Title page**Increased fundus autofluorescence and progression of geographic atrophy secondary to age-related macular degeneration. The GAIN study.***Authors:*Marc Biarnés^{1, 2}Luis Arias³Jordi Alonso^{2, 4, 5}Míriam Garcia¹Míriam Hijano¹Anabel Rodríguez¹Anna Serrano¹Josep Badal^{1, 6}Hussein Muhtaseb¹Paula Verdaguer¹Jordi Monés^{1, 7}

¹ *Institut de la màcula i de la retina (Centro Médico Teknon). C/ Vilana 12, Office 90 (08022 - Barcelona, Spain)*

² *Universitat Pompeu Fabra (UPF). Plaça de la Mercé, 10-12 (08002 - Barcelona, Spain)*

³ *Hospital Universitari de Bellvitge. C/ Feixa Llarga s/n (08907 - L'Hospitalet de Llobregat, Barcelona, Spain)*

⁴ *Health Services Research Unit, IMIM (Institut Hospital del Mar d'Investigacions Mèdiques). C/ Doctor Aiguader, 88, Edifici PRBB (08003 - Barcelona, Spain)*

⁵ *CIBER Epidemiología y Salud Pública (CIBERESP), Spain*

⁶ *Hospital Moisès Broggi. C/ Jacint Verdaguer 90 (08970 – Sant Joan Despí, Barcelona, Spain)*

⁷ *Barcelona Macula Foundation. C/ Horaci 41 (08022 - Barcelona, Spain)*

Corresponding author:

Marc Biarnés

Address: C/ Vilana 12, Office 90 (*Institut de la màcula i de la retina; Centro Médico Teknon*)

Telephone: +34.935.950.155

Fax: +34.935.950.345

e-mail: biarnes@oo.upc.edu

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Supplemental Material available at AJO.com

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Text

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 growth² 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 aging and, possibly, 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, such as N-retinylidene-N-retinylethanolamine (A2E). Some compounds of lipofuscin are autofluorescent, which contributes to identify its topographical distribution *in vivo* using fundus autofluorescence (FAF).⁵

In 2001, Holz et al⁶ 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),⁷ 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 pointed towards a causal relationship between lipofuscin accumulation and GA growth, supporting the development of visual cycle modulators, 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^{6,8–10} suggest that it has deleterious effects on RPE that may lead to cell death and GA progression, while other clinical^{11,12} and basic research findings^{13,14} do not support this hypothesis. Indeed, a visual cycle modulator did not meet the primary efficacy endpoint in a phase II clinical trial.¹⁵

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

Material and methods

Study design

The Characterization of geographic atrophy progression in patients with age-related macular degeneration (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 Ethics Committee of the Centro Médico Teknon 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 35° 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 mm²). Both eyes of each patient were eligible for the study. Eyes were excluded in the following situations: RPE atrophy secondary to other causes (macular dystrophy, high myopia, etc.); a history of wet AMD or any other macular disease thought to interfere with interpretation of FAF images in the study eye; contact between GA and peripapillary atrophy precluding the measurement of the lesion; lesion borders extending beyond the 30° field used for FAF imaging; a history of laser in the macula, intravitreal injection or intraocular surgery (aside from phacoemulsification) in the study eye; or poor image quality precluding 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 ($\lambda=480$ nm, approximate emission 500-700 nm) was acquired with Spectralis HRA+OCT[®] (Heidelberg Engineering, Heidelberg, Germany). High resolution (1536 x 1536 pixels), 30° x 30° 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.

All areas of atrophy in each image were 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.

Main outcome variable

The main outcome was growth of GA (mm²/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.

Main predictor and other independent variables

The predictor of primary interest in this study was FAF pattern. As originally described in the FAM study,⁷ 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. For analytic purposes, more simple classifications described below were used.

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 (**Figure 1**).

For multivariable analysis, the classification was further simplified into just 2 categories: low and high FAF. The low FAF category includes 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, 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 as **Supplemental Table 1**; Supplemental Material available at **AJO.com**).

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 equations¹⁶ 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 tests for comparison of continuous variables between groups, Spearman correlation coefficient (rho) when both variables were continuous and Fisher exact test for categorical variables. A stratified analysis of baseline area of atrophy on FAF patterns in relation to GA growth was conducted to inspect interactions.

