



Radiofrequency Current at 448 Khz For Female Pattern Hair Loss: Cellular Bases For Redensification Improvement

Naranjo Pablo^{1,2*}, Martínez-Pascual María Antonia³, López Andrino Rodolfo¹, Zarza Luciañez Daniel², Hernández-Bule María Luisa³

¹Elite Laser Clinic, Madrid, Spain

²Laser Department, Vithas Aravaca Hospital, Madrid, Spain

³Servicio de Bioelectromagnetismo, Hospital Universitario Ramon Y Cajal, IRYCIS, Madrid, Spain

***Corresponding Author:** Naranjo Pablo, Elite Laser Clinic, Madrid, Spain, Direction: Paseo de las Acacias nº 63, 28005 Madrid, Spain; E-mail: dr.naranjo@elitelaser.es

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Abstract

Objective: Capacitive-Resistive Electrothermal Therapies (CRET) have proven effective in tissue regeneration. This study analyzes the response to 448-kHz CRET treatment in 20 patients with Female Pattern Hair Loss (FPHL) and in woman's Dermal Papilla Cells (DPC).

Methods: Patients received ten 20-minute CRET sessions over an 8-week interval. Three months after the last session, the effects of the treatment were trichoscopically analysed. DPC were CRET stimulated intermittently for 12, 24 or 48 h and the effects on cell proliferation and expression of several proteins involved in cell proliferation were analysed.

Results: Trichoscopic data revealed generalized, statistically significant hair redensification (10 - 15 % over pre-treatment values) in all the treated scalp areas. *In-vitro* electrostimulation significantly increased DPC proliferation and expression of the proteins involved in cell proliferation. Since dysregulation of DPC proliferation is the main factor underlying abnormal hair loss, it is likely that electrically-induced DPC proliferation is involved in the redensifying effects obtained in the trichological study.

Conclusion: The results of the trichological study in FPHL patients and those of the experimental study in woman's DPC are consistent with each other and suggest that 448-kHz CRET could be effective both for hair redensification and hair loss prevention.

Keywords

Hair Growth; Hair Treatment; Cell Culture; Radiofrequency Therapy

Introduction

Decreased Capillary Density (DCD) can result from different etiologies that alter the normal pattern of the hair growth, which consists of the following phases whose duration varies according to the area of the scalp: active phase of hair formation (anagen), massive apoptosis phase (catagen) resting phase (telogen) that is followed by a phase of hair loss (exogen) and an interval of follicular void until the formation of the new follicle (kenogen) [1]. In women, the most common cause of DCD or Female Pattern Hair Loss (FPHL) is Female Pattern Androgenic Alopecia (FPAA), which is characterized by an increase in the frequency and duration of the kenogenic phase [2]. This condition, which affects approximately 40 % - 50 % of women throughout their lives, increasing its incidence with age, begins with a loss of hair at the level of the frontal and interparietal midline, and it is believed to be caused by an excess activity of the enzyme 5α -reductase, which converts testosterone to dihydrotestosterone [3].

Treatment options for DCD are varied, including the administration of drugs and nutritional supplements, as well as the use of microneedles to stimulate follicle healing and growth, stem cell treatments, hair transplant surgery, or physical therapies [4]. Among pharmacological treatments, androgen receptor antagonists (spironolactone and cyproterone acetate), 5α -reductase inhibitors (finasteride), antihypertensive vasodilators (minoxidil) or more recently, platelet-rich plasma have been tried [5,6]. However, currently only minoxidil has been approved by the FDA for the treatment of androgenic alopecia in women [7]. Although this treatment reduces hair loss, it is not capable of increasing the number of Hair Follicles (HF), which forces patients to undergo prolonged treatment that, in addition, can induce significant side effects such as sexual dysfunction, hypertrichosis and abnormalities in embryonic development [8]. Physical therapies include those using low power density laser or Low Level Laser Therapy (LLLT) and fractional lasers, which have the general drawback of not being applicable to all skin types. LLLT technology uses the non-thermal effect of red and infrared light to produce photostimulation. Although its mechanism of action has not yet been identified, it is believed that LLLT stimulates the reentry of telogen hair follicles into anagen and prolongs the duration of this phase, which would translate into an increase in the density

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and diameter of the hair [3]. Although knowledge about the efficacy of fractional laser technology is still limited, it has been proposed that it is capable of causing heat-induced microscopic lesions whose healing would stimulate hair growth [9]. In general, laser therapies do not present severe adverse effects, so there is consensus on their potential applicability to the treatment of CDC and on their safety when used correctly. However, the current paucity of double-blind, conflict-of-interest-free clinical studies does not yet allow a reliable determination of the true efficacy of this type of hair loss therapy [3,7].

Another modality of physical therapies for the treatment of alopecia is based on the use of Radio Frequencies (RF). These are electrothermal therapies that apply RF currents that, due to the resistivity of the adipose tissue to the passage of current, cause tissue thermal increases with or without ablative purposes. Non-ablative effects include vasodilation, activation of cell proliferation, neoangiogenesis, neocollagenesis or increased thickness of the dermis [10-13]. RF technologies can be applied to any skin type through non-invasive or minimally invasive procedures and with minimal risk of complications or side effects [14]. RFs have been applied successfully for aesthetic treatments such as sagging skin, wrinkles, body / skin rejuvenation, treatments for facial expression lines, recent and late fibrosis, scars and adhesions, cellulite, localized fat and alopecia [15].

Within electrothermal RF therapies, Capacitive Resistive Electrical Transfer (CRET) is a non-invasive technology that applies 448 kHz currents and has been used successfully in the regeneration of both muscle tissue and bone, as well as tendons and ligaments [16-18]. In aesthetic medicine, CRET has been shown to be potentially effective in reducing wrinkles [19,20]. However, with regard to the treatment of CDC, there are few clinical studies performed with RF therapies in general and with CRET treatments in particular, which limits the knowledge about the efficacy and the ideal conditions for the application of these technologies [21-24]. However, there is a large block of pre-clinical studies that show that, even applied under subthermal conditions, the CRET electrical stimulus has regenerative effects based on the promotion of proliferation and early chondrogenic differentiation of adipose derived human stem cells and human fibroblasts and keratinocytes, as well as of migration in fibroblasts and expression of heat shock proteins and can modulate adipogenic differentiation of human stem cells [11,12,25-27]. Taken together, the results of these studies are strongly indicative that the electrical stimulation can, by itself, induce different types of relevant cellular responses potentially involved in the effects of CRET electrothermal therapies when applied to the regeneration of various tissues.

Based on all of the above, the present study investigates the clinical efficacy of CRET electrothermal therapy in capillary redensification in women diagnosed with androgenic alopecia. Additionally, given that the proliferation of Dermal Papilla Cells (DPC) present in the human hair follicle is a fundamental phenomenon for the survival and growth of the capillary unit [28,29]. This study also focuses on the potential relevance of the electrical

component of the treatment by investigating the proliferative response of DPC from Caucasian woman exposed in vitro to the CRET electrical signal applied under subthermal conditions.

