UNIVERSITÀ DEGLI STUDI DI CATANIA

International PhD in Translational Biomedicine

XXXI cycle

DNA METHYLATION LANDSCAPE IN RETINAL DEGENERATIVE DISEASES: IMPLICATIONS FOR PUBLIC HEALTH AT THE CROSSROAD BETWEEN GENES AND DIET

PhD Thesis

Maugeri Andrea Giuseppe

PhD Coordinator:

Prof. Lorenzo Malatino

Tutor:

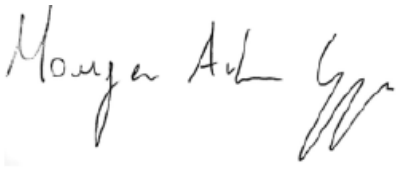
Prof. Antonella Agodi

a.a. 2017-2018

PhD Candidate

Andrea Giuseppe Maugeri

Signature



Tutor

Professor Antonella Agodi

Signature

Date

11th July 2018

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List of publications

This PhD thesis is based on the following original publications:

- Barchitta M, Maugeri A. Association between Vascular Endothelial Growth Factor Polymorphisms and Age-Related Macular Degeneration: An Updated Meta-Analysis. Dis Markers. 2016; 2016:8486406.
- Maugeri A, Kunzova S, Medina-Inojosa JR, Agodi A, Barchitta M, Homolka M, Kiacova N, Bauerova H, Sochor O, Lopez-Jimenez F, Vinciguerra M. Association between eating time interval and frequency with ideal cardiovascular health: Results from a random sample Czech urban population. Nutr Metab Cardiovasc Dis. 2018; pii: S0939-4753(18)30123-6.
- Maugeri A, Barchitta M, Agodi A. The association between complement factor H rs1061170 polymorphism and age-related macular degeneration: a comprehensive meta-analysis stratified by stage of disease and ethnicity. Acta Ophthalmol. 2018 Oct 2. doi: 10.1111/aos.13849.
- Maugeri A, Mazzone MG, Giuliano F, Vinciguerra M, Basile G, Barchitta M, Agodi A. Curcumin Modulates DNA Methyltransferase Functions in a Cellular Model of Diabetic Retinopathy. Oxidative Medicine and Cellular Longevity, vol. 2018, Article ID 5407482, 12 pages, 2018.doi.org/10.1155/2018/5407482.
- Maugeri A, Barchitta M, Mazzone MG, Giuliano F, Basile G, Agodi A. Resveratrol Modulates SIRT1 and DNMT Functions and Restores LINE-1 Methylation Levels in ARPE-19 Cells under Oxidative Stress and Inflammation. Int J Mol Sci. 2018 Jul 20;19(7). pii: E2118. doi: 10.3390/ijms19072118.
- Agodi A, Maugeri A, Kunzova S, Sochor O, Bauerova H, Kiacova N, Barchitta M, Vinciguerra M. Association of Dietary Patterns with Metabolic Syndrome: Results from the Kardiovize Brno 2030 Study. Nutrients. 2018 Jul 13;10(7). pii: E898. doi: 10.3390/nu10070898.
- Barchitta M, Maugeri A, Quattrocchi A, Barone G, Mazzoleni P, Catalfo A, De Guidi G, Iemmolo M, Crimi N, Agodi A. Mediterranean Diet and Particulate Matter Exposure Are Associated With LINE-1 Methylation: Results From a Cross-Sectional Study in Women. Front Genet. 2018 Oct 30;9:514. doi: 10.3389/fgene.2018.00514.

- Maugeri A, Barchitta M, Mazzone MG, Giuliano F, Agodi A. Complement System and Age-Related Macular Degeneration: Implications of Gene-Environment Interaction for Preventive and Personalized Medicine. BioMed Res Int. 2018. https://doi.org/10.1155/2018/7532507.

Acknowledgments

The research was carried out at the Department of Medical and Surgical Sciences and Advanced Technologies "GF Ingrassia" of the University of Catania (Catania, Italy) in collaboration with the Department of Ophthalmology of the University of Catania (Catania, Italy), the Research and Development Department of the SIFI SpA (Catania, Italy) and the International Clinical Research Center of the St Anne's University Hospital (Brno, Czech Republic).

The research activities were partially supported by the Department of Medical and Surgical Sciences and Advanced Technologies "GF Ingrassia", University of Catania (Piano triennale di sviluppo delle attività di ricerca scientifica del Dipartimento - 2016-18) and by the National Program of Sustainability II (MEYS CR) (no. LQ1605). I'm also grateful to SIFI SpA for technical and financial support.

Abbreviations

5mC	5-Methyl-cytosines
AA	Arachidonic acid
AGE	Advanced glycation end-products
AHEI	Alternate Healthy Eating Index
ALE	Advanced lipoxidation end products
ALR2	Aldose reductase gene
AMD	Age-related macular degeneration
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APOE	Apolipoprotein E
AREDS	Age-Related Eye Disease Study
ARIC	Atherosclerosis Risk in Communities
ARMS2	Age-related maculopathy susceptibility 2
ARPE-19	Human retinal pigment epithelial cells
BFM	Body fat mass
BMI	Body mass index
C2	Complement component 2
C3	Complement component 3
CAREDS	Carotenoids in Age-Related Eye Disease Study
CFB	Complement factor B
CFH	Complement factor H
CFI	Complement Factor I
CI	Confidence interval
CNV	Choroidal neovascularization
CR1	Complement receptor 1
DCCT	Diabetes Control and Complications Trial
DCF	2',7'- dichlorofluorescein
DCFDA	2',7' - dichlorofluorescin diacetate
DHA	Docosahexaenoic acid
DM	Diabetes mellitus
DME	Diabetic macular edema
DMEM	Dulbecco's Modified Eagle's medium
DNMT	DNA methyltransferase
DR	Diabetic retinopathy
EAR	Estimated Average Requirements
eNOS	Endothelial nitric oxide synthase
EPA	Eicosapentaenoic acid
FFQ	Food Frequency Questionnaire
GA	Geographic atrophy
GEO	Gene Expression Omnibus
GOx	Glucose oxidase
GST	Glutathione S transferase

GWAS	Genome Wide Association Study
HIF-2α	Hypoxia-inducible factor 2α
HTRA1	High temperature requirement factor A1
HWE	Hardy-Weinberg Equilibrium
IGF-1	Insulin-like growth factor 1
IL17RC	Interleukin-17 receptor
IQR	Interquartile range
LINE	Long Interspersed Nuclear Element
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MCP	Membrane cofactor protein
MDS	Mediterranean Diet Score
MET	Metabolic Equivalent of Task
MTT	Thiazolyl blue tetrazolium bromide
NF-kβ	Nuclear factor-kβ
NPDR	Non-Proliferative Diabetic retinopathy
OR	Odds ratio
ORF	Open reading frame
PCA	Principal component analysis
PDR	Proliferative Diabetic retinopathy
PLEKHA1	Pleckstrin Homology Domain-containing Protein family A member 1
PUFA	Polyinsatured fatty acid
RAGE	Receptor for advance glycation end-products
Rco	Correlation coefficient
RCT	Randomized controlled trial
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SD	Standard deviation
SE	Standard error
SINE	Short Interspersed Nuclear Element
SIRT1	Sirtuin 1
STZ	Streptozotocin
TET	Ten-eleven-translocation
TGF- β	Transforming growth factor β
UKPDS	UK Prospective Diabetes Study
VEGF	Vascular endothelial growth factor
WHR	Waist to hip ratio

1 Introduction

1.1 Gene-diet interaction

"Let food be thy medicine and medicine be thy food" - this quote by Hippocrates, the father of western medicine, has inspired humans to understand how environment and foods affect individual's health since ancient times. However, in the last decades alone, researchers and health professionals have revealed that dietary habits play a crucial role in maintaining health and in disease prevention [1]. The research, especially after the conclusion of the Human Genome Project, raised questions whether gene-diet interaction may positively or negatively influence human health. With this question in mind, researchers coined the term "Nutrigenomics", referring to the study of dietary influence on the Genome, Transcriptome, Proteome, and Metabolome [2]. Although several lines of evidence began to show the protective effect of dietary intervention on chronic degenerative diseases by modulating molecular functions [1], further research is needed to develop novel strategies for maintaining health and preventing diseases [3]. A specific area of research that elucidates mechanisms involved in gene-diet interaction is the Nutriepigenomics, the study of the impact of diet on changes in gene expression by modulating epigenetic mechanisms [3]. In 1939, Conrad H. Waddington was the first to define Epigenomics as the causal interactions between genes and their products which bring the phenotype into being [4]. This definition was later revised as the study of heritable changes in gene expression that occur without changes in DNA sequence [5]. Thus, the field of Nutriepigenomics is further revealing the nature of diet-gene interaction, providing support for the role of nutrition in preventing diseases[3].

1.1.1 DNA methylation

Epigenetic mechanisms - including DNA methylation, histone modifications, histone variants, chromatin remodelers and non-coding RNAs - regulate how and when genes are expressed without altering DNA sequence. These molecular processes characterize the epigenome which is dynamic in response to environmental signals, modifiable during normal cell differentiation and heritable in daughter cells [6]. Among these mechanisms, DNA methylation is one of the most extensively studied and best characterized. In mammals, DNA methylation is regulated by the activity of three DNA methyltransferases (DNMTs): while DNMT1 has a maintenance role, DNMT3a and 3b are *de novo* methylases. By contrast, the removal of methyl groups on DNA is mediated by the ten-eleven-translocation (TET) proteins [7]. DNA methylation almost exclusively occurs within *CpG islands* – short sequences in gene promoters and regulatory regions that typically contain about 5-10 CpG dinucleotides per 100 bp [8]. In humans, approximately 60% promoters include *CpG islands* and ~90% of them is unmethylated [9]. However, up to 80% CpG dinucleotides occur in repetitive sequences

scattered throughout the genome [10]. Some of these sequences act as retrotransposons, disrupting gene expression and eventually leading to genomic instability. To protect from this potential deleterious effect, DNMT1 works to maintain these sequences highly methylated [11,12]. DNMTs functions are associated with several key physiological processes, including genomic imprinting, X-chromosome inactivation, regulation of gene expression, maintenance of chromosome integrity through chromatin modulation, DNA stabilization and DNA-protein interactions [13]. Aberrant DNMT expression and activity are involved in several diseases including cardiovascular diseases, obesity, type-2 diabetes and cancer [14-16].

1.1.2 Long Interspersed Nuclear Elements 1

Human genome includes almost 3 billion non-coding base pairs, of which 50% is recognized as repetitive sequences. Most of these sequences - Long Interspersed Nuclear Elements (LINEs) and Short Interspersed Nuclear Elements (SINEs) - occur discontinuously as singular scattered copies in the genome [17]. LINE-1 elements - retrotransposons capable of independent and autonomous retrotransposition via RNA intermediate - comprise approximately 17% of the genome with more than 500,000 copies. Retrotransposition of LINE-1 may lead to chromosomal instability, DNA rearrangement and alteration of ectopic gene expression in cancerous tissues [18]. A full length LINE-1 sequence is approximately 6 kb long including bidirectional, non-canonical promoter and tow open reading frames (ORF1 and ORF2) [19]. After RNA polymerase II transcription, mRNA processing and its export to the cytoplasm, ORF1 and ORF2 encode two proteins (ORF1p and ORF2p) that preferentially associate with their encoding RNA [20]. The encoded ribonucleoprotein particles transfer the RNA to the nucleus, where ORF2p triggers target-primed reverse transcription to insert a copied DNA sequence at a new site in the host cell genome [21]. Although LINE-1 transcription is largely regulated by DNA methylation of about 50 promoters, most of LINE-1 elements are truncated and cannot be transcribed [20]. It follows that only one hundred LINE-1 elements are functionally capable of retrotrasposition and only a few contribute to the vast majority of retrotransposition events [22]. LINE-1, along with Alu sequences, are the most common transposable sequences and measurement of their methylation levels has been used as a surrogate marker of global genomic DNA methylation [23]. Although LINE-1 methylation is not an universally accepted marker of global methylation, aberrant methylation of these sequences was associated with cancer, cardiovascular and neurodegenerative diseases [24-26].

1.1.3 The effects of nutrients on DNA methylation

Environmental and lifestyle factors can potentially modify DNA methylation, leading to genome reprogramming in exposed individuals and in future generations [27]. Several classes of nutrients -

folate, polyphenols, selenium, retinoids, fatty acids, isothiocyanates and allyl compounds – modulate DNA methylation process via different mechanisms [28]. Folate, a fundamental methyl donor for cellular replication and maintenance, modulates DNA methylation, synthesis and repair. However, since folate status has been both positively and negatively associated with DNA methylation [29-35], its effect remains to be completely elucidated. While most of the studies found that global DNA methylation levels increased with increasing folate intake [29,31-35], others observed an inverse relationship [30]. Controversial results might be attributed to unmeasured factors such as ethnicity, genetic variants in one-carbon metabolism, lifestyles, physiological and pathological conditions, which in turn can affect DNA methylation process.

Other dietary compounds exhibit the potentiality to modulate DNA methylation. For instance, fatty acids have been suggested to affect DNA methylation process via mechanisms independent of 1-carbon nutrients. In fact, it has been demonstrated that global DNA methylation decreased with increasing intake of saturated fatty acids. Since few studies investigated the effect of n-3 and n-6 polyinsatured fatty acids (PUFAs) on DNA methylation - especially concerning eicosapentaenoic acid (EPA) [36], docosahexaenoic acid (DHA) [37] and arachidonic acid (AA) [38] - further research is recommended to elucidate the association between dietary fat intake and DNA methylation [35]. Furthermore, several lines of evidence suggest that dietary compounds present in fruit, vegetables and spices may modulate epigenetic signatures in human cells. For instance, it has been reported that dietary phytochemicals may repair DNA damage by enhancing histone acetylation and altering DNA methylation [39].

1.1.4 The effects of dietary patterns on DNA methylation

Previous studies focused on the consumption of specific foods or nutrients, but there is currently growing interest in determining how dietary patterns may affect global and local DNA methylation. A previous study - evaluating the association between diet and leukocyte DNA methylation in a cancer-free population - identified two dietary patterns: the prudent dietary pattern, characterized by a high intake of vegetables and fruits; the western dietary pattern, characterized by a high intake of energy-dense foods such as grains, meats, potato, oil, and dairy [35]. While only the intake of dark green vegetables seemed to be significantly associated with DNA methylation among specific food groups, the analysis of dietary patterns revealed a positive association between the prudent dietary pattern and global DNA methylation in a dose-response manner [35]. In line with this evidence, further studies observed that healthy women with high intake of vegetables and/or fruits had a lower risk of LINE-1 hypomethylation [32,35]. The biological explanation of this relationship could be attributed to the wide variety of nutrients and bioactive compounds provided by fruits and vegetables, including phytochemicals (phenolics, flavonoids, and carotenoids), vitamins (vitamin C, folate, and pro-vitamin

A), minerals (potassium, calcium, and magnesium), and fibres, which in turn modulate multiple pathways associated with epigenetic mechanisms [40,41].

1.2 Epigenetic hallmarks in aging and age-related diseases

If Genetics alone cannot explain the distinct patterns of aging nor different susceptibility to age-related diseases, for instance, between monozygotic twins [42], Epigenetics offers an explanation of these phenomena [43]. Senescence and aging are characterized by progressive loss of histones, transcriptional changes, losses and gains in heterochromatin, global hypomethylation and local hypermethylation, and chromatin remodelling (**Figure 1**). These changes are heavily influenced by environmental stimuli and nutrient availability, which in turn alter intracellular metabolite concentrations [44]. During aging, the overarching profile of DNA methylation changes, showing global DNA hypomethylation and local hypermethylation that may activate specific transcriptional pathways. Although this is consistent with the abovementioned changes that occur in aging cells, it remains unclear what specific genes are directly affected by aberrant DNA methylation. While hypermethylation mainly occurs at promoter CpGs, loss of methylation occurs in repetitive regions of the genome that correlate with constitutive heterochromatin. The analysis of DNA methylation status of 26,486 autosomal CpGs in a number of human tissues revealed hypermethylation of promoter CpGs and hypomethylation of those outside during aging [45]. The observed paradox - hypermethylation of promoters versus hypomethylation of repetitive regions - is likely due to differential expression and activity of DNMTs over repeat elements and key genes affecting lifespan [46]. The identification of DNA methylation markers associated with aging represents a milestone in this area of research: in a large study, Horvath and colleagues demonstrated that DNA methylation at 353 CpGs - termed clock CpGs - accurately predict age in more than 8000 samples, including healthy human tissue and cell types including liver, kidney, immune, and brain cells and cancer samples [47,48]. Consistently, an independent study on a population aged 19-101 allowed to build a predictive model of aging using a smaller set of 71 CpGs [49]. However, further advances require to understand the underlying mechanism of these epigenetic clocks in age-related diseases.

1.3 Age-related Macular Degeneration

1.3.1 Clinical features and epidemiology

Age-related macular degeneration (AMD) is a neurodegenerative disease which leads to the progressive destruction of the neurosensory macular area, involving retinal pigment epithelium (RPE), Bruch's membrane and choroid [50]. The reasons why AMD preferentially affects the central region of the retina are not well clarified. According to the severity of symptoms, the disease is classified into early, intermediate and advanced stages: while the early stage is characterized by the

aberrant pigmentation of the RPE and the accumulation of "drusen" - yellowish lipid-rich, protein-containing extracellular deposits accumulating between RPE and Bruch's membrane -, the advanced stage may manifest as non-exudative (dry) or exudative (wet) AMD. The first is characterized by the geographic atrophy (GA) of RPE and thinning of the retina, which lead to the gradual loss of photoreceptors and central vision [51]. The second is characterized by the growth of abnormal blood vessels from the choroid into the normally avascular sub-RPE and sub-retinal regions (choroidal neovascularization, CNV), which negatively affects central vision [52,53]. Although exudative AMD patients represent a small proportion of total AMD cases, they account for the majority of blindness associated with AMD [54]. Approximately 170 million individuals are affected by AMD worldwide, with a prevalence that ranges from 2% to 20% among elderly people. AMD is thereby the leading cause of blindness in the developed countries and the third leading cause globally [55]. Prevalence of early AMD is positively associated with age across all ethnicities, though this is most marked in Caucasians and Hispanics and to a lesser extent in Asians and Africans. Across all age strata, Africans had the lowest prevalence of early AMD, followed by Asians. For individuals aged under 55 years, a reasonable prevalence of early AMD among Caucasians, Hispanics, Asians, and Africans is 4%, 6%, 3%, and 3%, respectively; the occurrence increases to 24%, 22%, 13%, and 11% for persons older than 75 years. With regard to advanced AMD, it has been demonstrated an exponential age-related increase in Caucasians and Asians, a moderate increase in Hispanics and a slight increase in Africans. Although prevalence of advanced AMD in individuals younger than 55 years ranges from 0% to 0.2% across all ethnicities, its occurrence increases to 6.5%, 2.4%, 1.3%, and 0.6% among persons older than 75 years in Caucasians, Asians, Hispanics, and Africans, respectively [56]. As the aging population increases, the global prevalence of AMD is anticipated to spread to 288 million by the year 2040 [57], with direct healthcare cost due to AMD that is likewise expected to increase proportionately. In the last decades, several studies - especially on Caucasians - reported consistent AMD incidence rates across different populations [56]. The overall 10-year risk ranged from 11.1% to 23.7%, reflecting partial differences in study design, follow-up time, and variation in risk factors. There was no difference in incidence rate between men and women. AMD diagnosis and staging require the exam of fundus imaging of the retina for the visualization of drusen, pigmentary changes in the RPE, neural retinal degeneration, and/or proliferative events. Diagnosis of CNV is confirmed by fluorescein angiography, which marks blood vessels between choroid and RPE [56]. Clinicians classifies AMD progression using the Age-Related Eye Disease Study (AREDS) system [51], a standard grading scale from 1 to 4 (**Figure 2**):

- Non-AMD eyes (AREDS1);

- Eyes include early AMD cases in which symptoms were limited to small drusen <63 μm (also referred to as "hard" drusen), a single intermediate-sized druse 63–124 μm, and/or pigmentary changes;
- Eyes include those with more extensive drusen such that they have at least one large druse >124 μm ("soft" druse), multiple intermediate drusen, and/or GA not involving the central macula (AREDS3);
- Eyes exhibiting GA involving the central macula and/or CNV (AREDS4).

1.3.2 Major risk factors

The main causes of AMD are characterized by the interaction of oxidative stress, aberrant RPE activity and function, apoptosis and impaired immune system activation [58,59]. Smoking - leading to oxidative stress, ischemia, hypoxia and neovascularization - is the strongest modifiable risk factor [60]. Although both current and former smoking may increase AMD risk, a protective effect has been observed for time since smoking cessation [61]. In fact, subjects who had quitted smoking for more than 20 years were not at risk of advanced AMD [62,63]. Several lines of evidence suggested that - together with smoking - many other cardio-metabolic risk factors are also potential for AMD [64], including elevated total serum cholesterol [62] higher body mass index (BMI) [65] and hypertension [66,67]. The Beaver Dam Eye Study reported that higher pulse pressure and systolic blood pressure were associated with an increased risk of early AMD lesions and exudative AMD [67]. In addition, a recent meta-analysis concluded that excessive body weight was weakly associated with increased AMD risk - especially with advanced AMD - in a dose-dependent manner, indicating that maintaining normal body weight and avoiding weight gain may confer potential protection against this disease [68]. However, it is still unclear if the observed associations may be related to an overall unhealthy lifestyle instead of a direct effect on AMD susceptibility.

To date, the only well-established protective factor against AMD is a healthy diet, characterized by high intake of omega-3, lutein, zeaxanthin, and antioxidants [69-72]. Consistently, the AREDS2 formulation - a combination of zinc, b-carotene, vitamins C, and E supplements - has been shown to reduce the risk of progression to advanced AMD [73]. While AREDS formulation represents the only available treatment against non-exudative AMD, intravitreal injections of anti-vascular endothelial growth factor (VEGF) agents (i.e. ranibizumab, bevacizumab and aflibercept) may improve visual acuity in patients with exudative AMD [74-79].

1.3.3 Genetic Risk Factors

In addition to modifiable factors, genetic variants confer about 60% of the attributable risk [80], with at least 34 genomic loci implicated in AMD pathogenesis [81]. The main breakthrough has been achieved

after the discovery of genetic variants associated with AMD in the complement factor H (CFH) gene and in the 10q26 locus, which contains the age-related maculopathy susceptibility 2 (ARMS2) and high temperature requirement factor A1 (HTRA1) genes [82]. For the first time, these findings pointed out the crucial role of inflammation and oxidative stress pathways in the aetiology of AMD. Later, other genetic variants in complement genes, VEGF and apolipoprotein E (APOE) [83-86] were associated with AMD, although they confer a smaller effect.

1.3.3.1 Complement system and Complement Factor H

The refined equilibrium between activation and inhibition of complement system is one of the most critical regulatory mechanisms to prevent self-tissue damage [87,88]. In fact, on one hand, increased complement activity may be protective against chronic low-grade inflammation and infection in early life [89], on the other hand, lack of its inhibition is associated with several diseases, such as systemic lupus erythematosus [90], atypical haemolytic uremic syndrome [91], dense deposit disease [92], and AMD [93]. Therefore, complement system activity is strictly controlled by regulatory proteins, which mainly act by degrading complement components, increasing complement component 3 (C3) convertase decay and modulating the membrane attack complex (MAC) assembly [94-96]. The first is a function of Factor I (FI), which modulates both the classical and alternative pathways by cleaving C3 into inactive fragments [97]. To prevent non-specific degradation of complement components, the proteolytic activity of FI however requires several cofactors - such as complement receptor 1 (CR1), membrane cofactor protein (MCP), and CFH [97-101] - which accelerate C3 convertase decay [102,103].

The capacity of alternative pathway to distinguish between self and non-self is conferred by the recognition of glycosaminoglycans and sialic acid glycans (i.e. heparin-sulfate and N-Acetylneuraminic acid) on host cells [104-107]. Binding of CFH to the surface of necrotic cells and to apoptotic particles is mediated by CRP, Annexin II, DNA and histones [108-111]. An additional complement inhibitor is the decay-accelerating factor, which inhibits assembly of neo-formed C3 convertases and accelerates the decay of pre-existing convertases [96,112-116]. Lastly, the regulation of complement system may be also provided by inhibiting MAC formation via membrane bound (CD59) or fluid-phase (Vitronectin and Clusterin) inhibitors [117-122].

Several lines of evidence demonstrated that dysregulation of complement pathways - especially the alternative pathway - is involved in AMD pathogenesis. The major stressors for AMD development, such as aging, smoking, and oxidative stress, have been linked to the over-activation of the complement system (**Figure 3**). This has been also supported by immune-histological and proteomic studies identifying complement components as constituents of drusen [80,123-126]. Increased levels of activated complement components, which are released during complement activation, have been also observed in peripheral blood of AMD patients [127-129]. Consistently, complement regulators, such as

Vitronectin, Clusterin and MCP, are highly expressed in drusen and RPE cells adjacent to drusen [80,130,131]. Drusen are especially characterized by Amyloid beta accumulation, which in turn results in complement activation and chronic low-grade inflammation [132]. During RPE aging, it has also been observed the accumulation of lipofuscin and bis-retinoid component, which leads to the formation of lipid peroxidation products [133], apoptosis and complement activation [134,135].

In 2005, four independent genetic association studies revealed the CFH gene on chromosome 1q32 as the first one associated with AMD risk [125,136-138], with an effect that was mainly attributed to the rs1061170 polymorphism. This polymorphism leads to an amino acid change at position 402 of the CFH polypeptide (Y402H). Prevalence of the 402H risk variant varies across ethnicities [139], with an increased AMD risk of 2.5 times among heterozygous individuals, and 6.0 times among homozygotes [140]. This finding was confirmed by pooled analyses in both Caucasians [139] and Asians [141-143]. The rs1061170 polymorphism has been also recognized as a predictor of response to the anti-VEGF treatment: homozygotes patients were less likely to achieve a better outcome than those carrying wild type genotype, suggesting the need of more effective therapeutic strategies for this subgroup of patients [144]. By contrast, the rs800292 polymorphism - a coding variant in the SCR1 domain - has been found to be protective against AMD in both Caucasians and Asians [143]. This polymorphism, which leads to an amino acid change at position 62 of the CFH polypeptide (V62I), also conferred a better response to treatment of exudative AMD [144]. To date, the role of other CFH genetic variants is still under debate. A recent meta-analysis [145] aimed at resolving inconsistent findings about the effect of four coding and noncoding variants (rs1410996, rs1329428, rs2274700, rs3753394). Pooled results demonstrated that these polymorphisms are significantly associated with increased AMD risk, but none of them was related to response to treatment [146].

1.3.3.2 Genetic variants in other complement components

The C3 gene is located on chromosome 19p13.3-13.2 and consists of 41 exons encoding for 1663 amino acids and 13 functional domains. The encoded protein is biologically inactive until it undergoes to conformational changes that expose binding sites to pathogenic cell surface and other complement components [147]. Although emerging evidence proposed the association between C3 polymorphisms and AMD, findings are currently controversial [148-152]. The most commonly investigated is the rs2230199 polymorphism, which leads to the R102G substitution modulating both C3 binding capacity, cofactor activity and thereby extending convertase lifetime [153]. The effect of this polymorphism on AMD risk was confirmed in Caucasians but not in Asians [154]. More recently, a meta-analysis added to the current knowledge, suggesting, on one hand, the adverse effect of rs1047286 and rs11569536, and on the other hand the protective effect of the rs2250656 [155]. Instead, lack of evidence exists about the effect of C3 genetic variants on response to AMD treatment [156-159].

Particularly, the comparison of AMD Treatments Trials failed in demonstrating the effect of rs2230199 polymorphism on visual and anatomical outcomes, in patients treated with anti-VEGF drugs [160]. However, the analysis of changes in central macular thickness after ranibizumab treatment, showed that the minor allele of rs2250656 polymorphism was associated with improvement in retinal thickness and architecture [161].

The complement factor B (*CFB)* gene is located on chromosome 6p21 which includes the major histocompatibility complex class III region. Mounting evidence suggests that genetic variants in this region are associated with reduced AMD risk. In fact, previous meta-analyses confirmed the protective effect on AMD risk of the common rs641153 polymorphism - also known as R32Q - in different ethnic groups [86,162]. Located 500 bp upstream from *CFB* gene, there is also the complement component 2 (*C2)* gene, encoding for a serum glycoprotein that functions as part of the classical pathway. Two polymorphisms (i.e. rs9332739 and rs547154) have been directly associated with decreased AMD risk [86]. However, these variants may be indirectly linked to AMD risk due to linkage disequilibrium with *CFB*. Indeed, some common haplotypes, spanning *CFB* and *C2* genes, are considered highly protective against AMD [163]. Here again, lack of evidence exists about the effect of CFB and C2 genetic variants on response to intravitreal anti-VEGF injections, with no significant effects on patients with exudative AMD [146,164].

Located on chromosome 4q25, the CFI gene encodes for a precursor protein in hepatocytes, macrophages, lymphocytes, endothelial cells and fibroblasts. To obtain the active protein, the precursor is cleaved into heavy and light chains, which form a heterodimeric glycoprotein. This heterodimer can prevent the assembly of convertase enzymes by cleaving C4b and C3b. The association between *CFI* polymorphisms and AMD was firstly reported by Fagerness et al. [165], and further studies identified genetic variants that modulate gene expression and protein production [166-169]. Although the rs10033900 polymorphism is the most investigated, evidence of an association with AMD risk is still controversial. To date, an updated meta-analysis summarizes that carriers of rs10033900 polymorphism have a reduced risk of developing AMD in Caucasians, but not in Asians[170].

1.3.3.3 Age-related maculopathy susceptibility 2 locus

The susceptibility conferred by genetic variants at chromosome 10q26 was initially proposed by linkage studies [171-173]. Although it has been demonstrated a strong association between several genetic variants in the 10q26 locus and AMD, high linkage disequilibrium between three genes (Pleckstrin Homology Domain-containing Protein family A member 1 - PLEKHA1 -, ARMS2 and HTRA1 genes) made difficult to understand the source of genetic effect at this region. Mounting evidence supports the involvement of both ARMS2 and HTRA1 in the AMD pathogenesis, whereas PLEKHA1

seems to be weakly associated with AMD [174-178]. In the ARMS2 gene, the rs10490924 polymorphism – which leads to an A69S change - was associated with a ≈15-fold increased risk of AMD [174-178]. In addition, a deletion-insertion polymorphism (del443ins54; in/del) in the 3-UTR of ARMS2 was associated with AMD both in Caucasian and Asians [179-181]. Although molecular mechanisms underpinning the association between ARMS2 and AMD remain to be elucidated, evidence of disorganized mitochondrial membranes and decreased number of mitochondria in RPE of AMD patients pointed out the mitochondrial dysfunction in AMD pathogenesis [182,183]. In fact, ARMS2 may affect mitochondrial function, leading to the production of reactive oxygen species, which in turn cause apoptosis and increased AMD risk [182,183].

In the HTRA1 gene, the rs11200638 polymorphism is associated with an increased risk of AMD, as confirmed by a recent meta-analysis [184]. Interestingly, subgroup analyses revealed that the polymorphism is significantly associated with CNV but not with GA, and that the effect is stronger in Caucasians than in Asians [184,185]. Several lines of evidence demonstrated that the rs11200638 risk allele is associated with higher levels of HTRA1 mRNA and protein levels. Particularly, HTRA1 may inhibit signalling of transforming growth factor β (TGF- β) proteins, which have been reported to act as negative growth regulators in the retina and RPE [186,187]. In addition, HTRA1 promotes the degradation of extracellular matrix - through enhanced expression of matrix metalloproteases - and may affect the integrity of Bruch's membrane and RPE layer. Common haplotypes encompassing both the ARMS2 and the HTRA1 genes have also been associated with AMD risk. Among these, a common haplotype TAT tagged by rs10490924, rs11200638, and rs2293870 significantly predisposed to AMD, while the haplotype GGG significantly reduced the risk of AMD [188]. Similarly, the haplotype T-G-Wt-G tagged by rs2736911, rs10490924, in/del/Wt, and rs11200638, appeared to be protective against AMD both in Caucasian and Asians [189], while the in/del and the rs11200638 risk allele by itself were insufficient to modify HTRA1 expression levels [189].

1.3.4 Nutrition and age-related macular degeneration

A research area of increasing interest concerns the potential role of diet, antioxidant and/or mineral supplementation in preventing and delaying AMD progression. In fact, in the last decades, it has been consistently demonstrated that an adequate intake of omega-3 fatty acids, lutein, zeaxanthin, and other antioxidants, represents the only well-known protective factor against AMD onset and progression [69-72]. Among these, antioxidants have been proposed to protect the macula against the oxidative stress that leads to photoreceptor damage. A meta-analysis, including 65,250 participants from four randomized controlled trials (RCTs), evaluated the effect of antioxidants (i.e. lutein, zeaxanthin, and vitamins C and E) and/or minerals (i.e. zinc and selenium) supplementation, alone or in combination, versus placebo control subjects. Findings from this work demonstrated a no significant effect of

antioxidant supplementation on AMD onset [190]. However, when examining evidence related to the role of nutrition in AMD, it is worth highlighting the necessity to differentiate between disease onset and progression. In line with this need, AREDS I [191] and AREDS II [73] - two large multicentre RCTs sponsored by the National Eye Institute - have been designed to evaluate safety and efficacy of vitamins and other nutrients supplementation for altering the natural history in patients with established disease.

The AREDS I was conducted from 1992 to 2006 to evaluate whether systemic antioxidant supplementation might ameliorate clinical aspects, natural history, and risk factors associated with AMD. AREDS I demonstrated that, in patients with at least intermediate AMD, daily long-term high-dose supplementation of 500 mg vitamin C, 400 IU vitamin E, 15 mg beta-carotene, 80 mg zinc oxide, and 2 mg cupric oxide) reduced the risk of progression to advanced AMD at 5 years from 28 to 20% [191]. Next, observational studies pointed out the potential beneficial role of higher dietary intakes of the retinal carotenoids (i.e. zeaxanthin and lutein) and omega-3 long-chain polyunsaturated fatty acids (i.e. DHA and EPA) [71,192]. Lutein and zeaxanthin are xanthophyll carotenoids that have been recognized to improve antioxidant protection, filtration of short-wavelength light, maintenance of structural integrity of cell membranes, and modulation of signal transduction pathways within the retina. DHA is one of the structural components of lipid membranes in retinal photoreceptors [193], and its status has been found to influence the phototransduction cascade [194]. DHA and EPA show also crucial retino-protective effect by modulating gene expression [195], cellular differentiation [196], and cell survival [196]. Thus, the AREDS II rationale was based partly on these findings suggesting that retinal carotenoids and omega-3 fatty acids influence the biological processes that have been implicated in AMD pathogenesis. Accordingly, AREDS II aimed at investigating whether the addition of 10 mg lutein and 2 mg zeaxanthin, alone or in combination with 350 mg DHA and 650 mg EPA, further reduced the risk of progression to late AMD in subjects with at least intermediate disease [197]. AREDS II demonstrated that the addition of lutein + zeaxanthin, DHA + EPA, or both components to the AREDS formulation did not further reduce the risk of progression from intermediate to late AMD, compared with the original AREDS supplement [73]. Although a couple of small RCTs suggested the potential for carotenoid supplements to enhance visual function in AMD patients [198,199], design limitations of these studies raise the need of high-level evidence to corroborate these findings.

Given the results from AREDS, diet has been proposed as a potentially modifiable factor that may influence both the development and progression of AMD. In fact, foods and nutrients may interact with each other [200]. Examining dietary pattern rather than single nutrient intake may better account for the relationships among different diet components [200]. A case control study of ≈ 700 subjects demonstrated that high adherence to a healthy diet - assessed by using the Alternate Healthy Eating

Index (AHEI) - significantly decreased the odds of AMD [200]. This is consistent with evidence from the Carotenoids in Age-Related Eye Disease Study (CAREDS), showing that women which reflected the healthiest smoking, physical activity and dietary habits had 71% lower odds of early AMD compared to those with unhealthy lifestyle [201]. In spite of this couple of studies, there is still the need of further research exploring the effect of healthy dietary pattern, such as Mediterranean diet, on AMD onset and progression.

1.3.4.1 Gene-Diet interaction in age-related macular degeneration

While antioxidants supplementation clearly decreases the progression from early to advanced AMD [202], evidence about the effect of their intake through the diet is still controversial, probably due to different genetic susceptibility and/or other unmeasured effect modifiers. The Rotterdam study found a synergic interaction between CFH rs1061170 polymorphism and dietary intake of antioxidants, demonstrating that higher intake of zinc, omega-3 fatty acids, β-carotene, lutein and zeaxanthin might reduce the incidence of early AMD in subjects at higher genetic risk [203]. This is in line with pooled analysis of Blue Mountains Eye and Rotterdam cohorts, showing that dietary intake of lutein and zeaxanthin protected against the risk of early AMD, only in concurrence with at least two risk alleles of CFH rs1061170 and ARMS2 rs10490924 polymorphisms [204]. Conversely, in absence of genetic susceptibility, higher intake of lutein and zeaxanthin was associated with higher incidence of early AMD [204]. The analysis of the Atherosclerosis Risk in Communities (ARIC) study added to these controversial findings, founding that higher lutein and zeaxanthin intake was associated with lower AMD prevalence among carriers of the heterozygous CFH genotype, higher prevalence among carriers of the homozygous risk genotype, and no statistically significant effect among those with no-risk genotype [205].

In the AREDS II, increased intake of DHA and EPA was associated with decreased risk of non-exudative AMD, after adjusting for behavioural factors and genetic variants, including polymorphisms in CFH, ARMS2/HTRA1, CFB, C2, C3, and CFI genes [206]. Moreover, the Blue Mountain Eye Study found that weekly consumption of fish was associated with lower risk of advanced AMD, only among patients with the CFH homozygous risk genotype [207]. Findings from a sub-sample of the AREDS II also demonstrated a significant interaction between folate intake and the rs2230199 C3 polymorphism. In fact, the protective effect of folate intake was evident among subjects with homozygous non-risk genotype, but not in those carrying the risk allele. By contrast, no significant effect on AMD progression was observed for dietary intake of thiamin, riboflavin, niacin, and vitamins B6 and B12 [208]. With regard to dietary pattern, the study by Merle and colleagues - including participants of the AREDS - was the first evaluating the interaction between genetic risk factors and overall diet [209]. Particularly, they demonstrated that adherence to the Mediterranean diet

was associated with lower risk of progression to advanced AMD among patients with non-risk genotype, but not among those with the homozygous risk genotype [209]. The significant association, in absence of genetic susceptibility, might be explained by the protective effect of Mediterranean diet on immune and inflammatory responses.

The effect of the interaction between nutrient supplements and genetic susceptibility on the progression to advanced AMD is currently under debate. In 2008, Klein et al. demonstrated that the effect of supplementation with antioxidant and zinc on the AMD progression was higher among subjects with non-risk genotype for the CFH rs1061170 polymorphism than in high-risk subjects [210]. In addition, Seddon et al. reported that antioxidant and zinc supplementation reduced the risk of progression to the exudative AMD but not to non-exudative AMD [211]. Next, Awh et al. found that zinc supplementation reduced the progression to advanced AMD, among subjects with no risk alleles for CFH and at least one risk allele for ARMS2 [212]. Further, the same researchers demonstrated a distinct effect on disease progression according to the number of risk alleles for these polymorphisms: while zinc supplementation was protective against the harmful effect of the ARMS2 risk allele, it increased the risk posed by CFH allele [212]. These findings are supported by current knowledge about physiologic implication of zinc binding to CFH, which might counteract the ability to inactivate C3 convertase [213-215]. This, together with functional consequences of CFH rs1061170 polymorphism, might cause the detrimental effect associated with concurrence of CFH risk genotypes and zinc supplementation [216].

1.3.5 DNA methylation landscape in age-related macular degeneration

Given the abovementioned evidence, a typical gene-environment interaction has been proposed in the context of retinal degenerative disorders [217]. In fact, it seems reasonable that environmental effects may be under genetic control, as well as environmental risk factors may trigger the disease in genetically susceptible subjects [82]. Retinal cells show altered gene expression in response to environmental factors (i.e. nutrient intake, light, and oxidative stress) and internal cellular signals (i.e. reactive oxygen species - ROS), calcium concentration, and DNA damage) [218]. Epigenetic mechanisms may represent the events modulating the interaction between genetic factors, life experiences and environmental exposures [5]. Among these, DNA methylation and histone modifications are strictly related to gene expression [219,220] and genome stability [18]. The key pathological features of AMD are associated with several risk factors (e.g. smoking, low omega-3 diet, excessive retinal iron levels and ageing) which induce high oxidative stress [221]. In a recent genome-wide methylation study in post-mortem RPE and choroid from AMD patients, the hypermethylation of the promoter regions of two glutathione S transferase isoforms (GSTM1 and GSTM5) was identified [222]. The epigenetic downregulation of these detoxification enzymes may

increase the susceptibility to oxidative stress in AMD [41]. The cellular redox state of the retina and aging might also involve the activation of sirtuin 1 (SIRT1). SIRT1 upregulates the expression of hypoxia-inducible factor 2α (HIF-2α), VEGF and erythropoietin, activating both hypoxia and angiogenesis mechanisms [223].

