A liposome-based formulation containing equol, dihomo-γ-linolenic acid (DGLA), and propionyl-L-carnitine to prevent and treat hair loss: a prospective investigation.

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Conflict of interest

The authors declare that they have no conflict of interest. Francesca Consolaro and Monica Campisi are employees of Fidia Farmaceutici S.p.A. All the authors state, however, that Fidia Farmaceutici S.p.A. did not participate in the decision to submit this manuscript for publication.

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Abstract Hair loss is a common aesthetic disorder that can be triggered by genetic, inflammatory, hormonal, and environmental factors acting on hair follicles and their life cycle. There are several types of hair loss that differ in causes, symptoms, and spatial and temporal progression. Androgenic alopecia, a common form of hair loss, is the consequence of a decreased microcirculation of the scalp as well as the toxic action of elevated dihydrotestosterone levels on the hair bulbs. In the present study, the lotions TRINOV Lozione Anticaduta Uomo and TRINOV Lozione Anticaduta Donna, containing dihomo-γ-linolenic acid (DGLA), S-equol, and propionyl-L-carnitine, were tested on 30 men and 30 women (mean age of men was 46.6 ± 6.4 years; mean age of women was 49.5 ± 9.0) with signs of androgenic alopecia, respectively. DGLA is a precursor of the prostaglandin PGE1, which acts by improving microcirculation, S-equol inhibits 5α-reductases, thus preventing the transformation of testosterone into dihydrotestosterone, and propionyl-L-carnitine promotes lipid metabolism, stimulating energy production. These three molecules are loaded into liposomes for their effective transdermal delivery. Daily topical applications of the lotions resulted in a hair count that significantly increased for women and marginally increased for men after 6 months of treatment. Furthermore, significant increase in anagen hair and a significant decrease in telogen hair were observed starting from 3 months in male and 1 month in female patients. Thus, the formulations under investigation were effective in attenuating androgenic alopecia-related hair loss in men and women .

Key words: Hair loss, androgenetic alopecia, liposomes, S-equol, dihomo-γ-linolenic acid (DGLA), propionyl-L-carnitine

Introduction

Hair loss is a frequent aesthetic disorder, with male and female pattern hair loss affecting 50% of men by 50 years of age and nearly 50% of women (Rogers et al., 2008). Humans are usually born with approximately 5 million follicles, and no new follicles are thought to be added after birth. The hair follicle cycle includes three stages: anagen, telogen, and catagen. In the anagen stage, the hair undergoes growth; follicular keratinocytes then undergo apoptosis in the catagen stage, leaving a club hair. Telogen is the resting period within the two stages; during this period, the club hair is shed, new anagen hair replaces it, and the cycle is resumed (Wolff et al. 2009; Habif 2010). The hair follicle cycle is not synchronous, and at a given moment, different hair follicles may be in different phases (Krause et al, 2006; Paus et al., 1999).

Hair loss is consequent to disorders affecting the hair follicles and the hair follicle cycle (Qi et al., 2014), caused by genetic, inflammatory, hormonal, and environmental factors, usually acting in combination (Breitkopf et al., 2013). In androgenic alopecia, for example, follicles undergo progressive miniaturization, with a decrease in anaphase duration and a corresponding increase in the duration of the telogen stage because of increased dihydrotestosterone levels (Piérard-Franchimont et al., 2001; D'Ovidio et al., 2014). In telogen effluvium, an increased proportion of follicles enter the telogen stage because of a variety of possible environmental triggers (Malkud, 2015). In alopecia areata, hair loss is thought to be caused by an autoimmune response to hair follicles in the anagen stage (Perera et al., 2015).