Material and Methods

Clinical Study

Patient enrollment and inclusion/exclusion criteria

The study group consisted of 25 female volunteers who were recruited from patients diagnosed of DCD who attended the Elite Laser Clinic (Madrid, Spain) and the Laser Department of Vithas Aravaca Hospital (Madrid, Spain). These patients had skin phototypes 2-4 of the Fitzpatrick classification and were between 30 and 50 years of age. All volunteers showed capillary thinning in the frontal and medial interparietal region located behind the first hairline: grade 1 of the Ludwig classification of androgenic alopecia [30,31]. No alterations were revealed by the analysis of their hormonal profile and digital trichoscopy did not show alopecia-inducing diseases. Menopausal women were excluded from the study, as well as those who declared to be aware of an active loss of hair during the last 12 months prior to diagnosis, those who during that period had been subjected to some type of treatment against DCD or who during the period of realization of this study had planned to start some type of treatment for alopecia.

Equipment for RF treatment

Non-invasive electrothermal stimulation takes place by treating the target areas with RF currents that start from an active electrode applied to the scalp and reach a self-adhesive, rectangular return electrode plate with a surface area of 132 cm² (mod. F7805P, FIAB, Vicchio, Italy), placed in the interscapular region when the patient is treated in the supine position, or at the abdominal level when she is treated in the prone position. It was used a capacitive monopolar radiofrequency equipment, model HairWave (INDIBA, S.A.U., C/ Moianés, 13, Pol.Ind. Casablanques, 08192 Sant Quirze del Vallés, Barcelona, Spain) that generates an electrical current of sinusoidal signal with a frequency of 448 kHz and densities between 115 and 125 $\mu\text{A}/\text{mm}^2$, which induced in the treated scalp areas a moderate thermal increase, the applied energy during the sessions was in the range of 5 - 7 of a subjective 11-point analogue scale that the participants used to self-report thermal sensing (0, no thermal sensing; 10, worst possible thermal sensing) according to the manufacturers' recommended safety instructions [32]. Depending on the surface of the region to be treated, different curved active electrodes were used in capacitive mode. In the cervical and frontoparietal areas, a 1750 mm² electrode

was used (Mod. ELE0148; INDIBA, S.A.U., Barcelona, Spain). In the cranial area 600 mm² and 1652 mm² electrodes (Mod. ELE0147 and ELE0154, respectively) were used.

Study Design

A prospective, open and non-randomized multicenter study design was applied. All recruited patients voluntarily submitted to clinical treatment, which they received free of charge after signing an informed consent. To guarantee permanence in the study, the patients posted a bond that was reimbursed to them when they completed the treatment. All procedures followed the principles described in the current revised version of the Helsinki declaration, good clinical practices and compliance with all Spanish applicable laws and relevant regulatory requirements for the use of medical devices. Data collection and management was carried out following Organic Law 15/1999 on the Protection of Personal Data and with Regulation (EU) 2016/679 by the European Parliament and of the Council regarding the protection of individuals in relation to the processing of personal data and the free circulation of these data [33,34].

The treatment was applied following the manufacturer's recommendations and using active electrodes with surfaces between 600 and 1750 mm² that are put in contact with the scalp through an inert electroconductive medium in the form of a cream (in hairless areas) or lotion (in hairy areas). In each case, the choice of electrode used was determined by the cranial curvature and the surface of the area to be treated, so that the maximum possible contact with the scalp is achieved. Each volunteer received 10 treatment sessions, at a rate of 2 times during the first week and once a week for the next 8 weeks. Each session consisted of two phases:

- Phase I, of electrothermal pre-treatment (IAS 5 - 7) of the cervical (supine position) and frontoparietal (prone position) regions in order to increase blood flow in the areas adjacent to the targeted cranial region. After applying the electroconductive cream (Proionic Care Cream; INDIBA, S.A.U., Barcelona, Spain), the corresponding active electrode was applied for 5 minutes per session
- Phase II, electrothermal treatment (IAS 6-8) of the cranial region: vertex and occipital (in prone position) and frontal and interparietal areas (in supine position). Previous application of hair lotion (INDIBA, S.A.U., Barcelona, Spain). The duration of the treatment was 15 minutes per session

In all sessions, both phases I and II of the treatment were applied by the same therapist. No complementary treatments were applied, and the patients agreed not to use harmful maneuvers (scalp traction or others) for hair health during the study.

Overall efficacy assessment

To evaluate the efficacy of the treatment, a model FotoFinder Trichoscale Pro System trichoscope (FotoFinder Systems GmbH, Germany) equipped with a Medicam 1000 camera (FotoFinder) was used. A total of 32 digital trichoscopic images were taken of each patient: 16 (4 for each of the 4 areas studied) immediately before the first session and 16 post-treatment, 3 months after the last session. The images were taken following the instructions of the trichoscope manufacturer and each image photographed a 0.903 cm^2 area of the corresponding region. The images were independently evaluated using TrichoLAB Suite software by three different laboratories (TrichoLAB, Warsaw, Poland), which evaluated the following parameters: Average number of hairs (No./cm²), Average hair shaft thickness (μm), Thin hairs $<30\mu\text{m}$ (%), Mid hairs $30\text{-}50 \mu\text{m}$ (%), Thick hairs $> 50 \mu\text{m}$ (%), Single follicular units (%), Double follicular units (%), Triple follicular units (%), Cumulative hair thickness (mm/cm²) and Number of follicular units (No./cm²). Additionally, the general redensifying effect was assessed using the derived Sinclair scale, a standard procedure for classifying hair loss degree in women, which assesses the width of the mid parting line of the central hair [35]. In this scale, the negative values of the comparison between the trichoscopic images before and after the treatment are indicative of redensification.

Assessment of individual responsiveness to the treatment

In order to assess the degree of individual response in the four treated areas, four fundamental parameters were selected to evaluate the redensifying effect of the treatment: number of hair follicles, cumulative thickness, number of follicular units and triple follicular units, and for each patient and parameter, it was considered that a post-treatment value greater than 10 % on the pre-treatment value is a favorable effect. This value is an arbitrary threshold adopted from the analysis of the average efficacy results, which showed statistically significant favorable effects for average increases $< 10 \%$ over the pre-treatment values in several of the parameters analyzed.

Satisfaction questionnaire

After the fifth and tenth treatment sessions, as well as in the inspection carried out 3 months after the last session, the patients responded to a satisfaction questionnaire of four questions on their perception of improvement in terms of softness, fall, quality and capillary volume.

Statistical Analysis

For the calculation of the sample size, data on sizes previously used for other similar studies were used [21,22,24].

To assess the effects of the treatment on hair redensification, the change in each parameter was evaluated using absolute values and the change between pre and post treatment was expressed in percentage of change.

The percentage of change for each parameter/variable was calculated for each patient using the following equation:

$$\% \text{ of change} = (\text{Value in Post-treatment} - \text{Value in Pre-treatment}) / \text{Value in Pre-treatment} * 100$$

For all the variables, descriptive statistics were calculated. The comparison between pre and post treatment was performed using the non-parametric Wilcoxon Test for paired samples (recommended for small sample size comparisons) and the mean difference between post and pre evaluations, and Cohen's d size effect, were also computed.

