Lasers in Surgery and Medicine 53:1208–1219 (2021)

Low-Level Light Therapy Downregulates Scalp Inflammatory Biomarkers in Men With Androgenetic Alopecia and Boosts Minoxidil 2% to Bring a Sustainable Hair Regrowth Activity

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Background and Objectives: Low-level light therapies using visible to infrared light are known to activate several cellular functions, such as adenosine triphosphate and nitric oxide synthesis. However, few clinical observations report its biological consequences for skin and scalp homeostasis. Since scalp inflammation was recognized as a potential physiological obstacle to the efficacy of the reference hair regrowth drug Minoxidil *in vivo* and since perifollicular inflammation is the hallmark of about 50%–70% follicular units in androgenetic alopecia, we decided to investigate whether the anti-inflammatory activity of LLLT/GentleWaves® device were assigned to L'Oréal by Light BioScience L.L.C., Virginia Beach, VA (US) could enhance hair regrowth activity of Minoxidil.

Study Design/Materials and Methods: We conducted a first experimental clinical study on 64 men with androgenetic alopecia using LLLT/GentleWaves®, 590-nm predominant wavelength 70 seconds, specifically pulsed once per day, for 3 days, and we performed a whole-genome analysis of treated scalp biopsies. In a second clinical study, including 135 alopecic volunteers, we evaluated the hair regrowth activity in response to the upgraded LLLT/GentleWaves® device and Minoxidil.

Results: In the first clinical study, whole-genome analysis of treated scalp biopsies showed downregulation of scalp inflammatory biomarkers, such as AP1/FOSB messenger RNA (mRNA) and mir21, together with the disappearance of CD69 mRNA, specific to scalp-infiltrating T cells of about 50% of the studied volunteers prior to the LLLT/GentleWaves® treatment. In the second clinical study, we observed that LLLT/GentleWaves® was able to boost the hair regrowth activity of a Minoxidil 2% lotion to the extent of the highest concentration (5%) in terms of efficacy, number of responders, and perceived performance.

Conclusions: Altogether, these observations suggest the potential benefit of LLLT/GentleWaves® as a non-invasive adjunctive technology for skin and scalp conditions, where a mild perifollicular inflammation is involved. Lasers Surg. Med. Copyright © 2021 Wiley Periodicals LLC

Key words: androgenetic alopecia; hair regrowth; inflammation; LLLT; Minoxidil; perifollicular fibrosis; whole genome

INTRODUCTION

Scientific Background on Alopecia, Inflammation, and Perifollicular Fibrosis

Since the mid-70s, several clinical data [1–5] have highlighted the presence of distinct inflammatory biological biomarkers and subclinical signs related to hair follicle physiology and described the presence of an active inflammatory perifollicular infiltrate in the scalp of about 30% to 50% of subjects with androgenetic alopecia (AGA). Recently, Michel et al. [6], by studying gene expression alteration in male AGA evidenced again activation of immune and inflammatory responses in AGA subjects [6]. It was also demonstrated that production of interleukin-1α (IL1α) from plucked hair follicles, found highly expressed in about one-third of the subjects with AGA, could also be envisioned as a reliable prognostic factor for hair bulb inflammatory status and progressing alopecia [4].

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and have disclosed the following: Dr. MAHE reports that he is L'Oréal full-time employee and that the authors Dr. S. de BERNARD and Dr. L. Buffat have received grants from L'Oréal. In addition, Dr. MAHE reports that the following patents, all in the name of L'Oréal, are relevant to this study: patent FR2000163 filed on 08 Jan 2020 pending, patent EP2912509B1 issued, patent EP2861203B1 issued, and patent US6936044 issued.

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Accepted 27 February 2021
Published online 11 May 2021 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/lsm.23398

Furthermore, this pro-inflammatory cytokine was shown to directly inhibit hair growth *in vitro* and induce hair bulb degradation at very low physiological concentration [4,7,8]. This degradation of the hair bulb was suspected to occur through the secondary triggering of inflammatory proteases, such as MMP-9, which was found to be enzymatically activated in the inner root sheath of inflamed hair follicle upon pro-inflammatory (IL1 or TNF) treatments [9]. At the same time, it was reported that IL1α or IL1α + IL1 receptor type I overexpression in the epidermal compartment could dramatically alter hair growth in transgenic mice models [10,11], further highlighting the possible contribution of an inflammatory cytokines cascade to hair growth arrest and involution. At the clinical level, in humans, visible superficial peripilar signs on the scalp, such as brown halo and cupula, have been reported to be linked to deeper invisible perifollicular inflammatory parameters. In early alopecic stages, there was indeed a strong correlation between the intensity of those peripilar clinical signs and the extent of perifollicular inflammation based on both lymphocytic histological features and levels of specific lymphocytic biomarkers levels [5]. Aiming at integrating those data in a dynamic manner, it has been thus hypothesized [12,13] that this perifollicular inflammation (we named it "micro-inflammation" since it is subclinical) was the prerequisite of the well-described perifollicular fibrosis phase. This later phase was thought to be involved in hair follicle miniaturization [2] against which the anti-hair loss molecule Aminexil (2,4-diamino pyrimidine 3-oxide) was acting through limiting lysyl-hydroxylase activity [14]. It is therefore tempting to speculate that limiting propagation of inflammation in the scalp and the pilosebaceous unit might have an additional beneficial effect by reducing the progression of hair loss in the phase, where a perifollicular infiltrate is observed, prior to perifollicular fibrosis taking hold and the subsequent hair bulb miniaturization [15].

Low-level light therapy (LLLT) uses laser diode or light-emitting diode (LED) sources that emit in the visible and infrared spectra in the range 500–1100 nm (the so-called optical window of tissue) and deliver fluences of 1–10 J/cm^2 with a power density of 3–90 mW/cm^2 [16]. LLLT is more and more widely used in dermatology to help fight acute inflammatory disorders, such as post-operative, post-peeling, burning, and post-UV-induced inflammation, as well as to accelerate wound healing [16–18]. It is thus widely considered by clinicians as precious adjunctive therapy with potential anti-inflammatory activity in the field of skin dermatology and also hair regrowth [16,19–22]. However, only a few data are available that could explain the precise biological and physiological mechanisms through which this technology can lead to anti-inflammatory activities *in vivo* and *in vitro*.

In this work, we have evaluated the ability of LLLT/GentleWaves® to modulate the inflammatory cascade (Study 1) and confirmed *in vivo* and *in vitro* that GentleWaves® could significantly alter the inflammatory cascade propagation in scalp cells as initially demonstrated by Weiss et al. for skin cells [19–21]. In the second step (Study 2), we have performed a clinical evaluation of the induced hair growth in the scalp of a panel of 135 volunteers, demonstrating the boosting of performance of Minoxidil 2% generated by LLLT.

