Stem Cell Surgery and Growth Factors in Retinitis Pigmentosa Patients: Pilot Study After Literature Review

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Running title: Stem cell surgery in RP patients.

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Abstract: To evaluate whether autologous mesenchymal cells, adipose derived stem cells and platelet-rich plasma, grafted into the supracoroideal space by surgical treatment according to Limoli retinal restoration technique (LRRT), can produce growth factors in order to exert a beneficial effect in retinitis pigmentosa (RP) patients. Twenty-one eyes underwent surgery and divided based on retinal foveal thickness ≤ 190 or >190 μm into group A and group B, respectively. The specific LRRT triad was grafted in a deep scleral pocket above the choroid of each eye. At 6-month follow-up, group B showed an improvement in residual close-up visus and sensitivity at microperimetry compared to group A. After an in-depth review of molecular biology studies concerning degenerative phenomena underlying the etiopathogenesis of RP, it can be confirmed that further research is needed on tapeto-retinal degenerations both from a clinical and molecular point of view to obtain better functional results. In particular, it is necessary to increase the number of patients, extend observation times, and treat subjects in the presence of still trophic retinal tissue to allow adequate biochemical and functional catering.

Keywords: autograft; embryonic stem cells (ESC); growth factor (GF); hereditary retinal disease; induced pluripotent stem cells (iPSCs); Limoli retinal restoration technique (LRRT); mesenchymal stem cell (MSC); retinitis pigmentosa; spectral domain-optical coherence tomography (SD-OCT)

1. Introduction

Retinitis pigmentosa (RP) comprises a heterogeneous group of hereditary retinal diseases characterized by progressive degeneration of photoreceptors. It primarily and severely affects the rods with subsequent involvement of cone functions [1-3].

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Although the ethiology is quite variable, the final pathway is progressive photoreceptor cell death by apoptosis with subsequent retinal atrophy. The prevalence of RP is approximately 1:4000, affecting more than 1 million individuals worldwide [4].

In X-linked patients, who account for approximately 5-15% of all cases, the phenotype of the disease generally tends to be the most severe. Conversely, patients with autosomal recessive RP, comprising 50-60% of cases, and patients with autosomal dominant RP, which is responsible for 30-40% of cases[6,7], show a better visual prognosis, a slower progression of the disease, and longer maintenance of central vision. A large number of mutations in more than eighty different genes are known to be the major cause of RP [1-4].

The etiopathogenesis of RP is not explained by genetics alone, because there are other mechanisms that cover various biological aspects: trophism, oxidation, inflammation, immune, vascularization, and apoptosis [5].

In the majority of cases visual impairment usually begins with night blindness and progresses to the restriction of the visual field peripheral vision. Macular degeneration usually occurs only at the very end stage of the disease and may also culminate in the loss of central vision [1,2,6].

The suspect of the disease, caused by visual concerns, can be confirmed by specific examinations as visual field testing, full-field electroretinogram (ERG), and optical coherence tomography (OCT) [7-8].

To date, the disease has no curative treatment, but new therapeutic options are being actively developed, involving implanted retinal prosthetic system devices, gene therapy, and cell therapy to replace or restore defective cells. Cell preservation is being actively investigated, especially as regards the neurotrophic, antiapoptotic, haemorheologic and immunomodulatory actions of growth factors (GFs) and cytokines, which can be used directly or in a cell-mediated way, targeting the residual retinal cells [9-13].

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The therapeutic aim is to slow down or prevent the death of photoreceptors [10,14-17].

This objective can be achieved by delivering embryonic stem cells (ESC), induced pluripotent stem cells (iPSCs), and mesenchymal stem cells (MSCs) to precise target locations in the eye [14,18-21].

ESC, iPSC, and MSCs are capable of self-renewal and display multipotency, i.e., the ability to differentiate into all cells derived from any of the three germ layers

MSCs can be obtained from different sources: umbilical cord blood, peripheral blood, bone marrow and adipose tissue. The multipotent nature of MSC has been demonstrated in appropriate culture conditions with lineage-specific GFs that direct the differentiation of MSCs into specific cell types [22-23]. They therefore play a key role in organogenesis and remodeling as well as in tissue repair [24]. These cells can reactivate synaptic connections by means of GFs, and can therefore enhance the formation of new functional conditions. Other positive aspects are the immunosoppressant function and the inhibition of pro-inflammatory cytokine release. Hence, autologous and allogeneic transplantation can be performed. Stem cell-based therapy offers a new hope for RP treatment as a replacement, restoration or regeneration strategy. However, ESC and iPCS have generated much controversy concerning in particular ethical, immunological and oncological issues.

Taking into account the risk of transplant rejection, whether autologous (ESC) or allogenic (iPSC), medical research has been thoroughly investigating the rapidly changing field of MSC [25,26].

As demonstrated by clinical and preclinical studies [21,27-30], MSC administration is associated with a significant restoration of the visual system through cell-mediated therapeutic mechanisms, that include:

- Cellular differentiation processes that are able to replace damaged or senescent cells.

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- Paracrine action for cell repair and regeneration.
- Modulation of host antioxidative, immunitary, anti-inflammatory responses at the inflamed site.
- Hemorheological regulation.

In addition, MSC administration does not require immunosuppression nor does it induce neoplastic transformation.

Recently, Limoli retinal restoration technique (LRRT) has been developed as a potential therapy for currently untreatable retinal disorders. This technique is a variant of Pelaez's intervention wherein only orbital autologous fat is transplanted in the subscleral space [31-33]. This technique exploits the use of GFs to create an environment conducive to the neuroenhancement of still functioning retina. This, in turn, leads to the preservation and improvement of visual functions [34,35]. The source of autologous GFs in LRRT is an implant of certain cell types of mesenchymal origin, such as adipose stromal cells, adipose tissue-derived stem cells (ADSCs) contained in the stromal vascular fraction of adipose tissue and platelets (PLTs) obtained from the PLT-rich plasma (PRP) prepared from fresh whole blood by centrifugation [31-33]. This specific triad is grafted in the sclera, above the choroid, in order to exert its beneficial effect on the residual retinal cells [31-33]. In this eye surgery technique, the distance between grafted autologous cells and choroid is reduced by means of deep sclerectomy and the contact area between the stalk and choroid is expanded to promote paracrine autologous cell secretion into the choroidal flow (Figure 1). The photoreceptors also receive mediated trophic action from potentially improved conditions of Müller cells, retinal pigment epithelium (RPE) cells and retinal microcirculation.

The primary aim of this retrospective, pilot clinical study was to evaluate whether autologous stem cell transplantation in patients with RP, according to LRRT surgery, may be useful for

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retinal restoration. In order to evaluate the prognosis of treated RP patients, we postulated that the larger the residual cell number is, the greater the interaction between GFs and the membrane receptors of chorioretinal cells, cellular activity and, ultimately, the improvement of visual performance. Furthermore, the secondary aim was to evaluate prognostic factors to identify the time and tests needed to allow appropriate surgical intervention in those affected by RP.