On what concerns the assessment of hair softness, hair fall, hair quality and hair volume, the frequency of answer reporting a perception of improvement is reported as well as the change across time tested using the Q Cochran test.

Statistical significance limit was defined as 0.05. Nonetheless, statistically significant results were also indicated applying the Bonferroni correction for multiple tests performed for each region (for each region 11 statistical tests were performed resulting in $\alpha = 0.0045$ significance limit). All the analyses were performed with Jamovi V.1.8.4 Software (Jamovi project, 2021) or with SPSS V. 22.0 (IBM Corp. Released, 2013).

Linear regression analysis was applied for modelling the relationship between individual responsiveness to the treatment and patient's age using GraphPad Prism 6.01 software (GraphPad Software, San Diego, CA, USA).

In-vitro study

Cell culture

Human follicle Dermal Papilla Cells (HDPC) purchased from (PromoCell, cat. no. C-12071, Heidelberg, Germany) were isolated from lateral scalp dermis of a Caucasian, non-alopecic, healthy woman. The cells were incubated in appropriate cell growth medium (PromoCell, cat. no. C-26501) and maintained in a 5 % CO₂ atmosphere at a temperature of 37 °C inside CO₂ incubators (Thermo Fisher Scientific, Waltham, MA, USA). After detached with Detachkit (PromoCell, cat. no. C-41220), the cells were subcultured once a week and plated on the bottom

of 60 mm Petri dishes (Nunc, Roskilde, Denmark) except for immunofluorescence assays, in which the cells were seeded on glass coverslips placed on the bottom of the plates. Depending on the aim of the corresponding experiment, a total of 4 to 10 Petri dishes were plated per experimental replicate.

***In-vitro* electric treatments**

The procedure and materials for *in-vitro* Capacitive Resistive Electric Therapy (CRET) treatments have been described in previous studies [11,36]. Briefly, 3 or 4 days after seeding, depending on the experiment, pairs of sterile stainless-steel electrodes designed ad hoc for *in-vitro* stimulation were inserted in all Petri dishes and connected in series. Only the electrodes corresponding to plates intended for electrical stimulation were energized using a signal generator (Indiba Activ HCR 902, INDIBA®, Barcelona, Spain) adapted to supply a 448 kHz, sinusoidal signal current identical to that applied in the treatment of patients. The remaining, unenergized plates were sham-exposed simultaneously inside an identical, separate CO₂ incubator. The intermittent stimulation pattern consisting of 5-minute pulses of the 448 kHz current delivered at a subthermal density 100 $\mu\text{A}/\text{mm}^2$, separated by 235 min interpulse lapses and administered for a total of 12 h, 24 h or 48 h. Such exposure parameters have been shown to stimulate the proliferation of human stem cells, keratinocytes and fibroblast [11,12,25,27].

XTT proliferation assay

Cell proliferation was determined by XTT assay (Roche, Switzerland). The HFDPC cultures were seeded at densities of 5500 cells/cm² and incubated for 3 days. After 48 h of CRET- or sham-treatment, the cells were incubated for 3 hours with the tetrazolium salt XTT in a 37 °C and 6.5 % CO₂ atmosphere, as recommended by the manufacturer. The metabolically active cells reduced XTT into coloured formazan compounds that were quantified with a microplate reader (TECAN, Männedorf, Switzerland) at a 492 nm wavelength. Three experimental replicates of the experiment were conducted.

Immunofluorescence for proliferation marker Ki67

The immunofluorescence assay for Ki67 was carried out on cells grown on coverslips. At the end of the 48-hour treatment the cells were fixed with 4% paraformaldehyde and permeabilized with 95/5 ethanol/acetic acid. The cells were incubated overnight with monoclonal primary antibody anti-Ki67 [SP6] (1:250, cat. No. ab16667; Abcam; Cambridge, UK) at 4 °C. Then, the secondary antibody Alexa Fluor® 488 goat anti-rabbit IgG (1:500; cat. No. A11034; Life Technologies, Oregon, USA) was added and the samples were incubated at room temperature

for 1 h. The preparations were counterstained and mounted in ProlongTM Gold antifade reagent with DAPI (cat. No. P36941; Thermo-Fisher, Oregon, USA) and studied through an inverted fluorescence microscope (Nikon Eclipse Ts2R, Japan) attached to a digital camera DS-Ri2 (Nikon, Japan). In each experimental repeat, fifteen microscope fields per coverslip were photographed and analyzed. Images from three experimental replicates were recorded and Ki67+ cells and total cells were counted through NIS-Elements Br image software (version 4.40, Nikon, Japan). Ki67+ cell identification was based on fixed thresholds of fluorescence determined and automated at the beginning of the analysis.

Immunoblot for ERK1/2, p-ERK1/2, Cyclin D1 and GAPDH expression

At the end of each experimental run, the cell samples were centrifuged and lysed in lysis buffer: 10 mM Tris HCl (Merck; Darmstadt, Germany), pH 7.6, 10 mM KCl (Merk), 1 mM dithiothreitol (Sigma), 1 mM EDTA (Bio-Rad, Richmond CA), 1 mM PMSF (Sigma), 10 µg/ml leupeptin (Sigma), 5 µg/ml pepstatin (Sigma), 100 mM NaF (Sigma), 20 mM β-glycerophosphate (Calbiochem, CA. USA), 20 mM sodium molybdate (Sigma), 0.5 % Triton x100 (ICN Biomedical, Germany) and 0.1 % SDS (Bio-Rad). The protein was quantified using Pierce BCA Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). The protein samples (50-60 µg protein aliquots) were separated in 10 % sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK) using a semi-dry system (Semi-Dryblotting, TRANSBLOT-SD; Bio-Rad).

The blots were incubated at 4°C overnight in the presence of mouse monoclonal primary antibody Cyclin D1 (1:1000, n°. cat. P2D11F11; Novocastra, Newcastle, United Kingdom), rabbit polyclonal primary antibody ERK1/2 (1:1000; n°. cat. 9102S, Thermo Fisher Scientific) and rabbit polyclonal primary antibody p-ERK1/2 (1:1000; cat n°. 44-680G, Thermo Fisher Scientific). Monoclonal mouse anti-GAPDH (1:1000; sc-47724, Santa Cruz Biotechnology, Dallas, Texas, USA) was used as loading control. To detect non-phosphorylated forms of ERK1/2, the membranes were stripped with 25 mM glycine at pH 2.0 for 30 minutes. The protein was detected using peroxidase-conjugated secondary antibodies (ECL donkey anti-rabbit, cat. No. NA934; or sheep anti-mouse, cat. No. NA931; IgG horseradish peroxidase-linked species specific whole antibody; GE Healthcare). ChemiDoc Imaging system (Bio-Rad) was used to detect ECL-chemiluminescence in the blots and the images were analyzed using the QuantityOne program (version 4.6.7, BioRad). Four petri dishes were used per treatment in each experiment. At least four replicates of each experiment were performed per protein and exposure time.

