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Evaluation Study

A randomized double-blind placebo-controlled clinical trial to evaluate the effect of an Annurca apple supplement formula in androgenic alopecia

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DISCLOSURE: **ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.**

ABSTRACT

Androgenic alopecia is the most common form of hair loss, affecting men and women at different ages. The role of natural bioactive compounds has gained increasing recognition as a potential means to address hair loss. There is great interest in oligomeric procyanidins, particularly procyanidin B2, which have been shown to possess hair-growing activity. Annurca (*Malus pumila* Miller cv Annurca) apple fruits have one of the highest amounts of oligomeric procyanidins, specifically of procyanidin B2, compared to more common apple cultivars. In this study, randomized double-blind controlled parallel group trial was performed to compare the efficacy of the Annurca apple fruit extract as nutraceutical AT HAIR-FUL AA® food supplement with a placebo in hair growth. AT HAIR-FUL AA® procyanidin B2 whole fruit (peel and pulp) is characterized by a complex mixture of polyphenolic compounds, especially chlorogenic acid (400 - 600 µg/g) and procyanidin B2 (60-100 µg/g). The products were assigned to 80 enrolled subjects with alopecia, divided into 2 groups: 40 subjects took AT HAIR-FUL AA® food supplement and 40 subjects took the alternative treatment (PLACEBO). Each group of volunteers underwent 180 days of treatment, with the intake of 2 capsules per day. After that, volunteers took nothing for 30 consecutive days (follow up). The AT HAIR-FUL AA® food supplement significantly affected hair loss: hair density and weight significantly increased and hair loss significantly decreased over time ($p<0.001$). Moreover, we observed a fairly good pleasantness and a good skin and gastro-intestinal tolerability of the product, confirming its compliance of use: the product did not have a significant effect on gastrointestinal disorder and stomach ache onset ($p=0.41$ and $p=0.25$, respectively). AT HAIR-FUL AA® food supplement could be used as potential agent to induce hair growth.

INTRODUCTION

Alopecia is characterized by hair loss mainly on scalp. It does not have many physically harmful effects, but psychological wellness and overall quality of life is impacted in individuals suffering from hair loss (1, 2).

The most common form of hair loss is androgenetic alopecia, affecting men and women at different ages (3, 4). It is a dihydrotestosterone-dependent process with continuous miniaturization of sensitive hair follicles (4).

Nutrition has been shown to play key roles in the risk of developing hair loss. Hair follicles have high turnover rates and high metabolic activity requiring an ample supply of energy from nutrients (5). For example, a primary component of hair is keratin, which relies on adequate protein intake or production stimulation to maintain sufficient levels in the body (6).

The use of food supplements with different bioactive compounds to prevent and manage hair loss could provide many benefits over current treatment options without producing any side effects (6).

Great interest has been aroused by oligomeric procyanidins, a family of condensed tannins, specifically the procyanidin B2, which have been shown to possess hair-growing activity both *in vitro* and in human by topical applications (7-12).

Takahashi *et al.* showed that grape seed proanthocyanidins stimulate proliferation of mouse hair follicle cells *in vitro*, with the maximum growth promoting activity for procyanidin B2, and convert hair cycle from telogen to anagen phase *in vivo* (7, 8). It was confirmed that also procyanidin B2 from apples acts as a growth-promoting factor in double-blind clinical trials (9-10). The hair-growing effects of topical application of 1% and 0.7% procyanidin B2 on the scalp and hair was investigated after sequential use for 4 and 12 months respectively (9-10). An increase in mean value of hair diameter and number of total hairs was observed (9-10).

In cultured murine hair epithelial cells, procyanidin B2 reduces the expression of isoforms of protein kinase C (PKC-α, -βI, -βII and -η), an enzyme acting as a negative hair-growing factor, and inhibits translocation of these isozymes to the particulate fraction of hair epithelial cells (11).

Kamimura *et al.* examined the hair-growing mechanisms of procyanidin oligomers and their relationship to the TGF- β signal pathway, a regulator of catagen induction in the hair cycle (12). Addition of TGF- β1 or TGF- β2 to murine hair epithelial cell cultures decreased cell growth and induced apoptosis; addition of procyanidin B2 and B3 to the culture counteracted the growth-inhibiting effects of both factors and protected the cells from apoptosis (12). Moreover, they observed that procyanidin B2 upregulates the expression of MAPK/extracellular signal regulated kinase kinase (MEK) in cultured murine hair epithelial cells (12).

Apple fruits cv Annurca have one of the highest amounts of oligomeric procyanidins, specifically of procyanidin B2, compared to more common apple cultivars, such as Red Delicious, Granny Smith, Pink Lady, Fuji and Golden Delicious (13, 14).

Annurca apple fruit, known since ancient Roman times, is one of the most important cultivars of Southern Italy (15). It is native to the Campania region (Southern Italy) and represents 60% of the Campania and 5% of Italian apple production (14, 16).

