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Morphological and functional evaluation of Intermediate Age-related Macular Degeneration in the scope of the Epidemiological Coimbra Eye Study

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MORPHOLOGICAL AND FUNCTIONAL EVALUATION OF INTERMEDIATE AGE-RELATED MACULAR DEGENERATION IN THE SCOPE OF THE EPIDEMIOLOGICAL COIMBRA EYE STUDY

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LIST OF ACRONYMS

AMD – Age-related macular degeneration

BCVA – Best-corrected visual acuity

BrM – Bruch's membrane

CES – Coimbra Eye Study

CFP – Color fundus photographs

ELM – External limiting membrane

ERM – Epiretinal membrane

ETDRS – Early Treatment Diabetic Retinopathy Study

FAF – Fundus autofluorescence

GCL – Ganglion cell layer

HF – Hyperreflective foci

ILM – Inner limiting membrane

INL – Inner nuclear layer

IPL – Inner plexiform layer

IR – Infra-red

IRL – Inner retinal layer

ONL – Outer nuclear layer

OPL – Outer plexiform layer

ORL – Outer retinal layer

PED – Pigment epithelial detachment

PRL – Photoreceptor layer

RNFL – Retinal nerve fiber layer

RPD – Reticular pseudodrusen

RPE – Retinal pigment epithelium

SDD – Subretinal drusenoid deposits

SD-OCT – Spectral-domain optical coherence tomography

VMA – Vitreomacular adhesion

RESUMO

Introdução: A degenerescência macular relacionada com a idade (DMI), uma das principais causas de perda irreversível da visão em adultos mais velhos, é atualmente considerada uma doença neurodegenerativa da retina. Através de análise histopatológica e de tomografia de coerência ótica de domínio espectral (SD-OCT), alguns estudos demonstraram que, para além das alterações existentes no complexo RPE/coriocapilar, também é possível identificar degenerescência ao nível da retina interna em estádios iniciais da doença. Desta forma, o objetivo do nosso trabalho é realizar uma avaliação morfológica quantitativa detalhada de todas as camadas da retina e coróide em olhos com estádios iniciais de DMI, bem como correlacioná-la com dados clínicos e microestruturais, numa coorte de participantes retirada do estudo de base populacional de Coimbra relativo à DMI (*Coimbra Eye Study-CES).*

Métodos: Estudo transversal de base populacional. Todos os doentes incluídos no estudo de Incidência da DMI do *CES* (NCT02748824) com DMI precoce (estádios 2a, 2b e 3 da classificação de Roterdão) foram elegíveis para a presente análise. Todos foram submetidos a avaliação por SD-OCT com *Spectralis HRA+OCT* (*Heidelberg Engineering, Germany*) e os exames foram enviados para um centro de leitura para classificação. Neste estudo, a segmentação automática de oito camadas da retina foi aplicada à imagem completa do volume macular recorrendo ao programa *Heidelberg Eye Explorer.* Todas as camadas segmentadas foram revistas e corrigidas manualmente quando necessário. A espessura média dos círculos maculares central, interno e externo da grelha *ETDRS*, bem como o volume macular, foram extraídos para cada camada retiniana. A espessura subfoveal da coróide foi medida manualmente no mesmo programa. Recorrendo a modelos mistos lineares, foram procuradas diferenças para cada camada retiniana ao longo dos estádios de DMI precoce. Adicionalmente, foram avaliadas possíveis associações entre a espessura das camadas retinianas e a camada RPE/Bruch, a melhor acuidade visual corrigida (MAVC) e alterações qualitativas da retina no SD-OCT, com recurso a análises de regressão linear multivariada corrigidas para a idade, género, estado refrativo e estádio de DMI.

Resultados: Uma coorte de 347 olhos obtida de 234 doentes foi elegível para análise. A idade média dos doentes foi de 74.5±7.0 anos e 63.7% (149) eram do sexo feminino. Para avaliar as diferenças na espessura das camadas retinianas nos estádios de DMI precoce todos os olhos foram incluídos: 286 (82.4%) no estádio 2a, 20 (5.8%) no 2b e 41 (11.8%) no estádio 3. Através de modelos mistos lineares e depois de testar para diferenças aos pares entre os diferentes estádios de DMI separadamente por círculo macular, encontrámos uma tendência global para a diminuição da espessura das camadas internas da retina com o aumento do estádio, especialmente quando comparado o estádio 3 com o 2a: GCL (círculo interno; p=0.003), IPL (círculo interno; p=0.002) and ONL (círculo central; p=0.002). Por outro lado, a espessura da camada RPE/Bruch foi superior no círculo central no estádio 3, comparando com os estádios 2a e 2b (p=0.0001, p=0.002). Foram encontradas associações entre as espessuras das camadas da neuroretina com a RPE/Bruch, depois de selecionado o pior olho por doente (234 eyes: GCL(β=0.299,p<0.001), IPL(β=0.387,p=0.044),

OPL(círculo central:β=-0.182,p=0.04; círculo interno:β=0.200,p=0.012), ONL(β=0.218,p<0.001) and PRL(β=1.098,p<0.001)). Os pseudodrusen foram associados a uma diminuição da espessura da camada RPE/Bruch e da coróide (p<0.05), enquanto que os focos hiperrefletivos se associaram a um aumento da espessura das camadas INL e RPE/Bruch (p<0.05). Um valor inferior de MAVC foi relacionado com uma menor espessura das camadas ONL e IPL, mas com uma maior espessura das camadas PRL e INL no círculo macular central (p<0.05).

Discussão/Conclusão: Demonstrámos, numa grande coorte de doentes, que as alterações na retina interna são observadas precocemente na DMI, para além das alterações expectáveis na retina externa. Foi detetada uma diminuição da espessura de diversas camadas da neuroretina nos estádios precoces, nomeadamente com a progressão do estádio, sugerindo que a avaliação quantitativa da retina interna pode constituir um biomarcador de progressão da doença. Estes achados sustentam a existência de neurodegenerescência já em estádios iniciais de DMI. As associações encontradas entre a espessura/volume de algumas camadas neuroretinianas com a camada RPE/Bruch, sugerem que estas se relacionam proporcionalmente. Confirmámos, ainda, que os pseudodrusen constituem biomarcadores de um fenótipo específico de DMI relacionado com coróides mais finas, enquanto que os focos hiperrefletivos estão associados a um aumento da espessura da camada RPE/Bruch. As repercussões funcionais estão presentes já em estádios iniciais de DMI.

Palavras-chave: Degenerescência macular relacionada com a idade; Análise microestrutural com tomografia de coerência ótica de domínio espectral; Espessura das camadas retinianas; Pseudodrusen; Coróide.

ABSTRACT

Introduction: Age-related macular degeneration (AMD), a leading cause of irreversible vision loss in older adults, is today regarded as a neurodegenerative disease of the retina. Studies using histopathological and SD-OCT analysis have shown that there is progressive degeneration of the inner retina in the early stages of the disease, along with the changes in the RPE-choriocapillaris complex. The purpose of our study is to accomplish a detailed quantitative morphological evaluation of all retinal layers and choroid in early AMD-staged eyes and to correlate with clinical and microstructural data, in a cohort of participants from the AMD Coimbra Eye Study.

Methods: Population-based, cross-sectional study. All patients included in the Incidence AMD Coimbra Eye Study (NCT02748824) and with early AMD (Rotterdam system stages 2a, 2b, and 3) were eligible for the present analysis. All were submitted to SD-OCT examination with Spectralis HRA+OCT (Heidelberg Engineering, Germany) and the exams sent to a centralized reading center for classification. In the present study, automatic segmentation of eight retinal layers was applied to the complete macular volume scan of the included eyes using the Heidelberg Eye Explorer software. All segmented layers were reviewed, and manual correction was performed whenever necessary. The mean retinal thicknesses from the central field, from the inner and outer circles of the ETDRS grid, as well as the macular volume, were obtained for each retinal layer. Subfoveal choroidal thickness was measured manually in the same software. Using linear mixed models, we tested for differences in each retinal layer thickness across early AMD stages. Additionally, associations between the thickness of retinal layers and the RPE/Bruch layer, best-corrected visual acuity (BCVA), and qualitative SD-OCT features, were assessed by stepwise multivariate linear regression analysis, while adjusting for age, gender, refractive status, and AMD stage.

Results: A cohort of 347 eyes from 234 patients was eligible for analysis. The mean age was 74.5±7.0 years, and 63.7% (149) were female. To assess for differences in retinal layers thickness across early AMD stages all eyes were included: 286 eyes (82.4%) in stage 2a, 20 (5.8%) in 2b, and 41 (11.8%) in stage 3. With linear mixed models and after testing for pairwise differences between AMD stages separately for each macular circle, we found a global tendency for lower thickness of inner layers with higher stage, especially when comparing stage 3 to stage 2a: GCL (inner circle; $p=0.003$), IPL (inner circle; $p=0.002$) and ONL (central circle; $p=0.002$). In contrast, the RPE/Bruch layer was thicker in the central circle in stage 3, compared to 2a and 2b ($p<0.001$, $p=0.002$). Associations between neuroretinal layers and RPE/Bruch layer thicknesses were found, after selecting the worst eye per subject (234 eyes: GCL($\beta=0.299$,$p<0.001$), IPL($\beta=0.387$,$p=0.044$), OPL(central circle:$\beta=-0.182$,$p=0.04$; inner circle:$\beta=0.200$,$p=0.012$), ONL($\beta=0.218$,$p<0.001$) and PRL($\beta=1.098$,$p<0.001$)). Pseudodrusen were associated with thinner RPE/Bruch layer and choroid ($p<0.05$). Hyperreflective foci were associated with thicker INL and RPE/Bruch ($p<0.05$). Lower BCVA was associated with thinner ONL and IPL and with thicker PRL and INL in the central circle ($p<0.05$).

Discussion/Conclusion: We demonstrated in a large cohort of patients that changes in the inner retina are observed early in AMD, in addition to the expected changes in the outer retina. Thinning

of several neuroretinal layers were detected in the early stages, and further decreased in thickness by stage severity, suggesting that quantitative analyzes of the inner layers may serve as biomarkers of disease progression. These findings support the existence of neurodegeneration in the early AMD stages. The associations found between thickness/volume of some neuroretinal layers and the RPE/Bruch layer suggest that they are proportionally related. Pseudodrusen were confirmed as biomarkers of an AMD specific phenotype related to thinner choroids, but also to thinner RPE/Bruch layer, and hyperreflective foci were associated with a thicker RPE/Bruch layer. Functional repercussions were also present in early AMD stages.