Inflammation is another hallmark of AMD that may be triggered by changes in histone acetylation and methylation status that involve the production of inflammatory cytokines and auto-inflammatory T cells [224]. A DNA methylation study, using peripheral blood mononuclear cells from one pair of monozygotic twins and two pairs of dizygotic twins with discordant AMD phenotypes, was performed [225]. Notably, the promoter region of interleukin-17 receptor (IL17RC) was hypomethylated in the twins with AMD, enhancing IL17RC expression and, hence, increasing the chronic inflammatory response in the macula [225].

Interestingly, it has been suggested that hypomethylation of the promoter of the clusterin gene – one of the major component of drusen - is an epigenetic signature of AMD, which leads to the upregulation of clusterin gene expression in cultured RPE cells derived from AMD patients compared to age-matched healthy donors [226].

1.4 Diabetic Retinopathy

1.4.1 Clinical features and Epidemiology

Diabetic retinopathy (DR) is a specific microvascular complication of diabetes mellitus (DM) which results in the damage of small blood vessels and neurons of the retina. The earliest stages include narrowing of the retinal arteries followed by the reduction of retinal blood flow, dysfunction of the neurons of the inner retina, changes in the function of the outer retina, which in turn are associated with changes in visual function and dysfunction of the blood-retinal barrier [227]. In the advanced stages, the basement membrane of the retinal blood vessels clots and capillaries degenerate, leading to reduced blood flow and progressive ischemia. These events cause the formation of microscopic aneurysms - balloon-like structures jutting out from the capillary walls - which in turn recruit inflammatory initiating the degeneration of the retinal neurons and glial cells. DR is one of the leading cause of vision loss in middle-aged economically active people, accounting for 4.8% of the number of cases of blindness (37 million) worldwide [228]. The prevalence of DR ranges from 10 to 50% dependent of population, type and duration of DM [229]. It is worth underlying that, with the increasing number of people with diabetes, the number of DR - which includes severe non-proliferative DR, proliferative DR (PDR) and diabetic macular edema (DME) - has been estimated to rise to 191 million by 2030 [230]. An European multicenter study reported that DR prevalence among T1DM patients ranges from 25% to 60% [231]. Notably, 50% of type I DM patients with no DR had been shown to develop retinopathy by 5 to 7 years, and 9% with mild non PDR (NPDR) would

develop PDR by 5 years [232]. The burden of DR appears to be lower in type II DM patients, with prevalence that ranges from 25% in United Kingdom to 40% in Italy [233-236]. In United Kingdom, the 5-year cumulative DR incidence in type II DM patients was 4%, rising to 16.4% after 10-years follow-up [237]. In the last decades, there have been made major advances in preventing DR development and progression. The increased awareness of DR risk factors and the access to community screening programs led to a decline in the prevalence and incidence of DR in the developed countries [238]. Moreover, RCTs showed that early treatment can reduce the risk of severe visual loss by 57% [239].

1.4.2 Major risk factors

The risk factors of DR can be broadly classified into modifiable (i.e. hyperglycaemia, hypertension, hyperlipidaemia and obesity) and non-modifiable factors (i.e. duration of diabetes, puberty, pregnancy and genetic susceptibility). The Diabetes Control and Complications Trial (DCCT) showed that glycaemic control (i.e. HbA1c value less than 7%) could reduce the risk of DR development and progression in type I and type II DM patients, respectively [233]. Notably, it has been proposed that the protective effect of immediate intensive treatment of hyperglycaemia continues regardless of glycaemia in the later course of diabetes [240]. The long lasting effect of glycaemic control - also known as metabolic memory - could counteract hyperglycaemia-induced pathological processes, such as enhanced oxidative stress and glycation of cellular proteins and lipids [241]. The Action in Diabetes and Vascular Disease trial proposed a HbA1c threshold for micro-vascular events of 6.5%: above this threshold, every 1% increase in HbA1c level was associated with a 40% higher risk of a micro-vascular complications [242]. Although there is no evidence of achieving additional benefit for microvascular events by reducing HbA1c level below these thresholds, it is not even demonstrated a harmful effect.

Despite several observational studies failed in demonstrating that blood pressure is a risk factor for DR incidence and progression [243,244], several RCTs indicated the benefits of blood pressure control as a major modifiable factor for DR management. Among these, the UK Prospective Diabetes Study (UKPDS) was the first to point out the importance of tight blood pressure control in reducing DR [233,240]. After 9 years of follow-up, tight blood pressure control reduced the risk of DR progression by 34%, while for every 10mmHg increase in systolic blood pressure the risk of early DR and PDR increased by 10% and 15%, respectively [245,246].

Findings on the effect of lipid on the development and progression of DR are currently controversial [247-249]. While DCCT showed that the severity of DR increased with increasing triglycerides and decreasing high-density lipoprotein (HDL) levels [250], the Multi-Ethnic Study of Atherosclerosis [251] and the Chennai Urban Rural Epidemiology Study Eye Study [249] showed no association between total cholesterol and DR. However, of the subset in the lipid panel, triglycerides seemed to be associated

with DR and the LDL appeared to be related to DME [249]. These findings were confirmed by the Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Genetic Study, which demonstrated that high serum LDL and high cholesterol ratio were associated with DME [248].

With regard to obesity, recent studies found a positive correlation between BMI, waist to hip ratio (WHR) and the risk of DR [252-254]. Consistently, two European prospective studies independently demonstrated the positive association of BMI and WHR with severity of DR [252,255].

Overall, although evidence on the risk of DR related to hypertension, hyperlipidaemia and obesity is still inconclusive or controversial, it is crucial for people with diabetes to maintain an optimal cardio-metabolic status to help prevent both development and progression of DR and other diabetes-related complications.

1.4.3 Genetic Risk Factors

More recently, several twin studies [256], family studies [257-263], candidate gene studies [264-268], linkage studies [259,268] and small-scale Genome Wide Association Study (GWAS) attempted to identify genes in the development of DR. For instance, concordant twins with type II DM seemed to have same severity of DR severity compared to twins with type I DM [256].

Moreover, familial aggregation studies found that siblings or relatives of diabetic patients with DR showed a three-fold increased risk of developing DR than those with no DR [258-261]. Among genetic factors associated with DR onset, it is worth mentioning chromosome 1p [259], chromosomes 3 and 9 [269], aldose reductase gene (ALR2) [270,271], receptor for advance glycation end-products (RAGE) gene [272], TGF-β1 gene [273], VEGF gene [274], endothelial nitric oxide synthase (eNOS) gene [274], vitamin D receptor and insulin-like growth factor 1 (IGF-1) gene [275]. However, it is not possible to draw any conclusion since these findings are weak, inconsistent and lacking of standardization across different populations. The same limitations should be considered when interpreting results from linkage analysis and GWAS approaches. For instance, although genetic variants in chromosomes 1,3 and 12 were related to DR in Pima Indians and Mexican Americans [259,269], none of these regions reached statistical significance. Similarly, a GWAS found five novel chromosomal regions (i.e. chromosome 1p, 10p, 10q, 13q and 5q) associated with DR [276], but none of the regions reached genome-wide statistical significance.

1.4.4 Dietary risk factors

Even if diet is a well-established factor involved in the development of DM, its role in DR pathogenesis should be better clarified. A post hoc analysis of an RCT demonstrated that adherence to the Mediterranean diet is associated with a decreased risk of DR compared to a low-fat control diet [277]. The reasons of this relationship rely on the protective effect of Mediterranean diet against type II

DM [278], obesity and cardiovascular risk factors in people with DM [279]. The Mediterranean diet is characterized by the intake of fruit, vegetables, whole grains, plant proteins, fish, and low-fat dairy products. In line with previous evidence, high fruit, vegetable and oily fish intake have been found to confer protective effects against the onset of DR [280-282]. The protective effect of fruit and vegetables may depend on their antioxidant content, vitamins C and E, carotenoids or polyphenols. However, evidence about the effect of vitamins C, E and carotenoid intake is not entirely consistent. Although several cross-sectional and prospective studies supported the beneficial effect of vitamin C intake against DR [281,282], others failed in demonstrating this relationship [283,284]. Overall, vitamin E seemed to be not associated with DR, but rather it appeared to increase the risk among subjects not treated with insulin [284], those taking oral hypoglycaemic agents [283], and those with poor glucose control [283]. With regard to carotenoids, while lutein intake was not associated with DR [285], its serum concentration was lower in diabetic patients with NPDR compared to those without DR [286]. Consistently, a cross-sectional study demonstrated the beneficial effect of combined lutein/zeaxanthin and lycopene plasma concentration against DR risk [287]. Fish may exert its protective effects through its omega-3 content and not to vitamin D, as neither intake nor supplements were associated with DR. Several lines of evidence supported PUFA intake as beneficial in helping prevent DR [280,288]. Particularly, supplementation with n3-PUFA decreased the number of retinal acellular capillaries associated with diabetes and inflammatory markers in animal models of diabetes [289,290].

In spite of controversial findings, several RCTs demonstrated that some polyphenols may inhibit the onset of retinopathy [291], while antioxidant supplementation reduced retinal oxidative stress and slowed DR progression in patients with NPDR [292,293]. However, preclinical and clinical studies are warranted to better evaluate potential strategies against DR based on dietary intervention and/or supplementation.

1.4.4.1 Biological mechanisms of dietary risk factors

While poor glycaemic control is one of the main causes of DR, the intake of healthy foods and/or nutrients appears to be protective. Several studies confirmed that hyperglycaemia, along with the DM duration, is one of the strongest risk factors of DR [294,295]. Hyperglycaemia-induced pathways that influence the development of DR include non-enzymatic protein glycation, activation of protein kinase C, activation of the hexosamine pathway, production of reactive oxygen species (ROS), and induction of HIF [294,295]. Particularly, non-enzymatic protein glycation accelerates the accumulation of advanced glycation end-products (AGEs), which are not only implicated in the loss of the retinal capillary pericytes, but also in inflammation, oxidative stress and activation of VEGF [294,295]. VEGF play an important role in DR, since it is the key driver of neovascularization in the proliferative diabetic retinopathy [296]. The abovementioned pathways seem to be correlated: while the activation of

HIF and protein kinase C upregulates VEGF expression [294], the hexosamine pathway increases the level of AGEs [294] and ROS. The diet, on one hand, participates in the glycaemic control, on the other hand may affect DR onset and progression as an exogenous source of AGEs. In fact, food rich in fat and protein are the greatest contributors of AGEs [297]. Moreover, the accumulation of long-chain fatty acids may affect DR via increasing flux through the protein kinase C pathway [298]. The retina is extremely subject to oxidative stress and lipids accumulation, which in turn can result in lipid peroxidation and in advanced lipoxidation end products (ALEs). ALEs, along with AGEs, can accumulate in tissues triggering a sustained response of the pro-inflammatory transcription nuclear factor-kβ (NF-kβ), and diminishing antioxidant defences. If animal-derived foods can be considered detrimental for DR, fruit and vegetables may exert protective effects through their polyphenol content that improves glucose homeostasis and insulin resistance, and reduces inflammation [291]. Moreover, their antioxidant activity - due to the high content of vitamins C, E, and carotenoids - acts to reduce neovascularization, to restore retinal blood flow, and to scavenge ROS [299].

1.4.5 DNA methylation landscape in diabetic retinopathy

Emerging evidence suggests that a complex gene-environment interaction is involved in the pathogenesis of diabetes-related microvascular complications [300]. Epigenetic mechanisms - including DNA methylation, histone modifications, and miRNAs regulation - contribute to the dysregulation of signalling pathways involved in oxidative stress, inflammation, apoptosis, and aging, and modulate the expression of several key genes in DM [301,302]. Several lines of evidence describe distinctive methylation profiles in diabetes-associated cardiovascular complications [303-306], suggesting that hyperglycaemia-induced oxidative stress is an important mediator of these events [307,308]. Differential DNA methylation of genes involved in the natural killer cell-mediated cytotoxicity pathway was described in DR [309]. Moreover, retinal endothelial cells exposed to high glucose concentration showed impaired mitochondrial integrity and functions probably due to increased mitochondrial DNA methylation [310] and to the imbalance between methyl-cytosine and hydroxyl methylation of Matrix metalloproteinase-9 gene [311]. However, in spite of considerable evidence to hypothesise that hyperglycaemia might affect retinal DNA methylation, the current knowledge about the effect of high glucose on DR needs to be implemented.

1.5 Rationale and specific aims

In line with the current state of the art, Epigenetics is becoming an increasingly important area of biomedical research, with mounting evidence showing that epigenetic alterations influence the common pathophysiological responses in aging and age-related disease. However, it has been also demonstrated that both genetic susceptibility and diet - by participating in a typical gene-diet

interaction – modulate the risk of retinal degenerative diseases. Given this scenario, we designed and developed an integrated multiple approach to answer the following questions:

Could the diet - especially the Mediterranean diet – modulate DNA methylation in healthy people?
Although several studies suggested that the intake of specific foods and nutrients is associated with changes in DNA methylation, inconclusive evidence exists on the effect of a healthy dietary pattern such as the Mediterranean diet. In the context of a wide project [312], we filled this lack evaluating whether the adherence to Mediterranean Diet, as well as the intake of specific foods and nutrients, may influence LINE-1 methylation in healthy women living in the urban area of Catania.

How do dietary habits affect risk factors of retinal degeneration in healthy people?
Hyperglycaemia, hypertension, hyperlipidaemia, and obesity are among the strongest risk factors of retinal degeneration regardless its aetiology. Thus, we aimed at evaluating how dietary habits - in terms of quality and timing - could affect cardio-metabolic factors that increase the risk of AMD and DR. To address this issue, we used data from the Kardiovize Brno 2030 study - which recruited a randomly selected sample of residents from the urban population of Brno, Czech Republic [313] - to characterize the main dietary patterns and to investigate their relationship with hyperglycaemia, hypertension, hyperlipidaemia and indices of obesity.

How does genetic susceptibility contribute to AMD risk?
The association between the rs1061170 polymorphism in CFH gene and AMD risk has been extensively investigated, but prevalence of the polymorphism varies between ethnicities and the strength of its association with AMD risk could differ within AMD subtypes. Therefore, we carried out a comprehensive systematic review and an updated meta-analysis to assess the association between the rs1061170 polymorphism and AMD, stratifying for stage of disease and ethnicity.

While the effect of CFH polymorphisms on AMD risk has been consistently demonstrated, the role of genetic variants in the VEGF gene remains controversial. Thus, we conducted a comprehensive systematic review and an updated meta-analysis to summarize the current published studies on the associations between four common VEGF gene polymorphisms (i.e. rs833061, rs1413711, rs3025039, and rs2010963) and AMD risk, stratifying for stage of disease and ethnicity.

How do oxidative stress and inflammation influence epigenetic mechanisms in the retina and how can we counteract these changes?
Oxidative stress and chronic inflammation are crucial pathophysiological conditions in retinal degeneration. Since it is becoming evident that these conditions may affect gene expression and genome stability [219,220] via modulating DNA methylation and histone modification, we investigated their effect on epigenetic enzymes functions and LINE-1 methylation in cellular models of retinal

stress. Next, we evaluated whether treatment with resveratrol - a flavonoid with antioxidant and anti-inflammatory properties - may restore changes in LINE-1 methylation by modulating epigenetic mechanisms.

Is LINE-1 methylation a hallmark of AMD?

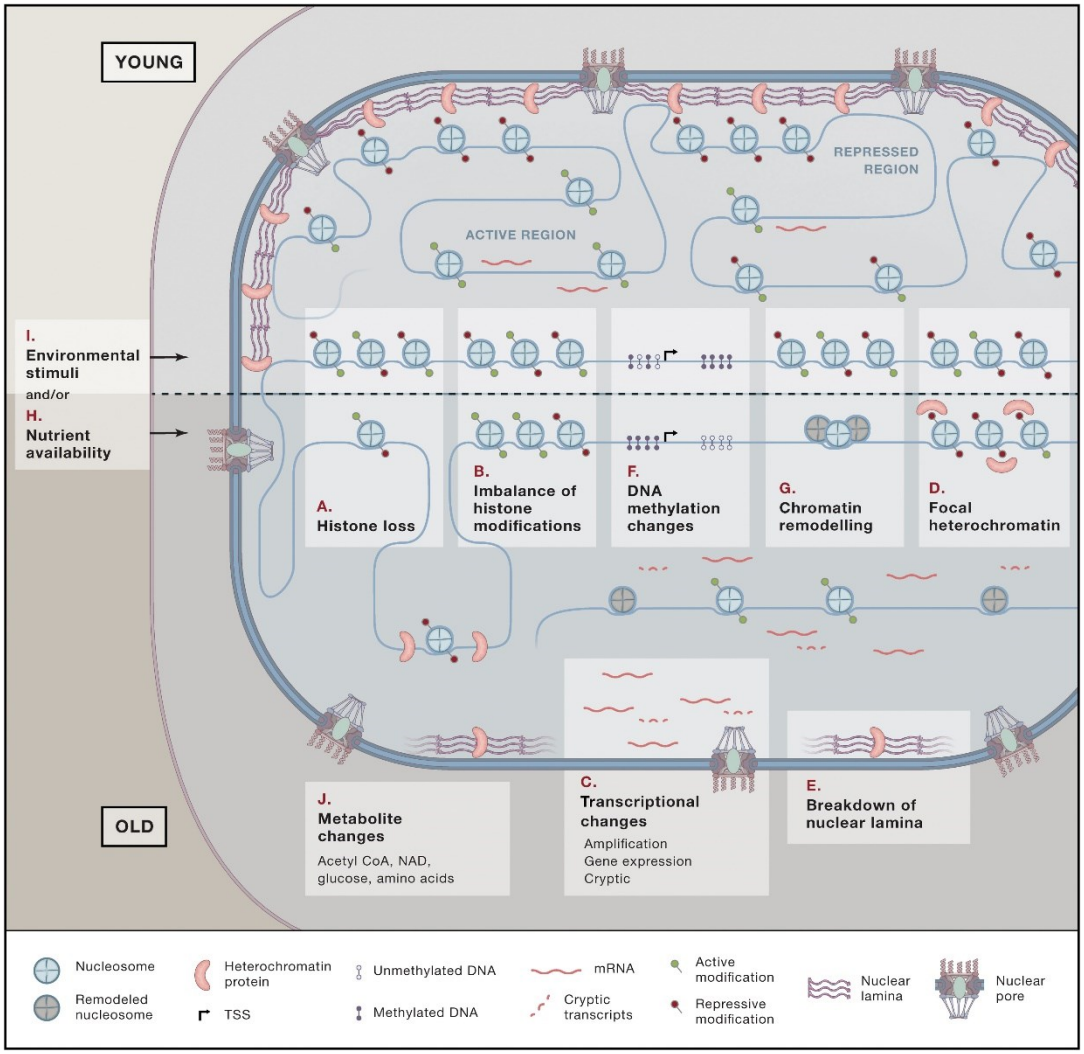
To solve these questions, we designed and conducted a cross-sectional study aimed at investigating novel epigenetic biomarkers in a population at risk of AMD. Particularly, we compared LINE-1 methylation level - widely used as a surrogate marker of global methylation in aging and age-related disease [25,314-316] - between AMD patients and age- and sex-matched controls.

Does hyperglycaemia affect epigenetic mechanisms in the retina?

Regardless of the type of DM, hyperglycaemia-induced oxidative stress has been identified as the major contributing factor to retinal degeneration. Several lines of evidence proposed that retinal oxidative stress alters both histone modifications and DNA methylation [317], which have been further recognized as potential epigenetic mechanisms involved in the pathophysiology of DR [310,318-320]. Accordingly, we compared the expression levels of DNMTs in retina of diabetic and non-diabetic mice, using the Gene Expression Omnibus (GEO) datasets. Next, using a cellular model of DR, we analysed the time-related effect of hyperglycaemia on oxidative stress and DNMTs functions. Finally, we evaluated whether the antioxidant properties of curcumin - a natural phenol from the rhizome of Curcuma longa - may restore hyperglycaemia-induced changes in DNMTs function.

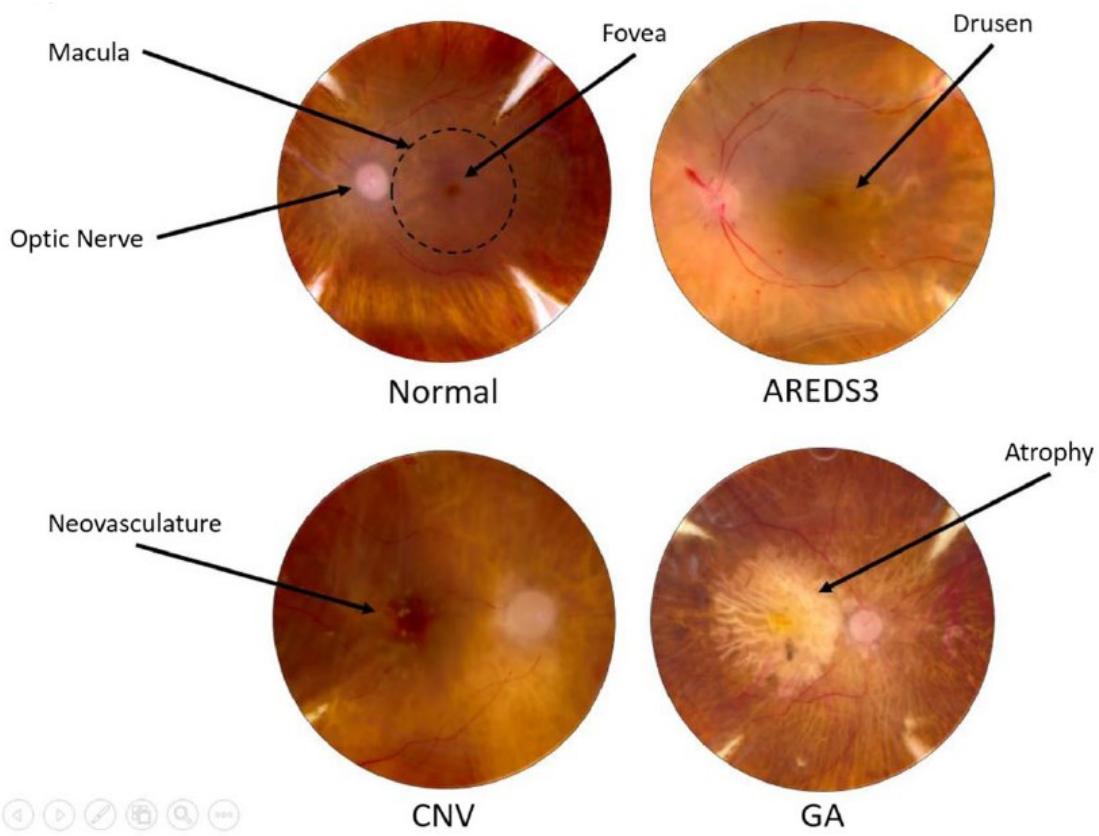
Figures

Figure 1. The epigenetic hallmarks of aging.



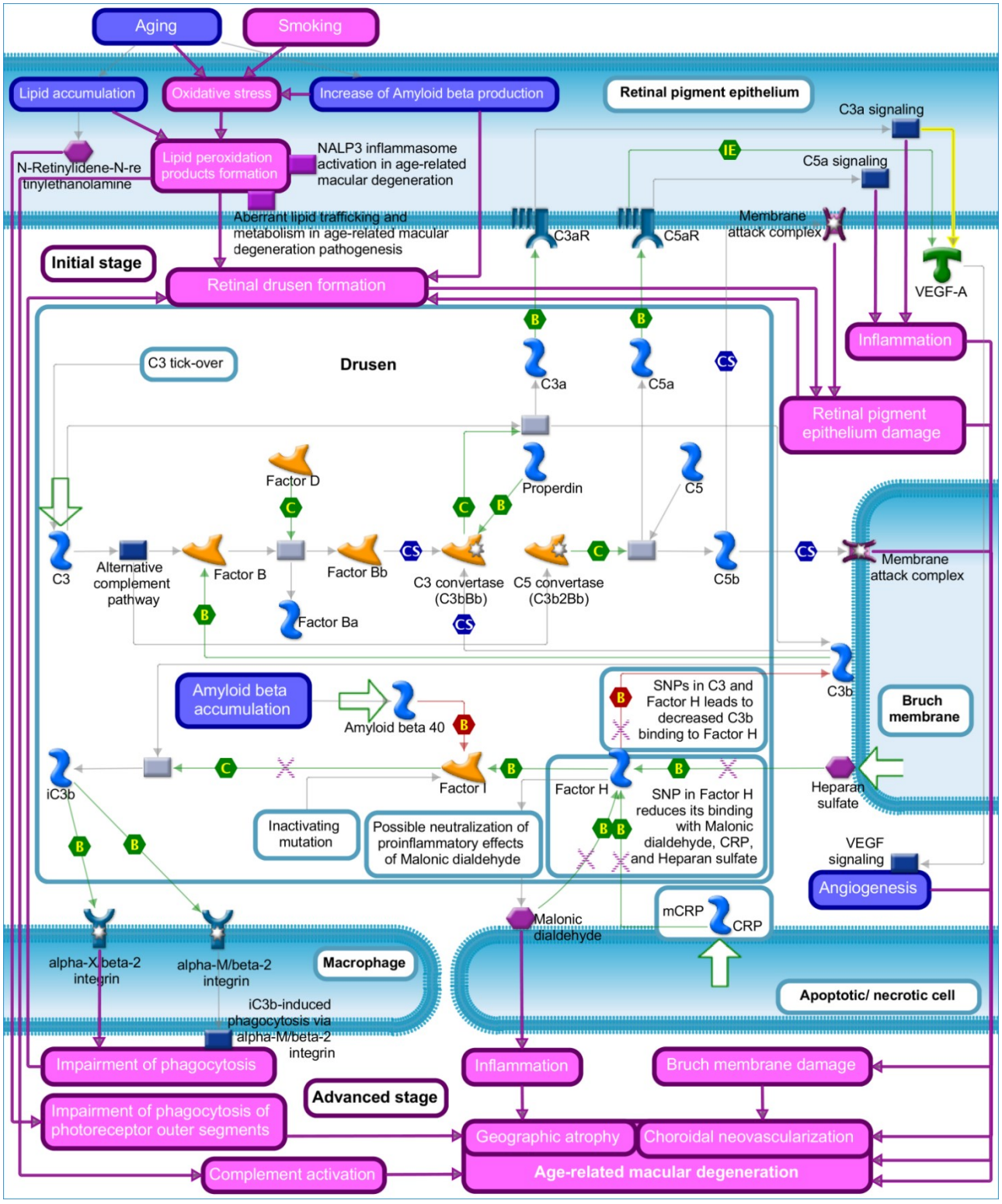
Aging is characterized by (A) loss of histones, (B) imbalance of activating and repressive modifications, (C) transcriptional changes, (D) losses and gains in heterochromatin, (E) breakdown of nuclear lamina, (F) global hypomethylation and gene-specific hypermethylation, and (G) chromatin remodeling. These changes are influenced by (H) environmental stimuli, (I) nutrient availability and (J) intracellular metabolite concentrations. Cell 2016 166, 822-839. DOI:10.1016/j.cell.2016.07.050.

Figure 2. Progression of age-related macular degeneration.



Fundus images of normal, intermediate (AREDS3), and advanced AMD (CNV, choroidal neovascularization and GA, geographic atrophy) eyes. Eye and Vision 2016, 3:34. DOI:10.1186/s40662-016-0063-5.

Figure 3. Complement system dysregulation in age-related macular degeneration.



The dysregulation of complement pathways - especially the alternative pathway - is involved in AMD pathogenesis. The major risk factors of AMD (i.e. aging, smoking, and oxidative stress) are associated with the over-activation of the complement system, leading to retinal drusen formation, chronic inflammation, impairment of phagocytosis, geographic atrophy and choroidal neovascularization.

2 The effect of Mediterranean diet on LINE-1 methylation: a cross-sectional study in healthy women

2.1 Background

In the last years, it has been shown that environmental and lifestyle factors can potentially modify DNA methylation process, leading to altered gene expression and genome instability both in exposed individuals and in future generations [27]. In fact, DNA methylation regulates gene transcription and chromosome stability via the addition of methyl groups to cytosine residues. In mammals, 5-Methyl-cytosines (5mC) represent 2–5% of all cytosines that are mainly found on CpG islands. Although hypermethylation of CpG sites located in promoter regions downregulates gene expression [321], more than 90% of CpG sites are located within transposable repetitive elements [23]. Hypomethylation of these elements has been associated with chromosomal instability and aberrant genome function [322,323]. Since LINE-1 is among the most common transposable sequences, the assessment of its methylation status has been used as a surrogate marker of global DNA methylation in cancer [24,25], cardiovascular [26] and neurodegenerative diseases [324]. Several classes of nutrients - such as folate, polyphenols, selenium, retinoid, fatty acids, isothiocyanates and allyl compounds - can affect DNA methylation via different mechanisms [28]. Among these, folate is a methyl donor essential for cellular replication and maintenance via modulating DNA methylation, synthesis and repair. However, the role of this nutrient remains to be fully elucidated since it has been both positively and negatively associated with LINE-1 methylation [29-35].

To date, there is growing interest in investigating overall diet or dietary patterns, rather than focusing on single foods or nutrients, because dietary pattern analysis can summarize the potentially synergistic effect of food and nutrients. Recently, the adherence to a healthy diet characterized by a high intake of vegetables and fruits has been positively associated with LINE-1 methylation [32,35,325]. Despite this evidence, there are currently no studies investigating the effect of the Mediterranean Diet - widely recognized as the optimal diet for maintaining global health - on LINE-1 methylation. To fill this lack of knowledge, using data from a wide project [312], we evaluated whether the adherence to MD, as well as the intake of specific nutrients, might modulate LINE-1 methylation in healthy women living in the urban area of Catania.

2.2 Methods

2.2.1 Study Design

Women referred to two clinical laboratories of Catania (Italy) were enrolled in this cross-sectional study if they satisfied the following inclusion criteria: (i) non-pregnant women (ii) with no current or

previous self-reported history of severe diseases, (iii) who signed a written consent to participate in the study. The study protocol was approved by the ethics committee of the involved institution and performed according to the Declaration of Helsinki. Women were fully informed of the purpose and procedures of the study and an informed consent was signed.

Information on sociodemographic and lifestyle data were collected by trained epidemiologists using a structured questionnaire. Educational level was classified as low (≤8 years of school) and high (>8 years of school). Women were also classified as employed or unemployed (including students and housewives). BMI was calculated as weight (kg) divided by height (m2), based on criteria from the World Health Organization [326]. For smoking status, women were classified as no smokers (including ex-smokers) and current smokers. From each participant, a peripheral blood sample was collected into EDTA tubes for molecular analysis.

2.2.2 Dietary assessment

Dietary data were collected by using a 95-item semi-quantitative Food Frequency Questionnaire (FFQ), with the previous month as the reference period [327]. For each food item, women were asked to report the frequency of consumption and portion size using an indicative photograph atlas. Frequencies of food consumption were classified into twelve categories, ranging from "almost never" to "two or more times a day". The medium serving sizes were described by natural portions or standard weight and volume measures of the servings commonly consumed in the Italian population. Accordingly, portion size was classified into three categories: small (half a medium serving size), medium, and large (1.5 times or more than a medium serving size). The food intake for each item was calculated by multiplying the daily frequency of consumption with the portion size. Folate and total caloric intakes were calculated using the USDA Nutrient Database (http://ndb.nal.usda.gov/) adapted to the Italian food consumption. Intake of folic acid from supplements was specifically addressed as previously described [328]. Prevalence of folate deficiency was estimated by comparing folate intake with the Estimated Average Requirements (EAR) [329], taking into account the use of folic acid supplements.

2.2.3 Mediterranean Diet Score

Adherence to Mediterranean diet was assessed using the Mediterranean Diet Score (MDS) [330,331] which includes 9 components: fruits and nuts, vegetables, legumes, cereals, lipids, fish, dairy products, meat products, alcohol and the ratio of unsaturated to saturated lipids. For components that positively characterize Mediterranean diet (vegetables, legumes, fruits and nuts, cereals, fish, and a high ratio of unsaturated to saturated lipids), women whose consumption was below or equal to the median value of the population were assigned a value of 0, and a value of 1 was assigned otherwise.

For components that negatively characterize Mediterranean diet (dairy and meat products), women whose consumption was below the median were assigned a value of 1, and a value of 0 was assigned otherwise. A value of 1 was given to women consuming a moderate amount of alcohol (5 to <25 g per day). Accordingly, MDS ranges from 0 (no-adherence) to 9 (perfect adherence). MD adherence was categorized, according to the MDS, as follows: low adherence (MDS range: 0–3), medium adherence (MDS range: 4–6), or high adherence (MDS range: 7–9) [332].

2.2.4 DNA extraction and methylation analysis

Whole blood samples were centrifuged at 2500 rpm for 15 min. The buffy coat fraction was transferred to a cryovial and immediately frozen at -20 °C until use. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Italy) according to the manufacturer's protocol. LINE-1 methylation levels were measured by pyrosequencing-based methylation analysis, using the PyroMark Q24 instrument (Qiagen, Italy), as previously reported [32,333]. Briefly, bisulfite conversion and clean-up of DNA for methylation analysis of 30–40 ng of DNA were completed using the EpiTect Bisulfite Kit (Qiagen, Italy) and the converted DNA was eluted in 20 µl of Eluition Buffer. PCR was conducted in a reaction volume of 25 µl, using the PyroMark PCR Kit (Qiagen, Italy). According to the manufacturer's instructions, each reaction mixture contained 1.5 µl of bisulfite-converted DNA, 12.5 µl of PyroMark PCR Master Mix 2X, 2.5 µl of Coral Load Concentrate 10X, and 2 µl of the forward primer (5′-TTTTGAGTTAGGTGTGGGATATA-3′) and the reverse-biotinylated primer (5′-biotin-AAAATCAAAAAATTCCCTTTC-3′) (0.2 µM for each). HotStart PCR cycling conditions were 1 cycle at 95 °C for 15 min, 40 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30s, and a final extension at 72 °C for 10 min. Then, the PCR product underwent pyrosequencing using 0.3 mM of the sequencing primer (5′-AGTTAGGTGTGGGATATAGT-3′). All runs included 0% and 100% methylated human DNA as positive controls as well as a negative control. To confirm reproducibility every sample was tested two times and failed assays were repeated. Overall, intra-observer coefficient of variability between the two replicates of LINE-1 methylation measurements was 3.2% (SD=3.0%). LNE-1 methylation levels were calculated as percentage of methylated cytosines over the sum of methylated and unmethylated cytosines and reported for each CpG site as well as the average of the three CpG sites (GenBank Accession No. X58075).

2.2.5 Statistical analyses

Statistical analyses were performed using the SPSS software (version 22.0, SPSS, Chicago, IL). Descriptive statistics were presented as frequencies, means ± standard deviation (SD), median values and interquartile range (IQR). Prior to analysis, the normal distribution of all variables was checked using the Kolmogorov-Smirnov test. Since LINE-1 methylation exhibited non-normal distribution,

differences by population characteristics were tested using the Mann–Whitney U test or the Kruskal–Wallis test. To evaluate the association between MDS and LINE-1 methylation, we used the following linear regression models: the age-adjusted model; the multivariable model adjusted for covariates that were selected a priori (i.e. age, educational level, employment status, smoking, BMI and folate deficiency). Overall, results were reported as beta regression coefficient (β) expressing the change in LINE-1 methylation level associated with a standard deviation increase in MDS. A p-value of 0.05 was considered as statistically significant.

2.3 Results

2.3.1 Characteristics of study population

The main characteristics of study population (n=299) - aged from 15 to 80 years - are shown in **Table 1**. In summary, mean age was 38.99 years (SD= 16.53; median=35), 69.9% reported high educational level whereas 54.2% were unemployed. Focusing on lifestyle factors, 21.2% were current smokers and 37.9% were overweight or obese. Mean folate intake was 295.36 μg/day, and only 18.1% reported the use of folic acid supplements. Thus, taking into account the use of supplements, prevalence of folate deficiency was 47.5%.

2.3.2 LINE-1 methylation

LINE-1 methylation levels at the three CpG sites correlated with each other (r_{1-2} = 0.398; r_{1-3} = 0.940; r_{2-3} = 0.394; $p < 0.001$), with mean values of 80.47 (SD=3.14), 57.11 (SD=9.20) and 65.39 (SD=7.48), respectively. The average LINE-1 methylation level was 67.66 (SD=7.48). **Figure 1** shows that distribution of LINE-1 methylation levels was significantly different by age (p<0.001) and nutritional status (p=0.047), while no differences by educational level, employment status, smoking and folate deficiency were evident.

2.3.3 Mediterranean Diet Score and LINE-1 methylation

According to MDS (mean=4.64; SD= 1.78), adherence to Mediterranean diet has been classified as low (27.1%), medium (58.2%) and high (14.7%). Particularly, LINE-1 methylation levels increased with increasing MD adherence (p=0.004) (**Figure 2**). Results from linear regression analysis demonstrated that MDS was significantly and positively associated with LINE-1 methylation levels both in the age-adjusted (β= 0.606; p<0.001) and multivariable-adjusted model (β = 0.610; p<0.001). By contrast, age was significantly and negatively associated with LINE-1 methylation in both models (β= -0.787; p<0.001; β= -0.776; p<0.001).

2.4 Discussion

Although previous studies reported no effect of aging on LINE-1 methylation level [334-336], we demonstrated that it decreased with increasing age. This is consistent with other findings showing that methylation status in the repetitive elements changes over time [337-339]. Similarly, it has been proposed that folate status and the amount of other methyl donors could affect global DNA methylation. In our population, we observed that folate deficiency was negatively associated with LINE-1 methylation, partially confirming the majority of previous studies which demonstrated that global DNA methylation levels increased with increasing folate intake [29,31-35]. However, results are conflicting with other studies reporting an inverse relationship [30]. This controversy might be partially explained by differences in unmeasured factors such as ethnicity, genetic variants in one-carbon metabolism, lifestyle, physiological and pathological conditions, which in turn can affect DNA methylation mechanism.

To our knowledge, the present study demonstrated for the first time that adherence to Mediterranean diet is positively associated with LINE-1 methylation, after adjusting for age, educational level, employment status, smoking, BMI and folate deficiency. This is in line with previous studies reporting that a higher intake of vegetables and/or fruits decreased the risk of LINE-1 hypomethylation [32,35]. Several nutrients and bioactive compounds provided by Mediterranean diet - such as phytochemicals (phenolics, flavonoids, and carotenoids), vitamins (vitamin C, folate, and provitamin A), minerals (potassium, calcium, and magnesium), and fibres - may act on multiple signal transduction pathways and epigenetic mechanisms [40,41], explaining the observed relationship.

This study had some limitations. Although dietary assessment through FFQ does not preclude inaccuracies, the FFQ used in the present study has been previously developed and validated among the Italian population [327]. Moreover, the association between Mediterranean diet and LINE-1 methylation may be also affected by genetic factors (i.e. polymorphisms in the MTHFR gene), which in turn may interact with folate status and methylation process [340]. With regard to molecular analysis, reliability and flexibility have made pyrosequencing of bisulfite-treated DNA the “gold standard”, and a high-throughput and replicable methodology to evaluate LINE-1 methylation [341,342]. However, in the present study, the methylation analysis was performed on white blood cell DNA, including several cell type subsets. Previous studies reported small differences in LINE-1 methylation levels according to target CpG site and blood cell composition [338,343-346]. Thus, the distinctiveness of LINE-1 methylation patterns discourages the comparison between results from studies which evaluate LINE-1 methylation status at different CpG sites, and also reinforces the importance of accounting for cellular heterogeneity in future research.

In conclusion, for the first time, we described a positive association between adherence to Mediterranean diet and LINE-1 methylation in healthy women. Since the methylation level in the repetitive elements decreases over time, the adherence to Mediterranean diet could be proposed as a healthy behaviour to counteract the normal aging process. However, given that DNA methylation may contribute to the risk of cancer, metabolic disorders and degenerative diseases, further outcome-driven researches should prospectively evaluate the effect of healthy diet and lifestyle on LINE-1 methylation.

Tables and Figures

Table 1. Characteristics of study population.

Population Characteristics (N=299)	Mean (SD) or Proportion
Age, years	38.99 (16.53)
Low Educational Level	30.1%
Unemployed	54.2%
Current smokers	21.2%
BMI, kg/m^2	24.55 (5.04)
Underweight	4.7%
Normal Weight	57.4%
Overweight	24.0%
Obese	13.9%
Dietary Folate Intake, µg/day	295.36 (132.62)
Folic Acid Supplement Users	18.1%
Folate Deficiency	47.5%
MDS	4.64 (1.78)
Low Adherence	27.1%
Medium Adherence	58.2%
High Adherence	14.7%
LINE-1 Methylation Level, % 5mC	67.66 (7.48)

Abbreviations: SD, standard deviation; BMI, Body Mass Index, 5mC, 5-Methyl-Cytosine

Figure 1. Distribution of LINE-1 methylation by population characteristics.