MATERIALS AND METHODS

LLLT/GentleWaves® devices in both studies contains LEDs emitting pulsed light at 590 nm as the predominant wavelength and 870 nm as secondary wavelength (Fig. 1). Each treatment of 70 seconds consisted of a single exposure dose of 0.1 J/cm² with a specific sequence of pulses as previously reported [23–25].

Clinical Study 1. Treatment of Scalp *In Vivo* With GentleWaves® Device for Evaluating Whole Genomic Expression Modulation

This study was conducted in Carrollton, Texas, USA, at the Thomas J. Stephens & Associates, Inc. and approved by the medical committee IntegReview Ethical Review Board on the 16th of February, 2009 with the study number C09-D011 and the study name Evaluation of the Effect of GentleWaves® Pro Light Device and Other Energetic Treatments on Hair Follicle Cells by Mean of Gene Expression Profiles in Randomized Male Subjects With Mild to Moderate Androgenetic Alopecia.

Since LLLT is reported to exert several biological activities *in vivo* and *in vitro* [18,26], we decided to investigate the effects of our GentleWaves® device for the first time at the whole-genome level in order to further characterize its targets. We studied which of the approximate 30,000 genes potentially expressed in humans were downregulated in the alopecic scalp after LLLT treatment.

Sixty-four alopecic male volunteers aged 30 to 46 years were enrolled in this study. Scalp biopsies, 3.6 mm Ø, containing on average 3–5 follicular units each were collected from the frontal progressing zone of all alopecic volunteers (graded Hamilton 2–3 vertex) aged 38 ± 8 years at $t = 0$ before any treatment. Thirty-five volunteers were then specifically treated by the GentleWaves® device on the top of the head (including vertex, middle part, and frontotemporal regions). For this *in vivo* study with biopsies, the treatment consisted of a single exposure of 0.1 J/cm^2 70 seconds per day for 3 consecutive days.

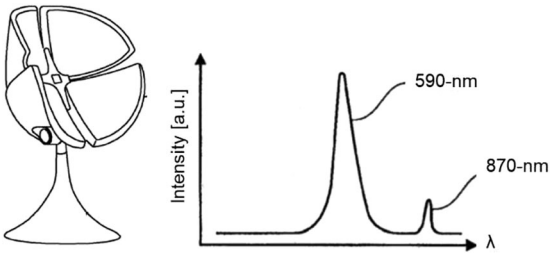

Fig. 1. Cartoon of the upgraded GentleWaves® device and its emission spectrum with a predominant peak at 590 nm (yellow-orange) and a secondary peak at 870 nm (infrared) (cfr. EP2912509B1, EP2861203B1).

To confirm these *in vivo* results, we performed an additional *in vitro* study with isolated cells on normal human skin keratinocytes (NHEK). The top plastic lid of the growth plate was removed during the LLLT exposure. For both *in vivo* and *in vitro* studies, biological samples, either scalp biopsies or growing cells in a culture medium without polyphenol red, were collected 18 hours after the last illumination and immediately freeze-dried before messenger RNA (mRNA) extraction. mRNA was extracted, reverse transcribed, and then the samples were evaluated using whole-genome Affymetrix transcriptome analysis to identify the occurrence of GentleWaves® modulated gene expression into the scalp and in the hair follicle unit (see Figs. 2 and 3). Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) [27] was then specifically performed for *in vitro* sample on a restricted array of selected skin inflammatory biomarkers, as reported in Figures 4 and 5.

Clinical Study 2. Treatment of Volunteers With GentleWaves® Device for Hair Regrowth Efficacy

This study was conducted in Bucharest, Romania, Europe at the Eurofins Evic Romania/S.C. Bio High Tech S.R.L. site, and approved by the independent ethical committee on December 08, 2016, with the study number ER 16/141 and the study name Evaluation of the Effect of Light-Emitting Diode (LED) GentleWaves Device on Hair Loss in Men Using Topical Minoxidil 2% Randomized, Single Center Open Clinical Trial of Efficacy and Safety Single Center Controlled, Randomized, Open, Study.

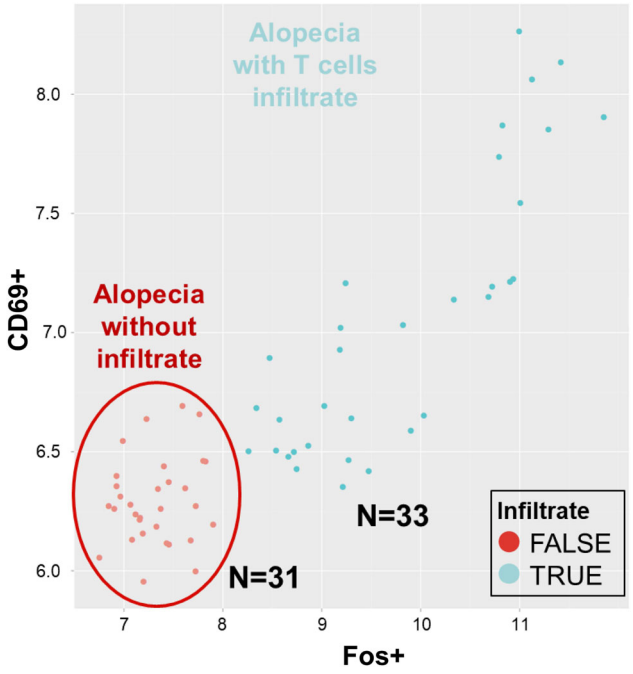

Fig. 2. Diagram of co-expression of inflammatory infiltrates ($CD69^+$) and inflammatory status (FOS^+) in the human scalp *in vivo* in 50% of the androgenetic alopecia men volunteers enrolled in the study (in cyan). Two groups and hence two stages of alopecia (i.e., inflammatory vs noninflammatory) can clearly be distinguished (red and cyan, respectively). Values represent the gene expression levels, as estimated by the Affymetrix arrays.

Nearly 130 alopecic male volunteers aged 20 to 50 years were enrolled in this study. One hundred and twelve completed the study (18 subjects decided to withdraw consent for personal reasons independent from the study) (see Table 1). Forty-five received full-head exposure of 0.1 J/cm^2, specifically pulsed, once per day during 70 seconds and used Minoxidil 2% twice daily for 5 consecutive days per week, during 6 months (group A); 45 used Minoxidil 5% twice daily, for 5 consecutive days per week, during 6 months (group B); 22 did not receive any treatment (control group, group C).

In groups A and B, Minoxidil was applied by the subject, one application in the evening and one in the morning. In group A, the exposure to GentleWaves® was performed at least 1 hour after Minoxidil applications. The unit was operated by a clinician, the head could be adjusted to fit the contour to cover the top of the subject's scalp (including vertex, middle part, and frontotemporal regions), and the distance from the scalp was kept fixed during the treatment (Fig. 1). A distance sensor helped to adjust the distance of the head from the scalp and to monitor its correct functioning during the treatment.

