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SAFETY AND EFFICACY OF SUBTHRESHOLD LASER IN THE TREATMENT OF RETICULAR PSEUDODRUSEN SECONDARY TO AGE-RELATED MACULAR DEGENERATION

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This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.

All the results presented here were obtained by myself, except for:

1. Genetic analyses (Results) were performed by the laboratory of Dott. G. Zerbini, San Raffaele Scientific Institute, Milan, Italy.

Part of this thesis was yet published during the last year of my PhD program. In detail,

- The results of the first pilot study PASCAL (Results, Section "Short-term safety and efficacy of SLT"; Material and Methods, Section "Short-term safety and efficacy of SLT") were published by myself in the following publication: "Querques, G., Sacconi, R. (co-first author), Gelormini, F., Borrelli, E., Prascina, F., Zucchiatti, I., Querques, L., & Bandello, F. (2021). Subthreshold laser treatment for reticular pseudodrusen secondary to age-related macular degeneration. Scientific reports, 11(1), 2193. https://doi.org/10.1038/s41598-021-81810-7";

- The methodology of the second clinical trial PASCAL (Material and Methods, Section "Long-term safety and efficacy of SLT treating the whole macula") was yet registered and published on the ClinicalTrial.gov (ID NCT04847635; link https://www.clinicaltrials.gov/ct2/results?cond=&term=NCT04847635&cntry=&state=&city=&dist=).

All sources of information are acknowledged by means of reference.

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Abstract

Background. Age-related macular degeneration (AMD) is a leading cause of visual decline in the elderly. Reticular pseudodrusen (RPD) represent a landmark of early/intermediate stages of AMD and they are associated with a higher risk of the AMD progression into geographic atrophy (GA) and/or neovascular AMD. To date, no effective treatments are available for the treatment of RPD.

Purpose. The aim of this thesis is to evaluate the safety and effectiveness of subthreshold laser treatment in patients with RPD secondary to AMD.

Methods. In the first pilot clinical trial, in order to test the safety, 20 eyes (20 patients) with RPD were prospectively enrolled and treated in an extrafoveal area with subthreshold laser treatment (PASCAL Synthesis 577nm). The patients were evaluated also at 1 month and 3 months after laser treatment. In the second multicentric, randomized, prospective clinical trial, 50 eyes (50 patients) with RPD were enrolled in 4 centers and randomized in the treated or sham group. Patients underwent a panmacular subthreshold laser treatment every 3 months for 12 months (long-term follow-up).

Results. No topical and/or systemic side effects were disclosed during both studies. Considering the retinal sensitivity (MS) of the whole macular area and of the treated area in the pilot study, no significant changes were reported ($p=0.152$). A trend of increase in MS was disclosed in the treated group in the long-term follow-up in opposite to the sham group. Interestingly, analyzing the efficacy outcomes using optical coherence tomography, the distribution of RPD among the RPD stages changed after the treatment. This data was suggested by the pilot-study and confirmed in the preliminary data of long-term follow-up. In detail, we observed a significant increase in Stage 1 RPD during the follow-up, associated with a significant reduction of Stage 3 RPD. Furthermore, we also observed a significant thickening of the outer nuclear layer during the follow-up in the pilot study ($p=0.001$), and no significant changes in long-term follow-up.

Conclusions. We reported the safety of subthreshold Pascal Synthesis 577nm in the treatment of RPD secondary to intermediate AMD. The results of the thesis also suggested that subthreshold laser treatment could induce a RPD anatomical regression and, for this reason, may reduce the rate of progression of RPD to late AMD.

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ACRONYMS AND ABBREVIATIONS

AMD: age-related macular degeneration.

BAF: blue autofluorescence.

BCVA: best-corrected visual acuity.

BLamD: Basal laminar Deposit in AMD.

CFP: color fundus photography.

ChT: choroidal thickness.

CMT: central macular thickness.

CNV: choroidal neovascularization.

EZ: ellipsoid zone.

FA: fluorescein angiography.

FAF: fundus autofluorescence.

GA: geographic atrophy.

ICGA: indocyanine green angiography.

IOP: intraocular pressure.

IR: infrared reflectance.

MNV: macular neovascularization.

MS: macular sensitivity.

OCT: optical coherence tomography.

OCTA: optical coherence tomography angiography.

ONL: outer nuclear layer.

PCV: polypoidal choroidal vasculopathy

PD: perfusion density.

PDT: photodynamic therapy.

PED: pigment epithelium detachment.

PEDF: pigment epithelium derived factor.

PHP: preferential hyperacuity perimeter.

PlGF: placental growth factor.

RAP: retinal angiomatous proliferation.

RPD: reticular pseudodrusen.

RPE: retinal pigment epithelium.

SDD: Subretinal Drusenois Deposits.

VEGF: vascular endothelial growth factor.

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INTRODUCTION

Age-related Macular Degeneration (AMD)

The macula is an area located in the middle of the posterior pole between, inside the retinal arcades. The diameter of the macula is 5-6 mm, and is responsible for the visual field of about central 20°. From an histological point of view, while the peripheral retina only shows a single layer of ganglion cells, the macula has more than one layer. Its inner layers are characterized by the presence of xanthophyll carotenoid pigments lutein and zeaxanthin, which are yellow, in much greater quantity than in the retinal out of the macula. For this reason, the full name of the macula is "macula lutea", that represents a yellow plaque.

Age-related macular degeneration is a progressive disorder affecting the macula. It is generally more common in older patients and results in an impairment of central vision caused by damages of the photoreceptor, retinal pigment epithelium (RPE), and choroidal complex. Visual loss occurs for the most part in the advance stages of the disorder which are classified into two different entities: neovascular AMD (or "wet" AMD) and geographic atrophy (GA) (or "dry" AMD). Neovascular AMD is characterized by the development of a choroidal neovascularization (CNV). CNV is localized in the neural retina, leading to various pathogenetic mechanisms such as: fluid leaking, lipids accumulation, hemorrhage, and ultimately to fibrous scarring. On the contrary, GA is characterized by progressive RPE atrophy, choriocapillaris, and photoreceptors.(Lim *et al*, 2012a)

Prevalence and Incidence

In a meta-analysis(Smith *et al*, 2001) the early-stage AMD was prevalent in 6-8% of white population of 40 years old or more, and late-stage AMD 1.5%. Meaning that almost 1.75 million people are affected in the US.

Data of prevalence in other ethnic populations have also been described. Late-stage AMD is up to 10 times more predominant in white than in black patients.(Friedman *et al*,

1999) Whereas, no differences were found in occurrence of late AMD between Asian and Caucasian people.(Kawasaki *et al*, 2010)

There is also indication that Asian people may present different neovascularization patterns, in fact many are affected by neovascular AMD present polypoidal dilation of the choroidal vessels, known as polypoidal choroidal vasculopathy (PCV). This form can account for up to 50% of neovascular AMD processes in Asians, but it accounts for only 8–13% in Caucasians.(Laude *et al*, 2010) Another variation of neovascularization is knowns as retinal angiomatous proliferation (RAP), accounting for 12–15% of neovascular AMD.(Gupta *et al*, 2010) These variants may have limited response to standard management of neovascular AMD.

To date, there are not many studies about the incidence of AMD. An American study described a 14.3% 15 years cumulative incidence for early-stage and 3.1% for late-stage AMD in adults aged 43–86 years old.(Klein *et al*, 2007) An Australian study showed similar data in adults older than 48 years.(Joachim *et al*, 2015) A 4-year incidence of AMD of 2.5% has been reported analyzing an Hispanics population older than 40 years.(Choudhury *et al*, 2011) In the same age group a Japanese study(Yasuda *et al*, 2009), reported a 10% of 9-year cumulative incidence for early-stage and 1.4% for late-stage AMD.

Given its incidence and the impact on the lives of the people affected, AMD is a great and relevant public health problem. There is a severe impact of AMD on the health system, compounding the public health dilemma. Initial AMD decreases the patient's quality of life (QUALYs) about 17% (similar to that seen in patients with moderate cardiac angina or acquired immunodeficiency syndrome), intermediate AMD decreases QUALYs about 32% (comparable to severe cardiac angina or femoral head fracture), severe AMD decreases QUALYs about approximately 53% (higher than renal hemodialysis) and finally very severe AMD decrease QUALYs about 60% (similar to end-stage cancer of prostate).(Brown *et al*, 2005) AMD is also responsible for a great economic burden, the total US annual cost has been estimated to be US$575–733 million, only accounting for direct costs.(Rein *et al*, 2006)

Risk factors

Various aetiological factors have been described for AMD in a complex interaction, involving polygenic, style of life and environmental factors(Bowling, 2016):

- Age: older age represents a greater risk factor. Indeed, analyzing people greater than 80 years old, more than 10% are affected by AMD(Smith *et al*, 2001);

- Genetic predisposition: various studies on families of people with AMD have produced evidence for a strong role of genetics in the development of the disease. Several genetic loci were linked to AMD, including 2 major loci in the complement factor H (*CFH*) gene, located on 1q32 and the *ARMS2/HTRA1* locus on the gene cluster 10q26. Complement pathway appears to be heavily involved in the pathogenesis of this disease as many related genes, as *C2*, *CFB*, *C2,* and *CFI* have been linked to this disease.(Edwards *et al*, 2005)·(Maller *et al*, 2007)·(Fagerness *et al*, 2009) Large genome-wide association studies, have shown association between HDL cholesterol pathway genes and AMD; in this pathway were implicated *LIPC, CETP*, *ABCA1, LPL,* and *APOE*.(Reynolds *et al*, 2010)·(Chen *et al*, 2010)·(Neale *et al*, 2010)(Mckay *et al*, 2011) Various genes of the collagen matrix and extracellular matrix pathway, *COL10A1, COL8A1* and *TIMP3* were linked with AMD.(Neale *et al*, 2010)·(Chen *et al*, 2010)·(Yu *et al*, 2011) Lastly, angiogenesis pathway genes (*VEGFA*) were associated to AMD in two AMD genome-wide association meta-analysis.(Yu *et al*, 2011) Not only is genetics associated with the development of the disease but early evidence suggests it can also influence a patient's response to treatments;

- Gender: Female sex is believed to be a risk factor, although this relationship has not been well established(Smith *et al*, 2001);

- Smoking: smoker activity was reported as a AMD risk factor (Chakravarthy *et al*, 2010; Khan *et al*, 2006; Seddon *et al*, 2006a; Fraser-Bell *et al*, 2006; Klein *et al*, 2008a);

- Cardiovascular risk factors: have a strong association with this disease.(Reynolds *et al*, 2010; Chakravarthy *et al*, 2010) People with AMD have a greater probability of developing cardiovascular disease and stroke.(Snow & Seddon, 2003)

- Cataract surgery: data is inconclusive as a meta-analysis(Chakravarthy *et al*, 2010) previously suggested that cataract surgery could be considered as a risk factor,(Chakravarthy *et al*, 2010) but this association was not confirmed when explored in a RCT(Chew *et al*, 2009);

- Iris colour: a darker iris pigmentation is protective(Chakravarthy *et al*, 2010);

- Body Mass Index: there is an adverse effect of being overweight/obese and the risk of late AMD(Chakravarthy *et al*, 2010);

- Sunlight exposure(Mitchell *et al*, 1998);

- Aspirin: Recent observational studies have linked aspirin use and AMD. A study reported a doubling incidence rate of late-stage AMD in subjects that used aspirin two times or more in a week for at least 10 years compared with subjects that did not.(Klein *et al*, 2012) Other studies have instead linked the use of aspirin to a lower risk of developing AMD.(Christen *et al*, 2001) In a meta-analysis including over 171,000 patients, aspirin was not shown to cause an increase of incidence of AMD.(Wang & Zhang, 2014) Given this body of evidence, patients who are taking aspirin should not discontinue their therapy;

- Biological Markers: there is an association between serum triglycerides and late AMD(Chakravarthy *et al*, 2010);

- Diet: low intake in the alimentation of vitamins (especially A, C and E), omega-3 fatty acids, zinc, and lutein. (Lim *et al*, 2012b);

Pathogenesis

The pathogenesis of AMD is thought to be triggered by various biological factors: such as senescence, demonstrated by lipofuscin accumulation in RPE, choroidal ischaemia, and oxidative damage.(Ding *et al*, 2009) However, although our understanding AMD pathophysiology has improved in recent years, the exact mechanisms of AMD are still relatively poorly understood. RPE/photoreceptor/Bruch's membrane complex has been the object of intensive research. The RPE is cellular layer with high metabolic activity that phagocytoses outer segments shed by the photoreceptors and continuously recycles and processes the materials used for the function of photoreceptors, thus supporting the function of the retinal photoreceptor. As these cells grow older, they tend to pile up residual bodies that contain lipofuscin, a process which we are able to investigate thanks to autofluorescence imaging. (Okubo *et al*, 1999) (Von Rückmann *et al*, 1997) Another function of these cells is to release material that is eliminated by the choriocapillaris; nevertheless, RPE cells disfunction and alterations of

Bruch's membrane permeability may cause the material accumulation between the RPE cells and the Bruch's membrane leading to genesis of drusen. Reduced clearance of such material may also play a role as various authors have reported a thinner choriocapillaris in patients affected by AMD.

Not only are drusen a mark of RPE malfunction but they are believed to be at the basis of RPE cells loss and, as a consequence, loss of photoreceptors. The progressive impairment of the RPE could lead to progressive degeneration of the Bruch's membrane, its rupture, together with increased VEGF could be at the basis of the growth of new neovessels starting from the choriocapillaris underneath the RPE. In fact, the accumulation of drusen and subsequent alteration of exchanges of metabolisms between the RPE and the choriocapillaris have been proposed as a mechanism for neovascular AMD. The following hypoxia stimulates the upregulation of VEGF-A and other factors of angiogenesis that lead to proliferation of new-vessels.(Stefánsson *et al*, 2011) These vessels, before evolving into a scar, go through a period of leakage and occasional bleeding; the formation of the macular fibrosis leads to a great impairment of central vision. Anomalous lipid exchanges of the RPE has also been theorized as a cause for drusen formation.(Fliesler & Bretillon, 2010) Similarly, these deposits seem to resemble atherosclerotic plaques in composition.(MacHalińska *et al*, 2012)

Data suggests that pathogenetic models should also take into account factors such as: oxidative stress, complement activation, local inflammation, and lipid homeostasis.(Gemenetzi & Lotery, 2016),(Stanton & Wright, 2014),(Pikuleva & Curcio, 2014) For all these reasons, the pathogenesis remain unclear.

Due to its role as therapeutic targets, the functions of VEGF was well studied in the last years; it is a key promotor of angiogenesis, and reduction in its function leads to halting the growth of neovessels and their regression. (Grisanti & Tatar, 2008) (Kondo *et al*, 1993) It was proven that the expression of VEGF could induce neovascularization of the choroid in animals. In humans, there are 4 differend isoforms of VEGF: VEGF-121, VEGF-165, VEGF-189, and VEGF-206, each one with different half-lives, the former being able to be present for more than 14 days, while the second is shortlived.(Rakic *et al*, 2003) This difference may account for the reduced efficacy of pegaptanib. Pegaptanib is a drug able to target only the isoform VEGF-165, while ranibizumab or bevacizumab (other anti-VEGF drugs) are able to target all isoforms. For this reason, pegaptanib is less

successful in treating CNV than ranibizumab or bevacizumab. Other potential therapeutic targets have been studied, such as endostatin, matrix metalloproteinases, and pigment epithelium-derived factor.(Grisanti & Tatar, 2008)

Classifications

Generally speaking, AMD has been classified into two different subtypes(Bowling, 2016):

1- "Dry" AMD (also known as non-exudative) is the most frequent form, accounting for up to 90% of patients. The advanced stage of dry AMD has been named Geographic Atrophy (GA); nonetheless, it has been suggested from several experts that the term 'dry AMD' should be used to refer to GA (one of advanced stages of AMD) and not to early and intermediate stages of the disease.

2- "Wet" AMD (also known as exudative or neovascular form) is less prevalent, nevertheless it is faster in the disease progression and it causes faster significant vision loss. The mark of wet AMD is the presence of choroidal neovascularization (CNV) in the sub-RPE space (Type 1) or in the sub-retinal space (Type 2). However, at least two additional conditions, RAP (or Type 3 MNV) and PCV have been added to the group of neovascular form of AMD by many authors.(Bowling, 2016)

Various AMD classifications, which are relevant for both research and clinical purposes, have been developed.(Seddon *et al*, 2006b),(Eye *et al*, 2015),(Spaide, 2017)

The majority of AMD classifications are based on color fundus photographs but to date there is no consensus of a unique classification, including a precise definition of the diagnosis and staging of different AMD phenotype. Of course, this is very relevant in both clinical or research settings.(Ferris *et al*, 2013)

The Age-Related Eye Disease Study (AREDS)(Eye *et al*, 2015) (Figure 1), in 2001, classified age-related macular degeneration into four categories:

- Category 1: no or a few small drusen (defined as drusen less than 63 μm in diameter), and visual acuity of 20/32 or better in both eyes;

- Category 2: are included patients with one or more of the following:

- Numerous small drusen
- Few intermediate drusen (defined as drusen from 63 to 124 μm in diameter)
- RPE abnormalities
- Visual acuity of 20/32 or better in both eyes

- Category 3: are included patients with one or more of the following:

numerous intermediate drusen

one or more large drusen (defined as drusen greater than 124 μm in diameter)

geographic atrophy sparing the foveal area

- Category 4: two scenario are possible. The first one is characterized by the presence of GA involving the foveal area. The second one is characterized by the presence of a CNV.

Grade	Largest Drusen Size	Drusen Area	Increased Pigment	Depigmentation	Geographic Atrophy	Predominance of Soft Indistinct Drusen
0	None	None, Q,* or <C-0	None	None	None	None
1	Questionable*	≥C-0, <C-1	Questionable*	Questionable*	Questionable*	Questionable*
2	<C-0	≥C-1, <C-2	<C-0	<I-2	<I-2	Present, not predominant
3	≥C-0, <C-1	≥C-2, <I-2	≥C-0, <C-1	≥I-2, <O-2	≥I-2, <O-2	Predominant in 1 of 3 zones
4	≥C-1, <C-2	≥I-2, <O-2	≥C-1, <C-2	≥O-2, <0.5 DA	≥O-2, <0.5 DA	2 of 3 zones
5	≥C-2	≥O-2, <0.5 DA	≥C-2, <O-2	≥0.5, <1.0 DA	≥0.5, <1.0 DA	3 of 3 zones
6	NA	≥0.5, <1.0 DA	≥O-2	≥1.0, <2.0 DA	≥1.0, <2.0 DA	NA
7	NA	≥1.0 DA	Unrelated to AMD	≥2.0 DA	≥2.0 DA	NA
8	Cannot grade	Cannot grade	Cannot grade	Cannot grade	Cannot grade	Cannot grade

Abbreviations: AMD, age-related macular degeneration; AREDS, Age-Related Eye Disease Study; DA, disc area; NA, not applicable.
*The "Questionable" category is chosen when the grader is at least 50%, but not 90%, sure that the abnormality is present.

Figure 1. AREDS grading scale.

The Figure illustrated the grading scale of AREDS classification. (ref. Davis, M. D., et al (2005). The Age-Related Eye Disease Study severity scale for age-related macular degeneration: AREDS Report No. 17. Archives of ophthalmology, 123(11), 1484–1498).

According to the AREDS study, the risk at 5 years of evolving late-stage AMD in one or both eyes was 1.3% in patients of category 2, 18.3% in those in category 3, 43.9% in those in category 4.(Eye *et al*, 2015)

Subsequently, in 2012, *Frederick L. Ferris et al.* developed another five stages clinical classification for AMD(Ferris *et al*, 2013) (Figure 2):

- Stage 1: no apparent aging changes
- Stage 2: Normal aging changes
- Stage 3: Early AMD
- Stage 4: Intermediate AMD

- Stage 5: Advanced AMD

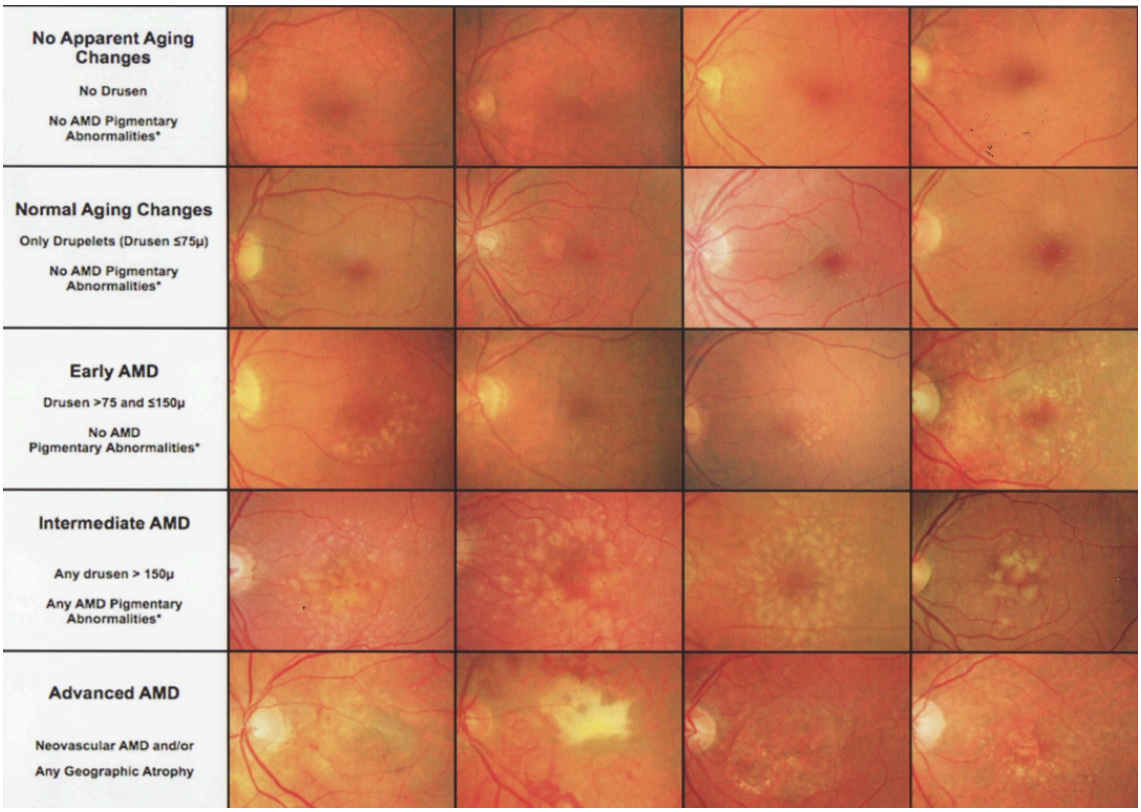

Figure 2. Ferris et al.'s classification of age-related macular degeneration.
(ref. Ferris, F. L., et al (2013). Clinical classification of age-related macular degeneration. Ophthalmology, 120(4), 844–851.)

The aim of this classification is to highlight two relevant topics:

- The size of drusen, that is a crucial parameter. There is the evidence that drupelets (i.e. drusen smaller 63 micron in diameter) was believed to represent a small risk of evolution to late AMD. This could mean that the drupelets presence only is not sufficient for the growth of large drusen and for the development of pigment abnormalities. Other factors are needed to set the development of the clinically dangerous phenotype in motion.

- Pigmentary abnormalities inside a distance of two disc-diameters starting from the fovea, if accompanied by medium drusen (greater than 63 but smaller than 125 micron), is linked with a higher risk to progression to late-stage disease. For this reason, these eyes are classified as early AMD, even if no large drusen (bigger than 125 micron) is present.(Ferris *et al*, 2013)

At last, in 2017, Spaide et al. expanded the concept of AMD classification(Spaide, 2017). The main feature of early AMD is proposed to be the accumulation Subretinal Drusenois Deposits (SDD). SDD are peculiar extracellular deposit in the form of drusen. Late-stage disease is instead divided into of Geographical atrophy or neovascularization. This classification has a prognostic value. SDD eyes are inclined to develop atrophy, especially if characterized by confluent SDD. However, also macular neovascularization (MNV) (a new conied term to refer to previously described CNV), and especially Type 3 MNV, are usually linked with SDD. Indeed, the regression of SDD can cause atrophy of the outer retina, which can then develop the atrophy of the RPE cells.