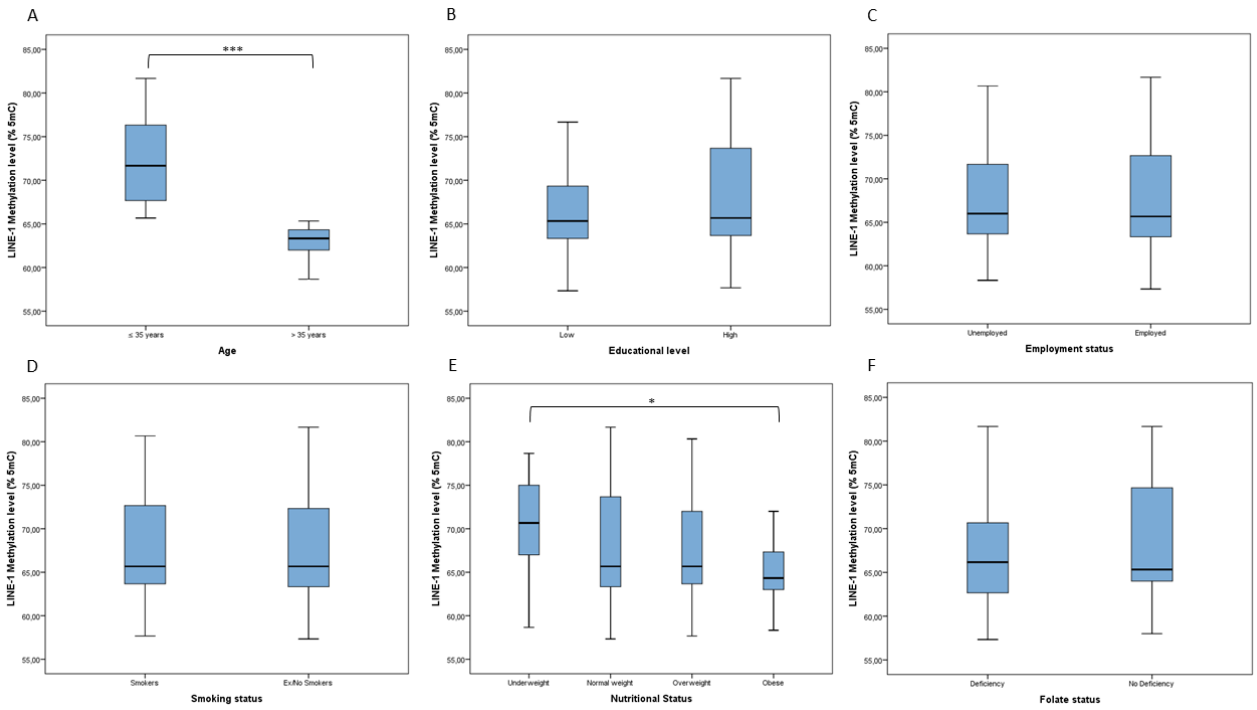
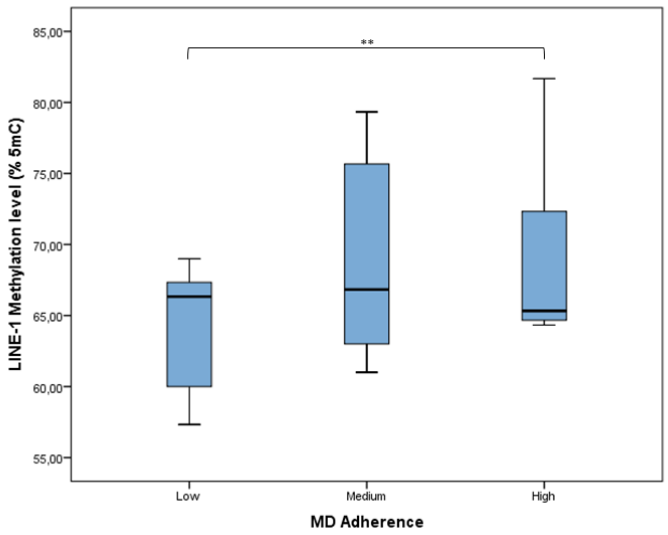


Figure 2. Distribution of LINE-1 methylation by categories of adherence to Mediterranean Diet



3 Association between dietary habits and risk factors of retinal degeneration: the Kardiovize Brno 2030 Study

3.1 Background

Although the most recognized risk factors of AMD are certainly age and smoking [55,347], the analogy between drusen deposition and the development of atherosclerosis [348] raised the question if cardiovascular disease and AMD share several of the same risk factors. However, while AMD patients showed an increased risk of stroke and coronary heart disease [349,350], the reverse has not been proven [351]. Among the most investigated cardiovascular risk factors, central obesity [352-354] and blood pressure (BP) [67,355,356] have received much attention for their potential role in AMD onset and progression. However, findings are currently controversial - particularly for BP - with some studies demonstrating that AMD risk increased with increasing blood pressure [355-357], while others failed in confirming this association [62,353,354,358,359]. Increasing body weight may cause physio-pathological changes such as oxidative stress, inflammatory response, and imbalance of blood lipids, which in turn are involved in AMD pathogenesis [354]. In addition, excessive body fat may affect the transport and deposition of carotenoids from blood to the retina, which ultimately cause a decline in the level of macular pigment [360]. In line with this evidence, some studies demonstrated an increased AMD risk with greater BMI, while others failed in confirming this association [67,355,356]. Controversial results were also reported for dyslipidaemia and diabetes [66,347,349,352,357,359].

DM and hyperglycaemia are certainly the strongest risk factors of DR, with glycaemic control and antidiabetic treatment exhibiting a protective role against DR development and progression in DM patients [233]. Despite several observational studies failed in demonstrating that blood pressure is a risk factor for DR incidence and progression [243,244,251], several RCTs pointed out the benefits of blood pressure control in DR management [245,246]. Findings on the effect of lipid on the development and progression of DR are currently controversial [247-249]. Some studies demonstrated that the severity of DR increased with increasing triglycerides and decreasing HDL levels [250], while others failed in confirming these associations [249]. As reported for AMD, two European prospective studies independently demonstrated the positive association of BMI and WHR with severity of DR [252,255].

One of the greatest challenges for Public Health is the management of risk factors - including obesity, fasting glucose, hypertension and higher lipid levels - through lifestyle interventions [361]. From this perspective, the promotion of healthy dietary habits represents one of the best approach. To explore the complex association of biological, environmental and behavioural risk factors with cardiovascular diseases, the Kardiovize Brno 2030 study - which recruited a randomly-selected sample of residents in an urban population of Brno, Czech Republic – has been recently designed [313]. The current cross-

sectional analysis, using data from the Kardiovize Brno 2030 cohort, aimed to evaluate the association of dietary habits - in terms of quality and frequency - with shared risk factors between cardiovascular disease and retinal degeneration.

3.2 Methods

3.2.1 Study design

Kardiovize Brno 2030 is a prospective cohort study on a random sample of residents of the city of Brno, Czech Republic [313]. Recruitment and baseline examinations were completed in 2014 with planned follow-up at 5-year intervals until 2030. The study was conducted in accordance with the Declaration of Helsinki and study protocol was approved by the ethics committee of St Anne's University Hospital, Brno, Czech Republic (reference 2 G/2012). Demographic socioeconomic information, lifestyle and medical history of diseases were collected through face-to-face comprehensive interviews. Anthropometric assessment was assessed by trained researchers according to standardized techniques, and previously described protocols were followed for physical examinations and laboratory analyses[313]. All data were stored in the web-based research electronic data capture (REDCap) [362]. For the current analysis, we excluded patients with previous/current cardiovascular disease and those who had no complete information about dietary habits.

3.2.2 Assessment of cardio-metabolic parameters

Anthropometric assessment was performed by trained professionals after overnight fasting according to previously described protocols [313]. Briefly, height and weight were measured to the nearest using a medical digital scale with meter (SECA 799; SECA, GmbH and Co. KG, Germany) and BMI was calculated as weight in kilograms divided by height in meters squared. Accordingly, subjects were classified as underweight (< 18.5 kg/m^2), normal weight (18.6–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obese (≥ 30 kg/m^2). Waist and hip circumferences were measured by using a manual measuring tape and WHR was calculated by dividing the waist circumference by hip circumference. Central obesity was defined as waist circumference ≥94 cm in men and ≥80 cm in women according to ethnic specific values. Body fat mass (BFM) was assessed using a direct segmental multi-frequency bioelectrical impedance analysis (InBody 370; BIOSPACE Co., Ltd., Korea).

Cardio-metabolic parameters were assessed following the standard study protocol [313]. Briefly, blood pressure was measured using a mercury sphygmomanometer (Baumanometer, W.A. Baum, Co., Inc., USA). Biochemical analyses were performed on 12-hour fasting full blood samples using a Modular SWA P800 analyzer (Roche, Basel, Switzerland) with total cholesterol, triglycerides, glucose and creatinine assayed by the enzymatic colorimetric method (Roche Diagnostics GmbH, Germany), HDL-cholesterol by the homogeneous method for direct measuring without precipitation (Sekisui

Medical, Japan). LDL-cholesterol was calculated according to the Friedewald equation only if triglyceride levels were lower than 4.5mmol/L; if higher, LDL-cholesterol was assayed by the homogeneous method for direct measuring (Sekisui Medical, Japan). Hypertension was defined as BP greater than or equal to 140/90 mmHg, or a prior diagnosis of hypertension or taking antihypertensive drugs. Hyperlipidaemia was defined as having either total cholesterol greater than or equal to 5.0 mmol/L, or LDL cholesterol greater than or equal to 3 mmol/L, or triglycerides greater than or equal to 1.7 mmol/L or taking lipid-lowering drugs. Diabetes mellitus was defined as a prior diagnosis of diabetes mellitus, a fasting glucose ≥7 mmol/L, or taking antidiabetic drugs.

3.2.3 Lifestyle assessment

Physical activity level for walking, moderate, and vigorous activities was assessed using the long version of the International Physical Activity Questionnaire (IPAQ-L) [363] translated in Czech, which includes the following domains: leisure time, work/commuting, home and garden/yard. Physical activity was reported as Metabolic Equivalent of Task (MET-min/wk) and classified as follows: high (vigorous-intensity activity on at least 3 days and accumulating at least 1500 MET-minutes/week or 5 or more days of any combination of walking, moderate-intensity or vigorous intensity activities achieving a minimum of at least 3000 MET-minutes/week); moderate (3 or more days of vigorous activity of at least 20 minutes per day or 5 or more days of moderate-intensity activity or walking of at least 30 minutes per day or 5 or more days of any combination of walking, moderate-intensity or vigorous intensity activities achieving a minimum of at least 600 MET-min/week); low (subjects who not meet criteria for categories 2 or 3) [363]. For smoking status, subjects were classified as no smokers (never being a smoker or having quit > 12 months) and current smokers (including both daily and occasionally smokers).

3.2.4 Dietary assessment

Dietary assessment was performed by a 43-item FFQ - using the previous week as reference period - and additional questions on specific dietary behaviors. The intake of alcohol drinks (i.e. wine, beer, dessert wines and spirits) was assessed using beverage-specific weekly recall. For each food item, participants indicated frequency of consumption classified into seven categories, ranging from “almost never” to “six or more times a day”. Standard portion sizes were attributed to each food item based on an individual dietary survey on the national level conducted on representative sample of the Czech population [364,365]. Food intakes were derived from the FFQ by multiplying frequency of consumption by standard portion size of each food item. Total energy intake was calculated using the NutriDan software. Food intakes were adjusted for total energy intake using the residual method [366]. To avoid the potential influence of outliers, subjects in the 5th and 95th percentiles of total energy

intake were excluded from further analyses. Participants were also asked to indicate the time of the day when they usually had main meals (i.e. breakfast, lunch and dinner) and snacks (i.e. at morning, afternoon and between dinner and bedtime). Participants were also asked to indicate number of eating occasions they usually consumed per day as the number of eating or drinking episodes containing calories. Accordingly, eating time interval was estimated as the elapsed time between the first and last eating occasion, while eating frequency was defined as the number of eating occasions per day.

3.2.5 Principal Component Analysis

A posteriori dietary patterns were derived using principal component analysis (PCA). Food items were classified into 31 predefined food groups, based on the similarity of nutrient profiles or culinary usage. Individual food items that constituted a distinct item on their own (e.g., pasta, pizza, or eggs, etc.) or that represent a particular dietary pattern (e.g., alcoholic drinks and fries, etc.) were preserved. Factors analysis was performed on energy-adjusted intakes followed by orthogonal transformation (varimax rotation), which maintains uncorrelated factors and facilitates interpretability. The number of dietary patterns was chosen according to eigenvalues >2.0, Scree plot examination, and interpretability. Factor loadings with absolute value ≥0.250 were retained to define food groups that characterized each dietary pattern. To confirm internal reproducibility, we separately performed factor analysis in two randomly selected subgroups by using the same abovementioned approach. For each dietary pattern, factor scores were computed by summing the products between observed energy-adjusted food group intakes and their factor loadings. For each dietary pattern, factor scores were categorized by tertiles (T1= low adherence; T2=medium adherence; T3= high adherence); the lowest tertile (T1) of each dietary pattern was used as the reference for further analyses.

3.2.6 Cluster analysis

To identify clusters of subjects with similar eating habits, the following variables were standardized and imputed in the cluster analysis: eating time interval and frequency; time of the first and last eating occasion; the minimum, maximum and average interval between eating occasions; skipping breakfast, morning snack, lunch, afternoon snack, dinner or after-dinner snack. The cluster analysis was conducted using the TwoStep Clustering method, an exploratory tool designed to reveal natural clusters within a dataset that would otherwise not be apparent. The optimal number of clusters was selected automatically by the clustering algorithm based on Schwarz's Bayesian Information Criterion. The algorithm employed by this procedure is able to handle categorical and continuous variables and to select the number of clusters by comparing the values of a model-choice criterion across different clustering solutions. Log-likelihood was chosen as a distance measure between individual data vectors [367].

3.2.7 Statistical analyses

All statistical analyses were conducted using the SPSS software (version 22.0, SPSS, Chicago, IL). The Kolmogorov-Smirnov test was used to assess the normal distribution of variables. Descriptive statistics were used to characterize the population, using frequency or mean and SD. Correlations between quantitative variables were evaluated by Pearson's and Spearman's correlation tests; results were reported as correlation coefficient (Rco). Comparisons between variables were analysed by the two-tailed Chi-squared test for categorical variables, and the Student's t test for continuous variables (data reported as mean and standard error, SE). The distribution of variables by categories of dietary habits was calculated and tested for linear trend, using the analysis of variance (ANOVA) for continuous variables and the Chi-squared linear trend for categorical variables. The analysis of covariance (ANCOVA) followed by Bonferroni correction was used to examine risk factors, adjusting for the effect of age, total energy intake and BMI. The adjusted p-values were obtained by multiplying the unadjusted p-value by the number of comparison. Binary logistic and linear regression models were used to estimate the odds ratio (OR) and the corresponding 95% confidence intervals (95%CI). The models were adjusted for age, sex, smoking, total energy intake, BMI, hypertension, hyperlipidaemia, diabetes mellitus and drug treatments. Eating meals categories, cluster 1 and T1 were chosen as reference. All statistical tests were 2-sided, and p values less than 0.05 were considered statistically significant.

3.3 Results

3.3.1 Characteristics of study population

A total of 2103 participants with a median age of 47 years (IQR=19 years; range= 25-65 years) were included in the current cross-sectional analysis. Approximately half the sample (55.5%) was female and 23.4% were current smokers. The majority of participants were married (61.8%) and employed (81.9%). Total energy intake and physical activity level were 2001 kcal (IQR=912) and 3363 MET-min/wk (IQR=4699), respectively. Half the sample was overweight or obese (48%), with 51.9% subjects meeting or exceeding the cut-offs for central obesity. The prevalence of hypertension was 39.7%, and median values of systolic and diastolic BP were 117.0 mmHg (IQR=19.5) and 79.0 mmHg (IQR=13.0), respectively. The prevalence of hyperlipidaemia was 66.7% and median values of HDL and triglycerides were 1.52 (IQR=0.50) and 0.99 (IQR=0.76), respectively. The prevalence of diabetes was 10% and median fasting glucose level was 4.8 (IQR=0.7).

3.3.2 Dietary patterns

Based on the Scree plot examination (**Figure 1**), we derived two major dietary patterns with eigenvalues ≥2.0 which explained 13.73% of total variance among 31 food groups. Figures 2a and 2b

show factor loadings characterizing each dietary pattern. The first dietary pattern - named western - was positively characterized by the intakes of white bread, processed meat, fries, hamburger, hot-dog and salty snacks. By contrast, the second dietary pattern - named prudent - was positively characterized by the intakes of cereals, jam and honey, fish, fruit, raw and cooked vegetables and nuts (**Figures 2 and 3**). The comparison of population characteristics by dietary pattern tertiles found that higher adherence to the western dietary pattern was associated with lower age and percentage of unemployed, as well as with higher total energy intake and percentage of male. No statistically significant differences were observed across tertiles of prudent dietary pattern.

3.3.3 Dietary patterns and cardio-metabolic parameters

Table 1 illustrates cardio-metabolic parameters by tertiles of dietary patterns. Notably, from T1 to T3 of western dietary pattern, we observed increasing weight, waist circumference, WHR, triglycerides and total cholesterol/HDL cholesterol ratio increased, while glycated hemoglobin and HDL cholesterol decreased (p-values<0.05). By contrast, from T1 to T3 of prudent dietary pattern, we observed that anthropometric measures (i.e. weight, BMI, BFM, waist and hip circumferences, prevalence of overweight/obese and abdominal obese subjects), as well as systolic and diastolic blood pressure decreased (p-values<0.05). Interestingly, after adjusting for age and sex, higher adherence to the western dietary pattern was associated with increased odds of abnormal blood pressure (systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg, or drug treatment for hypertension), triglycerides (≥1.7 mmol/L or drug treatment for lipid abnormality) and fasting glucose (≥5.6 mmol/L or treatment for type 2 diabetes) (p-trend=0.009; p-trend=0.005; p-trend=0.015, respectively). In fact, subjects in T2 and in T3 had higher odds of abnormal blood pressure (OR=1.55, 95%CI=1.17-2.05, p=0.002; OR=1.40, 95%CI=1.04-1.88, p=0.026; respectively), triglycerides (OR=1.44, 95%CI=1.04-2.00, p=0.029; OR=1.64, 95%CI=1.17-2.31, p=0.005; respectively) and fasting glucose (OR=1.72, 95%CI=1.07-2.78, p=0.026; OR=1.72, 95%CI=1.07-2.80, p=0.028; respectively), than those in T1. After adjusting for age, sex, marital status, employment, educational level, smoking, BMI, total energy intake, and physical activity, we confirmed that subjects in T3 had higher odds of abnormal triglycerides (OR=1.56, 95%CI=1.02-2.39, p=0.003).

By contrast, higher adherence to the prudent dietary pattern was associated with lower odds of central obesity and abnormal blood pressure, after adjusting for age and sex (p-trend=0.001 and p-trend=0.048, respectively), with subjects in T3 exhibiting lower odds of central obesity (OR=0.63, 95%CI=0.48-0.83, p=0.001), abnormal blood pressure (OR=0.76, 95%CI=0.57-0.99, p=0.049), and fasting glucose (OR=0.60, 95%CI=0.39-0.96, p=0.035) than those in T1. The multivariable-adjusted

model confirmed that subjects in T3 had lower odds of central obesity (OR=0.63, 95%CI=0.48-0.84, p=0.001) and abnormal fasting glucose (OR=0.58, 95%CI=0.35-0.94, p=0.039).

3.3.4 Eating timing, frequency and cardio-metabolic parameters

In a sub-sample of 1659 subjects who provided the detailed schedule of meal consumption, we assessed eating time interval and frequency. Particularly, the eating time interval was 11.00 hours (SD=2.07) and ranged from 1 hour, for subjects who ate only one meal a day, to 19 hours. According to Pearson correlation coefficients, we found that eating time interval was negatively correlated with BMI (Rco= -0.073; p=0.002), body fat mass (BFM; Rco= -0.113; p<0.001), total cholesterol (Rco=-0.097; p<0.001), triglycerides (Rco=-0.051; p=0.040), LDL cholesterol (Rco= -0.065; p=0.008), total cholesterol/HDL ratio (Rco= -0.059; p=0.016). The eating frequency ranged from one to six meals a day with a mean of 4.16 meals/day (SD=0.91). Consistently with eating time interval, eating frequency was negatively correlated with BMI (Rco= -0.097 p<0.001), WHR (Rco= -0.110; p<0.001), systolic and diastolic pressure (Rco= -0.077; p<0.001 and Rco= -0.084; p<0.001), BFM (Rco= -0.069; p=0.004), fasting glucose (Rco= -0.125; p<0.001), total cholesterol (Rco= -0.063; p=0.011), triglycerides (Rco= -0.081; p=0.001), LDL cholesterol (Rco= -0.059; p=0.016) and total cholesterol/HDL ratio (Rco= -0.075; p=0.002).

3.3.5 Skipping meals and cardio-metabolic parameters

The proportion of participants who skipped each eating occasion was 10.5% for breakfast, 48.9% for morning-snack, 2.4% for lunch, 29.8% for afternoon-snack, 3.9% for dinner and 89.0% for after-dinner snack. After adjusting for age, total energy intake and BMI, skipping breakfast was associated with higher LDL cholesterol levels (p=0.035); skipping lunch with higher diastolic pressure (p=0.002); skipping dinner with higher values of triglycerides (p<0.001) and total cholesterol/HDL ratio (p=0.003) (**Table 2**). After adjusting for age, sex, smoking, total energy intake, BMI, hypertension, hyperlipidaemia, diabetes mellitus and drug treatments), skipping breakfast was associated with abnormal cholesterol (OR=2.433; 95%CI= 1.720-3.442; p=0.001 and OR=1.687; 95%CI= 1.105-2.575; p=0.015, respectively); skipping the afternoon snack was associated with abnormal BMI and cholesterol (OR=1.635; 95%CI= 1.265-2.112; p=0.001; OR=1.523; 95%CI= 1.145-2.025; p=0.004; OR=1.648; 95%CI= 1.066-2.401; p=0.010, respectively); after-dinner snack skipping were less likely to be classified into abnormal BMI category (OR=0.589; 95%CI= 0.396-0.874; p=0.004).

3.3.6 Clusters of eating habits and cardio-metabolic parameters

Our hypothesis was that skipping meals as well as eating time interval and frequency could define distinct clusters of eating habits with specific effects on cardio-metabolic parameters. The TwoSteps

cluster analysis identified three clusters. Briefly, cluster 1 was characterized by late consumption of the first meal (8:51 a.m.) and early consumption of the last meal (6:25 p.m.), with an eating time interval of 9.35 hours (SE=0.07). Subjects belonging to this cluster ate 2.86 meals/day (SE=0.02), with an average interval between meals of 5.28 hours (SE=0.09). Compared to others, cluster 1 was characterized by the highest percentages of subjects who skipped meals. In cluster 2, subjects had the first meal at 7:55 a.m. and the last meal at 6:56 p.m., with an eating time interval of 11.03 hours (SE=0.03). Subjects belonging to this cluster ate 4.00 meals/day (SEM=0.03), with an average interval between each meal of 4.23 hours (SE=0.04). Compared to others, cluster 2 showed intermediate percentages of subjects who skipped meals. Cluster 3 was characterized by early consumption of the first meal (7:27 a.m.) and consumption of the last meal at 7:18 p.m., with an eating time interval of 11.52 hours (SE=0.03). Subjects belonging to this cluster ate 5.11 meals/day (SEM=0.01), with an average interval between meals of 3.56 hours (SE=0.04). Cluster 3 showed the lowest percentages of subjects who skipped meals. In summary, from cluster 1 to cluster 3, eating time interval and frequency increased (p-trends <0.001) whereas the intervals between eating occasions and the percentages of subjects who skipped meals decreased (p-trends <0.001). Distributions of cardio-metabolic parameters by clusters of eating habits are shown in **Table 3**; particularly, from clusters 1 to 3, BMI, WHR, as well as the prevalence of hypertension and hyperlipidaemia decreased. Similarly, BFM, systolic pressure, diastolic pressure, fasting glucose, triglycerides, cholesterol and cholesterol/HDL-cholesterol ratio decreased (p-trends<0.05). After adjusting for age, total energy intake and BMI, the difference in diastolic pressure between clusters remained significant. Adjusting for age, sex, smoking, total energy intake, BMI, hypertension, hyperlipidaemia, diabetes mellitus and drug treatments, subjects in clusters 3 were less likely to be classified into abnormal category of BMI and blood pressure (OR=0.660; 95%CI=0.465-0.936; p=0.020; OR=0.721; 95%CI=0.526-0.988; p=0.042, respectively).

3.4 Discussion

The present cross-sectional study investigated the possible impact of dietary habits - in terms of quality and timing - on shared risk factors between cardiovascular disease and retinal degeneration. Since these disorders show the same cardio-metabolic risk profile, then it follows that they should share similar pathophysiological features, such as central obesity, hyperglycaemia, hypertension and hypertriglyceridemia [368].

To evaluate the effect of dietary habits, we first derived two dietary patterns: the first pattern - named "western" - was characterized by high intake of white bread, processed meat, fries, hamburger, hot-dog and salty snacks; the second one - named "prudent" - was characterized by high intake of cereals, jam and honey, fish, fruit, raw and cooked vegetables, and nuts. We observed that weight, waist

circumference and WHR increased with increasing adherence to western dietary pattern, but we failed in replicating previous findings reporting that unhealthy dietary habits may increase the risk of central obesity [369-372]. After adjusting for age and sex, medium-high adherence to the western dietary pattern was associated with increased odds of abnormal blood pressure, triglycerides and fasting glucose, when compared to low adherence. This is in line with the evidence western dietary pattern was associated with blood pressure and serum lipids levels [373-375]. By contrast, other studies found that a diet rich in animal products affected HDL level but not blood pressure, LDL and triglycerides [376,377]. However, we confirmed a robust positive association between adherence to western dietary pattern and abnormal triglycerides, by adding to the regression model both socio-demographic (i.e. marital status, employment and educational level) and behavioural factors (i.e. smoking, BMI, total energy intake and physical activity).

In addition, we found that blood pressure and prevalence of central obesity decreased with increasing adherence to prudent dietary pattern. In fact, after adjusting for age and sex, subjects with high adherence had lower odds of central obesity, abnormal blood pressure and fasting glucose, than those with low adherence. We also confirmed a robust association of adherence to prudent dietary pattern with central obesity and abnormal fasting glucose, by adding to the model both socio-demographic (i.e. marital status, employment and educational level) and behavioural factors (i.e. smoking, BMI, total energy intake and physical activity). Here again, our results are in line with previous studies demonstrating the protective effect of healthy dietary patterns against the risk of central obesity [370,378-380], impaired fasting glucose [381], insulin resistance [382], and diabetes [383]. Since all these risk factors are related to retinal degeneration, it has been similarly proved that adherence to a prudent dietary pattern reduced the risk of AMD [209,384] and DR [277]. Beyond quality of diet, meal timing and frequency vary dramatically across cultures, change over time, and may profoundly affect cardio-metabolic parameters. Data from the National Health and Nutrition Examination Survey show that many Americans have moved away from the typical three-meal-a-day pattern and replaced it with a preference for snacking [385,386]. Particularly, energy intake consumed at breakfast is inversely associated with weight gain, elevated LDL-cholesterol, low HDL-cholesterol, and elevated blood pressure [387-389]. Our data showed that skipping breakfast increased LDL-cholesterol, adding to the well-established relationship between skipping breakfast and increased obesity, cardio-metabolic risk and chronic diseases [390-395]. Despite this evidence - especially in developed countries - the prevalence of eating breakfast and lunch has seen a great decline over the past decades [385,396], raising the need of preventive strategies to tackle these unhealthy habits.

Contrary to breakfast, lack of evidence exists about the effect of missing lunch on health and cardio-metabolic parameters. In our population, participants who skipped lunch showed higher diastolic

pressure than non-skippers. Blood pressure follows a nearly 24 hours rhythmicity that is lost in hypertension [397] and in obesity [398]. The role of meal timing in regulating the circadian patterns of metabolism has been investigated mainly by observational studies [399,400]. At the molecular level, circadian rhythms in gene expression synchronize metabolic processes with the external environment, allowing the organism to function effectively in response to physiological challenges [401-404]. In mammals, this daily timekeeping is driven by the biological clocks of the circadian timing system, composed of master molecular oscillators within the suprachiasmatic nuclei of the hypothalamus, pacing self-sustained and cell-autonomous molecular oscillators in peripheral tissues through neural and humoral signals [401-404]. A recent study showed that breakfast skipping adversely affects clock and clock-controlled gene expression and is correlated with increased postprandial glycaemic response in both healthy and diabetic individuals [405]. Our finding highlights the need for more in-depth study of the association between time-of-day of eating occasions and blood pressure.

There are suggestions about the effect of evening meals on BMI and weight control; however, the evidence is controversial and scarce in the matter of skipping dinner. Previous observational studies demonstrated a positive association between large evening intake and BMI [406-408], but others showed no association [409,410] or an inverse relationship [411]. We found evidence that skipping dinner increased triglycerides level and total cholesterol/HDL ratio, suggesting a potential impact on the risk of cardiovascular disease and retinal degeneration. Similarly, the relationship between snacking patterns and cardio-metabolic parameters is controversial[412]. An accepted definition of snack, to distinguish it from a meal, is that a snack contributes to <15% of total daily energy intake, while a meal provides ≥15% of total energy intake[413]. Eating more frequently is often recommended as a strategy for weight loss, because it is believed to reduce appetite and thus energy intake [414]. While snacking in a non-hungry state is certainly detrimental to energy regulation and likely leads to weight gain[393], other studies reported a significant impact of meal frequency on blood pressure: consuming 1 meal a day increased both systolic and diastolic blood pressures (1% increase from baseline) after 8 weeks of treatment; when these same individuals crossed over to a regimen of 3 meals a day, systolic and diastolic blood pressures decreased by 6% and 4%, respectively [415]. Moreover, an analysis of meal patterns in ~19000 adults participating in the 2001-2008 National Health and Nutrition Examination Survey showed that snacking patterns are not associated with cardio-metabolic risk factors, but that snack consumption was associated with better diet quality, compared to individuals consuming no snacks [416].

There are some limitations to our study. The cross-sectional design does not allow demonstrating the causality of the relationships. Moreover, food intakes were estimated using a FFQ with standard portion sizes, which does not preclude measurement error and may suffer from inaccuracies.

Nevertheless, standard portion sizes were obtained from an individual dietary survey on the national level, which involved age and gender representative sample of the Czech population [365]. Moreover, foods composition of our PCA-derived dietary patterns was consistent with those identified by previous studies across different European populations [371,376,417-419]. Another potential weakness is that meal timing and frequency may be directly associated with metabolic changes, but they may be also a proxy for other behaviours, such as physical activity, alcohol consumption or sleeping.

However, our study has also several strengths, including the large sample that was randomly selected from the urban population of Brno, Czech Republic. Therefore, these results - obtained from a nationally representative sample - can be extrapolated to the general Czech population. Moreover, we used clearly specified measurements of dietary practices and standard and validated protocols for cardio-metabolic and anthropometric assessment, which allowing comparisons with other well-designed studies. Given the high quality of our data, we were able to apply factorial analyses to derive dietary patterns/clusters - the most comprehensive approaches that have been successfully applied to understand the effect of dietary pattern on health and diseases [416,420-423] - and to confirm the robustness of our results adjusting for several socio-demographic and behavioural factors.

In conclusion, on one hand, we confirmed the deleterious effect of a western dietary pattern on several cardio-metabolic risk factors, on the other hand, we demonstrated that the consumption of a diet rich in cereals, fish, fruit and vegetables is associated with a healthier cardio-metabolic profile. Interestingly, we demonstrated that eating more frequently and consuming breakfast might be an effective long-term preventive tool to tackle weight gain and to maintain cardio-metabolic parameters at proper levels. Since cardiovascular disease and retinal degeneration exhibit the same metabolic risk profile - sharing similar pathophysiological features - public health professionals could benefit from our findings, developing and validating novel potential preventive strategies against cardiovascular disease, AMD and DR.

Tables and Figures

Table 1. Cardio-metabolic parameters of study participants by tertiles of dietary patterns.

Characteristics	Western Dietary Pattern				Prudent Dietary Pattern			
	T1 (N=668)	T2 (N=684)	T3 (N=661)	p-value[a]	T1 (N=669)	T2 (N=678)	T3 (N=666)	p-value[a]
Weight, Kg[b]	72.4 (19.8)	78.2 (24.0)	77.5 (23.7)	**0.002**	77.2 (22.5)	76.6 (24.7)	74.1 (22.1)	**0.006**
BMI, Kg/m^{2}[b]	24.5 (5.2)	25.2 (6.2)	24.8 (5.9)	0.128	25.1 (6.0)	25.2 (5.7)	24.3 (5.4)	**<0.001**
BMI categories (%)								
Underweight	3.9%	2.5%	2.3%	0.425	2.5%	1.6%	4.5%	**0.005**
Normal weight	49.7%	45.8%	48.9%		45.9%	45.9%	52.5%	
Overweight	32.8%	34.7%	34.0%		34.6%	35.6%	31.4%	
Obese	13.6%	17.0%	14.9%		17.1%	16.9%	11.6%	
Waist circumference[b], cm	85.0 (18.0)	87.5 (21.0)	88.0 (21.0)	**0.001**	88.0 (19.8)	87.0 (20.0)	85.0 (18.0)	**0.001**
Hip circumference[b], cm	102.0 (11.0)	102.0 (10.8)	101.0 (10.0)	0.236	102.0 (10.0)	103.0 (11.0)	101.0 (9.0)	**0.001**
WHR[b]	0.83 (0.13)	0.85 (0.14)	0.87 (0.14)	**<0.001**	0.86 (0.14)	0.85 (0.13)	0.84 (0.15)	0.075
Central Obesity (%)	52.8%	54.6%	50.3%	0.401	56.3%	55.9%	45.8%	**0.001**
Body Fat Mass[b], Kg	18.4 (12.4)	18.6 (13.0)	17.5 (11.4)	0.121	19.3 (12.7)	18.7 (12.2)	16.9 (10.8)	**<0.001**
Systolic Blood Pressure, mmHg[b]	116.5 (19.5)	117.5 (18.0)	116.5 (19.5)	0.341	117.5 (19.5)	117.5 (20.3)	115.5 (18.5)	**0.022**
Diastolic Blood Pressure, mmHg[b]	78.3 (12.5)	80.0 (13.0)	79.0 (14.0)	0.087	79.5 (13.5)	80.0 (13.3)	78.0 (12.0)	**0.021**
Glycated Haemoglobin, nmol/mol[b]	40.0 (5.0)	39.0 (5.0)	39.0 (5.0)	**0.002**	39.0 (6.0)	39.0 (5.0)	39.0 (6.0)	0.279
Fasting Glucose, nmol/l[b]	4.8 (0.6)	4.9 (0.7)	4.8 (0.6)	0.187	4.8 (0.7)	4.9 (0.7)	4.8 (0.7)	0.157
Creatinine, nmol/l[b]	73.0 (17.3)	75.0 (18.8)	78.0 (18.0)	**<0.001**	75.0 (19.0)	76.0 (17.5)	74.0 (18.0)	0.281
Triglycerides, nmol/l[b]	0.9 (0.7)	1.0 (0.9)	1.1 (0.8)	**0.001**	1.0 (0.7)	1.0 (0.8)	1.0 (0.9)	0.278
Total Cholesterol, nmol/l[b]	5.1 (1.4)	5.2 (1.4)	5.1 (1.3)	0.061	5.1 (1.3)	5.2 (1.3)	5.1 (1.3)	0.687
HDL Cholesterol, nmol/l[b]	1.6 (0.5)	1.5 (0.5)	1.4 (0.5)	**<0.001**	1.5 (0.5)	1.5 (0.5)	1.5 (0.5)	0.206
LDL Cholesterol, nmol/l[b]	3.1 (1.3)	3.0 (1.2)	3.0 (1.2)	0.388	3.0 (1.2)	3.1 (1.2)	3.0 (1.3)	0.761
Total Cholesterol/HDL-ratio[b]	3.2 (1.3)	3.3 (1.4)	3.4 (1.6)	**0.001**	3.3 (1.4)	3.3 (1.4)	3.3 (1.4)	0.578

Abbreviations: T tertile; IQR Interquartile range. BMI body mass index; WHR waist-to-hip ratio.

[a] Statistically significant p-values ($p < 0.05$) are indicated in bold font. [b] Data reported as median (IQR).

Table 2. Adjusted means of cardio-metabolic parameters according to categories of skipping meals*

CVD risk factors	Breakfast			Morning snack			Lunch			Afternoon snack			Dinner			Late-night dinner		
	Skippers (n=175)	Non-skippers (n=1484)	p-value†	Skippers (n=811)	Non-skippers (n=848)	p-value†	Skippers (n=40)	Non-skippers (n=1619)	p-value†	Skippers (n=495)	Non-skippers (n=1164)	p-value†	Skippers (n=64)	Non-skippers (n=1595)	p-value†	Skippers (n=1476)	Non-skippers (n=183)	p-value†
Systolic pressure (mmHg)	121.60 ±1.07	120.61 ±0.36	0.377	122.15 ±1.30	122.16 ±1.53	0.797	123.96± 2.18	120.69 ±0.35	0.139	121.02 ±0.62	120.61 ±0.41	0.584	117.62± 1.86	120.89 ± 0.35	0.084	120.76 ±0.36	120.64 ±1.06	0.917
Diastolic pressure (mmHG)	80.07 ±0.68	79.88 ±0.23	0.785	79.12 ±0.83	78.18 ±0.97	0.670	84.05 ±1.37	79.83 ±0.22	**0.002**	80.19 ±0.39	79.77 ±0.26	0.379	78.73 ±1.37	79.88 ±0.22	0.293	79.94 ±0.23	79.67 ±0.67	0.696
Body fat mass (Kg)	20.93 ±0.27	20.89 ±0.09	0.903	21.00 ±0.12	20.84 ±0.13	0.344	21.24 ±0.56	20.93 ±0.09	0.576	21.10 ±0.16	20.81 ±0.11	0.126	20.91 ±0.48	20.93 ±0.09	0.966	20.93 ±0.09	20.75 ±0.27	0.524
Visceral fat area (cm^2)	85.06 ±1.51	87.59 ±0.51	0.113	87.89 ±0.68	86.72 ±0.68	0.229	91.47± 3.07	87.30 ±0.49	0.179	88.22 ±0.87	88.88 ±0.58	0.199	88.85 ±2.62	87.33 ±0.49	0.569	87.35 ±0.51	86.86 ±1.49	0.757
Glycated haemoglobin (nmol/mol)	40.33 ±0.51	40.38 ±0.17	0.920	47.69 ±0.53	48.09 ±0.60	0.609	39.77 ±1.04	40.41 ±0.16	0.549	40.25 ±0.29	40.41 ±0.20	0.638	39.58 ±0.85	40.42 ±0.16	0.334	40.33 ±0.17	40.89 ±0.50	0.293
Glucose (nmol/l)	5.15 ±0.07	5.03 ±0.02	0.098	6.10 ±0.07	6.25 ±0.08	0.159	5.18 ±0.15	5.04 ±0.02	0.327	5.05 ±0.04	5.04 ±0.27	0.729	4.92 ±0.12	5.05± 0.02	0.297	5.04 ±0.02	5.12 ±0.07	0.232
Creatinine (nmol/l)	74.38 ±1.12	76.38 ±0.35	0.091	76.96 ±1.41	74.53 ±1.59	0.240	75.52 ±2.35	76.17 ±0.36	0.785	76.12 ±0.64	76.21 ±0.43	0.907	76.11 ±1.91	76.17 ±0.36	0.976	76.24 ±0.38	75.34 ±1.11	0.436
Cholesterol total (nmol/l)	5.27 ±0.09	5.12 ±0.01	0.136	5.19 ±0.04	5.10 ±0.04	0.095	5.25 ±0.19	5.14± 0.03	0.557	5.12 ±0.05	5.15 ±0.04	0.559	5.44 ±0.16	5.13 ±0.03	0.054	5.15 ±0.03	5.06 ±0.09	0.338
Triglycerides (nmol/l)	1.28 ±0.13	1.28 ±0.04	0.997	1.31 ±0.06	1.23 ±0.06	0.319	1.32± 0.26	1.28 ±0.04	0.889	1.25 ±0.08	1.29 ±0.05	0.596	2.18 ±0.22	1.24 ±0.04	**<0.001**	1.28 ±0.04	1.29 ±0.13	0.920
HDL-cholesterol (nmol/l)	1.59 ±0.03	1.53 ±0.01	0.135	1.54 ±0.01	1.54 ±0.01	0.968	1.56 ±0.06	1.53 ±0.01	0.621	1.54 ±0.02	1.54 ±0.01	0.999	1.57 ±0.05	1.53 ±0.01	0.395	1.54 ±0.01	1.49 ±0.03	0.067
LDL-cholesterol (nmol/l)	3.21 ±0.07	3.08 ±0.02	**0.038**	3.12 ±0.03	3.07 ±0.03	0.354	3.12 ±0.14	3.10 ±0.02	0.886	3.08 ±0.04	3.10 ±0.03	0.560	3.06 ±0.12	3.10 ±0.02	0.764	3.10 ±0.02	3.03 ±0.07	0.347
Cholesterol total/HDL-cholesterol	3.64 ±0.10	3.58 ±0.03	0.595	3.62 ±0.05	3.56 ±0.05	0.346	3.55 ±0.21	3.60 ±0.03	0.841	3.55 ±0.04	3.62 ±0.04	0.321	4.10 ±0.17	3.58 ±0.03	**0.003**	3.59 ±0.03	3.64 ±0.10	0.634

*Adjusted means for age, total energy intake and BMI by analysis of covariance (ANCOVA); †p-values<0.05 are in bold font

Abbreviations: SEM, standard error of the mean; HDL, high density lipoprotein; LDL, low density lipoprotein.

