Increased fundus autofluorescence, a biomarker of lipofuscin content, as a risk factor for the progression of geographic atrophy secondary to age-related macular degeneration

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Dedicat a la meva mare

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Abstract

Lipofuscin is a non-degradable intracellular compound that accumulates with age in post-mitotic cells and presumably interferes with its function. Increased autofluorescence can be used to identify its spatial distribution *in vivo* using fundus autofluorescence imaging (FAF). Lipofuscin build-up within retinal pigment epithelium cells of patients with geographic atrophy (GA, the advanced form of dry age-related macular degeneration) is well-known, but its role in disease pathogenesis is controversial.

We conducted a prospective, natural history study in patients with GA to identify factors associated with its progression, with particular attention to increased FAF as a biomarker of lipofuscin build-up. We confirmed that the distribution of increased FAF in GA followed distinct patterns (or "phenotypes"), but the interobserver agreement in their evaluation was suboptimal. These patterns were later found to be a consequence, not a cause, of disease growth. We identified time, baseline area of atrophy and FAF patterns as variables independently associated with disease progression, but the effect of FAF patterns was modest and strongly confounded by baseline area of atrophy. Additionally, patients with GA and foveal sparing showed thinning of subfoveolar photoreceptor outer segments and thickening of Henle's fiber layer on optical coherence tomography in an otherwise normal-looking fundus.

These results suggest that it is disease progression which actually induces changes in the distribution of FAF and that lipofuscin may have a smaller influence in GA pathogenesis than previously thought.

Resum

La lipofuscina és un compost intracel-lular no degradable que s'acumula amb l'edat en cèl.lules postmitòtiques i que presumiblement interfereix amb les seves funcions. L'augment de l'autofluorescència es pot utilizar per identificar la seva distribució espaial *in vivo* utilitzant l'autofluorescència del fons d'ull (AFU). L'acumulació de lipofuscina a les cèl-lules de l'epiteli pigmentari de la retina en pacients amb atròfia geográfica (AG, la variant avançada de la degeneració macular seca associada a l'edat) és un fet conegut, però el seu paper en la patogènia de la malaltia és controvertit.

Vàrem dissenyar un estudi prospectiu sobre la historia natural de l'AG per identificar factors de risc associats amb la seva progressió, amb especial atenció a l'augment de l'AFU com a biomarcador de l'acumulació de lipofuscina. Vàrem confirmar que la distribució de l'augment de l'AFU segueix diferents patrons (o "fenotips"), però que l'acord interobservador en la seva avaluació és imperfecte. Aquests patrons semblen una conseqüència, no una causa, del creixement de la malaltia. El temps de seguiment, l'àrea d'atròfia basal i els patrons d'AFU estan associats de manera independent amb el creixement de l'AG, però l'efecte dels patrons va ser moderat i estava marcadament confós per l'àrea d'atròfia basal. D'altra banda, els pacients amb AG i respecte foveal van mostrar un aprimament del segment extern dels fotoreceptors subfoveolars un engruiximent de la capa de fibres de Henle en la tomografia de coherència òptica, en un fons d'ull, per la resta, aparentment normal.

Aquests resultats suggereixen que és la pròpia progressió de l'AG la que indueix canvis en la distribució de l'AFU i que la lipofuscina té una menor influència el la patogènia de la malaltia del que es pensava prèviament.

Preface

The current work was conducted from September 2009 to August 2013 at the Institut de la Màcula i de la Retina (Centro Médico Teknon, Barcelona). Since its inception in 2007, this center has been devoted to provide stat-of-the-art ophthalmologic care to patients with retinal diseases and to conduct research for blinding disorders. The group is led by Dr Jordi Monés.

Since 2009, I had the opportunity to work in investigator-driven research projects and in international, phases I to IV clinical trials in age-related macular degeneration (AMD) and allied disorders as study coordinator and photographer. I learned many of the methods used in this Thesis through courses at the Fall Institute of the Johns Hopkins School of Public Health and the University of Freiburg, amongst others.

Geographic atrophy (GA), the advanced form of dry AMD, is a retinal disease for which no treatment is available yet. Excessive lipofuscin accumulation has been linked to the progression of GA, but its role remains controversial due to conflicting results from both, basic and clinical research.

In this context, our aim was to contribute to clarify this issue with the prospective collection of high quality clinical data and a focused question: is high autofluorescence, assumed to represent excessive lipofuscin accumulation within retinal pigment epithelium cells, a risk factor for GA progression? Patients with GA had been classified according to the peculiar distribution of high autofluorescence around areas of atrophy in several categories (patterns or phenotypes). We evaluated the consistency of this

classification and the feasibility of a new, simplified one. This analysis turned out to demonstrate unexpected results regarding the underlying causes of the distribution of high autofluorescence on which the original classification was based.

As will be thoroughly discussed, these findings have implications for visual cycle modulation, a therapeutic strategy aimed at limiting the rate of lipofuscin build-up within retinal pigment epithelium cells.

Table of contents

Abbreviations

A2-DHP-PE	A2-dihydropyridine-phosphatifylethanolamine
A2E	N-retinyledene-N-retinylethanolamine
A2PE	Phosphatidyl-pyridinium
ABCA4	ATP-binding cassette transporter, sub-family A, member 4 gene
AGEs	Advanced glycation end products
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
ApoE	Apolipoprotein E gene
AREDS	Age-Related Eye Disease Study
ARMS2	Age-related maculopathy susceptibility 2 gene
ARVO	Association for Research in Vision and Ophthalmology
BAA	Baseline area of atrophy
BCVA	Best corrected visual acuity
BDES	Beaver Dam Eye Study
BMES	Blue Mountains Eye Study
BMI	Body mass index
CATT	Comparison of AMD Treatments Trial
CEP	Carboxyethylpyrrole

CI	Confidence interval
CFB	Complement Factor B gene
CFH	Complement Factor H gene
CNTF	Ciliary neurotrophic factor
CNV	Choroidal neovascularization
cSLO	Confocal scanning laser ophthalmoscope
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DRCR.net	Diabetic Retinopathy Clinical Research network
ERG	Electroretinography
ETDRS	Early Treatment Diabetic Retinopathy Study
EUREYE	European Eye Study
FA	Fluorescein angiography
FAF	Fundus autofluorescence
FAM	Fundus Autofluorescence in age-related Macular degeneration study
FDA	Food and Drug Administration
FP	Fundus photography
GA	Geographic atrophy
GAP	Geographic Atrophy Progression study
GWAS	Genome-wide association study
HFL	Henle's fiber layer

HR	Hazard ratio
HuGENet	Human Genome Epidemiology Network
ICGS	International Classification and Grading System for age-related maculopathy and age-related macular degeneration
IL	Interleukin
LCA	Latent class analysis
MALDI-IMS	Matrix-assisted laser desorption-ionization imaging Mass spectrometry
MEM	Mixed-effects model
Mt	Mitochondrial
NLRP3	NOD-like receptor family, pyrin domain containing 3
OR	Odds ratio
PCA	Principal component analysis
PUFA	Polyunsaturated fatty acids
RIP	Receptor-interacting protein kinases
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SD OCT	Spectral domain optical coherence tomography
SEE	Spanish Eyes Epidemiologic study

SNP	Single nucleotide polymorphism
STROBE	Strengthening The Reporting of Observational studies in Epidemiology
VCM	Visual cycle modulators
VEGF	Vascular endothelial growth factor
WARMGS	Wisconsin Age-Related Maculopathy Grading System

Introduction

1. Brief reminder of retinal and macular anatomy

The retina is a light-sensitive tissue that covers the innermost part of the eye (**Figure 1**, left), whose function is converting light energy into an electric signal in a process known as phototransduction. This signal is transmitted through the optic nerve to the brain, where this information is converted into vision.

The macula (**Figure 1**, middle) is the central part of the retina and harbors the maximum capacity of discrimination (visual acuity). It is defined clinically as an oval area of 5 mm of horizontal diameter temporal to the optic nerve. In its center is located the fovea, where the concentration of cones (the photoreceptors that provide the maximum visual acuity and color vision) is highest. Age-related macular degeneration (AMD) is a disease that affects this critical area of the retina.

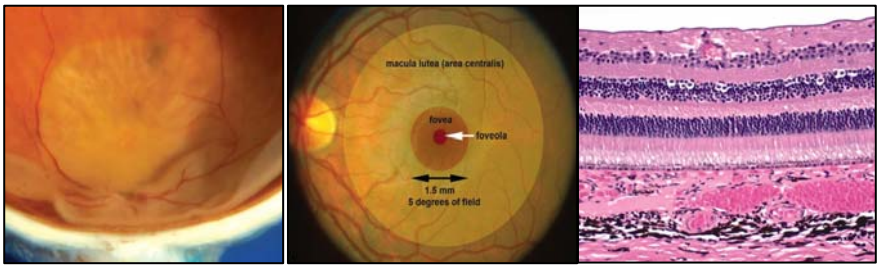

Figure 1. *Left*, posterior pole of an enucleated eye with geographic atrophy. The retina is the innermost layer, which is artifactually detached here. *Middle*, the location of the macula and the fovea on a fundus photograph of the retina. *Right*, histologic section showing the multilayered, highly organized architecture of the retina; its thickness is ¼

of a millimeter (from: Eagle RC, Jr. Eye pathology, 2nd edition. Ed LWW, 2011. Used with permission).

A cross-section of an extrafoveal area of the retina shows its ten-layered structure (**Figure 1**, right). The innermost part of the retina (from bipolar to ganglion cells) arranges and conducts the information generated in the photoreceptors to the midbrain through the optic nerve. In the outer retina there is another type of photoreceptor intermixed with cones: rods, which allow vision in dim light. The outermost part of the retina is the retinal pigment epithelium (RPE), a monolayer of cells with several fundamental functions for proper photoreceptor survival and retinal homeostasis (1).

Bruch´s membrane lies external to the retina and adjacent to the RPE and provides a semipermeable filtration barrier through which metabolic exchange occurs (2). Finally, the choriocapillaris is a thin layer of vessels of the choroid external to Bruch´s membrane and provides nutrients to the outer third of the retina (RPE and photoreceptors). Retinal vessels nourish the inner retina.

Photoreceptors, RPE, Bruch´s membrane and choriocapillaris form a functional unit, and damage to one cell type has deleterious effects on the others. These are the structures damaged in geographic atrophy (GA).

2. Definition and classification of age-related macular degeneration and its advanced atrophic form, geographic atrophy

AMD is the first cause of legal blindness in developed countries and the third cause worldwide, after cataract and glaucoma (3-6). It is a disorder of the macula characterized by one or more of the following lesions: drusen, hyper or hypopigmentation of the RPE, GA of the RPE and choriocapillaris, and neovascular maculopathy (7). These lesions vary widely in their ophthalmoscopic aspect and relevance in terms of impact on visual function. AMD is bilateral, relatively symmetric and painless.

In its early and intermediate stages, AMD is characterized by drusen, yellowish deposits located between the RPE and Bruch's membrane that are the hallmark of the disease. These lesions can be accompanied by areas of hyper or hypopigmentation of the RPE (first column in **Figure 2**). The advanced or late forms of the disease and major causes of vision loss are GA and neovascular AMD. In GA (also called advanced dry or atrophic AMD), large areas of RPE atrophy, photoreceptor and choriocapillaris loss ensue (second column in **Figure 2**). This induces scotomas, irreversible blind spots that can severely decrease visual acuity if the fovea is affected. Neovascular (wet, exudative) AMD is caused by growth of new blood vessels from the choriocapillaris towards the retina, where they bleed and leak lipid and fluid that destroy the retinal architecture, causing severe acute vision loss (third column in **Figure 2**). Neovascular AMD can be successfully managed by

intravitreal injection of antiangiogenic drugs (8-10). Unfortunately, there is no treatment for GA.

The most common classification systems for AMD are the AREDS (Age-Related Eye Disease Study) (11), the ICGS (International Classification and Grading System for age-related maculopathy and age-related macular degeneration) (12) and the WARMGS (Wisconsin Age-Related Maculopathy Grading System) (13). The definition of GA in each is rather similar (**Table 1**).

In this study, GA was defined as an area of complete RPE depigmentation or atrophy equal or larger than 0.5 DA (1.27 mm^2) as seen on 35º non-stereoscopic fundus photography (FP) that met at least two of the following: sharp borders, approximate oval shape or increased visibility of choroidal vessels. This definition is probably the most restrictive (with higher specificity) and it is used in many clinical studies. Patients fulfilling it will probably have GA by all other classification systems.

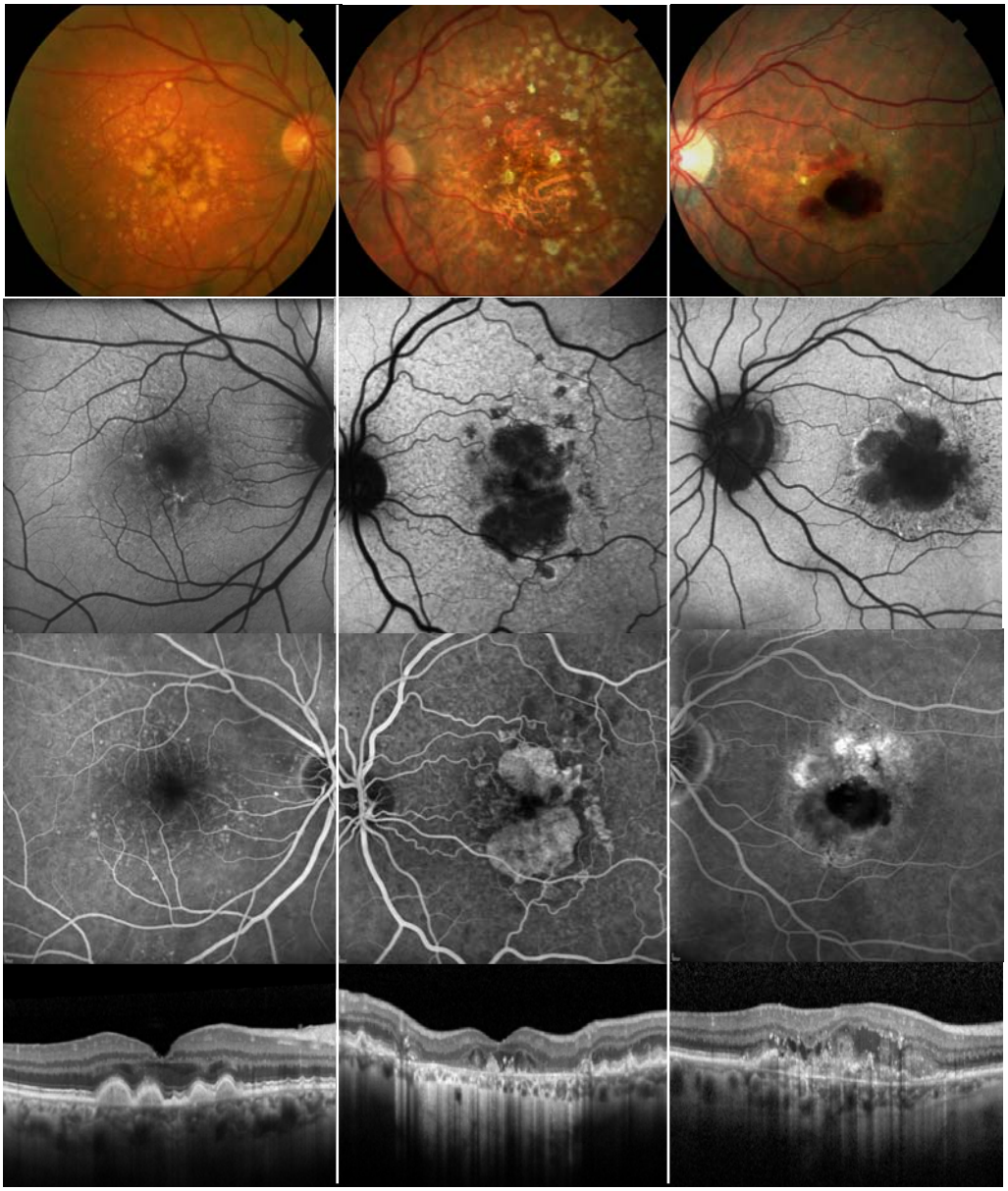

Figure 2. Patients with drusen, GA and neovascular AMD (first to third columns, respectively) as seen with different imaging methods (FP, FAF, FA and SD OCT in each row). *Left*, drusen are identifiable in FP as yellowish deposits in the outer retina, which correspond to RPE elevations on SD OCT; they are more difficult to identify on FAF and FA. *Middle*, GA is an area of sharp RPE atrophy, which is more clearly delineated on FAF

as a dark area (due to the lack of lipofuscin caused by absence of RPE) and bright on FA (window defect). Lack of RPE and photoreceptors cause retinal thinning and increased signal from the choroid on SD OCT. *Right*, neovascular AMD is seen here as an hemorrhage accompanied by a few exudates, which are hypoautofluorescent on FAF; there is leakage on FA and retinal thickening by sub/intraretinal fluid on SD OCT. AMD: age-related macular degeneration; FA: fluorescein angiography; FAF: fundus autofluorescence; FP: fundus photography; GA: geographic atrophy; RPE: retinal pigmented epithelium; SD OCT: spectral domain optical coherence tomography.

Table 1. Definition of geographic atrophy according to different grading systems (AREDS: Age-Related Eye Disease Study; ICGS: International Classification and Grading System for Age-related Maculopathy and Age-related Macular Degeneration; RPE: retinal pigment epithelium; WARMGS: Wisconsin Age-related Maculopathy Grading System).

AREDS	A sharply demarcated, usually circular zone of partial or complete depigmentation of the retinal pigment epithelium, typically with exposure of the underlying large choroidal blood vessels, that must be as large as 1/8 disk diameter in diameter [an approximate diameter of 187.5 μm].
ICGS	Any sharply delineated roughly round or oval area of hypopigmentation or depigmentation or apparent absence of the RPE in which choroidal vessels are more visible than in surrounding areas that must be at least 175 μm in diameter [1/8.6 disk areas].
WARMGS	A sharply defined area of dropout of the RPE, exposing choroidal blood vessels […]. The area of atrophy must be at least as large as standard circle I_1 to be considered definitely present [an approximate radius of 175 μm].

3. Epidemiology of geographic atrophy

Population-based studies conducted in many countries around the world for the past 20 years have provided a large amount of data on the epidemiology of AMD and GA in different geographic locations, age ranges and ethnic groups, and have begun to provide information on secular trends.

a. Prevalence and disease burden

To get a wide perspective of the disease, it is useful to first review briefly the prevalence of AMD. According to the meta-analysis conducted by Friedman et al in 2004 (14), the estimated prevalence of late AMD in the population of the United States aged 40 years or older is 1.47% (95% confidence interval -CI-, 1.38% to 1.55%), equivalent to 1.75 million individuals with AMD. Of these, 0.81% (almost 1 million) had GA in at least one eye. Additionally, more than 7 million had large drusen and therefore were at risk for incident late AMD.

Patients with advanced AMD report low levels of quality of life, equivalent to those experienced by subjects with severe medical conditions such as coronary heart disease or stroke (15,16). They suffer a higher prevalence of depressive symptoms (17), cognitive impairment (18) and increased risk of falls than age-adjusted controls (19). An increased risk of mortality has also been reported in some (20,21) but not all (22) studies, presumably through shared vascular risk factors. The economic burden of atrophic AMD

(drusen, RPE abnormalities and/or GA) in the United States is enormous: $26.100 million annually in one study (23), and approximately 0.22% of its Gross Domestic Product in 2010 in terms of wage loss from untreated disease in another report (24).

GA increases steeply with age: it is unusual before 55 years old, and it raises to 3.5% in people aged 75 years or older, and to 22% in those older than 90 years (25-27). It also varies between ethnic groups, being more common in Caucasians (28,29). Overall, there is a similar prevalence of GA in developed countries: 1.2% (95% CI, 0.8% to 1.7%) in the European Eye Study (EUREYE) (30) and 1.5% (95% CI, 0.9% to 2.1%) in the Spanish Eyes Epidemiological (SEE) Study Group (31). A notable exception is found in Northern Europe, where most (32,33) (but not all (34)) studies have shown a strikingly high prevalence: 9.2% (95% CI, 5.6% to 12.7%) in the Reykjavik Eye Study, in Iceland (35). These results were seen in slightly older age groups (>70 years) than most other studies (≥40 years in the meta-analysis of Friedman et al, ≥65 years in EUREYE and SEE), but age or methodology do not seem to be the only factors explaining these differences.

b. Incidence

The 15-year cumulative incidence of late AMD in the Beaver Dam Eye Study (BDES) was 3.1%, 1.3% of which corresponded to GA (36). The incidence of pure GA increased with age (from 0% amongst those between 43 and 54 years old to 3.2% in those 75-86 years). Increasing drusen area, soft indistinct drusen and pigmentary changes at baseline were identified as retinal risk

factors for GA incidence. Again, the 5-year incidence of GA in the Reykjavik Eye Study was larger, 4.6% (95% CI, 1.2% to 7.9%) (37).