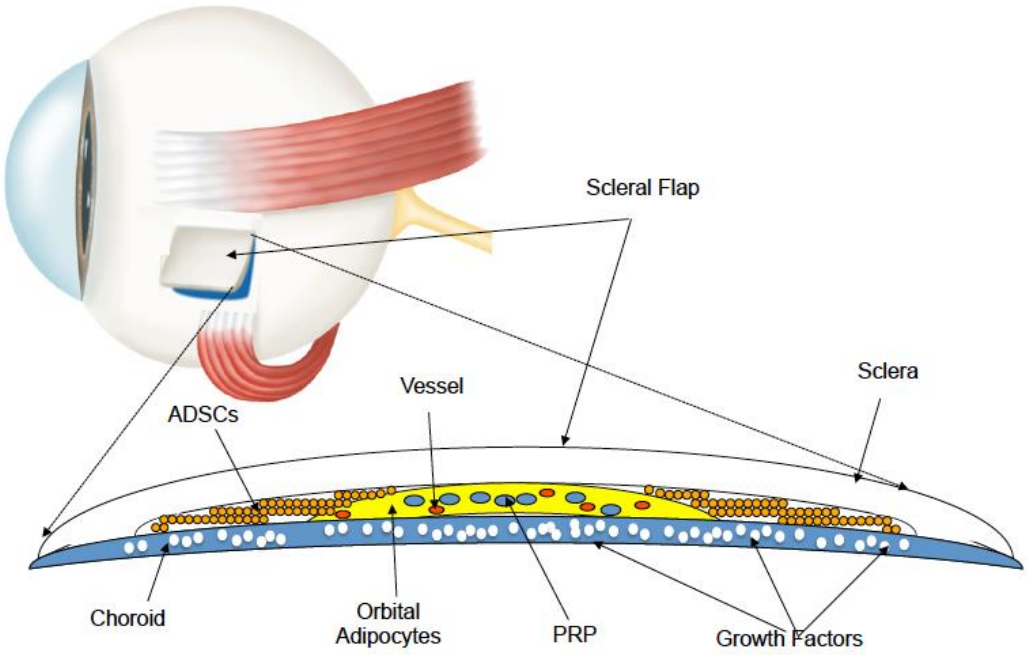

Figure 1. The suprachoroidal autograft obtained by Limoli Retinal Restoration Technique (LRRT) allows placing fat stromal cells, adipose tissue-derived stem cells (ADSCs) and platelets (PLTs), obtained from PLT-rich plasma (PRP), close to the choroid. The production of growth factors (GFs), typical of these cells, is poured directly into the choroidal flow, helping to maintain retinal cell trophism.

2. Results

A total of 21 eyes of 15 patients affected by RP, 9 males and 6 females (mean age 52.06 years

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±19.31 SD, range 19–86 years) were enrolled in the study (Table 1). The visual functional and anatomical parameters and the average values recorded at baseline (T0) and 6 months (T180) after surgery are shown in Table 2.

Table 1. Demographic data of retinitis pigmentosa (RP) patients with foveal thickness (FT) ≤ 190 µm or >190 µm.

Patients	**Group A FT ≤ 190 µm**	**Group B FT > 190 µm**	**Total**
Number/eyes	6/8	9/13	15/21
Age years (± SD)	40.33 (13.98)	59.88 (18.93)	52.06 (19.31)
Range	19-54	32-86	21-82
Female/Male	3/3	3/6	6/9
Eye: right/left	2/6	7/6	9/12

Table 2. Descriptive characteristics of analysed parameters in the two groups according to the foveal thickness (FT): A-FT ≤ 190 µm (n=8) and B-FT > 190 µm (n=13), at baseline (T0) and after 6 months (T180); mixed model results.

			Time			**Mixed model results**	
			T0	**T180**	**%**	**Time effect *p* value**	**Group effect *p* value**
logMAR	**A-FT ≤ 190 µ**	mean ± sd	1.02 ± 0.76	1.01 ± 0.77	+1.76		
		min-max	0.10-2.70	0.10-2.70			
	B-FT > 190 µ	mean ± sd	0.47 ± 0.21	0.45 ± 0.18	+4.51		
		min-max	0.15-0.70	0.15-0.79		0.562	0.051
Pts	**A-FT ≤ 190 µ**	mean ± sd	25.88 ± 20.29	26.13 ± 21.03	-0.97		
		min-max	8-64	7-64			
	B-FT > 190 µ	mean ± sd	15.15 ± 5.86	12 ± 4	+20.79		
		min-max	7-26	7-18		0.269	0.08
dB	**A-FT ≤**	mean ± sd	5.45 ± 6.8	6.29 ± 8.11	+15.41	0.003	0.535

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MAIA	**190 µ**	min-max	0-16	0-18.2			
	B-FT >	mean ± sd	3.15 ± 6.45	4.18 ± 7.79	+32.70		
	190 µ	min-max	0-19.4	0-21.8			
Cµ	**A-FT ≤**	mean ± sd	140.75 ± 37.42	133.88 ± 54.28	-0.05		
	190 µ	min-max	49-160	0-161			
	B-FT >	mean ± sd	275.46 ± 88.1	275.08 ± 89	0.00		
	190 µ	min-max	195-462	187-471		0.303	<0.001
μ^2	**A-FT ≤**	mean ± sd	7.03 ± 1.39	7.67 ± 0.45	+0.09		
	190 µ	min-max	4.6-8.7	7.3-8.6			
	B-FT >	mean ± sd	8.92 ± 1.38	8.79 ± 1.48	-0.01		
	190 µ	min-max	6.5-10.7	6.5-11		0.806	0.023
Aµ	**A-FT ≤**	mean ± sd	202.49 ± 23.4	212.86 ± 12.75	+0.05		
	190 µ	min-max	164.9-240	202-239			
	B-FT >	mean ± sd	247.62 ± 38.69	244.15 ± 40.4	-0.01		
	190 µ	min-max	179-299	181-305		0.949	0.023

LogMAR: logarithm of the minimum angle of resolution; Pts: close-up visus in points; Cµ: thickness of central fovea (in µm); Aµ: average of retinal thickness (in µm); dB: deciBel.

Based on foveal thickness (FT), 8 of the 21 eyes were classified in Group A (FT ≤ 190 µm) and the remaining 13 were classified in Group B (FT > 190 µm). All 15 patients completed the 6-month follow-up and none of them had systemic complications intra-operatively and post-operatively throughout that period. Mean values of the intraocular pressure recorded before and after surgery did not change significantly. The mixed model results showed a significant difference between the two groups in close-up visus in points (pts). Specifically, group A-FT ≤ 190 µ showed mean higher values than the group with > 190 µ (group effect p=0.031). While group B- FT > 190 µ showed significantly higher mean values than group A-FT in central fovea thickness (Cµ), μ^2 and average retinal thickness (Aµ) (Table 2). In all models, the interaction Time/Group had no significant effect (Table 3).

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Table 3. Variation between time at baseline and after 6 months (T180 and T0) estimated by mixed model in two groups according to the foveal thickness (FT): A-FT ≤ 190 µm (n=8) and B-FT > 190 µm (n=13).

Variation (T180-T0)		**A-FT ≤ 190 µ n=8**	**B-FT > 190 µ n=13**	**Interaction effect *p* value**
logMAR	mean ± sd	-0.02 ± 0.07	-0.02 ± 0.04	0.971
Pts	mean ± sd	0.25 ± 3.76	-3.15 ± 1.24	0.390
dBMAIA	mean ± sd	0.84 ± 0.59	1.02 ± 0.53	0.818
Cµ	mean ± sd	-6.88 ± 6.71	-0.38 ± 1.59	0.346
μ^2	mean ± sd	0.35 ± 0.37	-0.12 ± 0.18	0.248
Aµ	mean ± sd	5.66 ± 5.63	-3.46 ± 4.66	0.212

LogMAR: logarithm of the minimum angle of resolution; Pts: close-up visus in points; dB: decibel MAIA: microperimetry; Cµ: thickness of central fovea (in µm); Aµ: average of retinal thickness (in µm).