Table 3. Adjusted means of cardio-metabolic parameters according to clusters of eating habits.

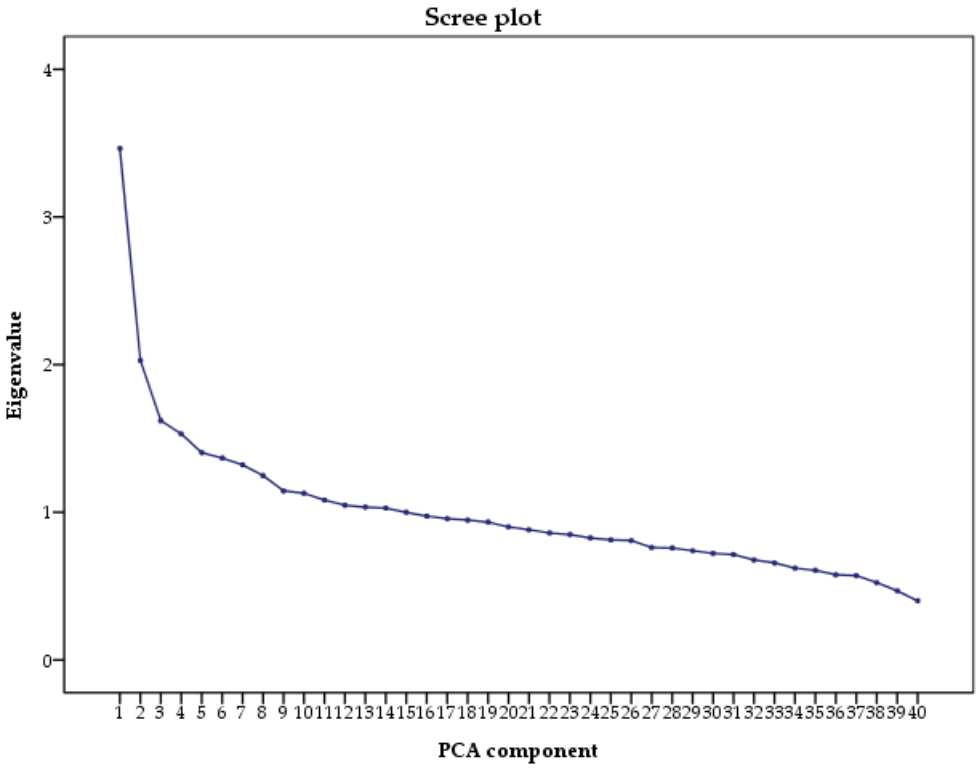
Characteristics	Adjusted means*†			
	Cluster 1 (n=388)	Cluster 2 (n=637)	Cluster 3 (n=634)	p-trend‡
Body fat mass (Kg)	20.76±0.44	20.29±0.42	20.95±0.43	0.129
Visceral fat area (cm^2)	86.76±2.45	86.22±2.33	88.47±2.39	0.211
Systolic pressure (mmHg)	126.45±1.72	122.36±1.63	121.61±1.67	**0.018**
Diastolic pressure (mmHg)	80.77±1.10	78.49±1.04	78.49±1.07	0.356
Glycated haemoglobin (nmol/mol)	46.19±0.65	49.09±0.61	47.20±0.62	0.742
Glucose (nmol/l)	6.01±0.09	6.17±0.09	6.17±0.08	0.065
Creatinine (nmol/l)	75.41±1.77	77.91±1.73	74.42±1.73	0.220
Cholesterol total (nmol/l)	4.79±0.14	4.52±0.14	4.51±0.14	0.232
Triglycerides (nmol/l)	1.53±0.21	1.27±0.20	1.40±0.20	0.671
HDL-cholesterol (nmol/l)	1.53±0.05	1.46±0.04	1.50±0.04	0.875
LDL-cholesterol (nmol/l)	2.58±0.10	2.52±0.10	2.45±0.10	0.310
Total Cholesterol/HDL-cholesterol	3.36±0.16	3.27±0.16	3.23±0.16	0.497

Results are reported as mean ± standard error

† adjusted means for age, total energy intake and BMI by analysis of covariance (ANCOVA)

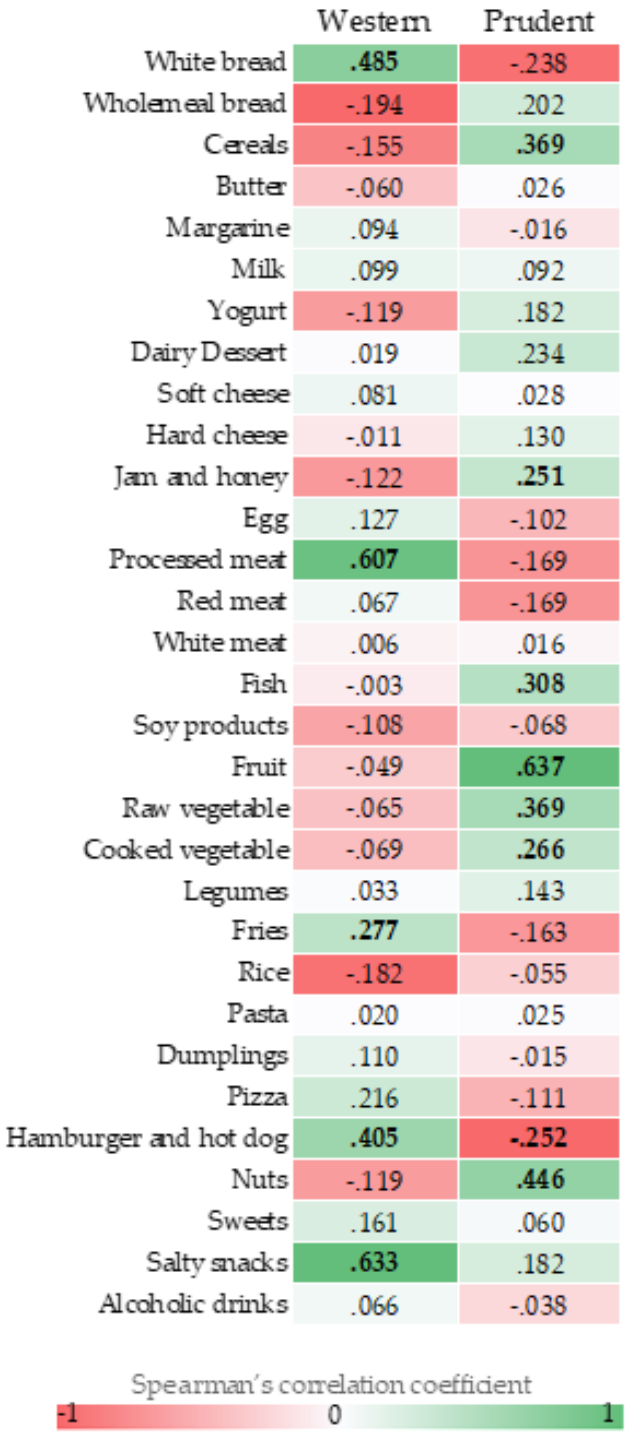
‡p-values<0.05 are in bold

Figure 1. Scree plot of the eigenvalues.



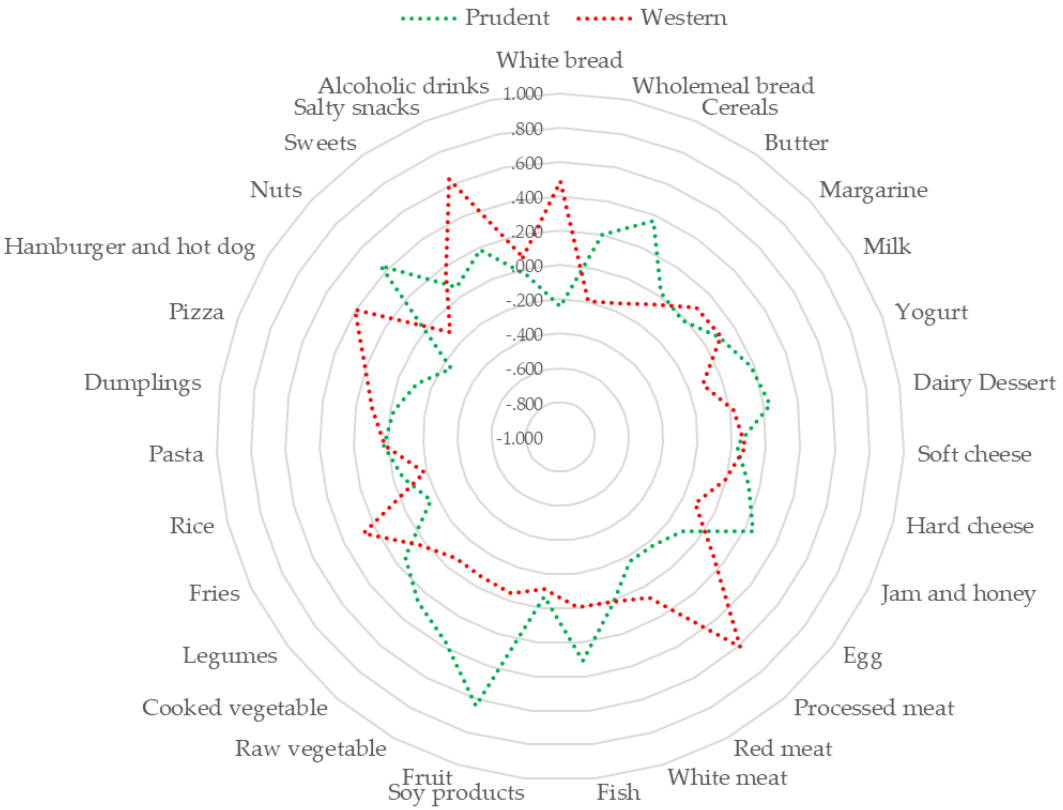
The scree plot examination was used to determine the appropriate number of dietary pattern. Scree plot represents the partitioning of the total variation (i.e. eigenvalue) accounted for each principal component, against the PCA component number.

Figure 2. Correlation matrix of factor loadings characterizing each dietary pattern.



Food groups that negatively characterize the dietary patterns are indicated in red. Food groups that positively characterize the dietary patterns are indicated in green. Factor loadings ≥|0.25|are in bold font.

Figure 3. Radar graph of factor loadings characterizing each dietary pattern.



Red line indicates factor loadings related to the western dietary pattern. Green line indicates the factor loadings related to the prudent dietary pattern.

4 Association between Complement Factor H rs1061170 polymorphism and age-related macular degeneration: a systematic review and meta-analysis

4.1 Background

AMD is a complex disorder which implicates socio-demographic (age and race), environmental (cigarette smoking, light exposure and unhealthy diet) and genetic risk factors [53]. Genetic variants confer at least the 60% of the attributable risk [424], with 34 genomic loci implicated in disease pathogenesis [81], especially those involved in complement system activity, lipid metabolism and angiogenesis [81,83,425]. Among the polymorphisms associated with the risk of developing AMD, the rs1061170 in the CFH gene has been extensively investigated via genetic and molecular approaches, providing strong evidence for disease association in a plausible biological context [52]. The polymorphism leads to an amino acid change at position 402 of the factor H polypeptide (Y402H), which substitutes a tyrosine residue with histidine. However, its prevalence varies between ethnicities and the strength of its association with AMD risk could differ between disease subtypes. Therefore, we performed a comprehensive systematic review and an updated meta-analysis to assess the association between the rs1061170 polymorphism and AMD, stratifying for stage of disease and ethnicity.

4.2 Methods

4.2.1 Search strategy

A systematic literature search in the PubMed-Medline, EMBASE and Web of Science databases, was conducted to identify relevant epidemiological studies, published before September 2017, investigating the association between rs1061170 polymorphism and AMD risk. The search strategy comprised the terms ("complement factor H" or "CFH") and ("age-related macular degeneration" or "AMD" or "ARMD" or "age-related macular disease" or "age-related maculopathy" or "ARM"). The search was limited to human studies and no language restrictions were imposed. Moreover, the reference lists from selected articles were checked to search for further relevant studies. After exclusion of irrelevant studies through reading titles and abstracts, full texts of eligible articles were reviewed to determine whether they provided relevant data on the topic of interest. The preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines were followed [426].

4.2.2 Inclusion/Exclusion Criteria and Data Extraction

Two of the investigators independently assessed eligibility of articles and extracted data from each study. Any inconsistencies were resolved through discussion. Studies were included in the meta-analysis only if they satisfied the following criteria: i) they used a case-control design or provided baseline data from prospective analysis; ii) AMD was diagnosed using a validated method; iii) they evaluated the associations between rs1061170 polymorphism and AMD; iv) they contained at least 2 comparison groups (a control group with no signs of AMD and a case group that included AMD); v) they provided sufficient genotype data to estimate odds ratios (ORs) and 95% confidence intervals (95%CIs). By contrast, exclusion criteria were as follow: i) no case-control or cross-sectional design; ii) inadequate case or control group; iii) insufficient or lacking data to estimate ORs and 95%Cis. If there were multiple publications from the same population, the article reporting the most comprehensive data was chosen for meta-analysis. Two of the investigators independently reviewed all the eligible studies and abstracted the following information in a standard format: first Author's last name, year of publication, country where the study was performed, ethnicity, the mean age and gender ratio of the participants, phenotype of the cases evaluated, sample size of subjects with and without AMD, the methods for genotyping , the genotype distributions in cases and controls, and P values for Hardy-Weinberg equilibrium (HWE) in controls. If additional data were needed, the Authors of retrieved articles were contacted.

4.2.3 Quality assessment

The quality of studies included in the meta-analysis was independently assessed by two of the investigators using the Newcastle-Ottawa scale (NOS) for case-control study [427]. In this scale, three perspectives were carefully checked and scored: selection (0 to 4 points); comparability (0 to 2 points); and exposure (0 to 3 points). Total scores ranged from 0 (worst) to 9 (best), and a study with a score ≥ 7 indicates good quality. Disagreements were adjudicated by a third Author.

4.2.4 Statistical analyses

For each study, the Chi-square test was used to determine if genotype distribution in the control groups deviated from the Hardy-Weinberg Equilibrium (HWE). The strength of the association between the rs1061170 polymorphism and AMD was estimated as ORs (95% CIs) under the allelic model (C vs. T), the homozygous model (CC vs. TT) and the heterozygous model (CT vs. TT). The significance of pooled OR was determined by the *Z* test. Heterogeneity across studies was measured using the Q test, considering significant statistical heterogeneity as $p < 0.1$. As the Q test only indicates the presence of heterogeneity and not its magnitude, we also reported the I^2 statistic, which estimates the percentage of outcome variability that can be attributed to heterogeneity across studies. An I^2

value of 0% denotes no observed heterogeneity, whereas, 25% is “low”, 50% is “moderate” and 75% is “high” heterogeneity [428]. We also estimated the between-study variance using tau-squared (t) statistics [429]. According to heterogeneity across studies, we used the fixed-effects model (Mantel–Haenszel method) when heterogeneity was negligible or the random-effects models (DerSimonian-Laird method) when heterogeneity was significant. Furthermore, we conducted subgroup analyses by AMD subtypes and ethnicity. To explore the source of heterogeneity and the effect of continuous variables on the estimate of effect size, we performed a meta-regression analysis, using mean age of the subjects and percentage of males as covariates [430]. To confirm the stability of findings, sensitivity analysis, removing each study one at a time, was performed. The extent of publication bias was shown with a funnel plot and assessed by the Begg and Mazumdar rank correlation test and the Egger’s regression asymmetry test [431,432]. Except for the Q test, $p< 0.05$ was considered statistically significant, and all tests were 2-sided. All statistical analyses were performed using the *Comprehensive Meta-Analysis* software (Version 2.0).

4.3 Results

4.3.1 Search Findings and Studies Characteristics

The detailed steps of the search strategy are given as a PRISMA flow diagram in **Figure 1**. Briefly, a total of 7165 articles were retrieved from the databases and 3153 duplicates were excluded. Among the 4012 potentially eligible articles, 3842 were excluded after reading titles and/or abstracts. Thus, 170 articles were subjected to a full-text review, 75 of which met our inclusion criteria. Since the article by *Rivera et al.* investigated the association between rs1061170 polymorphism and AMD in two populations [175], the meta-analysis ultimately included data from 76 studies. Characteristics and genotype distribution from each individual study are listed in **Table 1**.

As indicated in the inclusion criteria, all studies had a case–control design or provided baseline data of prospective analysis. Overall, the meta-analysis included genotype data from 27418 AMD patients and 32843 controls; mean age ranged from 57.3 to 81.0 years and percent of males ranged from 28.3% to 81.8%. Twenty-one studies grouped genotype data of cases in a group that included all AMD subtypes, whereas 55 studies reported data according to specific AMD subtypes. Among these, early and advanced AMD (wet and/or dry AMD) were investigated by 15 and 51 studies, respectively. According to advanced AMD subtypes, genotype data were provided by 38 studies from wet AMD patients and 14 studies from dry AMD patients. Thirty-nine studies were performed in participants of Caucasian ethnicity, 27 studies were conducted among Asians, 3 studies among Africans, and 7 studies have enrolled subjects of mixed ethnicity. All studies, except eight, observed HWE. The quality scores ranged from 4 to 9 and 50 studies were considered good quality.

4.3.2 Meta-analysis

We initially performed a meta-analysis of the relationship between CFH rs1061170 variant and AMD, combining data of all AMD subtypes into the case group. The polymorphism showed a significant association with AMD under an allelic model (C vs. T; OR: 2.15; 95%CI 1.96-2.37). The Q test and I^2 statistics indicated significant heterogeneity across the studies ($p<0.001$; $I^2= 90.47\%$,).

We also found a significant relationship under the heterozygous model (CT vs TT; OR: 2.12; 95% CI, 1.90-2.38) and under the homozygous model (CC vs TT; OR: 4.66; 95%CI, 3.81-5.69), with evidence of heterogeneity ($p<0.001$; $I^2= 83.07\%$ and $p<0.001$; $I^2= 88.15\%$, respectively).

To explore the source of heterogeneity, we performed an univariate meta-regression analysis based on the random-effects model (methods of moments). There was no statistically significant effect on the summary OR by mean age of study subjects ($p= 0.610$) and percentage of males ($p= 0.360$).

4.3.3 Subgroup analysis by stage of disease and ethnicity

In the subgroup analysis by AMD subtypes, the rs1061170 showed a significant association with early AMD (OR: 1.41; 95%CI 1.25-1.60) and advanced AMD (OR: 2.35; 95%CI 2.14-2.59), under an allelic model (**Figure 2**). The Q test and I^2 statistics indicated significant heterogeneity across studies ($p<0.05$; $I^2= 83.52\%$ and $p<0.05$; $I^2= 69.05\%$, respectively).

With regard to advanced AMD subtypes, the rs1061170 showed a significant association with dry AMD (OR: 2.52; 95%CI 1.78-3.57) and wet AMD (OR: 2.35; 95%CI 2.09-2.65), under an allelic model (**Figure 3**). The Q test and I^2 statistics indicated significant heterogeneity across studies ($p<0.05$; $I^2= 79.61\%$ and $p<0.05$; $I^2= 66.26\%$, respectively).

Then, we also performed a subgroup analysis by ethnicity. This analysis was limited to Caucasians and Asians because of the few studies available for Africans and the undetermined ethnicity of mixed populations. In Caucasians, the rs1061170 showed a significant association with early AMD (OR: 1.44; 95%CI 1.27-1.63) and advanced AMD (OR: 2.47; 95%CI 2.24-2.72), under an allelic model. The Q test and I^2 statistics indicated significant heterogeneity across studies ($p<0.001$; $I^2= 81.93\%$ and $p<0.001$; $I^2= 59.90\%$, respectively).

With regard to advanced AMD subtypes, the rs1061170 showed a significant association with dry AMD (OR: 2.90; 95%CI 1.89-4.47) and wet AMD (OR: 2.46; 95%CI 2.15-2.83), under an allelic model. The Q test and I^2 statistics indicated significant heterogeneity across studies ($p<0.001$; $I^2= 74.63\%$ and $p=0.003$; $I^2= 56.34\%$, respectively).

In Asians, the rs1061170 showed a significant association with advanced AMD (OR: 2.09; 95%CI 1.67-2.60), but not with early AMD (p-value for Z test=0.315), under an allelic model. The Q test and I^2 statistics indicated significant heterogeneity across studies ($p<0.001$; $I^2= 73.32\%$ and $p=0.001$; $I^2= 81.909\%$, respectively).

With regard to advanced AMD subtypes, the rs1061170 showed a significant association with wet AMD (OR: 2.24; 95%CI 1.81-2.77), but not with dry AMD (p-value for Z test=0.264), under an allelic model. The Q test and I^2 statistics indicated significant heterogeneity across studies ($p<0.001$; $I^2= 69.64\%$ and $p<0.001$; $I^2= 92.30\%$, respectively).

4.3.4 Sensitivity analysis

To evaluate the robustness of the relationship between rs1061170 and AMD, a sensitivity analysis was performed by removing each study once at a time in every genetic model and recalculating the pooled OR. The summary OR remained stable under the allelic model, indicating that the association between rs1061170 and AMD subtypes was not driven by any single study. The sensitivity analysis also suggested that this finding was robust also under the heterozygous model and the homozygous model.

4.3.5 Publication Bias

To determine the possible extent of publication bias, the symmetry of funnel plots was assessed by the Begg and Mazumdar rank correlation test and the Egger's regression asymmetry test. With regard to advanced AMD, symmetrical funnel plot was obtained under the allelic model (**Figure 4**). Accordingly, neither the Begg and Mazumdar rank correlation test nor the Egger's test suggested publication bias for the association between rs1061170 polymorphisms and advanced AMD (p-value for Begg's test=0.739; p-value for Egger's test= 0.524). Regarding early AMD, the funnel plot indicated moderate asymmetry (**Figure 5**), suggesting that publication bias cannot be completely excluded. Accordingly, the Egger's test but not the Begg and Mazumdar rank correlation test suggested publication bias for the association between rs1061170 polymorphisms and early AMD (p-value for Egger's test= 0.015; p-value for Begg's test=0.656).

4.4 Discussion

The discovery of genetic variants in components of the complement system and findings that drusen are characterized by complement proteins and regulators [124,130,131,433] supported the investigation of chronic local inflammation in the AMD pathogenesis [132]. Although reduced complement system activity is frequently associated to decreased AMD risk, changes in its efficiency may be a dangerous compromise. In fact, some AMD related genetic variants may also modulate the susceptibility to infections [89]. Among these, the rs1061170 polymorphism in the *CFH* gene confers survival advantage against streptococcal infections in early life [434]. Accordingly, evidence that a combination of genetic variants may alter both systemic and local complement activation needs accurate investigations.

The rs1061170 polymorphism, located within a binding site for heparin and C-reactive protein, results in a malfunctioning CFH protein that is not able to inhibit the complement cascade. The relationship

between this polymorphism and AMD has been well investigated, but the relatively small sample sizes in several studies have frequently hindered the assessment of the association. Moreover, these investigations show several differences with regard to population characteristics, disease subtypes and ethnic groups under examination. It has been observed that the strength of association between rs1061170 polymorphism and AMD seems to be lower when studies move from the West to the East. The compelling association, observed in European cohorts, was not as relevant to AMD risk in populations of Asian ancestry [142]. Individual studies have been performed in Asian populations, but most of these reported lack of association in Korean, Japanese and Chinese [435-441]. To some extent, this controversy may be due to the low minor allele frequency in the Asian population. In European populations, there was strong evidence for association between the rs1061170 polymorphism and AMD with a 2.5 increased risk in individuals carrying at least one copy of the risk allele [140]. More recently, several meta-analyses reported the evidence that the rs1061170 polymorphism is a risk factor for AMD also in Asian populations [142,442,443].

The high variability in the strength of association may be due to an increased genetic effect with age, but it may also represent a variation between early and advanced AMD. While some studies have reported that the rs1061170 polymorphism was similarly associated with AMD subtypes [444], others showed a stronger association with exudative AMD [137]: in Asians, the meta-analysis by Wu et al. suggested that the effect might be more relevant for exudative AMD but the related mechanism still remained to be elucidated [142].

Our study reported results of a more comprehensive meta-analysis and provides subgroup analyses in order to evaluate the effect of the rs1061170 polymorphism on different AMD subtypes and ethnicities. Overall, we combined genotype data from 76 case–control studies, including 27418 AMD patients and 32843 controls. Consistently with previous meta-analyses [140,142,442,443], our results showed a significant association between the rs1061170 polymorphism and the risk of all AMD subtypes, with a summary allele OR of 2.15. However, our analysis underlined a significant between-study heterogeneity, with an I^2 measure that ranged from 81.2% to 92.4% in any genetic model. To explore the source of heterogeneity, we initially performed an univariate meta-regression analysis, which revealed no statistically significant effect of demographics variables (mean age and percentage of males) on the summary ORs. Moreover, we performed subgroup analyses by AMD subtypes and ethnicity.

In Caucasians, the risk associated with the polymorphism was lower for early AMD (OR of 1.44 under the allelic model) than advanced AMD (OR of 2.47). Particularly, the mutant allele conferred a 2.9-fold increased risk of dry AMD and a 2.5-fold increased risk of wet AMD. In Asians, the rs1061170 polymorphism was significantly associated with advanced AMD, but not with early AMD.

It is worth noting that the mutant allele conferred a 2.2-fold increased risk of wet AMD, but it was not associated with the risk of dry AMD.

As previously mentioned, the controversy between Caucasian and Asian populations may be due to the low minor allele frequency in the Asians. However, it is well established that the genetic effects of disease-associated variants are usually similar across ethnicities, regardless of divergent allelic frequency between different populations [445]. Thus, controversial results raise the need for the reliable assessment of the association between rs1061170 polymorphism and AMD, particularly in the Asian populations.

The main limitation of this meta-analysis is the high heterogeneity across studies. To take into account this issue, data of individual studies were combined through a random effects model but the pooled ORs should be interpreted with caution. Moreover, to explore the source of heterogeneity, a meta-regression was conducted and pooled ORs were calculated in more homogeneous subsets of studies through subgroups analyses. In addition, the possible existence of a publication bias was considered and the symmetry of funnel plots was assessed by the Begg's test and Egger's regression asymmetry test. No publication bias was detected under any genetic model. Finally, AMD is a complex disorder with sociodemographic, environmental, and genetic risk factors. Although potential confounding factors and gene-environment interactions should be considered, not all included studies provided adjusted ORs. Thus, the present meta-analysis combined crude ORs from each study and hence the effect of confounders cannot be completely excluded. To partially overcome this weakness, we performed a meta-regression, adjusting for age and gender, and then we stratified our analysis by ethnicity.

In conclusion, our work provides the most comprehensive meta-analysis of studies investigating the effect of rs1061170 polymorphism on AMD risk. These findings not only improve the comprehension of AMD pathogenesis, but also constitute a scientific background to be translated into clinical practice for AMD prevention [446]. Because of the genetic discoveries in AMD, several prediction models, able to predict AMD risk, are now widely available. However, findings from the evaluation of these models are not encouraged, since the same subject can receive controversial results [447,448]. To overcome this issue, genetic tests for AMD should be based on a more accurate assessment of disease risk. Although convincing evidence shows that the rs1061170 polymorphism is a risk factor for all AMD subtypes in Caucasians, well-designed studies with larger sample and more ethnic groups size are required. In Asians, in fact, the polymorphism seems to be closely associated with exudative AMD. Thus, given the above-mentioned limitations, further investigations are needed to better clarify the effect of genetic susceptibility in the development of AMD and its perspectives for disease prevention.

Table and Figures

Table 1. Characteristics and genotype distribution of studies included in the meta-analysis.

First Author, year	Ethnicity	Genotyping method	Mean age	Male (%)	Type of AMD	Total cases	CC	TC	TT	Total controls	CC	TC	TT	HWE	Quality score
Abbas & Azzazy 2013	African	RFLP	63,0	56,5	All	20	5	11	4	15	0	5	10	Yes	7
Almeida et al. 2013	Mixed	Real-time PCR-based (TaqMan)	75,0	53,9	All	161	54	74	33	290	71	66	153	No	7
Babanejad et al. 2016	Asian	RFLP	NA	60,0	All	100	32	58	10	100	9	40	51	Yes	7
Baird et al. 2006	Caucasian	Direct sequencing	67,8	29,5	All Early Advanced Wet Dry	236 117 119 93 26	91 39 52 38 14	109 56 53 45 8	36 22 14 10 4	144	14	79	51	No	7
Bonyadi et al. 2016	Asian	RFLP	74,3	59,2	Advanced Wet Dry	254 175 79	80 52 28	133 102 31	41 21 20	164	34	68	62	Yes	7
Brantley et al. 2007	Caucasian	Direct sequencing	74,7	62,0	Advanced Wet	188 155	57 50	93 76	38 29	189	18	93	78	Yes	7
Buentello-Volante et al. 2012	Mixed	Direct sequencing	75,2	30,5	Advanced	159	18	57	84	152	3	24	125	Yes	5
Chakravarthy et al. 2013	Caucasian	Competitive alle specific PCR SNP genotyping	73,2	45,0	All Early Advanced	2275 2136 139	386 347 39	1056 983 73	833 806 27	2058	244	977	837	Yes	8
Chen et al. 2006	Asian	Real-time PCR-based (TaqMan)	74,3	49,6	Wet	163	1	17	145	244	0	19	225	Yes	7
Chen et al. 2013	Asian	Real-time PCR-based (TaqMan)	61,6	28,3	Early	158	146	12	0	157	144	13	0	Yes	7
Chowers et al. 2008	Mixed	MALDI-TOF MS	75,7	NA	Wet	240	58	127	55	118	15	54	49	Yes	7
Chu et al. 2008	Asian	RFLP	67,2	54,8	Wet	144	1	34	109	126	1	11	114	Yes	7

Conley et al. 2005	Caucasian	RFLP	73,1	41,7	All	168	65	81	22	108	14	40	54	Yes	6
Delcourt et al. 2011	Caucasian	Real-time PCR-based (TaqMan)	80,0	36,6	All Early Advanced Wet Dry	273 228 45 24 21	40 33 7 5 2	123 101 22 15 7	110 94 16 4 12	523	51	222	250	Yes	8
Despriet et al. 2006	Caucasian	Real-time PCR-based (TaqMan)	68,7	40,5	All Early Advanced	2062 1984 78	364 333 31	910 875 35	788 776 12	3619	417	1644	1558	Yes	9
Dong et al. 2011	Asian	RFLP	70,4	59,1	Wet	136	4	56	76	140	2	21	117	Yes	7
Droz et al. 2008	Caucasian	PCR followed by DHPLC	75,6	36,3	All Early Advanced Wet Dry	420 156 264 208 56	139 47 92 70 22	210 83 127 100 27	71 26 45 38 7	50	4	27	19	Yes	9
Edwards et al. 2005	Caucasian	Real-time PCR-based (TaqMan)	72,7	54,2	All	395	124	186	85	190	26	83	81	Yes	5
Fisher et al. 2006	Caucasian	Direct sequencing	72,6	27,7	All	155	38	69	48	150	24	59	67	Yes	6
Fourgeux, 2012	Caucasian	RFLP	76,1	34,8	Advanced	1388	389	708	291	487	65	234	188	Yes	7
Fuse et al. 2006	Asian	Direct sequencing	70,0	57,9	Dry	80	0	7	73	192	2	24	166	Yes	5
Gangnon et al. 2012	Caucasian	NA	65,0	43,3	All Early Advanced	2901 2607 294	446 375 71	1465 1285 180	990 947 43	8474	1089	3903	3482	Yes	8
García et al. 2014	Caucasian	Real-time PCR-based (TaqMan)	75,8	41,6	Advanced	130	41	66	23	96	11	44	41	Yes	5
Goverdhan et al. 2008	Caucasian	Real-time PCR-based (TaqMan)	73,8	42,4	All	557	167	258	132	551	75	261	215	Yes	7
Gu et al. 2009	Caucasian	Direct sequencing	NA	NA	All Early Advanced	788 262 526	244 75 169	384 123 261	160 64 96	381	48	172	161	Yes	8
Haas et al. 2009	Caucasian	Direct sequencing	76,8	48,0	Advanced Wet	75 66	18 17	40 35	17 14	75	10	29	36	Yes	7

Habibi et al. 2013	African	Direct sequencing	70,9	53,4	Dry All Advanced Wet fibrovascular Dry	9 127 117 105 10 12	1 51 48 45 3 3	5 64 59 51 5 8	3 12 10 9 2 1	135	10	60	65	Yes	8
Hageman et al. 2005	Caucasian	Direct sequencing	73,9	NA	All	952	306	454	192	403	53	169	181	Yes	8
Hao et al. 2015	Asian	RFLP	61,7	59,5	All	109	1	20	88	165	1	16	148	Yes	6
Hautamäki et al. 2015	Caucasian	Direct sequencing	NA	34,8	Advanced Wet Dry	329 301 28	129 109 20	162 154 8	38 38 0	41	13	59	41	Yes	6
Hayashi et al. 2010	Asian	Real-time PCR-based (TaqMan)	57,3	57,7	Wet	401	7	75	319	1342	8	160	1174	Yes	8
Huang et al. 2014	Asian	MALDI-TOF MS	68,0	52,0	Wet	312	8	52	252	461	1	57	403	Yes	7
Nazari Khanamiri et al. 2014	Asian	Direct sequencing	71,2	48,7	All	70	24	33	13	86	15	36	35	Yes	7
Kim et al. 2008	Asian	Direct sequencing	67,4	46,8	Wet	114	1	22	91	187	1	22	164	Yes	6
Kim et al. 2013	Asian	RFLP	NA	NA	Wet	114	0	26	88	240	2	34	204	Yes	6
Klein et al. 2005	Caucasian	microarray	80,0	47,4	Advanced	95	42	39	14	48	6	25	17	Yes	5
Lau et al. 2006	Asian	RFLP	76,1	81,8	Wet	163	6	25	132	232	0	13	219	Yes	6
Lin et al. 2008	Asian	Real-time PCR-based (Melting curve analysis)	70,5	54,3	Early	133	8	19	106	180	0	16	164	Yes	7
Losonczy et al. 2011	Caucasian	RFLP	76,1	52,5	All Early Advanced	105 48 57	38 15 23	38 16 22	29 17 12	95	7	49	39	Yes	7

Magnusson et al. 2005	Caucasian	Real-time PCR-based (TaqMan)	NA	NA	All	1330	422	644	264	1265	182	613	470	Yes	6
Marioli et al. 2009	Caucasian	Direct sequencing	77,7	37,0	All Early Advanced	100 40 60	36 10 26	49 23 26	15 7 8	115	23	39	53	No	6
Mori et al. 2007	Asian	Real-time PCR-based (TaqMan)	69,6	65,7	All	188	3	27	158	139	0	15	124	Yes	7
Narayanan et al. 2007	Caucasian	Direct sequencing	76,0	NA	Advanced Wet Dry	66 51 15	21 13 8	34 29 5	11 9 2	58	7	22	29	Yes	5
Ng et al. 2008	Asian	Direct sequencing	74,3	50,3	Wet	163	1	17	145	155	0	9	146	Yes	7
Okur et al. 2015	Mixed	Direct sequencing	67,6	52,7	Advanced Wet Dry	87 45 42	25 11 14	44 25 19	18 9 9	80	12	37	31	Yes	7
Pei et al. 2009	Asian	Direct sequencing	69,9	53,0	Wet	123	2	28	93	130	0	12	118	Yes	5
Pulido et al. 2007	Caucasian	RFLP	NA	NA	Wet	89	32	31	26	230	25	104	101	Yes	4
Reynolds et al. 2009	Caucasian	MALDI-TOF MS	81,0	50,0	Advanced Wet Dry	103 51 52	40 21 19	43 22 21	20 8 12	56	10	18	28	No	9
Ricci et al. 2009	Caucasian	Real-time PCR-based (TaqMan)	71,3	44,7	Wet	101	27	55	19	100	5	48	47	Yes	6
Rivera et al. 2005 (population A)	Caucasian	MALDI-TOF MS and sequencing	76,3	36,2	All	793	288	368	137	611	70	327	214	No	5
Rivera et al. 2005 (population B)	Caucasian	MALDI-TOF and sequencing	71,8	37,3	All	373	149	162	62	335	46	148	141	Yes	5
Schaumberg et al. 2006	Caucasian	Real-time PCR-based (TaqMan)	NA	NA	All	111	25	49	37	401	55	166	180	Yes	7

Schaumberg et al. 2007	Caucasian	Real-time PCR-based (TaqMan)	60,2	35,8	All	437	130	208	99	1015	131	462	422	Yes	8
Seddon et al. 2006	Caucasian	MALDI-TOF MS	68,4	46,3	Advanced Wet Dry	574 429 145	228 166 62	250 190 60	96 73 23	280	41	126	113	Yes	8
Seddon et al. 2010	Caucasian	MALDI-TOF MS	NA	NA	All Advanced	244 69	74 32	115 30	55 7	209	30	89	90	Yes	8
Sharma et al. 2013	Asian	Real-time PCR-based (TaqMan)	63,4	65,3	Advanced Wet Dry	109 80 29	44 31 13	45 33 12	20 16 4	59	6	8	45	No	7
Simonelli et al. 2006	Caucasian	Real-time PCR-based (TaqMan)	72,4	45,96	All	104	39	41	24	131	21	61	49	Yes	7
Soheilian et al. 2016	Asian	RFLP	73,3	61,3	Wet	137	44	90	3	92	6	37	49	Yes	6
Souied et al. 2005	Caucasian	Direct sequencing	74,4	40,0	Wet	141	48	63	30	91	11	33	47	Yes	8
Soysal et al. 2012	Mixed	RFLP	68,2	54,0	All	147	44	71	32	105	15	45	45	Yes	6
Sundaresan et al. 2012	Asian	Real-time PCR-based (TaqMan)	NA	49,1	All Early Advanced	1634 1594 40	164 158 6	663 642 21	807 794 13	1862	187	804	871	Yes	8
Tanimoto et al. 2007	Asian	Real-time PCR-based (TaqMan)	73,5	70,1	Wet	95	3	20	72	99	2	13	84	Yes	7
Tedeschi-Blok et al. 2007	Caucasian	RFLP	58,3	NA	Early	285	12	93	180	570	14	165	391	Yes	9
Teixeira et al. 2010	Mixed	Direct sequencing	72,4	37,1	All	119	44	46	29	152	20	71	61	Yes	7
Teper et al. 2012	Caucasian	Direct sequencing	NA	NA	Wet	90	28	47	15	40	22	3	15	No	5
Tian et al. 2012	Asian	MALDI-TOF MS	66,6	53,8	All Early Advanced Wet	489 62 427 420	9 1 8 8	89 6 83 81	391 55 336 331	445	1	49	395	Yes	7
Uka et al. 2006	Asian	Real-time PCR-based (TaqMan)	72,4	58,5	Wet	67	3	13	51	107	3	18	86	Yes	7

Velissari et al. 2015	Caucasian	Real-time PCR-based (Melting curve analysis)	77,5	55,6	Wet	120	39	54	27	103	16	51	36	Yes	7
Wang et al. 2015	Asian	RFLP	66,0	36,2	All	119	0	33	86	99	0	10	89	Yes	6
Wegscheider et al. 2007	Caucasian	RFLP	77,6	39,9	Wet	179	63	92	24	163	14	68	81	Yes	6
Xu et al. 2008	Asian	RFLP	66,1	54,9	Wet	121	1	23	97	132	0	21	111	Yes	7
Yang et al. 2010	Asian	RFLP	64,8	46,7	Wet	110	2	22	86	150	2	22	126	Yes	7
Yücel et al. 2012	Mixed	RFLP and sequencing	69,4	56,6	Advanced Wet Dry	95 26 69	0 0 0	67 20 47	28 6 22	87	0	47	40	No	7
Zareparsi et al. 2005	Caucasian	Direct sequencing	77,8	37,8	All	616	219	311	86	275	25	136	114	Yes	7
Zerbib et al. 2011	Caucasian	Real-time PCR-based (TaqMan)	76,1	34,8	Wet	1093	309	553	231	396	54	188	154	Yes	7
Ziskind et al. 2008	African	RFLP	NA	NA	Early	16	5	7	4	98	13	56	29	Yes	5

[a] Hardy-Weinberg equilibrium in control group

Abbreviations: AMD, age-related macular degeneration; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; NA, not available; SNP, single nucleotide polymorphism; MALDI-TOF MS, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; DHPLC, denaturing high-performance liquid chromatography