In that paper, Spaide et al. also differentiate the entity of pachydrusen from the "classic" drusen. Pachydrusen are drusen present in eyes with thick choroids, and are linked to the presence of Type 1 MNV or PCV. Another difference between pachydrusen and drusen is the rate of progression to atrophy, Pachydrusen usually are not associated to atrophy development. On the other hand, "classic" drusen could regress causing atrophy development. (Spaide, 2017)

Sign and symptoms

AMD is characterized by the following signs:

- Drusen. As explained before, this entity is characterized by extracellular deposits below the RPE (between the RPE and the Bruch's membrane). Their composition varies depending on the size and it entails many substances; they are thought to be generated as a result of metabolic and immune-mediated processes in the pigment epithelium. Their exact function in the pathogenesis of AMD has not been clearly established, but correlates with the diameter of the lesions and with the presence/absence of pigment anomalies. Drusen are uncommon before 40 years of age, but tend to appear more frequently after the fifth decade. There is great variability in their distribution, as they may be limited to the fovea, or they might surrond it or form a band around the periphery of the macula. Drusen could be also be located in the periphery and mid-periphery fundus(Bowling, 2016);

- Pigmentary abnormalities;
- Atrophy areas;
- Choroidal NeoVascularization (CNV) or Macular NeoVascularization (MNV);
- Retinal pigment epithelial detachment (PED);
- Haemorrhages;
- Hard exudates (lipid) within the macula, and not caused by other vascular diseases of the retina;
- Epiretinal, intraretinal, subretinal or sub-RPE fibrosis;
- Retinochoroidal anatastamosis and RAP (or type 3 MNV);
- Retinal pigment epithelial tear(Bowling, 2016).

Recognizing symptoms which may be caused by AMD should be a priority and clinicians would refer the patient to a retinal specialist for a comprehensive evaluation and for eventually need and timing of therapy. In a people with good visual acuity and who are older than 55 years, worrying symptoms include:

- Progressively or suddenly decreased vision, which cannot be corrected
- Central field defect
- Metamorphopsia, micropsia, or macropsia
- Difficulties in carrying out daily life activities(García-Layana *et al*, 2017)

One of the key symptom to assess in the diagnostic work-up of AMD is metamorphopsia. This is a fundamental finding characterizing patients affected by various maculopathies. This sign is easily evaluated using a fast test, the Amsler grid test, or M-charts.(Simunovic, 2015)·(Nowomiejska *et al*, 2013) In the preferential hyperacuity perimeter (PHP) test, there is a single straight dotted line with a few dots out of alignment across different macular loci, and the subject could easily touch the screen to indicated the locus of distorted line, the software then proceeds to show a map reporting the intensity of metamorphosia and the area of such distortion. Although PHP has been shown to be superior in AMD patients to the Amsler grid,(Loewenstein, 2005) they have similar performance in the screening of neovascular AMD.(Faes *et al*, 2014) Nevertheless, PHP is very expensive, and it is not applicated to all patients with AMD. Contrary, the macular

mapping test (*MacuFlow*)(Frisén, 2009) cannot be universally used as a screening tool because, although it can be used at home, the cooperation of a individual with good VA and without cognitive impairment is required. Considering the limitations of other tests, the joint use of near vision and reading stereotypes with the Amsler grid has been demonstrated to be an useful tool for AMD patients to early self-diagnose the neovascular form.(Díaz-Llopis *et al*, 2010)

Below will be analyzed the five clinical stages of AMD(Ferris *et al*, 2013) (Ferris et al.) and the respective symptoms and signs.

- Normal aging changes

The presence of only small drusen (i.e. drupelets) characterizes changes related to the normal aging.

Drupelets, also named 'hard' drusen, are well-defined, small (less than 63 micron) uniformly white–yellow deposits located under the RPE.(Bowling, 2016)(Kassoff *et al*, 2001b)(Bird *et al*, 1995) These are drusenoid deposits which can be classified a normal consequence of age,(Rudolf *et al*, 2008) though AMD frequently begins with this type of deposits.

In a recent study, Rudolf et al divided drusen into categories based on their histological appearance(Rudolf *et al*, 2008). Hard drusen are classified as round deposits, with very sharp borders filled with homogeneous hyalinised material. Hard drusen are much more compact in comparison to soft drusen, even if both are eosinophillic, amorphous, and express PAS-positivity. As opposed to other types of deposit, hard drusen are associated with a RPE cell loss. Hard drusen that are located in the center of the macular area are larger, associated with a thicker Basal laminar Deposit in AMD (BLamD) and had a more heterogenous substructure with more amyloid assemblies in comparison to hard drusen located in the periphery.(Khan *et al*, 2016) Moreover, hard drusen located in the macular area are usually calcific earlier in comparison to hard drusen of the periphery.(Ozaki *et al*, 1999)(Osusky *et al*, 1997)(Anderson *et al*, 1999)

Usually, hard drusen are seen as small sub-RPE deposits, hyperreflective on Optical Coherent Tomography. (Figure 3)

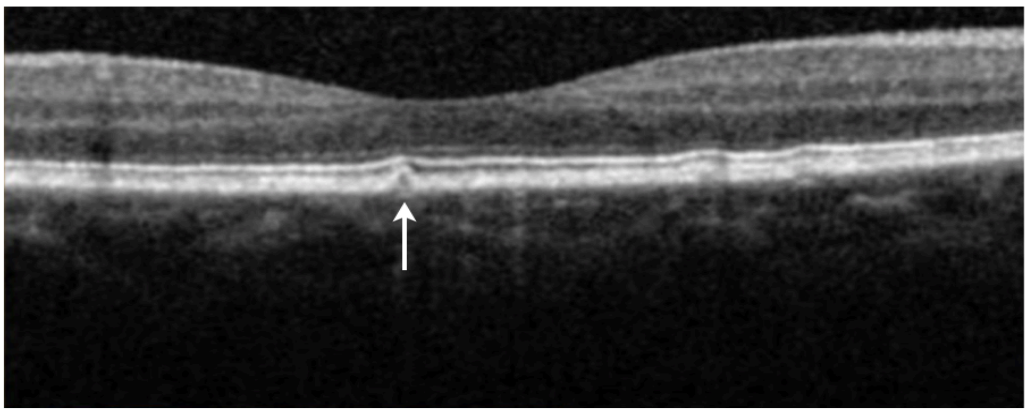

Figure 3. Hard druse visualization on SD-OCT.

- Early AMD

Early AMD is not linked to visual loss or other symptoms, but the evidence of medium-sized drusen (greater than 63 and smaller than 125 μm) allows the diagnosis to be made.

- Intermediate AMD

The intermediate stage of the disease is characterized by development of large drusen drusen (diameter of 125 μm or more), abnormalities of the retinal pigment, or both. This type of drusen is generally located inside the vascular arcade, more often in the central macula. Furthermore, they have a predisposition for the superior and temporal quadrants of the macula.(Wang *et al*, 1996) The appearance of large drusen is less demarcated than the hard phenotype and their central portion may seem whiter than their yellow edge.(Spaide & Curcio, 2010) Soft drusen are composed by a a relevant percentage of esterified cholesterol and they have been proposed to have a more cholesterol less lipoprotein than hard drusen.(Malek *et al*, 2003) It has been speculated that if there is the presence of drusen causing a splitting of the RPE to the Bruch's membrane, this could cause the development of choroidal neovessels within this cleavage plane, explaining the link between the presence of soft drusen and the higher risk to develop advanced-stage AMD.

When observed on Optical Coherence Tomography they appear as as moderately hyperreflective sub-RPE deposits (Figure 4).

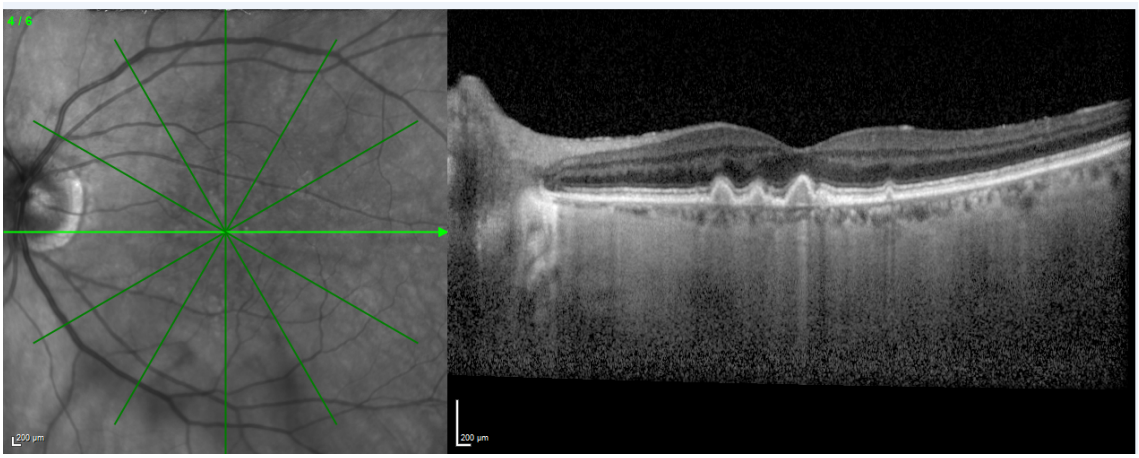

Figure 4. Soft drusen visualization on SD-OCT

The presence of drusen alters the function of Bruch's membrane as a barrier, and reduces the function of the RPE cells in transporting waste products through the Bruch's membrane. Patients with early- or intermediate stage disease tend to have a choriocapillaris of reduced density, and signs of inflammation inside the outer retina (i.e. signs of activated microglia cells).

Although people who develop intermediate AMD have a higher risk of progressing to both forms of late-stage AMD, they are usually asymptomatic.

Advanced AMD

Late AMD usually causes a central vision damage which is due to a damage to the macula. This stage is represented by two clinical forms:

Geographic atrophy (GA) (also named 'dry' or 'non-exudative' AMD) shows a progressive and loss of three different components linked each-other: the RPE, photoreceptor cells, and the choriocapillaris. The development of such damage leads to a reduced visual function.

Symptoms are generally characterized by a gradual impairment of vision and usually both eyes experience damage, although the damage could be asymmetric. Vision may oscillate, but it is generally improved in bright light.

Generally speaking signs follow this progression: development of several large drusen with a confluence of the drusen themselves; alterations (hypo- or hyper- pigmentation) of the RPE cells; development of localized atrophy of the RPE, that could be associated with the regression of drusen; enlargement and confluence of atrophic areas. In this stage, larger choroidal vessels may become visible at fundus examination. Furthermore, rarely CNV may develop in an area of GA).

Neovascular AMD (also named 'exudative' or 'wet' AMD) is characterized by the presence of Choroidal Neovascularization, in which immature vessels grow and invade the retina starting from the choroid. Given the immature nature of these vessels they tend to be leaky and lead to accumulation of fluid and blood under the retina, ultimately causing detachment of the retina or RPE and scarring. This process can cause vision loss that can be very rapid and evolve within weeks or even days if not treated.

The pathogenesis of CNV has not been completely understood. Any insult to the Bruch membrane or the RPE can be a cause for CNV, in fact, this process can be considered as a phisiological response to a damage to the RPE. The development of CNV is thought to be regulated by a balance of angiogenic and antiangiogenic molecules: vascular endothelium growth factor (VEGF) is an established pro-angiogenic factor involved in CNV, whereas pigment epithelium derived factor (PEDF), was shown to inhibit ocular neovascularization. VEGF upregulation is known to occur due to various stimuli, such as high glucose and protein kinase c activation , hypoxia, reactive oxygen species, advanced glycation end products, activated oncogenes, and several types of cytokines, although the precise cause for its increase in CNV hasn't been established yet. Histopathologic specimens show the presence of VEGF in CNV; in addition, many authors were able to overexpress VEGF thus inducing CNV formation. VEGF activates various signal transduction pathways by binding to its tyrosine kinase receptors, usually expressed on endothelial cells, these pathways lead to increase the permeability of the vessels, the proliferation of the endothelial cells, and the migration of cells. The final result is the development of a neovascular network.

The neovascular network could growth in the sub-RPE space (Gass type 1 or occult CNV) (Figure 5) or upper to the RPE in the subretinal space (Gass type 2 or Classic CNV). Especially during the growth of neovessels, bleeding and exudation occur, resulting in visual symptoms for the patients.

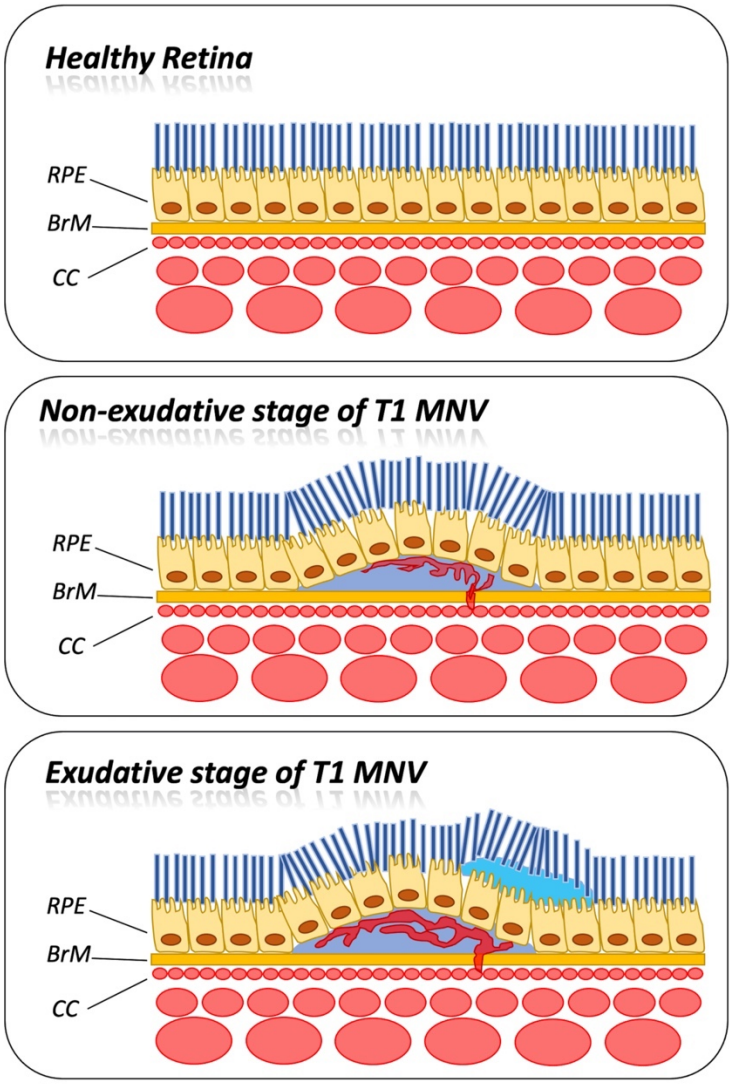

Figure 5. Schematic representation of the different stages characterizing the development of type 1 macular neovascularization.
BrM: Bruch's membrane; CC: choriocapillaris; RPE: retinal pigment epithelium.

There is another subtype of neovascularization, namely RAP, or type 3 MNV. In this case, the abnormal neovessels originate from the retinal circulation, especially from the deep capillary plexus, and growth down to the RPE (Figure 6). (Freund *et al*, 2008)

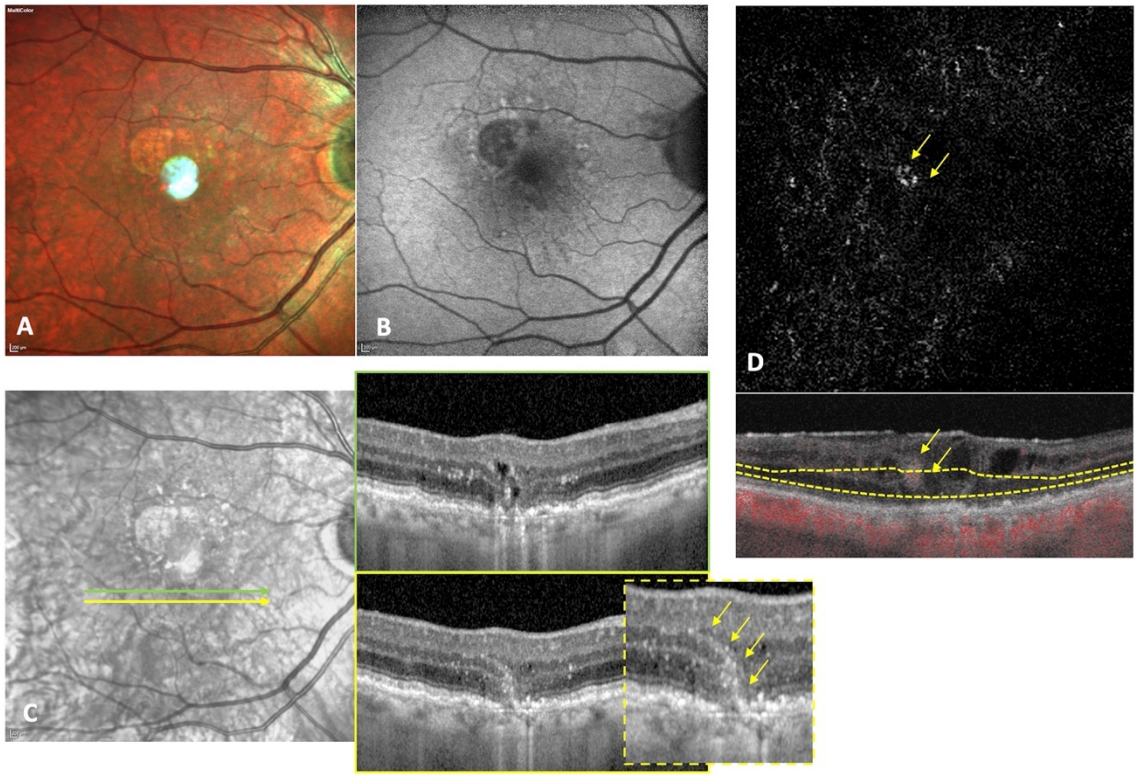

Figure 6. Multimodal imaging evaluation of a 80-year-old patient affected by type 3 macular neovascularization (MNV) in the right eye.

Multicolor image (A) and short-wave fundus autofluorescence (B) showing the presence of an atrophic area, drusen, and retinal pigment epithelium (RPE) alterations. Combined infrared reflectance and horizontal structural optical coherence tomography (OCT) showing the presence of intraretinal cysts with increase in retinal thickness (upper structural OCT, passing through the green arrow), and the presence of hyperreflective lesion growing from the inner plexiform layer to the RPE (lower structural OCT, passing through the yellow arrow). En-face OCT-angiography of the avascular slab and b-scan with flow showing the presence of flow inside the hyperreflective lesion detected by structural OCT, confirming the diagnosis of type 3 MNV.

While type 1 NV and 2 NV originate from the choroidal circulation, the vast majority of type 3 lesions originate from the retinal vascularization.(Jung *et al*, 2014; Chen *et al*, 2016)·(Su *et al*, 2016)

Symptoms consists of painless blurring of vision which can be acute or subacute, usually associated with metamorphopsia. Haemorrhage can also cause a positive scotoma.

Signs may vary: the CNV itself can be seen as a grey–green or pinkish-yellow lesion; medium and large drusen are a typical finding in the same or contralateral eye;intra and

subretinal lipid deposition (sometimes extensive); localized subretinal fluid; haemorrhage is common; different kinds of PED (serous, drusenooid, fibrovascular, or haemorrhagic); subretinal fibrosis in an evolved or treated lesion.

Diagnostic Workup

Retinal imaging and clinical examination are a fundamental part of patient management and are essential to make a diagnosis and to monitor response to therapy.

In fact the eye specialist may request a series of painless useful exams wich will reveal if there is any damage or alterations in your eyes:

- Fundoscopy or ophthalmoscopy: a light source allows to examine the fundus of the eye
- Colour Fundus Photographs
- Optical Coherence Tomography (OCT): this is technique based on laser rays to take photographs of your retina and therefore measure and map its thickness
- Autofluorescence: autofluorescence is a technique used to determine the area affected by RPE alterations and/or atrophy
- Fluorescein Angiography (FA) and IndoCyanine Green Angiography (ICGA): still today they represent the gold standard for the diagnosis and classification of CNV.(Do *et al*, 2012) Angiographs are dynamic tests that show the filling of retinal and choroidal vessels, both physiological and pathological, with possible phenomena of hyperpermeability (leakage), accumulation (pooling), and impregnation (staining) of tissues
- Optical Coherence Tomography Angiography (OCT-A)

Fundoscopy or ophthalmoscopy

The slit lamp biomicroscopy is a key part of ophthalmologic examination. This tool is able to investigate the condition of fundus and can confirm the presence of AMD. Biomicroscopy is suitable to detect drusen, pseudodrusen, pigment abnormalities, atrophy and hemorrhages.

Colour Fundus Photographs

Fundus photography reports on a two-dimensional support the three-dimensional retinal structures, particularly the macula, using a fundus camera. Localisation of the pathological process is possible thanks to the stereoscopic images, which are taken at 35 degrees of the posterior pole using reflected Red Green Blue (RGB) spectrum and light sensor.

Optical Coherence Tomography

OCT is a fast and non-invasive technology that allows acquisition of high resolution cross-sectional (tomographic) images of the neurosensory retina and deeper structures.(Schmitt, 1999) It functions by the measurement of the reflected light waves that is scattered by different tissue, allowing evaluation and assessment of anatomical changes associated with different retinal diseases (similar to the mechanism of ultrasonography).

OCT is based on a near infrared (820 nm) coherent radiation to scan the eye. Given the short wavelength, this technique can create images with higher resolution than those of ultrasonography.

However, the use of light gives some technical challenges, which have to be overcome to produce a viable image. For example, the light speed is very high and thus measuring optical ‘‘echoes’’ is not possible, this has been resolved by using a technique named interferometry, where a light beam is split into two rays, the measuring and the reference beam, respectively.(Keane *et al*, 2012) The mismatch between the measuring beam (reflected by the tissues) and the reference beam (reflected by a reference path) is used to generate the OCT image.

OCT has come to be the most frequently used diagnostic tool in ophthalmology and has brought forth a new paradimg of clinical imaging for diagnosis and disease management in most retinal disease ,including AMD.

OCT provides high-resolution imaging of neurosensory retina and subretinal space and is able to visualize qualitative features such as subretinal fluid, intraretinal cystoid fluid, disruption or changes in thickness of retinal layers and the status of the vitreomacular interface.(Mowatt *et al*, 2014)·(Dansingani *et al*, 2016) Furthermore, it can provide information on central retinal thickness (CRT) and detailed retinal thickness map (RTM) and can show morphological changes in response to therapy after AMD treatments. For all this reasons, OCT is widely used for screening, classification, monitoring and treatment evaluation of AMD.