The main evaluation criterion in this study was the change in hair density measured by Tiff counting method; this phototrichogram method consists of taking a photograph from preshaved area of the scalp of about 1 cm^2. New photographs are taken of the same precisely delineated area after 1.5, 3, 4, and 6 months of treatment (Fig. 6). Subjects with mild to moderate hair loss grade II–III and III Vertex on the Hamilton scale amended by Norwood, evaluated by the dermatologist, and presenting Tiff counting hair density between 200 hair/cm^2 and ≤350 hair/cm^2 were enrolled in the study (Table 2).

Change in hair-loss parameters was evaluated by the clinician and by the subject after 1.5, 3, 4, and 6 months of treatment, at the end of the study on the same photographs (the clinician was not aware of the subject's randomization group). A 7-point scale was used (−3: greatly decreased, −2: moderately decreased, −1: slightly decreased, 0: no change, 1: slightly increased, 2: moderately increased, 3: greatly increased) (see Fig. 7). Tolerance also was monitored during the study. Intrasubject and intragroup comparisons were performed to evaluate the efficacy of the treatment using GentleWaves® in combination with Minoxidil 2% compared with Minoxidil 5% alone.

The impact of the active treatments and nontreated control, and their comparison during the 6-month treatment period were analyzed in change and relative change for the primary criterion (hair density) from baseline, using generalized linear mixed models for repeated longitudinal data (for ordinal data) and linear mixed model for repeated longitudinal data (for quantitative data) with repeated time, and with treatment, time, and the interaction treatment × time as fixed effects. Within and between treatment groups' comparisons were performed using contrasts (Benjamini Hochberg's adjustments for

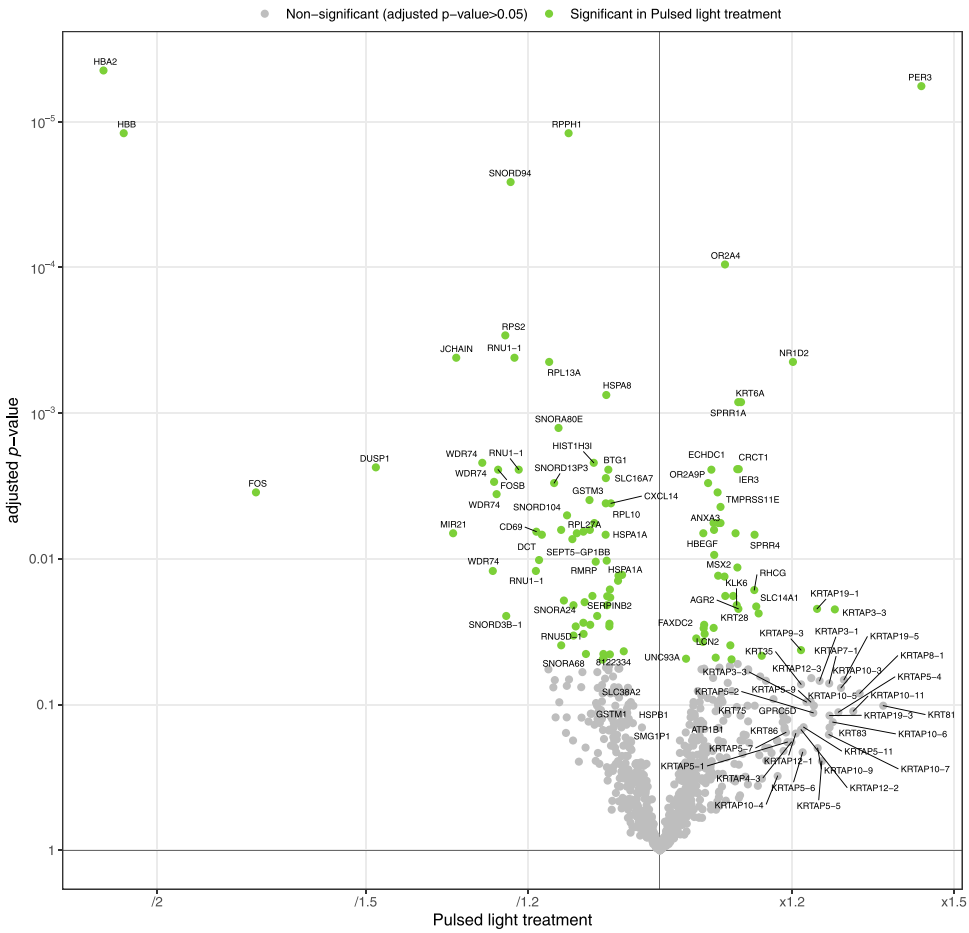

Fig. 3. Detection of GentleWaves® significantly downregulated (left panel) and moderately upregulated (right panel) genes *in vivo* in the scalp from men with androgenetic alopecia based on transcriptional whole expression study. HBA2 and HBB (hemoglobin A2 and hemoglobin B), FOS, mir21, CD69^{+}, and DUSP1 are the most downregulated genes, while keratins are slightly induced by GentleWaves®. Fold changes after pulsed light treatment (x axis) are expressed in log2 base, which means, for example, that Hba and Hbb are decreased more than $2^{2.2}$ (i.e., divided by $4.4 = \times 0.22$); FOS is downregulated $2^{1.7}$ (i.e., divided by $3.4 = \times 0.29$); CD69^{+} decreased by 2^{1} (i.e., divided by $2 = \times 0.5$), etc. Genes in green were deemed significant by the statistical model.

primary criterion at endpoint time). All efficacy results were performed on Per Protocol Set. All hypothesis tests comparing the treatment were performed using two-sided tests with a level of significance set to 5%.

Equivalence test was applied for the comparison between the three groups on the main evaluation criterion: equivalence between groups was confirmed when the following two conditions were both met: Relative change group X versus group Y difference is between +2% and −2% and 95% confidence interval (95% CI) is [−0.07; +0.07] (no *P*-value is generated from this statistical test) (Table 3).

RESULTS

Clinical Study 1

As shown in Figure 2, whole-genome transcriptional expression study of 3.6-mm Ø scalp biopsies from the 64 alopecic men volunteers revealed as expected from previous clinical observations [1–4,6], the presence of an established inflammatory status evidenced here by the overexpression of a lymphocyte-specific strictly perifollicular biomarker CD69^{+} and of a ubiquitous inflammatory biomarker FOS^{+} transcript. This was detected in 50% of alopecic volunteers (33 of the 64 volunteers in cyan). Consequently, two distinct stages of alopecia (i.e., with and without inflammatory infiltrates, in red and in blue, respectively) were thus detected by means of this whole-genome transcriptional study, using those two inflammatory biomarkers.