Keywords: Early age-related macular degeneration; Microstructural spectral-domain optical coherence tomography analysis; Retinal layers thickness; Pseudodrusen; Choroid.

BACKGROUND

Age-related macular degeneration (AMD), a leading cause of irreversible vision loss and blindness in older adults, is a neurodegenerative disease of the retina associated with aging.(1-6) The global prevalence of AMD is estimated to be 8.7% in people aged between 45 and 85 years, and the burden is expected to increase with estimates of 288 million affected individuals by 2040.(7-9)

AMD is characterized by the progressive degeneration of the photoreceptors, retinal pigment epithelium (RPE) and choriocapillaris complex in the macular region of the retina.(1, 2, 6, 10) In asymptomatic early stages, the main identified clinical lesions are drusen - extracellular yellow deposits located between the RPE and Bruch's membrane (BrM) - and pigmentary changes.(1, 3, 5, 6, 10) More recently, reticular pseudodrusen (RPD) were described, and they correspond to deposits located above the RPE, hence, also being called subretinal drusenoid deposits (SDD). RPD are more commonly seen in older patients and in eyes with thin choroid.(3, 11-13)

With progression of disease, development of areas of geographic atrophy or choroidal neovascularization in the macula might arise, both corresponding to late AMD. At this stage, there is impairment of central vision, perceived by the patient as a decrease in vision and/or distortion of images (*i.e*. metamorphopsia). Areas of low or absent retinal sensitivity (*scotomata)*, may develop in the central visual field, making face recognition or tasks such as reading or driving a car extremely difficult.(14) Symptoms may be apparent only when both eyes are affected, but usually the progression to late stage is asymmetric. According to Joachim *et al.*, on average, 20–25% of unilateral any AMD cases, and up to 50% of unilateral late AMD cases, progress to bilateral involvement within 5 years.(15) Therefore, timely diagnosis and patient awareness are of extreme importance for early recognition of fellow eye involvement.(14)

The understanding of the pathophysiological mechanisms underlying AMD represents a challenge by its multifactorial nature, with multiple genetic and environmental risk factors already identified or in study. Smoking is the main modifiable risk factor,(15) and age and family history are the main non-modifiable risks.(5) Gender, nutrition, cardiovascular diseases, and hypertension are also thought to play a role in the development of the disease, but with conflicting reports.(16)

Several staging systems of AMD severity have been developed, however, none has been accepted as universal and no global consensus still exists.(17) The Rotterdam staging system is one of the most used in major epidemiologic studies, being also applied in the Coimbra Eye Study (CES), the only study regarding AMD prevalence and incidence in a Portuguese cohort.(5, 16, 18)

The diagnosis of AMD was conventionally based on clinical examination and assessment of color fundus photographs (CFP), which is to date the basis of staging systems in AMD. However, the introduction of spectral-domain optical coherence tomography (SD-OCT) and fundus autofluorescence (FAF) imaging in AMD management provided improved ability to detect lesions in earlier stages, even when not clinically observed by ophthalmoscopy.(14, 19) The SD-OCT, a non-invasive approach, has become the standard imaging modality to diagnose and guide treatment for AMD, as it allows *in vivo* quantitative and qualitative analyses of the macula and its layers individually.(4, 6) Automatic

segmentation of different retinal layers by OCT is now possible and has been applied in some studies, however, semi-automatic segmentation is a more adequate term, as corrections are often needed.(6)

Traditionally, AMD is regarded as a disease that predominantly affects the outer retina.(2) More recently some studies using histopathological analyses or SD-OCT analysis have demonstrated that there are progressive degenerative changes in the inner retina as well, and that this happens in the early/intermediate stages of disease.(2, 4, 6, 10)

The purpose of this study is to accomplish a detailed quantitative morphological evaluation of all retinal layers and choroid in early AMD-staged eyes and to correlate with clinical and microstructural data, in a cohort of participants from the AMD *Coimbra Eye Study.*

SUBJECTS AND METHODS

Study design and selection of participants

Information on the identification and description of the study population has appeared in previous reports.(5, 16, 18, 19) Briefly, the CES is a single-center population-based study whose cohort included two geographically distinct populations aged ≥55 years for the estimation of AMD prevalence: one from a coastal town (Mira), and the second from an inland town (Lousã). The subsequent AMD Incidence Study(NCT01298674) conducted 6.5 years later, only included the subjects recruited from the primary health care unit in Mira.(18, 19) This latter population was extensively characterized from a clinical, demographic, and imagiological perspective. For inclusion in the present analysis, patients staged with early AMD by multimodal imaging (stages 2a, 2b, and 3 of Rotterdam classification) in the AMD Incidence study were identified.

Signed informed consent was obtained for all participants in the scope of the AMD incidence study. The study adhered to the tenets of the Declaration of Helsinki (2008) and was approved by the Association for Innovation and Biomedical Research on Light and Image (AIBILI) Ethics Committee.

Data collection and AMD staging

All CES participants underwent a detailed questionnaire-based interview on demographic, clinical and lifestyle related information, as well as complete bilateral ophthalmological examination including BCVA, tested with Early Treatment Diabetic Retinopathy Study (ETDRS) charts.(5, 16, 18)

After an initial generic analysis of all imaging exams collected, patients with AMD diagnosis were selected for grading, and AMD staging was performed using the Rotterdam staging system (Table 1). Early AMD was defined as stages 2a, 2b and 3, and Late AMD as stage 4. In brief, and as reported elsewhere, staging was established using a multimodal approach including CFP, OCT, FAF, and infra-red (IR) imaging.(18, 19) CFP image grading was supported by *RetmarkerAMD Research* software (Retmarker SA, Coimbra, Portugal), that assists manual grading of lesions according to the International Age-Related Macular Epidemiological Study Group Classification.(17) This was performed while analysing the correspondent SD-OCT, IR, and FAF exams using the Heidelberg Eye Explorer software (version 1.10.4.0). All imaging data were collected in the context of the AMD Incidence Study.(18, 19) This assessment was performed at a centralized reading center (Coimbra Ophthalmology Reading Center, AIBILI), by 4 senior medical retina specialist graders trained according to study protocol.

SD-OCT segmentation and quantitative assessment

SD-OCT of both eyes was acquired with *Spectralis* HRA+OCT (Heidelberg Engineering, Heidelberg, Germany) according to the methodology previously described.(18, 19) All OCT exams were sent to the centralized reading center, and imported into the Heidelberg Eye Explorer software for

grading. The EDI Macular Volume Scan was selected for analysis (20ºx20º, 49 or 97 B-scans, 16 frames per scan), and segmentation of all retinal layers was performed.

Table 1. Rotterdam staging system for AMD.

STAGES		DEFINITION
0	a	No signs of AMD at all
	b	Hard drusen (<63 µm) only
1	a	Soft distinct drusen (≥63 µm) only
	b	Pigmentary abnormalities only, without soft drusen (≥63 µm)
2	a	Soft indistinct drusen (≥125 µm) or reticular drusen only
	b	Soft distinct drusen (≥63 µm) with pigmentary abnormalities
3		Soft indistinct drusen (≥125 µm) or reticular drusen with pigmentary abnormalities
4		Atrophic or neovascular AMD

First, automatic segmentation of each retinal layer was applied to the complete volume scan. This was performed using the in-built *Spectralis* mapping software Heidelberg Eye Explorer (version 1.10.4.0). Then, all layers were reviewed for segmentation and manual correction was performed whenever necessary, using appropriate tools of the software. When, after maximum effort, a scan had poor quality and a layer could not be segmented, it was automatically subtracted from the map. Decentration of the macular volume was also manually rectified if necessary. Scans where the foveal region was not captured by the macular cube were excluded. Eyes with concomitant retinal disease distorting the macular layers or compromising accurate thickness assessment were also excluded (e.g. significant epiretinal macular membrane). Automatic segmentation and the necessary manual corrections were conducted by a trained grader (ALS), certified by the reading center according to the specific study protocol developed for this analysis, and supervised by a Senior Grader (medical retina specialist, CF). Doubtful or difficult cases in the opinion of the grader were discussed and revised by the supervisor.

The *Spectralis* segmentation software nomenclature includes seven distinct layers: retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL) and RPE/Bruch layer (RPE/BrM) (Figure 1A). Besides the thickness of the previous layers, the software also provides information on three additional composite layers: (1)the combined inner retinal layer (IRL), which extends from the inner limiting membrane (ILM) to the external limiting membrane (ELM), (2)the outer retinal layer (ORL), extending from ELM to Bruch's membrane (BrM), and (3)the total retinal thickness, ranging from ILM to BrM (Figure 1B). Since the software does not provide the retinal thickness of inner and outer segments of the photoreceptors, and as these are of paramount interest in AMD pathophysiology, we have calculated the thickness and volume values for an additional layer, the "photoreceptor" layer (PRL), subtracting the RPE/BrM layer from the ORL, as described by Brandl *et al.*(6)

After verification of each individual B-scan and correction of the segmentation errors, the thickness from the nine macular fields, as determined by the ETDRS grid available in the software, and the volume for each of the segmented retinal layers were registered (Figure 1B). The mean retinal thicknesses in inner and outer circles (3 and 6 mm diameter, respectively) were calculated by averaging the thickness measurements of the inner and outer superior, inferior, nasal and temporal fields of the grid.(6, 20, 21)

Besides the retinal thickness, central choroidal thickness beneath the fovea was manually measured using the caliper tool available in the *Spectralis* software, using the 1:1 µm viewing mode, in the horizontal scan passing through the center of the fovea. Choroidal thickness was defined as the vertical distance from the hyperreflective line of the BrM to the innermost hyperreflective line of the