The ophthalmologic evaluation included the measurement of visual acuity for far and near distance: Best Corrected Visual Acuity (BCVA) measured by early treatment diabetic retinopathy study (ETDRS) charts at 4 meters in logarithm of the minimum angle of resolution (logMAR). Mean BCVA before the treatment was 1.02 ± 0.76 logMAR (20/200) in group A-FT ≤ 190 µ (n=8) and 0.47 ± 0.21 logMAR (20/200) in group B-FT > 190 µ (n=13). Specifically, BCVA in group A varied from 1.02 to 1.01 logMAR (+1.76%) and from 0.47 to 0.45 logMAR (+ 4.51%) in group B (Figure 2).

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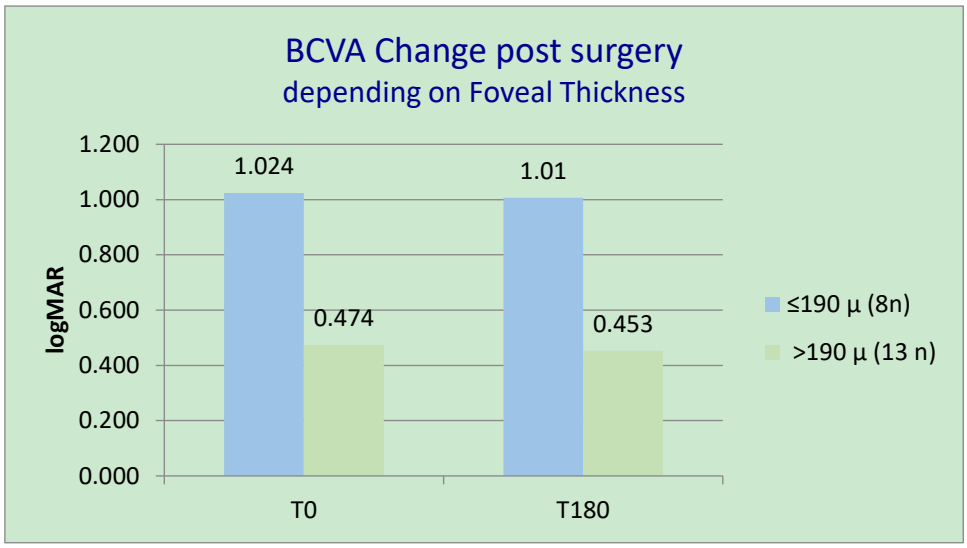

Figure 2. The best corrected visual acuity (BCVA) was stable after suprachoroidal autograft or increased (+4,51%) in patients with foveal thickness (FT) > 190 µm.

No patient showed a reduction in BCVA at 6-month follow-up. There was no statistically significant difference in visual acuity from baseline within the same group and between the two groups after 6 months (1.01 ± 0.77 *vs.* 0.45 ± 0.18, respectively). Percentage variation was lower in A (-1.76%) than in B (-4.43%).

Close-up visus in points (pts): At baseline, mean close-up visus was 25.88 pts ± 20.29 in group A-FT ≤ 190 µ (n=8) and 15.15± 5.86 pts in group B-FT > 190 µ (n=13).

At 6-month follow-up visit, it decreased to 26.13 pts in group A, whereas it increased to 12.00 pts in group B, showing that there was a trend towards significance in the latter group. Percentage variation was negative in A (-0.97%), conversely it was greatly increased (+20.79%) (Figure 3).

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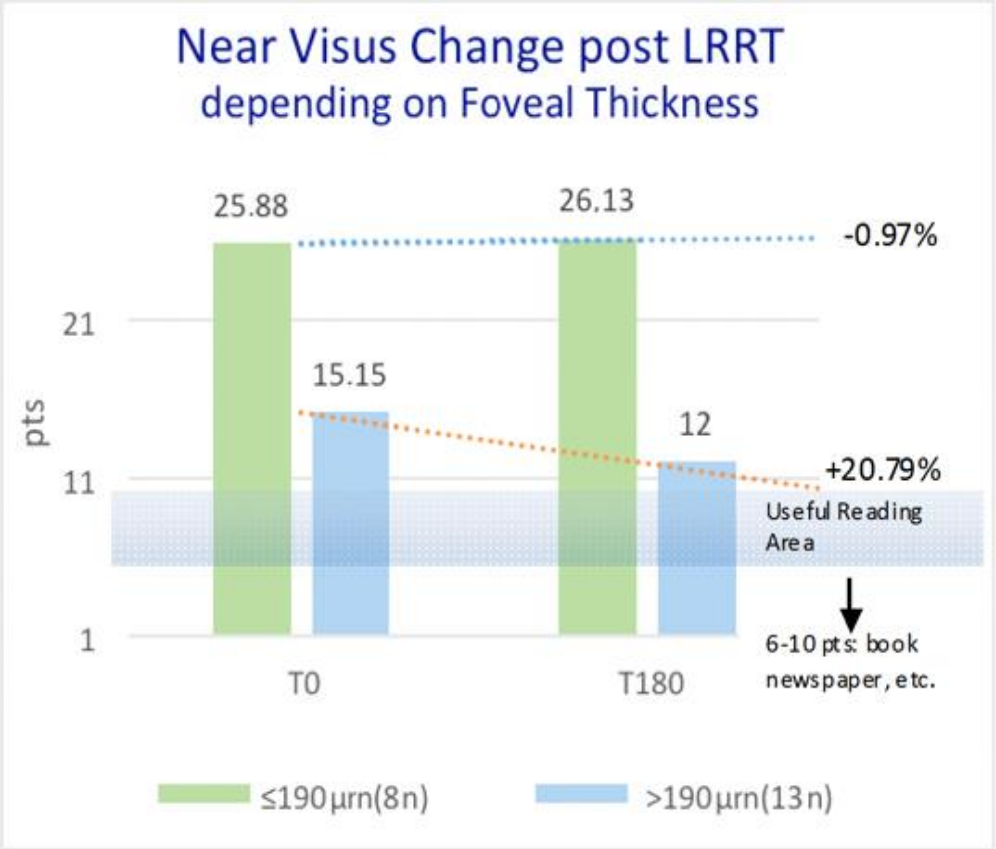

Figure 3. Residual close-up visus change post Limoli retinal restoration technique (LRRT) depending on foveal thickness (FT). Six months after surgery (T180), close-up visus was stable if FT was ≤ 190 µm, or increased by +20.79% in the group where FT was > 190 µm.

The average threshold sensitivity by microperimetry (MY) at baseline was 5.45 deciBel (dB) ± 6.8 in group A-FT ≤ 190 µ (n=8) and 3.15 ± 6.45 in group B-FT > 190 µ (n=13). In the 6-month follow-up it increased in both groups (6.29 ± 8.11 *vs.* 4.18 dB ± 7.79, respectively). Percentage improvement in retinal sensitivity was lower in group A (+15.41%) than in group B (+32.70). Despite the improvement in retinal sensitivity, it was not significant within the same group and between them (Figure 4-6).

