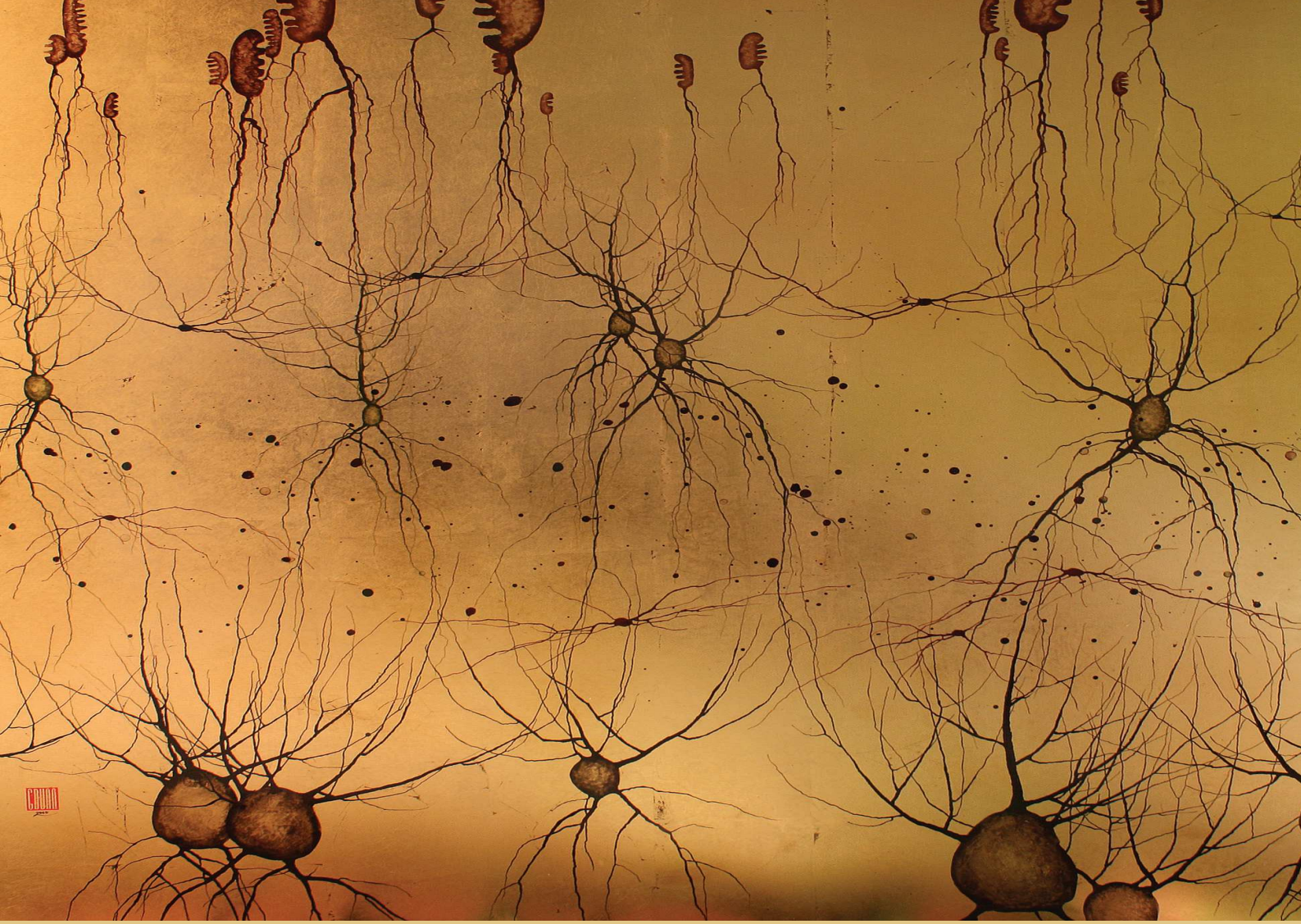

Sandrina Gonçalves Nunes

INVESTIGATOR-DRIVEN CLINICAL RESEARCH. IMAGING BIOMARKERS OF PROGRESSION IN DIABETIC RETINOPATHY AND AGE-RELATED MACULAR DEGENERATION. CONTRIBUTION FOR EVIDENCE-BASED CLINICAL EYE RESEARCH.

Doctoral Thesis in Health Sciences Branch Biomedical Sciences, supervised by Maria Conceição Lopes Lobo da Fonseca and Rufino Martins da Silva and presented in the Faculty of Medicine of the University of Coimbra

June 2014

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Universidade de Coimbra

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RESUMO

A Investigação Clínica da Iniciativa do Investigador (ICII) desempenha um papel importante na promoção do conhecimento científico tanto na área do diagnóstico como na área das abordagens terapêuticas, contribuindo como tal para a medicina baseada em evidências bem como para a investigação orientada para o doente. A ICII tem como objectivo a identificação de novos biomarcadores e/ou de novas metodologias que permitam a identificação precoce de doentes com maior risco, a melhoria das atuais abordagens diagnósticas e/ou terapêuticas, a melhoria das estratégias de tratamento e/ou acompanhamento dos doentes, bem como a melhoraria da qualidade, acessibilidade e relação custo-eficácia do sistema de saúde.

A investigação clínica em ciências da visão representa menos de 5% da investigação clínica realizada em todo o mundo. A Retinopatia Diabética (RD) e a Degenerescência Macular Relacionada com a Idade (DMI) representam cerca de 20% dessa investigação. Estas duas doenças da retina são a principal causa de perda da visão em todo o mundo tendo, como tal, um elevado impacto socioeconômico na população ativa. A detecção precoce da RD e da DMI, bem como a identificação precoce dos doentes que estão em risco de progredir, são portanto grandes desafios da investigação clínica na área das ciências da visão. A identificação e validação de biomarcadores baseados em imagem, capazes de identificar/prever os doentes com maior risco, são assim de grande importância.

Nesta tese são apresentados três estudos de ICII que permitiram a caracterização e/ou validação de biomarcadores baseados em imagem como estimadores de risco de progressão da RD e da DMI. O estudo em RD demonstra a existência de três fenótipos diferentes de progressão da RD (fenótipos A, B e C), caracterizados por dois biomarcadores retinianos de progressão da RD, quantificáveis usando técnicas não-invasivas de imagiologia: a presença de edema macular, com base em tomografia de coerência ótica (fenótipo B), e a atividade dos microaneurismas (MA), com base na fotografia do fundo ocular (fenótipo C). Os doentes classificados nos fenótipos B e C apresentam um maior risco de desenvolver Edema Macular Clinicamente Significativo (EMCS). O primeiro estudo de ICII em DMI mostra que, através de imagiologia multimodal, a presença de: áreas hiperfluorescentes em Angiografia de Indocianina Verde (Indocyanine Green Angiography – ICG) nas fases precoces e tardias; áreas hipofluorescentes em ICG nas fases precoces; áreas de derrame identificadas com Retinal Leakage Analyser (RLA); são fatores preditivos para a neovascularização da coróide. No segundo estudo de ICII a prevalência da DMI na região Centro de Portugal foi estimada com base em biomarcadores retinianos obtidos em fotografias do fundo ocular. Este segundo estudo decorreu em duas unidades de cuidados de saúde, o centro de

saúde de Mira e a unidade de saúde familiar da Lousã. Os resultados preliminares mostram uma prevalência da DMI tardia inferior ao que esta publicado, ou seja, cerca de 80% da população não tem, ou tem poucas alterações morfológicas (sem drusens ou com drusens < 63 µm de diâmetro), cerca de 19% tem DMI precoce e cerca de 1% tem DMI tardia.

Os estudos de ICII apresentados nesta tese demonstram a relevância da ICII para a investigação clínica orientada para o doente bem como a importância dos biomarcadores baseados em imagem para a investigação clínica na área das ciências da visão. Ao classificar os doentes com RD num dos três fenótipos de progressão da RD, podemos identificar precocemente os doentes com maior risco de desenvolver EMCS, bem como, otimizar o acompanhamento clínico desses doentes. A identificação de biomarcadores para a DMI é também um grande desafio. A identificação de lesões da retina e/ou da coróide associadas com um maior risco de conversão é de grande relevância para retardar a perda de visão nos doentes com DMI.

Com base nos estudos apresentados, novos estudos de ICII foram desenhados, e com o apoio de uma nova infraestrutura de apoio à ICII, o Centro de Coimbra de Coordenação de Investigação Clínica (4C), foram implementados estudos clínicos multicêntricos a nível europeu, para caracterizar as alterações que ocorrem na retina e/ou coróide nas fases precoces das doenças, e/ou para confirmar os resultados obtidos anteriormente em diferentes populações. A ICII é uma atividade complexa e demorada como tal, para poder promover a ICII foi criada em 2009 com o apoio do Centro Coordenador da rede europeia de centros de investigação clínica em ciências da visão (European Vision Institute Clinical Research network – EVICR. net), a infraestrutura de apoio aos investigadores 4C. A identificação e/ou validação de biomarcadores baseados em imagem na RD e na DMI irá contribuir para a identificação dos doentes em risco, a monitorização da progressão da doença, bem como a avaliação da eficácia de novas abordagens terapêuticas.

ABSTRACT

Investigator-Driven Clinical Research (IDCR) plays a major role, for the promotion of scientific knowledge on diagnostic and/or therapeutic approaches, and for the contribution to evidence-based medicine and to patient-oriented research. IDCR aims to identify new biomarkers, and/or new methodologies, that can allow for an early identification of patients at risk; improve the existing diagnostic and/or therapeutic approaches; improve therapies and patient-care strategies; and improve the quality, accessibility and cost-effectiveness of the health-care system.

Clinical eye research represents less than 5% of the clinical research performed worldwide. Diabetic Retinopathy (DR) and Age-Related Macular Degeneration (AMD) represent approximately 20% of the clinical eye research. These two retinal diseases are the major causes of visual impairment and have a high socioeconomic impact in the working population. The early detection of DR and AMD, and the early identification of the patients that are at risk of progression and sight threatening, are therefore major challenges in clinical eye research. The identification and validation of imaging biomarkers, able to predict patients at risk for vision impairment, are therefore of major importance in clinical eye research.

In this thesis three IDCR that characterized and/or validated imaging biomarkers as risk estimators of DR and AMD progression are presented. The IDCR on DR demonstrates the existence of 3 different phenotypes of DR progression (phenotypes A, B and C), characterized by two retinal biomarkers of DR progression/worsening, quantifiable using non-invasive imaging techniques (Optical Coherence Tomography – OCT, and Colour Fundus Photography – CFP): the presence of macular edema based on OCT (phenotype B); and the microaneurysms (MA) turnover based on CFP (phenotype C). Patients from phenotypes B and C show a higher risk for the development of Clinically Significant Macular Edema (CSME). The first IDCR on AMD shows, using multimodal imaging, that the presence of early and late Indocyanine Green Angiography (ICG) hyperfluorescent spots, early ICG hypofluorescent spots and leakage detected with Retinal Leakage Analyser (RLA), are predictive factors for the conversion to wet AMD (Choroidal Neovascularization – CNV). In the second IDCR the prevalence of AMD in the Central region of Portugal was estimated using retinal biomarkers assessed on CFP. This second IDCR was conducted in two healthcare units, Mira and Lousã. The preliminary results showed a lower prevalence for the late stage AMD, when compared to other studies, i.e., approximately 80% of the population had no or minimal morphological changes (no drusens or small drusens < 63 μm in diameter), approximately 19% had early AMD and approximately 1% had late AMD.

The IDCR presented in this thesis show the relevance of IDCR for patient-oriented research and the importance of imaging biomarkers for evidence-based clinical eye research. By classifying DR patients according to the different phenotypes of DR progression, patients showing a higher risk for CSME development can be identified earlier and followed more closely. The identification of biomarkers for AMD is also a challenging task, with a high impact for the management of AMD patients. The identification of specific retinal and/or choroidal lesions, that are associated with a higher risk of conversion, is of major importance to delay vision loss in these patients.

Based on these studies new IDCR were designed, and with the support of the newly created infrastructure for IDCR support, the Coimbra Coordinating Centre for Clinical Research (4C), multicentre IDCR were setup at the European level to characterize more precisely changes occurring in the retina and/or choroid in the early stages of the diseases, and/or to confirm the results obtained previously in different populations. IDCR is a complex and time-consuming activity, and therefore to promote IDCR a new infrastructure was created in 2009 with the collaboration of the Coordinating Centre of the European Vision Institute Clinical Research network (EVICR.net), to support investigators for the different activities involved in clinical research, the 4C. The identification and/or validation of DR and AMD imaging biomarkers will contribute for the identification of the patients at risk, the monitoring of the disease progression, and the assessment of the efficacy of new therapeutic approaches.

Abbreviations

AIBILI	Associação para Investigação Biomédica em Luz e Imagem (Association for Innovation and Biomedical Research on Light and Image)
AMD	Age-related Macular Degeneration
AREDS	Age-Related Eye Disease Study
ARS	Administração Regional de Saúde
ARVO	Association for Research in Vision and Ophthalmology
BCVA	Best-Corrected Visual Acuity
BRB	Blood-Retinal barrier
CA	Competent Authority
CEC	Centro de Ensaios Clínicos (Centre for Clinical Trials)
CEIC	Comissão de Ética para a Investigação Clínica
CES	Comissão de Ética para a Saúde
CFP	Colour Fundus Photography
CHUC	Centro Hospitalar e Universitário de Coimbra (Coimbra University Hospital)
CNPD	Comissão Nacional de Protecção de Dados
CNV	Choroidal Neovascularization
CONSORT	CONsolidated Standards of Reporting Trials
CORC	Coimbra Ophthalmology Reading Centre
CRA	Contract Research Associate
CRF	Case Report Form
CRIO	Centro de Responsabilidade Integrado de Oftalmologia (Integrated Responsibility Centre of Ophthalmology)
CRO	Contract Research Organization
CSME	Clinically Significant Macular Edema
CTA	Clinical Trial Application
C-TRACER	Champalimaud Translational Centre for Eye Research
DGS	Direção-Geral da Saúde
DM	Diabetes Mellitus
DME	Diabetic Macular Edema
DMI	Degenerescência Macular Relacionada com a Idade
DMP	Data Management Plan

DR	Diabetic Retinopathy
EC	European Commission
ECRIN	European Clinical Research Infrastructure Network
EEC	European Economic Community
EEIG	European Economic Interest Grouping
e.g.	*exempli gratia* (for example)
EMA	European Medicines Agency
EMCS	Edema Macular Clinicamente Significativo
EORTC	European Organisation for Research and Treatment of Cancer
ERIC	European Research Infrastructure Consortium
ESF	European Science Foundation
ESFRI	European Strategy Forum on research Infrastructures
et al.	*et alii* (and others)
ETDRS	Early Treatment Diabetic Retinopathy Study
EU	European Union
EUREC	European Network of Research Ethics Committees
EUROCONDOR	European Consortium for the Early Treatment of Diabetic Retinopathy
EVI	European Vision Institute
EVICR.net	European Vision Institute Clinical Research Network
E3	European Eye Epidemiology network
FA	Fluorescein Angiography
FAF	Fundus Autofluorescence
FCT	Fundação para a Ciência e Tecnologia (Foundation for Science and Technology)
FDA	Food and Drug Administration
FMUC	Faculdade de Medicina da Universidade de Coimbra
FPFV	First Patient First Visit
FPLV	First Patient Last Visit
FP7	7th Framework Programme
GA	Geographic Atrophy
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice
HbA_{1C}	Glycosylated Haemoglobin A1c

HDL	High-Density Lipoprotein
HRA	Heidelberg Retina Angiograph
IB	Investigator Brochure
IBILI	Instituto Biomédico de Investigação da Luz e Imagem (Institute for Biomedical Imaging and Life Sciences)
ICF	Informed Consent Form
ICG	Indocyanine Green Angiography
ICH	International Conference on Harmonization
ICH-GCP	International Conference on Harmonization – Good Clinical Practice
ICII	Investigação Clínica da Iniciativa do Investigador
ICNAS	Instituto de Ciências Nucleares Aplicadas à Saúde (Nuclear Sciences Applied to Health)
IDCR	Investigator-Driven Clinical Research
i.e.	*id est* (that is)
IEC	Independent Ethics Committee
IMP	Investigational Medicinal Product
INE	Instituto Nacional de Estatística
INFARMED	Autoridade Nacional do Medicamento e Produtos de Saúde, I.P.
IQR	Interquartile Range
IRB	Independent Review Board
LDL	Low-Density Lipoprotein
LPFV	Last Patient First Visit
LPLV	Last Patient Last Visit
MA	Microaneurysm
MMI	Multimodal Macular Imaging
MS	Member State
MWA	Medical World Association
NIH	National Institutes of Health
NPDR	Nonproliferative Diabetic Retinopathy
OCT	Optical Coherence Tomography
OR	Odds Ratio
PDR	Proliferative Diabetic Retinopathy
PDT	Photodynamic Therapy

PtCRIN	Portuguese Clinical Research Infrastructure Network
RA	Regulatory Authority
RCT	Randomized Clinical Trials
RD	Retinopatia Diabética
RLA	Retinal Leakage Analyser
RNEC	Registo Nacional de Estudos Clínicos
RPE	Retinal Pigment Epithelium
SAP	Statistical Analysis Plan
SCT	Society for Clinical Trials
SD	Standard Deviation
SmPC	Summary of Product Characteristics
SNP	Single Nucleotides Polymorphisms
SOP	Standard Operating Procedure
STRONG	European Consortium for the Study of a Topical Treatment of Neovascular Glaucoma
UNESCO	United Nations Educational, Scientific and Cultural Organization
USA	United States of America
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VHP	Voluntary Harmonized Procedure
VICT	Vision and Imaging Consortium for Translational Research
WHO	World Health Organization
4C	Centro de Coimbra de Coordenação de Investigação Clínica (Coimbra Coordinating Centre for Clinical Research)
95%CI	95% Confidence Interval

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1 INTRODUCTION

Clinical Research study the safety and/or efficacy of medicines, medical devices, diagnostic methods and/or new therapeutic approaches in humans. When promoted by individual researchers, scientists or physicians, universities, or other entities whose purpose is the scientific knowledge and the improvement of the current diagnostic or therapeutic approaches, the Clinical Research is referred to as Investigator-Driven Clinical Research (IDCR). IDCR promotes scientific knowledge and innovation in diagnostics and/or therapeutics independently of commercial interests, playing an important role in patient-oriented clinical research.

Clinical eye research represents less than 5% of the clinical research performed worldwide. Diabetic Retinopathy (DR) and Age-Related Macular Degeneration (AMD) represent approximately 20% of the clinical eye research. These two retinal diseases are the major causes of visual loss having a high socioeconomic impact in the working population.

The early detection of DR and AMD and the early identification of the patients that are at risk of progression and sight threatening are therefore major challenges in clinical eye research. In this context, imaging biomarkers, i.e., biomarkers detected and measured objectively on imaging methods, are clinically useful biomarkers that can be used for the early diagnosis and management of DR and AMD. Retinal biomarkers are therefore a target in clinical eye research, since they can assist in elucidating the mechanisms of the diseases, determining the risk of the disease and the disease progression, and can serve as outcome parameters in clinical eye research [1].

In this thesis one IDCR performed in DR and two IDCR performed in AMD will be presented. In these IDCR imaging biomarkers (retinal biomarkers) were characterized and/or validated as risk estimators of the diseases progression, i.e., for the development of Clinically Significant Macular

Edema (CSME) for the IDCR in DR and for the development of AMD and progression to wet AMD for the two IDCR in AMD. The relevance of these retinal biomarkers for patient-oriented clinical research will be discussed.

Also, to show the relevance of IDCR for evidence-based clinical eye research and to show how infrastructures focused on IDCR, can help the investigators in the different stages of the clinical research and how this support may contribute for IDCR efficiency and competiveness, and for more patient-oriented clinical research (in Europe and more precisely in Portugal), my contribution to the development and consolidation of a new infrastructure for IDCR, the Coimbra Coordinating Centre for Clinical Research (4C), will be also presented.

Therefore, this thesis will be composed by the following chapters:

INVESTIGATOR-DRIVEN CLINICAL RESEARCH

To perform patient-oriented IDCR, with a high clinical evidence, the investigators need a qualified team and a specialized infrastructure to help them with the design, submission and conduction of the clinical study, according to the regulations and legal requirements. Infrastructures focused on IDCR are needed to help the investigators to overcome the difficulties faced when trying to perform a clinical research. The problems and difficulties faced by the investigators are presented and discussed by the European Science Foundation (ESF) in the report on “Investigator-Driven Clinical Trials” [2], and by the European Strategy Forum on Research Infrastructures (ESFRI) in the “Strategy Report on Research Infrastructures – Roadmap 2010” [3]. Infrastructures focused on IDCR are lacking in Europe and particularly in Portugal. In the last decade a significant decrease of the number of IDCR was observed, due to the burden of work related with the different clinical research activities. Given this decrease and the needs identified by the investigators for high level independent academic clinical research, new infrastructures were recently created, at the European and at the National levels, to support IDCR both in academic and hospital environments.

In this context, I will introduce, in the first chapter of this thesis, IDCR and more precisely IDCR in clinical eye research; I will describe the main activities related with Clinical Research, explain how to perform these activities (e.g., design, submit and perform a clinical study), and explain how the existing infrastructures dedicated to IDCR can help the investigators. Some practical examples will be presented, helping the investigators to understand better the different phases involved in the clinical research as well as the different steps needed for the design, submission and conduction of IDCR.

DEVELOPMENT OF AN INFRASTRUCTURE FOR INVESTIGATOR-DRIVEN CLINICAL RESEARCH - THE COIMBRA COORDINATING CENTRE FOR CLINICAL RESEARCH (4C)

In this chapter my contribution to the development of a new infrastructure for IDCR in eye research, the 4C, will be presented. The 4C was created to support IDCR in the field of eye research in 2009 in the Association for Innovation and Biomedical Research on Light and Image (AIBILI), member of the Vision and Imaging Consortium for Translational Research (VICT)[1].

This infrastructure arose from the increase of the IDCR performed at AIBILI, and from the growing of the European Vision Institute Clinical Research Network (EVICR.net), an eye-oriented network of European Ophthalmological Clinical Research Sites that has its Coordinating Centre at AIBILI.

CONTRIBUTION TO CLINICAL EYE RESEARCH IN DIABETIC RETINOPATHY

In this chapter the IDCR entitled "Validation of a predictive model to estimate the risk of conversion to clinically significant macular edema and/or vision loss in mild nonproliferative retinopathy in diabetes type 2" will be presented. In this study two retinal biomarkers, obtained using non-invasive imaging methods, were tested as estimates of DR progression to CSME. The importance and relevance of these imaging biomarkers for the characterization of the disease and for the identification of the patients that are at risk of DR progression to CSME will be discussed.

CONTRIBUTION TO CLINICAL EYE RESEARCH IN AGE-RELATED MACULAR DEGENERATION

In this chapter two IDCR in AMD will be presented. The first study entitled "Characterization of early Markers of Choroidal Neovascularization (CNV-MARKERS)", explored several imaging biomarkers, based on multimodal imaging, in order to identify retinal biomarkers of AMD progression to wet AMD. The second study entitled "Epidemiological study of the Prevalence of Age-related Macular Degeneration in Portugal", and that was the first IDCR fully supported by the new infrastructure 4C (i.e., from the study design, set-up in the primary healthcare units,

[1] The VICT is a consortium signed in June 3rd 2013 between: AIBILI; the Institute for Biomedical Imaging and Life Sciences (Instituto Biomédico de Investigação de Luz e Imagem – IBILI) of the Faculty of Medicine of the University of Coimbra (FMUC); the Institute for Nuclear Sciences Applied to Health (Instituto de Ciências Nucleares Aplicadas à Saúde – ICNAS) of the University of Coimbra; and the Integrated Responsibility Center of Ophthalmology (Centro de Responsabilidade Integrado de Oftalmologia – CRIO), of the Coimbra University Hospital (Centro Hospitalar e Universitário de Coimbra – CHUC).

coordination and statistical analysis), aimed to determine the prevalence of the disease in the Portuguese population aged over 55 years based on retinal biomarkers obtained using non-invasive imaging methods. The importance and relevance of the different imaging biomarkers for the characterization of the disease, and for the identification of the patients that are at risk for AMD progression to wet AMD, will be discussed.

2 INVESTIGATOR-DRIVEN CLINICAL RESEARCH

2.1 Clinical Research

Clinical research is a branch of the medical sciences that aims to study the safety and/or efficacy of medicines, medical devices, diagnostic methods and/or new therapeutic approaches in humans. Clinical research aims therefore to contribute to the prevention, diagnosis, treatment, and establishment of new clinical approaches representing therefore an added value for the patient.

According to the National Institutes of Health (NIH) of the United States of America (USA) clinical research can be divided in two types of studies, observational studies and non-observational studies (i.e., interventional studies or clinical trials)[2]. In the observational studies, the investigators aims to assess health outcomes in groups of participants that may, or not, receive interventions, e.g., investigational medicinal products (IMP) (i.e., drugs) or devices, and for which all the procedures performed by the participants are part of their routine medical care (i.e., according to the normal clinical practice). In the non-observational studies, or clinical trials, participants receive specific interventions according to a study protocol created by the investigators. Usually the investigators aim to assess the safety and efficacy of the interventions by measuring specific health outcomes. Clinical trials are design and conducted usually for drug development.

The distinction between observational and non-observational studies is also assumed by the new regulation adopted by the European Commission (EC) in April 2014 and that defined [4]:

[2] From www.clinicaltrials.gov (accessed on January 10th 2014).

- Clinical studies: any investigation intended "to discover or verify the clinical, pharmacological or other pharmacodynamics effects of one or more medicinal products; to identify any adverse reactions to one or more medicinal products; or to study the absorption, distribution, metabolism and excretion of one or more medicinal products; with the objective of ascertaining their safety or efficacy".
- Clinical trials: clinical studies in which "the IMP are not authorised; […] the IMP are not used in accordance with the terms of the marketing authorisation of the Member State concerned; the assignment of the subjects to a particular therapeutic strategy is decided in advance and does not fall within normal clinical practice of the Member State concerned; the decision to prescribe the IMP is taken together with the decision to include the subject in the clinical study"; or in which the "diagnostic or monitoring procedures in addition to normal clinical practice are applied to the subjects".
- Low-intervention clinical trials: clinical studies in which "the IMP are authorised; the IMP are used in accordance with the terms of the marketing authorisation or their use is a standard treatment in any of the Member States concerned"; or in which "the additional diagnostic or monitoring procedures do not pose more than minimal additional risk or burden to the safety of the participants compared to normal clinical practice in any Member State concerned".
- Non-interventional studies: clinical studies "other than clinical trials".

Clinical research includes therefore: epidemiological studies (or screening studies), non-interventional observational clinical studies (when the study follow the normal clinical practice, this includes registry studies); interventional observational clinical trials (when the study does not follow the normal clinical practice, i.e., when the study includes additional examinations, or when the frequency of the examinations is changed); interventional clinical trials, carried out with drugs and/or medical devices; preventive clinical trials, performed with medicines, vaccines, vitamins/minerals or by changes in lifestyle; diagnostic clinical trials; treatment studies that test new combination of treatments, or new surgical techniques.
Clinical research allows therefore obtaining clinical evidences that may be used to establish new clinical strategies in the healthcare services that will improve patients' care.

Evidence-based medicine, or evidence-based healthcare, combines the clinical expertise of the physicians, or paramedics, and the clinical evidences that result from the clinical research, according to predefined criteria. Evidence-based medicine is therefore a support for the clinical

practice, allowing for a faster update of the clinical knowledge and for a continuous improvement of the healthcare quality.

The clinical evidences obtained by the clinical studies[3] can have however different levels of recommendation depending on the purpose of the research and the methodology used. In order to have a patient-oriented clinical research, the clinical studies should be controlled, and based on homogeneous groups of patients who are representative of the population under study. Therefore, by improving the methodologies used in clinical research, using for example randomized controlled clinical trials (RCT), or meta-analyses studies, the clinical evidences obtained by the clinical studies can be improved [5, 6].

While the first clinical study record dates from the sixteenth century with Ambroise Paré (1545)[4], the first attempt to improve the existing methodology allowing for stronger clinical evidences emerged only in the eighteenth century with the study conducted by James Lind (1747). In Lind's study, twelve sailors with scurvy were grouped two by two and each group received one of the six possible treatments. This study allowed to demonstrate the therapeutic superiority of citrus, contributing for the eradication of the disease.

It was only in the twentieth century, that the clinical research methodology suffered its first major turning point with the introduction of the randomization concept by Ronald Fisher (1926) and Bradford Hill (1937). The first randomized clinical trial was conducted in 1948 by the Medical Research Council (United Kingdom). This was the first study using random numbers to allocate the study participants into groups (in this case into control and experimental groups). Currently randomized and double-blind clinical trials, i.e. clinical trials in which the participant' allocation is unknown (blind) to the participant and to the investigator, constitute the standard methodology in clinical research when the purpose is to compare different therapeutic approaches. This methodology is still the methodology that provides the greater degree of clinical evidence, being frequently required in clinical research to justify the use of treatments or interventions.

The second major turning point of the clinical research methodology occurred after World War II being related with the ethical aspects of the clinical research in humans. The Nuremberg Code, in 1947, sets for the first time the ethical principles for clinical research in humans making

[3] In the context of this thesis, clinical studies include observational and non-observational studies, i.e. clinical trials.

[4] Ambroise Paré compared two groups of subjects with gunshot wounds, one treated with boiling elderberry oil and cauterization (conventional treatment), and one treated with a recipice made of egg yolk, oil of roses and turpentine (an antiseptic).

mandatory the voluntary consent of the human subject participating in a clinical research. In 1962, after the thalidomide case[5], the Food and Drug Administration (FDA) sets also as mandatory, before conducting any clinical research, the existence of evidences on the safety and efficacy of the IMP to be used.

These concerns, related with participants' safety and consent for participating in the clinical research, were enforced by the Medical World Association (MWA) in the Declaration of Helsinki in 1964[6]. The Declaration of Helsinki sets the major ethical aspects that need to be considered when conducting clinical research. These aspects are based on the fundamental principle of participants' respect, participants' right to self-determination and right to make an informed decision regarding participation in the clinical research, before and during the course of the clinical research [7].

To establish standards for clinical research, the USA created in 1980 the Society for Clinical Trials (SCT), composed by different specialists, such as investigators, academics, industry representatives, physicians, paramedics, biostatistics, members of the decision-making institutions and members of the government. Rules and methodologies for good clinical practice in clinical research were established, such as the use of randomization when appropriate and ethically acceptable, and the need for continuous data verification allowing stopping the clinical study earlier if the preliminary results showed that there is no need to continue with the clinical study.

In 1990 the European Union (EU), USA and Japan formed the International Conference on Harmonisation (ICH) to standardize the requirements for drug development, promoting clinical studies efficiency and facilitating the mutual acceptance of clinical data by the regulatory authorities in these jurisdictions. In 1996 the ICH elaborated a set of rules for Good Clinical Practice (ICH-GCP) [8], defining the rights and responsibilities for the different stakeholders in the clinical research (e.g., investigators, sponsor, regulators and participants), and standardizing the development and conduction of clinical studies.

[5] Thalidomide was marketed in 1957 as a sedative and hypnotic drug with few side effects. The pharmaceutical industry believed that the drug was safe and suitable for prescribing to pregnant women to treat morning sickness. Tests conducted in rats revealed no teratogenic effects. Thalidomide was prescribed to thousands of women and distributed to all parts of the world. A few years later, the first cases of congenital malformations were identified in Germany, United Kingdom and Australia. In 1962, there were over 10000 children with birth defects. Thalidomide was then removed from the list of drugs prescribed for such condition.

[6] The Declaration of Helsinki was reviewed in October 2013 by the 64th MWA General Assembly (Declaration of Helsinki, seventh revision, Fortaleza, Brazil).

In 2001 an European Directive was issued based on the ICH-GCP guidelines and the Declaration of Helsinki (Directive 2001/20/EC[7]) laying down the requirements for conducting clinical studies, more specifically for clinical trials [9]. The European Directive 2001/20/EC was transposed into national legislations in the 25 Member States (MS) of the EU, being transposed in Portugal in 2004 (Decreto Lei 46/2004) [10]. The Portuguese law was recently reviewed in order to include also the dispositions of the European Directive on medical devices (Directive 2007/47/EC[8]) [11] being released in April 2014 a new law (Lei 21/2014) [12].

Apart from the national legislations, the ICH-GCP guidelines and the Directive 2001/20/EC are the main documents that should be followed when planning a clinical study in Europe.

[7]Directive 2001/20/EC - on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use.

[8] Directive 2007/47/EC – amending Council Directive 90/385/EEC on the approximation of the laws of the Member States relating to active implantable medical devices, Council Directive 93/42/EEC concerning medical devices and Directive 98/8/EC concerning the placing of biocidal products on the market.

2.2 Good Clinical Practice Guidelines

Clinical studies must conform to the ICH-GCP guidelines that defines standards to ensure adequate protection to the clinical research participants and to ensure that all clinical study activities and data are accurately documented and reported [8].

The ICH-GCP guidelines are an international ethical and scientific quality standard for designing, conducting, recording and reporting clinical studies that involve the participation of human subjects. Compliance with this standard provides assurance that the rights, safety and well-being of study participants are protected; consistent with the principles that have their origin in the Declaration of Helsinki; and that the clinical study data is credible. The guidelines were developed with consideration of the current good clinical practice in EU, USA and Japan, as well as those in Australia, Canada, Nordic countries and those of the World Health Organization (WHO).

The ICH-CGP guidelines should be followed when conducting clinical trials that are intended to be submitted to regulatory authorities. These principles may also be followed in other clinical studies that may have an impact on the safety and well-being of human subjects [13].

Therefore, before initiating a clinical study the following aspects should be considered:

- Foreseeable risks and inconveniences should be weighed against the anticipated benefit for the study participants. The study should be initiated and continued if and only if the anticipated benefits justify the risks.
- The rights, safety, and well-being of the study participants should prevail over interests of science and society.
- Non-clinical and clinical information on the IMP should be adequate to support the proposed clinical study.
- The clinical study should be scientifically comprehensive, and described in a clear, detailed study protocol.
- The study should be conducted in compliance with the protocol that received prior approval by:
    - the institutional review board (IRB) and/or an independent ethics committee (IEC) and,
    - the national competent authority (CA), for IMP, and
    - the national data protection committee, if applicable.

- Each element of the research team involved in the conduction of the clinical study should be qualified, trained, and experienced to perform his or her respective task(s).
- Informed consent should be obtained from every participant prior to inclusion in the clinical study.
- All clinical study information should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation and verification.
- The confidentiality of records that could identify study participants should be protected, respecting the privacy and confidentiality rules in accordance with the applicable legal and/or regulatory requirement(s).
- IMP should be manufactured, handled, and stored in accordance with applicable good manufacturing practice (GMP)[9] [14] and should be used in accordance with the study protocol.

[9] Directive 2003/94/EC - laying down the principles and guidelines of good manufacturing practice in respect of medicinal products for human use and investigational medicinal products for human use.

2.3 European Directive for Clinical Research

The European Directive 2001/20/EC establishes specific provisions regarding the conduction of interventional clinical studies involving IMP, i.e., clinical trials, including multicentre trials [9][10].

The objective of the Directive 2001/20/EC is to harmonize the European regulatory systems, to improve the protection of study participants, to optimize the use of safety information, and to ensure the quality of clinical studies and the credibility of data. The Directive 2001/20/EC was adopted on 2001 and was transposed into national legislation between 2004 and 2006 in the different European MS. This European legislation increased the responsibility of the clinical trial sponsors; led to shared responsibilities between ethics committees and competent authorities; and improved the patients' protection. The European Directive contributed for the improvement of the safety and the ethics of the clinical research, for the improvement of the data reliability, and for the strengthening of the scientific validity of the clinical research in Europe.

Through harmonisation of the regulatory framework of clinical research, the Directive was expected to stimulate multinational collaboration, to make European clinical research more competitive and the European Union more attractive for industry-sponsored clinical trials. However, due to the application of the same rules to all types of clinical trials and due to the divergent transposition of the Directive's principles into pre-existing national legislations, the Directive partly missed its facilitation and harmonisation objectives. Moreover, the Directive is considered to impose unnecessary administrative burden and costs being problematic for IDCR [15]. Therefore industry and academic stakeholders frequently claim for changes in the regulatory framework of the clinical research. Between 2007 and 2011, the number of clinical trials submissions decreased by 25% in Europe [16, 4].

In October 2007 the EC and the European Medicines Agency (EMA) organized a conference to discuss the possible changes to be brought to the Directive. In 2009 the EC put forward a legislative proposal to revise the Clinical Trials Directive 2001/20/EC. To assess the impact of this revision, a public consultation was held from 9 October 2009 to 8 January 2010 (the

[10] When conducting clinical research with medical devices additional directives should be followed. The two main directives that should be considered are: Directive 2001/83/EC - on the Community code relating to medicinal products for human use [153]; and Directive 2007/47/EC - – amending Council Directive 90/385/EEC on the approximation of the laws of the Member States relating to active implantable medical devices, Council Directive 93/42/EEC concerning medical devices and Directive 98/8/EC concerning the placing of biocidal products on the market [11].

'2009/10 public consultation'), being released in 2012 a new proposal to replace the actual Directive 2001/20/EC [4][11]. One of the major improvements proposed is related with the simplification of the submission process by proposing a common platform for the electronic submission of the clinical trials for both the CA and the IEC, simplifying significantly the submission process of multinational clinical trials. The European Parliament approved this new proposal in April 2014 and a new regulation for clinical trials is being finalized for publication. It is expected to have this new regulation in place in Europe by mid-2016.

[11] Also, after the Poly Implants Prothèse case, known as the PIP case in 2011, regulatory gaps were identified in the medicinal devices framework. In 2012 a new proposal for the Directive 2007/47/EC was also released for public discussion [154].

2.4 Regulatory and Legal Issues for Clinical Research

To perform a clinical research, the protection of the study participants, participants' rights and dignity, with regard to the research, should be sought. The protection of the study participant should be evaluated through risk assessment based on previous studies (e.g., for IMP based on toxicological experiments) and the foreseeable risks and inconveniences should be weighed against the anticipated benefit for the study participant.
The protection of the study participants should be reviewed and approved by an IEC; when an IMP is used, by a CA; and according to the national rules for data protection [9, 17].
Therefore, before initiating a clinical study, the sponsor[12] should submit the clinical study to the appropriate authorities, according to the applicable regulatory and legal requirements, i.e.: IEC and/or IRB, CA and data protection committee (when applicable) [8, 9]. The clinical study can initiate if and only if appropriate approvals from the IEC and/or IRB, CA and data protection committee, have been obtained.

Depending on the type of the clinical study, the regulatory and legal requirements may be different from one country to the other. In all the European countries, any clinical research should be reviewed and approved by an ethics committee. If the clinical study is observational non-interventional, the study may be submitted to the local IEC, or to an IRB. If the study is observational interventional, the study should be submit to a central IEC, and if the study involved an IMP, the study should also be submit to the national CA. For any type of study, approval for data collection should be sought, in some countries, such as for Portugal, the study needs approval from the national data protection committee (Comissão Nacional de Protecção de Dados – CNPD).
The list of the European IEC can be found in the European Network of Research Ethics Committees (EUREC) website (www.eurecnet.org, accessed on January 10th 2014) while the list of the European CA can be found in the EMA website (www.ema.europa.eu, accessed on January 10th 2014).

[12] The sponsor of a clinical study is "an individual, company, institution, or organization which takes responsibility for the initiation, management, and/or financing of a clinical trial", [8]. When the sponsor is "an individual who both initiates and conducts, alone or with others, a clinical trial, and under whose immediate direction the investigational product is administered to, dispensed to, or used by a subject" the sponsor is referred as sponsor-investigator. "The obligations of a sponsor-investigator include both those of a sponsor and those of an investigator", [8].

The requirements for the submission of a clinical study to the regulatory authorities of the participating clinical centres, will depend on the type of the clinical study and the national/local requirements.

To initiate a clinical study, the sponsor must therefore have all the regulatory and legal approvals. The major steps for the process of submission are summarized in Figure 1.

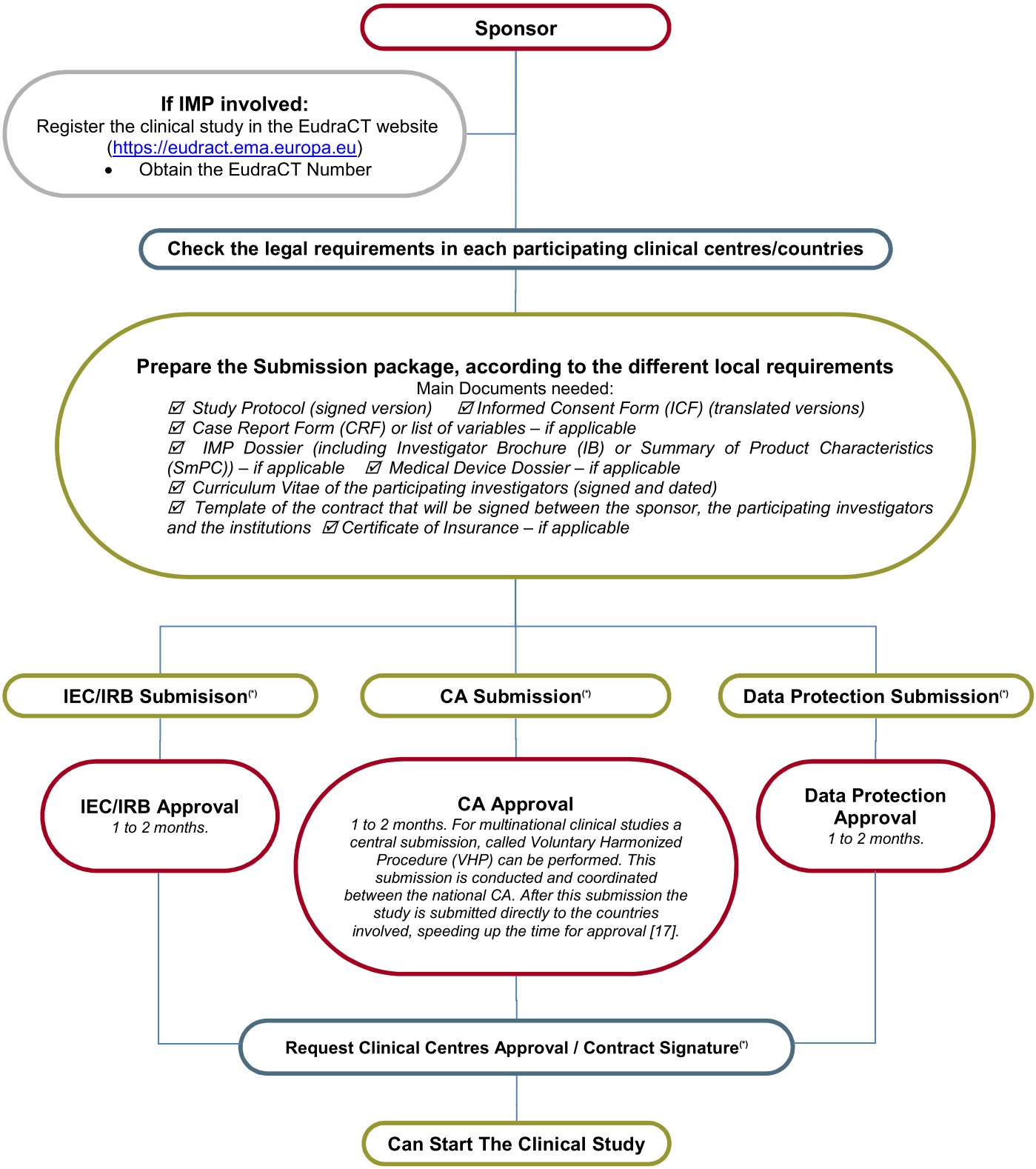

Figure 1. Clinical study submission steps. *(*) In Portugal the new law, Lei 21/2014, published in April 2014 and that should be in force by June 2014, sets timelines for Regulatory Authorities (RA) and contracts approvals, being now established 30 days for RA approvals and 15 days for contracts approval by the clinical centres. The submission process was also changed, being now planned a centralized submission of the clinical studies through a national electronic platform, the Registo Nacional de Ensaios Clínicos (RNEC) platform).*

2.5 Clinical Research Methodology

The scientific quality and validity of a clinical study is determined mainly by the study design. The study design must be planned very carefully prior any activity, being difficult to correct any inconstancy afterward.

To develop a clinical study the sponsor/sponsor-investigator has to [18]:

- Perform a systematic literature review to increase knowledge and familiarity with the research area.
- Learn about current trends and technological advances on the area.
- Use the FINER and PICOT criteria when developing the clinical research hypothesis, i.e., by answering the following questions:
    - FINER criteria:
        - *Feasible*: Is the clinical question answerable? Does the investigator have access to all the materials need for the study? Does the investigator have access to enough subjects? Will the investigator have enough time and money to conduct the study? Does the investigator have the expertise to perform the study or can he collaborate with someone who does?
        - *Interesting*: Is the question interesting to the investigator, and to others?
        - *Novel*: Has this study been done before? Does it add to the current medical knowledge?
        - *Ethical*: Can the study be done without risks to the participant? Will an IRB and/or IEC review and approve the study?
        - *Relevant*: Will the study further medical science? Will the results change clinical practice, health policy or point towards further possibilities of research?
    - PICOT criteria:
        - *Population* (patients): What specific patient population is the investigator interested in?
        - *Intervention* (for interventional studies only): What is the investigational intervention?

- *Comparison* group: What is the main alternative to compare with the intervention?
- *Outcome* of interest: What does the investigator intend to accomplish, improve or affect?
- *Time*: What is the appropriate follow-up time to assess the outcome?

- Develop a research hypothesis from the research question.
- Develop a clear and well-defined primary objective and, if applicable, a clear and well-defined secondary objective.
- Ensure that the clinical research question and objectives are answerable, feasible and clinically relevant.

To help sponsors/sponsor-investigators, ensuring that no critical scientific or ethical issue is left behind, the guidelines of the ICH-GCP E6 for the development of the clinical study protocol should be followed [8]. These guidelines describe each aspect that should be considered in the planning, designing and reporting of a clinical study. More information is available in the ICH-GCP E3 guidelines for reporting clinical studies results [19], and on the ICH-GCP E9 for statistical considerations (statistical planning, analysis and sample size estimation) [20].

The main information that should be considered when planning, designing and reporting a clinical study and that should be fully described in the clinical study protocol, is summarized in Table 1.

Table 1. Information needed when writing a clinical study protocol (based on the ICH-GCP E6 [8], the ICH-GCP E3 [19], and for statistical issues the ICH-GCP E9 [20]).

Clinical Study Protocol		Information to be included / Description
1.	General information	⦻ Sponsor details (for IDCR name of the investigator and name of the institution). ⦻ Study title and Protocol identifier (i.e., protocol number). ⦻ Identification of the participating investigators and the clinical centres.
2.	Background information	⦻ Scientific background for the clinical study (including scientific references).
3.	Study purpose and objectives	⦻ Main purpose of the study. ⦻ Description of the primary objective. ⦻ Description of the primary outcomes/endpoints[13].

[13] In clinical research outcomes, and endpoints are often used as equivalents [18]. According to the NIH, outcomes are a "specific key measurement(s) or observation(s) used to measure the effect of

Table 1. Information needed when writing a clinical study protocol (based on the ICH-GCP E6 [8], the ICH-GCP E3 [19], and for statistical issues the ICH-GCP E9 [20]).

Clinical Study Protocol		Information to be included / Description
		⦾ Description, if applicable, of the secondary objectives. ⦾ Description, if applicable, of the secondary outcomes/endpoints.
4.	Study design	⦾ General description of the study population (disease, pathology, condition to be analysed). The population to be recruited, or study sample, should be representative of the general population in order to make right conclusions. ⦾ Description of the primary outcomes/endpoints of the study (based on the primary objective). ⦾ Description, if applicable, of the secondary outcomes/endpoints that will be measured during the study.
5.	Subjects selection and withdrawal	⦾ Type/design of the study (i.e., retrospective, prospective, cross-over, parallel, etc.). ⦾ Description of the method used to select patients (i.e., invited directly by the physician, invited by a patients' organization, etc.). ⦾ Full description of the population to be included (i.e., definition of the inclusion and exclusion criteria, to avoid bias). ⦾ Number of patients needed: Sample size estimation for the primary study objective. The correct estimate of the sample size is crucial for an adequate power of the study and therefore for a valid clinical study. Details on sample size considerations can be found in the ICH-GCP E9 guidelines [20]. Some of the major considerations are [21, 22]: study objective, study design, primary variables, statistical test to be used, allocation ratio, expected results (e.g., treatment difference), types I and II errors, and expected rate of withdrawals (e.g., drop-out rate). ⦾ Description of the measures taken to minimize bias (e.g., randomization and binding procedures). ⦾ Description of the study procedures (i.e., description of the study visits, frequency, procedures to be performed, diagnostic and therapeutic procedures). This description should include the description of the number of visits, the sequence and duration of all study periods, including pre-randomisation and post-treatment periods, therapy washout periods and single- and/or double-blind treatment periods. ⦾ Description of the study treatment, if applicable. Description of the specific drugs, doses and procedures. For studies with IMP, the dose, dosage regimen, dosage form, packaging and labelling must be described. ⦾ Description of the stopping rules or discontinuation criteria for individual participant and for the entire study. ⦾ Description of the procedure for withdrawn patients (e.g., post-study

experimental variables in a study, or for observational studies, to describe patterns of diseases or traits or associations with exposures, risk factors or treatment" (www.clinicaltrials.gov, accessed on January 10th 2014). Outcomes may also be defined as "response variables […] measured during the course of the trial" defining and answering the questions raised by the clinical research [19]. On the other hand, according to the ICH-GCP E8, endpoints are "response variables that are chosen to assess drug effects that are related to […] efficacy and safety" and that "should reflect clinically relevant effects" being "selected based on the principal objective of the study" [20]. Based on these different and overlapping definitions, outcomes are usually called endpoints, as stated by the CONsolidated Standards Of Reporting Trials (CONSORT) group: "outcome variable of interest in the trial (also called an endpoint)" (www.consort-statement.org, accessed on January 10th 2014).

Table 1. Information needed when writing a clinical study protocol (based on the ICH-GCP E6 [8], the ICH-GCP E3 [19], and for statistical issues the ICH-GCP E9 [20]).

Clinical Study Protocol		Information to be included / Description
		contact, discharge visit procedures, etc.). ⦻ Description of the randomization process, for randomized studies, and, if applicable, description of the blinding procedures. ⦻ Detailed description of the level and method used for blinding/masking and for unblinding, for blind studies. ⦻ Description of the patients' allocation procedure (e.g., randomization, stratification methods).
6.	Treatments	⦻ Full description of the treatments to the performed and their frequency: technical procedures, drug information (name of the finished product and name of the active ingredient), test product (dose and mode of administration). ⦻ Description of the reference therapy, if any. ⦻ Description of the rescue treatment, if any. If, for safety reasons, a rescue treatment is planned, the sponsor should decide if patients treated with the rescue treatment should continue in the study.
7.	Efficacy assessment	⦻ Description of the parameters used to assess the study efficacy. ⦻ Definition of how these parameters will be monitored during the study.
8.	Safety assessment	⦻ Description of the parameters used to assess the study safety. ⦻ Definition of how these parameters will be monitored during the study.
9.	Statistics *(see ICH-GCP E9 guidelines for statistical considerations [20])*	⦻ General description of the statistical methods to be used for the primary and secondary objectives. A Statistical Analysis Plan (SAP) is usually elaborated with the full details on the statistical analysis. This section of the study protocol should describe the statistical hypotheses of the study, the variables to be collected and analysed, such as how missing data will be handled. Sample size estimates should also be provided.
10.	Access to source data/documents	⦻ Description of the measures that will be followed to guarantee access to the source documents (i.e., who will have access to the study source documents, and who will keep these documents in the participating centres).
11.	Quality control and quality assurance	⦻ Description of the measures that will be followed to assure study quality (i.e., measures to control the access to the study documents, persons qualified to introduce and keep the data).
12.	Ethics	⦻ Descriptions on how will the safety and well-being of the study participant be guarantee. The detail description of the ethical consideration for the study participant is usually write-down on the Inform Consent Form – ICF. This document should contain the following issues: subject voluntary participation and freedom of withdraw; study objectives, design, procedures, treatments and expected duration; risks and potential adverse reactions; expected benefits for the participation; alternative treatments; measures to guarantee the confidentiality of the data and rights to access them; economic compensation for the participants, the investigators and the institutions; information of the sponsor, investigators and institutions; contacts for more information; contacts of the IEC and/or IRB that reviewed and approved the study.
13.	Data Handling and Record Keeping	⦻ Description of the data/information that should be considered as source data/documents. ⦻ Description of who will handle and keep source data/documents. ⦻ Description of who will be responsible to enter the data into the study

Table 1. Information needed when writing a clinical study protocol (based on the ICH-GCP E6 [8], the ICH-GCP E3 [19], and for statistical issues the ICH-GCP E9 [20]).

Clinical Study Protocol		Information to be included / Description
		case report form (CRF) (the CRF is a document created and used to capture clinical data from each study participant and to transfer it to the study database. The CRF contains all of the protocol-designated information that will be recorded on every study visit, including safety information, adverse events, serious and non-serious, in compliance with regulatory requirements. In addition the CRF ensure accurate documentation of the study data, as well as a high quality of the data).
14.	Financing and Insurance	◌ Details on the financial issues related with the study (who will pay for the study, who will pay the participating centres, the investigators and the study participants expanses). ◌ Description of the person/institution responsible for the study insurance (mandatory to cover any expanses related with the study, i.e., in the case of any injury suffered by the study participants).
15.	Publication Policy	◌ Details of the policy for study publication (persons allowed to use the study data).
16.	Supplements	◌ Description of how will study protocol supplements handled.

The scientific, clinical and ethical aspects of the study design are not the only issues to be considered. The study financial, organizational, logistical aspects and the research team should also be considered and documented by the sponsor when planning the clinical study.

Section 8 of the ICH-GCP E6 describes the essential documents needed to set-up and to perform a clinical study. These documents are usually kept in two files: the “Study Master File”, established at the beginning of the study design and kept by the sponsor; and the “Site Investigator File”, established before starting the clinical phase, i.e., before recruiting patients, and that is kept with the participating investigator/centre. The final close-out of a clinical study can only be done by the sponsor when all the documents, both in the sponsor and the investigator/centre files has been reviewed and confirmed. This task is usually performed by a contract research assistant (CRA), or study monitor, designed by the sponsor.

