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ORIGINAL RESEARCH

A randomized blinded retrospective study: the combined use of micro-needling technique, low-level laser therapy and autologous non-activated platelet-rich plasma improves hair re-growth in patients with androgenic alopecia

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ABSTRACT

Introduction: Mini-invasive therapies based on autologous non-activated Platelet-Rich Plasma (ANA-PRP), Low-Level Laser Therapy (LLL-T), and Micro-Needling Technique (MN-T) used in combining for hair re-growth need to be standardized.

Objectives: The work aims to show *in vivo* outcomes resulted from retrospective case-series study in which ANA-PRP + MN-T + LLL-T were used in combined in patients affected by Androgenic alopecia.

Methods: 23 patients were treated, of which 13 males were classified in stage I–V by the Norwood–Hamilton scale, and 10 females were classified in stage I–III by the Ludwig scale. Assessment of hair re-growth was evaluated with photography, physician's and patient's global assessment scale, and standardized phototrichograms during a follow-up: T0 – baseline, T1 – 12 weeks, T2 – 23 weeks, T3 – 44 weeks, T4 – 58 weeks.

Results: Interesting outcomes represented by a hair density increase of 81 ± 5 hairs/cm^2 and 57 ± 7 hairs/cm^2 respectively at T1 and T2 compared with baseline (173 ± 5 hairs/cm^2 at T1 and 149 ± 9 hairs/cm^2 at T2 versus 92 ± 2 hairs/cm^2 at baseline) were observed using computerized trichograms.

Expert Opinion: The main limitation in the autologous regenerative therapies and biotechnologies in hair-regrowth is the extreme variability of PRP products used, in the absence of standardized protocols and widely shared. Appropriate PRP preparations have to be pick after carefully thinking about their bio-molecular specifications and intended indications for use in patients. This approach will aid in matching the optimal PRP product to specific patient factors, leading to improved outcomes and the elucidation of the cost-effectiveness of this treatment.

The combined use of biotechnologies as the association of PRP with micro-needling and low-level laser therapy may improve the results in terms of hair count and hair density compared with those obtained by alone PRP. All the procedures must be performed in the full respect of international and local rules.

Conclusions: The effect of the combined use of MN-T, LLL-T, and ANA-PRP has been demonstrated.

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Hair re-growth PRP; platelet-rich plasma; PRP; micro-needling; low-level laser therapy; LLL-T therapy; regenerative plastic surgery

1. Introduction

A clinical want exists for the development of biotechnologies to enhance regenerative tactics in hair loss (HL). To acquire this, advances in tissue engineering (TE) have to revolve around the improvement of mini invasive-technology. During this case, combined use Autologous Non-Activated Platelet-Rich Plasma (ANA-PRP), Micro-Needling Technique (MN-T) and Low-Level Laser Therapy (LLL-T) to enhance hair re-growth (HR-G) appears to be fundamentals.

The growth factors (GFs), contained in ANA-PRP, may find application in TE through their action to improve cellular differentiation, proliferation, and neo-angiogenesis, thereby aiding the wound healing (WH) process [1–3] and HR-G [4]. ANA-PRP incorporates many signaling proteins and GFs like basic fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and insulin-like growth factor-1 (IGF-1) that are released after platelet activation [4]. Every one of these primary GFs is engaged in a bio-molecular pathway during HR-G and WH [5].

Several existing approaches for the preparation of ANA-PRP based on the diverse centrifugation time and *g*-force used, platelet quantity and GFs, and chemokines availability have been described [5]. A biological (between patients) and temporal (day to day) variation of platelets and GFs has been reported [6]. As such, it's hard to assess which PRP procedure is excellent and which is worst [7]; several PRP products may be more, or then less effective within the treatment of diverse types of tissues and pathologies. The efficacy of PRP generally appears to be known also if an identical process of preparation has not yet been established [8]. Therefore, appropriate PRP preparations have to be pick after carefully thinking about their bio-molecular specifications and intended indications for use in patients [9]. As reported greater

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times [1–4], it's feasible to set up different varieties of PRP based on their cellular content and/or fibrin architecture like Leukocyte-poor PRP (LP-PRP), PRP and Leukocyte (L-PRP), Leukocyte poor platelet-rich fibrin (LP-PRF), Leukocytes and platelet-rich fibrin (L-PRF). Anti-apoptotic efficacy of ANA-PRP is suggested because of the major contributing factors stimulating hair growth (HG) through the activation of the Bcl-2 protein (anti-apoptotic regulator) and Akt signaling, prolonging the survival of dermal papilla cells (DPCs) for the duration of the hair cycle (HC) [10].

MN-T may be considered a minimally invasive dermatological technique during which fine needles are rolled over the skin to puncture the corneum stratum. This therapy is employed to result in collagen formation, neo-vascularization and GFs manufacturing of treated areas [11]. MN-T creates small holes within the skin and multiple micro-channels increasing transdermal penetration of medication and ANA-PRP. It's been utilized in a good range of hair disorders, including Androgenic Alopecia (AGA) and Alopecia Areata (AA) [11]. Superficial mechanical skin trauma produced with MN-T would induce long-term HR-G at handled sites [12].

Red light and laser therapies at 660 nm have been confirmed effective for HL, and therefore the use of LLL-T, such as phototherapy with light-emitting diodes (LEDs) [13], has been intensified to market HG in AGA [14,15]. For years, LED phototherapy has been offered as a good and safe device for the treatment of the skin, mucous and scalp affected inflammatory component, being employed efficaciously within the treatment of acne [16], vaginal atrophy [17], facial aging [18,19], and additionally in disorders associated with HL [20,21]. The innovative use in combining LLL-T, LEDs, and magnetic flux technology for the treatment of AGA seems to be the latest advancement. The synergy of emissions, including spectrum, infrared, soft laser, and magnetic flux, helps to density the hair by activating the cellular metabolism of hair follicles (HFs) and improving the standard and density of the prevailing hair.

This work aims to tell about the impact of various mini-invasive procedures on HR-G. The characteristics and therefore the results of the procedures analyzed are reported, aspiring to clarify any doubts regarding the chance to standardize the utilization of those methods combined.