This cultivar has shown its potential as effective nutraceutical in many biological contexts (14, 16-20). Its beneficial health effects seem to depend particularly on the content of procyanidin B2 (20).

Recent preclinical evidence and clinical trials have shown an Annurca apple extract as a nutraceutical able to influence keratin biosynthesis *in vitro* and *in vivo*, affecting hair growth (18, 20).

In this study it was assessed whether the AT HAIR-FUL AA® food supplement has an efficacy in reducing hair loss, improving hair density and weight, and a good skin and gastrointestinal tolerability by comparison with the placebo. Moreover, a series of sensory evaluations perceived by the enrolled subjects were collected, with the aim of evaluating the effect and pleasantness of use of the product.

MATERIALS AND METHODS

1. Chemicals

Water (HPLC PLUS) and acetonitrile (HPLC GOLD Ultragradient Grade) were purchased from Carlo Erba (Rodano, Milan, Italy). Glacial acetic acid was obtained from VWR International Srl (Milan, Italy). Methanol (puriss. p.a., ACS reagent) was purchased from Honeywell International (San Donato Milanese, Milan, Italy). Chlorogenic acid and phlorizin dihydrate were from Sigma-Aldrich (St. Louis, USA). Procyanidin B2, (−)-epicatechin and (−)-catechin were obtained from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

2. Apple Collection

Annurca (*Malus pumila* Miller cv Annurca) apple fruits were acquired from farms located in the province of Caserta (Campania, Italy). Annurca apples were harvested manually in September-October when fruits had green peel and reddened, following a specific treatment for about 1 month (15); then the red fruits are stored or processed.

3. Preparation of AT HAIR-FUL AA, Annurca apple extract for supplement

The Annurca apple has been extracted using water. The apple extract solution has been filtrated and concentrated; then the water extract has been spray-dried in combination with maltodextrins, obtaining a fine powder with constant actives concentration, named AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp).

AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp) is characterized by a complex mixture of polyphenolic compounds, especially chlorogenic acid (400 - 600 µg/g) and procyanidin B2 (60 - 100 µg/g).

The Annurca apple supplement used in this study consisted of capsules containing AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp) (exclusive EVRA S.r.l. extract of *Malus pumila* Miller cv Annurca) (400 mg/cps) and anti-caking agent (4 mg silica). The placebo capsules contained maltodextrin and anti-caking

agent (4 mg silica). Commercial production of active supplements and placebo supplements was manufactured by Evra S.r.l (Lauria – Potenza - Italy).

4. HPLC Analysis of Polyphenols

HPLC separation and quantification of phenolic compounds in AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp) were performed according to procedures previously described (21, 22, 23) with some modifications.

AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp) phenols were extracted with methanol: water (80:20 v/v); the mixture was vortexed for 1 min, sonicated in the dark, at room temperature, for 30 min and centrifuged at 3000 rpm for 10 min. This procedure was repeated twice pooling the supernatants. The extracts were filtered through 0.22 μm nylon filters (Sartorius, Goettingen, Germany), prior to injection onto the column. Chromatographic analysis was performed through a Shimadzu Prominence HPLC system (Shimadzu, Japan), equipped with a binary pump (LC-20AT) and an UV detector (SPD-20A). Phenols were separated on a Kinetex® 5μm C18 100 Å column (250 mm × 4.6 mm) (Phenomenex, Torrance, CA, USA) at a flow rate of 1 ml/min; solvent A was 2% acetic acid and solvent B was 0.5% acetic acid in acetonitrile and water (50:50, v/v). After a 5 min hold at 10% solvent B, elution was performed according to the following conditions: from 10% (B) to 55% (B) in 50 min and to 95% (B) in 10 min, followed by 5 min of maintenance. Chlorogenic acid, (-)-catechin, (-)-epicatechin, procyanidin B2 and phlorizin were detected at 280 nm.

5. Participants

Study participants were recruited by Bio Basic Europe S.r.l. (Milan, Italy), following inclusion criteria: 50% women - 50% men for the group that tested the active product and 50% women - 50% men for the group that tested the placebo product; age between 18 and 60 years; with androgenic alopecia (men: Hamilton I / II / III, Women: Ludwig I); good general health status/absence of psychological and/or cognitive disorders; absence of dermatological and allergological pathologies (cosmetological or to other specific excipients) or other pathologies (such as irritative reactions of unknown origin); absence of ongoing pharmacological treatments which may affect the outcome of the test; non-participation in other clinical trials in the previous 30 days; informed consent shall be obtained.

Exclusion criteria were as follows: sensibility to one of the food supplement components; volunteers who do not consent to the use of personal data; smoking; obesity (BMI> 30 kg/m^2); diabetes, liver disease, kidney disease, heart disease, familiarity with chronic disease; subjects undergoing therapy or taking supplements for hair growth, subjects in drug therapy or who are taking supplements containing apple polyphenols; people who practice intense exercise (> 10 h/week); pregnant women, women with a probability of pregnancy, women who are trying to get pregnant, women in the period of breastfeeding; subjects with birch pollen allergy; subjects who took vitamins/minerals 2 weeks before taking part in the study; subjects who donated blood less than 3 months before the start of the study.