Figure 1. PRISMA flow diagram of study selection

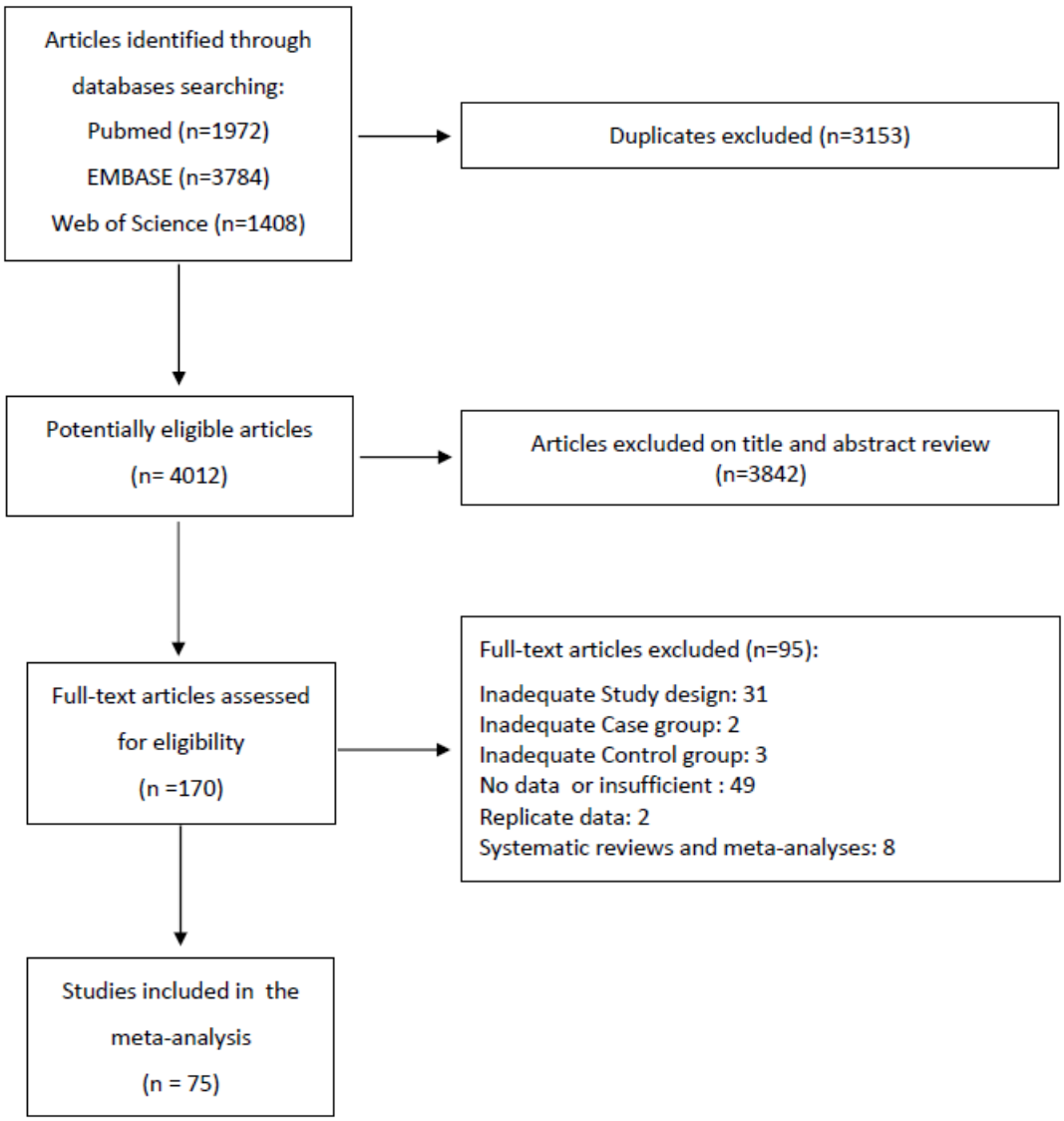
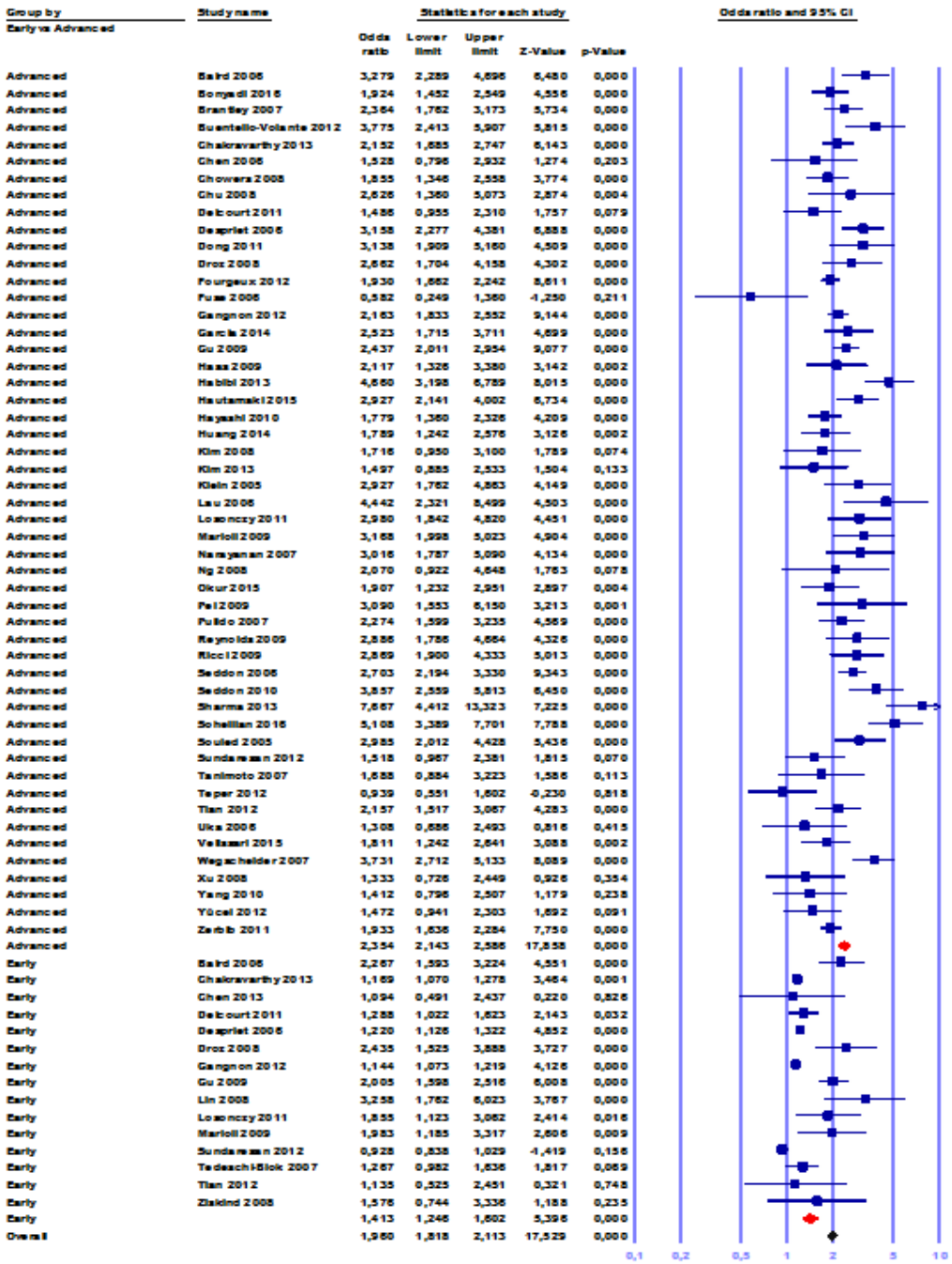
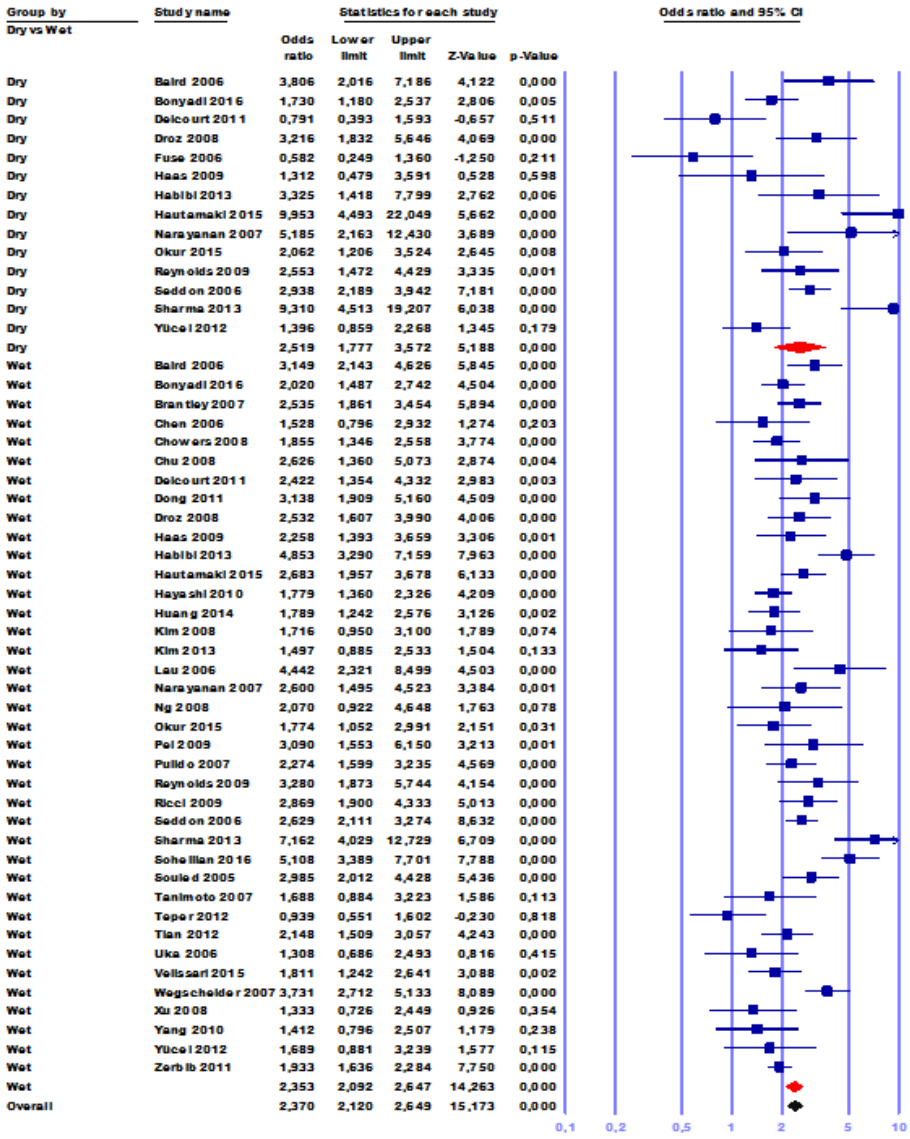


Figure 2. Association between rs1061170 and Age-Related Macular Degeneration under an allelic model



Subgroup analysis by Age-Related Macular Degeneration subtypes based on the random effects model.

Figure 3. Association between rs1061170 and advanced Age-Related Macular Degeneration under an allelic model



Group by Dry vs Wet	Study name	Odds ratio	Lower limit	Upper limit	Z-Value	p-Value
Dry	Baird 2006	3,806	2,016	7,186	4,122	0,000
Dry	Bonyadi 2016	1,730	1,180	2,537	2,806	0,005
Dry	Delcourt 2011	0,791	0,393	1,593	-0,657	0,511
Dry	Droz 2008	3,216	1,832	5,646	4,069	0,000
Dry	Fuse 2006	0,582	0,249	1,360	-1,250	0,211
Dry	Haas 2009	1,312	0,479	3,591	0,528	0,598
Dry	Habibi 2013	3,325	1,418	7,799	2,762	0,006
Dry	Hautamaki 2015	9,953	4,493	22,049	5,662	0,000
Dry	Narayanan 2007	5,185	2,163	12,430	3,689	0,000
Dry	Okur 2015	2,062	1,206	3,524	2,645	0,008
Dry	Reynolds 2009	2,553	1,472	4,429	3,335	0,001
Dry	Seddon 2006	2,938	2,189	3,942	7,181	0,000
Dry	Sharma 2013	9,310	4,513	19,207	6,038	0,000
Dry	Yücel 2012	1,396	0,859	2,268	1,345	0,179
Dry		2,519	1,777	3,572	5,188	0,000
Wet	Baird 2006	3,149	2,143	4,626	5,845	0,000
Wet	Bonyadi 2016	2,020	1,487	2,742	4,504	0,000
Wet	Brantley 2007	2,535	1,861	3,454	5,894	0,000
Wet	Chen 2006	1,528	0,796	2,932	1,274	0,203
Wet	Chowers 2008	1,855	1,346	2,558	3,774	0,000
Wet	Chu 2008	2,626	1,360	5,073	2,874	0,004
Wet	Delcourt 2011	2,422	1,354	4,332	2,983	0,003
Wet	Dong 2011	3,138	1,909	5,160	4,509	0,000
Wet	Droz 2008	2,532	1,607	3,990	4,006	0,000
Wet	Haas 2009	2,258	1,393	3,659	3,306	0,001
Wet	Habibi 2013	4,853	3,290	7,159	7,963	0,000
Wet	Hautamaki 2015	2,683	1,957	3,678	6,133	0,000
Wet	Hayashi 2010	1,779	1,360	2,326	4,209	0,000
Wet	Huang 2014	1,789	1,242	2,576	3,126	0,002
Wet	Kim 2008	1,716	0,950	3,100	1,789	0,074
Wet	Kim 2013	1,497	0,885	2,533	1,504	0,133
Wet	Lau 2006	4,442	2,321	8,499	4,503	0,000
Wet	Narayanan 2007	2,600	1,495	4,523	3,384	0,001
Wet	Ng 2008	2,070	0,922	4,648	1,763	0,078
Wet	Okur 2015	1,774	1,052	2,991	2,151	0,031
Wet	Pol 2009	3,090	1,553	6,150	3,213	0,001
Wet	Pulido 2007	2,274	1,599	3,235	4,569	0,000
Wet	Reynolds 2009	3,280	1,873	5,744	4,154	0,000
Wet	Ricci 2009	2,869	1,900	4,333	5,013	0,000
Wet	Seddon 2006	2,629	2,111	3,274	8,632	0,000
Wet	Sharma 2013	7,162	4,029	12,729	6,709	0,000
Wet	Soheilian 2016	5,108	3,389	7,701	7,788	0,000
Wet	Souied 2005	2,985	2,012	4,428	5,436	0,000
Wet	Tanimoto 2007	1,688	0,884	3,223	1,586	0,113
Wet	Teper 2012	0,939	0,551	1,602	-0,230	0,818
Wet	Tian 2012	2,148	1,509	3,057	4,243	0,000
Wet	Uka 2006	1,308	0,686	2,493	0,816	0,415
Wet	Vellissari 2015	1,811	1,242	2,641	3,088	0,002
Wet	Wegscheider 2007	3,731	2,712	5,133	8,089	0,000
Wet	Xu 2008	1,333	0,726	2,449	0,926	0,354
Wet	Yang 2010	1,412	0,796	2,507	1,179	0,238
Wet	Yücel 2012	1,689	0,881	3,239	1,577	0,115
Wet	Zerbib 2011	1,933	1,636	2,284	7,750	0,000
Wet		2,353	2,092	2,647	14,263	0,000
Overall		2,370	2,120	2,649	15,173	0,000

Subgroup analysis by Age-Related Macular Degeneration subtypes based on the random effects model

Figure 4. Funnel plot of random-effect meta-analysis of the association between rs1061170 and early Age-Related Macular Degeneration under an allelic model.

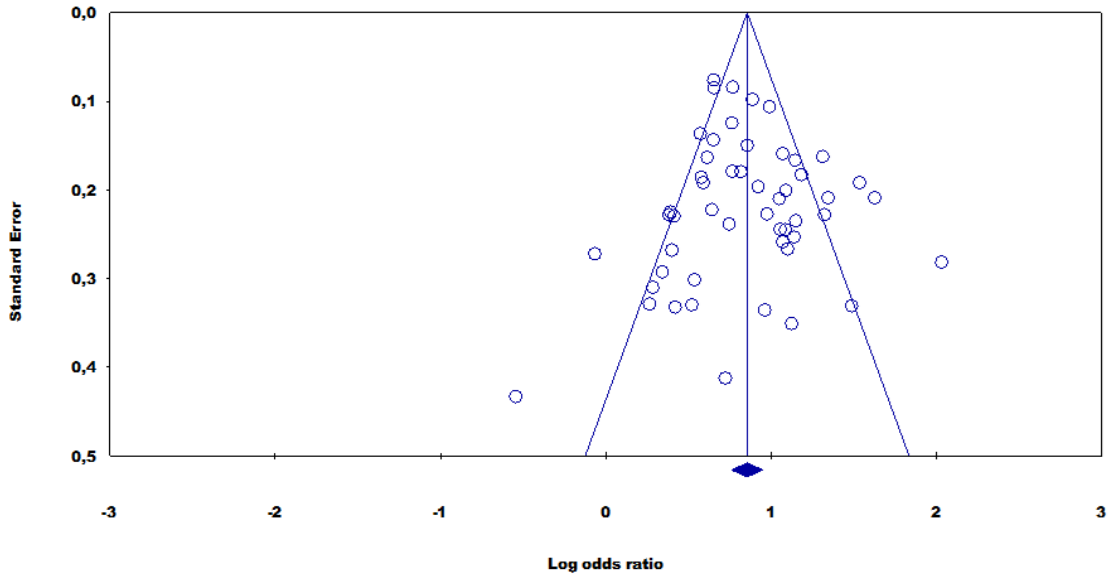
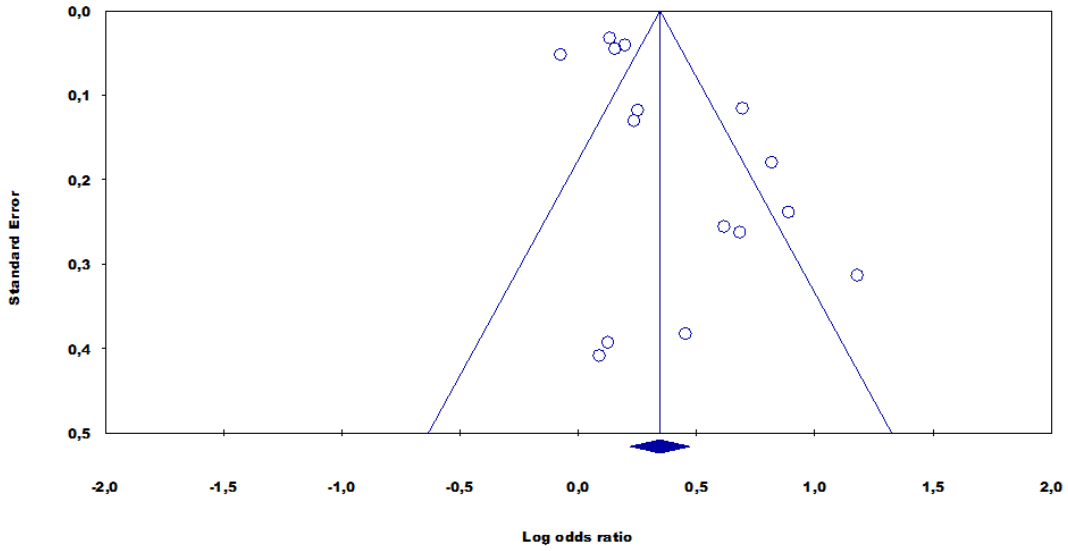


Figure 5. Funnel plot of random-effect meta-analysis of the association between rs1061170 and advanced Age-Related Macular Degeneration under an allelic model.



5 Association between Vascular Endothelial Growth Factor polymorphisms and age-related macular degeneration: a systematic review and meta-analysis

5.1 Background

VEGF has a key role in promoting angiogenesis, vasculogenesis, and lymphangiogenesis in normal and pathological cells [449]. The VEGF family molecules (i.e. VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E) are involved in development, survival and maintenance of vessels which are essential for retinal health [450], but also in inducing vascular leakage and inflammation [449,451].The human VEGF gene is located on chromosome 6p21.3 and contains seven introns and eight exons [452,453]. Recently, several studies focused on the association between polymorphisms in the *VEGF* gene and AMD risk. Among these, the rs833061 (−460T/C) in the promoter region, the rs1413711 (+674C/T) in intron 1, the rs3025039 (+936C/T) in the 3'-untranslated region and the rs2010963 (+405G/C) in the 5′-untranslated region were found to be associated with AMD susceptibility. Although genetic variability of the *VEGF* gene may have a critical role in determining AMD risk, current evidence remains inconclusive - as confirmed by previous meta-analyses [454-456] - since the majority of studies were conducted on small to moderate sample sizes. Herein, we carried out a comprehensive systematic review and an updated meta-analysis in order to summarize the current published evidence and to evaluate the associations between four common VEGF gene polymorphisms (i.e. rs833061, rs1413711, rs3025039, rs2010963) and AMD risk, also stratifying for stage of disease and ethnicity.

5.2 Methods

5.2.1 Search strategy and selection criteria

A systematic review of original articles, published before June 2016, was conducted by searching in the Medline database in order to collect data from epidemiological studies investigating the association between VEGF polymorphisms and AMD risk. The literature search, limited to studies written in English, was independently conducted by the two Authors, using the following key words: ("VEGF" or "vascular endothelial growth factor") AND ("variant" or "SNPs" or "polymorphism") AND ("age-related macular degeneration" or "AMD"). Moreover, the references from retrieved articles were also checked to search for additional studies.

The selection criteria were as follow:(1) studies must employ a case-control or cohort design; (2) and must evaluate the associations between VEGF polymorphisms (rs833061, rs1413711, rs3025039, rs2010963) and AMD; (3) genotype data of patients and controls must be available in order to estimate odds ratios (ORs) and 95% confidence intervals (95%CIs). Furthermore, exclusion criteria

were: (1) studies that did not provide genotype data in AMD patients and/or in control subjects and (2) review articles. The meta-analysis was conducted according to the preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines [457].

5.2.2 Data extraction and quality assessment

The following information were extracted: first Author's last name, year of publication, country and ethnicity, sample size of subjects with and without AMD, genotype distribution of case and control groups, subtypes of AMD cases and controls (dry AMD and wet AMD), and P value for Hardy–Weinberg equilibrium test in subjects without AMD.

5.2.3 Statistical analyses

The Review Manager 5.2 software, provided by the Cochrane Collaboration (http://ims.cochrane.org/revman), was used to estimate the association between VEGF polymorphisms and AMD risk. For each polymorphism, the wild-type allele was set as 1 and the risk allele as 2 and the Chi-square test was performed to determine if the genotype distribution of the control subjects is deviated from the HWE ($p<0.05$ was considered significant). To calculate pooled odds ratios ORs and 95% confidence intervals (95%CIs), the following genetic models were adopted for each polymorphism: the homozygote model (22 versus 11), the dominant model (22 + 12 versus 11), the recessive model (22 versus 12 + 11), the heterozygote model (12 versus 11) and the allelic model (1 versus 2). The significance of pooled OR was determined using the *Z* test, and a $p<0.05$ was considered significant. Heterogeneity across studies was assessed using the Q-test based on the $\chi 2$ statistic ($p <0.1$ was considered statistically significant). To quantify heterogeneity, the I^2 value was calculated and interpreted as follows: an I^2 value of 0% indicates "no heterogeneity", whereas 25% is "low", 50% is "moderate" and 75% is "high" heterogeneity [428]. The between-study variance was estimated using tau-squared (t) statistic [429]. According to heterogeneity across studies, the fixed-effects (Mantel–Haenszel method) or random-effects models (Der Simonian-Laird method) were used to calculate pooled effect estimates.

Furthermore, subgroup analyses by subtypes of AMD and ethnicity (Asian and Caucasian) were conducted. The leave-one-out sensitivity analysis, by the omission of a single study at a time, was performed in order to assess whether a particular omission could affect the overall OR value and the heterogeneity across studies. To identify potential publication bias, the asymmetry of the funnel plots, in which ORs were plotted against their corresponding standard errors, was examined.

5.3 Results

5.3.1 Search findings and studies characteristics

The detailed steps of the systematic review and meta-analysis process are given as a PRISMA flow chart in **Figure 1**. A total of 115 articles were retrieved from the database and 86 records were excluded after reading titles and/or abstracts. Thus, 29 studies were subjected to a full-text review and selected according to the selection criteria. Among these, 17 studies evaluated the association between VEGF polymorphisms and AMD risk by a case-control or cohort design, but five studies were excluded for insufficient data. Consequently, 12 articles, published between 2006 and 2015, were included in the systematic review and their main characteristics are summarized in **Table 1**. Particularly, three studies investigated the role of more than one polymorphism: the rs833061 and the rs3025039 were respectively analysed in six studies (rs833061: 1431 cases and 806 controls; rs3025039: 1396 cases and 1326 controls); the rs1413711 in four studies (554 cases and 551 controls), and the rs2010963 in three studies (614 cases and 454 controls).

5.3.2 Meta-analysis

For the rs833061 polymorphism, the meta-analysis showed a significant association with AMD under a homozygote model (CC *vs.* TT: OR = 1.56, 95%CI 1.15–2.13); a dominant model (CT + CC *vs.* TT: OR = 1.66, 95%CI 1.04–2.65); and an allelic model (C *vs.* T: OR = 1.31, 95%CI 1.08–1.58). Pooled ORs, under a heterozygote model (CT *vs.* TT: OR = 1.63, 95%CI 0.97–2.72) and a recessive model (CC *vs.* TT + CT: OR = 1.22, 95%CI 0.94–1.59), were not statistically significant. Subgroup analysis by ethnicity confirmed that: in Asians, the polymorphism was associated with AMD under the homozygote (OR = 2.15, 95%CI 1.07–4.31), the recessive (OR = 2.04, 95%CI 1.03–4.04) and the allelic (OR = 1.28, 95%CI 1.00–1.65) models; in Caucasians, the polymorphism was associated with AMD under the homozygote (OR = 1.44, 95%CI= 1.02–2.03) and the allelic (OR = 1.33, 95%CI 1.00–1.77) models. Subgroup analysis by subtypes of AMD confirmed that this polymorphism was associated with wet AMD under a homozygote model (CC *vs.* TT: OR = 1.48, 95%CI 1.07–2.04) (**Figure 2**); a dominant model (CT + CC *vs.* TT: OR = 1.58, 95%CI 1.00–2.51) (**Figure 3**); and an allelic model (C *vs.* T: OR = 1.27, 95%CI 1.04–1.56) (**Figure 4**). However, there was no association between this polymorphism and the risk of wet AMD in any other of the genetic models.

For the rs1413711 polymorphism, the meta-analysis showed no significant association between the polymorphism and AMD under a homozygote model (TT *vs.* CC: OR = 1.50, 95%CI 0.71–3.16); a dominant model (CT + TT *vs.*CC: OR = 0.98, 95%CI 0.65–1.49); a heterozygote model (CT *vs.* CC: OR = 0.98, 95%CI 0.65–1.49); a recessive model (TT *vs.* CC + CT: OR = 1.69, 95%CI 0.98–2.89); and an allelic model (T *vs.* C: OR = 1.15, 95%CI 0.86–1.56). However, the stratified analysis

indicated that this polymorphism was associated with wet AMD under a recessive model (TT *vs.* CC + CT: OR = 1.64, 95%CI 1.14–2.36) (**Figure 5**). Subgroup analysis by ethnicity showed no significant association between the polymorphism and AMD in the Asian populations; the stratified analysis was not performed for the Caucasians because only the study by Churchill et al. (2006) [458] reported genotype data of a Caucasian population.

For the rs3025039 polymorphism, the meta-analysis showed that no significant association between the polymorphism and AMD was assessed under a homozygote model (TT *vs.* CC: OR = 1.39, 95%CI 0.71–2.73); a dominant model (CT + TT *vs.* CC: OR = 1.07, 95%CI 0.91–1.27); a heterozygote model (CT *vs.* CC: OR = 1.05, 95%CI 0.89–1.25); a recessive model (TT *vs*. CC + CT: OR = 1.39, 95%CI 0.71–2.71); and an allelic model (T *vs.* C: OR = 1.10, 95%CI 0.96–1.27). Further subgroup analyses, by subtypes of AMD and ethnicity, confirmed that there was no association between this polymorphism and the risk of AMD in any of the genetic models.

For the rs2010963 polymorphism, the meta-analysis showed that no significant association between the polymorphism and AMD was assessed under a homozygote model (CC *vs.* GG: OR = 0.81, 95%CI 0.49–1.32); a recessive model (CG + GG *vs.* CC: OR = 0.88, 95%CI 0.58–1.35); a heterozygote model (CG *vs.* GG: OR = 0.85, 95%CI 0.62–1.17); a dominant model (GG *vs.* CC + CG: OR = 0.85, 95%CI 0.63–1.16); and an allelic model (C *vs.* G: OR = 0.96, 95%CI 0.73–1.11). Further subgroup analyses, by subtypes of AMD and ethnicity, confirmed that there was no association between this polymorphism and the risk of AMD in any of the genetic models.

5.3.3 Sensitivity analysis

The leave-one-out sensitivity analysis was performed in order to investigate the sources of heterogeneity across studies.

For the rs833061 polymorphism, the Q-test and I^2 statistics showed no significant heterogeneity across studies under the homozygote and recessive models (p-values >0.1). Conversely, significant heterogeneity across studies was reported under the heterozygote ($p<0.001$; $I^2=83\%$), dominant ($p<0.001$; $I^2=82\%$) and allelic ($p=0.09$; $I^2=47\%$) models. The sensitivity analysis found that the study by Janik-Papis et al. [459] affected the heterogeneity across studies. When this study was omitted, the between studies heterogeneity decreased under the heterozygote ($p=0.02$; $I^2=67\%$), dominant ($p=0.03$; $I^2=62\%$) and allelic ($p=0.47$; $I^2=0\%$) models. Particularly, a significant association with AMD was confirmed under an allelic model (OR = 1.19, 95%CI 1.02–1.38).

In the stratified analysis of wet AMD, significant heterogeneity across studies was reported under the dominant ($p<0.001$; $I^2=78\%$) and allelic ($p=0.09$; $I^2=48\%$) models. When the study by Janik-Papis et al. [459]was omitted, the between study heterogeneity decreased under the dominant ($p=0.03$; $I^2=64\%$)

and allelic (p=0.34; I^2=11%) models. However, no significant association was confirmed between the rs833061 polymorphism and wet AMD under both genetic models.

In the subgroup of Caucasians, the Q-test and I^2 statistics showed a significant heterogeneity across studies under the allelic model (p=0.03; I^2=67%). When the study by Janik-Papis et al. [459] study was omitted, the between studies heterogeneity decreased to I^2 = 24% (p=0.27) but no significant association was confirmed between the rs833061 polymorphism and AMD, in Caucasians, under the allelic model (OR = 1.16, 95%CI 0.94–1.44). Considering both populations, no significant heterogeneity across studies was reported in any other of the genetic models (p-values>0.1).

For the rs1413711 polymorphism, no significant heterogeneity across studies was reported under the heterozygote model (p=0.13). Conversely, significant heterogeneity across studies was reported under the homozygote (p=0.01; I^2=71%), dominant (p=0.06; I^2=60%), recessive (p=0.08; I^2=55%) and allelic (p=0.05; I^2=62%) models. The sensitivity analysis found that the study by Almeida et al. (2012) affected the heterogeneity across studies under the homozygote and recessive models. When this study was omitted, the between study heterogeneity decreased under the homozygote (p=0.10; I^2=57%) and recessive (p=0.43; I^2=0%) models. With regard to the dominant and allelic models, the sensitivity analysis found that the study by Churchill et al. (2006) [458] affected the heterogeneity across studies. When this study was omitted, the between study heterogeneity decreased under the dominant (p=0.47; I^2=0%) and allelic (p=0.20; I^2=38%) models. Particularly, a significant association with AMD was confirmed under the allelic model (OR = 1.29, 95%CI 1.01–1.64). In the stratified analysis of wet AMD, significant heterogeneity across studies was reported under the homozygote (p=0.02; I^2=68%), dominant (p=0.05; I^2=61%) and allelic (p=0.06; I^2=59%) models. When the study by Churchill et al. [458] was omitted, the between study heterogeneity decreased under homozygote (p=0.08; I^2=59%), dominant (p=0.39; I^2=0%) and allelic (p=0.26; I^2=26%) models. However, no significant association was reported between the rs833061 polymorphism and wet AMD under these genetic models. In the subgroup analysis by ethnicity, no significant heterogeneity across studies was reported in any of the genetic models (p-values>0.1).

For the rs3025039 polymorphism, the Q-test showed no significant heterogeneity across studies under the heterozygote, dominant, recessive and allelic models (p-values>0.1). Conversely, significant heterogeneity across studies was reported under the homozygote model (p=0.06; I^2=56%). The sensitivity analysis found that the study by Tian et al. [144] affected the heterogeneity across studies. When this study was omitted, the between study heterogeneity decreased (p=0.31; I^2=16%) and a significant association with AMD was showed under the homozygote model (OR = 1.92, 95%CI 1.14–3.22). In the stratified analysis of wet AMD, significant heterogeneity across studies was reported under the homozygote (p=0.004; I^2=78%), heterozygote (p=0.06; I^2=57%), recessive

(p=0.007; I^2=75%), dominant (p=0.01; I^2=69%) and allelic (p=0.002; I^2=77%) models. Particularly, when the study by Tian et al. [144] was omitted, under the recessive model, the between studies heterogeneity decreased (p=0.17; I^2=44%) and a significant association was reported between the rs833061 polymorphism and exudative form of AMD (OR = 2.49, 95%CI 1.11–5.60). In the subgroup analysis by ethnicity, no significant heterogeneity across studies was reported in any of the genetic models (p-values>0.1).

For the rs2010963 polymorphism, the Q-test showed no significant heterogeneity across studies under the homozygote, heterozygote, dominant, and allelic models (p-values>0.1). Conversely, significant heterogeneity across studies was reported under the recessive model (p=0.06; I^2=63%). The sensitivity analysis found that the study by Lin et al. [460] affected the heterogeneity across studies. When this study was omitted, the between study heterogeneity decreased (p=0.50; I^2=0%) but no significant association with AMD was showed under the recessive model. In the subgroup analyses by subtypes of AMD and ethnicity, no significant heterogeneity across studies was reported in any of the genetic models (p-values>0.1).

5.3.4 Publication bias

The funnel plots of the pooled analyses were quite symmetric. The Begg's rank correlation method and Egger's weighted regression method showed that no obvious publication bias for these polymorphisms was found (data not shown).

5.4 Discussion

VEGF is a naturally occurring growth factor selective for endothelial cells which regulates angiogenesis, vascular permeability, and plays a leading role in the retinal tissue of AMD. In developed countries, exudative AMD - characterized by the formation of sub-retinal CNV - is the major cause of severe vision loss and blindness in the elderly [461]. The association between CNV and increased VEGF expression provides a strong reason for exploring *VEGF* polymorphisms that can contribute to the risk of AMD. Results from several studies, which evaluated the role of *VEGF* polymorphisms in the AMD development, remain ambiguous as stated by three previous meta-analyses [454-456]. The first one by Lu et al. did not demonstrated an association between rs833061, rs1413711 and rs2010963 polymorphisms and the risk of AMD. However, the subgroup analysis showed a significant association for rs833061 and rs1413711 polymorphisms in Caucasians, and for rs1413711 in Asians [454]. Further, Huang et al. suggested that CC genotype for rs833061 and TT genotype for rs1413711 were associated with an increased risk of AMD, with a more pronounced effect on exudative AMD. By contrast, no associations with AMD risk were reported for rs2010963

and rs3025039 polymorphisms [455]. Finally, Liu et al. confirmed that VEGF polymorphisms are associated with increased or decreased risk of AMD, especially with exudative AMD [456].

Our study, critically reviewing twelve studies, reported results of a more comprehensive meta-analysis and provided a stratified analysis for the exudative AMD. The results, using different genetic models, confirmed the overall effects of VEGF polymorphisms on the risk of AMD. The T allele of the rs833061 (−460 T/C) polymorphism, located in the promoter region, was associated with a decreased activity of the gene promoter [462]. We showed that subjects who carried the C allele had an increased risk of AMD under the homozygote dominant and allelic models. However, the sensitivity analysis, omitting the study by Janik-Papis et al. [459], did not confirm this association under the dominant model. Subgroup analysis by ethnicity confirmed that, in Asians, who carried the C allele had an increased risk of AMD under the homozygote, recessive and allelic models. In Caucasians, the polymorphism was associated with AMD risk under the homozygote and allelic models. However, when the study by Janik-Papis et al. [459] was omitted, the association under the allelic model was not confirmed. Results from the stratified analysis reported that who carried the C allele had an increased risk of exudative AMD under the homozygote, dominant and allelic models.

For the rs1413711 (+674C/T) polymorphism, located in intron 1 of the VEGF gene, we showed no association with overall AMD risk. The sensitivity analysis found that the study by Janik-Papis et al. [459] was a source of heterogeneity across studies. Accordingly, omitting this study, a significant association with AMD was confirmed under the allelic model. Furthermore, the stratified analysis indicated that who carried the TT genotype had an increased risk of exudative AMD under the recessive model. At the best of our knowledge, no evidence is available regarding the effect of this polymorphism on functional activity and further analyses are warranted to clarify the effects on gene expression and protein activity.

For the rs2010963 (+405G/C) polymorphism, we firstly showed no association with AMD risk. However, sensitivity analysis identified the study by Tian et al. [144] as the main source of heterogeneity between studies. Interestingly, when this study was omitted, a significant association with overall AMD was evident under the homozygote model. Particularly, subjects who carried the CC genotype had an increased risk of exudative AMD. With regard to the rs3025039 (+936C/T) polymorphism, we failed in demonstrating an association with AMD in general and exudative AMD in particular.

The present study had some limitations. First, the number of studies included in the meta-analysis is modest and some relevant articles were excluded due to insufficient data. Accordingly, the polymorphisms rs833061 and rs3025039 were analysed in six studies, the polymorphism rs1413711 in four studies, and the polymorphism rs2010963 in three studies. Second, the heterogeneity across studies, which existed for some polymorphisms, must be considered and, although the random effects

model and the sensitivity analysis were appropriately performed, the pooled estimates should be interpreted with caution. Finally, since AMD is a complex disorder with socio-demographic, environmental and genetic risk factors [52,463], adjusted analyses should be performed, taking into account the confounding factors and gene–environment interactions.

In conclusion, the present study constitutes an useful guide for readers to study AMD and adds novel evidence to the growing literature on the role of four common VEGF polymorphisms on the risk of AMD. Significant associations with AMD risk were evident for rs833061, rs1413711 and rs3025039 VEGF polymorphisms but not for rs2010963. However, given the abovementioned limitations, further studies are recommended to better clarify the effect of genetic susceptibility on the development of AMD.