Approaches for treating hair diseases aim to directly or indirectly return an individual's hair follicle size, density, and growth cycles to within normal parameters (Breitkopf et al., 2013). Drug treatment currently involves the use of minoxidil or finasteride as the primary treatments (Rogers et al., 2008; Nusbaum et al., 2013). Minoxidil, (2,4-diamino-6-piperidinopyrimidine-3-oxide) acts by stimulating angiogenesis in the follicular hair anagen stage (Meidan et al., 2001). Finasteride, administered orally, is a synthetic 4-azasteroid compound acting as a competitive and specific inhibitor of dihydrotestosterone receptors (Gupta et al., 2014). Use of minoxidil is associated with adverse dermatological (Spindler et al., 1988) and non-dermatological effects (Wu et al., 2016), while adverse effects of finasteride include decreased libido and erectile dysfunction in men (McClellan et al., 1999; Libecco et al., 2000; Gupta et al., 2014). Finasteride is also contraindicated in pregnant women or women who wish to become pregnant (Blumeyer et al., 2011; Kanti et al., 2018). As effective and side-effect free adjuvant treatments for hair loss are still lacking, alternative or supplemental treatments are currently being extensively investigated (Herman et al., 2017;

Herman et al., 2016; Kelly et al., 2016; Famenini et al., 2014: Lourith et al., 2013). Many nutritional supplements have been used traditionally to treat hair disorders, aiming to provide anti-oxidative benefits or increase vitamin support, but evidence of their performance in non-deficient patients is limited (Finner et al., 2013; Soleymani et al., 2017; Rinaldi et al., 2017). Some cosmetic products are available on the market, including shampoos and lotions based on different herbal extracts (Bussoletti et al., 2018; Herman et al., 2017), yet robust evidence of their effectiveness in preventing and treating hair loss is still lacking.

Recently, two new hypoallergenic cosmetic formulations, one for men and the other for women, have been devised for preventing and treating hair loss. Both contain S-equol, dihomo-γ-linolenic acid (DGLA), and propionyl-L-carnitine, embedded into liposomes functioning as carriers, yet the formulation for men contains double the quantity of equol than that for women due to the fact that men have higher levels of testosterone. Equol (7-hydroxy-3[4'hydrozyphenyl]-chroman) is the main metabolite of daidzein, an isoflavone abundant in soybeans, acting as a phytoestrogen; it exists in two enantiomeric forms, S- and R-equol (Setchell et al., 2010). S-equol has long been used for reducing menopausal symptoms by oral administration (Thomas et al., 2014; Taylor et al., 2015). It also binds dihydrotestosterone, inhibiting the hormone in vivo (Lund et al., 2011), and it may inhibit 5α-reductases (Hiipakka et al., 2002; Lund et al., 2011; Bae et al., 2012). DGLA is a 20-carbon ω-6 polyunsaturated fatty acid deriving, in humans, from linolenic acid. It is converted by the lipid-peroxidizing enzyme COX into various bioactive metabolites, comprising prostaglandins PGE1 and PGE2 (Wang et al., 2012). PGE1 has been long used in the treatment of peripheral vascular disorders (Murota et al., 2008) due to its effects, including vasodilation, inhibition of leukocyte adhesion, inhibition of platelet aggregation, and suppression of inflammation (Kerins et al., 1991). DGLA contained in the formulations may therefore increase the local levels of PGE1 and PGE2, favoring microcirculation, and acting only as a precursor of these bioactive molecules, not as a drug. Propionyl-L-carnitine is a naturally occurring derivative of carnitine that protects tissues from oxidative damage as a result of its antioxidant properties (Mingorance et al., 2011) and favors carbohydrate and lipid metabolism, increasing adenosine triphosphate (ATP) generation, thus stimulating energy production (Wiseman et al., 1998). Liposomes have been extensively used as effective carriers for transdermal delivery of different medications, and liposomes made of phospholipids, like those of the two formulations of interest, have been shown to be effective especially for topical applications (Ashtikar et al., 2016: Franzé et al., 2017; Irby et al., 2017). The aim of the present investigation was to assess the safety and efficacy of these new cosmetic formulations through a prospective clinical study on both men and women suffering from pattern

hair loss.