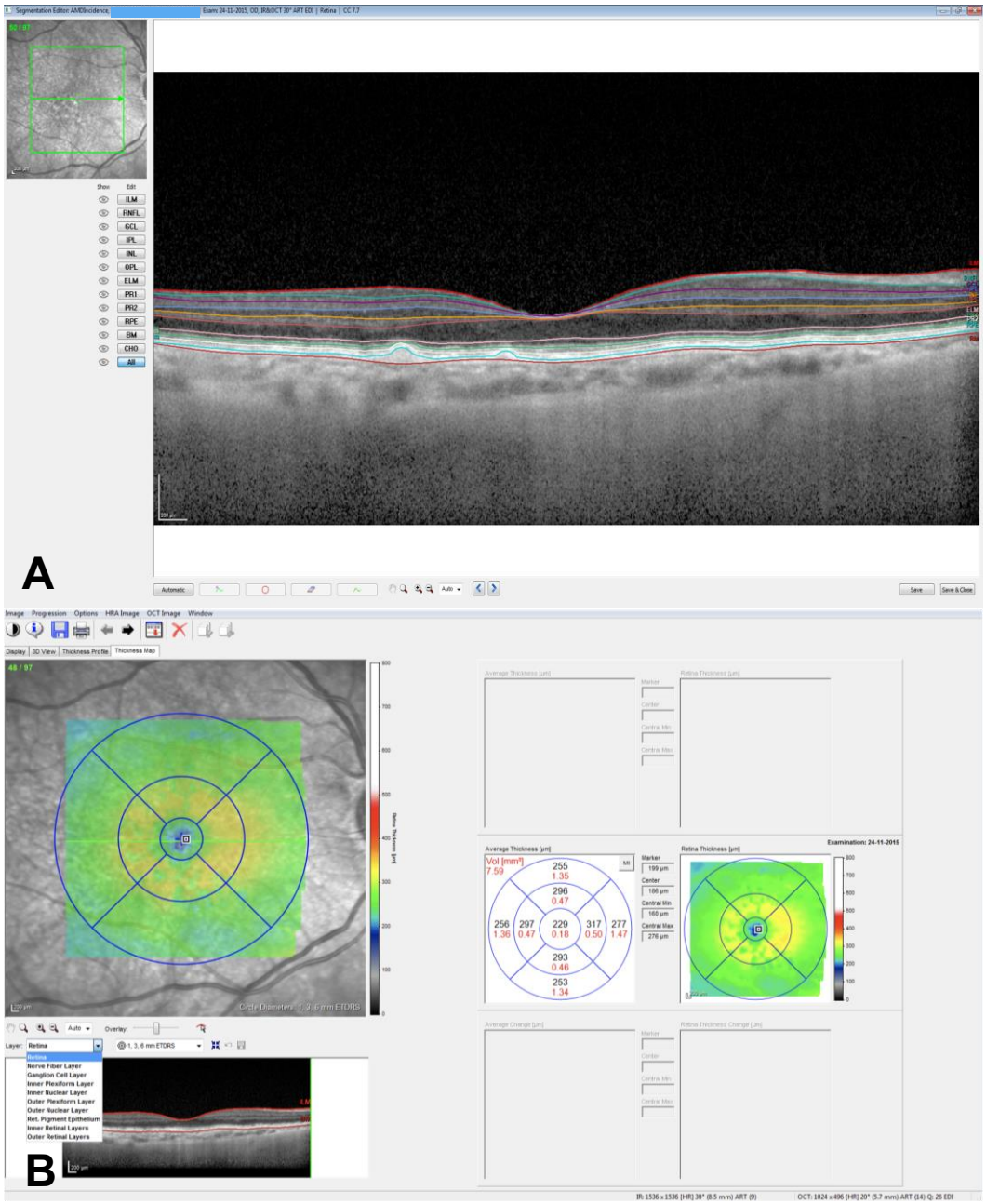

Figure 1. (A) Representation of semi-automatic segmentation of retinal layers in a horizontal foveal SD-OCT B-scan, performed in the *Spectralis software*. **(B)** Depicted are the retinal layers available for segmentation, as well as the information of thickness (ETDRS grid) and volume provided.

chorioscleral interface. If the hyperreflective line of the BrM was not separated from the RPE, the choroid was measured from beneath the outermost hyperreflective line of RPE/BrM layer to the innermost hyperreflective line of the chorioscleral interface.

SD-OCT qualitative assessment of retinal features

An extensive qualitative analysis of the SD-OCT scans was performed in all AMD patients included in the AMD Incidence Study according to recently proposed "European Eye Epidemiology spectral-domain optical coherence tomography classification of macular diseases for epidemiological studies".(22)

Again, this assessment was performed by 4 trained senior medical retina specialist graders and several features were graded according to presence and location to the fovea:

- Assessment of the neuroretina and RPE/BrM layer: soft drusen, RPD, pigment epithelium detachment (including drusenoid PED), RPE atrophy, hyperreflective foci (HF), intraretinal cystoid edema, subretinal fluid, outer retinal tubulations, and subretinal hyperreflective material.
- Assessment of the vitreomacular interface: vitreomacular adhesion (VMA), vitreomacular traction and epiretinal membrane (ERM).

Statistical Analyses

Data were analysed using STATA software (StatCorp., College Station, Tex., USA), version 12.1 SE.

Demographic and clinical characteristics were summarized using descriptive methods. Normal distribution of data was assessed by Shapiro-Wilk test.

Two distinct statistical methodologies were performed in this study to properly analyze not only the differences between retinal layers by stage, but also the associations between them and to other functional and qualitative morphological features in the population.

To test for differences in retinal layer thickness within eyes with early AMD stages, we used a more complex approach that allowed the inclusion of all eyes in the analysis. Differences in retinal layer thickness were therefore assessed using a linear mixed model for each layer separately, this is, the eight distinct layers: RNFL, GCL, IPL, INL, OPL, ONL, PRL and RPE/BrM. For each layer, the dataset consists of multiple measurements per study participant, eyes, and macular circle. This correlation structure was taken into account by including a participant-specific random intercept and a nested random intercept for each eye per participant into each model. The dependent variable of each model was the natural logarithm of manually corrected retinal layer thickness by circle (central, inner, and outer) and by eyes. The independent variables were age (linear), gender, circle and AMD stage (2a, 2b, 3). Age and gender were integrated as potential cofounders, due to significant variation in thickness of retinal layers in normal eyes depending on these variables.(6, 20). An interaction effect of the AMD stage with macular circle was included to account for differing retinal layer thickness by circle and to

estimate potentially differing effects of the disease stages on retinal layer thickness in the different circles. To test for pairwise differences we used approximate F-tests based on the Kenward-Rogers approach. The p-values were adjusted by Bonferroni method for 3 tests. The significance level was set as 0.05/24=0.002 to consider the p-values adjustment for 24 = (3 macular fields x 8 distinct retinal layers) tests.

For the remaining statistical analysis, we included only the worst-AMD staged eye to not exclude more severe stages that were less represented in the total sample. The correlations between the different retinal layers and the RPE/BrM layer, regarding thickness and volume, were tested using Spearman correlation due to the non-normal distribution of the sample, followed by a stepwise backward multivariate regression analysis to adjust for possible confounders (age, gender, refractive status, and AMD stage). The same statistical methodology was applied to test the association between the BCVA and the retinal layers thicknesses in the central circle and to assess the relation between the SD-OCT qualitative retinal features more prevalent in this population and the retinal layers thicknesses across each macular circle.

RESULTS

Study population

Early AMD (stages 2a, 2b, and 3) was established in a multimodal manner including CFP, OCT, FAF, and IR imaging, as described above. From the original cohort of the AMD incidence study, early AMD was found in 360 eyes from 237 patients. From these, 347 eyes from 234 patients (96.39%) were eligible for segmentation analysis and included in the study (Figure 2). Study eyes with the following criteria were excluded(n=13): poor image quality for accurate segmentation, absence of SD-OCT scans in the macular volume, and concomitant retinal disease distorting the retinal layers preventing reliable manual segmentation correction.

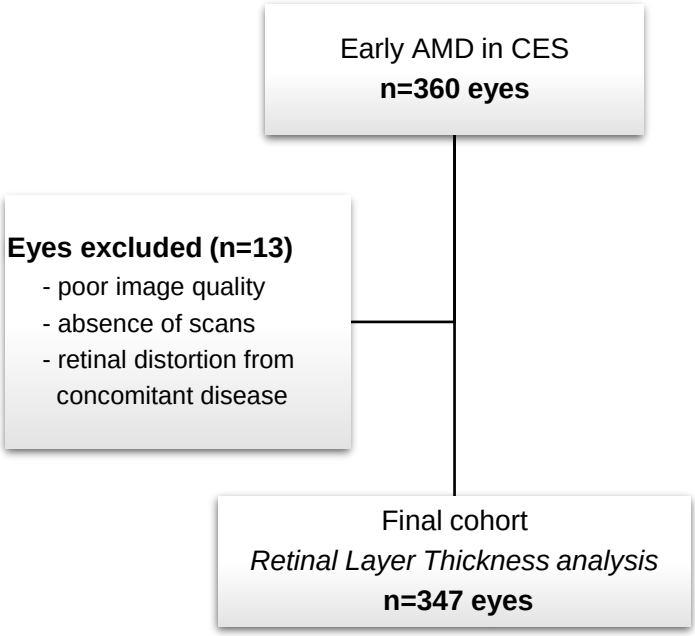

Figure 2. Chart of the study eyes selected and included in the *Retinal Layer Thickness* (RLT) analysis.

Clinical and Demographic characterization

The demographic and clinical characteristics from included CES patients are presented in Table 2. The mean age was 74.5±7.0 years (range, 61-88 years) and 80.4% had between 65 and 84 years old. Most patients with early AMD were women (63.7%) and Caucasian (98.3%).

Regarding risk-factors, the majority were nonsmokers (86.2%, n=200) and had no family history of AMD (89.3%, n=209). As for comorbidities, 12.8%(n=30) had diabetes, 56.8%(n=133) had hypertension, and 50.0%(n=117) had hypercholesterolemia/hyperlipidemia.

Ophthalmologic examination revealed a mean BCVA of 76.2±9.8 letters (range, 33-90), and a mean spherical equivalent of +0.58±1.60 diopters (range, -6.00 to +4.63) in this population.

Table 2. Demographic and clinical characteristics of the study population.

Characteristics	N (%)
Gender	
Male, n (%)	85 (36.3)
Female, n (%)	149 (63.7)
Age	
55-64 years, n (%)	26 (11.1)
65-74 years, n (%)	94 (40.2)
75-84 years, n (%)	94 (40.2)
≥ 85 years, n (%)	20 (8.6)
Ethnicity	
Caucasian	230 (98.3)
African	4 (1.7)
Smoking	
Smoker	5 (2.2)
Ex-smoker	27 (11.6)
Nonsmoker	200 (86.2)
Familiar history of AMD	
Yes	2 (0.9)
No	209 (89.3)
Does not know	23 (9.8)
Diabetes	
Yes	30 (12.8)
No	200 (85.5)
Does not know	4 (1.7)
Hypertension	
Yes	133 (56.8)
No	95 (40.6)
Does not know	6 (2.6)
Hypercholesterolemia	
Yes	103 (44.0)
No	114 (48.7)
Does not know	17 (7.3)
Hyperlipidemia	
Yes	14 (6.0)
No	193 (82.5)
Does not know	27 (11.5)
BMI, mean ± SD (min-max)	27.9 ± 4.9 Kg/m^2 (17.2 – 47.0)
BCVA, mean ± SD (min-max)	76.2 ± 9.8 letters (33 – 90)
Spherical equivalent, mean ± SD (min-max)	+0.58 ± 1.60 diopters (-6.00 – +4.63)

BMI, body mass index; BCVA, best-corrected visual acuity; SD, standard deviation; min, minimum; max, maximum.