A multivariable analysis using a hierarchical mixed-effects regression model was fitted to evaluate the independent contribution of each variable on GA progression. The model accounted for the increased correlation between the two eyes of a given patient. We also determined the relative contribution of FAF patterns and baseline area of atrophy on GA growth under the current hypotheses using mediation analysis,¹⁷ with hierarchical mixed-effects regression models. Finally, Bland-Altman 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.¹⁸

Results

Figure 2 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 familial 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 main characteristics of patients included in the study are shown in **Table 1** (a more detailed description is provided in **Supplemental Table 2**; Supplemental Material available at **AJO.com**).

Median follow-up time was 18 months (range, 6 to 42). The median (mean) growth was 1.62 (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 $\leq 20/200$ 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 3**).

In other bivariate analyses, GA growth was also correlated with baseline area of atrophy ($\rho=0.38$, $p<0.0001$), time of follow-up ($\rho=-0.19$, $p=0.05$) and age ($\rho=0.23$, $p=0.02$). 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 ($p\geq 0.08$).

The association of FAF patterns 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 baseline area of atrophy ($p=0.0001$). Patterns characterized by larger areas with hyperautofluorescence (banded and diffuse) had larger baseline area of atrophy 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 baseline area of atrophy, while the opposite was true for patterns with high FAF (banded or diffuse; **Figure 4**).

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 ($p\geq 0.06$). The complete set of bivariate analyses is provided in **Supplemental Table 3** (for the relationship with the main outcome, GA growth) and **Supplemental Table 4** (for the relationship between FAF patterns and other independent variables; Supplemental Material available at **AJO.com**).

Stratified analysis

Within FAF patterns, median GA growth increased with increasing baseline area of atrophy size for all pattern groups (**Figure 5**). Eyes with the pattern none had the smallest progression, while the others experienced a rather similar rate of growth.

Multivariable analysis

The results of multivariable analysis are shown on **Table 3**. Baseline area of atrophy ($\beta=1.02$), FAF patterns ($\beta=0.54$) and follow-up time ($\beta=1.62$) were independently associated with GA growth. On the other hand age, pseudophakia, extrafoveal location of atrophy and CRT, which were associated with GA growth on bivariate analyses, were no longer significant.

Figure 6 shows the hypothetical relationship between FAF patterns, baseline area of atrophy and GA growth that served as the basis for analysis of mediation.^{17,19} The purpose of mediation analysis was to understand the relationship between FAF patterns and GA growth considering baseline area of atrophy, and to determine the relative contribution of each variable to the enlargement of GA. The analysis consists of two parts. First, all associations among the variables must be statistically significant (**Table 4**): Step 1) FAF patterns were associated with GA growth without inclusion of baseline area of atrophy in the model ($p=0.002$); Step 2) Baseline area of atrophy was associated with GA growth independently of FAF patterns ($p<0.001$); and Step 3) FAF patterns were associated with baseline area of atrophy ($p<0.001$). This suggests that the relationship shown in **Figure 6** is a possible scenario. Then, the percent change in the coefficient of FAF patterns when baseline area of atrophy was included in the model was evaluated; it decreased from 3.30 (in Step 1 on mediation analysis, representing its total effect) to 0.54 (in the multivariable analysis in **Table 3**, representing its direct effect only). This implies strong confounding of baseline area of atrophy on the relationship between FAF patterns and GA growth. A large percentage of the effect of FAF patterns on GA growth (83.6%, $[(3.30-0.54)/3.30]*100$) was presumably mediated (caused) through baseline area of 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-Altman plot (see **Supplemental Figure**; Supplemental Material available at **AJO.com**).

Discussion

We found that baseline area of atrophy, FAF patterns, and time of follow-up are all independently associated with GA growth. Our study suggests that it is the enlargement of atrophy which induces changes in FAF and that baseline area of atrophy is the main known driver of GA growth. This is in contrast with other current hypothesis, in which high FAF levels are caused by elevated intracellular lipofuscin, which in turn induce RPE cell death and GA progression.