Statistical analysis

The Student's T-test for unpaired data was applied using GraphPad Prism 6.01 software (GraphPad Software, San Diego, CA, USA). Differences $p < 0.05$ between experimental groups were considered statistically significant.

Results

Five out of the 25 participating patients failed to complete the treatment: two of them fell ill with COVID-19, one changed residence and two did not regularly attend the sessions. The treatment caused no detectable adverse effects in any of the patients throughout the study, which lasted up to 3 months after the last treatment session.

Treatment results in FPHL patients

General effects on hair redensification: Derived Sinclair scale

Fig. 1 shows representative images of redensification in the four regions studied in a patient, before the first treatment session and three months after the last session. On the Derived Sinclair scale, the negative values of the comparison between the trichoscopic images before and after the treatment are indicative of hair redensification. The corresponding data, represented in Fig. 2, reveal that, in the whole sample of patients, electrothermal treatment induced significant capillary redensification, both in the areas initially diagnosed as affected by DCD (frontal and interparietal / temporal) and in those considered unaffected (non-DCD: occipital and vertex).

Effects on parameters related to the number and morphology of hairs

The data summarized in Fig. 3A shows that CRET treatment induced significant increases in the mean number and cumulative thickness of hairs in all treated areas, both DCD and non-DCD. Regarding the parameters related to hair morphology, the average shaft thickness was significantly reduced in the DCD areas (frontal and temporal), but not in the non-DCD areas. The values corresponding to the remaining morphological parameters: percentages of thin, mid and thick hairs, the deviations with respect to the respective means were high and did not present significant responses to the treatment, therefore they are not shown in the figure.

Effects on parameters related to the number and morphology of hair follicles

The data summarized in Fig. 3B shows that the treatment induced significant increases in the number of follicular units present in the DCD areas. In these areas, significant increases were recorded in the percentages of triple bigger follicular units, accompanied by a reduction in single and double follicular units. This same effect occurred in the occipital non-DCD area, but not in the vertex. The treatment did not induce significant changes in the number of follicular units in either of the two non-DCD areas.

Assessment of individual response in the four treated areas

The data in Fig. 4 show the number of patients, out of the total sample ($n = 20$), who presented favorable responses in the different treated areas (post-treatment values greater than 10 % over the corresponding pre-treatment values) in four essential parameters in redensification: average number of hairs, cumulative hair thickness, number of follicular units and number of triple follicular units. Regarding the average number of hairs, the results indicate that the individual response to treatment was more evident in the frontal and temporal DCD areas, in which 15 and 14 patients, respectively, showed increases greater than 10% in this parameter, while the total of patients who showing increased hair number were less in the occipital area (11 patients) and in the vertex (6 patients). There were also more patients with increases greater than 10 % in the number of follicular units in the frontal and temporal areas (10 and 8, respectively) than in the occipital area and in the vertex (4 and 5 patients, respectively). However, there were no notable differences between the four treated areas in the number of patients with increases in cumulative hair thickness (9 - 12 patients) and triple follicular units (10 - 15 patients).

Taken together, the results of the analysis of individual responses indicate that a majority of the patients had favorable effects in several of the treated areas. In fact, considering again the 4 selected parameters and maintaining the criterion of the difference greater than 10 % over the pre-treatment values, it was found that all the patients registered some improvement in one or more parameters in at least two of the treated areas, and up to 15 of the 20 patients showed some improvement in the 4 areas (Fig. 5).

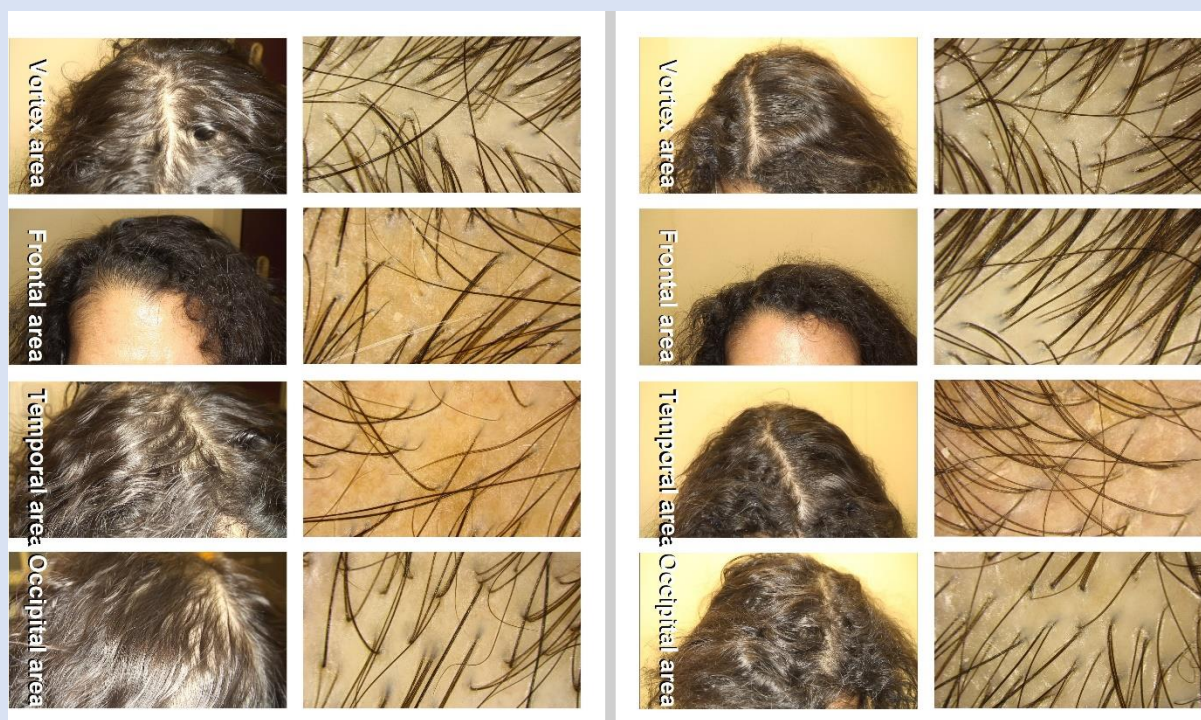


Figure 1: Capillary redensification in a patient (affected by DCD). Representative images. From left to right: Before the first session: the four areas studied in a patient before the first session (Column 1) and representative trichoscopic images of the corresponding areas (Column 2). Three months after the last session: the four areas in the same patient (Column 3) and trichoscopic images of the same surfaces photographed in column 2 (Column 4).

