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*International PhD in Translational Biomedicine*

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## ROLE OF EPIGENETIC AND FOOD INTAKE IN THE DEVELOPMENT OF TYPE 2 DIABETIC RETINOPATHY: A CROSS-SECTIONAL STUDY

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*PhD Thesis*

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## TABLE OF CONTENTS

# 1 INTRODUCTION

## 1.1 Diabetes mellitus

Diabetes mellitus (DM) is a widespread disease whose prevalence has continued to increase over the past decades representing a burden on clinical and public health systems. Globally, more than one in ten adults are living with diabetes: the current prevalence of DM is estimated to be about 540 million cases (10% of the population), rising, without sufficient action to counteract this chronic pandemic, to 780 million in the next two decades (12% of the population). Moreover, there are several countries where DM affects one-in-five of the adults (Figure 1) [1]. The American Diabetes Association (ADA) estimated the annual cost related to DM in $237 billion for direct health care and an additional $90 billion in lost productivity [2]. Recent estimates of the International Diabetes Federation indicate that 1 in 3 people with DM – about 230 million subjects worldwide - are undiagnosed [1].

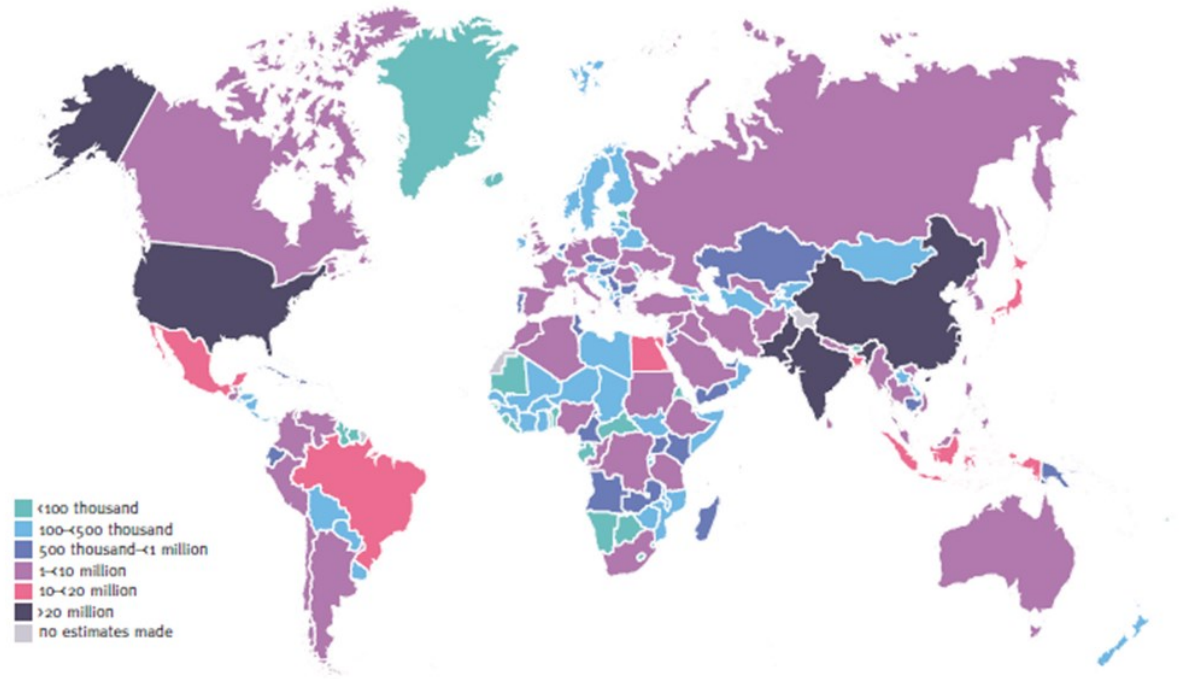


**Figure 1.** Estimated total number of adults (20–79 years) with diabetes in 2021 [1].

Type 2 diabetes mellitus (T2D) is the predominant form of diabetes, accounting for 90% to 95% of cases worldwide, involving both developed and developing countries. The remaining 5% to 10% is represented by type 1 diabetes (T1D), specific types of DM due to different causes, (e.g., monogenic diabetes syndromes, diseases of the exocrine pancreas, and drug induced DM), and gestational diabetes mellitus [3].

All types of DM are characterized by a multifactorial pathogenesis whose mail findings are an insufficient or absolute lack of insulin secretion and insulin resistance, leading to progressive vascular complications such as cardiovascular diseases (CVD), nerve damage, kidney damage, lower-limb amputation, and retinal disease. An appropriate management of DM is mandatory to prevent or delay these disabling and life-threatening complications: the overall life expectancy is about 11 to 13 years shorter for people with T1D and 7 to 10 years shorter for people with T2D compared to people without DM [4,5]. T2D accounting for over 90% of all subjects with DM represents both the main determinant of these chronic vascular complications and one of the most worrying health problems due to its socio-economic burden on public health.

## 1.2 Type 2 diabetes

### 1.2.1 Risk factors and pathogenesis

The complex pathogenesis of T2D involves the interaction between genetic and environmental factors. The most common forms of T2D are polygenic and an interaction between multiple genes and environment, as well as epigenetic factors, contributes to this disease (Figure 2) [6]. Insulin production, release, and action have to accurately meet the metabolic demand, thus the molecular mechanisms and pathways involved in these processes are tightly regulated.

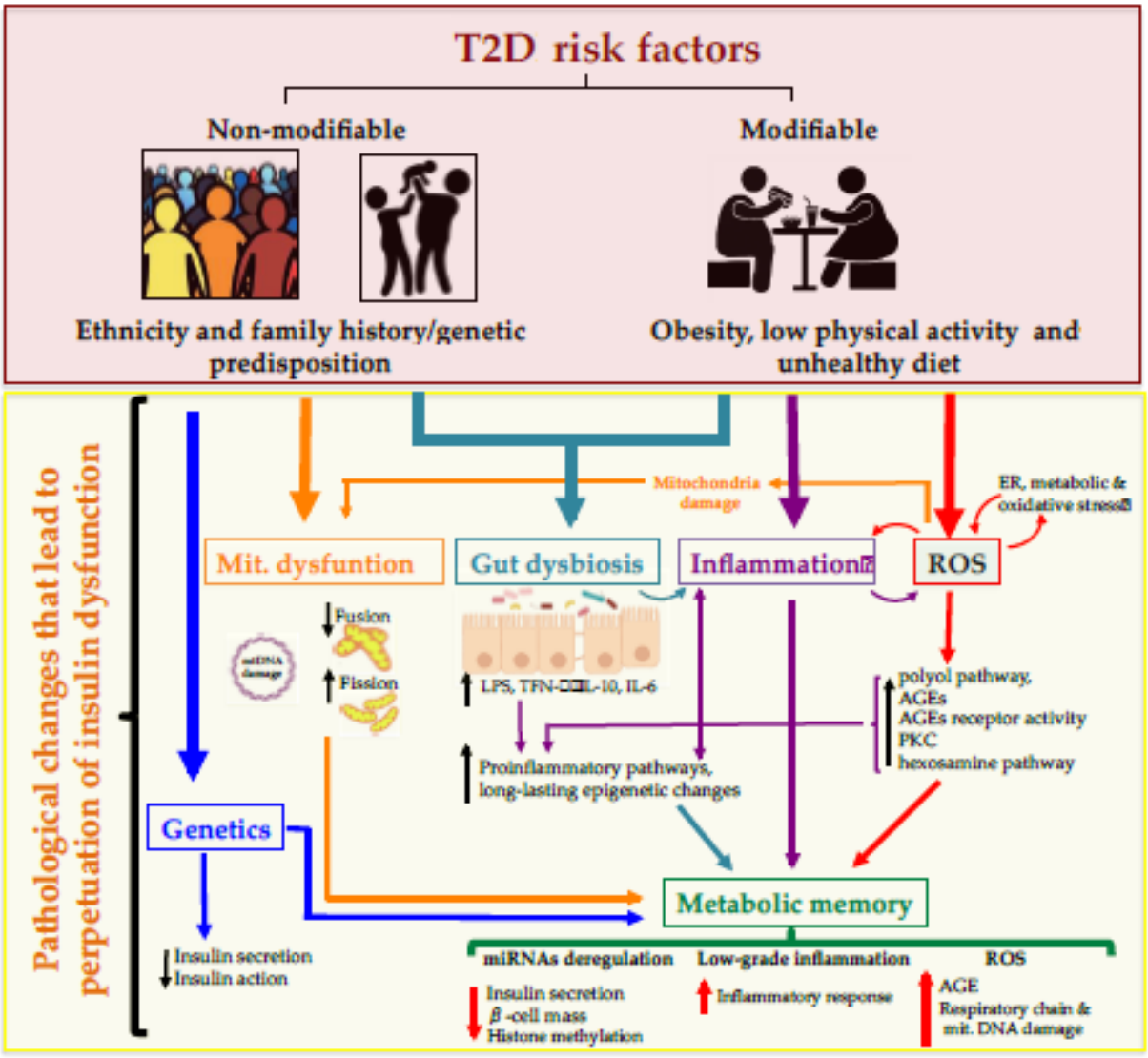


**Figure 2.** Type 2 diabetes risk factors and the pathological changes leading to insulin dysfunction. Complex combinations of genetic, metabolic and environmental factors that interact with both non-modifiable (ethnicity and family history/genetic predisposition) and modifiable risk factors (obesity, low physical activity and unhealthy diet) [7].
ROS: reactive oxygen species; ER: endoplasmic reticulum; AGEs: advanced glycation end products; PKC: protein kinase C; LPS: lipopolysaccharide; miRNA: microRNA.

Despite more than 100 genes play a role in T2D pathogenesis, the exact mechanisms and the environment interactions remain not fully understood [8]. Common genetic variants for T2D genetic only account for 10% of total trait variance, suggesting that unknown variants contribute to the pathogenesis of the disease [9]. Several studies of genome-wide association demonstrated

that most of the loci linked to T2D risk impair insulin secretion, and a minority act reducing insulin action [10]. Observational studies and clinical trials observed that the impact of each genetic variant is modulated by the interaction with environmental factors [11]. Genetic features exert their effect together with exposure behavioural factors, such as, sedentary lifestyle and high-calorie intake. The prevalence of T2D vary widely according to ethnicity and geographical region: Japanese, Hispanics and Native Americans having the highest risk for T2D [12,13].

Although the non-modifiable predisposition to T2D (genetic predisposition, ethnicity, family history) due to risk factors has a strong genetic basis, several studies suggests that many cases of T2D can be prevented by improving the main modifiable risk factors [14]. Several environmental factors play a critical role in the development of T2D. The main drivers of the T2D epidemic are the global rise of obesity, sedentary lifestyles, high caloric diets and population aging [9]. In the last decades, the spread of westernised unhealthy and sedentary lifestyles, leading to an imbalance between excessive caloric intake and reduced expenditure, favoured body fat storage and weight increase.

Overweight, obesity and the consequent metabolic derangement are the strongest risk factor for T2D [15] .

Clinically, T2D is a heterogeneous syndrome, characterised by a wide variability in age at onset, grade of hyperglycaemia and obesity. From a pathophysiologic standpoint, T2D subjects demonstrate the following metabolic abnormalities (Figure 3) [16,17]:

- Insulin resistance in peripheral tissues, particularly in muscle, fat, and liver;
- Impaired insulin secretion, particularly in response to a glucose intake, although insulin levels may be high, low, or normal depending on the phase of the natural history of the disease;
- Increased liver glucose production, responsible of fasting hyperglycaemia;

- Hyperglucagonemia, alterations in the incretin axis, accelerated fat lipolysis, gut microbiome alterations, increased glucose renal reabsorption, and abnormalities in brain metabolism.

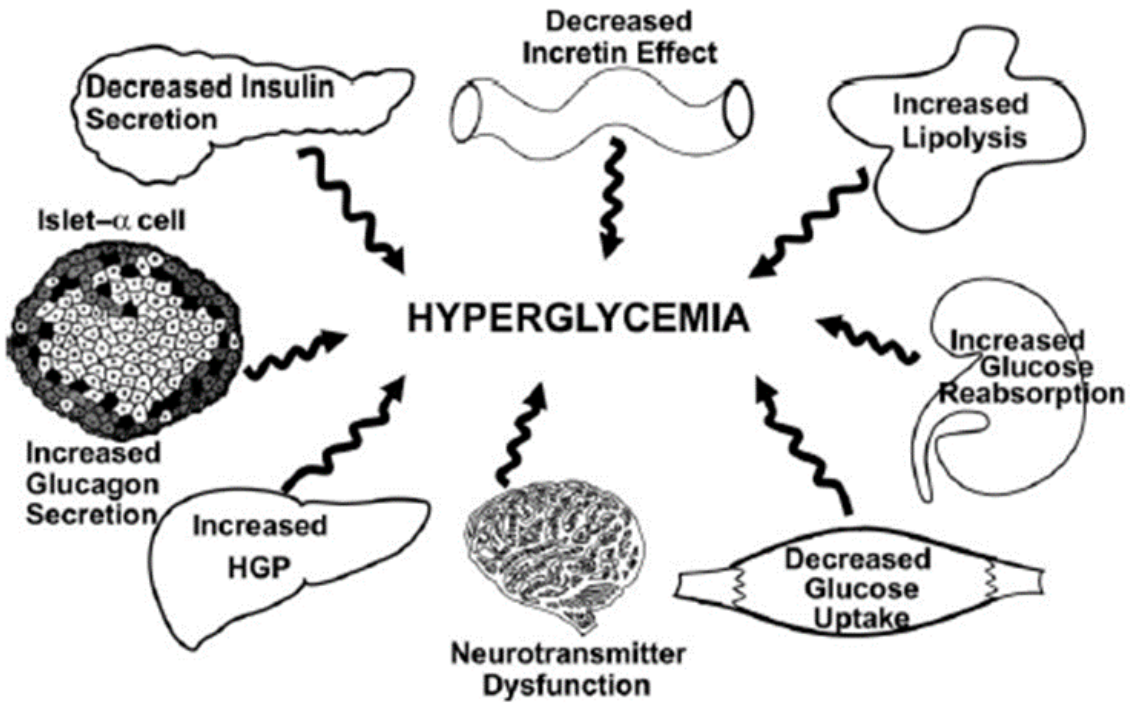


**Figure 3.** The ominous octet involved in type 2 diabetes pathogenesis [17].

Each of these dysfunctions, involving either single cell or tissue and inter-organ networks, have a significant role in the development of T2D: although muscle and liver insulin resistance and β-cell failure represent the core of T2D pathophysiology, other organs are involved, such as, adipose tissue, small intestine, kidneys, and brain [6,17].