There is a very highly significant relationship between FAF patterns and area of atrophy ($p=0.0001$) and it is very difficult to disentangle which is the cause and which is the consequence. However, our results offer some hints. **Table 2** and **Figure 4** 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 the 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”)²⁰ rather than true phenotypes. In fact, transitions between FAF subtypes have been reported in Stargardt disease, a phenotypically similar disease.²¹

Baseline area of atrophy has been previously found to be associated with GA growth.^{22,23} In fact, baseline area of atrophy seems more important than FAF patterns in disease progression. Stratified analysis showed an increasing rate of median growth by increasing quartiles of baseline area of atrophy in each FAF pattern, and a similar distribution of growth when comparing focal, banded and diffuse patterns. It is the different distribution of baseline area of atrophy within FAF patterns which seems to account for their ability to predict GA growth. Also, even if we hypothesize that FAF patterns determine baseline area of atrophy and GA growth (**Figure 6**), we found that most of the effect of FAF patterns on disease progression was mediated through baseline area of atrophy. These findings strongly suggest that baseline area of atrophy, not lipofuscin (through FAF patterns) is the main known factor causing GA growth. The impact of the square root transformation of baseline area of atrophy on its relationship with GA growth²⁴ will be evaluated in another study.

The FAM study was the first to report an association between FAF patterns and GA growth based on bivariate analyses,⁷ but not on multivariable analysis.¹¹ The authors suggested that small sample size was the likely cause for the latter, 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.

Aside from baseline area of atrophy, other morphological features of GA associated with its rate of growth are multifocal^{24,25} and irregular-shaped²⁶ lesions. Large, multifocal and irregular areas of atrophy have in common that their perimeter is larger than that of corresponding small, unifocal or rounded lesions. The perimeter of a lesion represents, amongst others, the number of atrophic RPE cells in direct contact with healthy cells. Is it possible that the enlargement of GA involves the transmission of toxic compounds from diseased

to healthy cells? This mechanism has been recently shown to occur in other neurodegenerative diseases.²⁷ If this were the case, then the deleterious pathogen should be capable of inducing RPE cell death and also it should demonstrate the ability of transmission through cell-to-cell contact.

Lipofuscin compounds (notably, A2E) have shown a wide range of deleterious effects on RPE physiology on in vitro and animal studies.^{9,28–30} However, these findings 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,^{13,31} suggesting that A2E cannot be responsible for increased FAF at the macula. On the other hand, Rudolf et al¹⁴ 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). This mechanism offers an alternative explanation for increased FAF.

We hypothesize that as atrophy enlarges in a centrifugal manner,³² RPE cells at the borders of atrophy become disorganized and vertically aligned,³³ inducing high FAF levels.¹⁴ On the other hand, the normal distribution of FAF increases with retinal eccentricity, reaching a maximum at 3 mm from the fovea.³⁴ With growth, large lesions eventually reach this area, where intraretinal migration of RPE cells with physiologically higher autofluorescence would induce widespread high FAF, clinically identified as diffuse or banded patterns.

Nonetheless, lipofuscin may play a role on GA pathogenesis. The presence of any degree of increased FAF (patterns focal, banded or diffuse) was associated with increased and rather similar median growth of atrophy as compared with no FAF (**Figure 5**). 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, visual cycle modulators may have a small but relevant effect on GA growth.

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.³⁵ Residual confounding due to unmeasured or poorly measured factors is another potential concern in all observational studies, but it seems unlikely that a biased measure of baseline area of atrophy or another, as yet unidentified factor, may significantly dilute the role of baseline area of atrophy on GA growth. However, a modification of the relative contribution of the different variables may occur as progress in this area is made. Also, we assumed that slight deviations from the normal distribution of GA growth could be accepted when using repeated measures mixed models, as previously shown.¹¹ Finally, GAIN participants may not be representative of all GA patients, but representativeness is not required to address causality.³⁶

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.

Many questions remain to be answered. The search for the missing fluorophore appears as a priority, 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.

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. Approaches that consider the role of baseline area of atrophy (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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Figure captions

Figure 1. Examples of fundus autofluorescence patterns. (Top left) None, (Top center) Focal, (Top right) Patchy. (Bottom left) Banded, (Bottom center) Diffuse, (Bottom right) Undetermined. No patient with the patchy pattern was found in the study. This classification is based on the “Fundus autofluorescence in age-related macular degeneration” study.⁷

Figure 2. Flow diagram of patient selection in the Characterization of geographic atrophy progression in patients with age-related macular degeneration 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.