Patients and methods

Study design

This was a prospective pilot study which involved a single private center in Italy (ISPE, Institute of Skin and Product Evaluation, Milan, Italy). The study was carried out in compliance with the ethical principles of the Declaration of Helsinki and the Good Clinical Practices International Conference on Harmonization (ICH) Guidelines. All subjects were healthy and volunteered for the study. All subjects provided their informed consent.

Selection of study population

Subjects underwent recruitment during a screening visit. Both men and women presented a hair loss pattern typical of androgenic alopecia. Inclusion criteria were: a) male or female Caucasian subjects characterized by a Norwood-Hamilton scale (Norwood et al., 1975) (males) class 2 to 4 or a Ludwig scale (Ludwig et al., 1977) (females) class I or II of hair loss pattern; b) 18 to 55 years of age (males) or 30 to 60 years of age (females); c) capable of following all study directions and willing to attend follow-up visits for the entire study duration. Subjects were excluded if: a) having a history of unusual skin reactions to skin care products or cosmetics; b) displaying sensitivity to any of the formulation components; c) taking topical or systemic drugs that could affect the test results (anti-inflammatory drugs, corticosteroids, etc.); d) presenting systemic diseases or skin disorders (eczema, psoriasis, severe acne) that could affect the test results or pose a concomitant risk for the subject; e) participating in other anti-hair loss investigations within the last 30 days prior to this study; or f) using any other topical or systemic anti-hair loss product.

Volunteers were asked to wash their hair 48 hours before each visit and to refrain from applying any product on their hair after washing. The formulations under investigation (TRINOV Lozione Anticaduta Uomo and TRINOV Lozione Anticaduta Donna, Fidia Farmaceutici S.p.A., Abano Terme, Italy) were provided to the volunteers as a liquid lotion in unmarked containers. Subjects were instructed to apply the lotion (approximately 1 ml) on the portion of the scalp showing more hair thinning or loss, and then spread it all over while applying a gentle massage to favor absorption. Application was to be carried out once a day for 6 months. Subjects were examined at baseline (T0), and at 1 (T1), 3 (T3), and 6 (T6) months thereafter.

Study endpoints and their assessment

Study endpoints were: a) the changes, as well as the relative number of patients showing improvement, in the total number of hairs, and the changes in: b) the number of anagen and telogen hairs in a given scalp area; c) the resistance of hair to traction when measured according to a semi-quantitative scale; d) the number of hairs lost after washing under controlled conditions; e) the hair diameter. Other endpoints were: f) the self-reported scores evaluating itching and burning; g) the

experimenter-reported scores evaluating the presence of dandruff, seborrhea, and the level of erythema; and h) an overall tolerability score. At the end of the study, subjects also provided their subjective opinion on the efficacy and the pleasantness of the treatment. The null hypothesis of the study was that the lotion was not beneficial, implying that significant differences would not be observed for any endpoint at the 1, 3, and 6 controls with respect to baseline.

The number and percentage of anagen and telogen hairs in a given scalp area were measured using a phototrichogram technique. In short, a 20-fold magnified photograph of a given (0.728-cm^2) hair-clipped area of the scalp was taken using an epiluminescence microscope system (FotoFinder Dermoscope, FotoFinder Systems GmbH, Bad Birnbach, Germany) and analyzed using a dedicated image-analysis software (TrichoSchan, DermoScan GmbH, Regensburg, Germany) (Hoffmann et al., 2002) that identifies and counts hairs in their telogen (length $\leq$ 0.65 mm) or anagen (length > 0.65 mm) phases. The 0.728 cm^2 area is the maximum area that the Trichoscan software can analyze. For this analysis, hair of subjects with blond hair was dyed to enhance contrast and facilitate software recognition. The hair-clipped area was positioned in an apical zone of the head presenting the most thinned hair pattern. In order to ensure that the analyzed area was the same at different time points, the measurements were taken using the 'anatomical landmarks' method (we took the coordinates in cm from the attachment of the ear, from the nose, etc.) to find again exactly the same area, Additionally, a picture of the area was taken from above in order to further facilitate the selection of the same area at different timepoints. Resistance of hair to traction was measured according to a pull-test evaluation: a cluster of 40 to 70 hairs was grasped adjacent to the scalp between the ends of the thumb and ring finger, a slow constant traction was exerted on the hair, and the number of hairs being removed was counted (McDonald et al., 2017). The assessment was carried out in three scalp areas: the temporal area, located 3 cm above the back auricle-line; the frontal area, located on the median line, 4 cm from the frontal hairline; and the occipital area, located on median longitudinal line, 4 cm from the back hairline. A semi-quantitative score was assigned according to the total number of hairs removed in the 3 areas as follows: 0, >6 hairs; 1, 6-4 hairs; 2, 3-1 hairs; 3, no hair. The number of hairs lost after washing was measured by placing a gauze in the sink and counting the hair collected by the gauze after washing (wash test). Volunteers were asked to shampoo only once using their habitual shampoo and to rinse their hair accurately. Hair diameter was measured on 3 separate hairs, collected at each visit, using scanning electron microscopy (SEM).