Autofluorescence

The adventum of confocal scanning laser ophthalmoscopy (SLO) has implemented the use of autofluorescence in retinal diseases. This technique uses the signal produced by the excitation of fluorophores, mainly the lipofuscin (a by-product of the visual cycle found in RPE cells). This imaging tool can thus be used to assess the status of RPE. If the RPE becomes atrophic this appears on AF as a distinct dark area, due to absence of lipofuscin-containing cells, on the contrary, failing RPE can be signaled by an increase in autofluorecence, representing accumulation of lipofuscin. Several patters were reported in different AMD stages. However, the exact utility of autofluorescence is still being investigated. For example, central scotoma patterns may be explained by the finding of areas of absent autofluorescence, this tool may also be useful to monitor GA progression (Figure 7).(Göbel *et al*, 2011)

Similarly, autofluorescent patterns seen with a longer wavelength (near-infrared instead of blu/green) are starting to be described.(Theelen *et al*, 2009) This technique uses a higher excitation wavelength, similar to that of ICG angiography. This longer wavelength seems to generate a signal from the melanin instead of the lipofuscin, which is present both in the choroid and the RPE.(Keilhauer & Delori, 2006)

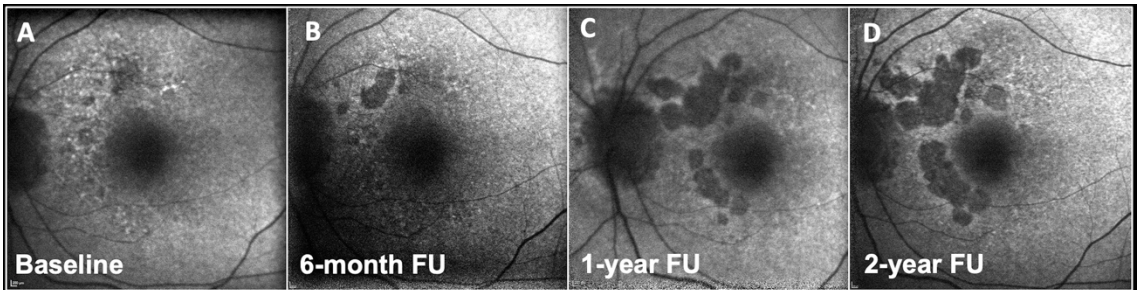

Figure 7. Serial short-wave fundus autofluorescence (FAF) images during the time of a 79-year-old patient affected by geographic atrophy.

Short-wave FAF image showing the presence of a small area of retinal pigment epithelium atrophy at the baseline (A). During the time, geographic atrophy showed great enlargement with the confluence of the atrophic lesions (B, C, and D).

Fluorescein Angiography (FA)

FA is the gold standard in the diagnosis of type 2 CNV (according to Gass classification) in 'wet' AMD.

This technique allows examination of the circulation of the retina using a fluorescent dye. Sodium fluorescein is injected into a peripheral vein, the retina was irradiated by a blue light (wavelenght of 488 nm). The FA image is the result of the fluorescent green light that is emitted by the irradiated dye inside retinal vessels. The images are taken at 40-50 seconds after the injection to capture the critical phases of early transit, at 2-3 minutes to capture the intermediate phase and finally at 5-6 minutes to capture the late phase.

The different CNVs behave differently in FA:

- Type 1 CNV (occult CNV, not visible at FA): Type 1 CNVs are the most common form of neovascular AMD, almost 50% of all neovascularizations. They appear at the FAG as pin-points in the early stages and as dye leakage in the late phase, without the possibility of identifying the source of this hyper-permeability.
- Type 2 CNV (classic CNV): account for about 10% of the forms of neovascular AMD. The FAG allows to identify this type of lesions very well, such as staining and leakage that reflects the shape of the neovascular network.
- Type 3 CNVs: appear as hot-spots in late phases.

Finally FA of atrophic areas (GA) shows a window defect caused by the unmasking of choroidal fluorescence, provided that the choriocapillaris is still intact. Exposed sclera may exhibit late staining.

IndoCyanine Green Angiography (ICGA)

ICGA is currently the gold standard for diagnosing type 1 CNV (according to Gass classification) in 'wet' AMD.

Indocyanine green (ICG) is a dye soluble in water with a near-total protein-bound fraction (98%) when injected, this means that it cannot diffuse through the small choriocapillaris fenestrations. Because of this, ICG is the ideal tool to view choroidal blood circulation, due to its retention and low permeability; the dye is then metabolized in the liver and eliminated through the bile.

As ICG emits light in the near-infrared spectrum (800 nm), it can be injected before, together, or after the sodium fluorescein. The fluorescent intensity of ICG is 4% in comparison to sodium fluorescein dye. For this reason, only dedicated tools are able to see the fluorescence of ICG (i.e. infrared video angiography using modified fundus cameras, or a SLO camera). The wavelength of the diode used in ICGA is set on an output of 805 nm, whereas the barrier filters is set from 500 to 810 nm.

Theoretically ICG fluoresces better than fluorescein over pigment, fluid, lipid, and hemorrhage because due to the longer wavelength, thus reducing the probability of signal blockage by hyperplastic RPE or subretinal hemorrage, and allowing improved imaging in various situations as occult CNV and PED.

Indocyanine green is administered intravenously and the images are taken at 40-50 seconds to 3 minutes after the injection to capture the critical phases of early transit, at 15 minutes to capture the intermediate phase and finally at 30-40 minutes to capture the late phase.

The different CNVs behave differently in ICGA:

- Type 1 CNV (occult CNV, not visible at FA): ICGA shows a "plaque" staining in late phases, corresponding to the neovascular texture.(Costanzo *et al*, 2016)

- Type 2 CNV (classic CNV): ICGA shows a neovascular network in both in early phase and in late phase.

- Type 3 CNV: appear as hot-spots which increases fluorescence in late phases

Lastly, atrophic areas (GA) appear as discrete hypofluorescent areas with loss of background fluorescence caused by the associated atrophy of the choriocapillaris, moreover outlining the area of atrophy seems to be more difficult than with FA. Nonetheless, deep and large choroidal vessels can still be visualized.

Optical Coherence Tomography Angiography (OCT-A)

Optical coherence tomography angiography (OCT-A) has established itself as a non-invasive technique in the world of imaging of the retinal and choroidal blood vessels, allowing to segmented independently the various retinal and choroidal vascular plexus.(Nikolopoulou *et al*, 2018)·(Malamos *et al*, 2017) It is a relatively new technique, in fact, the first clinical studies have been published in 2014.(Fingler *et al*, 2008)

OCT-A does not need intravascular dyes as it uses laser light reflected by erythrocytes' surface to detect and render vessels with a great degree of accuracy.(Koustenis *et al*, 2017) The OCT scan works by compiling various A-scans of the retina into B-scan to provide cross-sectional structural information; various scans of the same section are repeated at different times and differences are analyzed to evaluate flow based on the mismatch between two scans, thus detecting high flow rates (greater changes) and slower, or no-flow (with minor changes) that will show similarities among scans.(Spaide *et al*, 2015)

Light could be produced through either a swept-source OCT (SS-OCT), using a wavelength of 1050nm, or a spectral domain OCT (SD-OCT), using a shorter wavelength, near to 800nm. As mentioned before, shorter wavelengths have a lower tissue penetrance due to the screen effect of EPR.(de Oliveira Dias *et al*, 2018) Two protocols of motion detection are employed: amplitude decorrelation or phase variance. The first one identifies differences in amplitude between OCT B-scans taken at different times. The

latter instead, measures emitted light waves properties, and the variation of phase when they are reflected by objects in motion. In order to reduce noise, which may be caused by normal small eye movements, thus allowing for a clearer image, two methods of average (based on split spectrum amplitude decorrelation technique and volume averaging) were developed.(Jia *et al*, 2012)·(Gorczynska *et al*, 2016) OCT-A produces an image (3 mm2 to 12 mm2) that is usually segmented into four areas: the choriocapillaris, the outer retina, the deep retinal plexus, and the superficial retinal plexus.

Shorter acquisition time and its non-invasiveness are the main advantages of this technique, which has been recognized as a useful tool in the diagnosis and understanding of AMD:

- Dry AMD: a decrease in choriocapillaris flow is observable, usually in a broader area than that of atrophy. Devices using SS-OCT usually provide better-defined images of choroidal vessels changes(Cicinelli *et al*, 2018a)·(Choi *et al*, 2015).

- Wet AMD: OCT-A allows for quantitative and qualitative analysis of choroidal neovascular membranes, their classification, and follow-up on anatomical changes after treatment. This tool could potentially detect neovascular complexes when exudation is absent, which is a difficult task using conventional imaging such as SD-OCT or FA, and thus lead to a more effective and closer follow-up.(Khan *et al*, 2017; Huang *et al*, 2015)

The CNVs have different OCTA patterns. The OCTA is able to identify the plot of type 1 CNV Ffigure 8), which have an "umbrella" or "medusa" morphology, with an "interlacing" or "tangled" pattern depending on the number and complexity of the terminal anastomoses of neovascularization.(Ma *et al*, 2017)

In the type 2 CNV OCTA shows a lesion with a "medusa" or "a glomerulus" morphology, generally formed by a dense high-flow network.(El Ameen *et al*, 2015) Crossing the Bruch's membrane and developing into the sub-retinal space, the new vessels can be well visualized in the plexus outer retina, contrary to what happens for type 1 lesions, in which the "choriocapillaris" slab is particularly important for the diagnosis.

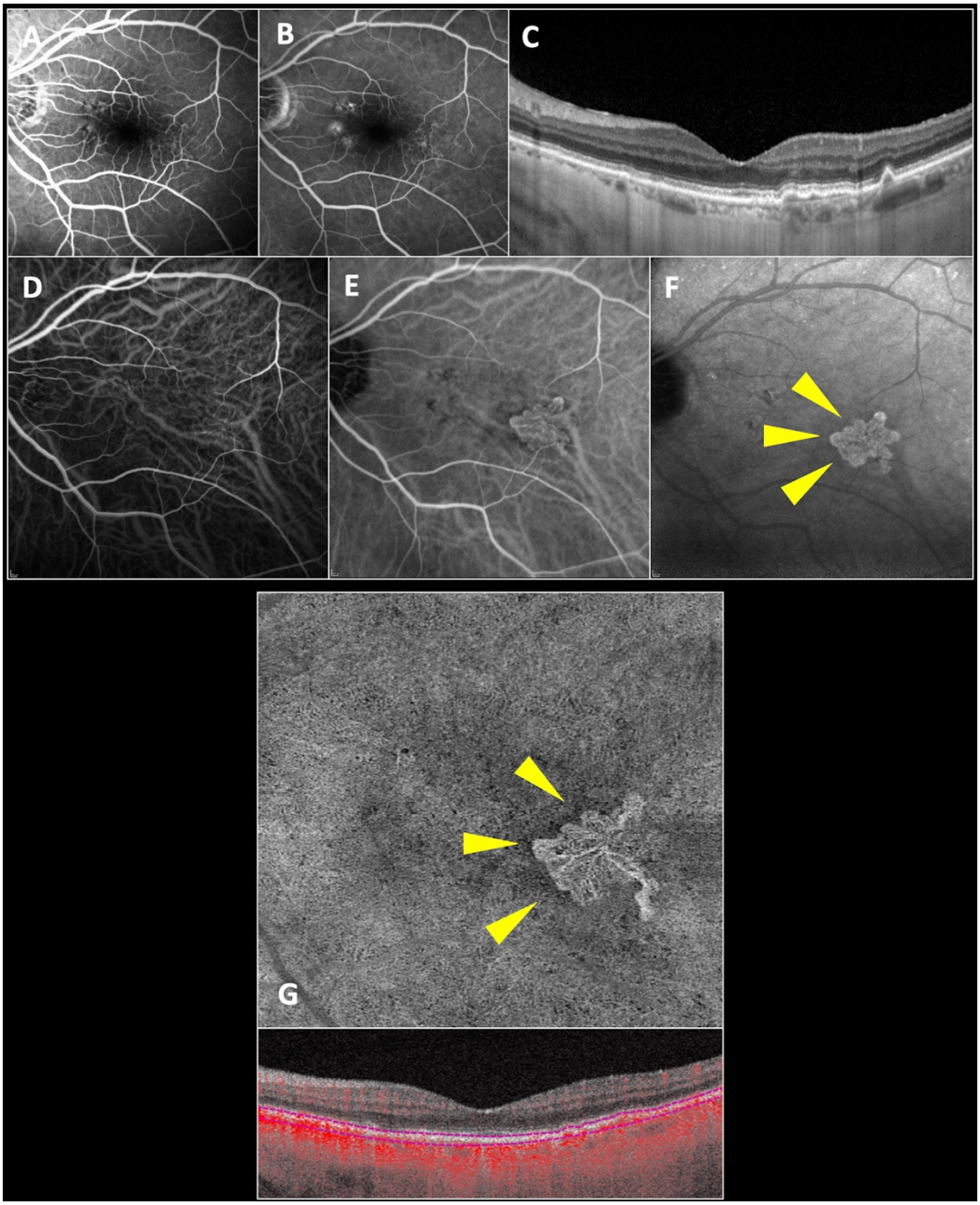

Figure 8. Multimodal imaging evaluation of a 76-year-old patient affected by type 1 non-exudative macular neovascularization (MNV) in the left eye.

Early (A) and late phases (B) of fluorescein angiography showing the presence of mixed hypo- and hyper-fluorescent area in the macular area, with pin-points inferotemporal to the fovea. Horizontal structural optical coherence tomography (OCT) passing through the fovea (C) showing the presence of a flat irregular pigment epithelium detachment with no signs of exudation (i.e. intra- or sub-retinal fluid, or subretinal hyper-reflective material). Early (D), intermediate (E), and late phases (F) of indocyanine green angiography showing the neovascular network with a hyperfluorescent plaque in the late phases of the examination (yellow triangles). En-face OCT-angiography and b-scan with flow (G) showing the presence of a neovascular network, matching non-exudative type 1 MNV (yellow triangles).

The active phase of type 2 CNV is characterized by an area of hypoperfusion at the level of the choriocapillaris surrounding the lesion.(Kuehlewein *et al*, 2015)

Finally, the type 3 CNV are characterized by the presence of anomalous flow in the deep capillary plexus. The choriocapillary plexus can show in some cases the presence of a neovascular lesion below the EPR, which would indicate a choroidal origin of the lesion.(Bansal *et al*, 2018)

Therapeutic Strategies

Several treatments have been proposed, their usage are different based on the stage of the disease and, recently, there has been a number of breakthrough in the management of macular degeneration.

Early AMD

The oral intake of a mix of minerals, antioxidant, and vitamins has been proposed as a tool to avoid progression from early AMD to more advanced stages, although there wasn't enough evidence support this claim, and they were not able to reduce the transition to the intermediate stage of AMD. So, no evidences are present suggesting the intake of this supplementation for patients that are in the early stages of the disease. In fact, in early AMD a small percentage of patients (1.3%) progress to advanced-stage of AMD during a 5-year follow-up.

Intermediate AMD

The AREDS (Age-Related Eye Disease Study) included 4757 subjects that were followed for 6 years after a randomization in two groups: a first group of people who took a mix of antioxidant, vitamins, and minerals, and a second group of people who took a placebo. (Kassoff *et al*, 2001a) In the AREDS, a daily dose of 15mg beta-carotene, 80 mg zinc, 2 mg copper, 500 mg vitamin C and 400 UI vitamin E was used. In AREDS2 instead, the beta-carotene was replaced with lutein (10 mg), zinc oxide was reduced and zeaxanthin (2 mg) was added. For this study, 4203 participants who had large drusen in both eyes or only in one eye with advanced-stage AMD in the fellow eye, were included.

This population was identified in the AREDS study to have higher risk group of progressing to more advanced-stages of AMD.(Davis *et al*, 2005) In this study, there was a randomization of the subjects in two groups: the first group was composed of patients who took the original supplementation; the second group was composed of patients who took the original supplementation plus lutein, zeaxanthin, and more omega- 3. a second randomization was performed to assign patients to a modified formula which variations included removal of beta-carotene, lower zinc levels, or both. The evidence yielded by AREDS2 suggests that replacement of beta-carotene using lutein (10 mg) and supplementation of zeaxanthin (2 mg) leads to an even lower risk of evolving to advanced-stage of AMD.

In these studies, participants affected by intermediate stage AMD in both eye, or only in one eye with advanced-stage AMD in the fellow-eye benefited from AREDS/AREDS2 supplementation. This treatment also reduced the probability of evolving in the advanced-stage of AMD by 25% at 5 years for participants with a defined profile (several medium sized drusen, at least one large drusen, presence of non-subfoveal geographic atrophy or advanced AMD in one eye). Supplementation also reduced the risk of losing vision of three or more lines (which is double the MAR) by almost 20%. Although both zinc alone or antioxidants alone reduced progression, the combination therapy yielded the strongest results in terms of reduction of both vision loss and disease progression.

The AREDS2 study results demonstrated that of omega-3 had no additional benefit, and that there was no difference in progression risk between the different formulations for patients at high risk for progression. Moreover, for patients in the lowest quartile for lutein and zeaxanthin intake, supplemental lutein and zeaxanthin was protective. This, together with the evidence that the use of beta-carotene, together with lutein and zeaxanthin reduces the absorption of the nutrients, and the increased in the incidence of lung cancer in the patients of the beta-carotene group, led to the conclusion that lutein and zeaxanthin represent a safe and valuable alternative to beta-carotene in the supplementation. Lastly, reducing the zinc intake (25 mg) doesn't lead to a greater risk of progression.

'Wet' AMD

Along with the posterior ocular segment diagnosis, neovascular AMD therapy is one of the fields of ophthalmology that has shown the most progress over the last few years. Several treatments have been tested in large prospective, randomized and controlled clinical trials, including conventional laser photocoagulation, photodynamic therapy (PDT) and anti-VEGF drugs.(Bressler & Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study Group, 2001)·(Schachat *et al*, 1994; Gragoudas, 2004)

Currently, anti-VEGF drugs administered by injection into the vitreous chamber are the reference therapy in most forms of neovascular AMD, in the absence of systemic or local contraindications.(Schmidt-Erfurth *et al*, 2014a)

The first anti-VEGF drug to be introduced and approved by the Food and Drug Administration (FDA) was Pegaptanib (Macugen; Eyetech/Valeant Pharmaceuticals) in 2004, an RNA oligonucleotide able to selectively block isoform 165 of VEGF.(Gragoudas, 2004) However, in relation to the limited percentage of patients (about 6%) with a significant improvement in visual acuity, this treatment was later overcome by the use of more effective drugs, such as Ranibuzumab, Bevacizumab and Aflibercept, capable of also inhibit other VEGF isoforms.(Schmidt-erfurth *et al*, 2017)

Thanks to the MARINA and ANCHOR studies, the FDA authorized Ranibizumab as a treatment for neovascular AMD in 2006.(Brown *et al*, 2006) This molecule is a monoclonal antibody fragment capable of inhibiting the main VEGF isoforms.(Chen *et al*, 1999) The low molecular weight, associated with a shorter intravitreal half-life, guarantees ranibizumab a high local and systemic safety, greater than that reported for bevacizumab, which instead consists of the complete antibody.(Shih & Lindley, 2006) In fact, compared to ranibizumab, bevacizumab has a longer half-life and greater effects on systemic VEGF levels when used intravitreally, as well as a lower VEGF binding capacity.(Schmidt-Erfurth *et al*, 2014a) Despite the most unfavorable pharmacokinetic and pharmacodynamic profile, this compound has often been used as a cheaper alternative molecule in the therapy of neovascular AMD.(Stein *et al*, 2014) The evidence for the usage of Bevacizumab in neovascular AMD is mainly derived from the comparative non-inferiority study compared to ranibizumab (CATT, conducted on 1208 patients), and from the IVAN and GEFAL trials.(Kodjikian *et al*, 2013; Chakravarthy *et al*, 2012)

As far as ranibizumab is concerned, fixed-scheme treatment regimens have been evaluated, in addition to the aforementioned MARINA and ANCHOR studies, also in the PIER and EXCITE trials, demonstrating excellent clinical efficacy.(Abraham *et al*, 2010; Schmidt-Erfurth *et al*, 2011) In particular, the MARINA study has included 716 patients with occult or minimally classical CNVs who received injections of Ranibizumab 0.3, 0.5 mg or sham every month for 24 months. At the end of the study, 90% of the eyes treated with 0.5 mg of Ranibizumab had maintained a stable visual acuity (loss <15 letters) compared to 53% in the control group, with an average gain of about 7 letters. In particular, a large clinical and functional response was observed in the first months, with a subsequent maintenance of the gain obtained until the end of the follow-up. The ANCHO study investigated the efficacy and safety of Ranibizumab in 423 patients with classic CNVs, who were given monthly injections at doses of 0.3 or 0.5 mg, and compared with a control group subjected to PDT. 90% of patients receiving Ranibizumab 0.5 mg had a lesser functional loss than patients undergoing PDT. Furthermore, 41% of patients treated with Ranibizumab 0.5% had gained more than 15 letters compared to 6% of those undergoing PDT.

Flexible treatment regimens, on the other hand, have been evaluated by subsequent studies such as the Pronito, the CATT, the SECURE and the HARBOR.(Csaky, 2010; Silva *et al*, 2013; Busbee *et al*, 2013) Flexible treatment modalities involve injections based on the finding of neovascular activity (Pro Re Nata-PRN) or injections based on a progressive extension of the treatment interval, even in the absence of signs of neovascular activity (treat and extend).(Abedi *et al*, 2014) Regardless of the treatment regime adopted, it has been shown that the higher efficacy of anti-VEGF drugs in terms of functional gain is obtained in cases where the starting visual acuity is higher; therefore, the role of an early diagnosis is fundamental, for initial treatment before a substantial loss of sight has already occurred. In clinical practice, this often does not happen, due to the lower efficacy of anti-VEGF drugs in real-life, associated with a lower number of injections per year, compared to randomized controlled trials.

A relatively new resource in the therapy of neovascular AMD is represented by the Aflibercept. Unlike the two previous molecules, Aflibercept is not a monoclonal antibody, but a recombinant fusion protein composed of the Fc portion of IgG1 bound to

fragments of VEGF receptors.(Holash *et al*, 2002) This composition guarantees a very high affinity for VEGF, higher than that of native receptors.(Papadopoulos *et al*, 2012) Aflibercept can also block the placental growth factor (PlGF), a further growth factor expressed by the endothelium.(Stewart & Rosenfeld, 2008) The approval of the use of this molecule in neovascular AMD occurred through two parallel clinical trials, the VIEW1 and VIEW 2, which included more than two thousand patients.(Schmidt-Erfurth *et al*, 2014b) The primary objective of the studies was that of non-inferiority of the Aflibercept compared to Ranibizumab in the maintenance of visual acuity, achieved with a 96-week follow-up. The suggested treatment regimen is monthly intravitreal injections for the first three months, followed by injections every 8 weeks.

Several new anti-VEGF drugs are being added to the current molecules. Among these, Brolocizumab has received the FDA and EMA approval for the AMD treatment. Brolocizumab is a single chain antibody fragment, of extremely low molecular weight, composed only of fragments that bind VEGF. The phase 2 and phase 3 studies that tested the efficacy and safety of the drug were HAWK and HARRRIER in the United States and Europe, respectively. In them, brolucizumab 6 mg administered every 12 weeks was shown to be non-inferior in terms of visual gain compared to aflibercept administered every 8 weeks.(Dugel *et al*, 2017)

'Dry' AMD

To date, no therapy is available to avoid or treat the enlargement of geographic atrophy. However, many trials are underway but up to now no treatment has been approved.

Reticular Pseudodrusen (RDP)

In the early 90s, Mimoun et al. firstly reported retinal lesions with an approximative diameter of 100mm, describing them as "pseudodrusen visible en lumière bleue", because they were lesions, similar to drusen, that were visible only using blue reflectance fundus photography. (Mimoun et al., 1990). These lesions were originally described a variation of "classic" drusen and similarly they were thought to originate from beneath the RPE.