Any documents addressed in these ICH-GCP guidelines may be subject to, and should be available for, audit by the sponsor and inspection by the regulatory authority(ies).

Table 2 summarizes the essential documents needed before initiating the clinical phase of a clinical study.

Table 2. Documents needed before and after the conduction of a clinical study (according to the ICH-GCP E6 [8]).

	Documents	Documents to be included / Description
1.	Approved clinical study protocol/ amendments	⦻ To document sponsor's approval and responsibilities, the signed version of the clinical study protocol should be kept. ⦻ To document investigators' acceptance and responsibilities, the signed clinical study approval page, by the participating investigators, should be kept.
2.	Participants' ICF and any document/ advertisement for recruitment	⦻ To document participants' informed consent, a sample of the ICF, and any translated versions, should be kept by the sponsor. ⦻ To document that the subjects received appropriate written information (content and wording), one copy signed by the study participant should be kept in the clinical centre. ⦻ To document that recruitment measures were appropriate and not coercive, any document issued to recruit patients must also be kept.
3.	Financial and agreements issues	⦻ To document the financial agreement for the clinical study and to document the responsibilities of the sponsor, the participating investigator and the clinical centre, the signed study agreement between the sponsor, the participating investigator and the participating centre, should be kept by the sponsor. ⦻ To document the responsibilities of other partners, any agreement celebrated with a third party (e.g., contract research organizations – CRO, central laboratories, central reading centres, etc.) should be kept by the sponsor.
4.	Certificate of insurance	⦻ To document that compensation related to injury to study participants will be available, the insurance certificate should be kept. Insurance is a legal requirement to perform a clinical research.
5.	Approval from the IRB and/or IEC	⦻ To document that the study was subject to IRB/IEC review and receive approval/favourable opinion the study approval should be kept. ⦻ To document that the IRB/IEC that evaluates the study was established in agreement with ICH-GCP E6 a copy of the members of the IRB/IEC should also be kept.
6.	Approval from the CA *(if applicable)*	⦻ To document CA authorisation (approval/notification), prior to study initiation, the CA study approval should be kept.
7.	Approval from other RA – Data Protection Committee *(if applicable)*	⦻ To document appropriate authorisation by other RA, such as a Data Protection Committee, the respective study approvals should be kept.
8.	Curriculum Vitae and/or other relevant documents evidencing investigators qualifications	⦻ To document qualifications and eligibility of the research team to conduct the study and/or to provide medical supervision of the study participants, a signed and updated copy of the research team Curriculum Vitae should be kept by the sponsor.
9.	Technical Procedures and Normal values/ranges	⦻ To support reliability of the results (e.g., staff and/or equipment certification, quality assessment, etc.) standard operating procedures for all the procedures performed during the clinical study must be elaborated and approved by the sponsor before initiating the clinical study.

Table 2. Documents needed before and after the conduction of a clinical study (according to the ICH-GCP E6 [8]).

	Documents	Documents to be included / Description
		⦻ To document normal values and/or ranges for the procedures/tests a copy of the normal values/ranges should be kept.
10.	IMP information	⦻ To document relevant and current scientific information about the investigational product (investigational drug), the Investigator Brochure (IB) has to be requested by the sponsor to the IMP manufacturer, and must be provided to the participating investigators. When the IMP has already a Marketing Authorization the IB may be replaced by a Summary of Product Characteristics (SmPC), available at the EMA website.
11.	IMP label sample	⦻ To document compliance with applicable labelling regulations (e.g., with the GMP guidelines) and appropriateness of instructions provided to the subjects, a sample of the label attached to the IMP container must be kept by the sponsor.
12.	Instructions for handling IMP and other study-related materials	⦻ To document instructions needed to ensure proper storage, packaging, dispensing and disposition of IMP products and study-related materials, a procedure, elaborated and approved by the sponsor, should be provided to the participating investigators.
13.	Shipping records for IMP and other study-related materials	⦻ To document shipment dates, batch numbers and method of shipment for IMP and study-related materials, records should be kept by the participating investigators. These records will allow for tracking of product batch, review of shipping conditions, and accountability.
14.	Certificates of analysis of IMP	⦻ To document identity, purity, and strength of IMP to be used in the study, the certificated of analysis should be required and kept by the sponsor and the participating investigators.
15.	Unblinding procedures	⦻ To document how, in case of an emergency, to unblind a participant without breaking the blind for the remaining participants, an unblinding procedure should be elaborated and approved by the sponsor before initiating the clinical study.
16.	Randomization List	⦻ To document the method used for randomisation of study participants, a randomization list should be elaborated and kept by the sponsor.
17.	Data Management Plan (DMP)	⦻ To document how the study data will be collected, kept, monitored and exported, in order to assure data quality, a DMP should be elaborated before initiating the clinical study. To assure the safety of the study participants and the validity of the data, the procedure for safety data management should also be included in the DMP.
18.	Statistical Analysis Plan (SAP)	⦻ To document how the study data will be handled and analysed, a SAP should be elaborated before analysing the data. More details can found in the ICH-GCP E9 [20].
19.	Monitoring Plan	⦻ To ensure that regulatory requirements are met and that the study procedures are performed in compliance with the study protocol, a monitoring plan should be elaborated and approved by the sponsor before initiating the study. The monitoring plan should describe the frequency of the monitoring, the percentage of source data verification, and the

Table 2. Documents needed before and after the conduction of a clinical study (according to the ICH-GCP E6 [8]).

	Documents	Documents to be included / Description
		method used for monitoring, i.e., central monitoring or on-site monitoring. The plan should be shared with the participating investigators.
20.	Monitoring Reports	◌ To document that the participating centres are suitable for the study, a pre-initiation visit (on-site or central) should be performed. The site initiation report should be kept by the sponsor. ◌ To document that, during the clinical study, the study procedures were followed and reviewed by the participating investigators and by the research team, monitoring visits (on-site or central) must be performed by the sponsor. The monitoring reports should be kept by the sponsor. Any finding (e.g., missing data, protocol deviation, etc.) should be analysed by the sponsor and the investigators and solved/followed in order to guarantee the study quality.

The “Guide for Investigator Initiated Trials” [23] provides a good summary of the steps, procedures and documents that are needed to perform a clinical study. Several templates are provided that may help the sponsor in the design, planning, submission and initiation of clinical studies. Moreover, several lists of actions, for the different phases of the conduction of the clinical study, may be found in the “Guide for Clinical Trial Staff” [24]. These two guides offer useful tools for both sponsor/sponsor-investigator and participating investigators/research team.

2.6 Investigator-Driven Clinical Research

Investigator-Driven Clinical Research (IDCR) is any clinical research promoted by individual researchers, scientists or physicians, universities, or other entities whose purpose is the scientific knowledge and the improvement of the current diagnostic or therapeutic approaches [2]. IDCR promotes scientific knowledge and innovation in diagnostics and/or therapeutics independently of commercial interests. As examples are the proof-of-concept studies, the rare diseases studies, the comparative studies for diagnostic and/or therapeutic intervention, and the studies conducted to test new surgical procedures or new indications of already registered drugs. IDCR has a greater scientific range and impact when compared to the research initiated by industry, being a key part in the patient-oriented clinical research, allowing for the creation of solid bases for the continuous improvement of the diagnostic and/or therapeutic approaches and for the clinical management of patients.

IDCR plays therefore a major role for evidence-based medicine by obtaining new clinical evidences or by supporting the existing ones. The strengthening of these clinical evidences will depend on the methodology used in the clinical research and, on the system used to categorize these evidences. Given the present needs and methodologies, the current system for the hierarchization of the clinical evidences needs to be reviewed. This system should go beyond the categorization of the study design and the accuracy of the results. The analysis of the risk-benefit ratio should be related to the type of research, since different types and methodologies of investigations are needed to address different clinical questions [25].
The present system of hierarchization of clinical evidences places RCT methodology on the top. RCT are the clinical studies that produce clinical evidences with the highest degree and, observational studies are the clinical studies that produce clinical evidences with the lowest degree [26]. Even if the observational studies are more heterogeneous and may have a higher degree of bias, these studies are important and complementary, providing valuable information on specific populations and on the effectiveness of specific therapies and/or procedures in the long term and in the everyday clinical practice, since the effectiveness of specific treatments and/or procedures depends on the patient's decision and action, on the concomitant therapies used and, on the patients' treatment adherence [27]. The awareness of these issues and the increase in new therapeutic approaches stimulated the interest of the investigators for wide-range clinical studies on treatments and/or procedures effectiveness. The challenges raised by

the methodology used in observational studies for the analysis and interpretation of the results, and the lack of criteria for assessing the quality of these studies, have limited the practical use of observational research. In this sense a new hierarchy of evidence for observational research is currently being developed, which can be used by researchers and decision makers, which includes the definition of issues and methods for clinical data collection, analysis, interpretation and presentation, in accordance with the ICH-GCP E6 guidelines [28, 29, 30].

Considering the clinical studies that are registered in the public database ClinicalTrials.Gov (total of 158806 clinical studies, Figure 2): 18.6% (29568) are observational studies; 81.0% (128491) are non-observational studies; 0.1% (229) are expanded access studies; and 0.3% (518) are not defined. Moreover, 61.2% of the clinical studies (97165) are initiated by the investigators (e.g., clinical research networks, governments, national institutes of health, etc.) and 38.8% (61641) are initiated, or are performed in collaboration with the industry.

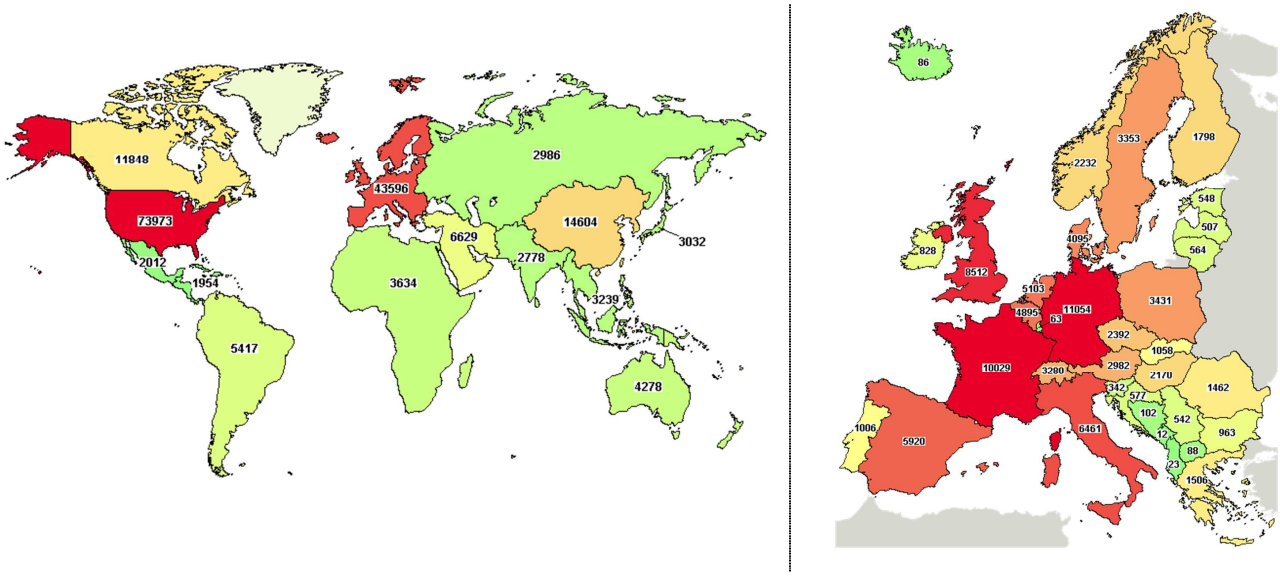

Figure 2. Number of clinical studies registered in the public database ClinicalTrials.Gov on January 10th 2014 (left: number of clinical studies in the World; right: number of clinical studies in Europe).

When considering only non-observational studies, i.e., clinical trials, the number of IDCR, when compared to the number of clinical trials initiated by the industry, decreases significantly, due essentially to the higher complexity of these studies and the higher costs associated with the submission and conduction. Every year, about 4400 new clinical trials are submitted in Europe,

and approximately 60% of these clinical trials are initiated by the industry, while 40% are initiated by institutions, governments, universities or investigators [4].

The smaller number of clinical trials initiated by the investigators is well demonstrated in Portugal in which only 13.3% of the clinical trials submitted to the National Competent Authority (CA) (Autoridade Nacional do Medicamento e Produtos de Saúde I.P. – INFARMED) are initiated by the investigators (Figure 3).

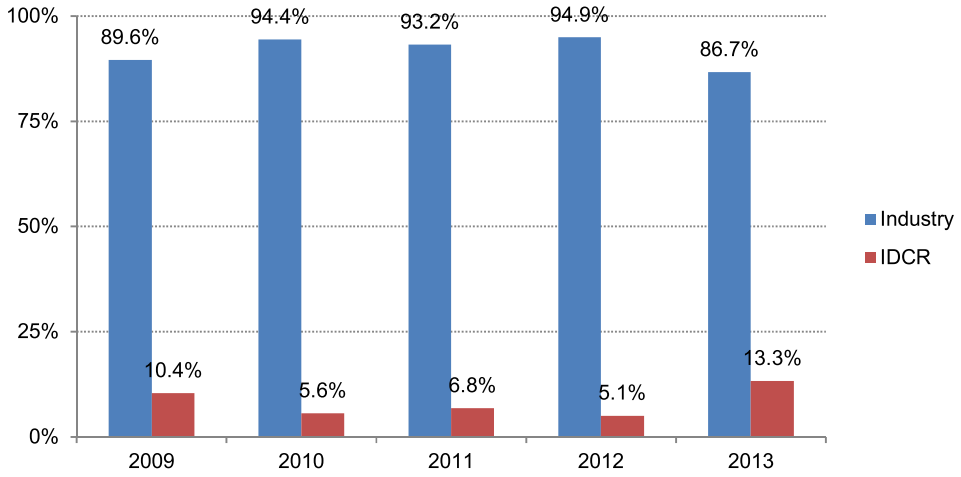

Figure 3. Percentage of clinical trials initiated by the investigators (red) and industry (blue) in Portugal (according to the Portuguese Competent Authority website, www.infarmed.pt, accessed on January 10th 2014).

Due to the burden of work related with the different clinical research activities, IDCR is usually less complex than clinical research initiated by the industry. Also, it has been shown that most of the clinical studies initiated by the investigators have an inadequate sample size, use an insufficient or inappropriate methodology [31, 2], and are performed in one single centre [32, 31]. The creation of infrastructures that can support the investigators, technically, administratively and logistically, may therefore contribute for the increase of IDCR both at the national and international levels, by implementing methodologies and/or techniques that will increase the level of clinical evidence [32, 33], promoting therefore a more transversal and international clinical research [34].

2.7 Investigator-Driven Clinical Research in Eye Research

Clinical studies in eye research represent 3.8% of the total number of the clinical studies registered in the world[14]. The proportion of observational versus non-observational studies in eye research is similar to the overall picture of the clinical studies in the world, i.e. 21.8% are observational; 78.1% are non-observational; and 0.1% are not defined. In eye research less studies are initiated by the investigators, only 56.6% of the clinical studies are initiated by the investigators (e.g., clinical research networks, governments, national institutes of health, etc.) versus 43.4% initiated, or performed in collaboration with the industry.

A total of 1444 pathologies/conditions (ocular and non-ocular) are associated with "Eye Diseases", being some on these pathologies/conditions included in the same clinical study.
Ninety-seven (97) pathologies/conditions have more than 100 clinical studies registered (Table 3 and Figure 4). Forty-nine (49) have more than 200 clinical studies, 33 more than 300 clinical studies, 22 more that 400 clinical studies and 11 more than 500 clinical studies. Retinal diseases (retinal and macular degeneration) and glaucoma/hypertension are the ocular pathologies/conditions with more clinical studies registered (Figure 5).

Table 3. Pathologies/conditions associated with "Eye Diseases" with 100 or more clinical studies registered (data from ClinicalTrials.Gov on January 10th 2014).

Pathologies / Conditions associated with Eye Diseases and number of clinical studies		
Retinal Diseases 1743 studies	Diabetic Retinopathy 291 studies	Glucose Metabolism Disorders 157 studies
Retinal Degeneration 1023 studies	Diabetes Complications 282 studies	Conjunctivitis, Allergic 156 studies
Vascular Diseases 939 studies	Diabetic Angiopathies 272 studies	Respiratory Tract Infections 156 studies
Macular Degeneration 935 studies	Brain Diseases 271 studies	Nose Diseases 151 studies
Hypertension 915 studies	Respiratory Tract Diseases 268 studies	Eye Neoplasms 150 studies
Glaucoma 884 studies	Hypersensitivity, Immediate 267 studies	Rhinitis 150 studies
Ocular Hypertension 884 studies	Mental Disorders 255 studies	Blindness 144 studies
Syndrome 767 studies	Autoimmune Diseases 254 studies	Embolism 140 studies
Conjunctival Diseases 616 studies	Psychotic Disorders 253 studies	Embolism and Thrombosis 140 studies
Conjunctivitis 565 studies	Neovascularization, Pathologic 245 studies	Thrombosis 140 studies
Corneal Diseases 534 studies	Metabolic Diseases 243 studies	Wet Macular Degeneration 138 studies
Endocrine System Diseases 483 studies	Cranial Nerve Diseases 237 studies	Retinal Vein Occlusion 137 studies
Cataract 478 studies	Vision Disorders 231 studies	Venous Thromboembolism 137 studies
Uveal Diseases 461 studies	Sensation Disorders 229 studies	Venous Thrombosis 137 studies

[14] According to the public database ClinicalTrials.Gov from the 158806 clinical studies registered in January 10th 2014, 6087 studies are associated with "Eye Diseases" (from theses 6087 studies, only 4929 are registered in the predefined field "Condition" as "Eye Diseases").

Table 3. Pathologies/conditions associated with "Eye Diseases" with 100 or more clinical studies registered (data from ClinicalTrials.Gov on January 10th 2014).

Pathologies / Conditions associated with Eye Diseases and number of clinical studies		
Glaucoma, Open-Angle 460 studies	Otorhinolaryngologic Diseases 221 studies	Lacerations 135 studies
Edema 449 studies	Neoplasms, Nerve Tissue 201 studies	Rupture 135 studies
Lens Diseases 449 studies	Chorioretinitis 196 studies	Inflammation 132 studies
Refractive Errors 432 studies	Uveitis 196 studies	Neoplasms, Glandular and Epithelial 131 studies
Pigment-dispersion Syndrome 428 studies	Neoplasms, Germ Cell and Embryonal 195 studies	Congenital Abnormalities 127 studies
Diabetes Mellitus 420 studies	Neuroectodermal Tumors 195 studies	Stomatognathic Diseases 122 studies
Neurologic Manifestations 414 studies	Neuroepithelioma 195 studies	Respiratory Hypersensitivity 120 studies
Macular Edema 410 studies	Eye Diseases, Hereditary 191 studies	Neurodegenerative Diseases 116 studies
Dry Eye Syndromes 377 studies	Choroidal Neovascularization 187 studies	Astigmatism 115 studies
Keratoconjunctivitis Sicca 377 studies	Neoplasm Metastasis 187 studies	Neuroendocrine Tumors 112 studies
Lacrimal Apparatus Diseases 355 studies	Metaplasia 185 studies	Melanoma 110 studies
Keratitis 349 studies	Panuveitis 185 studies	Mouth Diseases 110 studies
Central Nervous System Diseases 329 studies	Skin Diseases 173 studies	Pathological Conditions, Anatomical 110 studies
Choroid Diseases 319 studies	Musculoskeletal Diseases 172 studies	Nevus 109 studies
Retinitis 311 studies	Eye Infections 171 studies	Nevus, Pigmented 109 studies
Keratoconjunctivitis 307 studies	Optic Nerve Diseases 166 studies	Connective Tissue Diseases 103 studies
Hypersensitivity 305 studies	Uveitis, Posterior 165 studies	RNA Virus Infections 102 studies
Myopia 301 studies	Choroiditis 163 studies	
Genetic Diseases, Inborn 300 studies	Virus Diseases 159 studies	

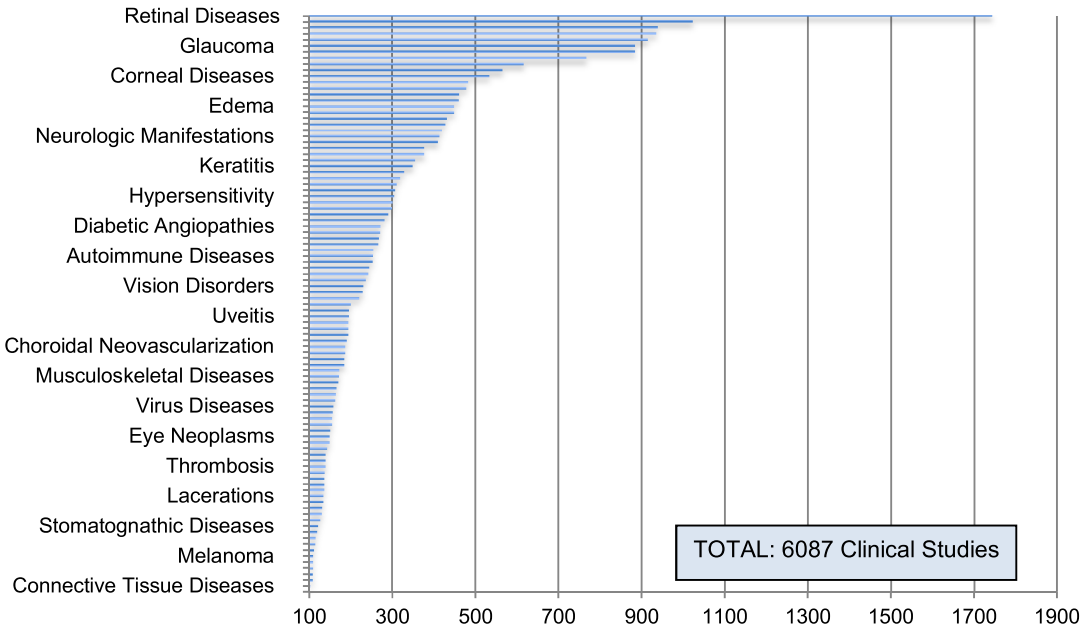

Figure 4. Number of clinical studies in eye research registered in the public database ClinicalTrials.Gov by pathology/condition (only pathologies/conditions with 100 or more clinical studies are shown) (data available from ClinicalTrials.Gov on January 10th 2014).

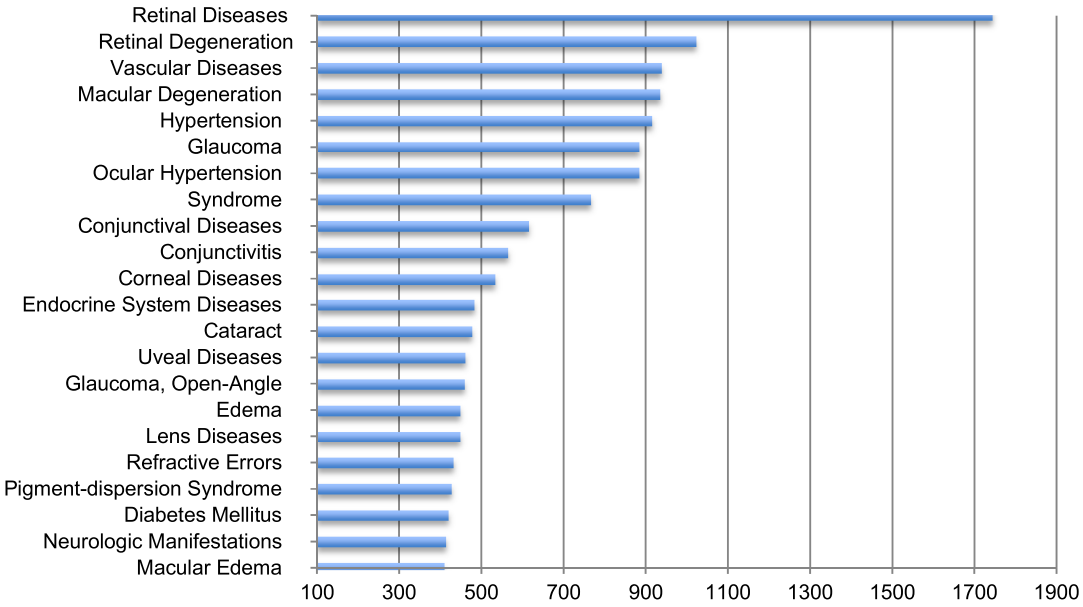

Figure 5. Pathologies/conditions with more than 400 clinical studies registered in the public database ClinicalTrials.Gov (data available from ClinicalTrials.Gov on January 10th 2014).

When looking at the Portuguese situation, and considering only non-observational studies (i.e., clinical trials), since these are the only clinical studies that need to be submitted to the national ethics committee (Comissão de Ética para a Investigação Clínica – CEIC) and, when applicable, to the national competent authority (INFARMED), being therefore the only clinical studies for which information is available in a national public database[15], clinical trials in eye research, i.e., ophthalmology, represents 8.0% of the clinical trials submitted in Portugal (Figure 6). If we consider the number of clinical trials submitted to INFARMED in 2013 (90) (Figure 7) and extrapolated to the number of clinical trials that may be submitted in ophthalmology (8.0%), approximately 7 clinical trials in eye research (i.e., ophthalmology) may have been submitted in 2013, from which 1 was probably an IDCR.

[15] With the new Portuguese law (Lei 21/2014), that should be in force by June 2014, it is expected to have in the near future all the clinical studies registered in a national public database.

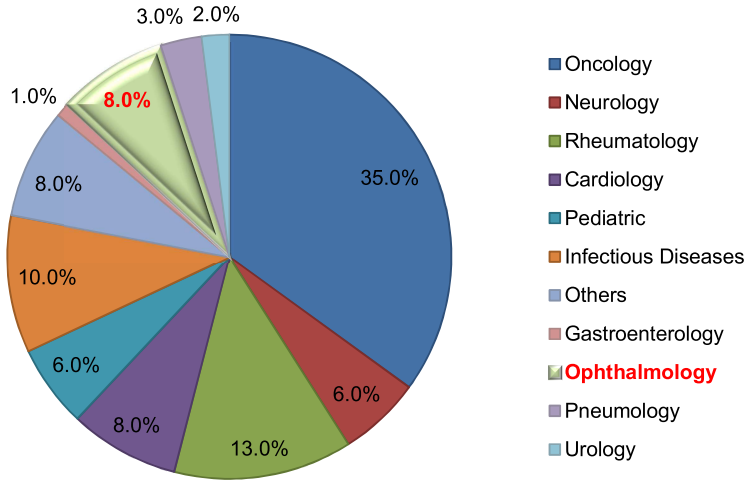

Figure 6. Clinical trials submitted in 2013 to the Portuguese IEC (CEIC) by clinical area (97% of the clinical trials submitted in 2013 were approved, according to the Portuguese IEC website, www.ceic.pt, accessed on March 1st 2014).

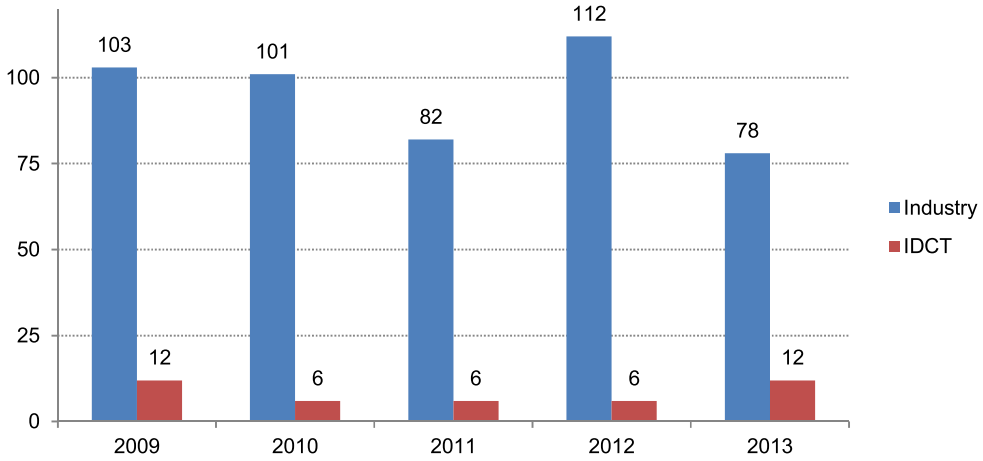

Figure 7. Number of clinical trials initiated by the investigators (red) and initiated by the industry (blue) in Portugal (according to the Portuguese Competent Authority website, www.infarmed.pt, accessed on January 10th 2014).

The small number of IDCR in eye research at the national level demonstrates well the importance and the urgent need for infrastructures specialized in IDCR, to help and support the investigators in this clinical area.

2.8 Disease-Oriented Networks for Investigator-Driven Clinical Research

Disease-oriented, or thematic networks, play an important role for the promotion of the scientific and medical excellence in disease-oriented research. These networks composed by scientists, healthcare providers (in hospitals and/or private practice), patient organizations and/or regulatory bodies, integrates the existing expertise on disease-oriented prevention, diagnosis and treatment, allowing for a multidisciplinary cooperative medical research, improving scientific competitiveness, and resulting in a better patient-oriented healthcare.

In Europe, these networks promote pan-European expansion, capacity building, and partnership with other regions of the world, improving also the attractiveness for industry-driven studies.

They are thousands of disease-oriented networks, being therefore impossible to list all of them.

One of the first disease-oriented network created in Europe is the European Organisation for Research and Treatment of Cancer (EORTC), created in 1962. With over 50 years and over 300 universities or affiliated hospitals in more than 30 countries, this network dedicated to IDCR and translational research, has an impressive contribution for the establishment of state-of-the-art treatments and in ensuring that new cancer agents are developed and tested efficiently with the aim of minimizing delays between laboratory discovery and their therapeutic benefit for the patients.

2.8.1 Network for Clinical Eye Research: The European Vision Institute Clinical Research Network – EVICR.net

The European Vision Institute Clinical Research Network (EVICR.net)[16], is a eye-oriented network of European Ophthalmological Clinical Research Sites (clinical centres and reading centres) dedicated to perform clinical eye research with the highest standards of quality, following the European and International Directives for Clinical Research, and like the EORTC Network that exists for over 50 years, the EVICR.net aims to contribute for the development of IDCR, making the European clinical eye research more competitive and efficient.

EVICR.net was created in 2004 within the European Vision Institute (EVI), but became independent by establishing itself in 2010 as an independent European Economic Interest Grouping (EEIG), in accordance with the Council Regulation. At present, EVICR.net has 81 Clinical Sites Members from 16 European Countries (Figure 8).

[16] EVICR.net: www.evicr.net (accessed on January 10th 2014).

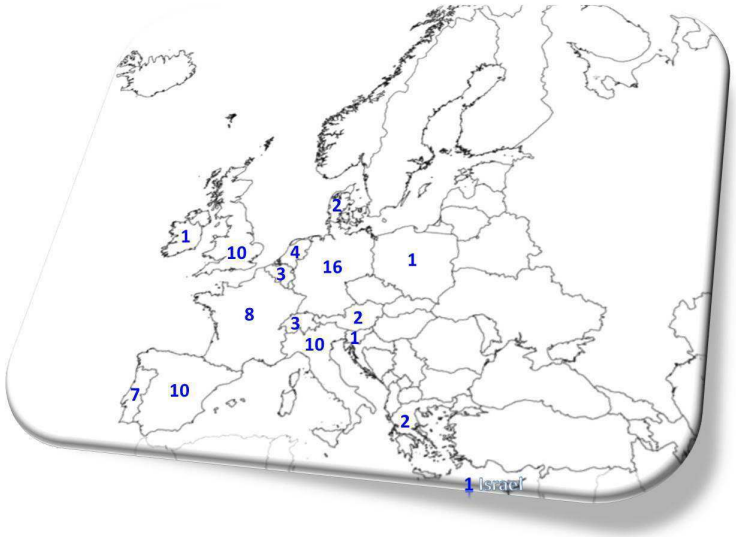

Figure 8. Number of clinical sites members of the EVICR.net per country (from EVICR.net website, accessed on January 10th 2014).

To apply to the EVICR.net network the clinical centre, or reading centre, must fulfil basic requirements such as having a dedicated space to perform clinical activities, having a qualified and experienced team, having experience in multicentre clinical studies and to agree to implement organizational Standard Operating Procedures (SOP) according to the ICH-GCP guidelines.

This network serves as a fundamental resource for the development of translational research in Europe [35, 36].

2.9 *Infrastructures for Investigator-Driven Clinical Research*

To perform more patient-oriented IDCR and with higher degree of clinical evidence, the investigators needs a qualified team and a specialized infrastructure to help them with the design, submission and conduction of the research. The main needs of the investigators are: support for the study design and selection of the methodology to be used (according to the purpose of the research); administrative and logistical support to conduct the study in compliance with the regulations and the legal requirements; support for the management and coordination of the study, providing more time for the investigators-clinicians in their clinical practice; training in good clinical practices; and finally, access to funding, or partnership, allowing for a higher quality of the research nationally and internationally [2, 34].
Such infrastructures, focused on IDCR are lacking in Europe and particularly in Portugal.

Recently, with the funding of the EU and the ESFRI (European Strategy Forum on Research Infrastructures [3]), an European infrastructure for IDCR was created, the ECRIN[17] (European Clinical Research Infrastructure Network). ECRIN was created to support IDCR promoting more and better clinical research in Europe [37]. This infrastructure consists of national networks of the MS, which allow performing and conducting IDCR at the European level. The Portuguese national network PtCRIN[18] (Portuguese Clinical Research Infrastructure Network), is currently being created. This network consists of several clinical and research centres, and some infrastructures already created to support IDCR across Portugal. AIBILI through one of its units, the Coimbra Coordinating Centre for Clinical Research (4C) is the first infrastructure created to support IDCR in the central region of Portugal. AIBILI is also the Coordinating Centre of the EVICR.net, an European network of clinical centres dedicated to vision research. The Clinical Trials Centre of AIBILI (Centro de Ensaios Clínicos – CEC) and the Coimbra University Hospital (Centro Hospitalar e Universitário de Coimbra – CHUC) are 2 of the active members of this network.
The existence of such infrastructures is critical to stimulate and to perform clinical research in Europe.

[17] ECRIN: www.ecrin.org (accessed on January 10th 2014).
[18] PtCRIN: web.fcm.unl.pt/ptcrin (available on January 10th 2014).

2.9.1 European Clinical Research Infrastructure Network – ECRIN

The ECRIN is a not-for-profit infrastructure created in 2004, dedicated to support multinational clinical research in Europe for any clinical area. In November 2013, ECRIN was officially awarded the Community legal framework for an European Research Infrastructure Consortium (ERIC). This infrastructure provides consulting and services to investigators and sponsors, for the preparation and conduction of multinational clinical studies being financially supported by the different European health and legislative systems.

ECRIN is composed by national networks of clinical research centres and clinical trials units that are able to provide support and services to multinational clinical studies (Figure 9). Consultancy is provided by experts from the national ECRIN partners and coordinated by the European Correspondent. Services are provided by national ECRIN partners at not-for-profit rates. To apply for ECRIN support the investigators needs to submit the clinical study protocol to the ECRIN Scientific Board for review and approval. In 2013 the Scientific Board of ECRIN recommended 8 multinational IDCR for support, from the 47 studies that were submitted. Presently 7 on-going multinational IDCR are supported by ECRIN (according to the information available on the ECRIN website on January 10th 2014).

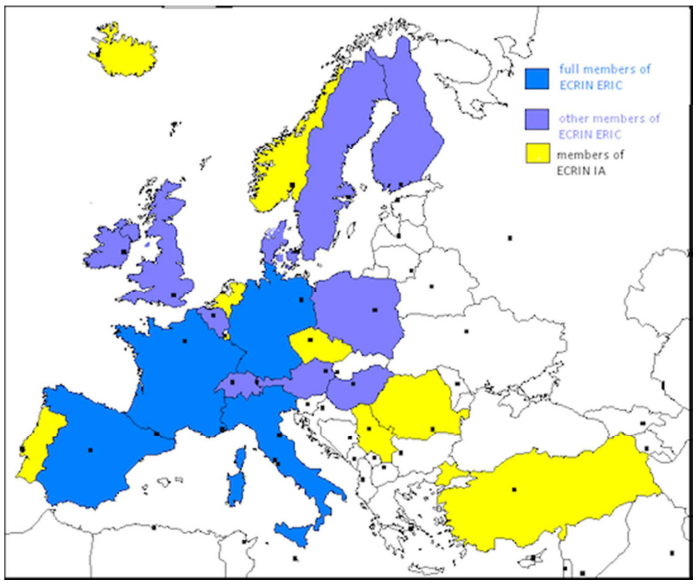

Figure 9. ECRIN partners (from ECRIN website, accessed on January 10th 2014).

2.9.2 Portuguese Clinical Research Infrastructure Network – PtCRIN

The PtCRIN is a Portuguese infrastructure with the purpose of facilitating and improving the quality of the clinical research in Portugal and promoting national and international collaboration. Its mission is to develop and organize a Portuguese clinical research infrastructure to provide coordination of the activities involved in clinical research, for IDCR mainly. PtCRIN was created in 2012 and links several existing centres (Figure 10) forming a hub for national and international multicentre IDCR managed by ECRIN.

This infrastructure concentrates efforts to promote clinical research in Portugal, contributing therefore for clinical research innovation.

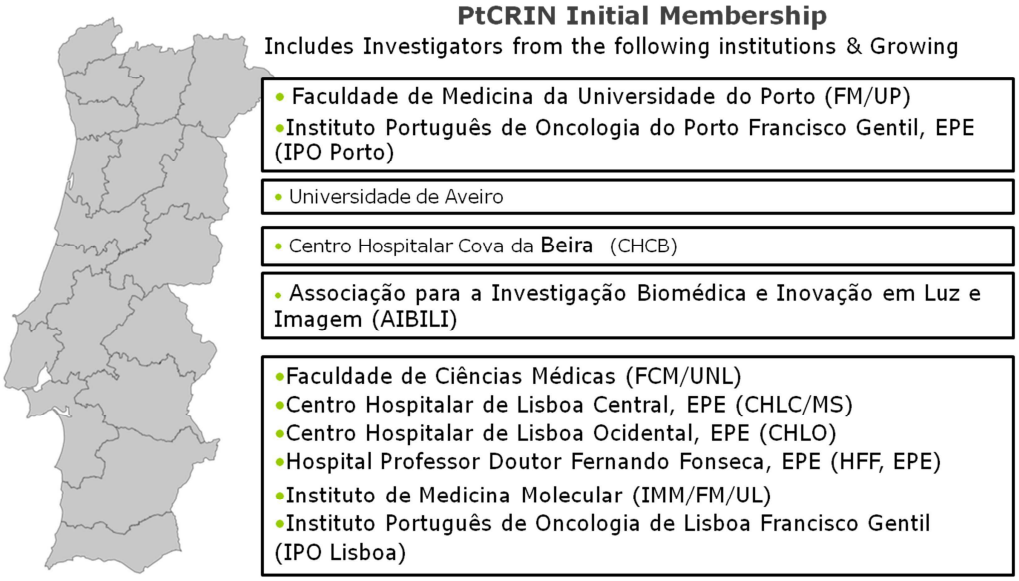

Figure 10. PtCRIN members (from ECRIN website, accessed on January 10th 2014).

2.9.3 Infrastructure for Clinical Eye Research: The Coimbra Coordinating Centre for Clinical Research – 4C

The Coimbra Coordinating Centre for Clinical Research (4C) from AIBILI was created in 2009 to support the design, submission and conduction of clinical studies in eye research, more precisely in ophthalmology. This centre starts as a small infrastructure working closely with the investigators of the Clinical Trial Centre of AIBILI (CEC) and with the investigators of the Coimbra University Hospital (CHUC).

The first studies supported by 4C in 2009, performed in AIBILI-CEC, were prospective, observational studies on diabetic retinopathy (DR) and age-related macular degeneration (AMD). By mid-2009 4C supported the elaboration and submission of its first prospective, multicentre, clinical trial, performed in 7 Portuguese clinical centres, and in 2010, 4C supported the design and coordination of its first prospective, observational, multinational study, conducted in 19 clinical centres from the EVICR.net.

4C has at the present a prominent place in clinical eye research, at national and international levels having skills as an academic CRO. 4C provides services for study design and statistical planning; protocol development; elaboration of study documents, such as the ICF and the CRF; development of operational and technical SOPs for the conduction of the study; study submission to regulatory authorities (RA); clinical sites feasibility; study coordination and implementation in the clinical sites; study monitoring and quality control; data management; reporting to the sponsor and/or to the RA; statistical analysis and final study report; support for scientific publication; and training for the research teams. 4C is ISO 9001 certified for Planning, Coordination, and Monitoring of Clinical Research Activities since 2011.
As the Coordinating Centre of the EVICR.net 4C has strengthened gradually its skills.
Presently 4C, through AIBILI, is part of the PtCRIN network, is one of the Champalimaud Translational Centre for Eye Research (C-TRACER) from the Champalimaud Foundation, and member of the VICT consortium.

3 DEVELOPMENT OF AN INFRASTRUCTURE FOR INVESTIGATOR-DRIVEN CLINICAL RESEARCH: THE COIMBRA COORDINATING CENTRE FOR CLINICAL RESEARCH – 4C

3.1 Implementation of an Infrastructure for Investigator-Driven Clinical Research

The Coimbra Coordinating Centre for Clinical Research (4C) was created in 2009 to support IDCR performed at the national level (mainly at AIBILI), and at the European level, i.e., within the network EVICR.net.

One of the initial IDCR that was performed at AIBILI, to study retinal changes in DR, was the "Prospective study of the initial changes occurring in the retinal microcirculation in diabetes by multimodal imaging of the eye fundus". This study included 40 patients with mild nonproliferative DR and received financial support from the national agency for science and technology (the Fundação para a Ciência e Tecnologia – FCT) (grant number POCTI/CBO/35866/1999). Conducted from November 2000 to May 2004, this IDCR allowed for the identification of different patterns of progression of DR using multimodal imaging techniques [38, 39]. Due to the small number of patients included, and the large number of parameters used, the results obtained needed to be validated, i.e., to be confirmed in a different and larger sample. Therefore, in 2007, a new clinical study was designed to validate the results of the previous study, and a sample of 400 patients was estimated to be necessary in order to achieve statistically significant results. This new IDCR, "Validation of a predictive model to estimate the risk of conversion to clinically significant macular edema and/or vision loss in mild nonproliferative retinopathy in diabetes type 2", was funded by the FCT (grant number

PDTC/SAU-OSM/72635/2006). Due to its dimension and scientific relevance, a supportive structure had to be created. This structure was composed by:

- A specialized team for the conduction of the clinical study that includes 400 type 2 diabetic patients with 3 visits per patient and with the acquisition of 2 different imaging techniques;
- A specialized team for statistical planning, data management and statistical analysis, to assure a good study design and a good coordination in order to achieve valid and solid results;
- A specialized team for image processing and analysis.

As member of the research team and responsible for the statistical planning of the study, its coordination and its analysis, this IDCR is one of the IDCR presented and discussed in this thesis (section 4.3 and section 4.4). This IDCR allowed for the identification and characterization of different phenotypes of DR progression based on imaging biomarkers assessed using two non-invasive methods.

Along with this study, other IDCR that were conducted at AIBILI in the area of DR and AMD, needed support for statistical planning and analysis. One of these IDCR was a prospective observational study that was designed to characterize early marker of neovascular AMD using multimodal imaging techniques. This IDCR is also one of the IDCR presented and discussed in this thesis (section 5.3), being responsible for the exploratory statistical analysis. This study highlighted the relevance of the multimodal imaging techniques for the correct and precise classification of retinal lesions in the early stages of the AMD, a disease that is responsible for 14 to 40% of the cases with vision impairment in Europe [40].

In Portugal the prevalence of this disease is unknown, and therefore, an epidemiologic study was planned to estimate the prevalence of AMD in Portugal and to characterize retinal lesions. AMD is characterized and classified based on the presence of some imaging biomarkers. The reproducibility of using a new semi-automated system for AMD lesions identification, and AMD grading is discussed in this thesis (section 5.3).

To assess the true prevalence of AMD in the Portuguese population aged 55 years or more, a collaboration was established in 2008 between AIBILI and the regional health administration (Administração Regional de Saúde – ARS do Centro). To step-up this IDCR in primary healthcare units of the central region of Portugal, a grant was requested to the industry. This study was the first IDCR fully supported by the 4C, i.e., from the study design, set-up in the primary healthcare units, coordination and statistical analysis. As member of the research team

and responsible for this support, in all the different phases of the study, this IDCR is presented and discussed in this thesis (section 5.5).

In the meantime, the EVICR.net registered in the last years a significant increase of its members that was followed by an increase of the requests for IDCR support within the network. This increases led the Coordinating Centre of the EVICR.net to develop within AIBILI an infrastructure to support multinational IDCR.

As a result, in 2009, a new unit of AIBILI, the Coimbra Coordinating Centre for Clinical Research – 4C, was created to support IDCR in their different stages, i.e. from the study design, submission, coordination, and final analysis. This new infrastructure was initiated by me, working closely with the EVICR.net Coordinating Centre and the Quality Management department of AIBILI.

The following areas of expertise were defined and developed:

- Study design;
- Study submission;
- Study implementation;
- Study coordination;
- Data management;
- Data analysis;
- Study publication.

For each of these areas different SOPs were elaborated to ensure that all the activities performed by the 4C, listed in Table 4, are ICH-GCP and ISO 9001 compliant.

Table 4. Activities performed by the 4C.

Pre-Study	In-Study	Post-Study
- Study Design	- Study Management and Coordination	- Data Analysis
- Protocol Design	- Monitoring (Initiation & Close-out)	- Final Study Report
- Inform Consent Form	- Auditing	- Regulatory Reports
- Sites feasibility	- Data Management	- Scientific Publication
- SOP Development	- Reporting to the Sponsor and/or RA	
- Submission to the RA	- Pharmacovigilance	
- Contracts Elaboration	- Data Validation	

With the consolidation of this infrastructure and involvement of other personnel, 4C evolved to a full unit of AIBILI, being certified in 2011 for Planning, Coordination and Monitoring of Clinical Research Activities. This infrastructure is the first national academic CRO dedicated to clinical eye research, providing services to the national network PtCRIN, and to the members of the VICT (i.e., IBILI, ICNAS and CRIO). The 4C has now 8 full-time persons, 5 shared persons and 4 medical consultants (Figure 11).

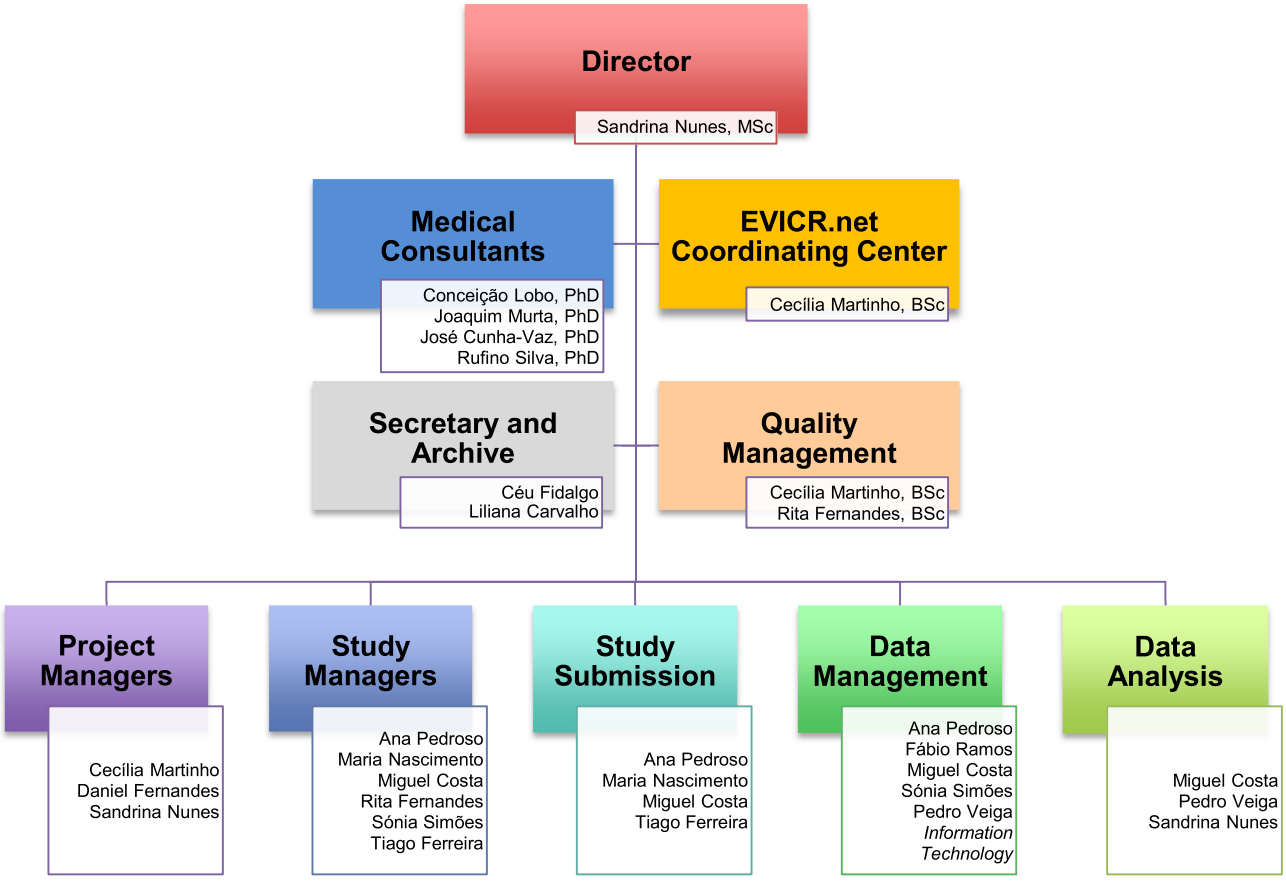

Figure 11. Organogram of the Coimbra Coordinating Centre for Clinical Research (4C) (organogram in April 10th 2014, more details are available at www.aibili.pt).

3.2 Activity of the Coimbra Coordinating Centre for Clinical Research – 4C

In 2009 4C was involved in the design, submission, coordination and/or data analysis of 6 national IDCR. Currently, 4C is responsible for the design, submission, coordination and/or data analysis of 16 IDCR (9 nationals and 7 internationals) (Figure 12).

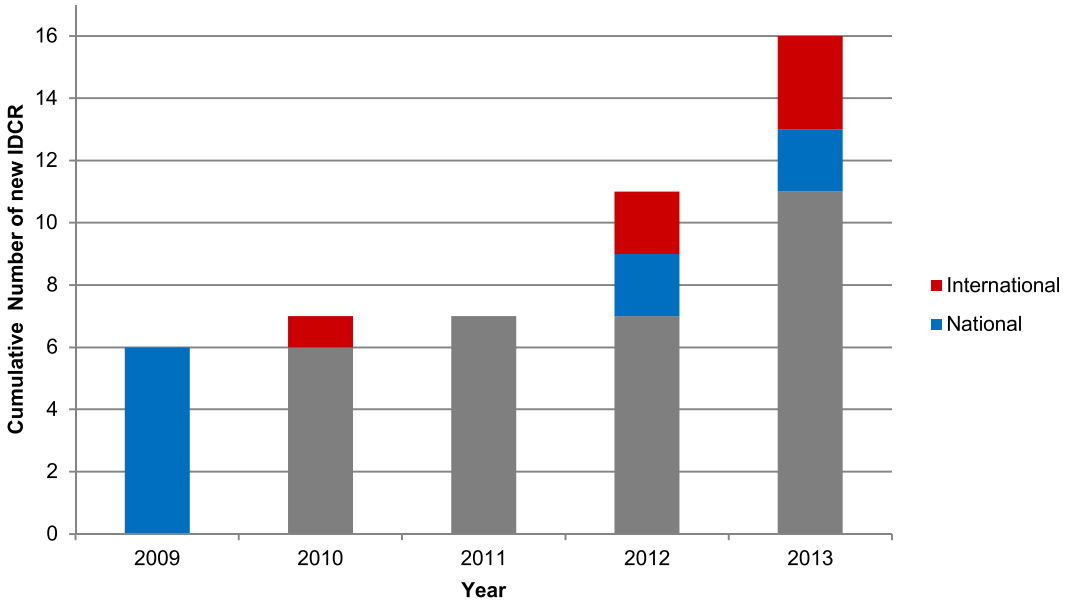

Figure 12. Cumulative number of new IDCR designed, submitted, coordinated, and/or analysed by 4C, from 2009 to 2013.

Based on the IDCR "Validation of a predictive model to estimate the risk of conversion to clinically significant macular edema and/or vision loss in mild nonproliferative retinopathy in diabetes type 2", 4 new IDCR were designed: one with the collaboration for the FMUC (NCT01228981); one with the network EVICR.net (NCT01145599); one with the Foundation Champalimaud and the two C-TRACERs (C-TRACER Coimbra, Portugal and C-TRACER Hyderabad, India) (NCT01607190); and one with the FMUC (IBILI) and ICNAS (NCT01440660).

The clinical study NCT01228981, conducted in collaboration with the FMUC, (entitled "Observational Study to Assess Genotypes/Phenotypes Correlations in Type-2 Diabetic Retinopathy"), was designed to identify genotypes associated with the 3 phenotypes of DR progression, identified in the first IDCR. A cross-sectional observational study was designed to

collect blood samples in the patients included in the first IDCR (NCT00763802). One hundred and seventy four (174) single nucleotides polymorphisms (SNP), from 11 candidate genes, are being analysed in order to identify correlations between the 3 different phenotypes of DR progression and the candidate genes.