Obesity and visceral fat storage are associated with a significant decline in insulin sensitivity, but glucose level, at this early stage, remains almost normal because of the compensatory increase in insulin secretion, although β-cell function is already abnormal. An imbalanced and excessive nutritional intake, favours, by means of higher glucose and lipids levels, insulin-

resistance and chronic inflammation. High-caloric Western diet based on fats and carbohydrates elevates blood glucose and circulating very-low-density lipoproteins (VLDLs), chylomicrons (CMs) and their remnants rich in triglycerides. This induces the production of reactive oxygen species whose increase in steady-state levels significantly contributes to T2D by increasing insulin resistance [18]. Similarly, low physical activity and sedentary behaviours are associated with increased low-grade systemic inflammation: pro-inflammatory molecules, such as, interleukin 6, c-reactive protein, tumour necrosis factor-α, released both into the bloodstream and specific tissues induces a metabolic inflammation [19,20]. Beta cells are exposed to inflammatory, oxidative, and amyloid stress leading to a progressive loss integrity and secretory capacity. Over time, the disruption of islet integrity/organization, consequent β-cells disruption, and insulin secretory dysfunction make difficult ensure a large compensatory amount of insulin [17]. On the other hand, insulin resistance contributes to increase the liver glucose production and decrease glucose uptake in the muscle, liver and adipose tissue. The traditional concepts of gluco- and lipo-toxicity have been expanded to include all nutrients nutri-toxicity. Increased lipolysis, the storage of intermediary lipid metabolites, high free fatty acid and adipokine levels, further increase the glucose output, insulin resistance, and impair β-cell function. The latter, exposed to this metabolic, inflammatory, oxidative, and amyloid stress, loss their integrity and secretory function. When both β-cell dysfunction and insulin resistance are present, hyperglycaemia is exacerbated leading to overt T2D [21].

Other impaired metabolic pathways are involved in T2D pathogenesis. An inappropriate glucagon release from the pancreatic α-cells has been demonstrated, particularly in the post-prandial period. Abnormalities in the incretin axis, responsible of insulin secretion after nutrients intake, play a key role in T2D β-cell failure. Gastrointestinal peptides stimulate insulin secretion at physiological concentrations. Glucagon like peptide 1 (GLP-1) and glucose-

dependent insulin trophic polypeptide (GIP) are the main peptide involved in the incretin effect. In T2D, a deficiency of GLP-1 and a pancreatic resistance to GLP-1 and GIP have been observed [17].

The gut microbiome is composed of several microbial species that participating in different biological arrangements (e.g., immunity, inflammation, metabolism, metabolites) also influences the physiopathology of the diseases including T2D. Recent evidence indicates that changes in dysbiosis favours T2D onset promoting a low-grade inflammation state and insulin resistance and T2D [22].

Moreover, intestinal dysbiosis reduces short-chain fatty acid synthesis that promotes gut barrier integrity, pancreatic β-cell proliferation and insulin synthesis [23].

## 1.3 DIABETIC RETINOPATHY

### 1.3.1 Epidemiology

Diabetic retinopathy (DR), a specific microvascular complication of both T1D and T2D, is one of the leading causes of vision loss in middle-aged economically active people, accounting for about 5% of cases of legal blindness worldwide [24]. The increasing incidence of DM will lead to about 200 million of people with DR in the next decade [24]. DR involves three out of four diabetic after 15 years of disease duration. The individual lifetime risk for DR is estimated to be over 90% in T1D and 50–60% in T2D [25]. Currently, the prevalence of DR ranges from 25% to 60% in T1D and 25% to 40% in T2D [26–28]. The majority of subjects who develop DR have no symptoms until the late stages of DR, thus, the risk assessment, community-screening programs, early and accurate diagnosis, and appropriate treatments are critical to reduce the health burden related to DR [29,30]. Over the past decade, the advancements in retinal imaging technology and the development of new therapies improved the evaluation, treatment, and

visual outcomes of subjects affected by DR. Nonetheless, proliferative diabetic retinopathy (PDR) and diabetic macular oedema (DMO) are the main causes of vision loss in most developed countries: from 1990 to 2010, the visual impairment and blindness related to DR increased by 64% and 27% respectively [31].

### 1.3.2 Risk factors

The development and progression of retinal damage in diabetic subjects is a multifactorial process involving multiple mechanisms in its pathogenesis. Although the level of glucose control and diabetes duration have a major effect on the development of DR, several other factors, modifiable (e.g., hypertension, hyperlipidaemia, obesity, and cigarette smoke), and non-modifiable (e.g., puberty, pregnancy and genetic susceptibility) are involved [32–34].

The most important modifiable risk factor for DR is hyperglycaemia. In RCT involving both T1D and T2D subjects, elevated glycated haemoglobin (HbA1c) levels increase the risk for DR onset and progression [35]. The Diabetes Control and Complications Trial (DCCT) recruited 1441 T1D subjects and found that intensive glucose control, compared to usual care, reduced both the risk of developing DR by 76% and, in subjects with pre-existing retinopathy, the progression of DR by 54%, assessing an average reduction by 44% for every 10% reduction in HbA1c [36]. In a subgroup of 2856 T2D subjects of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study, the progression rate of DR, after a 4 years follow-up, was 7.3% in the intensive care group vs. 10.4% in the standard care group. Interestingly, four years after the trial ended, DR progressed in 5.8% of the intensive treatment group vs. 12.7% in the standard treatment group confirming the role of metabolic memory [37]. Although hyperglycaemia is the strongest risk factor for the development and progression of DR, HbA1c accounted for only 11% of the risk of retinopathy [36]. In subjects affected by DM, in particular in T2D, the presence

of other comorbidities, such as, hypertension, dyslipidaemia, non-alcoholic fatty liver disease, and adiponectin and homocysteine levels further damage retinal health [33].

Hypertension enhances glycaemia-induced oxidative stress, thus worsening the course of DR [38]. In diabetic subjects with hypertension, RCT of lowering blood pressure have not shown clear beneficial effects on DR. The study Action in Diabetes and Vascular Disease-Preterax and Diamicron Controlled Evaluation (ADVANCE) examined the effect of blood pressure control in 1241 subjects with T2D, randomized to blood pressure lowering drugs or placebo and followed for about 4 years [39]. Fewer subjects in the treated group experienced a new onset or progression of DR compared to placebo, although this difference was not statistically significant [39]. In the ACCORD study, evaluating T2D subjects for the effect of intensive vs. standard blood pressure control, the progression of DR was not different in the two groups [37]. A recent meta-analysis of RCT revealed a statistically significant decrease in the risk of DR when treatment for hypertension was initiated at systolic pressure lower than 140 mmHg and stopped for values lower than 130 mmHg [40].

Lipid-lowering therapy is not specifically used for the treatment or prevention of retinopathy, nevertheless, most T2D subjects with type 2 diabetes will require treatment with statins or other lipid lowering drugs. The benefit of lipid reduction for the prevention of diabetic retinopathy has not well clarified and the evidence on this concern are currently conflicting. [7,21,22]. Despite several retrospective studies indicated a lower risk for DR related to statin use, very few evidence derived from RCT are available to confirm this data [41,42]. In the ACCORD study there was a significant lower rate of DR progression in subjects treated with fenofibrate [37].

Considering that HbA1c, blood pressure, and lipids, together accounted for only 9 to 10% of the risk of retinopathy in the Wisconsin Epidemiologic Study of Diabetic Retinopathy it is clear that the prevention and treatment of DR should include other factors [43].

Unhealthy lifestyles, such as dietary intake, physical activity, tobacco consumption negatively could influence the course of DR [44,45]. The increase in physical activity in independently related with the risk reduction for both the onset and progression for DR, although diabetic subjects with PDR should avoid high-intensity aerobic and resistance exercise due to the risk of vitreous haemorrhage and retinal detachment [46].

Several clinical studies confirm that a healthy diet could reduce the risk of DM and DR. This risk decreases by 30-60% in individuals who improve physical activity and dietary style [38,47]. Preclinical and clinical studies suggest the involvement of vitamins and micro or macro-nutrients intake, through both usual feeding and dietary supplementation, in the course of DR [48,49]. However, current evidence is heterogeneous and controversial, and the role of diet in DR pathogenesis should be better clarified.

Even if diet is a well-established factor involved in the development of DM, its role in DR pathogenesis should be better investigated [50]. The Mediterranean diet (MD) is based on the following milestones: 1) elevated consumption of bread, cereals, olive oil, fruits, legumes, and vegetables, with low saturated fat consumption; 2) moderate-to-high intake of chicken and fish; 3) moderate intake of cheese, yogurt, and wine; (4) low red meat consumption [51]. Mediterranean diet is related to a lower risk for obesity, T2D, and CVD. Its beneficial effects on health motivated its appointment as a lifestyle and cultural heritage for humanity of the United Nations Educational, Scientific and Cultural Organization (UNESCO) [51]. Evidence from RCT indicated a risk reduction for DR in subjects adherent to the MD - based on fruit, vegetables, whole grains, plant proteins, fish, and low-fat dairy products - compared to a low-fat diet [52]. Nevertheless, the mechanisms involved in DR protection are not fully clarified and should be further investigated to confirm this association [53].

Fruit, vegetable and oily fish have been found to confer protective effects against the onset of DR probably due to their antioxidant content vitamins C and E, carotenoids or polyphenols [54–56]. Oxidative stress reduction is a cornerstone in the approach to DR. Vitamin E could play a role in DR by reducing serum malondialdehyde (MDA) and ROS level, as observed in T2D subjects with DR [57,58] . In addition, high-dose vitamin E (1800 IU) has demonstrated to improve both retinal blood flow and central macular thickness [58,59]. The potential role of lutein/zeaxanthin and lycopene in DR prevention derives form cross-sectional evidence, which observed a beneficial effect of combined plasma concentration of these molecules against DR risk [60]. The intake of polyunsaturated fatty acid (PUFA), through dietary fish, could reduce the risk for DR, in particular supplementation with n3-PUFA improved the number of abnormal retinal capillaries inflammatory markers in animal models of diabetes [54,61,62]. However, the studies designed to demonstrate the association between these antioxidant substances and DR protection led to conflicting results, often failing in demonstrating this relationship [55,63,64].

Both observational and intervention studies investigated the role of vitamins B in DR. Most of these studies evaluated the complex of vitamins B, while few investigations observed the effect of single vitamin B on retinal outcomes of diabetic subjects, making difficult to detect each vitamin size effect [65]. In observational studies, lower plasma level of vitamin B1 and B12 was related to a higher occurrence of DR, while the intervention with vitamin B12 determined improvements of the macular bioelectrical responses, neuro-retinal degeneration, and microvascular damage probably mediated by ROS reduction and release of neurotrophic and neuroprotective growth factors [66–69]. Several observational studies concerning the association between vitamin D serum level and the risk for DR highlighted a higher risk of this complication in subjects with lower vitamin D level [65]. In fact, the deficiency of vitamin D led to β-cell dysfunction, insulin resistance, chronic inflammation, and endothelial dysfunction [70].

Nevertheless, the results are not always univocal and should be confirmed by intervention studies.

Recent strides in comprehension of risk factors for DR showed that genetic components play an important role in DR incidence, progression, and response to treatment [71]. In the last decade, a large body of research approaches focused on the investigation of DR genetic determinants: genetic linkage analyses, candidate gene studies, genome-wide association studies, and next-generation sequencing have all brought several information concerning genes and loci related to the development of DR [72].

These studies support the hypothesis of the role of genetics in DR. In particular, familial clustering studies have consistently shown the involvement of genetics in DR whose contribution has been estimated to be 27% for DR and 52% for PDR [72]. Studies involving non-insulin-dependent diabetic twins revealed 95% agreement in the degree of DR [73]. In the DCCT study, diabetic first-degree family members of recruited subjects who progressed to severe NPDR or PDR had a significantly higher risk for progression compared to subjects who did progressed [74]. Moreover, differences in the prevalence and severity of DR have been observed among different ethnic populations [75,76].

Many candidate genes have been related to the onset of DR: vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1-alpha (HIF-1A), and erythropoietin (EPO) genes. Other genes, such as, glucose transporter 1 (SLC2A1), receptor for advanced glycation end product (RAGE), aldose reductase (AKR1B1), angiotensin-1 converting enzyme (ACE), nitric oxide synthase 3 (NOS3), and intracellular adhesion molecule-1 (ICAM1), related to glucose metabolism, blood pressure regulation, and inflammation have been identified [77,78]. However, these results are not conclusive since these associations are often weak and lacking of standardization between studies.

Genome-wide association study (GWAS) identified hundreds of genetic variants by the screening of single nucleotide polymorphisms (SNPs) across the whole genome. Recently, GWAS identified a relation between genetic variations near the GRB2 gene (chromosome17q25.1) and sight-threatening DR [79]. These results are the first to be confirmed and reproduced in independent cohorts, while previous GWAS produced variable results[74].