Figure 3. Median growth of geographic atrophy according to each fundus autofluorescence pattern in the Characterization of geographic atrophy progression in patients with age-related macular degeneration study. The classification using 5 categories was used ($p=0.0013$, Kruskal-Wallis test).

Figure 4. Number of eyes presenting each fundus autofluorescence pattern by quartiles of baseline area of atrophy in the Characterization of geographic atrophy progression in patients with age-related macular degeneration study. B: Banded; D: Diffuse; F: Focal; FAF: Fundus autofluorescence; N: None.

Figure 5. Bar chart showing stratified analysis. Median growth of geographic atrophy by quartiles of baseline area of atrophy within levels of each fundus autofluorescence pattern as classified in the Characterization of geographic atrophy progression in patients with age-related macular degeneration study.

Figure 6. Current hypothesized relationship between FAF patterns, baseline area of atrophy and GA growth. FAF patterns are presumably causally related to GA growth and also determine baseline area of atrophy when the patient is first seen (patients with FAF patterns characterized by faster progression will show, on average, larger baseline areas of atrophy when first diagnosed than patients with FAF patterns with slower progression). In addition, baseline area of atrophy may have a direct effect on GA growth not mediated by FAF patterns. The relationships depicted here are used for mediation analysis. FAF: Fundus autofluorescence; GA: geographic atrophy.

Table 1. Main baseline characteristics of patients meeting eligibility criteria in the Characterization of geographic atrophy progression in patients with age-related macular degeneration study

<i>Characteristic</i>	<i>Value</i>
Age (years)	80 (74.5 to 84.5)
Female, %	66.7
Follow-up (months)	18 (10 to 31)
Baseline area of atrophy (mm ²)	6.85 (3.14 to 11.88)
Fundus autofluorescence pattern, %:	
None	4.7
Focal	27.1
Banded	25.6
Fine Granular	17.8
Branching	14.0
Trickling	3.1
Reticular	3.1
Fine granular with peripheral punctate spots	1.6
Undetermined	3.1
Best corrected visual acuity (letters)	71 (59 to 79)
Pseudophakia, %	48.8
Antioxidant use, %	72.9
Current smokers, %	9.4
Configuration of atrophy, %:	
Unifocal foveal	22.5
Multifocal foveal	22.5
Unifocal extrafoveal	3.9
Multifocal extrafoveal	20.2
C shaped	4.7
C shaped multifocal	17.1
Annular	9.3
Quantitative variables (age, follow-up, baseline area of atrophy and best corrected visual acuity) are summarized by median (interquartile range). All	

other variables are expressed as percentage.

Table 2. Baseline area of atrophy in each pattern of fundus autofluorescence in the Characterization of geographic atrophy progression in patients with age-related macular degeneration study

<i>Fundus autofluorescence patterns (n)</i>	<i>Baseline area of atrophy in mm² Median (Mean)</i>	<i>Interquartile range</i>	<i>p-value</i>
None (6)	2.67 (2.67)	1.84 – 3.74	0.0001
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	

Table 3. Association of geographic atrophy growth (dependent variable) with clinical characteristics (independent variables) in the multivariable random-effects model ^a of the Characterization of geographic atrophy progression in patients with age-related macular degeneration study

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

BAA: Baseline area of atrophy; CI: Confidence interval; FAF: Fundus autofluorescence; SE: Standard error.

^a Model includes: age, body-mass index, best-corrected visual acuity and central retinal thickness as random effects (not shown).

Table 4. Steps involved in mediation analysis ^a in the Characterization of geographic atrophy progression in patients with age-related macular degeneration study

<i>Step</i>	<i>Variables</i>	<i>Coefficient (β)</i>	<i>SE</i>	<i>95% CI</i>	<i>p-value</i>
1	<i>FAF patterns</i>	3.30	1.06	1.22 to 5.39	0.002
2	<i>BAA</i>	1.02	0.01	1.00 to 1.05	<0.001
3	<i>FAF patterns</i>	3.86	0.55	2.79 to 4.94	<0.001

BAA: Baseline area of atrophy; CI: Confidence interval; FAF: Fundus autofluorescence; SE: Standard error.