The clinical study NCT01145599, conducted with the network EVICR.net, (entitled "Identifying progression of retinal disease in eyes with NPDR in type 2 diabetes using non-invasive procedures"), was the first IDCR conducted within the network EVICR.net. The aim of this study is the identification and characterization of DR progression in patients with diabetes type 2 and nonproliferative DR (NPDR) using colour fundus photography (CFP) and optical coherence tomography (OCT) (non-invasive procedures). This clinical study, conducted in 19 European clinical centres, began in 2010 and will end by mid-2014. Four hundred and forty six (446) patients were screened between September 2010 and April 2012. From the 400 type 2 diabetic patients with NPDR planned initially, 374 were included. Three hundred and thirty one (331) patients completed the study (i.e., completed the 12-month visit or developed CSME during the study).

The inclusion rate was 6.5% below the expected, due to the lower number of participating centres. From the initial 23 clinical centres only 19 could initiate this study without funding. The main reasons being administrative and/or ethics, because this clinical study was the first IDCR performed within the network; had no financial support; and was considered, by some clinical centres, non-observational, due to some of the procedures required, mainly CFP and OCT. The drop-out rate, on the other hand, was 11.5%, i.e., below the normal drop-out rate for this type of study and type of patients (i.e., approximately 30% for a one-year follow-up period [41]). This low drop-out rate reduced the impact of the lower inclusion rate, allowing for an adequate statistical power of the study. It should be pointed-out that this lower drop-out rate could only be possible due of the active involvement of the EVICR.net clinical centres' research team and the EVICR.net Coordinating Centre (4C).

At the moment the study data is under analysis for the final study report, which is expected by mid-2014.

The clinical study NCT01607190, conducted in the two C-TRACERs, C-TRACER Coimbra (Portugal) and C-TRACER Hyderabad (India), (entitled "Biomarkers of Diabetic Retinopathy Progression"), was the first study conducted within the C-TRACERs with support from the Foundation Champalimaud. The aim of this study is to validate the predictive model of DR

progression (i.e., the 3 different phenotypes of DR progression) in two different populations of type 2 diabetic patients (i.e., Portugal and India). This clinical study, conducted in Portugal and India, began in 2012 and will end by 2015. The recruitment ended in March 2014. From November 2012 (First Patient First Visit – FPFV) to March 2014 (Last Patient First Visit – LPFV) 205 patients were included (101 in Portugal and 104 in India) (from the 200 originally planned, 100 in each centre).

The recruitment period was extended over 1 year to overcome the difficulties observed in the Indian centre. Several issues explain the slow recruitment rate. First, the principal investigator left the centre and therefore a new submission was needed to the local ethics committee. After the approval by the local ethics committee a new contract was signed between the Sponsor-Investigator (AIBILI), the clinical centre (Institute LV Prasad) and the new principal investigator. These processes delayed significantly the start of the recruitment. Moreover, difficulties in recruiting DR patients in India were reported by the investigators. These difficulties are related mainly to the fact that DR patients with mild NPDR, i.e., without visual complaints, do not appear frequently in hospital; the fact that most of the patients that appears in the hospitals have cataract and/or maculopathy (exclusion criteria for this study) and due to the deficient public transport services in India.

At the present patients are being followed. The clinical phase of the study should ended by March 2015 (Last Patient Last Visit – LPLV), and the final study results should be ready by December 2015.

The clinical study NCT01440660, conducted with the FMUC and ICNAS, (entitled "Phenotypes of nonproliferative diabetic retinopathy in DM 2 patients Identified by OCT, CFP, RLA and mfERG (DIAMARKER)"), aims to characterise phenotypes of NPDR progression using ophthalmological, psychophysical and brain multimodal testing/imaging procedures. This clinical study began in 2012 and should end in 2014. From January to July 2012, 22 type 2 diabetic patients were included, from the 20 patients initially planned. Two additional patients were included to compensate two patients that withdrawn the study prematurely. The study is still on-going, the LPLV is expected by July 2014, and the final study results by December 2014.

Based on the epidemiologic study for the determination of the prevalence of AMD in the Portuguese population, one new IDCR was designed to identify nutritional and lifestyle risk factors for AMD, i.e., in the population included in the epidemiologic study (entitled “Life Style and Food Habits Questionnaire in the Portuguese Population Aged 55 or More”). This study

started in 2012 and is expected to be concluded by 2015. At the moment 1000 participants were included. This study will provide a population-based sample in which precise and contemporary information on the frequency of nutritional and lifestyle risk factors will be collected, enabling to evaluate whether the separate and/or joint effects of these factors are likely to have a major impact in terms of AMD.

Also, in 2009 4C was involved in the elaboration, submission and coordination of 2 national clinical trials, both in proliferative DR. Both IDCR aimed to assess the efficacy and safety of intravitreal injections of anti-VEGF. The clinical trial entitled: "Prospective, Randomized, Open Label, Phase II Study to Assess Efficacy and Safety of Macugen® (Pegaptanib 0.3 mg Intravitreal Injections) Plus Panretinal Photocoagulation (PRP) and PRP (Monotherapy) in the Treatment of Patients With High Risk Proliferative Diabetic Retinopathy (PDR)", was performed in the Clinical Trial Unit of AIBILI (CEC), being at the moment under the elaboration of the study publication (NCT01281098). The other clinical trial entitled "Prospective, Randomized, Multicentre, Open Label, Phase II Study to Access Efficacy and Safety of Lucentis® Monotherapy Compared With Lucentis® Plus Panretinal Photocoagulation (PRP) and PRP in the Treatment of Patients With High Risk Proliferative Diabetic Retinopathy", was conducted in 7 national clinical centres, being at the moment under data analysis.
Based on these two clinical trials, mainly on the second one, one international, multicentre, clinical trial was planned within the EVICR.net (entitled "Prospective, randomized, multicentre, open label, phase II / III study to assess efficacy and safety of ranibizumab 0.5 mg intravitreal injections plus panretinal photocoagulation (PRP) versus PRP in monotherapy in the treatment of subjects with high risk proliferative diabetic retinopathy. (PROTEUS)"). This clinical trial, on-going in 12 clinical centres from 4 European countries, received a grant from industry and started recently (FPFV – May 2014) (NCT01941329).

Since 2012 AIBILI, through the EVICR.net Coordinating Centre, is participating in 2 European projects funded by the European Commission under the 7th Framework Programme (FP7), the projects EUROCONDOR ("European Consortium for the Early Treatment of Diabetic Retinopathy") (NCT01726075) and STRONG ("European Consortium for the Study of a Topical Treatment of Neovascular Glaucoma") (FP7 Proposal number 305321, study synopsis not yet published online). Both projects involved the performance of clinical trials, for the EUROCONDOR project, 11 clinical centres are participating in the clinical trial on DR, and for

the STRONG project, 35 clinical centres are planned to participate in the clinical trial on neovascular glaucoma (a rare condition).

In 2013, 2 prospective studies were initiated, one observational study, to be conducted in one clinical centre (CEC) (entitled “Characterization of Eyes with Diabetic Macular Edema that show different Treatment Response to Intravitreal anti-VEGF (CHARTRES)”), and one clinical trial, to be conducted in 9 European clinical centres (entitled “Intraocular pressure and tolerability Study of Preservative Free Bimatoprost 0.03% Unit Dose (BUDPF) or preservative free Latanoprost 0.005% Unit Dose (LUDPF) (Monoprost®) in patients with Ocular hypertension or glaucoma: A Randomized, single masked, 3 month cross-over, Investigator led, European multicentre Trial. (SPORT)”).

All these studies demonstrate recognition of the competence and the capacity of the 4C as an infrastructure for IDCR, both at the national and the international level (through EVICR.net).
At the national level the 4C contributed significantly for the increase of the number of IDCR in eye research. According to the data available on the ClinicalTrials.Gov website, on January 10th 2014, from the 1006 clinical studies registered in Portugal (Figure 2), 219 are IDCR (21.8%). From these IDCR, 23 (10.5%) are on clinical eye research, AIBILI participates in 18 (78.3%) and the 4C supported or is supporting 12 (52.1%).
With a greater support for IDCR in clinical eye research it is expected to increase the number of clinical studies in this area, strengthening therefore the level of clinical evidence of the current medical practice, and contributing for a more and better patient-oriented research.

The list of the IDCR supported by the 4C is shown in Table 5 (clinical studies synopses are available on the ClinicalTrials.Gov website).

Table 5. IDCR supported by the 4C since 2009 for elaboration, submission, coordination and/or data analysis (until January 10th 2014).

Study type	Status	Study Title Study Identifier (ClinicalTrials.Gov) Number of Clinical Sites / Planned Number of Participants	Grant/Support
NATIONAL IDCR			
Epidemiological	Completed	"Epidemiological Study of the Prevalence of Age-Related Macular Degeneration in Portugal" NCT01298674 Clinical Sites: 2 / Participants: 6000	Industry
Survey	On-going	"Life Style and Food Habits Questionnaire in the Portuguese Population Aged 55 or More" NCT01715870 Clinical Sites: 2 / Participants: 2000	Industry
Prospective observational	Completed	"Validation of a Predictive Model to Estimate the Risk of Conversion to Clinically Significant Macular Edema and/or Vision Loss in Mild Nonproliferative Diabetic Retinopathy in Diabetes Type 2 (CPM)" NCT00763802 Clinical Sites: 1 / Participants: 400	FCT
Cross-sectional observational	Completed	"Observational Study to Assess Genotypes/Phenotypes Correlations in Type-2 Diabetic Retinopathy" NCT01228981 Clinical Sites: 1 / Participants: 400	FCT
Prospective observational	On-going	"Phenotypes of Nonproliferative Diabetic Retinopathy in DM 2 Patients Identified by OCT, CFP, RLA and mfERG (DIAMARKER)" NCT01440660 Clinical Sites: 1 / Participants: 20	National Strategic Reference Framework
Exploratory prospective observational	Completed	"Characterization of Early Markers of Choroidal Neovascularization (CNV-MARKERS)" NCT00801541 Clinical Sites: 1 / Participants: 60	AIBILI
Prospective clinical trial	Completed	"Prospective, Randomized, Open Label, Phase II Study to Assess Efficacy and Safety of Macugen® (Pegaptanib 0.3 mg Intravitreal Injections) Plus Panretinal Photocoagulation (PRP) and PRP (Monotherapy) in the Treatment of Patients With High Risk Proliferative Diabetic Retinopathy (PDR)" NCT01281098 Clinical Sites: 1 / Participants: 34	Industry
Prospective clinical trial	Completed	"Prospective, Randomized, Multicentre, Open Label, Phase II Study to Access Efficacy and Safety of Lucentis® Monotherapy Compared With Lucentis® Plus Panretinal Photocoagulation (PRP) and PRP in the Treatment of Patients With High Risk Proliferative Diabetic Retinopathy" NCT01280929 Clinical Sites: 7 / Participants: 54	Industry
Prospective observational	On-going	"Characterization of Eyes with Diabetic Macular Edema that show different Treatment Response to Intravitreal anti-VEGF (CHARTRES)" NCT01947881 Clinical Sites: 1 / Participants: 70	Industry
INTERNATIONAL IDCR			
Prospective observational	Completed	"Identifying Progression of Retinal Disease in Eyes With NPDR in Diabetes Type 2 Using Non-invasive Procedures (RET02)" NCT01145599 Clinical Sites: 23 / Participants: 450	EVICR.net
Prospective clinical trial	On-going	"Trial to Assess the Efficacy of Neuroprotective Drugs Administered Topically to Prevent or Arrest Diabetic Retinopathy (EUROCONDOR)" NCT01726075 Clinical Sites: 12 / Participants: 450	European Union

Table 5. IDCR supported by the 4C since 2009 for elaboration, submission, coordination and/or data analysis (until January 10th 2014).

Study type	Status	Study Title Study Identifier (ClinicalTrials.Gov) Number of Clinical Sites / Planned Number of Participants	Grant/Support
Prospective clinical trial	On-going	"Prospective, randomized, multicentre, open label, phase II / III study to assess efficacy and safety of ranibizumab 0.5 mg intravitreal injections plus panretinal photocoagulation (PRP) versus PRP in monotherapy in the treatment of subjects with high risk proliferative diabetic retinopathy. (PROTEUS)" NCT01941329 Clinical Sites: 12 / Participants: 94	Industry
Prospective clinical trial – rare disease	On-going	"European Consortium for the Study of a Topical Treatment of Neovascular Glaucoma – the STRONG Study" FP7 Proposal number 305321 *Study synopsis not yet published online* Clinical Sites: >20 / Participants: 333	European Union
Prospective observational	On-going	"Biomarkers of Diabetic Retinopathy Progression" NCT01607190 Clinical Sites: 2 / Participants: 200	Champalimaud Foundation
Prospective clinical trial	On-going	"Intraocular pressure and tolerability Study of Preservative Free Bimatoprost 0.03% Unit Dose (BUDPF) or preservative free Latanoprost 0.005% Unit Dose (LUDPF) (Monoprost®) in patients with Ocular hypertension or glaucoma: A Randomized, single masked, 3 month cross-over, Investigator led, European multicentre Trial. (SPORT)" NCT01975714 Clinical Sites: 9 / Participants: 67	Industry
Prospective observational – rare disease	On-going	"Clinical and genetic examination of Usher syndrome patients' cohort in Europe (EUR-USH)" – E-Rare2 project "European young investigators network for Usher Syndrome" NCT01954953 Clinical Sites: 2 / Participants: 400	European Union and FCT

4 CONTRIBUTION TO CLINICAL EYE RESEARCH IN DIABETIC RETINOPATHY

4.1 Investigator-Driven Clinical Research in Diabetic Retinopathy

From the 6087 clinical studies performed in eye research worldwide (according to the public database ClinicalTrials.Gov), 595 (9.8%) are on diabetic retinopathy (DR). Three hundred and eighty (380, 63.9%) are initiated by the investigators (e.g., clinical research networks, governments, national institutes of health, etc.), and 215 (36.1%) are initiated, or performed in collaboration with the industry. Also, 133 studies (22.3%) are observational, 461 (77.5%) are non-observational and 1 (0.2%) is expanded access.
One third of the clinical research performed in DR is on diabetic macular edema (27.1%), one of the major complication of DR and principal cause of vision loss. From the 161 clinical studies performed on diabetic macular edema, 54.0% are IDCR (87) while 46.0% are initiated, or performed in collaboration with the industry (74). The main areas of clinical research in DR are listed in Table 6.

Regarding the primary outcomes used for DR, the primary outcomes most frequently used are the visual acuity (271 studies, 45.5%) and the retinal thickness, assessed using OCT (208 studies, 34.9%). Fluorescein angiography and CFP are used for the primary outcomes assessment in less than 10% of the clinical studies (38 studies used fluorescein angiography and 15 studies used CFP).

Table 6. List of conditions studied in DR (84 studies are registered only as DR being not listed below and some of the conditions are studied in the same clinical study) (data from the ClinicalTrials.Gov website, accessed on January 10th 2014).

DR Conditions (total 595 clinical studies)	Number of Studies	Percentage
Diabetic macular edema	161	27.1%
Diabetes mellitus (not specified)	120	20.2%
Macular degeneration	63	10.6%
Retinal vein occlusion	20	3.4%
Cardiovascular diseases	18	3.0%
Glaucoma	15	2.5%
Retinal disease (not specified)	13	2.2%
Choroidal neovascularization	12	2.0%
Vitreous hemorrhage	11	1.8%
Uveitis	9	1.5%
Cataract	8	1.3%
Retinal detachment	7	1.2%
Vision disorders (not specified)	7	1.2%
Vitrectomy	7	1.2%
Retinal neovascularization	5	0.8%
Retinitis	5	0.8%
Inflammation	4	0.7%
Macular hole	4	0.7%
Diabetic neuropathy	3	0.5%
Myopia	3	0.5%
Tractional retinal detachment	3	0.5%
Severe diabetic retinopathy	2	0.3%
Retinal artery occlusion	2	0.3%
Vitreous detachment	2	0.3%
Hemorrhage	2	0.3%
Pregnancy	2	0.3%
Central serous retinopathy	2	0.3%
Telangiectasia	2	0.3%
Retinal degeneration	1	0.2%

4.2 Diabetic Retinopathy

Diabetes Mellitus (DM) is a multifactorial disease whose prevalence and incidence is increasing [42]. Estimates from the WHO reported the existence in 2000 of 171 million people with DM, with an expected increase in the number of cases to 366 million by the year of 2030 [43].

Diabetic retinopathy (DR), is one of the complications associated with DM. DR is a chronic disorder of the retina characterized by progressive changes in the retinal microvasculature. The development and progression of this disease varies with the type of diabetes (type 1, or insulin dependent and type 2, non-insulin dependent) as well as from patient to patient, since not all the patients progress to the proliferative form of DR [44].

DR is currently one of the leading causes of vision loss in the working population in the Western world, being responsible for 4.8% of the blindness in the world [43] and accounting for 10% of the new cases of blindness each year [42]. Most diabetic patients will develop RD, showing the initial form of the disease, i.e., nonproliferative DR (NPDR), or the more advanced form of DR, i.e., proliferative DR (PDR) [45].

The development of DR depends, among other factors, of the type and duration of DM. Thirteen percent (13%) of the patients with DM type 1 and with less than 5 years of DM will develop DR. When the duration of diabetes is higher than 10 years the prevalence of DR increase to 90% [43]. For patients with DM type 2, a quarter of the patients with less than 5 years of DM will develop DR (24%). The prevalence of DR doubles when the duration of diabetes is higher than 15 years (53%) and when the patient is insulin-dependent [43]. For patients taking insulin, the incidence of DR increase to 40%, when the duration of DM is less than 5 year, and 80%, when the duration of DM is higher than 15 years [43, 46, 47, 48]. After 15 to 20 years of DM, more than 90% of the type 1 diabetic patients develop DR, 40% of which develop PDR. For type 2 diabetic patients 60% of the patients will develop diabetic macular edema (DME), while 20% of the patients will develop PDR. Smoking, insulin, blood pressure, lipid levels, and glycosylated haemoglobin A_{1C} (HbA_{1C}) levels are also associated with the progression of DR [49, 46, 50]. However, these factors, that characterize the progression of the disease, cannot explain the variability observed in different diabetic patients. The identification of genetic and environmental factors [51, 52] that may contribute to a more or less rapid progression of DR is still a major challenge [44].

It is in the early stages of the disease that DR treatment is more effective for the reason that it is in the early stages that some biological processes, involved in DR progression, are still

reversible. To prevent, reduce and/or stop the progression of the disease, providing to the patients a safer and healthy life, it is crucial to identify and implement a model able to identify in the early stages of the disease, patients with higher risk of vision loss and that may respond positively to the treatment. It is therefore important to identify biological markers (anatomical, physiological, biochemical and/or molecular parameters) designated as biomarkers [53, 54], that are associated with the presence and/or severity of DR and that can be used for early detection and monitoring of the disease progression.

4.3 Biomarkers of Diabetic Retinopathy Progression

Duration of DM, glycemic and blood pressure control are risk factors for the development and progression of DR [55, 56], however these factors can only explain partially the variance observed in DR patients, since some patients with good glycemic control develop rapidly progressive forms of DR, while some patients with a relatively poor glycemic control take several years to manifest signs of DR. Therefore, it is difficult to predict the clinical course and to identify which eyes/patients will develop vision-threatening retinopathy complications. The identification of biomarkers of disease progression is therefore of major importance for patient-oriented clinical care and for better understanding the pathogenesis of DR involved in disease progression. Biomarkers are clinically useful when preceding the development of sight threatening outcomes, serving as a risk factor, or when acting as a surrogate for an outcome that should be avoided. The identification of biomarkers for DR progression, namely the identification of retinal biomarkers, is therefore expected to contribute for the early diagnosis of DR, for the clinical monitoring and for a better risk stratification among patients with DM.

Any retinal biomarker of DR progression should be associated with vision loss, the most commonly accepted clinically outcome in DR. However, vision loss only occurs when approximately 50% of the neuronal component of the macula is damaged, which is clearly in the advanced stages of the retinal disease. Vision loss is associated with two major complications of DR, CSME[19] and PDR, and does not occur before these two complications. However, CSME and PDR, which are also clinically meaningful outcomes, are also associated with the advanced stages of the DR. There is therefore a clear need for the identification of retinal biomarkers of DR progression, associated with the retinal alterations that occur in the early stages of the disease and that can predict development of these clinical outcomes.

The initial stages of DR are characterized by four major retinal alterations: presence of microaneurysms/haemorrhages, alterations of the blood-retinal barrier (BRB) permeability, capillary closure and changes in the neuronal and glial cells of the retina [57, 44]. The

[19] CSME is defined by the Early Treatment Diabetic Retinopathy Study (ETDRS) based on the following criteria: a) Thickening of the retina located less than 500 μm from the center of the macula; OR b) Hard exudates (with thickening of the adjacent retina) located less than 500 μm from the center of the macula; OR c) A zone of retinal thickening 1 disc area or larger in size, located less than 1 disc diameter from the center of the macula [102].

pathological changes are located in the small vessels of the central retina (macula) and include damage of the endothelial cells and pericytes in the vessels. Some of these changes can be detected using imagiology techniques. Microaneurysms, associated with changes occurring in the vascular walls, can be identified on colour fundus photographs, detected as small red dots (small haemorrhages, themselves visible as small red spots can be confused with microaneurysms), or on fluorescein angiographs, detected as small hyperfluorescent dots [58]. Changes of the BRB permeability, associated with changes occurring in the retinal vascular endothelium, can be detected by fluorometry, fluorescein angiography (FA) and/or retinal leakage analyser (RLA), that allow for the visualization and/or quantification of the fluorescein injected in the DR patient that leak from the retina to the vitreous. An indirect way to identify and quantify BRB permeability changes is through the visualization and/or quantification of the retinal thickness increase, since increase in retinal thickness is associated with the presence of fluid within the retinal layers, i.e., with retinal edema [59].
Candidates for retinal biomarkers of DR progression are therefore the microaneurysm turnover and the central retinal thickness.

4.3.1 Microaneurysms Activity

The presence of microaneurysms (MA), identified as red-dots, is one of the initial retinal changes in DR, being visible on ophthalmoscopic examination [44]. MA activity, formation and disappearance, is a major biomarker of the disease severity and progression [44, 60, 61].
MA formation and disappearance are dynamic processes. In a 2-year follow-up study of 24 type 1 diabetic patients with mild DR, Hellstedt and Immonen [62] observed, using FA, 395 new MA and the disappearance of 258 MA previously identified. The disappearance of a MA is not usually a reversible process and indicates vessel closure and progressive vascular damage. Therefore, to assess progression of DR, MA counting should take into account not only every newly developed MA identified in a new location but also the disappearing ones. MA disappearance is most probably due to thrombotic phenomena leading to subsequent rerouting of capillary blood flow and progressive remodelling of the retinal vasculature in diabetes [63]. The presence and number of MA and their rates of formation and disappearance are, therefore, good candidates as biomarkers of retinal vascular remodelling and may be good indicators of DR progression. The higher the MA activity the higher is the risk for CSME development [64, 65, 66].

4.3.1.1 Microaneurysms Turnover assessed based on Colour Fundus Photography

MA counting on colour fundus photographies and MA counting on fluorescein angiographies have been proposed as predictive factors for DR progression [67]. CFP is a non-invasive imaging technique consisting of colour photographs of the eye fundus in which some retinal structures are visible. The photographs, or retinographs, can be obtained by a conventional camera or by a digital camera. Due to the non-invasive nature of this technique, efforts had been made to obtain a reliable biomarker for the MA activity based on CFP.

The identification of MA requires some training of the clinicians responsible for earmarking the MA (graders). In 2009 a new semi-automatic method for MA earmarking was developed at AIBILI to count automatically MA by mapping their locations through image co-registration [68]. MA were defined as small lesions that appears as a round or ovoid red spots of 20-200 μm in diameter with regular borders and located within the superior and inferior arcades. The identification of the exact location of an individual MA is particularly important because a new MA is considered to develop only once in a specific location, its disappearance being generally associated with capillary closure, leaving in its place mainly remnants of basement membrane [69, 70].
To validate this new methodology, 235 colour fundus photographs, from 47 patients/eyes with 5 visits each, were graded by 3 trained ophthalmologists. Despite the good agreement found between graders for the number of MA (inter-grader agreement), only 60% of the MA detected by one grader were detected by a second grader. The inter-grader agreement improved significantly when computing the MA turnover (i.e., the MA formation and disappearance rates), which is possible by using this new methodology that registers the exact location of the MA and projects it on the same fundus reference [68].

This methodology was used in a retrospective 10 years follow-up study of 113 type 2 diabetic patients with mild-to-moderate NPDR [64] (see section 4.3.1.3). Patients were followed up for 2 years as controls in DR clinical trials, and thereafter by usual care at the same institution. MA turnover from the initial 2 years and the occurrence of CSME during the following 8 years were analysed.
At the end of the 10-year follow-up period, 17 out of the 113 patients developed CSME needing photocoagulation.

Patients that developed CSME presented at baseline HbA_{1C} levels significantly higher (8.5 ± 1.2 %) than the group of patients that did not develop CSME (7.3 ± 1.2 %; P = 0.001). No statistically significant differences were found at baseline between CSME and non-CSME eyes for blood pressure, cholesterol, high-density and low-density lipoproteins (HDL and LDL) and triglycerides levels.

When counting the total number of MA over the first 2 years of the follow-up, a significant increase in the number of MA was found for the CSME eyes (P = 0.002), while for the non-CSME eyes the number of MA remained relatively constant (P = 0.647). Also, both MA formation and disappearance rates were found to be significantly higher in the eyes that developed CSME (P < 0.001). A MA formation rate of at least 2 MA per year was found in 12 of the 17 eyes that developed CSME (70.6%), whereas this was only found in 8 of the 96 eyes that did not develop CSME during the 10-year follow-up period (8.3%).

This study showed that in the initial stages of DR a MA formation rate ≥ 2 MA per year is a good surrogate outcome for CSME development (sensitivity and specificity of 70.6% and 91.7%, respectively, [64]), and that the MA turnover, i.e., MA formation and disappearance rates, computed based on the precise location of each MA on CFP, can be used as a retinal biomarker of DR activity and progression to CSME [66].

4.3.1.2 Automated Microaneurysms Turnover assessment

To overcome the human intra-grader variability for the MA detection a software was developed by Critical Health and AIBILI, the RetmarkerDR. The RetmarkerDR software (Critical Health SA, Coimbra, Portugal), developed based on a patent registered by AIBILI[20], is a medical device that was certified for automatic MA earmarking.

The automated computer-aided diagnostic system, RetmarkerDR, consists of a software that earmarks MA and vascular lesions. It includes a co-registration algorithm that allows comparison within the same retinal location between different visits for the same eye. Its performance is not affected by fatigue, stress, ambient light conditions, or other factors that may influence a human grader. The algorithm detects the presence of MA and red-dot like lesions. To detect these pathologies, the images are initially converted to a processing size and subject afterwards to a contrast normalization and enhancement based on principal component analysis. Dark objects of a given size are thereafter detected and used as candidates.

[20] System for Analysing Ocular Fundus Images, United States Patent n.º 7,856,135 (www.uspto.gov, accessed on September 15th 2013).

For each of these candidates, features such as area, shape, intensity distribution, and gradient magnitude distribution are extracted. Next, a state of the art classifier, based on support vector machines is used to classify the candidates as true or false. The training of this classifier was done with a specific dataset in which an human grader was asked to earmark only small lesions that appeared as a round or ovoid red spots of 20-200 μm in diameter with regular borders and located within the superior and inferior arcades. Since by ophthalmoscopy or CFP MA are identified as deep red-dots, sometimes they are difficult to differentiate from punctuate haemorrhages or localized vascular abnormalities. Punctuate haemorrhages typically have irregular borders, if the borders happen to be more regular they may be wrongly classified as MA [71, 64].
Images from field 2 are then co-registered [72] in the central 3000 μm circle of the macula. Image co-registration is achieved by extracting a retinal vascular tree, which is used for landmarks during the registration process. A rigid registration estimates the translation based on fovea displacement. This rigid transformation is then adjusted to obtain exact pairings of selected landmarks.

The RetmarkerDR software allows therefore for the automatic detection of the MA in colour fundus photographs, allowing, by a co-registration process of images acquired in a given subject over time, to identify automatically new MA such as MA that disappear over time [65, 66]. The following parameters are automatically computed (Figure 13):

- Number of MA in each visit;
- MA formation rate (i.e., for a given period of time the number of new MA); and
- MA disappearance rate (i.e., for a given period of time the number of MA that disappeared).

To validate the RetmarkerDR software the results obtained by Nunes et al. (2009) [64] in the 10 years follow-up study were replicated. The software was used in the 113 type 2 diabetic patients, and the results obtained confirmed the previous results, i.e., that 70.6% of the eyes that developed CSME showed a MA formation rate ≥ 2 MA per year. In a different study with 160 type 2 diabetic eyes followed-up during 5 years [73], it was also possible to demonstrate that a MA formation rate ≥ 2 MA per year in the early stage of DR is present in 71.4% of the eyes that developed CSME.

Figure 13. MA detection in colour fundus photographs using the RetmarkerDR software (case 10003 with 5 visits of follow-up – top right).

The RetmarkerDR was used more recently in a prospective, observational 2-year study of 410 eyes/patients with mild NPDR (Early Treatment Diabetic Retinopathy Study - ETDRS levels 20 and 35) [65] (see section 4.3.1.3). Men and women, with type 2 DM, aged 40 to 80 years with best-corrected visual acuity (BCVA) ≥ 95 ETDRS letters, underwent in the 3 study visits (baseline, month-6 and month-24), a complete eye examination, which included CFP. Of the 410 eyes/patients, 348 were used for the RetmarkerDR analysis because they reached either the study endpoint, development of CSME (26 eyes/patients) or completed the 24-month visits without developing CSME (322 eyes/patients).

MA turnover (i.e., the sum of the MA formation and disappearance rates) was 11.2 ± 11.2 in the 26 eyes/patients that developed CSME and 5.0 ± 5.2 in the remaining 322 eyes ($P < 0.001$). The MA turnover showed a higher predictiveness for CSME than the remaining MA parameters (with an area of the Receiver Operating Characteristics (ROC) of 0.695). A MA turnover of 9 or more showed a sensitivity for CSME development of 57.7% and a specificity of 81.2%. In this study, it was found that eyes with a MA turnover ≥ 9 during the initial 6-month period showed a higher risk for CSME development than eyes with a lower MA turnover, Odds Ratio (OR) of 5.886; 95% CI (2.503-13.844). Furthermore, eyes that developed CSME before the 24 months visit presented a higher MA turnover (26.6 ± 15.9) when compared to the eyes in which CSME was detected only at month-24 (12.8 ± 3.6; $P = 0.018$), indicating an association between higher MA turnover values and the risk for the development of CSME for eyes with the same level of

DR. A multivariate analysis, Poisson Regression Analysis, showed that the MA turnover is predictive of CSME development independently of the HbA_{1C} level.

The results obtained with the RetmarkerDR for the automatic MA assessment, based on the precise identification of the location of each MA on CFP in diabetic eyes, suggest that MA formation and disappearance rates (i.e., MA turnover) may be an appropriate retinal biomarker of disease activity and DR progression to CSME (clinical outcome that is associated with vision loss).

4.3.1.3 Scientific Contribution

The following contributions were published:

- S. Nunes, I. Pires, A. Rosa, L. Duarte, R. Bernardes, and J. Cunha-Vaz. Microaneurysm turnover is a biomarker for diabetic retinopathy progression to clinically significant macular edema: Findings for type 2 diabetics with nonproliferative retinopathy. Ophthalmologica, 223:292–297, 2009.
- L. Ribeiro, S. Nunes, and J. Cunha-Vaz. Microaneurysm turnover at the macula predicts risk of development of clinically significant macular edema in persons with mild nonproliferative diabetic retinopathy. Diabetes Care, 36:1254–1259, 2013.

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Microaneurysm Turnover Is a Biomarker for Diabetic Retinopathy Progression to Clinically Significant Macular Edema: Findings for Type 2 Diabetics with Nonproliferative Retinopathy

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Key Words
Microaneurysm count · Microaneurysm turnover · Microaneurysm formation rate · Microaneurysm disappearance rate · Diabetic retinopathy · Image co-registration · Color fundus photography

Abstract
Purpose: To examine the relationship between microaneurysm turnover (formation rate), using a new semi-automatic method (MA-Tracker) based on color fundus photographs, and diabetic retinopathy (DR) progression to clinically significant macular edema (CSME). ***Methods:*** In total, 113 patients/eyes with nonproliferative DR (NPDR) were followed up every 6 months for 2 years as controls of the DR clinical trials, and by conventional general and ophthalmological care for the next 8 years (over a total of 10 years' follow-up). Microaneurysm turnover for the 2 first years was computed using the MA-Tracker. ***Results:*** The 17 patients that developed CSME over the 10 years of follow-up presented a microaneurysm formation rate of 9.2 ± 18.2 microaneurysms/year (mean ± SD) during the first 2 years, which was statistically higher than the eyes that did not develop CSME (0.5 ± 1.2 microaneurysms/year, $p < 0.001$). These 17 patients also presented higher HbA_{1C} levels at baseline (8.5 ± 1.2%) compared to the patients who did not develop CSME (7.3 ± 1.2%, $p = 0.001$). ***Conclusions:*** A high microaneurysm formation rate on color fundus photographs appears to be a good biomarker for DR progression to CSME in type 2 diabetic patients with NPDR.

Introduction

Diabetic retinopathy (DR) is one of the major causes of visual loss in the western world. This disease affects the retinal microvasculature, and is characterized in its initial stages by the presence of microaneurysms and/or dot hemorrhages [1].

Several studies have already shown that even the presence of only 1 or 2 microaneurysms [2] (primarily microaneurysm dynamics in the early stages of diabetic retinopathy [3]) indicate an increased risk of disease progression to the level requiring laser photocoagulation [2, 4–6].

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It is now widely accepted that DR lesions may be reversed and that retinopathy progression can be slowed, but only in the early stages of the disease. The detection of these initial lesions using noninvasive methods of fundus imaging, mainly of microaneurysms and small hemorrhages, is expected to open new opportunities for improved management of retinopathy [2].

In a previous study, our group has shown that microaneurysm formation and disappearance rates (microaneurysm turnover) obtained from color fundus photographs using proprietary software, the MA-Tracker, show a very good agreement between graders in the initial stages of the DR.

Using this new methodology in this study, we analyzed data from a group of 113 type 2 diabetic patients with mild-to-moderate nonproliferative DR (NPDR), followed up for 2 years as controls in DR clinical trials, and thereafter by usual care at the same institution.

Microaneurysm turnover from the initial 2 years and the occurrence of clinically significant macular edema (CSME) during the following 8 years were analyzed in this retrospective 10-year follow-up study.

Patients and Methods

Patients

One hundred and thirty-four patients with type 2 diabetes and NPDR were followed up for 2 years as controls of DR clinical trials (81 men and 53 women with ages ranging from 41 to 70 years, mean ± SD: 55.6 ± 6.3 years, and with a diabetes duration ranging from 1 to 20 years, mean ± SD: 7.9 ± 4.4 years). Patients were maintained under acceptable metabolic control during this period, and underwent ophthalmological examinations (including color fundus photography) every 6 months.

Patients were diagnosed with type 2 diabetes according to the World Health Organization criteria. All patients were followed up by the same diabetologist and treatment was given to maintain stabilized metabolic control. Medication for diabetes was initiated with oral agents only, and patients with a blood pressure reading over 130/80 mm Hg were treated with angiotensin-converting enzyme inhibitors (ramipril) alone or associated with hydrochlorothiazide and calcium channel blockers (nifedipine).

At baseline, all patients showed mild-to-moderate retinopathy, and were classified as levels 20 (microaneurysms only) or 35 (microaneurysms/hemorrhages and/or hard exudates) according to the Early Treatment of Diabetic Retinopathy Study (ETDRS) grading scale, based on the 7-field stereoscopic fundus photographs. No evidence of CSME was found. Best-corrected visual acuity was 20/20 of the ETDRS visual acuity chart in every enrolled eye.

These patients/eyes were then followed in the Retina Unit of AIBILI by conventional general and ophthalmological care.

Ophthalmological and systemic data were collected retrospectively and microaneurysm turnover for the 2 first years of follow-up was computed using a new semi-automatic method for microaneurysm earmarking (MA-Tracker). Only 1 eye per patient was used for the final data analysis: either the treated eye for patients that developed CSME needing photocoagulation, or, alternatively, either the right or the left eye.

During the second period of follow-up (8 years), 3 patients died and 10 were lost to follow-up. Another 8 patients had missing data, leaving 113 patients/eyes to be analyzed from the initial cohort of 134 patients/eyes. The baseline characteristics of these 113 patients are shown in table 1.

Table 1. Baseline characteristics (n = 113)

	Min.; max.	Mean ± SD
HbA_{1C}, %	5.1; 10.2	7.5 ± 1.3
Systolic blood pressure, mm Hg	108; 180	137.7 ± 15.0
Diastolic blood pressure, mm Hg	60; 100	81.0 ± 8.1
Cholesterol, mg/dl	114; 325	213.2 ± 40.9
HDL, mg/dl	28; 97	51.2 ± 13.5
LDL, mg/dl	41; 230	123.7 ± 35.1
Triglycerides, mg/dl	42; 712	145.6 ± 102.2

Informed consent was obtained from all patients for this observational retrospective study. The tenets of the Declaration of Helsinki were followed and approval from the institutional review board was obtained (clinical trial registration number: NCT00840541).

Color Fundus Photography

Stereoscopic color fundus photography was performed according to the ETDRS protocol. The 7-field photographs were obtained at 50° using a Kowa fundus camera (FX-500S; Kowa, Tokyo, Japan) for classification according to the Wisconsin grading.

For the current study, only one field-2 color fundus photograph per eye and per visit was analyzed.

These images, originally available on color slides, were digitized to produce 2,867 × 1,911 (width/height) pixel images (with each pixel corresponding approximately to 13 μm of the eye fundus).

Clinically Significant Macular Edema

CSME was identified by retinal thickening within 500 μm of the center of the fovea or by the presence of exudates within 500 μm of the center of the fovea, or by adjacent thickening or thickening of at least 1 disc area of any part within a 1-disc diameter of the center of the fovea [7].

The treatment for macular edema followed the ETDRS guidelines [7–9].

Microaneurysm Turnover (MA-Tracker)

To help graders to earmark microaneurysms on color fundus photographs, a new method (MA-Tracker) was developed, which performs microaneurysm counting while taking into account the specific location of each microaneurysm by registering its exact coordinates in the eye fundus.

To compute the microaneurysm turnover, the grader was asked to earmark only small lesions that appeared as a round or ovoid red spot of 20–200 μm in diameter with regular borders and located within the superior and inferior arcades, since by ophthalmoscopy or color fundus photography microaneurysms are identified as deep red dots, sometimes difficult to differentiate from punctate hemorrhages.

The MA-Tracker involves 3 steps: preprocessing (ROI segmentation and image enhancement), image co-registration (each visit to the baseline visit) and manual microaneurysm earmarking, as described elsewhere [this issue, pp. 284].

Color fundus photographs are presented to the grader at random and anonymized. The grader is then asked to earmark each microaneurysm by pointing and clicking over the respective location in the eye fundus using the computer mouse. Each microaneurysm is therefore recorded by its specific location (image coordinates) at each visit. Through the image co-registration procedure, each microaneurysm at any visit can be projected onto the baseline image and labeled according to the visit number where it was detected.

Any microaneurysm projected onto the baseline visit image that falls within 5 pixels of a previously detected one (since the first (baseline) visit) is considered to be the same one.

In this way, it is not only possible to identify new microaneurysms, the ones appearing for the first time since the baseline (not within the 5-pixel vicinity of a previous one when projected onto the baseline image), but to also identify in which visit a particular microaneurysm was detected, the number of new microaneurysms at each visit and microaneurysms that had disappeared since a previous visit.

With these parameters at hand, microaneurysm turnover (i.e. microaneurysm formation and disappearance rates) can be computed for each patient eye.

Data Analysis

The following parameters were collected, computed and compared between patients that developed CSME needing photocoagulation treatment (CSME group) and the patients that did not developed CSME (non-CSME group): age, diabetes duration, HbA_{1C}, blood pressure, cholesterol, HDL, LDL and triglyceride levels, all at baseline, and the number of microaneurysms and the microaneurysm turnover (formation and disappearances rates) for the first 2 years of follow-up.

Due to the skewed distribution for the parameters under analysis, for the CSME and the non-CSME groups, non-parametric tests were used.

Statistically significant differences between CSME and non-CSME eyes were tested using the Mann-Whitney test (for continuous variables), and/or the χ^2 test with the Yates' continuity correction for 2 × 2 tables (for nominal variables).

Statistically significant differences over the 2-year follow-up period for the number of microaneurysms were tested using the paired Friedman test, while statistically significant differences between microaneurysm formation and disappearance rates were tested using the paired Wilcoxon test.

To assess the reliability of the number of microaneurysms earmarked and the microaneurysm turnover, a second grader was asked to earmark a subset of color fundus photographs using the MA-Tracker. The agreement between the 2 graders was assessed for the number of microaneurysms and for the microaneurysm turnover, using the intraclass correlation coefficient (ICC) – defined as the ratio of the between-grader variance to the total variance (i.e. between- and within-grader variance) [10]. A two-way mixed effects model was used to compute the ICC between graders, i.e. grader effects were considered random while measure effects (microaneurysm number and/or turnover) were considered fixed. ICC values over 0.75 were considered as a good agreement between graders (based on the Landis and Koch criteria [11]).

All statistical analyses were performed using the SPSS software version 13.0 (SPSS, Chicago, Ill., USA). p values <0.05 were considered as statistically significant results.

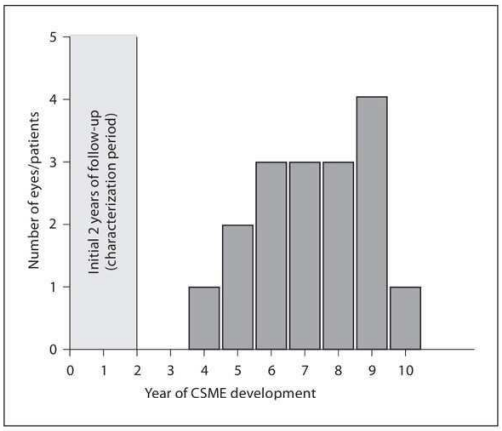

Fig. 1. Year (since baseline) of the CSME development needing photocoagulation treatment (n = 17 patients/eyes, within the 8 years after the initial characterization period).

Results

At the end of the 10-year follow-up period, 17 out of the 113 patients developed CSME needing photocoagulation.

The first patient that developed CSME needing photocoagulation developed CSME at year 4, while the last patient developed CSME at year 10 (fig. 1), respectively 2 and 8 years after the initial follow-up period.

At baseline, patients that developed CSME presented HbA_{1C} levels significantly higher (mean ± SD: 8.5 ± 1.2%) than the group of patients that did not develop CSME (mean ± SD: 7.3 ± 1.2%, p = 0.001; table 2). No statistically significant differences were found between CSME and non-CSME eyes for blood pressure, cholesterol, HDL, LDL and triglyceride levels at baseline.

Table 2. Baseline characteristics of CSME and non-CSME patients/eyes (n = 113)

	CSME (n = 17)	non-CSME (n = 96)	p
Sex (M/F)	7/10	62/34	0.120
Age, years	56.5 ± 6.0	55.2 ± 6.3	0.283
Diabetes duration, years	7.9 ± 5.5	7.8 ± 4.4	0.840
HbA_{1C}, %	8.5 ± 1.2	7.3 ± 1.2	0.001
Blood pressure, mm Hg			
Systolic	141.0 ± 14.4	137.2 ± 15.1	0.289
Diastolic	80.2 ± 5.4	81.0 ± 8.6	0.535
Cholesterol, mg/dl	208.1 ± 51.2	214.0 ± 39.2	0.432
HDL, mg/dl	51.8 ± 6.0	51.2 ± 14.3	0.473
LDL, mg/dl	129.2 ± 50.7	122.9 ± 32.7	0.881
Triglycerides, mg/dl	151.9 ± 131.9	144.6 ± 97.2	0.604

Table 3. Number of microaneurysms and microaneurysm turnover for the first 2 years of the follow-up (n = 113)

	CSME (n = 17)	non-CSME (n = 96)	p
Microaneurysms			
0 months	4.9 ± 10.9	0.3 ± 0.9	0.003
6 months	4.6 ± 10.1	0.4 ± 1.2	<0.001
12 months	5.0 ± 11.5	0.4 ± 1.1	0.009
18 months	7.9 ± 13.7	0.4 ± 1.1	<0.001
24 months	8.2 ± 14.3	0.3 ± 0.9	<0.001
Turnover, microaneurysms/year			
Formation rate	9.2 ± 18.2	0.5 ± 1.2	<0.001
Disappearance rate	7.5 ± 16.6	0.5 ± 1.2	<0.001

When counting the total number of microaneurysms over the first 2 years of the follow-up, a significant increase in the number of microaneurysms was found for the CSME eyes (p = 0.002), while for the non-CSME eyes the number of microaneurysms remained relatively constant (p = 0.647).

When computing the microaneurysm turnover for the same period of time, a higher microaneurysm turnover was found in the group of patients/eyes that developed CSME (higher microaneurysm formation and disappearance rates). Formation and disappearance rates of 9.2 ± 18.2 and 7.5 ± 16.6 microaneurysms/year, respectively, were found for the eyes that developed CSME while rates of 0.5 ± 1.2 and 0.5 ± 1.2 microaneurysms/year were found for the non-CSME eyes (p < 0.001; table 3).

A microaneurysm turnover of at least 2 microaneurysms/year was found in 12 of the 17 eyes that developed CSME (70.6%), whereas this was only found in 8 of the 96 eyes that did not develop CSME during the 10-year follow-up period (8.3%).

A statistically significant difference was found in the eyes that developed CSME between the microaneurysm formation and disappearance rates, the microaneurysm formation rate being significantly higher than the microaneurysm disappearance rate (p = 0.022). No statistically significant difference between these 2 variables was registered in the non-CSME eyes (p = 0.675).

A very good agreement was found between 2 independent graders for the number of microaneurysms earmarked (ICC: 0.836, 95% CI: 0.804–0.862) and for the microaneurysm turnover, i.e. for the microaneurysm formation rate (ICC: 0.922, 95% CI: 0.884–0.948) and for the microaneurysm disappearance rate (ICC: 0.873, 95% CI: 0.810–0.915).

Discussion

This study shows that in the initial stages of DR higher microaneurysm counts and microaneurysm turnover obtained from color fundus photography are good indicators of retinopathy progression and development of CSME needing photocoagulation.

It is widely accepted that it is crucial for improved management of DR to characterize the early alterations occurring in the retina and their progression, because there is accumulated evidence that they may be reversible and respond to treatment in their initial stages.

The available classifications of DR have contributed tremendously to our present understanding of diabetic retinal disease, but have 2 major shortcomings. One is the lack of information on the initial stages of the disease. ETDRS report number 12 clearly states that 'because few eyes were included in the ETDRS that have very mild retinopathy the definitions of the final scale regarding the lower range of NPDR could not be examined' [12]. The second is the fact that the ETDRS classification was constructed on the basis that DR, given time, uniformly progresses to proliferative retinopathy. It is now apparent, from the data available from a variety of longitudinal studies and from clinical experience, that the evolution and progression of DR varies between different individuals and does not necessarily progress in every patient to the terminal stage of proliferative retinopathy [13].

On ophthalmoscopic examination and fundus photography, microaneurysms and small hemorrhages are the initial changes detected in the diabetic retina. They may be counted, and retinal microaneurysm counting has been suggested as an appropriate marker of retinopathy progression [4, 14].

In a previous study using the MA-Tracker, the total number of microaneurysms detected on color fundus photographs showed lower sensitivity in detecting progression of the retinopathy when compared with the determination of the microaneurysm turnover taking into account the exact location of new microaneurysms in successive fundus photographs taken at 6-months intervals, apparently because the regressed microaneurysms are constantly being balanced by the new ones.

In summary, we found that differences between successive visits using microaneurysm counts are, therefore, less reliable than microaneurysm formation rates, which take into account mainly newly formed microaneurysms and give more accurate information on activity of the retinopathy. Furthermore, we previously found much better agreement between graders when determining microaneurysm turnover than microaneurysm counts [this issue, pp. 284].

Recently, Sharp et al. [15] found that the microaneurysm turnover varied widely between eyes of the same retinopathy level. This is also consistent with our findings. Microaneurysm turnover has been shown in this study to vary between patients that were classified with the same retinopathy level. Particularly relevant is the finding that the patients who have higher microaneurysm turnover values are the ones that go on to develop CSME within a period of 10 years and show a more rapid retinopathy progression, particularly in association with poor metabolic control demonstrated by higher HbA_{1C} values.

The observation that in this group of patients with diabetes type 2 the level of metabolic control, given by HbA_{1C} values, correlates with retinopathy progression confirms previous reports [16]. It is interesting that other systemic variables, such as blood pressure and blood lipid levels, did not appear to be relevant in this relatively well controlled group of patients.

Microaneurysms are a key lesion in the early stages of DR. Our work, presented in the first paper of this series, demonstrates consistency in the determination of microaneurysm turnover values [this issue, pp. 284]. This study demonstrates that it is not the absolute total number of microaneurysms at a certain point in time that may provide the best indication of retinopathy progression, but the rate of microaneurysm turnover in successive visits during a 1- or 2-year period.

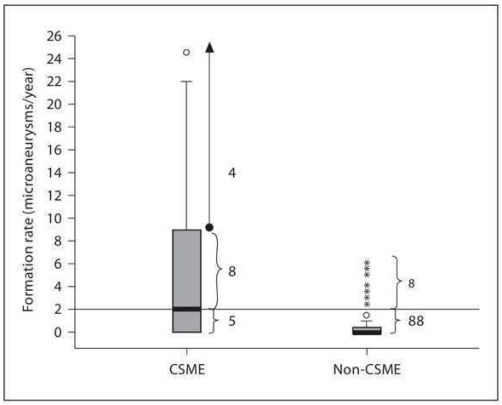

Fig. 2. Boxplot for the microaneurysm formation rate for CSME and non-CSME eyes, and number of eyes for the different values of the microaneurysm formation rate.

It appears that it is possible to use microaneurysm turnover computed from noninvasive color fundus photographs as a biomarker to identify eyes/patients at risk of progression to CSME. A microaneurysm formation rate of at least 2 microaneurysms/year in eyes with mild NPDR and diabetes type 2 appears to identify patients at risk of progression to CSME well. In our study, with a 10-year follow-up of 113 eyes/patients, the percentage of false negatives (eyes that developed CSME with a low microaneurysm formation rate) was 29.4% (5/17) and the percentage of false positives (eyes that did not develop CSME with a high microaneurysm formation rate) was 8.3% (8/96), resulting in a sensitivity in predicting CSME development of 70.6% and a specificity of 91.7%. The high negative predictive value for CSME (94.6%, 88/93) indicates that a low microaneurysm turnover, i.e. less than 2 microaneurysms/year, identifies particularly well the eyes/patients that are not expected to progress to CSME within a 10-year period (fig. 2).

It is considered of major importance that microaneurysm turnover can be determined in diabetic retinas using only noninvasive color fundus photographs, without the need for fluorescein angiography.

In the United Kingdom, it is estimated that there are 1,100 new cases of blindness every year secondary to DR [15]. Early detection and identification of the eyes/pa-

tients that show actively progressing retinal vascular disease, based on computer-assisted detection of microaneurysm turnover using digital fundus images, will be a major step forward. These eyes/patients should be followed more closely, concentrating limited resources with more aggressive management of metabolic control. Similarly, the testing of new drugs in clinical trials may be economically feasible by testing the efficacy of a drug on a select group of patients for shorter periods of time, i.e. in eyes/patients with higher microaneurysm turnover and poor metabolic control.

These observations, if confirmed, will have a major impact on clinical trial design and the feasibility of smaller clinical trials to test the efficacy of treatments on DR progression in the early stages of DR disease.

The validation of digital fundus cameras as a tool for following DR progression is a promising development. Digital fundus imaging is ideally suited to quality assurance and will allow more efficient utilization in widespread screening programs. Automated analysis techniques offer the advantages of repeatability and consistency, and, although not better in absolute terms, they avoid the variability inherent to individual human graders who have their own varying internal reference standards.

This study shows, for the first time, that a digital system based on computer-assisted analysis of fundus photographs can detect the progression of DR.

The results of this study show that MA turnover obtained from noninvasive color fundus photography, based solely on field 2, may be able to identify the eyes/patients that are at risk of progression to CSME and vision loss, and this now needs to be confirmed in a larger numbers of patients.

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Pathophysiology/Complications
ORIGINAL ARTICLE

Microaneurysm Turnover at the Macula Predicts Risk of Development of Clinically Significant Macular Edema in Persons With Mild Nonproliferative Diabetic Retinopathy

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OBJECTIVE—To examine the relationship between microaneurysm (MA) turnover using automated analysis of fundus photographs (RetmarkerDR; Critical Health SA) and development of clinically significant macular edema (CSME) in nonproliferative diabetic retinopathy (NPDR).

RESEARCH DESIGN AND METHODS—A prospective, monocenter, observational study was designed to follow eyes/patients with type 2 diabetes and NPDR (Early Treatment Diabetic Retinopathy Study levels 20 and 35) with no prior laser treatment for 2 years or until development of CSME. A total of 410 patients, one eye per patient, fulfilled the inclusion/exclusion criteria and were included in the study. Ophthalmologic examinations including best corrected visual acuity, fundus photography, and optical coherence tomography were performed at baseline, 6 months, and at the last study visit (24 months or before laser treatment).

RESULTS—A total of 348 eyes/patients performed the 24-month visit or developed CSME. Of these 348 eyes/patients, 26 developed CSME. HbA_{1c} levels at baseline and MA turnover (i.e., the sum of the MA formation and disappearance rates) computed during the first 6 months of follow-up were found to be independently predictive factors for development of CSME. MA turnover was 11.2 ± 11.2 in the 26 eyes/patients that developed CSME and 5.0 ± 5.2 in the remaining 322 ($P < 0.001$). Higher MA turnover values correlated with earlier development of CSME. MA turnover predictive values for CSME development were, for the positive predictive value, 20% and for the negative predictive value, 96%.

CONCLUSIONS—MA turnover calculated with the RetmarkerDR predicts development of CSME in eyes with NPDR. Low MA turnover values identify well the eyes that are less likely to develop CSME in a 2-year period.

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Diabetic retinopathy (DR) is a common and serious condition. It is the leading cause of blindness among working-age adults in the United States (1). Vision loss related to eye disease among people with diabetes is an important disability that threatens independence and can lead to depression, reduced mobility, and reduced quality of life (2).