Nevertheless, it has been observed that some diabetic subjects without these modifiable and non-modifiable risk factors for DR can likewise develop this complication, suggesting the involvement of other, less well-known, pathogenetic elements.

Emerging evidence suggests the role of gene–environment interaction in the pathogenesis of diabetes-related microvascular complications, including DR [80]. Epigenetics explored how behaviors and environment could affect gene expression but differently from genetics, in a reversible manner [34]. Notably, epigenetic mechanisms, including DNA methylation, histone modifications, and miRNAs and long non-coding RNA (lnc-RNA) regulation, contribute to the dysregulation of oxidative balance, inflammation, apoptosis, and aging, and modulate the expression of several key genes in several diseases [81,82]. In vitro, in vivo and clinical studies demonstrated that histone modifications, post-transcriptional RNA regulation, and DNA methylation, could play a role in the pathogenesis of diabetes-related microvascular complications by regulating specific molecular pathways [34]. The majority of clinical studies evaluating the influence of epigenetic in T2D retinopathy investigated specific epigenetic biomarkers in subjects with or without DR and in a healthy control group. A higher risk for DR was related to the dysregulation of some miRNAs, such as, miR-15a, miR-93, miR-93-5p, miR-126, miR-150-5p, miR-184, miR-320, hsa-let-7a-5p, hsa-miR-novel chr5_15976, hsa-miR-28-3p. In addition, for other miRNAs (miR-20b, miR-21, miR-29b, miR-122, miR-155, miR-221, miR-423), an association with the grade of DR was also found. Moreover, the dysregulation of

miR-21, miR-93, and miR-221, was directly related to fasting plasma glucose (FPG), HbA1c, and insulin resistance index, by providing an epigenetic explication of the role of glucose control and insulin resistance in the course of DR [83–86]. Concerning the role of lnc-RNA and DNA methylation in T2D retinopathy, initial evidence is available on the possible role of the lnc-RNA MALAT1 dysregulation and MTHFR gene promoter hypermethylation [87–90]. Nevertheless, the clinical application of these epigenetic biomarkers encounters several challenges due to the low level of the evidence available, the lack of confirms in independent cohort, and the need to standardise laboratory procedure.

### 1.3.3 Pathogenesis

A variety of mechanisms underlying DR have been identified. New insights into retinal physiology suggest that the retinal neurovascular unit, composed by blood vessel, pericytes, neurons, and glia, is dysfunctional in DR (Figure 4) [32].

This neuro-vascular network regulates energy homeostasis, neurotransmitter regulation and promote the formation of the blood–brain and blood–retina barriers involved in the control of the flux of fluids and blood borne metabolites [33]. Nevertheless, whether DR begins as a vasculopathy or neuropathy is not well understood. The loss of pericytes from the retinal capillaries is an early lesion in DR histopathology of diabetic retinopathy. The normal contractile function of pericytes, containing smooth muscle around the capillary endothelium, regulates capillary blood flow, thus pericytes death is followed by the loss of capillary endothelial cells [91]. Retinal ischemia results in tissue hypoxia and capillary hypoperfusion, and represents a strong inducer of VEGF release and subsequent angiogenesis and impaired vascular permeability.

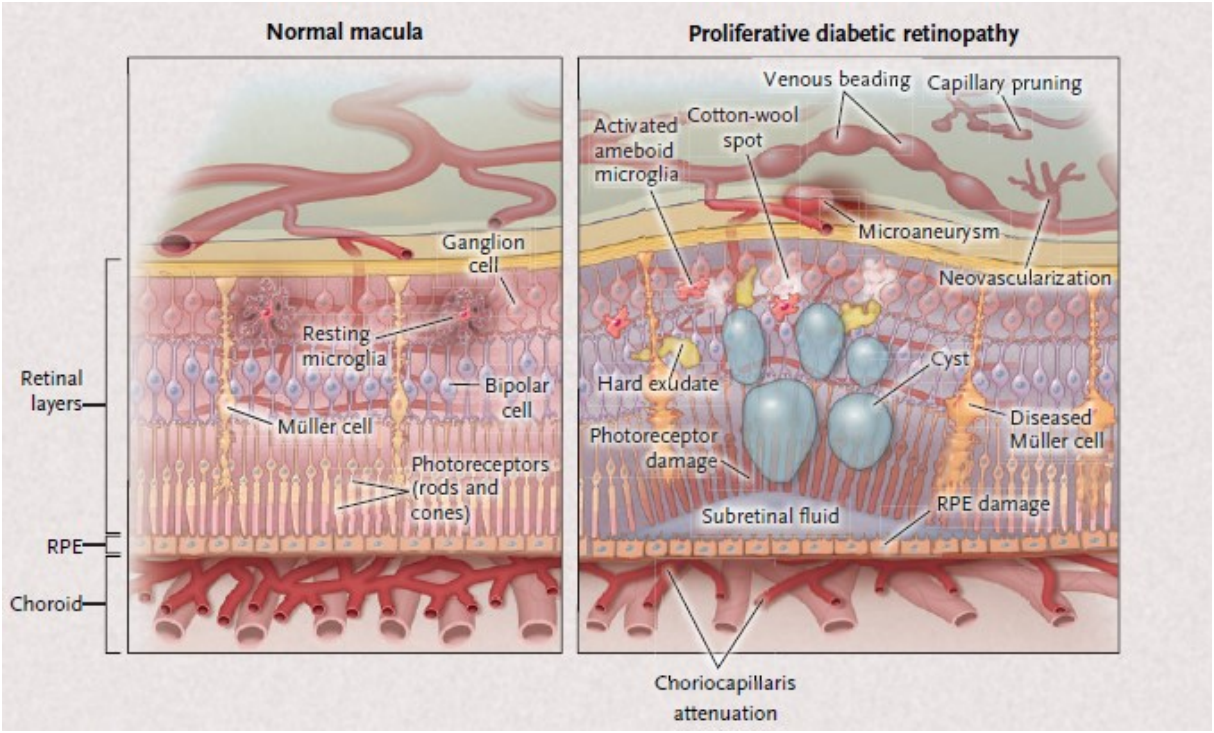


**Figure 4**. Normal macula (left panel) compared to macular structural abnormalities of proliferant diabetic retinopathy with macular oedema (DMO) (right panel): microaneurysms, venous beading, retinal neovascularization, and cotton-wool spots. Cysts, subretinal fluid, hard exudates, and thickening adjacent to the fovea are evidence of DMO [32].
RPE: retinal pigment epithelium.

Vision-threatening complications are due to increased retinal vascular permeability, neovascularization, and extensive vascular loss in the central retina[32]. New vessels can extend and bleed in the vitreous cavity, can cause tractional retinal detachments from the contractile fibrous tissue, resulting in visual acuity reduction [91].

Several biochemical mechanisms have been proposed for the development and progression of DR (Figure 5) [71].

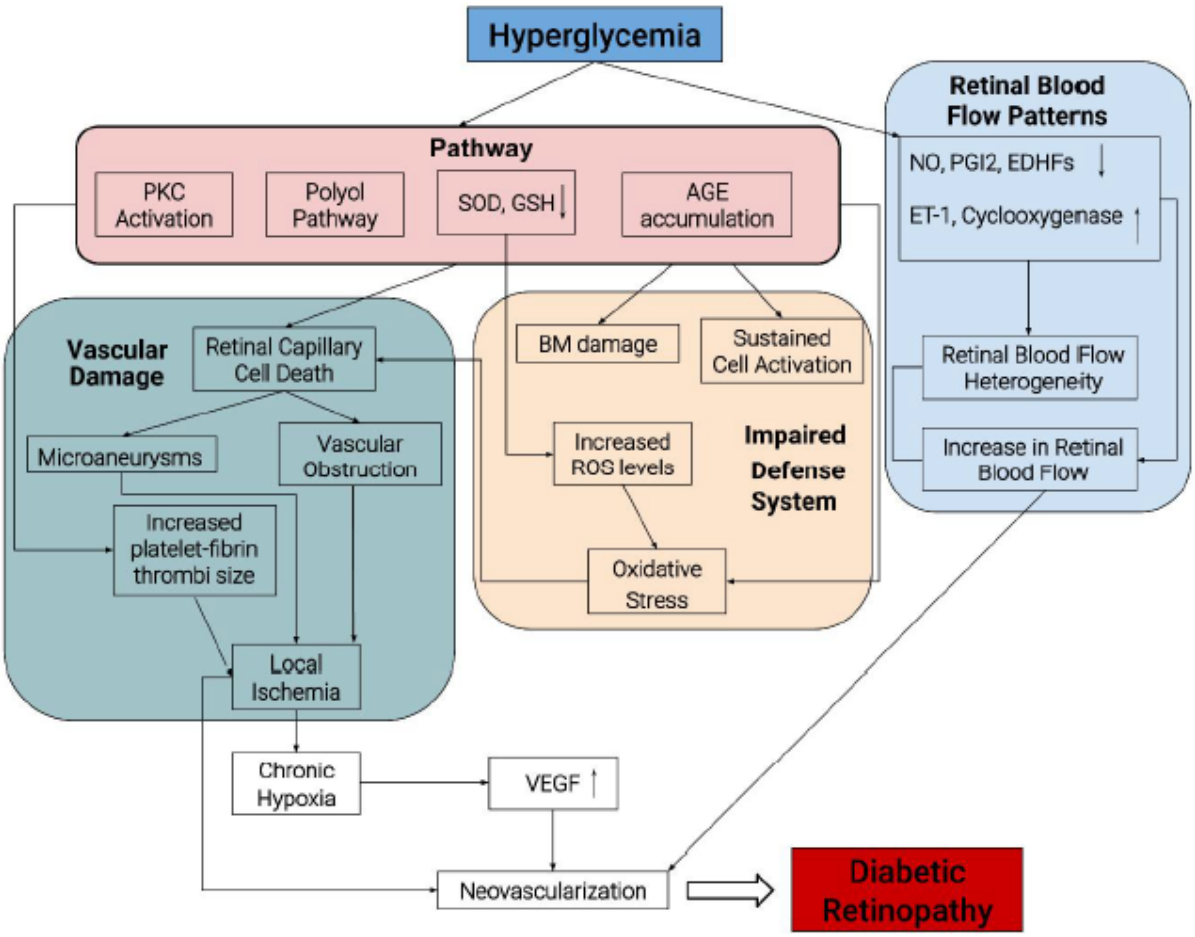


**Figure 5.** Schematic view of pathogenic mechanisms of diabetic retinopathy [71].
PKC: protein kinase C; SOD: superoxide dismutase; GSH: glutathione; AGE: advanced glycation end-product; VEGF: vascular endothelial growth factor; NO: nitric oxide; PGI2: prostaglandin I2; EDHFs: endothelium-derived hyperpolarizing factors; ET-1: endothelin-1.

Hyperglycaemia favours the retinal accumulation of sorbitol with consequent altered Na/K-ATPase activity, impaired phosphatidylinositol metabolism and protein kinase C (PKC) isoforms activity, increased prostaglandin production. PKC mediate the activity of VEGF as well as regulate vascular permeability, and it may cause the further accumulation of sorbitol [92,93]. The production of VEGF in response to hypoxia play a central role in the development of neovascularization by activating protein kinase C-ß2 [93]. Moreover, the excessive advanced glycation end products (AGEs) production and the crosslinking of basement membrane proteins contribute to increased vascular permeability and endothelial cell proliferation. Through the

last three decades, several data shown the role of reactive oxygen species (ROS) in DR. Hyperglycaemia favours alternative pathways leading to the formation of ROS (e.g. superoxide anion, hydrogen peroxide, and hydroxyl radical) even at the point of surpassing the antioxidant capacity (e.g., catalase, glutathione peroxidase, superoxide dismutase, hemoxygenase-1), a state known as oxidative stress affecting retinal integrity. Oxidative stress leads to a neuro-vascular retinal damage by activating secondary pathways like the polyol, PKC, and hexosamine, thus producing inflammation, mitochondrial dysfunction, cell death, apoptosis, and neurodegeneration leading to neural, vascular, and retinal tissue damage [94].

### 1.3.4 Screening

Screening programs for DR are mandatory because sight-threatening retinopathy may be asymptomatic until the disease progresses and advanced stages occur. Moreover, the treatment to counteract the risk of vision loss in diabetic subjects is most effective when early initiated, before severe vision loss has occurred.

According to the ADA recommendations, adults with T1D should receive an initial dilated and comprehensive eye examination by an ophthalmologist within 5 years after the diagnosis of diabetes because retinopathy is estimated to take at least 5 years to develop. Subjects with T2D should start screening procedures for DR at diabetes diagnosis due to the possible years of undiagnosed diabetes [95]. If there is no evidence of DR for one or more annual eye exams and glycaemia is well controlled, the screening should be performed every 1–2 years, while in case of any level of DR a subsequent dilated retinal examinations should be repeated at least annually o more frequently if retinopathy is progressing or visual loss occurs [95].

The morphologic structures of the retina can be easily explored through non-invasive imaging procedures. The initial approach to the assessment of DR relies on retinal fundus photography,

which visualizes approximately one third of the retinal surface (posterior retina), although newer technique of ultra wide-field photography allows to reach more than 80% of the retinal surface. Fluorescein angiography (FAG), in which an intravenous fluorescent dye is used to assess vascular structure and permeability [96]. In addition, optical coherence tomographic (OCT) allow evaluating retinal structures in both faces and in cross section and provides information on the extent and location of retinal thickening and morphologic changes in the neural retina. A more recent advance, the OCT angiography, detecting blood cell movement, produces a map of perfusion of the three layers of retinal vessels, thus providing the visualization and morphologic evaluation of perfused retinal vessels [97].

Over the past few years, the diagnostic accuracy of artificial intelligence systems represent an alternative to traditional screening approaches [98].