Tables and Figures

Table 1. Characteristics of the studies included in the meta-analysis

Polymorphism	First Author, Year	Country	Ethnicity	Typeof AMD	Genotype frequency in cases				Genotype frequency in controls				HWE[a]
					Sample size	11	12	22	Sample size	11	12	22	
rs833061	Richardson, 2007 [464]	Australia	Caucasian	Total AMD	566	154	259	153	157	40	78	39	0.937
				Dry AMD	100	27	43	30	157	40	78	39	
				Wet AMD	336	92	158	86	157	40	78	39	
	Lin, 2008 [465]	China	Asian	Total AMD	190	116	66	8	180	116	60	4	0.239
				Dry AMD	104	60	38	6	180	116	60	4	
				Wet AMD	86	56	28	2	180	116	60	4	
	Janik-Papis, 2009 [459]	Poland	Caucasian	Total AMD	265	48	191	26	134	60	63	11	0.323
				Dry AMD	88	13	67	8	134	60	63	11	
				Wet AMD	177	35	124	18	134	60	63	11	
	Szaflik, 2009 [466]	Poland	Caucasian	Wet AMD	100	14	78	8	104	37	56	11	0.129
	Qu, 2011 [467]	China	Asian	Wet AMD	159	81	58	20	140	81	50	9	0.733
	Cruz-González, 2013 [468]	Spain	Caucasian	Wet AMD	151	31	77	43	91	24	44	23	0.754
rs1413711	Almeida, 2012 [469]	Brazil	Mixed	Total AMD	160	65	66	29	140	67	65	8	0.127
				Dry AMD	36	14	14	8	140	67	65	8	
				Wet AMD	124	51	52	21	140	67	65	8	
	Churchill, 2006 [458]	UK	Caucasian	Wet AMD	45	17	18	10	94	19	54	21	0.147
	Lin, 2008 [465]	China	Asian	Total AMD	190	57	80	53	180	50	85	42	0.617
				Dry AMD	104	29	46	29	180	50	85	42	
				Wet AMD	86	28	34	24	180	50	85	42	
	Qu, 2011 [467]	China	Asian	Wet AMD	159	81	58	20	140	81	50	9	0.733
rs3025039	Galan, 2010 [470]	Finland	Caucasian	Total AMD	226	175	48	3	248	190	54	4	0.942
	Qu, 2011 [467]	China	Asian	Wet AMD	159	114	33	12	140	92	40	8	0.205
	Jiang, 2013 [471]	China	Asian	Total AMD	200	132	50	18	200	138	55	7	0.603
				Dry AMD	49	32	13	4	200	138	55	7	
				Wet AMD	99	66	25	8	200	138	55	7	

	Gonçalves, 2015 [472]	Brazil	Mixed	Wet AMD	88	69	19	0	95	76	19	0	0.279
	Tian, 2012 [144]	China	Asian	Total AMD	533	341	179	13	463	302	143	18	0.835
				Wet AMD	462	299	153	10	463	302	143	18	
	Lin, 2008 [465]	China	Asian	Total AMD	190	120	58	12	180	134	42	4	0.742
				Dry AMD	104	75	27	2	180	134	42	4	
				Wet AMD	86	45	31	10	180	134	42	4	
rs2010963	Lin, 2008 [465]	China	Asian	Total AMD	190	40	132	18	180	34	116	30	<0.001
				Dry AMD	104	24	70	10	180	34	116	30	
				Wet AMD	86	16	62	8	180	34	116	30	
	Janik-Papis, 2009 [459]	Poland	Caucasian	Total AMD	265	164	84	17	134	85	44	5	0.813
				Dry AMD	88	47	30	11	134	85	44	5	
	Qu, 2011 [467]	China	Asian	Wet AMD	159	54	70	35	140	39	74	27	0.442

[a] p-value for Hardy–Weinberg equilibrium in controls

Figure 1. Flow diagram of study selection

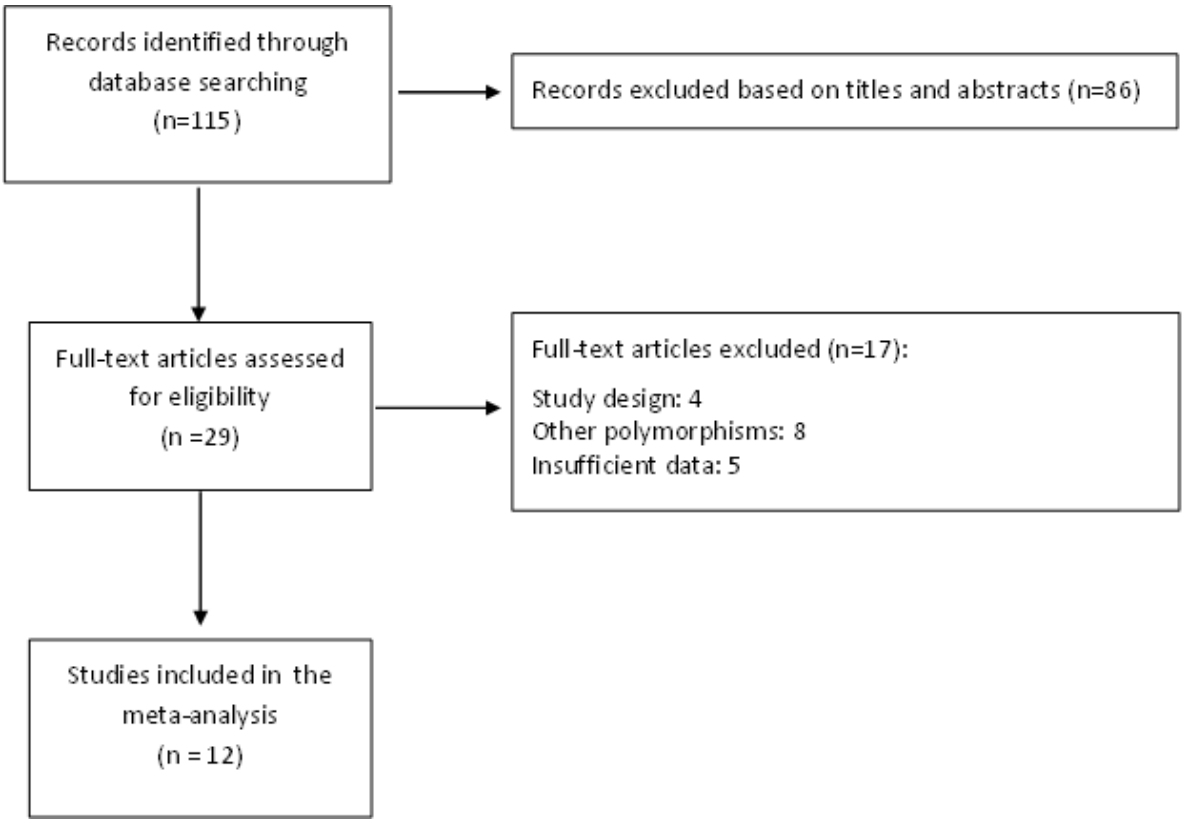


Figure 2. Forest plot of the association between rs833061 polymorphism and wet AMD under a homozygote model (CC *vs.* TT)

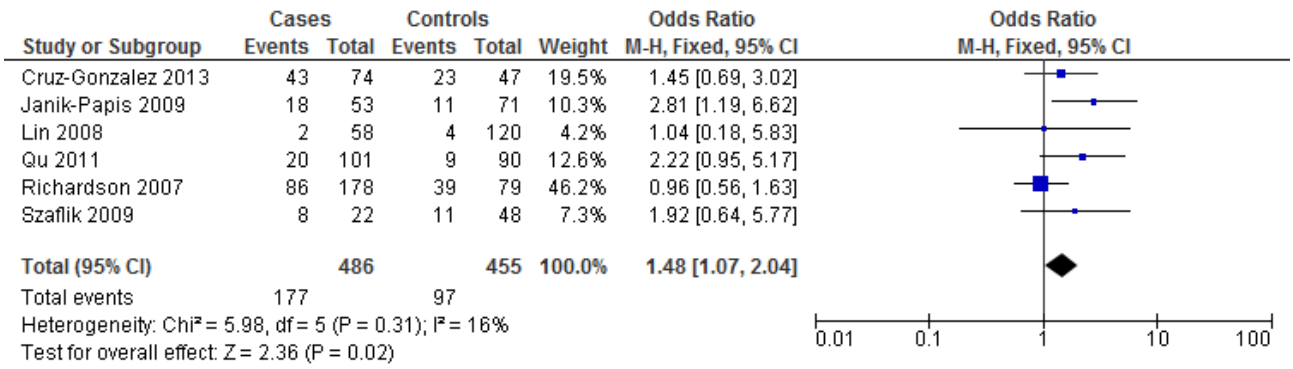


Figure 3. Forest plot of the association between rs833061 polymorphism and wet AMD under a dominant model (CT + CC *vs.* TT)

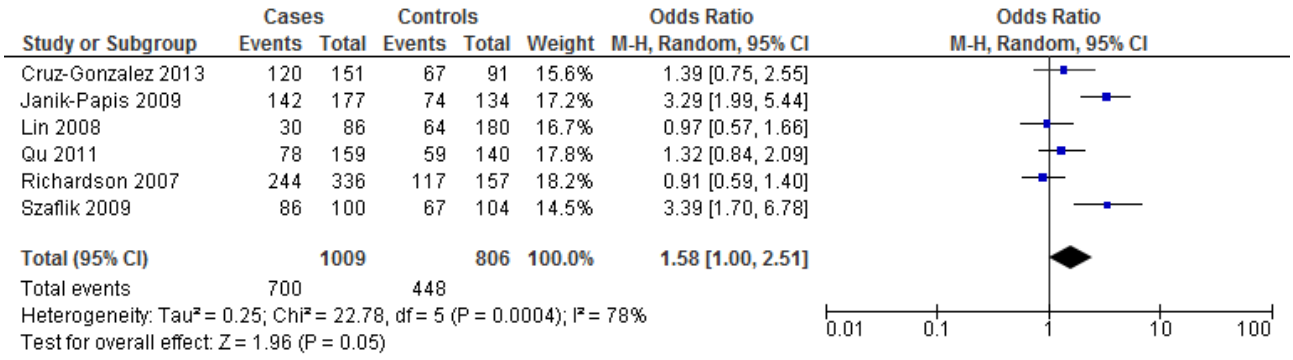


Figure 4. Forest plot of the association between rs833061 polymorphism and wet AMD under an allelic model (C *vs.* T)

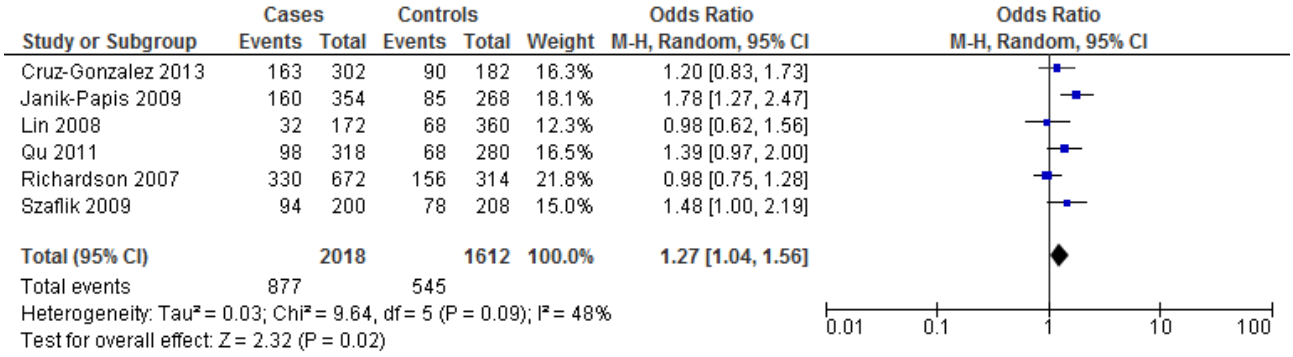
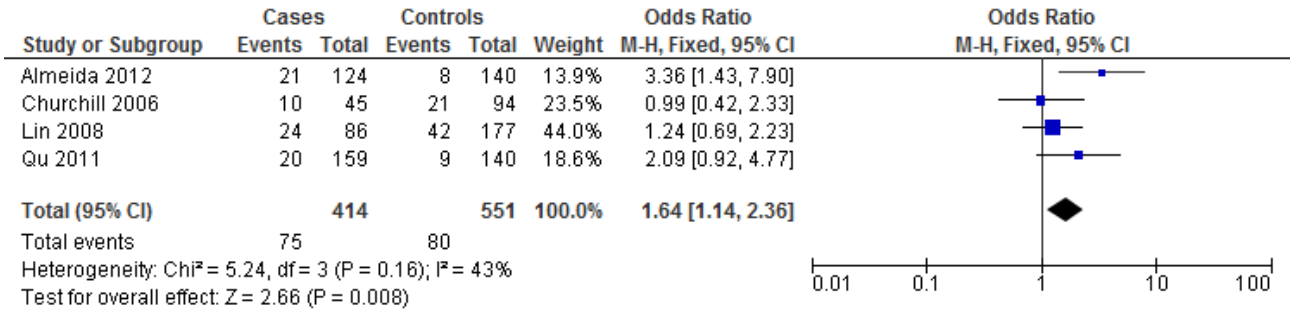


Figure 5. Forest plot of the association between rs1413711 polymorphism and wet AMD under a recessive model (TT *vs.* CC + CT)



6 SIRT1 regulates DNMTs functions and LINE-1 methylation in cellular models of retinal degenerative diseases: the role of resveratrol

6.1 Background

AMD is one of the most investigated multifactorial diseases with several socio-demographic (age and race) [473], environmental (cigarette smoking, light exposure and nutrient intake) [60,71,72,474,475] and genetic risk factors [81,83,425,476] that can act together leading to chronic inflammation and oxidative stress [221]. Moreover, a typical gene–environment interaction has been also proposed [217], with retinal cells showing altered gene expression in response to exogenous and endogenous exposures [218]. Given this scenario, epigenetic mechanisms, especially DNA methylation and histone modifications, might modulate the interaction between genetic factors and environmental exposures [5], affecting both gene expression and genome stability [219,220]. However, the significance of epigenetic alterations in the pathogenesis of AMD has been pending so far.

The methylation process is carried out by DNMTs, out of which only DNMT1, DNMT3A and DNMT3B are catalytically active [8]. In mammals, the methylation process almost occurs at short DNA sequences (i.e. CpG islands) which typically contain around 5-10 CpGs per 100 bp. Up to 80% of CpG islands is localized in non-coding regions scattered throughout the genome that mainly contribute to the global methylation status [8]. LINE-1 sequences, accounting for ≈18% of human genome, are widely used as a surrogate marker of global methylation in aging and age-related disease [25,314-316].

SIRT1, one of the seven mammalian homologs (SIRT1–SIRT7) of yeast silent information regulator 2, is a NAD+-dependent histone deacetylase with multiple roles in aging, apoptosis, DNA repair, inflammation, and oxidative stress [477]. Although DNA methylation and histone deacetylation are distinct biochemical processes that independently control gene expression, SIRT1 regulates the activity of DNMT1, the enzyme responsible for maintenance of DNA methylation [478].

Resveratrol (2,3,4′-trihydroxystilbene), a flavonoid associated with the cardiovascular benefits of red grapes and wine, has been shown to interact with NAD+ and the acetylated substrate, increasing the SIRT1 activity [479,480]. More recently, due to its antioxidant, anti-inflammatory, and anti-angiogenic properties, resveratrol has been also proposed as a candidate for the treatment of ocular diseases [481].

The present study investigated the effect of oxidative stress and inflammation on DNMTs and SIRT1 functions, as well as on LINE-1 methylation levels, in RPE cells. Therefore, we evaluated whether treatment with resveratrol may modulate DNMTs and SIRT1 functions and restore changes in LINE-1 methylation.

6.2 Methods

6.2.1 Cell culture and treatments

The human retinal pigment epithelial cells (ARPE-19), purchased from the American Type Culture Collection (Manassas, VA), were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Gibco BRL), 100 U⁄ml of penicillin and 100 µg⁄ml of streptomycin (Gibco BRL). Cells between 6-10 passages were used in all experiments and incubated at 37 °C and 5% CO2. Medium was changed every 48 hours.

To mimic conditions of oxidative stress and inflammation, cells reaching 80%–90% of confluence were starved in serum-free DMEM and treated with 25 mU/ml glucose oxidase (GOx) or 10 µg/ml lipopolysaccharide (LPS; type Escherichia coli, serotype 0127:B8; Sigma Chemical) for 24 hours, respectively. The concentrations of GOx and LPS were chosen according to previously published studies that used the same cell line [482,483]. To investigate whether resveratrol might restore changes induced by treatment with GOx and LPS, treated and untreated cells were also incubated with 10 µM resveratrol for 24 hours. This concentration was chosen according to results of cell viability after incubation with increasing concentrations (1–10 µM) of resveratrol for 24 hours.

6.2.2 Determination of cell viability

After treatment with GOx, LPS and resveratrol, alone or in combination, we evaluated cell viability by using the Thiazolyl blue tetrazolium bromide (MTT) assay. Briefly, treated and untreated cells were seeded at a density of 2.0×10^4 cells/well in a 96-well plate and incubated for 24 hours. Then, cells were incubated with MTT (1.6 mg/ml) at 37 °C for 4 h. After removing the solution, cells were re-suspended in 100 µl of dimethyl sulfoxide and optical density was read at 540 nm with an optional reference wavelength of 670 nm. Cell viability was reported as percentage of control.

6.2.3 Determination of reactive oxygen species

Intracellular ROS levels were determined using the Abcam cellular ROS detection assay kit according to manufacturer's instructions (Abcam plc, Cambridge, UK). Briefly, cells were seeded at a density of 2.0×10^4 cells/well in a dark, clear bottom 96-well microplate. Cells were rinsed with 100 µL/well of 1X Buffer and stained by adding 100 µL/well of the redox-sensitive fluoroprobe 2',7' – dichlorofluorescin diacetate (DCFDA) for 45 minutes at 37°C in the dark. After removing DCFDA, 100 µL/well of 1X Buffer were added and fluorescence was measured at Ex/Em=485/535 nm. ROS production was reported as percentage of control.

6.2.4 Nuclear protein extraction

Nuclear proteins were extracted using the Nuclear Extraction Kit according to manufacturer's instructions (Abcam plc, Cambridge, UK). Briefly, cell pellet (2×10^6 cells) was obtained by trypsinization and centrifugation of cells at 70-80% of confluence following standard protocols. Cell pellet was re-suspended in 200 µL of pre-extraction buffer and incubated on ice for 10 minutes. After centrifugation, nuclear pellet was re-suspended in 400 µL of extraction buffer and incubated on ice for 15 minutes. Finally, the suspension was centrifuged for 10 minutes at 14,000 rpm at 4°C and the supernatant was transferred into a new vial to measure the protein concentration of the nuclear extract. Nuclear proteins quantification was performed by the Qubit fluorimeter (Invitrogen) using the Qubit Protein Assay Kit according to manufacturer's instructions.

6.2.5 DNMTs activity quantification

Total DNMTs activity was quantified using the colorimetric DNMTs Activity Quantification Kit (Abcam plc, Cambridge, UK) according to manufacturer's instructions. Briefly, 7.5 ng of nuclear extracts were diluted in 50 µl/well of reaction solution and incubated at 37°C for 120 min, including blank and positive control. After removing the reaction solution, each well was rinsed with wash buffer for three times, and 50 µl/well of the diluted capture antibody were added. The plate was covered with aluminium foil and incubated at room temperature for 60 min. After removing the capture antibody, each well was rinsed with wash buffer for three times, and 50 µl/well of the diluted detection antibody were added. The plate was covered with aluminium foil and incubated at room temperature for 30 min. After removing the detection antibody, each well was rinsed with wash buffer for four times, and 50 µl/well of the enhancer solution were added. The plate was covered with aluminium foil and incubated at room temperature for 30 min. After removing the enhancer solution, each well was rinsed with wash buffer for five times, and 100 µl/well of the developer solution were added. Finally, the plate was covered with aluminium foil and incubated at room temperature for 10 min, away from direct light. When the positive control turned to medium blue, 100 µl/well of stop solution were added to stop the reaction. OD was read within 2-10 min at 450 nm with an optional reference wavelength of 655 nm. DNMTs activity was reported as percentage of control.

6.2.6 SIRT1 activity quantification

SIRT1 activity was quantified using a Sirt1 activity assay kit (Abcam plc, Cambridge, UK) according to manufacturer's instructions. The reaction mixture containing 30 µL ddH2O, 5 µL fluoro-substrate peptide, 5 µL NAD, 5 µL developer, and 7.5 ng nuclear extract was mixed thoroughly, and the fluorescence intensity was measured at Ex/Em= 350-450 nm for 30 to 60 minutes at 1-2 min interval. SIRT1 activity was reported as percentage of control.

6.2.7 Real-time polymerase chain reaction

Total cellular RNA was extracted using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to single-stranded cDNA using the SuperScript III Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. mRNA levels were determined by qPCR with TaqMan Gene Expression Assays (Life Technologies, Monza, Italy) using the QuantStudio™ 7 Flex System (Applied Biosystems, Foster City, CA, USA). Specific primers were used to detect DNMT1 (assay no. Hs00945875_m1), DNMT3a (Hs01027162_m1), DNMT3b (Hs00171876_m1), and SIRT1 (Hs01009006_m1). Data were normalized to the values of GAPDH expression (Hs02758991_g1). Relative RNA quantification was performed using the $2^{-\Delta\Delta CT}$ method [484].

6.2.8 DNA extraction and LINE-1 methylation analysis

DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Milan, Italy) and quantified using the Qubit dsDNA High Sensitivity Assay Kit (Life Technologies, Monza, Italy) according to the manufacturer's protocols. Methylation analysis of three CpG sites in the LINE-1 promoter (GeneBank accession no. X58075) was performed by pyrosequencing of bisulfite-converted DNA using PyroMark Q24 instrument (Qiagen, Milan, Italy), as previously reported [32,333]. Briefly, 20 µg of DNA extracted from each sample were converted by bisulfite treatment using the Epitect Bisulfite kit (Qiagen, Milan, Italy). Converted DNA was eluted in 20 µl of Elution buffer and stored at -80 C until used.

A reaction volume of 25 mL was amplified by polymerase chain reaction (PCR), using the PyroMark PCR Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. Briefly, each reaction mixture contained 12.5 µl of PyroMark PCR Master Mix 2X, 2.5 µl of CoralLoad Concentrate 10X, 2 µl of the forward primer (5'-TTTTGAGTTAGGTGTGGGATATA-3') and the reverse-biotinylated primer (5'-biotin-AAAATCAAAAAATTCCCTTTC-3') (0.2 µM for each) and 1.5 µl of bisulfite-converted DNA. HotStart PCR cycling conditions were 1 cycle at 95°C for 15 min, 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30s, and a final extension at 72°C for 10 min.

The biotinylated PCR product was purified and made single stranded using the Pyrosequencing Vacuum PrepTool (Biotage, Inc., Charlottesville, VA, USA). The biotinylated single-stranded product was annealed to the pyrosequencing primer (5' AGTTAGGTGTGGGATATAGT-3') and then subjected to sequencing using an automatically generated nucleotide dispensation order. The pyrogram was analysed using allele quantification mode to determine the proportion of methylated and unmethylated cytosines. LINE-1 methylation level was reported as the average of the three specific CpG sites.

6.2.9 Statistical analyses

All experiments were performed in triplicate for three times. Results were reported as MD ± SE unless otherwise indicated. Statistical differences in the data were evaluated by Student's t-test using the control group as reference. All the analyses were conducted using GraphPad Version 6.0 with a significance level of 0.05.

6.3 Results

6.3.1 Oxidative stress and inflammatory conditions affect cell viability in RPE cells

ARPE-19 cells were treated with GOx to mimic a condition of oxidative stress through the continuous production of H2O2, which leads to ROS production and a cytotoxic effect of less than 50%. Consistently with a previous study [482], compared to untreated cells, treatment with 25 mU/ml GOx for 24 hours reduced cell viability by 35.8% (p=0.004) increasing ROS production by 50.1% (p<0.001) (Figure 1).

Similarly, ARPE-19 cells were treated with LPS (type Escherichia coli, serotype 0127:B8) to mimic an inflammatory condition [485], which leads to a cytotoxic effect of less than 50%. Consistently with a previous study [483], treatment with 10 µg/ml LPS for 24 hours reduced cell viability by 24.2% (p=0.035). Interestingly, treatment with 10 µg/ml LPS for 24 hours significantly increased ROS production by 32.6% than in untreated cells (p=0.004) (**Figure 1**). According to these results, treatments of ARPE-19 with 25 mU/ml GOx or 10 µg/ml LPS for 24 hours were applied for further experiments.

6.3.2 Oxidative stress affects DNMTs and SIRT1 functions and LINE-1 methylation in RPE

To determine whether oxidative stress may affect DNA methylation process, we firstly evaluated DNMTs functions in ARPE-19 cells treated with 25mU/ml GOx for 24 hours (**Figure 2**). Compared to untreated cells, GOx treatment decreased DNMT1, DNMT3a and DNMT3b expression levels (FC=0.63, FC=0.47 and FC=0.46, respectively; p-values <0.05). Accordingly, total DNMTs activity was reduced by 28.5% in GOx treated cells than in untreated ones (p<0.0001). Since DNMTs functions, especially DNMT1, are regulated by SIRT1, we hypothesised that GOx treatment might also affect SIRT1 expression and activity. Interestingly, we demonstrated that GOx treatment decreased SIRT1 expression (FC=0.53; p=0.002) and activity (-29.0%; p<0.0001) compared to untreated cells (**Figure 3**). To evaluate the effect on global DNA methylation, we measured methylation levels of LINE-1, a surrogate marker of global DNA methylation. In line with reduced DNMTs and SIRT1 functions, LINE-1 methylation levels were lower in GOx treated cells compared to untreated ones (69.6% ± 0.1 vs. 72.6% ± 0.1; p<0.0001) (**Figure 4**).

6.3.3 Inflammatory condition affects DNMTs and SIRT1 functions and LINE-1 methylation in RPE

To determine whether inflammatory condition may affect DNA methylation process, we firstly evaluated DNMTs functions in ARPE-19 cells treated with 10 μg/ml LPS for 24 hours (**Figure 5**). Previous studies reported that treatment with LPS of RPE cells increased the expression of pro-inflammatory cytokines IL-6 and IL-8 [483,485]. Our study added to the current knowledge, demonstrating that LPS treated cells exhibited lower DNMT1 expression level (FC= 0.50; p=0.004), while DNMT3A and DNMT3B expression seemed to be unaffected. Consistently, treatment with LPS reduced total DNMTs activity by 14.9 % (p=0.007). Compared to untreated cells, we also showed that LPS treatment decreased both SIRT1 expression (FC=0.57; p=0.003) and activity (-20.1%; p=0.002) (Figure 6). In line with these results, treated cells exhibited lower LINE-1 methylation levels compared to untreated ones (69.7% ± 0.4 vs. 72.6% ± 0.1; p<0.0001) (**Figure 4**).

6.3.4 Resveratrol ameliorates viability and ROS production in cells under oxidative and inflammatory conditions

We also aimed at demonstrating the antioxidant and anti-inflammatory effect of resveratrol against GOx- and LPS-induced changes in ARPE-19 cells. Firstly, we determined viability of cells exposed to various concentrations of resveratrol (1–10 μM) for 24 hours. In line with a previous study [486], we found that treatment with 1-10 μM resveratrol for 24 hours did not affect viability of ARPE-19 cells. Similarly, resveratrol treatment (1-10 μM) of control cells did not induce changes in ROS production (data not shown). However, **Figure 1** shows that treatment with 10 μM resveratrol for 24 hours was able to ameliorate cell viability and to alleviate ROS production in ARPE-19 cells upon oxidative stress and inflammatory conditions (p-values>0.05 vs. control).

6.3.5 Resveratrol restores DNMTs/SIRT1 functions and LINE-1 methylation in cells under oxidative and inflammatory conditions

Finally, we evaluated whether resveratrol may modulate DNMTs and SIRT1 functions and restore changes in LINE-1 methylation. We demonstrated that treatment with 10 μM resveratrol for 24 hours restored both the expression and activity of DNMTs (**Figure 2**) and SIRT1 (**Figure 3**) in ARPE-19 cells upon oxidative stress condition (p-values>0.05). Similarly, resveratrol increased DNMT1 expression and total DNMTs activity (**Figure 5**), as well as SIRT1 expression and activity (**Figure 6**) in cells upon inflammatory condition (p-values>0.05). In line with these results, resveratrol also restored LINE-1 methylation levels in cells upon oxidative stress (72.4% ± 0.1; p>0.05) and inflammatory (72.3% ± 0.1; p>0.05) conditions (**Figure 4**).

6.4 Discussion

The functional mechanisms involved in the aging of the retina are not clearly elucidated and the discovery of both genetic and environmental risk factors begs the question whether there is an interaction in the pathogenesis of AMD. In general, this hypothesis has been raised by genome-wide association studies that have failed to explain the incomplete genetic heritability in complex diseases such as AMD [487]. Although it is becoming evident that epigenetic mechanisms - including DNA methylation and histone modification - might explain how interactions between genetics and the environment result in particular phenotypes, the extent to which DNA methylation contributes to AMD is not currently clarified. Evidence that methylation of repetitive elements changes over time points out LINE-1 methylation as a surrogate marker of global methylation in aging and age-related disease [25,314-316]. Efforts to understand the mechanisms underpinning the multifactorial nature of AMD have led us to explore DNA methylation process in RPE cells upon oxidative and inflammatory conditions, two of the major causes of retinal degeneration [488]. To our knowledge, the present work demonstrated for the first time that oxidative stress and inflammatory conditions modulate DNMTs and SIRT1 functions - some of the main enzymes involved in epigenetic mechanisms – and reduce LINE-1 methylation in RPE cells. Particularly, decreased LINE-1 methylation leads to genomic instability and plays a crucial role in the development of chronic degenerative disease. In patients with AMD, the degeneration of RPE layer is a progressive process with severe consequences on visual pigment regeneration, synthesis and remodelling of the interphotoreceptor matrix, transport of nutrients, ions and waste products, absorption of light via the pigmentation, and adhesion to the retina [50]. Interestingly, the effect of oxidative stress and inflammatory conditions on retinal DNMTs functions and LINE-1 methylation was similar, suggesting it as a convergence point during the pathogenesis of AMD. However, while inflammatory condition seemed to affect only DNMT1 expression - the maintenance DNMT - oxidative stress also downregulated de novo DNMTs (i.e. DNMT3a and DNMT3b), which in turn enable key epigenetic modifications for cellular differentiation, transcriptional regulation, heterochromatin formation, X-inactivation, imprinting and genome stability [489]. In support of the interplay between inflammation and oxidative stress, we also observed that RPE cells treated with LPS - to mimic an inflammatory condition with increased expression of pro-inflammatory cytokines [483,485] - also exhibited higher ROS production compared to untreated cells. In fact, a chronic inflammatory status might be exacerbated over time by the accumulation of oxidation products, which in turn cause tissue damage and impairment of central vision [488].

Several lines of evidence proposed the relationship of oxidative stress and inflammation with DNA methylation in retinal degenerative diseases, especially in AMD. Oxidative stress occurs when ROS

levels exceeds the detoxifying capacity of antioxidants or molecular chaperones [490]. A previous study, comparing DNA methylation between AMD patients and age-matched controls, revealed that GSTM1 and GSTM5 undergo epigenetic repression in AMD RPE/choroid via promoter hypermethylation, which in turn decreased mRNA and protein levels [222]. These enzymes play an important role in the detoxification of electrophilic compounds, including products of oxidative stress, by conjugation with glutathione; reduced activity of GSTM1 and GSTM5 could affect protection from genome-damaging oxidants with increased vulnerability to oxidative insults. Genome-wide differences in DNA methylation between three pairs of twins (both monozygotic and dizygotic) with discordant AMD were assessed by Wei and colleagues [225]. Their results, further validated in discordant siblings for AMD and in a case–control study, reported a significantly decreased level of IL17RC promoter methylation in AMD patients, which in turn led to increased expression of its protein and mRNA in peripheral blood and in the affected retina [225]. The IL17RC gene encodes for an essential subunit of the IL-17 receptor complex that modulates activity of proinflammatory IL-17A and IL-17F. Although these findings were not confirmed in a subsequent study [491], the putative epigenetic mechanism by which proinflammatory stimuli could promote AMD pathology should be investigated.

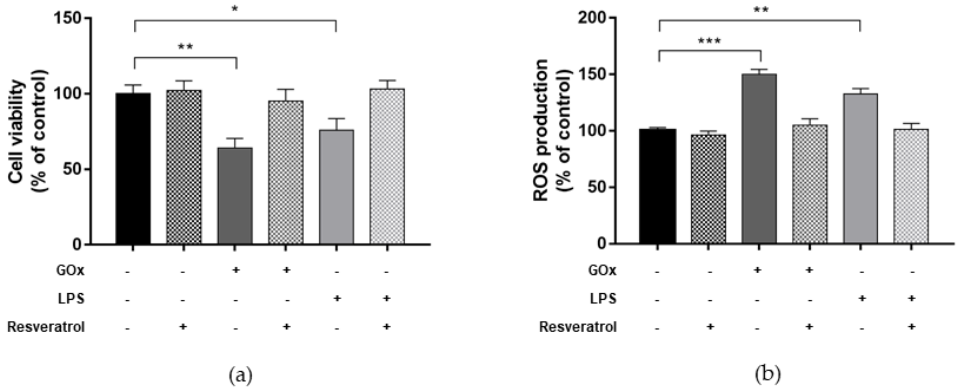
Recently, several lines of evidence suggested that SIRT1, a NAD+-dependent histone deacetylase, protects RPE cells against apoptosis and counteracts changes in RPE functions induced by oxidative stress and chronic inflammations. In fact, SIRT1 might be involved in the AMD pathogenesis by modulating cell senescence, DNA damage repair and apoptosis [477]. Previous studies demonstrated that SIRT1 expression significantly decreased with increasing age in retinal stem cells, and that it was down-regulated in human AMD retina compared to non-AMD donors [492]. Consistently, we reported for the first time that RPE cells upon oxidative and inflammatory conditions exhibited decreased SIRT1 expression and activity compared to untreated cells. Since SIRT1 regulates the activities of DNMTs, especially DNMT1 [478], this result partially explains how oxidative stress and inflammation might affect DNA methylation mechanism. Interestingly, SIRT1 also attenuated changes induced by Amyloid beta, a known constituent of drusen which induces chronic inflammation [493]. In fact, treatment with a SIRT1 agonist (i.e. SRT1720) restored Aβ-induced upregulation of IL-6, IL-8, and matrix metalloproteinase-9 (MMP- 9); this inhibitory effect was abolished in SIRT1 knockdown cells [493]. In addition, a mutual effect between SIRT1 and NF-κB has been established: if, on one hand, SIRT1 inhibits the activation of NF-κB-mediated inflammatory pathway, on the other hand, NF-κB signaling and inflammatory response can suppress SIRT1 activity [494]. Whilst it appears evident that SIRT1 is crucial for maintaining integrity and function of RPE cells under oxidative stress and chronic inflammation, results about the role of SIRT1 during neovascularization are controversial [495,496]. In

fact, a previous study, founding higher SIRT1 expression level in CNV membranes than in healthy donor eyes, demonstrated that SIRT1 inhibition with nicotinamide decreased the secretion of proangiogenic factors, such as VEGF-A, platelet-derived growth factor BB and angiogenin in ARPE-19 cells [497]. By contrast, others revealed that treatment with resveratrol, a natural SIRT1 activator, restored VEGF secretion in RPE cells upon oxidative and inflammatory conditions [498]. Resveratrol stimulates SIRT1 activity through allosteric interaction and increases SIRT1 affinity for both NAD+ and the acetylated substrate [479,480]. This is consistent with another study indicating that resveratrol downregulated VEGF expression and inhibited hypoxic-induced choroidal vascular endothelial cell proliferation, via modulating the SIRT1 pathway [223]. However, there is also evidence that resveratrol might inhibit angiogenesis both in vivo and in vitro by a SIRT1-independent pathway [499]. The present study adds to the current knowledge, demonstrating that treatment with 10 μM resveratrol for 24 hours not only ameliorated cell viability and ROS production in ARPE-19 cells upon oxidative stress and inflammatory conditions, but also counteracted the detrimental effect on LINE-1 methylation via increasing DNMTs and SIRT1 functions. However, further studies should be performed to elucidate controversies regarding the role of SIRT1 in AMD pathogenesis in general, and in CNV in particular. Moreover, it is worth investigating whether the protective effect of resveratrol on RPE cells relies on SIRT1 activation rather than on its antioxidant and anti-inflammatory properties.

In conclusion, we demonstrated for the first time that oxidative stress and inflammatory conditions negatively affect both DNMTs and SIRT1 functions, and LINE-1 methylation in RPE cells. Interestingly, treatment with 10 μM resveratrol for 24 hours counteracts these detrimental effects. Further studies should be encouraged to explore the perspectives of resveratrol as a suitable strategy for the prevention and/or treatment of retinal degenerative diseases.

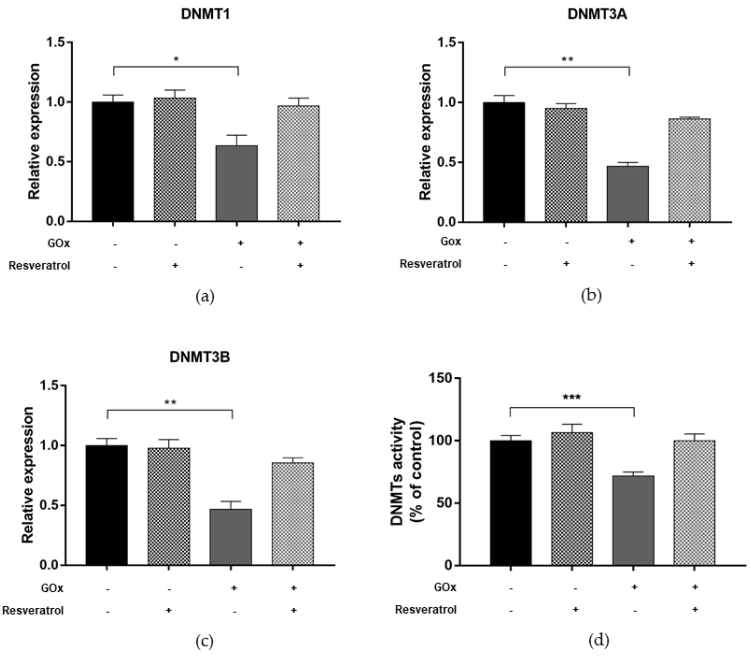
Tables and Figures

Figure 1. Cell viability and ROS production in ARPE-19 cells upon oxidative stress and inflammatory conditions.



(a) MTT assay showed that treatment with 25 mU/ml GOx or 10 µg/ml LPS for 24 hours reduced cell viability by 35.8% (p=0.004) and 24.2% (p=0.035), respectively. (b) The determination of ROS demonstrated higher ROS production in GOx- and LPS-treated cells compared to controls (50.1%, p<0.001; 32.6%,p=0.004; respectively). Resveratrol restores viability (a) and ROS production (b) in cells upon oxidative and inflammatory conditions. The experiment was performed in triplicate and repeated three times. Bar graphs show mean ± SE. * p<0.05, ** p<0.01, *** p<0.001 vs. control.

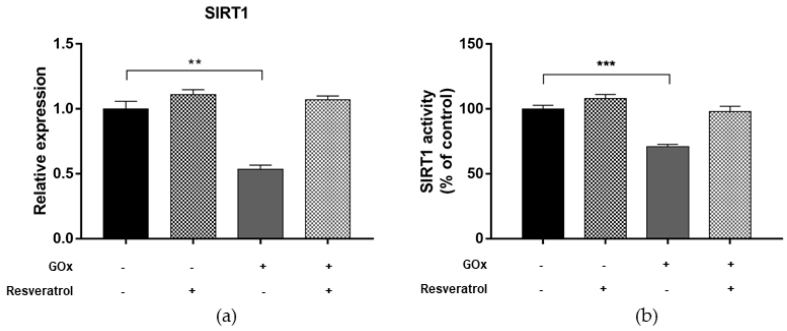
Figure 2. DNMTs expression and activity in ARPE-19 cells upon oxidative stress.



(a)(b)(c) Analysis of gene expression showed that treatment with 25 mU/ml GOx for 24 hours downregulated DNMT1, DNMT3A and DNMT3b expression levels (FC=0.63, FC=0.47 and FC=0.46, respectively; p-values <0.05). (d) Analysis of total DNMTs enzymatic activity using a colorimetric assay confirmed that total DNMTs activity was reduced by 28.5% in GOx treated cells compared to controls (p<0.0001). Treatment with 10 µM resveratrol for 24 hours restores DNMTs functions in cells upon

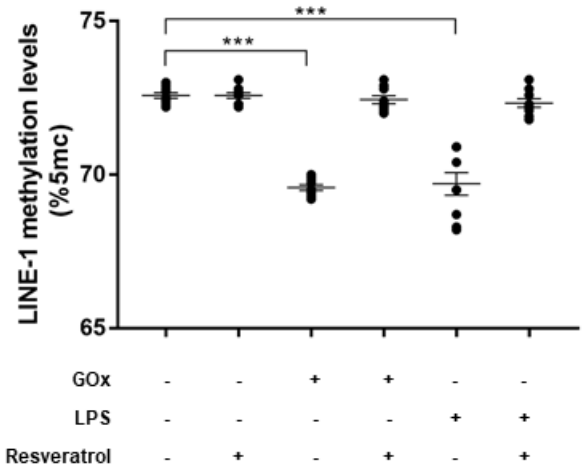
oxidative. The experiment was performed in triplicate and repeated three times. Bar graphs show mean ± SE. * p<0.05, ** p<0.01, *** p<0.001 vs. control based on the Student's t-test.

Figure 3. SIRT1 expression and activity in ARPE-19 cells upon oxidative stress.



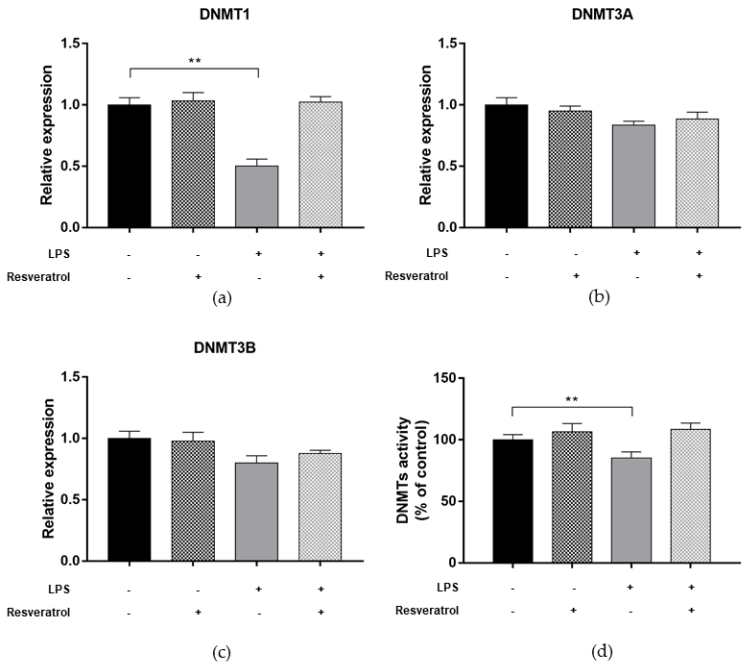
(a) Analysis of gene expression showed that treatment with 25 mU/ml GOx for 24 hours downregulated SIRT1 expression level (FC=0.53; p=0.002). (d) Analysis of SIRT1 enzymatic activity using a fluorimetric assay confirmed that total SIRT1 activity was reduced by 29.0% in GOx treated cells compared to controls (p<0.0001). Treatment with 10 μM resveratrol for 24 hours restores SIRT1 functions in cells upon oxidative stress. The experiment was performed in triplicate and repeated three times. Bar graphs show mean ± SE. ** p<0.01, *** p<0.001 vs. control based on the Student's t-test.

Figure 4. LINE-1 methylation levels in ARPE-19 cells upon oxidative stress and inflammatory conditions.