^a Models in each step are adjusted by: time of follow-up, familiar history, foveal atrophy and age.

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Biosketch Marc Biarnés

Marc Biarnés, OD, is master in Public Health and PhD in Biomedicine from Universitat Pompeu Fabra in Barcelona, Spain. He works at the Institut de la màcula i de la retina as clinical epidemiologist. His main interests include macular diseases, retinal imaging and methods in epidemiology.

Biosketch Jordi Monés

Dr. Jordi Monés MD, PhD, is an ophthalmologist, macula and vitreoretinal specialist. He is Director of the Institut de la Màcula i de la Retina, Director of the Barcelona Macula Foundation and researcher of the Networking Research Centre of Bioengineering, Biomaterials and Nanomedicine (Spain). His fields of interest are new therapies for degenerative disorders such as age-related macular degeneration, retinitis pigmentosa and Stargardt disease, and he currently conducts phases I-III clinical trials.

Supplemental material Table 1. Definition of variables measured in the Characterization of geographic atrophy progression in patients with age-related macular degeneration study.

<i>Variable</i>	<i>Definition</i>
Growth of geographic atrophy (dependent variable)	Change in area of GA from one visit to the next as measured with FAF. Time of follow-up between visits (in years) was used as an independent variable. A semiautomatic software included in the Spectralis HRA+OCT [®] was used to make the measures, the Region Finder [®] , version 2.4.3.0 (Heidelberg Engineering; Heidelberg, Germany).
Sex	Male/female
Age	Determined at each visit (years)
Race	White, Afro-American, Asian, Hispanic or other
High blood pressure	Determined at each visit (no/yes). High blood pressure was considered present when the patient reported being hypertensive upon request or using antihypertensive medication
Smoking status	Determined at each visit (never/ever). We defined current smokers as those who reported smoking one or more cigarettes, cigars, pipes or other forms of tobacco at least once a day in the last 12 months. We considered former smokers as those who had smoked (according to the previous definition) for at least 12 months and quit the habit, at least, 12 months before the visit (or began smoking less than 12 months before the study). Non-smokers were defined as those who had never smoked or who had smoked very infrequently during a short period of time
Antioxidant intake (supplements)	Determined at each visit, no/yes. We considered as antioxidant consumers

	those patients who had been taking regularly any commercially available ocular antioxidant supplementation for, at least, 2 years before the beginning of the study. This cutoff was chosen because this was the minimum time seen in the AREDS study (1) to show a beneficial effect of antioxidants on incidence of late age-related macular degeneration (see Supplemental material table 3)
Baseline body/mass index	Kg/m ² . Height was measured and weight was reported by the patient
Familiar history of AMD	As reported by the patient and only in first degree siblings (no/yes)
Diagnosis of the fellow eye	It is the diagnosis of the retinal condition of the non-study eye according to the ophthalmic exam. Determined at each visit
Manifest refraction and best corrected visual acuity	Measured by certified optometrists using an Early Treatment Diabetic Retinopathy Study chart. Determined at each visit
Lens status	Phakic/pseudophakic. Determined at each visit
Eye	Right/left
Central retinal thickness	Thickness of the retina (from internal limiting membrane to the outer limit of the retinal pigment epithelium). It was measured in a circular area of 1 mm of diameter centered on the foveola. Manual reposition of the 3D exam (formed by 19 horizontal B-scans spaced 240 µm on a 20°x15° field of view, with an automatic real time of 10, in high resolution mode, coupled with 30° FAF imaging of the fundus, - or infrared, in case of excessive light sensitivity-) and correction of inaccurate segmented borders was done for every scan. The instrument used was the Spectralis HRA+OCT [®] (Heidelberg Engineering; Heidelberg,

	Germany). Determined at each visit
FAF pattern	According to the description of 10 FAF phenotypes made by Holz et al (2). From this detailed classification, we assigned a General classification (with 6 categories) and a Simplified one (with 3 categories). For modeling purposes, we used this Simplified classification, which was more reproducible. (3) Since there were only 4 cases in one of the categories (Undetermined + Patchy), those patients were excluded in all multivariable analysis. Therefore, we compared the categories low FAF (None + Focal) vs high FAF (Banded + Diffuse). Two observers independently evaluated the images; in case of disagreement, a senior retinal specialist arbitrated. A consensus was reached in all cases
Description of configuration of atrophy	Foveolar involvement (unifocal or multifocal central) or without foveolar involvement (unifocal or multifocal extrafoveal, C-shaped unifocal or multifocal, annular). Determined at each visit
Patient referred from another physician	No/yes
AMD: Age-related macular degeneration; AREDS: Age-related eye disease study; FAF: Fundus autofluorescence; GA: Geographic atrophy.	