Furthermore, a recent study by Narayan et al. (3) demonstrated that diabetes prevalence in the United States is likely to increase dramatically through 2050, given recent increases in the incidence of diagnosed diabetes, decreases in diabetes-related mortality, and expected changes in the age of the population.

The Eye Diseases Prevalence Research Group classified DR into two major outcomes: any DR, as any DR consisting of mild, moderate, or severe DR; and vision-threatening DR (VTDR), as DR likely to result in vision loss on the absence of treatment, consisting of proliferative DR, clinically significant diabetic macular edema (CSME), or both (4).

This concept is crucial and a promising way to address the issue of management of DR in order to prevent vision loss and to identify which patients will progress to VTDR (i.e., to CSME and/or proliferative DR).

It is now clear that systemic markers of diabetes such as duration of the disease, poor glycemic control, increased blood pressure, and lipid levels are relevant factors, but they do not identify DR worsening (5). It is a well-established fact that patients under good metabolic control may worsen rapidly and develop VTDR before other patients with poor metabolic control. These observations led to the identification of three different DR phenotypes of progression (6) based on the characteristics of the retinal lesions.

It is, therefore, fundamental to identify lesions, their number, and dynamics in the earlier stages of DR and correlate their occurrence to the worsening of any stage of DR to VTDR (5).

Detection of red lesions by fundus photography has for a long time been suggested as a potential marker of DR progression. In the early stages of DR, several studies have shown that even the presence of only one or two microaneurysms (MAs) (7), but primarily MA dynamics, increases the risk of disease worsening to DR level requiring laser photocoagulation (i.e., VTDR) (8,9).

MAs appear and disappear in the retina of diabetic patients over time, disappearing by closing down due to thrombosis with new ones appearing in different locations of the vascular tree. This turnover indicates a dynamic process and disease activity and has been suggested as a predictive factor for disease worsening.

Recently, our group found in a retrospective study that MA turnover obtained

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from color fundus photographs using manual earmarking is a good predictor of DR worsening to CSME (10).

We report in this study a prospective 2-year study of a cohort of type 2 diabetic patients with mild nonproliferative DR (NPDR) analyzed using automated images analysis for MA turnover performed in color fundus photographs.

RESEARCH DESIGN AND METHODS

—The study was a prospective, observational study designed to follow eyes/patients with mild NPDR (Early Treatment Diabetic Retinopathy Study [ETDRS] grades 20 and 35) for a period of 2 years or at the time of development of a VTDR complication, CSME needing laser photocoagulation. The sample size was computed based on the results of Nunes et al. (10) for a statistical power of 90%, an α level of 0.05, and a dropout rate of 10% (i.e., 60% of the patients with mild NPDR and high MA turnover will develop CSME, and patients with high MA turnover represent only 20% of the patients with mild NPDR).

Four hundred ten patients, men and women, were included with the following inclusion criteria: diagnosed adult-onset type 2 diabetes, age 40–75 years, mild NPDR (grades 20 and 35 of ETDRS classification), without clinical evidence of macular edema, best corrected visual acuity (BCVA) ≥95, ETDRS letters (20/25), and refraction with a spherical equivalent less than 5D. Exclusion criteria included the presence of cataract or other eye disease that may interfere with fundus examination, glaucoma, other retinal disease, previous intraocular surgery, dilatation of the pupil <5 mm, and previous laser therapy or intravitreal injections. All patients gave written informed consent. Tenets of the Declaration of Helsinki were followed, and approval from the institutional review board was obtained (clinical trial registration number: NCT00763802).

At the baseline visit (V0), patient's body weight, height, blood pressure, and concomitant medications were recorded. A physical examination by a diabetologist was also performed.

Laboratory analyses were performed at baseline (V0), at 6-month (V6) and at 24-month (V24) visits, or at the pretreatment visit. Laboratory analyses included glucose and HbA_{1c} concentration, red blood cell count, white blood cell count, platelet amount, hemoglobin concentration, and packed cell volume. Metabolic control was also assessed by measuring in the plasma concentrations of HbA_{1c} and lipid fractionation identifying total cholesterol, HDL, LDL, and triglycerides.

One eye per patient was selected as the study eye by the physician based on the inclusion/exclusion criteria. When both eyes fulfill the same criteria, one of the eyes was selected by choosing sequentially the right or the left eye.

At the three study visits, V0, V6, and V24 (or pretreatment visit), the study eyes underwent a complete eye examination, which included BCVA, as tested in the ETDRS, slit-lamp examination, intraocular pressure measurements, fundus photography, and optical coherence tomography (OCT).

CSME

CSME was identified on clinical examination by retinal thickening within 500 μm of the center of the fovea, the presence of exudates within 500 μm of the center of the fovea, or adjacent thickening or thickening of at least one disc area of any part within 1 disc diameter of the center of the fovea (11).

Color fundus photography

Color fundus photography was performed according to the ETDRS protocol. The 7-field photographs were obtained at 30° using a Zeiss FF450 camera (Carl Zeiss Meditec, Dublin, CA) for DR classification according to ETDRS grading.

The field-2 color fundus images (macula) were subjected to automated MA analysis.

The automated computer-aided diagnostic system, RetmarkerDR (Critical Health SA, Coimbra, Portugal), was used to automatically detect MA on the field-2 color fundus images. This automated computer-aided diagnostic system consists of software earmarking MAs and vascular lesions; it includes a coregistration algorithm that allows comparison within the same retinal location between different visits for the same eye. The algorithm detects the presence of MAs and red dot–like lesions. For this purpose, the images are initially converted to processing size. Next follows contrast normalization and enhancement based on principal component analysis. Then, dark objects of a given size are detected and used as candidates. For each of these candidates, features such as area, shape, intensity distribution, and gradient magnitude distribution are extracted using a region covariance descriptor. Next, a state of the art classifier, based on support vector machines, is used to classify the candidates as true or false (classifier's training was done using a dataset of color fundus images in which graders were asked to earmark only small lesions that appeared as a round or ovoid red spot of 20–200 μm in diameter with regular borders and located within the superior and inferior arcades).

The images from field-2 are coregistered (12) to indicate disease activity in the central 3,000-μm circle of the macula. Image coregistration is achieved by extracting a retinal vascular tree, which is used for landmarks during the registration process. A rigid registration estimates the translation based on fovea displacement. The rotation is estimated using a polar representation of the vascular tree. This rigid transformation is then adjusted to obtain exact pairings of selected landmarks (13).

The RetmarkerDR (Critical Health SA) therefore computes for each eye/patient the number of MA in each visit and the number of MA that appears and/or disappears from one visit to the other, allowing calculation of the number of MA appearing and/or disappearing per time interval (i.e., the MA formation rate and the MA disappearance rate, respectively). The MA turnover is computed as the sum of the MA formation and disappearance rates.

For example, a patient who has three MA on the first visit, and those same three MA remain unchanged on the second visit, will have a formation rate of zero, indicating no disease activity. In contrast, a patient can have the total of three MA on the first and second visits, but if those MA are all registered in different retinal locations, an MA formation rate of 3 and a MA disappearance rate of 3 will be calculated, indicating an MA turnover of 6 per time interval.

Previous work from our group (10,14) showed a good intergrader agreement for the total number of MAs earmarked and the MA turnover for three independent human graders. The RetmarkerDR (Critical Health SA) shows a similar intergrader agreement for the total number of MAs and the MA turnover (when compared with a human grader, intraclass correlation coefficients were 0.857 and 0.806, respectively) while showing no intragrader variability as opposed to human graders, being, therefore, a reliable tool for MA assessment.

OCT

OCT scans were performed using the Stratus OCT (Carl Zeiss Meditec).

To obtain a more detailed retinal thickness map in the central macular region, proprietary software for increased-resolution OCT maps was used (15). To compute the new increased-resolution OCT maps (composed by 124 equal areas), two acquisition protocols were performed in each eye, the Fast Macular acquisition protocol, acquiring six radial scans 30° apart, 6 mm long, and the Fast RNFL acquisition protocol, acquiring five circular scans within the central 6 mm in diameter area.

Data analysis

The following parameters were collected at baseline, computed, and compared between eyes/patients that developed CSME needing photocoagulation treatment (CSME group) and the eyes/patients that did not develop CSME (non-CSME group): age, diabetes duration, HbA_{1c}, blood pressure, cholesterol, HDL, LDL, and triglyceride levels, macular retinal thickness (in the central 500 and 1,500 μm in diameter area), number of MA, MA formation and disappearance rates, and MA turnover.

Changes in the number of MA in field-2, including MA formation rate, disappearance rate, and turnover, were computed for the first 6-month period of follow-up, V0, and V6.

A univariate analysis was performed to test for statistically significant differences between CSME and non-CSME eyes. Due to the skewed distribution for the parameters under analysis, for the CSME and the non-CSME groups, the nonparametric Mann–Whitney test was used.

To analyze the predictiveness of the different MA parameters, a receiver operating curve (ROC) analysis was also performed. Cutoff values for CSME and non-CSME eyes were identified for the parameter that showed simultaneously the higher sensitivity and specificity (i.e., the higher ROC area). The odds ratio for the cutoff values was thereafter computed.

A multivariate analysis, considering the entire set of parameters at baseline (i.e., sex, age, diabetes duration, BCVA, diabetes treatment, systolic and diastolic blood pressure, glycose, cholesterol, HDL and LDL, triglycerides, central retinal thickness [in the 500 and 1,500 μm in diameter area], number of MA, and MA turnover [from baseline to month 6]), was also performed using a Poisson regression analysis to identify predictive factors for CSME development considering patient's time of follow-up.

All statistical analyses were performed using the STATA software version 12.0 (StataCorp LP, College Station, Texas), and P values ≤0.05 were considered as statistically significant results.

RESULTS—Three hundred forty-eight eyes/patients were considered for analysis because they reached the study end point, CSME needing laser photocoagulation, or performed the last study visit (V24) (Fig. 1). Baseline characteristics for the 410 patients included are shown in Table 1 (no statistically significant differences were found between excluded and included eyes/patients except for the cholesterol and LDL levels).

Of these 348 eyes/patients, 26 were diagnosed during the 2-year period of follow-up as having CSME and treated with laser photocoagulation. The other 322 eyes/patients completed the last study visit of follow-up without developing CSME (V24). Eyes/patients characteristics by CSME and non-CSME are shown in Table 2.

Fifteen eyes/patients progressed to more advanced ETDRS levels. Fourteen eyes progressed to moderate NPDR (11 with 43A and 3 with 43B), and 1 eye progressed to moderate proliferative DR (65B).

The average number of MA was 6.2 ± 5.4 in the 26 eyes/patients that developed CSME and 3.3 ± 3.7 in the remaining 322 eyes/patients ($P < 0.001$).

The MA turnover was 11.2 ± 11.2 in the 26 eyes/patients that developed CSME and 5.0 ± 5.2 in the remaining 322 eyes/patients ($P < 0.001$).

The MA turnover shows a higher predictiveness for CSME than the remaining MA parameters (the ROC area was for the MA turnover, 0.695; for the number

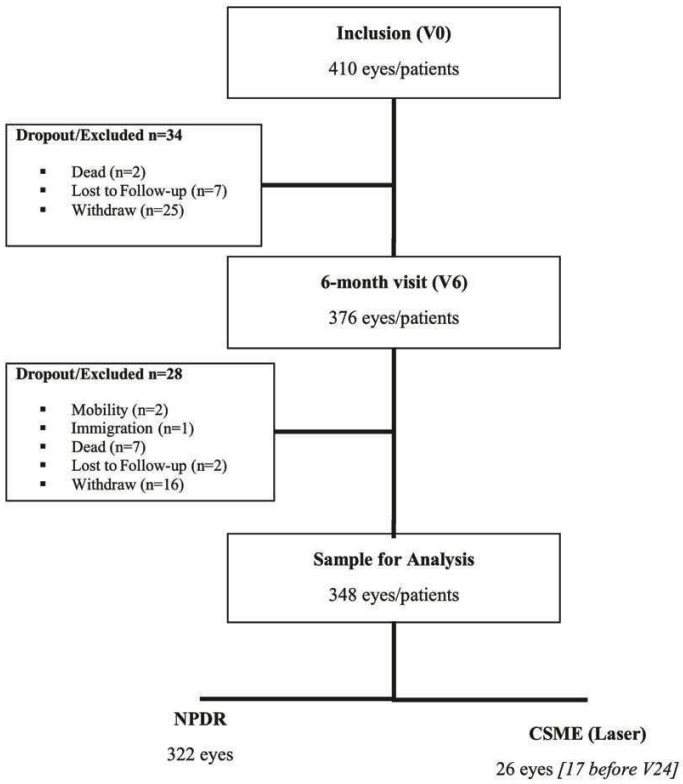

Figure 1—*CONSORT flow chart.*

Table 1—*Baseline characteristics of the patients in the study*

	Dropout patients (n = 62)		Patients included in the analysis (n = 348)		
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	P value
Males/females[1]	41 (65.1%)/22 (34.9%)		219 (62.9%)/129 (37.1%)		NS
Patients taking insulin[1]	14 (22.6%)		103 (29.6%)		NS
Age (years)	62.8 ± 8.3	63.5 (58.0–69.0)	60.9 ± 8.3	62.0 (55.0–67.0)	NS
Duration of diabetes (years)	9.3 ± 5.1	10.0 (5.0–14.0)	10.1 ± 5.0	10.0 (6.0–14.0)	NS
HbA_{1c} (%)	8.0 ± 1.7	7.9 (6.5–9.0)	7.9 ± 1.5	7.7 (6.9–8.9)	NS
Cholesterol (mg/dL)	209.2 ± 53.8	208.5 (173.0–228.0)	195.3 ± 39.2	193.0 (167.0–219.0)	0.016
HDL (mg/dL)	52.4 ± 13.1	51.5 (43.0–59.0)	50.8 ± 12.8	49.0 (42.0–57.0)	NS
LDL (mg/dL)	137.9 ± 43.9	132.0 (108.0–156.0)	126.4 ± 29.9	124.0 (105.0–146.0)	0.010
Glycose (mg/dL)	184.0 ± 85.0	168.5 (113.0–230.0)	175.6 ± 73.3	163.0 (119.0–226.0)	NS
Triglycerides (mg/dL)	151.4 ± 77.3	132.0 (104.0–192.0)	179.3 ± 121.1	147.0 (104.0–220.0)	NS
Systolic blood pressure (mmHg)	152.8 ± 24.8	152.0 (135.0–170.0)	151.5 ± 20.9	151.0 (137.0–164.0)	NS
Diastolic blood pressure (mmHg)	76.2 ± 9.4	75.0 (72.0–82.0)	75.8 ± 10.8	76.0 (69.0–82.5)	NS
BCVA (letters)	98.4 ± 2.3	100.0 (95.0–100.0)	98.8 ± 2.1	100.0 (95.0–100.0)	NS
Retinal thickness in the central 500 μm (μm)	178.4 ± 25.4	174.0 (158–197.3)	182.5 ± 25.5	180.1 (165.3–119.8)	NS
Retinal thickness in the central 1,500 μm (μm)	238.9 ± 24.2	237.9 (221.9–257)	242.3 ± 21.2	244.2 (228.3–255.5)	NS
Number of MA	4.1 ± 5.2	2.0 (1.0–6.0)	3.5 ± 4.0	2.0 (1.0–5.0)	NS

[1]Frequency (%). IQR, interquartile range, first and third quartiles; NS, $P > 0.05$. P value for statistically significant differences between dropout and included patients.

of MA, 0.676; for the MA formation rate, 0.658; and for the MA disappearance rate, 0.656).

For an MA turnover cutoff value of ≥9, a sensitivity of 57.7% and a specificity of 81.2% was achieved (i.e., 79.4% of the eyes are correctly classified).

Eyes with an MA turnover >9 during the initial 6-month period showed a higher risk for CSME development than eyes with a lower MA turnover (odds ratio 5.886 [95% CI 2.503–13.844]). The MA turnover predictive values for CSME development were: for the positive predictive value, 20.0%; and for the negative predictive value, 95.9%, showing that a low MA turnover value is associated with less likelihood for CSME development in a 2-year period.

Furthermore, considering only the eyes with an MA turnover ≥9, eyes that

Table 2—*Patient characteristics at baseline comparing the two patient groups, the ones that did not develop CSME and the ones that developed CSME*

	Non-CSME (n = 322)		CSME (n = 26)		
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	P value
Age (years)	60.8 ± 8.2	62.0 (55.0–67.0)	62.3 ± 8.9	63.0 (54.0–70.0)	NS
Duration of diabetes (years)	10.0 ± 5.1	10.0 (6.0–14.0)	11.0 ± 4.8	11.5 (7.0–15.0)	NS
HbA_{1c} (%)	7.9 ± 1.4	7.6 (6.9–8.8)	8.6 ± 1.8	8.1 (7.3–10.0)	0.022
Cholesterol (mg/dL)	195.2 ± 39.3	193.0 (167.0–218.0)	197.2 ± 38.9	197.0 (164.0–228.0)	NS
HDL (mg/dL)	50.9 ± 12.9	49.0 (42.0–57.0)	50.1 ± 11.9	48.0 (44.0–55.0)	NS
LDL (mg/dL)	126.1 ± 30.0	124.0 (104.0–146.0)	129.3 ± 30.2	121.5 (105.0–150.0)	NS
Glycose (mg/dL)	175.2 ± 73.3	162.0 (119.0–225.0)	180.9 ± 74.5	171.0 (128.0–239.0)	NS
Triglycerides (mg/dL)	180.8 ± 123.9	147.0 (104.0–220.0)	161.0 ± 77.2	142.0 (112.0–199.0)	NS
Systolic blood pressure (mmHg)	151.5 ± 21.0	151.0 (137.0–164.0)	151.6 ± 20.0	147.5 (135.0–168.0)	NS
Diastolic blood pressure (mmHg)	75.9 ± 10.9	76.0 (69.0–82.0)	74.5 ± 11.5	71.5 (65.0–84.0)	NS
BCVA (letters)	98.8 ± 2.1	100.0 (100.0–100.0)	98.8 ± 2.1	100.0 (100.0–100.0)	NS
Retinal thickness in the central 500 μm (μm)	181.5 ± 24.8	179.6 (164.8–199.3)	193.2 ± 32.6	186.2 (174.5–206.3)	0.025
Retinal thickness in the central 1,500 μm (μm)	241.6 ± 21.0	243.4 (227.6–255.4)	250.0 ± 22.3	250.5 (233.5–260.6)	NS
Number of MA	3.3 ± 3.7	2.0 (1.0–4.0)	6.2 ± 5.4	4.5 (2.0–11.0)	<0.001
MA formation rate	2.5 ± 3.5	1.0 (0.0–3.0)	6.3 ± 8.3	3.5 (1.0–8.0)	<0.001
MA disappearance rate	2.5 ± 2.8	2.0 (0.0–3.0)	4.8 ± 4.7	3 (2.0–7.0)	<0.001
MA turnover	5.0 ± 5.2	4.0 (1.0–7.0)	11.2 ± 11.2	9.0 (3.0–16.0)	<0.001

P value for statistically significant differences between CSME and non-CSME eyes. IQR, interquartile range, first and third quartiles; NS, $P > 0.05$.

developed CSME before the 24-month visit presented higher MA turnover values (26.6 ± 15.9) when compared with the eyes in which CSME was detected only at month 24 (12.8 ± 3.6) (P = 0.018), indicating again that there is a correlation between high turnover values and risk for the development of CSME for eyes with the same ETDRS retinopathy level.

Considering the central retinal thickness values at baseline, a statistically significant difference was found for the central 500 μm in diameter area (P = 0.025) but not for the central 1,500-μm area (P = 0.052).

For BCVA, no statistically significant differences were found between CSME and non-CSME eyes at baseline (P = 0.499) or in the last study visit (P = 0.593). However, there was an overall decrease in BCVA in both CSME and non-CSME ($P < 0.018$).

When considering the systemic parameters examined, the eyes that developed CSME during the 2-year study period had higher HbA_{1c} level at baseline (P = 0.022). The other parameters examined such as blood pressure (systolic and diastolic) and other blood lipids (triglycerides, cholesterol, HDL, and LDL) did not show any correlation with occurrence of CSME (Table 2). No statistically significant changes in the systemic parameter were found between the baseline and 6-month examinations.

The multivariate analysis (Poisson regression analysis) shows that MA turnover values and HbA_{1c} levels are independently predictive for CSME development in the early stages of NPDR (Table 3).

Combining the two factors MA turnover and HbA_{1c} to identify eyes at risk for CSME development, using <9 and ≥9 for the MA turnover (sensitivity and specificity of 57.7 and 81.2%, respectively) and <9 and ≥9 for the HbA_{1c} levels (sensitivity and specificity of 32.6 and 77.0%, respectively), the sensitivity for CSME development when considering MA turnover <9 and HbA_{1c} levels <9 increased to 76.9%, decreasing the specificity to 64.9%.

CONCLUSIONS—This 2-year prospective, longitudinal study of patients with diabetes type 2 and mild NPDR (ETDRS levels 20 and 35 at baseline) shows that MA turnover in field-2 is a good indicator of retinopathy worsening and development of CSME needing photocoagulation.

On ophthalmoscopic examination and color fundus photography, red-dot lesions, including MA and small hemorrhages, are the initial changes detected in the diabetic retina. They may be counted, and retinal MA counting has been proposed as an appropriate marker of retinopathy worsening (16).

In a previous study by our group (10), determination of MA formation rates taking into account the exact location of new MA in successive color fundus photographs showed higher sensitivity in predicting worsening of retinopathy for a 10-year period of follow-up than the simple counting of MA at one time. Moreover, we found that MA formation rates obtained during an initial 2-year follow-up period gave more accurate information on the activity of the retinopathy and that there was much better agreement between graders when determining MA formation rates than MA counts (14).

MA turnover values in this study show a high SD relatively to the mean, indicating that MA turnover values vary widely between eyes having similar ETDRS grading level. This confirms previous observations by Sharp et al. (17) and may indicate that MA turnover is an indicator of different vascular disease activity in different eyes.

Of particular interest in this study is the observation that MA turnover values, including both the MA formation rate and MA disappearance rate, determined over a period of only 6 months predicts with a high degree of confidence the eyes that do not go on to develop CSME for at least a period of 2 years. This observation is interpreted as indicating that eyes with low MA turnover have less disease activity and therefore less risk of worsening. It should be realized that retinal thickness was only measured in the central 6,000 μm in diameter area, within the vascular arcades, corresponding to ETDRS field-2. Therefore, any retinal edema outside of this central area was not considered.

The observation that, in this group of patients with diabetes type 2, the level of metabolic control, given by HbA_{1c} values, correlates with retinopathy worsening confirms a previous report (10). It is also of major interest that at these early retinopathy stages, other variables, such as blood pressure and blood lipid levels, do not appear to be associated with the development of CSME. However, cholesterol fractions were not measured.

It is also considered of major interest that MA turnover can be determined in diabetic retinas using only noninvasive color fundus photographs, without the need for fluorescein angiography (18). The identification of the eyes that show very slow worsening of retinopathy and low disease activity with low risk for development of vision-threatening complications, based on computer-assisted detection of MA turnover using digital color fundus images, is considered of potential relevance for management of diabetic retinal disease.

Table 3—*Multivariate Poisson regression analysis*

	IRR	95% CI	*P* value
Males/females	0.601	0.239–1.511	0.279
Age (years)	1.03	0.976–1.098	0.251
Duration of diabetes (years)	1.008	0.931–1.092	0.842
Patients taking insulin	0.957	0.355–2.582	0.932
HbA_{1c} (%)	1.400	1.039–1.886	0.027
Cholesterol (mg/dL)	0.990	0.949–1.033	0.651
HDL (mg/dL)	1.002	0.949–1.058	0.934
LDL (mg/dL)	1.016	0.967–1.068	0.521
Glycose (mg/dL)	0.996	0.988–1.004	0.339
Triglycerides (mg/dL)	0.998	0.993–1.004	0.597
Systolic blood pressure (mmHg)	0.996	0.970–1.022	0.759
Diastolic blood pressure (mmHg)	1.007	0.960–1.056	0.779
BCVA (letters)	1.010	0.816–1.251	0.924
Retinal thickness in the central 500 μm (μm)	1.012	0.985–1.039	0.398
Retinal thickness in the central 1,500 μm (μm)	1.008	0.971–1.046	0.680
Number of MA	1.023	0.908–1.151	0.709
MA turnover	1.085	1.014–1.160	0.018

Incidence rate ratios (IRR) and 95% CI for the IRR. *P* value for predictive parameters for CSME eyes.

The validation of digital color fundus cameras as a tool for following DR worsening to VTDR is considered a valuable development, taking into account their cost and availability. Quality assurance of digital color fundus imaging is straightforward and will allow more efficient utilization of widespread screening and prevention programs. Automated analysis techniques offer advantages of repeatability and consistency, and, although not better than trained graders, particularly in identifying all MA, they avoid the variability inherent to human grading, which has its own subjective reference standards. It is also considered relevant that MA turnover calculated by the RetmarkerDR (Critical Health SA) is much less time consuming than MA counting by an expert grader.

The observations reported in this article are also expected to have impact on clinical trial design. Excluding from the trials eyes/patients that show little disease activity and are not expected to progress during the trial period would offer a better chance for development of VTDR in the placebo control eyes, thus creating the conditions to identify efficacy of the drug being tested.

Limitations of this study include the relatively short duration of the study (2 years) and the lack of more detailed information on the systemic parameters such as lipid stratification.

The results of this study confirm that in a prospective study of a relatively large number of patients, MA turnover values obtained from noninvasive color fundus photography based solely on field-2 images may help to identify the eyes/patients at risk for developing CSME and potential vision loss.

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M.L.R. and S.G.N. work at the Association for Innovation and Biomedical Research on Light and Image. J.G.C.-V. is President of the Administration Board of the Association for Innovation and Biomedical Research on Light and Image and Emeritus Professor of the Faculty of Medicine of the University of Coimbra. J.G.C.-V. is also a consultant at Allergan, Alimera Sciences, Bayer, Critical Health, Fovea Pharmaceuticals, Gene Signal, Novartis, Pfizer, and Roche. No other potential conflicts of interest relevant to this article were reported.

M.L.R. researched data and reviewed and edited the manuscript. S.G.N. analyzed data, contributed to the discussion, and reviewed and edited the manuscript. J.G.C.-V. designed the study, researched data, wrote the manuscript, and contributed to the discussion. J.G.C.-V. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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4.3.2 Macular Edema

Retinal edema in the macula, or macular edema, results frequently from the accumulation of fluid in the retinal layers as a response to the inflammatory process originated by changes in the BRB permeability [59]. The degree of macular edema may therefore be an indicator of the degree of the BRB breakdown. The accumulation of fluid in the central area of the macula, i.e., central macular edema, may interferes with the functional activity of the cells located in the fovea and can result, in some cases, in vision loss [74].

With the development and improvement of the methodologies for objective assessment of the macular edema, the relationship between the degree of the macular edema and the visual acuity can be studied. The strength of the correlation between the central retinal thickness and the visual acuity is still not consensual. Correlations between the central retinal thickness and the visual acuity are generally present when the central macular area is already affected. Even though, this correlation is only observed in half of the patients [74] (see section 4.3.2.2).

4.3.2.1 Retinal Thickness Assessed Using Optical Coherence Tomography

The clinical evaluation of macular edema is difficult. Direct and indirect ophthalmoscopy may reveal nothing but an alteration of the foveal reflexes. Slit-lamp biomicroscopy demonstrate changes in retinal thickness in the macular area but it is dependent on the observer. These parameters are now well identified using OCT.

OCT is a non-invasive diagnostic technique that was first demonstrated in 1991 by Huang et al. [75] and which allow for the visualization and quantification of the different retinal layers with a longitudinal and axial resolution of some micrometres [76, 77] (Figure 14). This technique allows studying the morphological changes that occur in the different retinal layers, measuring the thickness and volume of these layers, and mapping the retinal surface.

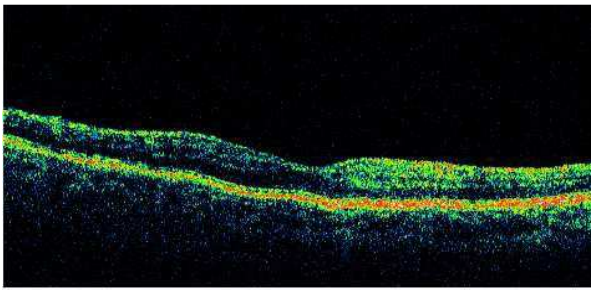

Figure 14. OCT B-scan centred in the fovea for a patient with NPDR (case MMS33).

The assessment of the macular thickness using OCT has been shown to be an objective and reliable method for the evaluation of the degree of macular edema [78].

OCT brought new insights about morphological changes of the retina in DR and DME. It showed that macular edema may assume different morphologic patterns [79, 80]. In addition, a quantitative characterization of macular edema became feasible, as determined by measurements of retinal thickness and volume. OCT has been demonstrated to be more sensitive than slit-lamp biomicroscopy in detecting small changes in retinal thickness [78, 81, 82, 83] and is clearly less subjective.

The quantification of the retinal thickness by OCT is based on the distance between the anterior and posterior hyper-reflective boundaries of the retina [84]. OCT images of DME show the presence of low intraretinal reflectivity, due to fluid accumulation in the extracellular space of the retina [85, 86]. Recently it was shown that BRB alterations could be measured with OCT [87] and that the alteration of the BRB can be quantified in the initial stages of edema.

Currently, the gold standard to diagnose CSME is still based on the ETDRS protocol, i.e. based on the clinician's subjective assessment of the eye fundus, using fundus biomicroscopy and/or stereo fundus photography, and therefore, OCT is not required to diagnose CSME. However, OCT has gained general acceptance as an additional technique to help identifying and evaluating macular edema. It was shown that OCT performs well compared with stereo CFP or biomicroscopy to diagnose CSME [88]. The meta-analysis performed by Virgili et al. [88] shows that CSME is usually diagnosed based on an OCT central macular thickness cutoff value between 250 and 300 μm (being the cutoff value of 300 μm the one that allows for a better sensitivity for CSME). Based on the clinical evidences of this meta-analysis it may be advised to treat CSME when the central retinal thickness is ≥ 300 μm and not treat when the central retinal thickness is ≤ 250 μm.

Moreover, several studies have been performed using OCT to identify and characterize DME in its early stages. Some studies have observed that OCT can detect macular thickening earlier than the clinical examination [89]. Here the definition of subclinical DME gains importance. The establishment of reference values that allows for the early identification of DME, before it can be clinically identified, will open news perspectives for the management of DR patients. The Diabetic Retinopathy Clinical Research Network (DRCR.net) performed in the last years several studies to identify biomarkers for CSME development based on retinal thickness measurements using OCT. Bressler et al. [90] verified that patients with DM and minimal or no DR showed a central macular thicknesses on OCT similar to the one observed in healthy subjects. Based on

the central macular thickness observed in a healthy population, a reference value of 225 µm for the central macular thickness was identified as a cutoff value for the presence of subclinical DME. This value was obtained considering the normal mean value of the central macular thickness plus 2 standard deviations (SD), corresponding to the central macular thickness value observed in 97.7% of the normal population. Recently, Bressler at al. [91] showed that approximately one quarter of the patients with subclinical DME, i.e., with a central macular thickness > 225 µm, will progress to CSME within a 2-year period, demonstrating that the presence of increased central macular thickness may be a good biomarker for the development of CSME [91].

Today the only method that allows for an objective follow-up of subclinical DME is based on OCT. OCT allows a clear identification of the intraretinal fluid distribution and the presence or absence of vitreous traction. Furthermore, OCT allows a quantitative diagnosis of DME, as it is used to obtain numerical representation of the retinal thickness.

In a recent 2-years prospective, observational study, with 410 eyes/patients with mild NPDR (ETDRS levels 20 and 35) [92, 93], changes in the central retinal thickness were investigated using OCT (see section 4.3.2.2). Men and women, with type 2 DM, aged 40 to 80 years with a BCVA ≥ 95 ETDRS letters, underwent in the 3 study visits (baseline, month-6 and month-24), a complete eye examination, which included OCT.

In this study the Stratus OCT equipment from Zeiss (Carl Zeiss Meditec, Dublin, USA) was used, being the equipment available at the time of the study with the highest resolution. Stratus OCT allows for an axial and longitudinal resolution lower than 10 µm (currently new OCT machines are available with higher resolutions, lower that 5 µm).

The Stratus OCT Fast Macular acquisition protocol was used in this study. This acquisition protocol allows obtaining maps of the retinal thickness in the macula, by using six radial scans of 6 mm each (B-scans), 30º apart. Ten (10) retinal thickness values are obtained (Figure 15):

- One central retinal thickness value in the fovea, or Central Point Value Thickness (average value at the intersection of the 6 scans).
- One central retinal value in the central 1000 µm in diameter area (mean value centred on the fovea).
- Four retinal thickness values in the area within 1000 and 3000 µm (parafoveal retinal thickness values in the nasal, temporal, superior and inferior areas).

- Four retinal thickness values in the area within 3000 and 6000 μm (perifoveal retinal thickness values in the nasal, temporal, superior and inferior areas).

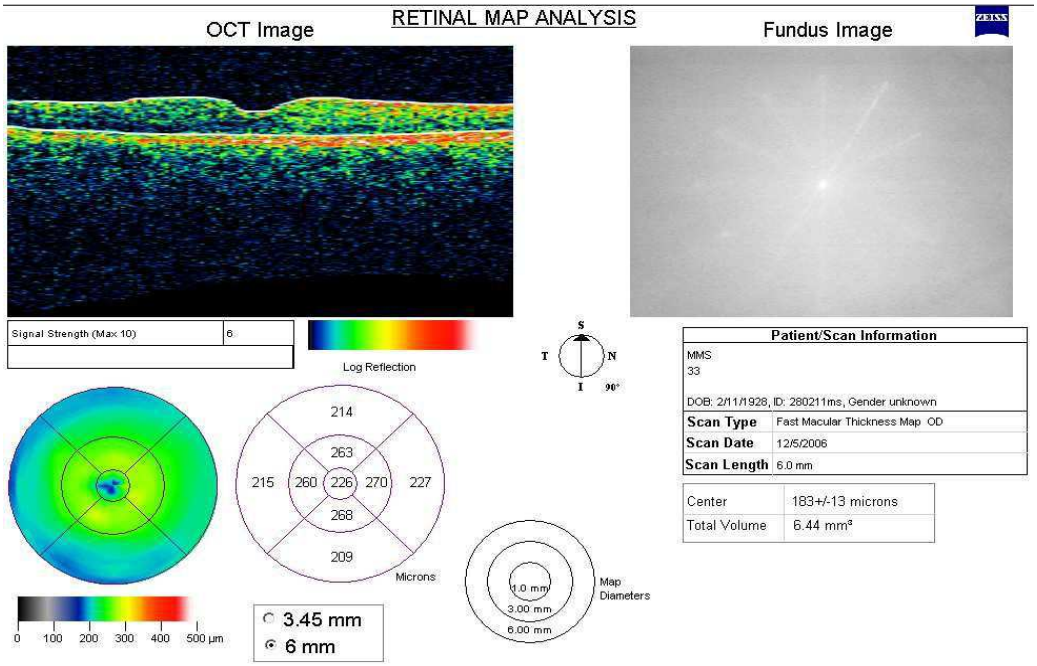

Figure 15. Retinal thickness map of a patient with NPDR obtained using Stratus OCT (case MMS33).

To identify macular areas with increased retinal thickness, i.e., with macular edema, the same acquisition protocol (Stratus OCT Fast Macular) was performed in an age-matched healthy population, since retinal thickness values changes with age [94, 92].

Twenty nine (29) volunteers were included (36% men and 64% women) aged ranging from 40 to 77 years, mean and SD of 53.4 ± 8.7 years.

One volunteer was excluded for showing extreme values on the retinal thickness (outlier).

To ensure the representativeness of the healthy volunteers included, as well as to ensure the independence of the sample considered for the normative database (eyes/volunteers), retinal thickness values obtained for the two eyes from each volunteer (56 eyes from 28 volunteers) and retinal thickness values considering only one eye from each volunteer (28 eyes from 28 volunteers, selecting alternatively the right or left eye), were compared. Since no statistically significant differences were found (Student's t-test for the mean values and Levene test for the variance values), reference values for the retinal thickness were established considering the values obtained in the 28 eyes from the 28 healthy volunteers.

The following reference values were therefore considered for this study [92]:

- Average retinal thickness in the fovea: 171.3 ± 20.8 µm
    - reference values (mean - 2 SD and mean + 2 SD): 130 to 210 µm
- Average retinal thickness in the central area (1000 µm): 201.1 ± 18.9 µm
    - reference values (mean - 2 SD and mean + 2 SD): 160 to 240 µm

No statistically significant differences were found between these reference values and the ones provided by Carl Zeiss Meditec for Stratus OCT [92].
Also, no statistically significant differences were found between men and women. In the fovea men had an average value of 172.0 ± 18.0 µm and women 171.6 ± 25.0 µm (P = 0.967) and in the central area men had an average value of 201.8 ± 15.4 µm and women 201.4 ± 21.8 µm (P = 0.962) [92].

By using Stratus OCT on patients with NPDR, and by comparing the retinal thickness values with the normal reference values, it was possible to show that DR patients with mild NPDR presents an increased retinal thickness in the central macular area indicating the presence of macular edema in the early stages of the disease. Eighteen percent (17.6%) of the eyes/patients with NPDR showed a central retinal thickness over the normal reference values, indicating the presence of macular edema and therefore the breakdown of the BRB; while 2.9% of the eyes/patients showed a central retinal thickness below the normal reference values, indicating the presence of a neuronal degeneration [92].
Moreover, it was shown that patients presenting already and increased retinal thickness in the central macular area, i.e., eyes/patients with subclinical macular edema (defined as an absence of foveal edema in slit-lamp examination and a central point retinal thickness on Stratus OCT between 225 and 299 µm [91]), are associated with a 3.7-fold increased risk for CSME development within a 2-year period [92, 93].

Increase in retinal thickness measured by OCT appears to predict the development of CSME. A potential retinal biomarker for CSME development is therefore, the increased macular thickness measured using OCT.

4.3.2.2 Scientific Contribution

The following contributions were published:

- S. Nunes, I. Pereira, A. Santos, R. Bernardes, and J. Cunha-Vaz. Central retinal thickness measured with HD-OCT shows a weak correlation with visual acuity in eyes with CSME. British Journal of Ophthalmology, 94(9):1201, 2010.
- I. Pires, A. Santos, S. Nunes, and C. Lobo. Macular thickness measured by stratus optical coherence tomography in patients with diabetes type 2 and mild nonproliferative retinopathy without clinical evidence of macular edema. Ophthalmologica, 229:181–186, 2013.
- I. Pires, A. Santos, S. Nunes, C. Lobo. and J. Cunha-Vaz. Subclinical Macular Edema as a Predictor of Progression to Clinically Significant Macular Edema in Type 2 Diabetes. Ophthalmologica, 230:201–206, 2013.

Central retinal thickness measured with HD-OCT shows a weak correlation with visual acuity in eyes with CSME

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Central retinal thickness measured with HD-OCT shows a weak correlation with visual acuity in eyes with CSME

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ABSTRACT

Aims To investigate the correlation between increased retinal thickness (RT) measured with spectral domain high-definition optical coherence tomography (OCT) (Cirrus HD-OCT (Carl Zeiss Meditec, Dublin, California, USA)) and best-corrected visual acuity (BCVA) in eyes with clinically significant macular oedema (CSME) and type 2 diabetes.

Methods Seventy eyes with CSME were included in this observational study. Sixty-two eyes were considered for analysis and were classified as having/not having retinal thickening in the central fovea (central 500-µm-diameter circle) by Cirrus HD-OCT. RT measurements were computed and correlated with BCVA. For comparison purposes, the Stratus OCT (Carl Zeiss Meditec, Dublin, California, USA) central point thickness was also obtained in these eyes.

Results In the 19 eyes with CMSE identified by Cirrus HD-OCT without increased RT in the central fovea (500-µm-diameter circle), no correlation was found between RT and BCVA (R=0.062; 95% CI −0.404 to 0.502). In the 43 eyes where the Cirrus HD-OCT identified an increased RT in the central fovea (central 500-µm-diameter circle), only a moderate correlation between RT and BCVA was found (R=−0.459; 95% CI −0.667 to −0.184).

Conclusion Correlations between RT and BCVA in CSME are only present when the central 500-µm-diameter circle is involved. However, even in this circumstance, a correlation was found in only 48.8% of the cases. RT cannot, therefore, be used as a surrogate outcome for visual acuity changes.

Diabetic macular oedema (DME) has been considered the most frequent cause of visual loss in patients with diabetes. This concept is due mainly from the perception that a decrease in macular oedema obtained by treatment is associated with an improvement in visual acuity.

Objective methods of measuring retinal thickness (RT) have recently become available, and studies are being performed to investigate the relationship between the degree of DME and visual acuity.

Different studies have reported different results, ranging between 0.28 and 0.73 for the correlation between optical coherence tomography (OCT)-measured RT and visual acuity.[1–5]

The verification of such a correlation, or the lack of it, has obvious clinical relevance. Is increased RT, which equals macular oedema, a good indicator of visual acuity loss? There are, now, commercially available improved versions of the OCT with increased sampling allowing a clear identification of the central 500-µm-diameter circle, such as the high-definition spectral domain Cirrus HD-OCT (Carl Zeiss Meditec, Dublin, California, USA).

In this paper, we compared best-corrected visual acuity (BCVA) with RT measurements, performed with the recently available Cirrus HD-OCT, in a series of patients with type 2 diabetes classified on stereocolour fundus photography, at an independent reading centre, as having clinically significant macular oedema (CSME) using the Early Treatment Diabetic Retinopathy Study (ETDRS) classification.

MATERIALS AND METHODS

Patients and study design

Seventy eyes with CSME from 37 type 2 diabetic patients, classified according to the ETDRS definition, were included in this observational study.

All eyes were classified as having CSME at an independent reading centre using stereocolour fundus photography according to the ETDRS definition. Eyes receiving this classification comply with one or more of the following criteria[6 7]:

1. thickening of the retina at or within 500 µm of the centre of the macula;
2. hard exudates at or within 500 µm of the centre of the macula, if associated with thickening of the adjacent retina;
3. a zone or zones of retinal thickening one disc diameter or larger, of which any part is within 1 disc diameter of the centre of the macula.

In addition to the CSME classification, according to the ETDRS criterion of "thickening of the retina at or within 500 µm of the centre of the macula", these eyes were also classified as being with or without retinal thickening in the central fovea based on Cirrus HD-OCT measurements and stereocolour fundus photography.

Exclusion criteria included eyes with photocoagulation treatment within the 3 months before inclusion in the study and eyes with cataract or any other eye disease that may interfere with fundus examination.

The tenets of the Declaration of Helsinki were followed, and approval from the institutional ethics and review board was obtained. Informed consent was obtained from all the patients (clinical trial identifier at http://www.clinicaltrials.gov/: NCT00797134).

All 70 eyes underwent RT measurements using the Cirrus HD-OCT, BCVA according to the ETDRS score and seven-field stereocolour fundus photography. Stratus OCT (Carl Zeiss Meditec, Dublin, California, USA) central point thickness was additionally collected.

Methods

Best-corrected visual acuity

BCVA in each eye was assessed according the ETDRS protocol[6 8] using Precision Vision charts "R", "1", and "2" (Precision Vision, Bloomington, Illinois, USA) at a distance of 4 m.

Retinal thickness

Retinal thickness was assessed by stereocolour fundus photography, for CSME classification according to the ETDRS criteria, and objectively measured by Cirrus HD-OCT and by Stratus OCT (central point thickness only).

Stereocolour fundus photography

Stereocolour fundus photography was performed according to the ETDRS guidelines for CSME classification—field 2 of seven-field stereocolour fundus photography.

Cirrus HD-OCT

A major advantage of the Cirrus HD-OCT, as compared to the Stratus version and besides the increase in sampling (65 536 readings for the Macular Cube 512×128 Combo Protocol), is the availability of two distinct fundus references: a C-scan computed from the tomographic data and a reference obtained by scanning the eye fundus through scanning laser ophthalmoscopy. Although none of these two references excels in image quality, both outperform the Stratus OCT fundus reference that is unusable for the large majority of scans.

To increase the accuracy in detecting the centre of the fovea, this location was identified in colour fundus photographs of the same eye. Through an image coregistration procedure (projective transformation), Cirrus HD-OCT fundus references were coregistered to the respective colour fundus photographs. This procedure allows the centre of the fovea to be identified in the thickness map based on its easier identification in the respective colour fundus image. In addition, because of sampling, the average RT for the 500-µm-diameter circle, centred at the identified foveal location, was computed resorting to thin plate spline interpolation,[9 10] a procedure that allows data to be interpolated by defining a smooth surface passing through all control points.

A Cirrus HD-OCT map over the fundus reference can be seen in figure 1.

To establish a reference value for the central 500-µm-diameter circle, an age-matched control population of 29 eyes, from healthy volunteers with ages ranging from 40 to 85 years (mean (SD) 52.9 (10.7) years), underwent RT measurements with the Cirrus HD-OCT. The reference value, that is, the value above which RT was considered increased, was defined as the mean (2 SD). Based on this thickness-value reference, RT in the central fovea was considered increased whenever the measured RT in the central 500-µm-diameter circle was >261.8 µm (ie, 231.5 + 2×15.1 µm).

Cirrus versus Stratus OCT

Whereas for the Stratus OCT the average RT for the central 1000-µm-diameter circle and the central point thickness values are the only options available, when using the six radial line-scans (Fast Macular Protocol), for the Cirrus HD-OCT a detailed RT map is provided, allowing access to detailed information. The number (density) of RT measures by Stratus and Cirrus HD-OCT for the central 1000-µm-diameter circle is 128 and >5600 readings, respectively (ie, a sampling factor >40).

Statistical analysis

To analyse the correlation between RT measured with the Cirrus HD-OCT in the central 500-µm-diameter circle and BCVA, a regression analysis was performed, with the BCVA as the dependent parameter.

Regression analyses were performed for the eyes with and without retinal thickening in the central fovea (increased RT in the central 500-µm-diameter circle based on the Cirrus HD-OCT RT classification or based on the stereocolour fundus photography classification). The normal distribution of the different parameters, tested using the Kolmogorov–Smirnov test, was ensured by removing four outliers and by applying the logarithmic function to the RT parameters (logRT), which initially presented a skewed distribution.

The correlation between the central RT values and patients' age, diabetes duration and haemoglobin A_{1C} levels was also analysed using the Pearson correlation coefficient.

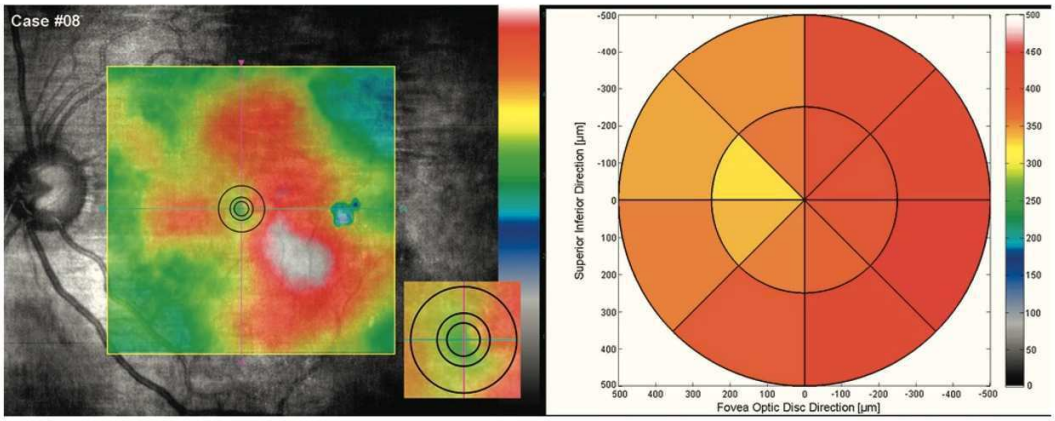

Figure 1 Retinal-thickness maps for case 08. (Left) Retinal-thickness map from the Cirrus HD-OCT where the circles indicate the central circles 300 (the foveola), 500 and 1000 µm in diameter centred on the fovea, as identified from the respective colour fundus photograph. (Right) Proprietary retinal-thickness map with circles of 500 and 1000 µm in diameter, each split into eight equal areas. Retinal thickness (in micrometers) can be deciphered through the colour bar on the right.

Nunes S, Pereira I, Santos A, *et al*. *Br J Ophthalmol* (2010). doi:10.1136/bjo.2009.165662

The agreement between the Cirrus HD-OCT and the stereocolour fundus photography classifications, for retinal thickening in the central fovea (ie, for the presence of an increased RT in the central 500-µm-diameter circle), was assessed using the Cohen's κ coefficient.

In addition, Stratus OCT central point thickness was correlated with the BCVA.

Statistical analyses (estimates and 95% confidence interval (CI)) were performed using the SPSS software V.13.0 (SPSS Inc., Chicago, Illinois, USA). Good agreements were considered for κ values >0.75 (based on the Landis and Koch criteria[11]). For the Pearson correlation coefficient (R), from the regression analyses between logRT and BCVA, the criteria defined by Swinscow and Campbell were used.[12]

RESULTS

From the initial 70 eyes included in this study, 8 eyes were excluded, 4 eyes with segmentation errors on Cirrus HD-OCT and another 4 considered as outliers on the statistical analysis.

Increased RT, in the central 500-µm-diameter circle (RT >261.8 µm) was detected in 43 eyes using the high-definition Cirrus HD-OCT. Therefore, 43 eyes were considered to have increased RT in the central fovea (central 500-µm-diameter circle) and 19 eyes were considered as not having increased RT in the central fovea.

Based on stereocolour fundus photograph grading, 40 of the 62 eyes were considered, by the independent reading centre, to have retinal thickening in the central fovea and 22 eyes were considered as not having retinal thickening in the central fovea.

Table 1 provides demographic and clinical characteristics for patients and study eyes.

No correlations were found between Cirrus HD-OCT RT in the central 500-µm-diameter circle and patients' age (R=0.044; 95% CI −0.208 to 0.291), diabetes duration (R=−0.055; 95% CI −0.301 to 0.197) and haemoglobin A_{1C} levels (R=0.031; 95% CI −0.220 to +0.279).

RT versus BCVA

Considering, separately, the eyes with and without increased RT in the central fovea by Cirrus HD-OCT (central 500-µm-diameter circle), the correlation between logRT and BCVA is absent for the eyes without increased RT in the central fovea and is only moderate for the eyes with increased RT in the central fovea (table 2).

For eyes with increased RT in the central fovea (central 500-µm-diameter circle), the correlation coefficient is R=−0.459 (95% CI −0.667 to −0.184) (figure 2), whereas for eyes without increased RT in the central fovea, the correlation coefficient is R=0.062 (95% CI −0.404 to 0.502).

The moderate correlation in the eyes with increased RT in the central fovea is well demonstrated by the fact that only 48.8% of the eyes (21 out of the 43) are within the 99% mean prediction interval for the regression line.

The lack of a correlation between the more increased values of RT and worst BCVA is shown in table 3 (number of eyes with an observed BCVA different, better or worse, than the predicted value, that is, than the BCVA predicted by the regression line).

Table 1 Patients' characteristics

Patients/eyes	
Patients/eyes (n/n)	36/62
Sex (men/women) (n/n)	22/14
Age (years), mean (SD) (min; max)	64.1 (8.7) (44; 79)
Diabetes duration (years), mean (SD) (min; max)	10.8 (6.8) (1; 30)
HbA_{1C} (%), mean (SD) (min; max)	8.4 (1.8) (5.9; 12.8)
BCVA (letters), mean (SD) (min; max)	73.3 (11.0) (45; 92)
RT—Cirrus HD-OCT	
Ø 500 µm area (µm), mean (SD) (min; max)	326.2 (100.1) (169.6; 621.7)

BCVA, best-corrected visual acuity; HbA_{1C}, haemoglobin A_{1C}; HD-OCT, high-definition optical coherence tomography; RT, retinal thickness.

Table 2 Pearson correlation coefficient and 95% CI for the regression analysis between Cirrus HD-OCT retinal thickness in the 500-µm-diameter circle (logRT) and BCVA

	Retinal thickening in the central fovea based on	
Central fovea (Ø 500 µm)	Cirrus HD-OCT	Stereocolour fundus photography
With increased RT	−0.459 (−0.667 to −0.184) (n=43)	−0.342 (−0.591 to −0.034) (n=40)
Without increased RT	+0.062 (−0.404 to +0.502) (n=19)	−0.005 (−0.426 to +0.417) (n=22)

BCVA, best-corrected visual acuity; HD-OCT, high-definition optical coherence tomography; RT, retinal thickness.

Foveal involvement classifications: Cirrus HD-OCT versus stereocolour fundus photography

A moderate agreement was found between the classifications for CSME with or without increased RT in the central fovea (central 500-µm-diameter circle) based on the Cirrus HD-OCT (ie, RT in the 500-µm-diameter circle >261.8 µm) and the stereocolour fundus photography (κ=0.673; 95% CI 0.478 to 0.868).

Fifty-three of the 62 eyes with CSME (85.5%) received the same classification by both Cirrus HD-OCT and stereocolour fundus photography. Thirty-seven eyes (59.7%) were classified with increased RT in the central fovea (central 500-µm-diameter circle) by Cirrus HD-OCT and stereocolour fundus photography, and 16 eyes (25.8%) were classified without increased RT in the central fovea by both methods.

When considering the stereocolour fundus photography classification for retinal thickening in the central fovea, the strength of the correlation between logRT and BCVA decreases. For these eyes, the correlation becomes weak (R=−0.342; 95% CI −0.591 to −0.034), whereas for eyes without retinal thickening in the central fovea, the correlation remains absent (R =−0.005; 95% CI −0.426 to +0.417) (table 2).

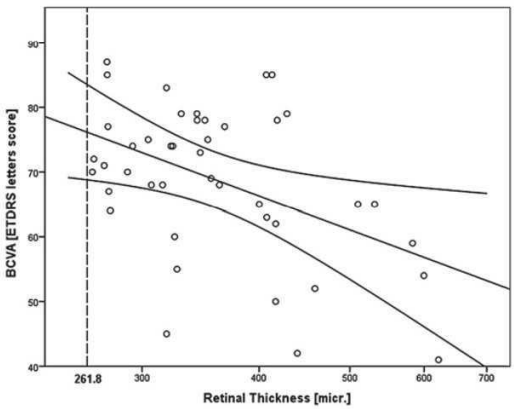

Figure 2 Retinal thickness in the 500-µm-diameter circle (logarithm scale) versus BCVA for the eyes with increased RT (based on Cirrus HD-OCT) in the central fovea (n=43; regression line with 99% mean prediction interval).