Of note, GA incidence in the BDES was 4 times as high as that of neovascular AMD in people ≥85 years old (8.2% vs 1.9%) (36). However, these results should be approached with care because few patients of this age range were followed for at least 5 years and the number of events was low.

c. Risk factors

Factors associated with incident GA have been more extensively studied than factors related to disease progression. However, since these factors may be related, a brief summary of the most relevant associations found for new GA is provided.

The most consistent risk (or prognostic) factors for development of new GA are older age (26,29), smoking (odds ratio -OR-, 2 to 3) (26,29,38) and specific genetic polymorphisms (see *Genetics of geographic atrophy*, on page 35). Soft confluent drusen precede areas of retinal atrophy (39) and, actually, GA is considered by some authors to be the end-stage of drusen life cycle (40). Reticular drusen have also been associated with an increased risk of incident GA (41,42) and its progression (43), but these lesions are ubiquitous in GA, and therefore their impact on disease growth is difficult to evaluate. Other factors associated with GA are ethnicity/race (with Caucasians having higher risk) (44), high body-mass index (BMI) (29), cardiovascular disease (29) and cataract

surgery (29). Basic and clinical research have suggested a potential beneficial role with the supplementation of antioxidants (45), ω-3 polyunsaturated fatty acids (PUFA) (46) or the macular pigments lutein and zeaxanthin (47), with point estimates in the protective direction in most published observational studies. However, results from systematic reviews have been inconclusive (48-50), and the two largest randomized, placebo-controlled, double-blind clinical trials to date, AREDS (51) and AREDS2 (52), have shown no protective effect of these compounds on incident central GA (see *Treatment of geographic atrophy. Antioxidants*, on page 45). Smoking, BMI, cataract surgery and dietary factors are especially important since they are potentially modifiable.

On the other hand, the prognostic/risk factors for progression of prevalent GA, the focus of the current Thesis, have been less consistently investigated. Sunness et al (53) reported that knowledge of prior rates of enlargement in the previous 2 years in a given individual was the major predictor of subsequent growth rates. In other words, spread of atrophy was subject-specific rather than random. However, using FP as the imaging method, they could not identify which factors drove progression, since baseline size of atrophy (BAA, an initial predictor of growth) lost its significance when considering of prior rates of enlargement (53). Yehoshua et al (54) reported years later that performing square root transformations on the area of atrophy measured in mm^2 made growth of atrophy constant (slope near 0) and independent of BAA.

In 2001, Holz et al used fundus autofluorescence (FAF) to follow three patients with GA for 3 years and showed that enlargement of atrophy was confined exclusively to areas that previously showed

increased levels of autofluorescence (55). In 1995, Delori et al (56) had attributed increased levels of FAF to lipofuscin, a byproduct of the visual cycle (see *Lipofuscin of the retinal pigment epithelium*, on page 17), and therefore a causal link was proposed between lipofuscin as seen by hyperautofluorescence and progression of the disease.

However, in 2006 Hwang et al (57) performed digital imaging analysis in 6 patients (8 eyes) with GA, and found that the positive predictive value of increased FAF in relation to development of new areas of GA was not greater than chance, thus challenging the hypothesis that increased FAF is a risk factor for GA. Of note, the same group reached the same conclusions after evaluating patients with Stargardt disease, a macular dystrophy phenotypically similar to GA: areas of hyperautofluorescence were not more likely than background to develop future hypoautofluorescence (atrophy) (58). A recent histologic study conducted in 10 donors with GA and 3 age-matched control eyes attributed the increase in FAF not to increased lipofuscin content within individual RPE cells, but to vertically superimposed cells or cellular fragments migrating into the retina, and found no difference in intracellular lipofuscin content in RPE with different grades of pathology (59), again challenging the explanation of lipofuscin-induced maculopathy.

Motivated by their previous findings, Holz et al initiated a prospective, multicenter, longitudinal natural history study in patients with GA, the Fundus Autofluorescence in age-related Macular degeneration (FAM) study (60). One of its most striking findings was the discovery of specific “patterns” or phenotypes in GA patients seen on FAF. The definition of these patterns was

based on the distribution of increased FAF around the areas of atrophy: some patients had no increased FAF, other showed focal increased FAF at the borders of the area of atrophy, others had an annular configuration of increased FAF at the junction, others had a more widespread distribution of high FAF, etc (**Figure 3**). The original description involved up to 10 categories, but different subclassifications were proposed by the same group to simplify the findings. These patterns had a high degree of interocular symmetry (61).

Most important, it was also found that FAF patterns were associated with progression of GA, whereby those patterns characterized by larger areas of increased FAF (that is, increased accumulation of lipofuscin) experienced a greater rate of growth. This relationship seems akin to that occurring between drusen and increased risk of AMD, where large, soft drusen show an increased risk of late AMD than small, hard drusen (62). Actually, it would be desirable to measure the area of elevated FAF and/or its intensity while controlling for variations in laser power, photoreceptor bleaching or other sources of autofluorescence, such as that of the aging lens. Ongoing progress is being made in this area (63).

The results of the FAM study can be summarized as follows: (1) FAF patterns were associated with disease progression and may represent GA phenotypes; and (2) lipofuscin was causally associated with GA enlargement.

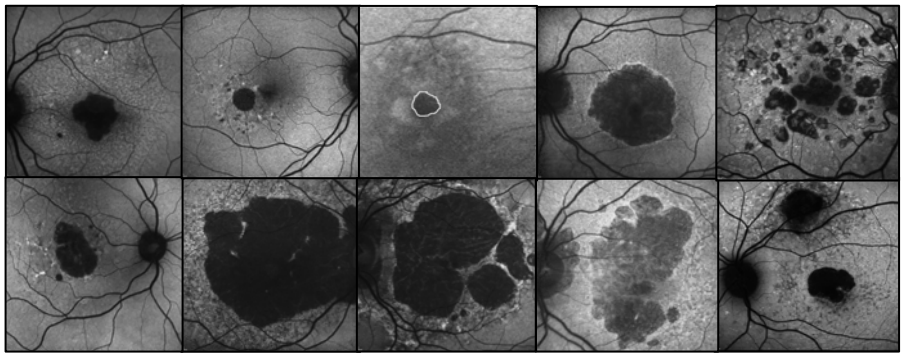

Figure 3. FAF patterns as described in the FAM study (60). First row, from left to right: None (no increased FAF at the borders of atrophy), Focal (individual small spots of increased FAF around atrophy), Patchy (laminar, increased FAF), Banded (ring-like increased FAF adjacent to atrophy) and Undetermined (patients unclassifiable by any definition). Second row, from left to right: Reticular (linear increased FAF with radial orientation), Fine granular (grainy FAF), Branching (ramified FAF), Trickling (grayish atrophy with increased FAF that fades towards the periphery) and Fine granular with peripheral punctate spots (grainy FAF at the junction with numerous peripheral focal increased FAF lesions). Patterns in the second row were collapsed into a single category called Diffuse for simplification. FAF: fundus autofluorescence (image from the Patchy pattern taken from: Holz FG et al. Progression of geographic atrophy and impact of fundus autofluorescence patterns in age-related macular degeneration. Am J Ophthalmol 2007; 3: 463-72. Used with permission).

However, FAF patterns and other variables collected in the FAM study were not associated with GA progression in multivariable models (64,65). The authors suggested that low power was

responsible for the negative findings, but confounding in the bivariate association was also a possible explanation.

Genetic associations may provide further insights in disease pathogenesis. In the BDES, polymorphisms in the age-related maculopathy susceptibility 2 gene (ARMS2; see *Genetics of geographic atrophy* on page 35) were nominally associated with a faster progression of GA, but not with progression to central GA or incident GA in the contralateral eye (66). This association was not replicated by Scholl et al (67). In the Blue Mountains Eye Study (BMES) there was an association between faster progression of atrophy and smoking at baseline, pseudophakia and gene variants in complement factor H (CFH) and ARMS2, (42) while Caire et al recently reported faster growth in patients with several variants in the complement pathway in a Spanish cohort (68).

Some factors may not truly confer an increased risk *per se* but may have prognostic value. A multifocal configuration of GA relative to unifocal lesions and an irregular shape as compared with a rounded atrophy have been associated with faster progression in different studies (40,43). Fleckenstein et al (69) found that the status of the fellow eye (classified as early AMD, neovascular AMD or GA) could predict progression of atrophy in the study eye: eyes with bilateral GA progressed faster than eyes with early AMD in the contralateral eye when GA size at baseline was 1 disk area or more (it must be noted that growth of atrophy is remarkably similar in both eyes in patients with bilateral GA (53,70)). In addition, some features identified by SD OCT, such as intraretinal migration of hyperreflective foci (71) and splitting of the RPE/Bruch's membrane complex at two junctional borders (72), have also been associated

with incident GA or greater enlargement rates, respectively. These results underscore the potential role of novel imaging technologies as biomarkers of disease progression. While these findings do not convey therapeutic opportunities, they may be useful for prognostic modeling.

In summary, progression does not seem to be random. Rates of enlargement in the previous 2 years, multifocal lesions, status of the fellow eye and certain SD OCT characteristics have been identified as potential prognostic factors of GA progression. Results from genetic studies have been contradictory, and the sporadic associations involving smoking and pseudophakia, mechanisms likely related to oxidative stress, are difficult to interpret. FAF patterns have emerged as a risk factor in bivariate but not in multivariable analysis, but they are regarded as the most important risk factor and are used as an eligibility criterion in many clinical trials. The identification of risk factors in GA progression remains a fundamental piece of the puzzle that needs to be addressed.

4. Lipofuscin of the retinal pigment epithelium

a. *What is lipofuscin?*

Lipofuscin is an intracellular material made of lipids, proteins and vitamin A derivatives stored in the lysosomal compartment of non mitotic cells. It is not amenable to degradation and therefore

accumulates with time, becoming a marker of age (**Figure 4**) (73,74). It may interfere with cell homeostasis.

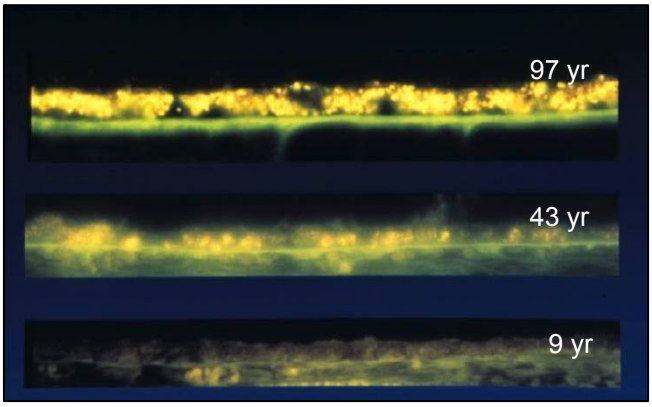

Figure 4. Age-related increase in autofluorescence caused by lipofuscin within the retinal pigment epithelium seen on fluorescence microscopy from 9 (bottom), 43 (middle) and 97 (top) year-old donors (from: Boulton ME. Ageing of the retina and retinal pigment epithelium. In: Holz FG et al (eds). Age-related macular degeneration, 2nd ed. Springer, Berlin, 2013, p 51, Fig 3.4.b. Used with kind permission from Springer Science+Business Media B.V.).

The formation of lipofuscin and its main bisretinoid, N-retinyledene-N-retinylethanolamine (A2E), occurs in the visual cycle. Light activates rhodopsin, which involves the isomerization of 11-*cis*-retinal into all-*trans*-retinal. All-*trans*-retinal is released in the photoreceptor membrane by ABCA4 protein (ATP-binding cassette transporter, sub-family A, member 4, whose polymorphism leads to Stargardt disease), where it binds to other compounds, eventually becoming A2E (75). A2E is transported to the RPE through phagocytosis of the photoreceptor outer segment, where it is

captured together with other chemically modified residues in the lysosome, forming lipofuscin (76,77). Therefore, its composition is derived mainly from metabolites of the visual cycle. Once lipofuscin and A2E are formed, they are not eliminated from the RPE (78). Other lipofuscin bisretinoids are isoA2E, A2-DHP-PE (A2-dihydropyridine-phosphatifylethanolamine), A2-PE (phosphatidyl-pyridinium), and its oxidated forms, peroxy-A2E and furano-A2E. Additional photosensitizing molecules remain to be identified (79).

Therefore, A2E is formed in the photoreceptor and lipofuscin within the RPE. Since light exposure and visual cycle dynamics determine their rate of accumulation, modulation of visual cycle is a rational therapeutic strategy if these compounds are involved in GA pathogenesis.

Using matrix-assisted laser desorption-ionization imaging mass spectrometry (MALDI-IMS), Ablonczy et al (80) reported a lack of spatial correlation between lipofuscin (which was found mostly in the macula) and A2E (concentrated in the far periphery) on human RPE, while their distribution was similar in mouse (81). If these results were confirmed, they would call into question the role of A2E in AMD pathophysiology in man. Sparrow et al have argued that this may be a consequence of photodegradation of macular lipofuscin (82).

b. Effects of lipofuscin

Most studies evaluating the effects of lipofuscin are based on *in vitro* and animal models. According to this preclinical data,

lipofuscin damages the RPE by interfering with the ATPase-dependent proton pump located in the lysosomal membrane (83), which induces an alkalinization of lysosomes, bleb extrusion and leakage of lysosome content into the RPE cytoplasm. It also causes deoxyribonucleic acid (DNA) damage, in part through oxidative base modifications (84). When stimulated by light (especially in the blue range), A2E induces apoptosis (85), probably through formation of reactive oxygen species (ROS). A2E also induces a low-grade inflammation that is able to trigger the complement (86,87) and activate the inflammasome (88), which have been implicated in GA. A 1989 histological study showed that the number of photoreceptors overlying areas of increased lipofuscin was reduced (89), a finding that was not confirmed in a recent study (90).

Lipofuscin is autofluorescent (56), and clinical studies have shown reduced retinal sensitivity in areas of increased FAF (91,92). These results suggest that an age-related increase in lipofuscin in the RPE in susceptible individuals may induce a loss in photoreceptors that decreases retinal sensitivity. Further damage may lead to atrophy of the RPE (GA) with permanent hypoautofluorescence and absolute scotoma (55).

Nonetheless, this view has been disputed (93-95). The increased FAF has been attributed to vertically superimposed RPE cells, not to increased intracellular lipofuscin content (59). There is no correlation between levels of increased FAF and reduction of retinal sensitivity, with similar levels of FAF showing different sensitivity and vice versa. Also, if lipofuscin increases with age and is toxic, why the number of RPE cells remains relatively constant

throughout life in healthy subjects (96)? How can RPE cells at the junction between GA and healthy retina acquire lipofuscin so fast? The disparate spatial distribution of A2E and lipofuscin in humans poses a novel question that was thought to be solved years ago. Clearly, there is a need to better characterize the relationship between lipofuscin accumulation and retinal dysfunction.

c. *Lipofuscin is the main (but not the only) source of fundus autofluorescence*

Lipofuscin has autofluorescent properties due to the presence of chromophores in its composition. Delori et al (56) demonstrated that high FAF originated from lipofuscin due to its spectral characteristics, topographic distribution and relationship with age. Therefore, increased FAF is considered a biomarker of increased lipofuscin content. Although A2E has long been presumed to be the main responsible for lipofuscin high FAF, this view is questioned (80,81). In this case, the main chromophore would remain to be identified.

The excitation of lipofuscin is maximal between 480 to 510 nm, and its emission ranges from 480 to 800 nm, peaking at 610 nm. Its distribution roughly follows that of rod photoreceptors: low concentration at the fovea (due to a slower lipofuscin formation in cones compared to rods and to macular pigment absorption), with a maximum at 7º-13º from the fovea (mostly superotemporally) and a decrease towards the periphery. FAF increases until the 70's; a decrease has been reported thereafter, presumably caused by removal of RPE cells (97).

Of note, photoreceptors can be another source of markedly increased FAF (98). Under conditions of RPE dysfunction (GA) or photoreceptor impairment (retinitis pigmentosa), mishandling of lipofuscin precursors in the photoreceptor outer segment (for example, increased phosphatidyl-pyridinium -A2PE- formation or deficient bisretinoid transport) would result in elevated FAF (98). This may explain the increase in FAF in a previously hypoautofluorescent area in GA: RPE atrophy would induce an area of low FAF (due to the loss of lipofuscin), which may be followed by increased FAF at the same location (due to increased bisretinoid formation by impaired photoreceptors) (82). Photooxidation of lipofuscin and stimulation of other fluorophores with an emission spectra similar to that identifiable by FAF imaging are other potential causes of high FAF (98).

d. Methods used to visualize fundus autofluorescence in vivo

The methods used to obtain FAF images in clinical practice are the confocal scanning laser ophthalmoscope (cSLO) and the fundus camera with filters.

The cSLO was originally developed by Webb et al in 1987 (99). The optical system consists of a laser beam projected onto the retinal surface that sequentially scans the fundus in x and y directions by means of oscillating mirrors. The reflected light is registered by a detector that generates a bidimensional image. Contrast can be enhanced by use of confocal apertures, pinholes located in a plane conjugated with the focused area that effectively

suppress light originated out of focus (for example, from the crystalline).

The use of cSLO to visualize FAF is credited to von Rückmann et al, in 1995 (100). The low signal originated by FAF requires the creation of mean images from several frames, which also improves the signal-to-noise ratio. The device used in the present study, the Spectralis HRA+OCT ® (Heidelberg Engineering; Heidelberg, Germany), is a cSLO which automatically generates mean images.

Spaide pioneered the use of filters in adapted fundus cameras to obtain FAF (101,102). To avoid the nuisance originated by the lens fluorescence, modifications in the emission and absorption spectra of the original filters were developed (excitation 535 to 580 nm, barrier 615 to 715 nm (102)) that achieved brighter and less noisy images. Since these filters do not match those used in fluorescein angiography (FA), FAF can be performed after this exploration with the fundus camera. Additionally, due to its decreased absorption by the luteal pigment, the fovea is less hypofluorescent than with a cSLO, making it easier to determine the extent of atrophy in the fovea. Nonetheless, FAF patterns are more difficult to identify with a modified fundus camera (103) and the final images suffer the deleterious effects of scattering and decreased contrast.

5. Clinical features of geographic atrophy

Many cases of incipient GA appear as multifocal, small areas of RPE depigmentation around the fovea. These lesions coalesce with time, forming a horseshoe-shaped band of atrophy that progressively becomes annular in shape before the fovea is finally consumed by the atrophic process. These areas of GA are devoid of RPE, photoreceptors and choriocapillaris (104) (see *Histology of geographic atrophy*, on page 38), and therefore correspond to absolute (and permanent) scotomas (105).

However, the damage caused by the disease process extends well beyond the area of atrophy: ophthalmoscopically normal, junctional (perilesional) areas show a decreased sensitivity on microperimetry (91,106,107) and a selective scotopic sensitivity loss as seen with fine matrix mapping (92). These areas usually show increased autofluorescence (60), and areas of increased FAF show severe alterations in outer retinal layers on SD OCT more frequently than areas with normal FAF (108). Again, since increased FAF has been attributed to lipofuscin (56), this compound is regarded as key in the pathogenesis of GA.

GA is the cause of blindness in, at least, 20% of patients with AMD (109), but best corrected visual acuity (BCVA) is a potentially misleading measure of the functional impact of the disease. The tendency of the areas of atrophy to respect the foveola (104,110) entails a typically conserved visual acuity until late in the course of the disease, the so-called foveal sparing. Instead, GA patients usually complain of difficulty reading, driving, recognizing faces or

vision in dim light (111,112). Contrast sensitivity is also poor, even with good visual acuity (111). The reasons for the preferential involvement of the perifoveal area (or the protection of the foveola) are incompletely understood, but selective vulnerability of rods (113), protection from the macular pigment (114) and the unique blood supply from the choroid (115) have been proposed.

Although BCVA has significant limitations in the evaluation of visual function in GA, it is the parameter most commonly used. Sunness et al (116) reported that the rate of moderate visual acuity loss (≥15 letters) was 31% in 2 years and 53% in 4 years, which represents a higher decline than that experienced by patients with untreated diabetic macular edema (117). This rate of loss was even greater in those with better baseline visual acuity (>20/50).

Sudden visual acuity loss should raise the suspicion of conversion to wet AMD, which appears in 18% of patients with fellow eye-choroidal neovascularization (CNV) after 2 years, and in 34% after 4 years (118). The risk is lower in patients without contralateral CNV (2% and 11% in the same period, respectively). Foveal GA is an important cause of limited functional improvement after treatment with anti-vascular endothelial growth factor (VEGF) in patients with CNV (119). It has been reported that frequent treatment with antiangiogenic therapy may be associated with faster GA enlargement in the CATT (Comparison of AMD Treatments Trials) study (120), and virtually all eyes treated with VEGF inhibitors go on to develop macular atrophy over the long term (121), which reinforces the need to develop therapies to manage GA.

The consequences of impaired visual function on everyday tasks can be measured through standardized quality of life questionnaires. Unfortunately, this issue has not been reported in a population of patients with pure GA.

The condition is bilateral in roughly half of patients (116), but baseline atrophy size may be asymmetric (61). Even in these cases, the growth of atrophy tends to be similar between eyes (70), with high interindividual variability. In fact, some patients may experience a median growth as low as 0.38 mm^2/year, while others suffer from a fast expansion of 3.02 mm^2/year (60). Sunness et al found that the strongest predictor of future growth of atrophy was growth in the previous 2 years (53). As previously stated, similar intraindividual growth rates coupled with high interindividual variation suggest that the spread of atrophy is not random. The factor/s may be environmental or genetically determined, but are largely unknown (see *Epidemiology of geographic atrophy*, on page 9).