2. Materials and methods

2.1. Study overview

This randomized, blinded, retrospective, observational case-series study has been performed following the principles reported in the Declaration of Helsinki and internationally consented ethics in clinical research [22]. A high-quality assessment was carried out based on the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist [23]. All patients received, understood, and signed detailed informed consent before any study procedure, about the protocol, including the risks, benefits, and alternative therapies. The study has been the object of a research contract with the University of Rome 'Tor Vergata' approved by rectoral decree R.D n. #1467/2017.

2.2. Rules

ANA-PRP must be identified as emo-component. To raised understand the context of current European Rules, it's necessary to differentiate among minimal manipulation of the blood aimed to obtain emo-components for topical use (as ANA-PRP) and cellular therapies, based on extensive manipulation that involves complex bioprocessing procedures of the therapeutic cells. Specifically, in Italy, preparation of ANA-PRP must be performed respecting the '*Decree of the Blood, 2 November 2015*', which details dispositions associated with quality and safety parameters of blood and emo-components, within which all patients must receive, understand and signs a detailed consent about the study, including the risks, side effects, benefits, and alternative strategies, in line with transfusion service. Additionally, the platelets number in ANA-PRP obtained from all participants has been microscopically counted respecting this rule, in which it had been necessary to get a degree of 1×10^6 platelets per μL.

2.3. Micro-needling device

Before each ANA-PRP injection, an MN-T was done within the targeted areas (TA) employing a sterile micro-needling of 1.0 millimeter (mm) stamp Genosys® (DTS MG, Co., LTD., Seongsui-ro, Seongdong-gu, Seoul Republic of Korea, CE medical device) (Figure 1(a,b)).

For each patient, the scalp affected by HL was divided into four areas (frontal, parietal, vertex, and occipital). Patients with HL localized to the frontal and parietal areas were treated with the MN-T and ANA-PRP only on the frontal areas (identified as TA), while the parietal area was treated with placebo (injection of saline solution). Patients with HL in the parietal and vertex areas were treated with the MN-T and ANA-PRP only in the parietal part of the scalp (identified as TA), while the vertex area was treated with placebo. The same number of injections was repeated in the scalp half treated with MN-T and ANA-PRP (TA) and in the half treated with placebo.

The micro-needling procedure aimed to enhance the vascularization of the areas to be injected with ANA-PRP and, at the identical time, determining follicle disruption producing collagen and GFs. The MN-T was done just before every PRP and it was repeated every 15 days after each infiltration for a total of 3 times.

2.4. ANA-PRP devices

Three different devices have been used and previously analyzed: C-Punt® (Biomed Device, Modena, Italy, 41,126), i-Stem® Preparation System (i-Stem, Biostems, Co., LTD., Seoul, Korea, 138–843), MAG-18® (DTS MG Co., Ltd., Seoul, Korea, #B108-147).

C-Punt® was consisting of a kit with a 60 mL syringe in which blood (55 mL) was collected using Anticoagulant Citrate Dextrose (ACD). The syringe has been centrifuged at 1200 rpm for 10 min and after, the amount of platelet pellet obtained (23 mL), represented by Platelet-Poor Plasma (PPP) and Platelet-rich plasma (PRP), has been inserted in a selector