Figure 3 illustrates the marked downregulatory effect of the GentleWaves® on several inflammatory biomarkers, such as FOS, DUSP1, CYR61, mir21, and of the one specific from tissue inflammatory T cells infiltrates (CD69) (FOS and co-regulated in the blue circle). It is noteworthy that this sudden downregulated effect of light

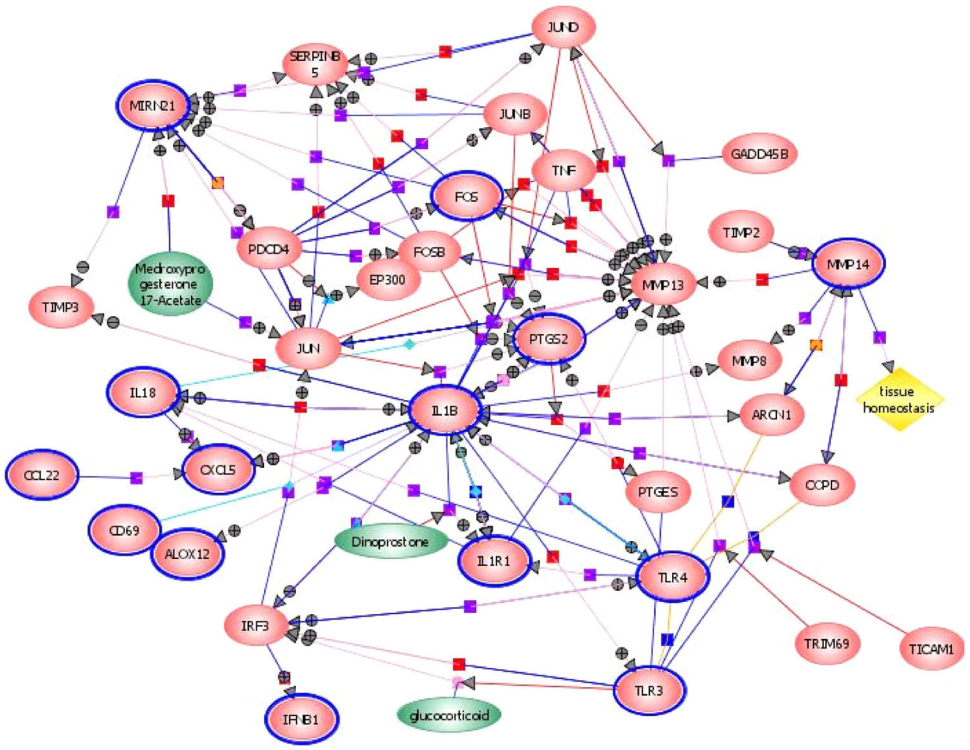

Fig. 4. Interactive map of inflammatory genes based on transcriptional whole expression study in 3.6-mm Ø scalp biopsies and QRT-PCR expression on cultured cells *in vitro* and *in vivo*: the not downregulated genes are shown in red ellipses, modulating molecules in green ellipses, and the genes downregulated in red ellipses circled in blue. The fold changes obtained from RT/PCR studies are as follows CCL22 (×0.45); IL1B (×0.19); IL1R1 (×0.36); 12Lipoxygénase (×0.38); MMP14 (×0.23); CXCL4 (×0.41); PTGD2S (×0.32); PTGS2 (×0.25); TLR3 (×0.16); TLR4 (×0.18); INFB1 (×0.47).

Gene name	Skin biopsy	Scalp biopsy	Plucked Hair	NHEK
CRY1	7,1	7,0	6,6	6,8
CRY2	7,5	7,4	7,1	6,8
GNAT2	5,8	5,9	5,9	5,5
OPN1LW	5,2	5,3	5,6	4,7
OPN1SW	5,3	5,4	5,7	5,2
OPN3	6,8	7,1	6,8	7,6
OPN4	6,6	6,6	6,9	6,2
OPN5	5,5	5,3	5,8	5,0
RGR	5,8	5,8	6,0	5,9
RHO	5,8	5,9	5,9	5,5
RRH	5,6	5,4	5,6	4,5

Expression level
High
Middle
Low

Fig. 5. Comparative expression of photoreceptors in human skin and hair and normal human epidermal keratinocytes (NHEK). CRY1, cryptochrome circadian regulator 1; CRY2, cryptochrome circadian regulator 2; GNAT2, G protein subunit alpha transducin 2; OPN1LW, long-wave-sensitive opsin 1; OPN1SW, short-wave-sensitive opsin 1; OPN3, opsin 3; OPN4, opsin 4; OPN5, opsin 5; RGR, retinal G-protein-coupled receptor; RHO, rhodopsin; RRH, retinal pigment epithelium-derived rhodopsin homolog.

treatment on both $CD69^{+}$ cells and pro-inflammatory genes and mRNA expression is observed, following only three successive sessions, once per day at 0.1 J/cm² of GentleWaves®. Apart from those downregulated inflammatory biomarkers, the most downregulated genes following GentleWaves® are those coding for the Hemoglobins Hba/2 and Hbb (left panel). Interestingly, both Hba1/2 and Hbb were recently described as putative forensic biomarkers that could sign an ischemic status of the heart tissue in sudden cardiac death [28]. It thus may also be taken into account in scalp biopsies as a potential signature of the ischemic status of the alopecic scalp that is potentially normalized to baseline after treatment with the GentleWaves®.

To put all these inflammatory biomarkers into an interactive perspective, an initial list of the downregulated inflammatory genes in response to GentleWaves® treatment *in vivo*, was created based on transcriptional whole expression study in 3.6-mm Ø scalp biopsies and QRT/PCR expression data from our cultured cells *in vitro* study with GentleWaves®. Afterward, we built the shortest path allowing the functional connection between them. The links between the entities were extracted using a text-mining algorithm from public literature, followed by a manual curation by Altrabio [29]. We thus identified a set of 14 biomarkers as *in vivo* and *in vitro* targets and genomic signatures of the biological effects of GentleWaves®. The mRNAs reported in the path and downregulated *in vivo* are: CD69, FOS, mir21, IL-18, while the mRNAs downregulated *in vitro* are: CCL22, IL1B, IL1R1,

TABLE 1. Summary of the Number of Subject Among the Three Groups

Withdrawal reason	Number of subjects
Completed the study	112
Clinical and/or biological adverse event	0
Total consent withdrawal	18
Consent withdrawal subjects from group A	6
Consent withdrawal subjects from group B	9
Consent withdrawal subjects from group C	3
Lost to follow-up	0
Protocol deviation	0

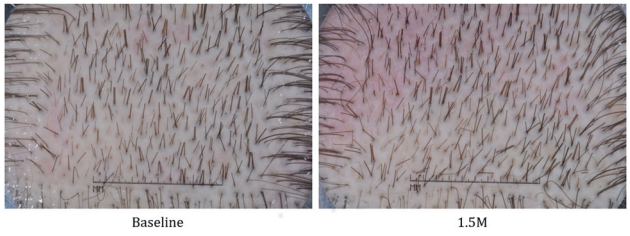

Fig. 6. Typical photos of the same subject of group A (GentleWaves® + Minoxidil 2%) obtained by the phototrichogram at baseline and at 1.5 months. Scale bar: 1 cm. In this example, a mean increase in hair density of +4.94% was observed in group A between baseline and 1.5 months (see also Table 5).