To date, five natural history studies have described the growth of GA: Sunness et al (53), FAM (60), BDES (40), AREDS (122) and the Geographic Atrophy Progression (GAP) studies (123). Mean GA growth ranged between 1.3 and 2.6 mm^2/year, as measured by either FP or FAF. An overview of the main findings of these studies is shown in **Table 2**, as adapted from Fleckenstein et al (124). An increase in the size of atrophy was associated with poorer reading speed (111) but not with visual acuity loss, presumably because of foveal sparing.

Table 2. Summary of the main findings in the natural history studies on geographic atrophy (adapted from Fleckenstein et al) (124). For a complete list of references, please see text. AREDS: Age-Related Eye Disease Study; BDES: Beaver Dam Eye Study; FAF: Fundus AutoFluorescence; FAM: Fundus Autofluorescence in age-related Macular degeneration; FP: Fundus Photography; GAP: Geographic Atrophy Progression; Yr: Years.

	Sunness (2007)	*FAM (2007)*	*BDES (2008)*	*AREDS (2009)*	*GAP (2010)*
Eyes	212	195	53	251	413
Patients	131	129	32	181	413
Median yr follow-up	4.3	1.8	5	6	≤1.5
Imaging	FP	FAF	FP	FP	FAF
Mean growth rate (range), mm^2/yr	2.6 (0-13.8)	1.74 (0-7.7)	1.3 (n/a)	1.71 median (0-2.27)	1.77 (0.1-7.3)

6. Measurement of progression of geographic atrophy

The progression of GA can be monitored by functional or structural methods. Functional measures such as visual acuity and contrast sensitivity reflect solely foveal involvement (which may take years to ensue), while other measures are sophisticated, lengthy and require stable foveal fixation to provide reliable results (visual fields, microperimetry). Other techniques (reading speed (125), foveal dark-adapted sensitivity (126), photopic and scotopic fine matrix mapping (92)) are beyond the scope of common clinical practice. Therefore, functional methods have important limitations to track the evolution of GA.

The most consistently used structural measure in GA is the size of atrophy. It is moderately correlated with visual function, minimally demanding for the patient and already available in the clinical setting. It has been recently accepted by the Food and Drug Administration (FDA) as a clinical trial endpoint (127). Therefore, it is the preferred measure to monitor GA progression. The area of atrophy can be measured with the following imaging techniques (see **Figure 1**):

a. Fundus photography

The availability of photographic digital systems in the mid eighties and their widespread use since then allowed the quick, manual

delimitation and quantification of the area of atrophy. Since then, many studies (40,53,122) have used this method to monitor GA progression. However, FP requires operator-driven demarcation of the borders of atrophy in all lesions, which may be difficult in patients with low pigmentation, and challenging in eyes with many drusen and small satellite lesions (128). Its reproducibility has been shown to be moderate to good (129-131).

b. Fluorescein angiography

FA can be used to delineate the extent of atrophy due to the increased fluorescence from the choroid through an absent RPE (window defect). However, its invasive nature precludes its widespread use for monitoring GA.

c. Spectral domain optical coherence tomography

A pilot study reported a high correlation in the area of atrophy between the *en-face* reflectance image on SD OCT (caused by an increased signal from the exposed choroid) and FAF (132). A longitudinal study confirmed the feasibility of this method for measuring GA (54). Also, the use of a new, automated polarization-sensitive OCT has shown high reproducibility and agreement with infrared, FAF and SD OCT (133). An additional advantage of OCT over all other imaging techniques is the simultaneous cross-sectional visualization of drusen, RPE, photoreceptors, CNV and GA, and the possibility to obtain quantitative information on drusen area and volume, retinal thickness and GA area in a single exam.

Unfortunately, the lesion must be contained within the 6x6 mm used in the 3D macular cube (which precludes the inclusion of larger or eccentric areas of atrophy) and the software is not commercially available yet.

d. Fundus autofluorescence

The use of conventional FAF (excitation λ=488 nm, absorption >500 nm) to measure the area of atrophy is based on the assumption that hypoautofluorescent (black) areas represent absence of RPE (atrophy). The FDA has accepted the progression of atrophy as a primary outcome in clinical trials of GA, and FAF is regarded as a valid tool to measure these areas (127). Hypoautofluorescence in FAF can be measured with a cSLO or a fundus camera (see *Methods used to visualize fundus autofluorescence in vivo*, on page 22).

A dedicated, semi-automatic software in the cSLO, the Region Finder ® (Heidelberg Engineering; Heidelberg, Germany; **Figure 5**), provides more reproducible results than with a fundus camera (134-136).

Using this method, the GRADE Reading Center reported excellent intraobserver (-0.06 mm^2; 95%CI, 0.07 to -0.20) and interobserver (0.08 mm^2; 95%CI, 0.25 to -0.09) reproducibility (137). Lesions were 30% larger when measured with FP than with FAF due to the inherent difficulty in measuring small areas of atrophy with the fundus camera (138). The progression rates measured with FAF are remarkably similar to those found with FP and SD OCT.

Another advantage of FAF is that it simultaneously identifies areas of hyperautofluorescence that are thought to represent lipofuscin build-up. The resultant phenotypic patterns are a potential risk factor for GA progression (60), as previously seen.

The limitations of FAF include the presence of other causes of fundus hypoautofluorescence (such as crystalline drusen, blood or increased RPE melanin content) and the physiologic hypoautofluorescence in the foveal area due to absorption by the luteal pigment. These limitations may require the side-by-side use of other methods to identify the causes of low FAF. As with other techniques, media opacities can compromise image quality.

Figure 5. Region Finder ® screenshot. The user clicks on the hypoautofluorescent locations and the software automatically identifies regions with similar grey-scale levels (orange color on right-hand image), and converts the number of pixels identified into mm^2. In case of software error, the user can define the borders of atrophy manually (red line on right-hand image).

7. Pathogenesis of geographic atrophy

Development of GA is attributed to a complex interplay between oxidation, inflammation and possibly other processes that manifest in the outer retina/ inner choroid. Its late-onset, slowly evolving nature and the lack of adequate *in vitro* and animal models have hampered the understanding of its pathogenesis.

Noell et al were the first to describe that light induces photoreceptor and RPE apoptosis, and that this effect is mediated by light absorption by rhodopsin and cone pigments, especially in the blue range of the spectrum (for a review, see Remé) (139). This phototoxic reaction involves the generation of ROS, whose production is increased with aging, inflammation and certain pollutants (i.e., tobacco). The macula is particularly prone to ROS-induced oxidative damage because its lifelong exposure to light, high O_2 tension, the presence of PUFA (molecules rich in double bounds particularly susceptible to oxidative damage) (140) and a low concentration of antioxidants (141). In this scenario, oxidative processes are very likely.

With age, PUFA in the photoreceptor outer segment undergo lipid peroxidation and generate lipofuscin. Lipofuscin deposition increases with age, reaching 20% of the cytoplasmatic volume by age 80 years (142). It can induce oxidative stress (73,143), and biomarkers of oxidative damage identified by indirect immunofluorescence staining are more abundant in the retina of donors with GA as compared with controls of similar age or even patients with CNV (45).

Inflammation has been implicated in the pathogenesis of AMD for a long time. The development of drusen in the fundus of patients with a complement-mediated disorder (dense deposit disease) (144), the identification of inflammatory markers in drusen and the presence of macrophages at the borders of GA (145) suggested a role for inflammation in the disease. The complement is a part of the innate immune system that helps ("complements") antibodies to clear pathogens. In 2005, the discovery of the association between CFH, a complement downregulator, and AMD (146-148) confirmed the relevance of inflammation in the disease. Since then, other molecules in the complement have been associated with GA (see *Genetics of geographic atrophy*, on page 35). As stated previously, A2E can activate the complement (86,87), establishing a vicious circle between oxidative stress and inflammation.

An integrated approach to the pathogenesis of dry AMD has been proposed and tested in mice by Hollyfield et al (149,150). Briefly, light incidence in the eye would cause lipid peroxidation and formation of carboxyethylpyrrole (CEP) adducts, uniquely generated from oxidation of docosahexaenoic acid (DHA). These deposits would not be recognized by the self and would trigger a low-grade inflammatory response in the outer retina in susceptible patients (those with complement deregulation -genetic polymorphisms-, low antioxidant capacity -smoking, decreased luteal pigment density-, etc.), fostering GA development. In line with this findings, it was found that mean plasmatic levels of CEP were 3-fold greater in patients with AMD than in controls (151) and complement protein levels in peripheral blood were also higher in patients than controls (152), suggesting that AMD is a systemic

disease with manifestations in the outer retina due to the decreased regulatory capacity of the macula.

Vascular changes may also promote GA. Progressive choroidal arteriosclerosis with aging induces a concomitant decrease in blood flow (153) which, coupled with thickening of Bruch's membrane, may compromise transport of nutrients and metabolic substances to and from RPE and choriocapillaris. This may lead to accumulation of RPE-derived lipoproteins, contributing to GA.

Aging is associated with mitochondrial (mt) DNA dysfunction caused by ROS, which damages the mitochondria and diminishes energy production efficiency reducing cell metabolism below the required threshold for normal function. In fact, GA-like changes were induced in mouse by depletion of mt-manganese superoxide dismutase (mt-SOD), a potent antioxidant (154). Dysregulation of autophagy, a key process in cell homeostasis used to remove damaged organelles, is common in aged retinas and could also contribute to GA through overproduction of ROS (155). Lipofuscin can be derived not only from phagocytosis (defined as degradation of extracellular substrates) of photoreceptor outer segments, but also from autophagy (degradation of intracellular substrates), notably mitochondria (156).

Finally, Kaneko et al (157) recently found that the enzyme DICER1 is reduced in GA. DICER1 degrades Alu ribonucleic acid (RNA). DICER1 underexpression leads to Alu RNA-induced upregulation of the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome, which drives interleukin (IL) -18 secretion. Together with the release of proinflammatory cytokine IL-1β from RPE lysosomal destabilization (158), these processes lead to RPE-cell

death (see an excellent review by Ambati et al (159)). Although the triggering events are unknown, it is important to note that A2E is capable to stimulate IL-1 production mediated by the inflammasome (88). Since DICER1 and AluRNA malfunction seem to be a common hub where multiple processes converge, their regulation emerge as an appealing therapeutic target.

8. Genetics of geographic atrophy

AMD (and, by extension, GA) is a complex disease in which the subject's susceptibility to its development depends on the interaction between genetic and environmental factors. Unfortunately, the first genetic associations reported in the late nineties, involving the ABCA4 (160) and ApoE (apolipoprotein E) (161) genes, showed a population attributable risk below 5%.

In April 2005, three landmark studies (146-148) led to a major step forward. The Tyr402Hys single nucleotide polymorphism (SNP) in 1q31 involving CFH, a downregulator of the alternative pathway of the complement system, underscored the role of inflammation in early and late AMD. Other genes in the complement pathway have since then been involved in the disease (162-164).

The other major locus in AMD (GA) is the ARMS2 (previously known as LOC387715) in 10q26, which involves the SNP Ala69Ser (165,166). It encodes a protein of unknown function, but possibly

related to mitochondrial homeostasis or extracellular matrix remodeling.

CFH and ARMS2 are responsible for more than half of the cases of late AMD (and possibly GA), and it is estimated that up to 70% of the cases of the disease have a genetic cause. Predictive models have shown that phenotype and some environmental factors (age, smoking) are pivotal to predict the incidence of GA (167), but some studies showed that inclusion of genotype (CFH, ARMS2) improved sensitivity (168).

In a genome-wide association study (GWAS) involving more than 17.000 cases and 60.000 controls, the AMD Gene Consortium identified 19 loci associated with AMD, including 7 new genes (169). Oxidative stress and inflammation were directly or indirectly related to most of them, and several SNP were involved in lipid metabolism. SNP associated with GA in one or more studies (170-175) are shown in **Table 3**.

These genes are associated with prevalent GA, but the relationship with its progression has been more elusive. Klein et al found that CFH and ARMS2 could predict individuals at risk of progressing from early to late AMD (176), they but did not address the growth of GA. In a subsequent study, they reported a nominally significant association between GA progression and ARMS2 (66), but it was not supported by other measures of GA growth (progression from extrafoveal to central GA and incidence of bilateral GA in those with unilateral disease at baseline). Scholl et al did not find any association between disease enlargement and CFH, ARMS2 or C3 in a case-control study (67), but a recent study in a smaller sample

Spanish population found a role for CFH and complement factor B (CFB) in faster GA progression (68).

Table 3. Genes that have shown statistically significant associations with geographic atrophy in one or more studies. For a list of references, please see text. Chr: chromosome.

ABCA1/4: ATP-binding cassette transporter sub-family A, member ¼ (chr 1)	*CFD*: Complement factor D (chr 19)
ApoE: Apolipoprotein E ε2 and ε4 (chr 19)	*CFH*: Complement factor H (chr 1)
ARMS2 (LOC387715)/HTRA1: Age related maculopathy susceptibility protein 2 (chr 10)	*CFI:* Complement factor I (chr 4)
C3: Complement component 3 (chr 19)	*CX3CR1*: Chemokine (C-X3-C motif) receptor 1 (chr 3)
CETP: Cholesteryl ester transfer protein (chr 16)	*LIPC*: Hepatic lipase (chr 15)
CFB/C2: Complement factor B/C2 (chr 6)	*MtDNA*: Mitochondrial DNA, haplogroups J, T and U
	TLR3/4: Toll-like receptor 3/4 (chr 4)

Thus, the role of genetics in prevalent GA is becoming well established, but its relevance in progression is not conclusive. Unraveling its influence on GA growth will be necessary to fully explode the emerging field of pharmacogenomics. FAM

researchers planned to study associations between FAF phenotypes (patterns) on GA and genetic variants, but results have not been published yet.

9. Histology of geographic atrophy

a. Photoreceptors:

With normal aging, there is a decrease of 30% in the number of rods and a relatively constant number of cones (113). In GA, almost all photoreceptors are lost in the area of atrophy (177,178), and those remaining are cones. At the borders of the lesion, a marked variation in the number of photoreceptors has been reported (90), but most are TUNEL-positive (apoptotic) (179). In fact, some authors believe that these cells may be the target of damage in GA (90), while others consider that primary insult occurs in the RPE (180-182). The remaining neural retina shows less dramatic changes (181), but loss of bipolar and ganglion cells is common in longstanding cases.

b. Retinal pigment epithelium:

In the aged RPE there is a decrease in melanin granules and an increase in the density of lipofuscin (183) from undigested photoreceptor outer segments (184,185). The amount of lipofuscin

increases with age within the cell and is inversely related to the number of RPE cells (184,186). There is also an increase in iron deposition (187) and advanced-glycation end products (AGEs) (188). In GA, the RPE is lost within the area of atrophy (104,182) (**Figure 6**) and hypertrophic at the borders (182). RPE hypertrophy and its abnormal morphologic features (layering, vertically superimposed cells) may contribute to the increased hyperautofluorescence observed clinically at the borders of atrophy (59), which may not be necessarily caused by actual increased lipofuscin content within individual cells.

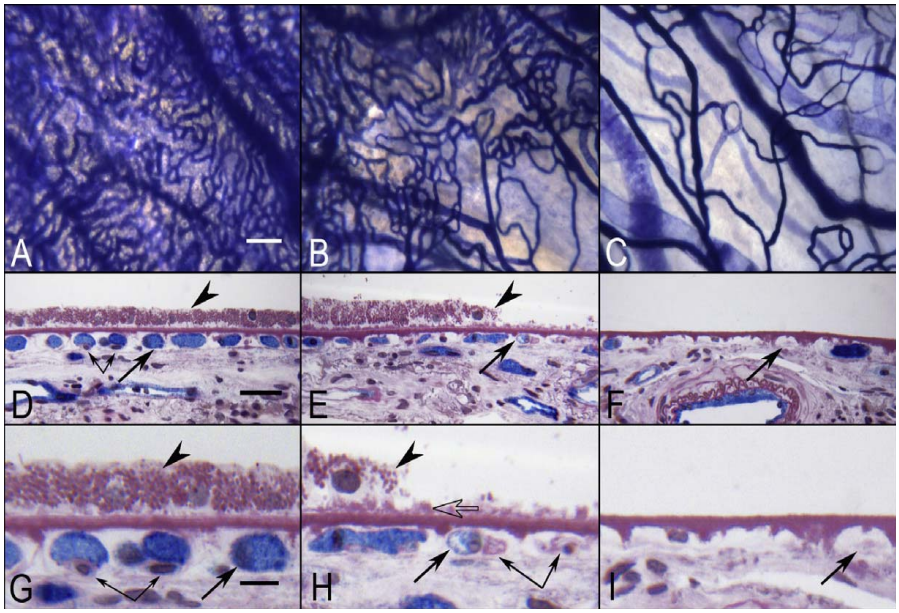

Figure 6. A patient with geographic atrophy in non-atrophic (first column), junction (second column) and atrophic area (third column), showing a progressive loss of vessels from normal to atrophic regions in flat (first row) and transverse (second and third rows) sections. The loss of retinal pigment epithelium (E, F, H and I) seems to precede the loss of choroidal

vessels (from: Bhutto et al. Understanding age-related macular degeneration: relationships between the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex. Mol Aspects Med 2012; 33: 295-317. Used with permission).

c. Bruch's membrane:

Bruch's membrane thickens with age (2). This leads to a decreased permeability, with deposition of drusen, AGEs and basal linear and laminar deposits that increase the susceptibility to GA (as reviewed by Bhutto et al (189)). Bruch's membrane is not fragmented, but macrophages can be found at the vicinity of healthy and diseased retina (145), signaling towards inflammatory processes in the expansion of atrophy.

d. Choriocapillaris:

With age, choroidal thickness decreases (190). Similar changes are seen in the choriocapillaris in GA, with development of sclerotic vessels (191) and a reduction in capillary density (190). McLeod et al (182) found that the progressive loss of the RPE in GA from the normal to the atrophic area was accompanied by a progressive reduction in the vascular area of the choriocapillaris, increased constriction of remaining capillaries and loss of their fenestrations (**Figure 6**). This was attributed to the decreased expression of VEGF from the basal side of the RPE, which would decrease the clearance rate of debris done by the choroid, fostering the formation of drusen.

10. Treatment of geographic atrophy

There is a paucity of treatments to prevent incident GA. Two large trials showed a decrease in the incidence of late AMD (defined as central GA or wet AMD) in patients at high risk when using an oral supplementation with vitamins C and E, beta-carotene and zinc (AREDS) (51), or lutein/zeaxanthin added to this formulation (AREDS2) (52). However, the benefits were driven by a reduction only in CNV. Neither formulation showed benefits in decreasing central GA (OR 0.75; 99%CI, 0.45-1.24 in AREDS; hazard ratio -HR- 0.92; 95%CI, 0.78-1.07 in AREDS2). On the other hand, observational studies suggest that quitting smoking (192) or following a diet with a high consumption of fish, nuts and dark green leafy vegetables (193) may decrease the risk of incident GA, with the possible addition of protection from sunlight (194) by the use of sunglasses or hats. These recommendations are sound even from the perspective of general health, and therefore represent the best advice that can be provided to patients at risk for incident GA.

There is no known treatment for slowing the progression of prevalent GA. This is an area of intense research and many strategies are being evaluated (195), as shown in **Figure 7**. The rationale behind each line of research is discussed briefly (141,196) with a particular emphasis on visual cycle modulators (VCM), which are built on the presumed toxic effect of lipofuscin on RPE health.

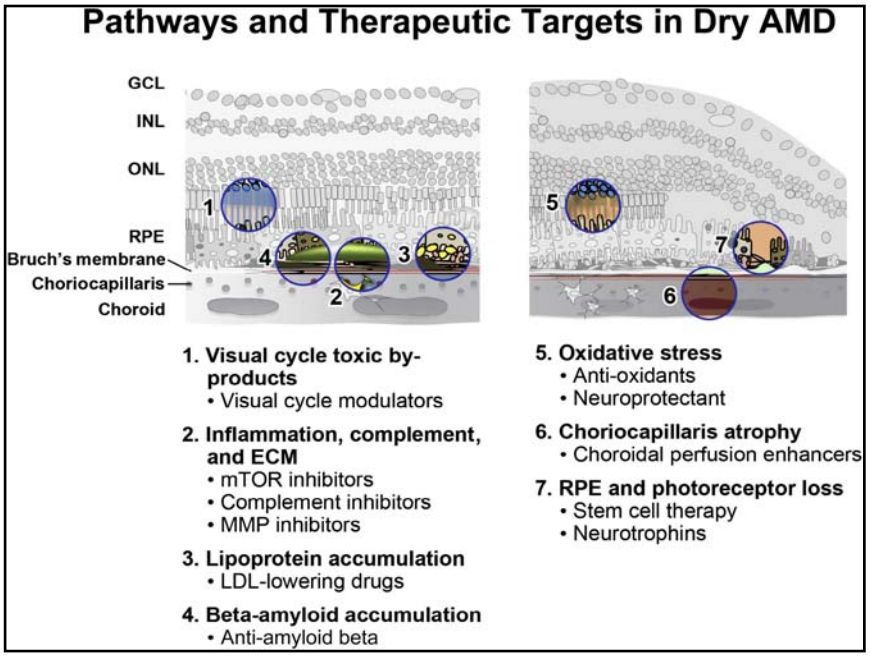

Figure 7. Potential therapeutic targets for dry age-related macular degeneration. Except for lipoprotein accumulation, all these strategies are also being evaluated for geographic atrophy (from: Holz FG et al. Geographic atrophy. Clinical features and potential therapeutic targets. Ophthalmology 2014. Used with permission).

a. Visual cycle modulators

VCM slow the normal visual cycle to decrease the accumulation of toxic by-products, such as lipofuscin main metabolite, A2E. Fenretinide (ReVision Therapeutics, Inc., San Diego, CA) and emixustat hydrochloride, previously known as ACU-4429 (Acucela Inc., Bothell, WA, and Otsuka Pharmaceutical Co., Ltd., Otsuka, Japan) are examples of drugs in this category (141).