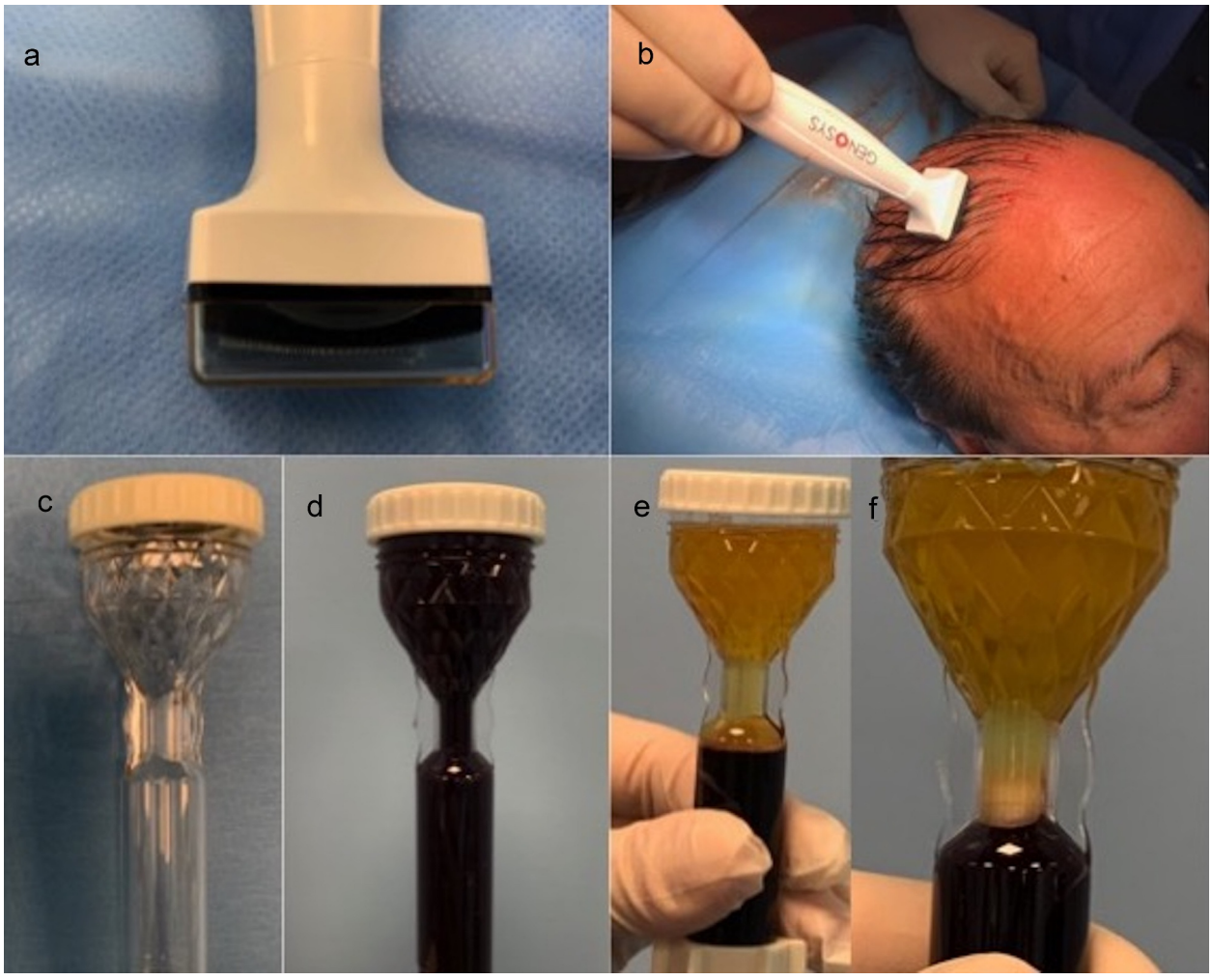

Figure 1. (a) Microneedles stamp Genosys®. (b) The micro-needling technique (MN-T) performed in the targeted areas (patient showed in Figure 3), before each ANA-PRP infiltration, using a sterile micro needles stamp Genosys®. (c) The MAG-18® PRP Kit; (d) ANA-PRP using MAG-18® PRP Kit prepared from 18 mL of blood and 1 mL of ACD; (e) 18 mL of PPP and PRP suspension obtained after the first spin (3000 rpm per 6 min), below the red blood cells (RBC) suspension; (f) 1 mL of PPP and 3 mL of RBC were removed before the second centrifugation (3000 RPM per 2 min); PPP and ANA-PRP concentrated in the superior and central portion of hourglass. The lower part of the hourglass was screwed allowing the concentration of the ANA-PRP in the central narrowing of the hourglass as a gel pellet.

device. At the end of the procedure, 9 mL of ANA-PRP were collected [4].

The i-Stem® kit was consisting of an hourglass system, in which peripheral blood (17.7 mL) was drawn and, ACD (2.2 mL) was employed as an anticoagulant. After the primary spin (3000 rpm per 6 min), 1 mL of the PPP and 2 mL of RBC (Red blood cells) were removed; therefore the suspension obtained was centrifuged a second time for 3 min. At the end of the procedure, 15 mL of ANA-PRP was obtained [24].

Mag-18 PRP® (Figure 1(c-f)) was an hourglass system in which 18 mL of peripheral blood and 1 mL of ACD were collected. The hourglass was centrifuged the first time, at 3000 rpm per 6 min. In the end, 3 mL of the PPP and 3 mL of RBC were removed. The suspension obtained was centrifuged a second time at 3400 rpm per 2 min. Screwing slowly the lower plug of the hourglass system, it was possible to concentrate 1,5 mL of ANA-PRP as 'gel pellet' within the middle portion of an hourglass [25]. Details of all systems tested have been reported in Table 1.

Table 1. PRP kits and device tested. Abbreviations: leucocyte platelet-rich fibrin (L-PRF); leucocyte platelet-rich plasma (L-PRP); pure platelet-rich fibrin (P-PRF); Pure platelet-rich plasma (P-PRP).

Type	Activation	System	Device	Manufacturer	Fields	Centrifugation	Ref
L-PRP	ANA-PRP	Closed	i-Stem®	(i-Stem, Biostems, Co., LTD., Seoul, Korea, 138–843)	Hair regrowth	1008 $g \times 10$ min (1st) 1295 $g \times 10$ min (2nd)	[24]
L-PRP	ANA-PRP	Closed	Mag-18 PRP®	(DTS MG Co., Ltd., Seoul, Korea, #B108-147)	Hair regrowth	1008 $g \times 10$ min (1st) 1295 $g \times 10$ min (2nd)	[25]
L-PRP	ANA-PRP	Closed	C-Punt®	(Biomed Device, MO, Italy, 41,126)	Hair regrowth	161 $g \times 10$ min (1st) 287 $g \times 15$ min (2nd)	[4]

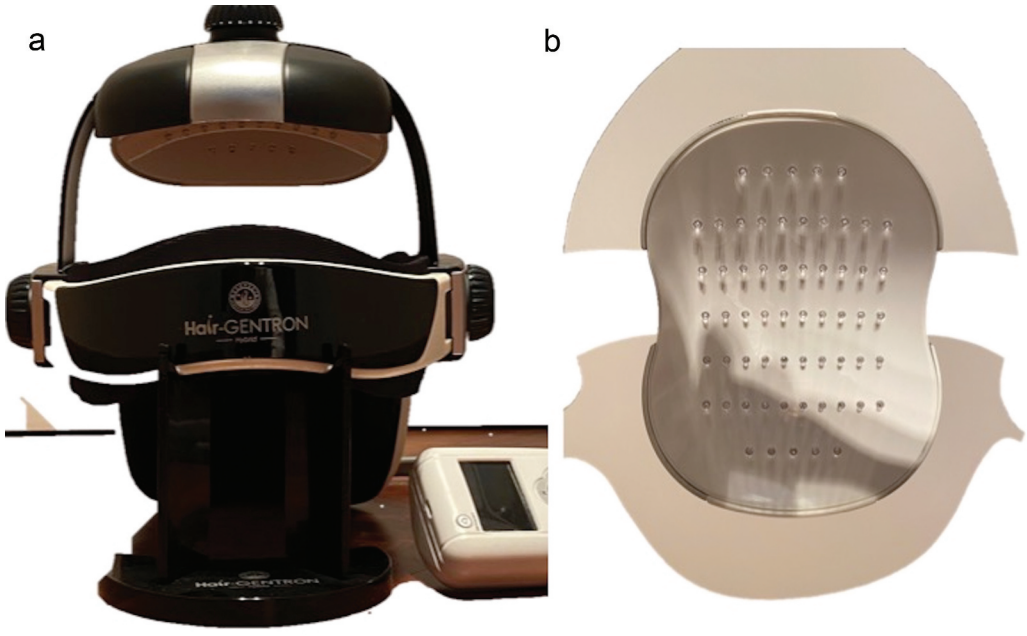

Figure 2. (a, b) Low-level laser therapy (LLL-T) represented by Hair Gentron (DTS MG Co., Ltd., Seoul, Korea, #B108-147).

2.4.1. Mechanical and controlled ANA-PRP injections

ANA-PRP injections (0.2 mL * cm^2) were performed in the targeted area at 5 mm deep using a mechanical and controlled infiltration through a mesotherapy gun (Ultim gun – Anti-Aging Medical Systems, Montrodat, France) equipped with a 30-gauge, 10 mL Luer lock syringe.