12 Lipoxygenase, MMP14, CXCL5, PTGD2S, PTGS2, TLR3, TLR4, INFβ1. The full interactive map coming out of this analysis is shown in Figure 4, where the down-regulated genes are indicated in blue circled red ellipses. The average range of fold-change levels observed in those two distinct technologies varied from a quasi-full inhibition of expression in full genome studies as observed for FOS (×0.06) to ×0.47; see legend of Figure 4 for gene-specific fold changes. Those biomarkers could thus be used as precious diagnostic tools to follow the beneficial anti-inflammatory effects of LLLT in other tissues and clinical procedures.

To further understand through which pathways and light receptors our GentleWaves® device could transduce this beneficial anti-inflammatory signal into the scalp, transcriptomic analysis using Affymetrix arrays was performed *in vivo* on human skin, hair biopsies or plucked hairs and *in vitro* on normal human epidermal keratinocytes (NHEK). We established a fine expression profiling of known potential photoreceptors in both skin and hair keratinized tissues reported in Figure 5. The highest expressed photoreceptors, with middle to high expression level are CRY1 (up to 7.1 in skin biopsy), CRY2 (up to7.5 in skin biopsy), OPN3 (up to 7.6 in NHEK), and OPN4 (up to 6.9 in plucked hair) both in skin and hair biopsies, as well as in NHEK, suggesting a preferential epidermal expression of those photoreceptors in the skin and hair.

Whether those receptors already found surprisingly expressed at least in the skin and hair keratinocyte cells are indeed involved and fully equipped to transduce the LLLT signaling to the cells remains, however, to be established.

Finally, at the same time and by contrast with an inhibitory effect of inflammatory and ischemic biomarkers, we observed the upregulation of numerous hair keratins, such as KRT83 and KRT81, *in vivo*, suggesting indirectly a concomitant pro-elongating/pro-keratogenic effect of GentleWaves® treatment on hair shaft formation, which is also detectable early at the transcriptional level. Thus, following these encouraging data indicating both an anti-inflammatory and pro-keratogenic signature of the GentleWaves® treatment, we decided to evaluate the long-term effects of GentleWaves® treatment on hair regrowth parameters in a second clinical study lasting up to 6 months (see "Clinical Study 2" section).

Clinical Study 2

Phototrichogram and clinical evaluation. Figure 8 and Table 4 illustrate the mean change from baseline in total hair density measured over time for the three groups of volunteers using phototrichogram: applying Minoxidil 2% twice daily in combination to a single daily exposure to GentleWaves® (group A, blue), applying Minoxidil 5% alone twice daily (group B, red), no treatment-control (group C, green).

We can observe that hair density change significantly increases in the 3 groups between baseline and 1.5 months. Total hair density mean change is comparable for group A and group B at 3 and 4 months, while in group C the change is moderate. While at 6 months there is no more increase in the control group, a significant increase is still observed in group B and group A. It is noteworthy that while the rate of increase starts diminishing in the Minoxidil 5% group (group A) it is still sustained in the GentleWaves® + Minoxidil 2% group (group B) (see Table 5

TABLE 2. Summary of Hair Density at Baseline Using Phototrichogram Among the Three Groups

Group	*n*	Mean (SD) (hair/cm^2)	Median (hair/cm^2)	Min.; Max (hair/cm^2)	95% CI
A. GentleWaves® + Minoxidil 2%	45	260 (42.70)	262.86	177.14; 338.57	247.17; 272.83
B. Minoxidil 5%	45	253 (51.86)	245.71	165.71; 398.57	237.40; 268.56
C. Control	22	265 (43.81)	249.29	201.43; 377.14	245.77; 284.62

CI, confidence interval.

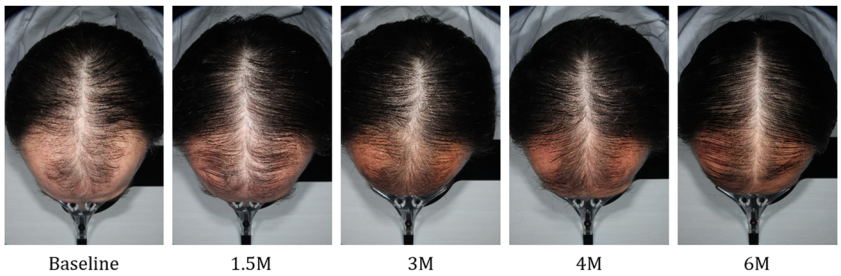

Fig. 7. Typical photos were obtained by global photographs of the same subject of group A (GentleWaves® + Minoxidil 2%) at baseline, 1.5, 3, 4, and 6 months. These photos were used for self-assessment by the subject and for evaluation by a clinician of scalp coverage. Note an increase in scalp coverage as observed by the subject and by the clinician from baseline up to 6 months. Also, note the increase in density of the central line that has been measured for the group as follow: +4.94% at 1.5 months, +7.33% at 3 months, +6.82% at 4 months, and +5.22% at 6 months (see also Table 5).

TABLE 3. Summary of Phototrichogram Findings After 1.5, 3, 4, and 6 Months of Treatment Among the Three Groups (For Equivalence Between 2 Groups, Difference Between Groups (Estimate) Should be Between +2% and −2%, and 95% Confidence Interval [CI]: [−0.07; +0.07])

	1.5 months		3 months		4 months		6 months	
Contrast	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
GentleWaves® + Minoxidil 2% vs Minoxidil 5%	−4.3%	[−0.07; −0.02]	−1.4%	[−0.04; 0.01]	−0.4%	[−0.03; 0.02]	1.7%	[−0.01; 0.04]
GentleWaves® + Minoxidil 2% vs Control	1%	[−0.02; 0.04]	5.8%	[0.03; 0.09]	6.7%	[0.03; 0.10]	5.6%	[0.02; 0.09]
Minoxidil 5% vs Control	5.3%	[0.02; 0.09]	7.2%	[0.04; 0.10]	7.2%	[0.04; 0.11]	3.9%	[0.01; 0.07]

In conclusion: (i) GentleWaves® + Minoxidil 2% is *equivalent* to Minoxidil 5% after 3 months and up to 6 months; (ii) GentleWaves® + Minoxidil 2% is *equivalent* to Control after 1.5 months but not equivalent after 3 and 6 months; (iii) Minoxidil 5% is not *equivalent* to Control at any time.