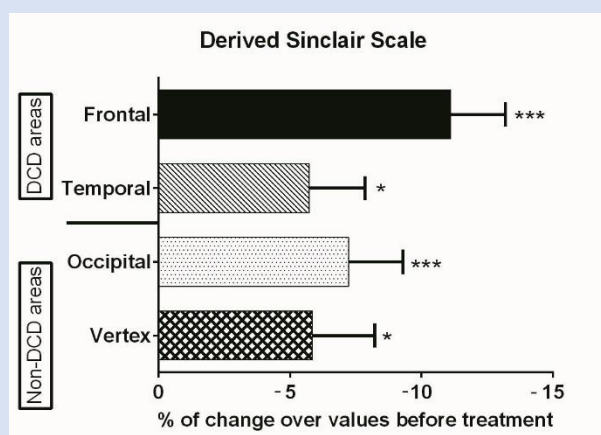


Figure 2: Evaluation of capillary redensification by derived Sinclair scale. Values are means $\pm$ SE of percentage of change between pre- and post-treatment in the four areas studied. *: $p < 0.05$; ***: $p < 0.001$: Cohen's d and Wilcoxon tests, followed by Bonferroni correction for multiple tests.

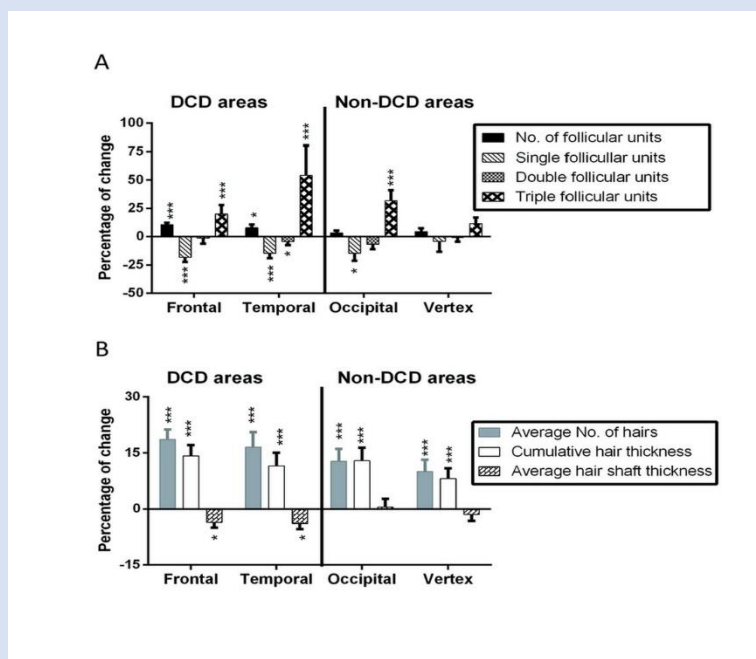


Figure 3: Effects on parameters related to the number and morphology of hairs (3A) and hair follicles (3B). Values are means $\pm$ SE of percentage change between pre- and post-treatment in the four areas studied. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; Cohen's d and Wilcoxon tests, followed by Bonferroni correction for multiple tests.

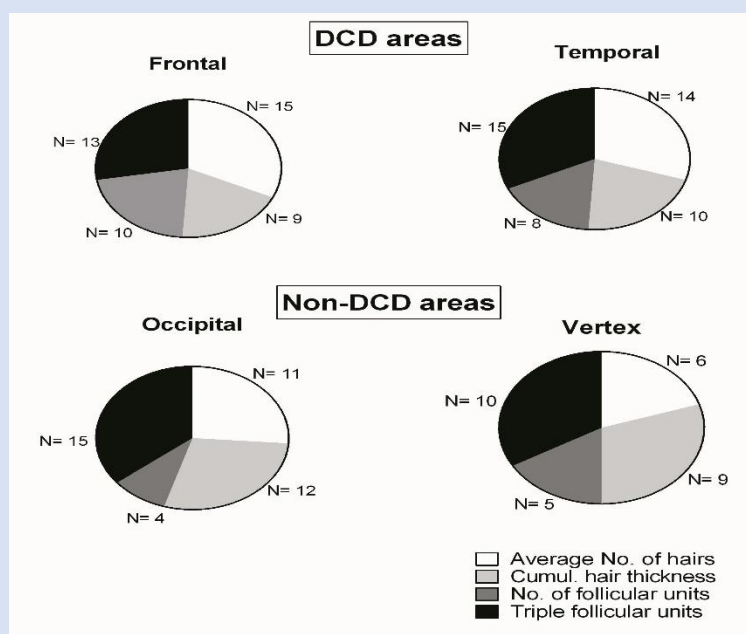


Figure 4: Number of patients (N) that showed favorable effects (>10% on pre-treatment values) in one or more of the parameters considered in the 4 areas studied, alopecic and non-alopecic.

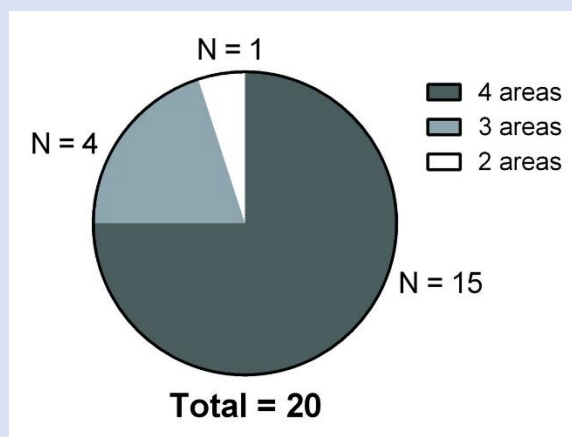


Figure 5: Number of patients (N) who showed some improvement (>10% over pre-treatment values) in one or more of the selected parameters. All patients recorded some improvement in at least two of the treated areas.

Assessment of individual responsiveness to the treatment as a function of age

The data in Fig. 6 represent for each of the patients the relationship between her age and the total number of parameters that showed favorable responses to treatment in the total of the four areas studied. No statistically significant correlation was found between both variables.

Results of the satisfaction surveys

The analysis of subjective assessment interview reveals that patients perceive some decrease in hair softness and hair fall, since the end of session five (middle of treatment) until three months after the tenth (and final) treatment session. However, these tendencies are not significant statistically. On what concerns hair quality, a significant increase is observed between the middle and end of the treatment that persists 3 months after the treatment. Finally, on what refers to hair volume perception, a significant increase is observed over time, presenting the maximum values 3 months after the last session of treatment (See Table 1).

	Middle Treatment	End treatment	3M after treatment	Q Cochran p-value
Softness	65%	55%	45%	0.513
Hair fall	65%	60%	60%	0.926
Quality	35%	70%	65%	0.020*
Volume	30%	45%	80%	0.001***

Table 1: Percentage of patients with positive perception. (*: $p < 0.05$; ***: $p < 0.001$; Cohen's d and Wilcoxon tests, followed by Bonferroni correction for multiple tests).

Effects of CRET electrical stimulation on dermal papilla cell cultures

Effects on cell proliferation

The XTT assay revealed that intermittent electrical stimulation for 48 h induced a significant increase, 20 ± 7.6 % over sham-exposed controls, in DPC proliferation (Fig. 7). This proliferative response was confirmed and reinforced by the results of the immunofluorescence assay with the proliferation marker Ki67, which showed a statistically significant increase (32 ± 8.5 %) compared to the controls in the amount of Ki67+ cells in the electrically treated group (Fig. 7).