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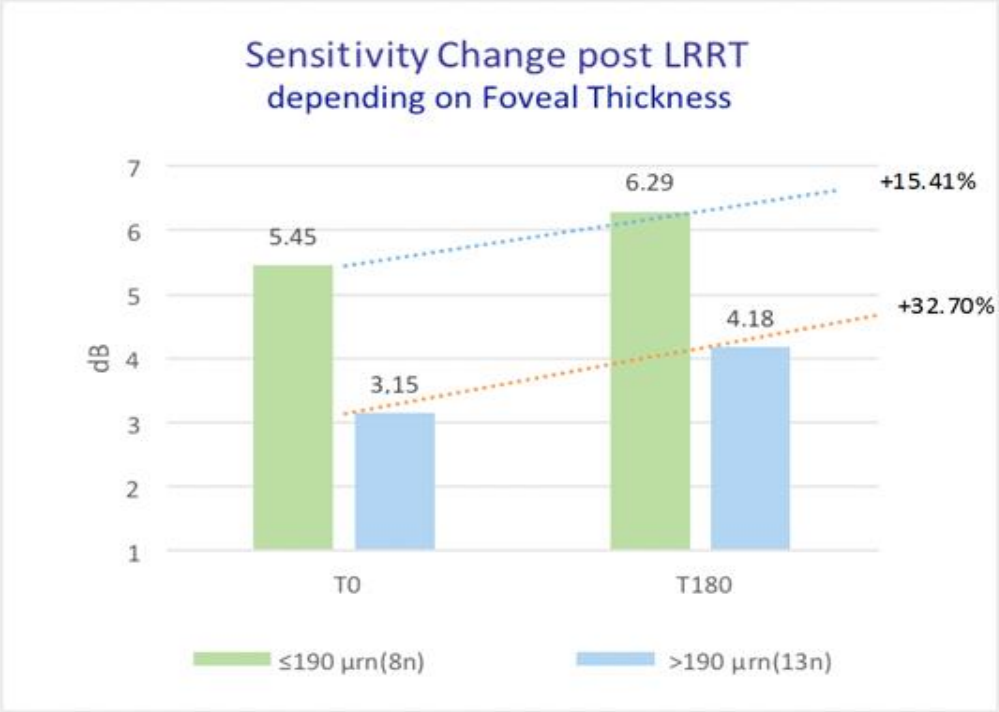
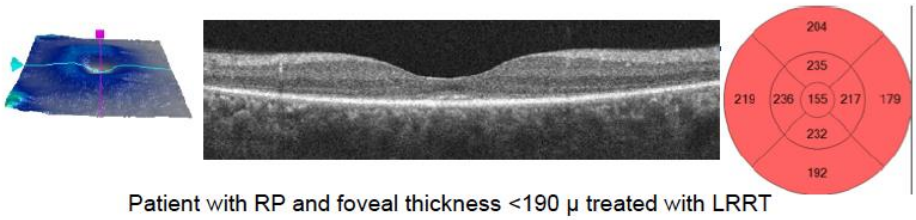
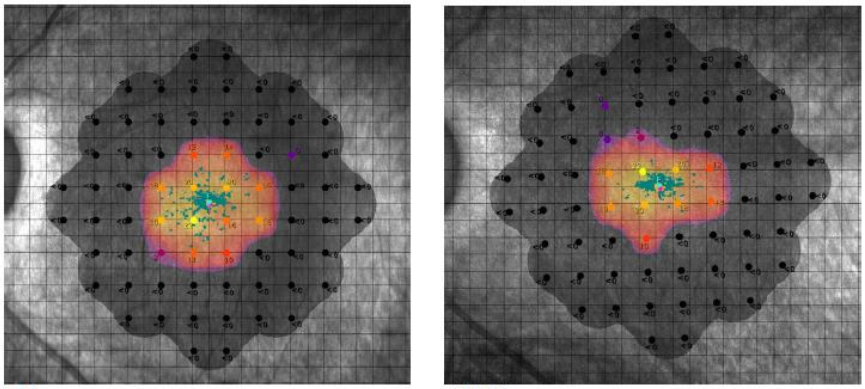


Figure 4. After Limoli Retinal Restoration Technique (LRRT), there was a more relevant change for sensitivity in the group with foveal thickness (FT) > 190 µm.



Patient with RP and foveal thickness <190 µ treated with LRRT



T0
0,097 logMar (0,8) 8 pts dB 2 cERG 3,97

T180
0,155 logMar (0,7) 8 pts dB 1,4 **cERG 25,24**

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Figure 5. A subtle foveal thickness (FT) <190 µm (Group A), as computed in our study, means that the retinal cell population is small, foveal structures are often dystrophic and the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex is no longer recognizable. Retinitis pigmentosa (RP). Limoli Retinal Restoration Technique (LRRT). Photopic electroretinogram (cERG).

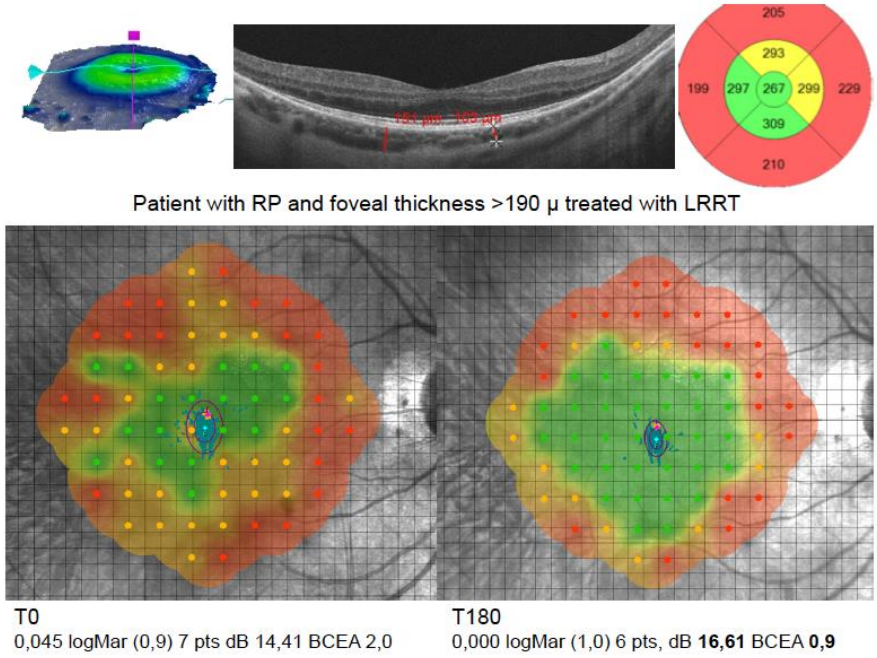

Figure 6. A considerable foveal thickness (FT) >190 µm (Group B), as computed in our study, means that the retinal cell population is still large, foveal structures are still intact and the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex is recognizable. Mesenchymal cell administration showed the ability to exert a positive influence over functional parameters six months after Limoli Retinal Restoration Technique (LRRT). Retinitis pigmentosa (RP). Bivariate contour ellipse area (*BCEA*) was used for fixation stability evaluation using *microperimetry* device.

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Surveying the subjective experience of all patients at 6 months post surgery with Patient Compliance Analysis, it was reported that visual performances increased in 15 eyes out of 21 (71.43%), were unvaried in 4 eyes (19.05%), and worsened in 2 eyes (9.52%) (Table 4).

Table 4. Compliance analysis after 6 months (T180) from the surgery in two groups according to the foveal thickness (FT): A-FT≤ 190 µm, and B-FT> 190 µm.

Compliance	**A-FT ≤ 190 µm n=8**		**B-FT > 190 µm n=13**	
Improved	4	50.00%	11	84.62%
Unchanged	3	37.50%	1	7.69%
Worse	1	12.50%	1	7.69%

However, examining patient feedback according to foveal thickness, the perception of improvement would be greater for patients with FT > 190 µ (11 eyes, 84.62%), rather than for patients with FT ≤190 µ (4 eyes, 50%) (Figure 7). If we considered only the improved group, 11 eyes (73.33%) belonged to group B and 4 (26.67%) to group A (Figure 7).