AMD Staging

Of the 347 eyes included, 82.42%(n=286) were in stage 2a, 5.76%(n=20) in stage 2b and 11.82%(n=41) in stage 3 (Table 3). The prevalence of different stages of early AMD was similar regarding the laterality (RE vs LE) (Table 3).

Table 3. Prevalence of different stages of early AMD (stages 2a, 2b and 3), by eye.

AMD STAGE	ALL EYES	OD	OS
2a	82.42% (286)	48.60% (139)	51.40% (147)
2b	5.76% (20)	45.00% (9)	55.00% (11)
3	11.82% (41)	46.34% (19)	53.66% (22)
All eyes	100% (347)	48.13% (167)	51.87% (180)

Frequencies in parenthesis.

Retinal Layers and Choroidal Thicknesses and Volumes

Thickness values of eight distinct retinal layers and three additional combinations (inner, outer, and overall retinal layers), were obtained from all 347 analyzed eyes, for the central, inner, and outer circles. The macular volume of these layers was also acquired. The median thicknesses and volumes of each retinal layer are presented in Table 4.

Table 4. Retinal layers thickness and volume in a population aged 55+ with early AMD, in the central, inner and outer circles.

Retinal layers	Central circle	Inner circle	Outer circle	Volume
RNFL	12.00 (11.00–14.00)	20.50 (19.25–22.25)	34.50 (31.50–37.75)	0.87 (0.80–0.96)
GCL	14.00 (12.00–16.00)	48.00 (43.75–51.00)	33.00 (30.50–36.00)	1.02 (0.94–1.09)
IPL	20.00 (18.00–22.00)	40.0 (37.50–42.50)	28.50 (26.38–30.25)	0.87 (0.81–0.92)
INL	20.00 (17.00–24.00)	40.75 (38.75–43.50)	32.50 (31.00–34.25)	0.96 (0.91–1.01)
OPL	25.00 (22.00–29.00)	31.50 (29.25–34.25)	26.25 (25.25–27.75)	0.78 (0.74–0.82)
ONL	95.00 (87.00–102.00)	70.25 (65.00–77.25)	55.25 (50.25–60.00)	1.69 (1.54–1.83)
PR	69.00 (67.00–72.00)	65.25 (64.00–66.25)	65.00 (63.50–65.75)	1.84 (1.80–1.86)
RPE	16.00 (14.00–17.00)	14.75 (13.50–15.75)	13.0 (12.00–14.00)	0.38 (0.35–0.41)
IRL	186.00 (174.00–201.00)	252.5 (240.75–264.00)	210.25 (200.75–220.25)	6.20 (5.90–6.49)
ORL	86.00 (83.00–89.00)	80.50 (78.25–82.00)	77.75 (76.00–79.25)	2.23 (2.17–2.27)
Overall Retina	272.00 (260.00–288.00)	331.75 (320.50–344.75)	287.75 (277.50–298.38)	8.41 (8.11–8.72)

The table above provides median thickness(µm) and volume values(mm^3) and, in parenthesis, the IQR (25^{th} to 75^{th} quartile) of the eight distinct retinal layers plus three combinations (IRL, ORL, and overall retina) in all 347 eyes analyzed, after manual correction of autosegmentation. IQR, interquartile range.

The retinal layers thicknesses by early AMD stage and macular circle are presented in Annex I, while the retinal layers' volume by stage is given in Annex II.

Additionally, retinal layers thicknesses and volumes separated for men and women as well as younger and older than 74.7 years (median age of the population in analysis) are presented in Annexes III-V. In descriptive comparisons between men and women, we found a general thinning of retinal layers

in female eyes across all circles, except for four layers (that is, RNFL, GCL, IPL and RPE) that showed a thickening in the outer circle (Annex III). Overall, the volume of the retinal layers in female eyes was also slightly reduced (Annex V). Comparing descriptively the “younger” (≤74.7 years) with the “older” eyes (>74.7 years), we noticed a thickening of inner retinal layers, but a thinning of outer retinal layers in “older” eyes regarding the central circle (Annex IV). The inner and outer circles were predominantly thinner in “older eyes” (Annex IV). We identified a slight decrease in the volume of the retinal layers in “older” eyes (Annex V).

The median subfoveal choroidal thickness was 238.0 µm, with an interquartile range (IQR) of 177.0 to 291.0 µm. The median subfoveal choroidal thickness values by early AMD stage, gender, and median age are shown in Annex VI. Through descriptive comparisons, we noticed that choroidal thickness was lower in stage 3 (229.0 µm, IQR 152.0–311.0), compared to stages 2a (237.5 µm, IQR 171.0–289.0) and 2b (268.5 µm, IQR 228.0–317.0). Besides, we observed a considerable choroid thinning in female eyes, as well as in “older eyes” (median age > 74.7 years).

Differences between stages for each retinal layer

To assess the differences in retinal layers thicknesses within early AMD stages all 347 eyes were considered: 286 eyes in stage 2a, 20 eyes in stage 2b, and 41 eyes in stage 3. The analysis was controlled to age and gender, as previously mentioned, and given the differences described above. Corrected estimates of expected retinal layers thicknesses by AMD status and macular circle are shown in Annex VII and illustrated in Figure 3.

In descriptive comparisons, we could notice that RNFL, GCL, IPL, INL, and OPL, had generally lower thickness in stage 3 in the inner and outer circles. The thinning noticed for the ONL and PRL in stage 3 was presented across all circles, but with a more prominent difference in the central circle. In contrast, the RPE/BrM layer showed increased thickness in stage 3 in the central and inner circles.

We tested for pairwise differences in retinal layer thickness measurements between AMD stages and separately for each macular circle (Table 5).

Considering inner layers, we found statistically significant differences when comparing stage 3 to stage 2a, and generally, the thicknesses were inferior in stage 3. Thus, the GCL and IPL thicknesses were inferior in the inner circle (GCL,$p=0.003$; IPL,$p=0.002$) in stage 3. As for the ONL, we also have found a significant reduction from stage 2a to stage 3 in the central circle ($p=0.002$). Taking into account the outer layers, the RPE/BrM layer was significantly increased in the central circle for stage 3, compared to both stage 2a ($p<0.0001$) and stage 2b ($p=0.002$).

No differences were observed regarding the RNFL, INL, OPL, and PRL, but considering the global thinning tendencies across stage observed in Figure 3, they seen to be nonetheless, clinically relevant.

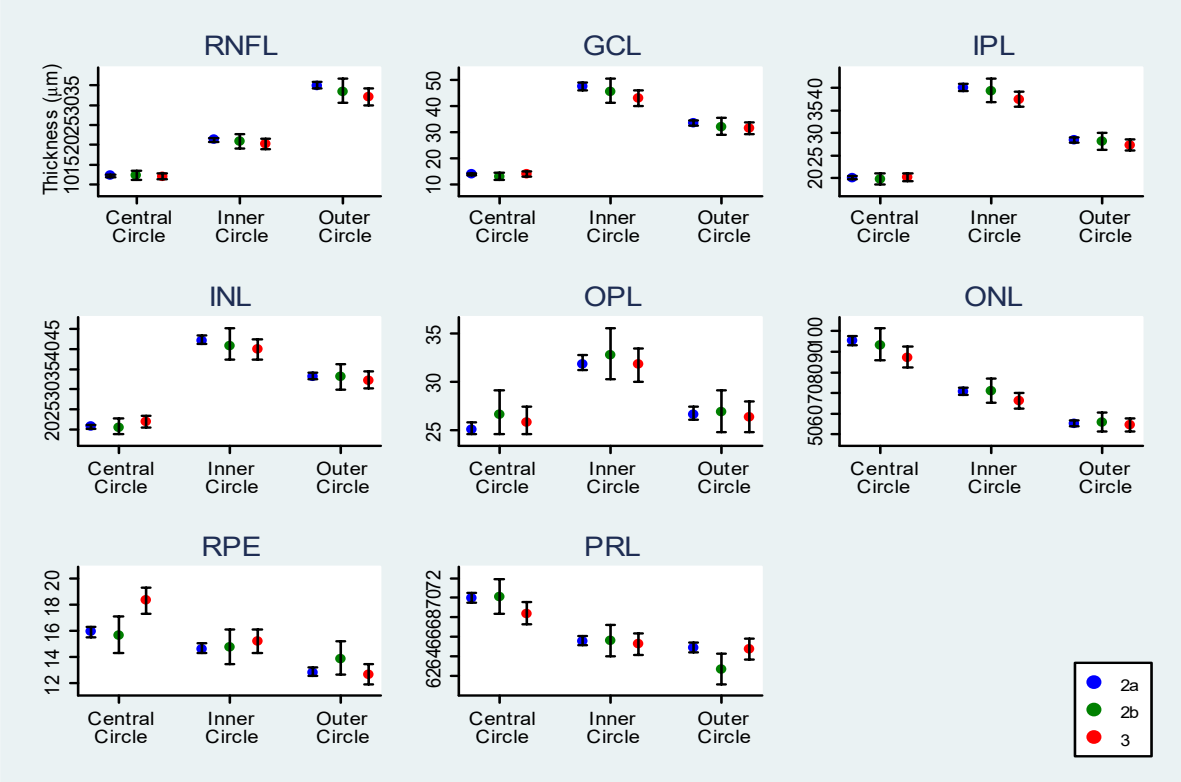

Figure 3. Model-based expected retinal layer thicknesses by early AMD stage, regarding central, inner, and outer circles. Estimates are derived from linear mixed models of 347 eyes with stage 2a (n=286), stage 2b (n=20), and stage 3 (n=41) of early AMD, adjusted for linear age and gender and nested random effects for within-person and within-eye correlations. The expected retinal layer thicknesses (µm) are illustrated above.