2.5. Low-level laser therapy (LLL-T) device

LLL-T utilized in this work has been represented by helmet Hair Gentron® (DTS MG Co., Ltd., Seoul, Korea, #B108-147). The participation period for every patient was six months, including the screening/baseline/first treatment visit up to the 48th treatment visit and a follow-up visit at six months. The whole treatment course included 48 sessions employing a home LLL-T helmet (Figure 2(a, b)) conducted twice per week for twenty-four weeks (ws). Patients were reevaluated six months after the last treatment session (1 year after the primary treatment). The helmet, once applied and fixed, maintaining the same distance between the scalp and the led offers a more rigorous application of LLL-T compared with the brush that depends on the use of the hand.

Hair Gentron® is constituted by lights with various wavelengths ranging from 423 nm to 640 nm. Three color lights (red, blue, and infrared) that emits an output of less than 1 W (Low-Level LED Light) were used:

- Red light, wavelength 640 nm, skin penetration depth 1–6 mm, promotes cell metabolism, improves blood circulation, promotes nutrition supply to capillaries and strengthen hair strands, promotes the alignment of the cuticles, promote pain relief;
- Blue light, wavelength 423 nm, skin penetration depth 1 mm, activates the keratin present in the hair shaft, increases water retention of hair, acts in the microbiological control, diminishes the sebaceous gland, reducing the grease of the scalp;
- Infrared light promotes cell metabolism and improves blood circulation.

Additionally, the device offers scalp massaging and heating functions to improve blood flow, provide more nutrients & oxygen to HFs.

All the patients affirmed in a declaration signed during the follow-up, to have respected the use recommendation of the helmet during homecare.

2.6. Patients

This study has been performed enlisting a complete of 23 patients, treated since 2015, aged 20–71 years. All patients were Caucasian. Of these, 13 were males displaying AGA in stage I–V according to the Norwood-Hamilton scale and 10 were females with AGA in stage I–III according to the Ludwig scale. All patients have been treated with ANA-PRP infiltrations in 3 sessions spaced 30 days average. Patient characteristics were reported in Table 2. Assessment of HR-G was evaluated with photography, physician's and patient's global assessment scale, and standardized phototrichograms during a follow-up: T0 – baseline, T1 – 12 weeks, T2 – 23 weeks, T3 – 44 weeks, T4 – 58 weeks. Exclusion criteria have been divided into two types, local and general. General exclusion criteria included platelet disorders, thrombocytopenia, anti-aggregating therapy, the employment of pharmacological therapeutics targeting on AGA (Finasteride®, similar drugs, and/or anti-androgens) within the earlier year, bone marrow aplasia, uncompensated diabetes, sepsis, cancer, and autoimmune disorders. Local exclusion criteria included a male pattern hair loss (MPHL),

Table 2. Patient characteristics.

Patients	Gender	Hamilton-Norwood degree	Ludwig degree	Targeted Area	Age	Platelet concentration
1	male	III–V	-	parietal	53	386.000 µL baseline 1.123.000 µL (PRP)
2	male	II	-	frontal	21	353.000 µL baseline 1.109.000 µL (PRP)
3	male	V	-	parietal + vertex	67	201.000 µL baseline 1.003.000 µL (PRP)
4	male	I	-	frontal	20	254.000 µL baseline 1.054.000 µL (PRP)
5	male	IV	-	parietal + vertex	71	237.000 µL baseline 1.049.000 µL (PRP)
6	male	III	-	parietal	40	241.000 µL baseline 1.051.000 µL (PRP)
7	male	IIa	-	frontal	33	206.000 µL baseline 1.011.000 µL (PRP)
8	male	IIa	-	frontall	31	298.000 µL baseline 1.081.000 µL (PRP)
9	male	III–V	-	parietal	41	267.000 µL baseline 1.069.000 µL (PRP)
10	male	III	-	parietal	38	301.000 µL baseline 1.092.000 µL (PRP)
11	male	IV	-	parietal + vertex	62	382.000 µL baseline 1.119.000 µL (PRP)
12	male	IIa	-	frontal	28	223.000 µL baseline 1.035.000 µL (PRP)
13	male	III–V	-	parietal	44	310.000 µL baseline 1.046.000 µL (PRP)
14	female	-	II	parietal	47	323.000 µL baseline 1.065.000 µL (PRP)
15	female	-	II	parietal	59	345.000 µL baseline 1.070.000 µL (PRP)
16	female	-	I	frontal	40	222.000 µL baseline 1.030.000 µL (PRP)
17	female	-	III	parietal + vertex	69	302.000 µL baseline 1.095.000 µL (PRP)
18	female	-	I	frontal	26	298.000 µL baseline 1.087.000 µL (PRP)
19	female	-	II	parietal	45	233.000 µL baseline 1.043.000 µL (PRP)
20	female	-	II	parietal	35	210.000 µL baseline 1.011.000 µL (PRP)
21	female	-	I	frontal	23	227.000 µL baseline 1.033.000 µL (PRP)
22	female	-	III	parietal + vertex	61	204.000 µL baseline 1.003.000 µL (PRP)
23	female	-	II	parietal	55	201.000 µL baseline 1.002.000 µL (PRP)

over V degree, the employment of topical medicines for AGA (lotions as Minoxidil®, prostaglandin analogs, retinoids, or corticosteroids) within the earlier year. Of the 23 patients enrolled, 1 has been excluded.

2.6.1. Allocation sequence and quality assessment

The patient's allocation sequence was created using a web randomization generator (*https://www.randomizer.org*) and was concealed by an individual unrelated to the trial management group. The patients, surgical team, and evaluators have been all blinded to treatment allocation, and blinding was maintained until all data had been analyzed. In detail, all told patients treated, quality checks were performed respecting the subsequent criteria:

- Platelets number evaluation (in all patients the ANA-PRP suspension had a platelet concentration equal to 1×10^6/ μL ± 20%);
- Blood volume was withdrawn (within 55 mL for every patient);
- The quantity of ANA-PRP obtained (variable in keeping with the degree and also the size of the targeted area full of AGA);
- Labeling of every sample of PRP for every patient for laboratory control;
- Adverse reaction signaling (did not occur).