Based on Bio Basic Europe institute experience, by considering the type of product, the objectives of the trial and taking into account any possible drop-out, the sample size was composed of 80 eligible subjects.

6. Ethical considerations

This trial was evaluated and approved by the Technical Scientific Committee (TSC) of "Bio Basic Europe S.r.l." (clinical trial report number is 2025F29FC-1), whose functions are comparable to those of IRB (Institutional Review Board). TSC is an independent and impartial working group, established according to interdisciplinary criteria having its own decision-making autonomy, which has the aim and responsibility of verifying the conformity of the methodologies and validating scientific and clinical research protocols. TSC makes practical proposals and advisory opinions on each research project performed by Bio Basic Europe

S.r.l., carried out at CDC Dermo-Clinical Research Institute of Milan (Italy), microbiological, chemical-physical and *in-vitro* laboratories in the Parco Tecnico Scientifico research centre within Pavia University (Italy), as well as in laboratories, medical offices and Hospital Institutes collaborating with the company. TSC acts in accordance with the ethical principles of Helsinki Declaration, Good Clinical Practices and any applicable national and European legislation, as well as recommendations and guidelines issued by competent international bodies and institutions.

All volunteers are adequately informed of the aims, methods, clinical trial details, anticipated benefits and potential undesirable effects of the study.

7. Study design

A randomized double-blind controlled parallel group trial was performed to compare the efficacy of the tested product with a placebo.

The test performed was a double-blind study: the tested subjects, the medical specialist and the operators involved in the study were unaware of the type of products (active or placebo), which were identical in packaging, labels, odor, shape, taste and method of use.

40 eligible subjects were assigned to the group to receive AT HAIR-FUL AA® food supplement and other 40 to the placebo group.

A simple randomization derived from random number tables was performed, for the assignment of the product (active product) and the placebo. In particular, two randomizations were performed, one within the group of 40 women and one in the group of 40 men. In the group of women, 20 volunteers were assigned the active and 20 the placebo, randomly; likewise, in the group of men, 20 volunteers were assigned the active and 20 the placebo, randomly. In order to limit the potential biases on the subject recruitment and product assignment, which represent an effect of subject knowledge about the treatment, the randomization data were kept strictly confidential; none of the people involved in the trial had access to any information.

Volunteers were instructed to take two capsules of AT HAIR-FUL AA® per day for 180 consecutive days. After that, volunteers took nothing for 30 consecutive days (follow up).

The following rules were imposed for a possible withdrawal of subjects during the trial: breach of one of the inclusion/exclusion criteria; development of adverse effects.

Eligible participants were recruited from July 2020 to February 2021. All subjects underwent basal evaluations (T0, before product use) and evaluations after 60 days (T60), 120 days (T120) and 180 days (T180). Evaluations were also collected 30 days after taking the last capsule (T210). Fig. 1 reports the study flowchart.

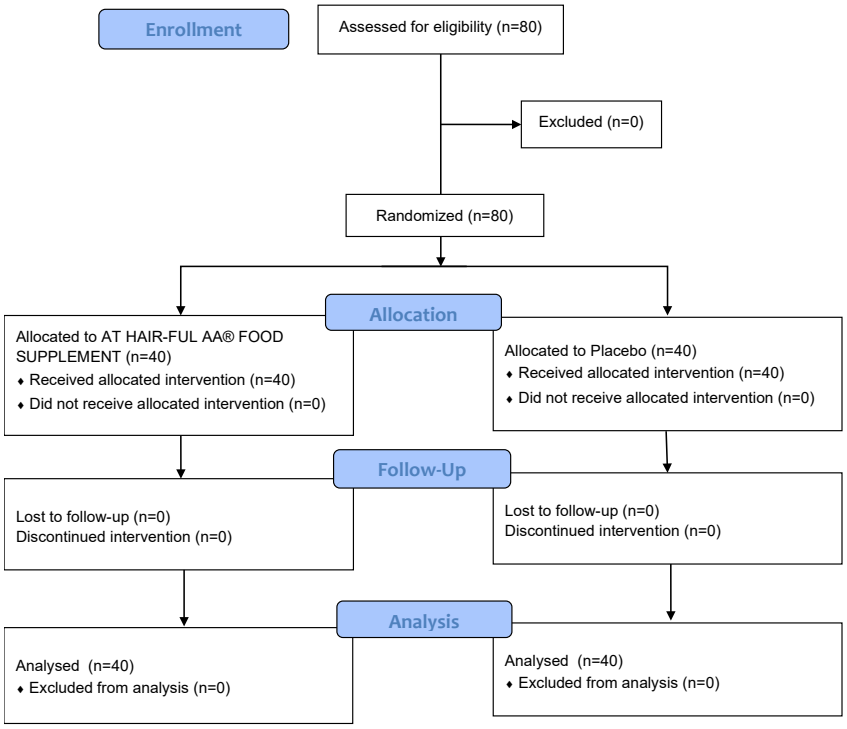

Fig. 1. *Study flow diagram.*

8. Primary endpoints

Primary endpoints measured were the hair density (quantitative discrete endpoint), number of hairs lost with pull test (quantitative discrete endpoint), number of hairs lost with wash test (quantitative discrete endpoint), skin tolerability (qualitative endpoint), gastrointestinal tolerability (qualitative endpoint) (24-26).