Table 3 Number of eyes with increased RT in the central fovea (central 500-µm-diameter circle) (n=43), with an observed BCVA different (worse, or better) than the predicted value (from the regression line)

Central 500-µm-diameter circle retinal thickness (measured by cirrus HD-OCT)	Number of eyes with a BCVA worse/better than the value predicted by the regression line	
RT range (µm)	Worse	Better
261.8–300	6	4
300–350	5	8
350–400	2	3
400–450	5	4
>450	3	3
Total	21	22

BCVA, best-corrected visual acuity; HD-OCT, high-definition optical coherence tomography; RT, retinal thickness.

Stratus OCT central point

The correlation between logRT and BCVA, when using the Stratus OCT central point thickness, was found to be moderate (as well) for the eyes with increased RT in the central fovea (R=−0.460; 95% CI −0.668 to−0.185), that is, in the same range of the correlation found for the Cirrus HD-OCT.

DISCUSSION

Central RT, as measured by OCT Stratus, has been proposed by several authors as a surrogate outcome for visual function, whereas recent findings reported by the DRCR.net in a large series of 251 eyes with DME, before and after laser treatment,[1] documented only a modest correlation between BCVA and Stratus OCT-measured central point thickness.

We examined a series of 62 eyes with DME, correlating BCVA with high-resolution spectral domain Cirrus HD-OCT measurements of RT for the inner 500-µm-diameter circle.

Evaluation of the central 500-µm-diameter circle is particularly relevant taking into consideration the accepted definition of CSME and the current understanding that visual acuity values are mainly dependent on the function of the central fovea.[6 7]

The separation of the eyes into two different groups based on retinal thickening in the central fovea, using the Cirrus HD-OCT RT maps (allowing a clear identification of the central 500-µm-diameter circle), offers a better insight into any correlation between RT and BCVA.[13]

This study confirms the DRCR.net study findings,[1] which showed only a moderate correlation between Stratus OCT thickness values and BCVA, even when considering the central 500-µm-diameter circle.

Correlations between RT, measured by the Cirrus HD-OCT, and BCVA were only identified in eyes with retinal thickening in the central fovea (central 500-µm-diameter circle).

No clear association could be found between higher ranges of increased thickness and worst visual acuities, nor the reverse. Worst than expected visual acuities were predominant in the group of eyes that had oedema of the central 500-µm-diameter circle <300 µm and in the group that had increased RT values in the central macula between 400 and 450 µm.

It is interesting that stereocolour fundus photography classification of retinal thickening in the central 500-µm circle performed by trained graders showed only a moderate agreement with the classification based on the Cirrus HD-OCT thickness in the 500-µm-diameter circle.

Our findings show that OCT RT measurements, although able to quantify the height and volume of macular oedema, cannot be used alone as a reliable indicator of visual acuity loss.

Assessment of macular thickness using OCT is clinically useful and demonstrates objectively the degree of macular oedema. Macular thickness, however, does not, per se, correlate well with visual acuity in eyes with DME.

The degree of macular oedema may only represent the degree of the breakdown of the blood–retinal barrier (BRB) and the associated inflammatory response, and, as such, is just a sign of only one component of disease progression. Subclinical macular oedema may be present from the earliest stages of diabetic retinal disease as soon as there is an alteration of the inner BRB that is not compensated by the pumping activity of the outer BRB.

It is necessary to reach a scientific consensus on the definition of the different subtypes of DME. Demonstration of increased RT, with an objective method such as OCT, should be mandatory. Furthermore, the presence, or absence, of increased RT values in the central 500-µm-diameter circle of the retina should also be indicated. Other factors that should be taken into account are the total area of the retina that shows increased RT, the finding of large "cystic" spaces in the retina suggesting large accumulations of extracellular fluid, the duration of the oedema and its response to treatment. Another piece of information that may become particularly relevant is the status of the retinal photoreceptors (rods and cones) within the area of oedema.[13]

It is realised that the next step is to correlate RT measurements with changes in BCVA over a time interval, before and after treatment, using procedures similar to the ones used in the present study.

Competing interests None.

Ethics approval This study was conducted with the approval of the Association for Innovation and Biomedical Research on Light and Image-Comissão de Ética para a Saúde, Coimbra, Portugal.

Provenance and peer review Not commissioned; externally peer reviewed.

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Macular Thickness Measured by Stratus Optical Coherence Tomography in Patients with Diabetes Type 2 and Mild Nonproliferative Retinopathy without Clinical Evidence of Macular Edema

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Key Words
Retinal thickness · Optical coherence tomography · Macular edema · Diabetes type 2 · Diabetic retinopathy

Abstract
To evaluate macular thickness in eyes with mild nonproliferative diabetic retinopathy (NPDR), patients with diabetes type 2, NPDR level 20 or 35, and without evidence of clinical macular edema underwent best-corrected visual acuity assessment, color fundus photography and Stratus optical coherence tomography. Mean center point thickness (CPT) and mean central subfield (CSF) thickness were compared with those of a healthy control population. 410 eyes/patients aged 61.2 ± 8.3 years, and with glycosylated hemoglobin of 7.9 ± 1.5% were included. Mean CPT and CSF were 186.6 ± 28.4 and 215.2 ± 25 μm, respectively, significantly increased compared to healthy subjects ($p < 0.001$). CSF thickness was abnormally increased in 17.6% of the patients, with values within the normal range in 79.5%, and abnormally decreased in 2.9%. CPT and CSF thickness were significantly thicker in men. No systemic factors showed a significant association. A significant increase in the macular thickness was found in eyes/patients with mild NPDR without clinical macular edema; however, only 17.6% of the eyes/patients had abnormally increased values and less than 3% abnormally decreased values.

Introduction

Macular thickness assessment is essential to evaluate and manage patients with diabetic retinopathy (DR), as diabetic macular edema is the major cause of severe visual impairment in this population [1, 2]. Slitlamp biomicroscopy of the fundus has been the standard method used for the clinical detection of diabetic macular edema [3], a process highly dependent on observer skill and experience, relatively insensitive to small degrees of foveal thickening [4–11]. Over the last few years, optical coherence tomography (OCT) imaging gained popularity, as it provides objective and reproducible measurements of retinal thickness (RT) [6, 12–14] and allows visualization of intraretinal morphology, similar to histological sections [15].

In the literature, reports on macular thickness measurements obtained with OCT in the early disease stages have been contradictory. In diabetes type 2, a decrease in central

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subfield (CSF) thickness was found by Oshitari et al. [16], in patients without DR, and by Asefzadeh et al. [17], in a subgroup of patients without DR or with mild DR. These findings were atributed to a neurodegenerative process in the diabetic retina, preceding vascular abnormalities [16, 17]. Other studies including patients with both types of diabetes, and absence of DR or mild DR lesions, showed no differences in RT measurements between diabetics and nondiabetics [18–20]. Finally, thicker central maculas in subjects with mild nonproliferative diabetic retinopathy (NPDR) were reported in studies including both types of diabetic patients, suggesting the occurrence of subclinical macular edema in the initial stages of DR [6, 8, 9].

The effect of age and gender on RT mean values has also been analyzed. Few studies found no significant correlation betweeen RT and aging, in normal [7, 21] and in diabetic subjects [6–8], whereas most studies have documented higher RT measurements in healthy men [19, 21–23] and in diabetic males with mild NPDR [7, 20, 24].

To address these issues, we used time domain Stratus OCT to measure RT in a large cohort of diabetic subjects (n = 410) with mild NPDR (Early Treatment Diabetic Retinopathy Study, ETDRS, levels 20 and 35), and compared the results with those obtained from a healthy control population. Stratus OCT was used because it was the most widely available equipment when the study was initiated and to allow comparison with previous reports in the literature. The effect of age and gender, duration of diabetes, glycosylated hemoglobin, blood pressure and serum lipid levels on macular thickness measurements was analyzed.

Materials and Methods

This study was conducted at the Association for Innovation and Biomedical Research on Light and Image (AIBILI), Coimbra, Portugal, and it reports the baseline values of a prospective study, designed to evaluate the 2-year progression of eyes with mild NPDR to clinically significant macular edema in diabetes type 2. Informed consent was obtained from each patient after explanation of the nature of the study and before any study procedure. The study followed the tenets of the Declaration of Helsinki and was approved by the local institutional review board and the local ethics committee (trial registry NCT00763802, ClinicalTrials.gov).

Study Population

Patients with adult-onset diagnosed type 2 diabetes, aged between 40 and 80 years, and no history of renal, liver or pancreatic disease in the medical records, were prospectively recruited. Eyes were eligible if they met the following inclusion criteria: (1) absence of foveal thickening on funduscopy; (2) mild NPDR – ETDRS levels 20 and 35, graded on color fundus photography by the AIBILI Reading Center; (3) best-corrected visual acuity (BCVA) ≥20/25 (≥79 letters), assessed with ETDRS charts; (4) refraction with spherical equivalent less than 5 dpt.

Patients with cataract or other eye disease that may interfere with fundus examination or any study procedure, glaucoma, vitreous syneresis or posterior vitreous detachment, other retinal vascular disease, recent intraocular surgery, previous laser therapy or any other previous treatment modality for macular edema were excluded from participation in the study.

Study Procedures

Clinical and Ophthalmological Examination

Demographic data, including age (age at the time of examination) and gender, duration of diabetes and current treatment, and concomitant medications were recorded. Biochemical analysis of blood samples was performed on the day of the clinical examination for glycosylated hemoglobin, total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol and triglycerides. Systolic and diastolic blood pressures were evaluated after the participants had been seated for 5 min with legs uncrossed. A total of 3 measurements were taken and averaged.

All patients underwent ophthalmological examination with determination of BCVA, by certified technicians, using ETDRS procedures and standardized refraction, slitlamp biomicroscopy, Goldmman applanation tonometry and ophthalmoscopy. Color fundus photographs of the 7 standard fields were obtained and graded for the presence and severity of DR, at the AIBILI Reading Center, using the ETDRS protocol, a modification of the Arlie House classification scheme [25].

Only one eye per subject was enrolled; when both eyes of the same patient were eligible, the right or left eye was randomly chosen.

OCT Measurements

The eyes from each subject were imaged using Stratus OCT (Carl Zeiss Meditec Inc., Dublin, Calif., USA), version 4 system, after pupil dilation, with tropicamide 1% and phenylephrine hydrchloride 2.5%, by a certified and experienced technician. RT was measured employing the standard fast macular thickness protocol of the Stratus OCT. This protocol generates retinal images in 1.92 s from 6 cross-sectional scan lines, 6 mm in length, in a radial spoke pattern centered on the fovea, at equally spaced angular orientations (30°). Each image had a resolution of 10 μm, axially, and 20 μm, transversally. The software places segmentation lines at the vitreoretinal interface and the junction between the inner and outer segments of the photoreceptors. The location of these boundaries is automatically determined by a thresholding algorithm that detects changes in reflectivity at each of these interfaces. RT is the distance measured between the two boundaries. Each line scan consists of 128 A scans; therefore, RT is measured at 768 points along 6 intersecting lines. This feature decreases the total acquisition time but sacrifices resolution. Scan analysis was performed using the Stratus OCT hardware with the Zeiss commercial scan analysis software. The machine printout comprises automated RT measurements in several macular locations, including the central point (center point thickness, CPT), the mean thickness at the point of intersection of the 6 radial scans, CSF, central circle area with 1-mm diameter, and in 4 inner and 4 outer subfields, located in 2 concentric rings, 1–3 mm (inner ring) and 3–6 mm (outer ring), from the center of the fovea, respectively.

Table 1. Age-matched healthy control group (n = 28)

	Mean	SD	Reference value (mean – 2 SD, mean + 2 SD)
Mean CPT, µm	171.3	20.0	130–210
Mean CSF thickness, µm	201.1	18.9	160–240

SD = Standard deviation.

Macular scans were visually inspected at AIBILI Reading Center for centration and image quality. Scans with signal strength <5 were excluded from the study. No manual measurements or corrections were done.

OCT Normative Database

An aged-matched healthy control group, composed by 28 normal eyes from 28 nondiabetic healthy volunteers, 11 men (39.3%) and 17 women (60.7%), with a mean age (±SD) of 53.4 ± 8.7 years (range: 40–77) was included. Control subjects had BCVA of 20/20, refraction with spherical equivalent of less than 3 dpt, unremarkable slitlamp biomicroscopy findings, normal intraocular pressure and absence of macular/retinal lesions or optic nerve abnormalities on funduscopy. These eyes were imaged using the same OCT device used in diabetic subjects, the Stratus OCT (Carl Zeiss Meditec), version 4 system, using the same acquisition protocol.

Mean (±SD) CPT and CSF thickness were 171.3 ± 20 and 201.1 ± 18.9 µm, respectively (table 1). No statistically significant differences were found between genders (males: mean CPT and CSF thickness ± SD were 172.0 ± 18.0 and 201.8 ± 15.4 µm, and females: mean CPT and CSF thickness ± SD were 171.6 ± 25.0 and 201.4 ± 21.8 µm, $p > 0.05$). Reference values were within 130–210 µm for CPT, and 160–240 µm for CSF thickness (i.e. mean – 2 SD and mean + 2 SD).

Due to the relatively small number of healthy controls, reference values were compared with those in the Stratus OCT normative database, obtained by Carl Zeiss Meditec from 350 healthy subjects, aged from 20 to 80 years. In this database, reference values are within 135–210 µm for CPT, and 160–240 µm for CSF thickness. In their normative database no statistically significant differences were found between gender and ethnicities [26, 27].

No statistically significant differences were found between the age-matched healthy control group included in this study (n = 28) and the Carl Zeiss Meditec normative database (CPT, $p = 0.309$, and CSF, $p = 0.507$). Therefore, because the RT measurements obtained in our age-matched healthy control population are comparable to those reported in the Carl Zeiss Meditec normative database, we used it for comparison with the diabetic patient population.

Statistical Methods

Statistically significant differences between genders were tested using the Mann-Whitney test and correlations between CPT, CSF thicknesses and BCVA were assessed using the Pearson correlation coefficient.

To identify predictive factors for the RT parameters (CPT and CSF), a linear regression analysis was performed considering age, gender, and systemic parameters as predictive factors.

Statistical analyses were performed using the STATA software version 12.1, and p values lower than 0.05 were considered as statistically significant results.

Results

Four hundred and ten eyes from 410 subjects with diabetes type 2 and mild NPDR were enrolled in this study. The study group was composed by 259 (63.2%) men and 151 (36.8%) women, with a mean ± SD age of 61.2 ± 8.3 years (range: 40–78). Diabetes duration was on average 10.0 ± 5.0 years (range: 1–21). Two hundred and ninety-three (71.5%) subjects were using diabetic oral agents, and 117 (28.5%) were taking insulin (61 insulin alone and 56 insulin combined with oral agents). The mean (±SD) level of glycosylated hemoglobin was 7.9 ± 1.5% (range: 5.0–13.0). Averaged blood pressure (systolic and diastolic), serum total, high-density lipoprotein and low-density lipoprotein cholesterol and triglyceride levels are shown in table 2. The BCVA letter score (and approximate Snellen equivalent) was 85.6 ± 4.1 letters (20/25; range: 80–95). Table 2 shows demographic and systemic parameters of the study population. Men had a significantly better BCVA and higher diastolic blood pressure, while women had significantly higher levels of cholesterol and high-density lipoprotein.

Overall, mean (±SD) CPT and CSF thickness were 186.6 ± 28.4 and 215.2 ± 25 µm, respectively. On average, diabetic subjects had a significant thickness increase in the central point ($p < 0.001$) and the CSF ($p < 0.001$), as compared with the control population. Nevertheless, using the normal reference values for the CPT (135–215), the numbers (and percentages) of eyes within, below and above these limits were 338 (82.4%), 9 (2.2%) and 63 (15.4%), respectively. For the CSF (normal reference values: 168–239), these numbers were 326 (79.5%), 12 (2.9%) and 72 (17.6%).

In our diabetic population, retinas from men were significantly thicker than retinas from women, in the central point (men: 190 ± 26.6 µm; women: 180.8 ± 30.4 µm, $p < 0.001$) and in the CSF (men: 219.2 ± 23.2 µm; women: 208.4 ± 26.6 µm, $p < 0.001$). Table 3 shows the ophthalmological characterization of the whole group considering genders for BCVA and OCT measurements.

CPT and CSF measurements showed a very strong correlation ($r = 0.934$; $p < 0.001$). No correlations were found between BCVA and RT, for the CPT $r = -0.015$ ($p = 0.672$) and for the CSF $r = 0.029$ ($p = 0.408$).

Based on the regression analysis, gender and age were the only parameters that showed an association with the central RT. Male gender was associated with higher RT

Table 2. Diabetic study population: demographic and systemic parameters (n = 410)

Variable		Mean	SD	Median	IQR
Age, years		61.2	8.3	62	55–67
	♂/♀	60.8/61.8	8.3/8.3	62/63	55–67/56–68
Diabetes duration, years		10	5	10	6–14
	♂/♀	9.9/10.2	5.0/5.1	10/10	6–14/6–14
HbA_{1c}, %		7.9	1.5	7.7	6.9–8.9
	♂/♀	7.8/8.1	1.5/1.6	7.6/7.9	6.7–8.9/7–8.9
SBP, mm Hg		151.7	21.5	151	136–164
	♂/♀	152.5/150.5	21.3/21.8	152/150	137–165/135–164
DBP, mm Hg		75.9	10.6	76	69–82
	♂/♀	76.9/74.3*	10.2/11.2	76.5/75	70–83/66–81
Cholesterol, mg/dl		197.5	42	194	168–222
	♂/♀	193.9/203.5*	44.1/37.5	190.5/201	162–223/177–222
HDL, mg/dl		51.1	12.8	49	42–58
	♂/♀	48.3/55.8*	11.6/13.6	47/52	40–55/47–62
LDL, mg/dl		128.1	32.6	125	105–148
	♂/♀	127/130.1	34.2/29.8	123/126	102–152/111–141
Triglycerides, mg/dl		175.1	115.9	145	104–214
	♂/♀	181.5/164.1	126.5/94.4	152.5/139	106–216/101–212

SD = Standard deviation; IQR = interquartile range; ♂ = men; ♀ = women; HbA_{1c} = glycosylated hemoglobin A_{1c}; SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL = high-density lipoprotein; LDL = low-density lipoprotein. * $p < 0.05$: statistically significant difference between genders.

Table 3. Ophthalmological characterization of the study population (BCVA and OCT) (n = 410)

Variable		Mean	SD	Median	IQR
BCVA, letters		85.6	4.1	85	85–90
	♂/♀	86.1/84.6*	4/4	85/85	85–90/80–90
CPT, µm		186.6	28.4	185	166–204
	♂/♀	190/180.8*	26.6/30.4	189/176	168–208/160–197
CSF thickness, µm		215.2	25	215.5	198–233
	♂/♀	219.2/208.4*	23.2/26.6	220/204	205–235/191–227

SD = standard deviation; IQR = interquartile range; ♂ = men; ♀ = women. * $p < 0.05$: statistically significant difference between genders.

measurements, both in the central point ($p = 0.002$) and in the CSF ($p < 0.001$). Age showed a positive association with CPT ($p = 0.034$; in the CSF, $p = 0.291$). After adjusting for gender, no systemic factors were significantly associated with RT in the central macula.

Discussion

In this study we performed macular thickness measurements in a large group of 410 patients with mild NPDR (ETDRS levels 20 and 35) and diabetes type 2. Only eyes with no evidence of macular edema on slitlamp examination or previous treatment for macular edema were included in the study (1 eye per patient, the study eye).

In this population of patients with diabetes type 2, mean macular CPT was 186.6 ± 28.4 µm, and mean macular CSF thickness was 215.2 ± 25.0 µm, both significantly increased over nondiabetic controls. It is generally recognized that CPT measurements show a greater variability than CSF thickness measurements and are, therefore, less reliable [14, 28].

Our CSF values are in agreement with measurements previously reported by Oshitari et al. [16] (220.1 ± 33.6 µm)

and Asefzadeh et al. [17] (218.0 ± 29.0 μm), in similar populations, with mild NPDR and diabetes type 2. Other studies, which included subjects with both types of diabetes and eyes without retinal vascular changes or with mild NPDR, reported lower thickness measurements in the CSF [18, 20, 24], similar to values found in healthy, non-diabetic controls.

Diabetic men showed, in our population, significantly thicker maculas than diabetic women. This gender imbalance has, indeed, been reported previously both in normal, healthy subjects [19, 21, 29] and diabetic patients with mild NPDR [16, 20, 24].

In addition to duration of disease, other risk factors for development and progression of diabetic maculopathy have included higher glycosylated hemoglobin levels [30, 31], higher blood pressure levels [32, 33] and higher serum lipid levels [34–36]. We found no significant association between any of these systemic parameters and macular thickness measurements, in our population, as in other reports [17, 20].

The presence of increased macular thickness measurements in the absence of clinical evidence of macular edema on slitlamp examination is not surprising, considering previously published reports. Brown et al. [4] found a poor agreement in macular thickness assessments between slitlamp examination and OCT imaging when macular thickness values remain below 300 μm. Thus, the term subclinical macular edema was introduced to describe diabetic subjects without a diagnosis of edema, on slitlamp examination, but showing abnormally increased macular thickness in OCT [4, 24]. More recently, the DRCR.net defined subclinical macular edema as absence of signs of edema, on slitlamp examination, and a CPT measurement on Stratus OCT greater than 225 μm and less than 299 μm [37]. Using this definition, we detected in our group of patients 38 (9%) eyes/patients that meet the criteria of subclinical macular edema.

A review of the literature shows variable results of RT measured by OCT in eyes with mild NPDR. Reports of abnormal retinal thinning have been associated with the presence of a neurodegenerative disease process preceding retinal vascular disease [16, 17].

Increased RT in diabetes, in the initial stages of clinical DR, has been associated with the presence of an early alteration of the inner blood-retina barrier and resultant fluid accumulation in the retina [38]. There have also been reports of retinal ganglion cell death and axonal degeneration occurring in the initial stages of DR, particularly in diabetes type 1 patients [39, 40].

Comparing our population of 410 patients with diabetes type 2 and mild NPDR, considering only 1 eye per patient, with our CSF thickness reference values and Carl Zeiss Meditec normative database, we found that retinal thinning was present in only 2.9% of the diabetic eyes, 79.5% had RT values within the normal range and 17.6% had abnormally increased values, showing definite retinal thickening. It is possible that both disease processes, neuronal degeneration and breakdown of the inner blood-retina barrier, occur in variable degrees in different patients in the initial stages of DR. Neuronal degeneration causing retinal thinning could be masked by increased fluid accumulation, resulting in different RT values in different patients. A more sophisticated analysis of the different inner retinal layers of the retina using spectral domain OCT, as performed by van Dijk et al. [41], will certainly contribute to answer this question.

Future studies should also consider separately diabetes type 1 and diabetes type 2 patients. Finally, more information on subclinical macular edema is clearly needed, particularly regarding its predictive value for the development of clinically significant macular edema.

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Disclosure Statement

Isabel Pires, Ana Rita Santos, Sandrina Nunes and Conceição Lobo work at AIBILI and have no other conflict of interest to declare.

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Subclinical Macular Edema as a Predictor of Progression to Clinically Significant Macular Edema in Type 2 Diabetes

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Key Words
Subclinical diabetic macular edema · Center point thickness · Optical coherence tomography · Clinically significant macular edema · Nonproliferative diabetic retinopathy · Type 2 diabetes

Abstract
Objective: To examine the relationship between subclinical diabetic macular edema (DME) and the development of clinically significant macular edema (CSME) in nonproliferative diabetic retinopathy (NPDR) in patients with type 2 diabetes. ***Methods:*** A prospective, monocenter, observational study was designed to follow patients/eyes with type 2 diabetes and NPDR (Early Treatment Diabetic Retinopathy Study levels 20 and 35) with no prior laser treatment for 2 years or until development of CSME. Ophthalmologic examinations, including best-corrected visual acuity, fundus photography and optical coherence tomography (OCT), were performed at baseline, 6 months and a final visit. ***Results:*** A total of 348 patients completed study follow-up; 26 eyes developed CSME. Six out of 32 eyes/patients presenting subclinical DME at baseline developed CSME (18.7%), while 20 out of 316 eyes without subclinical DME developed CSME (6.3%). Eyes/patients with subclinical DME presented a risk for DME progression 3.686 times higher than that of eyes/patients without subclinical DME (95% confidence interval 1.221–7.988). ***Conclusions:*** Subclinical DME in eyes with NPDR identified by center point thickness measured on a Stratus OCT is a good predictor of CSME development.

Introduction

Optical coherence tomography (OCT) allows objective and quantitative evaluation of diabetic macular edema (DME), providing cross-sectional images of the internal microstructures of the retina and reproducible retinal thickness measurements [1, 2].

OCT has improved investigators' ability to follow macular edema changes, allowing detection of increased retinal thickness, i.e. edema, even before clinical detection by slit-lamp examination [3]. The term subclinical DME has been proposed to describe these early stages of macular edema [4–6]. Nevertheless, there are few data in

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the literature regarding the natural history of eyes with subclinical DME. Browning and Fraser [7] found progression to clinically significant macular edema (CSME) in 48 of 153 eyes with subclinical DME over a median follow-up period of 14 months. More recently, the Diabetic Retinopathy Clinical Research Network (DRCR.net) [5] showed that one quarter to one half of eyes with subclinical DME would progress to CSME within 2 years after its identification.

We designed a prospective study to follow patients with type 2 diabetes and mild nonproliferative diabetic retinopathy (NPDR) lesions, during 2 years, with repeated clinical and OCT examinations. Eyes with subclinical DME at baseline were identified, and the rate of progression to CSME was investigated. We also analyzed the systemic and ocular features that may be associated with progression to CSME. The definition of subclinical DME proposed by the DRCR.net [5], consisting in an absence of edema involving the center of the fovea on slit-lamp examination and a center point thickness (CPT) measurement on Stratus OCT (Carl Zeiss Meditec, Dublin, Calif., USA) of ≥225 and ≤299 μm, was used.

Materials and Methods

This was a prospective, monocenter, observational study conducted at the Association for Innovation and Biomedical Research on Light and Image (AIBILI), Coimbra, Portugal, between September 2007 and December 2011. The study was designed to follow eyes in subjects with type 2 diabetes and NPDR, without prior treatment for DME, for 2 years or until development of CSME for which treatment is indicated. CSME was identified on clinical examination by retinal thickening within 500 μm of the center of the fovea, or by the presence of hard exudates within 500 μm of the center of the fovea associated with adjacent thickening of the retina, or thickening of at least 1 disc area, any part of which is within 1 disc diameter of the center of the fovea [8].

Written informed consent was obtained from each patient after explanation of the nature of the study and before any study procedure. Patients received no fee for their participation in the study. The study followed the tenets of the Declaration of Helsinki and was approved by the local institutional review board and the local ethics committee (trial registry NCT00763802, ClinicalTrials.gov).

Subjects with adult-onset type 2 diabetes aged between 40 and 80 years were eligible if they had a best-corrected visual acuity (BCVA) letter score ≥79 (approximate Snellen equivalent of 20/25 or better) and refraction with spherical equivalent less than 5 diopters; absence of foveal thickening on stereoscopic fundus examination, and mild NPDR [Early Treatment Diabetic Retinopathy Study (ETDRS) levels 20 and 35], graded on clinical examination and confirmed by the reading center on color fundus photography (CFP). Patients were excluded from participation in the study if they had any of the following: cataract or other eye disease that may interfere with fundus examination or any study procedure; glaucoma; vitreous syneresis or posterior vitreous detachment; other retinal vascular disease; recent intraocular surgery; previous laser therapy, or any other previous treatment modality for macular edema. Only one eye per subject was enrolled; when both eyes of the same patient were eligible, the right or left eye was randomly chosen.

Eyes of patients who fulfilled the inclusion criteria were screened for subclinical DME, defined as an absence of foveal edema on slit-lamp examination and a Stratus OCT-based CPT between 225 and 299 μm, according to the definition of the DRCR.net [5].

Study patients were followed for up to 2 years, with examinations performed at baseline and after 6 and 24 months (3 visits in total), or until development of CSME for which treatment was indicated, at the discretion of the investigator. When CSME was detected before the 24-month visit and treatment was performed, for analysis purposes, the final study visit was the visit at the time of treatment.

During the study, ophthalmological examinations included, in all visits, determination of BCVA with ETDRS charts by certified technicians, using standardized refraction procedures, Goldmann applanation tonometry, and slit-lamp examination before and after pupil dilation, to assess foveal thickening. CFP of the 7 standard ETDRS fields was obtained at 30°, with a Zeiss FF450 camera (Carl Zeiss Meditec), and graded for the presence and severity of diabetic retinopathy (DR) at the Coimbra Ophthalmology Reading Center at AIBILI, using the ETDRS protocol, a modification of the Arlie House classification scheme [9]. CFP was performed at baseline, to determine study eligibility, and at the final visit, to evaluate progression of DR severity.

At the 3 visits, a certified and experienced technician performed macular thickness scans with a Stratus OCT (Carl Zeiss Meditec) version 4 system. Retinal thickness was measured using the fast macular thickness protocol of the Stratus OCT, which acquires 6 consecutive macular scans, 6 mm in length, centered on the fovea, at equally spaced angular orientations (30°), in 1.92 s of scanning. Retinal thickness measurements were based on the map analysis printout. This map displays mean (±SD) retinal thickness in several macular regions, including at the central point, central subfield (central area with 1-mm diameter) and in 4 sectors at 2 concentric rings, the inner and the outer ring, 1–3 and 3–6 mm from the center of the fovea, respectively. CPT is the mean thickness at the point of intersection of the 6 radial scans, i.e. mean thickness at the very center point of the fovea.

Macular scans were visually inspected at the Coimbra Ophthalmology Reading Center at AIBILI for centration and image quality and to ensure that the foveal depression was evident in the center of the scan. Additionally, scans were reviewed by a retina specialist for abnormalities, such as vitreoretinal traction, retinoschisis and lamellar macular holes. Scans with artifacts (boundary errors, decentration) and/or signal strength <5 were excluded from the study. No manual measurements or corrections were made.

At baseline, the patient's body weight, height and concomitant medications were recorded. On the day of the clinical examinations, patients also underwent biochemical analysis of blood samples for glycosylated hemoglobin (HbA_{1c}), total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol and triglyceride levels and evaluation of systolic and diastolic blood pressure (BP), with a digital automatic BP monitor. Three mea-

Table 1. Baseline demographic, systemic and ophthalmological characteristics of the study participants (n = 410)

Variable	Min.	Max.	Mean	SD	Median	IQR
Age, years	40	78	61.2	8.3	62.0	55–67
Diabetes duration, years	1	21	10.0	5.0	10.0	6–14
HbA_{1c}, %	5	13	7.9	1.5	7.7	6.9–8.9
SBP, mg/dl	96	217	151.7	21.5	151	136–164
DBP, mg/dl	39	106	75.9	10.6	76	69–82
Glucose, mg/dl	38	468	176.9	751	165	119–226
Cholesterol, mg/dl	99	382	197.4	42.0	194	168–222
HDL cholesterol, mg/dl	24	104	51.1	12.8	49	42–58
LDL cholesterol, mg/dl	59	280	128.1	32.6	125	105–148
Triglycerides, mg/dl	49	1,126	175.1	115.9	145	104–214
BCVA, ETDRS letters	80	95	85.6	4.1	85	85–90
OCT-based CPT, µm	112	280	186.6	28.4	185	166–204

At baseline, 38 patients (9.3%) had subclinical DME. Min. = Minimum; Max. = maximum; IQR = interquartile range; SBP = systolic BP; DBP = diastolic BP; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

surements were taken and averaged after the participants were seated for 5 min with legs uncrossed.

To identify predictive factors for the development of CSME, a logistic regression analysis was performed.

Statistically significant differences between groups were compared using the Mann-Whitney test, and statistically significant differences between visits were tested using the Wilcoxon test.

Statistical analyses were performed using STATA software (version 12.1). Statistically significant results were considered for p values ≤0.05.

Results

Four hundred and ten eyes from 410 subjects with type 2 diabetes and mild NPDR (ETDRS levels 20 and 35) were included in this prospective study and screened for subclinical DME between September 2007 and December 2009. The study group was composed of 259 men (63.2%) and 151 women (36.8%), with a mean ± SD age of 61.2 ± 8.3 years (range 40–78). Mean ± SD diabetes duration and mean ± SD HbA_{1c} level were, respectively, 10.0 ± 5.0 years (range 1–21) and 7.9 ± 1.5% (range 5.0–13.0). Averaged BP (systolic and diastolic) and serum lipid levels are shown in table 1. The mean ± SD BCVA letter score was 85.6 ± 4.1 letters (range 80–95; approximate Snellen equivalent 20/20). At baseline, 38 eyes (9.3%) met the definition of subclinical DME chosen for this study, consisting in an absence of foveal thickening on slit-lamp examination and a Stratus OCT-based CPT between 225 and 299 µm. Table 1 shows baseline demographic, systemic and ophthalmological characteristics of the study participants.

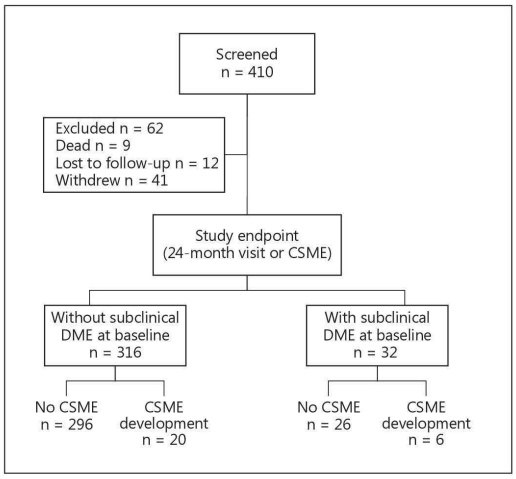

Fig. 1. CONSORT flowchart for eyes/patients enrolled in the study.

Overall, 376 patients attended the 6-month visit, and 348 completed study follow-up; 331 attended the 24-month visit (CSME detected in 6 eyes) and 17 reached the primary study outcome before the end of the study, i.e. developed CSME and received treatment. In total, 62 patients were lost to follow-up (fig. 1).

During follow-up, 15 eyes, 2 with subclinical DME at baseline, progressed on the ETDRS DR severity scale

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from level 35 (mild NPDR) to level 43 (moderate NPDR). In this group of eyes, no significant differences in BCVA letter score were found between baseline and the final study visit (p = 0.427). Similarly, at the final study visit, no significant differences in mean BCVA were found between eyes that showed increased severity on ETDRS levels versus those without DR level change (p = 0.490). Four of these eyes developed CSME, none of which had subclinical DME at baseline.

Of the 38 eyes/patients with subclinical DME at baseline, 35 eyes/patients attended the 6-month visit. At that time, BCVA had decreased by ≥5 letters in 21 eyes (60%), was maintained in 7 (20%) and increased by ≥5 letters in 7 (20%). CPT decreased in 45.7% of eyes (n = 16; below the subclinical DME definition limits), was within subclinical limits in 51.4% (n = 18) and exceeded 300 μm in 2.9% (n = 1), although without a clinical diagnosis of CSME. Thirty-two eyes/patients completed the study follow-up, i.e. reached the primary study outcome (between the 6- and 24-month visits) or attended the 24-month visit (with or without reaching the outcome). Among these, progression to CSME was clinically detected in 6 eyes, in 3 between month 6 and month 24 and in the other 3 at the 2-year visit.

In the regression analysis, patients with subclinical DME at baseline showed a risk of progression to CSME 3.686 times higher than that of patients without subclinical DME at baseline (95% confidence interval 1.261–10.771; p = 0.017). Moreover, for each 1% increase in HbA_{1c} level, the risk of developing CSME increases by a factor of 1.567 (95% confidence interval 1.120–2.191; p = 0.009). No other patient or systemic factor, such as age, gender, systolic or diastolic BP, or cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol or triglyceride levels, predicted progression to CSME (table 2).

In the group of patients without subclinical DME at baseline (n = 372), 341 attended the 6-month visit (21 presenting subclinical DME and 1 eye with CPT >300 μm), and of the 316 that completed follow-up, 296 (93.7%) showed no evidence of CSME on clinical evaluation at either visit. CSME was detected in 14 eyes between the 6- and 24-month visits and in 6 eyes at 2 years. Eyes that developed CSME showed a decrease in mean BCVA, from 86 ± 4.5 to 82.8 ± 11.1 letters (p = 0.054), and an increase in mean CPT, from 188.2 ± 19.4 to 227.6 ± 135.8 μm (p = 0.681), at the baseline and final visit, respectively.

At the final visit, eyes with subclinical DME at baseline (n = 32) showed a more significant decrease in BCVA when compared with those without subclinical DME (n = 316; p = 0.012).

Table 2. Logistic regression analysis for CSME development

	Odds ratio	95% confidence interval		p
		lower limit	upper limit	
Age (years)	1.012	0.954	1.074	0.68
Gender (male/female)	0.831	0.335	2.060	0.69
Diabetes duration (years)	1.009	0.927	1.098	0.83
HbA_{1c} (%)	1.567	1.120	2.191	0.009
SBP (mm Hg)	1.001	0.976	1.027	0.91
DBP (mm Hg)	0.991	0.944	1.040	0.72
Glucose (mg/dl)	0.994	0.987	1.002	0.19
Cholesterol (mg/dl)	0.996	0.952	1.041	0.85
HDL cholesterol (mg/dl)	0.981	0.927	1.038	0.51
LDL cholesterol (mg/dl)	1.013	0.960	1.068	0.64
Triglycerides (mg/dl)	0.996	0.990	1.002	0.24
Subclinical DME (yes/no)	3.686	1.261	10.771	0.02
Constant	0.009	0.000	3.801	0.13

SBP = Systolic BP; DBP = diastolic BP; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

Discussion

In a large cohort of patients with type 2 diabetes, mild NPDR and ETDRS levels of 20 or 35, considering only 1 eye per patient, without prior treatment for DME, we found an overall prevalence of subclinical DME, defined as an absence of foveal edema on slit-lamp examination and a Stratus OCT-based CPT between 225 and 299 μm, of 9.3%. Follow-up of these patients during a period of 2 years or until development of CSME allowed a better understanding of the influence of systemic and ocular characteristics with regard to progression of retinal disease to CSME. Our results suggest that the presence of subclinical DME at baseline and the patient's metabolic control, as evaluated by HbA_{1c} levels, are predictors of progression to CSME.

Prior to our study, the DRCR.net found a prevalence of subclinical DME of 4.8% in 582 participants with type 1 or 2 diabetes and mild NPDR (ETDRS retinopathy severity level ≥35) [5]. This study group proposed the definition of subclinical DME that was used in this study. In this definition, the limit of retinal thickness above which eyes are classified as having subclinical DME (225 μm) was based on previous retinal thickness measurements obtained in eyes without DR or with mild DR, being 2 SDs above the mean CPT of those diabetics [10]. The upper limit (299 μm) corresponds to what was considered the threshold for clinical detection [3].

When analyzing the eyes with subclinical DME, in the interval between baseline and the 6-month visit, 60.0% lost BCVA ≥5 letters, while 20.0% maintained the initial visual acuity score and 20.0% improved by ≥5 letters. CPT decreased in 45.7% of these eyes and remained within subclinical DME criteria in 51.4%.

Among the 32 eyes/patients with subclinical DME at baseline who completed the study follow-up, 6 progressed to CSME and received treatment (3 between the 6- and 24-month visits and the other 3 at the 24-month visit). When this group of 6 eyes with subclinical DME at baseline reached the outcome (CSME), there was an overall BCVA decrease and CPT increase, but these differences were not significant. Even though there is a trend favoring vision loss and an increase in central foveal thickness, on OCT, the results were not significant, probably due to the small sample size, which was insufficient to identify major differences. However, it is noteworthy that, when considering all eyes with subclinical DME at baseline, these eyes, when compared with eyes without subclinical DME at baseline, showed a significant decrease in visual acuity, indicating that the presence of subclinical DME is associated with future vision loss.

Disease progression, represented by a change in the level of DR severity, was evaluated clinically and on CFP of the 7 fields, using the ETDRS scale. Retinal level progression was detected in 15 eyes, from level 35 (mild NPDR) to level 43 (moderate NPDR), with only 2 of these showing subclinical DME at baseline. In this subset of eyes, there was no significant vision loss, and their final BCVA letter score was not different from that of the eyes that did not progress on the ETDRS scale. Vision loss observed in this study was, therefore, associated with retinal edema, identified by increased foveal thickening. This is in agreement with prior evidence from trials of ruboxistaurin, an orally administered inhibitor of protein kinase C-β, in DME patients, which showed that the most probable cause of sustained moderate vision loss in the eyes in their study was the presence of increased retinal thickening involving the center of the macula [11, 12]. It is generally accepted that, in the initial stages of clinical DR, increased retinal thickness is associated with an early alteration of the inner blood-retinal barrier [13, 14]. The resultant accumulation of fluid in the retinal layers around the fovea may lead to vision loss by altering the functional cell relationship in the retina and/or by promoting an inflammatory reparative response [14].

Furthermore, there have also been reports of retinal ganglion cell death and axonal degeneration occurring in the initial stages of DR [15]. It is possible that both disease processes, neuronal degeneration and breakdown of the inner blood-retinal barrier, occur in variable degrees in different patients in the initial stages of DR. Therefore, it is possible that increased thickness may be masked in some eyes that have more neuronal degeneration.

This study showed that eyes with subclinical DME at baseline have a 3.686-fold increased risk of progressing to CSME within a period of 2 years after its detection. Two previous studies have reported the natural course of eyes with subclinical DME. In the DRCR.net study [5], 75.0% of the eyes with subclinical DME met primary study outcome criteria by 1 year, and an estimated 38.0% by 2 years. Browning and Fraser [7], using a different definition of subclinical DME (macular edema clinically less than the CSME threshold, but detected by OCT – abnormal thickness in 1 of 9 OCT zones, abnormal total macular volume or abnormal comparison of 2 OCT zones), found, over a median follow-up of 14 months, progression to CSME in 48 of 153 eyes (31.4%). They also found spontaneous improvement of macular edema over time in a substantial fraction of eyes, as we did [7].

We found that for every 1% increase in HbA_{1c} level, the risk of developing CSME increases by a factor of 1.567. In this study, no other systemic factor predicted progression to CSME.

Subclinical DME is a relatively uncommon finding [5]. Nevertheless, when detected, the management of the patient should change. Early detection of DME is highly desirable, with the aim of preserving photoreceptors at early disease stages and retaining central visual acuity. Timely treatment for DME may be able to prevent loss of visual acuity associated with prolonged structural damage. These patients should, at least, be monitored more frequently to determine when the threshold for intervention is reached.

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Disclosure Statement

I. Pires, A.R. Santos, S. Nunes, C. Lobo and J. Cunha-Vaz work at AIBILI.

J. Cunha-Vaz is President of the Administration Board of AIBILI and Emeritus Professor of the Faculty of Medicine of the University of Coimbra. He is also a consultant for Allergan, Alimera Sciences, Bayer, Critical Health, Fovea Pharmaceuticals, Gene Signal, Novartis, Pfizer and Roche.

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4.4 Phenotypes of Diabetic Retinopathy Progression

The initial stages of DR are characterized by four major retinal alterations: presence of MA/haemorrhages, alterations of the BRB permeability, capillary closure and changes in the neuronal and glial cells of the retina [57, 44]. These retinal changes, even in the early stages of the disease, can be monitored by multimodal macula imaging (MMI) [95, 96]. MMI involves the combination of different imaging methodologies. It has already contributed for the identification of different phenotypes of NPDR [97]. Three phenotypes were identified by Lobo et al. using MMI (phenotypes A, B and C) [97]. These phenotypes are characterized by different patterns of progression. Phenotype A is characterized by the absence of BRB changes (no leakage on FA, i.e., no macular edema), phenotype B is characterized by changes of the BRB and phenotype C is characterized by the presence of ischemia in the central area of the macula. In a retrospective study [98] it was possible to show that only two of these phenotypes are associated with the development of CSME. It was shown that 11% of the patients classified as phenotype B and 30% of the patients classified as phenotype C developed CSME after 2 years of follow-up [98]. The existence of three different phenotypes of DR progression was also observed in a different group of patients using mathematical methods for data segmentation (i.e., using cluster analysis) [39, 99]. In this study a clear association was found between phenotypes I, II and III and phenotypes A, B and C. Despite the apparent correlation between the phenotypes previously identified by Lobo et al. [97] and the ones identified more recently by Nunes et al. [39, 99], a risk model for DR progression in the early stages of the disease could not be establish.

The identification of phenotypes of DR progression, associated with a higher or lower risk for CSME development, ideally based on non-invasive examinations, easy to repeat regularly in the clinical practice, will have therefore a significant impact both for the clinical management of diabetic patients, improving the services provided to these patients, and for the design of new clinical trials to evaluate new drugs or to define new therapeutic approaches.

To characterize and predict patients at higher risk of DR progression, more precisely to CSME, a prospective 2-year follow-up study, was designed to identify phenotypes of DR progression in patients with mild NPDR based on non-invasive imaging biomarkers. This study was conducted in the Clinical Trial Unit (CEC) of AIBILI.

4.4.1 Study Design

For the primary objective of creating and validating a predictive model for DR progression to CSME and/or vision loss, using non-invasive imaging techniques that allows for the quantitative assessment of the biomarkers associated with DR progression, a 2-years prospective observational clinical study was designed to include a cohort of 400 type 2 diabetic patients, males and females with ages ranging from 40 to 80 years, with:

- Mild NPDR (ETDRS severity levels 20 or 35, [100])
- BCVA ≥ 80 ETDRS letters score (Snellen equivalent of 20/25 or better) [101]

The study consist of an initial period of 6 months for the identification and characterization of patients' phenotypes (composed by 2 visits at months 0 and 6), plus one final evaluation visit to characterize patients after 2 years of follow-up (24-month visit).

Patients who developed CSME during the initial period (before the 6-month visit) were excluded from the study. Included patients were followed for 2 years, or until development of CSME (study endpoint).
Between month-6 and month-24 patients were followed according to the normal clinical practice. The presence of CSME was determined clinically based on the ETDRS definition [102]:

- Thickening of the retina located less than 500 μm from the centre of the macula; OR
- Hard exudates (with thickening of the adjacent retina) located less than 500 μm from the centre of the macula; OR
- A zone of retinal thickening 1 disc area or larger in size, located less than 1 disc diameter from the centre of the macula.

Considering the retinal changes occurring in the early stages of DR [57, 44], the following non-invasive ophthalmic procedures were performed to assess the two imaging biomarkers of DR progression, i.e., the MA activity and the macular thickness (macular edema):

- CFP – for the detection and quantification of the MA activity [58];
- OCT – for the assessment and quantification of retinal thickness and consequently for the assessment and quantification of macular edema (associated with the changes of the BRB permeability [59]).

The following procedures were also performed:

- Physical assessment: height, weight and blood pressure;

- Systemic Evaluation: laboratory analyses to evaluate the level of creatinine, HbA_{1C}, cholesterol, HDL, LDL, triglycerides and glucose;
- Ophthalmologic evaluation: complete ophthalmological examination with slit-lamp; review of the medical history and record of concomitant medications; BCVA, slit-lamp biomicroscopy and measurement of the intraocular pressure.

Only one eye per patient was considered for the study to reduce bias in the selection of the sample, being selected by the physician at baseline based on the inclusion and exclusion criteria. When both eyes meet the inclusion/exclusion criteria, the study eye was chosen alternatively by selecting the right or left eye.

4.4.1.1 Sample Size Estimation

The study sample size calculation was based on the results obtained in previous studies [98, 97, 39, 99], namely:

- Three different phenotypes of DR progression can be identified in patients with NPDR [98, 97, 39, 99]:
    - 50% of the patients should belong to one phenotype;
    - 30% of the patients should belong to a second phenotype;
    - 20% of the patients should belong to a third phenotype.
- Patients from two of these phenotypes are at higher risk for CSME development in a 2-year period [98]
    - 11% of the patients classified in the second phenotype will develop CSME;
    - 30% of the patients classified in the third phenotype will develop CSME.

Based on these results, to identify phenotypes of DR progression and to show an increased risk for CSME development for patients classified into two of these 3 phenotypes, 400 diabetic patients with NPDR need to be included.

The inclusion of 400 patients (one eye per patient):

- Will allow for the conclusion of the study with 360 patients (assuming a drop-out rate of 10%):
    - 180 patients should be classified in the first phenotype – none of these patients should develop CSME;
    - 108 patients should be classified in the second phenotype – 11 to 12 patients may develop CSME (11%);

  - 72 patients should be classified in the third phenotype – 21 to 22 patients may develop CSME (30%).
- Will allow for a statistical power of 80% and an alpha level of 0.05 for the detection of different risks for CSME development.

4.4.1.2 Study Outcomes for DR

To identify phenotypes of DR progression the following outcomes were considered:

- MA activity assessed on CFP. CFP were acquired using the digital retinal camera model FF450 from Zeiss (Carl Zeiss Meditec, Dublin, USA). Retinal images centred in the macula (30º images) were acquired and exported for RetmarkerDR processing. The following parameters were computed:
  - Number of MA in each visit;
  - MA formation rate (i.e., for a given period of time the number of new MA);
  - MA disappearance rate (i.e., for a given period of time the number of MA that disappeared); and
  - MA activity, or MA Turnover (computed manually as the sum of the MA formation and disappearance rates for a given period of time).

- Central retinal thickness measured by OCT. The Stratus OCT equipment from Zeiss (Carl Zeiss Meditec, Dublin, USA) was used, being the equipment available at the time of the study with the highest resolution. The Stratus OCT Fast Macular acquisition protocol was used in this study. This acquisition protocol allows obtaining maps of the retinal thickness in the macula, by using six radial scans of 6 mm each (B-scans), 30º apart.

4.4.2 Study Synopsis

The study protocol was elaborated and submitted to the RA for approval. Being an observational clinical study, the study protocol was submitted to the local Ethics Committee of AIBILI (Comissão de Ética para a Saúde – CES), and to the National Data Protection Committee (CNPD). This study was registered in the public database ClinicalTrials.Gov under the number NCT00763802. The study synopsis is provided in Table 7.

Table 7. Study synopsis ("Validation of a predictive model to estimate the risk of conversion to clinically significant macular edema and/or vision loss in mild nonproliferative diabetic retinopathy in diabetes type 2").

Study Synopsis (based on the Annex I of the ICH-E3 [19]).	
Sponsor	J. Cunha-Vaz (AIBILI).
Finished Product/Active Ingredient	None.
Title of Study	Validation of a predictive model to estimate the risk of conversion to clinically significant macular edema and/or vision loss in mild nonproliferative diabetic retinopathy in diabetes type 2.
Investigators	Prof. Dr. José Cunha-Vaz.
Study Centres	CEC-AIBILI, Coimbra, Portugal.
Studies periods (years)	2 years follow-up.
Study type / Phase of development	Prospective observational.
Objectives	To validate a predictive model of diabetic retinopathy progression to clinically significant edema (CSME) needing photocoagulation treatment and/or vision loss using non-invasive imaging techniques.
Methodology	Type 2 diabetic patients with mild NPDR will be followed during 24-months with a maximum of 3 study visits (Month-0, Month-6 and Month-24). Patient will performed in each visit: • Ophthalmic examination; • Laboratory analysis; • BCVA; • CFP for ETDRS assessment and for MA count; • OCT. Patients will be recruited at the Clinical Trial Centre, CEC-AIBILI, Azinhaga de Santa Comba – Celas, Coimbra, Portugal.
Number of patients	400 type 2 patients with mild NPDR.
Diagnosis and main criteria for inclusion	Patients of both sexes and any race with diagnosed diabetes type 2 will be included according to the inclusion/exclusion criteria. . Inclusion criteria: 1. Diabetes type 2 according to 1985 WHO criteria; 2. Age between 40 and 80 years; 3. Mild NPDR (based on ETDRS criteria); 4. BCVA ≥ 80 ETDRS letters score (Snellen equivalent 20 /25 or better); 5. Refraction with a spherical equivalent less than 5 diopters; 6. Inform consent. Exclusion criteria: 1. Cataract or other eye disease that may interfere with fundus examinations; 2. Glaucoma; 3. Vitreous syneresis or posterior vitreous detachment; 4. Other retinal vascular disease; 5. Recent intraocular surgery; 6. Previous laser therapy; 7. Dilatation of the pupil < 5 mm.
Treatment Duration	Not applicable.
Test product/Reference Therapy	Not applicable.
Criteria for evaluation Efficacy and Safety	The primary endpoints are CSME needing photocoagulation and/or vision loss of 2 lines (ETDRS scale). Efficacy parameters: BCVA; Retinal thickness (using OCT); MA (using CFP) Safety parameters: BCVA

4.4.3 Statistical Methods

To identify phenotypes of mild NPDR a hierarchical cluster analysis was performed using MA parameters (number of MA at baseline and MA turnover from baseline to month-6) and central retinal thickness parameters (central subfield retinal thickness at baseline and changes from baseline to month-6). Cluster analysis is an unsupervised segmentation technique that allows for the identification of homogenous groups of patients based on the dispersion observed in the patients' data [103]. This technique group data sharing some similarity measure or feature [104], being hierarchical when the number of underlying clusters is unknown or non-hierarchical when the number of underlying clusters is known *a priori*. To identify phenotypes of NPDR, i.e., homogeneous groups (clusters) underlying the study population, the hierarchical Ward's method was used. Ward's method promotes the minimization of the within-cluster dispersion being therefore one of the methods most frequently used in clinical research [105, 106, 107]. To determine the best number of clusters the Calinsky-Harabasz pseudo-F statistics was computed [108]. Before performing the clustering process data was normalized since the range of values for the different parameters (MA and retinal thickness) had one order of magnitude.
The identified clusters, i.e., phenotypes, were thereafter fully characterized, based on the original parameters and on the remaining parameters: age, diabetes duration, creatinine, HbA_{1C}, blood pressure, cholesterol, HDL, LDL, glucose and triglycerides levels. Statistically significant differences between phenotypes were tested using the Kruskall-Wallis test. Moreover, to validate the phenotypes of DR progression, the risk for CSME development was computed and compared between the identified phenotypes (OR). To establish rules for phenotypes' classification a decision and classification tree was performed.