Table 5. Retinal layer thicknesses with significant differences across early AMD stages.

	Expected Retinal Layer Thicknesses, µm			**Effect Estimates / Pairwise Test p-value**		
Retinal layers	**2a** (n=286)	**2b** (n=20)	**3** (n=41)	**2a vs. 2b**	**2a vs. 3**	**2b vs. 3**
GCL, inner circle	47.5	45.6	42.9	0.043 / 0.953	**0.099 / 0.003**	0.056 / 0.768
IPL, inner circle	40.1	39.3	37.5	0.018 / 1.000	**0.067 / 0.002**	0.048 / 0.420
ONL, central circle	95.4	93.1	87.3	0.023 / 1.000	**0.088 / 0.002**	0.065 / 0.353
RPE, central circle	16.0	15.6	18.4	0.013 / 1.000	**-0.140 / <0.001**	**-0.153 / 0.002**

The table above provides Expected Retinal Layer Thicknesses (µm), effect estimates (on log scale), and p-values of pairwise tests, obtained from layer-specific linear mixed models. The analysis includes 347 eyes with early AMD (stages 2a, 2b, and 3), obtained from the original cohort of AMD incidence study. P-values were judge at Bonferroni-corrected significance level, $p < 0.05/24=0.002$. Significant results are highlight with bold text.

Association between retinal layers and RPE/BrM layer

To analyze the correlation between the RPE/BrM thickness and the thickness of the neuroretinal layers (RNFL, GCL, IPL, INL, OPL, ONL, and PRL) for each macular circle, separately, only the worst eye of each subject was included, resulting in a total of 234 eyes, being 77.8%(n=182) in stage 2a,

7.3%(n=17) in stage 2b and 15.0%(n=35) in stage 3 (Table 6). The ORL and IRL were excluded from the analysis, as they are collinear with other retinal layers and are not independent.

Table 6. Correlation between the RPE/BrM layer thickness and volume to the neuroretinal layers, age, gender, spherical equivalent, and AMD stage.

Variable	RPE/BrM Thickness			RPE/BrM Volume
	Central circle	Inner circle	Outer circle	
RNFL	0.032 (0.626)	-0.115 (0.081)	-0.086 (0.193)	-0.073 (0.264)
GCL	0.044 (0.507)	0.046 (0.488)	0.039 (0.561)	0.056 (0.397)
IPL	0.055 (0.405)	0.039 (0.556)	0.063 (0.343)	0.050 (0.451)
INL	-0.032 (0.623)	0.009 (0.891)	0.079 (0.233)	0.102 (0.119)
OPL	-0.115 (0.080)	0.030 (0.645)	0.009 (0.893)	0.082 (0.212)
ONL	0.060 (0.359)	**0.247 (<0.001)**	0.060 (0.369)	**0.156 (0.017)**
PRL	-0.039 (0.555)	**0.227 (<0.001)**	**0.218 (<0.001)**	**0.254 (<0.001)**
Age	-0.015 (0.818)	**-0.189 (0.004)**	-0.079 (0.231)	-0.109 (0.097)
Gender	-0.100 (0.126)	0.006 (0.923)	0.070 (0.287)	0.062 (0.344)
Spherical equivalent	0.023 (0.761)	-0.032 (0.676)	-0.113 (0.138)	-0.099 (0.191)
Stage	**0.241 (<0.001)**	**0.162 (0.013)**	**0.131 (0.046)**	**0.164 (0.012)**

The table above provides the Spearman correlation coefficient and, in parenthesis, the p-value. These results are based on 234 eyes - worst eye *per* subject. Statistically significant correlations are highlighted with bold text ($p<0.05$).

Regarding thickness, a weak positive correlation was found between the RPE/BrM and the ONL in the inner circle ($r_S=0.247$,$p<0.001$), and with the PRL in inner ($r_S=0.227$,$p<0.001$) and outer circles ($r_S=0.218$,$p<0.001$) (Table 6). Concerning the layers' volume, we found a positive correlation with the ONL ($r_S=0.156$,$p=0.017$) and the PRL ($r_S=0.254$,$p<0.001$) (Table 6). Furthermore, we also identified positive correlations between RPE/BrM layer and the stage both for thickness and volume, and a negative correlation with age ($p<0.05$) (Table 6). Although this was not the objective of the present analysis, we could observe that, in general, age was negatively correlated with the retinal layers' thicknesses and volumes, while the spherical equivalent and gender was not correlated.

Afterwards, stepwise backward multivariate linear regression analysis was performed. Most associations remained statistically significant after adjusting for age, sex, refractive status and AMD stage, and new ones were established. Statistically significant results are shown in Table 7.

With the increasing thickness of the RPE/BrM layer, the OPL at the central circle became thinner, while the GCL became thicker. The OPL and ONL at the inner circle and the IPL and PRL at the outer circle were also positively associated with the RPE/BrM layer thickness. Similarly, the increasing volume of this layer was related to a greater ONL and PRL volume. Furthermore, with the increase of the stage, there was an increment in the thickness and volume of the RPE/BrM layer.

Table 7. Multivariate linear regression analysis of thickness and volume between RPE/BrM layer and retinal layers.

Variable	RPE/BrM Thickness			RPE/BrM Volume
	Central circle	Inner circle	Outer circle	
GCL	**0.299 (<0.001)**	–	–	–
IPL	–	–	**0.387 (0.044)**	–
OPL	**-0.182 (0.040)**	**0.200 (0.012)**	–	–
ONL	–	0.218 (<0.001)	–	0.118 (0.046)
PRL	–	–	1.098 (p<0.001)	0.760 (<0.001)
Gender	–	–	**0.037 (0.033)**	–
Stage	0.113 (<0.001)	0.051 (<0.001)	0.026 (0.018)	0.038 (<0.001)

The table above provides the regression coefficient β and, in parenthesis, the p-value, derived from stepwise backward multivariate linear regression analysis separately for each macular layer. Only significant results are presented, and are based on the set of 234 eyes - worst eye *per* subject. All retinal layer thicknesses were log-transformed and adjusted for age, gender, refractive status, and AMD stage. Variables with p-value>0.1 were removed from the model. New associations are highlighted with bold text.

Qualitative SD-OCT analysis and relation to retinal layer and choroidal thickness

The presence of qualitative retinal features obtained in the SD-OCT grading from de AMD Incidence Study was also considered and major findings are presented in Annex VIII. The RPD and ERM were the most prevalent features in this population, being present in 24.93% and 18.84% of the studied eyes. The HF and VMA were also common, existing in 11.30% and 10.43% of the eyes, respectively. Very few eyes presented drusenoid PED (1.16%).

To assess the relation between each retinal layer thickness, by macular circle, and choroidal thickness with the qualitative retinal features, stepwise multivariate linear regression analysis was performed. Statistically significant results are presented in Table 8. As the prevalence of drusenoid PED in the studied population was low, they were excluded from the analysis.

Eyes with RPD were significantly associated a thinner RPE/BrM layer (central and inner circles) and thinner subfoveal choroid. When comparing the retinal layers and choroidal thicknesses, between pseudodrusen and non-pseudodrusen eyes, significant differences were found (Annex IX). Eyes with HF were associated to thicker INL (inner circle) and RPE/BrM layer (central and inner circles).

Association with Best-Corrected Visual Acuity

To assess the functional repercussions of early AMD, correlations between the retinal layer thicknesses in the central circle and the BCVA were evaluated. Only the worst eye of each patient was included in this analysis(n=234). The BCVA was positively correlated with the ONL thickness at the central circle (r_S=0.155,p=0.018) and negatively with age (r_S=-0.357,p<0.001). Afterwards, stepwise backward multivariate linear regression analysis was performed, and significant associations are shown in Table 9.

Table 8. Statistically significant results from the association between retinal layers and choroidal thicknesses with the presence of qualitative retinal features (RPD, ERM, HF).

Outcomes	Reticular pseudodrusen (β/p-value)	Hyperreflective foci (β/p-value)	Epiretinal membrane (β/p-value)
Central RNFL	–	–	0.126/0.002
Inner RNFL	–	–	0.144/<0.001
Outer RNFL	–	–	0.137/<0.001
Central GCL	0.142/0.004	–	0.189/<0.001
Central IPL	0.067/0.042	–	0.093/0.004
Central INL	–	–	0.137/0.007
Inner INL	–	0.062/0.008	–
Central RPE	-0.103/0.029	0.130/0.036	0.092/0.042
Inner RPE	-0.110/<0.001	0.096/0.005	–
Choroid	-63.902/<0.001	–	–

The table above provides the regression coefficient β and the p-value, derived from stepwise backward multivariate linear regression analysis. Only significant results are presented, and are based on the set of 234 eyes - worst eye *per* subject. All retinal layer thicknesses were log-transformed, except the choroid due to its normal distribution, and adjusted for age, gender, refractive status, and AMD stage. Variables with p-value>0.1 were removed from the model.

Table 9. Multivariate linear regression analysis of retinal layers thicknesses (central circle) and the Best-Corrected Visual Acuity (only significant associations are presented).

Variables	Regression Coefficient, β	p-value
IPL	0.311	<0.001
INL	-0.104	0.032
ONL	0.233	<0.001
PRL	-0.513	0.011
Age	-0.007	<0.001

The table above provides the regression coefficient β and, in parenthesis, the p-value. These results are based on 234 eyes, the worst eye *per* subject. All retinal layer thicknesses were log-transformed and adjusted for age, gender, refractive status, and AMD stage. To statistical significance, p-value was established at 0.05.