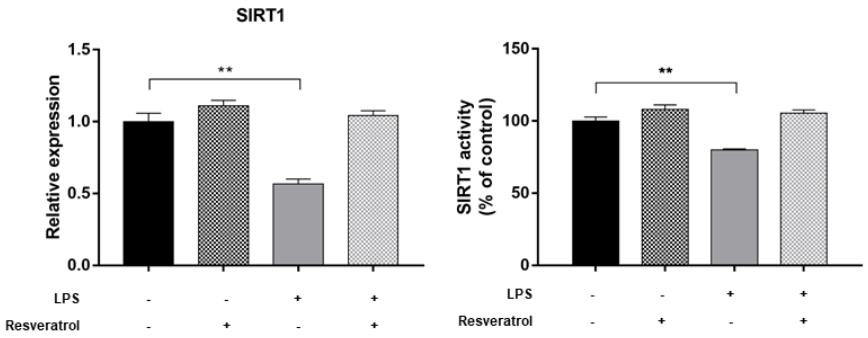
Compared to controls (72.6% ± 0.1), LINE-1 methylation levels were lower in GOx (69.6% ± 0.1; p<0.0001) and LPS (69.7% ± 0.4; p<0.0001) treated cells. Treatment with 10 μM resveratrol for 24 hours restores LINE-1 methylation levels in cells upon oxidative stress (72.4% ± 0.1; p>0.05) and inflammatory (72.3% ± 0.1; p>0.05) conditions. The experiment was performed in triplicate and repeated three times. *** p<0.001 vs. control based on the Student's t-test.

Figure 5. DNMTs expression and activity in ARPE-19 cells upon inflammatory condition.



(a) Analysis of gene expression showed that treatment with 10 µg/ml LPS for 24 hours downregulated DNMT1 expression level (FC= 0.50; p=0.004), (b)(c) while DNMT3A and DNMT3B expression seemed to be unaffected. (d) Analysis of total DNMTs enzymatic activity using a colorimetric assay confirmed that total DNMTs activity was reduced by 14.9 % in LPS treated cells compared to controls (p=0.007). Treatment with 10 µM resveratrol for 24 hours restores DNMTs functions in cells upon inflammatory condition. The experiment was performed in triplicate and repeated three times. Bar graphs show mean ± SE. ** p<0.01 vs. control based on the Student's t-test.

Figure 6. SIRT1 expression and activity in ARPE-19 cells upon inflammatory condition.



(a) Analysis of gene expression showed that treatment with 10 µg/ml LPS for 24 hours downregulated SIRT1 expression level (FC=0.57; p=0.003).(d) Analysis of SIRT1 enzymatic activity using a fluorimetric assay confirmed that total SIRT1 activity was reduced by 20.1% in LPS treated cells compared to controls (p=0.002). Treatment with 10 µM resveratrol for 24 hours restores SIRT1 functions in cells upon inflammatory condition. The experiment was performed in triplicate and repeated three times. Bar graphs show mean ± SE. ** p<0.01 vs. control based on the Student's t-test.

7 Characterization of SIRT1/DNMTs functions and LINE-1 methylation in age-related macular degeneration: a cross sectional study

7.1 Background

Growing body of evidence suggests a possible role for epigenetic changes in AMD aetiology. In general, Epigenetics refer to stable and heritable patterns of gene expression and genomic functions that do not involve changes in DNA sequence [5]. In particular, DNA methylation - the most investigated epigenetic hallmark - is a reversible mechanism that affects genome function and chromosomal stability by the addition of methyl groups to cytosine located in CpG islands to form 5mC. AMD patients displayed elevated homocysteine, vitamin B12 and folate deficiency, suggesting a dysregulation in the S-adenosylmethionine cycle that contributes methyl donors for DNA methylation [500]. In the last few years, aberrant epigenetic patterns have been described in several pathophysiological processes - including aging, oxidative stress, inflammation, and angiogenesis - that are related to the pathogenesis of AMD and DR [501,502]. Although previous studies focused on DNA methylation patterns in genes affecting AMD aetiology, the role of methylation of repetitive elements in peripheral blood leukocytes of AMD patients has never been investigated so far. Repetitive elements - including Alu and LINE-1 sequences - represent a large genomic portion and contain numerous CpG dinucleotides. Therefore, methylation status of these sequence has been used as a surrogate marker of global genomic DNA methylation to explore the association with several age-related diseases [25,314-316]. The current cross-sectional study aimed at investigating DNMTs functions and LINE-1 methylation levels in peripheral blood samples of AMD patients and controls.

7.2 Methods

7.2.1 Study design

From July 2017 to July 2018, subjects referred to the Department of Ophthalmology, University of Catania (Italy), were enrolled in a cross-sectional study. The study protocol was approved by the ethics committee of the involved institution and performed according to the Declaration of Helsinki. Subjects were fully informed of the purpose and procedures of the study and an informed consent was signed. During the routine eye exam, AMD was diagnosed by dilated retinal exam, optical coherence tomography and fluorescein angiography. The following inclusion criteria were applied: i) AMD patients ii) aged over 50 years iii) with no previous/current ocular diseases of the posterior segment except AMD. Patients with cardiovascular disease, diabetes mellitus, autoimmune disorders

and history of cancer were excluded. Age- and sex-matched controls were enrolled among patients who underwent cataract surgery fulfilling the inclusion and exclusion criteria.

Information on sociodemographic and lifestyle data were collected by trained epidemiologists using a structured questionnaire. Educational level was classified as low (≤8 years of school) and high (>8 years of school). Subjects were also classified as employed or unemployed (including students and housewives). BMI was calculated as weight (kg) divided by height (m^2), based on criteria from the World Health Organization [326]. For smoking status, subjects were classified as no smokers (including ex-smokers) and current smokers. From each participant, a peripheral blood sample was collected into EDTA tubes for molecular analysis.

7.2.2 Nuclear protein extraction

Nuclear proteins were extracted from peripheral blood samples using the Nuclear Extraction Kit according to manufacturer's instructions (Abcam plc, Cambridge, UK). Nuclear proteins quantification was performed by the Qubit fluorometer (Invitrogen), using the Qubit Protein Assay Kit according to manufacturer's instructions.

7.2.3 DNMTs activity quantification

Total DNMTs activity was quantified using the colorimetric DNMTs Activity Quantification Kit (Abcam plc, Cambridge, UK) according to manufacturer's instructions. Briefly, 7.5 ng of nuclear extracts were diluted in 50 µl/well of reaction solution and incubated at 37°C for 120 min, including blank and positive control. After removing the reaction solution, each well was rinsed with wash buffer for three times, and 50 µl/well of the diluted capture antibody were added. The plate was covered with aluminium foil and incubated at room temperature for 60 min. After removing the capture antibody, each well was rinsed with wash buffer for three times, and 50 µl/well of the diluted detection antibody were added. The plate was covered with aluminium foil and incubated at room temperature for 30 min. After removing the detection antibody, each well was rinsed with wash buffer for four times, and 50 µl/well of the enhancer solution were added. The plate was covered with aluminium foil and incubated at room temperature for 30 min. After removing the enhancer solution, each well was rinsed with wash buffer for five times, and 100 µl/well of the developer solution were added. Finally, the plate was covered with aluminium foil and incubated at room temperature for 10 min, away from direct light. When the positive control turned to medium blue, 100 µl/well of stop solution were added to stop the reaction. OD was read within 2-10 min at 450 nm with an optional reference wavelength of 655 nm. DNMTs activity was reported as percentage of control.

7.2.4 SIRT1 activity quantification

SIRT1 activity was quantified using a Sirt1 activity assay kit (Abcam plc, Cambridge, UK) according to manufacturer's instructions. The reaction mixture containing 30 µL ddH2O, 5 µL fluoro-substrate peptide, 5 µL NAD, 5 µL developer, and 7.5 ng nuclear extract was mixed thoroughly, and the fluorescence intensity was measured at Ex/Em= 350-450 nm for 30 to 60 minutes at 1-2 min interval. SIRT1 activity was reported as percentage of control.

7.2.5 Real-time polymerase chain reaction

Total cellular RNA was extracted using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to single-stranded cDNA using the SuperScript III Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. mRNA levels were determined by qPCR with TaqMan Gene Expression Assays (Life Technologies, Monza, Italy) using the QuantStudio™ 7 Flex System (Applied Biosystems, Foster City, CA, USA). Specific primers were used to detect DNMT1 (assay no. Hs00945875_m1), DNMT3a (Hs01027162_m1), DNMT3b (Hs00171876_m1), and SIRT1 (Hs01009006_m1). Data were normalized to the values of GAPDH expression (Hs02758991_g1). Relative RNA quantification was performed using the $2^{-\Delta\Delta CT}$ method [484].

7.2.6 DNA extraction and LINE-1 methylation analysis

Peripheral blood samples were centrifuged at 2500 rpm for 15 min. The buffy coat fraction was transferred to a cryovial and immediately frozen at -20 °C until use. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Italy) according to the manufacturer's protocol. LINE-1 methylation level was measured by pyrosequencing-based methylation analysis, using the PyroMark Q24 instrument (Qiagen, Italy), as previously reported [32,333]. Briefly, bisulfite conversion and clean-up of DNA for methylation analysis of 30–40 ng of DNA were completed using the EpiTect Bisulfite Kit (Qiagen, Italy) and the converted DNA was eluted in 20 µl of Eluition Buffer. PCR was conducted in a reaction volume of 25 µl, using the PyroMark PCR Kit (Qiagen, Italy). According to the manufacturer's instructions, each reaction mixture contained 1.5 µl of bisulfite-converted DNA, 12.5 µl of PyroMark PCR Master Mix 2X, 2.5 µl of Coral Load Concentrate 10X, and 2 µl of the forward primer (5′-TTTTGAGTTAGGTGTGGGATATA-3′) and the reverse-biotinylated primer (5′-biotin-AAAATCAAAAAATTCCCTTTC-3′) (0.2 µM for each). HotStart PCR cycling conditions were 1 cycle at 95 °C for 15 min, 40 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30s, and a final extension at 72 °C for 10 min. Then, the PCR product underwent pyrosequencing using 0.3 mM of the sequencing primer (5′-AGTTAGGTGTGGGATATAGT-3′). All runs included 0% and 100% methylated human DNA as positive controls as well as a negative control. To confirm reproducibility

every sample was tested two times and failed assays were repeated. Overall, intra-observer coefficient of variability between the two replicates of LINE-1 methylation measurements was 3.2% (SD=3.0%). LNE-1 methylation levels were calculated as percentage of methylated cytosines over the sum of methylated and unmethylated cytosines and reported for each CpG site as well as the average of the three CpG sites (GenBank Accession No. X58075).

7.3 Results

7.3.1 Characteristics of study population

Forty AMD patients (mean age=69.8 years; 55% male) and 10 age- and sex-matched controls (mean age= 68.9 years; 55% male) were enrolled in this cross-sectional study. The comparison of sociodemographic characteristics and lifestyle between AMD patients and controls revealed no significant differences.

7.3.2 DNMTs and SIRT1 functions in AMD patients

The expression of DNMT1 and DNMT3b was upregulated in AMD patients compared to controls (p=0.003 and p=0.018, respectively), and was in line with increased DNMTs activity (48%; p=0.005) (**Figure 1**). Instead, no difference was observed in DNMT3a expression. Since DNMTs functions are regulated by SIRT1 [478], we hypothesised that AMD patients also exhibited altered SIRT1 expression and activity. However, we did not find statistically significant difference between AMD patients and controls in SIRT1 expression and activity (**Figure 2**).

7.3.3 LINE-1 methylation in AMD patients

To evaluate the effect of increased DNMTs function on global DNA methylation, we measured methylation levels of LINE-1, a surrogate marker of global DNA methylation. Overall, the average LINE-1 methylation level was 68.42% and no differences according to age, sex, educational level, employment status, nutritional status, and smoking were evident. In line with increased DNMTs function, LINE-1 methylation levels were higher in AMD patients compared to controls (69.10 % ± 0.68 vs. 65.73 % ± 0.59; p =0.020) (**Figure 3**).

7.4 Discussion

To our knowledge, the present study is the first investigating LINE-1 methylation in peripheral blood leukocytes of AMD patients. Previous studies have focused on DNA methylation changes in genes affecting AMD aetiology. For instance, Hunter and colleagues found that GSTM1 and GSTM5 genes undergo epigenetic repression in AMD RPE/choroid via promoter hypermethylation [222]. These enzymes play an important role in the detoxification of electrophilic compounds including products of oxidative stress. Wei et al. analysed genome-wide differences in blood DNA methylation between

three pairs of twins [225], demonstrating that methylation level in the IL17RC gene was lower in those affected by AMD than in healthy twins [225]. Although, they further validated these findings in discordant siblings for AMD and in an AMD case–control cohort [225], others failed in confirming this evidence [491]. It is worth mentioning that the IL17RC gene encodes for an essential subunit of the IL-17 receptor complex that modulates activity of proinflammatory IL-17A and IL-17F. Overall, these results are in line with the well-established role of oxidative and inflammatory pathways in AMD pathogenesis. Our study adds to this knowledge, demonstrating that LINE-1 methylation was higher in blood leukocytes of AMD patients when compared to healthy controls. Consistently, AMD patients also exhibited increased DNMTs and SIRT1 functions, suggesting that global DNA methylation changes in AMD might be modulated by a DNMTs/SIRT1 dependent pathway. As mentioned above, this is the first study evaluating LINE-1 methylation in blood samples from a cohort of AMD patients and healthy matched controls. However, the increased LINE-1 methylation in AMD patients is in agreement with LINE1 hypermethylation reported by previous studies on patients with Alzheimer disease [503,504]. AMD and Alzheimer disease share some environmental risk factors (i.e. smoking, systemic hypertension, and hypercholesterolemia) and histopathologic features such as the deposition of amyloid-β in ocular drusen and senile plaques [505]. Moreover, aging - recognized as the most important risk factor for developing both AMD and Alzheimer disease [506] - has been linked with global DNA hypomethylation and hypermethylation of CpG islands [43]. In peripheral blood cells, it has been reported a time-dependent change in global DNA methylation within the same individual over time [507]. However, we did not observe a significant association between age and LINE-1 methylation, either in healthy volunteers or in AMD patients, probably due to the small number and age range of our population. Results from this cross-sectional study diverge from previous report of a LINE-1 loss of methylation in RPE cells under oxidative and inflammatory stress (**see chapter 6**), further supporting the notion that methylation profiles in leukocytes might differ from those in the retina.

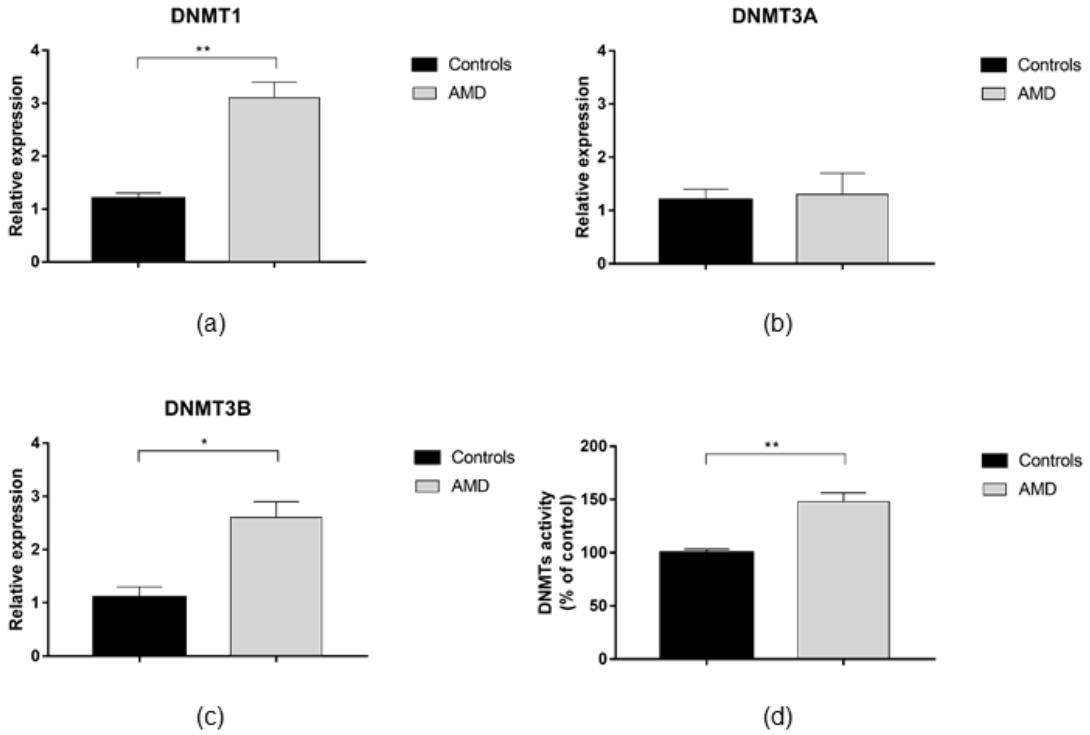
The present study had some limitations. First, the cross-sectional design does not allow to understand if changes in LINE-1 methylation are a cause or a consequence of AMD. Second, LINE-1 methylation levels can vary across target CpG sites and tissue type [344]. Thus, the distinctiveness of LINE-1 methylation levels discourages the comparison between results from studies which evaluate LINE-1 methylation status at different CpG sites [508]. Third, previous studies reported that differences in blood cell composition could lead to variation in methylation levels [509]. Although DNA was extracted from leukocytes, differences in the subtype composition could represent a weakness of our study, reinforcing the importance of accounting for cellular heterogeneity in clinical practice and research [510]. Finally, AMD and epigenetic biomarkers are both influenced by environmental and/or

endogenous factors including smoking, nutritional imbalance (folate/B6/B12 deficiency, over-nutrition), sunlight exposure and oxidative stress.

In conclusion, we demonstrated for the first time that AMD patients exhibited increased DNMTs and SIRT1 functions compared to healthy subjects. Interestingly, these changes are associated with higher LINE-1 methylation level in blood samples. However, further prospective researches are encouraged to understand if the modulation of these pathways is a cause or a consequence of AMD.

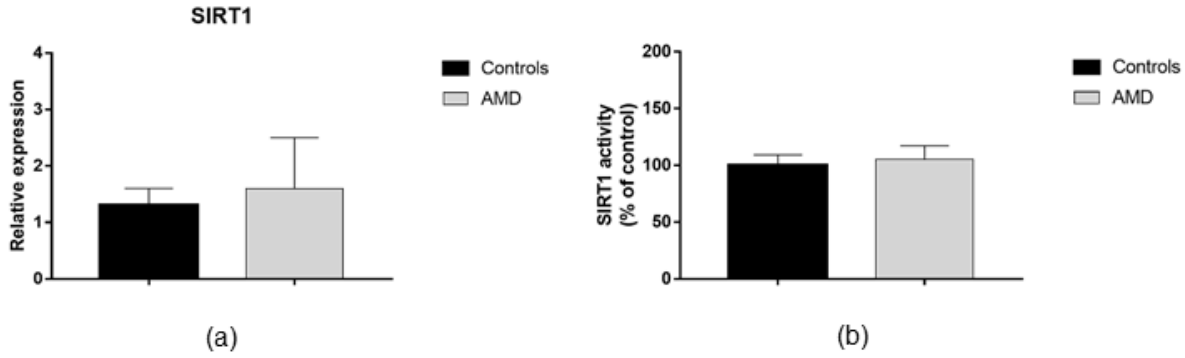
Figures

Figure 1. DNMTs expression and activity in AMD patients and matched controls.



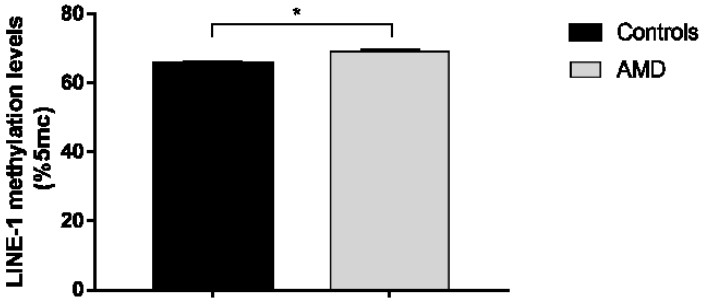
The analysis of gene expression showed that (a) DNMT1 (p=0.003) and (c) DNMT3b (p=0.018) were upregulated in AMD patients (n=40) compared to controls (n=10). (b) No difference was observed in DNMT3a expression. (c) The analysis of total DNMTs activity showed higher levels in AMD patients than in controls (48%; p=0.005). Bar graphs show mean ± SE. * p<0.05, ** p<0.01 vs. control based on the Student's t-test.

Figure 2. SIRT1 expression and activity in AMD patients and matched controls.



The analysis of gene expression (a) and enzymatic activity showed no differences between AMD patients (n=40) controls (n=10).

Figure 3. LINE-1 methylation levels in AMD patients and matched controls.



8 DNMTs functions and LINE-1 methylation in a cellular model of hyperglycaemia-induced retinopathy: the role of curcumin

8.1 Background

The growing incidence of DM and longer life span in the aging population point towards an increase in patients with DR. The incidence of DR appears to be higher in patients suffering from type 1 than in those with type 2 DM [511]. However, regardless of the type of diabetes, both hyperglycaemia and hyperglycaemia-induced oxidative stress have been recognized as the major contributing factors in DR aetiology [512,513]. It has been also demonstrated that DR progression continues even if normal glycaemic control is restored, suggesting that the detrimental effect depends on both the duration and the severity of hyperglycaemic insult [514]. Oxidative stress has been shown to alter histone modifications and DNA methylation [317], which have been further identified as potential epigenetic mechanisms involved in the pathophysiology of DR [310,318-320].

The effects of curcumin - a natural phenol from the rhizome of Curcuma longa - have been determined in animal models and in vitro systems [515]. Several lines of evidence have shown that curcumin significantly decreases lipid peroxidation, increases intracellular antioxidant amount, regulates antioxidant enzymes, and scavenges hyperglycaemia-induced ROS production [516,517]. Particularly, treatment with curcumin reduced ROS production both in RPE cells [518] and in the retina of diabetic rats [519]. However, the relying antioxidant activity of curcumin on epigenetic mechanisms has not been completely elucidated.

The present study investigated the effect of hyperglycaemia and high glucose-induced oxidative stress on retinal DNMTs activity and expression, as well as on LINE-1 methylation levels. To achieve this objective, we compared the expression levels of DNMTs in retina of diabetic and non-diabetic mice, using the Gene Expression Omnibus (GEO) datasets. In RPE cells, we analysed the time-related effect of high glucose condition on ROS production, DNMTs activity and expression. Finally, we evaluated whether the antioxidant properties of curcumin may restore high glucose-induced changes of DNMTs function in RPE cells.

8.2 Methods

8.2.1 Microarray data

Microarray datasets were retrieved and downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo) of the National Center for Biotechnology Information (NCBI), using the keyword “diabetic retinopathy”. The GSE12610 dataset included expression microarray profiling data derived from whole retina of adult CD1 streptozotocin (STZ)-induced diabetic mice

(3-week and 5-week) and age-matched controls. Mice with glucose levels above 250mg/dL were considered diabetic from the date of the last injection. To get adequate amounts of RNA, four retinas (2 animals) for each group were pooled. Total RNA was extracted and processed for being hybridized on the GPL1261 platform of Affymetrix Mouse Genome 430 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA). The GSE19122 dataset compared expression microarray profiling data derived from whole retina of eight C57BL/6J STZ-induced diabetic mice and nine insulin-deficient $Ins2^{Akita}$ mice after 3 months of hyperglycaemia, with those derived from eight controls. Total RNA was extracted and processed for being hybridized on the GPL6885 platform of Illumina MouseRef-8 v2.0 expression Beadchip (Illumina, Inc., San Diego, USA) [520]. The dataset GSE55389 included expression microarray profiling data derived from whole retina of four 8-week old db/db diabetic mice and four age-matched lean non-diabetic controls. Total RNA was extracted and processed for being hybridized on the GPL6246 platform of Affymetrix Mouse Gene 1.0 ST Array (Affymetrix, Inc., Santa Clara, CA, USA) [521]. Due to skewed distribution, for each dataset, raw data of DNMT1, DNMT3a and DNMT3b were extracted and signal values from the selected genes were log-transformed and normalized using the MeV free software online. Difference in log-transformed DNMTs expression levels between diabetic mice and controls was compared using the student's t-test and reported as absolute mean difference (MD).

8.2.2 Cell culture

ARPE-19 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% foetal bovine serum (FBS; Gibco BRL), 100 U⁄ml of penicillin and 100 µg⁄ml of streptomycin (Gibco BRL). Cells maintained at the following glucose conditions were used:

- Cells maintained at 5.5 mM glucose for 3 weeks (Normal glucose - NG - condition);
- Cells maintained at 25 mM glucose for 3 weeks (Chronic high glucose - HG - condition);
- Cells maintained at 5.5 mM glucose for 3 weeks, and then transferred to 25 mM glucose medium for 24 hours (Acute HG condition).

Normal glucose condition (5.5 mM) corresponded to fasting plasma glucose level of diabetes-free subjects, while high glucose condition (25 mM) reflected 2 h after-meal plasma glucose level in diabetic patients [522-524]. To rule out the potential effect of hyperosmotic stress, cells maintained in 25 mM mannitol medium were used as osmotic control. Cells between 6-10 passages were used in all experiments and incubated at 37 °C and 5% CO2. Medium was changed every 48 hours.

8.2.3 Curcumin treatment

The effect of curcumin on ROS production, DNMTs activity and expression, and LINE-1 methylation was evaluated in ARPE-19 cells. In brief, cells were maintained either at 5.5 mM or 25 mM glucose concentrations or maintained at 5.5 mM glucose and then transferred to 25 mM glucose medium. After 24 hours, cells were exposed to 25 μM curcumin (Sigma Aldrich, St. Louis, MO) for 6 hours, and then processed to further analyses.

8.2.4 Determination of cell viability

To evaluate the effect of curcumin on cell viability, the MTT assay was performed. Cells, maintained either in normal or high glucose conditions, were seeded at a density of 2.0×10^4 cells/well in a 96-well plate. Cells were then exposed to increasing concentrations (1–50 μM) of curcumin for 6 h. To determine the time-dependent effect of curcumin treatment on cell viability, cells were also exposed to 25 μM curcumin for 1 to 24 hours. MTT (1.6 mg/ml) was added to the cells in each well, followed by a further incubation at 37 °C for 4 h. After removing the solution, cells were resuspended in 100 μl of dimethyl sulfoxide (DMSO). Optical density was read at 540 nm and background was subtracted at 670 nm. Cell viability (%) was reported as (OD of the treated samples/OD of the control)×100.

8.2.5 Determination of reactive oxygen species

The intracellular ROS level was determined using the Abcam cellular ROS detection assay kit (Abcam plc, Cambridge, UK). The redox-sensitive fluoroprobe 2',7' –dichlorofluorescin diacetate (DCFDA) is a fluorogenic dye that measures hydroxyl, peroxyl and ROS activity within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively. Briefly, ARPE-19 cells were seeded at a density of 2.0×10^4 cells/well in a dark, clear bottom 96-well microplate. After removing the media, cells were rinsed with 100 μL/well of 1X Buffer and stained by adding 100 μL/well of DCFDA Solution. Cells were incubated with DCFDA Solution for 45 minutes at 37°C in the dark. After removing DCFDA Solution, 100 μL/well of 1X Buffer were added and fluorescence was immediately measure (Ex/Em=485/535 nm).

8.2.6 Nuclear protein extraction

Nuclear proteins were extracted from ARPE-19 cells using the Nuclear Extraction Kit (Abcam plc, Cambridge, UK). In brief, cells were grown to 70-80% confluency and removed by trypsinization following standard protocols. Cell pellets (2×10^6 cells) were resuspended in 200 μL of pre-extraction

buffer and incubated on ice for 10 minutes. After centrifugation, nuclear pellet was resuspended in 400 µL of extraction buffer and incubated on ice for 15 minutes, with vortexing every 3 minutes. Finally, the suspension was centrifuged for 10 minutes at 14,000 rpm at 4°C; the supernatant was transferred into a new microcentrifuge vial to measure the protein concentration of the nuclear extract. Nuclear proteins quantification was performed by the Qubit fluorometer (Invitrogen) using the Qubit Protein Assay Kit.

8.2.7 DNMTs activity quantification

Quantification of DNMTs activity was performed using the colorimetric DNMTs Activity Quantification Kit (Abcam plc, Cambridge, UK), suitable for measuring total DNMTs activity according to manufacturer's instructions. In brief, 7.5 ng of nuclear extracts were diluted in 50 µl/well of reaction solution. The 96-well plate, including blank and positive control, was covered and incubated at 37°C for 120 min. After removing the reaction solution, each well was washed with 150 µL of wash buffer for three times, and 50 µl/well of the diluted capture antibody were added. The plate was covered with aluminium foil and incubated at room temperature for 60 min. After removing the capture antibody, each well was rinsed with 150 µL of the wash buffer for three times, and 50 µl/well of the diluted detection antibody were added. The plate was covered with aluminium foil and incubated at room temperature for 30 min. After the detection antibody was removed, each well was rinsed with 150 µL of the wash buffer for four times, and 50 µl/well of the enhancer solution were added. The plate was covered with aluminium foil and incubated at room temperature for 30 min. After removing the enhancer solution, each well was rinsed with 150 µL of the wash buffer for five times, and 100 µl/well of the developer solution were added. The plate was covered with aluminium foil and incubated at room temperature for 10 min, away from direct light. When the positive control turned to medium blue, 100 µl/well of stop solution were added to stop enzyme reaction. Absorbance was read on a microplate reader within 2 to 10 min at 450 nm with an optional reference wavelength of 655 nm. DNMTs activity was reported as percentage of control.

8.2.8 Real-time polymerase chain reaction

Total cellular RNA was extracted using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) and RNA was reverse transcribed to single-stranded cDNA using the SuperScript III Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. mRNAlevels were determined by qPCR with TaqMan Gene Expression Assays (Life Technologies, Monza MB,) using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Specific primers were used to detect DNMT1 (assay no. Hs00945875_m1), DNMT3a (Hs01027162_m1), and DNMT3b (Hs00171876_m1). Threshold cycle values in each sample were used to calculate the

number of cell equivalents in the test samples. The data were normalized to the values for GAPDH expression (Hs02758991_g1).

8.2.9 DNA extraction and LINE-1 methylation analysis

DNA was extracted using the DNeasy Blood and Tissue kit and quantified using the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's protocols. Methylation analysis of LINE-1 promoter (GeneBank accession no. X58075) was investigated by pyrosequencing-based methylation analysis, using the PyroMark Q24 instrument (Qiagen), after DNA bisulfite conversion. Bisulfite treatment of 20 μg of DNA extracted from each sample was completed using the Epitect Bisulfite kit (Qiagen) and the converted DNA was eluted with 20 μl Elution buffer. The bisulfite-modified DNA was stored at -80 C until used.

A reaction volume of 25 mL was amplified by polymerase chain reaction (PCR), using the PyroMark PCR Kit (Qiagen). According to the manufacturer's instructions, each reaction mixture contained 1.5 μl of bisulfite-converted DNA, 12.5 μl of PyroMark PCR Master Mix 2X, containing HotStart Taq DNA Polymerase, 2.5 μl of CoralLoad Concentrate 10X, 2 μl of the forward primer (5'-TTTTGAGTTAGGTGTGGGATATA-3') and the reverse-biotinylated primer (5'-biotin-AAAATCAAAAAATTCCCTTTC-3') (0.2 μM for each)[32,333]. HotStart PCR cycling conditions were 1 cycle at 95°C for 15 min, 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30s, and a final extension at 72°C for 10 min. Electrophoresis of the PCR products was performed on a 2% Seakem Agarose (Lonza, ME, USA). Gels were stained with GelRed (Biotium, Inc, Hayward, CA, USA) in order to visualize the amplified DNA fragment of 290bps.

The biotinylated PCR product was purified and made single stranded to act as a template using the Pyrosequencing Vacuum PrepTool (Biotage, Inc., Charlottesville, VA, USA). The biotinylated single-stranded product was annealed to the pyrosequencing primer (5' AGTTAGGTGTGGGATATAGT-3') and then subjected to sequencing using an automatically generated nucleotide dispensation order for sequences to be analysed corresponding to each reaction. The pyrograms were analysed using allele quantification mode to determine the proportion of cytosine/thymine and, hence, methylated and unmethylated cytosines at the targeted position(s). The degree of methylation was evaluated at three specific cytosine followed by guanine (CpG) methylation sites, as well as the average percent methylation of the three CpG sites.

8.2.10 Statistical analyses

All experiments were performed in triplicate for three times. Results were reported as MD or fold change of control. Differences were assessed by ANOVA followed by the Bonferroni post hoc test

for multiple comparison, or by student's t-test for comparison of two groups. All the analyses were conducted using GraphPad Version 6.0 with a significance level of 0.05.

8.3 Results

8.3.1 Analysis of DNMTs expression using GEO datasets

Differences in the expression levels of DNMT1, DNMT3a and DNMT3b, between diabetic mice and controls at different time-points, were evaluated in three distinct GEO datasets. Since DR incidence is higher in type 1 diabetes patients [511], we firstly analysed microarray data of type 1 diabetes mouse models. No significant difference was revealed analysing the GSE12610 dataset, which compared adult CD1 STZ-induced diabetic mice, after 3-week and 5-week from induction, to non-diabetic mice (**Figure 1a**). The GSE19122 dataset reported microarray data of two type I diabetes mouse models, after 3 months of hyperglycaemia, and non-diabetic controls. Data analysis revealed that insulin-deficient $Ins2^{Akita}$ mice, but not STZ-induced diabetic mice, showed lower DNMT1 (MD=-0.28, $p<0.001$) and DNMT3a (MD=-0.31, $p<0.001$) expression levels compared to non-diabetic controls (**Figure 1b** and **Figure 1c**). We also analysed the GSE55389 dataset, which compared 8-week old db/db Type 2 diabetic mice to non-diabetic controls. However, no significant difference in the expression levels of DNMT1, DNMT3a and DNMT3b was reported (**Figure 1d**).

8.3.2 High glucose-induced oxidative stress precedes upregulation of DNMTs functions in RPE cells

One of the common feature of both type 1 and 2 DM is hyperglycaemia-induced oxidative stress in the retina [512,513]. Hence, we evaluated the time-dependent effect of high glucose on ROS production in ARPE-19 cells, seeded in 6-well plates and maintained either in normal (5.5 mM) and high glucose (25 mM) condition for 5 days. Under normal glucose condition, ROS production remained stable with increasing values after 96 hours, probably due to cell confluency. Under high glucose condition, ROS production immediately increased after 2 hours, maintaining stable high levels from 2 to 24 hours, and then slightly decreased. At each time-point, ROS production was higher in cells maintained at high glucose compared to normal glucose condition (**Figure 2a)**.

Since the analysis of GEO datasets suggested a possible time-related effect of hyperglycaemia on DNMTs expression levels, we performed a time-course analysis of DNMTs activity and expression in ARPE-19 cells maintained either in normal and high glucose condition for 5 days. DNMTs activity remained stable under normal glucose condition, whereas it showed a negative peak at 24 hours and a positive peak at 120 hours under high glucose condition (**Figure 2b**). Particularly, significant differences between normal and high glucose conditions were revealed after 24 (MD=-35.44, $p<0.05$) and 120 hours (MD=61.93, $p<0.001$). This result was partially confirmed by time-course analysis of

DNMT1 expression (**Figure 2c**). After 48 hours, DNMT1 expression level increased in cells under high glucose condition (FC= 1.25 at 72 hours, FC= 2.21 at 96 hours and FC=2.33 at 120 hours, respectively) but not in those under normal glucose condition. No significant difference and time-related effect were reported for DNMT3a and DNMT3b expression level (data not shown). Overall, results from time-course analysis demonstrated that high glucose-induced oxidative stress precedes upregulation of DNMTs expression and activity, suggesting that high glucose-induced changes in DNMTs function could be mediated by oxidative stress.

8.3.3 Effect of curcumin on viability and ROS production in RPE cells

Consistently with previous studies [516-518], we aimed to evaluate antioxidant effect of curcumin on high glucose-induced oxidative stress in RPE cells. Firstly, we determined cytotoxicity of curcumin in ARPE-19 cells, grown in 96-well plate and then exposed to various concentrations of curcumin (1–50 μM) for 6 hours or to 25 μM curcumin for various durations of exposure. No significant cytotoxic effect was observed with 1–25 μM curcumin, while treatment with 50 μM curcumin for 6 h resulted in 39% decrease in cell viability ($p<0.01$) (**Figure 3a**). Moreover, treatment with 25 μM curcumin for up to 12 hours had no significant effect on cell viability. However, cell viability was reduced by 22% ($p=0.179$) and 36% ($p<0.01$) of the untreated controls after 25 μM exposure for 12 and 24 h, respectively (**Figure 3b**). To avoid potential cytotoxicity, treatment with 25 μM curcumin for 6 hours was chosen for further experiments. The effect of curcumin on ROS production was evaluated in ARPE-19 cells maintained at normal glucose or exposed to acute and chronic high glucose condition. Similar to time-course analysis, exposure to acute and chronic high glucose condition increased the intracellular ROS levels compared to normal glucose ($p<0.05$ and $p<0.01$, respectively). However, ROS production was restored by treatment with 25 μM curcumin for 6 hours both in cells under acute and chronic high glucose condition (**Figure 4a**).

8.3.4 Curcumin restores DNMTs functions in RPE cells under hyperglycaemic conditions

We also evaluated the effect of curcumin on DNMTs activity and expression. Compared to cells at normal glucose concentration, we confirmed a 35% decrease in DNMTs activity under acute high glucose condition ($p<0.05$); in contrast, chronic high glucose exposure led to 70% increase in DNMTs activity. However, DNMTs activity was restored by treatment with 25 μM curcumin for 6 hours in both conditions (**Figure 4b**). With regard to DNMT1 expression level, chronic high glucose exposure up-regulated mRNA expression levels compared to cells at normal glucose concentration (FC= 2.01; $p<0.05$). However, consistent with results on DNMTs activity, treatment with 25 μM curcumin for 6 hours restored DNMT1 expression level (**Figure 4c**). No significant effect of high glucose and curcumin were reported for DNMT3a and DNMT3b expression levels (**Figure 4d** and **Figure 4e**).

8.3.5 LINE-1 methylation analysis

The effect of high glucose exposure and/or curcumin treatment on LINE-1 methylation, a surrogate marker of global DNA methylation, was evaluated using the bisulfite-converted DNA. Consistent with higher expression level of the maintenance DNMT1, exposure of ARPE-19 cells to acute or chronic high glucose condition did not affect LINE-1methylation levels. Similarly, no significant effect of treatment with 25 μM curcumin for 6 hours on LINE-1 methylation levels was reported (**Figure 4f**).

8.4 Discussion

Emerging evidence suggests that pathogenesis of diabetes-related microvascular complications relies on a complex gene-environment interaction [300]. Epigenetic changes, such as DNA methylation, histone modifications, and miRNAs regulation, contribute to the dysregulation of signalling pathways (i.e. oxidative stress, inflammation, apoptosis, and aging), modulating the expression of several key genes in diabetes mellitus [301,302]. Uncovering epigenetic changes involved in microvascular complications could improve our knowledge of pathophysiology and therapeutic management of these diseases. The role of DNA methylation in vascular complications of diabetes has been recently reviewed [525] and several lines of evidence described distinct methylation patterns in diabetes-associated cardiovascular complications [303-306], suggesting that high glucose-induced oxidative stress is an important mediator [307,308]. Moreover, both in vitro and epidemiological studies concluded that altered promoter methylation led to the dysregulation of several genes in diabetic nephropathy [526-528]. In diabetic retinopathy, differential DNA methylation of genes involved in the natural killer cell-mediated cytotoxicity pathway was described [309]. Moreover, retinal endothelial cells exposed to high glucose concentration showed increased mitochondrial DNA methylation [310] and an imbalance between methyl-cytosine and hydroxyl methylation of Matrix metalloproteinase-9 gene [311], impairing mitochondrial integrity and functions. However, in spite of substantial findings to suggest that hyperglycaemia might affect DNA methylation in the retina, the limited knowledge about the effect of high glucose in RPE needed to be explored. In this study, we first evaluated whether differences in the retinal DNMTs expression levels existed between diabetic and non-diabetic mice, using microarray data of three distinct GEO datasets. Since DR incidence is higher in patients suffering from type 1 diabetes [511], we first analysed microarray data of type 1 diabetes mouse models. Data analysis did not reveal dysregulation of DNMTs expression levels in 3-week, 5-week and 3-month STZ-induced diabetic mice. Similarly, inconclusive results have been recognized analysing DNMTs expression level of 8-week old db/db diabetic mice, a genetic mouse model of type 2 diabetes. By contrast, three months of hyperglycaemia in insulin-deficient $Ins2^{Akita}$ mice resulted in

downregulation of DNMT1 and DNMT3a expression. The Ins2^{Akita} mouse, harbouring a missense mutation in the Insulin 2 gene, is a model for type 1 diabetes [529]. However, a previous study reported that non-obese Ins2^{Akita} mice also developed type 2 diabetes phenotypes, such as peripheral and hepatic insulin resistance and cardiac remodelling, suggesting long-term intermediate complications between type 1 and type 2 diabetes [530].

When we evaluated the time-dependent effect of high glucose in ARPE-19 cells, ROS production immediately increased after 2 hours of exposure, maintaining stable high levels from 2 to 24 hours, and then slightly decreased. Particularly, at each time-point, ROS production was higher in cells maintained at high glucose compared to normal glucose condition.