Statistically significant results were considered for $P < 0.05$. Statistical analyses were performed using the STATA software version 12.1 (StataCorp, College Station, TX, USA). The SPSS software version 13.0 (SPSS LEADS Technology Inc., USA) was used to compute the decision and classification tree.

4.4.4 Study Results

From the 410 eyes/patients included in the study, 376 completed the first 6-months of follow-up (2 patients died between the baseline and the 6-months visits, 25 withdraw from the study, and

7 were lost of follow-up). Three hundred and twenty two (322) eyes/patients performed the last study visit, 24-month visit, without developing CSME and 26 eyes/patients developed CSME needing treatment (Figure 16). No statistically significant differences were found between the 348 eyes/patients that reached the study end-point or that performed the last study visit and the 62 eyes/patients that dropped-out except for the cholesterol, the LDL levels and the BCVA.
For cluster analysis, based on the MA and retinal thickness parameters, 376 eyes/patients were considered (eyes/patients that performed the 6-month visit).

Three (3) clusters were identified by the hierarchical Ward's clustering method (corresponding to the higher Calinsky-Harabasz pseudo-F statistics). The results were published by Nunes et al. [109]. The first cluster, phenotype A, composed by 181 eyes/patients (48.1%) was characterized by low values for both the MA and the retinal thickness parameters. The second cluster, phenotype B, composed by 87 eyes/patients (23.2%) was characterized by a higher central subfield retinal thickness ($P < 0.001$), by a lower BCVA ($P < 0.027$, corresponding only to 1 ETDRS letter difference), and by being composed mainly by males ($P = 0.015$ when compared with phenotype A) and by older subjects ($P < 0.011$). The third cluster, phenotype C, composed by 108 eyes/patients (28.7%) was characterized by higher MA parameters, number and turnover ($P < 0.001$), by higher HbA_{1C} values ($P = 0.043$ when compared with phenotype A) and by lower LDL values ($P < 0.038$).

From the 26 eyes/patients that developed CSME needing laser photocoagulation, 3 were from phenotype A (i.e., 1.8%), 7 from phenotype B (i.e., 8.5%) and 16 from phenotype C (i.e., 16.2%). Eyes/patients from phenotypes C and B showed a higher risk for CSME than eyes/patients from phenotype A. For phenotype C the OR was 3.536, 95%CI (1.917-6.524) ($P < 0.001$); and for phenotype B the OR was 2.802, 95%CI (1.445-5.434) ($P = 0.002$). Phenotype C showed also a higher risk for CSME when compared to phenotype B (OR = 1.994, 95%CI (1.144-3.477); $P = 0.015$).

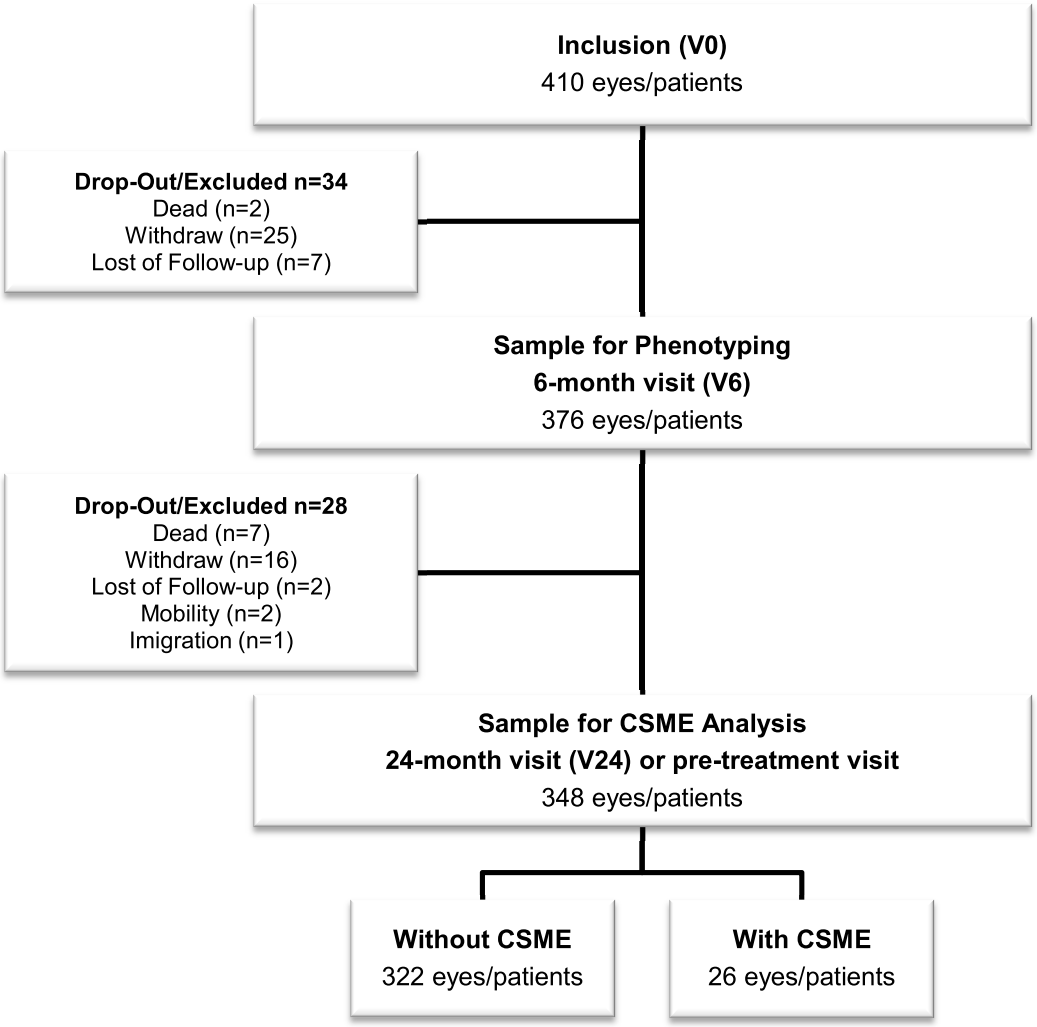

Figure 16. CONSORT flow chart for the patients included in the study NCT00763802.

Using a decision and classification tree 73.5% of the eyes/patients in phenotype A (133 out of the 181 eyes/patients) were characterized by a MA turnover < 6 and a central retinal thickness < 217 µm; 98.8% of the eyes/patients in phenotype B (86 out of the 87 eyes/patients) were characterized by a MA turnover < 6 and a central retinal thickness ≥ 217 µm; 97.2% of the eyes/patients in phenotype C (105 out of the 108 eyes/patients) were characterized by a MA turnover ≥ 6. The risk estimate for the decision and classification tree was 12.5%.

Based on these thresholds, and on the clinically meaningful parameters, the following rules were established to classify eyes/patients into one of the 3 phenotypes of NPDR progression:

- Phenotype A: MA turnover < 6 and normal retinal thickness values (central subfield retinal thickness < 220 µm, i.e., normal mean of the normative database + 1 SD).
- Phenotype B: MA turnover < 6 and increased retinal thickness values (central subfield retinal thickness ≥ 220 µm).
- Phenotype C: MA turnover ≥ 6.

Using these rules phenotype B shows a sensitivity and a specificity for CSME development of 88.9% and 60.5%, respectively (when compared to phenotype A), and phenotype C shows a sensitivity and a specificity of 94.4% and 55.9%, respectively (when compared to phenotype A).

4.4.5 Study Discussion

This IDCR demonstrated the existence of 3 different phenotypes of DR progression, characterized by two imaging biomarkers of DR progression/worsening, the MA turnover and the presence of macular edema, both quantifiable using non-invasive imaging techniques, CFP and OCT, respectively [109] (see section 4.4.6).

The potential of these two biomarkers was demonstrated independently in two sub-studies conducted by Ribeiro et al. [65] and Pires et al. [92, 93].

In the first sub-study, presented previously in section 4.3.1, we founded that the MA turnover shows a higher predictiveness for CSME than the remaining MA parameters, i.e., the number of MA and the MA formation and disappearance rates [66]. Moreover, the high standard deviation observed for the MA turnover values, when compared to the mean value, indicates that MA turnover values vary widely between eyes having similar ETDRS level, indicating that MA turnover may be a good indicator of different vascular disease activity in different eyes.

In the second stub-study, presented previously in section 4.3.2, the study population was characterized based on the presence of central retinal thickness abnormalities. The central retinal thickness was compared to a normative database and it was found that: 17.6% of the eyes/patients included had a central retinal thickness over the normal reference values, indicating the presence of macular edema and therefore the breakdown of the BRB; and, 2.9% of the eyes/patients included had a central retinal thickness below the normal reference values, indicating the presence of a neuronal degeneration [92]. Moreover, it was also found that eyes/patients with subclinical macular edema at baseline, defined as an absence of foveal edema in slit-lamp examination and a central point retinal thickness on Stratus OCT between 225 and 299 µm (according to the DRCR.net criteria), are associated with a more accentuated

vision loss, and showed a 3.7-fold increased risk for CSME development within the 2-year period [93].
These 2 sub-studies confirm the two processes that occur during DR, the breakdown of the BRB and the neuronal degeneration. These two processes occur in variable degrees in different patients in the initial stages of the disease [92].

Regarding the 3 phenotypes of DR progression, identified with cluster analysis in 376 patients with NPDR, these phenotypes were found to be comparable to those proposed previously by Lobo et al. [97]. One of the 3 phenotypes, phenotype B, is characterized by the presence of macular edema, being therefore associated with changes of the BRB permeability, and a second phenotype, phenotype C, is characterized by a high activity of the MA.

The identification of different phenotypes of DR progression, characterized by different retinal changes and by different risks of progression to CSME, opens new perspectives for a more personalized clinical management of the diabetic patients with DR. If patients with higher risk of progression (phenotypes B or C) and with the greatest potential to benefit from a particular treatment can be identified earlier, in a stage of the disease in which there is still no significant changes in the patient' visual acuity, we may significantly reduce the number of cases of blindness due to DR.
The results of this study may have also a significant impact on prospective interventional clinical research since it will contribute for a better selection of the patients, given that 50% of the patients with NPDR will have a slow progression phenotype. Therefore, interventional studies (i.e., clinical trials) of short duration (less than 2 years), may avoid the inclusion of patients presenting a slow progression phenotype since these patients may bias the results of these studies.

4.4.6 Scientific Contribution

The results of this study were published in 2013:

- S. Nunes, L. Ribeiro, C. Lobo, and J. Cunha-Vaz. Three different phenotypes of mild nonproliferative diabetic retinopathy with different risks for development of clinically significant macular edema. Investigative Ophthalmology and Visual Science, 54:4595–4604, 2013.

Retina

Three Different Phenotypes of Mild Nonproliferative Diabetic Retinopathy With Different Risks for Development of Clinically Significant Macular Edema

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Purpose. To identify different phenotypes of nonproliferative diabetic retinopathy (NPDR) and their progression to clinically significant macular edema (CSME).

Methods. A prospective observational study was designed to follow eyes/patients with diabetes type 2 and NPDR with no prior laser treatment for 2 years or until development of CSME. A total of 410 patients, one eye per patient, fulfilled the inclusion/exclusion criteria and were included in the study. Ophthalmological examinations, including BCVA, fundus photography with Retmarker analysis, and optical coherence tomography (OCT), were performed at baseline, month 6 and month 24, or before laser treatment. Hierarchical cluster analysis was used to identify homogeneous subgroups and clinically significant thresholds of the data collected.

Results. A total of 376 eyes/patients performed the 6-month visit and were considered for cluster analysis. This mathematical method identified three different phenotypes based on statistically significant differences for the microaneurysm (MA) turnover and for the central retinal thickness (RT): phenotype A (low MA turnover and normal RT, 48.1%); phenotype B (low MA turnover and increased central RT, 23.2%); and phenotype C (high MA turnover, 28.7%). From the 348 eyes/patients that reached the study end point or completed the 24-month visit, 26 developed CSME: 3 from phenotype A (1.8%), 7 from phenotype B (8.5%), and 16 from phenotype C (16.2%). Eyes/patients from phenotype C showed a higher risk for CSME development (OR = 3.536; $P < 0.001$).

Conclusions. Hierarchical cluster analysis identifies three different phenotypes of NPDR based on MA turnover and central macular thickness. Eyes/patients from phenotype C show a higher risk for the development of CSME. (ClinicalTrials.gov number, NCT00763802.)

Keywords: cluster analysis, phenotypes, diabetic retinopathy

Diabetic retinopathy (DR) is a common and serious ophthalmic condition. It is the leading cause of blindness among working-age adults in the United States.[1] Vision loss related to eye disease among people with diabetes is an important disability that threatens independence and can lead to reduced quality of life.[2]

Furthermore, a recent study by Narayan et al.[3] demonstrated that diabetes prevalence in the United States is likely to increase dramatically through 2050, given recent increases in the incidence of diagnosed diabetes, decreases in diabetes-related mortality, and expected changes in the age of the population. This perspective is perceived to occur in other regions of the world.[4]

It is well recognized that the duration of diabetes and the level of metabolic control condition the development of the retinopathy, but these risk factors do not explain the great variability that characterizes the evolution and rate of progression of the retinopathy in different diabetic patients. There are many diabetic patients who after many years with diabetes never developed sight-threatening retinal changes, maintaining good visual acuity. However, there are also other patients that even after only a few years of diabetes show a retinopathy that progresses rapidly and may not even respond to available treatments.

In a prospective 3-year follow-up study of 14 patients with type 2 diabetes and mild nonproliferative diabetic retinopathy (NPDR) followed using multimodal macula mapping, we found marked individual variations in the progression of DR and activity of retinal disease.[5] In this study, we were able to identify three major patterns of DR progression. The first one, identified as pattern A, consisted of eyes in which the retinal changes progressed slowly and showed little disease activity over the 3-year period of follow-up. The second, identified as pattern B, or wet form, was characterized by alterations of the blood-retinal barrier and the presence of edema. The third, identified as pattern C, or ischemic form, was characterized by increased retinal vascular remodeling and capillary closure. These different phenotypes were associated with different risks for DR worsening and development of clinically significant macular edema (CSME). Shortcomings of this study were the elaborate imaging methodology used and the small sample size.

In this study, we report the identification of similar phenotypes based on hierarchical cluster analysis in a prospective study of a large cohort of type 2 diabetic patients

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with mild NPDR using only noninvasive imaging methodologies that are easily used in routine clinical practice, digital color fundus photography (CFP), and optical coherence tomography (OCT) and their 2-year progression to CSME.

Methods

Patients

The study was a prospective, observational study designed to follow eyes/patients with mild NPDR (20 and 35 of Early Treatment Diabetic Retinopathy Study [ETDRS] classification) for a period of 2 years or until the time of development of CSME needing treatment.

A total of 410 patients were included between September 2007 and December 2009, men and women with diagnosed adult-onset type 2 diabetes, age 40 to 75 years, mild NPDR (20 and 35 of ETDRS classification), best corrected visual acuity (BCVA) as tested in the ETDRS[6] of 80 or higher ETDRS letters score (Snellen equivalent $\geq$20/25) and refraction with a spherical equivalent less than $\pm$5 diopters (D). Exclusion criteria included the presence of cataract or other eye disease that may interfere with fundus examination, glaucoma, other retinal disease, previous intraocular surgery, dilatation of the pupil less than 5 mm and previous laser therapy or intravitreal injections. All patients gave written informed consent. The tenets of the Declaration of Helsinki were followed and approval was obtained from the institutional review board (clinical trial registration number: NCT00763802).

At the baseline visit (V0) patients' body weight, height, blood pressure, and concomitant medication were recorded. A physical examination by a diabetologist was also performed.

One eye per patient was selected by the physician at baseline as the study eye based on the inclusion/exclusion criteria. When both eyes fulfilled the same criteria, one of the eyes was selected by choosing sequentially the right or the left eye.

At the three study visits, V0, V6, and V24 (or pretreatment visit), the study eyes underwent a complete eye examination, which included BCVA, slit-lamp examination, IOP measurements, digital CFP, and OCT.

During the period of the study and outside of the study visits, patients were followed in our institution according to usual clinical practice. Patients diagnosed as having CSME (identified on clinical examination by retinal thickening within 500 μm of the center of the fovea or by the presence of exudates within 500 μm of the center of the fovea, or by adjacent thickening or thickening of at least 1 disc area of any part within 1 disc diameter of the center of the fovea[7]) were referred to the principal investigator of the study (JC-V) for a full visit before treatment. Laser photocoagulation treatment for CSME was the first choice treatment at the time when the study was performed.

Laboratory analyses were performed at baseline (V0) and at the 6-month (V6) and at the 24-month (V24) visits or at the pretreatment visit. Laboratory analyses included creatinine, glucose and HbA_{1C} concentration, red blood cell count, white blood cell count, platelet amount, hemoglobin concentration, and packed cell volume. Metabolic control was also assessed by measuring in the plasma concentrations of lipid fractionation identifying total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides.

Baseline characteristics are presented in Table 1.

Table 1. Baseline Characteristics for the Patients Included in the Study ($n = 410$)

	Patients Included in the Study, $n = 410$
Males/Females, frequency (%)	259 (63.2)/151 (36.8)
Taking insulin, yes/no, frequency (%)	117 (28.5)/293 (71.5)
Age, y	62.0 (55.0–67.0)
Duration of diabetes, y	10.0 (6.0–14.0)
Creatinine, mg/dL	0.89 (0.78–1.01)
HbA_{1C}, %	7.7 (6.9–8.9)
Cholesterol, mg/dL	194 (168–222)
HDL, mg/dL	49 (42–58)
LDL, mg/dL	125 (105–148)
Glucose, mg/dL	165 (119–226)
Triglycerides, mg/dL	145 (104–214)
Systolic blood pressure, mm Hg	151 (136–164)
Diastolic blood pressure, mm Hg	76 (69–82)
BCVA, letters	85 (83–89)
No. of MAs	2 (1–5)
Central subfield retinal thickness, central 1000 μm, μm	215.5 (198.0–233.0)

Values are median and IQR, or frequency and percentage.

Color Fundus Photography

CFP was performed according to the ETDRS protocol. The seven-fields photographs were obtained at 30° using a Zeiss FF450 camera (Carl Zeiss Meditec, Dublin, CA) for DR classification according to ETDRS grading.

The field-2 images were subjected to automated microaneurysm (MA) analyses using the RetmarkerDR (Critical Health SA, Coimbra, Portugal). This automated computer-aided diagnostic system consists of software earmarking MA and vascular lesions in the macula; it includes a coregistration algorithm that allows comparison within the same retinal location between different visits for the same eye.[8,9] The algorithm detects the presence of MA and red dotlike lesions (i.e., small lesions that appeared as a round or ovoid red spot of 20–200 μm in diameter with regular borders and located within the superior and inferior arcades).

The RetmarkerDR computes for each eye/patient the number of MAs in each visit, the number of MAs that appear and/or disappear from one visit to the other, allowing calculation of the number of MAs appearing and/or disappearing per time interval (i.e., the MA formation rate and the MA disappearance rate, respectively). The MA turnover is computed as the sum of the MA formation and disappearance rates.

In a previous work from our group,[10,11] a good intergrader agreement was found for the total number of MAs earmarked and the MA turnover for three independent human graders. The RetmarkerDR shows a similar intergrader agreement for the total number of MAs and the MA turnover while showing no intragrader variability as opposed to human graders being, therefore, a reliable tool for MA assessment.[12]

Optical Coherence Tomography

OCT was performed using the Stratus-OCT (Carl Zeiss Meditec).

The *Fast Macular* acquisition protocol, acquiring six radial scans 30° apart, 6 mm long, was used to assess the central retinal thickness (RT).

Due to the strong correlation found between the central point RT and the central subfield RT ($r = 0.948$; $P < 0.001$), and due to the higher variability found in central point RT measurements,[13] only the central subfield RT was considered in this study.

Data Analysis

Patients are usually characterized by estimated values of the parameters under analysis (e.g., mean or median values). To analyze the homogeneity of the distribution of the data, dispersion measures of the parameters considered can be used (e.g., coefficient of variation when using the mean or coefficient of dispersion when using the median). The higher the measures of dispersion, the more heterogeneity the group of patients analyzed has.

Cluster analysis is a mathematical method that, based on the data dispersion observed in a group of patients, identifies more homogeneous subgroups (i.e., smaller groups of patients with the lowest dispersion). Cluster analyses are therefore unsupervised segmentation techniques that build models of the observed data in order to identify and create homogeneous groups.[14] These techniques group data that share some similarity measure or feature,[15] being hierarchical or nonhierarchical (i.e., when the number of underlying clusters is unknown or known a priori). In this work, the hierarchical method was used to identify the number of homogeneous groups (clusters) underlying the data set.

The Ward's method was used for hierarchical clustering. This method creates clusters by agglomeration (i.e., starting by considering initially the existence of as many clusters as patients and keeping agglomerating clusters until it achieves a single one enclosing all of the patients). Ward's method promotes the minimization of the within-cluster dispersion being therefore one of the methods more frequently used in clinical sciences.[16–18] Along the agglomerative process, the dissimilarity measure between the grouped clusters is computed. The analysis of this dissimilarity measure along the agglomeration process allows the number of homogeneous clusters in the data set to be identified. Moreover, to determine the best number of clusters, the Calinsky-Harabasz pseudo-F statistic was also computed.[19]

To identify phenotypes of mild NPDR using noninvasive procedures, a hierarchical Ward's cluster analysis was performed using MA parameters (number of MA at baseline and MA turnover from baseline to month 6) and central subfield RT parameters (central subfield RT at baseline and changes from baseline to month 6). Since the range of values for the different parameters had one order of magnitude, a data normalization was performed before clustering. In this way, the normalization was achieved through a *Z*-distribution, thus having a zero mean and unitary standard deviation (*Z*-score values).

The identified clusters (i.e., phenotypes) were thereafter fully characterized, based on the original parameters and on the remaining parameters: age, diabetes duration, creatinine, HbA_{1C}, blood pressure, cholesterol, HDL, LDL, glucose, and triglyceride levels. Statistically significant differences between phenotypes were tested using the Kruskall-Wallis test. The risk for CSME development (odds ratio [OR]) was computed and compared between the identified phenotypes of mild NPDR.

Once the phenotypes were identified (based on the cluster analysis), a decision and classification tree was used to establish rules for phenotype classification. This method allows for the establishment of rules based on cut-off values.

Statistical analyses were performed using the STATA software version 12.1 (StataCorp LP, College Station, TX), and *P* values less than or equal to 0.05 were considered as statistically significant results.

To identify threshold values for the phenotypes, a decision and classification tree was performed using the parameters considered in the cluster analysis. The CART algorithm with a 10-fold cross-validation was used (SPSS version 13.0; IBM SPSS, Inc., Chicago, IL).

Results

From the 410 eyes/patients included in the study, 376 completed the first 6 months of follow-up (2 patients died between the baseline and the 6-month visit, 25 withdrew from the study, and 7 were lost to follow-up). Only 348 eyes/patients reached either the study end point, CSME needing treatment (26 eyes/patients), or performed the last study visit (24-month visit) without developing CSME (322). There were a total of 62 drop-outs from the study (9 patients died, 44 withdrew from the study, and 9 were lost to follow-up) (Fig. 1). Of the 348 eyes/patients that completed the study 15 eyes (4.3%) progressed to more advanced ETDRS levels: 14 progressed to moderate NPDR (11 with level 43A and 3 with level 43B), and 1 progressed to moderate proliferative DR (level 65B). No statistically significant differences were found between the 348 eyes/patients that reached the study end point or that performed the last study visit and the 62 eyes/patients that dropped out, except for the cholesterol, the LDL levels, and the BCVA (Table 2).

For the cluster analysis, based on the MA and RT parameters, the 376 eyes/patients that performed the 6-month visit were considered.

Furthermore, to assess increased retinal thickness an age-matched healthy population was used. The normal mean and SD for the central subfield RT were 201.1 ± 18.9 μm. No statistically significant differences were found between sexes (the mean central subfield RT for males was 201.8 ± 15.4 μm and for females 201.4 ± 21.8 μm; $P > 0.05$).

Phenotypes of Mild NPDR

The hierarchical Ward's clustering method identified the existence of three clusters, corresponding to the higher Calinsky-Harabasz pseudo-*F* statistics (Fig. 2) (for the three clusters solution the Calinsky-Harabasz pseudo-*F* statistics was 91.9, whereas for the four clusters solution the Calinsky-Harabasz pseudo-*F* statistics was 69.7). The first cluster/phenotype was composed of 181 eyes/patients (48.1%), the second cluster/phenotype was composed of 87 eyes/patients (23.2%), and the third cluster/phenotype was composed of 108 eyes/patients (28.7%).

The three phenotypes result from the statistically significant differences for the MA and the RT parameters ($P < 0.001$, Table 3). Statistically significant differences between phenotypes were also found for sex ($P = 0.028$), age ($P < 0.001$), HbA_{1C} ($P = 0.050$) and LDL levels ($P = 0.049$), and for the BCVA ($P = 0.001$) (Table 3).

Phenotype B is characterized by a higher central subfield RT ($P < 0.001$). This phenotype shows a lower BCVA ($P < 0.027$, corresponding only to one ETDRS letter difference) and is composed mainly of males ($P = 0.015$ when compared with phenotype A) and older subjects ($P < 0.011$). Phenotype C is characterized by higher MA parameters, number, and turnover ($P < 0.001$). This phenotype also showed higher HbA_{1C} values ($P = 0.043$ when compared with phenotype A) and lower LDL values ($P < 0.038$).

Representative cases for the three phenotypes are shown in Figure 3.

Phenotypes of Mild NPDR and Risk for CSME

From the 348 eyes/patients that reached the study end point or that completed the 24-month visit, 26 developed CSME needing laser photocoagulation, 3 (1.8%) from phenotype A, 7 (8.5%) from phenotype B, and 16 (16.2%) from phenotype C (Fig. 4).

CSME eye/patient characteristics are shown in Table 4. Statistically significant differences between eyes/patients that did not develop CSME and eyes/patients that developed CSME

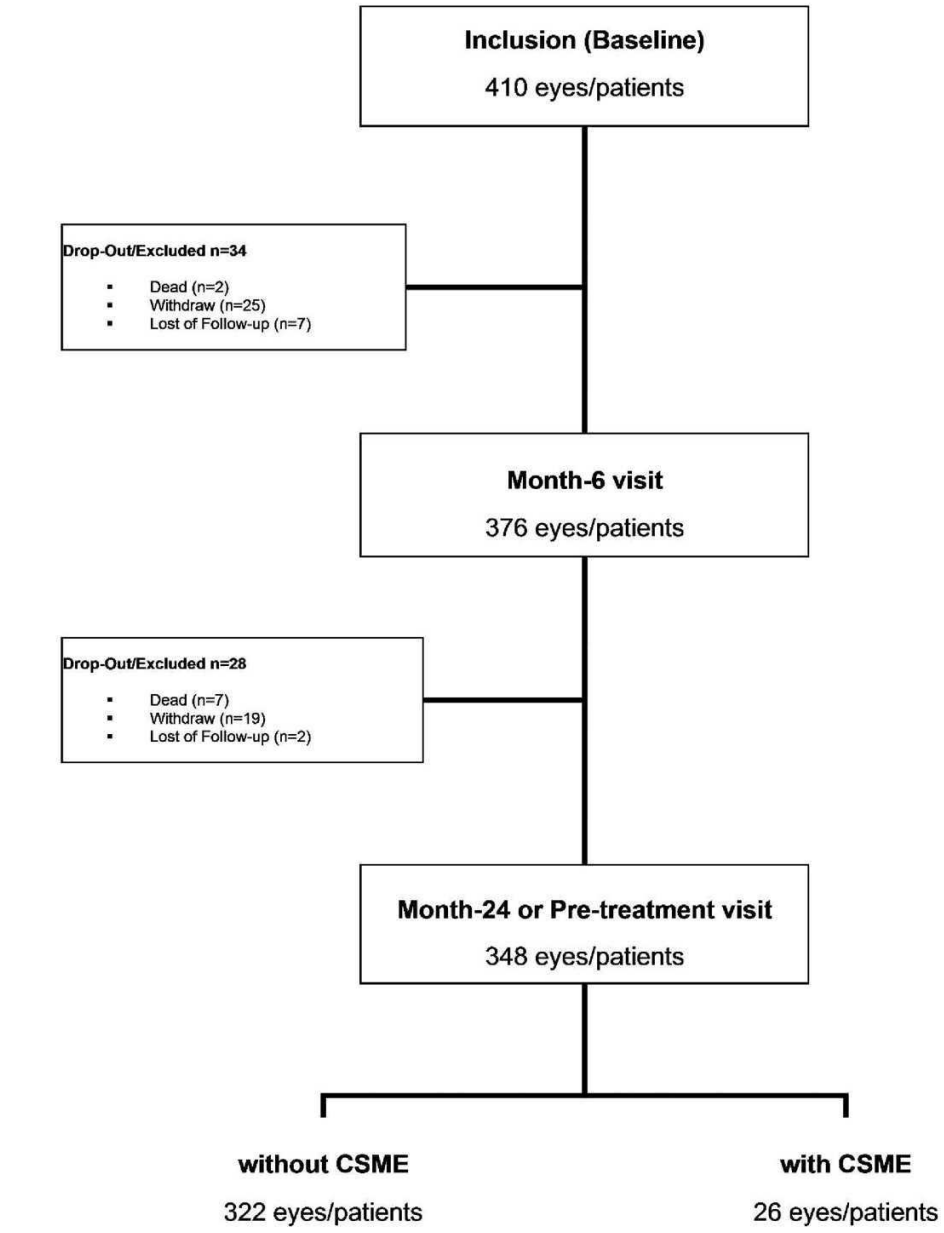

FIGURE 1. Composition of the patients included in the study over the study period: CONSORT flowchart.

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TABLE 2. Baseline Characteristics of the Eyes/Patients That Reached the Study End Point or That Performed the Last Study Visit

	Drop–Out Patients, $n = 62$	Patients Included in the Analysis, $n = 348$	P
Males/females	41 (65.1)/22 (34.9)	219 (62.9)/129 (37.1)	NS
Age, y	63.5 (58.0–69.0)	62.0 (55.0–67.0)	NS
Duration of diabetes, y	10.0 (5.0–14.0)	10.0 (6.0–14.0)	NS
Patients taking insulin	14 (22.6)	103 (29.6)	NS
Creatinine, mg/dL	0.89 (0.79–1.02)	0.89 (0.78–1.01)	NS
HbA_{1C}, %	7.9 (6.5–9.0)	7.7 (6.9–8.9)	NS
Cholesterol, mg/dL	208.5(173.0–228.0)	193.0 (167.0–219.0)	0.016
HDL, mg/dL	51.5 (43.0–59.0)	49.0 (42.0–57.0)	NS
LDL, mg/dL	132.0 (108.0–156.0)	124.0 (105.0–146.0)	0.010
Glucose, mg/dL	168.5 (113.0–230.0)	163.0 (119.0–226.0)	NS
Triglycerides, mg/dL	132.0 (104.0–192.0)	147.0 (104.0–220.0)	NS
Systolic blood pressure, mm Hg	152.0 (135.0–170.0)	151.0 (137.0–164.0)	NS
Diastolic blood pressure, mm Hg	75.0 (72.0–82.0)	76.0 (69.0–82.5)	NS
BCVA, letters	84 (80–88)	85 (84–89)	0.010
No. of MAs	2 (1–6)	2 (1–5)	NS
Central subfield RT, central 1000 μm, μm	211 (192–229)	216 (199–233)	NS

Values are median and IQR, or frequency and percentage; P value for statistically significant differences between eyes/patients. NS, not significant, $P > 0.05$.

were found only for the MA parameters ($P \leq 0.002$) and for the RT parameters ($P \leq 0.035$).

Eyes/patients from phenotypes C and B showed a higher risk for CSME than eyes/patients from phenotype A. For phenotype C, the OR is 3.536, 95% confidence interval (CI) 1.917–6.524 ($P < 0.001$); and for phenotype B the OR is 2.802, 95% CI 1.445–5.434 ($P = 0.002$). Phenotype C shows also a higher risk for CSME when compared with phenotype B (the OR is 1.994, 95% CI 1.144–3.477; $P = 0.015$).

Threshold Values for Phenotypes

Based on the three phenotypes, threshold values were identified using a decision and classification tree (Fig. 5): 73.5% of the eyes/patients in phenotype A (133 of the 181 eyes/patients) were characterized by an MA turnover less than 6 and a central RT less than 217 μm; 98.8% of the eyes/patients in phenotype B (86 of the 87 eyes/patients) were characterized by an MA turnover less than 6 and a central RT greater than or equal to 217 μm; 97.2% of the eyes/patients in phenotype C (105 of the 108 eyes/patients) were characterized by an MA turnover greater than or equal to 6.

The resulting classification tree presents a risk estimate of 12.5%, classifying correctly 88.6% of the eyes/patients in one of the three phenotypes.

Considering these thresholds, and based on the clinically meaningful parameters, that is, MA turnover and presence of edema (i.e., central RT increase over the normal reference

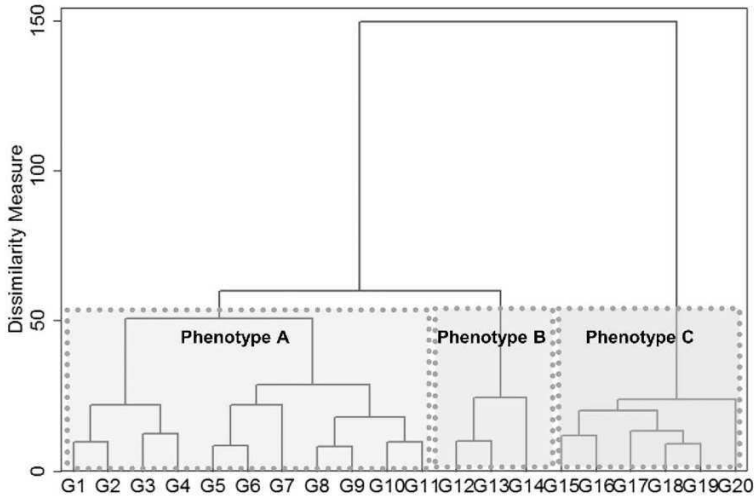

FIGURE 2. Dendrogram for the Ward's clustering method showing the dissimilarity measure that is computed in each iteration of the hierarchical agglomerative process (the clustering starts with 376 clusters, i.e., as many groups as eyes/patients, and ended with one single cluster). To simplify the graphical representation, the dissimilarity measures are shown from the 20 clusters solution (G1 to G20).

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TABLE 3. Characteristics of Each Phenotype ($n = 376$)

	Phenotype A $n = 181$	Phenotype B $n = 87$	Phenotype C $n = 108$	P
Males/females, frequency (%)	101 (55.8)/80 (44.2)	62 (71.3)/25 (28.7)	72 (66.7)/36 (33.3)	**0.028**
Taking insulin, yes/no, frequency (%)	50 (27.6)/131 (72.4)	30 (34.5)/57 (65.5)	31 (28.7)/77 (71.3)	0.502
Age, y	62 (54–68)	64 (59–70)	59 (53–65)	**<0.001**
Duration of diabetes, y	10 (6–13)	10 (6–15)	10 (6–14)	0.645
Creatinine, mg/dL	0.89 (0.78–1.01)	0.90 (0.80–1.03)	0.87 (0.76–1.01)	0.279
HbA_{1C}, %	7.7 (6.7–8.8)	7.6 (6.9–9.0)	7.9 (7.0–9.1)	**0.050**
Cholesterol, mg/dL	196 (169–218)	200 (177–222)	182 (161–215)	0.058
HDL, mg/dL	49 (42–57)	50 (41–58)	48 (42–56)	0.909
LDL, mg/dL	127 (107–148)	128 (110–149)	116 (101–136)	**0.049**
Glucose, mg/dL	165 (118–226)	166 (110–219)	162 (127–226)	0.742
Triglycerides, mg/dL	157 (109–236)	145 (106–208)	138 (99–189)	0.088
Systolic blood pressure, mm Hg	153 (136–166)	149 (141–165)	148 (134–159)	0.159
Diastolic blood pressure, mm Hg	75 (68–82)	75 (70–82)	76 (69–83)	0.783
BCVA, letters	85 (83–89)	84 (82–88)	87 (84–89)	**0.001**
No. of MAs	3 (1–4)	1 (1–2)	9 (6–12)	**<0.001**
MA turnover, no. per 6 mo	3 (1–5)	1 (0–2)	9 (7–14)	**<0.001**
Central subfield RT, central 1000 μm, μm	204 (192–212)	234 (227–245)	218 (198–235)	**<0.001**
Central subfield RT at month 6, central 1000 μm, μm	204 (190–219)	235 (222–247)	216 (202–231)	**<0.001**

Values are median and IQR, or frequency and percentage; *P* values for the three phenotypes (bold indicates values that are statistically significant).

value, 201.1 ± 18.9 μm), the following rules can be used to classify eyes/patients into one of the three phenotypes of NPDR progression:

- Phenotype A: MA turnover < 6 and normal RT values (central subfield RT < 220 μm, i.e., normal mean + 1 SD).
- Phenotype B: MA turnover < 6 and increased RT values (central subfield RT ≥ 220 μm).
- Phenotype C: MA turnover ≥ 6.

From the 133 eyes/patients with an MA turnover less than 6 and a central RT less than 220 μm, 1 eye/patient developed CSME (0.7%); from the 94 eyes/patients with an MA turnover less than 6 and a central RT greater than or equal to 220 μm, 8 eyes/patients developed CSME (8.5%); and from the 121 eyes/patients with an MA turnover greater than or equal to 6, 17 eyes/patients developed CSME (14.5%).

Using these rules to estimate the risk for CSME development, phenotype B shows a sensitivity and a specificity of 88.9% and 60.5%, respectively (when compared to phenotype A), and phenotype C shows a sensitivity and a specificity of 94.4% and 55.9%, respectively (when compared to phenotype A).

Phenotype A shows a negative predictive value for developing CSME of 99.2%.

DISCUSSION

The aim of this study was to identify patterns of progression and retinal disease activity in patients with diabetes type 2 and NPDR with noninvasive procedures used in routine clinical practice.

This study shows that using the mathematical method of hierarchical cluster analysis and only noninvasive procedures (CFP and OCT), three different phenotypes of NPDR can be identified, which show different risks of progression to CSME.

These three phenotypes are in agreement with the number of patterns of DR progression proposed previously by Lobo et al.[5] in a different and smaller sample. In this original study, three phenotypes of progression of mild NPDR were characterized: phenotype A as slow progression, phenotype B as "leaky," and phenotype C as ischemic. Similar categorization was found in this study as a result of hierarchical cluster analysis, phenotype B with predominance of edema, and phenotype C with predominance of MA turnover (i.e., with increased rates of MA formation and disappearance).

The main initial alterations occurring in the early stages of NPDR are MA formation and disappearance, capillary closure, and alteration of the blood-retinal barrier with associated retinal edema.[20] Digital CFP and OCT alone, both noninvasive procedures, appear to be adequate to document these initial alterations. MA and small hemorrhages can be detected by CFP, and their turnover (i.e., disease activity) can be quantified using a novel software for automatic analysis of the fundus images, the RetmarkerDR. MA turnover is a composite of MA formation rate, that is, the number of new MA per time interval and MA disappearance rate (i.e., number of disappearing MA per time interval). Both parameters represent microvascular disease activity and more specifically, the MA disappearance rate is considered a sign of capillary closure.

On the other hand, increased retinal thickness quantified by OCT identifies the presence of edema, which is a direct result of the alteration of the blood-retinal barrier. Fluorescein angiography was not used because the aim of the study was to identify patterns of progression using noninvasive procedures that can be easily repeated in clinical practice.

It was striking to find that 348 eyes/patients with similar levels of ETDRS classification (20–35) showed such a large interquartile range (IQR) both in MA turnover and retinal thickness values, demonstrating the wide range of values for each of these alterations. Hierarchical cluster analysis showed that these initial changes occur in different levels of intensity in different groups of patients and that different groups of patients can be characterized by levels of intensity of these retinal changes.

Our findings show that increased activity of microvascular disease in the macular region (field 2), well demonstrated by increased rates of MA turnover that characterize phenotype C, is associated with higher risk for development of CSME in the relatively short period of 2 years. This phenotype represents approximately 30% of the patients.

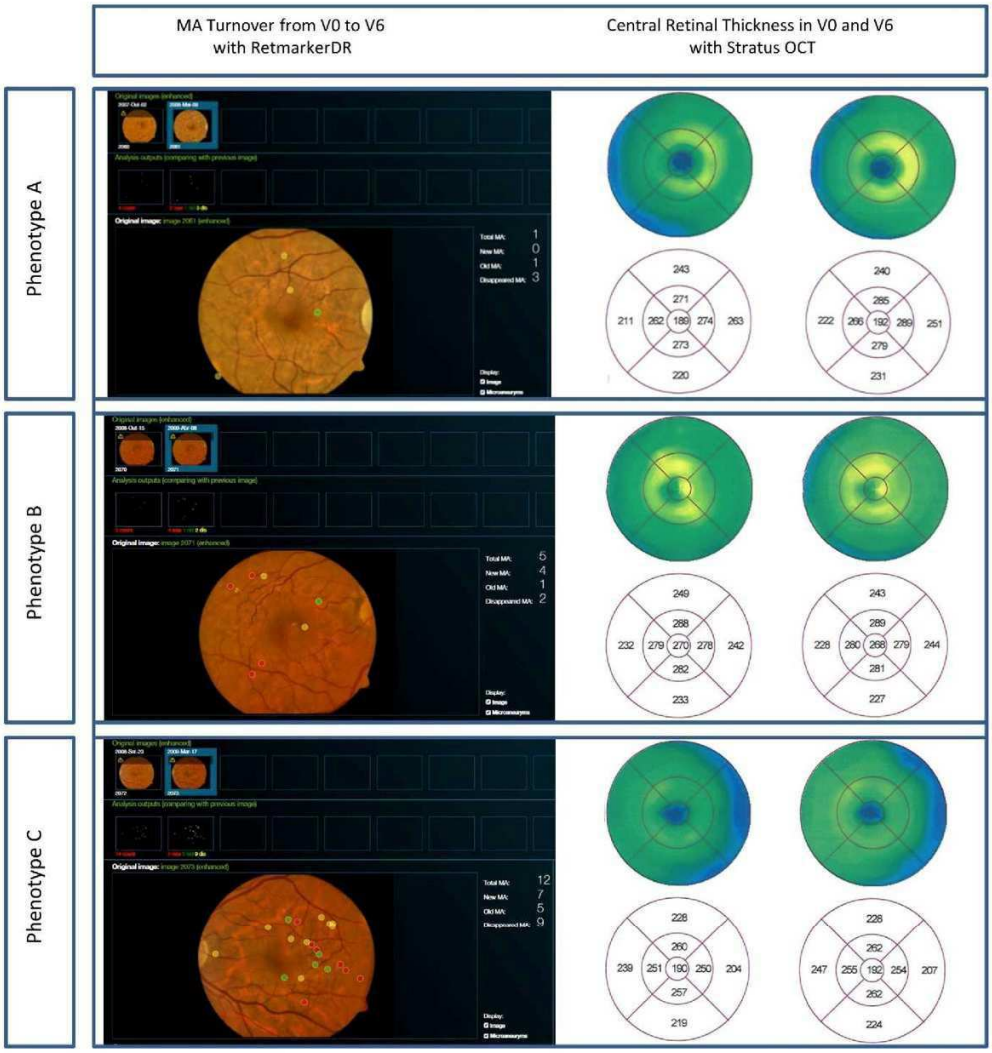

FIGURE 3. Representative cases for the three phenotypes of DR progression (*right side*: color fundus image in V6 with the MA earmarked using the software RetmarkerDR: *red dots* are new MA, *yellow dots* are MA that disappeared from V0 to V6, and *green dots* are MA that were present in both visits; *left side*: central macular thickness maps obtained with the Stratus OCT in V0, *left*, and in V6, *right*). Phenotype A images are from case 007 (OD), the MA Turnover (number of MA appearing plus number of MA disappearing) was 3 MA in 6 months and the central retinal thicknesses in V0 and V6 were 180 μm and 192 μm, respectively. This patient had a BCVA of 84 letters at baseline. Phenotype B images are from case 104 (OD), the MA Turnover was 6 MA in 6 months and the central retinal thicknesses in V0 and V6 were 270 μm and 268 μm, respectively. This patient had a BCVA of 82 letters at baseline. This patient developed CSME 6 months after V6. Phenotype C images are from case 210 (OS), the MA Turnover was 16 MA in 6 months and the central retinal thicknesses in V0 and V6 were 190 μm and 192 μm, respectively. This patient had a BCVA of 84 letters at baseline. This patient developed CSME in the last study visit.

Of relevance is also the finding that on the other hand, phenotype A, which is characterized by low MA turnover and also no signs of retinal edema (representing approximately 50% of the patients), has the lowest risk for development of CSME, predictive negative value of 99.2%. This observation has clear implications for management of DR. Furthermore, this observation indicates that a large proportion of the eyes with mild NPDR will progress very slowly, suggesting that these

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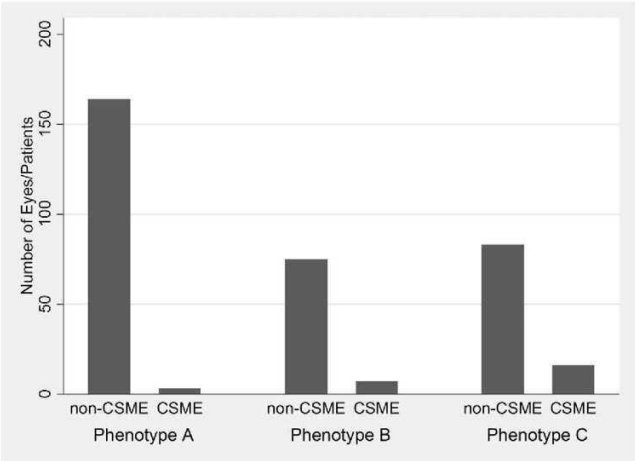

FIGURE 4. Phenotypes distribution by non-CSME and CSME eyes (number of eyes/patients for each phenotype that completed the 24-month visit without developing CSME – non-CSME, and number of eyes/patients for each phenotype that developed CSME needing treatment – CSME).

eyes should not be included in clinical trials because of their slow rates of progression. New OCT methodologies, such as swept-source OCT or even Doppler OCT, have the potential to achieve finer characterization of the different DR phenotypes.

Various systemic risk factors have been proposed to influence progression of DR. Our study shows that progression to CSME from the initial stages of the retinopathy is apparently mainly correlated with HbA_{1C} (i.e., glycemic metabolic control). Blood pressure measurements did not show any correlation with the risk for development of CSME, and from the creatinine and blood lipids levels analyzed, only LDL showed a correlation: lower LDL values are present in phenotype C. It appears that these risk factors may play a role only in more advanced stages of the disease when vision-threatening complications, such as CSME and proliferative retinopathy, are present.

It is of note that phenotype C was identified in a younger population. Because the duration of diabetes was similar in the three phenotypes suggests that eyes/patients with earlier onset of their diabetes have increased activity of their microvascular disease, which characterizes phenotype C.

The identification of different retinopathy phenotypes characterized by different dominant retinal alterations and different rates of progression to CSME opens new perspectives for personalized management of DR. If the patients with the greatest risk of progression and with the greatest potential to benefit from treatment can be identified, fewer patients will need to be followed closely to prevent one case of blindness.

TABLE 4. Characteristics of the Eyes/Patients That Did Not Develop CSME ($n = 322$) and That Developed CSME ($n = 26$)

	Eyes/Patients That Did Not Develop CSME, $n = 322$	Eyes/Patients That Developed CSME, $n = 26$	P
Males/females, frequency (%)	204 (63.3)/118 (36.7)	15 (57.7)/11 (42.3)	0.565
Taking insulin, yes/no, frequency (%)	94 (29.2)/228 (70.8)	9 (34.6)/17 (65.4)	0.560
Age, y	62 (55–67)	63 (54–70)	0.341
Duration of diabetes, y	10 (6–14)	11 (7–15)	0.281
Creatinine, mg/dL	0.88 (0.78–1.01)	0.89 (0.78–1.00)	0.987
HbA_{1C}, %	7.6 (6.9–8.8)	8.1 (7.3–10.0)	0.071
Cholesterol, mg/dL	193 (167–218)	197 (164–228)	0.850
HDL, mg/dL	49 (42–57)	48 (44–55)	0.809
LDL, mg/dL	124 (104–146)	121 (105–150)	0.743
Glucose, mg/dL	162 (119–225)	171 (128–239)	0.551
Triglycerides, mg/dL	147 (104–220)	142 (112–199)	0.671
Systolic blood pressure, mm Hg	151 (137–164)	147 (135–168)	0.963
Diastolic blood pressure, mm Hg	76 (69–82)	71 (65–84)	0.399
BCVA, letters	85 (84–89)	85 (84–89)	0.454
No. of MAs	3 (1–6)	8 (3–12)	**0.002**
MA turnover, no. per 6 mo	4 (1–7)	9 (3–16)	**<0.001**
Central subfield RT, central 1000 μm, μm	214 (198–233)	227 (208–239)	**0.035**
Central subfield RT at month 6, central 1000 μm, μm	214 (199–231)	226 (212–260)	**0.005**

Values are median and IQR, or frequency and percentage; P values for non-CSME versus CSME eyes/patients (bold indicates values that are statistically significant).

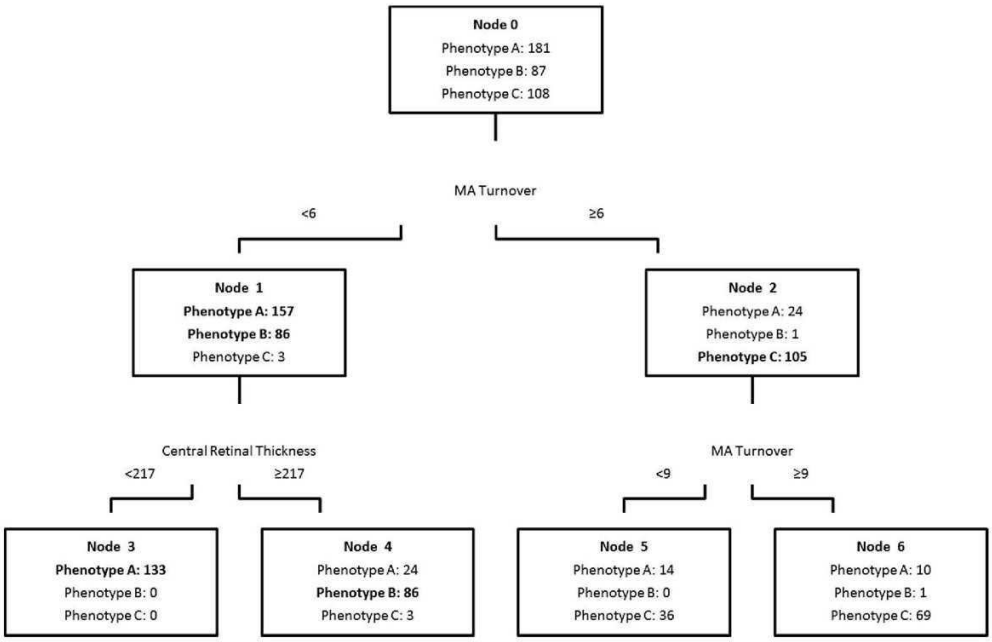

FIGURE 5. Decision and classification tree for the characterization of the three phenotypes. Nodes showing the higher number of eyes/patients from one single phenotype were used for phenotype characterization (for phenotype A characterization node 3 was considered; for phenotype B characterization node 4 was considered; and for phenotype C characterization node 2 was considered).

This is of extreme importance at a time in which scarce resources must be focused and concentrated on the individual cases that need close follow-up and timely treatment.

Limitations of this study are the relatively short duration of the follow-up period, only 2 years, the relatively short number of visits, and the option for noninvasive procedures.

In summary, eyes in the initial stages of DR show three different phenotypes that can be identified by noninvasive procedures and that have different risks for short-term development of CSME.

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5 CONTRIBUTION TO CLINICAL EYE RESEARCH IN AGE-RELATED MACULAR DEGENERATION

5.1 Investigator-Driven Clinical Research in Age-Related Macular Degeneration

From the 6087 clinical studies performed worldwide (according to the public database ClinicalTrials.Gov), 617 (10.1%) are on age-related macular degeneration (AMD). Three hundred and one (301, 48.8%) are initiated by the investigators (e.g., clinical research networks, governments, national institutes of health, etc.), and 316 (51.2%) are initiated, or performed in collaboration with the industry. Also, 130 studies (21.1%) are observational, 485 (78.6%) are non-observational and 2 (0.3%) are expanded access.
Approximately 20% of the clinical research performed in AMD is on neovascular AMD, the most sight threating condition of AMD. Forty one percent (41.7%) of the clinical studies on neovascular AMD are IDCR (45) while 58.3% are initiated, or performed in collaboration with the industry (63). The main areas of clinical research in AMD are listed in Table 8.

Regarding the primary outcomes used for AMD, the primary outcomes most frequently used are visual acuity (295 studies, 47.8%) and retinal thickness, assessed using OCT (150 studies, 24.3%). Fluorescein angiography, contrast sensitivity and CFP are used for the primary outcomes assessment in less than 10% of the clinical studies (59 studies used fluorescein angiography, 30 studies used contrast sensitivity and 25 studies used CFP).

Table 8. List of conditions studied in AMD (321 studies are registered only as AMD being not listed below and some of the conditions are studied in the same clinical study) (data from the ClinicalTrials.Gov website, accessed on January 10th 2014).