### 1.3.5 Classification

The classification of DR is based on the absence or presence of retinal abnormal new blood vessels, into two major forms: non-proliferative diabetic retinopathy (NPDR) and PDR. Retinopathy progresses from mild abnormalities to moderate and severe NPDR (characterised by progressive retinal capillary leakage or loss resulting in retinal ischaemia) to PDR (characterised by the development of new vessels on the optic disc and retina) (Figure 6) [33]. Subjects with NPDR are usually asymptomatic. The subsequent level of visual acuity is dependent on the degree of damage to critical structures that has occurred by that point. Vision loss, usually related to PDR, may occurs both gradually, if diabetes macular DMO develops and suddenly, due to a vitreous haemorrhage [99].

Non-proliferative retinopathy consists of a display of nerve-fiber layer infarcts (cotton wool spots), intraretinal haemorrhages, hard exudates and microvascular abnormalities

(microaneurysms, occluded vessels, and dilated or tortuous vessels) in the macula and posterior retina [100].

Proliferative retinopathy is characterised by neovascularization (disc and/or retinal vessels), preretinal and vitreous haemorrhage, fibrosis, and traction retinal detachment [100].

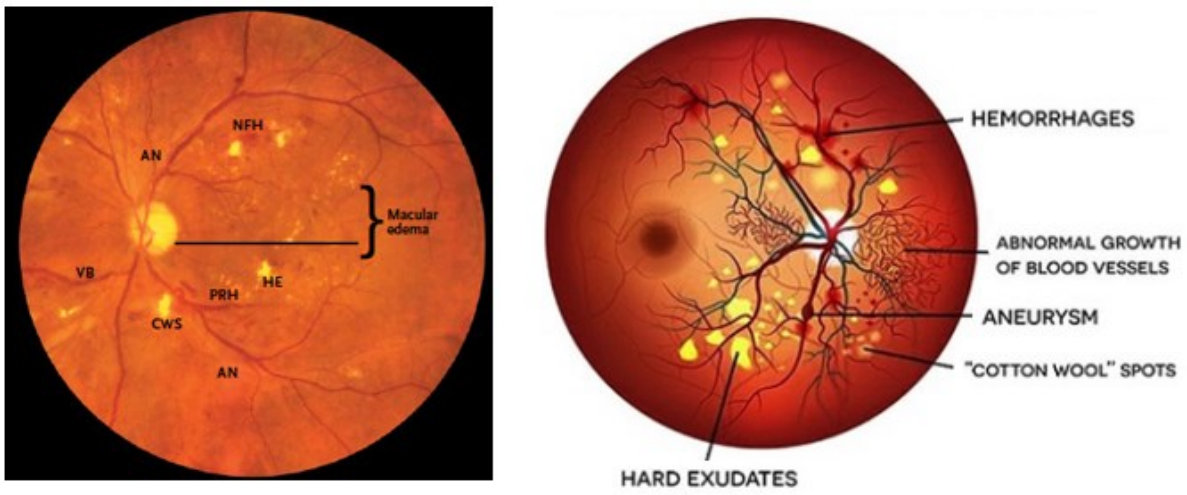


**Figure 6.** Fundus ocular image (left panel) and schematic representation (right panel) of the main alterations of diabetic retinopathy. The fundus image shows notable features including arteriolar narrowing (AN), nerve-fiber haemorrhage (NFH), hard exudates (HE), cotton-wool spots (CWS), venous beading (VB), preretinal haemorrhage (PRH), and foveal macular oedema [33].

The more detailed classification of The International Clinical Disease Severity Scale for DR, based on the results of the Wisconsin Epidemiologic Study of Diabetic Retinopathy and the Early Treatment of Diabetic Retinopathy Study, classified DR into five stages (Figures 7 and 8) [100,101].

- No apparent retinopathy: as the name implies there are no retinal alterations;
- Mild NPDR: presence of a few microaneurysms;
- Moderate NPDR: more than just microaneurysms, intraretinal haemorrhages but less than severe non-proliferative diabetic;

- Severe NPDR: more than 20 intraretinal haemorrhages in each of 4 quadrants or venous beading in more than 2 quadrants or prominent intraretinal microvascular abnormalities in at least one quadrant, with no signs of PDR;
- PDR: neovascularization of the disc, retina, iris, angle, vitreous haemorrhage or tractional retinal detachment.

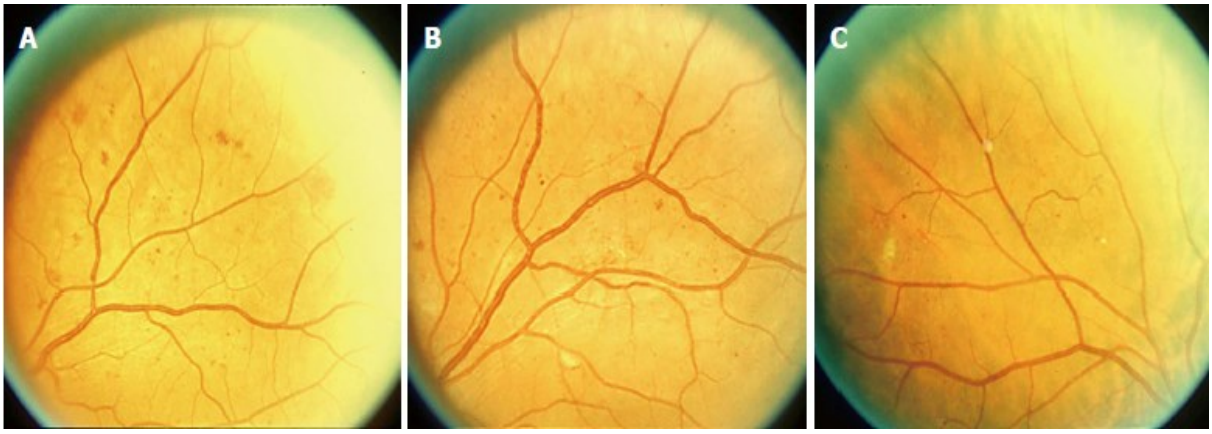


**Figure 7.** Fundus ocular images of different retinal abnormalities in non-proliferant diabetic retinopathy. A: intraretinal haemorrhages; B: venous beading; C: intraretinal microvascular abnormalities (IRMA) [101].

Macular oedema is present or absent. The severity of DMO is classified as mild, moderate and severe depending on the distance of the exudates and thickening from the centre of the fovea (Figure 8) [33,101]:

- Mild: some retinal thickening or hard exudates in posterior pole but far from the centre of the macula;
- Moderate: retinal thickening or hard exudates involving the centre of the macula but not involving the centre;
- Severe: retinal thickening or hard exudates involving the centre of the macula.

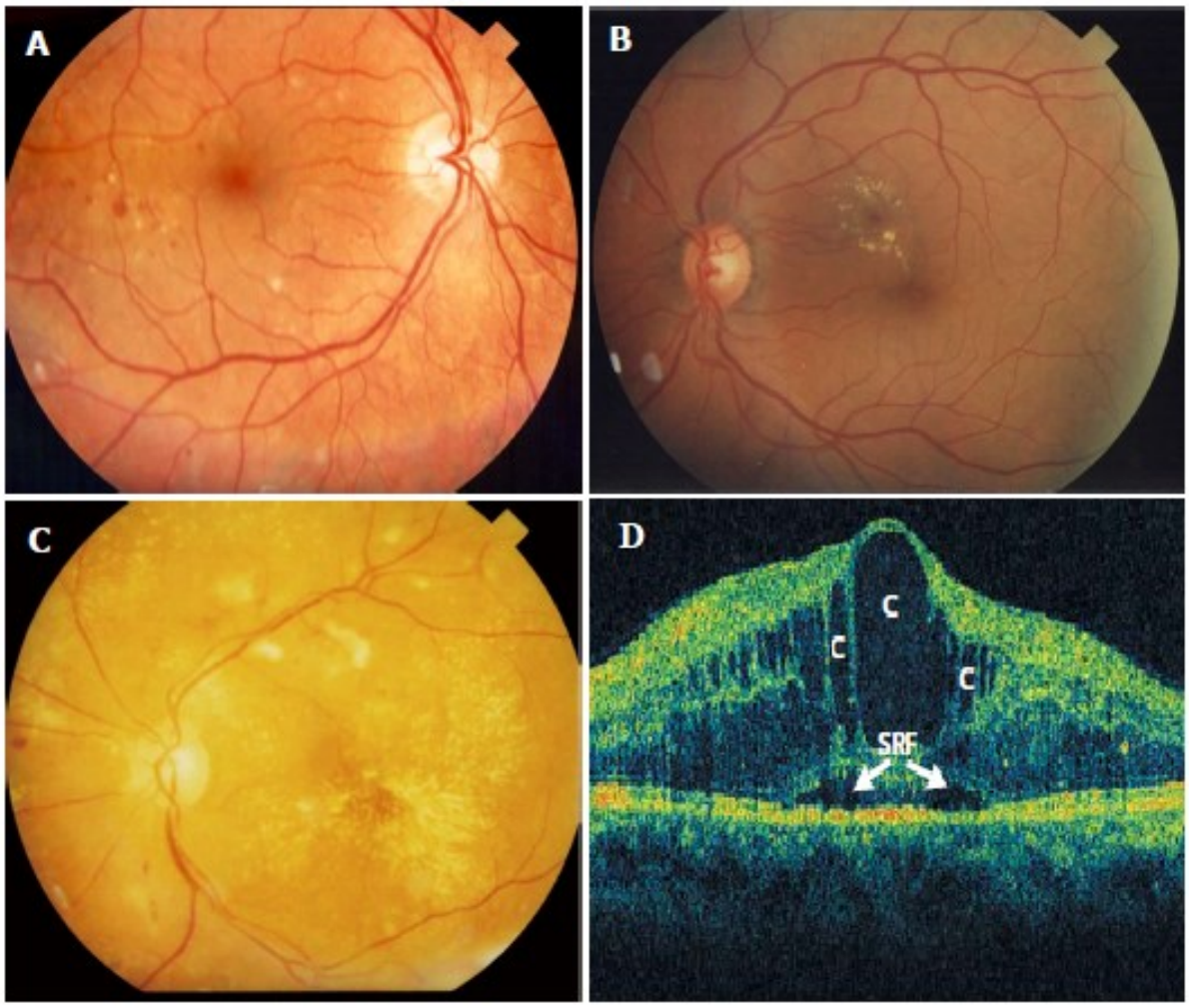


**Figure 8.** Fundus ocular images (panel A, B, C) and optical coherence tomographic (OCT) (panel D) scan of retinal abnormalities in proliferant diabetic retinopathy with macular oedema DMO. A: mild DMO, hard exudates located far from the centre of the fovea; B: moderate DMO, hard exudates located closer to the centre of the fovea; C: severe DMO, the centre of the fovea is involved with hard exudate and thickening; D: OCT horizontal scan through the central fovea reveals marked thickening and edema of the macula with cysts (C) and subretinal fluid (SRF) [33,101].

This stratification is tightly related to the risk of progression from NPDR to the more serious PDR, targeting both follow-up intervals and treatment strategies. While the one-year risk of progression to PDR for mild and moderate NPDR is 5-15%, the severe and very severe stages have one-year risk of 52-75%. Untreated PDR has a 60% risk of severe vision loss at five years [102].

Macular oedema may occur in either NPDR or PDR and refers to retinal thickening and oedema involving the macula, visualized by fundus exam with stereoscopic viewing, fluorescein angiography, and, most directly, by optical coherence tomography (OCT).

### 1.3.6 Treatment

The goals of treatment of DR include preservation and improvement of vision and the reduction in the rate of progression and frequency of retinopathy, vitreous haemorrhage, and DMO.

The optimization of glucose control, blood pressure, and serum lipid levels together with proper scheduled dilated eye examinations and an early intervention with both surgical and pharmacologic therapies can significantly decrease the risk of vision loss related to DR [95].

The marked reduction in the prevalence and incidence of retinopathy and vision impairment over the past few decades reflects improved management of glycaemia, blood pressure, and lipid levels.

Evidence from randomised controlled trials (RCT) supported the use of laser panretinal photocoagulation (PRP). The Diabetic Retinopathy Study evaluated the efficacy of PRP in subjects with PDR demonstrating a risk reduction for severe visual loss of 50% or more compared to subjects not treated with PRP [99]. In addition, the Early Treatment for Diabetic Retinopathy Study group suggested that PRP can be considered in subjects with clinically significant DMO and in severe NPDR but should be avoided in mild and moderate NPDR [99].

Although the exact mechanisms are not fully understood, PRP improves the outcome of DR by reducing the ischemia-related production of growth factors, in particular the VEGF and increasing oxygenation from the choroid to the inner retina that occurs through the laser scars due to thinning of the retina in the treated area [103]. Currently, PRP is recommended in subjects with high-risk PDR and in some cases of severe NPDR [95]. In case of PDR complicated by

refractory vitreous haemorrhage, tractional retinal detachments associated to severe fibrovascular proliferations, the vitrectomy of pars plana is considered the standard treatment [104].

The gold standard for the treatment of centre-involved DMO are intravitreal pharmacotherapeutics, including anti-VEGF drugs (e.g. pegaptanib, aflibercept, bevacizumab, ranibizumab, and faricimab) whose administration has a significant effect on the reduction of visual loss [105]. The synthesis of VEGF increases with tissue hypoxia, pro-inflammatory mediators, and growth factors. Several RCT demonstrated that drugs that bind soluble VEGF restore the integrity of the blood-retinal barrier, resolve macular oedema, and improve vision in most subjects with DME [106]. Intravitreal injections of anti-VEGF are indicated as first-line treatment for DMO involving the foveal centre with vision loss and represent an alternative to PRP for some subjects with PDR [95]. The addition of intravitreal glucocorticoid therapy to anti-VEGF treatment improves retinal thickening without improving visual outcomes [107].

The macular focal/grid laser photocoagulation could be effective in clinically significant DMO but is considered a second-line treatment for this condition [95].