2.6.2. The risk mitigation measures

Risk Evaluation and Mitigation Strategies (REMSs) with Elements to Assure Safe Use (ETASU) have been used for the MN-T, LLLT, and PRP procedures with significant safety risks reduction. ETASU characteristics were represented by the adoption of the same applicative protocol for all patients, the identification of exclusion and inclusion criteria, the use of the medical devices with CE marks. All

risks were identified before the clinical treatments. Ineffective results were the most frequent risks. Most REMSs required training healthcare providers, patient enrollment via allocation sequences, and suitable evaluation of the selected patients.

The main rationale for ETASU was to provide patients with safe access to the procedures. The authors evaluated the individual characteristics of REMSs with ETASU searching the FDA website (*http://www.accessdata.fda.gov/scripts/cder/rems/index.cfm*) and specifically identifying them in:

- Informed consent for all patients in which have been reported the risks and complications of the procedures;
- A specific training plan to healthcare providers;
- A communications plan of side effects;
- The need for CE marks for the medical devices used.
- The need to enroll patients through inclusion and exclusion criteria.

2.6.3. Trichoscopy evaluation of the targeted area

Phototrichograms were taken of all scalps by a trained evaluator using Fotofinder video-epiluminescence microscopy (FotoFinder Systems; *http://www.fotofinder.de*) in combination with the Trichoscan digital image analysis (Tricholog GmbH and Datinf GmbH; *http://trichoscan.com*). TrichoScan is a digital software-supported epiluminescence technique for measuring hair count (number of hairs per 0.65 cm^2), hair density (number of hairs per cm^2), hair diameter, anagen-to-telogen ratio, and vellus hair to terminal hair ratio. To determine the quality of hair leading to an increased hair density, it is important to differentiate the number of terminal and vellus hairs. In TrichoScan analysis, all hairs with a diameter > 40 μm are categorized as terminal hairs; those with lesser diameter are categorized as vellus hairs. In all patients, in both the treatment and control half-heads, two TA of HL were defined and marked with a semi-permanent tattoo for the subsequent trichogram. In the TA, hairs were clipped and dyed brown for 10 minutes to improve the hair contrast for the analytic software. The evaluator of the computerized trichogram analysis was blinded regarding the treatment and control areas of the scalp and was not involved in the administration of treatment.

3. Results

3.1. In vivo evaluation using trichoscopy analysis

The short-term outcomes showed at T1 after 12 ws (12 ws vs. 0 ws), represented by 65 ± 5 hairs/cm^2 constituted a 31 ± 2% increase in hair density (HD) when ANA-PRP treatment was performed in the TA, with a statistically significant difference in HG ($p = 0.0029$) compared with baseline (157 ± 5 hairs/cm^2 at T1 versus 92 ± 2 hairs/cm^2 at baseline), while the control area (CA) displayed a mean decrease of 1.3 hairs/cm^2 (control vs. treatment: $p < .0001$).

The long-term outcomes with this therapy, showed an improvement in the mean HD at T4 after 58 ws (58 ws vs. 0 ws) of 23.3 ± 3 hairs/cm^2, in the TA compared with baseline (115 ± 5 hairs/cm^2 at T4 versus 92 ± 2 hairs/cm^2 at baseline), while the CA displayed a mean decrease of 0.7 hairs/cm^2 (control vs. treatment: $p < .0001$). No statistically significant differences in vellus HD among the TA and the CA at T1 and T4 have been reported. Comparing the outcomes obtained during the follow-up evaluation, 12 ws, 23 ws, 44 ws, and 58ws after the last treatment, HD improvements for patients treated with ANA-PRP were 65 ± 5 hairs/cm^2 at T1, 28 ± 2 hairs/cm^2 at T2, 25 ± 3 hairs/cm^2 at T3, 23 ± 3 hairs/cm^2 at T4 compared with baseline (157 ± 5 hairs/cm^2 at T1, 120 ± 5 hairs/cm^2 at T2, 117 ± 5 hairs/cm^2 at T3, 115 ± 5 hairs/cm^2 at T4 versus 92 ± 2 hairs/cm^2 at baseline).

The use of ANA-PRP with MN-T in combining, where MN-T was performed before each injection, showed interesting results represented by an HD increase of 73 ± 5 hairs/cm^2 and 50 ± 7 hairs/cm^2 respectively at T1 and T2 compared with baseline (165 ± 5 hairs/cm^2 at T1 and 142 ± 9 hairs/cm^2 at T2 versus 92 ± 2 hairs/cm^2 at baseline) (control vs. treatment: $p < .0001$).

Additionally, very promising and interesting outcomes represented by an HD increase of 81 ± 5 hairs/cm^2 and 57 ± 7 hairs/cm^2 respectively at T1 and T2 compared with baseline (173 ± 5 hairs/cm^2 at T1 and 149 ± 9 hairs/cm^2 at T2 versus 92 ± 2 hairs/cm^2 at baseline) (control vs. treatment: $p < .0001$) were observed performing the combined use of MN-T, LLL-T, and ANA-PRP as showed in Figures 3(a-e) and 4(a,b).

4. Discussion

In vitro, antiapoptotic effects of ANA-PRP have been identified as the most important contributing factors stimulating HG via the Bcl-2 protein activation (antiapoptotic regulator) and Akt signaling, promoting the survival of DPCs during the HC. Specifically, the up-regulation of FGF-7/b-catenin signaling pathways, with ANA-PRP therapy, has been suggested to stimulate HG by inducing Human Follicle Stem Cells (HFSC) differentiation also as promoting the anagen phase of the HC [4]. It also seems to extend the perifollicular vascular plexus via the rise of VEGF and PDGF levels, which have an angiogenic effect [4].