Hair density was analyzed by HIROX – RX 2000 3D (number of hairs/cm^2). HIROX is a digital three-dimensional microscope system. After having captured images of the scalp, hair density was analyzed by manually counting the number of hairs. Hair count was performed on 5 different 0.01 cm^2 areas. The average number of hairs is then calculated.

During the pull test, a small (about 1 cm) hair strand was grasped and held between thumb and forefinger. A constant traction was carried out to the distal part. This procedure was repeated on 3 different scalp areas: temporal area, frontal area and occipital area.

During the wash test, voluntary hair was washed thoroughly in a sink or a container. The sink drain was covered with a gauze so that hair trapped in the sink bottom or gauze can be collected and counted. The subject was instructed to avoid shampooing for 2 days before the set test date.

Skin tolerability was evaluated by the appearance of erythema, oedema, inflammation according to judgment scale in the Table I.

Table I. *Skin tolerability judgment scale.*

Skin tolerability (skin erythema, skin oedema, skin inflammation)	
No erythema No oedema No skin inflammation	0
Slight erythema (hardly visible) Slight oedema (hardly visible) Slight skin inflammation (hardly visible)	1
Clearly visible erythema Clearly visible oedema Clearly visible skin inflammation	2
Moderate erythema Moderate oedema Moderate skin inflammation	3
Serious erythema (dark red with possible formation of light eschars) Serious oedema (extended swelling even beyond the application area) Serious skin inflammation	4

Gastro-intestinal tolerability was evaluated by the onset of stomach ache, nausea, vomiting, gastro-intestinal disorders, flatulence, diarrhea according to the following judgment scale: absence of the symptom (0), very slight symptom (1), slight symptom (2), moderate symptom (3) and serious symptom (4).

All measurements and evaluations were carried out following a rest period of at least 20 minutes in an air-conditioned room with controlled and regulated temperature and relative humidity (temperature = 21°C+/-2°C and humidity 40%-60%).

9. Secondary endpoints

Secondary endpoints measured were hair weight (quantitative endpoint) and subjective evaluations (quantitative discrete endpoint).

Hair weight was measured with ANALYTICAL SCALE KS ABS220-4 (gr). Each measurement taken was the average weight of the hairs extracted by pull-test.

All measurements and evaluations were carried out following a rest period of at least 20 minutes in an air-conditioned room with controlled and regulated temperature and relative humidity (temperature = 21°C+/-2°C and humidity 40%-60%). Subjective evaluations were self-evaluations about the questions 1 to 12 and were collected according to the VNS scale, with values from 0 to 10, where 0 is the minimum value and 10 the maximum value (discrete quantitative endpoint). Responses ≥7 were considered as fully positive and responses ≥6 as positive.

10. Statistics

Hair weight, Hair density, Pull test, Wash test

The data of the quantitative endpoints were described using the usual position and dispersion measurements: mean and standard deviation. The normality of the quantitative endpoint variables was verified by using Shapiro-Wilk test.

When the assumptions were fulfilled, a parametric one-way repeated measures analysis of variance model was applied in order to evaluate the effect of the treatment over time. When the sphericity assumption was violated, the Greenhouse-Geisser correction was applied. When the result was significant, post-hoc tests were performed. In particular, the most appropriate parametric (Student's t test) or non-parametric test (Wilcoxon signed rank test/Sign test) with Bonferroni corrections were used to compare the observation times of interest. When the assumptions were violated, a non-parametric approach was applied.

A non-parametric repeated measures analysis of variance model (Friedman test) was used to evaluate the effect of the treatment over time. When the result was significant, *post-hoc* tests were performed. In particular, after having verified the symmetry of the distribution of the differences between the paired evaluations, the most appropriate non-parametric test for paired data (Wilcoxon signed rank test/Sign test with Bonferroni corrections) was applied to compare the observation times of interest.

The homogeneity of the variances of the endpoint variables in the comparison groups was assessed by using the Levene test. Furthermore, the independence of the observations was verified. For each quantitative endpoint a mixed model of analysis of variance with a parametric approach was then applied with a factor component with two categories (product) and a repeated component (time), in order to compare the studied products and their effect over time. In case of violation of the sphericity assumption for the repeated component of the model, the Greenhouse-Geisser correction was applied. In case of a significant interaction between product and time variables, the results of the two main effects (product and time) were not taken into account and considerations regarding the outcome of the analysis were drawn. A significance level of <0.05 was considered. Analyses were performed using RStudio Version 1.3.959 © 2009-2020 RStudio, PBC.