In 1991 the Wisconsin age-related maculopathy grading system included these lesions as "ill-defined networks of broad, interlacing ribbons" and Klein described the reticular pattern at blue reflectance fundus photography.(Klein *et al*, 1991) Despite the increasing interest of ophthalmological community, their pathological and clinical significance was still unknown. Newer imaging techniques reconsidered the nomenclature and the interpretation of this retinal changes. Smith et al. proposed the name "reticular macular disease", while Zweifel et al. suggested the term "subretinal drusenoid deposits" (SDD).(Smith *et al*, 2009; Zweifel *et al*, 2010b) Multimodal imaging allowed to detect and report disparity between reticular pseudodrusen and subretinal drusenoid deposit, and over the years these lesions have gradually been gradually characterized with peculiar and unique imaging features.(Grewal *et al*, 2014; Sohrab *et al*, 2011)(Heiferman *et al*, 2015)

Epidemiology

Different studies focused on the assessment of prevalence of RPD. Extended, prospective, long-term follow-up studies mainly focused on elder population.(Finger *et al*, 2016; Joachim *et al*, 2014a; Klein *et al*, 2008b) RPD prevalence was described to be 0.41%–1.95% and 3.0%–4.0% at baseline and 15 years in the aforementioned studies, respectively. Nevertheless, those studies employed color fundus photography (CFP) as detection imaging technique, with lower sensitivity and specificity compared to new imaging examinations (ie, structural spectral-domain optical coherence tomography [SD-OCT], infrared reflectance [IR] images, and multicolor images). Prevalence assessment of RPD based on these novel modalities has been reported to be higher indeed.(Bats *et al*, 2016) Accordingly to Rotterdam Eye Study, CFP combined to near-infrared reflectance (NIR) established the RPD prevalence to be 5% among participants (Buitendijk *et al*, 2016), and greater (13.4%-32%) with employment of multimodal imaging techniques.(Chan *et al*, 2016; Zarubina *et al*, 2016) Moreover, RPD seem to be more frequent in Caucasian rather than Asian population.(Joachim *et al*, 2014b)RPD tend to be bilateral in more than a half of patients, with bilateral involvement ranging from 50% to 84% of cases.(Alten *et al*, 2012a; Sohrab *et al*, 2011; Smith *et al*, 2009)

RPD represent a risk factor for AMD in elder population, with an increased risk of 3.4-fold.(Zarubina *et al*, 2016) Patients with AMD present higher prevalence of RPD than age-matched general population, although the percentage of involvement depends from the imaging examination used and the AMD stage that was considered, displaying great variability in literature, from 16.8% to 79%.(Bats *et al*, 2016; Zarubina *et al*, 2016)·(Cohen *et al*, 2007) Most importantly, prevalence of RPD was reported greater (85%) in the histological specimen of postmortem retina in subjects affected by AMD.(Curcio *et al*, 2013) Furthermore, different studies focused on the prevalence of RPD according to the type of concurrent macular neovascularization (MNV). As reported, RPD are indeed more frequently discovered in those eyes affected by type 3 MNV (from 68.4% to 83%). Concurrently, RPD showed lower prevalence (9%–13.9% and 2%–3.4%) in eyes with type 1 and 2 neovascular AMD or PCV, respectively.(Ueda-Arakawa *et al*, 2013a; Wilde *et al*, 2015; Kim *et al*, 2015b) An association with geographic atrophy has also been established, and prevalence of RPD was shown to be 29%-50%.(Smith *et al*, 2009; Ueda-Arakawa *et al*, 2013a). Acquired vitelliform detachment has also been associated to RPD, highlighting the possible role of RPE dysfunction in pathogenesis.(Querques *et al*, 2011; Zweifel *et al*, 2011b)

RPD are not only related to AMD, but also to other retinal diseases, i.e. including early-onset drusen,(De Bats *et al*, 2013) pseudoxanthoma elasticum,(Gliem *et al*, 2016a; Zweifel *et al*, 2011a) Sorsby macular dystrophy(Gliem *et al*, 2015) and adult-onset foveomacular vitelliform dystrophy.(Wilde *et al*, 2016) There are also some reports about the presence of RPD in patients with late-onset macular degeneration,(Cukras *et al*, 2016) vitamin A deficiency(Aleman *et al*, 2013) and IgA nephropathy.(Lally & Baumal, 2014)

Risk factors

As previously reported, different features of AMD have been correlated with an increased risk of RPD. Subjects with greater risk to develop RPD are those with late AMD: the risk is progressively greater in early and intermediate stages and even higher in GA, over the MNV phenotype.(Pumariega *et al*, 2011; Schmitz-Valckenberg *et al*,

2011) Focal pigmentary changes and large drusen have been addicted as supplemental factor of risk of presence of RPD. Many studies focused on systemic and demographic risk factors, namely older age,(Finger *et al*, 2016; Klein *et al*, 2008b) female sex,(Finger *et al*, 2016; Klein *et al*, 2008b) current smoking (Joachim *et al*, 2014a) and high body-mass-index.(Joachim *et al*, 2014a). B-vitamin complex use, history of use of topical steroid eye drop and a previous diagnosis of glaucoma represent other reported risk factor for RPD.(Klein *et al*, 2008b) On the other hand, patients with diabetes presents lower prevalence of RPD and AMD .(Klein *et al*, 2008b)

Beaver Dam Eye Study(Klein *et al*, 2008b) addicted RPD as independent risk factor for overall survival, and higher prevalence of RPD has been recently reported in subjects affected by coronary artery disorder.(Cymerman *et al*, 2016) Patients with RPD showed a worse cardiovascular profile, with higher percentage of hypertension, angina pectoris and mortality than rates reported in patients with AMD without RPD.(Rastogi & Smith, 2016) Nevertheless, the association linking the presence of RPD to cardiovascular disease is debated in the literature, since other publications did not found the aforementioned correlation.(Puche *et al*, 2013) Decreased renal function has also been associated to RPD.(Leisy *et al*, 2017)

Thanks to novel imaging techniques, especially enhanced depth imaging (EDI) and swept-source OCT (SS-OCT), choroidal imaging has recently provided further insights in patients with RPD. Patients with RPD displayed lower subfoveal choroidal thickness (SCT) compared both to healthy subjects and patients with AMD without RPD.(Garg *et al*, 2013; Querques *et al*, 2012b; Thorell *et al*, 2015) Choroidal thinning is not focally limited to the macula, but extends to other areas, such as the peripapillary region, supporting the idea of a general involvement of the choroid.(Haas *et al*, 2014; Yun *et al*, 2016) A more consistent representation of the stromal area was shown in patients with AMD and RPD, compared to patients without RPD.(Corvi *et al*, 2016) These considerations suggest an association between RPD and choroidal vascular depletion with fibrotic replacement.

Supporting the emerging idea of choroidal vasculopathy, Zheng et al(Zheng *et al*, 2016) showed remarkably choroidal perfusion density (PD) impairment in patients affected by RPD. Specifically, the areas with lowest PD are not located in correspondence to the area with thinnest choroid. Thus, the idea of a global choroidal dysfunction in

patients with RPD could be better explained with the hypothesis of choriocapillaris hypoperfusion.(A Martillo *et al*, 2012)

Even potential genetic susceptibility for RPD has been largely investigated, little is still known and data are controversial. Large studies pointed out a linkage between RPD and 2 major AMD risks alleles: the complement factor H (CHF, rs1061170) 402H on chromosome 1q32 and the age-related maculopathy susceptibility 2 (ARMS2, rs10490924) 69S on chromosome 10q26. (Klein *et al*, 2008b)(Joachim *et al*, 2014a)(Ueda-Arakawa *et al*, 2013a)(Jakobsdottir *et al*, 2005; Rivera *et al*, 2005). Specifically, ARMS2 and CHF 402H variant were found to be a risk and protective factor, respectively.(Smith *et al*, 2011) The Melbourne Collaborative Cohort Study,(Finger *et al*, 2016) addicted the ARMS2 single-nucleotide-polymorphism (SNP) rs10490924, HTRA1 SNPs rs11200638 and rs3793917, and CFH SNPs rs393955, rs1061170 and rs2274700 as independent risk factors for RPD. In addition, Buitendijk et al(Buitendijk *et al*, 2016) reported that CFH, C2/FB, ARMS2, and C3 are associated with the presence of RPD.

However, literature data are still controversial, and there is not univocal consensus on genetic susceptibility and its impact in pathogenesis of RPD. Other studies did not find indeed any correlation between risks allele and RPD. (Puche *et al*, 2013)(Boddu *et al*, 2014)

Histology

Histology helps differentiating RPD from drusen. While drusen are lipidic deposits in the Bruch's membrane, RPD are made of material gathering under the neuroretina, in the subretinal space, growing to the outer segment and that can reach the outer nuclear layers.(Greferath *et al*, 2016; Khan *et al*, 2016) RPE polymegathism, impairment of the photoreceptors and reactive fibrosis were described as associated with RPD, attesting the involvement of RPD surrounding structures.(Greferath *et al*, 2016)

RPD composition has been extensively investigated, and differences between RPD and drusen have been pointed out. Even if lipid, amyloid, cholesterol, complement factor, and membranous debris are commonly findings both in drusen and RPD composition, RPD present some peculiarities. Indeed, RPD are characterized by greater levels of

unesterified cholesterol, vitronectin, and photoreceptor pigments (including precursors of A2E and lipofuscin).(Curcio *et al*, 2005; Johnson *et al*, 2001) Moreover, an inflammatory setting has been suggested in RPD, since immune cells, have been found in histologic specimens.(Greferath *et al*, 2016) However, despite the efforts to determine the precise composition of RPD, further insights are still needed.

Location (X-Y)

RPD are commonly found in the perifoveal region. Specifically, superior retina seems to be the area most frequently and severely affected.(Curcio *et al*, 2013) Conversely, basal linear deposit (BlinD) is a common finding within the fovea, where cones are maximally represented.(Curcio *et al*, 2013) These anatomical preferences suggest that rod and cone pathophysiology could partially explain RPD and BlinD aetiology. Recent studies based on novel imaging techniques reported that RPD disposition commonly follows choroidal vasculature, supporting that choroid plays a key role in pathogenesis of RPD.(Grewal *et al*, 2014)

Imaging

Several imaging modalities variably combined were used to detect and describe RPD, including IR, FAF (short-wave or NIR), CFP, multicolor, structural b-scan and en face OCT, OCT-A, FA, ICGA, and adaptive optics (Figure 9).

RPD are visualizable with higher sensitivity using IR, FAF, SD- and SS- OCT. Moreover, ICGA in the late-phases, confocal blue reflectance (CBR) and blue channel of CFP demonstrated the highest specificity.(Ueda-Arakawa *et al*, 2013b; Smith *et al*, 2009) Since gold standard for PRD detection has not yet been established, the combined use of at least 2 imaging techniques has been suggested.(Alten & Eter, 2015) Particularly, the combination of examination with higher sensitivity (ie, SD-OCT and IR) as screening test with a high specific one (i.e., ICGA, CBR) as confirmation has been highly recommended.(Ueda-Arakawa *et al*, 2013b)

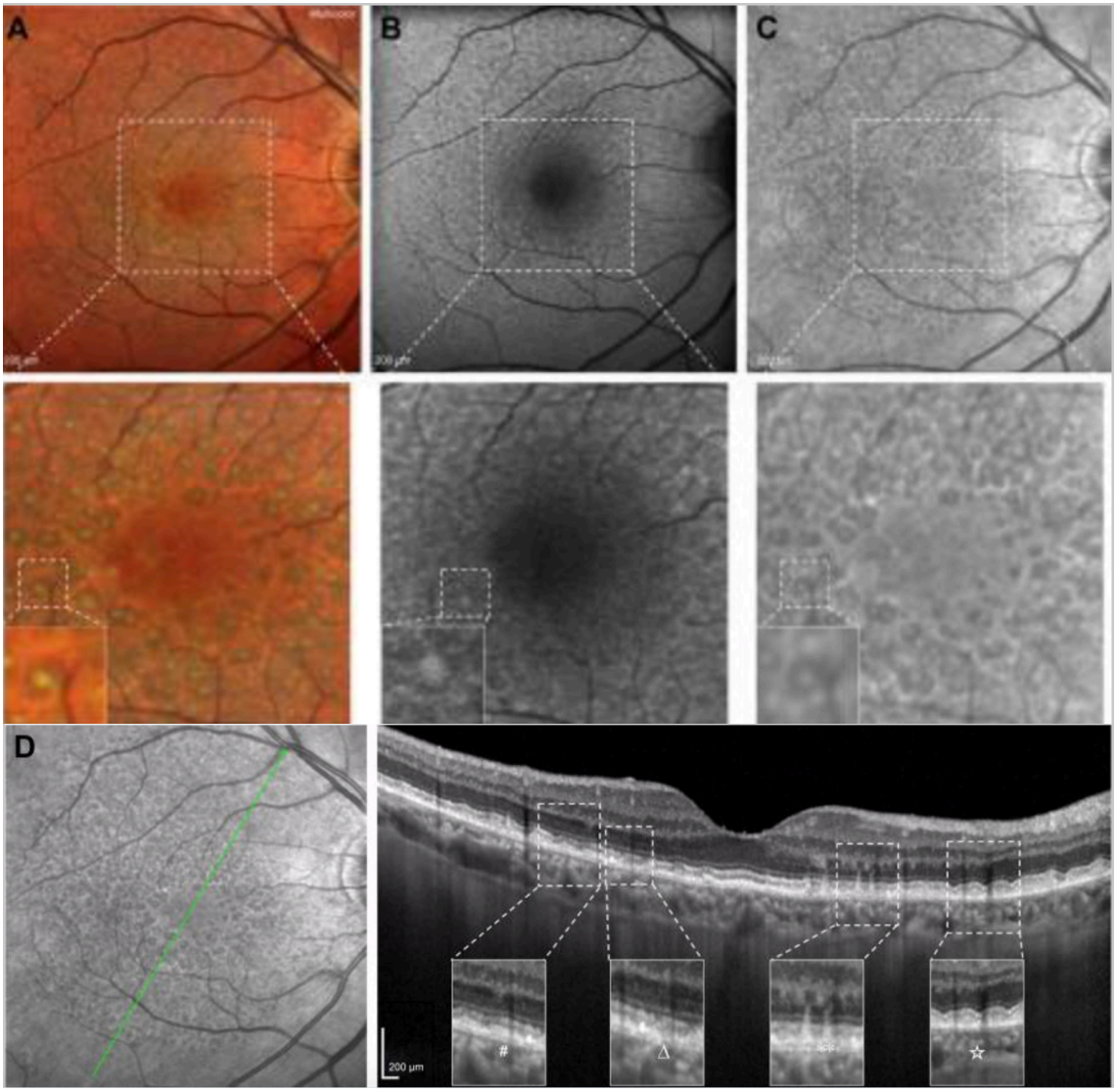

Figure 9. Multimodal imaging of a patient with RPD secondary to intermediate AMD. (A) Multicolor imaging revealing the presence of a lots of RPD with a target aspect. (B) Short-wave fundus autofluorescence revealing RPD as hyperautofluorescent dots surrounded by a faint hypoautofluorescent halo . (C) Infrared reflectance (IR) image revealing the target aspect of RPD. (D) Combined IR and structuralOCT B-scan passing through the fovea revealing different stages of RPD. (ref. Rabiolo, A., Sacconi, R., et al (2017). Spotlight on reticular pseudodrusen. Clinical ophthalmology. 11, 1707–1718.)

Color fundus photograph (CFP)

At CFP RPD appear as a network of large and interlacing yellowish ribbons, which progressively becomes thin and punctate as it approaches closer to the fovea.(Khan *et al*, 2016) Better identification of RPD is allowed by blue channel of CFP: RPD mainly reflect

the SW of blue light, which is well absorbed by the RPE surrounding the RPD, thanks to the presence of melanin. These differences in blue light absorption enhance indeed the contrast between RPD and the surrounding area.(Ueda-Arakawa *et al*, 2013b) Although different studies(Finger *et al*, 2016; Joachim *et al*, 2014a) employed this technique to detect RPD, the diagnostic reliability of CFP is globally unsatisfactory, mainly due to a lack of sensitivity.(Ueda-Arakawa *et al*, 2013b) On the other hand, an extremely high specificity, virtually reaching 100%, justifies the use of CFP as a confirmatory test. (Ueda-Arakawa *et al*, 2013b)

Fundus autofluorescence

RPD appear hypofluorescent using SW-FAF (excitation in the blu-light, 488 nm, and emission of light greater than 500 nm).(Smith *et al*, 2006; Schmitz-Valckenberg *et al*, 2009) .It has been postulated that the absorption of the blu light from the RPD avoid reaching the RPE of the light itself , and it could explain this typical hypofluorescent appearance of RPD at SW-FAF. (Ueda-Arakawa *et al*, 2013b)

However, several deposits show an isofluorescent nucleus, giving a characteristic target aspect due to the surrounding hypofluorescent edge.(Querques *et al*, 2011) A possible explanation is the accumulation of lipofuscin-like material in the RPD core. Others have suggested that the ellipsoid zone (EZ) break as cause of isofluorescence.(Querques *et al*, 2011) The aspect of a "target" can be also absent in some RPD, and this could be due to heterogeneous arrangement or confocal SLO properties.(Querques *et al*, 2011) Moreover, in a few subjects RPD appear as high hyperfluorescent lesions. Initially, these hyperfluorescent RPD have been reported to have a weaker correlation with advanced-stages of AMD, even if further analysis did not replicated this assumption. Others have proposed that hyperfluorescent RPD could be small foci of acquired vitelliform material or a transient form of pseudodrusen .(Lee & Ham, 2014) The area of RPD progressively extends over time, with a mean annual growth rate of 4.4 mm^2.(Steinberg *et al*, 2013) Quantitative FAF (qFAF) showed that RPD patients have lower values of qFAF in comparison with patients affected by soft or cuticular drusen. Reduced qFAF values could be explained by lower lipofuscin accumulation because of an impairment of the choroid-BM-RPE complex.(Gliem *et al*,

2016b) Thanks to the high sensitivity and specificity (respectively 86% and 92-95%) SW-FAF is considered one of the most reliable imaging modalities to detect RPD. On the other hand, NIR-FAF has not good diagnostic abilities. Thanks to the high sensitivity and specificity, respectively 86% and 92-95%, SW-FAF one of the most reliable imaging modality to identify RPD. NIR-FAF however has not valuable diagnostic abilities, because of different physical characteristics (excitation using a 787 nm light; emission using a 800 nm light). (Ueda-Arakawa *et al*, 2013b)

IR image and Multicolor

By means of IR, RPD usually display an hyporeflective reticular pattern with an aspect of "target", characterized by an hyporeflective lesion surrounding an isoreflective core.(Querques *et al*, 2011; Schmitz-Valckenberg *et al*, 2009) Suzuki et al(Suzuki *et al*, 2014) classified 3 subtypes of RPD (dot, ribbon, and midperipheral RPD, respectively), basically distinguished by different features pointed out through multimodal imaging. The first type is characterized by perifoveal hyporeflective dots with the aspect of "target" at IR; in the second subtype, IR reveals perifoveal faint hyporeflective ribbons; in the third type, IR shows presents midperipheral hyper-reflective spots as hallmark feature. These three distinct RPD subtypes may present different progression of the disease.(Suzuki *et al*, 2014) Because IR presents great sensitivity and reliability, this technique was proposed to be the screening test for RPD detecion.(Ueda-Arakawa *et al*, 2013b)

MultiColor imaging creates a unique pseudocolor image through a mege of 3 laser channels in the Spectralis machine (infrared reflectance, with a wave-length of 820 nm; green reflectance: with a wave-length of 515 nm; and blue reflectance with a wave-length of 488 nm). RPD present a peculiar yellowish-green reticular pattern and, close to IR, they may assume a target aspect with a hyperreflective yellow-greenish core surrounded by a hyporeflective halo. MultiColor presents a similar detection rate in comparison to IR and FAF.(Alten *et al*, 2014)

Structural b-scan and en face OCT, and OCT-A

RPD appear at structural OCT as deposits of hyperreflective lesion located over the RPE, particularly in the subretinal space.(Querques *et al*, 2012a) Nowadays, it is extensively assumed that the abovementioned RPD correspond to the hyperreflective lesion visualizable using SD-OCT.(Zweifel *et al*, 2010b) Zweifel et al(Zweifel *et al*, 2010b) proposed 3 different stages of the development of RPD: 1) deposition of hyper-reflective material above the RPE and under the EZ, 2) accumulation of material causing a distortion of the EZ profile, 3) focal disruption of EZ due conical amassing. Moreover, Querques et al(Querques *et al*, 2012a) explained that RPD are characterized by progressive and dynamic accumulation of subretinal material; most importantly, the Authors demonstrated that drusenoid material can be reabsorbed and migrate into the inner retinal layers, enhancing the idea of dynamism of these structures. This reabsorbing and migrating dynamic phase was referred to as stage 4. The dynamism of RPD has been subsequently confirmed through further studies.(Auge *et al*, 2014)(Schick *et al*, 2014) Advanced stages of RPD have been related to the degeneration of the RPE and deneration of photoreceptors. According to literature, RPD disappearance could occur during the time, even unilaterally and asymmetrically. This phenomena is followed either by focal reduction of choroidal thickness or by outer retinal atrophy, causing a novel phenotype of late AMD, which is not yet described in the most used classification of AMD.(Spaide, 2013) Specifically, RPD regression may lead to outer retinal atrophy, involving RPE and choriocapillaris and resulting in GA.(Spaide, 2017)

Many studies reported vascular loss of choroidal vessels and fibrotic changes in patients with RPD, accompanied by a global thinning of the choroid. Mrejen and Spaide(Mrejen & Spaide, 2014) reported a lower prevalence of RPD in patients with thinner choroid (for example eyes affected by pathologic myopia). They suggested that RPD development is not consequence of a choroidal dysfunction, but rather ascribed to other factors. Higher risk of RPD formation was described in case of choroidal atrophy due to aging, characterized by pigmentary changes and choroidal thickness reduction, suggesting a pathological alteration of choriocapillaris as a possible aetiology.(Mrejen & Spaide, 2014)

This assumption was supported by OCT-A findings, reporting a decrease in choriocapillary vessel density , which is more pronounced in eyes with RPD.(Alten *et al*, 2016; Cicinelli *et al*, 2018a)

SD-OCT showed high reliability in detection of RPD.(Ueda-Arakawa *et al*, 2013b) En face OCT can detect RPD with similar diagnostic reliability of conventional multimodal imaging.(Schaal *et al*, 2017)

RPD thickness has been demonstrated to be a strong predictor of GA growth(Niu *et al*, 2016), in association with EZ disruption, (Giocanti-Auregan *et al*, 2015) There is still a need to identify further biomarkers able to predict vision loss progression.(Schaal *et al*, 2016)

FA and ICGA

Early frames in FA show the presence of a defect in the filling of choriocapillaris matching to RPD.

At ICGA, RPD are typically hypocianescent spots in the middle and late-phases of examination. Since they are located in the same area of alterations seen at FAF and IR, a pathologic alterations inside the RPE have been proposed.(Arnold *et al*, 1997) Combining ICGA and IR, Querques et al(Querques *et al*, 2012b) demonstrated that RPD are usually located in the stromal area between choroidal vessels, sparing the area just above the major choroidal vessels, reinforcing the pathogenetic role of an alteration of the choroidal filling. In the same way, it has been reported that RPD co-localize in the areas of choroidal watershed, suggesting a possible role of hypoxic alterations of choroidal vasculature. (Alten *et al*, 2013) Because of its invasive nature, ICGA is not considered a first-line tool in the RPD diagnosis (sensitivity: 73%, specificity: 100%).(Ueda-Arakawa *et al*, 2013b) However, ICGA and FA have a key role into the diagnosis of CNV associated with RPD.