## 2 AIM OF THE THESIS

This study aimed to exploring the role of epigenetics and dietary habits – with a particular focus on MD adherence – on the course of T2D retinopathy. The impact of DR on quality of life was also investigated.

# 3 SUBJECTS AND METHODS

## 3.1 Study design

This cross-sectional study enrolled 129 Caucasian subjects affected by T2D, recruited, during their routine visit, at the Diabetes Centre of the Garibaldi-Nesima Medical Centre (Catania, Italy) from January to July 2021.

We included T2D subjects aged over 40 years, with DM duration of at least 15 years, and HbA1c at recruitment between 7.0-9.5%. Exclusion criteria were as follows: ocular diseases (except DR), immune system diseases, active cancers, systemic therapies in the past three months. Criteria for T2D diagnosis followed the American Diabetes Association guidelines [3].

The study was conducted according to the Declaration of Helsinki, all subjects provided written informed consent for participating in the study, and the Institutional Ethics Committee (Catania 2) approved the protocol (protocol number 601/CECT2).

## 3.2 Subjects' data collection

At recruitment visit, all subjects' data concerning medical history, the current anti-hyperglycaemic treatment, the status of diabetes related micro- and macro-vascular complications, and other comorbidities were gathered. Trained epidemiologists, using a structured questionnaire, collected sociodemographic and lifestyle data: educational level was classified as low (≤8 years of school) or high (>8 years of school). Subjects were also classified as employed or unemployed (including students and housewives) and smokers or non-smokers (including ex-smokers).

Weight, height, body mass index (BMI), waist circumference (WC), and systolic (SBP) and diastolic (DBP) blood pressure were measured. BMI was calculated by dividing the weight in kilograms by height in square meters ($kg/m^2$), to classify subjects as normal weight (BMI 18.5–

24.9 kg/m2), overweight (BMI 25.0–29.9 kg/m2 or obese (BMI ≥ 30 kg/m2) [108]. According to the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III), WC was measured at the iliac crest and considered abnormal when >88cm in women and >102cm in men [108].

The following blood laboratory data were obtained: HbA1c, cholesterol total, high-density lipoprotein (HDL), low-density lipoprotein (LDL) (calculated with Friedewald formula if triglycerides value was lower than 400 mg/dL: cholesterol total-HDL-triglycerides/5 [109]), triglycerides, aspartate transaminase (AST), alanine aminotransferase (ALT), creatinine. The estimated glomerular filtration rate (eGFR) was calculated with the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [110]. Albumin to–creatinine ratio (UACR) in random spot urine collection was recorded: according to the ADA criteria, microalbuminuria was defined as an UACR between 30 and 299 mg/g urine creatinine, while macro-albuminuria as an UACR ≥ 300 mg/g urine creatinine [110]. Chronic kidney disease staging was assigned according to ADA standards of medical care in diabetes considering eGFR and albuminuria [110].

### 3.3 Ocular assessment

#### 3.3.1 Instrumental examinations

Based on subjects' retinal status, the recruited cohort was divided into the following three groups: NDR (subjects without DR), NPDR (subjects with NPDR), PDR (subjects with PDR). All participants underwent a complete ophthalmological examination including visual acuity and ocular pressure assessment, retinal fundus photography, FAG, and OCT. Retinal images were blindly analysed by two ophthalmologists with proved experience in DR diagnosis of the Oculistic Centre of the Garibaldi-Nesima Medical Centre (Catania, Italy), and classified

according to classification of The International Clinical Disease Severity Scale for DR (see section 1.3.5) [100,101].

### 3.3.2 National Eye Institute Visual Function Questionnaire (NEI-VFQ 25)

To assess the vision-related quality of life (VRQoL) we used the 25-item National Eye Institute Visual Function Questionnaire (NEI-VFQ) [111,112]. The VFQ-25 takes approximately 10 minutes to administer to subjects and consists of a base set of 25 vision-targeted questions representing 11 vision-related constructs, plus an additional single-item general health-rating question. The VFQ-25 generates the following vision-targeted subscales: global vision rating, difficulty with near vision activities, difficulty with distance vision activities, limitations in social functioning due to vision, role limitations due to vision, dependency on others due to vision, mental health symptoms due to vision, driving difficulties, limitations with peripheral and colour vision, and ocular pain. In addition, the VFQ-25 contains an extra question about the general health rating which has been shown to be a robust predictor of future health and mortality.

To calculate the VFQ-25 score, we recoded the original values from the survey according to specific scoring rules so that a high score represents better visual functioning. Each item was converted to a 0 to 100 scale, in order that the scores represent the achieved percentage of the total possible score (e.g., a score of 50 represents 50% of the highest possible score) (Table 1).

**Table 1. Recoding of the 25 of the Visual Function Questionnaire**

| Item Numbers | Original response category | Recoded value |
|---|---|---|
| 1,3,4,15c[(a)] | 1 | 100 |
| | 2 | 75 |
| | 3 | 50 |
| | 4 | 25 |
| | 5 | 0 |
| 2 | 1 | 100 |
| | 2 | 80 |
| | 3 | 60 |

| | | |
|---|---|---|
| | 4 | 40 |
| | 5 | 20 |
| | 6 | 0 |
| 5,6,7,8,9,10,11,12,13,14,16,16a | 1 | 100 |
| A3,A4,A5,A6,A7,A8,A9[(b)] | 2 | 75 |
| | 3 | 50 |
| | 4 | 25 |
| | 5 | 0 |
| | 6 | * |
| 17,18,19,20,21,22,23,24,25, | 1 | 0 |
| A11a,A11b,A12,A13 | 2 | 25 |
| | 3 | 50 |
| | 4 | 75 |
| | 5 | 100 |
| A1,A2 | 0 | 0 |
| | to | to |
| | 10 | 100 |

*[(a)] Item 15c has four-response levels, but is expanded to a five-levels using item 15b. Note: If 15b=1, then 15c should be recoded to "0"If 15b=2, then 15c should be recoded to missing. If 15b=3, then 15c should be recoded to missing.*

*[(b)] "A" before the item number indicates that this item is an optional item from the Appendix. If optional items are used, the NEI-VFQ developers encourage users to use all items for a given sub-scale. This will greatly enhance the comparability of sub-scale scores across studies.*

**Response choice "6" indicates that the person does not perform the activity because of non-vision related problems. If this choice is selected, the item is coded as "missing."*

Thus, items within each sub-scale were averaged together to create the 12 sub-scale scores. To calculate the overall composite score for the VFQ-25, we averaged the vision-targeted subscale scores, excluding the general health-rating question (Table 2).

**Table 2. Recoding of the 25 of the Visual Function Questionnaire**

| Scale | Number of items | Items to be averaged |
|---|---|---|
| General Health | 1 | 1 |
| General Vision | 1 | 2 |
| Ocular Pain | 2 | 4, 19 |
| Near Activities | 3 | 5, 6, 7 |
| Distance Activities | 3 | 8, 9, 14 |
| Vision Specific: | | |
| Social Functioning | 2 | 11, 13 |
| Mental Health | 4 | 3, 21, 22, 25 |
| Role Difficulties | 2 | 17, 18 |

| | | |
|---|---|---|
| Dependency | 3 | 20, 23, 24 |
| Driving | 3 | 15c, 16, 16a |
| Color Vision | 1 | 12 |
| Peripheral Vision | 1 | 10 |

### 3.4 Dietary Assessment

Dietary data were recorded by using a 95-item semi-quantitative Food Frequency Questionnaire (FFQ), which refers to the previous month dietary habits, adapted from a 46-item FFQ validated for the assessment of folate intake in Italian women of childbearing age [113]. During the interview, subjects were invited to indicate their consumption frequency (stratified into 12 categories from "almost never" to "two or more times a day") and meal size (low, medium or large), indicating he medium size by describing the standard weight or volume measures usually consumed in the Italian population, while small and large sizes were half a medium meal size or 1.5 times or more larger than a medium meal, respectively. Moreover, a photograph atlas was used to estimate the amount of each food and to minimize inaccuracies. Food intakes were estimated by multiplying the consumption frequency by the daily portion size of each food group adjusted for total energy intake using the residual method [114].

#### 3.4.1 Mediterranean Diet Score

The adherence to MD was investigated by using the MD Score (MDS), which refers to the ideal or poor consumption of nine food categories: fruits and nuts, vegetables, legumes, cereals, lipids, fish, dairy products, meat products, alcohol and unsaturated to saturated lipids ratio [115,116]. For vegetables, legumes, fruits and nuts, cereals, fish and unsaturated to saturated lipids ratio, subjects whose consumption was below or equal to the median value of the population were assigned the value of 0, otherwise the value of 1. For dairy and meat products, subjects whose consumption was below the median were assigned a value of 1, otherwise the value of

0. To subjects consuming 5 to 25 g of alcohol per day, a value of 1 was assigned. Finally, MDS ranged from 0 (non-adherence) to 9 (perfect adherence). The adherence was further categorized as follows: low (MDS: 0–3), medium (MDS: 4–6), or high adherence (MDS: 7–9) [117].

### 3.5 Sample collection and molecular analysis

For each participant, a peripheral blood sample was collected into EDTA tubes for molecular analysis. First, blood samples are collected and anticoagulated using EDTA. The blood samples were then centrifuged to separate the plasma or serum from the cellular fraction. Next, the plasma or serum is loaded onto a QIACUBE cartridge containing silica-based columns. The QIACUBE then automated the binding, washing, and elution steps of the miRNA purification protocol using a series of pre-programmed protocols and reagents. Total RNA was extracted using the miRNeasy Serum/Plasma Kit (Qiagen). Recently, preclinical and clinical evidence showed a promising role for miR-320 in the pathogenesis of DR, being involved in several biological pathways, such as insulin secretion and resistance, fatty acid metabolism, lipotoxicity, endothelial dysfunction, and cardiac damage [118–122].

For the miRNA-320 quantitative analysis, reverse transcription was carried out using the TaqMan™ MicroRNA Reverse Transcription Kit (Thermofisher). Quantitative real-time PCR (qPCR) was carried out using the TaqMan MicroRNA Assays (Thermofisher) according to the manufacturer's instructions. miRNA expression was normalized using the detection of U6. The primer sequences for miRNA-320 and U6 amplification were (forward) 5'-ACACTCCAGCTGGGAAAAGCTGGGTTGAGAG-3' and (forward) 5'-CTCGCTTCGGCAGCACATA-3', respectively. The relative expression was calculated using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method.

### 3.6 Statistical analysis

The distribution of the continuous variables was analysed by using the Shapiro-Wilk with Lilliefors significance correction and Kolmogorov-Smirnov tests. Graphic analyses of histogram and Q-Q normality graph and asymmetry/standard error or kurtosis/standard error ratio supported the exploration of the distribution of continuous variables.

Continuous variables were expressed as median (interquartile range, IQR) and compared using the Mann–Whitney U test or Kruskal–Wallis test, the latter in case of comparison between three or more independent groups. Categorical variables were expressed as frequency (percentage), and compared by using the Yates' chi-square or Fisher exact test to detect a percentage difference between groups.

Multivariable logistic regression models have been created to consider the effect of possible confounders on each outcome occurrence and to identify independent determinants of the ideal consumption of each food category and of medium-to-high adherence to MD. We calculated the adjusted odds ratios of the event after correcting for potential confounders.

A two-sided P value of $< 0.05$ was considered statistically significant. Statistical analyses were performed using the IBM SPSS software (version 21.0, SPSS, Chicago, IL, USA).

#### 3.6.1 Sample size calculation

Based on the primary study endpoint, the study sample size calculation defined a minimum number of 98 subjects (32 cases with DR and 64 controls without DR) to detect a mean difference of 2 points (Standard Deviation = 5) in the MDS between cases and in study controls, with $\alpha$ significance level of 0.05 and a statistical power of 80%.

# 4 RESULTS

## 4.1 Overall study population

One hundred twenty-nine participants with T2D, 65 male (50.4%) and 64 female (49.6%), were enrolled between 28 January 2021 and 8 July 2021. The characteristics of the study population are illustrated in Table 3.

The median age at enrollment and at T2D diagnosis was 70 (IQR 65-74) and 53 (IQR 46-58) years, respectively, with a median diabetes duration of 17 (IQR 14-19) years. Merely one-tenth of subjects had a normal weight, while most of them were overweight (41.9%) or obese (45.0%) and had an above normal WC (80.6%) (Table 3).

Median HbA1c was 8% (IQR 7-8), 41.9% of subjects were insulin treated, in addition, for most of them, to other oral or injective diabetes drugs. More than two-thirds were also treated with lipid lowering (76.7%), anti-hypertensive (82.2%), or anti-platelets drugs (71.2%) (Table 3).

At ophthalmological evaluation, DR was found in 44 out of the 129 subjects (34.1%), the majority of them (n=36) was affected by NPDR and eight by PDR (Table 4). A high prevalence of CKD (75.2%) was detected, with most of subjects having a mild-moderate (stage 2-3) CKD (44.2% and 24.8%, respectively), while no subjects had a kidney failure requiring kidney replacement therapy (stage 5). An impairment of kidney tubular function, demonstrated by micro- or macro-albuminuria, was found in 18.6% and 0.8% of subjects. Concerning chronic macrovascular complications, 21 out of 129 subjects (16.3%) had a history of any kind of coronary ischemic disease and 10 of them (7.8%) had a stroke or a critical ischemia of lower limb requiring revascularization treatment (Table 4).