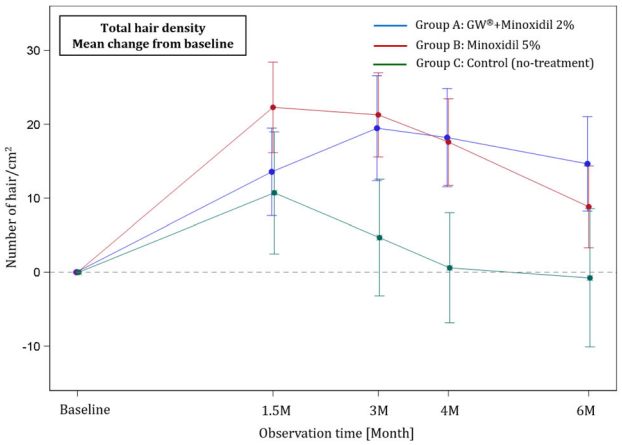

Fig. 8. Total hair density (number of hair per cm^2) using phototrichogram. Mean change from baseline (y axis) at different time points (x axis, baseline, 1.5 months, 3 months, 4 months, 6 months) for the three groups of volunteers: applying Minoxidil 2% twice daily in combination to GentleWaves® single daily exposure (group A, blue line), applying Minoxidil 5% alone twice daily (group B, red line), no treatment (group C, green line).

for statistical analysis). These observations showed that GentleWaves® + Minoxidil 2% was *equivalent to* Minoxidil 5% after 3 months and up to 6 months (Table 3).

The clinician (single-blind study) observed an improvement in scalp coverage compared with baseline after 3 and 4 months, for GentleWaves® + Minoxidil 2% and Minoxidil 5% groups. There is no improvement at 6 months visit in the alopecic control group; the clinician observed a decrease in scalp coverage as the one observed with subjects' self-assessment. We observe a significant difference of 1 score between both GentleWaves® + Minoxidil 2% and Minoxidil 5% groups versus control after 3 months (Fig. 9). No side effects were detected in any of the included subjects.

Self-Assessment. Figure 10 illustrates the mean value of the self-evaluation by the subject of the scalp coverage. Subjects in GentleWaves® + Minoxidil 2% group (group A) perceived the same efficacy than the subjects in group Minoxidil 5% alone (group B) throughout the treatment. Moreover, they perceived a significant efficacy (improvement by 1 score) on scalp coverage as early as 4 months, while the subjects in the Minoxidil 5% group observed an improvement by 1 score in scalp coverage after 6 months. There is, by

TABLE 4. Summary of the Changes in Total Hair Density From Baseline in the Three Groups Using Phototrichogram

Group	Time (months)	n	Mean (SD) (hair/cm^2)	Median (hair/cm^2)	Min; Max. (hair/cm^2)	95% CI (hair/cm^2)
A. GentleWaves® + Minoxidil 2%	1.5	45	13.56 (19.67)	11.423	−22.86; 65.71	7.65; 19.47
	3	45	19.46 (23.65)	18.57	−27.14; 82.86	12.36; 26.57
	4	45	18.19 (22.13)	15.71	−30.00; 74.29	11.54; 24.84
	6	45	14.64 (21.26)	12.86	−17.14; 70.00	8.25; 21.02
B. Minoxidil 5%	1.5	45	22.29 (20.38)	18.57	−10.00; 72.86	16.16; 28.41
	3	45	21.27 (18.98)	14.29	−5.71; 77.14	15.57; 26.98
	4	45	17.59 (19.54)	18.57	−31.43; 61.43	11.72; 23.46
	6	45	8.83 (18.36)	5.71	−35.71; 42.86	3.31; 14.34
C. Control	1.5	22	10.71 (18.60)	5.00	−15.71; 47.14	2.47; 18.97
	3	22	4.68 (17.77)	1.43	−25.71; 58.57	−3.21; 12.56
	4	22	0.58 (16.75)	−2.86	−30.00; 41.43	−6.84; 8.01
	6	22	−0.78 (21.08)	−3.57	−32.86; 37.14	−10.12; 8.57

contrast, a decrease in scalp coverage effect observed by the subjects in the alopecic control group (group C). The growth scored by the clinician was perceived by the volunteers as early as only 1.5 months after the beginning of treatment. This perceived growth effect is enhanced throughout the treatment.

It is noteworthy that, while the higher efficiency of GentleWaves® in combination with Minoxidil 2% compared with Minoxidil 5% is measured after 4 months of treatment (Fig. 8, group A), the volunteers enrolled in this group perceived higher efficacy as early as only 1.5 months and throughout the treatment (Fig. 10, group A). This suggests that GentleWaves® brings shorter term and higher perceived efficacy as well as a quantitative boost to Minoxidil 2% hair regrowth efficacy in the long term, which might be due to healthier looking and thicker hairs. As such, GentleWaves® shortens the time to long-term quantitative efficacy by bringing shorter-term perceived efficacy. Moreover, when Minoxidil is used in combination with GentleWaves® at a lower concentration, it may solve potential issues (intolerance and discomfort) of higher concentrated lotions (i.e., 5%).

DISCUSSION

GentleWaves® as Anti-Inflammatory Adjunctive Strategy for the Treatment of Alopecia

Until now, very few scientific articles show how inflammation modulators might modulate these inflammatory signs in the scalp and correlate with alopecia. Indeed, both alopecia areata and alopecia totalis are thought to be related to an autoimmune disease that affects the lower hair bulb. By contrast, the upper and pilosebaceous duct areas are thought to be affected in AGA. It is only very recently that both alopecias were very successfully treated with an oral anti-inflammatory treatment initially designed to fight Rheumatoid arthritis by means of the Janus Kinase Inhibitor Tofacitinib [30]. These findings showed, for the first time, that a

TABLE 5. Summary of the Changes in Total Hair Density Compared with Baseline in Each Groups Using Phototrichogram

	A. GentleWaves®+Minoxidil 2%			B. Minoxidil 5%			C. Control		
Time	Estimate (SEM)	P value	Effect size	Estimate (SEM)	P value	Effect size	Estimate (SEM)	P value	Effect size
1.5 months	+4.94%	<0.0001	0.802	+9.22%	<0.0001	1.497	+3.87%	0.0185	0.629
3 months	+7.33%	<0.0001	1.190	+8.68%	<0.0001	1.409	+1.56%	0.34	0.253
4 months	+6.82%	<0.0001	1.107	+7.25%	<0.0001	1.177	+0.50%	0.97	0.009
6 months	+5.22%	<0.0001	0.848	+3.48%	0.0026	0.565	−0.41%	0.80	−0.067

Total hair density increases in the three groups after 1.5 months, with the greatest increase rate for group B (+9.22%). In group A, the total hair density increase rate continues to rise after 3, 4 months and is maintained after 6 months. In group B, it slightly decreases after 3, 4, and 6 months. In Control group it weakens after 3, 4, and 6 months. Effect size (strength of the phenomenon) scales (e): Very important effect, $e > 2$; Important effect, $1.5 < e \leq 2$; Moderate effect, $0.8 < e \leq 1.5$; Weak effect $0.5 < e \leq 0.8$; No effect $0 \leq e \leq 0.5$. P values (the two-sided significance threshold will be set at 5%) interpretation and scales (P): Significant, $P < 0.05$; Trend, $0.05 < P \leq 0.1$; No effect, $P > 0.1$.