AMD Conditions (total 617 clinical studies)	Number of Studies	Percentage
Neovascular AMD	108	17.5%
Diabetic retinopathy	29	4.7%
Geographic atrophy	19	3.1%
Diabetic macular edema	17	2.8%
Retinal vein occlusion	15	2.4%
Macular edema	14	2.3%
Choroid (not specified)	9	1.5%
Uveitis	9	1.5%
Retinal degeneration	9	1.5%
Retina (not specified)	7	1.1%
Diabetes	7	1.1%
Retinal detachment	6	1.0%
Cardiovascular disease	5	0.8%
Hereditary eye diseases	5	0.8%
Central serous chorioretinopathy	4	0.6%
Histoplasmosis	3	0.5%
Macula (not specified)	3	0.5%
Retinal artery occlusion	3	0.5%
Myopia	3	0.5%
Retinal dystrophies	3	0.5%
Choriditis	2	0.3%
Genetics	2	0.3%
Trauma	2	0.3%
Retinoschisis	2	0.3%
Ocular disorder (not specified)	2	0.3%
Cancer	1	0.2%

5.2 Age-Related Macular Degeneration

Age-Related Macular Degeneration (AMD) is the leading cause of severe visual loss in the elderly population. Twenty five (25) to 30 million people worldwide presents severe visual loss due to AMD [40]. In Europe, visual impairment due to AMD represents 14 to 40% of the cases with visual impairment [40]. The prevalence of the disease increases with age being higher in the oldest population. A prevalence of 36.3% in subjects with 55 years or more was estimated in the Rotterdam study, for any type of AMD [110], being the incidence of 0.12% [111, 112].

AMD can be briefly defined as early, intermediate or late AMD[21] [113, 114]. *Early AMD* is characterized by the presence of medium-sized drusens under the retina (i.e., drusen size <125 μm), and usually patients do not have vision loss. *Intermediate AMD* is characterized by large drusens under the retina, pigment changes in the retina, or both, and patients have usually some vision loss, but do not experience any symptoms. *Late AMD*, on the other hand, is characterized by other macular lesions, in addition to drusens. There are two forms of late AMD: the wet AMD form (neovascular/exudative AMD) and the dry AMD form (geographic atrophy). The wet form of the disease is characterized by the growth of abnormal blood vessels from the choroid underneath the macula (also called choroidal neovascularization – CNV). This form of AMD represents 10 to 15% of the cases with AMD and accounts for more than 90% of the cases with severe visual loss. Patients with bilateral wet AMD report also a substantially lower quality of life, poorer vision-related functioning, greater anxiety and depression, more frequent falls and fractures, and greater dependency [40]. The dry form of the disease, on the other hand, is characterized by a potential visual loss secondary to drusen accumulation, Bruch's membrane thickening, retinal pigment epithelium (RPE) lipofuscin accumulation, macular degeneration of RPE cells and finally photoreceptors. The dry form may finish in atrophic AMD called geographic atrophy (GA). This form of AMD represents approximately 80 to 90% of the cases with AMD and progress more slowly to vision loss. The aetiology of the disease is not yet very well defined. There are several risk factors such as age, family history and smoking habits. Lack of vitamins and trace elements, intense sunlight and ultraviolet (UV) radiation exposure, hypertension and arteriosclerosis are considered as potential risk factors, but their role was not yet confirmed [115, 116, 117].

[21] According to the NIH – National Eye Institute (www.nei.nih.gov/health/maculardegen/ armd_facts.asp #1, accessed on January 10th 2014).

The socioeconomic burden associated with AMD patients, mainly with the dry form of the disease, strengthen the importance for the early detection of AMD and for new therapies that can slow-down the progression of the disease. Treatments, such as anti-vascular endothelial growth factors (VEGF) intravitreal injections, laser photocoagulation, and photodynamic therapy (PDT) have been shown to improve patients' quality of life [118, 114]. However clinical studies are still needed to identify early biomarkers that can contribute for the improvement of the existing therapeutic approaches and the improvement of the patients' quality of life.

To characterize retinal biomarkers based on imaging techniques, morphological and functional, that may predict wet AMD conversion, an exploratory, prospective, observational study was conducted in the Clinical Trial Unit (CEC) of AIBILI with patients with wet AMD in one eye and early AMD in the other eye. Furthermore, to assess the prevalence of the disease in subjects with 55 years or more in Portugal an epidemiologic study was conducted.

5.3 Potential Biomarkers of Age-Related Macular Degeneration

AMD is a complex disease. The epidemiological studies conducted in the past 25 years have helped identifying major risk factors, being some of them related with the lifestyle. Smoking is the best-characterized lifestyle risk factor [119]. It is currently known that smokers, when compared to non-smokers, have approximately a 3-fold increased risk for late stages AMD [120]. The exact mechanism by which smoking increases the risk for AMD is still unclear, it may include oxidative stress, inflammation and decreased macular pigment [117]. Moreover, recent studies have shown that the risk for AMD is particularly high in smokers bearing at-risk polymorphisms in some genes, demonstrating that some environmental factors, such as smoking, can modulate the genetic susceptibility for AMD [121, 122, 123, 115]. Other factors, such as systemic hypertension, obesity, diabetes, plasma lipids or alcohol may also be associated with an increased risk for AMD, but here the results of the epidemiological studies have been inconsistent [124].

The retina is particularly susceptible to oxidative stress because of the high level of in-site reactive oxygen species production, due in particular to light exposure and high metabolic activity [125]. The role of light exposure is not yet well established. Light exposure may have deleterious effect on the eye, in particular through the production of reactive oxygen species [126]. Intense blue light exposure has been shown to induce retinal damage, and the macular pigment, which absorbs blue light, is thought to protect the macula against phototoxic damage [127], however, studies are still needed to demonstrate the involvement of light exposure in AMD. Also, recent epidemiological studies have shown that antioxidants may play a protective role in AMD [128]. The Age-Related Eye Diseases Study (AREDS), a randomized clinical trial performed in the United States with almost 5000 subjects followed-up during 5 years, showed a reduction of 25% in the incidence of the late stages of AMD in patients taking antioxidants and zinc supplements [129]. This significant reduction was also observed in the POLA study, a population-based study conducted in France with approximately 2500 participants, where a 80% decreased risk for late stage AMD was observed in subjects with higher plasma vitamin E [130]; and in the Rotterdam study, a population-based study conducted in the Netherlands with approximately 8000 participants, where a decreased risk for AMD was found in subjects with a high dietary intake of vitamin E or zinc [131].

To diagnose and characterize AMD stages, and to monitor AMD progression, the identification and validation of functional and/or morphological imaging biomarkers are of major relevance. The identification of imaging biomarkers is also of particular importance for patient-oriented treatment [132].

The different stages of AMD are characterized by the presence of drusens, pigmentary changes, CNV, Bruch's membrane thickening, RPE alterations, and GA according to Table 9.

Table 9. Stages of AMD.

Early AMD	Intermediate AMD	Late AMD	
		wet AMD	dry AMD
– Medium-sized drusens	– Large drusens – Pigmentary changes	– CNV	– Medium-sized drusens accumulation – Bruch's membrane thickening – RPE lipofuscin accumulation – RPE degeneration – Photoreceptors degeneration – GA

The presence of medium-large size drusens is one of the major biomarkers of AMD. Retinal cells overlying both soft and hard drusen exhibit structural and molecular abnormalities indicating photoreceptor degeneration and Muller glial activation. These abnormalities resemble the degenerative effects common to many forms of retinal degeneration, but are confined to areas directly overlying drusen. This suggests that photoreceptor cell function is compromised as a consequence of drusen formation [133].

The AREDS study developed a severity scale based on the grading system developed by the Wisconsin Age-Related Maculopathy Grading System [134] which is based on the presence, location, and/or severity of two morphologic retinal findings that are characteristic of AMD, i.e., drusens (size and area) and pigmentary abnormalities [135, 136]. The presence of these lesions, that are associated with a higher risk of the disease progression, is easily identified on CFP, being therefore the imaging method mostly used for AMD grading [136].

5.3.1 Drusens and Pigmentary Changes assessed based on Colour Fundus Photography

A severity scale was developed by the AREDS based on stereoscopic colour fundus photographs collected during a 5-year follow-up period in more than 3000 participants [135, 136]. The severity scale was developed based on the presence or absence of two retinal features that are characteristic of AMD and that are clinically easily identified (drusens and

pigmentary abnormalities). These two features are highly associated with the progression to late stages AMD, especially when the status of the two eyes is considered [136].

This severity scale, based on a non-invasive imaging technique, provides a clinically useful risk-assessment for the development of late stages AMD in patients with early AMD.

The AREDS severity scale assigns 1 risk factor for the presence of large drusens and 1 risk factor for the presence of pigmentary abnormalities. Each of these risk factors is added for the two eyes resulting in a severity scale with 5 levels (starting with 0, no risk factor, and ending with 4, when both eyes present large drusens and pigmentary abnormalities). When a patient does not present large drusens, the presence of intermediate drusens in both eyes is counted as 1 risk factor. The 5-year risk for late stages AMD increases with the level of severity, increasing: 0.5% for patients with 0 risk factor; 3% for patients with 1 risk factor; 12% for patients with 2 risk factors; 25% for patients with 3 risk factors; and 50% for patients with 4 risk factors [136].

Apart from this severity scale, that is useful in clinical practice and in clinical research to identify patients at risk for AMD progression and vision-threatening, other retinal findings were also identified as risk factors for the progression of AMD such as the drusen area [137] and the drusens location [138, 139], as defined by the International Classification System for AMD [140]. The assessment of these findings requires more time from the examiners, since they need to identify and aggregate the amount of drusens in a given macular region [138].

Presently there are several scales published for AMD classification [140, 141]. The one that is accepted and used by most of the European epidemiology studies, including the European Eye Epidemiology (E3) network, is the Rotterdam classification [111] that is described in Table 10.

Table 10. Rotterdam Classification of mutual exclusive stages of AMD [111].

Stage	
0	No signs of AMD OR Hard drusen (< 63 µm) only
1	a. Soft distinct drusen (≥ 63 µm) only
	b. Pigmentary abnormalities only (no soft drusen ≥ 63 µm)
2	a. Soft indistinct drusen (≥ 125 µm) OR reticular drusen only
	b. Soft distinct drusen (≥ 63) WITH pigmentary abnormalities
3	Soft indistinct drusen (≥ 125 µm) OR reticular drusen WITH pigmentary abnormalities
4	Atrophic OR Neovascular AMD

To characterize the different stages of AMD all these retinal features need to be considered [140, 142, 111] and therefore, semi-automated systems are need to help the examiner identifying and counting these retinal features and to improve the quality of the data gathered.

5.3.1.1 Semi-automatic detection of the AMD Retinal lesions

AMD grading systems are based on colour fundus images, which were in the past acquired using slides. Presently digital colour fundus imaging is widely used and therefore, most of the grading systems are based on digital colour fundus photographs [143]. The reproducibility of using digital colour fundus imaging for AMD grading was demonstrated by the AREDS group in over 4000 subjects [144], allowing to compare the results obtained in different studies.

AMD grading based on digital colour fundus images allows for a higher quality of the grading, by allowing the use of image enhancement tools and by reducing bias since the fundus images can be masked to the graders. Moreover, colour fundus images can be acquired in the common clinical practice and since this non-invasive methodology provides fundus images most similar to the view of the physician, it allows for the extrapolation from clinical research to clinical practice.

Automated and semi-automated lesions detection has been used with some success to assess lesions area and location, decreasing costs for grading and reducing the intra-grading variability. Such computer-assisted systems are of major importance for AMD characterization [144, 145].

5.3.1.1.1 The RetmarkerAMD Research

RetmarkerAMD Research is a new software developed by Critical Health in collaboration with AIBILI for the assisted grading of colour fundus images of patients with AMD. This software provides a tool for AMD lesions earmarking and for the automatic assessment of the lesions number and areas, per retinal subfield.

The system was developed and fine-tuned to process 35º colour fundus images, centred on the macula [146]. Once the images are imported in the RetmarkerAMD Research software the first step is to calibrate the image, i.e., to adjust the International Classification Grading grid in the image. To calibrate the image, the borders of the optic disc and the centre of the fovea are earmarked (the software assumes that the average optic disc diameter is 1.5 mm). Then, the system overlaps the International Classification Grading grid (i.e., standard ETDRS grid) dividing the eye fundus image in 10 subfields: field 1 (central circle centred in fovea); fields 2 to 5 (inner circle); fields 6 to 9 (outer circle); and field 10 (outside area) (Figure 17).

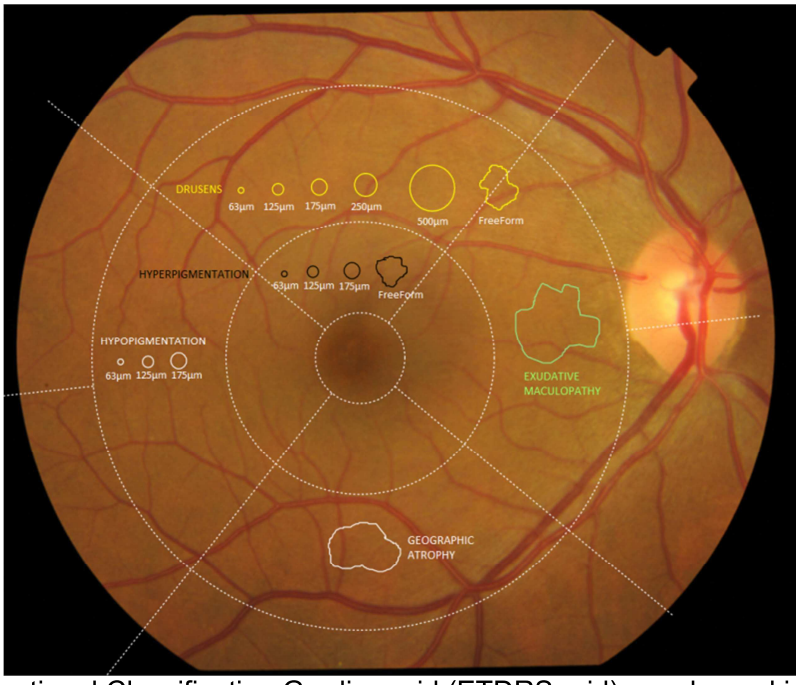

Figure 17. International Classification Grading grid (ETDRS grid), overlapped in the eye fundus image after calibration.

The RetmarkerAMD Research software allows the graders to manually earmark (draw-over) the following AMD features:

- Drusens, using 5 circles with standard dimensions (Figure 17 and Figure 18):
  - C0: < 63 µm;
  - C1: ≥ 63 µm and < 125 µm;
  - C2: ≥ 125 µm and < 175 µm;
  - C3: ≥ 175 µm and < 250 µm; and
  - C4: ≥ 250 µm and < 500 µm.
- Pigmentary abnormalities (hypo- and hyperpigmentation), using 5 circles with standard dimensions (C0 to C4, Figure 17 and Figure 18);
- Geographic Atrophy (GA) (using freehand drawing tools, Figure 19 - left); and
- Exudative Lesions (CNV) (using freehand drawing tools, Figure 19 - right).

For each type of lesion the system use a unique colour-code representation. Also, different filters can be used to better visualize retinal lesions. The results are displayed to the grader in real time.

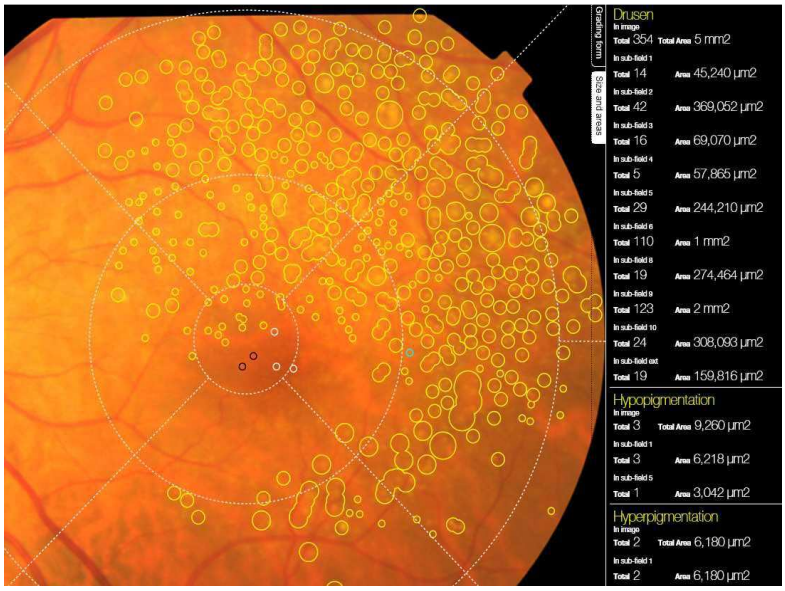

Figure 18. Drusens (yellow), hypopigmentation (white) and hyperpigmentation (black) earmarking.

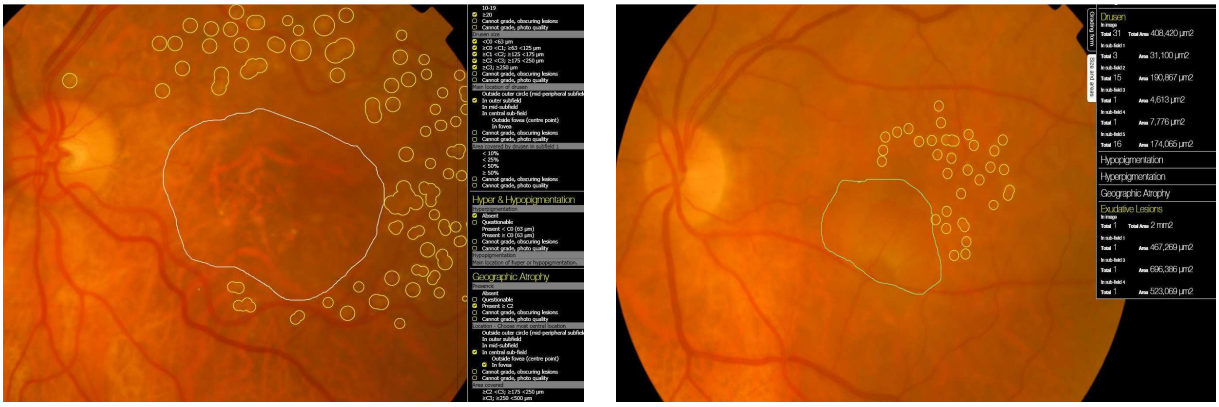

Figure 19. Left: Geographic atrophy (white freehand form). Right: Wet AMD (CNV) (green freehand form).

To test the RetmarkerAMD Research software, the detection of AMD lesions based on printed images and based on digital images (using the RetmarkerAMD Research software) were compared. Three senior ophthalmologists were asked to earmarked 6 CFP with 35°, acquired using a colour fundus camera model FF450 from Zeiss (Carl Zeiss Meditec, Dublin, USA).

The images were previously selected based on the presence of identifiable AMD lesions (i.e., 2 images with less than 10 lesions, 2 with more than 10 but less than 50 lesions, and 2 with more than 50 lesions). Images were acquired with a resolution of 3872 x 2592 pixels, zoomed to 210 x 297 mm (A4 format) and printed afterwards on photo quality paper for grading. For each of

these images, with size and quality simulating images as viewed on a 15" laptop screen (Figure 20), a grid with 10 subfields was printed in acetate. The grid was calibrated with the image in order to be superimposed on the printed image allowing for the localisation of the AMD lesions. A ruler of standard circles with diameters of 63 µm, 125 µm, 175 µm, 250 µm, 500 µm, 1000 µm and 3000 µm, was also built in the scale of the printed images to measure the identified lesions.

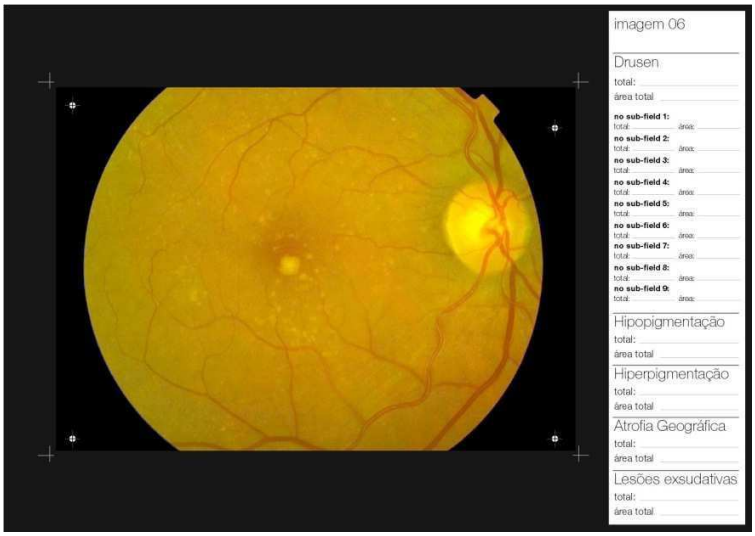

Figure 20. Colour fundus images were enlarged and printed on photographic paper (the sheet has an A3 size (420 x 297mm), while the image has an A4 size (210 x 297 mm)).

For lesions earmarking in digital images, using the RetmarkerAMD Research, a 15" laptop was used. The images were previously calibrated with the same settings as for the printed images, i.e., the subfields grid was in the same position and dimension relative to the background image. The images were presented in full screen mode.

Both images (printed and digital) were graded by 3 senior ophthalmologists, aged between 40 and 65 years, and with a vast experience in AMD. The space and light conditions in which the grading took place were not subject to any specific treatment. A standard room with mixed light (natural and artificial light) was used. For the grading on printed images a table lamp was used to increase the lighting level. This lamp remained turned off during digital images' grading. The graders were asked in a first session to identify lesions on the 6 printed images and, in a second session, to identify lesions on the same images, using the RetmarkerAMD Research software. The graders worked in independent sessions and under similar conditions.

More drusens were identified by the graders when earmarking digital CFP using RetmarkerAMD Research (Figure 21). A total of 670 more drusens were earmarked by the graders when using digital images than when using printed images (Figure 22). By using digital images the graders were able to document approximately 32% more lesions (2087 drusens earmarked when using printed images *versus* 2757 drusens earmarked when using digital images).

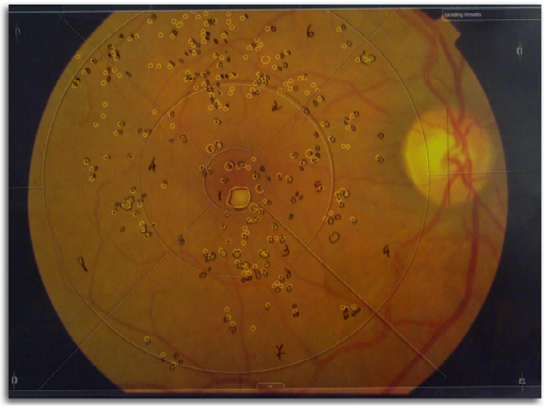

Figure 21. Overlap of the drusens identified by one grader on printed images (dark grading) and digital images (yellow grading).

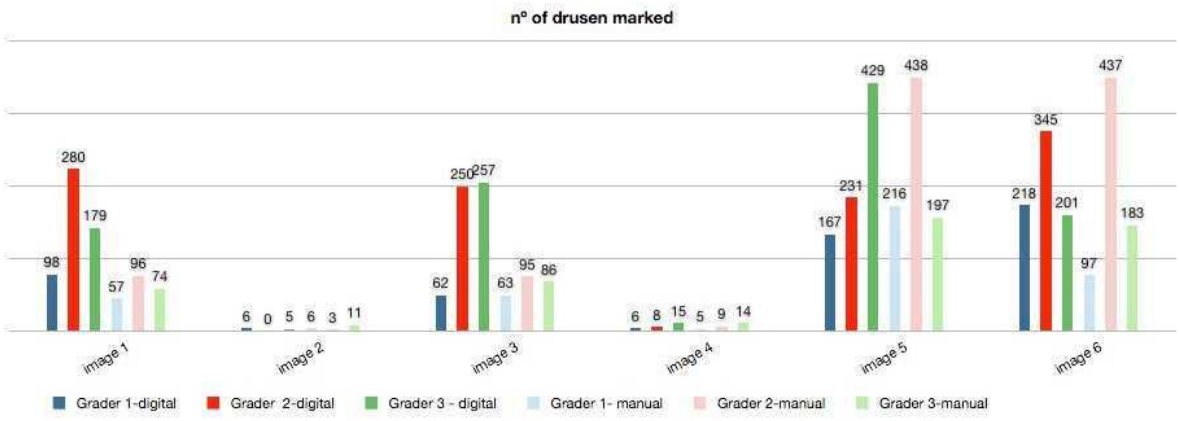

Figure 22. Number of drusens identified by the 3 graders when using digital and printed CFP.

The 3 graders took on average 6.1 minutes per image to earmark AMD lesions on printed images and 9.3 minutes per image to earmark AMD lesions on digital images. The graders spent with the digital images about 54% more time than the time used on printed images. On the other hand, when considering the time spent by the graders to count the lesions and to compute the corresponding areas, the average time spent per grader was approximately 35%

less when using the RetmarkerAMD Research software. When grading was performed on printed images the average time per grader was 62.6 minutes, and, when the grading was performed using the RetmarkerAMD Research software the average time was 40.6 minutes).

The data collected, even in a small sample, suggested some differences in the number of drusens identified by the 3 experienced graders. When comparing lesions earmarked by the 3 graders on digital CFP it was found a consistent agreement between graders in identifying most of the AMD lesions present in the images. All graders showed a trend to represent the same type of lesion, with similar dimensions, in the same areas (Figure 23). The major disagreement was found for the detection of areas of hypopigmentation.

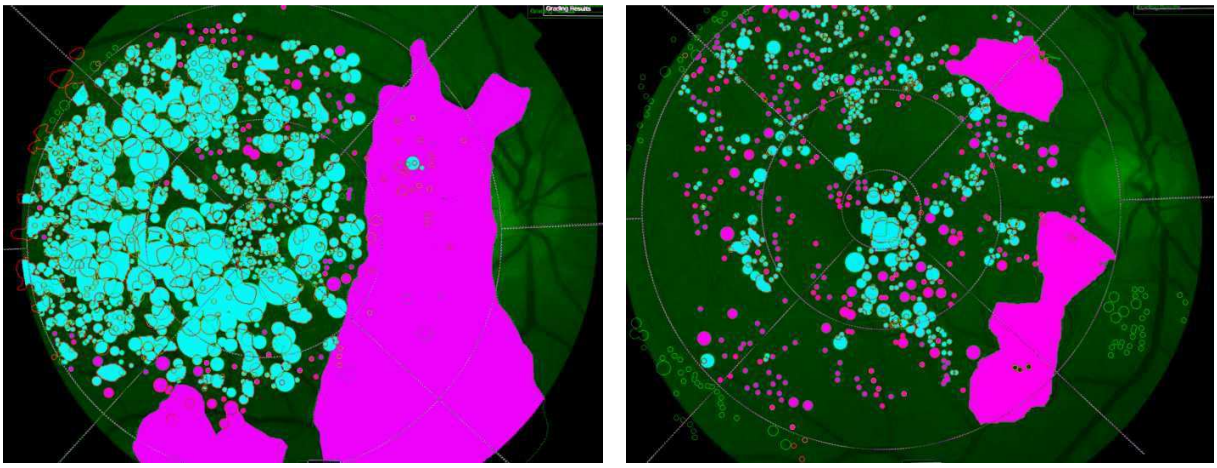

Figure 23. Lesions identified by the 3 graders in two images (Blue: areas earmarked by the 3 graders; Pink: Areas earmarked by 1 grader only).

The RetmarkerAMD Research software was found to be an effective tool to compute the number and size of the lesions earmarked manually by the graders, allowing for a significant time saving.

5.3.1.1.2 Grading Reproducibility using RetmarkerAMD Research

To assess the reproducibility of using the RetmarkerAMD Research as a new computer-assisted grading tool for AMD grading based on digital colour fundus images, 50 colour fundus photographs with 35º were acquired in 50 AMD patients using a digital mydriatic camera model

TRC-50DX (Topcon, Tokyo). Grading was performed independently by 2 senior ophthalmologists using the RetmarkerAMD Research software.
Additionally 3 junior ophthalmologists were asked to earmark a subset of 12 colour fundus images to assess the inter-grader variability in less experienced and trained graders.

The following parameters were graded by the graders:

- Number of drusens
    - Absent
    - Questionable
    - 1-9
    - 10-19
    - ≥ 20
- Number of drusens:
    - < 63 µm
    - ≥ 63 and < 125 µm
    - ≥ 125 µm
        - 0
        - 1-9
        - 10-19
        - ≥ 20
- Predominant drusen type within outer circle
    - Absent
    - Questionable
    - Hard Drusen (< 63 µm)
    - Intermediate, soft drusen (> 63 and ≤ 125 µm)
    - Large, soft distinct drusen (> 125 µm)
    - Large, soft indistinct drusen (> 125 µm)
        - Crystalline/calcified/glistening
        - Semisolid
        - Serogranular

- Pigmentary abnormalities
    - Hyperpigmentation
    - Hypopigmentation
        - Absent
        - Questionable
        - Present < 63 µm
        - Present ≥ 63 µm
- Geographic atrophy
    - Absent
    - Presence questionable
    - Present ≥ 125 µm
- Neovascular AMD
    - Absent
    - Presence questionable
    - Present ≥ 125 µm

The following parameters were computed automatically by RetmarkerAMD Research:

- Total Area occupied by drusens
- Area covered by drusens in subfield 1
- Total area occupied by drusens in the inner circle
- Total area occupied by drusens in the outer circle
    - 0%
    - < 1%
    - < 10%
    - < 25%
    - < 50%
    - ≥ 50%
- Confluence of drusens
    - No
    - < 10%
    - < 50%
    - ≥ 50%

The AMD severity levels were afterwards computed based on the Rotterdam classification [111] (Table 10).

The agreement between graders was tested using the Weighted Kappa coefficient for the following parameters:

- Number of drusens
- Number drusens < 63 µm
- Number drusens ≥ 63 and < 125 µm
- Number drusens ≥ 125 µm
- Predominant drusen type within outer circle
- Total area occupied by drusens
- Area covered by drusens in subfield 1
- Area covered by drusens in the inner circle
- Area covered by drusens in the outer circle
- Confluence of drusens
- Hyperpigmentation
- Hypopigmentation
- Geographic Atrophy
- Neovascular AMD
- Stage of AMD

A good overall agreement was found between the two senior ophthalmologists (average Kappa = 0.712). A good to excellent agreement was found for all the parameters assessed (Kappa values ranging from 0.669 for the stage of AMD, 95%CI (0.494-0.844), to 0.884 for the presence of hypopigmentation, 95%CI (0.830-0.938)), except for the confluence of drusens where the agreement was poor (Kappa = 0.220; 95%CI (0.667-1.107) (Table 11).

Table 11. Agreement between 2 senior ophthalmologists using the RetmarkerAMD Research for a set of 50 digital colour fundus images.

Parameter	Kappa	95%CI	
Number of drusen	0.801	0.705	0.897
Number drusens < 63 µm	0.777	0.667	0.887
Number drusens ≥ 63 and < 125 µm	0.769	0.655	0.883
Number drusens ≥ 125 µm	0.672	0.499	0.845
Predominant drusen type within outer circle	0.759	0.639	0.879
Total area occupied by drusens	0.716	0.571	0.861
Area covered by drusens in subfield 1	0.797	0.698	0.896
Area covered by drusens in the inner circle	0.670	0.496	0.844
Area covered by drusens in the outer circle	0.811	0.720	0.902
Confluence of drusens	0.220	0.667	1.107
Hyperpigmentation	Not Computed (all cases graded as "Absent")		
Hypopigmentation	0.884	0.830	0.938
Geographic Atrophy	Not Computed (all cases graded as "Absent" with the exception of 1 case that was graded as "Present ≥ 125 µm")		
Neovascular AMD	Not Computed (all cases graded as "Absent")		
Stage AMD	0.669	0.494	0.844

The results of this study, for the agreement between experienced and trained graders (senior ophthalmologists), are in agreement with the results published by the AREDS group [144, 129]. The AREDS studies found a good agreement between graders for the presence, type and area of drusens (Kappa = 0.680, 0.690 and 0.770, respectively, [129]); for the presence of geographic atrophy and neovascular AMD (Kappa = 0.790 and 0.860, respectively, [144]); and for the AMD severity scale (Kappa = 0.760, [144]). For the pigmentary abnormalities it is found that these are the lesions more dependent on the image quality, and here the use of digital colour fundus images can be a major advantage, since digital images can be adjusted to optimize lesions detection. Danis et al. [144] found a good agreement between graders for the presence of hyperpigmentation based on digital colour images (Kappa = 0.710), while years before the AREDS Report number 8 [129] reported only a fair agreement between graders (Kappa = 0.540), which shows that digital colour funds images allows for a more precise detection of the AMD lesions.

To demonstrate the importance of training, our group asked 3 junior ophthalmologists to earmark AMD lesions in a set of 12 digital colour fundus images. After a first intensive training section with one of the senior ophthalmologists, only one grader achieved a moderate agreement (averaged Kappa = 0.428). For the other 2 graders the agreement was poor (average Kappa=0.109 and 0.358, respectively, Table 12). After a second training section the average agreement for these 2 graders increased to moderate (Kappa=0.481 and 0.490, respectively).

Table 12. Agreement between junior ophthalmologists and senior ophthalmologists using the RetmarkerAMD Research for a set of 12 digital colour fundus images.

Parameter	Grader 1	Grader 2	Grader 3
Number of drusen	0.776	0.000	0.454
Number drusens < 63 µm	0.589	0.000	0.520
Number drusens ≥ 63 and < 125 µm	0.812	0.467	0.226
Number drusens ≥ 125 µm	0.750	0.056	0.143
Predominant drusen type within outer circle	0.700	0.000	0.368
Total area occupied by drusens	0.189	0.027	0.158
Area covered by drusens in subfield 1	0.500	0.077	0.500
Area covered by drusens in the inner circle	0.043	0.167	0.062
Area covered by drusens in the outer circle	0.189	0.000	0.158
Confluence of drusens	0.226	0.280	0.640
Hyperpigmentation	0.333	0.429	0.429
Hypopigmentation	0.294	0.000	0.000
Geographic Atrophy	N/A	N/A	N/A
Neovascular AMD	N/A	0.000	N/A
Stage AMD	0.164	0.027	1.000

The RetmarkerAMD Research showed to be a good tool for AMD grading based on digital CFP, allowing for a more precise quantification and localization of the different AMD lesions. This system is found to be more effective and less time consuming, being therefore a good tool for clinical research and epidemiologic studies.

It should also be point out that the performance of the graders is highly dependent on the grader's experience and training. When new graders (junior ophthalmologists) are asked to grade AMD lesions, the average agreement is usually poor increasing significantly after a second training section. It is know that AMD graders should perform regular training sections in order to guarantee a good performance [144].

AMD grading is a complex and time-consuming activity. To improve the reliability of the grading activity new tools that help the graders to identify and earmark the different AMD lesions are needed, like the RetmarkerAMD Research software. Moreover, graders' experience and training are a key element for the grading performance.

5.4 Characterization of early markers of Neovascular Age-related Macular Degeneration

AMD is a bilateral disease. Approximately 10 to 20% of patients with early AMD will develop the wet form of AMD [147]. The type and number of drusens, and the pigmentary changes in colour fundus images have being shown to be good biomarkers for the risk of progression of the disease [1].
The presence of large-size drusens (≥ 125 μm), soft indistinct drusens, pigmentary changes and large areas of drusens have already been found to be predictive of late stages AMD [136]. These retinal changes, detected on CFP, are used to classify AMD severity and risk of conversion [136], since approximately 50% of the patients with dry AMD in one eye and CNV in the other eye will develop bilateral CNV within a 5 years period [148]. The severity scale developed by the AREDS group, based on the drusens size and the presence of pigmentary abnormalities in both eyes, allows for the scoring for AMD conversion into 4 levels [136].
In AMD, retinal biomarkers showed to be important to determine risk of disease onset, to measure disease progression or to assess the disease stage of a particular patient allowing for a patient-oriented treatment [147]. The identification of biomarkers for the conversion to wet AMD will contribute for the disease diagnosis and treatment, allowing for the development of new therapies and contributing for the prevention of vision loss.

To identify other morphological and/or functional biomarkers of disease progression, specifically predictive factor for the conversion to wet AMD, a 3-year exploratory, prospective, observational study was performed, in the fellow eye of patients with exudative AMD, using multimodal imaging.

5.4.1 Study Design

To identify alterations occurring in the chorioretinal interface during progression of AMD from dry AMD to sight-threatening CNV, a 3-year prospective observational study was designed to include patients aged over 50 years and diagnosed with exudative (wet) AMD in one eye and non-exudative (dry) AMD in the other eye (i.e., eye with intermediate and/or large/confluent drusens, with or without hyperpigmentation, and no CNV or GA) (study eye).

Each patient was followed with a minimal time interval of 6-month. Unscheduled visits were performed in the presence of complaints of metamorphopsia or any visual acuity disturbance. Patients developing sight-threatening CNV in the study eye withdrew the study in order to be treated according to the normal clinical practice.

Each 6 months the following imaging methods were performed to characterize retinal features, or predictors, of AMD conversion to sight-threatening CNV:

- Fluorescein angiography (FA);
- Indocyanine green angiography (ICG);
- Colour fundus photography (CFP);
- Retinal leakage analyser (RLA);
- Fundus autofluorescence imaging (FAF); and
- Optical coherence tomography (OCT).

5.4.1.1 Sample Size Estimation

Being an exploratory study with different imaging methodologies, no sample size could be computed. A sample of 60 patients was estimated based on a conversion rate of: 10% (for patients with intermediate and/or confluent drusens, score 2); 25% (for patients with large drusens or hyperpigmentation, score 3); and 40% (for patients with large drusens and hyperpigmentation, score 4) after the 3rd year of follow-up [136]. Considering these conversion rates and a dropout rate of 20% for the 3-year follow-up period, 5 to 19 patients are expected to convert at the end of the study and may be used to identify, in an exploratory manner, risk factors for conversion.

5.4.1.2 Study Outcomes for AMD Conversion

To identify risk factors (or features) associated with the conversion to CNV, patients performed each 6 months the following imaging methodologies:

- FA using the HRA II (Heidelberg Retina Angiograph version II, Heidelberg Engineering, Dossenheim, Germany) with a 30º field-of-view centred on the macula.
    - FA images were used as gold standard to identify conversion to wet AMD.

- ICG using the HRA II with a 30º field-of-view centred on the macula.
    - ICG images were used to evaluate the presence of: CNV; early and/or late hyperfluorescent spots; and early and/or late hypofluorescent spots.

- FAF using the HRA II with a 30º field-of-view centred on the macula.
    - FAF images were classified according to the International Fundus Autofluorescence Classification Group (i.e., predominant FAF pattern) [149].

- OCT using the Stratus OCT (Carl Zeiss Meditec, Dublin, USA) using the Fast Macular acquisition protocol centred on the macula.
    - OCT was performed to measure retinal thickening and to evaluate intra- and subretinal fluid.

- RLA was performed using the HRA I (Heidelberg Retina Angiograph version I, Heidelberg Engineering, Dossenheim, Germany) following the methodology described by Bernardes et al. [72].
    - RLA was used to map the BRB function and to identify alterations of the BRB.

- CFP was performed using the FF450 digital fundus camera (Carl Zeiss Meditec, Dublin, USA) with a 30º field-of-view centred on the macula.
    - CFP images were graded according to the International Classification Grading System guidelines [140] using the RetmarkerAMD Research software.

5.4.2 Study Synopsis

The study protocol was elaborated and submitted to the RA for approval. Being an observational clinical study, the study protocol was submitted to the local Ethics Committee of AIBILI (CES), and to the National Data Protection Committee (CNPD). This study was registered in the public database ClinicalTrials.Gov under the number NCT00801541. The study synopsis is provided in Table 13.

Table 13. Study synopsis ("Characterization of early markers of choroidal neovascularization (CNV-Markers)").

Study Synopsis (based on the Annex I of the ICH-E3 [19]).	
Sponsor	R. Silva (AIBILI).
Finished Product/Active Ingredient	None.
Title of Study	Characterization of Early Markers of Choroidal Neovascularization (CNV-Markers).
Investigators	Prof. Dr. Rufino Silva.
Study Centres	CEC-AIBILI, Coimbra, Portugal.
Studies periods (years)	3 years follow-up.
Study type / Phase of development	Prospective observational.
Objectives	To identify alterations occurring in the chorioretinal interface during progression of AMD from "dry" AMD to sight-threatening chorioretinal neovascularization (CNV) (wet AMD).
Methodology	Patients with evidence of exudative – wet AMD in one eye and non-exudative – dry AMD in the fellow eye (study eye) (i.e., eye at risk for progression to wet CNV) will perform each 6 months different imaging methodologies in order to characterize markers or predictors of conversion to sight-threatening CNV (i.e., CNV classic or occult, or CNV within 2500 µm of the foveal centre, as evidenced by FA). Patients will exit the study at the time of developing sight-threatening CNV in the study eye and will be treated at the discretion of the physician according to the normal clinical practice. The following procedures will be performed: BCVA; FA; ICG; FAF; OCT; RLA; and CFP.
Number of patients	60 patients with wet AMD in one eye and dry AMD in the other eye.
Diagnosis and main criteria for inclusion	Patients of either sex, any race, aged over 50 years, diagnosed with wet AMD in one eye and dry AMD in the other eye (study eye, at risk for progression to wet AMD) and that meet all the inclusion and exclusion criteria. <u>Inclusion Criteria:</u> 1. Written informed consent; 2. Age over 50 years; 3. Any race and any sex; 4. Clinical diagnosis of exudative AMD in one eye and the presence of the following characteristics in the second eye (study eye): ○ At least 5 or more intermediate (> 63 µm) or larger soft drusen AND/OR ○ Confluent drusen within 3000 µm of the fovea centre AND/OR ○ Hyperpigmentation. <u>Exclusion Criteria:</u> 1. History of medical condition that would preclude scheduled study visits; 2. History of ophthalmic disease in the study eye other than AMD; 3. Clinical signs of myopic retinopathy, or refraction higher than -8 diopters; 4. Intraocular surgery in the study eye within 60 days prior enrolment; 5. Evidence of past or present CNV in the study eye.
Treatment Duration	Not applicable.

Table 13. Study synopsis ("Characterization of early markers of choroidal neovascularization (CNV-Markers)").

Study Synopsis (based on the Annex I of the ICH-E3 [19]).	
Test product/Reference Therapy	Not applicable.
Criteria for evaluation **Efficacy and Safety**	This is an exploratory study to identify morphological and functional risk factors of conversion to sight-threatening CNV, based on the following imaging methodologies: FA; ICG; FAF; OCT; RLA; and CFP.

5.4.3 Statistical Methods

Being an exploratory study the different features, as identified in the different imaging methodologies, were compared between eyes that progress to sight-threatening CNV and eyes that did not progress. Therefore, statistically significant differences for conversion were tested using the Pearson χ^2 test and for the parameters that showed a statistically significant difference, the OR and 95%CI were computed.

Statistically significant results were considered for $P < 0.05$. Statistical analyses were performed using the SPSS software version 13.0 (SPSS LEADS Technology Inc., USA).

5.4.4 Study Results

Fifty-two (52) patients completed the 3 years follow-up, 26 males (50.0%) and 26 females (50.0%), age ranging from 56 to 92 years (mean ± SD: 76.0 ± 6.0) (Figure 24).

Twenty four (24) patients converted during the 3-year follow-up period: 29.2% after the first year of follow-up (7); 45.8% after the second year of follow-up (11); and 25.0% after the third year of follow-up (6).

From the different retinal lesions only the presence of retinal leakage (assessed using RLA) (OR = 5.0; 95%CI (1.5-16.4); P = 0.006); the presence of hyperfluorescent spot on ICG for the early (OR = 7.2; 95%CI (2.0-25.7); P = 0.002) and late (OR = 4.7; 95%CI (1.4-15.4); P = 0.009) phases; and the presence of hypofluorescent spot on ICG for the early phases (OR = 3.7; 95%CI (1.2-12.1); P = 0.025), showed a statistically significant difference between eyes that progress and eyes that do not progress during the 3-year follow-up period.

Pattern of FAF; drusen number, size, type and area; hyper- and hypopigmentation showed no statistically significant differences.

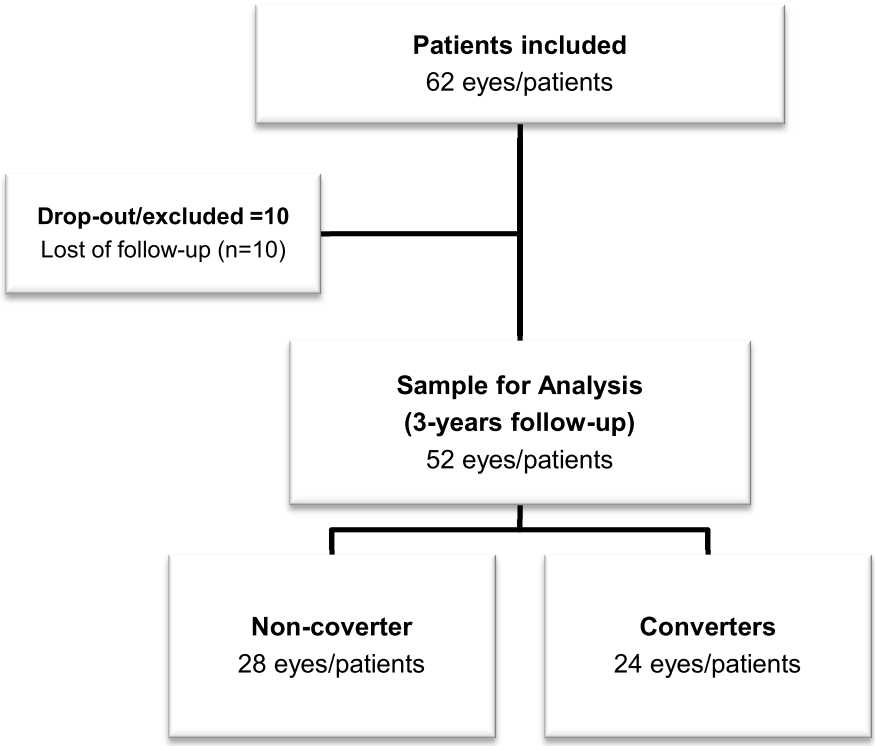

Figure 24. CONSORT flow chart for the patients included in the study NCT00801541.

5.4.5 Study Discussion

This IDCR showed, using multimodal imaging, that early and late ICG hyperfluorescent spots, early ICG hypofluorescent spots and leakage detected with RLA before conversion are predictive factors of CNV conversion [150]. Other lesions, such as drusens size and pigmentary changes, showed no predictive value for the conversion of AMD (see section 5.4.6).

Only the results obtained for the hyperfluorescent spots (ICG early phases) allowed for a statistical power of 90.8% (for the remaining results the statistical power was below 65.8%). Therefore, the results obtained in this study need to be confirmed in a larger population and with a longer follow-up. Also, possible confounding factors, such as high blood pressure, age and smoking habits should be considered.

AMD is a complex disease with both genetic and environmental factors. Future studies should consider not only retinal biomarkers but also genetic and environmental factors. The identification of morphological, functional and genetic biomarkers in the early stages of the disease is crucial for the early diagnosis of AMD. By detecting AMD in the early stages of the disease the number of patients with severe vision loss due to AMD may be reduced significantly. Furthermore, the improved knowledge of these biomarkers may contribute for the development of new therapeutic approaches for both early and late stages of the disease.

5.4.6 Scientific Contribution

The results of this study were published in 2011:

- R. Silva, M. Cachulo, P. Fonseca, R. Bernardes, S. Nunes, N. Vilhena, and J. Faria de Abreu. Age-related macular degeneration and risk factors for the development of choroidal neovascularisation in the fellow eye: A 3-year follow-up study. Ophthalmologica, 226:110–118, 2011.

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Age-Related Macular Degeneration and Risk Factors for the Development of Choroidal Neovascularisation in the Fellow Eye: A 3-Year Follow-Up Study

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Key Words
Age-related maculopathy · Drusen · Indocyanine green angiography hot spots · Risk factors · Exudative age-related macular degeneration · Computer-assisted grading · Retinal leakage analyzer

Abstract
Introduction: The presence of large-sized drusen (≥125 μm), soft indistinct drusen, pigmentary changes, a large area of drusen and a choroidal neovascular membrane in one eye have been found to be predictive risk factors of late exudative age-related macular degeneration (AMD). Multimodal imaging potentially increases the possibility of indentifying further potential risk factors of developing wet AMD. ***Purpose:*** To identify morphological and/or functional baseline risk factors for the development of choroidal neovascularization (CNV) in a multimodal set of images from fellow eyes of patients with exudative AMD. ***Methods:*** Single-center, prospective, observational, longitudinal 2-year plus 1-year extension study of 62 patients with neovascular AMD in one eye (the nonstudy eye) and early age-related maculopathy (ARM) in the fellow eye (study eye). Best-corrected ETDRS visual acuity, fluorescein angiography (FA) and indocyanine green angiography (ICG), fundus photography, retinal leakage analysis, fundus autofluorescence imaging and optical coherence tomography (OCT Stratus 4.0.2, Carl Zeiss Meditech Inc.) were performed at baseline and every 6 months in order to identify both conversion to CNV as well as possible predictive features present before conversion. A semiautomated computer-assisted grading system was used for classifying fundus color images. Only eyes with 3 years of follow-up were considered for statistical analysis. ***Results:*** Fifty-two patients completed the 3-year study follow-up: 26 men and 26 women aged from 56 to 92 years (mean ± SD: 76 ± 6 years). CNV confirmed with FA developed in 46% of the 52 study eyes during the 3-year follow-up (24 converted eyes: 7 in the first year, 11 in the second and 6 in the third). A significantly higher risk for conversion to wet AMD was found only for leakage on a retinal leakage analyzer (odds ratio, OR = 5.0; 95% confidence interval, CI = 1.5–16.4; p = 0.006) detected at least in one visit before the onset of exudative lesions, for baseline ICG hot spots (OR = 7.2; 95% CI = 2.0–25.7; p = 0.002), baseline late ICG hot spots (OR = 4.7; 95% CI = 1.4–15.4; p = 0.009) and baseline early ICG hypofluorescent spots (OR = 3.7; 95% CI = 1.2–12.1; p = 0.025). The total area of drusen, the area of drusen in subfield 1, inner circle or outer circle, the total number of drusen and the number of

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drusen ≥125 μm, fundus autofluorescence patterns, OCT findings and the severity of ARM at baseline did not show any correlation with an increased risk of conversion to wet AMD. ***Conclusion:*** At 3 years, progression from early to late exudative AMD was superior to the expected rate (44%). ICG early and late hyperfluorescent spots or areas, ICG early hypofluorescent spots or areas and early leakage detected with the retinal leakage analyzer, but not pigmentary changes, large drusen, number and area of drusen at any location or a greater severity of ARM at baseline, showed to be a predictive parameter of conversion to wet AMD.

Introduction

The presence of large-sized drusen (≥125 μm), soft indistinct drusen, pigmentary changes and a large area of drusen have been found to be predictive of late exudative age-related macular degeneration (AMD) [1–6]. According to the AREDS Study [6], the presence of large drusen, any pigmentary changes or choroidal neovascularization (CNV) in the other eye are particularly predictive of developing advanced AMD. In fact, it is widely accepted that the risk of conversion to wet AMD in the second eye is particularly increased [5, 6]. Data from the Macular Photocoagulation Study Group show that 42–58% of patients with dry AMD features in one eye and CNV in the fellow eye will develop bilateral CNV within 5 years [4, 5]. A simplified scale was developed providing convenient risk categories for the development of advanced AMD that can be determined by clinical examination or by photographic procedures [6]. In this risk scale, the presence of exudative AMD in one eye accounts for 2 risk factors (on a scale with a maximum value of 4 risk factors), and the risk of conversion in wet AMD can be as high as 10% a year [6]. The evaluations in these studies were normally performed with a 35-mm color film. However, digital fundus color photography has been considered reliable, and as good as the 35-mm film, for grading age-related maculopathy (ARM) features in epidemiological studies and in clinical assessment [7–9].

We performed a 3-year prospective study, in the fellow eye of patients with exudative AMD, using multimodal digital imaging.

The objective of our study was to identify morphological and/or functional predictors of conversion in wet AMD in the fellow eyes of patients with exudative AMD.

Materials and Methods

Single-center, prospective, observational, longitudinal 2-year plus 1-year extension study. Patients with neovascular AMD in one eye (the nonstudy eye) and early ARM in the fellow eye (study eye) were included. Inclusion and exclusion criteria and definition of methodology, already described [9], are briefly summarized. Inclusion criteria were: (a) age 50 or more years; (b) any race and either sex; (c) signed informed consent; (d) to be able to return for the required visits, and (e) clinical diagnosis of wet AMD in one eye (nonstudy eye) and the presence of the following characteristics in the second eye (study eye): (1) at least 5 or more intermediate drusen (≥63 and <125 μm), ≥1 large soft drusen ≥125 μm and/or confluent drusen, within 3,000 μm of the foveal center, and (2) with or without pigmentary changes. Exclusion criteria were: (a) other fundus diseases like vascular retinopathy, central serous chorioretinopathy, inflammation or non-AMD CNV; (b) current or past history of a medical condition that would preclude scheduled study visits or completion of the study; (c) clinical signs of myopic retinopathy or refractive power of ≥8 dpt or funduscopic evidence of degenerative myopia; (d) past history of intraocular surgery within 60 days prior to enrolling in the study, and (e) evidence of past or present CNV in the study eye.

Best-corrected ETDRS visual acuity, fluorescein angiography (FA), indocyanine green angiography (ICG), fundus photography, retinal leakage analysis (RLA), fundus autofluorescence imaging (FAF) and optical coherence tomography (OCT Stratus 4.0.2, Carl Zeiss Meditech Inc.) were performed at baseline and every 6 months, in order to identify both conversion to CNV as well as possible predictive features present before conversion. Unscheduled visits were performed in the presence of complaints of metamorphopsia or any visual acuity disturbance. All patients were followed during 3 years or up to 6 months after conversion to wet AMD.

Simultaneous fluorescein angiograms and ICG images were acquired using the HRA II Heidelberg Retina Angiograph (Heidelberg Engineering, Dossenheim, Germany) with the 30-degree field of view centered on the macula. FA was used as the gold standard for identifying conversion to wet AMD. Baseline ICG images were evaluated for the presence of early and/or late hyperfluorescent spots or areas and early and/or late hypofluorescent spots or areas and also for the presence of CNV by two senior independent graders (R.S., M.L.C.). Any disagreement was decided by a third one (J.R.F.A.).

