-β2.

Pyrus malus extract is a natural antioxidant, rich in flavonoids and chalcones that preserves the health and vitality of the skin, limiting the oxidation mechanisms of cellular proteins and enzymes, reducing *in vitro* the risk of DNA degradation.

Zanthalene, extracted from *Zanthoxylum bungeanum*, is an active ingredient that is capable of reducing wrinkles. The lipophilic hydroxylamines contained in the Zanthalene extract act transiently and reversibly on the nerve synaptic transmission of Na^+-dependent channels, affecting heat and tactile sensitivity, thus reducing skin discomfort such as itching.

Vitis Vinifera extract protects the skin from overexposure to UV rays, environmental pollutants, and adverse weather conditions. Its antioxidant efficacy is linked to the abundance of phenols, anthocyanins, and catechins present in the skin of red grapes, demonstrating an antimutagenic, antioxidant, anti-inflammatory and free-radical neutralizing action. *Vitis vinifera* extract possesses an inhibitory action on the metalloproteases responsible for dermal degeneration.

Experimental Design

The study involved 21 subjects aged between 26 and 49 years. They all signed informed consent to treatment and privacy data. All treatments were applied by means of a radio frequency device, known by its trade name Genotechnology-1®.

Genotechnology-1® device stimulates the regeneration of collagen fibers and the metabolism of fibroblasts at the dermal level through the application of medium frequency radio waves. The device is equipped with a specific bipolar handpiece, capable of delivering exogenous heat that, together with the endogenous heat generated by the passage through the dermis of the radio wave (the principle of radio frequency), makes it possible to increase the penetration of the active ingredients through the skin barrier [22]. The cosmetic compositions were selected on the basis of the degree of personal susceptibility to Chronoaging and Photoaging (Tables 3 and 4).

The first experiment evaluate the variation of skin properties induced by the personalized approach (Genotechnology) towards a standard radiofrequency treatment.

Nine subjects were treated in 10 sessions, one every 14-21 days; on one-half of the face were applied the cosmetic compositions chosen according to the patient's susceptibility, following an application procedure each session with the following order of application: preparatory gel (2 min), chronoaging gel (5 min); photoaging gel (5 min). After each step of treatment, the cosmetic composition was removed and replaced by the following one. The other half of the face was used as a control and was treated by radiofrequency using the same device (which allows the two treatment methods), by applying a standard ultrasound conductive gel (placebo) and using the same specific delivery methods as for the treated part. The choice of protocol based on the treatment of one-half of the face was made in order to eliminate individual variability, caused by exposure to different environmental pressures.

Skin properties measurements were taken using a Skin Tester Device (Selenia, Italia). Skin Tester uses ultrasound densitometry for the investigation and the measurement of facial skin properties:

Total H_2O (T_H_2O),

Intracellular H_2O (I_H_2O),

Extracellular H_2O (E_H_2O),

Skin elasticity (SE),

Thickness of the stratum corneum (SCT).

The device uses an ultrasound-emitted beam that is reflected by the dermal tissues, according to its stromal density and vascular tone, allowing the analysis of skin structure. Furthermore, the diagnostic device encompasses impedance variation as related to intracellular and interstitial water content. Therefore, total, extracellular and intracellular water can be detected [23].

Two measurements per subject were performed in the right cheek and in the left cheek, pre and post treatment. Average of the measurements was calculated. All statistical analyses were performed using the XLSTAT® (Addinsoft) software.

The second experiment evaluate the variation of phenotypic features (wrinkles) induced by the personalized approach (Genotechnology) towards a standard radiofrequency treatment.

In this case 6 subjects were treated in 6 sessions, one every 14-21 days using the cosmetic compositions chosen according to the patient's susceptibility, following an application procedure each session with the following order of application: preparatory gel (2 min), chronoaging gel (5 min); photoaging gel (5 min). After each step of treatment, the cosmetic composition was removed and replaced by the following one. The control group (n=6) was treated by radiofrequency using the same device (which allows the two treatment methods), by applying a standard ultrasound conductive gel (placebo) and using the same specific delivery times and methods as for the Genotechnology group.