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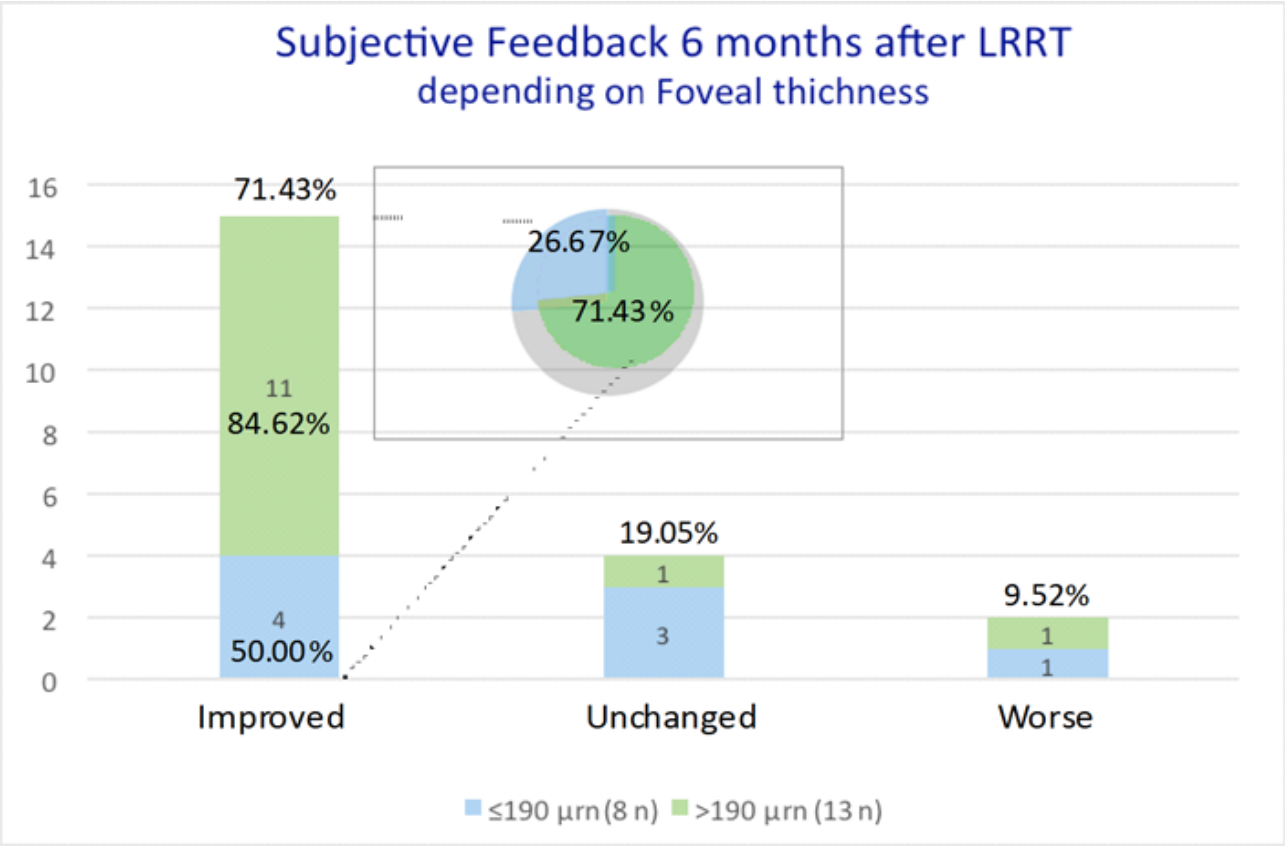

Figure 7. Retinitis pigmentosa (RP) patients compliance analysis post-Limoli Retinal Restoration Technique (LRRT) (T180) depending on foveal thickness (FT): the compliance was good in 71.43% of all cases (groups A and B). Patients reported to see better 6 months after LRRT, but the percentage reached 84.62% in those with FT > 190 µm. If we considered only the improved group, 11 eyes (73.33%) belonged to group B and 4 (26.67%) to group A.

3. Discussion

The main objectives of our suprachoroidal autograft technique, used for RP patients in this research, can be summarized as follows:

- Giving a regenerative start-up to the retinal cells, through the paracrine secretion of GFs in the retinal tissue;
- Promoting vascular pedicle fat engraftment with the underlying tissue;

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• Enhancing pedicle fat original vascularization to ensure its volume and survival.

LRRT cell therapy has been proven to have an impact on certain functional parameters after interaction with the residual cells. Close-up visus and retinal sensitivity improved in group B in which foveal thickness was greater, compared to A group where atrophy was greater and cellularity lower. Results of our study cast light upon the therapeutic potential of Cell-GF activity that therefore could be crucial for retinal degeneration. Given these findings, the group with a foveal thickness greater than 190 microns is associated with a better prognosis. While, in patients with thinner FT at OCT exam, the low cellular concentration could hinder the alleged beneficial interactions between GFs and membrane receptors. Hence, central thickness is an important parameter to understand the complex processes underlying RP progression. The surgical technique designed to graft three different types of autologous cells, which function as a natural reservoir of specific asset of GFs essential to the regenerative process and therefore exploited for regenerative medicine applications (Figure 8).

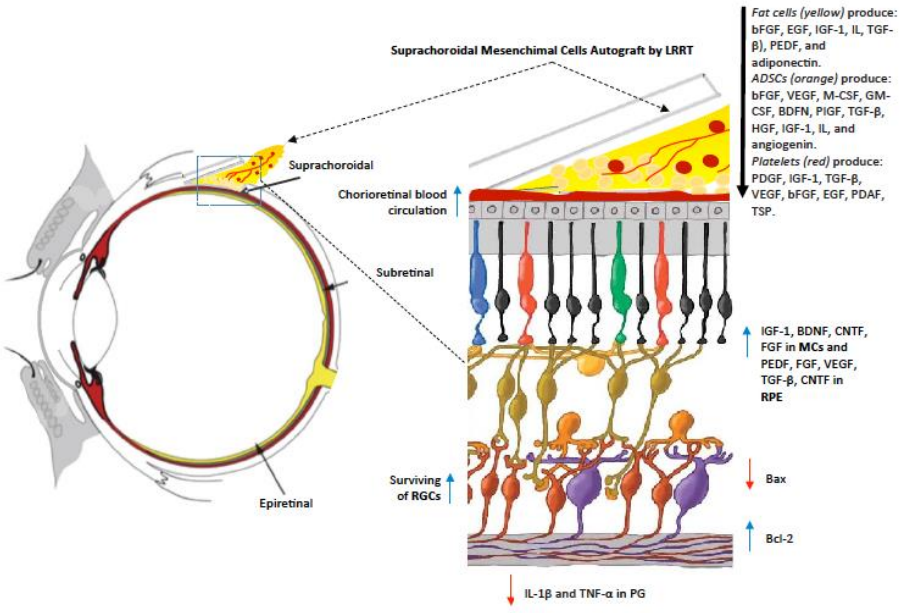

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Figure 8. A possible neuroprotective effect given by the incretion of growth factors (GFs) produced by mesenchymal cells implanted in the suprachoroidal space. These factors can act both directly on retinal cells, and indirectly, through the mediation of Müller cells (MCs) and retinal pigment epithelium (RPE), generating angiotrophic, neurotrophic, anti-inflammatory and antiapoptotic effects.