DISCUSSION

We report the findings from a detailed quantitative morphological evaluation of all retinal layers and choroid in early AMD-stages eyes (stages 2a, 2b, and 3) in a cohort of participants from the AMD *Coimbra Eye Study*.(18) We have demonstrated that changes in the inner retina are observed early in the disease process since the increasing stages were associated with thinner inner retinal layers, that is, CGL, IPL, and ONL. These outcomes support the existence of neurodegeneration already in early AMD stages. On the other hand, we also reproduced previous findings on the outer retina, since we have shown that within early AMD stages, the RPE/BrM layer becomes thicker, being consistent with the expected drusen volume increase as the disease progresses in early stages.(1, 6, 23, 24)

The demographic data of our study population is in accordance with previous reports for AMD, with a mean age of 74.5 years, and a higher prevalence of early AMD in women, which allows for comparisons with preceding studies.(18) The most prevalent risk factors in this population are hypertension, hypercholesterolemia, diabetes, and overweight, which may indicate that the AMD pathogenesis may have something in common with cardiovascular diseases.(25)

First, we have analyzed the retinal thickness and volume values of all retinal layers individually, by circle (central, inner, and outer) and by stage (2a, 2b, and 3), acquired after the semiautomatic segmentation. We compared our raw thickness results with reference values given by German AugUR Study, the only epidemiologic study, beside ours, that provides an extensive report on retinal layers thickness analysis in AMD.(6) When compared to the reference values for the general older population(70+ years), our retinal layers thicknesses showed similar values and tendencies across the central, inner, and outer circles.(6) However, when comparing our results with their thickness measurements for a population aged 70+ without changes in the CFP, we could notice the presence of a thinner IRL in our population consistent across all circles, which could be related to the involvement of inner layers and degeneration in early stages of the disease.(2, 4, 26)

The volume of the distinct retinal layers was also assessed. Concerning Lamin *et al.*(2) study, the retinal layers' volume of 71 eyes with early and intermediate AMD from an identical population regarding age and gender, was determined, and significant differences were reported when comparing to the control group: GCL+IPL and INL volumes were smaller in AMD eyes, where PRL and RPE/BrM volumes were greater in this population. Considering the retinal layers that showed significant differences in the above-mentioned report, our volume measures have shown similar values and tendencies when compared to their AMD group.

The median subfoveal choroidal thickness obtained is also comparable with previous studies.(27) Moreover, we could perceive that the raw value in stage 3 was lower, comparing to stages 2a and 2b of the disease. The finding that the choroidal thickness decreases as the stage of AMD increases has been reported in previous studies, and may be related to atrophy of underlying capillaries and medium-sized vessels.(13, 27) The increased value of choroid thickness in stage 2b is not consistent with previous studies and may be related to the fact that the sample size is considerably smaller(n=20) than in stages 2a(n=286) and 3(n=41).

The differences observed through descriptive comparisons in thickness and volume of the retinal/choroidal layers between "younger" and "older" eyes, as well as male and female eyes, highlight the necessity to adjust the inferential analysis for age and gender.

To assess for the presence and progression of possible degenerative changes in the inner and outer retina, we focused on the evolution of all retinal layer's thicknesses across early AMD stages using a linear mixed models' analysis. There were significant differences to be described. Overall, stage 3 of early AMD was significantly associated with lower thickness of GCL, IPL and ONL, and a greater thickness of RPE/BrM layer. Importantly, despite they have not reached statistical significance, almost all neuroretinal layers demonstrated a global tendency of thinning across early AMD stages.

The thickness variation observed in GCL and IPL in the inner circle, and not in the central circle, may be related to the fact that these inner layers are predominantly preserved in the central circle in early AMD eyes.(4) However, these retinal layers do not exist in the central fovea, meaning that possible changes in the central circle may be too small to be discernible in our analysis. As for the thickness decrease observed in the inner circle, there are several hypotheses to explain this variation of the inner retinal layers at this level of the disease progression. One is related to the reduction of the layers perfusion and subsequent ischemia developing from abnormal superficial retinal microvasculature, that is, loss of vascular density as well as vessel's caliber.(2, 4, 26, 28) Others refer to reducing transneuronal input resulting from the photoreceptors' loss or damage that could trigger the inner retina degeneration process.(2, 4, 26) Our finding of a thinner ONL, where the nuclei of the photoreceptor cell are located, in stage 3 supports the idea of a photoreceptor loss or damage in early AMD, and not only in advanced stages of the disease, where it is characteristic.(6) Despite the difference for the PRL thickness across early AMD stages did not reach significance, descriptively comparing we could notice that its thickness value was lower in stage 3 after correcting for age and gender, which sustains what was afore-mentioned. Thus, with these consistent results, we can suggest that inner retinal layers thinning are potential quantitative imaging biomarkers of neuroretinal degeneration in early AMD stages.

In our work, we estimated the drusen thickness and volume, a hallmark of early AMD, indirectly through the thickness of the RPE/BrM layer, as they are accumulated between the RPE and BrM. The finding of a thicker RPE/BrM layer in the central circle for stage 3, compared to 2a and 2b stages is comparable with previous studies and may be related to the drusen growth along early AMD stages.(1, 6, 23, 24) The positive correlations found between RPE/BrM layer thickness and volume with the AMD stage support this finding.

The significant thinning of ONL observed in stage 3 of early AMD may result from the squishing of this layer due to the drusen growth, along with the photoreceptor cell loss/damage already at early-disease stages.(1, 6) The associations for the RPE/BrM and ONL were only noticeable in the central circle, which is in agreement with the foveal region being the primary place of AMD pathology.(6) Therefore, these layers may also be potential quantitative biomarkers of early AMD, as it was suggested by Brandl *et al.*(6)

To better understand the relation between RPE/BrM layer with the neuroretinal layers, a correlation analysis followed by a multivariate regression analysis adjusted to age, gender, refractive

status, and early AMD stage was performed. Although several correlations have been found, the strength of the analysis was weak. The inverse association noticed with OPL thickness in the fovea may be related to the mechanical tension caused by the RPE/BrM enlargement, as advanced confluent drusen appear to thin both inner and outer retinal layers.(10, 24) Also, since the RPE is responsible, among other functions, for the photoreceptors phagocytosis (mainly outer segments), its disintegration and consequent lipid deposition on BrM ("oil spill hypothesis") with increasing drusen volume lead to the loss or damage of photoreceptor cell, resulting in a diminished transneuronal input and subsequent thinning of inner retinal layers as well.(23, 24) However, the findings over the GCL, IPL, and the OPL(inner circle) do not support the previous explanations. The positive associations observed in the ONL and PRL thickness and volume were also not expected, as these structures are usually affected earlier with the drusen growth process.(24) Thus, careful conclusions must be taken regarding this aspect.

Besides, we analyzed specific OCT qualitative features previously graded in our studied population and we explored their relationship with retinal layers and choroidal thicknesses. Considering the RPD our work confirms that their presence is strongly associated with a thinner choroid, and we also found that they are related to a thinner RPE/BrM layer, again pointing to their association with RPE atrophy in AMD. Both findings are consistent with previous studies showing a specific phenotype in AMD when RPD are present.(1, 13, 29, 30) Despite our results on the inner retinal layers being non-significant in multivariate analysis when searching for an association of RPD to inner retinal layers, we found that globally in our cohort, eyes with RPD had significantly thinner inner layers compared to eyes not having RPD. Again it is described that eyes with RPD are associated with retinal thinning along with the choroidal thinning.(13)

Considering the HF, several studies have suggested that its presence may represent the migration of activated RPE cells into the neurosensory retina, which may support the thicker INL noticed in our study.(31-33) With the drusen growth within early AMD stages, the RPE disarrangement increases the number of HF, which may explain our finding of a thicker RPE/BrM layer in eyes presenting this retinal feature.(1, 33) Previous studies had shown that the presence of HF was associated with disrupted PRL, however, no association was found in our work.(31, 33, 34)

To assess the functional repercussions of early AMD, the comparison between the BCVA with the retinal layers' thickness in the central circle was performed. Our results suggested that the thinning of ONL and IPL, along with the thickening of PRL and INL were associated with a lower BCVA. The thinning of ONL being associated with a diminished visual acuity was expected giving the fact that this layer contains the nuclei of the photoreceptor cell. The result of a thinner IPL being associated with a loss of visual acuity is reasonable, as this layer contributes to the continuous neuronal transmission across the neurosensory retina. However, the finding of a thicker INL and PRL being associated with a lower BCVA was unexpectable. Regarding PRL, this result is not consistent with some studies, where it was described that a decreased visual function was associated with a thinning of this layer.(6, 24, 35) However, this can be explained considering previous histopathological studies that revealed a displacement of nuclei from the ONL to the layer of rods and cones, that is, the PRL, at very early stages

of AMD, leading to an initial expansion of the PRL, but afterwards evolving to atrophy with PRL thinning.(2)

Our study's main limitations' can be described in the following key points. First, the presence of groups with different sample sizes may have underpowered our statistical analysis. Second, although hyperopia had shown to be associated with a higher risk of AMD and a thicker RPE/BrM, we were not able to use the spherical equivalent to adjust the refractive status in our retinal layer's analysis, which may have induced a bias in the analysis of retinal thickness across AMD stages. However, the impact of this confounder in the RPE/BrM thickness appears to be lesser comparing to the observed differences, as Brandl *et al.* concluded, and we support this idea since we found no association between them.(6) Third, another limitation of our work is that choroidal thickness was determined by a single-point measure taken at the foveal center. Finally, despite severe ERMs have been excluded, we should have included its presence as a covariate in our analysis, given their potential interference with the retinal layers thickness and BCVA.(36, 37)

The main strengths of our study are related to the fact that it is one of the few using a large population-based dataset to further analyze how early AMD changes by stage severity, specifically regarding the inner retinal layers, through complex multivariate analysis. Thus, we provided further support to the concept of AMD as a neurodegenerative disease involving the entire retina. Additionally, we have explored how the distinct retinal layers relate to RPE-BrM/drusen volume variation, SD-OCT qualitative markers, and functional status of the patients.

Regarding future work, more associations should be evaluated between the retinal layers and the choroid and correlations with the most prevalent risk factors should also be assessed.

CONCLUSION

In conclusion, despite the changes noticed in the outer retina, our data showed that quantitative changes in the inner retina are also observed in early AMD, and thinning of the neuroretina increases by stage severity. This suggests that quantitative analysis of inner layers may serve as biomarkers of disease progression and supports the existence of neurodegeneration in the early stages of AMD. Furthermore, the associations found between thickness/volume of some neuroretinal layers and the RPE/Bruch layer suggest that they are proportionally related, except for the OPL at the fovea, perhaps due to mechanic effect of the underlying, more centrally located, drusen. Additionally, pseudodrusen and hyperreflective foci, have shown to affect retinal and/or choroidal thickness. Our work also sustains the concept of a lower BCVA being related to a loss or damage of the photoreceptor cell already in early AMD stages. Thus, AMD is a degenerative disease affecting the entire retina since its very beginning.

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ANNEX I.

Retinal layer thickness values in a population aged 55+ by early AMD stage, regarding central, inner and outer circles.