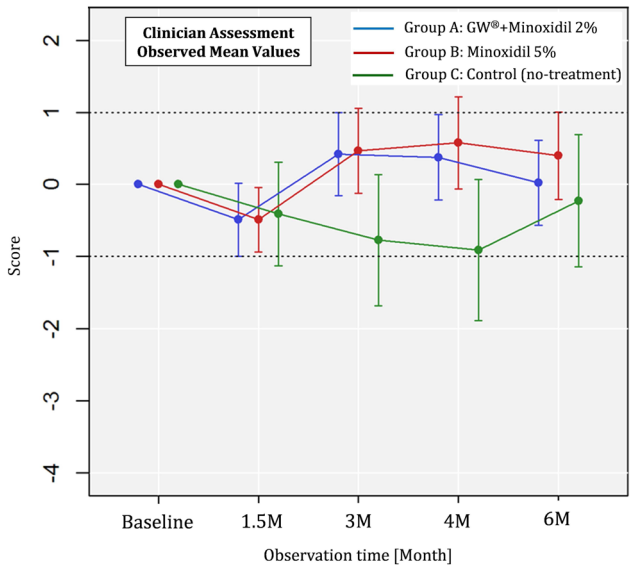

Fig. 9. Clinicians' single-blind evaluation of scalp coverage. Mean values (*y* axis) at each observation time point (*x* axis, 1.5 months, 3 months, 4 months, 6 months) for each group. A 7-point scale was used (−3: greatly decreased, −2: moderately decreased, −1: slightly, decreased, 0: no change, 1: slightly increased, 2: moderately increased, 3: greatly increased).

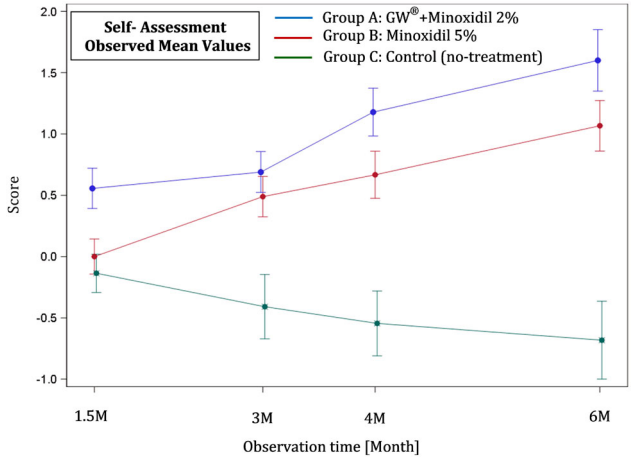

Fig. 10. Perceived efficacy. Mean values of self-assessment (*y* axis) at each observation time point (*x* axis, 1.5 months, 3 months, 4 months, 6 months) for each group. A 7-point scale was used (−3: greatly decreased, −2: moderately decreased, −1: slightly decreased, 0: no change, 1: slightly increased, 2: moderately increased, 3: greatly increased).

targeted anti-inflammatory pharmacological intervention could, without doubt, reverse a yet considered irreversible and long-standing hair bulb involution. This gives hope in the fight against hair loss by decreasing its inflammatory components. However, if acute treatment of scalp with corticosteroids or the newly developed Janus Kinase inhibitors family can give excellent results against immune/inflammatory hair disorders, such as alopecia areata and alopecia universalis or totalis, and a risk/benefit ratio, such as immune depletion, has to be estimated for long-term use. Furthermore, for so-called cosmetic disorders, such as mild or diffuse alopecia and AGA, a long-standing inflammatory/immune pharmacological shut down of the scalp cannot be accepted. There is thus room for new anti-inflammatory adjunctive therapies, where either a mild, localized, or transitory anti-inflammatory action is needed. In this case, light-based therapies could be used to prolong scalp homeostasis after a pharmacological treatment period to prevent the undesirable side effects of a long-lasting, strong pharmacological anti-inflammatory and long-term immunomodulation treatment.

Minoxidil 5% is the reference for the treatment of AGA. However, Minoxidil 5% is a life-lasting pharmacological treatment and features undesirable cosmetic limitations, such as stickiness, oiliness, odor, and most of all transitory hair fall that alter the compliance of its users.

We chose Minoxidil 2% because a lower concentrated solution features higher tolerance despite its lower efficacy. As such, the combination of Minoxidil 2% with GentleWaves® had the potential for more cosmetic treatment for AGA of at least the same efficacy as Minoxidil 5% alone, and that in addition could reverse the downward trend in user compliance.

As shown here, we could confirm that an adjuvant light therapy device was indeed able to boost Minoxidil 2% solution hair regrowth efficacy and even perceived efficacy to the level of the more concentrated reference (Minoxidil 5%) hair regrowth lotion. This boost was demonstrated by scientific clinical scoring as early as 4 months after the beginning of hair regrowth treatment, while volunteers perceived the efficacy as early as 1.5 months. Several clinical studies have described the better efficacy of a 5% lotion over a 2% lotion [31,32]. Furthermore, after the first 2 months of usage, the hair regrowth effect of Minoxidil is often transient or even halted due to telogen effluvium, often discouraging the patient from pursuing the treatment. We found the same pattern in our study in the Minoxidil 5% treated group after 3 months (regrowth rate slow down, red line drops) while in the GentleWaves® treated group, such brutal hair loss signature could not be observed even after 6 months. Indeed, in the GentleWaves® treated group, the hair growth effect is and more progressive, and at the same time, as expected (Fig. 8), the perceived efficacy is significantly better (Fig. 10). This suggests that the use of GentleWaves® in combination with Minoxidil 2% might change the difficult and well-known treatment path of all patients who become less compliant due to the unexpected transitory, counter-intuitive, and paradoxical hair loss effect of Minoxidil that they experience after 2-3 months of a daily and compliant use. Several other LLLT devices have proven such a significant effect on the increase of terminal hair density in alopecic volunteers, often comparable in efficacy to the one of Minoxidil 5% applied twice daily at least in so-called short-term (3–6 months) studies [19–22]. Fewer evaluations of combined or concomitant treatments of LLLT with Minoxidil have also been performed in female- and male-patterned hair loss [20,22]. Esmat et al. [20]