Self-evaluations

The data of the quantitative endpoints were defined using the median. For each self-evaluation question, the percentage frequencies of the answers were also calculated, at every observation time. Finally, the percentage frequencies of the responses were summarized: responses ≥ 7 and ≥ 6 were considered respectively as fully positive and positive. The conclusions of the self-assessment test were deduced by overall analysis of the medians of responses, as: insufficient pleasantness and perceived effect (overall median<6), sufficient pleasantness and perceived effect (overall median 6-6.5), fairly good pleasantness and perceived effect (overall median 7-7.5), good pleasantness and perceived effect (overall median 8-8.5), very good pleasantness and perceived effect (overall median 9-10).

For each self-evaluation question, a Kolmogorov-Smirnov test was used to assess that the distributions of the evaluations between the two comparison groups had the same shape. The most appropriate paired samples non-parametric test was then used (Mann-Whitney U test / Median test) to compare the data from the two groups. A significance level of <0.05 was considered. Analyses were performed using RStudio 2022.02.0 © 2009-2022 RStudio, PBC.

Qualitative endpoints

The data of the qualitative endpoints were defined using the normal position and dispersion measurements: median and interquartile range. If any adverse reactions regarding the skin and gastro-intestinal tolerability occurs, a non-parametric repeated measures analysis of variance model (Friedman test) is applied in order to evaluate the effect of the treatment over time. A significance level of <0.05 is considered. Analyses are performed using RStudio Version 1.3.959 © 2009-2020 RStudio, PBC.

RESULTS

1. Identification and quantification of polyphenols in the AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp).

AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp) polyphenols were analyzed by HPLC. Chlorogenic acid and phloridzin are the main phenolic compounds, as previously reported in Scafuri *et al*., 2016 (22). An example of AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp) HPLC chromatogram, at 280 nm, is shown in Fig. 2 and the list and content of compounds characterized in this extract is reported in Table II.

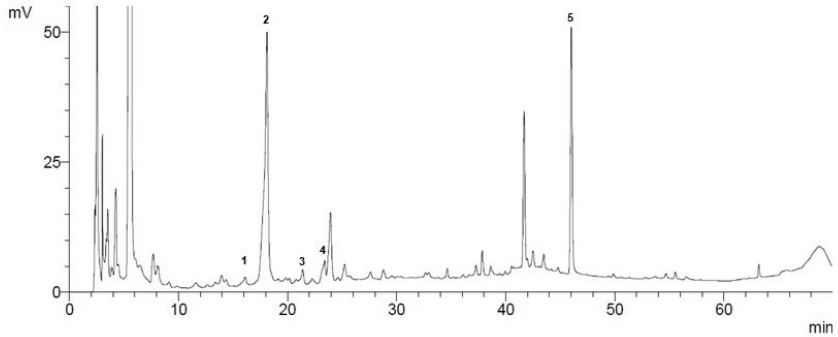

Fig. 2. *HPLC chromatogram of AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp) at 280 nm. Peaks are labeled according to Table II.*

Table II. *List and content of compounds characterized in AT HAIR-FUL AA procyanidin B2 whole fruit (peel and pulp).*

Peak	Compound	Content (µg/g)
1	(−)-Catechin	28.9±0.6
2	Chlorogenic acid	524.8±15.1
3	Procyanidin B2	71.5±4.9
4	(−)-Epicatechin	47.6±2.3
5	Phoridzin	145.2±8.1

Results are expressed as average (mean) concentration ± SD of triplicate.

2. Dietary and anthropometric assessments

The subjects were in good general health status and were asked to keep their dietary habits unchanged throughout the entire study. Volunteer features (sex, alopecia level, age, weight, height and Body Mass Index) are reported in Table III. Demographic and baseline characteristics were well balanced between study groups.

Table III. *Demographic and baseline clinical characteristics.*

Characteristic	Active (n=40)	Placebo (n=40)
Sex		
Male	20	20
Female	20	20
Alopecia level		
Hamilton I	7	9
Hamilton II	8	8
Hamilton III	5	3
Ludwing I	20	20
Age	44.5±12.6	43.3±11.6
Weight (kg)	68.1±11.3	69.6±11.6
Height (m)	1.70±0.1	1.72±0.1
BMI (kg/m^2)	23.5±2.7	23.5±2.3

Values are mean± standard deviation.

3. Enrollment and subject attrition

During the trial, no subject developed undesirable effects or breached the established inclusion/exclusion criteria. Furthermore, there were no cases of drop-out. Therefore, the analysis referred to a sample of 80 subjects.

4. Primary efficacy measure

Hair density

In the group treated with the active (AT HAIR-FUL AA® food supplement), compared to the baseline value (T0), an increase of hair density was observed as follows:

- 7% after 60 days of product treatment;

- 11% after 120 days of product treatment;
- 14% after 180 days of product treatment.

30 days after intake of the last capsule (T210), an increase of hair density by 12% was observed compared to the baseline value (T0). In the group treated with the placebo, hair density remained approximately the same as baseline at all observation times (Table IV).