Previous studies suggested that high glucose-induced oxidative stress might modulate epigenetic changes involved in the pathophysiology of DR [310,311,318-320,513,531]. This substantial evidence, together with findings from GEO datasets analysis, prompted us to determine the effect of high glucose on DNMTs function, taking into account the duration of insult. In ARPE-19 cells maintained at different glucose conditions, we demonstrated the time-related effect of high glucose exposure on DNMTs activity, as shown by the time-course analysis, with a negative peak after 24 hours and a positive peak after 120 hours. Consistently, the high glucose-induced effect on DNMTs expression was evident after 48 hours from the insult, with the upregulation of DNMT1. By contrast, we did not observe dysregulation of DNMT3a and DNMT3b expression.

Since DNMT1 is responsible for maintenance DNA methylation on hemimethylated DNA[8], we also evaluated the effect of high glucose exposure on global DNA methylation, using LINE-1 methylation level as a surrogate marker. Consistent with higher DNMT1 expression, we did not observe differences in LINE-1 methylation levels between cells maintained at normal glucose concentration and those exposed to acute or chronic high glucose condition. However, the potential effects on other repetitive sequences and/or on specific promoter regions cannot be completely excluded.

Our data were in line with previous studies demonstrating that hyperglycaemia significantly increased both DNMTs activity and DNMT1 expression in retinal endothelial cells [310,311,532]. These changes persisted even when glucose level is normalized, indicating that DNA methylation is probably involved in the metabolic memory of DR [320,532-534].

To our knowledge, this study is the first to demonstrate that high glucose-induced oxidative stress precedes upregulation of DNMTs expression and activity in RPE, suggesting that changes in DNMTs function could be mediated by oxidative stress via a potential dual effect. The early effect results in decreasing DNMTs activity, accompanied by the highest ROS production, while long-term oxidative stress increases DNMTs activity and DNMT1 expression. It is plausible that ROS production is involved in the activation of redox-sensitive enzymes, accelerating the reaction of DNA methylation

via deprotonating the cytosine molecule [535]. On the other side, it has also been demonstrated that inhibition of DNMTs, using the DNMTs inhibitor RG108 (RG), protected RPE from detrimental effects of oxidative stress by modulation of antioxidant enzyme gene expression [536]. Although the temporal relationship between high glucose-induced oxidative stress and changes in DNMTs function appears evident, further in vitro and in vivo studies, using antioxidants and DNMTs inhibitors, are recommended to better clarify molecular pathways involved in this mechanism.

Curcumin is considered - especially for its antioxidant properties - an interesting phytochemical candidate for the treatment of hyper-inflammatory wounds such as chronic diabetic wounds. Since it has been demonstrated that topical curcumin treatment of the wounds of diabetic rats showed enhanced angiogenesis [537], it will be interesting to evaluate the efficacy of topical curcumin on human diabetic wounds [538]. Extensive researches have increase the disease set for which curcumin may be valuable, and the identification of molecular targets will help future research in the development of curcumin as an important therapeutic agent [539]. In the present study, we also investigated whether antioxidant properties of curcumin might restore the high glucose-induced changes in RPE cells. Growing body of evidence demonstrated the pleiotropic effect of curcumin on several signalling pathways, via modulating the expression and activation of cellular regulatory systems, such as NFκB, AKT, growth factors, and Nrf2 transcription factor [540-547]. Consistent with previous works [548,549], we observed that treatment with 25 μM curcumin for up to 12 hours had no significant effect on cell viability. Interestingly, we demonstrated that curcumin treatment for 6 hours reduced ROS production associated with acute and chronic exposure to high glucose concentration. In turn, the normalization of intracellular ROS levels restored the DNMTs activity and DNMT1 expression. These results suggest that the antioxidant properties of curcumin might exerts a beneficial effect on high glucose-induced changes in DNMTs function. In line with this evidence, a previous work also demonstrated that curcumin downregulated the oxidative stress-induced expression of miR-302, an inhibitor of DNMT1[549]. However, further studies are needed to explore if curcumin modulates DNMTs function via an antioxidant effect, or if it reduces oxidative stress acting on DNMTs inhibition.

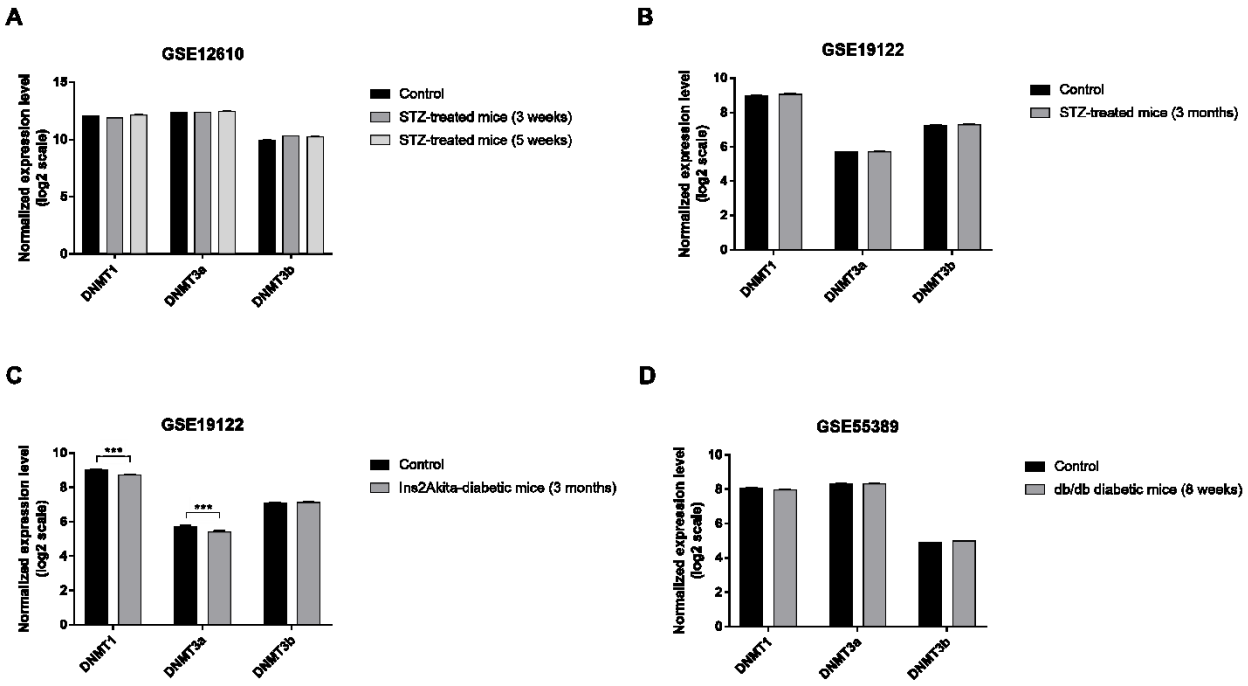
One of the main weaknesses of our study is that it is not evident if curcumin mainly acts as antioxidant or DNMTs inhibitor. Since curcumin treatment restored both ROS production and DNMTs functions, further experiments should evaluate whether the effect of curcumin depends on the oxidative and/or DNMTs pathways. Moreover, inconclusive evidence from in vivo studies exists. Whilst we did not reveal dysregulation of DNMTs expression using microarray data of short-term type 1 diabetes mouse models, three months of hyperglycaemia in insulin-deficient $Ins2^{Akita}$ mice resulted in downregulation of DNMT1 and DNMT3a expression. As reported, the $Ins2^{Akita}$ mouse is a model for type 1 diabetes, which also developed type 2 diabetes phenotypes. Overall, these findings suggest the long-term

intermediate effect of type 1 and type 2 diabetes on DNA methylation, but they also point out the need of additional in vivo studies. Finally, we observed that treatment with 25 μM curcumin (≈ 9.2 μg/ml) for up to 12 hours had no significant effect on cell viability, which was consistent with previous in vitro studies [548,549]. In addition, a previous clinical trial found that daily high-dose curcumin consumption - up to 3.6g - was not associated with toxicity and adverse outcomes [550]. However, pharmacokinetic studies of oral Curcuma extracts in rats showed poor absorption, rapid metabolism, and elimination, which in turn suggest a low oral bioavailability [551,552]. On the other hand, it is well-established that curcumin passes through the blood-brain barrier, and dietary supplementation (≈ 0.2% in diet) was found to be effective against retinal degeneration in in vivo model of light-induced retinal degeneration [553,554]. Accordingly, further studies should be encouraged to evaluate how much diet-supplemented curcumin reaches the human retina.

For the first time, we demonstrated that high glucose-induced ROS production precedes upregulation of DNMTs expression and activity in RPE, suggesting that changes in DNMTs function could be mediated by oxidative stress. Curcumin may represent an effective antioxidant compound to restore DNMTs expression and function. However, further in vitro and in vivo studies, and well-designed epidemiological studies are recommended to better clarify whether curcumin mainly acts as antioxidant or DNMTs inhibitor.

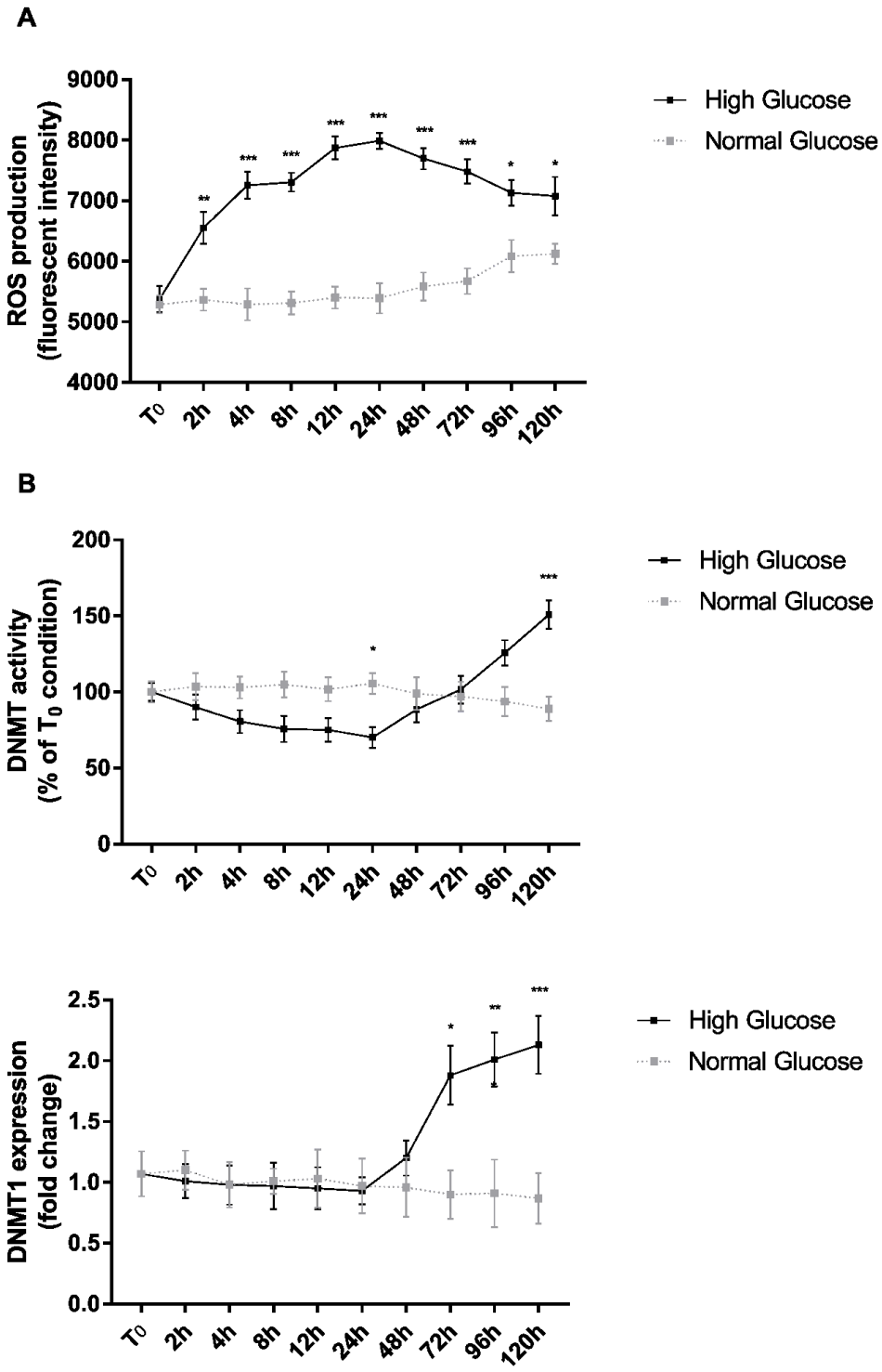
Figures

Figure 1. Comparison of DNMTs expression using GEO datasets of microarray profiling in mouse models of diabetic retinopathy.



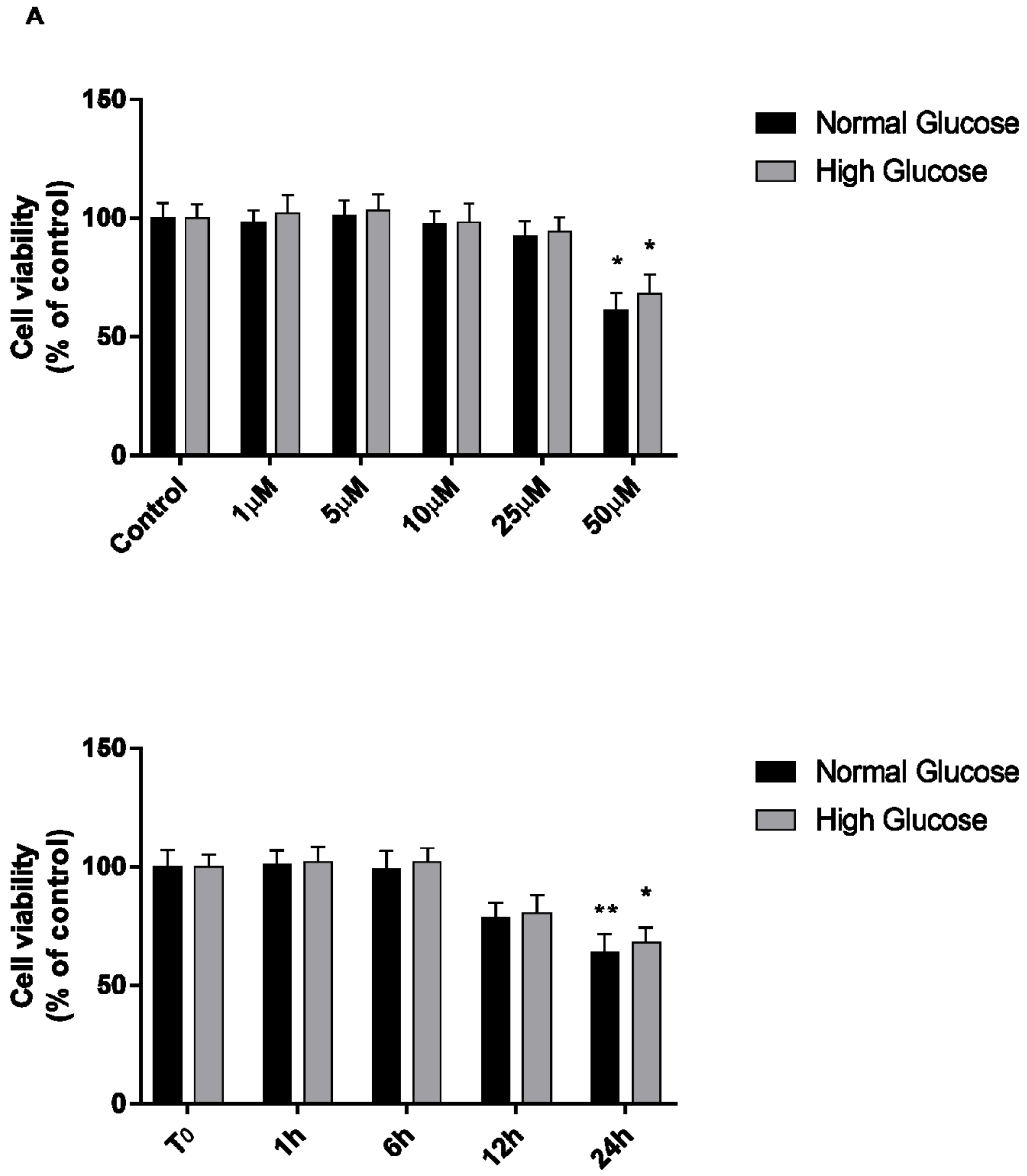
(A) Comparison of retinal DNMTs expression between adult CD1 streptozotocin (STZ)-induced diabetic mice (3-week and 5-week) and age-matched controls (4 retinas for each group were pooled) using the GSE12610 dataset; (B) Comparison of retinal DNMTs expression between eight C57BL/6J STZ-induced diabetic mice, after 3 months of hyperglycaemia, and eight controls, using the GSE19122 dataset; (C) Comparison of retinal DNMTs expression between nine insulin-deficient $Ins2^{Akita}$ mice, after 3 months of hyperglycaemia, and eight controls, using the GSE19122 dataset; (D) Comparison of retinal DNMTs expression between four 8-week old db/db diabetic mice and four age-matched lean non-diabetic controls, using the GSE55389 dataset.

Figure 2. Time-dependent effects of high glucose in ARPE-19 cells.



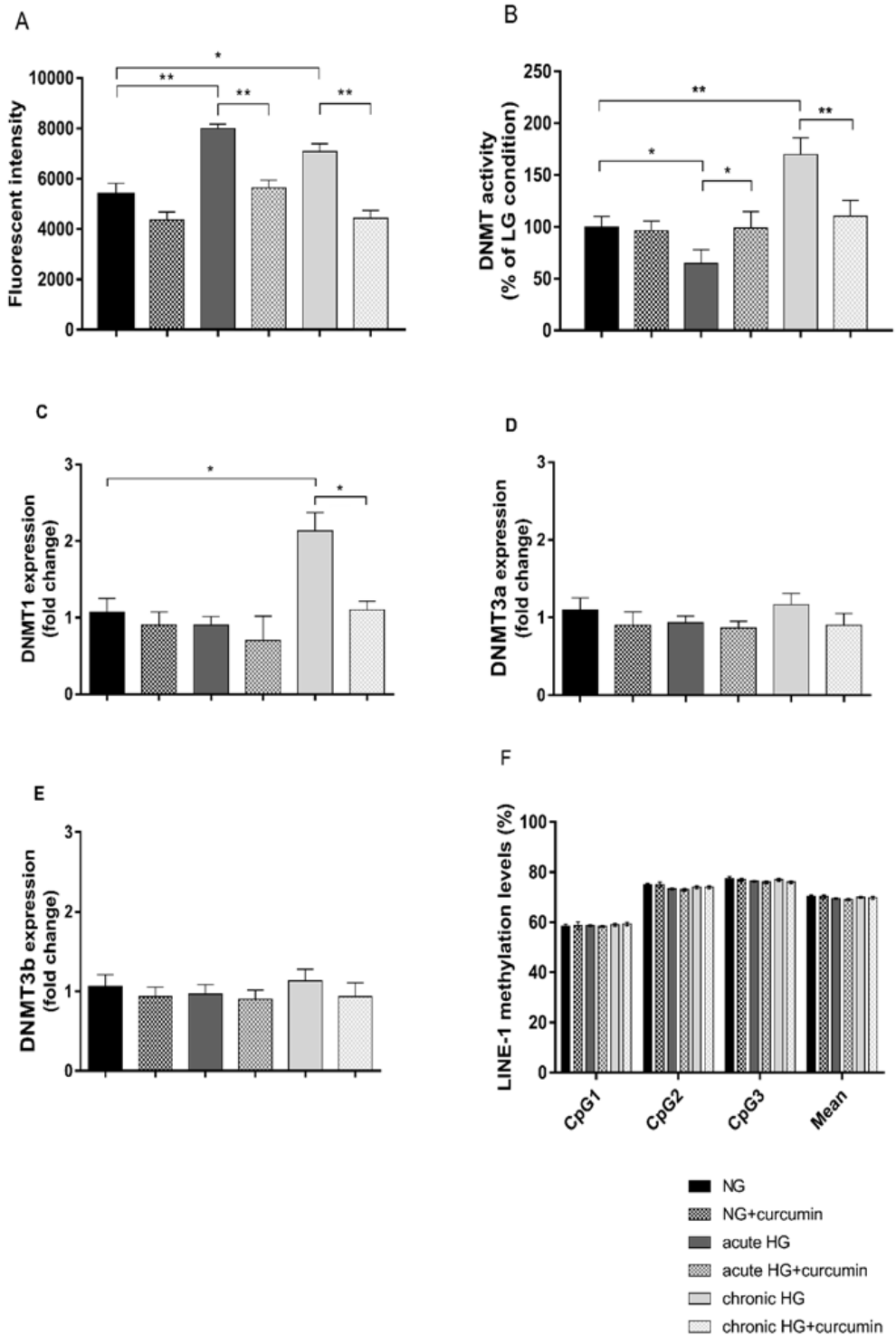
(A) Time-course analysis of ROS production in ARPE-19 cells maintained under normal and high glucose conditions; (B) Time-course analysis of total DNMTs activity in ARPE-19 cells maintained under normal and high glucose conditions; (C) Time-course analysis of DNMT1 expression in ARPE-19 cells maintained under normal and high glucose conditions.

Figure 3. Effect of curcumin on viability of ARPE-19 cells.



(A) Thiazolyl blue tetrazolium bromide (MTT) assay performed on ARPE-19 cells, maintained either in normal or high glucose conditions, and then exposed to increasing concentrations (1–50 μM) of curcumin for 6 h; (B) MTT assay performed on ARPE-19 cells, maintained either in normal or high glucose conditions, and then exposed to 25 μM curcumin for 1 to 24 hours.

Figure 4. Effects of curcumin on ROS production and DNMTs function in ARPE-19 cells.



(A) Comparison of ROS production in ARPE-19 cells, maintained either in normal or high glucose conditions (acute and chronic exposure), and then exposed to 25 μM curcumin for 6 hours; (B) Comparison of total DNMTs activity in ARPE-19 cells, maintained either in normal or high glucose conditions (acute and chronic exposure), and then exposed to 25 μM curcumin for 6 hours; (C-E) Comparison of DNMTs expression in ARPE-19 cells, maintained either in normal or high glucose conditions (acute and chronic exposure), and then exposed to 25 μM curcumin for 6 hours; (F) Comparison of LINE-1 methylation level in ARPE-19 cells, maintained either in normal or high glucose conditions (acute and chronic exposure), and then exposed to 25 μM curcumin for 6 hours.

9 Discussion and Conclusions

Retinal degenerative diseases are the leading causes of blindness and low vision among working-age and older adults worldwide, with 170 and 130 million individuals suffering from AMD or DR, respectively. Anti-VEGF therapies have proven to be effective in reducing visual loss in those with exudative AMD, while the supplementation of antioxidants has been shown to slow the progression of intermediate to advanced disease [555]. In fact, findings from the AREDS demonstrated that the disease progression can be impacted by nutritional interventions [555,556]. Until recently, the treatment of DR relied almost exclusively on glycaemic control until the severity of vascular lesions warranted laser surgery. More recently, the central role of VEGF in the vascular lesions has been recognized also in DR, and anti-VEGF therapies became an effective treatment for this debilitating disease. It is worth noting that these are useful strategies in slowing the progression of existing diseases but do not appear to prevent development of AMD and DR. Therefore, preventive measures through lifestyle modifications that might reduce or abolish modifiable risk factors are attractive strategies to tackle the likelihood that these diseases will develop or progress. However, retinal degenerative diseases - especially AMD - are complex disorders which implicates also genetic risk factors [53]. Genetic variants confer approximately 60% of AMD risk [424], with at least 34 genomic loci implicated in disease pathogenesis [81]. Among the polymorphisms associated with the risk of developing AMD, the rs1061170 polymorphism in the CFH gene has been extensively investigated, providing strong clues of association in a plausible biological context [52]. To summarize this mounting evidence, we performed a comprehensive meta-analysis - stratified for stages of diseases and ethnicities - of studies evaluating the effect of the rs1061170 polymorphism on AMD risk. Overall, we pooled genotype data from 76 case–control studies on 27418 AMD patients and 32843 controls. Consistently with previous meta-analyses [140,142,442,443], our results confirmed a significant association between the rs1061170 polymorphism and the risk of all AMD stages, with a summary allele OR of 2.15. In Caucasians, the risk conferred by the polymorphism was lower for early AMD (OR of 1.44 under the allelic model) than advanced AMD (OR of 2.47). Particularly, the mutant allele conferred a 2.9-fold increased risk of dry AMD and a 2.5-fold increased risk of wet AMD. In Asians, the polymorphism was significantly associated with exudative AMD, but not with early or dry AMD.

Uncovering the influence of genetic susceptibility on AMD risk might reveal why the protective effect of antioxidants intake through the diet is still under debate. Pooled analysis of Blue Mountains Eye and Rotterdam cohorts showed that dietary intake of lutein and zeaxanthin protected against the risk of early AMD, only in occurrence of at least two risk alleles of CFH rs1061170 and ARMS2 rs10490924 polymorphisms [204]. By contrast, in absence of genetic susceptibility, the risk of early

AMD increased with increasing intake of antioxidants [204]. Similarly, Merle and colleagues demonstrated that adherence to the Mediterranean diet was associated with lower risk of progression to advanced AMD among patients with non-risk CFH genotype, but not among those with the homozygous risk genotype [209]. Thus, in absence of genetic susceptibility, the Mediterranean diet is protective by modulating immune and inflammatory responses, but it fails in counteracting the effect of the rs1061170 polymorphism.

Other genetic variants, such as C3, C2/FB, CFI , VEGF and APOE, have been associated with AMD, even if they confer a slighter risk [83-86]. Among these, the role of VEGF polymorphisms in AMD development and progression remain ambiguous, as concluded by three previous meta-analyses [454-456]. Our study critically reviewed twelve studies and reported results of a more comprehensive meta-analysis. In summary, significant associations with AMD risk were evident for rs833061, rs1413711 and rs3025039 VEGF polymorphisms but not for rs2010963. In fact, subjects who carried the C allele of the rs833061 polymorphism had an increased risk of AMD - especially of exudative AMD - independent of ethnicity. For the rs1413711 polymorphism, we observed a significant association with AMD under the allelic model, while subjects who carried the TT genotype had an increased risk of exudative AMD under the recessive model. For the rs2010963 polymorphism, a significant association with overall AMD was evident under the homozygote model. Particularly, subjects who carried the CC genotype had an increased risk of exudative AMD. Overall, these findings not only improve the understanding of AMD pathogenesis, but also constitute a scientific background to be translated into clinical practice for AMD prevention and treatment [446].

After examining the role of genetic susceptibility in AMD, we focused on identifying modifiable risk factors that might benefit from lifestyle interventions to help reduce the burden of retinal degenerative disease. Interestingly, both AMD and DR share the same cardio-metabolic risk profile of cardiovascular diseases, so it follows that they exhibit similar pathophysiological features including central obesity, hyperglycaemia, hypertension and hypertriglyceridemia [368]. In line with this analogy, both central obesity [352-354] and blood pressure [67,355,356] have received much attention for their potential role in AMD onset and progression. Excessive body weight may exacerbate oxidative stress, inflammatory response, and imbalance of blood lipids [354], affecting the transport and deposition of carotenoids from blood to the retina [360]. Consistently, a couple of studies demonstrated an increased risk of AMD risk with increasing BMI [352-354] and/or blood pressure [355-357]. However, these findings are currently under debate, since others failed in confirming the association with obesity, blood pressure [62,353,354,358,359], and also with dyslipidaemia and diabetes [66,347,349,352,357,359]. As reported for AMD, two European prospective studies independently demonstrated the positive association of excessive body weight and central obesity with severity of DR [252,255]. Beyond the protective effect of

glycaemic control and antidiabetic treatment in type I and type II DM patients [233], several RCTs pointed out the benefits of blood pressure control in DR management [245,246]. Evidence on the effect of lipid on the development and progression of DR is currently controversial [247-249] with some studies demonstrating that the severity of DR increased with increasing triglycerides and decreasing HDL levels [250], while others failed in confirming these associations [249].

One of the greatest challenges for Public Health is the management of these risk factors through the promotion of healthy lifestyles which include smoking cessation, adequate physical activity and diet rich in fruits, vegetables, fish and antioxidants [361]. To elucidate the association between dietary habits and the cardio-metabolic profile that poses a threat for the development of retinal degenerative diseases, we used data from the Kardiovize Brno 2030 study [313]. In this cohort we distinguished two major patterns that characterize the dietary habits of the Czech population: the western pattern which provided a high intake of white bread, processed meat, fries, hamburger, hot-dog and salty snacks; the prudent pattern rich in cereals, jam and honey, fish, fruit, raw and cooked vegetables, and nuts. Consistently with previous studies [373-375], we found that high adherence to the western dietary pattern increased the likelihood of abnormal blood pressure, triglycerides and fasting glucose, after adjusting for age and sex. Notably, we confirmed a robust positive association with abnormal triglycerides by adding to the model both socio-demographic (i.e. marital status, employment and educational level) and behavioural factors (i.e. smoking, BMI, total energy intake and physical activity). By contrast, we observed that high adherence to the prudent dietary pattern decreased the odds of central obesity, abnormal blood pressure and fasting glucose, after adjusting for age and sex. We also confirmed a robust association with central obesity and abnormal fasting glucose, by adding to the model both socio-demographic and behavioural factors. Here again, our results are in line with previous studies demonstrating the protective effect of healthy dietary patterns against the risk of central obesity [370,378-380], impaired fasting glucose [381], insulin resistance [382], and diabetes [383]. Since all these risk factors are related to retinal degeneration, it has been similarly proved that adherence to a prudent dietary pattern reduced the risk of AMD [209,384] and DR [277].

Thus, quality of diet - along with meal timing and frequency - may profoundly affect cardio-metabolic parameters. Despite this evidence, the typical three-meal-a-day pattern has been replaced with a preference for snacking [385,386] in many developed countries. Our data showed that skipping breakfast increased LDL-cholesterol, adding to the well-established relationship between skipping breakfast and increased obesity, cardio-metabolic risk and chronic diseases [390-395]. Contrary to breakfast, lack of evidence exists about the effect of missing lunch on health and cardio-metabolic parameters. In our population, participants who skipped lunch showed higher diastolic pressure than non-skippers. There are suggestions about the effect of evening meals on BMI and weight control, however the

evidence is controversial and scarce in the matter of skipping dinner. Previous observational studies demonstrated a positive association between large evening intake and BMI [406-408], but others showed no association [409,410] or an inverse relationship [411]. We found evidence that skipping dinner increased triglycerides level and total cholesterol/HDL ratio, suggesting a potential impact on the risk of cardiovascular disease and retinal degeneration. Interestingly, an analysis of meal patterns in ~19000 adults participating in the 2001-2008 National Health and Nutrition Examination Survey concluded that snacking patterns are not associated with cardio-metabolic risk factors, but that snack consumption was associated with better diet quality, compared to individuals consuming no snacks [416]. Moreover, eating more frequently is often recommended as a strategy for weight loss, because it is believed to reduce appetite and thus energy intake [414]. In line with this, we observed that subjects who ate more frequently with snacking had a lower risk of obesity and abnormal blood pressure. Although we confirmed the protective effect of healthy dietary habits on several risk factors of retinal degeneration, lack of evidence still exists about the molecular mediators of this relationship. Efforts to understand the mechanisms underpinning the gene-diet interaction in AMD have led us to explore DNA methylation process in RPE cells upon oxidative and inflammatory conditions, two of the major causes of retinal degeneration [488]. Our results demonstrated for the first time that oxidative stress and inflammation modulate DNMTs and SIRT1 functions - some of the main enzymes involved in epigenetic mechanisms - and reduce LINE-1 methylation in RPE cells. Interestingly, the effect of oxidative stress and inflammatory conditions on retinal LINE-1 methylation and DNMTs functions was similar, suggesting it as a convergence point during the pathogenesis of AMD. However, while inflammatory condition seemed to affect only DNMT1 expression - the maintenance DNMT - oxidative stress also reduced mRNA levels of de novo DNMTs (i.e. DNMT3a and DNMT3b), which in turn enable key epigenetic modifications for cellular differentiation, transcriptional regulation, heterochromatin formation, X-inactivation, imprinting and genome stability [489]. In support of the interplay between inflammation and oxidative stress, we also observed that RPE cells treated with LPS - to mimic an inflammatory condition with increased expression of proinflammatory cytokines [483,485] - also exhibited increased ROS production. In fact, a chronic low-level inflammation status might be exacerbated over time by the accumulation of oxidation products, which in turn cause tissue damage and impairment of central vision [488]. Recently, it has been suggested that the NAD+-dependent histone deacetylase SIRT1 counteracts changes in RPE functions induced by oxidative stress and chronic inflammation. Consistently, we observed that RPE cells upon oxidative and inflammatory conditions exhibited decreased SIRT1 expression and activity compared to untreated cells. Since SIRT1 regulates the activities of DNMTs, especially DNMT1 [478], this result partially explains how oxidative stress and inflammation might affect DNA methylation mechanism.

Resveratrol - a flavonoid associated with the cardiovascular benefits of red grapes and wine - has been shown to significantly increase SIRT1 activity and its affinity for both NAD+ and the acetylated substrate, through allosteric interaction [479,480]. More recently, due to its antioxidant, anti-inflammatory, and anti-angiogenic properties, resveratrol has been also proposed as a candidate for the treatment of ocular diseases [481]. With this in mind, we evaluated whether treatment with resveratrol might counteract changes induced by oxidative stress and inflammation in RPE cells. Thus, we demonstrated that treatment with 10 μM resveratrol for 24 hours not only ameliorated viability and ROS production in ARPE-19 cells upon oxidative stress and inflammatory conditions, but also counteracted the detrimental effect on DNMTs/SIRT1 functions and LINE-1 methylation.

What we have seen in RPE cells differed from that observed in peripheral blood of AMD patients, further supporting the notion that methylation profiles in leukocytes might diverge from those in the retina. In fact, we demonstrated for the first time that AMD patients exhibited increased DNMTs and SIRT1 functions compared to healthy subjects. Interestingly, these changes were associated with higher LINE-1 methylation level in blood samples. However, the cross-sectional design of our study does not rule out causality, warranting further prospective researches to understand if the modulation of these pathways is a cause or a consequence of AMD. To our knowledge, the present study is the first investigating global DNA methylation in peripheral blood leukocytes of AMD patients. Previous studies focused on DNA methylation changes in genes affecting oxidative stress and inflammation. Hunter and colleagues found that GSTM1 and GSTM5 genes undergo epigenetic repression in AMD RPE/choroid via promoter hypermethylation [222]. These enzymes play an important role in the detoxification of electrophilic compounds including products of oxidative stress. Wei et al. analysed genome-wide differences in blood DNA methylation between three pairs of twins [225], demonstrating that methylation level in the IL17RC gene was lower in those affected by AMD than in healthy twins [225]. Although they further validated these findings in discordant siblings for AMD and in an AMD case–control cohort [225], others failed in confirming this evidence [491]. It is worth mentioning that the IL17RC gene encodes for an essential subunit of the IL-17 receptor complex that modulates activity of pro-inflammatory IL-17A and IL-17F. Overall, these results are in line with the well-established role of oxidative and inflammatory pathways in AMD pathogenesis.

Oxidative stress is also a pathological feature of DR, in fact both hyperglycaemia and hyperglycaemia-induced oxidative stress have been recognized as major contributing factors in DR aetiology regardless of the type of DM [512,513]. It has been also demonstrated that DR progression continues even if normal glycaemic control is restored, suggesting that the detrimental effect depends on both the duration and the severity of hyperglycaemic insult [514]. Oxidative stress has been shown to affect histone modifications and DNA methylation [317], which have been further identified as

potential epigenetic mechanisms involved in the pathophysiology of DR [310,318-320]. To address this issue, we first analysed microarray data of type 1 DM mouse models. However, this analysis did not reveal dysregulation of DNMTs expression in 3-week, 5-week and 3-month STZ-induced diabetic mice. Similarly, inconclusive results have been recognized analysing DNMTs expression level of 8-week old db/db diabetic mice, a genetic mouse model of type 2 DM. By contrast, three months of hyperglycaemia in insulin-deficient $Ins2^{Akita}$ mice resulted in downregulation of DNMT1 and DNMT3a expression. The $Ins2^{Akita}$ mouse, harbouring a missense mutation in the Insulin 2 gene, is a model for type 1 DM [529]. However, a previous study reported that non-obese $Ins2^{Akita}$ mice also developed type 2 DM phenotypes, such as peripheral and hepatic insulin resistance and cardiac remodelling, suggesting shared long-term intermediate complications between type 1 and type 2 diabetes [530]. When we evaluated the time-dependent effect of high glucose in RPE cells, ROS production immediately increased after 2 hours of exposure, maintaining stable high levels from 2 to 24 hours, and then slightly decreased. Particularly, at each time-point, ROS production was higher in cells maintained at high glucose compared to normal glucose condition. Previous studies suggested that high glucose-induced oxidative stress might modulate epigenetic changes involved in the pathophysiology of DR [310,311,318-320,513,531]. This substantial evidence, together with findings from microarray analysis, prompted us to determine the effect of high glucose on DNMTs function, taking into account the duration of insult. Our study is the first to demonstrate that high glucose-induced oxidative stress precedes upregulation of DNMTs expression and activity in RPE, suggesting that changes in DNMTs function could be mediated by oxidative stress via a potential dual effect. The early effect results in decreasing DNMTs activity, accompanied by the highest ROS production, while long-term oxidative stress increases DNMTs activity and DNMT1 expression. It is plausible that ROS production is involved in the activation of redox-sensitive enzymes, accelerating the reaction of DNA methylation via deprotonating the cytosine molecule [535]. Consistent with higher DNMT1 expression, we did not observe differences in LINE-1 methylation levels between cells maintained at normal glucose concentration and those exposed to acute or chronic high glucose condition. However, the potential effects on other repetitive sequences and/or on specific promoter regions cannot be completely excluded. Our data were in line with previous studies demonstrating that hyperglycaemia significantly increased both DNMTs activity and DNMT1 expression in retinal endothelial cells [310,311,532]. These changes persisted even when glucose level was normalized, indicating that DNA methylation is probably involved in the metabolic memory of DR [320,532-534]. In our study, we also investigated whether curcumin - a natural phenol from the rhizome of Curcuma longa which exhibits antioxidant and anti-inflammatory properties - might restore high glucose-induced changes in RPE cells. Consistent with previous works [548,549], we observed that treatment with 25 μM curcumin for up

to 12 hours had no significant effect on RPE cells viability. Interestingly, we demonstrated that curcumin treatment for 6 hours reduced ROS production associated with acute and chronic exposure to high glucose concentration. In turn, the normalization of intracellular ROS levels restored the DNMTs activity and DNMT1 expression. These results suggest that the antioxidant properties of curcumin might exerts a beneficial effect on high glucose-induced changes in DNMTs function. In line with this evidence, a previous work also demonstrated that curcumin downregulated the oxidative stress-induced expression of miR-302, an inhibitor of DNMT1[549]. However, further studies are needed to explore if curcumin modulates DNMTs function via an antioxidant effect, or if it reduces oxidative stress acting on DNMTs inhibition.

While our in vitro studies focused on the effect of specific dietary compounds on DNA methylation process, there is currently growing interest in determining how dietary patterns may affect global and local DNA methylation in humans. In the context of a wide project [312], we wondered if the adherence to Mediterranean diet might modulate LINE-1 methylation in healthy people. In this population, we observed that folate deficiency was negatively associated with LINE-1 methylation, partially confirming the majority of studies which demonstrated that global DNA methylation levels increased with increasing folate intake [29,31-35]. However, results are conflicting with other studies reporting an inverse relationship [30]. Notably, we also demonstrated for the first time that adherence to MD is positively associated with LINE-1 methylation, after adjusting for age, educational level, employment status, smoking, BMI and folate deficiency. This is in line with previous studies reporting that a higher intake of vegetables and/or fruits decreased the risk of LINE-1 hypomethylation [32,35]. The biological basis of this relationship could be attributed to the wide variety of nutrients and bioactive compounds provided by MD - including phytochemicals (phenolics, flavonoids, and carotenoids), vitamins (vitamin C, A and folate), minerals (potassium, calcium, and magnesium), and fibres - which may act on multiple signal transduction pathways and epigenetic mechanisms [40,41]. Since we previously demonstrated changes in DNA methylation both in RPE cells and blood from AMD patients, further outcome-driven researches should prospectively evaluate the effect of healthy diet and lifestyle on LINE-1 methylation.

In conclusion, this thesis reveals the role of DNA methylation process in the pathophysiology of retinal degenerative diseases, exploiting a multiple integrated approach. Findings from *in vitro* studies uncovered how pathological features of retinal degeneration - including oxidative stress, inflammation and hyperglycaemia - modulate DNMTs and SIRT1 functions, affecting LINE-1 methylation levels in retinal cells. However, what we have seen in retinal cells differed from that observed in peripheral blood of AMD patients. Thus, further research - which takes into account also genetic susceptibility - is needed to understand the mechanistic underpinnings of retinal degeneration

at the local and systemic level. In addition, we proved that resveratrol and curcumin may restore DNA methylation changes which occur in cells under oxidative, inflammatory or high glucose conditions. The use of these compounds along with the promotion of healthy diet represent promising preventive and therapeutic approaches to tackle the increased burden of retinal degenerative diseases. However, further advances require an understanding of how dietary habits may counteract the risk profile and the pathological features of these diseases at a molecular level, so that research findings can be translated into effective preventive strategies.

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