RETINAL LAYERS	CENTRAL CIRCLE			INNER CIRCLE			OUTER CIRCLE		
	2a	2b	3	2a	2b	3	2a	2b	3
RNFL	12.0 (11.0–14.0)	13.0 (11.0–14.5)	12.0 (11.0–14.0)	20.5 (19.5–22.3)	20.8 (18.8–22.3)	20.0 (18.8–21.3)	34.8 (31.8–37.8)	34.6 (29.1–39.3)	33.5 (28.5–37.8)
GCL	14.0 (12.0–16.0)	14.0 (12.0–15.0)	15.0 (12.0–16.0)	48.3 (44.0–51.3)	47.8 (44.8–50.5)	45.8 (39.9–50.3)	33.3 (30.8–36.0)	33.0 (30.0–35.4)	32.3 (28.8–34.5)
IPL	20.0 (18.0–22.0)	20.5 (19.0–22.0)	20.0 (18.0–23.0)	40.3 (38.0–42.5)	40.6 (38.1–41.5)	37.3 (34.9–41.3)	28.5 (26.5–30.5)	27.6 (26.3–29.4)	27.3 (25.5–29.3)
INL	20.0 (17.0–24.0)	20.5 (18.0–23.5)	23.0 (17.0–28.0)	41.0 (39.0–43.5)	40.4 (36.5–44.8)	40.3 (38.0–42.9)	32.5 (31.0–34.3)	32.9 (30.4–35.6)	32.3 (30.8–34.3)
OPL	25.0 (21.0–29.0)	26.0 (22.0–31.5)	25.0 (23.0–31.0)	31.5 (29.0–34.0)	32.0 (30.5–36.1)	31.5 (29.8–35.5)	26.3 (25.3–27.8)	27.0 (25.5–27.9)	26.3 (25.3–27.3)
ONL	95.0 (87.0–102.0)	94.0 (86.0–103.5)	93.0 (86.0–101.0)	70.3 (65.0–77.3)	73.8 (69.0–78.0)	69.8 (65.3–72.3)	55.3 (50.3–60.0)	56.8 (53.3–59.9)	55.3 (49.3–61.5)
PRL	69.5 (67.0–72.0)	70.0 (67.0–73.0)	68.0 (66.0–71.0)	65.3 (64.0–66.5)	65.5 (64.8–65.9)	65.5 (64.3–66.8)	65.0 (63.5–65.5)	64.9 (64.0–65.5)	64.8 (63.0–66.3)
RPE	15.0 (14.0–17.0)	16.5 (15.0–17.0)	17.0 (15.0–21.0)	14.8 (13.5–15.8)	14.9 (14.0–16.3)	15.3 (13.8–16.5)	13.0 (11.8–14.0)	13.6 (12.3–15.1)	13.3 (12.1–14.1)
IRL	186.0 (174.0–200.0)	192.0 (178.5–199.5)	189.0 (176.0–204.0)	253.0 (241.0–264.3)	252.4 (243.3–263.4)	245.8 (233.0–257.8)	211.8 (201.8–220.3)	210.5 (197.4–222.0)	206.3 (191.5–215.3)
ORL	86.0 (83.0–89.0)	86.0 (84.0–89.0)	87.0 (84.0–90.0)	80.0 (78.0–81.8)	81.3 (79.1–82.1)	81.5 (79.3–83.8)	77.6 (76.0–79.3)	78.6 (76.9–80.0)	78.5 (75.5–80.3)
Overall retina	271.0 (260.0–286.0)	275.5 (266.0–284.0)	276.0 (259.0–295.0)	332.0 (320.5–346.0)	333.6 (323.4–343.1)	326.5 (315.5–339.75)	288.5 (278.4–298.6)	288.9 (275.3–301.3)	285.0 (272.0–291.8)

The table above provides median thickness values (µm) and, in parenthesis, the IQR (25th to 75th quartile) of the eight distinct retinal layers plus three combinations (IRL, ORL, and overall retina) in all 347 eyes analyzed from 234 patients. Results are separated by early AMD stage as well as for central, inner, and outer circles. IQR, interquartile range.

ANNEX II.

Retinal layer volume values by early AMD stage.

LAYER	STAGES		
	2a	**2b**	**3**
RNFL	0.88 (0.81–0.96)	0.88 (0.75–0.98)	0.84 (0.74–0.94)
GCL	1.03 (0.94–1.10)	1.02 (0.94–1.07)	0.98 (0.87–1.06)
IPL	0.88 (0.81–0.93)	0.86 (0.82–0.90)	0.83 (0.78–0.89)
INL	0.96 (0.92–1.01)	0.98 (0.89–1.06)	0.94 (0.90–1.02)
OPL	0.78 (0.74–0.82)	0.80 (0.76–0.83)	0.77 (0.75–0.83)
ONL	1.69 (1.53–1.83)	1.75 (1.64–1.83)	1.68 (1.55–1.83)
PRL	1.84 (1.80–1.86)	1.85 (1.83–1.86)	1.84 (1.80–1.88)
RPE	0.38 (0.35–0.40)	0.39 (0.37–0.43)	0.39 (0.36–0.41)
IRL	6.23 (5.93–6.50)	6.16 (5.91–6.57)	6.05 (5.59–6.25)
ORL	2.22 (2.17–2.26)	2.25 (2.20–2.28)	2.25 (2.16–2.29)
Overall retina	8.44 (8.12–8.73)	8.40 (8.12–8.82)	8.28 (7.91–8.51)

The table above provides median volume values (mm^3) and, in parenthesis, the IQR (25th to 75th quartile) of the eight distinct retinal layers plus three combinations (IRL, ORL, and overall retina) in all 347 eyes analyzed, separated by early AMD stage. IQR, interquartile range.

ANNEX III.

Retinal layer thickness values in a population aged 55+ separated for men and women.

RETINAL LAYERS	Male eyes median thickness (IQR)			Female eyes median thickness (IQR)		
	Central circle (n=122)	Inner circle (n=122)	Outer circle (n=122)	Central circle (n=225)	Inner circle (n=225)	Outer circle (n=225)
RNFL	13.0 (12.0–15.0)	20.8 (19.5–22.8)	33.9 (30.4–37.3)	12.0 (10.0–13.0)	20.5 (19.3–22.0)	34.8 (32.0–38.3)
GCL	15.0 (13.0–18.0)	48.0 (43.0–52.3)	32.8 (30.6–35.6)	13.0 (12.0–15.0)	48.0 (44.0–50.8)	33.5 (30.3–36.0)
IPL	21.5 (19.0–23.0)	40.5 (37.0–43.0)	28.4 (26.5–30.4)	19.0 (18.0–21.0)	40.0 (38.0–42.0)	28.5 (26.3–30.1)
INL	24.0 (20.0–28.0)	42.5 (39.8–45.0)	32.8 (31.5–35.1)	19.0 (16.0–22.0)	40.3 (38.0–43.0)	32.3 (30.8–34.3)
OPL	25.0 (22.0–30.0)	31.5 (29.5–34.8)	26.3 (25.5–27.8)	25.0 (21.0–29.0)	31.5 (29.0–34.0)	26.3 (25.0–27.8)
ONL	96.0 (90.0–104.0)	71.8 (65.8–78.3)	57.5 (53.9–62.1)	93.0 (86.0–101.0)	70.0 (65.0–76.5)	54.5 (49.0–59.0)
PRL	69.0 (66.0–72.0)	65.8 (65.0–67.3)	65.5 (64.3–66.0)	69.0 (67.0–73.0)	65.0 (63.8–66.0)	64.5 (63.0–65.5)
RPE	16.0 (14.0–18.0)	14.5 (13.5–15.5)	12.9 (11.8–13.8)	16.0 (14.0–17.0)	14.8 (13.5–15.8)	13.0 (12.0–14.0)
IRL	194.0 (184.0–210.0)	257.1 (242.0–269.0)	213.0 (203.0–221.8)	180.0 (169.0–194.0)	249.8 (240.0–261.8)	209.0 (199.5–219.3)
ORL	86.0 (83.0–89.0)	80.9 (78.8–83.0)	78.5 (76.8–79.8)	86.0 (83.0–89.0)	80.0 (78.0–81.8)	77.5 (75.8–79.3)
Overall retina	280.5 (269.0–297.0)	336.6 (325.5–347.8)	291.3 (282.1–299.4)	268.0 (256.0–281.0)	329.5 (319.0–343.5)	287.0 (275.8–296.8)

The table above provides median thickness values (µm) and, in parenthesis, the IQR (25th to 75th quartile) of the eight distinct retinal layers plus three combinations (IRL, ORL, and overall retina) in all 347 eyes analyzed from 234 patients (122 male eyes, 225 female eyes). Results are separated for men and women as well as central, inner, and outer circles. IQR, interquartile range.

ANNEX IV.

Retinal layer thickness values in a population aged 55+ separated for “young” and “old”.

RETINAL LAYERS	Age participants ≤74.7 years median thickness (IQR)			Age participants >74.7 years median thickness (IQR)		
	Central circle (n=168)	Inner circle (n=168)	Outer circle (n=168)	Central circle (n=179)	Inner circle (n=179)	Outer circle (n=179)
RNFL	12.0 (11.0–13.0)	20.5 (19.3–21.8)	34.8 (30.6–38.1)	13.0 (11.0–14.0)	20.5 (19.5–22.5)	34.5 (31.8–37.8)
GCL	13.0 (12.0–16.0)	48.1 (45.3–52.0)	34.0 (31.8–36.9)	14.0 (12.0–17.0)	47.9 (42.8–50.5)	32.5 (30.0–35.0)
IPL	19.0 (18.0–22.0)	40.9 (38.1–42.5)	29.0 (27.0–30.8)	20.0 (18.0–22.0)	39.5 (37.3–42.0)	27.8 (25.5–29.3)
INL	19.0 (16.0–23.0)	40.9 (39.3–43.5)	33.0 (31.6–35.3)	22.0 (18.0–26.0)	40.8 (38.3–43.5)	31.8 (30.3–33.3)
OPL	25.0 (22.0–29.0)	31.9 (29.3–34.8)	26.5 (25.5–28.0)	25.0 (22.0–29.0)	31.0 (29.3–34.0)	26.0 (25.0–27.5)
ONL	93.0 (86.0–101.0)	70.3 (65.8–75.6)	55.3 (50.9–59.1)	96.0 (87.0–102.0)	70.3 (64.0–78.3)	55.5 (49.3–61.0)
PRL	71.0 (68.0–73.0)	65.6 (64.5–67.4)	65.0 (63.5–66.0)	68.0 (66.0–70.0)	65.0 (63.8–65.8)	64.8 (63.5–65.5)
RPE	16.0 (14.5–17.0)	14.8 (13.8–15.8)	13.0 (12.0–14.0)	15.0 (14.0–18.0)	14.5 (13.3–15.8)	13.0 (11.8–14.0)
IRL	183.0 (171.5–195.0)	254.9 (241.0–263.9)	212.8 (203.1–221.3)	189.0 (177.0–204.0)	249.8 (239.8–264.3)	208.0 (196.5–219.3)
ORL	87.5 (85.0–89.5)	81.0 (79.0–82.5)	78.0 (76.5–79.6)	85.0 (81.0–88.0)	79.5 (77.5–81.8)	77.5 (75.8–79.3)
Overall retina	271.0 (258.5–283.5)	334.4 (322.1–346.1)	289.8 (280.9–300.4)	274.0 (260.0–291.0)	330.0 (318.3–344.0)	286.0 (274.6–296.0)

The table above provides median thickness values (µm) and, in parenthesis, the IQR (25th to 75th quartile) of the eight distinct retinal layers plus three combinations (IRL, ORL, and overall retina) in all 347 eyes analyzed from 234 patients (168 “young” eyes, 179 “old” eyes). Results are separated for participants younger and older than 74.7 years (=median) as well as central, inner, and outer circles. IQR, interquartile range.