**Table 3. Demographic, anthropometric, clinical and biochemical characteristics of the 129 recruited subjects**

| | |
|---|---|
| Male gender, n (%) | 65 (50.4) |
| Age (years) | 70 (65-74) |
| Age at diabetes diagnosis (years) | 53 (46-58) |
| Duration of diabetes (years) | 17 (14-19) |
| BMI ($Kg/m^2$) | 29.8 (26.3-33.8) |
| BMI classification, n (%) | |
| Normal weight | 17 (13.2) |
| Overweight | 54 (41.9) |
| Obese | 58 (45.0) |
| WC (cm) | 105 (98-115) |
| High WC, n (%) | 104 (80.6) |
| HbA1c (%) | 8 (7-8) |
| Fasting glucose (mg/dL) | 133 (121-158) |
| Creatinine (mg/dL) | 1 (1-1) |
| GFR (mL/min) | 77.5 (58.4-91.4) |
| Microalbuminuria, n (%) | 24 (18.6) |
| Macroalbuminuria, n (%) | 1 (0.8) |
| Cholesterol total (mg/dL) | 164 (143-182) |
| HDL (mg/dL) | 46 (39-53) |
| LDL (mg/dL) | 90 (70-109) |
| Triglycerides (mg/dL) | 117 (89-151) |
| AST (U/L) | 18 (16-23) |
| ALT (U/L) | 19 (14-28) |
| SBP (mmHg) | 130 (120-145) |
| DBP (mmHg) | 75 (70-80) |
| Insulin treatment, n (%) | 54 (41.9) |
| Long-acting insulin analogues | 54 (41.9) |
| Short-acting insulin analogues | 19 (14.7) |
| Pro-Kg insulin daily dose (U) | |
| Long-acting insulin analogues | 0.26 (0.18-0.37) |
| Short-acting insulin analogues | 0.26 (0.21-0.41) |
| Total dose | 0.32 (0.21-0.50) |
| Other diabetes drugs, n (%) | |
| Metformin | 111 (86) |
| GLP-1 RA | 59 (45.7) |
| SGLT2-I | 31 (24) |
| GLP-1 RA or SGLT2-I | 81 (62.8) |
| DPP4-I | 13 (10.2) |

| | |
|---|---|
| Pioglitazone | 17 (13.2) |
| Acarbose | 3 (2.3) |
| Lipid lowering drugs, n (%) | 99 (76.7) |
| Anti-hypertensive drugs, n (%) | 106 (82.2) |
| Anti-platelets drugs, n (%) | 89 (71.2) |

BMI, body mass index; WC, waist circumference; GFR, glomerular filtration rate; SBP, systolic blood pressure, DBP, diastolic blood pressure; GLP-1 RA, glucagon like peptide 1 receptor agonists; SGLT2-I, sodium glucose transporter-2 inhibitors; DPP4-I, dipeptidyl peptidase-4 inhibitors.

**Table 4. Prevalence of diabetic retinopathy and other micro- and macro-vascular complications in the 129 recruited subjects**

| | |
|---|---|
| DR, n (%) | 44 (34.1) |
| NPDR, n (%) | 36 (27.9) |
| PDR, n (%) | 8 (6.2) |
| Chronic kidney disease, % | 75.2 |
| Chronic kidney disease stage, % | |
| Stage 1 | 5 (3.9) |
| Stage 2 | 57 (44.2) |
| Stage 3 | 32 (24.8) |
| Stage 4 | 3 (2.3) |
| Stage 5 | 0 (0.0) |
| Ischemic heart disease | 21 (16.3) |
| Stroke or lower limb revascularization | 10 (7.8) |

DR: diabetic retinopathy, NPDR, non-proliferant diabetic retinopathy; PDR, proliferant diabetic retinopathy

## 4.2 Groups with and without Diabetic retinopathy

### 4.2.1 Demographic, socio-behavioural, anthropometrical, and biochemical characteristics

The main characteristics of the study groups, classified based on the ocular examinations, are showed in Table 5, indicating no difference for gender, age at recruitment and HbA1c between the groups with and without DR at recruitment. These two groups were also similar for educational level, employment status, and smoking status (Table 5).

At multivariate analysis, subjects with DR had a significantly higher duration of diabetes compared to those not affected by DR (18 vs. 16 years, IQR 21.8 vs. 16, $p<0.01$, OR 1.12), while no difference was observed between NPDR and PDR (Table 5).

A trend for a higher BMI, close to reaching statistical significance, was observed in group with PDR compared to NPDR and NDR group (31.3 vs. 27.4 vs. 30.0 $Kg/m^2$, IQR 26-39 vs. 26-32 vs. 26-34, p=0.09) (Table X). A similar, not statistically significant trend was observed for WC, whose values were higher in subjects who develop PDR (Table 5).

The three study groups were similar for blood pressure, cholesterol total and LDL, and triglycerides, while HDL levels were significantly lower in subjects with PDR compared to NPDR and NDR group (40.0 vs. 50.0 vs. 45.0 mg/dL, IQR 33-36 vs. 43-59 vs. 38-52.8, p=0.09) (Table 5).

The use of lipid lowering, anti-hypertensive, and anti-platelets drugs were similar between the three groups (Table 5).

**Table 5. Demographic, anthropometric, clinical and biochemical characteristics of the 129 recruited subjects subdivided according to retinal status**

| | **NDR (N=85)** | **DR (N=44)** | **NPDR (N=36)** | **PDR (N=8)** | **p** ***(NDR vs. DR)*** | **p** ***(NDR vs. NPDR vs. PDR)*** |
|---|---|---|---|---|---|---|
| Male gender, n (%) | 38 (44.7) | 27 (61.4) | 26 (63.9) | 4 (50.0) | 0.07 | 0.16 |
| Age (years) | 70 (63.5-74) | 70 (65.3-76) | 71 (66-76) | 70 (64-75) | 0.46 | 0.65 |
| High educational level (%) | 28.2 | 20.4 | | | 0.34 | |
| Employed (%) | 14.1 | 9.1 | | | 0.41 | |
| Smoker or ex-smoker (%) | 55.3 | 47.7 | | | 0.41 | |
| Supplement use (%) | 14.1 | 25.0 | | | 0.12 | |
| Total energy intake (kcal/die) | 1756 (440) | 1755 (590) | | | 0.60 | |
| Age at diabetes diagnosis (years) | 53 (47-58) | 53 (45-56) | 54 (45-56) | 51 (40-59) | 0.31 | 0.57 |
| Duration of diabetes (years) | 16 (13-19) | 18 (16-21.8) | 18 (15-22) | 18 (17-21) | <0.01 | <0.01 |
| BMI ($Kg/m^2$) | 30 (26.4-34.3) | 28.1 (25.6-32.7) | 27.4 (26-32) | 31.3 (26-39) | 0.12 | 0.09 |
| BMI classification, n (%) | | | | | | |
| Normal weight | 10 (11.8) | 7 (15.9) | 6 (16.7) | 1 (12.5) | 0.20 | 0.38 |
| Overweight | 32 (37.6) | 22 (50.0) | 19 (52.8) | 3 (37.5) | 0.20 | 0.38 |
| Obese | 43 (50.6) | 15 (34.1) | 11 (30.6) | 4 (50) | 0.20 | 0.38 |
| WC (cm) | 105 (97-115) | 107 (101-114.8) | 106 (103-112) | 115 (105-125) | 0.53 | 0.29 |
| High WC, n (%) | 69 (81.2) | 35 (79.5) | 28 (77.8) | 7 (87.5) | 0.82 | 0.80 |
| HbA1c (%) | 7 (7-8) | 8 (7-8) | 8 (7-8) | 8 (7-8) | 0.92 | 0.23 |
| Fasting glucose (mg/dL) | 133 (121-156) | 132 (121-161) | 132 (121-161) | 131 (119-171) | 0.67 | 0.91 |
| Creatinine (mg/dL) | 1 (1-1) | 1 (1-1) | 1 (1-1) | 1 (1-1) | 0.70 | 0.83 |
| GFR (mL/min) | 79.8 (61.5-92) | 74.7 (57-87) | 71.4 (56.8-87) | 82.0 (72-96) | 0.41 | 0.34 |
| Microalbuminuria, n (%) | 15 (17.6) | 9 (20.5) | 6 (16.7) | 3 (37.5) | 0.71 | 0.36 |
| Macroalbuminuria, n (%) | 1 (1.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0.47 | 0.77 |
| Cholesterol total (mg/dL) | 162 (141.3-179) | 166 (144-187) | 169 (153-192) | 145 (120-152) | 0.56 | 0.04 |
| HDL (mg/dL) | 45 (38-52.8) | 49 (41-54) | 50 (43-59) | 40 (33-46) | 0.17 | 0.01 |

| | | | | | | |
|---|---|---|---|---|---|---|
| LDL (mg/dL) | 87.5 (66.3-109) | 90 (73-108) | 95 (80-112) | 78 (61-84) | 0.95 | 0.16 |
| Triglycerides (mg/dL) | 123 (89-151) | 110 (84.3-163.5) | 107 (84-164) | 127 (80-163) | 0.56 | 0.80 |
| AST (U/L) | 19 (16-24) | 18 (14-21) | 18 (14-21) | 18 (16-21) | 0.16 | 0.37 |
| ALT (U/L) | 20 (14-29) | 17 (14-24) | 17 (13-24) | 21 (16-33) | 0.19 | 0.26 |
| SBP (mmHg) | 130 (123-145) | 133 (120-144) | 133 (125-144) | 128 (120-144) | 0.91 | 0.83 |
| DBP (mmHg) | 75 (70-80) | 70 (66-80) | 70 (66-80) | 70 (66-78) | 0.10 | 0.20 |
| Insulin treatment, n (%) | 29 (36.2) | 23 (56.1) | 17 (50.0) | 6 (85.7) | 0.04 | 0.03 |
| Long-acting insulin analogues | 29 (34.1) | 23 (52.3) | 17 (47.2) | 6 (75.0) | <0.05 | <0.05 |
| Short-acting insulin analogues | 8 (9.4) | 11 (25.0) | 9 (25.0) | 2 (25.0) | 0.02 | 0.06 |
| Pro-Kg insulin daily dose (U) | | | | | | |
| Long-acting insulin analogues | 0.26 (0.18-0.38) | 0.27 (0.17-0.37) | 0.24 (0.16-0.30) | 0.4 (0.3-0.5) | 0.96 | 0.07 |
| Short-acting insulin analogues | 0.26 (0.17-0.57) | 0.26 (0.21-0.32) | 0.24 (0.18-0.37) | 0.27 (0.26-NA) | 0.60 | 0.76 |
| Total dose | 0.29 (0.20-0.46) | 0.39 (0.22-0.56) | 0.36 (0.24-0.52) | 0.51 (0.34-0.62) | 0.23 | 0.27 |
| Other diabetes drugs, n (%) | | | | | | |
| Metformin | 78 (91.8) | 33 (75.0) | 26 (72.2) | 7 (87.5) | <0.01 | 0.02 |
| GLP-1 RA | 38 (44.7) | 21 (47.7) | 16 (44.4) | 5 (62.5) | 0.74 | 0.62 |
| SGLT2-I | 16 (18.8) | 15 (34.1) | 13 (36.1) | 2 (25.0) | 0.054 | 0.13 |
| GLP-1 RA or SGLT2-I | 47 (55.3) | 34 (77.3) | 28 (77.8) | 6 (75.0) | 0.01 | <0.05 |
| DPP4-I | 11 (13.4) | 2 (4.5) | 2 (5.6) | 0 (0.0) | 0.13 | 0.28 |
| Pioglitazone | 11 (12.9) | 6 (13.6) | 6 (16.7) | 0 (0.0) | 0.91 | 0.45 |
| Acarbose | 3 (3.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0.21 | 0.45 |
| Lipid lowering drugs, n (%) | 68 (80.0) | 31 (70.5) | 25 (69.4) | 6 (75.0) | 0.22 | 0.45 |
| Anti-hypertensive drugs, n (%) | 72 (84.7) | 34 (77.3) | 28 (77.8) | 6 (75.0) | 0.30 | 0.57 |
| Anti-platelets drugs, n (%) | 56 (69.1) | 33 (75) | 28 (77.8) | 5 (62.5) | 0.49 | 0.54 |

NDR, no diabetic retinopathy; DR: diabetic retinopathy, NPDR, non-proliferant diabetic retinopathy; PDR, proliferant diabetic retinopathy; BMI, body mass index; WC, waist circumference; GFR, glomerular filtration rate; SBP, systolic blood pressure, DBP, diastolic blood pressure; GLP-1 RA, glucagon like peptide 1 receptor agonists; SGLT2-I, sodium glucose transporter-2 inhibitors; DPP4-I, dipeptidyl peptidase-4 inhibitors.

### 4.2.2 Diabetes therapy

Despite glucose control at recruitment, evaluated by means of FPG and HbA1, was not different between the study groups, subjects affected by PDR, compared to NPDR and DR group, had a significant higher percentage of insulin use, both for short- and long-acting analogues, (85.7% vs. 50.0% vs. 36.2%, p=0.03) (Table 5). Besides, subjects with PDR, compared to NPDR and NDR groups, had a higher, close to reaching statistical significance, pro-kilogram dosage of long-acting insulin (0.40 vs. 0.24 vs. 0.26 mg/dL, IQR=0.3-0.5 vs. 0.16-0.30 vs. 0.18-0.39, p=0.07), while the requirement of short-acting insulin did not differ between the groups (Table 5).

In subjects with DR, compared to those with no DR, we observed a higher prevalence of sodium glucose transporter-2 inhibitors (SGLT2-I) administration (34.1% vs. 18.8%, p=0.05) and a lower metformin use (75.0% vs. 91.8%, p<0.01) (Table 5).

No difference between groups was observed for glucagon-like peptide-1 receptor agonists (GLP1-RA), dipeptidyl peptidase-4 inhibitors (DPP4-I), pioglitazone, and acarbose, and none of the recruited subjects was in treatment with insulin secretagogues (sulphonylureas or glinides) (Tables 5).