Functional tests

Abnormality in retinal functional tests have been associated to early and intermediate AMD. AMD patients showed impaired low-luminance test and dark adaption, particularly in those patients with RPD because of rod function impairment.(Huisingh *et al*, 2016) Steinberg et al(Steinberg *et al*, 2015) reported a decrease of mean macular sensitivity at microperimetry, particularly pronounced in the scotopic analysis. Further

studies showed no changes considering mesopic, photopic, and low-luminance acuity, photopic light and contrast sensitivity in eyes affected by RPD or not.(Neely *et al*, 2017)

Eyes with RPD presented lower microperimetric sensitivity.(Forte *et al*, 2013)(Querques *et al*, 2014)(Wu *et al*, 2015)

At multifocal electroretinography (mfERG), Alten et al(Alten *et al*, 2012b) did not reported significant differences between RPD eyes and healthy subjects. Furthermore, an amplitude reduction was reported in affected areas compared to areas without pseudodrusen,.(Alten *et al*, 2012b)

Natural history

As previously explained, RPD are dynamic lesions and can even reabsorb. RPD represent a risk factor in patients affected by AMD for its progression to late-stages and development of GA (2-fold risk increase). Moreover, RPD were found in the 25% of elder subjects with healthy macula.(Huisingh *et al*, 2016)(Joachim *et al*, 2014a) (Pumariega *et al*, 2011) (Niu *et al*, 2016) However, RPD are strongly associated to type 3 CNV and to earlier and bilateral disease onset.(Chang *et al*, 2016) So similarly, type 3 CNV are highly associated to RPD. RPD were proposed indeed as a key feature to discern between type 3 MNV and other subtypes of MNV.(Ravera *et al*, 2016) Moreover, RPD alone are typically associated with type 2 CNV.(Naysan *et al*, 2016)

RPD constitute a risk factor for atrophy development after intravitreal anti-VEGF injections, without any interference with the response to the intravitreal therapy.(Cho *et al*, 2015) .(Nghiem-Buffet *et al*, 2017)

Therapy

Nowadays, there is no therapy for RPD, and the presence of RPD in patients with AMD does not affect the therapy. As RPD are included in the features of AMD, patients with early of intermediate AMD,could eventually have a benefit from vitamin supplementation following the AREDS/AREDS2 formulation. The supplementation with zeaxanthin, lutein and meso-zeaxanthin could cause an increase of the macular pigment optical density mainly in the RPD.(Corvi *et al*, 2017)

Laser in Ophthalmology

The introduction of laser photocoagulation to treat ocular disorders was a major advance in ophthalmology. Proper use of lasers in ophthalmic practice starts with a good understanding of how laser works.

LASER refers to Light Amplification by Stimulated Emission of Radiation. A laser derives indeed from the excitation by a photon of light, of the atoms falling back to a lower level and thereby emitting energy in the form of radiation.

The matter, composed of the atoms and molecules, at low temperatures is in the lowest and thereby the most stable level or "ground state". As the temperature increases, more and more atoms jump to the higher energy levels. This is described as the Boltzmann's energy distribution, according to which the higher energy level always has fewer molecules populating it than a lower level. Now if a light beam of a suitable wavelength is introduced into the medium, the beam will become attenuated and the photons will get absorbed by the atoms which get excited to a higher energy level. From here, the atoms spontaneously decay to the lower level and emit photons in random direction.

Here, it is essential to understand a phenomenon which is reverse of the Boltzmann's distribution: "population inversion", i.e. higher energy levels contain more atoms than are contained in lower energy level. It can be achieved by introduction of: (i) electrical discharge, and (ii) optical pump using a xenon arc lamp or another laser. In a medium with a population inversion, the introduction of a beam of light leads to subsequent emission of photons which are in phase and coherent with each other and also with the exciting light beam. So, the two prerequisites for the emission of a laser beam are: i.) population inversion of the medium, ii.) a light beam of correct wave length introduced to stimulate the excited atom into emitting light that is coherent with the exciting light beam.

Population inversion occurs when the proportion of pumping atoms in the higher energy level is larger than the decay into the lower energy level. This increases the rate of stimulated emission and is a prerequisite for laser emission. This chain reaction is

amplified by surrounding the medium with two mirrors, one of which is totally reflective and the other one typically partially reflective. To constrain the direction of radiation release, the excited atoms are contained in a laser cavity.

Laser System

The resonator is an optical cavity limited by mirrors at both sides and contains the lasering medium. Thanks to the mirror system, light reflects multiple times into the cavity in order to elicit radiation emission from excited atoms. Once emitted, laser is able to escape the cavity because of partial transmittance of one of the mirrors.

Lasering medium can be Solid (i.e. neodymium-doped yttrium aluminum-garnet, Nd:YAG), Liquid (i.e. Fluorescent dye) or Gas (i.e., Krypton and Argon).

Laser is monochromatic, therefore eliminates chromatic aberration, in phase, i.e. all the photons produced are in phase with each other unlike normal light beam where photons exist in random phases, is coherent, is collinear, highly energized and with limited divergence. The resultant beam of light is easy to focus to a small spot. The total amount of light produced depends on the volume of the optical cavity, not the surface area.

Choice of Wavelenght

A number of wavelengths are available to the surgeon.

The key pigments ocular tissues and investigated by laser are:

- Melanin, that easily absorbs multiple wavelength (red, yellow, green and infra-red)
- Macular xanthophyll, that mainly absorbs blue wavelength, with minimal absorbption of yellow or red wavelenghts
- Hemoglobin, that easily absorbs green, blue and yellow with low absorption of red wavelenght

The reasons to pick one wavelength over another are mostly theoretical, but the overriding concern is an attempt to increase the therapeutic index. One wavelength, argon

blue, should not be used as blue light is more likely to be scattered and has the potential to be absorbed by the xanthophyll in the macula causing unintended macular damage.

Different wavelength are absorbed selectively from different structures. Specifically, argon blue-green laser is composed 70% of blue light and 30% of green light (respectively 488 nm and 514nm), and it is absorbed from retinal inner and outer nuclear layer, retinal pigment epithelial layer (RPE), choriocapillaris and hemoglobin pigments. The possible adverse effects of laser use can be macular damage and intraocular scattering. In case of Bruch's membrane defects, a further adverse effect is the choroidal neovascularization.

Melanin, hemoglobin and trabecular meshwork also absorb yellow light (532 nm).

Yellow laser has low intraocular scatter, and minimal xanthophyll absorption. Thus, it is largely used for its reduced risk of photochemical damage. Vascular structures are successfully and safely destroyed. PASCAL (PAttern SCAn Laser) consists of a frequency-doubled Nd-YAG Laser 532 nm). It uses semi-Automated multiple pattern and produces several and accurate burn in a short duration (10-20 msec). Thanks of these features, many retinal affections are treated with PASCAL.

Red wavelengths (647 nm) are selectively absorbed by melanin with low intraocular scattering. Conversely, hemoglobin does not absorb red wavelengths: subretinal neovascular membrane, as well as choriocapillaris and choroidal affections, are thus successfully treated with red laser .

Diode 810 nm wavelengths are absorbed by melanin. It is characterized by deeper penetration in the retina and choroid. It represents the first line in treatment of Retinopathy of Prematurity (ROP). Moreover, ciliary body can be treated via trans-scleral with this kind of laser

Laser-tissue interaction

Interactions between laser and tissue have been largely investigated and can occur in different ways: there may be a photothermal effect (photocoagulation and photovaporization), a photochemical effect (photoablation and photoradiation) and a photoionizing effect (photodisruption).

Photothermal effects consists of photovaporization and photocoagulation.

Photocoagulation occurs when tissue's temperature rises (10° to 20°), provoking proteins denaturization, necrosis, focal coagulation, and hemostasis at the level of RPE, photoreceptors and Bruch membrane. Diode (810 nm), krypton, argon, and Frequency doubled ND:YAG lasers (532 nm) cause photocoagulation. Thus, they are extensively used for pan-retinal photocoagulation.

When high energy laser light hits a target tissue, it causes vaporization of both extracellular and intracellular water. This phenomena is commonly known as photovaporization. Thanks to photovaporization, blood vessels are also treated and a bloodless field is guaranteed. The carbon dioxide laser causes this type of effect.

Photochemical effects consists of photoablation and photoradiation. Photoradiation occurs when an intravenous photosensitizing agent is administered, causing a sensitization of a selected tissue. The sensitized tissue is then exposed to red laser light, producing free radicals with cytotoxic effect. If low wavelengths laser (<350) are used, polymeric molecules are typically broken into smaller volatile molecules. This phenomena is known as photoablation. Photodynamic therapy (PDT) and Excimer laser are examples of photoradiation and photoablative therapy respectively. Photoionization occurs when high-energy light hits a target tissue in a short time and causes an acoustic shock wave. ND:YAG laser (1064 nm) is and example of photodisruptive therapy, generally used for peripheral iridotomy or posterior capsulotomy.

Continuous Wave Laser, Pulsed Laser and Subthreshold laser

Conventional continuous wave lasers delivery energy in a continuous stream of photons and the tissue temperature rises considerably throughout the pulse.

The other way around repetitively pulsed lasers produce pulses that have a lot of short micropulses, commonly defined pulse train. This train of micropulses is defined by duty factor (the percentage of time that the laser is "on" during the pulse envelope) and frequency (repetition rate in Hz). (Figure 10). This mechanism prevents the thermal rise of the target.

Subthreshold laser does not cause a visible damage to the target tissue. It produces low energy light and limits its thermal effect to RPE.(Dorin, 2004; Framme *et al*, 2002) Two

approaches for subthreshold laser treatment are now available: Subthreshold Micropulse Diode (SMD) and Selective Retinal Therapy (SRT).

SRT consists of single continuous wave laser pulses. The duration varies from microseconds to nanoseconds.(Schuele *et al*, 2005) This brief pulse vaporizes only those RPE cells which are highly rich in melanin and can absorb about half of the light. SRT has been reported to promote migration and proliferation of surrounding healthy RPE cells.(Kim *et al*, 2015a)

Subthreshold micropulse diode (SMD) distributes energy into a multiple consecutive pulses, shorter than thermic relaxation time of target tissues. ‘‘On’’ (100–300 μs) and “off” (1700–1900 μs) time are respectively the duration of each micropulse and the interval between consecutive micropulses. A short duty cycle prevents thermal elevation collateral damages (i.e. necrosis). According to the so-called “reset to default” theory, SMD has been supposed to help and support RPE cells activity.(Luttrull *et al*, 2015a) SMD causes sub-lethal damages into RPE cells and the activation of heat shock proteins pathway and damage fixing.

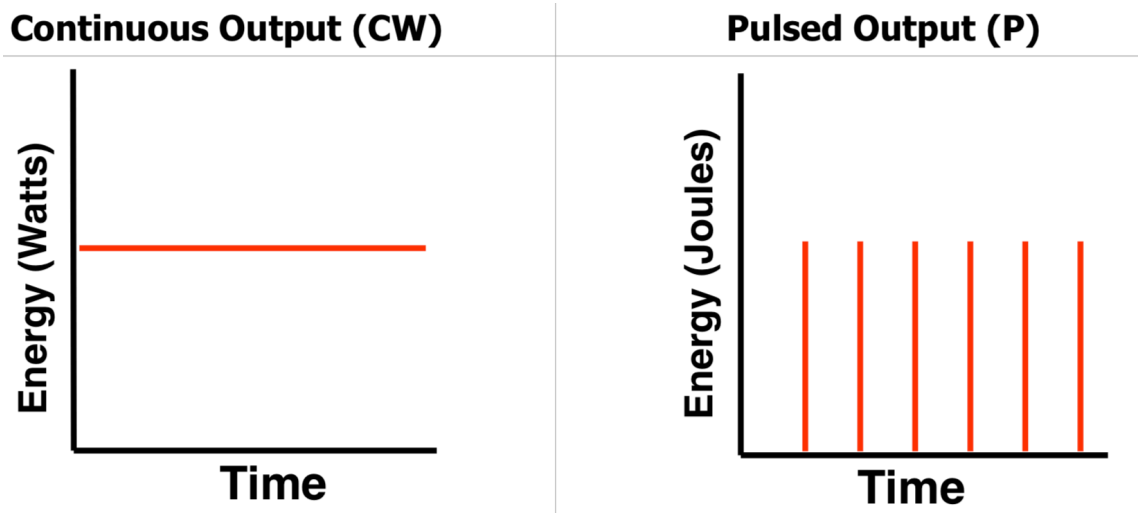

Figure 10. Different output of the laser.
The output of a laser may be a continuous constant amplitude output (known as continuous wave) (left); or pulsed (right).

Laser therapy in AMD

In recent years, different types of laser treatments have been proposed as a possible treatment of AMD.

Thermal Laser Photocoagulation and Photodynamic Therapy in 'Wet' AMD

According to the Macular Photocoagulation Study (MPS), the thermal laser photocoagulation has been considered for a long time as primary treatment of exudative AMD and extensively used to destroy CNV. MPS showed that photocoagulation of subfoveal, juxtafoveal and extrafoveal CNV contained the risk of severe reductions in visual acuity.

The presence of a classic CNV determined by FA was the inclusion criteria for photocoagulation treatment, but only 13-26% of all patients satisfied the abovementioned inclusion pattern. There were many doubts indeed about the benefits of photocoagulation in exudative AMD.(Argon Laser Photocoagulation for Senile Macular Degeneration: Results of a Randomized Clinical Trial, 1982; Macular Photocoagulation Study Group, 1991; Moisseiev *et al*, 1995) In addition, more than 50% of the treated patients presented recurrent or persistent CNV within 2 years of treatment.

Nowadays, thermal laser photocoagulation is limited to CNV far from the fovea and for the treatment of variants of exudative AMD, including polypoidal choroidal vasculopathy and retinal angiomatous proliferation (RAP).(Nishijima *et al*, 2004) Several ophthalmologists do not use laser photocoagulation indeed in the treatment of subfoveal CNV because of the induction of an immediate central blinding scotoma. Different methods have been investigated as an alternative approach with laser for the treatment of subfoveal CNV (i.e. feeder-vessel photocoagulation(Shiraga *et al*, 1998) and transpupillary thermotherapy(Reiche *et al*, 2005)). Despite many efforts have been spent, these laser alternatives are not widely employed.

Because of the central iatrogenic scotoma as collateral effect in the treatment of subfoveal CNV thermal laser, PDT has been proposed as an alternative. Verteporfin, a photosensitizing dye, is infused and then stimulated with a pre-established wavelength (689 nm) of light selectively directed at the target CNV. The stimulation promotes the

generation of free radicals, which induce platelet activation and thrombosis selectively in the vascular lesion.(van den Bergh, 2003)

Despite PDT is considered safe and avoids central blindness scotoma, it is not widely employed due to poor visual recovery and the need to repeat the treatment several times. Thus, other treatment approaches for subfoveal CNV have been investigated.

Laser Therapy in Dry AMD

In 1973 J. Donald Gass firstly proposed laser photocoagulation in patients with drusen.

There is currently no clear explanation of the mechanism behind drusen regression when they are treated with laser. A role of phagocytic cells differentiated from pericytes of the choriocapillaris has been proposed.(Duvall & Tso, 1985) Further studies supported the relationship between laser treatment and choroidal cells modifications, since changes in their processes and shape have been reported in human eyes after laser photocoagulation.(Guymer *et al*, 2001) Moreover, choroidal blood flow has been shown to increase with laser therapy.(Figueroa *et al*, 2006) Since choroid plays a key role removing waste products removal, the abovementioned changes could promote drusen reabsorption. Further hypothesis have been suggested, including a trophic benefit from RPE cells proliferation, a reduction of accumulation drusenoid material in the macula and lower outflow resistance of thicker Bruch membrane.(Little *et al*, 1997; Frennesson & Nilsson, 1998)

After Gass proposed laser photocoagulation as an alternative treatment, different reports and pilot trials focused on drusen regression with laser photocoagulation.(Figueroa *et al*, 1997; Sarks *et al*, 1996; Duvall & Tso, 1985) According to these trials, patients with intermediate AMD experienced low or no improvement of visual acuity, accompanied by a reduction in number of drusen.(Sternberg *et al*, 1998)(Maguire *et al*, 2003)

However, due to several limitations of the previous trials (i.e. short follow-up and heterogeneity in treatment strategy), multicenter prospective randomized clinical trials were conducted (Choroidal Neovascularization Prevention Trial and Drusen Laser Study).

They reported an increased risk of CNV in the contralateral eye after laser photocoagulation in patients with unilateral exudative AMD. Moreover, efficacy of laser has not been proven in those patients with bilateral drusen. (Owens *et al*, 2003; Maguire *et al*, 2003)

Complication of AMD Prevention Trial was a randomized trial conducted to clarify the role of laser therapy in case of bilateral drusen.(Maguire, 2004) More than 1000 patients with bilateral drusen have been included in different clinical centers. These studies showed no significant difference in long term visual acuity, CNV risk or GA development in patients treated with laser compared to the group of patients that did not receive laser treatment.

A recent systematic review concluded there is no benefit from photocoagulation to prevent CNV and GA.(Virgili *et al*, 2015)

Finally, the LEAD Randomized Controlled Clinical Trial proposed Subthreshold Nanosecond Laser (SNL) as a potential treatment in intermediate AMD (iAMD) in patients presenting bilateral large drusen without atrophy. At the end of this clinical trial, SNL did not showed efficacy preventing the progression to late AMD.(Guymer *et al*, 2019)

To clarify this point Rosenfeld and Feuer recommend not treating iAMD with laser therapy because of the potential risk of accelerating disease progression.(Rosenfeld & Feuer, 2019)

AIM OF THE WORK

AMD is a degenerative disease, that could be manifested in distinctive stages, reflecting the progression of the disease. Although the use in our practice of anti-VEGF drugs has completely changed the natural history and prognosis of patients affected by advance neovascular stage of AMD(Rosenfeld *et al*, 2006), to date there are no specific treatment available for the prevention of advance AMD stages development. It is well known that patients with reticular pseudodrusen are affected by greater risk of progression from intermediate AMD to late-stages of AMD (both neovascular form and geographic atrophy)(Querques *et al*, 2014)·(Rabiolo *et al*, 2019; Zweifel *et al*, 2010a), and RPD are characterized by a worse functional and anatomical features starting from the early signs of the disease(Querques *et al*, 2014; Borrelli *et al*, 2019; Sacconi *et al*, 2021; Cicinelli *et al*, 2018b; Rabiolo *et al*, 2019) For this reason, a new therapeutic strategy aimed to the resolution of reticular pseudodrusen would be of fundamental importance in order to prevent or delay the conversion from intermediate AMD to advance AMD in several patients.

Recently, the use in our practice of subthreshold laser treatment (SLT) has given us a new strategy for the therapy in several retinal diseases (Vujosevic *et al*, 2013; Parodi *et al*, 2006; van Dijk *et al*, 2018). The action mechanism of SLT is not complete clarified. However, the most accredited hypotheses highlighted that SLT works by "targeting, preserving, and normalizing" the RPE-cell function(Luttrull *et al*, 2015a). It was proven that the disfunction of RPE is one of the main driver at the basis of RPD development. For this reason, there is an important rationale in the use of SLT for the RPD treatment. To date, there are no studies that evaluate in a prospective way the safety and the effectiveness of SLT in RPD patients. The aim of this thesis is to study, first of all, the safety of SLT in RPD patients due to intermediate AMD. After that, we evaluated the effectiveness of the SLT in RPD patients from an anatomical and functional point of view.

RESULTS

Short-term safety and efficacy of SLT

Patient demographics and main clinical findings

As reported in our published study(Querques *et al*, 2021), twenty eyes of 20 patients (mean age 78.4±6.8 years, median 77.5, range 67-89) fulfilled the criteria of our study and were included. All patients were Caucasians, 15 were females and 5 males. The fellow eye of 15 patients was affected by nAMD, whereas the fellow eye of 5 patients was affected by dAMD. During the 3 months of the study protocol, patients were not treated with any intravitreal injections or other treatments for AMD in the fellow eye.

At the baseline, BCVA was between 20/25 and 20/32 Snellen equivalent (0.140±0.09 LogMAR; median 0.1; range, 0-0.4) and did not shown significant changes at 1-month and 3-month follow-up (BCVA 0.135±0.10 LogMAR; median 0.1; range 0-0.4 and 0.115±0.09 LogMAR; median 0.1; range 0-0.3 at 1-month and 3-month follow-up, respectively) (p=0.232). None of the included patients gained or lost more than 15 letters during the follow-up. IOP did not show significant changes during the follow-up (p=0.267) (Table 1). Furthermore, no significant changes were disclosed analyzing CMT and subfoveal ChT during the follow-up. In detail, CMT was 275±19 µm (median 275, range 229-305) at the baseline, 276±19 µm (median 276, range 237-320) at 1-month follow-up and 273±26 µm (median 273, range 209-324) at 3-month follow-up (p=0.725). Subfoveal ChT at the baseline was 223±110 µm (median 187, range 72-426) and did not show significant changes at 1-month and 3-month follow-up (223±112 µm; median 188; range 77-425 and 221±107 µm; median 184; range 72-433 at 1-month and 3-month follow-up, respectively) (p=0.730) (Table 1).

Functional changes in the treated area

Assessment of retinal sensitivity was performed using microperimetry. No significant changes were disclosed before and after the treatment analyzing the overall MS of the macular area and the MS of the treated area. Of note, the overall MS was 13.99±4.27 dB

at the baseline and did not significantly change during the follow-up (p=0.152)(Table 1, Figure 11). Analyzing the mean MS of only the treated area, it was 12.08±4.65 dB at the baseline, 11.08±5.06 dB at 1-month follow-up, and 11.45±5.70 dB at 3-month follow-up (p=0.404). Furthermore, the mean MS of only the control area was 12.01±4.67 dB at the baseline, 11.02±4.45 dB at 1-month follow-up, and 11.69±5.21 at 3-month follow-up (p=0.120) (Table 1, Figure 11).

Mean fixation percentage calculated within the central 2° (centered on the fovea) was 42.5±13.2% at the baseline and did not significantly change during the follow-up (p=0.649); also mean fixation percentage calculated within the central 4° (centered on the fovea) did not show significant changes during the follow-up (p=0.342) (Table 1).

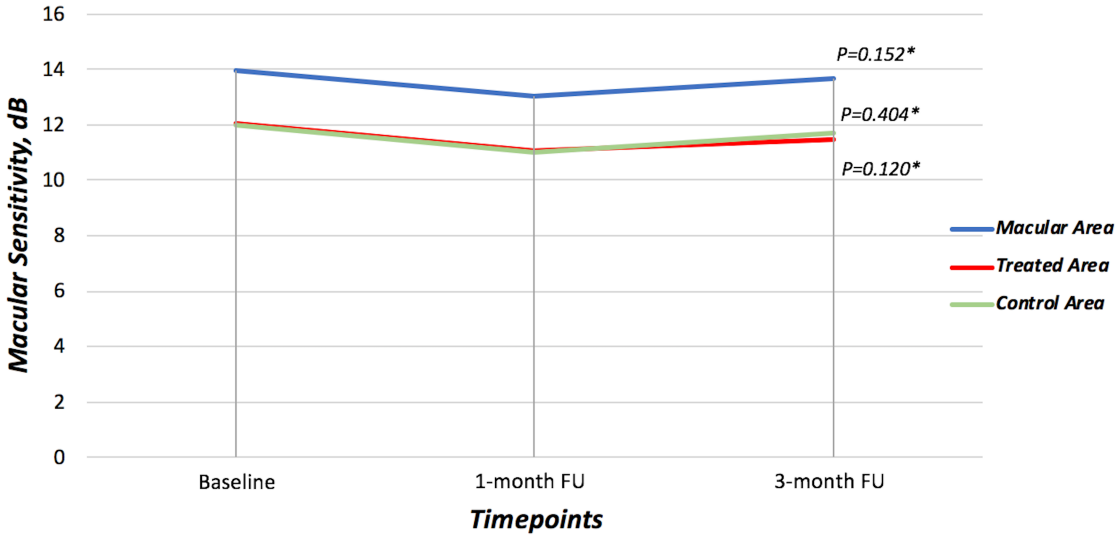

Figure 11. Results of microperimetry in terms of macular sensitivity of the whole macular area, of the treated area, and of the control area during the follow-up.

*: Analysis of Variance (ANOVA) for paired samples.