ANNEX V.

Retinal layer volume values in a population aged 55+ separated for man and women as well as "young" and "old".

RETINAL LAYERS	Median volume (IQR) by Gender		Median volume (IQR) (mm^3) by Age	
	Male eyes (n=122)	Female eyes (n=225)	≤74.7 years (n=168)	>74.7 years (n=179)
RNFL	0.86 (0.78–0.95)	0.87 (0.81–0.96)	0.88 (0.79–0.96)	0.87 (0.81–0.96)
GCL	1.02 (0.94–1.09)	1.03 (0.94–1.09)	1.04 (0.96–1.11)	1.00 (0.92–1.06)
IPL	0.87 (0.81–0.93)	0.87 (0.81–0.92)	0.89 (0.83–0.94)	0.86 (0.79–0.90)
INL	0.97 (0.94–1.03)	0.96 (0.90–1.00)	0.98 (0.93–1.03)	0.95 (0.89–1.00)
OPL	0.78 (0.74–0.83)	0.77 (0.73–0.82)	0.78 (0.75–0.84)	0.77 (0.73–0.82)
ONL	1.75 (1.62–1.88)	1.67 (1.52–1.81)	1.69 (1.57–1.81)	1.69 (1.52–1.87)
PRL	1.86 (1.83–1.88)	1.83 (1.79–1.86)	1.85 (1.81–1.88)	1.84 (1.80–1.86)
RPE	0.38 (0.35–0.40)	0.38 (0.35–0.41)	0.38 (0.36–0.41)	0.38 (0.34–0.41)
IRL	6.29 (5.98–6.58)	6.15 (5.86–6.46)	6.28 (5.99–6.52)	6.11 (5.80–6.46)
ORL	2.24 (2.18–2.27)	2.21 (2.17–2.26)	2.24 (2.19–2.27)	2.21 (2.15–2.25)
Overall retina	8.48 (8.25–8.75)	8.37 (8.08–8.68)	8.48 (8.20–8.77)	8.35 (7.99–8.68)

The table above provides median volume values (mm^3) and, in parenthesis, the IQR (25th to 75th quartile) of the eight distinct retinal layers plus three combinations (IRL, ORL, and overall retina) in all 347 eyes analyzed from 234 patients. Results are separated for man and women as well as participants younger and older than 74.7 years (=median). IQR, interquartile range.

ANNEX VI.

Median subfoveal choroidal thickness values in a population aged 55+, by stage, gender and median age.

CHOROID		Median thickness (µm)	25th quartile	75th quartile
Stage	2a	237.5	171.0	289.0
	2b	268.5	228.0	317.0
	3	229.0	152.0	311.0
Gender	Male	260.0	203.0	298.0
	Female	231.0	167.0	282.0
Age	≤74.7	261.0	205.0	311.0
	>74.7	225.0	148.0	279.0

The table above provides median subfoveal choroidal thicknesses (µm) with the IQR (25th to 75th quartile) of all 347 eyes analyzed. Results are separated by early AMD stage, gender, and age (=median). IQR, interquartile range.

ANNEX VII.

The expected retinal layer thicknesses (µm) and the comparison across early AMD stages, by circle.

	Expected Retinal Layer Thicknesses (µM)			**Effect Estimates / Pairwise Test p-value**		
	2a	2b	3	2a vs. 2b	2a vs. 3	2b vs. 3
RNFL, central circle	12.3	12.3	12.1	-0.006 / 1.000	0.012 / 1.000	0.018 / 1.000
RNFL, inner circle	21.3	20.9	20.3	0.023 / 1.000	0.051 / 0.187	0.028 / 1.000
RNFL, outer circle	34.8	33.4	32.1	0.043 / 0.810	0.087 / 0.005	0.044 / 0.992
GCL, central circle	14.0	13.2	14.2	0.061 / 0.464	-0.003 / 1.000	-0.064 / 0.557
GCL, inner circle	47.5	45.6	42.9	0.043 / 0.953	**0.099 / 0.003**	0.056 / 0.768
GCL, outer circle	33.4	32.1	31.5	0.032 / 1.000	0.055 / 0.193	0.023 / 1.000
IPL, central circle	20.0	19.7	20.2	0.015 / 1.000	-0.006 / 1.000	-0.021 / 1.000
IPL, inner circle	40.1	39.3	37.5	0.018 / 1.000	**0.067 / 0.002**	0.048 / 0.420
IPL, outer circle	28.4	28.1	27.3	0.012 / 1.000	0.038 / 0.168	0.026 / 1.000
INL, central circle	20.7	20.5	22.0	0.006 / 1.000	-0.059 / 0.133	-0.065 / 0.528
INL, inner circle	42.1	40.9	40.0	0.030 / 1.000	0.056 / 0.176	0.027 / 1.000
INL, outer circle	33.1	33.1	32.1	0.006 / 1.000	0.028 / 1.000	0.022 / 1.000
OPL, central circle	25.0	26.6	25.8	-0.061 / 0.268	-0.028 / 0.796	0.032 / 1.000
OPL, inner circle	31.8	32.8	31.8	-0.029 / 1.000	0.006 / 1.000	0.035 / 1.000
OPL, outer circle	26.6	26.8	26.3	-0.007 / 1.000	0.011 / 1.000	0.018 / 1.000
ONL, central circle	95.4	93.1	87.3	0.023 / 1.000	**0.088 / 0.002**	0.065 / 0.353
ONL, inner circle	70.7	70.9	66.2	-0.003 / 1.000	0.067 / 0.026	0.070 / 0.278
ONL, outer circle	55.3	55.7	54.7	-0.008 / 1.000	0.011 / 1.000	0.020 / 1.000
PRL, central circle	70.0	70.1	68.4	-0.002 / 1.000	0.023 / 0.006	0.025 / 0.140
PRL, inner circle	65.6	65.6	65.2	-0.0003 / 1.000	0.005 / 1.000	0.005 / 1.000
PRL, outer circle	64.8	62.7	64.7	0.034 / 0.004	0.002 / 1.000	-0.032 / 0.030
RPE, central circle	16.0	15.6	18.4	0.013 / 1.000	**-0.140 / <0.0001**	**-0.153 / 0.002**
RPE, inner circle	14.6	14.7	15.2	-0.003 / 1.000	-0.034 / 0.559	-0.031 / 1.000
RPE, outer circle	12.8	13.9	12.7	-0.076 / 0.138	0.017 / 1.000	0.093 / 0.102

The table above provides Expected Retinal Layer Thicknesses (µm), effect estimates (on log scale), and p-values of pairwise tests, obtained from layer-specific linear mixed models. The analysis includes 347 eyes with early AMD (stages 2a, 2b, and 3), obtained from the original cohort of the AMD Incidence study. P-values were judge at Bonferroni-corrected significance level, $p<0.05/24=0.002$. Significant results are highlighted with bold text.

ANNEX VIII.

Qualitative retinal features in the studied eyes, obtained in the SD-OCT grading from de AMD Incidence Study.

Feature	Present N (%)	Absent N (%)	Questionable N (%)	Non-gradable N (%)
Reticular pseudodrusen	86 (24.93)	253 (73.33)	6 (1.74)	-
Drusenoid pigment epithelial detachment	4 (1.16)	338 (97.97)	3 (0.87)	-
Intraretinal Hyperreflective Foci	39 (11.30)	300 (86.96)	5 (1.45)	1 (0.29)
Vitreomacular adhesion	36 (10.43)	308 (89.28)	1 (0.29)	-
Epiretinal membrane	65 (18.84)	274 (79.42)	5 (1.45)	1 (0.29)

ANNEX IX.

Comparison between pseudodrusen and non-pseudodrusen eyes regarding the retinal/choroidal layers thicknesses.

Variables	Pseudodrusen (n=48)	Non-pseudodrusen (n=181)	p-value
Inner IPL	38.63 (37.13–41.00)	40.50 (38.00–42.50)	0.035
Outer INL	31.75 (30.25–32.75)	32.75 (31.25–34.75)	0.013
Outer OPL	25.25 (24.5–27.0)	26.5 (25.5–28.0)	<0.001
Central ONL	89.5 (84.0–98.0)	96.0 (88.0–102.0)	0.015
Inner ONL	67.25 (61.0–72.38)	71.75 (66.0–78.25)	0.003
Outer ONL	52.13 (48.25–57.0)	55.63 (51.75–61.00)	0.005
Central PRL	67 (65.5–69.5)	70 (68.0–73.0)	< 0.001
Inner PRL	64.75 (63.00–65.75)	65.5 (64.5–67.0)	<0.001
Outer PRL	64.13 (62.5–65.25)	65.00 (63.75–65.75)	0.005
Central RPE/BrM	15.0 (13.5–16.0)	16.0 (14.0–17.0)	0.001
Inner RPE/BrM	13.75 (12.5–15.25)	14.75 (13.75–15.75)	<0.001
Choroidal Layer	186.1 ± 84.7	255.1 ± 76.0	<0.001

The table above provides median thickness values (µm) and, in parenthesis, the IQR (25th to 75th quartile) of the retinal layers, by circle (central, inner and outer) that showed significant differences between pseudodrusen and non-pseudodrusen eyes. Furthermore, the mean choroidal thickness (µm) with the standard deviation is also provided. To statistical significance, p-value was established at 0.05.