To evaluate the tolerability of the product, both the self-reported and the investigator-reported scores were given according to a 0 to 3 scale: itching and burning were graded as: 0, absent; 1, mild; 2, moderate; 3, severe. Tolerability was graded as: 0, poor; 1, mild; 2, moderate; 3, very good.

Finally, study participants provided their subjective evaluation of the efficacy and pleasantness of treatment, answering on a 4 point-scale (very effective, fairly effective, not very effective, not effective at all) to a 9-item satisfaction questionnaire.

Statistical analysis

Subjects' characteristics at baseline were summarized using descriptive statistics. Further statistical analyses were carried out separately for the male and female groups. To investigate whether the lotion was effective in reducing hair loss as well as in stimulating hair growth, total number of hair, percentages of anagen and telogen hair, and diameter of hair distribution was checked for normality using the Kolmogorov-Smirnov test; as they were found to be normal, further comparisons between data at the T1, T3, and T6 time points and those at baseline were carried out by means of repeated measures analysis of variance (ANOVA) followed by post-hoc Bonferroni tests; wash test, pull test and subjective/clinical evaluation results were compared by means of Friedman's ANOVA and Kendall's coefficient of concordance for non-parametric data. Data on the total number of hairs data were also analyzed by comparing each subject's results at 1, 3, and 6 months with baseline data and calculating the relative (%) number of subjects showing improvement. Questionnaire answers were analyzed by means of descriptive statistics. Statistical tests were regarded as significant if $p < 0.05$. All statistical calculations were performed using a standard statistical software (SAS, SAS Institute Inc., NC, USA).

Results

Thirty men and 30 women were recruited for the study. Mean age of men was 46.6 ± 6.4 years (range, 32-55); mean age of women was 49.5 ± 9.0 (range, 30-60). One woman dropped out for reasons unrelated to the study; accordingly, results concerning women refer to 29 subjects. Demographics and distribution of men and women according to the severity of their alopecia are provided in Supplementary Table 1. Pictures of two representative male and female subjects at baseline and T6 control visits are provided in Figure 1. Results concerning the total number of hairs as well as of anagen and telogen hair, the pull and the wash test, and the hair diameter are provided in Table 1. No significant differences were observed in men regarding the total number of hair at any control visits in comparison to baseline, yet men experienced a constant improvement. At the end of treatment (6 months) 63.3% of them had experienced an increase in total hair number and the percent change of total hair count after 6 months was +1.6% compared to baseline. A significant increase in anagen hair and a significant decrease in telogen hair were observed starting from 3 months (Table 1 and Supplementary Figure 1), together with a significant increase in the pull test score. The wash test provided a significantly better score starting from the first month. No significant increase in the hair diameter was observed at any time point (Table 1). Women experienced a significant increase in the total number of hairs after the first month of treatment; at 6 months, most (89.7%) had experienced improvement. The percent change of total hair count after 6 months was +1.1%, a significant increase ($p<0.0001$) compared to baseline. In women, a significant increase in anagen hair and a significant decrease in telogen hair were observed starting from the first month (Supplementary Figure 1). Pull test and wash test scores also improved starting from the first month of treatment. The hair diameter did not change significantly at any time point in women (Table 1). Results concerning the clinical evaluation of the scalp are shown in Supplementary Table 2. No significant changes were observed concerning men, except for a burning sensation that significantly decreased at 6 months; women experienced less erythema and itching than at baseline at all control visits.