Table 1. Comparisons of anatomical and functional variables between baseline, 1-month follow-up and 3 month follow-up after the treatment

	Baseline		*1-month follow-up*		*3-month follow-up*		
	mean ± SD	***P value****	**mean ± SD**	***P value*§**	**mean ± SD**	***P value*§**	***P value*#**
BCVA, LogMAR	0.14±0.09	0.232	0.135±0.10	1.000	0.115±0.09	0.288	0.311
IOP, mmHg	15.5±2.9	0.267	14.9±2.4	0.827	14.7±2.4	0.300	1.000
CMT, µm	275±19	0.725	276±19	1.000	273±26	1.000	1.000
Subfoveal ChT, µm	223±110	0.730	223±112	1.000	221±107	1.000	1.000
Retinal Thickness (treated area), µm	295±26	0.238	294±24	1.000	292±25	0.258	0.847
ChT (treated area), µm	197±88	0.450	195±94	1.000	201±88	1.000	0.613
ONL thickness (treated area), µm	59.30±13.50	0.001	64.75±12.31	0.012	67.75±15.52	<0.001	0.213
ONL thickness (control area), µm	51.61±10.91	0.199	53.39±12.29	0.645	53.56±12.31	0.204	1.000
MS (macular area), dB	13.99±4.27	0.152	13.05±4.19	0.148	13.65±4.70	1.000	1.000
MS (treated area), dB	12.08±4.65	0.404	11.08±5.06	0.538	11.45±5.70	1.000	1.000
MS (control area), dB	12.01±4.67	0.120	11.02±4.45	0.112	11.69±5.21	1.000	0.910
Fixation percentage (2°), %	42.5±13.2	0.649	39.3±15.1	1.000	40.5±17.3	1.000	1.000
Fixation percentage (4°), %	83.2±12.8	0.342	78.3±15.3	0.550	80.2±11.8	0.969	1.000

SD: standard deviation; BCVA: best-corrected visual acuity; IOP: intraocular pressure; CMT: central macular thickness; ChT: choroidal thickness; ONL: outer nuclear layer; MS: retinal sensitivity.
*: Analysis of Variance (ANOVA) for paired samples.
§: comparison with baseline using ANOVA for paired samples with Bonferroni post-hoc analysis.
#: comparison with 1-month follow-up using ANOVA for paired samples with Bonferroni post-hoc anaysis

Anatomical changes in the treated area

An area of 1.27 mm^2 was treated in all patients. The area was located at a mean distance of 1904±544 μm (median 1811.5, range 1127-3000) from the fovea. Using structural OCT, we identified a mean of 12.4±6.2 RPD (median 11.5, range 3-23) inside the treated area. The distribution of RPD among the RPD stages changed after the treatment ($p<0.001$). At the baseline, 0.35±0.59 RPD were classified as Stage 1, 8.60±4.79 as Stage 2, 3.30±4.35 as Stage 3, and 0.15±0.49 as Stage 4. At 1-month follow-up, 1.20±1.70 RPD were classified as Stage 1, 8.85±5.20 as Stage 2, 2.20±3.98 as Stage 3, and 0.15±0.49 as Stage 4. At 3-month follow-up, 2.30±2.18 RPD were classified as Stage 1, 8.75±4.79 as Stage 2, 1.15±2.56 as Stage 3, and 0.20±0.70 as Stage 4 (Figure 12) (Table 2). This accounted for a significant increase of Stage 1 RPD during the follow-up ($p=0.002$), no significant changes of Stage 2 RPD ($p=0.909$), but a significant decrease of Stage 3 RPD ($p=0.020$) and no significant changes of Stage 4 RPD ($p=0.630$). This improvement was mainly due to a general improvement in stage 2 and 3 RPD. In detail, 62% of stage 3 RPD (41 out of 66 stage 3 RPD of all patients) showed an improvement during the 3-month follow-up (28 out of 66 RPD from stage 3 to stage 2, and 13 out of 66 RPD from stage 3 to stage 1). Furthermore, 16% of stage 2 RPD (27 out of 172 of stage 2 RPD of all patients) showed an improvement to stage 1 during the 3-month follow-up.

Another area of 1.27 mm^2 with a similar distance from the fovea (1901±528 μm $p=0.689$) was selected as a control area (i.e. area without treatment). In the control area, we identified a mean of 9.6±4.6 RPD (median 9.5, range 4-20)($p=0.127$). Analyzing the distribution of RPD among the RPD stages in the control area (i.e. area without treatment), no significant changes were observed during the follow-up in different stages of RPD. In detail, at the baseline, 0.56±0.24 RPD were classified as Stage 1 (0.50±1.04 and 0.22±0.55 RPD at 1-month and 3-month follow-up, respectively; $p=0.157$). At the baseline, 5.61±3.15 RPD were classified as Stage 2 (5.67±3.61 and 6.06±3.56 RPD at 1-month and 3-month follow-up, respectively; $p=0.305$), 3.83±3.75 RPD were classified as Stage 3 (3.28±3.89 and 3.22±3.73 RPD at 1-month and 3-month follow-up, respectively; $p=0.251$), and 0.11±0.32 RPD were classified as Stage 4 (0.17±0.51 and 0.11±0.32 RPD at 1-month and 3-month follow-up, respectively; $p=0.331$).

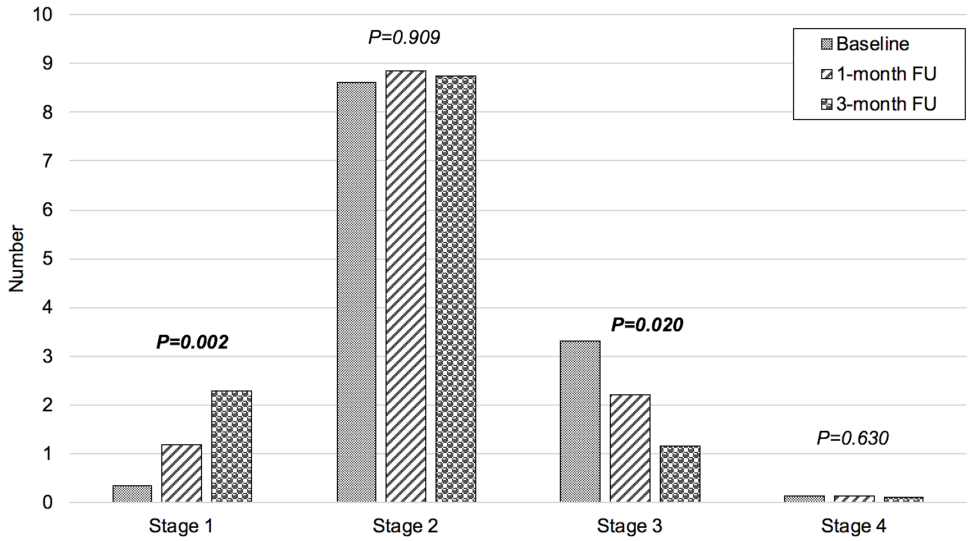

Figure 12. Number of reticular pseudodrusen (RPD) in the treated area according to the stage before and after subthreshold laser treatment

Stage 1 RPD significantly increased after the subthreshold laser treatment (first histogram) due to the significant regression of stage 3 RPD (third histogram). No significant differences were disclosed after the treatment in stage 2 and stage 4 RPD (second and fourth histograms, respectively).

Table 2. Number of reticular pseudodrusen (RPD) in the treated area according to the stage before and after subthreshold laser treatment and during the follow-up evaluation

	Baseline	**1 Month FU**	**3 Month FU**	**P value***
	mean ± SD	*mean ± SD*	*mean ± SD*	
Stage 1 RPD	0.35±0.59	1.20±1.70	2.30±2.18	0.002
Stage 2 RPD	8.60±4.79	8.85±5.20	8.75±4.79	0.909
Stage 3 RPD	3.30±4.35	2.20±3.98	1.15±2.56	0.020
Stage 4 RPD	0.15±0.49	0.15±0.49	0.20±0.70	0.630

RPD: reticular pseudodrusen; FU: follow-up.

*: Analysis of Variance (ANOVA) for paired samples.

Analyzing the treated area, the thickness of the ONL significantly increased during the follow-up (p=0.001). In detail, mean ONL thickness at the baseline was 59.30±13.50 μm (median 60.5, range 32-84) and increased to 64.75±12.31 μm (median 69, range 41-82) at 1-month follow-up (p=0.012) and to 67.75±15.52 μm (median 71, range 36-93) at 3-month follow-up (p<0.001) (Table 1) (Figure 13). On the other hand, analyzing the control area, the ONL thickness was 51.61±10.91 μm at the baseline (median 55.5, range 22-69) and it did not show any significant change during the follow-up (53.39±12.29 μm, median 54, range 23-65 [p=0.645], and 53.56±12.31 μm, median 58, range 17-66 [p=0.204] at 1-month and 3-month follow-up, respectively).

Interobserver variability between readers was excellent for all measurements (ICC = 0.965 [0.912-0.986]).

No significant changes during the follow-up were observed analyzing the mean retinal thickness and choroidal thickness in the treated area (p=0.238 and p=0.450, respectively) (Table 1).

Analyzing FAF images, no development/extension of atrophic lesions was observed during the follow-up. Furthermore, no changes in the appearance of RPD were observed.

Systemic Safety Analysis

No topical and/or systemic side effects were reported from the patients during the 3-month follow-up. None of the patients developed a MNV in the treated eye, and no eye developed atrophic lesion during the follow-up detected using FAF. No other retinal changes in the treated area were disclosed using multimodal imaging modality.

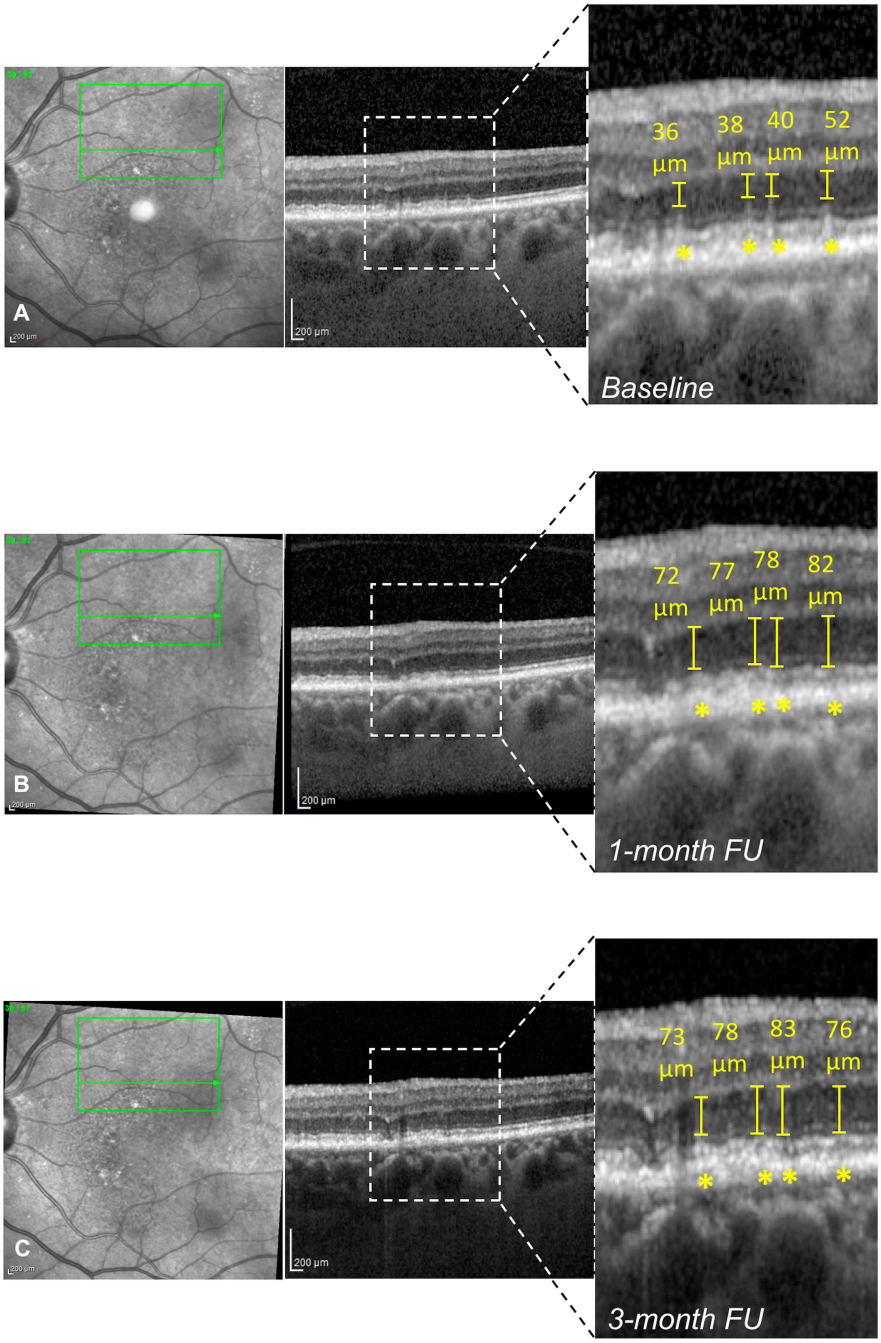

Figure 13. Structural optical coherence tomography (OCT) showing changes of the thickness of the outer nuclear layer (ONL) 1-month and 3-month after subthreshold laser treatment

A) Combined infrared reflectance (IR) and structural OCT showing the presence of 4 reticular pseudodrusen (RPD) in the treated area at the baseline (magnification). B-C) Combined IR and structural OCT showing the partial regression of RPD with increased ONL thickness at 1-month (B) and 3-month follow-up (C) (magnifications). [ref: Querques, G., Sacconi, R., Gelormini, F., Borrelli, E., Prascina, F., Zucchiatti, I., Querques, L., & Bandello, F. (2021). Subthreshold laser treatment for reticular pseudodrusen secondary to age-related macular degeneration. Scientific reports, 11(1), 2193]

Short-term safety of SLT on choriocapillaris: post-hoc analysis

A post-hoc analysis was performed in order to evaluate possible side effects of the SLT on the choriocapillaris perfusion density. The OCT-A images of the same cohort of the first study (Figure 14) were evaluated, after binarization of images. In detail, the percentage of the flow voids of the choriocapillaris was calculated (as explained in the Method's section) at the baseline, at 1-month follow-up, and at the end of the 3-month follow-up. The measurements were performed in three areas: in the total evaluated area, in the area of 1.27 mm^2 treated by SLT, and in the other area of 1.27 mm^2 with a similar distance from the fovea that was previously selected as a control area (i.e. area without treatment).

At the baseline, the mean percentage of flow voids of the total area was 24.60% ± 8.23% and showed no significant changes during the follow-up (p=0.167). In the treated area, the mean percentage of flow voids was 27.90% ± 12.17% and showed no significant changes during the follow-up (p=0.115). In the control area, the mean percentage of flow voids was 24.12% ± 8.11% and showed no significant changes during the follow-up (p=0.204) (Table 3).

Table 3. Percentage of flow voids of the choriocapillaris in the treated area, control area, and total area, measured at the baseline, 1-month follow-up and 3-month follow-up

	Baseline	**1 month FU**	**3 months FU**	**P value***
Flow Voids (%) Total area	24.60± 8.23	24.94 ± 8.56	27.83± 8.96	0.167
Flow Voids (%) Treated area	27.90 ± 12.17	28.76 ± 11.95	32.63 ± 13.86	0.115
Flow Voids (%) Control area	24.12 ± 8.11	24.27 ± 8.85	26.77 ± 8.66	0.204
P value§	0.641	0.574	0.464	

FU: follow-up.
*: Analysis of Variance (ANOVA) for paired samples.
§: ANOVA for independent samples.

Comparing the mean percentage of flow voids between different analyzed areas (i.e. total area, treated area, and control area), there was no statistical differences between the values was observed (Table 3).

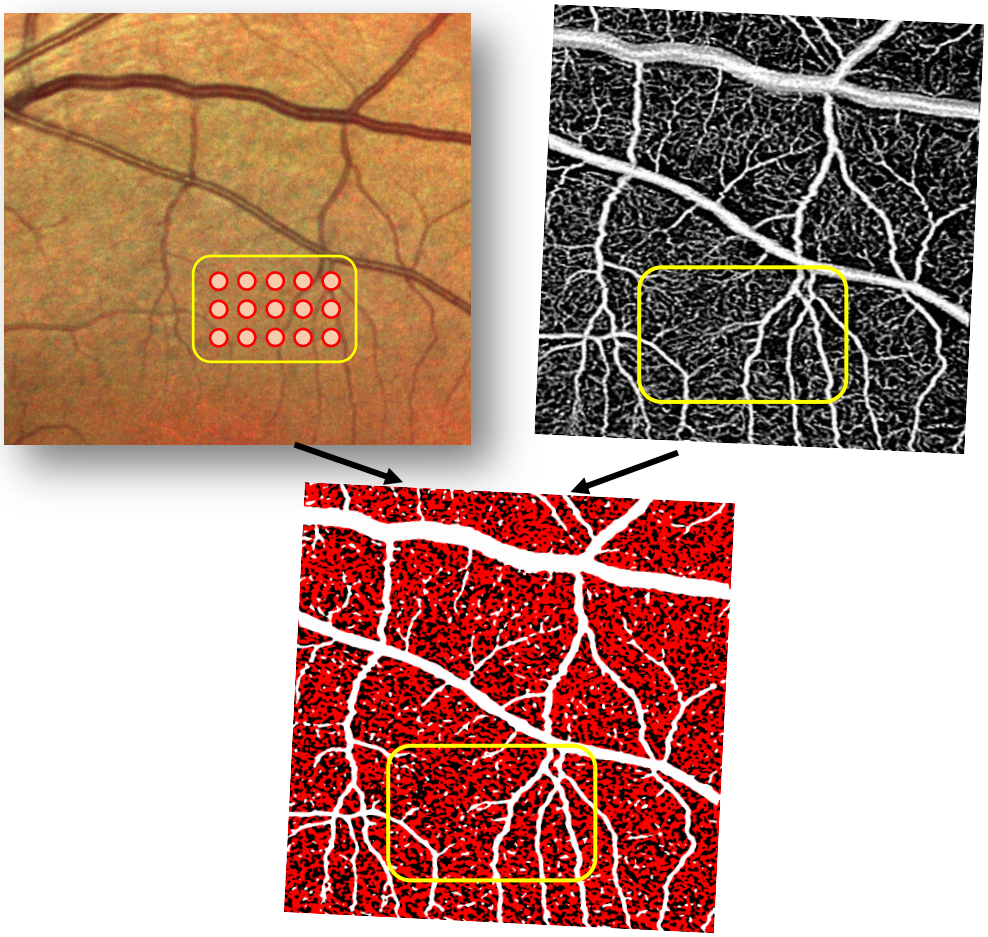

Figure 14. Analysis of the flow voids percentage of the choriocapillaris in the treated area

The treated area was selected based on the multicolor imaging (upper-left image) and transferred on the OCT-A image (upper right image). After that, the flow voids percentage of the choriocapillaris was evaluated as the percentage of black areas in comparison of total area, after binarization of the choriocapillaris image and exclusion of areas below the major retinal vessels (white areas).

Long-term safety and efficacy of SLT treating the whole macula

Patient demographics and main clinical findings

The enrollment of all patients in all 4 sites involved in the PASCAL2 clinical trial will be complete by the end of June 2022 (see Method's section). Here, I am reporting the preliminary data (6-month follow-up) of the all patients enrolled in the San Raffaele Scientific Institute.

Out of 50 patients, 10 eyes (10 patients) were included in the San Raffaele Scientific Institute (mean age 80.7±3.7 years, median 81, range 72-86). Seven patients were females, and 3 males. All patients were affected by neovascular AMD in the fellow eye. Four patients were randomized to the treatment group, whereas 6 patients were randomized in the sham group. Unfortunately, one patient of the sham group withdraw the study after 3 months due to personal problems not related to the study. For this reason, the analysis was performed on 9 patients (4 of the treated group and 5 of the sham group). At the baseline, no significant differences were disclosed between two groups in terms of BCVA, CMT, ChT, RPD number, ONL thickness, and MS ($p>0.1$ in all analyses).

At baseline examination, best-corrected visual acuity was about 20/25 Snellen equivalent (0.078±0.067 LogMAR), and did not showed significant changes at the end of the 6 months follow-up in both treated and sham groups (final BCVA of 0.120±0.044 LogMAR [p=0.465] and 0.075±0.050 LogMAR [p=0.500], respectively). All the demographics and main clinical feature were reported in Table 4 and 5 for the treated group and for sham group, respectively.

Table 4. Comparisons of anatomical and functional variables of the treated group between baseline, 3-month follow-up and 6 month follow-up

	Baseline		***3-month follow-up***	***6-month follow-up***
	mean ± SD	***P value****	**mean ± SD**	**mean ± SD**
BCVA, LogMAR	0.05±0.06	0.500	0.075±0.06	0.075±0.05
CMT, µm	279±17	0.024	282±11	275±16
Subfoveal ChT, µm	186±67	0.874	181±59	185±78
ONL thickness, µm	63.5±3.3	0.556	62.5±2.9	62.5±2.4
MS (macular area), dB	14.55±2.29	0.417	14.62±2.47	14.87±1.66
Fixation percentage (2°), %	47.2±7.3	0.164	47.5±15.2	36.25±19.3
Fixation percentage (4°), %	87.5±4.0	0.292	86.0±6.5	80.0±16.4

Table 5. Comparisons of anatomical and functional variables of the sham group between baseline, 3-month follow-up and 6 month follow-up

	Baseline		*3-month follow-up*	*6-month follow-up*
	mean ± SD	***P value****	**mean ± SD**	**mean ± SD**
BCVA, LogMAR	0.10±0.08	0.500	0.12±0.10	0.12±0.05
CMT, µm	271±30	0.399	269±25	274±26
Subfoveal ChT, µm	147±84	0.906	147±82	149±90
ONL thickness, µm	63.2±3.5	0.242	61.2±2.7	61.5±4.8
MS (macular area), dB	16.30±1.93	0.180	16.40±2.27	15.65±2.07
Fixation percentage (2°), %	32.5±6.6	0.164	36.0±9.4	40.0±9.4
Fixation percentage (4°), %	72.5±19.8	0.701	75.0±8.9	79.2±6.4

SD: standard deviation; BCVA: best-corrected visual acuity; IOP: intraocular pressure; CMT: central macular thickness; ChT: choroidal thickness; ONL: outer nuclear layer; MS: retinal sensitivity.
*: Analysis of Variance (ANOVA) for paired samples.

Functional changes after panmacular treatment

The primary outcome of the PASCAL2 trial was the efficacy of treatment at month 12, evaluated as functional changes in the retinal sensitivity on customized microperimetry. The preliminary results of our center at 6-month follow-up showed a trend of increase retinal sensitivity in the treated patients, whereas sham patients showed a trend of decrease retinal sensitivity. In detail, MS in the treated group was 14.55±2.29 dB at the baseline and 14.87±1.66 dB at the end of the follow-up (p=0.417), whereas MS in the sham group was 16.30±1.93 dB at the baseline and 15.65±2.07 dB at the end of the follow-up (p=0.180), (Table 4 and 5). Mean fixation % in 2° centered on the fovea was 47.2±7.3% and 32.5±6.6% for the treated and sham group, respectively, whereas in the 4° was 87.5±4.0% and 72.5±19.8% for the treated and sham group, respectively. No significant changes were observed at the end of the follow-up (Table 4 and 5).

Anatomical changes after panmacular treatment

In all patients, the whole macular area was treated sparing an anulus centered on the fovea of 1900 microns of radius. The evaluation of the RPD was performed in two ways. First of all, by means of structural OCT, the number of the RPD was evaluated in the whole treated area. RPD were counted as 47.2±5.9 at the baseline in the treated eyes, and 40.7±5.6 in the sham eyes. The distribution of RPD in the different stages was similar in both groups (Table 6 and 7). After the treatment, RPD in the treated eyes showed a different distribution in different RPD stages. In detail, there was a trend of reduction of stage 3 RPD, whereas an increased number of stage 1 RPD (table 6). The mean number of stage 3 RPD changed from 16.75±10.87 to 14.75±10.78 (p=0.057) The mean number of stage 1 RPD changed from 3.25±0.96 to 6.00±1.82 (p=0.214). On the other hand, no significant changes were disclosed in the sham group.

Analyzing the ONL thickness using the in-build software of Spectralis, no changes were disclosed in the treated eyes. In detail, mean ONL thickness was 63.5±3.3 μm at the baseline, and 62.5±2.4 μm at the end of the follow-up (p= 0.556). On the other hand, the sham eyes showed a slightly trend of reduction of the ONL thickness during the follow-up (from 63.2±3.5 to 61.5±4.8 μm, (p=0.242)).