Fenretinide [N-(4-hydroxyphenyl)retinamide] is an oral medication that binds to circulating serum retinol binding protein, blocking its association with retinol. This prevents the transport of retinol to the RPE, downregulating photoreceptor metabolism and decreasing the residual formation of ocular toxins from the visual cycle (197). The results of a phase II trial (198) were puzzling: the rate of GA growth at 24 months in the placebo, 100 mg and 300 mg of oral fenretinide was 2.03, 2.14, and 1.95 mm^2/year respectively; there were no differences in visual acuity loss between groups, more than 30% patients withdraw from the study and the rate of adverse events in active arms was around 20%. On the other hand, there was a mean reduction in GA growth of 0.33 mm^2/year in those who achieved levels of serum retinol ≤1μM (1.70 vs 2.03 mm^2/year, p=0.18) and a 45% decrease in the incidence of CNV relative to placebo (p=0.06). To complicate things further, a change in the manufacturing process in the middle of the trial precluded the review of data by the FDA (199). Apparently, further clinical investigation involving this drug has been arrested.

Emixustat hydrochloride is also in an oral formulation that inhibits the RPE65 isomerase, which prevents the conversion of all-*trans*-retinyl ester to 11-*cis*-retinol, decreasing the accumulation of A2E (200). Given that inhibits rather than decreases an intermediate step in the visual cycle, its effects should be long-lasting, but the risk of adverse events (dyschromatopsia, delayed dark adaptation) is theoretically greater. In a phase I study (201), 67% of patients experienced ocular adverse events, which were mild and disappeared after drug discontinuation. A phase IIb/III trial, the SEATTLE study, is ongoing.

ALK-001 (Alkeus Pharmaceuticals, Boston, MA) is a modified vitamin A molecule able to slow the formation of A2E. It was developed for Stargardt disease, but it may also benefit GA patients. It is currently in a preclinical stage.

The frequency and relevance of adverse events, coupled with the need to use sophisticated methods to monitor the response to therapy (electroretinography, ERG) underscore the complexities involved in VCM development.

b. Suppressors of inflammation

Since genetic (147,148,202,203), histopathologic (144) and epidemiologic (204) data has shown an association between GA and inflammation, the modulation of this process seems a rational approach. Broad strategies in this category involve the use of anti-inflammatory drugs (such as fluocinolone), the replacement of inefficient downregulators of this pathway (CFH) or the control of complement activation (at C3, C5 or factors D). Actually, results from MAHALO, a phase II trial evaluating lampalizumab (Genentech/Roche, South San Francisco, CA), a monoclonal antibody directed against complement factor D, have shown a 44% decrease in GA progression at 18 months in patients who tested positive for CFI as compared to controls ($p<0.005$), and possibly represents the most promising therapy under evaluation for GA. Nevertheless, Ambati et al (159) have called into question the role of complement inhibition in GA given that complement deposition is not marked in GA, the lack of genetic association between complement genes and disease progression and the resistance of

RPE to complement-induced cell death. The restoration of DICER1 levels or the regulation of the MyD88 effector (159) are other alternatives to modulate the immune response. In addition, sirolimus (an mTOR inhibitor) showed no beneficial effect on GA progression in a phase I/II trial, and patients experienced a greater loss in visual acuity in active vs control eyes (-21 vs -3 letter loss, respectively; p=0.03) (205).

c. Beta-amyloid suppressors

Beta-amyloid build-up occurs in Alzheimer's disease as well as on drusen and colocalizes with C3 and has been shown to activate the complement. Trials involving RN6G (Pfizer Inc, New York, NY) and GSK933776 (GlaxoSmithKline, Research Triangle Park, NC) are currently in phase II.

d. Antioxidants

Basic (149) and clinical research (51) support the role of oxidative stress on AMD through damage caused by free radicals, which promote lipid peroxidation and inflammation. Unfortunately, the trials AREDS (51) and AREDS2 (52) did not assess the effect of supplementation on progression of prevalent GA as the main outcome. The lack of positive results on incident central GA do not support the beneficial effect of these formulations on progression of the disease. Another antioxidant delivered topically, OT-551, was not beneficial in GA.

e. Choroidal perfusion enhancers

The disruption of adequate choroidal blood flow, responsible for nourishing the outer retina, may be detrimental to RPE and photoreceptors. Unfortunately, trimetazidine (currently licensed for angina pectoris) was not effective in slowing the progression of GA.

f. Photoreceptor and retinal pigment epithelium loss

This approach intends to preserve viable photoreceptors and/or RPE and limit the spread of visual loss. Neuroprotection is characterized by the use of proteins that promote rod and possibly cone survival, proliferation, differentiation and function. Ciliary neurotrophic factor (CNTF) delivered by encapsulated cell technology (Neurotech, Lincoln, RI) is in a phase III trial (206) after showing a significant increase in retinal thickness (presumably driven by enhanced photoreceptor survival) and a non-statistically significant visual acuity stabilization. CNTF did not show benefits in terms of GA growth. Also, an intravitreal implant of brimonidine tartrate (Allergan, Irvine, CA) is in phase II.

Replacement of lost tissue may be the only alternative for advanced cases. Allogenic fetal RPE cell transplant had limited success (207), macular translocation showed recurrence of GA at the new location (208,209), and autologous translocation of an RPE and choroidal graft showed a normal appearing outer retina overlying the graft on SD OCT, but poor functional results (210). Recently, transplantation of human embryonic stem cell-derived RPE has been evaluated with promising results (211).

Finally, visual rehabilitation should be considered in visual impaired patients (212). Given the frequency with which GA patients show foveal sparing, BCVA should not be used to decide when to send patients for low vision aids, and threshold for referral should be low. Unfortunately, perifoveal scotomas induce poor reading ability because different retinal loci are used to read single letters (fovea) or text (perifoveal area). Optical magnification can project words inside the atrophic area, making some tasks actually more difficult (112). Therefore, the prescription of low vision aids is specially challenging, but should be attempted in motivated patients.

Justification

AMD is the most frequent cause of visual impairment in developed countries, accounting for half of the registered cases of legal blindness. The late stages of the disease, GA and neovascular AMD, are largely responsible for the severe visual loss experienced by patients with the disorder. The impact of AMD is well beyond its obvious effects on vision, and has been associated with a decrease in quality of life, an increased rate of depression, risk of falls and even mortality, and remains a heavy burden from a personal, familiar, social, sanitary and economic standpoint.

Currently there is no treatment for GA, but much can be learned from the historical evolution of the treatments for its sister, the aggressive neovascular form of the disease. Physical therapies used for this condition before 2004 (thermal laser photocoagulation and photodynamic therapy with verteporfin) destroyed the obvious manifestation of the disorder, the choroidal neovascular complex, but the functional impact of these strategies was modest at best, since they only retarded the visual decline. An understanding of the role of vascular endothelial growth factor (VEGF), a key protein that mediates vascular proliferation and permeability, fueled the development of anti-VEGF therapies that have dramatically improved the prognosis of patients with wet AMD. Similarly, a thorough knowledge of the pathophysiology of GA should lay the foundation for the development of rational therapies for this condition.

Lipofuscin is an intracellular non-degradable by-product of cell metabolism that accumulates in several tissues and is considered a marker of aging. In the eye, lipofuscin is found within RPE cells as a result of the incomplete phagocytosis of photoreceptor outer

segments. In recent years, excessive lipofuscin has been linked to incident RPE atrophy and GA progression.

The toxic effects of lipofuscin are based mainly on results from basic research, althought some clinical studies have also supported this hypothesis. These effects can be summarized as follows:

- In humans, lipofuscin content within the RPE increases with age and occupies nearly 20% of its intracellular volume by the age of 80.

- Lipofuscin interferes with RPE phagocytic activity *in vitro* by inducing an acidic shift in the intralysosomal pH, which causes bleb extrusion.

- A2E in lipofuscin increases oxidative stress under blue light irradiation *in vitro*.

- A2E is able to trigger the complement and inflammation *in vitro*.

- A2E can induce apoptosis *in vitro*.

- The number of photoreceptors overlying RPE cells decreased linearly as the lipofuscin content in those RPE cells increased in one study.

- Elevated levels of FAF that signal the presence of high lipofuscin content have been shown to precede development of atrophy in some clinical studies.

- A larger retinal area covered by increased FAF (as identified through FAF patterns) was associated with a faster rate of progression of GA on bivariate analysis.

These results have fueled the development of the VCM, a class of drugs aimed to slow the visual cycle to decrease the rate of lipofuscin build-up in the RPE.

However, the evidence of the pivotal role of lipofuscin on GA progression has been challenged and remains to be firmly established. Some of the conflicting results are summarized herein:

- The peak physiologic concentration of lipofuscin in the fundus does not match the distribution of the earliest atrophic lesions seen in GA.

- The hyperautofluorescence originated from lipofuscin seen *in vivo* with FAF increases until 70-80 years old and then decreases almost linearly, while GA prevalence increases exponentially with age.

- It is not clear how RPE cells can accumulate lipofuscin so fast in GA, while it takes a lifetime to fill 20% of the cellular volume with this compound to mildly increase the background levels of FAF.

- A clinicopathologic study found that increased FAF was caused by vertically aligned RPE cells, not by increasing lipofuscin within individual cells.

- Photoreceptor loss was not associated with higher RPE autofluorescent characteristics in a recent histopathological study.

- Although A2E has been consistently implicated in AMD, a recent study found that its distribution in the fundus in humans involves preferentially the peripheral retina, not the macular area.

- The association between increased FAF and faster GA progression vanished on multivariable analysis, suggesting confounding, mediation and/or lack of power.

- If accumulation of lipofuscin (identified as hyperautofluorescence on FAF) is toxic to the RPE, it should be followed by hypoautofluorescence (RPE atrophy) in the long term. However, the positive predictive value of hyperautofluorescent spots regarding the development of hypoautofluorescence with time is *not* greater than that of surrounding, normal appearing retinal areas.

- The preliminary results of the trials involving VCM have not shown a significant benefit of this approach.

The purpose of this thesis is to determine the role of increased FAF, a biomarker of lipofuscin, as the predictor of main interest in the progression of GA. We conducted a prospective, longitudinal, natural history study in a cohort of patients with GA in whom demographic, medical and imaging data was collected from December 2009 to August 2013. These results may be relevant for patients with pure GA and also with neovascular AMD, since foveal

atrophy remains the main reason of limited functional improvement after successful treatment with anti-VEGF therapy. These reasons reinforce the need to understand the causes of GA, and determining the role of lipofuscin accumulation represents an important early step in this process.

Objectives and Working hypotheses

Objectives:

A. General objectives

- To describe the progression of geographic atrophy (GA) in a cohort of patients seen in a tertiary clinic in Barcelona, Spain.

- To identify factors associated with the progression of the disease. In particular, the role of increased fundus autofluorescence (FAF) as a biomarker of lipofuscin within the RPE.

B. Specific objectives

- To describe the progression of GA as measured with FAF.

- To evaluate the independent role of FAF patterns as a risk factor for progression of GA.

- To describe the macular morphologic changes of patients with foveal sparing secondary to GA.

- To develop a new, simplified classification of FAF patterns in GA.

- To assess if the FAF patterns arising from this classification are stable in a given eye of a patient through time.

- To evaluate the intra and interobserver reproducibility of the original classification of FAF patterns in GA.

Working hypotheses:

- The progression of GA is grossly similar to that reported in other studies despite the differences in the geographic area (Spain vs Germany or United States) and the methods used for patient recruitment (tertiary referral center vs a population-based sample).

- FAF patterns are associated with rates of growth of atrophy on bivariate analysis. The association on multivariable analysis may not reach statistical significance, but the point estimate is in the direction suggesting faster progression with higher FAF content.

- Foveal swelling is an artifact caused by parafoveal atrophy in patients with foveal sparing secondary to GA.

- It is possible to obtain a simplified classification of FAF patterns using an objective analytic approach.

- The emerging FAF patterns from the new approach are stable in a given individual, supporting the notion that they represent true phenotypes and not different stages of the disease.

- Intra and interobserver reproducibility of FAF patterns in GA is suboptimal.

General description of methods

a. Sample size

Although clearly desirable, sample size calculations were not conducted. There was little knowledge about previously reported effect sizes for risk factors in GA progression, as has been previously discussed. In this situation, a range of clinically significant plausible effect sizes for each risk factor could have been assumed and sample sizes or power could have been derived for any given scenario, but Stata does not perform these calculations for repeated measures beyond analysis of variance (ANOVA). The risk of an underpowered sample size (213) and false negative findings was a serious concern, and we planned two potential solutions: (1) increasing sample size by including patients with GA from other centers; and (2) following similar methods to those used in other landmark studies (ie, the FAM and GAP studies) (60) in terms of imaging methods, eligibility criteria, etc, to perform a meta-analysis between these studies and GAIN at a later stage.

We intended to establish a collaboration with the Hospital de Bellvitge, in L'Hospitalet de Llobregat, Barcelona. Unfortunately, this was not feasible because FAF, the imaging method from which the main outcome (GA growth) was derived, was based on different technologies in each center (cSLO vs fundus camera). Direct referral of patients to our center was deemed to be inadequate, and the collaboration was finally dismissed. The conduct of a subsequent meta-analysis was therefore considered and is discussed later, but was finally not required given the results found.

b. Basic design features

We conducted a prospective, longitudinal, open cohort study of a series of consecutive patients with pure GA secondary to dry AMD at the Institut de la Màcula i de la Retina (Centro Médico Teknon, Barcelona). The recruitment period began in December 21st, 2009 and finished in December 31st, 2012. The study finished gathering data on patients on August 9th, 2013. The study was registered at clinicaltrials.gov under the acronym GAIN (Characterization of Geographic Atrophy progressIoN in patients with age-related macular degeneration), with the identifier NCT01694095.

The timeline of the study and the corresponding dates of the submission of each paper are shown in **Figure 8**:

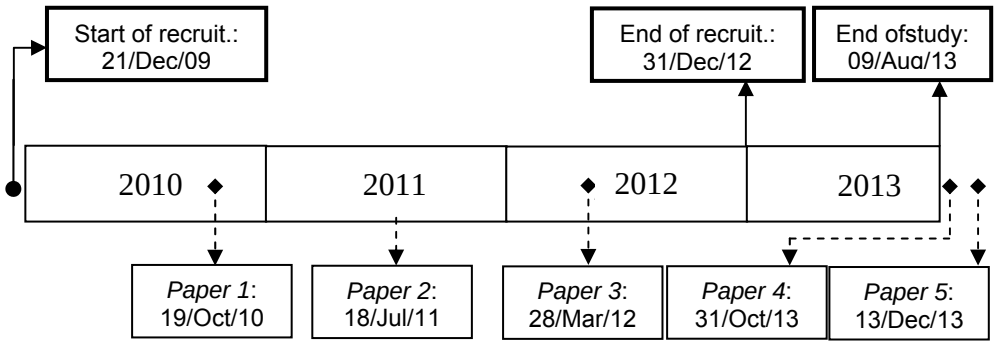

Figure 8. Description of recruitment period and date of submission of papers. Recruit.: recruitment.

c. Ethics

The study adhered to the tenets of the Declaration of Helsinki, was approved by the Ethics Committee of the Centro Médico Teknon and all patients signed an informed consent.

d. Eligibility criteria

The general criteria included pattients of both sexes 50 years or older with pure GA secondary to AMD in one or both eyes, irrespective of visual acuity level. Ocular comorbidity was not considered an exclusion criterion as long as it did not preclude the assessment of the extent of atrophy on FAF (ie, mild to moderate cataract) or it did not interfere significantly with visual acuity or macular anatomy (mild epiretinal membrane was allowed, but macular pucker was not). Other causes of retinal atrophy (high myopia, chronic central serous chorioretinopathy, toxic maculopathies) or intraocular surgery (aside from phacoemulsification) were considered exclusion criteria. The minimum follow-up period was established at 6 months, as in other similar studies (54,60). The specific criteria used in each study paper could vary. For example, contact between peripapillary and macular atrophy was an exclusion criteria if the study measured of the area of atrophy, but it was not when the study dealed with the identification of FAF patterns for intra and interobserver agreement. A linear growth of atrophy was assumed in the whole period, although uncertainty remains in the literature regarding the appropriateness of this assumption (40,53,64).

Patients were recruited in their first visit after data collection began. They were followed until their last visit prior to August 9th, 2013 or until the development of significant visual comorbidity (for example, CNV), whichever occurred first.

e. Field work

The study gathered information on the progression of GA when patients attended the clinic as part of their regular visual care visits. Therefore, the GAIN study did not imply more visits or ancillary testing than those required according exclusively to the ophthalmologist criteria (see below).

Most, but not all of the field work was done by the PhD candidate. Refraction procedures and visual acuity measures were also conducted by other experienced optometrists at the clinic (Míriam Garcia, Míriam Hijano or Anabel Rodríguez) and imaging was also performed by a nurse with full time dedication to retinal imaging (Anna Serrano). Additionally, all personnel at the clinic was certified by international organizations (Klinitrial, CertifEYED, Duke and Vienna Reading Center, etc.) in their corresponding roles.

The diagnosis of GA and the schedule of patient visits were decided on an individual basis by ophthalmologists specialized in vitreo-retinal diseases at the Institut de la Màcula i de la Retina (Drs Jordi Monés, Lluís Arias, Josep Badal, Ernesto Basauri, Hussein Muhtaseb or Fabio Trindade). For example, patients with fellow eye active wet AMD required monthly monitoring, while

patients with contralateral drusen could be followed at 6 or 12 month-visit intervals.

f. Definition of main variables

The main outcome variable was the growth of the area of atrophy. It was defined as change in area of GA measured in mm^2 with FAF. The Region Finder ®, version 2.4.3.0 (Heidelberg Engineering; Heidelberg, Germany) was used to make the measures, which were done by the PhD candidate.

Aside from operative variables (medical record number, number/order of visit, date, etc.), other independent variables included were: age, sex, race, eye, hypertension (those who reported being hypertensive or under antihypertensive medication), smoking habit (former smokers were defined as those reporting having smoked cigarettes, cigars, pipes or other forms of tobacco at least once a day for 12 months and having quitted the habit 12 months ago or more), use of antioxidant supplementation for the previous 2 years (to allow for an induction period), BMI (height was measured, weight was self-referred by the patient), family history of AMD (in first degree siblings), visual acuity (on an ETDRS chart), lens status (phakic/pseudophakic), GA pattern on FAF (as described by Holz et al (60)), foveal atrophy on fundus photography, FAF, infrared and SD OCT, central retinal thickness as measured on SD OCT (in the central area covering a diameter of 1 mm, with manual correction of inner and outer retinal borders if segmentation errors ocurred), concomitant ocular comorbidities, atrophy configuration (unifocal or multifocal central, unifocal or

multifocal extrafoveal, C-shaped unifocal or multifocal, annular) and diagnosis in the fellow eye.

Data were entered in the database used for statistical analysis, Stata IC versions 11.1 and 13.1 (StataCorp, College Station, TX, USA).

g. Specific methods used in the different publications

A general description of the methods used in each empirical article is provided (with the exception of the first paper, *Update on geographic atrophy in age-related macular degeneration*, which was a narrative review).

Paper 2. Intra and interoberver agreement in the classification of fundus autofluorescence patterns in geographic atrophy secondary to age-related macular degeneration.

The objective of this study was methodological: to evaluate the consistency of different observers in assigning a FAF pattern, as compared with themselves and with other evaluators. The practical implication was to determine if more than one observer was required to assign a pattern to each participant in the GAIN study.

We used a cross-sectional design in which all available patients with images of acceptable quality for FAF pattern assignment visited from June 2009 (date of availability of the Spectralis HRA+OCT ® instrument at the clinic) to May 2010 were eligible for inclusion. We took advantage from the fact that the protocol for

acquisition of FAF images was unchanged since the device purchase and all images had similar quality aside from the learning curve of the operator. Therefore, the inclusion of subjects visited prior the beginning of the GAIN recruitment was considered as an acceptable strategy to increase sample size. Four observers with similar level of experience in grading FAF imaging assigned a pattern to each of the 69 eyes (49 patients) included. Images were identified by the PhD candidate, were exported to a .jpg format from the Spectralis HRA+OCT ® and were imported to a power point presentation (2 images/slide). Afterwards, the slides were randomly presented to each of the 4 observers on 2 occasions spaced at least 1 month apart to avoid recall bias. The classification obtained on the second occasion was used for interobserver agreement.