FAF imaging was performed with the same retina angiograph scanning laser ophthalmoscope following a standard protocol [9]. Two independent graders classified FAF images (P.F. and R.S.), and any disagreement was decided by a third one (J.R.F.A.). All images were classified according to the International Fundus Autofluorescence Classification Group [10] for the description of the predominant FAF pattern, and the patterns obtained at baseline were correlated with fundus photograph findings at baseline and to CNV development. OCT was performed with the Stratus 4.0.2 OCT using the macula mapping fast mode centered on the macula with a total of 6 radial line scans, 30° apart from one another, and the cross hair mode. OCT was performed and compared for changes at baseline, namely for retinal thickening, intra- and subretinal fluid.

RLA was performed using the Heidelberg Retina Angiograph following closely the methodology previously described [11]. The aim of the RLA confocal scanning laser ophthalmoscope is to

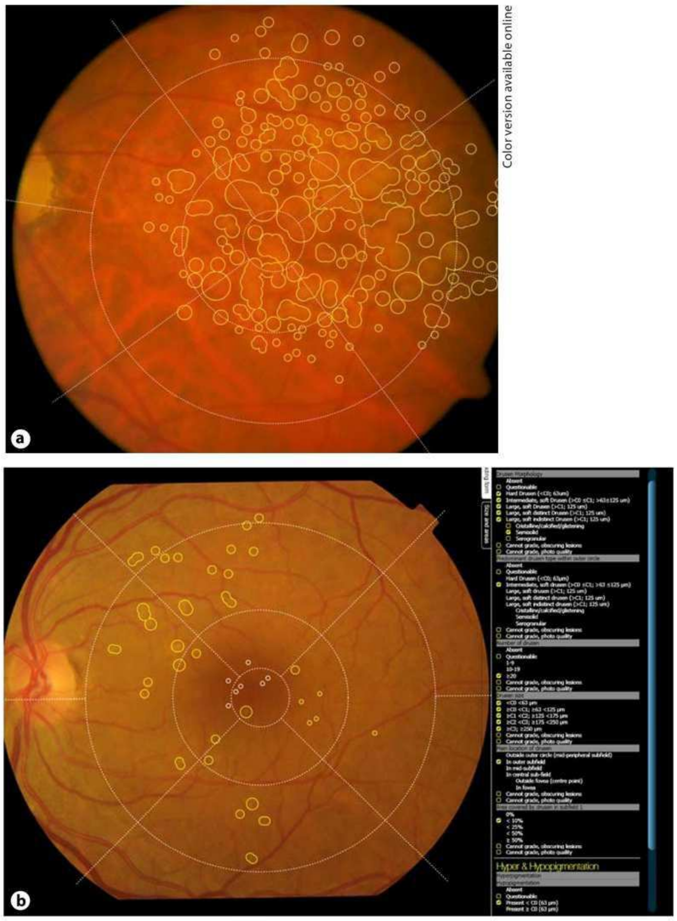

Fig. 1. **a** The computer-assisted grading system follows the International Classification guidelines for ARM [10]. A diameter of 1.5 mm is assumed for the optic disk. Circles of 1, 3 and 6 mm centered in the fovea are displayed over the image. Different predefined circles with <63, <125, <175, <250 and <500 μm are used for quantifying fundus features in fundus color photography, including drusen, pigmentary changes, geographic atrophy or exudative lesions. Free form measurements are also allowed. Using this semiautomated grading system, the results are displayed on screen in real time, and the final characterization of soft distinct, indistinct, reticular or crystalline drusen is performed by the operator (**b**).

map the blood-retina barrier function by measuring retinal fluorescein leakage from the blood stream into the human vitreous using a confocal scanning laser ophthalmoscope. This procedure allows measuring fluorescein leakage into the vitreous while simultaneously imaging the retina. With the RLA we expect to identify the occurrence of alterations of the blood-retina barrier before conversion to wet AMD.

The study design was approved by the ethics committee at the Association for Innovation and Biomedical Research on Light and Image, and informed consent was obtained from all patients before enrollment. The study was carried out in accordance with the Declaration of Helsinki. Only eyes with 3 years of follow-up were considered for statistical analysis.

Grading Fundus Color Photography

A Zeiss FF450 Fundus Camera equipped with a Nikon high-resolution camera was used for mydriatic fundus color photography acquisition. Fundus images centered both on the fovea and optic disk, with a 30-degree field of view, 1,594 × 1,326 pixel resolution (50% of maximum resolution), were obtained. Grading of fundus color photography was performed by using RetmarkerAMD (Critical Health SA), a computer-assisted grading system that follows the International Classification Grading System guidelines [12]. The system components allow to: (i) differentiate graded/nongraded images; (ii) assess images in full screen view; (iii) draw free forms of all the lesions' types over the images; (iv) draw preset circular objects, of different color codes and sizes (63–

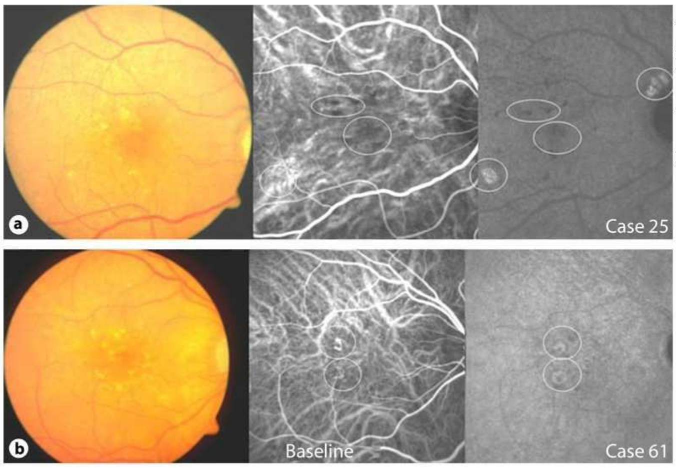

Fig. 2. Baseline findings in early and late ICG. **a** Case 25: early and late hypofluorescent spots and a late hot spot. **b** Case 61: early and late ICG hot spots.

500 μm) to represent drusen, hyperpigmentation, hypopigmentation, geographic atrophy and exudative lesions; (v) allow drusen marks to be confluent; (vi) zoom in/out the image or display an image area with a higher/lower degree of magnification; (vii) measure distances in the image and control RGB channels, brightness and contrast; (viii) superimpose a standard grid to identify eye subfields, and (ix) manually enter data for study protocol questionnaire.

Each image is calibrated, before any measurement is performed, using the optic disk diameter (1.5 mm) as a reference. This, along with the manual indication of the macula center (fovea), gives the software the necessary reference points to all measurements and to set up an overlaying reference grid. In this system, all ARM signs within a fixed area (diameter, 6,000 μm) around the fovea are recorded. The area is delineated by a grid consisting of 3 concentric circles and a right-angled cross at 45 and 135° to the horizontal, which is adjusted according to the previous calibration. The diameters of the central, inner and outer circles are 1,000, 3,000 and 6,000 μm, respectively, the same as used by the International Classification System [10]. The fundus signs that are allowed to be graded by this tool include: number of drusen <63, ≥63 to <125, and ≥125 μm; drusen type (hard; soft distinct; soft indistinct: semisolid, serogranular, crystalline); total area occupied by drusen (<1, <10, <25, <50, ≥50%, for central, inner and outer circles separately); cumulative real area occupied by drusen and area of each subfield, confluence of drusen (absent, <10, <50, ≥50%); hyperpigmentation (absent, <125, <175, ≥175 μm) and hypopigmentation of the retinal pigment epithelium (RPE; absent, <175, ≥175 μm), atrophic AMD; neovascular AMD. Using this semiautomated grading system, the results are displayed on screen in real time (fig. 1a, b), and the final characterization of soft distinct, indistinct, reticular or crystalline drusen is performed. Preliminary results from comparative tests have shown that RetmarkerAMD is more effective than 35-mm film manual grading allowing significant time savings (overall, it is 35% faster) and is more accurate (improved accuracy: overall 32% more lesions identified) [Silva R., oral presentation, Euretina 2010].

Statistically significant differences for conversion were tested using the Pearson χ^2 test. Additionally the odds ratio (OR) was computed for the predictive parameters of conversion in exudative AMD. Data was analyzed using the SPSS statistical software (SPSS 13.0 version, SPSS LEADS Technologies Inc., USA).

Results

Fifty-two patients completed the 3-year study follow-up: 26 men and 26 women aged from 56 to 92 years (mean ± SD: 76 ± 6 years). CNV confirmed with FA developed in 46% of the 52 study eyes during the 3-year follow-up (24 converted eyes: 7 in the first year, 11 in the second and 6 in the third). One third of the exudative lesions were retinal angiomatous proliferation. Stratus OCT was able to document the presence of intra- or subretinal fluid only at the time of conversion in all the 24 eyes. Similar dispersion of baseline FAF patterns in both groups, converter and nonconverter eyes, was found. All the study eyes presented large or intermediate drusen at baseline. A significantly higher risk for conversion to wet AMD was found for leakage on RLA (OR = 5.0; 95% confidence interval, CI = 1.5–16.4; p = 0.006) detected at least in one visit before the onset of exudative lesions; for baseline ICG hot spots (OR = 7.2; 95% CI = 2.0–25.7; p = 0.002),

Table 1. Variables tested for predictive value of conversion to exudative AMD

Variable	Nonconverters %	Converters %	p	OR
RLA (present)	31.8	68.2	0.006	5.0 (1.5–16.4)
FAF	–	–	0.384	
Predominant drusen type within outer circle	–	–	0.312	
Number of drusen (≥20)	55.2	44.8	0.338	
Area covered by drusen in subfield 1 (<10%)	55.5	44.4	0.381	
Hyperpigmentation (present)	64.7	35.3	0.652	
Hypopigmentation (present)	41.7	58.3	0.101	
Geographic atrophy (present)	100	0	0.202	
Number of drusen				
<63 μm (≥20)	53.8	46.1	>0.755	
63–<125 μm (≥20)	57.9	42.1	0.446	
≥125 μm (≥20)	50.0	50.0	0.337	
Total area occupied by drusen (≥50%)	100	0	0.866	
Total real area occupied by drusen	–	–	0.198	
Inner circle: total area occupied by drusen (≥50%)	100	0	0.198	
Outer circle: total area occupied by drusen	100	0	0.149	
Confluence of drusen (≥50%)	0	100	0.668	
Stage of ARM	–	–	0.600	
ICG hyperfluorescence				
Early phases (present)	26.3	73.7	0.002	7.2 (2.0–25.7)
Late phases (present)	34.8	65.2	0.009	4.7 (1.4–15.4)
ICG hypofluorescence				
Early phases (present)	37.5	62.5	0.025	3.7 (1.2–12.1)
Late phases (present)	47.5	52.5	0.100	3.9 (0.7–20.9)

Threshold of significance: $p \leq 0.05$; figures in parentheses indicate 95% CI. Only the presence of leakage at RLA, before conversion, baseline ICG early and late hyperfluorescence, and baseline early hypofluorescence were shown to be predictive parameters of CNV formation in the second eye.

baseline late ICG hot spots (OR = 4.7; 95% CI = 1.4–15.4; p = 0.009) and baseline early ICG hypofluorescent spots (OR = 3.7; 95% CI = 1.2–12.1; p = 0.025). The total area of drusen, the area of drusen in subfield 1, inner circle or outer circle, the total number of drusen and the number of drusen ≥125 μm, and the severity of ARM at baseline did not show any correlation with an increased risk of conversion to wet AMD (table 1).

Discussion

In our 3-year multimodal imaging prospective study, large soft drusen, area of drusen, severity of ARM or pigmentary changes showed no predictive value of developing exudative AMD. Risk markers were found only for ICG findings (early and late hyperfluorescent spots or areas; early hypofluorescent spots or areas) and for leakage detected by RLA (fig. 2–4). These are unexpected findings. In fact, it is widely accepted that large soft drusen, pigmentary changes (and exudative AMD in one eye) are the main known risk factors of developing CNV [6]. Several reasons may explain our findings. The most important is the small sample size. Only 52 patients concluded the study. Epidemiological and interventional studies [1–8] with a longer follow-up and a much larger number of patients are more powered to show an increased risk of developing exudative AMD in the first or in the fellow eye. However, we have chosen to evaluate potential predictive risk factors of developing exudative AMD in the second eye using multimodal imaging. The study was designed in order to quantify known risk factors not only in fundus color photography, but also to look for potential risk factors in ICG, OCT, RLA and FAF. ICG hyperfluorescent spots (or hot spots with <1 disk area) or plaques (≥1 disk area) may be associated with fibrin deposit but also with large drusen [13–16]. In fact, it has been shown that ICG has a high affinity for

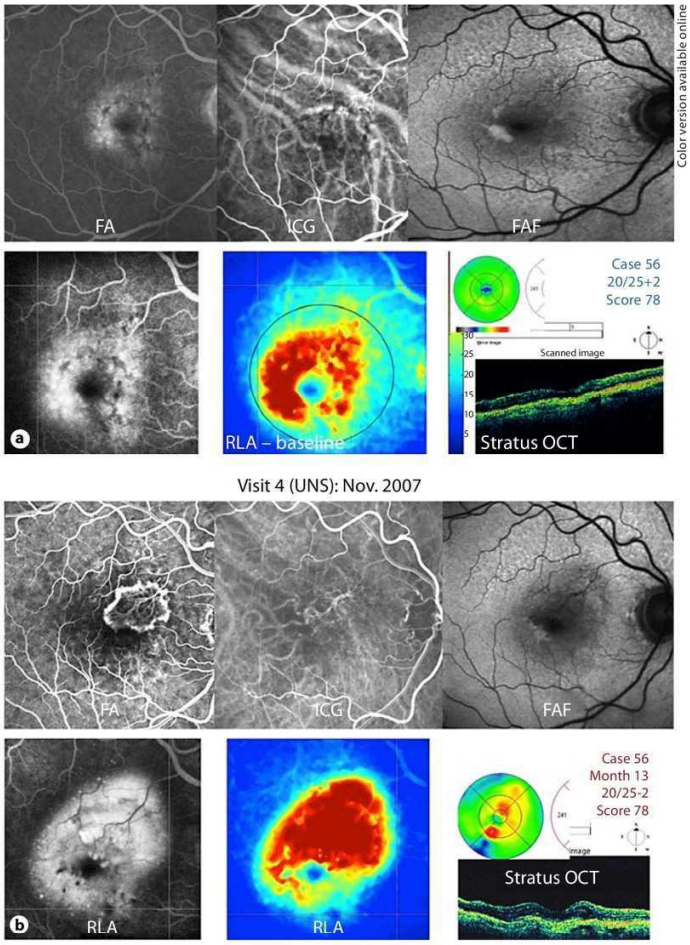

Fig. 3. Case 56. **a** Baseline. RLA shows baseline leakage. No signs of CNV are observed in FA, OCT or ICG. **b** The same case 13 months after baseline. Signs of CNV are observed in all the examinations.

some poorly vascularized intra-Bruch fibrovascular tissue containing fibrin [17] and for some drusen components including apolipoproteins B and E [18, 19]. Eyes with abnormal ICG hyperfluorescent drusen were also found to have a higher probability of developing exudative changes, compared with eyes with drusen and normal ICG [16]. In our study, some but not all of the hot spots were drusen related (fig. 3). In these cases with non-drusen-related hot spots, the hyperfluorescence is probably due to fibrin staining, like in ICG plaques, and an inactive CNV or a poorly vascularized fibrovascular tissue may be involved. Both the ICG hyperfluorescent drusen and the potential inactive CNV were shown to be predictive of conversion to wet AMD.

Early (but not late) ICG hypofluorescent spots or areas showed also to have a predictive value for conversion to wet AMD (fig. 2, 4). ICG hypofluorescence in ARM eyes may be due to a filling defect, serous or drusenoid pig-

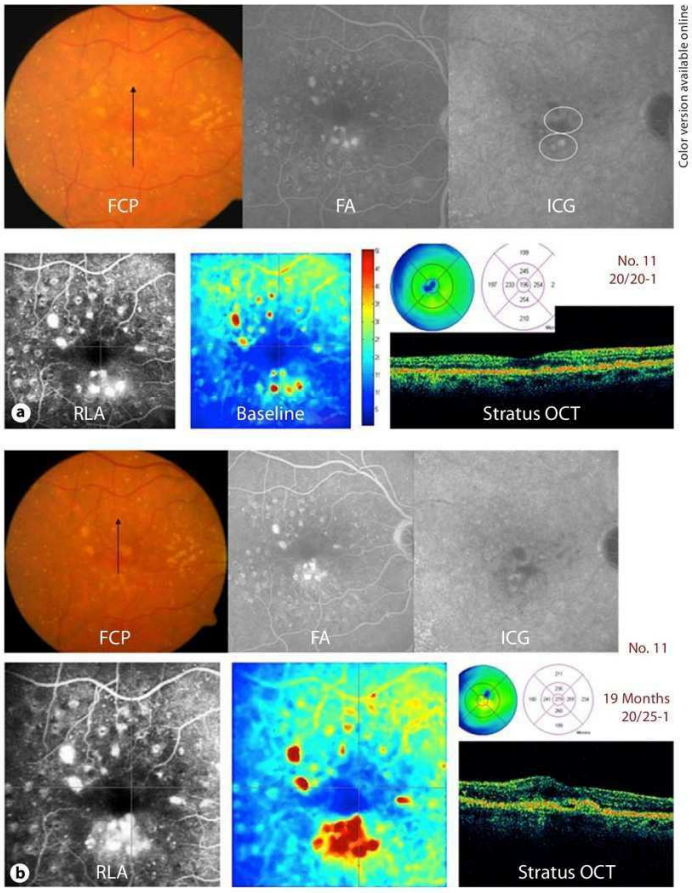

Fig. 4. a Baseline, case 11. Late ICG hot spots. **b** Nineteen months later, CNV is evident in FA, there is leakage in RLA and intraretinal fluid is observed in OCT. FCP = Fundus color photography.

ment epithelium detachment (PED), masking effect or drusen. In our series only drusenoid PEDs were found. In fact no serous PEDs were registered in OCT or FA. Observed filling defects were multiple but not representing extensive areas of ischemia in most cases. Even so, theoretically, they can be associated with an overexpression of vascular endothelial growth factor. PED and filling defects may show early and late ICG hypofluorescence more often than a masked effect or drusen which are, in many cases, associated only with late ICG hypofluorescence. This may explain why only early ICG hypofluorescence, more often related with drusenoid PED or filling defects (but not late ICG hypofluorescence), was associated with a higher risk of developing CNV.

FA was the gold standard for determining the occurrence of conversion to wet AMD. Stratus 4.0.2 OCT was able to identify exudative lesions in all the cases after conversion to wet AMD, showing subretinal and/or intraretinal fluid correlating with FA. However, it was useless for predicting conversion to wet AMD. Drusen or drusenoid PED, identified with Stratus OCT, did not show any predictive value for exudative AMD.

A previous study suggested an increased risk of CNV development of specific autofluorescence patterns at

baseline [20, 21]. In our series, different autofluorescence patterns were observed at baseline: 27% eyes had minimal changes, 27% a patchy pattern, 15% a reticular pattern, 13% a speckled pattern, 12% a focal increased pattern, 4% a focal plaque-like pattern and 2% a lace-like pattern. A linear pattern was not observed at baseline. No particular autofluorescence pattern was found to be correlated with a higher risk of conversion to wet AMD. For McBain et al. [22], increased autofluorescence was rarely seen in eyes with CNV and in fellow eyes, suggesting that increased autofluorescence, and thus RPE lipofuscin, may not play an essential part in the formation of CNV. Smith et al. [23] also found that in the neovascular case, a large group of fellow eyes have no focally increased autofluorescence abnormalities, suggesting that lipofuscin is not a major determinant of CNV. However, they also stated that 'reticular hypoautofluorescence, consistent with widespread inflammatory damage to the RPE, appears to be a highly sensitive imaging marker for the disease that determines reticular pseudodrusen and is strongly associated with CNV'. However, in our series, with a wide range of pattern dispersion, no specific pattern was associated with an increased risk of developing CNV.

Leakage detected with RLA was shown to be a good predictive parameter of conversion to wet AMD, in our series, with an OR of 5.0 (fig. 3). Leakage before conversion may be associated with RPE damage with relatively preserved choriocapillaries. RPE suffering is well documented in ARM with lipofuscin accumulation, RPE atrophy and cell death [23, 24]. This outer blood-retina barrier damage may explain the presence of leakage before CNV formation.

The rate of conversion can be as high as 10% a year. In our series, the rate of conversion was even higher. This is probably related with the high number of retinal angiomatous proliferation found. We have looked for other known risk factors potentially associated with progression to wet AMD, including age, smoking and high blood pressure, and they showed no correlation.

Fundus color photography features, including the number of large drusen (≥125 μm), the percentage of area occupied by drusen in the central 6 mm, the real area occupied by drusen, confluence of drusen, location of drusen and area in each subfield, the stage of ARM and the presence of pigmentary changes (table 1) did not show any predictive value for CNV formation. These were unexpected results. In fact in the MPS and AREDS Studies, drusen and pigmentary changes were clearly associated with a higher risk of developing CNV in the first or in the second eye [1–6]. Epidemiological studies found different risk factors but all of them associated with fundus color photography findings: the most important risk factors in the Rotterdam Study were ≥10% of the macular area covered by drusen, 10 or more large soft drusen and the presence of hyper- or hypopigmentation [25]. The incidence of CNV at 2 years was 20%, compared with 22% at 5 years in the Blue Mountains Study [26] and 20–30% in the Beaver Dam Study [27] at 5 years. In the Beaver Dam Study, the presence of soft indistinct drusen or pigmentary changes was associated with a higher risk of CNV. We have looked for all these variables. Our computer-assisted system is designed according to the International Classification of ARM [12] which includes: number of drusen <63, ≥63 to <125, and ≥125 μm; drusen type (hard; soft distinct; soft indistinct: semisolid, serogranular, crystalline); total area occupied by drusen (<1, <10, <25, <50, ≥50%, for central, inner and outer circles separately); real area occupied by drusen and area of each subfield, confluence of drusen (absent, <10, <50, ≥50%); hyperpigmentation (absent, <125, <175, ≥175 μm) and hypopigmentation of the RPE (absent, <175, ≥175 μm). Each one of these variables, including percentages or subgroups and real values, did not show any increased risk of progression to the exudative form of AMD. These were unexpected findings. Possible explanations for these results are certainly related with the small sample size and the duration of the study (3 years compared with 5 or 10 years in other large studies [6, 25–27]. Other confounding factors like high blood pressure, age or smoking were excluded.

In conclusion our multimodal imaging study showed that early and late ICG spots or plaques, early ICG hypofluorescent spots or areas and leakage detected with the RLA before conversion are predictive parameters of CNV formation in the second eye of AMD patients with an exudative lesion in one eye.

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5.5 Epidemiological Study on the prevalence of Age-Related Macular Degeneration

The incidence and prevalence of AMD increased in the last years due to the aging of the population, and to the improvement of the diagnostic methods. According to the Portuguese administration of health (Direção-Geral da Saúde – DGS), about 12% of the Portuguese population with 55 years or more have AMD[22]. Eighty five (85) to 90% of the AMD cases may have early stages AMD, i.e. approximately 310000 patients, while 10 to 15% may have late stages AMD, i.e. approximately 45000 patients with severe and irreversible loss of the central vision and with incapacity of reading (15000 with GA and 30000 with exudative AMD). Every year there are about 45000 new cases with early stages AMD and 5000 with late stages AMD. The risk for progression from the early stages to the late stages increases with the increase of age and with the seriousness of the lesions. The annual progression can achieve 10% but up to now no study was performed to assess the true prevalence of AMD in Portugal.

To assess the prevalence of AMD in the Portuguese population with 55 years or more and, to characterize the retinal lesions of the patients with this disease, an epidemiologic study was conducted. This study started in August 2009 and ended in January 2014. The study data is still under analysis.

5.5.1 Study Design

To assess the prevalence of AMD in the Portuguese population with 55 years or more, and to characterize retinal lesions associated with AMD, an observational study, with a single visit, was designed. The study was planned to be conducted in primary healthcare units in the Northern, Central and Southern regions of Portugal. The study visit consisted of one interview (to collect demographic data, medical history, family medical history and risk factors), and one visual examination (to assess visual acuity, tonometry and biomicroscopy, and to acquire CFP, for grading by the reading centre of AIBILI, the Coimbra Ophthalmology Reading Centre – CORC).

[22] Based on the information available at the DGS website (www.dgsaude.min-saude.pt/visao/html/dmi.html, accessed on January 10th 2014).

Initially 15000 patients were planned to be recruited, 5000 patients in 3 primary healthcare units distributed in the Northern, Central and Southern regions of Portugal. The study started in the Mira primary healthcare unit, in the Central region of Portugal. Due to financial and technical constraints, the primary healthcare units from the Northern and Southern regions of Portugal withdrew and the study design was reviewed in order to be performed only in the Central region of Portugal. For this purpose one additional primary healthcare unit from the Central region of Portugal was selected, and the sample size was adjusted accordingly.

5.5.1.1 Sample Size Estimation

Based on the European Eye Study results [151] the prevalence for the late stages of AMD should be approximately 3.3%. Therefore, to estimate the prevalence of AMD in Portugal, at least 5000 patients are needed (considering a confidence interval of 95% and a precision of 0.5%, [152]). Since only 3000 patients could be included in Mira, 3000 patients were planned for the second primary healthcare unit. Being Mira located near the sea, the second primary healthcare unit was chosen near the mountains allowing for the inclusion of two different subpopulations of the Central region of Portugal (located in Lousã). Moreover, with 3000 patients in each subregion statistically significant differences between AMD prevalence's in Mira and Lousã can be tested with a statistical power of 95% and an alpha level of 0.05 (considering a maximal difference between the two subpopulations' prevalence of 0.05%).

5.5.1.2 Study Outcomes for AMD Classification

CFP images with 35º were acquired using a mydriatic camera model TRC-50DX (Topcon, Tokyo). Images were graded based on the Rotterdam grading scale [111] using the RetmarkerAMD Research software.

To assess the prevalence of AMD by disease stage, senior ophthalmologists were trained and certified by the reading centre CORC. For each graded image the stage of AMD was assessed based on the Rotterdam classification i.e., based on the presence of the following retinal lesions: drusens, hyperpigmentation, hypopigmentation, geographic atrophy and exudative lesions, (Table 10).

5.5.2 Study Synopsis

The study protocol was elaborated and submitted to the RA for approval. Being an observational clinical study, the study protocol was submitted to the local Ethics Committee of AIBILI (CES), and to the National Data Protection Committee (CNPD). This study was registered in the public database ClinicalTrials.Gov under the number NCT01298674. The study synopsis is provided in Table 14.

Table 14. Study synopsis ("Epidemiological study of the prevalence of age-related macular degeneration in Portugal").

Study Synopsis (based on the Annex I of the ICH-E3 [19]).	
Sponsor	R. Silva (AIBILI).
Finished Product/Active Ingredient	None.
Title of Study	Epidemiological study of the prevalence of age-related macular degeneration in Portugal.
Investigators	Prof. Dr. Rufino Silva.
Study Centres	Health Care Units from Mira and Lousã.
Studies periods (years)	None.
Study type / Phase of development	Observational, cross-sectional.
Objectives	To assess the prevalence of AMD.
Methodology	Patients from the healthcare units of Mira and Lousã will be invited to participate in the study. Patients participating in the study will perform an ophthalmological examination including CFP. CFP will be graded by the central reading centre (CORC) for the presence of AMD, wet AMD, geographic atrophy, DR or other ocular pathology. Eyes with AMD, wet AMD or geographic atrophy will be subject to a second grading for retinal lesions assessment using the RetmarkerAMD Research software.
Number of patients	6000 participants (3000 from Mira and 3000 from Lousã).
Diagnosis and main criteria for inclusion	Participants with 55 or more years-old living in Mira or Lousã.
Treatment Duration	Not applicable.
Test product/Reference Therapy	Not applicable.
Criteria for evaluation Efficacy and Safety	Signs of Age-Related Macular Degeneration or other ocular disease (e.g. Diabetic Retinopathy, Glaucoma, Cataract).

5.5.3 Statistical Methods

The prevalence of AMD was analysed by stages, according to Table 10. The OR and 95%CI for the presence of AMD were computed for the following risk factors: smoking habits; relatives with AMD; other ophthalmological disease; diabetes and hypertension. Logistic regression analyses were performed to assess the association of AMD with age, gender and smoking.
Statistically significant results were considered for $P < 0.05$. Statistical analyses were performed using STATA software version 12.1 (StatCorp., College Station, TX, USA).
To compare the prevalence's of the two different subpopulations, the χ^2 test was used.

5.5.4 Study Results

The recruitment in the second primary health unit (Lousã) ended in October 2013, and the grading ended in January 2014. The database verification for the two healthcare units is still on-going and therefore only preliminary results are available, i.e., results for the two healthcare units separately.

5.5.4.1 Study Results for Mira healthcare unit

Of the 4370 individuals contacted in Mira, 3000 subjects were included in the study from August 2009 to April 2011. The participation rate was 68.6%, considering: the subjects that underwent the interview and the ophthalmologic examination; non-responders subjects (subjects initially selected but for which it was impossible to establish contact due to repeated absence, n = 29; 0.7%); subjects that declined to participate (either due to express refusal, n = 258; 5.9%, or due to their inability to attend the healthcare unit because of health problems, n = 157; 3.6%); and subjects who agreed to participate but did not attend the eye examination (after being contacted twice) (n = 926; 21.1%) (Figure 25). Patients' characteristics are shown in Table 15.

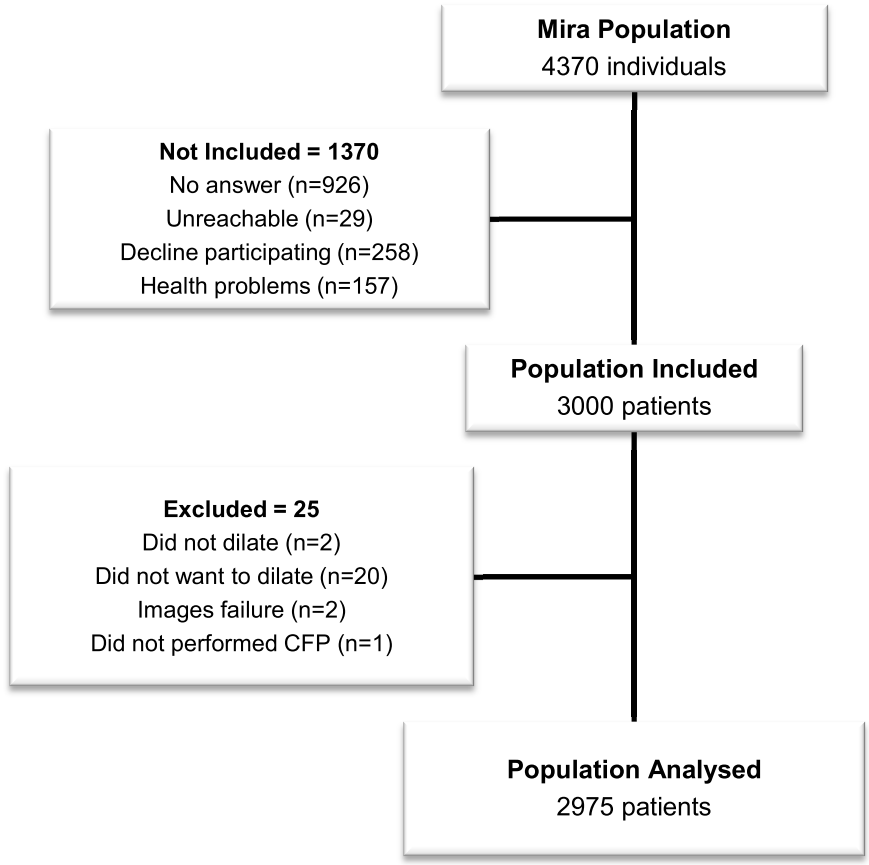

Figure 25. CONSORT flow chart for the patients included in Mira for the study NCT01298674.

Table 15. Patients' characteristics in Mira primary healthcare unit.

Gender	Male	1291 (43.4%)
	Female	1684 (56.6%)
Age	55-64	1051 (35.3%)
	65-74	1125 (37.8%)
	75-84	659 (22.2%)
	≥ 85	140 (4.7%)
Smoking	Smokers	63 (2.1%)
	Ex-Smokers	142 (4.8%)
Family History of AMD		339 (11.4%)
Diabetes		538 (18.1%)
Hypertension		1413 (47.5%)

The study sample was found to be representative of the original Mira's population (according to the Portuguese National Institute of Statistics, Instituto Nacional de Estatística – INE)[23], for age and gender, except for the older group. For the category 85 years-old and more, for both men and women, our study sample was less representative (P < 0.001).

The prevalence for the different stages of AMD is presented in Table 16.

Table 16. AMD Prevalence in Mira (% and 95%CI).

Stage 0		83.8 (82.5-85.1)
Stage 1	a	8.0 (7.1-9.0)
	b	0.5 (0.2-0.7)
Stage 2	a	6.1 (5.3-7.0)
	b	0.3 (0.1-0.4)
Stage 3		0.6 (0.3-0.8)
Stage 4		0.7 (0.4-1.0)

Most participants (83.8%) had no or minimal morphological changes on colour fundus images (no drusen or small drusen < 63 µm in diameter) in either eye (AMD grade 0), 15.5% had early AMD (grades 1 to 3) and 0.7% had late AMD (grade 4). Patients with early or late AMD were significantly older than those without AMD (P ≤ 0.001). No significant differences were found between the prevalence of early or late AMD among genders (P = 0.241) and among non-smokers/smokers (P = 0.466).

Controlling for age, gender and smoking, only age was significantly associated with the prevalence of early and late AMD (P ≤ 0.001 and P = 0.003, respectively). Per each year increase of age, there was an increase of 1.028 in the OR of having early AMD, 95%CI (1.026-1.039) and 1.086 in the OR of having late AMD, 95%CI (1.034-1.140). No significant association was found for gender and for current or previous smoking.

Regarding retinal lesions, drusen of any size were found to be common across all age groups and were present in 15.3% of the cases. Large soft drusen (≥ 125 µm) were found to increase with age, both in men and women, from 3.5% in women and 6.0% in men in those aged 55–64 years and from to 11.4% in women and 12.1% in men in those aged 75-84 years.

[23] Based on the information available at the INE website for the year 2009 (www.ine.pt, accessed on July 6th 2012).

Hyperpigmentation and hypopigmentation were found in 4.7% and 1.4% of the cases, respectively. Men had a significantly higher prevalence of pigmentary changes than women (1.9% and 0.9%, respectively, P = 0.017).
Retinal pigmentary abnormalities, distinct, indistinct soft drusen, or early AMD was significantly associated with age in both women and men (P < 0.001 and P < 0.015, respectively).
The total prevalence of neovascular AMD was, across all age groups, 0.4%. This prevalence increased with age from 0.0% to 1.2% in women (P = 0.003) and from 0.2% to 1.7% in men (P = 0.236). There was an overall higher prevalence of neovascular AMD in women than in men (0.5% compared with 0.3%), but this difference was not statistically significant (P = 0.357).
The total prevalence of geographic atrophy was, across all age groups, 0.3%. This prevalence increased with age in women (from 0.2% to 1.2%, P = 0.060) and in men (from 0.2% to 1.4% for subjects aged 75 to 84, P = 0.118 – for subjects aged 85 or more the prevalence was 0.0%). There was an overall higher prevalence of geographic atrophy in men than in women (0.5% compared with 0.1%), but this difference was not statistically significant (P = 0.071).

5.5.4.2 Study Results for Lousã healthcare unit

From the list of 8692 individuals registered in the primary healthcare unit of Lousã, 4999 were contacted and 3023 were included in the study from April 2012 to October 2013. One hundred and seventy two (1072) subjects declined to participate (either due to express refusal, n = 398; 8.0%, due to their inability to attend the healthcare unit, n = 325; 6.5%, or due to other reasons, n = 349; 7.0%); and 904 subjects agreed to participate but did not attend the eye examination (n = 386; 7.7%) or asked to be scheldued later (n = 518; 10.4%) (Figure 26). Patients' characteristics are shown in Table 17.

The study sample was found to be representative of the original Lousã's population (according to the Portuguese National Institute of Statistics, Instituto Nacional de Estatística – INE)[24], for age and gender, except for the older group. For the category 85 years-old and more, for both men and women, our study sample was less representative (P < 0.001).

The prevalence for the different stages of AMD is presented in Table 18.

[24] Based on the information available at the INE website for the year 2011 (www.ine.pt, accessed on May 10th 2014).

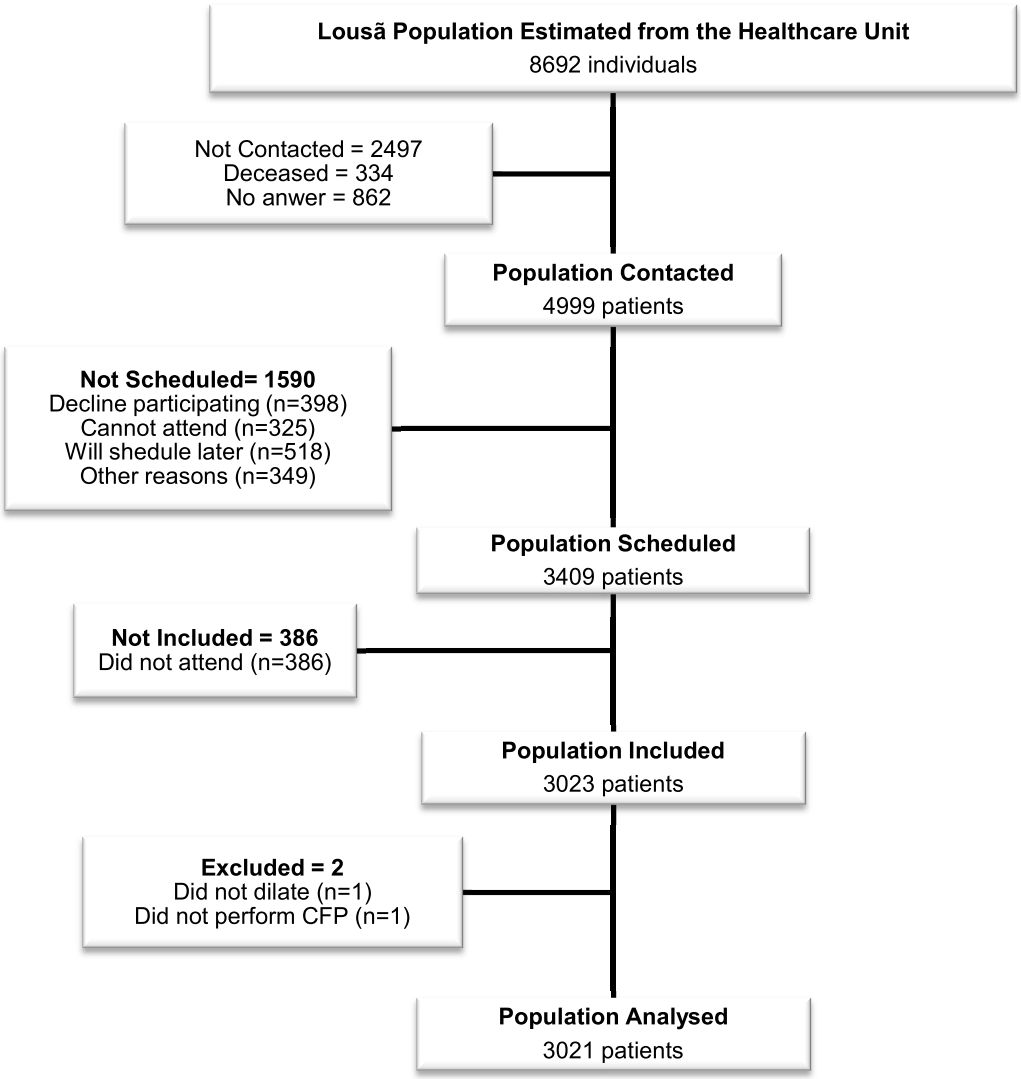

Figure 26. CONSORT flow chart for the patients included in Lousã for the study NCT01298674.

Table 17. Patients' characteristics in Lousã primary healthcare unit.

Gender	Male	1326 (43.9%)
	Female	1695 (56.1%)
Age	55-64	1136 (37.6%)
	65-74	1102 (36.5%)
	75-84	648 (21.4%)
	≥ 85	135 (4.5%)
Smoking	Smokers	202 (6.7%)
	Ex-Smokers	622 (20.6%)
Family History of AMD		193 (6.4%)
Diabetes		794 (26.3%)
Hypertension		1943 (64.3%)

Table 18. AMD Prevalence in Lousã (% and 95%CI).

Stage 0		79.0 (77.5-80.4)
Stage 1	a	3.6 (3.0-4.3)
	b	0.7 (0.4-1.0)
Stage 2	a	11.0 (9.9-12.1)
	b	1.8 (1.3-2.3)
Stage 3	a	2.6 (2.0-3.2)
Stage 4		1.3 (0.9-1.7)

Most participants (79.0%) had no or minimal morphological changes on colour fundus images (no drusen or small drusen < 63 µm in diameter) in either eye (AMD grade 0), 19.7% had early AMD (grades 1 to 3) and 1.3% had late AMD (grade 4).

Data from Lousã healthcare unit is still under analysis. The primary analyses indicate a prevalence of late AMD higher in Lousã than in Mira ($P<0.001$) and a higher risk for AMD in hypertensive patients ($P = 0.014$). No statistically significant associations were found for gender, smoking or diabetes ($P > 0.050$).

5.5.5 Study Discussion

Epidemiologic studies are important to know the prevalence of diseases such as AMD, in which vision loss is irreversible and in some stages of the disease sight threatening. In Portugal, no epidemiologic study was ever performed to assess the prevalence of AMD considering the different stages of the disease.

The preliminary results of this study showed a lower overall prevalence for the late stage AMD, when compared to other countries/studies.

This difference can be explained by several factors such as:

1. *The classification system used for the assessment of the AMD prevalence.*

   In this study the Rotterdam classification was used in order to compare the prevalence obtained in Portugal with other countries/studies. However, it is well known human grading systems have some degree of inter- and intra-variability and therefore some differences may be expected between AMD prevalence's. To reduce the inter- and intra-grader variability a new semi-automatic system for AMD grading, the RetmarkerAMD Research, was used.

2. *Risk factors for AMD.*

   The prevalence of AMD depends on several risk factors, such as: age, family history, and smoking habits; and some potential risk factors such as: lack of vitamins and trace elements, intense sunlight and UV exposure, hypertension and arteriosclerosis. Due to the two populations considered, i.e., one from a region from the centre of Portugal near the sea and the other near the mountains, it may be expected to found some lifestyle and food habits that may be different from other countries/studies, explaining therefore the lower prevalence observed for the late stages of AMD. As an example, smoking habits in the population of Mira were found to be significantly lower than in other countries (only 2.1% of the population of Mira and 6.7% of the population of Lousã smoke), which may explain the lack of association found between AMD and smoking habits (which have been reported in other studies). By comparing and analysing jointly the two primary healthcare units, we expect to be able to adjust the prevalence of AMD by some of these factors, since the two subpopulations may be different.

3. *Lower participation rate in elderly subjects.*

   The lower participation rate in the group of subjects aged 85 years or more, may contribute for the lower prevalence of late AMD in the elderly subjects.

4. *The anti-VEGF era.*

   The new era of anti-VEGF treatment for AMD may contribute for a lower participation rate since most of the patients with late AMD may be already followed in eye clinics and treated for their disease. In this study the patients' files from the subjects that declined participating were reviewed in order to identify already treated patients. No significant conclusions could be driven.

The knowledge of the true prevalence of the different stages of AMD in Portugal, and the characterization of the retinal lesions that are present in the initial stages of the disease, will open new perspectives for the management of the AMD patients.

5.5.6 Scientific Contribution

A manuscript with the results of the Mira healthcare unit is being written for publication. Submission of this manuscript is expected by mid-2014.

6 SUMMARY AND CONCLUSIONS

6.1 Summary

Biomarkers are important tools for disease detection and management playing therefore an important role in both clinical practice and clinical research. In clinical practice the use of biomarkers, that characterizes the stage of the disease and/or predicts the risk of disease progression, allows for the optimization of the patients' management. In clinical research biomarkers can be used as objective and valid outcomes for the stage and/or progression of the disease, and for the selection of the study population, by knowing the risk of the disease progression.
In clinical eye research, which represents only 3.8% of the clinical research performed worldwide, the identification and validation of imaging biomarkers, able to predict patients at risk of vision loss, is of major importance. In this thesis three IDCR in DR and AMD, two retinal diseases with a high socioeconomic impact in the working population, were presented.

The IDCR entitled "Validation of a predictive model to estimate the risk of conversion to clinically significant macular edema and/or vision loss in mild nonproliferative retinopathy in diabetes type 2" (NCT00763802), allows demonstrating the existence of 3 different phenotypes of DR progression (phenotypes A, B and C), characterized by two retinal biomarkers of DR progression/worsening: the presence of macular edema (phenotype B); and the MA turnover (phenotype C). Two retinal biomarkers quantifiable using non-invasive imaging techniques, OCT and CFP, respectively [109], and whose potential was demonstrated in two sub-studies [92, 93, 65].

In the first sub-study the MA turnover showed to be a good predictive factor for CSME development [66] being a good indicator of different vascular disease activity in different eyes. In the second sub-study it was showed that 17.6% of the eyes/patients with NPDR had a central retinal thickness over the normal reference values, indicating the presence of macular edema and therefore the breakdown of the BRB; while 2.9% of the eyes/patients had a central retinal thickness below the normal reference values, indicating the presence of a neuronal degeneration [92]. Moreover, it was also shown that eyes/patients with subclinical macular edema at baseline are associated with a 3.7-fold increased risk for CSME development within a 2-year period [93]. These 2 sub-studies confirm the two processes that occur during DR, i.e., the breakdown of the BRB and the neuronal degeneration, two processes that occurs in variable degrees in different patients in the initial stages of the disease [92].
The identification of different phenotypes of DR progression, characterized by different retinal changes and by different risks of progression to CSME, opens new perspectives for a more personalized clinical management of the diabetic patients with DR. If patients with higher risk of progression (phenotypes B or C) and with the greatest potential to benefit from a particular treatment can be identified earlier, in a stage of the disease in which there is still no significant changes in the patient' visual acuity, we may significantly reduce the number of cases of blindness due to DR. The results of this study may have also a significant impact on prospective interventional clinical research since it will contribute for a better selection of the patients, given that 50% of the patients with NPDR will have a slow progression phenotype.

The IDCR entitled "Characterization of early Markers of Choroidal Neovascularization (CVN-MARKERS)" (NCT00801541), showed, using multimodal imaging, that early and late ICG hyperfluorescent spots, early ICG hypofluorescent spots and leakage detected with RLA before conversion are predictive factors of CNV conversion in AMD patients [150]. Still, these results needs to be confirmed in a larger population and with a longer follow-up, due to the statistical power needed. The identification of morphological, functional and genetic biomarkers in the early stages of AMD is crucial for the early diagnosis of the disease. By detecting AMD in the early stages the number of patients with severe vision loss due to AMD may be reduced significantly. Also, the improved knowledge of these biomarkers may contribute for the development of new therapeutic approaches for both early and late stages of the disease.

The IDCR entitled "Epidemiological study of the Prevalence of Age-related Macular Degeneration in Portugal" (NCT01298674), is the first IDCR fully supported by the infrastructure

4C and the first epidemiological study performed in Portugal to assess the prevalence of AMD. Epidemiologic studies are important to know the prevalence of diseases such as AMD, in which vision loss is irreversible and in some stages of the disease sight threatening. This study was performed in two healthcare units from the Central region of Portugal Mira and Lousã, and used retinal biomarkers obtained from a non-invasive imaging method, CFP. AMD grading was performed using a semi-automatic system for AMD lesions earmarking, the RetmarkerAMD Research, which allows for a good inter-grader agreement for the detection of the AMD lesions, when graders are experienced and trained. The preliminary results showed a lower prevalence for the late stage AMD, when compared to other studies, i.e., 79.0 to 83.8% of the population had no or minimal morphological changes on colour fundus images (no drusens or small drusens < 63 µm in diameter) (AMD grade 0), 15.5 to 19.7% had early AMD (grades 1 to 3) and 0.7 to 1.3% had late AMD (grade 4). The classification system, the lower participation rate in subjects aged 85 years or more, and the era of anti-VEGF, may explain part of this difference. It is known that the prevalence of AMD depends on several risk factors, such as: age, family history and smoking habits. The involvement of factors such as: lack of vitamins and trace elements, intense sunlight and UV exposure, hypertension and arteriosclerosis, is still controversial. Due to the populations considered, i.e., one from a region from the centre of Portugal near the sea and the other near the mountains, it may be expected to found, in these populations, some lifestyle and food habits that may be different from other countries/studies, explaining therefore the lower prevalence observed. By comparing and analysing jointly the two primary healthcare units we expect to be able to adjust the prevalence of AMD by some of these factors, since these two subpopulations may be different.

The knowledge of the true prevalence of the different stages of AMD in Portugal, and the characterization of the retinal lesions that are present in the initial stages of the disease, will open new perspectives for the management of AMD patients.

The studies presented here demonstrate simultaneously the relevance of IDCR and the importance of imaging biomarkers for evidence-based clinical eye research and for patient-oriented clinical research.

While the two first studies were performed at AIBILI without any specific infrastructure, the third study could only be possible with the existence of an infrastructure focused on IDCR support, due to the burden of work related with it. This infrastructure, the 4C, was created in 2009 in AIBILI, to support IDCR in its different stages, i.e. from the design, submission and conduction of clinical studies in eye research, more precisely in ophthalmology, at the national and

international levels. The creation and development of this infrastructure was the result of the larger number of requests from the investigators for support and coordination of multicentre studies; the increased activity of the EVICR.net; the integration of AIBILI in the C-TRACER network; its integration in the VICT consortium; and its collaboration with ECRIN and PtCRIN.

This infrastructure was created in 2009, with the collaboration of the EVICR.net Coordinating Centre and the Quality Management department of AIBILI. SOPs were elaborated for the different activities ensuring that all the activities to be performed are ICH-GCP and ISO 9001 compliant. With the consolidation of this infrastructure and involvement of other personnel, 4C evolved to a full unit of AIBILI, being certified in 2011 for Planning, Coordination and Monitoring of Clinical Research Activities. From 2009 to 2013 this infrastructure supported the design and submission of 9 national clinical studies and 7 international clinical studies promoted by investigators (e.g., physicians), contributing for the increase of IDCR in clinical eye research, mainly in the area of ophthalmology.

This infrastructure is the first national academic CRO dedicated to clinical eye research, providing services at the national level (to the national network PtCRIN, and to the members of the VICT, i.e., IBILI, ICNAS and CRIO) and at the international level (to the EVICR.net Coordinating Centre, the ECRIN and the C-TRACERs).

With a greater support for IDCR in clinical eye research, in both academic and hospital environments, it is expected to increase the number of clinical studies in this area, strengthening therefore the level of clinical evidence of the current medical practice, and contributing for a more and better patient-oriented research.

6.2 Conclusions

Clinical eye research represents 3.8% of the clinical research performed worldwide. The identification and validation of imaging biomarkers, able to predict patients at risk of vision loss, is of major importance.

MA activity and central macular thickness, assessed using non-invasive methodologies are good biomarkers of DR progression.

Eyes/patients with an MA turnover ≥ 9 during a 6-month period are associated with a 5.9-fold increased risk for CSME development within a 2-year period [66].

Eyes/patients with subclinical macular edema are associated with a 3.7-fold increased risk for CSME development within a 2-year period [93].

- 17.6% of the eyes/patients with NPDR showed an increased retinal thickness, i.e., macular edema and therefore breakdown of the BRB while 2.9% of the eyes/patients with NPDR showed a decreased retinal thickness indicating the presence of a neuronal degeneration [92].

Three different phenotypes of DR progression can be identified in NPDR patients (phenotypes A, B and C), characterized by two non-invasive retinal biomarkers: the presence of macular edema (phenotype B); and the MA turnover (phenotype C) [109].

DR patients are characterized by three different phenotypes of DR progression, characterized by different retinal changes and by different risks of progression to CSME within a 2-year period. Patients from phenotype B are associated with a 2.8-fold increased risk for CSME development, while patients from phenotype C are associated with a 3.5-fold increased risk for CSME development.

The identification of different phenotypes of DR progression is expected to have a significant impact on prospective interventional clinical research since it will contribute for a better selection

of the patients, given that 50% of the patients with NPDR will have a slow progression phenotype.

Number and drusens' morphology; presence of pigmentary abnormalities; geographic atrophy; and neovascular AMD, are the main retinal biomarkers that characterize the severity of AMD.

AMD grading is based on colour fundus images, a non-invasive imaging technique, and can be improved by using semi-automated software that allows for the precise quantification and localization of the different AMD lesions.

AMD grading shows a good reproducibility. The inter-graders agreement is good for experienced and trained graders, being moderate for less experienced and/or trained graders.

The presence of late ICG hyperfluorescent spots, early ICG hypofluorescent spots and leakage detected with RLA, in AMD patients, are potential predictive factors for the conversion to CNV [150].

The preliminary results of the epidemiologic study showed a lower prevalence for the late stages AMD

- 79.0 to 83.8% of the population of the central region of Portugal had no or minimal morphological changes on colour fundus images (no drusens or small drusens < 63 μm in diameter) (AMD grade 0);
- 15.5 to 19.7% of the population of the central region of Portugal had early AMD (grades 1 to 3);
- 0.7 to 1.3% of the population of the central region of Portugal had late AMD (grade 4).

The knowledge of the true prevalence of the different stages of AMD in Portugal, and the characterization of the retinal lesions that are present in the initial stages of the disease, will open new perspectives for the management of AMD patients.

Multinational IDCR are needed to test and validate different imaging biomarkers and therefore structures, such as the 4C, are needed to support the investigators.

A greater support for IDCR in clinical eye research is expected to increase the number of clinical studies in eye research in Europe, strengthening the level of clinical evidence of the current medical practice, and contributing for a more and better patient-oriented research.

The creation and development of 4C was a response to the larger number of requests from the investigators for support and coordination of multicentre studies.

4C is the first national academic CRO dedicated to clinical eye research, providing services at the national level (to the national network PtCRIN, and to the members of the VICT, i.e., IBILI, ICNAS and CRIO) and at the international level (to the EVICR.net Coordinating Centre, the ECRIN and the C-TRACERs).

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