Immunoblot analysis of proteins involved in DPC proliferation: p-ERK, ERK and cyclin D1

Intermittent electrical stimulation maintained for 12 hours caused a significant increase in p-ERK1/2 expression (51.5 ± 18.3 % over their sham-treated controls). No changes were observed at longer times, when the treatment was maintained for 24 or 48 hours (Fig. 8). Regarding the non-phosphorylated form of ERK1/2, its expression was not significantly affected by the stimulation in any of the intervals tested. On the other hand, the expression of cyclin D1 was significantly affected by electrostimulation with CRET in all the intervals studied (Fig. 9), observing significant overexpression of cyclin at the end of the 12 h and 24 h treatment intervals (23.05 ± 9.3 % and 10.3 ± 2.8 % over the corresponding controls, respectively) and subexpression after 48 h of exposure (11.5 ± 5.0 % below controls).

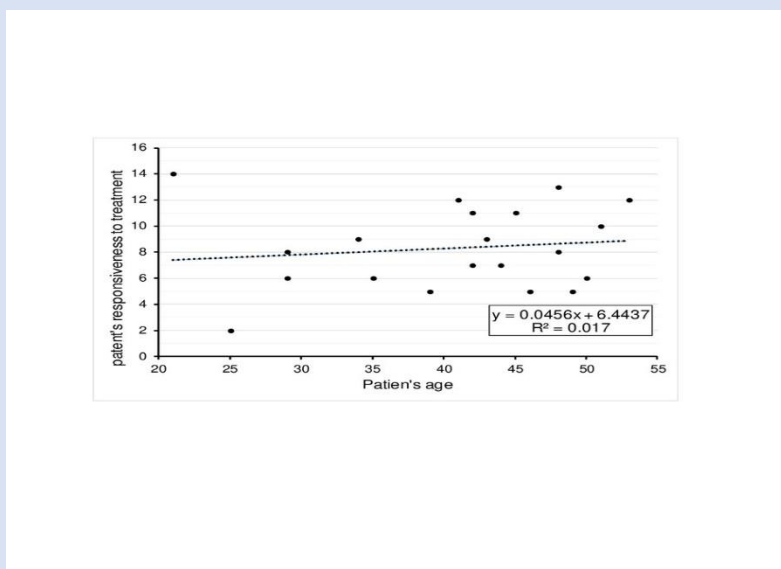


Figure 6: Correlation between the age of each patient and the total number of parameters that responded favorably (>10% over pre-treatment values) to treatment in the 4 areas studied.
Simple linear regression model.

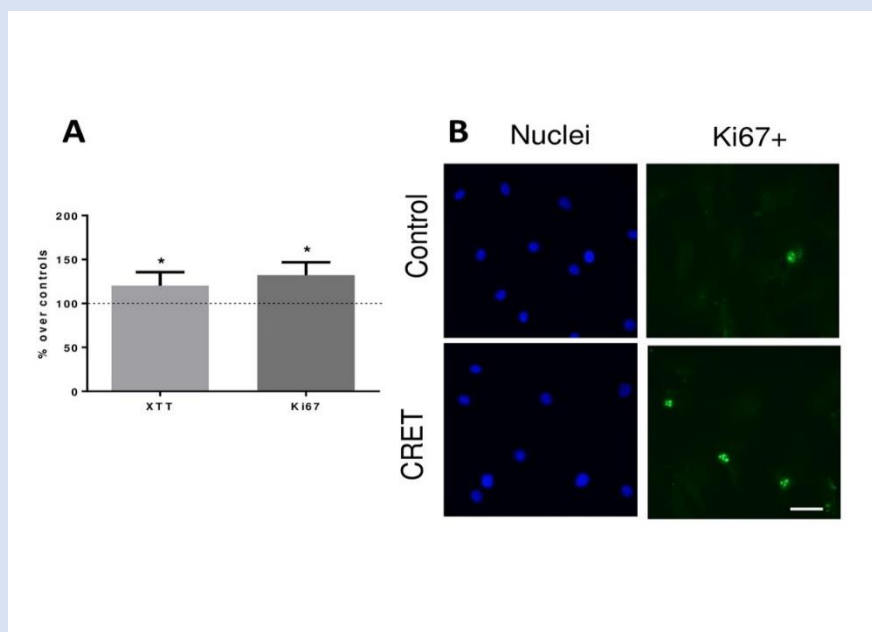


Figure 7: Proliferation assays. A. XTT assays and analysis of immunofluorescence for Ki67+ cells. The data, normalized over sham-treated controls, are the Means $\pm$ SEM of at least 3 experimental replicates. The treated samples were submitted to 48-h intermittent exposure to CRET at 100 μ A/mm². *: $p < 0.05$; Student's t-test. B. Representative images of immunofluorescence for Ki67. Green: Ki67-positive cells stained with anti-Ki67 antibody and Alexa Fluor® 488. Blue: cell nuclei stained with DAPI. Scale bar, 50 μ m. CRET: treated sample; C: sham-treated control.

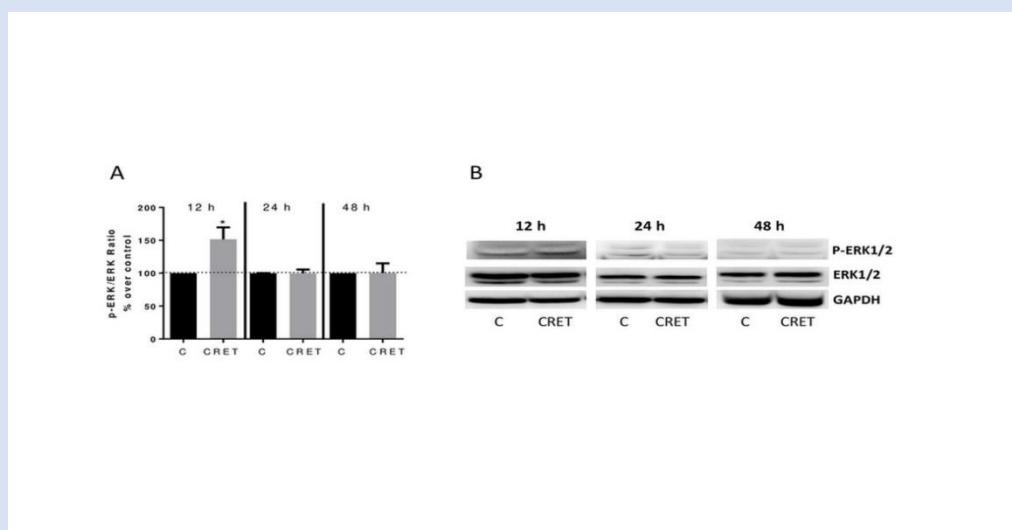


Figure 8: Immunoblot for p-ERK1/2 and ERK1/2. A. Analysis of p-ERK1/2 and ERK1/2 expression. The samples were treated with CRET (4 h intermittent exposure) for 12, 24 or 48 h. The data, normalized over sham-treated controls, are Means $\pm$ SEM of the ratio of p-

ERK1/2 over total ERK1/2 protein in at least 5 experimental replicates per treatment interval.