In the Table IV the result of the analysis of variance was reported: it shows a statistically significant effect of the interaction between product and time; therefore, it is possible to state that the evolution over time of the hair density is different between the group treated with the active AT HAIR-FUL AA® food supplement compared to the group treated with the placebo. In particular, in the group treated with the active supplement, a greater increase of this parameter starting from 60 days of treatment was observed, while in the group treated with the placebo, hair density seemed to remain constant (Table IV). 30 days after the end of the treatment, a decrease of the variable hair density of 2% can be observed, compared to the value obtained after 180 days of treatment (T180). However, a 12% increase of the variable hair density is maintained, compared to the baseline value (T0).

Table IV. *Hair density.*

	Active			**Placebo**		
Survey times	**Mean**	**±**	**Standard deviation**	**Mean**	**±**	**Standard deviation**
T0	113	±	17[a]	114	±	16[a]
T60	120	±	18[b]	113	±	16[a]
T120	125	±	17[c]	116	±	16[a]
T180	129	±	18[d]	116	±	17[a]
T210 (follow up)	126	±	17[c]	115	±	18[a]
Two-way mixed-design ANOVA						
Variability	**F**			***p*-value**		
time	45.70			<0.001		
product	4.25			0.042		
time*product	30.85			<0.001		

Significant differences are denoted by different letters (Sign test, $p<0.05$).

Number of Hairs Lost with Pull Test

In the group treated with the active (AT HAIR-FUL AA® food supplement), compared to the baseline value (T0), a reduction of the number of hairs lost with pull test was observed as follows:

- 13% after 60 days of product treatment;
- 20% after 120 days of product treatment;
- 26% after 180 days of product treatment.

30 days after intake of the last capsule (T210), a reduction of the number of hairs lost with pull test of 24% was observed compared to the baseline value (T0). In the group treated with the placebo, the number of hairs lost with pull test remains the same as baseline at all observation times (Table V).

In the Table V the result of the analysis of variance was reported: it shows a statistically significant effect of the interaction between product and time; therefore, it is possible to state that the evolution over time of the number of hairs lost with pull test is different between the group treated with the active AT HAIR-FUL AA® food supplement compared to the group treated with the placebo. In particular, in the group treated with the

active, a greater reduction of this parameter is observed starting from 60 days of treatment, while in the group treated with the placebo, the number of hairs lost with pull test seems to remain constant (Table V).

Table V. *Number of hairs lost with pull test.*

	Active			**Placebo**		
Survey times	**Mean**	**±**	**Standard deviation**	**Mean**	**±**	**Standard deviation**
T0	6	±	2^a	6	±	2^a
T60	5	±	1^b	6	±	1^a
T120	5	±	1^b	6	±	1^a
T180	4	±	1^c	6	±	2^a
T210 (follow up)	5	±	1^b	6	±	2^a
Two-way mixed-design ANOVA						
Variability	**F**			***p*-value**		
time	13.29			<0.001		
product	6.38			0.01		
time*product	8.39			<0.001		

Significant differences are denoted by different letters (Sign test, $p<0.05$).

Number of Hairs Lost with Wash Test

In the group treated with the active (AT HAIR-FUL AA® food supplement), compared to the baseline value (T0), a reduction of the number of hairs lost with wash test was observed as follows:

- 13% after 60 days of product treatment;
- 25% after 120 days of product treatment;
- 33% after 180 days of product treatment.

30 days after intake of the last capsule (T210), a reduction of the number of hairs lost with wash test of 31% was observed compared to the baseline value (T0). In the group treated with the placebo, the number of hairs lost with wash test remains approximately the same as baseline at all observation times (Table VI).

In the Table VI the result of the analysis of variance was reported: it shows a statistically significant effect of the interaction between product and time; therefore, it is possible to state that the evolution over time of the number of hairs lost with wash test is different between the group treated with the active AT HAIR-FUL AA® food supplement compared to the group treated with the placebo. In particular, in the group treated with the active, a greater reduction of this parameter is observed starting from 60 days of treatment, while in the group treated with the placebo, the number of hairs lost with the wash test seems to remain constant (Table VI).

Table VI. *Number of hairs lost with wash test.*

	Active			**Placebo**		
Survey times	**Mean**	**±**	**Standard deviation**	**Mean**	**±**	**Standard deviation**
T0	8	±	3[a]	8	±	2[a]
T60	7	±	3[b]	8	±	2[a]
T120	6	±	2[c]	7	±	2[b]
T180	6	±	2[c]	7	±	2[b]
T210 (follow up)	6	±	2[c]	7	±	2[b]
Two-way mixed-design ANOVA						
Variability	**F**			***p*-value**		
time	34.19			<0.001		
product	3.00			0.09		
time*product	18.06			<0.001		

Significant differences are denoted by different letters (Sign test, $p<0.05$).

Skin Tolerability

There were no cases of erythema, oedema and inflammation insurgence in the group treated with the active AT HAIR-FUL AA® food supplement and with the placebo product.