Percentage of agreement and unweighted kappa correlation coefficient (κ), along with 95% confidence intervals, were used for statistical analysis. It was decided not to use weighting for three reasons. First, there is no reference standard for the adjudication of FAF patterns, and hence some level of uncertainty remains in each assignment. Second, the weighting value is arbitrary. And third, unweighted κ provides more conservative results (lower κ values), and offers a benchmark against which all other results can be compared.

Paper 3. Optical coherence tomography assessment of apparent foveal swelling in patients with foveal sparing secondary to geographic atrophy.

The purpose of this paper was to determine if the foveola of patients with GA and foveal sparing was swollen as seen with SD OCT, as previously stated (214). This work contributes to improve our understanding the pathophysiology of foveal atrophy in GA and the mechanism driving cell death.

Although this study was not initially planned as a part of the thesis, the phenomenon was intriguing and the aforementioned paper (214) apparently contained some flaws: (a) that study was retrospective, and it is unclear if foveal swelling was an *a priori* hypothesis or a *post hoc* finding; (b) due to the retrospective design, some of the SD OCT scans did not cross exactly at the foveola, potentially biasing foveolar thickness; (c) the figures included in the paper showed in all cases the scans in a direction in which atrophy was found on both sides of the foveola, but images in which the atrophy was only found on one side, mitigating the apparent effect of swelling, were omitted; (d) some controls could not have been cases; and (e) in the statistical analysis, the authors used the Wilcoxon test, but cases and controls were not individually matched; therefore, unpaired tests should have been used instead.

We used a cross-sectional design nested within the GAIN cohort of patients visited from December 2009 to July 2011 who had foveal sparing and apparent foveal swelling according to the independent and blinded criteria of two retinal specialists. Therefore, only the most marked cases were included.

Non-cases were selected from the clinic in the same period and with the same eligibility criteria, aside from the fact that they could not have AMD and visual acuity had to be ≥20/25. The ratio of

cases to non-cases was 1:1. The tilting technique on SD OCT, described on March 2011 (215,216), was used to differentiate photoreceptors from Henle's layer. Since some cases had been visited before the description of this technique, they were contacted by phone to make a new appointment and repeat the scans. All patients agreed.

The analysis involved descriptive statistics and comparison of median thickness values of specific retinal layers between groups using the unpaired Mann-Whitney test (sample matching was used). Correction for multiple comparisons was only used for *post hoc* analysis using the method of Bonferroni.

Paper 4. Reappraisal of geographic atrophy patterns seen on fundus autofluorescence using a latent class analysis approach.

The FAM study described up to 10 different patterns of FAF in patients with GA (60). Intra and interobserver agreement in the adjudication of these patterns was moderate at best (paper 2), probably because there was a high number of categories. The main objective of this study was to evaluate if a more simple classification for FAF patterns could be devised from the categories described in the FAM study. As a secondary outcome, we evaluated if a patient was classified in the same (new) FAF pattern at follow-up; that is, if patterns were stable in a given individual. In case of transitions, we intended to study if these were predictable (from one specific pattern to another).

The 171 patients screened for the GAIN study were evaluated and those with a follow-up ≥1 year were included. The main inclusion

criterion was good FAF image quality. CNV beyond 1 year was not an exclusion criterion. Five observers independently evaluated FAF patterns at baseline and at the last follow-up. Review of the results of the first classification was not allowed.

We anticipated that the low to moderate interobserver agreement previously found in the evaluation of FAF patterns would induce undesirable noise. We approached this issue by: (1) using 5 evaluators who worked in different clinical settings, and (2) analyzing the results with an objective approach: latent class analysis (LCA) (217).

LCA allows relating a set of observed variables to a group of unobserved (latent, not initially obvious) characteristics. In this scenario, we expect that the emerging classes (new FAF patterns) will be able to summarize the fundamental information contained within patterns of the original classification, but hopefully summarizing the those aspects with a lower number of categories. The number of classes which maximized the responses provided by the observers (in terms of the Bayesian Information Criterion and entropy) was finally chosen. Cochran's Q index was used to evaluate potential transitions of the new classes in each individual.

Finally, we sought to describe the resulting classes. Demographic and a set of pre-specified FAF characteristics, considered appropriate to describe the main imaging features, were compared using the Kruskal-Wallis test for continuous and the likelihood-ratio chi-squared test for categorical variables.

Paper 5. Increased fundus autofluorescence as a risk factor for progression of geographic atrophy. The GAIN study.

The objective of the last paper was to evaluate the role of increased FAF in GA progression. Although the existence of true FAF patterns (phenotypes) was called into question in paper 4, high FAF is still considered a biomarker of elevated lipofuscin content and we were interested in evaluating the role of lipofuscin in GA growth. Therefore, the original description for FAF patterns in the FAM study was used as a surrogate marker for lipofuscin content.

All patients with pure GA, good image quality on FAF and a minimum follow-up of 6 months were included. Patients with significant concomitant disease, contact between GA and peripapillary area of atrophy or an area of atrophy not constrained within the 30º x 30º FAF field of view were excluded.

The main outcome was growth of GA as measured on FAF. Intraobserver agreement in GA area determination was evaluated by measuring twice 20% of a randomly selected sample, which was compared using Bland-Altmann plots.

The predictor of primary interest was FAF pattern as a binary variable, high (“banded” + “diffuse”) vs low (“none” + “focal”) FAF, as evaluated by 2 independent observers. A senior observer arbitrated in case of disagreement.

Given the close relationship between FAF patterns and BAA seen in paper 4, we also used mediation analysis to inspect the relative contribution of each to GA growth under the current, implicitly accepted hypothesis that states that FAF patterns induce GA

growth and determine BAA, and that BAA may also cause GA growth independently of FAF patterns.

The information of each eye of each patient on each visit was analyzed using mixed-effects models (MEM) (218). These models were selected because they can accommodate the correlated nature of data observed with repeated measures and because they can handle its hierarchal nature (one or two eyes nested within a given patient). In addition, they allow the inclusion of mixed ("fixed" and "random") effects. A variable was considered as fixed when data was collected on all possible values of that variable (for example, sex); a variable was included as a random effect when its values in our study were a sample of all the potential range of values that the variable could take (age).

Multiple imputation was used to estimate the missing values assuming a "missing at random" mechanism. We implemented an iterative chained equations approach, in which the variables with missing data are modeled conditional upon the rest of variables without missing values a number of times (and hence the name "iterative") until the coefficients in the model converge. Monte Carlo error was used to check the appropriateness of the 20 imputations conducted following Rubin's rules.

Publications

Paper 1: Biarnés M, Monés J, Alonso J, Arias L. “Update on geographic atrophy in age-related macular degeneration”. Optom Vis Sci 2011; 88: 881-9.

Link: http://journals.lww.com/optvissci/pages/default.aspx

Impact Factor (2011): 2.108

Rank: 20/58 (percentile 34)

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Rank: 20/59 (percentile 34)

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Impact Factor (2012): 5.563

Rank: 2/59 (percentile 3)

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Paper 4: Biarnés M, Forero CG, Arias L, Alonso J, Monés J. "Reappraisal of geographic atrophy patterns seen on fundus autofluorescence using a latent class analysis approach". Submitted for publication in Invest Ophthalmol Vis Sci.

Link: http://www.iovs.org/

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Rank: 5/59 (percentile 8%)

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Paper 5: Biarnés M, Arias L, Alonso J, Garcia M, Hijano M, Rodríguez A, Serrano A, Badal J, Muhtaseb H, Verdaguer P, Monés J. “Increased fundus autofluorescence as a risk factor for progression of geographic atrophy. The GAIN study”. Submitted for publication in Am J Ophthalmol.

Link: http://www.ajo.com/

Impact Factor (2012): 3.631

Rank: 4/59 (percentile 7)

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Increased fundus autofluorescence as a risk factor for progression of geographic atrophy. The GAIN study.

Marc Biarnés, Luis Arias, Jordi Alonso, Míriam Garcia, Míriam Hijano, Anabel Rodríguez, Anna Serrano, Josep Badal, Hussein Muhtaseb, Paula Verdaguer, Jordi Monés

Abstract

Purpose: To define the role of increased fundus autofluorescence (FAF), a surrogate for lipofuscin content, as a risk factor for progression of geographic atrophy (GA).

Design*:* Prospective, natural history, cohort study.

Methods*: Setting.* Single center study conducted in Barcelona, Spain. *Patients.* After screening 211 patients, 109 eyes of 82 patients with pure GA secondary to age-related macular degeneration and a minimum follow-up of 6 months were included. *Observation procedures.* Lipofuscin content was classified independently by two masked observers according to FAF patterns described previously. Bivariate, stratified and multivariable analyses (with a random-effects model) were used to explore the associations between GA growth and independent variables. Mediation analysis was used to evaluate the relative contribution of FAF patterns to GA progression. *Main outcome.* Progression of GA in mm^2/year as measured with FAF.

Results*:* Median follow-up was 18 months (range, 6 to 29) and median GA growth was 1.61 mm^2/year. FAF, BAA and time of follow-up were the only variables associated with GA progression (all $p<0.004$). However, FAF patterns and BAA were strongly associated ($p<0.0001$), suggesting potential confounding and bias. Mediation analysis suggested that approximately 85% of the effect of FAF patterns on GA growth was actually caused by BAA.

Conclusions*:* FAF patterns, BAA and time of follow-up were independently associated with GA progression. However, FAF patterns seem to be a consequence (not a cause) of enlarging atrophy and their effect on GA progression is mostly driven by BAA. Visual cycle modulators may have limited benefit in patients with GA.

Introduction

Geographic atrophy (GA), the advanced form of dry age-related macular degeneration (AMD), is characterized by enlarging areas of retinal pigment epithelium (RPE) atrophy. Regions affected by GA are devoid of RPE, photoreceptors and choriocapillaris, [1] and thus they cause absolute scotoma. It has been reported that antiangiogenic therapy in patients with neovascular AMD may foster GA growth, [2] and that most of these patients develop macular atrophy over the long term, [3] which is one of the main reasons for lack of visual improvement in this population. [4] These issues emphasize the need to understand disease pathogenesis to develop rational therapies.

Lipofuscin accumulation within the RPE is a well-known feature of GA. It is the result of incomplete phagocytosis of photoreceptor outer segments by the RPE. As such, lipofuscin is formed by a mixture of metabolites from the visual cycle, [5] such as N-retinyledene-N-retinylethanolamine (A2E). Some compounds of lipofuscin are autofluorescent, which helps to identify its topographical distribution *in vivo* using fundus autofluorescence (FAF). [6,7]

In 2001, Holz et al [8] reported that GA progression took place solely in areas that showed increased FAF. Some years later, the "Fundus autofluorescence in age-related macular degeneration" (FAM), [9] a prospective, multicenter, natural history study of GA, used FAF to classify patients with GA according to the distribution of increased autofluorescence around atrophy. The FAM reported 10 distinct categories (patterns or phenotypes), which showed a marked association with GA growth on bivariate analysis. These patterns became an important risk factor in GA progression and strengthened the causal relationship between lipofuscin accumulation and GA growth, supporting the development of visual cycle modulators (VCM), a therapeutic strategy aimed at slowing down the build-up of lipofuscin bisretinoids within the RPE.

Nonetheless, the role of lipofuscin in GA pathogenesis remains controversial. Most studies [8,10-14] suggest that it has deleterious effects on RPE that may lead to cell death and GA progression, but other clinical [15,16] and basic research findings [17-19] do not support this hypothesis. Indeed, a VCM drug did not meet the primary efficacy endpoint in a phase II clinical trial. [20]

We conducted a study to evaluate factors associated with progression of prevalent GA, in particular increased FAF (as a surrogate for increased lipofuscin) as the predictor of primary interest. The results may contribute to clarify the role of this compound on GA pathogenesis.

Material and methods

Study design

The Characterization of GA progression in patients with AMD (GAIN) study (NCT01694095) was a prospective, natural history study that aimed to identify risk factors associated with GA progression. It was conducted at the Institut de la Màcula i de la Retina (Centro Médico Teknon) in Barcelona (Spain). Recruitment began on December 21st, 2009 and finished on December 31st, 2012. Data collection was completed on August 9th, 2013. The study followed the tenets of the Declaration of Helsinki, was prospectively approved by the institution Ethics Committee and all patients signed an informed consent after explanation of the nature and possible consequences of the study.

Inclusion and exclusion criteria

The GAIN study included patients of either sex, 50 years or older, with GA secondary to AMD followed for at least 6 months. GA was defined as uni or multifocal areas of RPE atrophy on a 35º fundus photograph (TRC 50DX IA, IMAGEnet, Topcon Corporation, Tokyo, Japan); at least one of the atrophic lesions had to be larger than 0.5 disk areas (1.27 mm^2). Both eyes of each patient were eligible for the study. Eyes were excluded if RPE atrophy was

deemed to be secondary to other causes (macular dystrophy, high myopia, etc.); if there was a history of wet AMD or any other macular disease thought to interfere with interpretation of FAF images in the study eye; if there was contact between GA and peripapillary atrophy that precluded the measurement of the lesion; if the size of atrophy could not be measured in its entirety in the FAF imaging 30º field; if there was any history of laser in the macula, intravitreal injection or intraocular surgery (aside from phacoemulsification) in the study eye; or if poor image quality precluded the assignment of patient's eyes to a particular FAF pattern.

Procedures

All patients underwent a complete ophthalmic examination that included best-corrected visual acuity (BCVA), intraocular pressure, fundus biomicroscopy and imaging (fundus photography, infrared, FAF and spectral domain optical coherence tomography -SD OCT-) after pupil dilatation with 1.0% tropicamide and 10% phenylephrine. FAF imaging (λ=480 nm, approximate emission 500-700 nm) was acquired with Spectralis HRA+OCT ® (Heidelberg Engineering, Heidelberg, Germany). High resolution (1536 x 1536 pixels), 30º x 30º field of view images centered on the fovea with a minimum averaging of 10 frames were captured. Fluorescein angiography was performed when required according to medical criteria. Refraction and imaging were performed by certified optometrists and technicians.

Area of atrophy was measured with FAF by a single observer (MB) using the Region Finder software, version 2.4.3.0 (Heidelberg Engineering, Heidelberg, Germany). The observer was masked to previous measurements. To evaluate intraobserver agreement, a random sample of 20% of all images was drawn and the area was re-evaluated at least one month apart from the first measure.

FAF patterns were independently determined by two experienced observers (FT and MB) using the 10-item classification (see below). In case of disagreement, a senior observer (JM) arbitrated. A consensus was reached in all cases.

Definition of the main outcome variable

The main outcome was growth of GA (mm^2/year). For bivariate analysis, it was measured between the last and the first visit, divided by the time between them. For multivariable models, GA growth was expressed as the change in the area of atrophy from one visit to the next, including the time between visits as an independent variable.

Definition of main predictor and other independent variables

The predictor of primary interest in this study was FAF pattern. As originally described in the FAM study, [9] there are 10 different patterns: none (no increased FAF in the junctional zone of atrophy), focal (single or individual small spots of FAF at the junction), banded (an -almost- continuous ring of atrophy), patchy (laminar, homogeneous FAF around GA), reticular (linear structures with predominantly radial orientation), branching (ramified FAF), fine granular (FG; grainy, heterogeneous), fine granular with peripheral punctate spots (FGPPS; grainy at the junction and spot-like, well-defined FAF elsewhere), trickling (grayish atrophy, with high FAF at the margins that seeps towards periphery) and undetermined (FAF features different to those previously described). This

detailed classification was the one used by the observers to classify each eye, but the following, more simple classifications, were used for analytic purposes:

For bivariate analysis, we used a classification with 5 categories: none, focal, banded, diffuse and undetermined. The diffuse category gathers patterns characterized by the presence of FAF beyond the borders of atrophy (the reticular, branching, FG, FGPPS and trickling patterns). No patient was classified into the patchy pattern.

For multivariable analysis, the classification was further simplified into just 2 categories: low and high FAF. The low FAF category gathers patterns characterized by a small area of retina with hyperautofluorescence (patterns none and focal), while the high FAF assembles those with larger retinal areas with elevated FAF (patterns banded and diffuse). The category undetermined was excluded given the small number of patients involved.

Other independent variables were age, sex, race, baseline area of atrophy (BAA), time of follow-up, bilateral GA, eye, BCVA, lens status, central retinal thickness (CRT), high blood pressure, smoking status, body mass index (BMI), familiar history of AMD, antioxidant use, concomitant ocular diseases and atrophy description. Their definition is available in **Table 1** of **Supplemental material.**

Statistical analysis

Some data was not captured at some visits. Those values were assumed to be missing at random, and multiple imputation using iterative chained equations [21] was used to infer them.

Univariate and bivariate analyses were used to describe the characteristics of participants and the association between variables, respectively. Bivariate analyses were conducted using Mann-Whitney or Kruskal-Wallis for continuous and Fisher exact test for categorical variables. A stratified analysis of BAA on FAF patterns in relation to GA growth was conducted to inspect interactions.

A multivariable analysis using a mixed-effects regression model was fitted to evaluate the independent contribution of each variable on GA progression. We also determined the relative contribution of FAF patterns and BAA on GA growth under the current hypotheses using mediation analysis, [22] with linear and repeated-measures models. Finally, Bland-Altmann plots were used to evaluate intraobserver agreement of GA size measures.

Results were analyzed using Stata IC/13.1 (Stata Corp, College Station; Texas, USA). A two-tailed p-value≤0.05 was considered statistically significant. Since all analyses were prespecified and type I errors were preferred over type II errors, no correction for multiple comparisons was made. [23]

Results

Figure 1 shows the flow diagram of all screened patients. Longitudinal data was available from 109 eyes of 82 patients, after exclusion of 11.5% of patients who were lost to follow-up. A positive familiar history of AMD was the only statistically significant difference between patients remaining in the study and those lost to follow-up (30.5% vs 0% respectively, p=0.03). The description of

the main characteristics of patients included in the study is shown in **Table 1** (a detailed description is provided in **Table 2** of **Supplemental material**).

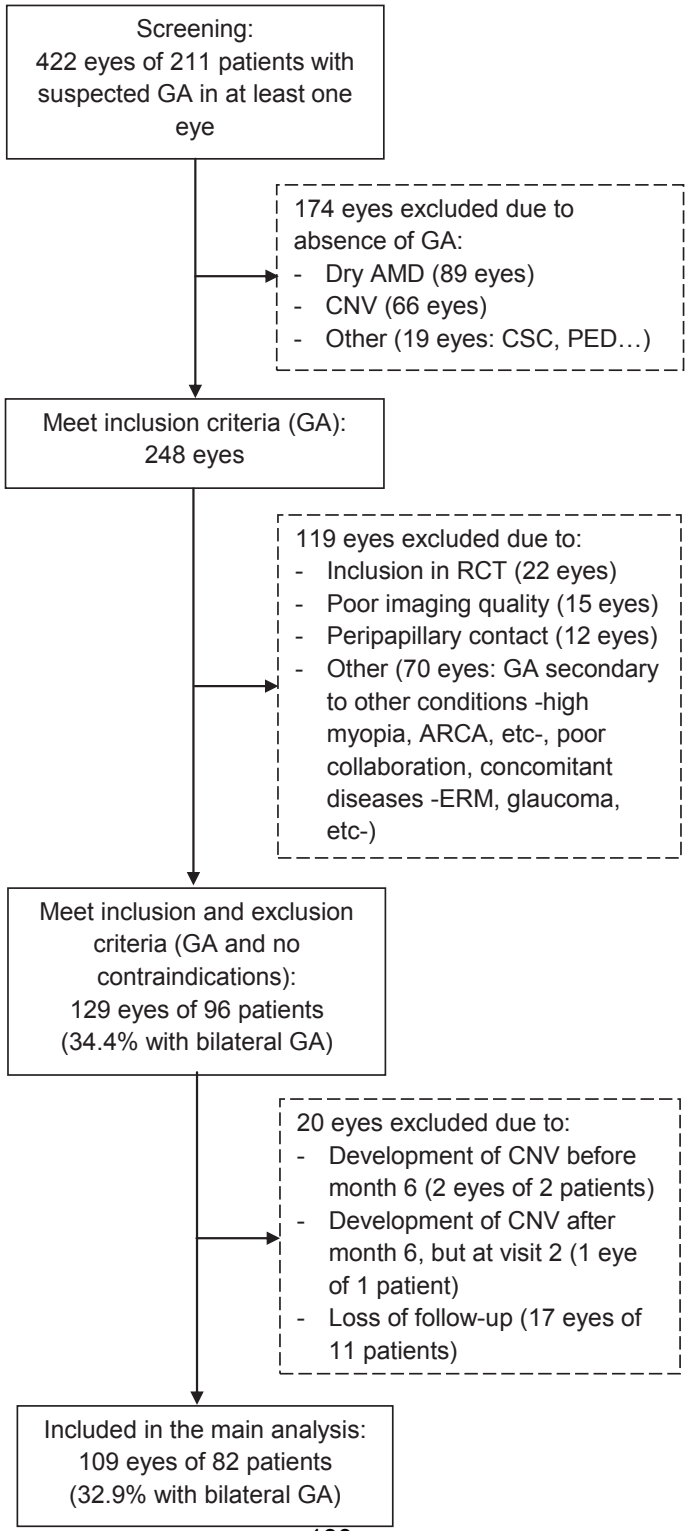

Figure 1. Flow diagram of patients included in the GAIN study. AMD: age-related macular degeneration; ARCA: age-related choroidal atrophy; CNV: choroidal neovascularization; CSC: central serous chorioretinopathy; ERM: epiretinal membrane; GA: geographic atrophy; PED: pigment epithelial detachment; RCT: randomized clinical trials.