By means of fundus autofluorescence, no changes were disclosed after the treatment. In particular, there was no development of geographic atrophy.

Table 6. Number of reticular pseudodrusen (RPD) according to the stage during the study follow-up, analyzing the treated eyes

	Baseline	**3 Month FU**	**6 Month FU**	**P value***
	mean ± SD	*mean ± SD*	*mean ± SD*	
Stage 1 RPD	3.25±0.96	6.00±1.82	6.00±1.82	0.214
Stage 2 RPD	23.75±9.11	23.75±8.14	23.50±9.04	0.912
Stage 3 RPD	16.75±10.87	15.50±10.54	14.75±10.78	0.057
Stage 4 RPD	3.50±1.73	2.75±0.96	2.75±1.50	0.250

RPD: reticular pseudodrusen; FU: follow-up.

*: Analysis of Variance (ANOVA) for paired samples.

Table 7. Number of reticular pseudodrusen (RPD) according to the stage during the study follow-up, analyzing the sham group

	Baseline	3 Month FU	6 Month FU	P value*
	mean ± SD	*mean ± SD*	*mean ± SD*	
Stage 1 RPD	4.25±3.30	4.00±2.45	4.00±3.16	0.900
Stage 2 RPD	22.75±2.36	23.00±1.63	22.25±2.06	0.673
Stage 3 RPD	11.75±3.59	12.00±3.83	11.75±2.87	0.963
Stage 4 RPD	2.00±0.82	2.50±1.73	3.00±1.41	0.630

RPD: reticular pseudodrusen; FU: follow-up.

*: Analysis of Variance (ANOVA) for paired samples.

Safety Analysis

No topical and/or systemic side effects were reported from the patients during the 3-month follow-up. None of the patients developed a MNV in the treated eye, and no eye developed atrophic lesion during the follow-up detected using FAF. No other retinal changes in the treated area were disclosed using multimodal imaging modality.

DISCUSSION

In this thesis, I have described the results of the short-term safety and efficacy of subthreshold laser treating eyes with a peculiar phenotype of intermediate AMD in one eye (i.e. presence of RPD) and the preliminary result about the long-term efficacy of this treatment. Generally, the results of our studies (both clinical trials and a post-hoc analysis) showed that the yellow subthreshold laser with the strategy of end-point management is a safe therapy in patients affected by RPD. In fact, no conversion to advance form of AMD (both neovascular AMD and geographic atrophy) happened during the follow-up of both clinical trials. Furthermore, from a functional point of view, no decrease of visual acuity and of macular sensitivity using microperimetry were disclosed in both clinical trials. Furthermore, anatomical data of the first pilot study, and the preliminary data of the second randomized clinical trial suggest a possible clinical effect of subthreshold laser treatment on RPD.

In 2005, Luttrull et al. reported for the first time the efficacy of "high-density/low-intensity" subthreshold laser treatment in patients with diabetic macular edeama.(Luttrull *et al*, 2005) By definition, subthreshold laser does not produce damage to the retina, and adverse effect related to the treatment are not know.(Parodi *et al*, 2006; Chen *et al*, 2008; Luttrull & Sinclair, 2014) This kind of laser selectively targets the RPE, and there are several evidences that it produce favorable outcomes in several diseases affecting the retina, as edema related to diabetes or to branch retinal vein occlusion, central serous chorioretinopathy, proliferative diabetic retinopathy and epiretinal membrane.(Parodi *et al*, 2006; Chen *et al*, 2008; Luttrull & Sinclair, 2014; Luttrull, 2020) Previous studies speculated that subthreshold laser act by "targeting, preserving, and normalizing" the RPE function. A recent publication of Luttrull et al(Luttrull & Sinclair, 2014) demonstrated that subthreshold laser could induce a restoration to the response of anti-VEGF injections in patients with neovascular AMD that previously developed a drug-tolerance to the treatment. Subthreshold laser on one hand perform this effect, on the other hand it do not cause any visible anatomical change to the RPE cells. In patients affected by diabetic macular edema involving the foveal area, subthreshold laser showed an excellent safety also in eyes with high visual acuity (20/20 Snellen equivalent) in which

the treatment was performed to minimize the risk of progressive visual decline due to the edema itself.(Luttrull *et al*, 2005) Furthermore, Vujosevic et al reported that no visible lesions are present after the treatment of diabetic macular edema with subthreshold laser using an infrared wavelength, and by subthreshold laser using a yellow wavelength (Vujosevic *et al*, 2015)

The paper of Luttrull et al.(Luttrull *et al*, 2015a) suggests that the effect of the subthreshold laser is due to an activation of the RPE function, suggesting a potential role in several macular diseases.

Starting from this point of view, we have conducted two different clinical trials. The first one was a pilot study, in which we wanted to primary analyze the safety of yellow subthreshold laser in patients affected by RPD. The second one was aimed to test the long-term effectiveness and safety of the same treatment, using a panmacular treatment and a randomization of the sample.

In the development of RPD, there is a great impairment of the RPE cells and of the ellipsoid zone (previously known as IS/OS interface) inducing a great reduction of the sensitivity of the retina starting from the first steps of the disease. In fact, Querques et al, comparing patients with intermediate AMD affected by RPD to patients with drusen, demonstrated that both groups showed high visual acuity, but RPD patients showed a reduced retinal sensitivity. (Querques *et al*, 2014) Moreover, RPD patients are characterized by a greater risk of progression to the late stages of AMD, both neovascular stage and geographic atrophy stage (Cicinelli *et al*, 2018b; Rabiolo *et al*, 2019; Zweifel *et al*, 2010a) The primary factor in the pathogenesis of RPD seems to be related to a RPE cell disfunction. Since subthreshold laser seems to induce an activation of the RPE function in AMD patients (Luttrull *et al*, 2012; K. Luttrull & Dorin, 2012; Chang & Luttrull, 2020) Luttrull *et al*, 2015b) and an alteration of the RPE function seems to be the primary factor in the RPD development, the use of subthreshold laser could be an effective treatment to induce a RPD regression. This kind of treatment use a lower energy in comparison to the “classic” thermal laser, avoiding to induce a damage to the tissue around the treatment.(Dorin, 2004; Framme *et al*, 2002) For this reason a PASCAL scanning technology was used. PASCAL 577 uses the advantage of a yellow light (577 nm of wavelength) and also the most advances patterns of modern laser setting. The

yellow wavelength gives the advantage to target in a more selective way the RPE cells in comparison to the 561 or 532 nm lasers. The advantage is that, during the treatment, the power of the laser is located in a small area, permitting to use lower power and faster pulse.

The trial safety and retinal sensitivity were evaluated as the primary outcomes of the first PASCAL clinical trial. The primary outcome was about the retinal sensitivity of the treated area. Non worsening in the retinal sensitivity was disclosed, confirming the safety of the treatment. There were any kind of side effects (local and/systemic) at 3-month follow-up after the of laser usage. In particular, during the protocol, we monitored the possible progression rate to neovascular AMD using SD-OCT and OCTA. No neovascular membranes were observed in the treated area after three month of laser treatment. On the other hand, no functional improvement was disclosed in terms of visual acuity and retinal sensitivity in the treated area. Nevertheless, two important issues should be kept in mind. First, the treated area was far to the fovea, and for this reason no changes in visual acuity were disclosed. Secondly, the treated are was small (in order to test the safety), and probably for this reason and for the short-term follow-up we were not able to disclosed significant improvement of retinal sensitivity. On the other hand, a short follow-up in the pilot-study was essential in order to disclosed as soon as possible adverse events.

In order to test another aspect of the safety, we also performed an analysis of the perfusion density changes of the choriocapillaris after the subthreshold laser treatment. It is well known that conventional laser causes a thermal effect, resulting in the loss of the choriocapillaris. However, in our post-hoc analysis, no changes in the perfusion density were disclosed after treatment in both the treated and control areas, confirming again the safety of the subthreshold laser, and the no visible effects.

The safety of the subthreshold laser treatment was confirmed also by the second clinical trial with a longer follow-up. Also in this study, we did not observed any systemic or local side effects. Furthermore, no conversion to late stages of AMD (neovascular stage or geographic atrophy stage) was disclosed, and no worsening in the retinal sensitivity was shown also using a panmacular treatment.

Considering the efficacy outcomes, we disclosed interesting retinal morphology changes caused by the subthreshold laser using multimodal imaging during follow-up of the first clinical trial, that were confirmed in the second trial. Qualitatively, we noticed a regression of the RPD third stage and simultaneously a RPD first stage increase after three months of laser treatment. Complementarily, from a quantitative point of view, we observed an increase thickness of outer retinal nuclear layer (ONL) probably due to the regression of the RPD which is partly reabsorbed by the laser stimulated EPR. Contrarily, the results showed no differences in the distribution of RPD among stages, and no differences in the thickness of ONL before and after the treatment in the area without treatment (i.e. the control treatment). Zweifel et al.(Zweifel *et al*, 2010b) defined a RPD grading system by dividing them into 3 stages: in stage 1 there is an hyper-reflective material accumulation above the EPR up to the inner segments (IS) and outer segments (OS) of the photoreceptors, in stage 2 the hyper-reflective material accumulate causing an alteration of the IS/OS interface and finally in stage 3 the material broke through the IS/OS interface. Subsequently Querques et al. described stage 4 of the RPD where there is a rupture of the RPD and release of hyper-reflective material inside the retinal layers followed, over time, by atrophy of EPR and choriocapillaris.(Querques *et al*, 2012b, 2011) Indeed, Spaide (Spaide, 2013) showed that patients with RPD regression due to the progression of RPD themselves were affected by outer retinal atrophy, matching a thinning of the ONL. Therefore the natural course of the RPD leads to atrophy of both the EPR and the choriocapillaris which in turn could be complicated by a neovascularization. Then, a downstaging of RPD induced by subtreshold laser with a concomitant thickening of the ONL seems to be related to a real regression of the disease, reducing the risk to develop a progression of the AMD staging to a neovascular form or to geographic atrophy.

The preliminary data of the long-term PASCAL clinical trials confirmed and expanded the previously suggested data of the pilot study. First of all, there is a trend in the retinal sensitivity changes during the treatment. Although not statistically significant due to the low number of patients included in the preliminary data (only our center out of 4 centers was included in this in interim analysis, sham patients showed a trend of reduction of retinal sensitivity of the macular area, instead treated patients showed a trend of slight

improvement of the retinal sensitivity. From an anatomical point of view, our data suggested a regression of the stage of RPD (mainly from stage 3 to stage 1) in the treated eyes, whereas no significant changes were disclosed in the sham eyes. Finally, no changes in the ONL was observed in the treated eye. This data could seem different from what was reported in the pilot study. However, the strategy of the measurement was different. In the pilot study, we manually measure the distance between RPD and the upper bord of the ONL. This was performed in that way because no automatic measurements were possible in the treated small extrafoveal area. On the other hand, in the second clinical trial, we used the automatic in-build software of the Spectralis in order to measure the ONL segmentation (from the upper bord of ONL to the external limiting membrane). This was possible because the treatment was perform in the whole macular area. In this second way, the reabsorption of the RPD played a marginal role in the ONL thickness of the whole area, and, thus, no changes were disclosed after treatment. However, no changes could be considered as a good result. Indeed, as discussed before, Spaide et al.(Spaide, 2013) reported that the progression of RPD is characterized by a decrease of ONL thickness, due to the atrophy of the outer retina. Also our data of the sham group confirmed this aspect, showing a trend of reduction of the ONL in the sham group during the time. Certainly, the final results of the clinical trial with the data of the whole sample and a longer follow-up are mandatory in order to confirm our preliminary analysis.

A recent study, the LEAD clinical trial, investigated a possible therapeutic role of subthreshold nanosecond laser (SNL) in patients affected by intermediate AMD.(Guymer *et al*, 2019) At the end of that study, the SNL demonstrated the safety of the therapy, but no efficacy in reducing the rate of the progression to advanced-stages of AMD in patients with bilateral drusen. Nevertheless, their post-hoc analyses demonstrated an increase rate of progression to advanced-stage of AMD in eyes displaying RPD.(Guymer *et al*, 2019) Being a post-hoc analysis and not a primary study outcome, this last data should be taken with caution. Moreover comparison between the LEAD study and our PASCAL and PASCAL2 studies are not possible because the laser treatment is completely different: LEAD used a subtreshold nanopulsed laser, whereas we used subtreshold continuous laser. The effect on RPD could be different due to the different mechanism of action used by the two different lasers.

Even if the first pilot study and the preliminary results of the long-term randomized clinical trial showed promising results, it is mandatory to show the limitations of our study. Limitations are mainly related to the relative small sample size, and the possible error of the "Heidelberg tracking system" used in the follow-up mode. Finally, we have investigate only a particular phenotype of intermediate AMD patients (i.e. only RPD without drusen). We have selected this peculiar phenotype in order to exclude possible confounding factors due to the presence of drusen. However, being the mixed phenotype (RPD plus drusen) more frequent, further studies are recommended to validate the effect of subthreshold treatment also on RPD in the contest of drusen appearance.

In conclusion, this thesis confirmed the safety of subthreshold Pascal Synthesis 577nm in the treatment of RPD and suggested a potential efficacy from an anatomic point of view. Indeed, Pascal Synthesis 577nm induced a RPD regression with no atrophic changes of the outer retinal layers. However, the final data of the clinical trials considering the patients of all 4 involved centers and the whole 1-year follow-up are mandatory to confirm our results.

MATERIALS AND METHODS

Short-term safety and efficacy of SLT

In order to test the safety of SLT in the treatment of patients affected by RPD secondary to intermediate AMD, we started performing the PASCAL clinical trial. This is a single-center, non-randomized, pilot study including adults admitted to the Department of Ophthalmology of University Vita-Salute San Raffaele in Milan, Italy, who are suffering from RPD secondary to dAMD.

The trial was conducted in accordance with the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of San Raffaele Hospital (approved on 05/05/2016). The trial was registered on ClinicalTrials.gov (ID NCT02800356, registered on 15/06/2016). All study participants provided written informed consent. The study was conducted in the Medical Retina & Imaging Unit of the Department of Ophthalmology of University Vita-Salute, IRCCS Hospital San Raffaele in Milan, Italy, between June 2016 and September 2019.

Briefly, we included patients aged more than 50 years old, with a diagnosis of dAMD and the presence of RPD. We excluded patients with evidence of GA or MNV in the included eye, any prior treatment for AMD in the included eye (aside from antioxidants), and opacities of the ocular media that not permit high-quality imaging examinations.

In cases where both eyes were eligible, the eye with the worse BCVA at baseline was selected as the study eye. If both eyes have the same BCVA, it was recommended to select the right eye as the study eye.

Subjects had the right to withdraw from the study at any time, for any reason, without jeopardizing their medical care.

Study protocol

The study protocol is summarized in Table 8. After obtaining informed consent and after the screening visit, at the Baseline (Day 0) all patients were evaluated with a complete ophthalmic examination, including Best Corrected Visual Acuity (BCVA) using Snellen charts, slit lamp examination, fundus examination (by indirect

ophthalmoscopy) and intraocular pressure measurement (IOP). As imaging protocol, all patients were evaluated using Spectral Domain Optical Coherence Tomography (SD-OCT), fundus autofluorescence (FAF) in the area later treated with laser, microperimetry in a customized area later treated with laser. An extrafoveal area of 1.27 mm^2 (½ of a disk area, disk area= 2.54 mm^2) was treated using yellow subthreshold laser (Pascal Synthesis 577 system, Topcon Corporation, Tokyo, Japan). SD-OCT and FAF images were performed using Spectralis HRA + OCT (Heidelberg Engineering, Heidelberg, Germany), whereas microperimetry was performed using MP-1 (Nidek Technologies, Padova, Italy).

All patients were evaluated at 1-month and 3-month follow-up, with a complete ophthalmic examination, including SD-OCT, FAF, and microperimetry.

Table 8. Timing of study assessment

VISIT	Screening	Treatment (baseline)	Follow-up	Follow-up
POINT OF TIME	Between -14 days and day 0	day 0	Month 1	Month 3
ASSESSMENT				
Inclusion/exclusion criteria	X			
Informed consent	X			
Demographic data	X			
Medical history	X			
Concurrent medications	X			
Ophthalmic history	X			
BCVA at 4 meter prior to dilation	X	X	X	X
Slit lamp examination	X	X	X	X
Fundus examination	X	X	X	X
IOP measurement	X	X	X	X
Macular sensitivity by microperimetry	X	X*	X	X
FAF by BAF	X	X	X	X
Structural SD-OCT	X	X	X	X

Subthreshold laser treatment	X		
Adverse events assessment	X	X	X

BCVA: Best-corrected visual acuity; IOP: Intra-ocular pressure; FAF: Fundus autofluorescence; BAF: Blue autofluorescence; SD-OCT: spectral-domain optical coherence tomography

* If not executed on screening visit

[ref: Querques, G., Sacconi, R., Gelormini, F., Borrelli, E., Prascina, F., Zucchiatti, I., Querques, L., & Bandello, F. (2021). Subthreshold laser treatment for reticular pseudodrusen secondary to age-related macular degeneration. Scientific reports, 11(1), 2193]

Subthreshold laser treatment

All the treatments were performed by an expert senior author (GQ). The treatment was performed using the Pascal Synthesis 577 system. During the treatment, the investigator identified the threshold layer within the vascular arcades but outside the central fovea. In detail, the threshold level output power was set to obtain barely visible burn at approximately 200-250mW using the titration mode. After that, the investigator identified an area inside the vascular arcades affected by RPD. The irradiation was conducted on this area after switching over to Endpoint Management (~30% of the power of the barely visible burn) with a pattern of 5 x 3 spots (area of 1.27 mm^2). Figure 15 shows a representative fundus schema and laser area of the treatment.

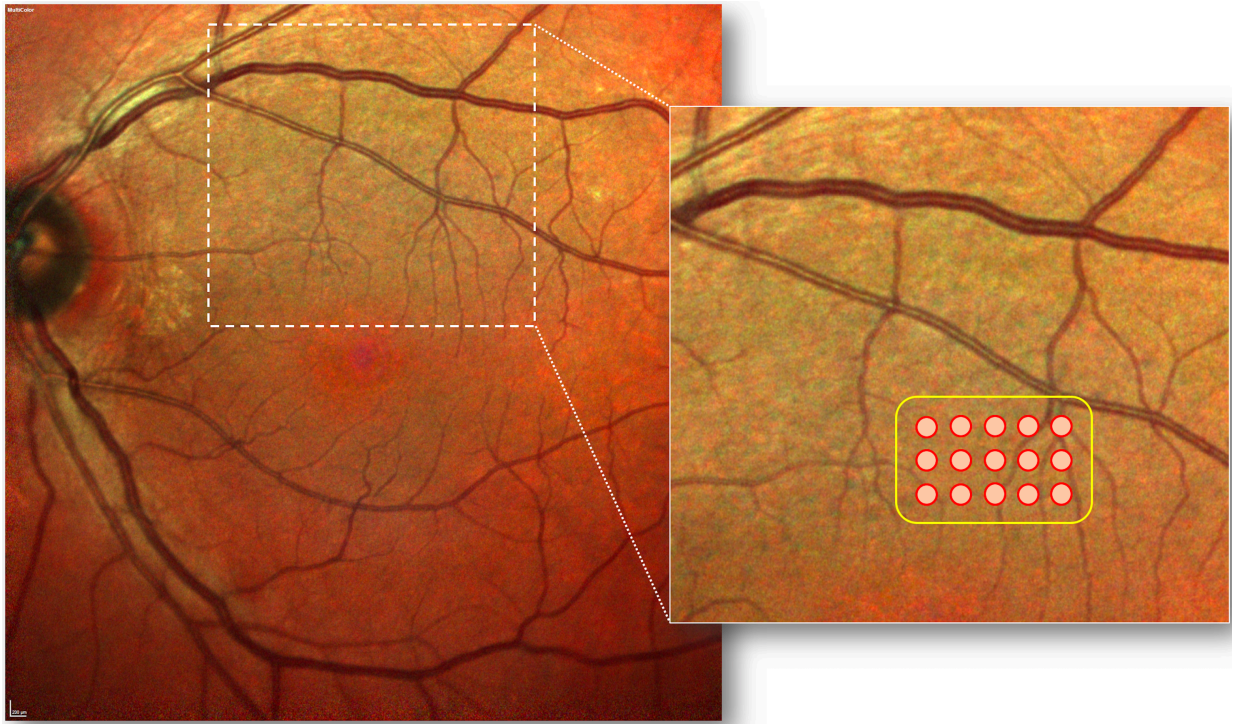

Figure 15. A representative case of an included patient affected by reticular pseudodrusen in the left eye.

An extrafoveal area of 1.27 mm2 (½ of a disk area, disk area= 2.54 mm2) was selected and treated using yellow subthreshold laser (Pascal Synthesis 577 system, Topcon Corporation, Tokyo, Japan). The treatment was performed using the Endpoint Management (~30% of the power of the barely visible burn) with a pattern of 5 x 3 spots (area of 1.27 mm2). [ref: Querques, G., Sacconi, R., Gelormini, F., Borrelli, E., Prascina, F., Zucchiatti, I., Querques, L., & Bandello, F. (2021). Subthreshold laser treatment for reticular pseudodrusen secondary to age-related macular degeneration. Scientific reports, 11(1), 2193]

Fundus autofluorescence and Spectral Domain Optical Coherence Tomography

FAF images were used to detect the possible appearance of GA (defined as a hypo-autofluorescence area) in the treated area during the follow-up. SD-OCT images were used to analyze the outer retinal morphology in the treated area, including evaluation of the stages of RPD at the baseline and during the follow-up as previously reported24, and the thickness of the outer nuclear layer (ONL) at the baseline and during the follow-up. Briefly, stage 1 RPD are characterized by diffuse deposition of hyperreflective material between the RPE and the inner/outer segments (IS/OS) boundary; stage 2 is characterized by accumulated material that alters the IS/OS boundary; stage 3 is characterized by thicker and conical appearance of deposited material passing through the IS/OS boundary; stage 4 is characterized by fading of the material due to the reabsorption and migration within the inner retinal layers(Querques *et al*, 2012a). The ONL thickness was manually measured in the correspondence of each RPD in the treated area; the mean value was considered for the statistical analysis. All measurements were performed by two independent and experienced readers (FG and EB). The grading of the RPD stage was performed by the same two expert readers. In those cases in which the two graders did not agree on a single consensus result, the final decision was performed by a senior author (GQ).

The retinal thickness and choroidal thickness (ChT) were also recorded. Retinal thickness was assessed in the central 1-mm-diameter circle of ETDRS thickness map [central macular thickness (CMT)] and in the treated area using the Spectralis Software (Heidelberg Eye Explorer, Version 1.10.4.0, Heidelberg, Germany). To achieve good choroidal visualization, enhanced depth imaging (EDI) structural OCT was used in all acquisitions. ChT was assessed in the subfoveal area and in the treated area by manually measuring the distance between Bruch's membrane and the sclerochoroidal interface to

identify the inner and outer boundaries of the choroid, respectively. To better investigate the treated area, a 49 horizontal raster dense linear B-scans, each composed by 16 averaged OCT B-scans (384 A-scans per line) at 30 μm intervals, covering an area of 15 degrees by 5 degrees was performed in each patient at the baseline, at 1-month and 3-month follow-up. All the sections were analyzed in the same place of the retina during the follow-up examinations. In detail, we have obtained the sections in the same place using the follow-up function available on the Spectralis Software (version: 1.10.4.0).

All the evaluations were also performed in another area of 1.27 mm^2 with a similar distance from the fovea that did not receive treatment. This area was used as a control area (i.e. area without treatment).