The myriad of bioactive factors released by the cell graft are as follows:

- Fat cells, which are contained in the pedicle grafted into the suprachoroidal space, produce basic fibroblast GF (bFGF), epidermal GF (EGF), insulin-like GF-1 (IGF-1), interleukin (IL), transforming GF (TGF), pigment-epithelium-derived factor (PEDF), and adiponectin [36-38].
- ADSCs produce bFGF, vascular endothelial GF (VEGF), macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), placental GF (PlGF), TGF, hepatocyte GF, IGF-1, IL, angiogenin, ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) [39,40].
- PLTs produce platelet-derived GF (PDGF), IGF-1, TGF, VEGF, bFGF, EGF, platelet-derived angiogenesis factor (PDAF), and thrombospondin (TSP) [41,42]).

Hence, the rationale behind this autograft lies in exploiting the stabilising effect exerted by cytokines and GFs released by the grafted cells. The direct contact of the autograft with the choroid enhances the incretion of these bioactive actors into the choroidal flow and consequently favors widespread dissemination thereof through the retinal tissue and in the vitreous body.

The binding of GF to its own specific receptor of the target cell is the initial step that triggers an intracellular signaling transduction cascade, activating second messangers. The latter can activate

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specific intracellular biochemical pathways generally by a series of phosphorylation events, with the ultimate aim of regulating enzyme activity or gene expression [43,44].

Notably, the activated transcription factors, entering the nucleus and binding directly or indirectly to DNA, regulate the expression of various genes with different mechanisms, promoting an increased synthesis of proteins including enzymes and cytokines [32].

The significance of GFs lies in their essential role of cell cycle regulation, since their presence triggers the cell transition from G_0 or quiescent phase to G_1 or growth phase, necessary to enter the cellular growth cycle. Moreover, they are also important for stimulating a wide range of cellular processes, including mitosis, cell survival, migration and cellular differentiation [45].

Mesenchymal cell graft into the sovrachoroidal space promotes a continuous incretion of GFs that are capable of interfering with the evolution of RP in several ways [46,47].

3. 1. Antioxidant activity

One cause of the deterioration of the cones is hyperoxia resulting in a more intense oxidation process and reactive oxygen species (ROS) formation. The mechanisms involved in hyperoxia are:

- Excessive amount of oxygen in the choroid that stems from the deterioration and death of the rods;
- Exposition of the foveal area to light;
- Concomitant lack of anti-oxidative enzymes, such as superoxide dismutase (SOD), glutathione peroxidase and catalase [48-50].

The bFGF and BDNF concentration within the photoreceptors has been shown to increase in response to stress in order to promote the retinal cell survival and to prevent the oxygen-induced photoreceptor cell death in the posterior retina. [51,52].

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Rod survival is essential for extending the life span of cones inasmuch as the paracrine secretion of rod cone viability factor (RdCVF) by rods is a pivotal trophic factor for cone survival [53,54]. It has been demonstrated that RdCVF has an antioxidant activity and decreases cone death in rd10 and P23H transgenic rat model [55].

3. 2. Antinflammatory activity

Another causal factor of apoptosis and death of photoreceptors is suggested by the triggering of an inflammatory microclimate that underpins the chronicity and progression of a vast number of neurodegenerative diseases, including RP. In fact, several studies have reported that the activation of microglia generally occurs simultaneously or just before the peak of apoptotic photoreceptor death in RP [56,57]. The eye is an immune-privileged organ and microglia and RPE cells are the frontline of retinal immune defense [58]. Not only does RPE perform a number of processes essential for retinal homeostasis and function, but RPE cells are capable of secreting a diversified panel of proinflammatory cytokines, e.g.: IL-6, IL-8, monocyte chemoattractant protein-1, interferon-β, as well as anti-inflammatory factors, such asIL-11 and TGF-β [59-61]. Furthermore, microglial cells normally exist in a quiescent state until they are activated by debris of dead or apoptic cells, lipopolysaccharides or ROS during the course of RP [62]. Upon activation, microglia adopt a larger, amoeboid morphology that is associated with enhanced functions of proliferation, motility and phagocytosis [63]. In addition, they express a unique set of proinflammatory cytokines and chemokines and they yield neurotoxic agents including hydrogen peroxide and superoxide anion [64,65]. Microglia can still return to the resting state, if the activating stimuli is removed promptly, otherwise the persistence of the stimuli makes the microglia bind to the neuronal surface and produce continuously the above factors, thus leading to the death of neuron cells.

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Cellular debris originating from endangered neurons together with the unceasing production of such factors display the ability to activate further microglial cells and promote their chemotactic recruitment and hence favor the chronicization of neuroinflammation [62].

Inhibition of microglia activation by minocycline or SC-560 in an rd10 mouse model of RP decreases microglia-mediated photoreceptor death. Therapeutic treatments that aim to prevent photoreceptor cell loss can therefore exploit the suppression of microglial activation [66].

Intravitreal administration of MSC has shown to have a remarkable effect on the host immune response by suppressing pro-inflammatory cytokine production, such as INF and tumor necrosis factor (TNF) through IL-1 receptor antagonist, and prostagandin E2 receptor activation [37].

Another study carried out by Guadagni et al. [17] has shown that a microenvironment supplemented with GFs can slow down the genetically determined photoreceptor death, concurrently reducing retinal inflammation, and thereby establish framework conditions for the viability of the overall cell population.

The MSC therapeutic effect has been also corroborated by the neurotrophic action of CNTF and BDNF. In culture of retinal ganglion cells (RGCs), under oxidative stress conditions, MSC excrete the latter factor that contributes towards reducing proinflammatory cytokine release, e.g. TNF-α and IL-1 [28]. Moreover, M-CSF, GM-CSF and IL exert an anti-inflammatory function and recruit macrophages by chemotaxis, thus contributing to remove intraretinal cell debris [67-69].

In fact, under defined experimental conditions a consistent increase in the number of macrophages in the aftermath of bFGF intravitreal injection has been reported [70].

3. 3. Antiapoptotic activity

Stress stimuli like those from GFs and toxic substances can induce the initiation of intracellular apoptotic signaling. Excess generation of ROS causes damage to membrane lipoproteins and

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cellular DNA thus leading to apoptosis and photoreceptor death [71]. Furthermore, since RPE and Müller cells are known to release GFs into the retinal citosol, their progressive loss hinders the incretion of bioactive agents, whose anti-apoptotic action is therefore prevented. Indeed, RPE cells produce a wide heterogeneity of factors, i.e., Fibroblast GFs (FGF-1, FGF-2 e FGF-5) [72,73], TGF-β [74], IGF-1 [75], CNTF [76], PDGF [77], VEGF [78], certain members of the IL family [79], and PEDF [79]. Mesenchymal cell administration can interfere with the apoptotic process involved in retinal degeneration, most notably in RP.

The GFs excreted by grafted mesenchymal cells perform a variety of functions; in particular, they can facilitate Bcl-2 gene expression in order to avoid the unrelenting cell death, regardless of any cause [21].

Bcl-2 family proteins are most notable for their regulation of apoptosis by interacting with caspases, a family of protease enzymes containing cystein aspartate-specific proteinasis [80]. More specifically, the process is orchestrated by regulatory cytokines by either inhibiting (anti-apoptotic) or inducing apoptosis (pro-apoptotic) by blocking inhibitory mediators [80,81].

In addition, cell apoptosis is triggered by the aggregation of Fas-associated proteins with death domain, and the ensuing formation of the pro-caspase 8 binding site [82]. This event induces the subsequent activation of caspase 8 [83]. The latter, in turn, cleaves and activates caspases 3, which play an essential role in the execution phase of apoptosis [84,85].