To better describe the various results obtained *in vivo* using ANA-PRP, it appears necessary to discuss and compare the foremost recent results in HD and hair count (HCO) obtained for this procedure with those obtained by HFSC injection. In detail, 12 ws after the last treatment (ANA-PRP has been performed every 30 days, three times), HD measurements for patients treated with ANA-PRP were 65 ± 5 hairs/cm^2. The results obtained constitute a 31 ± 2% increase in HD when ANA-PRP treatment is performed, with a statistically significant difference in HG ($p = 0.0029$) [4]. Differences among the 12 ws follow-up counts and the baseline count for these HG parameters were higher within the ANA-PRP treatment population than within the autologous activated Platelet-Rich Plasma (AA-PRP) treatment population as displayed within the previous trials performed by Gentile et al. [4]. 23 ws after the last treatment, HD measurements for patients treated with ANA-PRP and AA-PRP were 28 ± 2 and 15 ± 3 hairs/cm^2 respectively [4]. The improvement of HD and HCO parameters for ANA-PRP over AA-PRP may reflect the *in vivo* greater efficacy of body thrombin to activate platelets

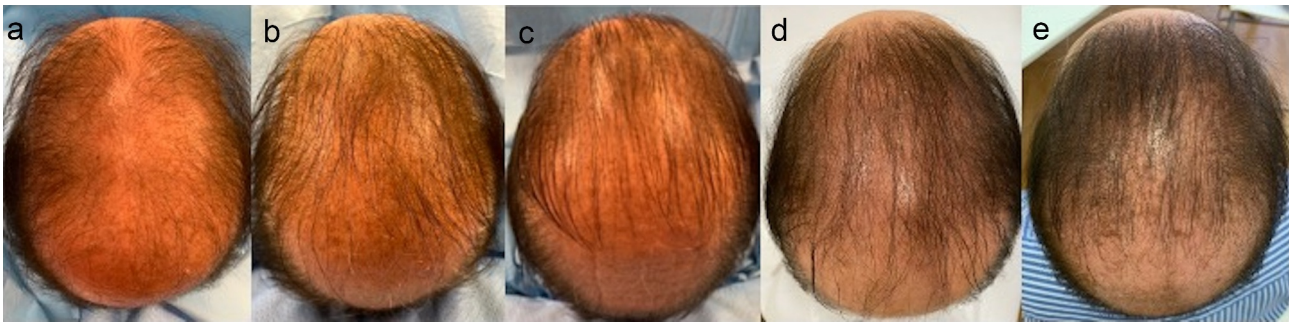

Figure 3. (a) Pre-operative view of the scalp of 67 years old male patient affected by AGA V according to Norwood Hamilton scale; (b) Post-operative view after 1 session of PRP, 1 session of MN-T and 4 sessions of LLL-T; (c) Post-operative view after 2 PRP, 2 MN-T and 12 sessions of LLL-T; (d) Post-operative view, after 3 sessions of ANA-PRP mechanical and controlled injection, 3 sessions of MN-T and 24 sessions of LLLT, at T1 (12 ws), constituted of 81 ± 5 hairs/cm2 increase in HD. (e) Post-operative view, after 3 sessions of ANA-PRP mechanical and controlled injection, 3 sessions of MN-T and 46 sessions of LLL-T application, at T2 (23 ws), constituted of 57 ± 7 hairs/cm2 increase in HD.

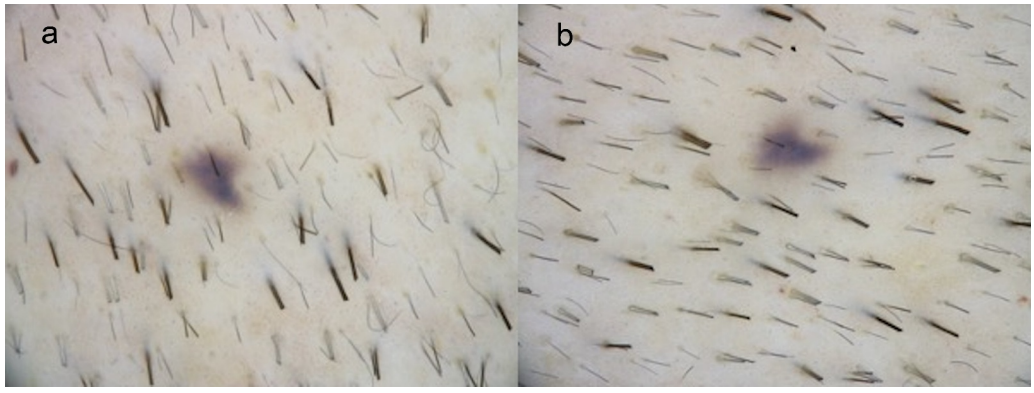

Figure 4. Trichoscan digital image analysis performed by Fotofinder in a patient shown in Figure 3. (a) At T0 pre-operative hair density was 92 ± 2 hairs/cm2 and proportions of telogen and anagen hairs were 38.8% and 51.3%, respectively; (b) At T2 post-operative hair density was 149 ± 9 hairs/cm2, and proportions of telogen and anagen hairs were 48.2% and 49.9%, respectively.

and to distribute the GFs discharged by activated platelets compared with *in vitro* calcium activation. Moreover, delivery of ANA-PRP may enable the assembly of thromboxane A2 (TXA2) by the platelets once they're activated *in vivo*, which might, in turn, activate additional platelets and amplify platelet aggregation [26]. Both patient populations treated with ANA-PRP and AA-PRP respectively displayed improvement within the number of follicular bulge cells and follicles, epidermal thickening, improved vascularization, and the next number of Ki67+ basal keratinocytes in PRP-treated scalp tissue compared with placebo (saline) [4]. Indeed, microscopical examination of ANA-PRP and AA-PRP treated scalp tissue from the authors' previous article [4] provides such *in vivo* evidence. The outcomes obtained with ANA-PRP and AA-PRP infiltration could also be considered the same as the HFSC treatment.