Microperimetry

Microperimetry was used to assess changes of retinal sensibility in the customized treated area. After training, all subjects underwent scotopic microperimetry examinations of the central retina in the study eye. Prior to testing, pupil dilatation and dark adaptation were performed. The eyes were fully covered with an opaque eye patch, followed by a waiting period of 30 minutes in a dark room (<0.1 lux). During the first examination, all patients underwent a fast test only for learning. The anatomical position of the fovea was determined by uploading the combined central infrared reflectance image and horizontal B-scan SD-OCT scan of the Spectralis to the MP-1S software. Using the optic nerve head and the major retinal vessels as landmarks for registration to the fundus real-time image of the MP-1S, test stimuli were placed around the fovea (Goldmann size V, 200 msec, 4-2 strategy, background luminance 0.0032 cd/m2, grid centered on the anatomical position of the fovea). Due to the testing under scotopic conditions, the fixation ring was not necessarily centered on the fovea. The following parameters were recorded in each timepoint: overall retinal sensitivity (MS) of the macular area and the MS of the treated area, fixation percentage calculated within the central 2° and 4°(Steinberg *et al*, 2015).

Clinical outcome measures

The primary outcome of the PASCAL trial was the safety of treatment measured as retinal sensitivity changes in the treated area 3 months after subthreshold laser treatment (i.e. change in retinal sensitivity).

Prespecified secondary outcomes included:

- Changes in the outer retinal morphology in the treated area using structural OCT during the follow-up;
- Change in mean BCVA during the follow-up;
- Change in the treated area using FAF during the follow-up;
- Adverse and Serious Adverse Events during the follow-up;
- Change in intraocular pressure during the follow-up.

Statistical Analysis

A sample size of 16 eyes has a greater than 80% power to identify a variation of 1.5 decibels in macular sensitivity between pre and post laser treatment assessments, with an estimated standard deviation of the change outcome of 2.0 and an alpha error of 0.05. Allowing an additional 20% of the estimated sample size in order to counter possible withdrawn patients, we estimated that 20 eyes would be required in our series.

All statistical analyses were performed using SPSS Statistics Version 20 (IBM, Armonk, New York, USA). In all patients, BCVA was converted to Logarithm of the Minimum Angle of Resolution (LogMAR) for statistical analysis. Categorical variables were expressed as count and percentage, whereas quantitative variables were expressed as mean±standard deviation. The agreement between individual measurements from both readers was performed using the intraclass correlation coefficient (ICC; 95% CI). The Gaussian distribution of continuous variables was verified with the Kolmogorov-Smirnov test. Comparisons of BCVA, CMT, subfoveal ChT, retinal and choroidal thickness in the treated area, number of RPD, ONL thickness, IOP, overall MS of the macular area and of the treated area, fixation percentage in the 2° and 4° between different time-points (baseline, 1-month follow-up and 3-month follow-up) were performed using the repeated measures Analysis of Variance (ANOVA) with Bonferroni post-hoc analysis. The comparison between stages of RPD at the baseline and at the end of the follow-up was performed using the Chi-squared test. In all analyses, p values <0.05 were considered statistically significant.

Short-term safety of SLT on the choriocapillaris: post-hoc analysis

Image analysis

In order to evaluate the safety of SLT on the choriocapillaris, we performed a post-hoc analysis or the PASCAL clinical trial. All patients were evaluated at the baseline and at 1-month and 3-month follow-up using OCT-A. In detail, OCT-A was performed using Swept-Source OCT-A PLEX®Elite 9000 (Carl Zeiss Meditec, Inc., Dublin, CA, USA). A 6x6 examination was performed centered to the treated area, and using a follow-up mode in the follow-up examination.

All acquisitions were performed using FastTrac™ retinal tracking technology to reduce motion artifacts. We used the automatic segmentation provided by the system software (20 μm thick starting 29 μm posterior to the Bruch's membrane reference) that was manually adjusted in those cases in which the fully automated algorithm failed to identify the correct location of Bruch's membrane. The minimum strength of OCT-A images was 7 out of 10.

All MultiColor and OCT-A images obtained at the baseline were exported into ImageJ 1.50 (National Institutes of Health, Bethesda, Maryland, USA) software. Using MultiColor images obtained at the baseline, the treated area was outlined using the polygon selection tool and transferred in baseline OCT-A images.

To evaluate the CC around the atrophic area, we analyzed two different regions at the baseline:

- The total evaluated area (the whole 6x6 image);
- The area of 1.27 mm^2 treated by SLT (i.e. the treated area);
- Another area of 1.27 mm^2 with a similar distance from the fovea that was previously selected as a control area (i.e. area without treatment).

The image-processing algorithm to investigate the CC variables in the 3 regions of interest (ROI) is illustrated in Figures 16. The 6x6 mm en face OCT-A images of CC were exported(Byon *et al*, 2019) (resolution of 1024x1024 pixels) and binarized using the Phansalkar method (radius, 15 pixels) for quantitative image analysis of the signal voids, as previously reported.(Chu *et al*, 2019; Rabiolo *et al*, 2018; Borrelli *et al*, 2018; Sacconi *et al*, 2018) After thresholding and binarization, the morphology of the vessels was investigated using the percentage of flow voids, defined as the ratio of the area spared by vessels (i.e. black area) divided by the total area using binarized images.

In order to exclude possible shadowing and projection artifacts confounding the analysis, we excluded from the analysis the CC directly beneath RPE elevations and under

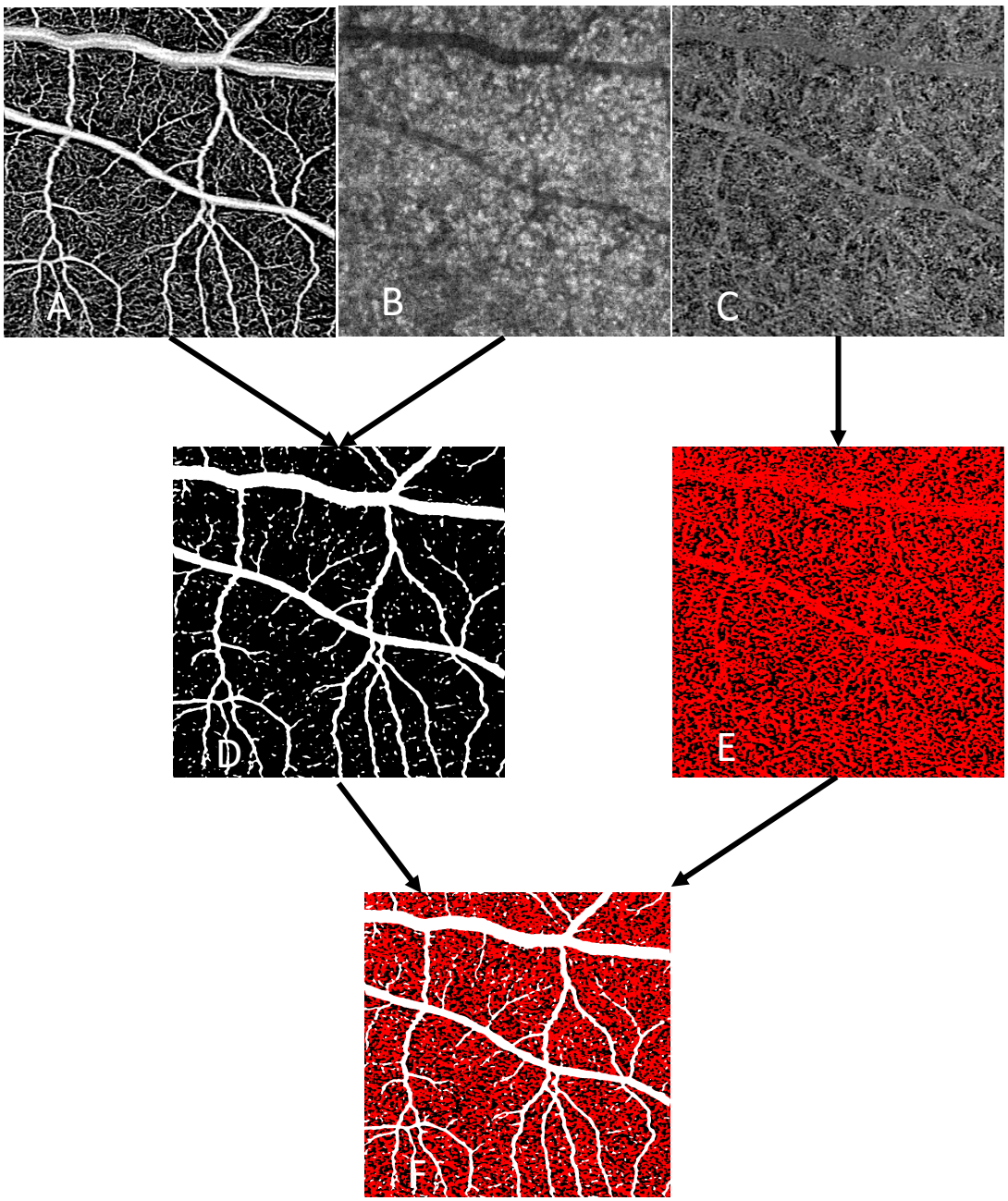

Figure 16. Representation of the OCT-A analysis.

En-face 6x6 OCT-A of the superficial capillary plexus (A) and en-face structural OCT of above the Ellipsoid Zone (~28 µm thick) were selected, binarized with the MaxEntropy threshold in order to visualize only the greater superficial retinal vessels and RPE elevations (D). En-face 6x6 OCT-A of the choriocapillaris slab (20 µm thick starting 29 µm posterior to the Bruch's membrane reference) (C) was selected and binarized using the Phansalkar method (radius, 15 pixels) (E). Finally, all obtained images were matched (F). Flow voids were calculated as the ratio

of the area spared by vessels (i.e. black area) divided by the total area using binarized images, after exclusion of white pixel from the analisis.

large superficial retinal vessels(Lane *et al*, 2016). To identify the RPE elevations, we created a map using en face structural OCT slab (~28 μm thick) above the Ellipsoid Zone (EZ). As previous reported(Zhao *et al*, 2017), this slab highlights elevation of the RPE (including drusen and subretinal drusenoid deposits) as hyperreflective lesions. The obtained image was enhanced with the "brightness/contrast improvement" function of ImageJ to increase the contrast between RPE elevation and the surrounding non-drusen area, and then binarized(Borrelli *et al*, 2018). To identify the greater superficial retinal vessels, the en face OCT-A image of the superficial capillary plexus (SCP) (starting from 3 μm below the internal limiting membrane to 15 μm below the inner plexiform layer) was imported in ImageJ, and the MaxEntropy threshold was applied to visualize only the greater superficial retinal vessels (Figure 16)(Borrelli *et al*, 2018).

Statistical Analysis

All statistical analyses were performed using SPSS Statistics Version 20 (IBM, Armonk, New York, USA). The Gaussian distribution of continuous variables was verified with the Kolmogorov-Smirnov test. Comparisons of flow void percentage between different time-points (baseline, 1-month follow-up and 3-month follow-up) were performed using the repeated measures ANOVA with Bonferroni post-hoc analysis. The comparison between flow voids percentage between the three analyzed areas (i.e. total area, treated area, and control area) were performed using ANOVA for independent samples. In all analyses, p values <0.05 were considered statistically significant.

Long-term safety and efficacy of SLT treating the whole macula

After the results of the first PASCAL study that confirmed the short-term safety of SLT, we started performing the PASCAL2 clinical trial. The objective of this study was to establish the effectiveness of subthreshold laser treatment in increase/prevent the decrease of the retinal sensibility in patients with reticular pseudodrusen, and to reduce the progression of RPD to atrophy.

This is a prospective, randomized, controlled, longitudinal, interventional multicentric study. All subjects were randomized in one of the two arms of the study (subthreshold laser group or sham group) in a 1:1 ratio. The randomization was performed by RS, not informing the patient about the assigned group. The randomization was performed before the beginning of the study, using a pre-specified sequential list of the ID subjects (from number 1 to number 50). In detail, a simple random sample was performed using SPSS Statistics Version 20 (IBM, Armonk, New York, USA). Twenty-five of the 50 subjects of the pre-specified list were extracted using the simple random sample method and were assigned to the subthreshold laser group before the beginning of the study. The other 25 subjects will be assigned to the sham group. The patient was not informed about the arm it was assigned (i.e. single blind study, for the patient). For sham group, the exact laser procedure will be performed, except that short bursts of the light from the retinal illumination system on the laser device will be used instead of the laser beam.

Four different centers were involved:

- Department of Ophthalmology, University Vita-Salute San Raffaele, Milan, Italy → 10 patients
- Fondazione Bietti, Rome, Italy → 20 patients
- Department of Ophthalmology, University of Genova, Italy → 10 patients
- Unit Patologie Retiniche - Fondazione PTV, University Tor Vergata, Rome, Italy → 10 patients

The trial was conducted in accordance with the Declaration of Helsinki and the study protocol was approved by the Ethics Committee of San Raffaele Hospital (approved on 10/02/2021) and of the local Ethics Committee of the other 3 involved centers. The promotor and coordinator center was the University Vita-Salute San Raffaele. This trial was registered on ClinicalTrials.gov (ID NCT04847635, registered on 19/04/2021). All study participants provided written informed consent. The inclusion started from 14/04/2021 in San Raffaele Hospital, and the enrollment will be complete in all 4 centers by the end of June 2022.

The inclusion criteria were:

1. 50 years or older;
2. Presence of RPD secondary to AMD;
3. BCVA between 20/20 and 20/400 inclusive;

4. Clear ocular media;
5. Ability to provide informed consent and attend all study visits;

The exclusion criteria were:

1. Presence of Geographic Atrophy;
2. Evidence of choroidal neovascularization in the treatment eye;
3. Any prior treatment for AMD, aside from antioxidants;
4. Any media opacity, which may interfere with viewing by the laser surgeon of the target structures;
5. Neovascular glaucoma or glaucoma caused by congenital angle anomalies;
6. Any other ocular condition that would progress in the study period and confound visual acuity assessment
7. Any intraocular surgery 3 months of entry;
8. Any prior thermal laser in the macula;
9. History of vitrectomy, filtering surgery, corneal transplant or retinal detachment surgery;
10. Participation in an investigational drug, biologic, or device study within 6 Months prior to Baseline;

In cases where both eyes are eligible, the eye with the worse BCVA at the baseline was selected as the study eye. If both eyes have the same BCVA, the eye was selected flipping a coin.

Subjects had the right to withdraw from the study at any time, for any reason, without jeopardizing their medical care.

Study protocol

After obtaining informed consent, the following study assessments were performed within 14 days prior to Day 0:

- BCVA at 4 meters prior to dilatation
- Slit lamp examination
- Fundus examination (by indirect ophthalmoscopy)

- Intraocular pressure (IOP)

- Spectral Domain Optical Coherence Tomography (SD-OCT) using Spectralis (Heidelberg engineering, Heidelberg, Germany)

- Blue autofluorescence (BAF) in the area later treated with laser using Spectralis (Heidelberg engineering, Heidelberg, Germany)

- Swept-Source OCT-angiography (OCT-A) using PLEX Elite 9000 (Carl Zeiss Meditec Inc., Dublin, CA, USA).

- Microperimetry in a customized area later treated with laser using MP-1 (Nidek Technologies, Padova, Italy).

After obtaining informed consent, the same procedure was performed at Day 0 (baseline). Of note, screening visit and Day 0 can be performed on the same day (in that case, the examination was performed once).

All subjects will be randomized in one of the two arms of the study (subthreshold laser group or sham group) in a 1:1 ratio (as reported before). Treatments were performed on the same day of baseline using the subthreshold laser Pascal Synthesis 577 (Topcon Corporation, Tokyo, Japan). For sham group, the exact laser procedure was performed, except that short bursts of the light from the retinal illumination system on the laser device will be used instead of the laser beam.

During the study, all patients underwent genotype determination in the AMD risk alleles. In detail, previous study demonstrated that rs10490924 (A69S variant of ARMS2) and rs11200638 (HTRA1) were associated with an increased risk of RPD, whereas rs1061170 (Y402H of CFH) was associated with a reduced risk of RPD(Lin *et al*, 2018). For this reason, these 3 AMD-associated SNPs were tested. However, due to technical problems related to the sequencing of all the samples together, we are still waiting for the results.

Follow-up visits and re-treatments were planned at: month 3 +/- 7 days , month 6 +/- 7 days, month 9 +/- 7 days, month 12 +/- 7 days. During the follow-up visits and re-treatments, the following procedures were performed:

- BCVA at 4 meters prior to dilatation

- Slit lamp examination

- Fundus examination (by indirect ophthalmoscopy)

- Intraocular pressure (IOP)

- Spectral Domain Optical Coherence Tomography (SD-OCT) using Spectralis (Heidelberg engineering, Heidelberg, Germany)

- Blue autofluorescence (BAF) in the area later treated with laser using Spectralis (Heidelberg engineering, Heidelberg, Germany)

- Swept-Source OCT-angiography (OCT-A) using PLEX Elite 9000 (Carl Zeiss Meditec Inc., Dublin, CA, USA).

- Microperimetry in a customized area later treated with laser using MP-1 (Nidek Technologies, Padova, Italy).

- Re-treatment using the same procedure of the baseline, in the same area (except in the case of contraindications or development of adverse events). Patients assigned to the treatment arm were treated by the laser in all follow-up evaluations. In patients assigned to the sham arm, the light from the retinal illumination system on the laser device were used instead of the laser beam in all follow-up evaluations.

At the final follow-up Visits (month 12 +/- 7 days), the same procedures were followed, expect of the re-treatment.

Subthreshold laser treatment

All treatments were performed by RS in San Raffaele Hospital, and by the local PI in the others centers. Treatments were performed using the subthreshold laser Pascal Synthesis 577 (Topcon Corporation, Tokyo, Japan) on a customized macular area, including an anulus centered of the fovea of 6000 microns of external diameter, and 3800 microns of internal diameter. The treatment was performed by 192 spots, of 200 microns of diameter (Figure 17). The investigator identified the threshold layer within the vascular arcades but outside the central fovea. In detail, the threshold level output power was set to obtain barely visible burn at approximately 200-250mW using the titration mode. The irradiation was conducted in the customized area after switching over to Endpoint Management (~30% of the power of the barely visible burn). Figure 17 shows a representative fundus schema and laser area of the treatment.

For sham group, the exact laser procedure was performed, except that short bursts of the light from the retinal illumination system on the laser device will be used instead of the laser beam.

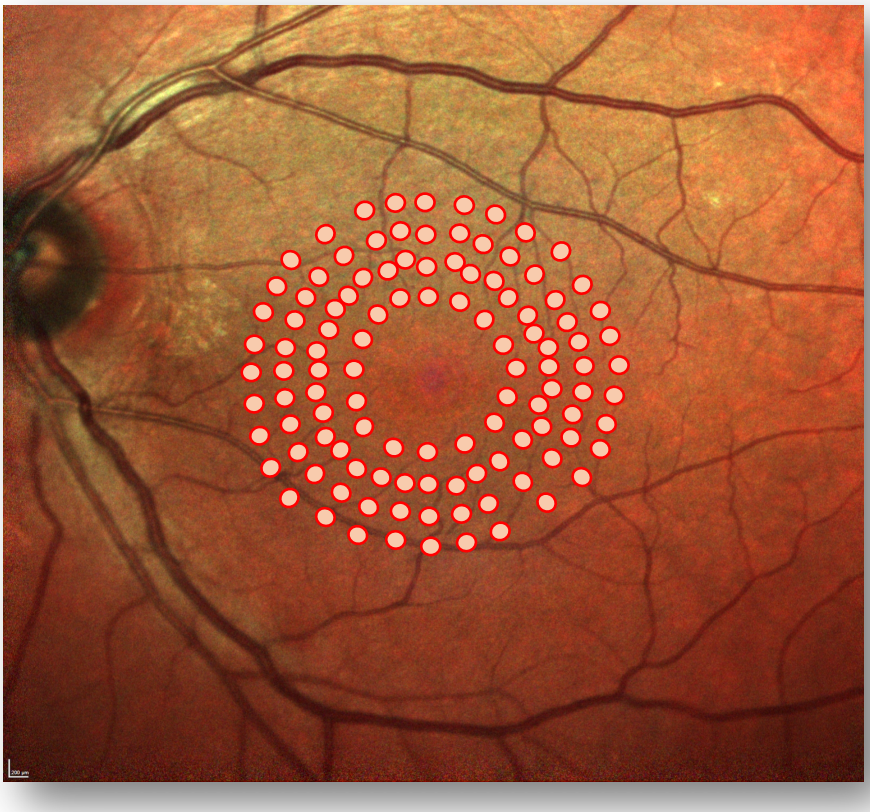

Figure 17. A representative case of an included patient affected by reticular pseudodrusen in the left eye.

An anulus centered of the fovea of 6000 microns of external diameter, and 3800 microns of internal diameter was selected. The treatment was performed by 192 spots, of 200 microns of diameter, using yellow subthreshold laser (Pascal Synthesis 577 system, Topcon Corporation, Tokyo, Japan). The treatment was performed using the Endpoint Management (~30% of the power of the barely visible burn).

Fundus autofluorescence and Spectral Domain Optical Coherence Tomography

FAF images were used to detect the possible appearance of GA (defined as a hypo-autofluorescence area) in the treated area during the follow-up. SD-OCT images were used to analyze the outer retinal morphology in the treated area, including evaluation of the stages of RPD at the baseline and during the follow-up as previously reported(Querques *et al*, 2012a), and the thickness of the outer nuclear layer (ONL) at the baseline and during the follow-up. The ONL thickness was automatically measured the area in the anulus between 1500 and 3000 microns of radius, using the in-build software of Spectralis (Heidelberg Eye Explorer, Version 1.10.4.0, Heidelberg, Germany). An expert senior author (RS) controlled the automatic segmentation provided by the software, and, eventually corrected the segmentation in case of error. The grading of the RPD stage was performed by two expert readers. In those cases in which the two graders did not agree on a single consensus result, the final decision was performed by a senior author (GQ).

The ChT and CMT analyses at the baseline, at 3-month, 6-month, 9-month, and 12-month follow-up were performed using the same protocol protocol of the first PASCAL pilot study, as reported before (see previous Section).

Microperimetry

Microperimetry was performed at the baseline, at 3-month, 6-month, 9-month, and 12-month follow-up using the same protocol of the first PASCAL pilot study, as reported before (see previous Section).

Clinical outcome measures

The primary outcome of the PASCAL2 trial was the efficacy of treatment at month 12. In detail the primary outcome is represented by functional changes in the retinal sensitivity on customized microperimetry (objective test, not influenced by the operator) from day 0 to month 12.

Secondary outcomes are:

1) anatomical changes using structural SD-OCT;

2) rate of progression to advance AMD (GA and/or neovascular AMD);

3) changes in BCVA;

4) changes in SW-FAF images;

5) changes in Perfusion Density of choriocapillaris analyzed using OCT-A;

6) presence of Adverse Events (e.g. development of CNV);

7) Association between AMD-associated SNPs and response to the treatment.

Finally, also the safety outcomes are analyzed:

1) Incidence of adverse events;

2) Serious adverse events;

3) Moderate or significant visual acuity loss;

4) Slit lamp findings;

5) Crystalline lens changes in phakic eyes;

6) Intraocular pressure;

7) Fundus findings.

Statistical Analysis

Fifty patients were randomized in two different arms: subthreshold laser group or sham group (25 subjects for each group). A sample size of 20 subjects for each group has a greater than 80% power to identify a variation of 1.0 decibel in retinal sensitivity between pre and post laser treatment assessments, with an estimated standard deviation of 1.0 and an alpha error of 0.05. Allowing an additional 20% of the estimated sample size in order to counter possible withdrawn patients, we estimated that 25 eyes for each group would be required in our series.

All statistical analyses were performed using SPSS Statistics Version 20 (IBM, Armonk, New York, USA). Categorical variables were expressed as count and percentage, whereas quantitative variables were expressed as mean±standard deviation. Comparisons of BCVA, CMT, subfoveal ChT, number of RPD, ONL thickness, overall MS of the macular area, fixation percentage in the 2° and 4° between different time-points (baseline, 3-month follow-up and 6-month follow-up) were performed using the repeated measures Analysis of Variance (ANOVA) with Bonferroni post-hoc analysis. In all analyses, p values <0.05 were considered statistically significant.

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