Gastrointestinal Tolerability

One case of very slight reaction insurgence of gastrointestinal disorders in the group treated with the active AT HAIR-FUL AA® food supplement was observed. The analysis of variance did not show a statistically significant effect of the time: the product use did not have a significant effect on gastrointestinal disorders insurgence (the averages of gastrointestinal disorders of the analyzed groups at the different observation times are not statistically different, p=0.41).

Two cases of very slight reactions of onset of stomach ache in the group treated with the active AT HAIR-FUL AA® food supplement were observed. The analysis of variance did not show a statistically significant effect of the time: the product use did not have a significant effect on stomach ache onset (the averages of stomach ache of the analyzed groups at the different observation times, are not statistically different, p=0.25).

There were no cases of diarrhea, flatulence, nausea and vomiting onset in the group treated with the active AT HAIR-FUL AA® food supplement.

There were no cases of gastrointestinal disorder, stomach ache, flatulence, nausea and vomiting onset in the group treated with the placebo product.

5. Secondary efficacy measure

Hair weight

In the group treated with the active (AT HAIR-FUL AA® food supplement), compared to the baseline value (T0), an increase of hair weight was observed as follows:

- 10% after 60 days of product treatment;
- 22% after 120 days of product treatment;
- 34% after 180 days of product treatment.

30 days after intake of the last capsule (T210) an increase of hair weight of 30% compared to the baseline value (T0) was observed. In the group treated with the placebo, hair weight remains approximately the same as baseline at all observation times (Table VII).

In the Table VII the result of the analysis of variance was reported: it shows a statistically significant effect of the interaction between product and time; therefore, it is possible to state that the evolution over time of hair weight is different between the groups treated with the active AT HAIR-FUL AA® food supplement compared to the group treated with the placebo. In particular, in the group treated with the active, a greater reduction of this parameter starting from 60 days of treatment is observed, while in the group treated with placebo the hair weight seems to remain constant (Table VII). 30 days after the end of the treatment, a decrease of the variable hair weight of 3% can be observed, compared to the value obtained after 180 days of treatment (T180). However, a 30% increase of the variable hair weight is maintained, compared to the baseline value (T0).

Table VII. *Hair weight.*

	Active			**Placebo**		
Survey times	**Mean**	**±**	**Standard deviation**	**Mean**	**±**	**Standard deviation**
T0	0.0009	±	0.0005[a]	0.0009	±	0.0005[a]
T60	0.0010	±	0.0006[b]	0.0010	±	0.0005[b]
T120	0.0011	±	0.0006[c]	0.0010	±	0.0005[b]
T180	0.0012	±	0.0007[d]	0.0010	±	0.0005[b]
T210 (follow up)	0.0011	±	0.0007[c]	0.0010	±	0.0005[b]
Two-way mixed-design ANOVA						
Variability	**F**			***p*-value**		
time	26.82			<0.001		
product	0.44			0.51		
time*product	19.95			<0.001		

Significant differences are denoted by different letters (Sign test, $p<0.05$).

6. Subject evaluation

In the Table VIII the percentage frequencies of positive and fully positive responses of self-evaluation were reported. There are statistically significant differences between the active treated group and placebo treated group for almost all self-evaluation responses (median test, $p<0.001$), except the variable sensation of digestibility of the product which is not statistically different between the two groups.

Table VIII. *Summary table of the percentage frequency of responses.*

Questions	Frequency % of responses			
	Active		Placebo	
	positive ≥6	fully positive ≥7	positive ≥6	fully positive ≥7
Do you think this supplement reduces hair loss?	93%	80%	58%	33%
Do you think this supplement reduces hair loss during washing?	93%	83%	58%	30%
Do you think this product reduces hair loss during brushing?	93%	85%	58%	30%
Do you think this product stimulates hair growth?	90%	73%	53%	25%
Do you think this product makes your hair stronger?	98%	85%	58%	40%
Do you think this product makes your hair thicker?	95%	83%	55%	35%
Do you think this product increases hair density?	88%	68%	50%	20%
Evaluate the digestibility of the product	100%	90%	100%	90%
Overall opinion on the product	98%	85%	55%	28%
Would you buy the product for its features?	98%	83%	48%	23%

7. Safety evaluation

Adverse event occurrence was assessed over the entire treatment period, including in the first 4 weeks after cessation of treatments. During the test, any adverse dermatological reaction, such as inflammation, erythema or eczema, and gastro-intestinal disorder, such as stomach ache, nausea, vomiting, flatulence and diarrhea, was evaluated.

DISCUSSION

The role of natural bioactive compounds has gained increasing recognition as a potential means to address hair loss (6, 18).

Nutraceuticals enriched in procyanidin B2, a dimeric procyanidin, have shown remarkable hair-growing activity both *in vitro* and *in vivo* (7-12).

Procyanidins are phenolic compounds present in plants as complex mixtures of oligomers ranging from dimers to pentadecamers, built of flavan-3-ol units (27, 28).