A recent investigation by Gentile et al. [27], displayed HD improvement for HFSC treatment 23 ws after the second injection (one injection was done every 60 days for 2 infiltration cycles) of 29 ± 5% hairs/cm^2 compared with 28 ± 2 hairs/cm^2 when ANA-PRP was used. Gentile et al. [27] reported, for the primary time within the literature (2017), preliminary data which autologous HFSC suspension, obtained by mechanical centrifugation of a 2 mm punch biopsy of the scalp, injected in patients suffered AGA. In 2019, the 'Gentile protocol' [28] based on mechanical and controlled injections of autologous micrografts containing Human Intra and Extra Dermal Adipose Tissue-Derived Follicle Stem Cells (HD-AFSCs), obtained by fragmentation, disaggregation, and centrifugation of two mm scalp's punch biopsy, with none commercial kit or device, showed a 33% ± 7.5% increase of HD increments, after 23 ws [28]. As briefly alluded to previously, GFs contained in PRP, in different concentrations depending on the procedure used (ANA-PRP or AA-PRP), may stimulate HG and tissue-regeneration, positively influencing the healing pathway. Every one of GFs is involved in an exceedingly specific biomolecular pathway during HR-G. In HR-G, evaluated in vitro, EGF stimulates migration and growth of follicle ORS cells by activation of Wnt/β-catenin signaling; b-FGF stimulates hairs' follicles development; VEGF stimulates perifollicular vascularization; TGF-β improves the signaling pathways that regulate hair cycle; IGF-1 stimulates migration, proliferation, and survival of follicle cells; IL-6 is involved in WIHN through STAT3 activation; IGFBP-1 to −6 regulates IGF-1 effects and its

interaction with extracellular matrix (ECM) proteins at the follicle level; PDGF and PDGFR-β/-α64 up-regulate the genes involved in follicle differentiation; PDGF and its receptors are essential for follicular development; Wnt3a is involved in follicle development through β-catenin signaling; PGE2 stimulates anagen in follicles; PGF2α and analogs stimulate the transition from telogen to anagen; BIO (GSK-3 inhibitor); PGE2 or inhibition of PGD2 or PGD2 receptor D2/GPR4477 stimulates follicle regeneration; BMP maintains DPC phenotype (crucial for stimulation of hair follicle stem cell); BMPR1a maintains the correct identity of DPCs (essential for specific DPC function); M-CSF and M-CSFR are involved in wound-induced hair re-growth [22].

Current medicines reporting the U.S. Food and Drugs Administration (FDA) approval for AGA include Minoxidil® and Finasteride®.

Minoxidil® (pyrimidine derivate) increases the anagen and improves HFs size via activation of prostaglandin endoperoxide synthase-1, which increases the quantity of prostaglandin E2 [29]. Minoxidil® prolongs the survival of DPCs by increasing the Bcl-2/Bax ratio and by activating ERK and Akt [30].

Finasteride® was identified as a type II 5-alpha-reductase-inhibitor which decreases dihydrotestosterone (DHT) of about 65% in serum, prostate, and scalp [31,32]. Oral Finasteride® also increases anagen, with the gradual improvement of hair thickness (HT) [31]. Finasteride® has been shown to scale back the pattern HL related to increased expression of caspases and apoptosis inhibitors, stimulating anagen phase which leading to HG [32,33]. Adil A et al. [34] realized a scientific review suggesting that Minoxidil®, Finasteride®, and LLL-T may be considered effective for promoting HG in men who suffer AGA while Minoxidil® was effective in women with AGA. To better describe the in vivo results obtained by the utilization of Minoxidil®, Finasteride®, ANA-PRP, MN-T, LLL-T, and HFSCs it's necessary to report the foremost recent outcomes in HD and HCO obtained for these different treatments.

Compared to previous studies based only on ANA-PRP or AA-PRP injections, within the present work, the authors intended to report, for the primary time, the combined use of MN-T and LLL-T with the mechanical injection of ANA-PRP in an exceedingly retrospective analysis, evaluating the outcomes during a long-term follow-up. Additionally, the outcomes obtained have been compared in terms of HCO and HD with other procedures like HFSCs, Adipose-derived mesenchymal stem cells (AD-MSCs), HD-AFSCs, Minoxidil®, and Finasteride® as displayed in Table 3.

In an investigation of Van Nestle et al. [35] 212 male patients, suffered from AGA were randomized to receive Finasteride® 1 mg daily or placebo, for 48 weeks. At baseline, mean total and anagen HCO within the Finasteride® group (FG) were 200 and 124 hairs, respectively (% anagen = 62%) and therefore the anagen to telogen ratio was 1.74 (geometric mean); while In the placebo group (PG), the respective values were 196 and 119 hairs (% anagen = 60%) and 1.57. At week 48, the FG had a net improvement (mean ± SE) compared with PG, in total and anagen HCO of 17.3 ± 2.5 hairs (8.3% ± 1.4%) and 27.0 ± 2.9 hairs (26% ± 3.1%), respectively ($p < 0.001$). Furthermore, the Finasteride® resulted in net improvement within the anagen to telogen ratio of 47% ($p < 0.001$) promoting the HG. In a recent investigation of Bao L. et al. [36], based on the use of Minoxidil® 5%, the mean improvement in total HD from baseline to 24 ws was 18.8/cm^2 in patients underwent topical application and 38.3/cm^2 in patients underwent electrodynamic micro-needle treatments with topical 5% Minoxidil® [36]. The results obtained and everyone data discussed were reported in Table 3.

Regarding the effectiveness/expenses ratio, the drugs discussed may be considered effective in AGA patients but they cause a dependence promoted by the requirement to use daily Finasteride® or to use Minoxidil® topically, for a protracted period, however not inferior to 12–24 months, consistent with the analyzed studies [35,36]. Regarding the MN-T, that the authors performed within the present study, Stoll et al. [12] have hypothesized, in a pre-clinical model, that superficial mechanical skin trauma applied with a micro-needling device would induce long-term HR-G at treated sites. 5 ws after the micro-needling, HR-G started, followed by hyperpigmentation of the treated skin. After 12 ws there was a 90% improvement in coat coverage at previously alopecic areas. One year after the treatment, coat conditions remained stable [12].

As reported by Ferting et al. [11] in a clinical review, the MN-T could be considered a minimally invasive dermatological procedure during which fine needles are rolled over the epidermis to puncture the horny layer with the aim to induce collagen formation, neovascularization and protein production. The MN-T has been employed in a large range of dermatologic conditions, including AGA and AA [11]. Some studies

Table 3. *In vivo* evaluation using trichoscopy analysis in terms of hair density (HD) (hairs/cm^2) and Hair Count (HC) (hairs/0.65cm^2) improvement.