During the study, no significant adverse events were observed. One female subject perceived a mild burning sensation immediately after each lotion application for the entire 6 months of treatment; however, the burning sensation disappeared within few minutes. Tolerability scores were high at all time points (men: T1, 3.0±0.0; T3, 3.0±0.0; T6, 3.0±0.0; women: T1, 3.0±0.2; T3, 3.0±0.2; T6, 3.0±0.2). Satisfaction with the efficacy and the characteristics of the lotion under investigation were addressed by means of a self-assessment questionnaire. For all items, most of subjects stated that they were very or fairly satisfied of the product efficacy and characteristics (Figure 2).

Discussion

Hair loss is a symptom of a variety of underlying conditions, including androgenetic alopecia, alopecia areata, and telogen effluvium, with androgenic alopecia being its most common cause (Lolli et al., 2017; Severi et al., 2003). Recognized treatment involves using drugs that are effective only following continued administration, and are not free from side effects, which may be so severe that they could affect the subjects' quality of life (Spindler et al., 1988; Wu et al., 2016; McClellan et al., 1999; Libecco et al., 2000; Gupta et al., 2014; Blumeyer et al., 2011). This has led to the investigation of alternative treatments, including physical therapies, cosmetic treatments, food supplements, and the use of herbal extracts (Kelly et al., 2016; Famenini et al., 2014: Lourith et al., 2013).

The lotion used in the present study has been designed and patented as cosmetic treatment, according to the rationale of providing the scalp microvessels with a precursor (DGLA) of PGE1 that favors circulation, while inhibiting the effects of dihydrotestosterone due to the inhibitory action of a natural flavonoid (S-Equol) on 5α-reductases, as well as the metabolism-enhancing effects of a derivative of carnitine. Delivery of the lotion components is ensured by embedding them into liposomes made of phospholipids, a well-known technique to achieve transdermal delivery in topical applications (Ashtikar et al., 2016; Franzé et al., 2017; Irby et al., 2017).

Results of the present study conducted on a group of male and a group of female subjects suffering from a hair loss pattern typical of androgenetic alopecia, show that these formulations were effective in reducing hair loss and favoring hair growth, while having a good safety profile. Response of men was slightly different than that of women: the lotion had a significant effect on the total hair number in women, and its effects on the number of the anagen and telogen hair as well as those on the pull test appeared later in men than in women. These differences might be due to the different hormonal profiles of the two sexes. At present, the role of androgens in female alopecia remains unclear (Olsen et al., 2001; Ioannides et al., 2015; Torres et al., 2015). Further studies are needed to investigate the differences observed in the present investigation concerning the response of women and men. Results of the present study show that the formulations under investigation were particularly effective in increasing resistance to pull test and, conversely, to reduce the number of hairs that may be lost. This should be the subject of further studies aimed at confirming these results and comparing them with those that may be achieved using other approaches. Noteworthy, the rate of adverse effects (not including transitory and unharmful burning sensation) in the present study was low, indicating that the lotions under investigation were quite well-tolerated. Further

studies should be aimed at comparing the efficacy and safety profiles of these formulations to those of other therapies. At the time of this manuscript's creation, it is unknown to the authors whether stopping the treatment causes the subjects to return to their pristine conditions and, if so, how long the process will take. Further studies with a longer follow-up should be carried out to elucidate this matter, as well as to define whether the dosing regimen used in the present investigation is the most effective. Additional studies should also contain placebo groups or comparative treatment arms (such as finasteride or minoxidil) in order to further investigate the lotions' efficacies. In addition, further studies should be carried out to assess whether the efficacy of these lotions varies in subjects of different ages and, most importantly, if they are effective to treat more severe forms of alopecia, including alopecia areata totalis.