Supplemental material Table 2. Complete list of baseline characteristics of patients meeting eligibility criteria in the Characterization of geographic atrophy progression in patients with age-related macular degeneration study

<i>Characteristic</i>	<i>Value</i>
Age (years)	80 (74.5 to 84.5)
Female, %	66.7
White, %	99.0
Baseline area of atrophy (mm ²)	6.85 (3.14 to 11.88)
Follow-up (months)	18 (10 to 31)
Bilateral geographic atrophy, %	34.4
Right eye, %	45.7
Fundus autofluorescence pattern, %:	
None	4.7
Focal	27.1
Banded	25.6
Fine Granular	17.8
Branching	14.0
Trickling	3.1
Reticular	3.1
Fine granular with peripheral punctate spots	1.6
Undetermined	3.1
Best corrected visual acuity (letters)	71 (59 to 79)
Pseudophakia, %	48.8
Central retinal thickness (μm)	219 (188 to 255)
Hypertension, %	59.4
Current smokers, %	9.4
Body mass index (kg/m ²)	26.7 (24.4 to 29.4)
Familiar history of age-related macular degeneration, %	27.1
Antioxidant use, %	72.9

Any concomitant disease, %	61.2
Configuration of atrophy, %:	
Unifocal foveal	22.5
Multifocal foveal	22.5
Unifocal extrafoveal	3.9
Multifocal extrafoveal	20.2
C shaped	4.7
C shaped multifocal	17.1
Annular	9.3
Referred, %	32.3
Quantitative variables (age, baseline area of atrophy, follow-up, best corrected visual acuity, central retinal thickness and body mass index) are summarized by median (interquartile range). All other variables are expressed as percentage.	

Supplemental material Table 3. Bivariate analyses between each independent variable and geographic atrophy growth, the main outcome.

<i>Independent variables</i>	<i>p-value^a</i>
Age	0.02
Sex	0.86
Baseline area of atrophy	<0.0001
Fundus autofluorescence patterns, Specific	0.0031
Fundus autofluorescence patterns, General	0.0013
Fundus autofluorescence patterns, Simplified	0.0007
Hypertension	0.86
Body mass index	0.57
Smoking	0.34
Familiar history of age-related macular degeneration	0.71
Antioxidant use	0.61
Best-corrected visual acuity	0.21
Lens	0.03
Central retinal thickness	0.08
Location of atrophy (extrafoveal)	0.002
Concomitant disease	0.08
Time of follow-up	0.05
^a Mann-Whitney or Kruskal-Wallis tests were used for comparison between groups and Spearman correlation coefficient for continuous independent variables.	

Supplemental material Table 4. Bivariate analyses between each independent variable and fundus autofluorescence patterns.	
<i>Independent variables</i>	<i>p-value^a</i>
Age	0.24
Sex	0.17
Baseline area of atrophy	0.0001
Hypertension	0.33
Body mass index	0.11
Smoking	0.20
Familiar history of age-related macular degeneration	0.05
Antioxidant use	0.21
Best-corrected visual acuity	0.36
Lens status	0.08
Central retinal thickness	0.13
Location of atrophy	0.03
Concomitant disease	0.13
Time of follow-up	0.06
^a Kruskal-Wallis test was used for comparison of continuous variables between groups and Fisher exact test for categorical variables.	

Supplemental material (caption page)

Supplemental material Figure. Intraobserver agreement in the measure of area of geographic atrophy using fundus autofluorescence. The cases represent a random sample of all eyes (approximately 20%). Mean difference in intraobserver agreement was -0.084 mm^2 , with 95% limits of agreement $(-1.124, 0.956)$, while 5/104 (4.81%) measurements fell outside the limits of agreement.