Phenotypic features were analyzed using Antera 3D (Miravex, Ireland), an optical skin scanning device able to evaluate the changes over the time of skin profiles. Anthera 3D is based on the acquisition of multiple images obtained with different lighting: diodes at different wavelengths illuminate the skin with the incident light at different illumination direction. The acquired data were used for spatial analysis and multi-spectrum for the reconstruction of the texture of the skin

and the analysis of its chromophores. This device employs a specific algorithm (Spot-On™) that automatically registers two or more images to one another, by correcting displacements due to different positions of the patient when capturing an image. This algorithm allows comparing "before-and-after" images (Figure 1) in an objective manner [24].

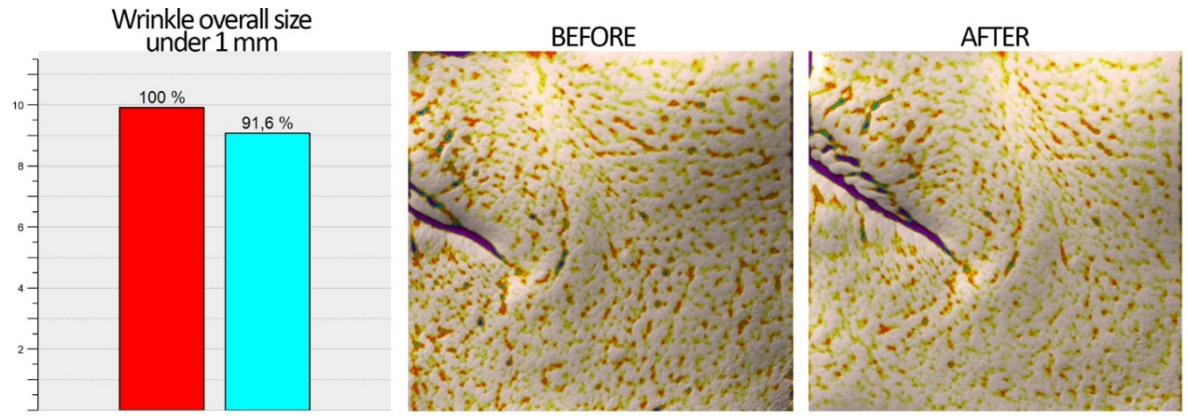


Figure 1: Example of Antera 3D® analysis output.

Five measurements were taken for each subject (Figure 2), and the mean variation of wrinkle dimension was calculated. Measurements were taken before the first treatment and after the sixth treatment. All statistical analyses were performed using the XLSTAT® (Addinsoft) software.

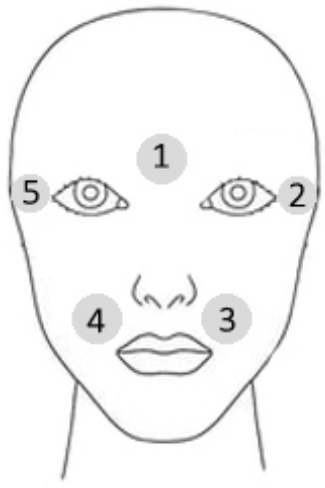


Figure 2: Wrinkles measurement areas.

Results

Regarding the effect of Genotechnology treatment on skin parameters, post treatment results show a statistically significant difference between the groups. A greater efficiency of the Genotechnology-1 treatment *vs.* radiofrequency has been shown in all the parameters examined (Figure 3). The differences between groups were assessed using Student's t-test and were all found to be highly significant (P<0.01%). The relative advantage of Genotechnology treatment range from a reduction of 40.1% more in stratum corneum thickness (from -3.6% to -5.1%) to an increase of 84.6% more in total H_2O content (from +5.1% to +9.4%).