Conclusions

Results of the present study show that the formulations under investigation were safe and effective to prevent and attenuate hair loss in men and women having a hair loss pattern typical of androgenetic alopecia. Further studies should be carried out to investigate how their effectiveness compares with other treatments and assess their dose-response profile as well as their long-term effect and their efficacy in treating different forms of alopecia.

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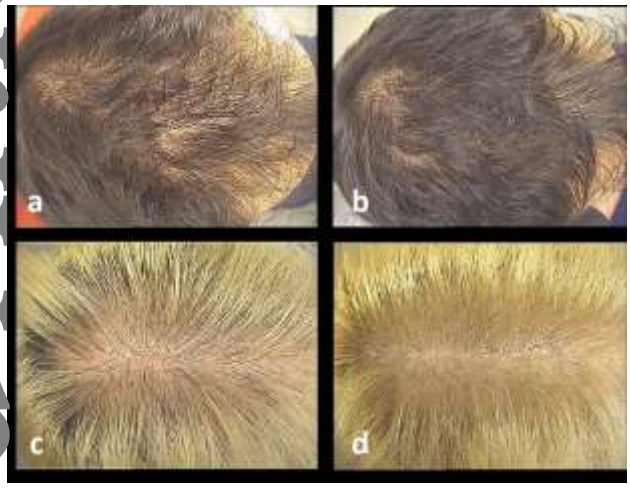
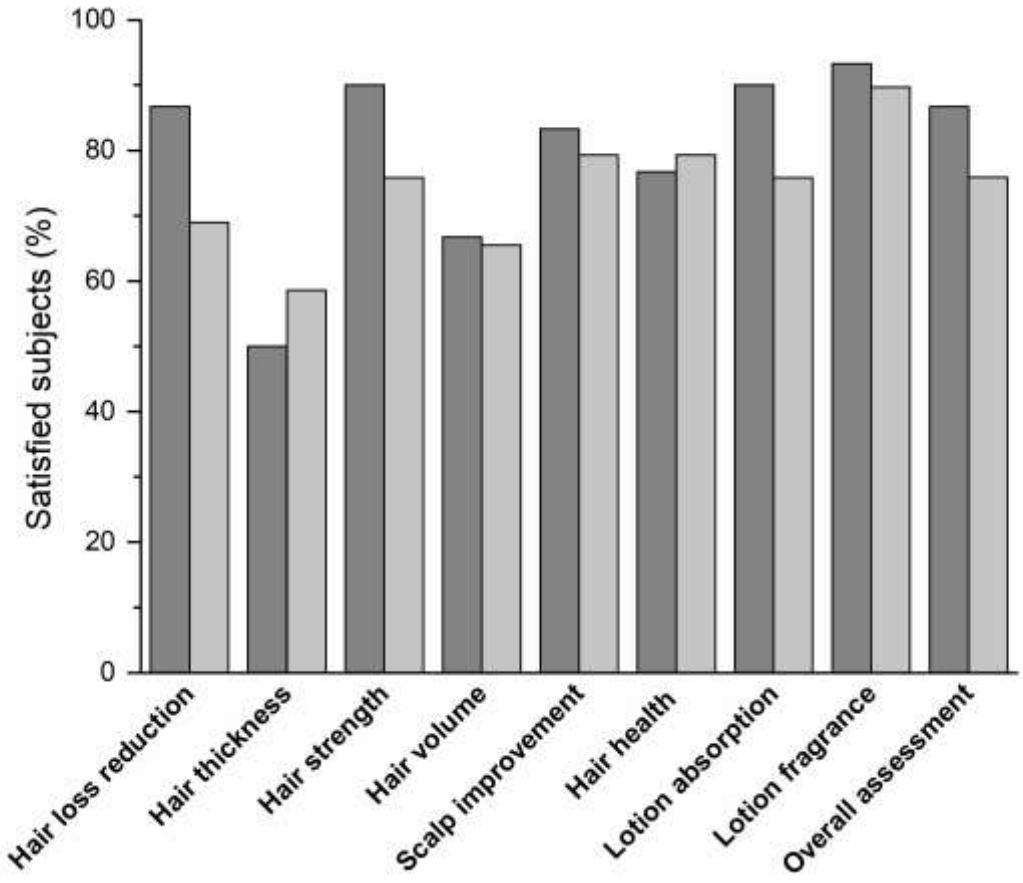
Figure legends