reported that statistically significant better results were obtained after 16 weeks with a combination of 5% Minoxidil and LLLT. Additionally, patients in the combined group occupied the top physician assessment position, with 90% of cases showing improvement and 100% of patients being satisfied. It is noteworthy that at the macroscopic level, hair follicles in the combined group were found to occupy deeper dermal levels into the scalp, suggesting a deeper anchorage of newly growing anagen hair follicles under combined treatment [20]. Both studies concluded with efficacy of LLLT alone comparable to the one of Minoxidil 5% at least on short-term regrowth with a decrease of efficacy of LLLT alone compared with Minoxidil 5% and even 2% on the long term (1-year use) and the need of a better understanding of molecular events underlining the adjuvant effect of LLLT treatments [19,20]. We thus investigated the molecular events in the scalp of LLLT-treated alopecic volunteers by using full genome expression analysis in follicular hair containing scalp biopsies to identify and understand the modulated genes and pathways in response to LLLT treatment. We found a strong downregulation of a large pro-inflammatory pathway in response to LLLT treatment. Whether this effect could be prolonged after long-term treatment remains to be established.

Altogether, our data suggest that LLLT/GentleWaves® is a very promising noninvasive and nonablative light-based adjunctive technology, which is able to down-regulate, at least transitorily, mild inflammatory cascade into the human scalp *in vivo* by lowering the level of $CD69^+$ T cells infiltrates and AP1 components. The presence of $CD69^+$ T cells in the scalp has previously been reported by Deeth et al. in patients with long-standing extensive alopecia areata [33]. We report here the significant presence of $CD69^+$ T cells in biopsies from scalps of about 50% of men with common AGA. Following exposure to GentleWaves®, their number is thought to decrease since the expression of CD69 mRNA was found nearly absent after LLLT exposure. It is noteworthy that this downregulatory effect of light on CD69 mRNA expression is observed following only three successive sessions once per day, to visible light at 590-nm as the predominant wavelength at a fluence of 0.1 J/cm^2, with a specific sequence of pulses. Apart from the activation of adenosine triphosphate (ATP) synthesis that we could measure *in vitro* (×2.0 to ×2.7; data not shown) and which is consensually thought to result from photonic absorption by mitochondrial chromophores, the expression of several photoreceptors suggests that skin and hair cells could indeed respond to photostimulation also through a common mechanism of G-protein-coupled phototransduction comparable, to some extent, to eye phototransduction. The highest expressed photoreceptors are CRY1, CRY2, OPN3, and OPN4, both in skin and hair biopsies, as well as in NHEK, suggesting a preferential epidermal expression of those photoreceptors in human skin. Cryptochromes (CRY1 and CRY2) are blue-light absorbing flavoproteins that are a core component of the mammalian circadian clock. In humans, their expression is not restricted to the retinal cone photoreceptor cells of the eye and has also been followed in the brain, dissected hairs, and in B-Lymphocytes in relation to the circadian cycle. While they are considered as blue-light photoreceptors in nonmammalian cells, few recent and preliminary data support a light-activated status of those photoreceptors in the eye tissue of several mammalian species, suggesting its functionality as a potentially light-activated photoreceptor in mammals too [34]. OPN3 and OPN4 were also found significantly expressed in both skin and hair biopsies and in epidermal NHEK. They belong to the large family of Opsins, which are light-sensitive heptahelical G-protein-coupled receptors. OPN3, despite being also reported as a blue-light photoreceptor, is considered as a nonvisual photoreceptor (Encephalopsin) involved in phototransduction in other tissues, such as human epidermal skin. OPN4 (Melanopsin) is expressed in a subset of retinal cells and is thought to mediate circadian photo-entrainment in mammals [35]. The expression levels of the other members of the Opsins family (OPNILW; OPN1SW; RGR; RHO/OPN2 and RRH), which have been described together with OPN3 by others in skin keratinocytes (OPN3; OPN1SW; OPN2; OPN5) or melanocytes (OPN2; OPN3; OPN4; OPN5) were found in our hands, however, very discrete with a preferential (but very low) expression in the epidermal compartment for GNAT2 and RHO and in hair follicles for OPN5; OPN1SW; RRH; RHO; RGR and GNAT2). Recently, Buscone et al., reported the preferential expression of the rhodopsin receptor, OPN2/RHO and OPN3 in human anagen hair follicles. They evidenced that OPN3 is mandatory for blue-light inducing effect on hair growth [36]. At the same time, they showed that red light alone (689-nm) is not sufficient to stimulate hair growth *in vivo*. These discrepancies in expression rates might result from distinct specificities of the primers used by us and other teams addressing different splices of the corresponding mRNAs. This relatively new paradigm remains to be fully explored to better optimize phototherapy and LLLT devices and protocols for the benefit of hair and skin conditions in the near future. It is probable that not only the eye but also the skin and perhaps hair exposure to light might also contribute *in vivo* to the whole regulation of the global circadian clock. Whether some of these receptors are involved in transducing the effects of GentleWaves® into hair, scalp, or skin remains, however, to be fully scientifically investigated.

CONCLUSION

We and others reported the involvement of a deleterious micro-inflammatory sequence in the complex process of hair loss for about 30% to 50% of individuals affected by the most common cause of hair loss (i.e., AGA). Since it has been proposed that perifollicular inflammation could alter the hair regrowth response to Minoxidil, we suggest that the anti-inflammatory effects of GentleWaves®, we

observed *in vivo* on the human scalp might improve the hair regrowth efficacy of a low concentrated Minoxidil lotion (2%). Our new results show that the device improves the efficacy of this lotion at least to the level of a stronger 5% concentrated solution. Since the effects of a 5% lotion on hair undesirable cosmetic parameters, such as stickiness, oiliness, odor and most of all transitory hair fall can alter the adhesion to the often considered life-lasting pharmacological treatment, GentleWaves® might prove to be a useful adjunctive tool not only for skin disorders but also to help overcome hair loss in AGA. Thus, GentleWaves® + Minoxidil 2% offers great potential as a combination as a hair regrowth treatment as it improves the tolerance of Minoxidil, brings short-term perceived efficacy with long-term clinical efficacy. As previously reported by Esmat et al. and Jimenez et al. [19,20], the importance of such short-term perception efficacy is critical to consumers' adhesion to the treatment. Furthermore, it may also help decreasing the dosage and intensive use of anti-inflammatory drugs in more severe immune/inflammatory dermatological affections, such as alopecia areata, by limiting their reported side effects (such as dermal atrophy and immunosuppression). It might also improve the efficacy of other anti-hair loss compounds which do not act on inflammatory targets, such as Aminexil [14,15], but that remains to be further explored.

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