Retinal Müller glia cells play a central role in triggering intraretinal signalling pathways that regulate retinal trophism [86] and enhance photoreceptor survival by neurotrophic factors [87]. Other functions of Müller cells are the regulation of the secretion of neuronal trophic substances and the removal of end products originating from neuronal metabolism [88]; the conversion of glucose into lactose, i.e., the preferred energy substrate for the oxidative metabolism of photoreceptors [89]; the stimulation of survival capacity in photoreceptors and ganglion cells

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[90]. RP progression and the depletion of the different cell types involved exacerbate residual cell death. The latter process can be avoided or at least delayed by the anti-apoptotic activation of the Bcl-2 gene induced by GFs derived from implanted mesenchymal cells. Basically, these factors replace those that should have been produced by retinal cells, which are quantitatively reduced and functionally impaired due to RP.

MSC exert a further therapeutic effect by secreting neurotrophic factors and amongst them IL-6, which can additionally promote MSC migration to the site of injury [91].

IL-6 activates the phosphatidyl-inositol-3-kinase/Akt signalling pathway [92]. The phosporilated Akt in turn activates the inhibitor complexed with the X-linked inhibitor of the apoptosis protein and the latter phosporilation finally inhibits caspase 3 activity. The multitude of the above bioactive factors released entails an extensive trophic action on surrounding structures [93].

3. 4. Citoprotective activity

It has been determined that GFs are involved in neuroprotection by regulating photoreceptor metabolic activity, which is physiologically intense and thoroughly impaired during RP.

The high metabolic demand for oxygen and glucose is met by RPE cells through choroidal vascularization. Oxidative phosphorylation, which from glycolysis ends with the ATP production required for neuronal function, is ensured by mitochondria. For this to happen, the mTOR signalling pathway needs to be triggered by the binding of the IGF, released at high levels from the autograft, to the insulin receptor, integrating insulin effects (Figure 9).

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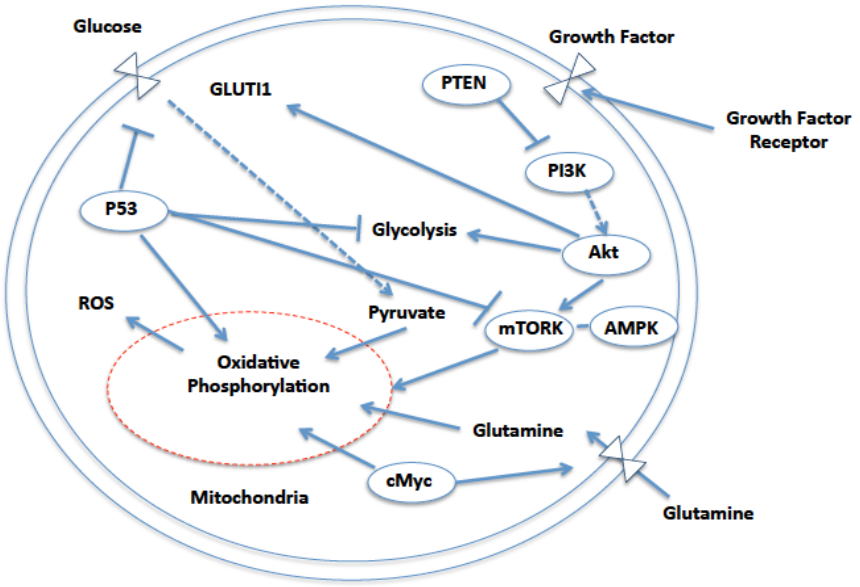

Figure 9. Metabolic signaling regulation. Glucose uptake in cells is regulated by Glut1 transporters that increase glycolysis and oxidative phosphorylation. This is enhanced by cMyc regulation of glutamine uptake in cells. Growth factors (GFs) (in particular, Insulin Like Factor) influence metabolism through activation of the PI3K/AKT/mTOR signaling pathway and contribute to increased glycolysis and oxidative phosphorylation. This is inhibited through PTEN. Under glucose limiting conditions, AMPK is activated inhibiting the mTOR signaling pathway. In addition, p53 activaiton inhibits glycolysis and the mTOR pathway but increases oxidative phosphorylation. ROS increases through inefficient oxidative phosphorylation in the mitochondria.

The subsequent phosphorylation, due to its oxidative nature, generates ROS as byproducts. ROS clearance is mediated by the concerted action of endogenous antioxidant enzymes including SOD, glutathione peroxidases and catalases [94,95].

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In addition, IGF facilitates DNA and RNA synthesis as well as numerical and dimensional cell increase. IGF is also involved in the regolation of neuronal growth and development through a variety of processes, i.e., neurogenesis, myelination, synaptogenesis, dendritic ramification, and neuroprotection as a consequence of neuronal injury. Finally, IGF promotes neuronal connections and inhibits neuronal death. Given these findings, IGF holds much potential especially in the treatment of neurodegenerative diseases [96].

In rat models with inherited retinal dystrophy, it has been shown that MCS contributes to visual function by the putative paracrine release of trophic cytokines that promote the clearance of dysmetabolic products of photoreceptors by RPE phagocytes [35].

MSC transplant has also been found to reduce damage to the retinal photoreceptor outer segment layer either by promoting cell regeneration through the paracrine release of hypoxia-inducible factor-1, or axonal regeneration through growth-associated protein-43 [97].

Data from another similar study provide evidence that neurotrophic factors, i.e., NGF, bFGF, and glial derived neurotrophic factor released by adipose tissue-derived MSCs, are involved both in maintaining the survival of RGCs and in reducing oxidative stress-related retinal damage [98].

Like bFGF, PEDF has been found to exert a neurotrophic action, inducing the overall survival of photoreceptors [99]. There are currently significant data suggesting that certain factors such as EGF and VEGF play an important role in enhancing the neuroprotective action of Müller cells, stimulating their intracellular transcription and expression of bFGF [100,101]. In addition, evidence has been building up that EGF is able to induce ADSC secretory activity [102].

VEGF released by PRP has been shown to stimulate the proliferation of ADSCs that hence promote the survival of grafted autologous fat and adipocytes [103]; bFGF is known to promote directly the survival of photoreceptors by binding the target receptors on their surface [104,105].

3. 5. Hemorheological activity

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In recent years, emphasis has been placed on retinal and choroidal vascolarization due to its involvement in the pathogenesis and progression of RP. Indeed, retinal blood flow appears to lack autonomic innervation, but is mainly controlled through an efficient autoregolatory process mediated by the local and endothelial release of vasoactive products. The progressive photoreceptor loss that occurs in RP has been identified as cause of microvascular dysfunction due to the release of cellular waste products secondary to apoptosis. In this case as well, the ensuing altered perfusion may end up in a vicious circle leading to the final loss of photoreceptors [106]. The choroid is a plexus of blood vessels, in which capillaries are more permeable than those of the microcirculation, and the choroidal blood flow is about fifty times greater than the retinal one. Blood flows through the choroid at high speed and delivers oxygen at a similar arterial content to veins. Unlike the retinal capillaries, the choroidal capillaries allow plasmatic proteins to quickly reach photoreceptors in order to meet their metabolic requirements. Decreased choroidal blood flow is now known to induce dysfunction of visual sensitivity [107]. Research publications across different study settings support that blood flow is decreased in RP. By proper monitoring of intraocular pressure (IOP), Langham and Kramer highlighted the association between choroidal ischemia and visual loss as well as RPE cell degeneration in RP patients [108]. Beutelspacher et al. proved that retinal blood flow is lower in RP patients than the control group, thus concluding that the ensuing reduction of retinal vessels is a typical feature of RP [109]. Turksever et al. demonstrated that retinal oxygen uptake in RP patients is decreased, having found increased venous oxygen saturation in the case group [110]. Ayton et al. showed that RP patients had a thinner choroid than the control group and observed that those patients were characterised by reduced visual acuity, thereby assuming that the choroidal thickness in RP can be a potential predictor of the therapeutic otucome [111].