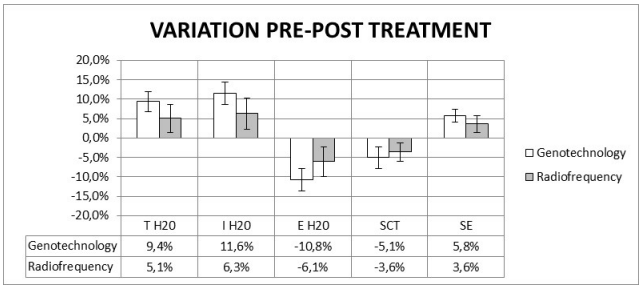
The Antera analysis shows an improvement of skin texture in both groups (Figure 4). The Genotechnology treated group show a greater decrease of wrinkles depth (<1 mm) respect to the Radiofrequency treated group (-20.7% *vs.* -6.1%). The difference was highly significant (P<0.01).

The results suggest a higher efficiency of Genotechnology in the anti-aging treatment.

Discussion and Conclusions

This is the first study that describes the application of a genetic personalized approach to the treatment of skin aging.

The use of genetic data to personalize medical therapies, based on the assumption that "one size does not fit all" has been demonstrated over the recent years in studies on gene-diet interactions [25], as well in pharmacogenetics [26].



	T H20	I H20	E H20	SCT	SE
Genotechnology	9,4%	11,6%	-10,8%	-5,1%	5,8%
Radiofrequency	5,1%	6,3%	-6,1%	-3,6%	3,6%

Figure 3: Post treatment results show a statistically significant difference between the groups. The data show increase/decrease in percentage of skin parameters after 10 treatments. Student's t-test highly significant (P<0.01%) for all parameters.

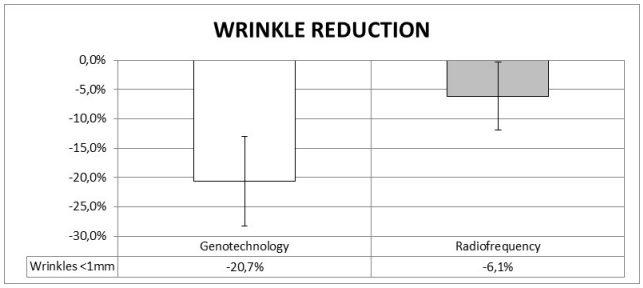


Figure 4: Wrinkle reduction results show a statistically significant difference between the groups after 6 treatments. The data show increase/decrease in percentage of wrinkles under 1mm depth. Student's t-test highly significant (P<0.01%).

Naval and colleagues [27] identified genetic clusters defined by genotypic variables linked with polymorphisms in genes associated with inflammation, oxidative stress and skin regeneration that contribute to a person's perceived age, suggesting the possibility to characterize human skin care and anti-aging needs based on individual's genetic signature. Starting from this approach, to better capture the complex relationships between genetics and skin aging, we used a multilocus genetic risk score approach [28].

Our results showed that the clusterization of subjects in different risk levels and the use of cosmetic composition according with individual genetic variability combined to a radiofrequency treatment, lead to a significant improvement of skin parameters as well to a significant decrease of wrinkles depth respect to a standard radiofrequency treatment.

Active ingredients used for the cosmetic composition are well known to act against metabolic impairments involved in accelerated skin aging. Identification of the better cosmetic composition to counteract metabolic mechanisms triggering skin aging was not the primary aim of this work; however, this pilot study was drawn up with the aim to evaluate whether genetic personalization may increase the efficacy of aesthetic treatment.

Limitations of our study include the modest sample size (n=21) and the limited number of SNPs included in the genetic analysis [8]. Only genetic variants with sufficiently described effects on skin properties were included for analysis. Although individually the impact of any one genotype on risk is modest, it has been suggested that when such risk-genotypes are common their combination may have a strong predictive power [29]. Several studies demonstrated that the aggregation of the contribution of multiple SNPs, selected from both candidate genes and genes identified through large-scale genomic association studies, into a single Genetic Risk Score (GRS) significantly increases the prediction power of the susceptibility to develop complex diseases like cardiovascular disease, type II diabetes, periodontitis or psoriasis [30-33].

Taking account these limitations, aware that further studies will be needed to confirm our data, this pilot study showed that genetic analysis applied to the prevention of chronoaging and photoaging may lead to a customized cosmetic method with significantly higher effectiveness compared to non-specific cosmetic treatments such as radiofrequency.

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