Procedure	T1 (12 Ws)	T2 (23 Ws)	T3 (44 Ws)	T4 (58 Ws)	Ref
ANA-PRP	65 ± 5 hairs/cm^2	28 ± 2 hairs/cm^2	25 ± 3 hairs/cm^2	23.3 ± 3 hairs/cm^2	[4]
ANA-PRP + MN-T	73 ± 5 hairs/cm^2	50 ± 7 hairs/cm^2	-	-	[25]
ANA-PRP + MN-T + LLL-T	81 ± 5 hairs/cm^2	57 ± 7 hairs/cm^2	-	-	[-]
AA-PRP	-	15 ± 3 hairs/cm^2	-	-	[4]
HFSCs	-	29 ± 5 hairs/cm^2	-	-	[27]
AD-HFSCs	-	33 ± 7.5 hairs/cm^2	-	-	[28]
Minoxidil 5%®	-	18.8 ± 0 hairs/cm^2 (24Ws)	-	-	[36]
Minoxidil 5%® + electro-MN-T	-	38.3 ± 0 hairs/cm^2 (24Ws)	-	-	[36]
Finasteride®	-	-	27.0 ± 2.9 hairs/0.65cm^2 (48Ws)	-	[35]

had investigated different laser and light sources with related treatment parameters for the management of alopecia, like LLL-T [37–42],, various wavelengths of LED light [43,44] and several other techniques combined, like LED – LLLT [45]. Although the available literature regarding phototherapy-based devices to treat AGA is restricted, study results regarding the first outcomes showed a more robust performance than those reported in previous studies.

Leavitt et al. (2009) [45] conducted a 26-week, randomized, double-blind, device-controlled, multicenter trial during which 110 male patients with AGA IIa – V were randomized for treatment with either the HairMax LaserComb® [45]. The primary efficacy endpoint was mean terminal HD. At 26 ws, HD had an average increase of 19.8% in patients of the study group. Kim et al. (2013) [38] performed a randomized, double-blind, sham device-controlled trial at two research centers that included 40 male and female subjects with AGA treated with a helmet-type 3 R LLL-T device with a lightweight source consisting of LEDs emitting wavelengths of 630 nm (3.5 mW, 24 units, L-513ECA) and 660 nm (2.5 mW, 18 units, L- 513LRC) and laser diodes (LDs) with wavelengths of 650 nm (4 mW, 27 units, DL3147 – 060) [38]. The primary efficacy endpoint was HD after 24 ws of treatment estimated with trichograms. 6 months after the last procedure, the mean percentage of increase in HD was 14.7%. Finally, Suchonwanit et al. (2018) [46] conducted a 24-week, prospective, randomized, double-blind, device-controlled run that included male subjects aged over 18 years with AGA treated with RAMACAP, a combat helmet-shaped device containing single-mode LDs, which emits at a wavelength of 660 ± 10 nm [46]. The first efficacy endpoint was HD and hair diameter in a TA at ws 8, 16, and 24, by photographing the TA with a Folliscope® and measuring it with Folliscope 2.8 software (LeadM Corporation, Seoul, Korea). Six months after treatment, the mean percentage of increase in HD was 9.1%.

Summarizing, the rationale of the combined use of these procedures (MN-T + LLL-T + ANA-PRP) was based on an increase in stimulating HG:

- Via the activation of the Bcl-2 protein and Akt signaling, prolonging the survival of DPCs for the length of the HC [10];
- The up-regulation of FGF-7/b-catenin signaling pathways, inducing HFSC differentiation;
- Increases the anagen phases of the HC.

Also, the use of HFSCs and Adipose-Derived follicle Stem Cells, as autologous cellular therapy minimally invasive, that promoting the release of GFs involved in HG, had an important role in the regenerative aspects of HG. Recently, on autologous cellular therapy minimally invasive, many investigations have appeared with the aim to promote regenerative medicine in different fields, additionally over of HR-G [47] for example, with the use of human periodontal stem cells in bone regeneration [48–52], in esthetic soft tissue improvement [53], in wound healing [54–56], and in reconstructive purposes [57–60], widening the horizons of regeneration to hitherto unexplored fields. In every case, as previously discussed, without a standardized use of innovative procedures and also cellular therapies with a specific control quality assessment, regenerative therapies would be difficult if not simply unreliable.

Other minimally invasive techniques such as the use of nanoparticles for clinical treatment of AGA were reported recently in the literature. Hatem et al. [61], reported the results of a study aimed to develop vitamin C based nanovesicles (Aspasomes) loaded with the antioxidant melatonin, as a novel cosmeceutical to be used for clinical treatment of AGA. The investigators tested clinically, melatonin aspasomes on AGA patients by evaluating the degree of improvement through conduction of hair pull test, histometric analysis, and dermoscopic evaluation. Results revealed that melatonin aspasomes showed favorable pharmaceutical properties in addition to clinically promising results compared to melatonin solution, manifested by increased HT, HD, and decreased HL, with photographic improvement in most patients. Improvement of HT, HD, and decreased HL have been also reported through the use of nanostructured lipid carriers (NLCs) exhibiting high skin deposition and high inherent antioxidant potential [62]. NLCs displayed good storage stability and they were able to increase the skin deposition of melatonin 4.5-folds in stratum corneum, 7-folds in the epidermis, and 6.8-folds in the dermis compared to melatonin solution, appearing as a new and minimally invasive technique to be studied in the future [62].

We are witnessing the definitive sunset of substitutive surgery born in this field with hair transplant and, at the same time, we find ourselves at the dawn of regenerative medicine, where the aim is to hair follicle regeneration.

5. Conclusions

The data reported obviously highlights the constructive effects of ANA-PRP, MN-T, and LLL-T on AGA, as demonstrated by clinical and trichoscopy analysis, without major side effects. Additionally, this combined therapy may be considered as a safe and effective alternative procedure to treat AGA. Combining ANA-PRP with other treatment modalities, such as LLL-T and MN-T has demonstrated synergistic effects, enhancing hair re-growth.

Authors' contributions

P.G. was the leader and principal author of this paper, performing the methodology, conceptualization, formal analysis, validation, investigation, data curation, writing—original draft preparation, writing—review and editing, acquisition funding and resources and project administration; B.D.A., and J. P. contributed resources and were involved in data curation and visualization; L.D. contributed analysis of European and Italian rules; S.G. contributed resources, data curation, validation, review, and editing. All authors made substantial contributions to concept and design of the study, revised the manuscript, and gave their approval to the final version of the manuscript.

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Declaration of interest

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