### 4.2.3 Extra-ocular vascular diabetes-related complications

Chronic kidney disease is a microvascular complication that share with DR risk factors and pathogenetic features. As expected, most of subjects recruited in this study, more than two-thirds, were affected by CKD, the majority having a mild-moderate renal involvement. The prevalence of CKD, in the whole population, resulted higher than DR (75.2% vs. 34.1) (Table 6). We explored the prevalence of CKD in the subgroups classified based on the ocular examinations. Although the percentage of any stage of CKD was slightly higher in subjects

with DR than NDR, this difference was not statistically significant (81.8% vs. 71.8%, p=0.21). Similar results were observed in subjects with and without DR stratifying the renal damage according to its severity (Table 6).

Type 2 diabetes is often associated to other CV risk factors, such as hypertension, dyslipidaemia, obesity, etc. We investigate, in all the recruited subjects, in subgroups classified based on DR, the presence of already known ischemic heart diseases or stroke, and lower limb vascular impairment: no significant difference was detected between subjects not affected by DR and those with different degree of DR (Table 6).

**Table 6. Prevalence of micro- and macro-vascular complications in the 129 recruited subjects subdivided according to retinal status**

| | **NDR (N=85)** | **DR (N=44)** | **NPDR (N=36)** | **PDR (N=8)** | **p** ***(NDR vs. DR)*** | **p** ***(NDR vs. NPDR vs. PDR)*** |
|---|---|---|---|---|---|---|
| Chronic kidney disease, % | 61 (71.8) | 36 (81.8) | 30 (83.3) | 6 (75.0) | 0.21 | 0.40 |
| Chronic kidney disease stage, % | | | | | | |
| Stage 1 | 4 (4.8) | 1 (2.3) | 1 (2.8) | 0 (0.0) | 0.39 | 0.48 |
| Stage 2 | 38 (45.2) | 19 (43.2) | 14 (38.9) | 5 (62.5) | 0.39 | 0.48 |
| Stage 3 | 18 (21.4) | 14 (31.8) | 13 (36.1) | 1 (12.5) | 0.39 | 0.48 |
| Stage 4 | 1 (1.2) | 2 (4.5) | 2 (5.6) | 0 (0.0) | 0.39 | 0.48 |
| Stage 5 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | NA | NA |
| Ischemic heart disease, % | 13 (15.3) | 8 (18.2) | 7 (19.4) | 1 (12.5) | 0.67 | 0.82 |
| Stroke or lower limb revascularization, % | 8 (9.4) | 2 (4.5) | 2 (5.6) | 0 (0.0) | 0.33 | 0.54 |

NDR, no diabetic retinopathy; DR: diabetic retinopathy, NPDR, non-proliferant diabetic retinopathy; PDR, proliferant diabetic retinopathy

### 4.2.4 Assessment of vision-related quality of life

Diabetic retinopathy, mostly in the advanced stages, could lead to vision threatening conditions often related to an impairment of quality of life. The administration of the 25-item NEI-VFQ to subjects involved in this study showed that the subjective perception of the disease and its impact on daily life activities worsened in accordance with the progression of DR stage. Interestingly, although no difference in the composite score and the specific items of the questionnaire was observed comparing the NDR and the DR group, the latter analysed without distinguish for DR severity (composite score: 99.1 vs. 99.1, IQR 95.9-99.5 vs. 97-99, p=0.47), opposite results were found also considering the severity of DR. Indeed, the composite score did not differs in NDR and NPDR groups, while a statistically significant reduction of this index, reflecting a worsening of vision-related quality of life, was observed in subjects affected by PDR (99.1 vs. 99.1 vs. 94.6 , IQR 95.9-99.5 vs. 97.6-99.1 vs. 85.7-98.0, p=0.04) (Table 7). A similar trend within the three groups was observed in the analysis of the specific items of the questionnaire, important measurements to estimate the burden of diseases: subjects affected by PDR, compared both NPDR and NDR groups, reported worst scores about mental health (p=0.01), ocular pain (p<0.01), near (p=0.02) and distance (p<0.05) activities, and driving (p=0.01) (Table 7).

**Table 7. Scores of the National Eye Institute Visual Function Questionnaire in the recruited subjects subdivided according to retinal status**

| | **NDR (N=85)** | **DR (N=44)** | **NPDR (N=36)** | **PDR (N=8)** | **p** ***(NDR vs. DR)*** | **p** ***(NDR vs. NPDR vs. PDR)*** |
|---|---|---|---|---|---|---|
| COMPOSITE SCORE | 99.1 (95.9-99.5) | 99.1 (97-99) | 99.1 (97.6-99.1) | 94.6 (85.7-98) | 0.47 | 0.04 |
| General health | 70.0 (60-77.5) | 65.0 (60.0-77.5) | 65 (60-75) | 72.5 (60-77.5) | 0.63 | 0.92 |
| General vision | 90.0 (70-95) | 87.5 (75-90) | 90 (82.5-90) | 72.5 (55-85) | 0.35 | 0.07 |
| Mental health | 100 (95-100) | 100 (100-100) | 100 (100-100) | 98 (83-100) | 0.17 | 0.01 |
| Ocular pain | 100 (88-100) | 100 (100-100) | 100 (100-100) | 88 (82-100) | 0.16 | <0.01 |
| Near activities | 100 (100-100) | 100 (100-100) | 100 (100-100) | 96 (63-100) | 0.62 | 0.02 |
| Distance activities | 100 (100-100) | 100 (100-100) | 100 (100-100) | 100 (81-100) | 0.44 | <0.05 |
| Peripheralvision | 100 (100-100) | 100 (100-100) | 100 (100-100) | 100 (88-100) | 0.69 | 0.17 |
| Social functioning | 100 (100-100) | 100 (100-100) | 100 (100-100) | 100 (96-100) | 0.77 | 0.10 |
| Color vision | 100 (100-100) | 100 (100-100) | 100 (100-100) | 100 (100-100) | 0.28 | 0.19 |
| Driving | 100 (100-100) | 100 (100-100) | 100 (100-100) | 96 (67-100) | 0.74 | 0.01 |
| Role difficulties | 100 (100-100) | 100 (100-100) | 100 (100-100) | 100 (100-100) | 0.15 | 0.14 |
| Dependency | 100 (100-100) | 100 (100-100) | 100 (100-100) | 100 (100-100) | 0.44 | 0.42 |

NDR, no diabetic retinopathy; DR: diabetic retinopathy, NPDR, non-proliferant diabetic retinopathy; PDR, proliferant diabetic retinopathy

### 4.2.5 Dietary intake and habits

Table 8 showed the level of different food categories in groups with and without DR. The total energy intake did not differ between subjects with and without DR (1755 vs. 1756 Kcal/die, p=0.60). The adherence rate to MD, estimated by using the MDS which allowed us to investigate the consumption of fruits and nuts, vegetables, legumes, cereals, lipids, fish, dairy products, meat products, alcohol and lipids, was overall similar in subjects with and without DR (4.0 vs. 4.0, IQR 3.0-5.0 vs. 3.0-5.0, p=0.89). Noteworthy, when we evaluated the consumption of each food category, we observed that subjects with DR introduce an amount of legumes significantly lower, at univariate analysis, than subjects who did not develop DR (34.0 vs. 40.3 g/die, IQR 21.8-53.1 vs.16.7-50.6, p<0.03) (Table 8). To better investigating this feature and evaluating the role of possible confounders, we created some models of multivariate logistic regression analysis. Subjects with a low legumes consumption, compared high legumes consumption, had a 2.5-fold increased risk for DR independently from gender, age, diabetes duration, BMI, WC, lipid and blood pressure levels (OR=2.5, 95% CI= 1.1–5.8, p=0.04) (Table XXAM). Instead, no difference in legumes intake between subjects with NPDR and PDR was observed (Table 8).

Besides, the daily fruits intake was lower in DR vs. NDR group (231.2 vs. 310.0 g/die, IQR 191.1-323.5 vs. 186.7-388.7), although this difference was not statistically significant (p=0.15). The intake of nuts, vegetables, cereals, lipids, fish, dairy products, meat products, alcohol and lipids was similar in the two groups. The consumption rate of supplement or nutraceuticals consumption did not differ between DR and NDR groups (25.0% vs. 14.1%, p=0.13) (Table 8).

**Table 8. Dietary intakes between patients with or without DR**

| Intake, g/die | NDR (N=85) | DR (N=44) | NPDR (N=36) | PDR (N=8) | p *(NDR vs. DR)* | p *(NDR vs. NPDR vs. PDR)* |
|---|---|---|---|---|---|---|
| Cereals | 168.3 (113.4-207.3) | 180.8 (136.7-219.5) | 186.7 (138.4-230.5) | 168.9 (107.2-184.3) | 0.19 | 0.21 |
| Fruits | 310.0 (186.7-388.7) | 231.2 (191.1-323.5) | 240.5 (192.1-365.4) | 212.3 (180.5-235.6) | 0.14 | 0.15 |
| Vegetables | 345.7 (225.3-483.8) | 331.0 (255.7-420.3) | 331.0 (245.8-427.3) | 329.0 (261.0-375.3) | 0.59 | 0.76 |
| Fish | 53.9 (26.9-87.3) | 51.4 (34.2-74.7) | 51.4 (39.2-71.4) | 41.9 (21.8-86.7) | 0.59 | 0.84 |
| Legumes | 40.3 (16.7-50.6) | 34.0 (21.8-53.1) | 32.2 (23.5-53.6) | 36.7 (27.6-47.7) | 0.03 | 0.08 |
| Meat | 61.3 (48.0-81.3) | 59.5 (48.2-75.7) | 57.2 (48.2-75.7) | 60.7 (50.8-77.2) | 0.93 | 0.96 |
| Dairy products | 278.3 (98.9-332.8) | 269.2 (91.7-330.5) | 208.5 (88.0-330.5) | 315.3 (179.3-345.9) | 0.96 | 0.69 |
| Alcohol | 0 (0-0.1) | 0.1 (0-0.1) | 0.1 (0-0.1) | 0.1 (0-0.1) | 0.19 | 0.30 |
| Unsaturated/saturated ratio | 2.4 (2.0-2.8) | 2.3 (2.0-2.9) | 2.3 (1.9-2.9) | 2.3 (1.9-2.9) | 0.53 | 0.19 |
| Mediterranean Diet Score | 4 (3-5) | 4 (3-5) | 4 (3-5) | 4 (3-6) | 0.89 | 0.35 |

NDR, no diabetic retinopathy; DR: diabetic retinopathy, NPDR, non-proliferant diabetic retinopathy; PDR, proliferant diabetic retinopathy.

### 4.2.6 Expression of miR-320

The serum expression of miRNA-320 was evaluated in the enrolled subjects and no significant difference was observed between subjects without DR vs. those with DR, regardless of the retinal injury stage (Figure 9).

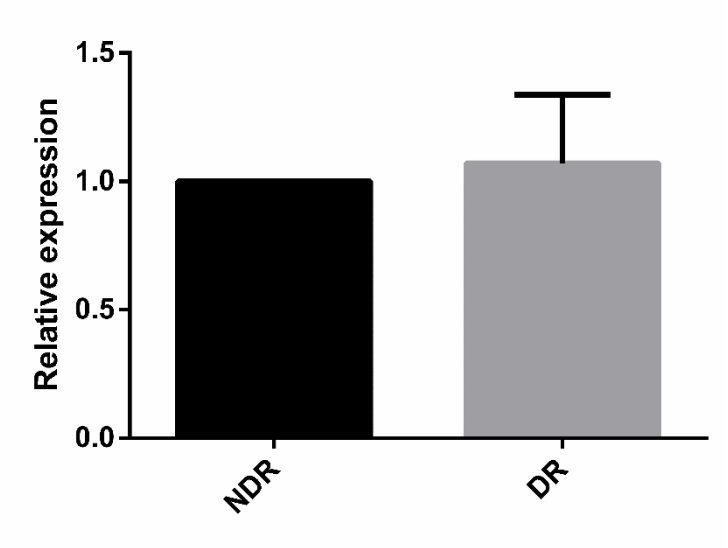


**Figure 9.** Relative expression of miR-320 in subjects with and without DR.

## 5 DISCUSSION

The current knowledge on risk factors and etiopathogenesis of DR did not provide a comprehensive view of this disease. Indeed, several diabetes subjects without the already known risk factors favouring the retinal damage could also develop DR. With the purpose to further investigating these critical features, in this study we explored the role of dietary habits - in particular the MD - and epigenetic dysregulations on the development of DR in T2D. The clinical expression of this highly specific diabetes-related neuro-vascular complication is

heterogeneous, ranging from asymptomatic to advanced vision threatening stages associated to visual acuity reduction, blindness and consequent difficulties in everyday life, social and work relationships. On this concern, we also investigated the burden on quality of life related to vision impairment in our cohort of T2D subjects affected by different degrees of DR.

Diabetic retinopathy occurs in three out of four diabetic subjects after 15 years of disease duration and the individual lifetime risk for DR in T2D is estimated to be 50–60% [25]. Our data confirmed the well-known detrimental role of diabetes duration on the risk of DR. Indeed, diabetes duration was significantly higher in subjects who developed DR, independently from the degree of the retinal damage, than in NDR group (18 vs. 16 years, IQR 21.8 vs. 16) (Table 5). At multivariate analysis, the model of logistic regression estimated a 12% increased risk for DR for each years of diabetes duration, adjusting for HbA1c, BMI, WC, lipid level, and blood pressure. The Epidemiology of Diabetes Interventions and Complications (EDIC) study, continuation of the DCCT study, observed, in 1214 diabetic subjects with thirty years of disease, a relation among diabetes duration and the prevalence of DR, with less than 10% of subjects free from DR at the end of the three decades of follow-up [123]. Recently, Wang and colleagues focused on the development of a nomogram to predict the risk of DR in subjects with T2D, identifying a progressive increased risk for DR according to diabetes duration, with a 5- and 12-fold increased risk after 5 and 10 years of diabetes [124].