It has been found that procyanidins B2 and B3 show evidence of protective action on induced apoptotic cell death in murine epithelial cells, neutralizing the growth-inhibition effects of both TGF- β1 and TGF- β2 (12). Hair-growing activity of procyanidin oligomers is probably linked to their inhibitory effects on TGF- β-

induced apoptotic cell death in hair epithelial cells through both MAPK/extracellular signal regulated kinase kinase (MEK) activation and their antioxidative action (12).

It has been reported that procyanidin B2 upregulates MEK in murine hair epithelial cells and activation of MAPKK (MEK) upregulates the expression of Bcl-$_{xL}$, an antiapoptotic factor (12, 29, 30). In addition, procyanidin oligomers could exert an inhibitory effect on TGF- β-induced apoptosis through blocking oxidative stress caused by this factor (12).

Moreover, some natural products have been reported to promote hair growth via activation of Wnt/β-catenin signaling (31, 32). In a previous study, hair follicle dermal papilla cells were treated with *Morus alba* root extract, containing chlorogenic acid and umbelliferone and eliciting the telogen-to-anagen transition (32). Chlorogenic acid is involved in the reduction of β-catenin phosphorylation and increase of cellular β-catenin content (32-34). β-catenin is a key regulator of hair follicular growth and is involved in anagen induction (35, 36).

Tenore *et al.* (18) reported that Annurca apple fruit polyphenolic extract supplementation *in vitro* promotes a remarkable upregulation of keratin expression in human keratinocytes, resulting in keratin accumulation within cells. These data are confirmed in a clinical trial: a keratin hair content increase of about 35% was observed in the group consuming the apple supplement more than in the untreated group, supporting the biological effects of Annurca polyphenolic extract on hair growth (18). In hair follicles, Annurca apple fruit polyphenolic extract re-programs their metabolism: glutaminolysis, pentose phosphate pathway, glutathione, citrulline and nucleotide synthesis are inhibited and mitochondrial respiration, β-oxidation and keratin production are stimulated (20). The metabolic shift induced by Annurca apple extract spares amino-acid from catabolism, keeping them available for keratin production (20).

In particular, Tenore *et al.* (18) directly compared two treatment groups (a supplement with Annurca apple polyphenolic extract microencapsulated with maltodextrins and a supplement with Annurca apple polyphenolic extract microencapsulated with maltodextrins biotin, selenium and zinc), without an official placebo group and a true double blind on placebo control and active supplement observations due to study protocol (4 weeks of placebo treatment, followed by 8 weeks of nutraceutical treatment).

Moreover, there are different commercial Annurca based products, most of them include other substances or extracts (e.g. biotin, selenium, zinc and bamboo extract). AT HAIR-FUL AA® food supplement comprises only Annurca apple extract and not other elements that could have effects on hair and skin.

In this study, for the first time, in a randomized double-blind placebo-controlled parallel group trial, the efficacy of the Annurca apple extract as nutraceutical AT HAIR-FUL AA® food supplement in supporting the hair growth was proved, considering density, weight and number of hairs lost with pull test and wash test. Analyzing results of this study (Tables IV-VII), we found that, after 180 days, hair density and weight, increased by 14% and 34%, respectively, and hair lost with pull test and with wash decreased by 26% and 33%, respectively. After treatment had been stopped, a slight decrease of the hair density and weight of the treated groups was observed. This loss of treatment-stimulated hair growth is expected, as reported in previous studies on hair loss, since the treatment does not alter the genetic predisposition for alopecia (4, 37-39).

The results obtained in this study demonstrate a good skin and gastro-intestinal tolerability of the product AT HAIR-FUL AA® food supplement, confirming a good compliance of use, and an efficacy in supporting the hair loss reduction and hair density increase; an improvement of all studied primary end-points after product use was observed. Besides this phenomenon, an increase of the hair weight and a fairly good pleasantness were also observed.

The main strength of the current study is the randomized double-blind placebo-controlled parallel trial design, applied for the first time to demonstrate the efficacy of an Annurca apple extract as safe alternative in hair growth support. However, the limitations of this study could be the short-term evaluation of hair loss treatment

for a disorder that could became long-term, and the lack of a fully-automated tool to evaluate the hair density and growth by phototrichography.

Annurca apple extract as nutraceutical AT HAIR-FUL AA® food supplement is characterized by a complex mixture of polyphenolic compounds, especially chlorogenic acid and procyanidin B2. It may promote hair growth thanks to a synergistic effect between chlorogenic acid and procyanidin B2 both involved in hair regeneration.

AT HAIR-FUL AA® food supplement consumption, thus, exerts a hair loss reducing activity and could be used as a potential agent to induce hair growth.

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Competing interests

Filomena De Biasio, Laura Ivaldi and Riccardo Salamone are scientific consultants for EVRA S.r.l. Antonella Ielpo and Luca Santarsiere are employees of EVRA S.r.l. Domenico Gorgoglione is a member of the board of directors of EVRA S.r.l.

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