Table 1. Main baseline characteristics of patients meeting eligibility criteria in the GAIN study (96 patients, 129 eyes). Quantitative variables are expressed as median (interquartile range) and categorical variables as percentages, which may not add to 100% because of rounding.

Characteristic	Summary measure	Characteristic	Summary measure
Age, yrs	80 (74.5 to 84.5)	**BCVA, letters**	71 (59 to 79)
Sex (female)	66.7	**Pseudophakia**	48.8
Follow-up, mo	17 (6 to 29)	**Antioxidant use (none)**	27.1
BAA, mm^2	6.85 (3.14 to 11.88)	**Smoking (current)**	9.4
FAF pattern:			
None	4.7	**Atrophy descr.:**	
Focal	27.1	**Unifocal fov.**	22.5
Banded	25.6	**Multifocal fov.**	22.5
Fine Granular	17.8	**Unifocal extraf.**	3.9
Branching	14.0	**Multif extraf.**	20.2
Trickling	3.1	**C shaped**	4.7
Reticular	3.1	**C shaped multif.**	17.1
FGPPS	1.6	**Annular**	9.3
Undetermined	3.1		

BAA: Baseline area of atrophy; BCVA: Best corrected visual acuity; Descr: Description; Extrafov: Extrafoveal; FAF: Fundus autofluorescence; FGPPS: Fine granular with peripheral punctate spots; Fov: Foveal; Mo: Months; Multif: Multifocal.

Median follow-up time was 17 months (range, 6 to 29). The median (mean) growth was 1.61 (1.76) mm^2/year (interquartile range, 1.01 to 2.44; range, 0.11

to 5.55). The only variable with missing values was central retinal thickness (CRT; 21/483, 4.3%). These values were imputed as previously described.

The median (mean) BCVA change from baseline to the last follow-up visit was a loss of 7 (9) letters, from 20/40+ to 20/50-, 17.4% of eyes (19/109) lost ≥3 lines and 18.4% (20/109) had a BCVA ≤20/200 at the last visit. Ten eyes developed neovascular AMD (10/129, 7.8% -including those lost to follow-up-) and were excluded from further analyses.

Bivariate analysis

The relationship between GA growth and FAF patterns (using the classification with 5 categories) was statistically significant ($p<0.0013$) and mirrored the distribution found in the FAM study (**Figure 2**).

In other bivariate analyses, GA growth was correlated with BAA (Pearson correlation coefficient $\rho=0.32$, $p=0.0007$), CRT ($\rho=0.25$, $p=0.01$), time of follow-up ($\rho=-0.22$, $p=0.02$) and age ($\rho=0.28$, $p=0.01$). Extrafoveal location of atrophy ($p=0.002$) and pseudophakia ($p=0.03$) were also associated with faster growth. No statistically significant relationship was found with other variables ($p\geq0.08$).

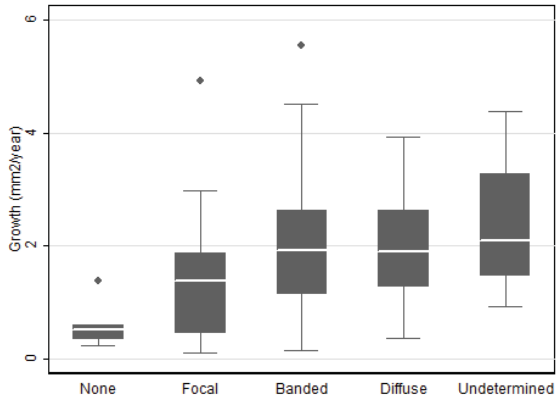

Figure 2. Boxplot showing the growth of geographic atrophy (in mm^2/year) by fundus autofluorescence patterns using the classification with 5 categories ($p=0.0013$).

The association of FAF patterns themselves with other independent variables was explored to assess the possibility that confounding by those variables may explain the relationship between FAF patterns and GA growth. FAF patterns were associated with BAA ($p=0.0001$), and patterns characterized by larger areas with hyperautofluorescence (banded and diffuse) had larger BAA than other patterns ($p=0.0001$; **Table 2**). In fact, the number of eyes with low FAF (patterns none or focal) decreased progressively with increasing BAA, while the opposite was true for patterns with high FAF (banded or diffuse; **Figure 3**).

Table 2. Size of baseline area of atrophy by fundus autofluorescence pattern. The differences were statistically significant (p=0.0001).		
FAF patterns (n)	**Median (mean) BAA, mm^2**	**Interquartile range**
None (6)	2.67 (2.67)	1.84 – 3.74
Focal (32)	3.25 (4.14)	1.59 – 4.78
Banded (27)	11.78 (12.58)	8.34 – 17.24
Diffuse (40)	8.26 (10.42)	4.57 – 13.10
Undetermined (4)	2.58 (2.39)	1.04 – 3.75
BAA: Baseline area of atrophy; FAF: Fundus autofluorescence.		

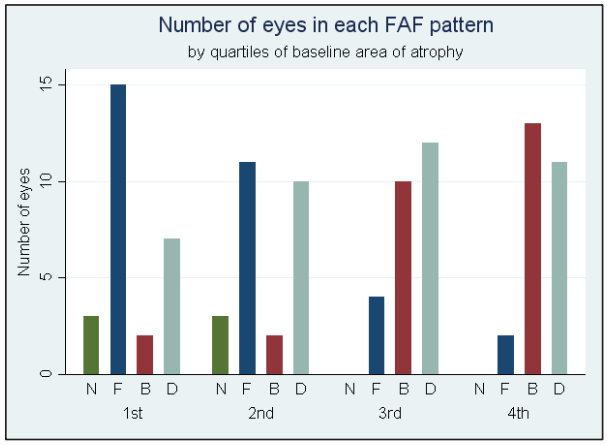

Figure 3. Number of eyes with each FAF pattern by quartiles of baseline area of atrophy. B: Banded; D: Diffuse; F: Focal; FAF: Fundus autofluorescence; N: None.

FAF patterns were also associated with location of atrophy (foveal vs extrafoveal; p=0.03) and familiar history of AMD (p=0.05), but not with other variables (p≥0.06).

Stratified analysis

Within FAF patterns, median growth of atrophy increased homogeneously by tertiles of BAA for focal, banded and diffuse groups (**Figure 4**). The pattern "none" experienced the smallest growth, without differences by tertiles of BAA.

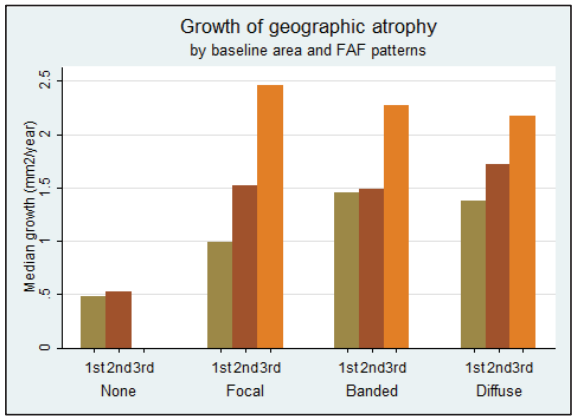

Figure 4. Median growth of atrophy by tertiles of baseline area of atrophy within levels of fundus autofluorescence pattern.

Multivariable analysis

The results of multivariable analysis are shown on top of **Table 3**. BAA (β=1.02), FAF patterns (β=0.54) and follow-up time (β=1.62) were the only variables independently associated with GA growth.

Figure 5 shows the hypothetical relationship between FAF patterns, BAA and GA growth that served as the base for analysis of mediation. [22] The analysis consists of two parts. First, each relationship must be confirmed on statistical grounds: the association between FAF patterns and GA growth (step 1, **table 3**), BAA on GA growth (step 2) and FAF patterns on BAA (step 3) were confirmed (p≤0.002), suggesting that this is a possible scenario. Second, the percent change in the coefficient of FAF patterns when BAA was included in the model was evaluated; it decreased from 3.30 to 0.53, implying strong confounding of BAA on the relationship between FAF patterns and GA growth. A large percentage of the effect of FAF patterns on GA growth (83.9%, [(3.30-0.53)/3.30]*100) was mediated (caused) through BAA.

Table 3. Top, multivariable model showing the association between several independent variables and GA growth (dependent variable). Age, BMI, BCVA and CRT were considered random effects (not shown). Bottom, results from mediation analysis. Variables used for adjustment were time of follow-up, familiar history, foveal atrophy and age.

Multivariable analysis (random-effects model)

Variables	*Coefficients (β)*	*SE*	*p-value*	*95% CI*
FAF patterns	0.54	0.20	0.007	0.15 to 0.94
Time (yrs)	1.62	0.13	<0.0001	1.36 to 1.89
Time ^{2}(yrs^2)	-0.03	0.05	0.52	-0.12 to 0.06
BAA	1.02	0.01	<0.0001	0.99 to 1.05
Familiar history	-0.14	0.20	0.51	-0.53 to 0.26
Sex	0.30	0.20	0.12	-0.08 to 0.69
Foveal atrophy	0.10	0.15	0.51	-0.19 to 0.38
HBP	0.10	0.19	0.61	-0.28 to 0.47
Smoking	-0.04	0.35	0.90	-0.72 to 0.64
Antioxidant use	0.09	0.21	0.68	-0.33 to 0.50
Lens status	0.13	0.21	0.53	-0.28 to 0.54
Concom. disease	-0.03	0.19	0.89	-0.39 to 0.34
Constant	-0.86	0.34	0.01	-1.54 to -0.19

Mediation analysis: BAA mediate the relationship between FAF patterns and GA growth

Steps	*Variables*	*Coefficients (β)*	*SE*	*p-value*	*95% CI*
1	*FAF patterns*	3.30	1.06	0.002	1.21 to 5.38
2	*BAA*	1.02	0.01	<0.001	1.00 to 1.05
3	*FAF patterns*	3.87	0.55	<0.001	2.79 to 4.94

BAA: Baseline area of atrophy; BCVA: Best corrected visual acuity; BMI: Body mass index; CI: Confidence interval; Concom: Concomitant; CRT: Central retinal thickness; FAF: Fundus autofluorescence; GA: Geographic atrophy; HBP: High blood pressure; SE: Standard error; Yrs: Years.

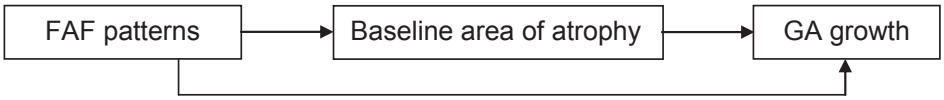

Figure 5. Current hypothesized causal relationship between FAF patterns, baseline area of atrophy and GA growth. The relationships depicted here are used for mediation analysis. FAF: Fundus autofluorescence; GA: geographic atrophy.

Intraobserver agreement in the determination of GA area showed a mean difference between measures of 0.084 mm^2, as seen in the Bland-Altmann plot (see **Supplemental material Figure 1**).

Discussion

We found that BAA, FAF patterns and time of follow-up were independently associated with GA growth. Current hypothesis states that high FAF levels are caused by elevated intracellular lipofuscin, which in turn induce GA progression. This has led to development of VCM. Nonetheless, this study actually suggests that it is the enlargement of atrophy which induces changes in FAF and that BAA is the main driver of GA growth, with a minor role of lipofuscin.

There is a tight relationship between FAF patterns and BAA (p=0.0001) and it is very difficult to dissect which is cause and which consequence, if any of them is truly the underlying cause. However, our results offer some hints. **Table 2** and **Figure 3** show that eyes characterized by low FAF (none, focal) have small areas of atrophy, while those with high FAF (banded, diffuse) are rarely small and are mostly large. We would expect that the number of eyes with high FAF patterns would be similarly distributed between small (those which are yet to grow) and large (those already grown) lesions, but this does not occur. This suggests that as lesion enlarges in a given patient, FAF patterns change from none and focal patterns towards diffuse and banded forms. In other words, FAF patterns seem to be a consequence of enlarging atrophy (an “epiphenomenon”) [24] rather than true phenotypes. In fact, transitions between FAF subtypes have been reported in Stargardt disease, a phenotypically similar disease. [25]

BAA has been previously found to be associated with GA growth. [26-27] In fact, BAA seems more important that FAF patterns in disease progression. Stratified analysis showed an increasing rate of median growth by increasing tertiles of BAA in each FAF pattern, and similar distribution of growth when comparing focal, banded and diffuse patterns. It is the different distribution of BAA within FAF patterns which seems to account for their ability to predict GA growth. Also, even if we hypothesize that FAF patterns determine BAA and GA growth (**Figure 5**), we found that about 85% of the effect of FAF patterns on disease progression was mediated through BAA. In other words: it seems that BAA, not lipofuscin (through FAF patterns), is the main driver of enlarging atrophy.

The FAM study was the first to report an association between FA patterns and GA growth on bivariate analysis, [9] but not on multivariable analysis. [15] The authors suggested that small sample size was the likely cause, but confounding is another potential reason. The independent association is of paramount importance, because it is this relationship that provides a causal explanation and therefore identifies potential therapeutic targets.

Certainly, lipofuscin compounds (notably, A2E) have shown a wide range of deleterious effects on RPE physiology on in vitro and animal studies. [10,12,28,29] However, these have not been demonstrated in vivo in humans. In fact, it has been recently reported that there is a disparate distribution between lipofuscin and A2E in human RPE, [18,30] suggesting that A2E cannot be responsible for increased FAF at the macula. On the other hand, Rudolf et al [17] reported that areas of increased FAF are caused by vertically aligned disorganized RPE cells at the borders of atrophy (not by increased lipofuscin within single cells), offering an intuitive reason for increased FAF.

We hypothesize that as atrophy enlarges in a centrifugal manner, [31] RPE cells at the borders of atrophy become disorganized and vertically aligned, [32] inducing high FAF levels (as explained by Rudolf et al). [17] Since the normal distribution of FAF increases with retinal eccentricity (reaching a maximum at 3 mm from the fovea), [33] large lesions eventually reach this area, where intraretinal migration of naturally higher rich lipofuscin-laden RPE cells would induce widespread high FAF, clinically identified as diffuse or banded patterns.

Nonetheless, lipofuscin may play a role on GA pathogenesis. The presence of little FAF (focal, banded or diffuse patterns) was associated with increased and similar median growth of atrophy as compared with no FAF (**Figure 4**). Also, the coefficient for FAF patterns is statistically significant ($p=0.007$) and may be clinically important ($\beta=0.54$), as seen on multivariable models. Thus, VCM may have a small but relevant effect on GA growth.

Many questions remain to be answered. The search for the missing fluorophore appears as a priority on the basic research agenda, while finding how single RPE cells can increase their levels of FAF so fast is necessary to support the lipofuscin theory. The evaluation of FAF images in particular patients over the long term would help to elucidate the transitions in patterns hypothesized to occur by the present study. Indeed, we recognize that some patterns (for example, the trickling) are so characteristic that it is difficult to explain them merely in terms of stages of growth. The effects of the interaction of lipofuscin with other exposures should also be explored. Finally, given the frequency of contradictory findings, replication of results (not just discovery) is required.

One limitation of this study is that clinical data may not always reflect the underlying cellular changes occurring in pathological states, making it necessary to use surrogate variables. Unfortunately, surrogate variables may be inaccurate constructs of the molecular changes. In our case, increased FAF can be caused by a heightened lipofuscin uptake within the RPE, but it may also reflect increased bisretinoid formation in the photoreceptor outer segment, amongst others. [34] Residual confounding due to unmeasured or poorly measured factors is another potential concern in all epidemiologic studies, but it is unlikely that a biased measure of BAA or another, as yet unidentified factor, may significantly dilute the role of BAA on GA growth. Finally, GAIN participants

may not be representative of all GA patients, but representativeness is not required to address causality. [35]

Study strength relies on its focused research question, prospective design, comprehensive data collection and efforts placed on maximize information. The results were robust irrespective of the analytic strategy used.

In summary, the GAIN study suggests that FAF patterns may be a consequence of enlarging atrophy and that the role of lipofuscin on GA growth is modest at best, which limits the potential benefits of VCM. Approaches that consider the role of BAA (through, for example, cell-to-cell contact) and, possibly, its interaction with other factors (presence of lipofuscin), may contribute to elucidate the causes of GA progression.

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SUPPLEMENTAL MATERIAL

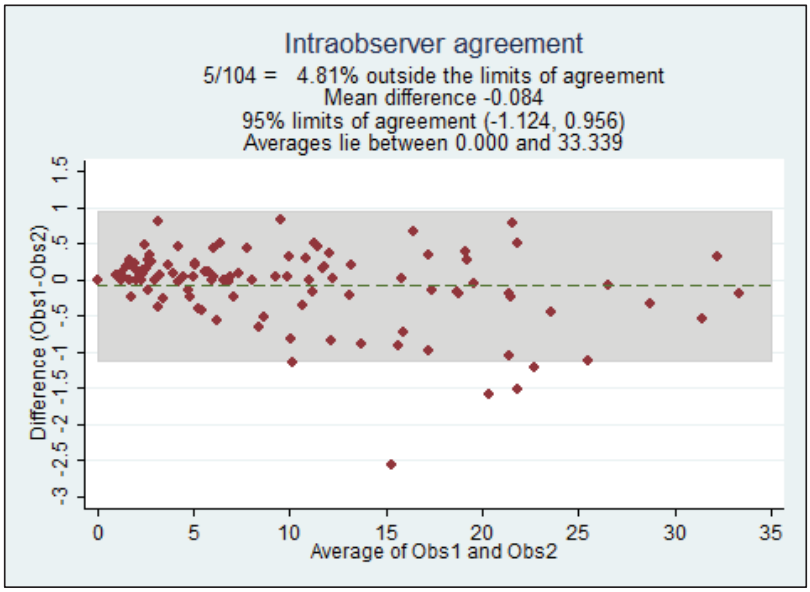
(**Supplemental material Table 1.** The description has not been provided herein due to space constraints. It has been provided in the Thesis main document)

Supplemental material Table 2. Full description of baseline characteristics of patients meeting eligibility criteria in the GAIN study (96 patients, 129 eyes). Quantitative variables are expressed as median (interquartile range) and categorical variables as percentages, which may not add to 100% because of rounding. AMD: Age-related macular degeneration; BAA: Baseline area of atrophy; BCVA: Best corrected visual acuity; BMI: Body mass index; CRT: Central retinal thickness; Descr: Description; Extrafov: Extrafoveal; FAF: Fundus autofluorescence; FGPPS: Fine granular with peripheral punctate spots; Fov: Foveal; Mo: Months; Multif: Multifocal. Pseudoph: Pseudophakic.

Characteristic	Summary measure	Characteristic	Summary measure
Age, yrs	80 (74.5 to 84.5)	**CRT (µm)**	219 (188 to 255)
Sex (female)	66.7	**Hypertension (yes)**	59.4
Race (Caucasian)	99.0	**Smoking (current)**	9.4
BAA, mm^2	6.85 (3.14 to 11.88)	**BMI, kg/m^2**	26.7 (24.4 to 29.4)
Follow-up, mo	17 (6 to 29)	**Familiar history of AMD (yes)**	27.1
Bilateral GA	34.4	**Antioxidant use (none)**	27.1
Eye (right)	45.7	**Concomitant disease (any)**	61.2

FAF pattern:			
None	4.7	**Atrophy descr.:**	
Focal	27.1	**Unifocal fov.**	22.5
Banded	25.6	**Multifocal fov.**	22.5
Fine Granular	17.8	**Unifocal extraf.**	3.9
Branching	14.0	**Multif extraf.**	20.2
Trickling	3.1	**C shaped**	4.7
Reticular	3.1	**C shaped multif.**	17.1
FGPPS	1.6	**Annular**	9.3
Undetermined	3.1		
BCVA, letters	71 (59 to 79)	**Referred (yes)**	32.3
Lens (pseudoph)	48.8	**-**	-

Supplemental material Figure 1. Intraobserver agreement in the measure of geographic atrophy using fundus autofluorescence (n=104).



Tables summarizing the main findings

Paper 2. Intra and interobserver agreement in the classification of fundus autofluorescence patterns in geographic atrophy secondary to age-related macular degeneration.

Intraobserver agreement in the classification of FAF patterns was good to excellent, while interobserver agreement was fair at best (**Table 4**). The use of more simple classifications improved reproducibility, but interobserver agreement remained below intraobserver agreement irrespective of the classification used.

Table 4. Summary of intra and interobserver agreement of fundus autofluorescence patterns according to the classification used.