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Finally, decreased macular blood flow may be associated with reduced visual sensitivity in RP patients [112]. Several factors, such as VEGF, bFGF, angiogenin, PDAF, PlGF, PDGF, EGF and TGF-β have been shown to promote endothelial regeneration and may therefore contribute to choriocapillaris reperfusion [113,114]. Moreover, others, including TSP and PEDF, inhibit neovascular processes [115,116].

Hence, the rationale for using PRP as a therapeutic tool lies in the belief that PLTs, through the ocular release of several different GFs, can subsequently enhance and speed up the natural healing process if applied directly to the surgical wound and can influence the success of cell engraftment [41]. PLTs, primarily known for their contribution to hemostatis, are also able to release factors that promote tissue repair and regeneration [5] and angiogenesis. In addition, they influence the course of inflammation and apoptosis [117], stimulate cell migration and adhesion in angiogenesis sites, and enhance the differentiation process of endothelial progenitor cells into mature endothelial cells [41]. In so doing, they play a key role in the formation or restoration of an adequate vascolar network.

PRP acts as a trigger for the early development of a new capillary plexus, facilitating oxygen and nutrient diffusion towards the grafted cells [113]. Subretinal injection of PRP in a neonatal mouse model has shown to promote the establishment of a denser vascular network [41]. PRP stimulates the survival of the grafted ADSCs [114]. A 40% diluted lipoaspirate combined with PRP enhances the proliferation ability of ADSCs by promoting a proper distribution of fat cells as well as a better growth of adipose tissue, favours intercellular interactions, stimulates ADSC differentiation, and exerts an anti-inflammatory activity locally [118]. It is considered that not only PLTs, but also MSCs have a manifold and regenerative potential for retinal vascularization. With respect to these findings, grafted cells have been extensively studied to promote the restoration of effective retinal perfusion.

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4. Materials and Methods

The tenets of the Declaration of Helsinki were observed, and written informed consent approval by the Ethics Committee of the Low Vision Academy was obtained. In this study, 15 patients with RP were included if they had:

- Clinical diagnosis of RP based on a history of night blindness, visual field constriction, abnormalities on ERG testing and specific ophthalmoscopic findings;
- Age ranging from 19 to 86 years;
- Normal intraocular pressure;
- Visual acuity for near (close-up) vision between 7 and 64 pts in order to avoid difficult evaluations for both low visus (> 64 pts) and normal visus (6 pts);
- Transparent lens;
- Acceptance of the clinical protocol by signing the informed consent.

The exclusion criteria were the following:

- Myopia or hypermetropy with spherical equivalent ≥ 6 diopters;
- Keratitis, keratoconos, cataract, cystoid macular edema, chorioretinal and neovascular membrane, macular pucker, uveitis, etc.;
- Other ocular disorders, such as glaucoma, optic neuritis, ocular trauma, high refractive errors, etc.;
- Insufficient compliance in individuals affected by medical conditions, such as Parkinson's disease, diabetes mellitus, hypertension, vasculitis, hypovitaminoses, multiple sclerosis, epilepsy, renal and hepatic diseases, gastrointestinal malabsorption, hypothyroidism, malignant neoplasias, and other systemic acute or chronic diseases.

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The ophthalmologic evaluation included the measurement of visual acuity for far and near distance: BCVA measured by ETDRS charts at 4 meters in logMAR units and close-up visus (pts); slit-lamp biomicroscopy with and without dilatation; applanation tonometry; and fundus examination.

All eyes recruited in the study cohort were retrospectively divided into two groups. The division was based on FT measured with spectral domain–OCT (SD-OCT). For this purpose, a cut-off ≤ 190 μm was used. Consequently, the subjects with FT ≤ 190 μm were included in group A, whereas subjects with FT > 190 μm were included in group B. At baseline (T0) and 6 months after surgery (T180), the ophthalmologic evaluation and the following exams were performed on each patient: SD-OCT, using the Cirrus 5000 SD-OCT (Carl Zeiss Meditec AG, Jena, Germany); MY using Maia 100809 (CenterVue S.p.A., Padua, Italy); ERG test recorded with ocular electrophysiology electromedical system, Retimax (C.S.O. S.r.l., Scandicci, Italy) according to the standards set in 2009 by the International Society for Clinical Electrophysiology of Vision (ISCEV) [7]. Comprehensive ophthalmic examination and LRRT surgery [31-33] were carried out for all patients by a single retinal specialist (PGL).

4. 1. Statistical analysis

Data were presented as mean ± standard deviation (SD); also minimum and maximum (min-max) values were reported. Mixed regression models with robust errors were applied to analyse the difference between the two groups (A-FT ≤ 190 μ, B-FT > 190 μ) at the two moments (at T0 and T180 months) considering that two eyes could be observed for one patient (patient as random effect). Also, the effect of the interaction between the group and time was evaluated.

A p-value <0.05 was considered statistically significant. All statistical analyses were performed by STATA v14 (Collage Station, Texas, USA).

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5. Conclusions

Obviously, the major limitation of our study was its lack of molecular characterisation of the sample considered. The study population was relatively small and the follow-up period was short, so further studies will be needed. However, the results of this study show that FT could be considered as a prognostic criterion for RP patients undergoing treatment by LRRT. Genetic diagnosis will surely become more relevant in coming years and it will be possible to ascertain the impact of MCS administration on different genetic groups of RP patients.Therefore, we can conclude that, despite the heterogeneity of the recruited subjects and the lack of molecular diagnostics, the transplant of autologous cytocomponents of mesenchymal origin placed into the suprachoroidal space could sustain retinal neuroenhancement especially in patients with adequate FT.

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Abbreviations

ADSCs: Adipose tissue-derived stem cells

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Aμ: Average of retinal thickness in micron

BCVA: Best corrected visual acuity

BDNF: Brain-derived neurotrophic factor

bFGF: Basic fibroblast growth factor

Cμ: Thickness of central fovea in micron

CNTF: Ciliary neurotrophic factor

dB: DeciBel

EGF: Epidermal growth factor

ERG: Electroretinogram

ESCs: Embryonic stem cells

ETDRS: Early treatment diabetic retinopathy study charts at 4 meters in logMAR

FT: Foveal thickness

GF: Growth factor

GM-CSF: Granulocyte-macrophage colony-stimulating factor

IGF-1: Insulin-like growth factor-1

IL: Interleukin

logMAR: logarithm of the minimum angle of resolution

iPSCs: Induced pluripotent stem cells

LRRT: Limoli retinal restoration technique

M-CSF: Macrophage colony-stimulating factor

MSCs: Mesenchimal stem cells

PDAF: Platelet-derived angiogenesis factor

PDGF: Platelet-derived growth factor

PEDF: Pigment-epithelium-derived factor

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PGE2R: Prostagandin E2 receptor

PlGF: Placental growth factor

PLTs: Platelets

PRP: Platelets rich plasma

pts: Points or print size

RdCVF: Rod cone viability factor

RGC: Retinal ganglion cell

ROS: Reactive oxigen species

RP: Retinitis pigmentosa

RPE: Retinal pigment epithelium

SD-OCT: Spectral domain–optical coherence tomography

SOD: Superoxide dismutase

TGF: Transforming growth factor

TNF-α: Tumor necrosis factor–α

TSP: Thrombospondin

VEGF: Vascular endothelial growth factor

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