* $P < 0.05$; Student's t-test. B. Representative immunoblots using GAPDH as the loading control.

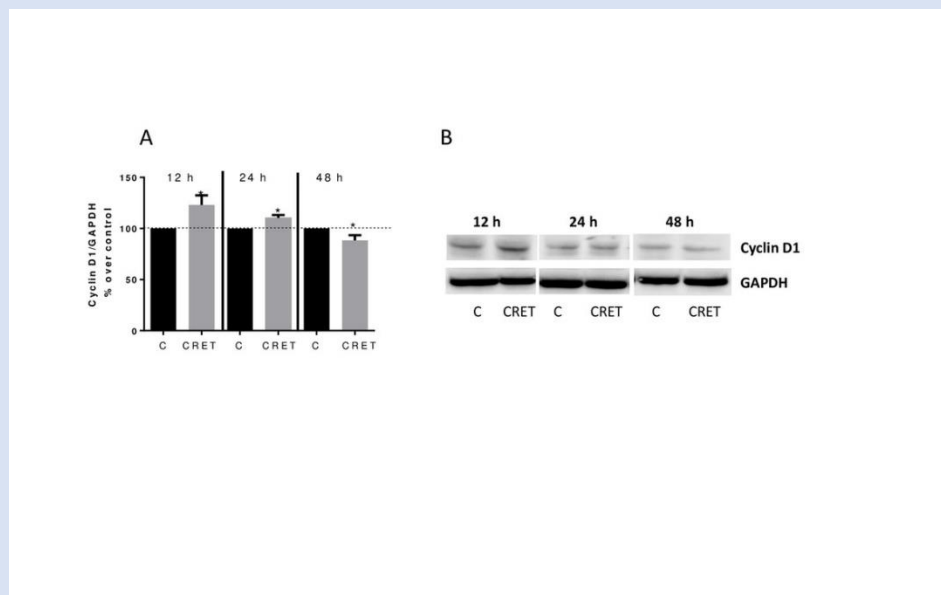


Figure 9: Immunoblot for Cyclin D1. A. Analysis of Cyclin D1 expression. The samples were treated with CRET (4 h intermittent exposure) for 12, 24 or 48 h. The data, normalized over sham-treated controls, are Means $\pm$ SEM of at least 4 experimental replicates per treatment interval. * $P < 0.05$; Student's t-test. B. Representative immunoblots using GAPDH as the loading control.

Discussion

Although there are a variety of therapies with varying degrees of efficacy in their application to the treatment of DCD, due to the deficiencies of some of them, the development of new therapies for the treatment of this disorder is considered essential. Recently, RF electrical therapies, alone or in combination with drugs, have generated promising results [21,22,24].

CRET electrothermal therapeutic technology has been shown to be effective for the regeneration of muscle, bones, tendons and ligaments and skin [16-19]. In the present study we have analyzed the response to CRET treatment at 448 kHz applied at current densities $j \geq 100 \mu\text{A}/\text{mm}^2$, which generate moderate hyperthermia in the target areas of the treatment, in a group of voluntary FPHL patients.

The analysis of the trichoscopic data according to the derived Sinclair scale reveals, three months after the end of the treatment, a generalized and statistically significant redensifying effect in the four treated areas of the scalp, despite the fact that the pre-treatment diagnosis had only detected a capillary density deficit in the frontal and interparietal areas. This electrothermally induced redensification would be related to the significant increases (approximately 10-15 % over the pre-treatment values) in the average number of hairs and the cumulative hair thickness registered in the four treated areas. Regarding the hair morphology, significantly reduced average shaft thickness was recorded in the frontal and temporal areas, diagnosed as deficient in capillary density at the beginning of the study, but not in the two remaining areas, with normal capillary density according to initial diagnosis. This confirms that the CRET electrothermal treatment exerts a hair growth promoting action. In fact, a reduction in the hair shaft thickness is a typical response in capillary redensification processes, since the incipient hairs have a reduced diameter, causing the general average of capillary diameter to decrease at the expense of the increase in the number of hairs and the capillary density.

The redensifying effect due to the formation of new follicular units was accompanied by increases in the number of follicular units, which were statistically significant in the areas characterized as affected by DCD, although not in the Non-DCD areas. Furthermore, trichoscopic analysis of the characteristics of these follicular units revealed in the frontal, temporal and occipital areas, but not in the vertex, significant increases in the rate of triple follicular units, at the cost of corresponding reductions in the rates of single and double follicular units. This is indicative of a potential capacity of the RF electrothermal treatment to increase the diameter of follicular subunits that had been miniaturized by the FPHL to diameters less than 30 microns, for which they were undetectable by the digital trichoscope in the pre-treatment analysis.

In general, these results of the set of effects observed in the pool of 20 patients are consistent with the results of the analysis of the individual response of each patient, summarized in Fig. 4. In fact, the number of patients who at the end the study showed increases greater than 10 % in the number of hairs and follicular units in the frontal and occipital areas was higher than that of patients who showed these effects in the temporal area and in the vertex. This would be indicative of a greater responsiveness to electrothermal treatment by the areas affected by DCD. On the other hand, the number of patients with increases in cumulative hair thickness and in the rate of triple follicular units was similar in the four treated areas, which confirms that also the areas with a capillary density considered normal according to the pre-treatment diagnosis are liable to respond to electrothermal stimulation. In fact, the combination of the individual responsiveness data (Fig. 5) reveals that 19 out of the 20 patients showed favorable effects in 3 or more of the treated areas for at least one of the four parameters considered.

However, the analysis of individual responses also revealed notable differences between patients in terms of the level of responsiveness to treatment. Indeed, while some patients showed improvements in several redensification parameters and in several treated areas, other patients showed improvements in fewer parameters and / or in fewer areas. The analysis of the records and histories of the patients did not reveal significant relationships between their degree of responsiveness to treatment and factors such as age (Fig. 6), characteristics of the hair follicle, characteristics of the scalp, previous pregnancies and previous diseases (data not shown).

Some of the results described above coincide with those of some studies that have applied RF electrical therapies for the treatment of alopecia. In a single blinded study carried out in a sample of 24 men, the application of non-ablative RF currents increased hair growth compared to the untreated group [21]. Another study with 19 male subjects diagnosed with androgenic alopecia has shown an improvement in the efficacy of the combined treatment of 1 MHz of bipolar RF currents and minoxidil compared to treatment with minoxidil alone [24]. In a study of 25 female patients with androgenic alopecia, treatment with fractional RF currents resulted in improvements in hair count and hair thickness, mainly in the frontal area, without causing any detectable discomfort or adverse effects [22]. Although the biological and physiological mechanisms involved in these responses to RF have not yet been determined, it has been proposed that RF exposure could promote the production of growth factors such as Insulin-Like Growth Factor 1 (IGF-1), Vascular Endothelial Growth Factor (VEGF) or Hepatocyte Growth Factor (HGF) involved in hair growth processes [37]. If so, it can also be proposed that at least part of the redensification effects observed in the present study could be due to increases in the production of such growth factors due to blood supply promotion induced the mechanical and thermal stimuli provided by the CRET treatment.