	Kappa (percentage of agreement)					
	Specific patterns		*General patterns*		*Simplified patterns*	
	Median	*Range*	*Median*	*Range*	*Median*	*Range*
Intraobserver	0.72 (76%)	0.5-0.8 (59-86%)	0.76 (83%)	0.6-0.9 (70-97%)	0.81 (93%)	0.7-0.9 (89-97%)
Interobserver	0.33 (42%)	0.3-0.6 (39-68%)	0.41 (59%)	0.2-0.7 (46-77%)	0.34 (78%)	0.1-0.8 (61-96%)

This study showed that, given the moderate interobserver agreement, more than one evaluator is required for assignment of FAF patterns, and that the use of more simple classifications is recommended to improve reproducibility. The results also implicitly suggested that a new, more intuitive classification of GA based on FAF imaging, would be desirable, and that the patterns described in a German population can also be extrapolated to a Spanish population.

Paper 3. Optical coherence tomography assessment of apparent foveal swelling in patients with foveal sparing secondary to geographic atrophy.

Although the qualitative inspection of SD-OCT images suggests that the foveola of patients with GA and foveal sparing seems swollen, the quantitative analysis does not support this impression (**Table 5**). This artifact is caused by thinning of perifoveal retina by the atrophic process relative to that of the foveola itself.

When retinal layers were manually segmented, the outer nuclear layer (ONL) seemed thicker in cases than in non-cases, but the effect waned when the tilting technique (215,216) was used to differentiate the ONL from Henle's fiber layer (HFL). In a *post-hoc* finding, HFL was thicker in cases than non-cases. Finally, the layer containing the photoreceptors outer segment was thinner in cases.

Table 5. Comparison of thickness of different retinal layers between patients with geographic atrophy and foveal sparing and non-cases. *: Bonferroni-corrected; HFL: Henle's fiber layer; ONL: Outer nuclear layer.

	Cases (median, µm)	*Non-cases (median, µm)*	*p-value*
Foveal thickness	226	227	0.56
Apparent ONL	125	114	0.02
ONL without HFL	74	73	0.82
Sub-ONL	83	106	0.0004
HFL	51	39	0.02*

In summary, the foveola of patients with GA and foveal sparing is not swollen, although individual layers show quantitative changes in thickness as compared to non-cases. These variations presumably reflect early changes at the cellular level in response to surrounding atrophy.

Paper 4. Reappraisal of geographic atrophy patterns seen on fundus autofluorescence using a latent class analysis approach.

After the classification performed by each observer using the 10 categories described in the FAM study (60), 5 new categories (classes) emerged from LCA. The characterization of these classes (**Table 6**) showed that they had different sizes of atrophy. This suggests that GA size is implicitly used to classify FAF patterns, whose definition was initially based solely on hyperautofluorescence features surrounding atrophy.

Table 6. Comparison of the main features of each resulting latent class.

	Class 1	*Class 2*	*Class 3*	*Class 4*	*Class 5*	*p-value*
Baseline area of atrophy (mm^2)	1.92	2.75	4.58	10.73	12.7	<0.001
Growth (mm^2/year)	0.43	1.11	1.75	1.77	1.79	0.002
% of high autofluorescence	14.3	77.8	90.0	95.7	100	<0.001

These results support the hypothesis that FAF patterns, as originally described in the FAM study (60), are not true phenotypes. They may be a consequence of the process of expanding atrophy

itself and, therefore, may represent different stages in the progression of atrophy.

Paper 5. Increased fundus autofluorescence as a risk factor for progression of geographic atrophy. The GAIN study.

On bivariate analysis, FAF patterns and BAA were strongly associated with GA growth and with each other. On stratified analysis, GA growth was similar within tertiles of baseline area of atrophy by FAF patterns, suggesting that the association between FAF patterns and GA growth is at least partially caused by an uneven distribution of areas of atrophy within FAF patterns, with patterns with high hyperautofluorescence (“banded” and “diffuse”) having a higher percentage of large areas of atrophy than patterns with low hyperautofluorescence (“none” and “focal”). On multivariable analysis, time of follow-up, BAA and FAF patterns were independently associated with progression of GA (**Table 7**), but the association of FAF patterns with GA growth was strongly confounded by BAA: the coefficient for FAF patterns decreased sixfold when BAA was included in the model. Analysis of mediation suggested that approximately 85% of the effect of FAF patterns on GA growth was actually mediated (caused) by BAA.

Table 7. Association between fundus autofluorescence patterns, baseline area of atrophy and time of follow-up on disease progression adjusted for potential confounders.

	Coefficient	*95% confidence interval*	*p-value*
Fundus autofluorescence patterns (high vs low)	0.54	0.15 to 0.94	0.007
Baseline area of atrophy (mm^2)	1.02	0.99 to 1.05	<0.0001
Time of follow-up (years)	1.62	1.36 to 1.89	<0.0001

Therefore, a clinically relevant effect of lipofuscin on GA growth cannot be excluded, but it seems small and is confounded by BAA. VCM may have a smaller effect on progression of GA than previously thought.

Discussion

a. Summary of main results

The main conclusions of this Thesis are that GA patterns as seen with FAF are not true phenotypes but rather a consequence of enlarging atrophy and that lipofuscin plays a smaller role on GA progression than previously thought. Additionally, we found no foveal swelling in patients with foveal sparing secondary to GA, but there are changes in specific cellular layers identifiable on SD OCT that are not visible on ophthalmoscopy.

As seen on LCA, different patterns of FAF implicitly reflect different areas of atrophy, different stages in the lifetime of a lesion. Therefore, we would expect that patients may change from one pattern to another as lesion grows. We could not confirm that in statistical terms (p=0.55). However, given the relatively slow growth of GA, 19 months of median follow-up may be a period too short to identify these hypothetical transitions.

Figure 9, taken from the Supplemental material provided in paper 5, gives further support to this hypothesis. It shows that the number of eyes with patterns characterized by small areas of elevated FAF as defined on the FAM study (60) ("none" and "focal") have almost invariably small areas of atrophy. The reverse is true for patterns with large areas of increased FAF ("banded" and "diffuse"): these patients are mostly found in the 3rd and 4th quartile of area of atrophy. Given the relatively slow growth of atrophy even in fast progressors, if FAF patterns were true phenotypes and they would not change with time, it would be reasonable to find a similar number of patients with the "banded" and "diffuse" patterns in each quartile of area of atrophy (patients with small, moderate, large and very large lesions), representing eyes whose lesions are yet to

grow as well as those in which the lesion has already become large. What we found is actually that the number of eyes with small FAF decrease with increasing size of atrophy and those with large FAF increase, which suggests that the first group of eyes may convert to the second as lesion enlarges. This provides an indirect explanation of transitions from small to large areas of hyperautofluorescence with ongoing GA progression. A hypothesis of the events leading to this phenomenon is provided later.

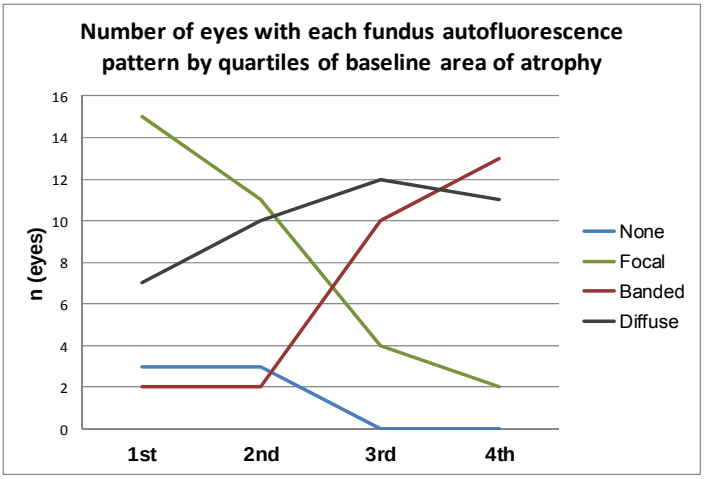


Figure 9. Number of eyes in each quartile of baseline area of atrophy by fundus autofluorescence patterns. The number of eyes with patterns corresponding to small areas of autofluorescence (none, focal) decrease with increasing size of atrophy, while the situation is reversed for patterns characterized by large areas of high autofluorescence (banded, diffuse).

The last paper showed that time of follow-up, BAA and FAF patterns were independently associated with GA growth. Data from stratified, multivariable and mediation analysis suggest that BAA is more relevant than FAF patterns on disease progression.

Stratified analysis showed that growth was similar within tertiles of BAA in each FAF pattern, suggesting that it is the different proportion of lesion sizes between each FAF pattern which confers them a prognostic value. On multivariable models, the (unstandardized) coefficient for FAF was smaller than that of BAA and decreased markedly when BAA was included in the model, which indicated strong confounding. Analysis of mediation suggested that 85% of the effect of FAF patterns on GA growth was attributable to BAA, and residual confounding may potentially decrease its effect even further. Therefore, although FAF patterns, a biomarker of lipofuscin accumulation, were an independent predictor of GA growth, they were not the main contributor.

Despite the aforementioned reasons, stratification analysis also showed that the mere presence of hyperautofluorescence (patterns “focal”, “banded” and “diffuse”) were associated with similar and larger growth than when no hyperautofluorescence was observed (“none”). Combined with the statistical significance and clinically relevant effect found in multivariable analysis, lipofuscin may contribute, albeit modestly, to GA progression.

b. Implications

The finding that FAF patterns are not phenotypes, but rather a consequence of disease enlargement, implies that GA may be a more uniform disease than previously recognized. Considering the difficulties encountered in finding genotypes that differentiate specific AMD phenotypes (neovascular AMD, GA, polypoidal vasculopathy, etc), the search for SNP associated with FAF patterns seems a futile endeavor that in the best scenario can lead to a waste of time and resources and, in the worst scenario, to false positive results.

Studies evaluating potential phenotypes or growth in GA should consider adjustment by area of atrophy due to its large influence on disease behavior. The marked difference in growth rates in patients with GA described in most studies may be a consequence of different lesion size instead of genuine intersubject heterogeneity.

In spite of these limitations, FAF patterns remain useful as predictors of disease growth because they are a good proxy for BAA, the main known prognostic factor. The study of the distribution of high FAF ("FAF patterns") is a useful adjunct for patient counseling (individual prognosis), as eligibility criteria for clinical trials and, possibly, for prognostic modeling. Therefore, the qualitative information provided by FAF in GA remains useful.

The fact that high FAF is only moderately associated with disease growth calls into question the predicted efficacy of VCM. This therapeutic strategy was developed to arrest the accumulation of presumed toxic metabolites from the visual cycle (A2E or other compounds of lipofuscin). It may have a minimal or moderate effect

on GA progression. Indeed, the phase II results from fenretinide, an oral VCM, did not provide statistically significant or clinically relevant benefits in slowing GA growth, despite the authors' claims of benefit on subgroup analyses (219), a strategy prone to false positive results (220).

Considering the complexities involved in this therapy, which require sophisticated, lengthy and not widely available examinations (ERG), with potentially relevant and frequent adverse events (rash, dyschromatopsia and altered dark adaptation, whose reversibility is not known if treatment is maintained over the long term), a better knowledge of lipofuscin role in GA progression seems required to avoid exposing patients in clinical trials to treatments with potentially serious consequences and uncertain benefit.

The role of BAA (53,221), multifocal lesions (40) or lesion irregularity (222) on GA progression may suggest that lesion perimeter has an impact on disease growth. This may reflect the fact that cell-to-cell contact is important. Neighbor-killing (157), retraction-from-free-edge (223) or other models may be, thus, rewarding. In fact, it has been shown that cell-to-cell transmission of pathogenic proteins operates in Alzheimer and other neurodegenerative disorders, which, as AMD, are characterized by the deposition of abnormal material (224).

It must be noted that small central GA lesions have a different growth behavior from the rest, as seen by different slopes of growth and stratification analysis (papers 4 and 5, respectively). Although this is highly speculative, it is possible that there are simply two types of GA lesions in terms of growth: small unifocal foveal lesions and the rest (see *Future directions*, on page 183).

Finally, the study of the morphological changes occurring in patients with foveal sparing showed that the atrophic process not only affects the specific area involved, but it also has consequences at the cellular level on nearby areas, even in normal appearing retina. The marked thinning of photoreceptor outer segments was an early signal of retinal damage. Given their relevance in the visual cycle and vision, the window of opportunity to restore visual function may begin to shorten well before ophthalmoscopic changes are evident, which underscores the need to use SD OCT or other high-resolution imaging methods (ie, adaptive optics) to evaluate these patients at an early stage. Thickening of HFL was unexpected, but had been previously described in murine models of AMD (225) and in a histochemical study in human donors (226). The morphological changes suggest a cell-death pathway involving necrosis or Müller cell hypertrophy in HFL. Necrosis was previously regarded as uncommon in AMD, in which apoptosis has been the most consistent mechanism of cell-death (179). This data must be interpreted with caution given the *post hoc* nature of the finding and it will need replication. If confirmed, it may open the opportunity to new therapeutic strategies, such as inhibitors of receptor-interacting protein (RIP) kinases. These results may also be useful in other atrophic disorders, such as Stargardt disease and high myopia.

This study also implicitly showed that SD OCT is sensible to detect subtle, early changes in normal-appearing retinal areas on ophthalmoscopy, and that the tilting technique described by Lujan (215) and Otani (216) is a useful method to differentiate adjacent layers with similar levels of reflectivity. It remains to be seen if this technique can be used to improve the automatic segmentation

algorithms that are being developed to quantify other retinal layers which may have a wide range of applications, from early glaucoma detection to the study of retinal dystrophies.

c. The role of lipofuscin on geographic atrophy progression in context and proposed hypothesis

Most preclinical findings suggest deleterious effects of lipofuscin of RPE cell function (for a review, see Sparrow et al (73)). Its toxic effects are attributed mainly to A2E (84,86,227). The fact that A2E distribution within the retina has been recently questioned (80) does not challenge the main hypothesis that lipofuscin confers a high risk for progression of GA, because high FAF has been unambiguously linked to both, lipofuscin (56,97) and GA growth (60). However, there are some reasons that warrant careful consideration of preclinical data:

- It is difficult to determine if the exposures used in the laboratory are similar to those that presumably induce GA. For example, how does an acute exposure to a light source with a broad band emission spectrum of 390 nm to 550 nm and an irrandiance of 2.8 mW/cm^2 for up to 144 hours compare with everyday life conditions in humans (85)? Do equivalent levels of exposure occur in real life?

- Laboratory conditions are highly controlled but many different exposures interact in a more complex milieu

like the living eye, which complicate the extrapolation of results found in that environment to actual conditions.

- The differences between animal and human species. For example, the retinal distribution of A2E in human and murine species has been recently shown to differ substantially (81).

Despite this caveats, it is reasonable to assume that a compound not amenable to degradation that fills progressively about 20% of the intracellular space in the aging RPE cell and that has shown many potentially toxic effects *in vitro* and in animal models may interfere with proper cell function.

The clinical evidence to support the lipofuscin theory is weaker. Researchers from the FAM study found an association in bivariate (60) but not multivariable (65) analysis. The authors blamed on lack of statistical power to justify the multivariable results. In fact, the tables in the paper showed that no single variable had complete data, which is necessary for MEM in most statistical packages; if no imputation method was used to infer the missing observations, sample size could have been severely reduced. Confounding and genuine lack of effect are also likely explanations for the absence of association. In addition, the FAM did not report a statistical association between FAF patterns and GA size in the main publication (60): "[…] In a combined analysis of the FAF pattern classification and the classification of baseline total atrophy, there was a trend of smaller GA size at baseline within the group of no abnormal and focal FAF pattern, but a statistical analysis could not abandon the thesis of the independence between both classification systems (P=0.070; Table 3) […]". They used the

Fisher exact test, but a reanalysis of their own data using an equally appropriated statistical method, the likelihood ratio chi-squared test, showed a p-value of 0.04. Whatever method is used, we do believe that data from FAM does not exclude a likely association between FAF patterns and BAA, which is a pivotal argument in the GAIN study.

Other groups have also found an association between increased FAF and faster growth using other classifications (228). We agree that they are related (FAF patterns predict growth), but we dispute that they are causally associated. Using imaging analysis, Hwang et al (57) found that the predictive positive value of increased FAF to become hypoautofluorescent was no greater that chance, suggesting that increased FAF does not increase the risk of incident atrophy.

While recognizing that GA and Stargardt are different diseases, they share phenotypic resemblances and lipofuscin build-up has been involved in the pathogenesis of both. Therefore, the results in one disorder may be relevant for the other. Fujinami et al (229) described that the 3 FAF patterns of Stargardt in which the patients were classified at baseline were correlated with progression of atrophy, as has been found in GA. Of note, they also found transitions between FAF patterns (ie, from type 1 to type 2, and from type 2 to 3), which means that FAF patterns change with time in a given individual as atrophy enlarges. This is also our hypothesis in GA. However, our median follow-up was 1.5 years while theirs was 9 years. Since Stargardt disease and GA show similar enlargement rates (229-231), a longer follow-up might be required to observe transitions in FAF patterns in GA. In another

study in an ABCA4$^{-/-}$ mouse model of Stargardt (232), lipofuscin accumulation did not have deleterious effects on retinal histology or function (ERG) as compared to wild type controls, although anatomical differences between species (humans have more cones) could have explained the discrepancies. It must be kept in mind that increased RPE lipofuscin load has been demonstrated in Stargardt disease and that it precedes RPE atrophy and vision loss (233) (in fact, the “dark choroid” sign on FA is thought to represent a consequence of lipofuscin deposition). These findings have not been reported in GA.

The aforementioned arguments provide plausible reasons to question the role of lipofuscin as the main driver of GA progression. We provide a unifying, conceptual framework of GA growth considering the results found in the present and other studies: a storyline of the progression of GA as seen on FAF.

For unknown reasons that possibly imply oxidative stress, low grade inflammation in the outer retina (150) or even increased lipofuscin deposition, GA ensues and grows, usually from small patches of foveal or perifoveal RPE atrophy. Anatomic or physiologic particularities at this location may underlie its increased susceptibility to incident GA. There, macular pigment absorption and physiological low levels of lipofuscin within RPE cells induce low levels of FAF around atrophy (clinically identifiable as patterns characterized by low hypoautofuorescence, “none” and “focal” (60)). As GA enlarges from pre-existing atrophy, there is RPE disturbance identifiable by alterations on the outer retina at the junction by SD OCT and increased FAF (234). Progressively increased FAF as lesion grows beyond the perifovea is explained

by RPE migration to the inner retina, as has been described from histological (104,145) and *in vivo* SD OCT (71) studies. The vertical alignment of RPE cells induces more markedly increased FAF there (59) because the physiological level of FAF is higher in that location (97). This is clinically recognized as patterns characterized by high FAF content, “banded” and “diffuse” (60). Also, this migration of RPE may induce a reduction in sensitivity (235,236) because photoreceptors are left without its support. The decreased sensitivity is more marked on scotopic conditions (92) by the increased vulnerability of rods over cones (93,113). Increased FAF will show different levels of sensitivity loss (235,236) depending on the degree of photoreceptor loss. Also, increased FAF will show different susceptibility to progression to GA (clinically, different susceptibility to show incident hypoautofluorescence) (57) depending on the number of RPE cells vertically aligned, their degree of degeneration and the likely involvement of photoreceptor damage in elevated levels of FAF (98), which may indicate a late stage of degeneration. FAF patterns are associated with progression of GA on bivariate analysis (60,228) because they are a consequence of disease enlargement and therefore the events leading to GA growth and high FAF are closely spatially and temporally related. This may also explain the relatively small association of high FAF (lipofuscin, through FAF patterns) with GA progression when other independent variables, mostly BAA, are included on multivariable analysis (64,65).

This hypothesis explains the increase of FAF with enlarging atrophy and some of the histological and functional findings reported in the literature, but other fundamental issues cannot not addressed. Clearly, the triggering event has not been identified; the

reasons for the increased susceptibility of the perifovea to atrophy are unknown, as are the factors providing protection to the fovea against GA progression. It is also not known why atrophy reaches a plateau near the arcades. On the other hand, this hypothesis provides an explanation for the sequence of events observed by FAF as GA grows and integrates results from other researchers, some of which had been used as arguments in favor of the role of lipofuscin on GA progression.

d. Replication is key

In this Thesis we have focused our attention mainly on three issues: the role of lipofuscin on GA progression, the classification of FAF patterns and the effects of surrounding atrophy on foveal integrity. Our results questioned previous findings on the last two (the first was inherently controversial). None of these two studies had been previously challenged, but some publications echoed their results, assuming their truthfulness (228,237-239).

Unfortunately, lack of replication of research results is common (240). Observational studies seem to be involved in most cases (241-244). In Genetic epidemiology, for example, Ioannidis et al (242) found that the first study reporting a novel association between a disease and a candidate susceptibility loci frequently showed the highest OR, with a descending secular trend in the following studies, approaching in many cases the null (**Figure 10**). This form of bias away from the null, frequently called the “winner’s curse” (245), arises from a combination of factors, namely selective reporting, underpowered samples (that inflate effect estimates),

conflicts of interest and publication bias (246). In addition, classic observational studies reporting therapeutic effects may persist to be cited even after being contradicted by results from clinical trials (247). Unfortunately, even clinical trials have been contradicted by subsequent trials (241,248).