Nevertheless, in T2D subjects the prevalence of DR could be significant already at diabetes diagnosis as demonstrated in a meta-analysis by Cai and colleagues which estimated a 15% prevalence of DR at diabetes onset in European T2D subjects, underlining the importance of effective strategies to promote an early diagnosis of T2D [125].

Obesity, representing a demonstrated risk factor for both diabetes and related comorbidities, such as hypertension and dyslipidaemia, all related to an increased risk of retinal impairment,

could influence the onset and progression of DR [126]. Consequently, we explored the potential effect of obesity on the occurrence of DR. Interestingly, while BMI was not different between NDR and NPDR groups, subjects with PDR had a higher BMI than the other two groups (Table 5). This difference, close to reach statistical significance (p=0.09), could reach the significance in a larger sample. It was reasonable considering the potential effect of obesity on the occurrence of DR. Nevertheless, the evidence deriving from observational studies are currently conflicting. In several studies, the increased risk for DR in patients with higher BMI was explained by its relation with higher level of blood pressure, cholesterol, leptin, oxidative stress parameters, and VEGF [45,127,128]. Differently, other studies indicated a null or inverse relation between BMI and the risk for DR [126,127,129]. Probably, the increased c-peptide level in overweight and obese subjects explain the protective effect of higher BMI values on DR [127]. Two meta-analysis were recently conducted to clarify how BMI influences the risk of DR without reaching concordant results [126,127]. Zhou et al. analyzed 27 cross-sectional and cohort studies and did not find any association between overweight or obesity and DR [126] . Nevertheless, the relevant heterogeneity between the included studies influenced the quality of the analysis and the obtained results. A meta-analysis of 13 prospective studies by Zhu et al. demonstrated a negative influence of obesity on the incidence of non-proliferative DR, mainly in T2D subjects [128].

Recently, we retrospectively evaluated, in a cohort of 168 subjects affected by both T2D and cancer, the retinal short-term effects of anticancer drugs [130]. Interestingly, BMI played the role of independent risk factor for early retinal worsening, evaluated within the six months after the anticancer drugs administration, determining a 45% increased risk of retinal impairment for one point of BMI increase.

Although well-conducted epidemiologic studies are necessary to better understanding this issue, weight reduction, due to its beneficial effects on insulin sensitivity, lipids and blood pressure, should be pursued in overweight and obese patients to reduce the risk of DR [131,132].

The evidence on the benefit related to intervention for lipid reduction in the prevention of DR are currently conflicting. In our cohort, we did not observed significant difference, between subjects with and without DR, in the level of cholesterol total, LDL, and triglycerides, and in the use of lipid lowering drugs. Instead, HDL was significantly lower in subjects with PDR compared to NPDR and NDR group (40.0 vs. 50.0 vs. 45.0 mg/dL) (Table 5). The influence of HDL on the course of DR is not well defined. Recently, the NO BLIND study analysed 2068 Italian patients with T2D who underwent fundus oculi exam observing a relationship between high HDL cholesterol and DR, estimated in 4% increased risk for DR with every increase in one unit of HDL cholesterol [133]. This finding was not confirmed by the previous literature, which did not report any effects of lipids in the pathogenesis of DR [43]. Few data demonstrated that a U-shaped association between HDL cholesterol and DR risk, already found for mortality and macro-vascular disease, could determine the increased risk for DR in subjects with high HDL cholesterol [133,134]. Further studies, with larger sample, are necessary to clarify this issue.

In T2D, insulin is used to counteract poor glucose control despite optimal non-insulin diabetes therapies. In our cross-sectional evaluation, we observed a higher prevalence of insulin use in subjects affected by PDR, compared to NPDR and DR group (85.7% vs. 50.0% vs. 36.2%, p=0.03) and a trend for a higher pro-kilogram dosage of long-acting insulin (0.40 vs. 0.24 vs. 0.26 mg/dL, IQR=0.3-0.5 vs. 0.16-0.30 vs. 0.18-0.39, p=0.07) (Table 5). Previous registry studies identified a significant higher risk for DR in insulin treated T2D subjects [135,136]. Grauslund and colleagues estimated that subjects who used insulin were 2.3 times more likely to have DR, and they had a 1.9–2.4 times higher risk for DR-development or progression,

suggesting insulin as a possible marker of present, incident and progressive DR [136]. Despite similar results were obtained in other studies, enrolling subjects with various ethnicity, indicating insulin therapy as risk factor for DR, a causal relationship between insulin use and DR should be demonstrated [135,137]. In fact, the pathophysiology of this phenomenon is not well understood and several mechanisms have been speculated. The fast improvement of glucose control with a rapid decrease of glycaemia and a transient reduction of retinal microcirculation could worsen retinal status in subjects with pre-existent DR [138]. Moreover, insulin could determine a co-synergistic effect with VEGF on retinal capillaries favouring vascular proliferation [138].

In our study, the percentage of metformin use was significantly lower in subjects with DR, compared to those with no DR (75.0% vs. 91.8%, $p<0.01$), despite a similar rate of CKD and GFR between these groups (Table 5). Metformin represents the oldest molecule for diabetes treatment but there is limited data from clinical trials regarding its in the development and course of DR. Retrospective studies suggest that metformin has a protective role against the development of DR and in reducing the severity of DR among subjects with underlying DR [139,140]. Metformin's role in DR could be explained by its anti-inflammatory, antiangiogenic, and anti-oxidant effect [141]. Nevertheless, further research is needed to clarify the role of metformin in DR.

Interestingly, we observed a higher prevalence of SGLT2-I administration in subjects with DR, compared to those with no DR (34.1% vs. 18.8%, $p=0.05$) (Table 5). Very limited evidence are available on the retinal influence of SGLT2-I. Few reports showed an improvement of DMO after SGLT2-I use, possible due to these molecules osmotic effect [142,143]. In a placebo-controlled crossover study ruled out in 59 patients with T2D, the treatment with dapagliflozin improved, after 6 weeks, the retinal capillary flow, arteriole remodelling, and arterial stiffness markers [144].

Empagliflozin, in a large, randomized clinical trial study, reduced, compared to glimepiride, the progression of DR in patients with DR at high risk for DMO [145]. Real world data suggest a significant protective role for of SGLT2-I, compared to DPP4-I, (HR 0.89) when started before the onset of DR, while no beneficial effects on DR progression were detected in subjects with DR pre-existent [146]. It has been postulate that similarly to their beneficial effects in the high oxygen consuming heart/kidney tissues, the SGLT2-I could improve the course of DR in the high oxygen consuming/hypoxia-prone diabetic retina through the production of chronic low-grade hyperketonaemia [147]. However, to validate the potential benefits of SGLT2-I in DR, we need evidence from well-controlled, adequately powered studies.

Diabetes mellitus is associated with multiple ocular complications, specifically DR and DMO, which are thought to severely impact on patients' vision and quality of life. Moreover, other minor visual dysfunctions related to alterations in refraction, contrast sensitivity, straylight and presbyopia could contribute to visual impairments of these subjects [148]. The prevalence of diabetes subjects suffering from moderate to severe vision impairment risen by 30.6% in the past decade, and the estimated prevalence vision-threatening DR reaches 56 million [149,150]. Thus, the evaluation of visual function should be part of the clinical approach to these subjects in both the ophthalmology and diabetes setting.

In our study, all subjects underwent the 25-item NEI-VFQ and a statistically significant worsening of vision-related quality of life and daily life activities was observed in subjects with more advanced stages of retinopathy. In particular, the mental health, ocular pain, near and distance activities, and driving were the most impaired items (Table 7).

Robust evidence are available on the NEI-VFQ accuracy for exploring the impact of ocular different ocular diseases. The essayed field of the questionnaire are reproducible and useful in multiple conditions of varying severity and in different population [112]. Some studies used the

25-item NEI-VFQ in Italian population similar to that enrolled in our cohort. In a prospective observational study, Rossi and colleagues enrolled non-hospitalized patients affected by DR and various other ocular diseases (cataract, age-related macular degeneration, glaucoma, and cytomegalovirus retinitis) to test the reliability and validity of the Italian translation of the 25-item NEI-VFQ. This study demonstrated the good validity, discriminatory power, internal consistency, and reliability of the Italian version of the questionnaire, concluding that it represents a valid and useful approach in both clinical and research setting as a specific measure of quality of life in subjects with chronic eye diseases [151]. Trento and colleagues tested the 25-NEI-VFQ in 196 Italian subjects (185 with T2D, 11 with T1D) who developed DR, the majority (80.6%) affected by PDR, with visual acuity less than 5/10 [152]. Similar to our results, they observed a decreased score for mental health, ocular pain, near and distance activities, and driving ability. The authors also found and impairment in general vision, social functioning, role difficulties and dependency, colour and peripheral vision, all items not impaired in our cohort. Nevertheless, compared to our study, Trento and colleagues did not enrol subjects with no DR but all subjects with DR and reduced visual acuity (<5/10), while we did not adopt this criteria including subjects with any acuity status. In 2017, a cross-sectional study including 123 Indian subjects, with type 1 or 2 diabetes, who underwent the 25- NEI-VFQ, and grouped based on the presence of DR, observed a considerable reduction of quality of life, relative to all the questionnaire items, in DR compared to NDR group with maximum effect as the severity of retinopathy increased, confirming the results of our study [153].

Our data confirmed and improved those already existing, suggesting the need of structured action protocols focused on the well-being of diabetes subjects affected by DR, mostly for those suffering from advanced stages of retinopathy.

Appropriate nutrition is mandatory in diabetes management; nevertheless, its role in DR development and progression of has not been clearly defined. The anti-inflammatory and antioxidant properties of the MD compounds (olive oil, red wine, fibre and cereals) were found to improve the glucose peripheral uptake and peripheral insulin resistance, thus preventing diabetic microvascular complications [154]. In our population, no difference was found in the adherence rate to MD between NDR and DR groups, nevertheless, a significant lower legume consumption in subjects affected by DR was detected. In particular, we observed a 2.5-fold increased risk for DR in subjects with a low legumes consumption, compared high legumes consumption (OR=2.5, 95% CI= 1.1–5.8, p=0.04) (Table 8). The monitoring of carbohydrate intake in diabetes subjects is important for improving postprandial glucose control and great focus on the quality of carbohydrates could prevent the development and progression of vascular complications [51]. A higher consumption of legumes, carbohydrates with low-glycaemic index, could protect against DR by improving glucose control and glycaemic variability in post-prandial phase [51,155]. Nevertheless, further studies are necessary to support these findings.

Recently, Sha and colleagues systematically reviewed the existing evidence on the relation between dietary intake and the risk for DR. They analysed 3 interventional, 17 prospective, 29 cross-sectional, and 5 case–control studies, founding that a MD and higher intakes of fruits, vegetables, fibers, fish, oleic acid, and tea have a protective effect against DR, while high intakes of soda, rice, and choline were associated with a higher risk of DR [156]. Prospective and interventional data derived from type 1 and 2 diabetes subjects reported a significant protective effect of the MD against the onset of DR with a 68% and 32-40% risk reduction in T1D and T2D, respectively [157,158]. A post hoc analysis of a cohort of subjects recruited for the PREvencion con DIeta MEDiterranea study, multicentre trial conducted on T2D subjects at

high CV risk, showed a reduced risk for DR, but not for nephropathy, in subjects who received a MD supplemented with extra-virgin olive oil compared to those who practiced a MD supplemented with mixed nuts or a low-fat diet [157]. However, considering the relatively low number of incident DR cases, these results should be interpreted with caution and further explored in prospective series.

The trend for a lower fruit intake observed in DR vs. NDR groups (231.0 vs. 310.0 g/die) of our cohort was in agreement with previous prospective data by Tanaka and colleagues, who described a reduced DR risk in subjects prone to a higher fruits consumption [159].

The identification of sensible DR biomarkers could improve the detection of DR at earlier stages, predict the treatments response, thus improving prognosis and subject's quality of life. Few evidence are available on the role of circulating miRNAs in DR. The miR-320 family and the related pathways are involved in several biological mechanisms, such as insulin secretion and resistance, fatty acid metabolism, lipotoxicity, endothelial dysfunction, and cardiac damage [118,120,122]. We did not find any difference in the expression level of miR-320 between NDR and DR groups. Differently from our results, Santovito and colleagues observed a significant upregulation of another member of the miR-320 family, namely, miR-320b, in T2D subjects with DR compared to those without, while changes in miR-320a-3p did not reach statistical significance [121]. In vitro experiments demonstrated that miR-320 over-expression restrained TGF-β1 signalling enhancing inflammation and oxidative stress in high glucose stimulated Müller cells [119]. These preclinical and clinical evidence, although encouraging need to be improved by multicentre and prospective studies to validate the diagnostic performance of these miRNAs as biomarker of DR.

## 6 CONCLUSION

This study reveals the association between a higher BMI and the risk for the advanced stages of DR. It is well known that the excess of body weight represents a risk factor for several diseases, such as hypertension and dyslipidaemia, all related to an increased risk of retinal impairment. Besides, we found that legumes consumption could protect against DR, both for the initial and advanced stages of this micro-vascular complication, as suggested by the higher daily intake of these low-glycaemic index carbohydrates in subjects without compared to those affected by DR. Although further studies are necessary to confirm these findings, we could speculate that the intake of these carbohydrates with a low-glycaemic index improves glucose control and glycaemic variability in post-prandial phase reducing the risk for retinal damage.

Our data reinforce the significance of a healthy, well-balanced diet, together with an appropriate lifestyle behaviour, to mitigate the fearful diabetes-related risk for retinal injury that is related to a significant worsening of vision-related quality of life and daily life activities - mental health, near and distance activities, and driving – mainly in subjects with more advanced stages of retinopathy.

Preventive and therapeutic strategies are mandatory to tackle the increased burden of DR. However, further advances require an understanding of how dietary habits may counteract the risk profile and the pathological features of retinal injury at a molecular level, in order to translate these findings into effective preventive strategies and action protocols focused on the well-being of these subjects.

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