On the other hand, the increase in the number of follicular units and hair follicles observed in patients treated with CRET may be due to stimulation of hair follicle growth. The dermal papilla, located at the base of the hair follicle, has been described as a key signaling center for the control of the hair growth cycle, which begins when the follicle in telogen or resting phase enters anagen or growth phase [38]. Dermal papilla cells are mesenchymal cells that interact with various types of epithelial cells, with the germ cells of the hair or with the stem cells of the hair follicles [28,39]. In fact, the process of hair growth activation begins with the proliferation of DPCs and is followed by the proliferation of stem cells from the bulge of the hair follicle [22]. Therefore, DPC proliferation plays a fundamental role in hair growth and in the regeneration and maintenance of hair follicles [28,29].

Previous studies by our group have shown that *in-vitro* exposure under subthermal conditions ($j \leq 150 \mu\text{A}/\text{mm}^2$) to electrical stimulation with a 448 kHz sinusoidal CRET signal is capable of promoting proliferation in a number of human cell types, including stem cells, fibroblasts or keratinocytes [11,12,26]. This experimental evidence constitutes the basis for the hypothesis

that at least part of the redensifying effects observed in the present study may be due to an increase in the proliferation of DPC present in the pre-existing or newly formed follicles of the patients treated with CRET. Such a proliferative effect, if it occurs, would be induced by the RF electrical stimulus, in the absence of the thermal and mechanical components of the CRET treatment. Therefore, to test this hypothesis, DPC cultures from woman have been exposed to electrical signal parameters equivalent to those used in the CRET treatment applied to the women diagnosed with DCD who participated in the medical study. The current density of 100 $\mu\text{A}/\text{mm}^2$, which causes thermal effects when applied to patients, did not induce a perceptible thermal increase ($\Delta T < 0.1^\circ\text{C}$) in the DPC cultures due to the low electrical resistivity of the culture medium to the passage of the current [36].

The results obtained reveal that 48 hours of intermittent exposure to the electrical signal under subthermal or normothermal conditions promotes DPC proliferation. These data support the proposed hypothesis and are indicative that at least part of the effects of capillary growth and densification observed in patients subjected to electrothermal and mechanical treatment with CRET, could be mediated by an early stimulation, exclusively electrical in nature, of the proliferation of DPC present in the hair follicles of these patients.

In order to investigate the nature of this electroinduced proliferative response, the expression of three proteins involved in cell proliferation was analyzed: Ki67, cyclin D1, and the mitogen-activated kinase ERK in its active (p-ERK1/2) and non-active (p-ERK1/2) forms. Ki67 protein is an excellent marker of proliferating cells, since it expresses during the active phases of the cell cycle (G1, S, G2 and mitosis), but not during the resting phase (G0) [40]. For its part, cyclin D1 expresses during phase G1 and is involved in the initiation of DPC cell cycle progression [41]. Regarding the MAPK / ERK pathway, its activation is crucial in the proliferation of different cell types, including dermal papilla cells and it has been described that several hair growth activators, such as the Endothelial Growth Factor (EGF), the Placental Growth Factor (PGF) or adenosine, exert their effects through the MAPK / ERK pathway [42,43].

The obtained experimental results reveal that, compared to the corresponding sham-exposed controls, subthermal RF electrostimulation with CRET induces significant increases in the expression of Ki67, observable after 48 h of exposure, as well as in the expression of cyclin D1, at 12 and 24 h, and in the phosphorylation of ERK1/2 at 12 h. It can be assumed that the stimulation of cyclin D1 expression and that of ERK1/2 phosphorylation are involved in the proliferative response detected by the XTT assay and Ki67 labeling after 48 h of exposure. The fact that after 12 h or 24 h of exposure no significant changes were detected in the expression of ERK1/2 or cyclin D1, respectively, indicates that the proliferative effect induced by CRET would take place through an early regulation of the expression of cyclin D1 and activation of the MAPK/ERK1/2 pathway. These results, which are coherent with others previously reported by our group, showing that ERK1/2 activation is also involved in proliferation of keratinocytes,

fibroblasts and human stem cells induced by subthermal electrostimulation at 448 kHz, add to the body of experimental evidence suggestive that RF exposure can stimulate hair growth [11,12]. Thus, it has been reported that subthermal exposure to electromagnetic fields of 1.763 MHz can promote Ki67 expression in matrix keratinocytes and increase hair shaft elongation in ex vivo hair organ culture [44], or that treatment with currents of between 1 and 2 MHz increases cell proliferation and expression of Wnt/b-catenin, Ki67, p-ERK and p-AKT in DPC [45].

Taken together, these data support the hypothesis that electrical stimulation alone contributes significantly to the hair redensifying effect of the CRET electrothermal treatment. The effect of the electrical stimulus would be exerted through the early induction of overexpression or activation of proteins directly involved in the control of the cell cycle of DPCs present in the hair follicles of DCD patients. Similar effects of early overexpression of cell cycle proteins have been reported in human fibroblasts and keratinocytes treated with CRET subthermal electrostimulation at 448 kHz [12]. There is experimental evidence indicating that the cellular response to the CRET electrical stimulus would be nonlinearly dependent on the signal frequency [46].

In short, the results of the study show that three months after the end of the CRET electrothermal treatment, the patients presented, as a whole, a significant increase in capillary redensification, as revealed by the analysis of the trichoscopic data in the derived Sinclair scale. This effect is due to the significant changes recorded in a variety of parameters involved in hair regeneration, including increases in the average number of hairs and in the number of follicular units,. Such changes were detected both in areas with deficient capillary density according to the pre-treatment diagnosis, and in those whose capillary density had been considered normal. Since the main mechanism underlying abnormal hair loss is the dysregulation of dermal papilla cell proliferation, the proliferative response of DPCs to the subthermal electric stimulus could be strongly implicated in some of the redensifying effects observed in the trichological study. In this way, the electrical stimulation could promote hair growth and follicular proliferation, as well as induce the entry and permanence of the hair follicles in anagen. This electroinduced effect could be added to those of the thermal and mechanical stimuli of the CRET treatment, whose potential contribution to the observed hair redensification has not yet been sufficiently characterized or quantified.

As a whole, the results of the trichological study in women with DCD and those of the experimental study in DPC of woman are consistent with each other and indicate that treatment with RF CRET at 448 kHz could be effective both in capillary redensification therapies and in treatments of prevention of hair loss. These data should be expanded by means of experimental studies and medical trials, double-blind and with the corresponding controls, which investigate the effects of the application of CRET in combination with other chemical or physical

techniques for the treatment of DCD, and allow identifying the factors involved in the individual reaction of patients to electrothermal treatment.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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