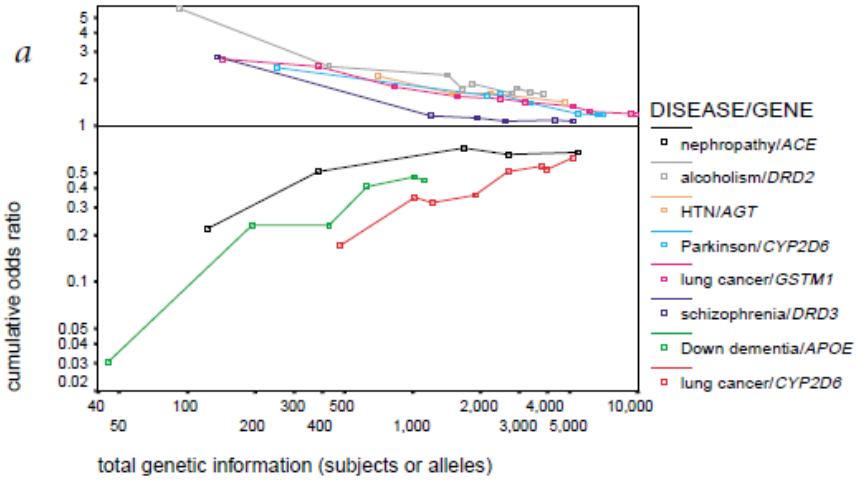


Figure 10. Changes in the odds ratio as data accumulate regarding the association between particular diseases and related genes. There is a marked overestimation of the first published studies that tends to decrease with time and approach the null (from: Ioannidis JP et al. Replication validity of genetic association studies. Nat Genet 2001; 29: 306-9. Used with permission).

Lack of replication also involves basic research, where only 11% of scientific findings in “landmark studies” in cancer could be replicated in the laboratory (249,250). In one study, only 5% of

highly promising drugs at the preclinical stage were licensed for clinical use 20 years later (251). Plans to enhance reproducibility have been a topic of discussion in the Association for Research in Vision and Ophthalmology (ARVO) Open Forum of 2014.

Overall, poor replication produces a distorted view of science (252) and discourages investments in research (253). And the problem is on the rise: the number of retractions (which include not only irreproducible results, but also honest error and misconduct) has increased 10-fold in the past decade, far more than the 0.5-fold increase in the number of publications (254).

This undesirable variability in research studies underlines one of the pillars of the scientific method: replication. The need for replication has been firmly established in disciplines such as Genetic epidemiology (which led to the creation of the Human Genome Epidemiology Network, HuGENet) or the field of Oncology (through the Breast Cancer Association Consortium (255), for example), and an ambitious program is ongoing, the Science Exchange Reproducibility Initiative (256). In the meantime, some measures can be undertaken to decrease the rate of false positives and to improve reproducibility of research findings: full disclosure of conflicts of interest, prespecified hypothesis and adequate correction for multiple testing, reporting of all results (irrespective of their statistical significance), online access to full databases, honest acknowledgment of study limitations and cautious interpretation of results (especially subgroup analysis) considering all available evidence.

e. Strengths and limitations

Study strengths are presented herein:

- The study design was the most adequate to answer the research question. In particular, its prospective nature is a desirable feature to collect data in a comprehensive manner and to establish the temporal relationship between cause (risk factors) and effect (GA progression).

- We had a focused main research question to decrease the chances of spurious results: the role of increased FAF, a biomarker of lipofuscin, as the predictor of primary interest on GA growth.

- The study was conducted in a single center, which allowed a highly uniform and standardized set of procedures that provided measurements of high quality and minimization of missing data.

- The personnel involved met high clinical standards. Aside from their corresponding professional qualifications, all people involved had experience with clinical research, all measures were taken by sanitary personnel certified by international organizations and four of them have followed a Good Clinical Practice course.

- State-of-the-art imaging and exploration methods were used: cSLO-based FAF, SD OCT, Region Finder ® software for determination of area of atrophy, etc.

- An explicit effort to minimize biases and to provide consistent results was made. An example of the first point is the use of LCA (an observer-independent statistical approach) and an *a priori* defined set of characteristics to define the presumed new patterns (classes). An example of the second point is the use of bivariate, stratified and multivariable analysis to identify the relationship between independent variables and GA progression.

On the other hand, this study has limitations that warrant some consideration and that pave the way for improvements in future research:

- Small sample size. We intended to establish a collaboration with another institution to increase the number of patients with GA, but technical and interim circumstances posed obstacles that finally prevented the consortia (see *General description of methods. Sample size*, on page 65).

- The possibility that some included patients considered to have GA had been misdiagnosed and actually presented a macular dystrophy or other entities cannot be absolutely ruled out. Although specific testing (genotyping, etc) was rarely used, we adopted a restrictive criterion for inclusion: a larger area of atrophy than most current guidelines (0.5 disk areas) and a low

threshold to exclude patients in case of doubt, such as those with age-related choroidal atrophy.

- At the same time, areas of atrophy undistinguishable from classic GA may be the end-stage of many retinal disorders unrelated to AMD (vitelliform lesions, retinal toxicity, etc). If, incidentally, drusen coexisted with these retinal lesions, these patients would have been inadvertently included in the study. This is probably the case also in most studies, and it is currently not known if atrophy in these patients behaves like classic GA. Studies including solely incident cases of GA, possibly in the setting of a large population-based study (BDES, BMES, etc), would avoid these concerns.

- We assumed that dark areas on FAF represented absence of RPE, but other causes of hypoautofluorescence also exist. We used fundus camera-based FAF to identify the extent of foveal atrophy, and infrared and SD OCT to help identify the perimeter of atrophy.

- We also assumed that areas of elevated FAF represented high lipofuscin content. There are other reasons for intense FAF, such as photoreceptor cell dysfunction, in some instances secondary to RPE atrophy. More work is required in this area to help differentiate the underlying cause of high FAF.

- Area of atrophy was measured by a single observer (the PhD candidate). We performed Bland-Altmann plots to

test intraobserver reproducibility, with very good results. However, interobserver reproducibility study was not conducted. Given the similar results of progression of GA in the GAIN (mean growth 1.76 mm^2/year) as compared with other studies involving Reading Centers (1.74 mm^2/year in FAM, 1.77 mm^2/year in GAP), we believe that the measurement of area of atrophy was not severely biased.

- The information from several variables may be imprecise or, in the worst scenario, biased. Smoking status, for example, was obtained by direct interrogation to the patient. To minimize errors in self-referred data, we conducted a structured series of questions for each self-reported variable.

- If GA progression is triggered by events that took place years ago, the risk factors proximal to disease progression may be uninformative or even erroneous. Again, a population-based study of incident cases of GA would be key.

- The minimum time of follow-up was arbitrarily chosen at 6 months. This cut-off defined patients lost to follow-up, which may in turn have an impact on selection bias. While we do not know how these changes in the definition could affect the results, this should be kept in mind.

- Indeed, selection bias is always a source of concern. For example, patients with bilateral foveal GA, for which

there is no treatment and small risk of further central vision loss, may be less prone to follow regular ophthalmic care. If the reasons for central GA development are related to disease progression, selection bias creeps in.

- The clinical behavior of patients in the GAIN study may differ to that experienced by GA patients in the general population in Spain (many patients with unilateral GA in GAIN had fellow eye CNV). Although this questions external validity, the main objective of this Thesis was to identify risk factors associated with GA progression, and causality does not require representativeness. Therefore, causal inference in this setting remains appropriate, while generalization may not.

- Some results were obtained as a *post hoc* finding, such as thickening of HFL in paper 3. This requires cautious interpretation of results.

- LCA has been only rarely used in Ophthalmology. It is a complex, unfrequent statistical method and therefore carries the potential to be regarded as a “black-box” analysis. This particular method was conducted by an experienced researcher in this field, Dr Carlos G Forero.

- Multivariable models used in GAIN assume a linear growth of atrophy. While this may not be true, it seems a reasonable assumption given the time frame of this study, a few years, and the relatively slow growth of GA.

Conclusions

a. The progression of GA as measured with FAF in a tertiary center in Spain is remarkably similar to that reported in other studies in other countries (mean growth in our study 1.76 mm^2/year; 1.74 mm^2/year in the FAM and 1.77 mm^2/year in the GAP studies).

b. On bivariate analysis, the factors associated with GA progression were time of follow-up, BAA, FAF patterns, age, central retinal thickness, extrafoveal location of atrophy and pseudophakia. On multivariable analysis, time of follow-up, BAA and FAF patterns were the only variables independently associated with disease progression. However, the relationship between FAF patterns and GA progression was strongly confounded by BAA, which emerged as a strong predictor of GA progression regardless of the analytical approach used. In fact, analysis of mediation suggested that 85% of the effect of FAF patterns on GA growth was mediated through BAA. We cannot rule out residual confounding as responsible for the remaining effect of FAF patterns on GA progression.

c. The foveola of patients with apparent (qualitative) foveal swelling secondary to foveal sparing in GA was not swollen. Nonetheless, there were statistically significant changes in thickness in specific layers: the photoreceptor outer segment layer was thinned, while, in a *post-hoc* finding, HFL was thickened.

d. A new, simplified classification of FAF patterns in GA was attempted. However, the results based on LCA suggested that FAF patterns are closely related to the area of atrophy. Therefore, they do not seem to represent genuine phenotypes,

but rather different stages in the continuum of disease enlargement. Based on these results, a new classification based on a qualitative description of increased FAF was considered not appropriate.

e. We did not find changes in a given patient from one “pattern” (class, in LCA nomenclature) to another in the time period evaluated (median follow-up 19 months, range 12 to 32). However, given the slow evolution of the disease, it is foreseeable that the study period may have been too short to study possible transitions.

f. Intraobserver agreement in the evaluation of FAF patterns was very good, while interobserver agreement was moderate at best.

Therefore, while many of our *a priori* hypotheses were confirmed, others were not. The most surprising finding was that FAF patterns in GA, which were regarded as disease phenotypes, were in fact a consequence of atrophy enlargement. This result indicates that GA may be a more homogeneous disease than previously thought.

We assumed that high FAF (classified afterwards into FAF patterns) is a biomarker of high lipofuscin content. The results from LCA coupled with a small independent effect of FAF patterns on GA progression, the strong confounding effect of BAA on their relationship with GA growth and the lack of independent association in other major clinical studies suggest that lipofuscin plays a moderate role at best on GA pathogenesis. These results call into question the effectiveness of VCM therapy in GA, whose

objective is to limit the build-up of lipofuscin bisretinoids within the RPE.

This study also implicitly stressed the importance of replication of research findings. Our reappraisal of FAF patterns and foveal swelling results contradicted previous reports. Although study design and various types of biases can induce an unduly influence on research findings, we believe that the use of small sample sizes is a major contributor to fluctuating results. To minimize this issue, efforts in developing multicenter studies or open access to full databases that allow the conduct of meta-analysis should be given a marked priority.

Future directions

In this this section we make suggestions spanning different disciplines with the aim to provide some ideas for future research that may contribute to advance our knowledge on GA.

a. Clinical research

A better characterization of the dynamics of growth of GA enlargement in its lifespan would be welcome. The growth of GA has been assumed to be linear (40,53,64) but, on the other hand, it has also been found to be associated with BAA in the current and other studies (53,122,123). Both statements are simultaneously untenable, since the second implies faster growth in larger lesions. GA growth in a given individual may probably be better described as “S-shaped”, that is, an initial exponential phase, followed by a linear growth that progressively flattens for larger areas of atrophy, becoming logarithmic. The evaluation of this hypothesis would require regular visits of many patients over a long period of time, which may be difficult to achieve in clinical practice. This goal may require the involvement of population-based studies.

A related issue is the metric used to measure GA. The most commonly used unit, mm^2/year, is easy to understand, but may be inappropriate for some purposes. For example, the square root transformation has been used to remove the dependence of growth on BAA (54). The percentage of growth relative to previous visits and the difference in growth between visits are other metrics whose utility remains to be established.

If these transformations achieve a specific shape in the growth-time relationship (linear, exponential, etc.), the appropriate modeling strategies may be applied, yielding more realistic results. Combined with a better description of the enlargement dynamics of GA, an accurate prognosis will be provided to individual patients and more consistent results would be obtained through improved study design and analysis. For example, if the natural history study using a specific metric turned out to describe an “S-shaped” curve, patients in a small clinical trial may need to be randomized with a stratified approach on BAA (using, for instance, minimization or blocked-randomization) to guarantee that study arms are balanced in terms of baseline GA size to reduce confounding. Crude and BAA-adjusted analysis of results may further increase their validity.

We did not observe actual transitions between FAF patterns (classes) over our study period, but we have seen examples of this in some of our patients (**Figure 11**). A study of patients with incident GA followed over a long period of time may contribute to solve this question. The study may be retrospective, but the evaluation of FAF patterns will need to be masked between visits.

Indeed, some FAF patterns seem true phenotypes. The “trickling” pattern has been consistently associated with greater rates of growth in different studies and it is morphologically different from other patterns on SD OCT and FAF (257). Therefore, the statement that GA is one single disease is not entirely satisfactory either. Given that the end-stage of many macular disorders is the development of atrophy, there may be a percentage of patients diagnosed as GA secondary to AMD who may actually primarily suffer from other entities. This may actually be the case in some

forms of small, central GA, which may have progressed from adult-onset foveomacular vitelliform dystrophy, subfoveal pigment epithelial detachments, late Stargardt or other conditions, even in the presence of incidental drusen. There is a need to accurately define if GA is one or many diseases. If different phenotypes were found using FAF, SD OCT or any other imaging method, their definition should be standardized to allow undertaking prognostic, therapeutic or genetic association studies.

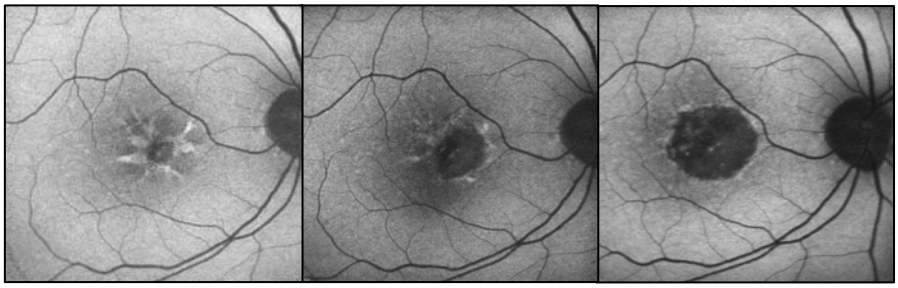


Figure 11. Fundus autofluorescence images of a patient with incident geographic atrophy after the collapse of a drusenoid pigment epithelial detachment taken, from left to right, on November 2009, May 2011 and June 2013. The corresponding fundus autofluorescence patterns could be defined as “patchy” (left), “reticular” (middle) and “banded” or “focal” (right) respectively, suggesting that, as atrophy enlarges, the distribution of high autofluorescence also changes.

There is also an emerging interest to evaluate the role of anti-VEGF therapy, as used in patients with wet AMD, on GA growth. However, it is difficult to evaluate GA size through retinal fluid, blood or fibrosis (120). The comparison of atrophy growth in

patients with mixed AMD and pure GA may have implications for management of patients with neovascular AMD and may also benefit patients with GA through improved knowledge of its pathogenesis.

b. Basic research

The characterization of lipofuscin composition is a hot topic in this area. The emerging field of omics will likely make important contributions in this regard. To support the role of lipofuscin in GA pathogenesis, it would be necessary to have histopathological evidence of an increase of lipofuscin content within individual RPE cells in GA patients as compared with healthy, age-matched controls. Also, it would be interesting to provide a sound explanation to support the presumed fast lipofuscin uptake by RPE cells at the junction.

A2E has been blamed for many of the toxic effects of lipofuscin *in vitro* and in animal models (84,86,227), but its peripheral location in humans (81) would make of it an unlikely candidate for triggering a disease of the posterior pole unless photodegradation decreases its macular accumulation (82). The search for the missing fluorophore becomes then an important issue.

There is an as yet unexplored possibility, that lipofuscin is truly associated with GA only under certain conditions. That is, lipofuscin induces GA growth only after it interacts with specific compounds, such as lipids in Bruch's membrane (82) or under excessive oxidative stress caused, for example, by a deficient concentration

of macular pigments. This hypothesis clearly poses great challenges for basic and clinical research alike. The list of potential effect modifiers is large and would require subgroup analysis in clinical studies, with the potential for false positives/negatives if hypotheses are not stated in advance, if effect modifiers are not correctly identified and measured, and if adequate sample sizes are not used. However, this possibility should not be dismissed (82).

A final word on safety. Short wavelengths have the potential to damage RPE cells, and the methods used for clinical FAF imaging have a peak emission in the blue range. Although FAF is considered to be safe, some concerns have been raised even at current exposure levels (258,259). Patients with prevalent disease may have an increased susceptibility to retinal damage and may require frequent exams in a short period of time, especially if they are included in clinical trials. In addition, the Spectralis HRA+OCT® uses an eye-tracking system, and therefore the same retinal area is exposed during all the exam, increasing the potential for phototoxicity. Standards for the safe use of FAF in susceptible individuals should be clarified, but until then, the indications for repeated FAF testing should consider the precautionary principle.

c. Imaging

Understanding FAF dynamics in health and disease and differentiating lipofuscin in the RPE from photoreceptor-induced increased autofluorescence is of paramount importance for this and other disorders. FAF has been quantified by noninvasive

spectrofluorometry, but the ability to use the cSLO or the fundus camera to allow the clinical quantification of autofluorescence would be a major step forward towards achieving this goal. Promising results have been published by the group of Delori et al (63,260).

The development of software capable of measuring the perimeter of atrophy on FAF, SD OCT or FP would be required to validate the hypothesis that cell-to-cell contact may be important in GA growth.

d. Miscellaneous

As discussed previously, sample size is a major determinant of results in clinical research (213). Although we have not been able to find any study addressing this particular issue, it seems that sample sizes in the field of Ophthalmology are particularly small. An informal review of 4 of the major journals in clinical ophthalmology corresponding to the last number of 2013 revealed that the median number of patients in the journal Ophthalmology was 176, 84 in Investigative Ophthalmology and Vision Science, 63 in American Journal of Ophthalmology and 60 in Archives of Ophthalmology. The corresponding numbers for general medicine journals were 227 in New England Journal of Medicine, 130 in JAMA, 5164 in BMJ and 514 in The Lancet. This does not intend to be a serious study to represent actual numbers, but it reflects the main point: there is a need to find ways to increase sample size.

The most obvious solution is to establish collaborations with other centers with similar interests, resources and time devoted to clinical

research. A paradigmatic initiative towards the implementation of multicenter studies is the Diabetic Retinopathy Research Network (available at www.drcr.net, accessed on March 1st, 2014), a collaborative effort dedicated to facilitate research on this vascular disease which has gathered 109 sites and 320 investigators in United States and Canada since 2002.

The standardized collection of data in participant sites could ideally be defined at the study design stage to facilitate the conduct of meta-analysis (261,262). A high efficiency is a positive feature of this approach, despite the known limitations of meta-analysis, such as publication bias. Unfortunately, natural history/prognostic studies are difficult to identify in database searches; the development of guidelines to ease study retrieval beyond STROBE (Strengthening the reporting of observational studies in epidemiology) appears necessary. Another major step forward would be the availability of full databases used to analyze primary studies. Journals have the capabilities to do so through online content and they should be access-free, at least for studies conducted using public funds.

There are other ways to increase power: reducing the number of variables in multivariable analysis or summarizing the information of different variables in a single one. Unfortunately, in most instances this last approach is not so simple, but strategies such as principal component analysis (PCA) can be used. PCA converts a group of observations of possibly correlated covariates into a set of values grouped in variables that are not linearly correlated (263). The resulting component scores (new variables from PCA) summarize all the information contained in the values originally

used, with (usually) a lower number of component scores than original variables. In the GAIN study, we planned the use of PCA as a secondary analysis in case of negative findings to get a summary of the main information contained in the variables included.

Ophthalmology has been permeable to outputs from other fields with success: the story of Oncology and VEGF therapy is an excellent example. The same reasoning can be applied to Genetic epidemiology and statistical methods.

The introduction of modern platforms for DNA analysis produced a vast array of data for Genetic epidemiology that made conventional statistical strategies prone to false positive findings (264). Through the establishment of consortia (HuGENet, Wellcome trust case-control consortium, etc), more stringent criteria to define statistical associations, full availability of analysis and standardized, preplanned study designs to favor replication, the field as a whole has benefited and has markedly reduced spurious findings. Although the precise reasons differ (massive data availability in Genetic epidemiology, use of small sample sizes in Ophthalmology), these approaches might be positive also for the study of eye disorders. The importation of methods from Statistics (multiple imputation, LCA, PCA) or models from Neurology or transmissible diseases, that explain the rational for cell-to-cell contact spread of disease, may be applied to Ophthalmology to provide a fresh and, hopefully, fruitful look at GA.

Finally, the relevance of replication cannot be overemphasized. There is a need to find ways to incentivate it. This will probably require the implication of several actors, such as researchers,

universities, funding agencies, peer-reviewers and editors. Otherwise, false positive results will keep plaguing the literature and will remain a recurrent concern.

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