Figure 1. A representative subject of the male (upper part) and female (lower part) group at baseline (a, c) and 6 months (b, d). A visible increase in the hair number is observed after 6 months of treatment.

Figure 2. Percentage of men (gray) and women (light gray) being very or fairly satisfied with the efficacy and the characteristics of the lotion under investigation. Most (≥ 50%) subjects are fairly or very satisfied.





Tables

Endpoint	*Gender*	**T0**	**T_1**	**T_3**	**T_6**	**T_1 – T0 Variation (%) Test significance**	**T_3 – T0 Variation (%) p-level**	**T_6 – T0 Variation (%) p-level**
Total number of hair	M	144.5 ± 29.0	144.7 ± 30.9	146.4 ± 30.0	146.8 ± 30.5	+0.2 (+0.1%)	+1.9 (+1.3%)	+2.3 (+1.6%)
	F	161.6 ± 44.4	163.1 ± 43.7	163.1 ± 44.0	163.3 ± 44.2	+1.5 (+0.9%) ***	+1.5 (+0.9%) ***	+1.7 (+1.1%) ****
Anagen hair	M	64.3 ± 10.5	66.4 ± 10.5	67.5 ± 10.3	69.3 ± 11.0	+2.1	+3.2 **	+5.0 ****
	F	59.9 ± 11.5	64.2 ± 9.7	67.1 ± 9.3	70.2 ± 8.6	+4.3 ****	+7.2 ****	+10.3 ****
Telogen hair	M	35.7 ± 10.5	33.6 ± 10.5	32.5 ± 10.3	30.7 ± 11.0	-2.1	-3.2 **	-5.0 ****
	F	40.1 ± 11.5	35.8 ± 9.7	32.9 ± 9.3	29.8 ± 8.6	-4.3 ****	-7.2 ****	-10.3 ****
Pull test	M	1.5 ± 0.8	1.6 ± 0.7	2.0 ± 0.8	2.2 ± 0.7	+0.1	+0.5 ****	+0.7 ****
	F	0.1 ± 0.3	0.7 ± 0.5	0.9 ± 0.3	1.2 ± 0.4	+0.6 ****	+0.8 ****	+1.1 ****
Wash test	M	98.7 ± 44.9	86.9 ± 41.5	74.3 ± 33.8	61.3 ± 26.7	-11.8 (-12.0%) ***	-24.4 (-24.7%) ****	-37.4 (-37.9%) ****
	F	78.1 ± 11.9	62.4 ± 9.8	57.1 ± 10.4	52.0 ± 11.0	-15.7 (-20.1%) ****	-21.0 (-26.9%) ****	-26.1 (-33.4%) ****
Hair diameter	M	0.0595± 0.0111	0.0586± 0.0127	0.0644± 0.0176	0.0651± 0.0181	-0.0009 (-1.5%)	+0.0049 (+8.2%)	+0.0056 (+9.4%)
	F	0.0678± 0.0131	0.0709± 0.0147	0.0714± 0.0151	0.0723± 0.0138	+0.0031 (+4.6%)	+0.0036 (+5.3%)	+0.0045 (+6.6)

Table 1. Study results concerning main endpoints. Asterisks show significance of statistical tests comparing results to baseline levels. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001