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DEPARTMENT OF SURGERY

FACULTY OF MEDICINE AND ODONTOLOGY

ANALYSIS OF GENE EXPRESSION IN RELATION TO CONCENTRATIONS OF ANTIOXIDANTS, VITAMIN B12, FOLATE AND HOMOCYSTEINE IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

Doctoral Thesis 2017

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Certifican**:**

Que el trabajo de investigación titulado "**Analysis of gene expression in relation to concentrations of antioxidants, vitamin B12, folate and homocysteine in patients with type 2 diabetes mellitus**" que presenta D. KIAN SHOAIE-NIA, licenciado en Medicina, fue realizado bajo nuestra dirección, reuniendo todos los requisitos necesarios para ser defendido ante un tribunal y optar al grado de doctor.

Y para que así conste, firmamos el presente certificado en Valencia, a 28 de octubre de 2017.

Prof. Manuel Díaz Llopis Prof. MªDolores Pinazo Durán Prof. Vicente Zanón Moreno

To my wonderful wife, Natasha

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TABLE OF CONTENT

TERMINOLOGY

AACE: The American Association of Clinical Endocrinologists

ABTS: 2,2'-Azino-di-3-ethylbenzthiazoline sulphonate

ACTID: Early Activity in Diabetes trial

ADA: American Diabetes Association

AGE: Advanced Glycation End-products

AHA: American Heart Association

Ang: Angiopoietins

ANOVA: Analysis Of Variance

BCA: Bicinchoninic Acid

BCVA: Best Corrected Visual Acuity

bFGF: Basic Fibroblast Growth Factor

BMI: Body Mass Index

BSA: Bovine Serum Albumin

cDNA: complementary DNA

CRISP: Comprehensive Read analysis for Identification of Single Nucleotide Polymorphisms from Pooled sequencing

CRP: C Reactive Protein

CTRL: Control Group

DBP: Diastolic Blood Pressure

DCCT: Diabetes Control and Complication Trial

DM-DR: Diabetes mellitus type 2 without diabetic retinopathy

DM: Diabetes Mellitus

DM+DR: diabetes mellitus type 2 with diabetic retinopathy

DM1: Diabetes Mellitus Type 1

DM2: Diabetes Mellitus Type 2

DME: Diabetic Macular Edema

DNA: Deoxyribonucleic Acid

DR: Diabetic Retinopathy

EASD: European Association for the Study of Diabetes

EGIR: European Group for the Study of Insulin Resistance

ELISA: enzyme-linked immunosorbent assay

ETDRS: Early Treatment Diabetic Retinopathy Study

EtOH: Ethanol

FA: Folic Acid

GAD-antibodies: Glutamic Acid Decarboxylase Autoantibodies

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Glu: Glucose

GP: Glutathione Peroxidase

GR: Glutathione Reductase

GSH: Glutathione

GWAS: Genome wide association study

HbA1c: Glycosylated Hemoglobin

HCl: Chlorhydric acid

Hct: Hematocrit

Hcys: Homocysteine

HDL-Ch: HDL cholesterol

HDL: High Density Lipoprotein

HGNC: HUGO Gene Nomenclature Committee

HRP: Horseradish Peroxidase

IDF: International Diabetes Federation

IFG: Interferon Gamma

IGF: Insulin-like Growth Factor

IOP: Intraocular Pressure

IRMA: Intraretinal Microvascular Abnormalities

LADA: Latent Autoimmune Diabetes in Adults

LDL-Ch: LDL cholesterol

LDL: Low Density Lipoprotein

LO: Left Orbit

logMAR: Logarithm of the Minimum Angle of Resulution

MDA: Malonildialdehyde

MIDD: Mitochondrial diabetes

MMACHC: Methylmalonicaciduria and homocystinuria type C protein

MMADHC: Methylmalonicaciduria and homocystinuria type D protein

MMP9: Proteases of the matrix metalloproteinase

MODY: Maturity-Onset Diabetes of the Young

mRNA: messenger Ribonucleic Acid

NCEP ATP III: National Cholesterol Education Program Adult Treatment Panel III)

NDDG: National Diabetes Data Group

NEI: National Eye Institute

NHLBI: National Heart, Lung, and Blood Institute

O.D.: Optical Density

OCT: Optical Coherence Tomography

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PKC: Protein Kinase C

R72P: Arginine 72 Protein

RBP1: Retinol Binding Protein 1

RNS: Reactive Nitrogen Species

RO: Right Orbit

ROS: Reactive Oxygen Species

RT: Room Temperature

SBP: Systolic Blood Pressure

SDF-1: Stromal Derived Factor-1

SDS: Sodium Dodecil Sulfate

SHBG: Sexual Hormone Binding Globulins

SLC: Solute carrier family

SLC23A2: Solute carrier family 23 member 2

SNP: Single Nucleotide Polymorphism

T-Ch: Total Cholesterol

TAC: Total Antioxidant Capacity

TAS: Total Antioxidant Status

TBA: Thiobarbituric Acid

TBARS: Thiobarbituric acid and its reactive species

TCDB: Transporter Classification Database

TG: Transglutamase

TG: Triglycerides

TGF-ß1: Transforming growth factor beta 1

THRA: Thyroid Hormone Receptor Alpha

TMB: Tetramethylbenzidine

TMCO1: Transmembrane and coiled-coil domain-containing protein 1

TNF: Tumor Necrosis Factor

TP53: Tumor Proteins 53

TRP53: Transformation-Related Protein 53

VEGF: Vascular Endothelial Growth Factor

VSDR: Valencia Study on Diabetic Retinopathy

WHO: World Health Organization

ABSTRACT

Summary

Diabetes Mellitus (DM) is one of the most important causes of vision loss and decrease of quality of life worldwide. It presents a large number of complications in its development. One of the most important complications is the Diabetic Retinopathy (RD). At present, the DM is considered a pandemic, since its prevalence does not stop increasing.

DR is a growing ocular pathology and a cause of blindness worldwide with a prevalence of 34.6%. People with DR often do not realize changes in their vision during the early stages of the disease, but as the pathology progresses, it causes significant loss of vision and even blindness. Early diagnosis is, therefore, essential to promote good visual prognosis and prevent blindness caused by this disease.

In this context, we propose a study about the relationship between plasma concentrations of folic acid (FA), vitamin B12, homocysteine (Hcys), total protein, C reactive protein (CRP), oxidative stress markers (malondialdehyde (MDA), glutathione Peroxidase (GPx), total antioxidant status (TAS) and the expression of certain genes related to these molecules in type 2 diabetic patients (DM2) and to analyse their correlation with the presentation and progression of DR in a Mediterranean population.

For this, we carried out a multicentre epidemiological study of cases and controls that included 81 subjects, 49 with DM2 (14 +DR, 35 -DR) and 32

controls. The concentrations of the aforementioned molecules were determined and the gene expression was analysed. The data obtained were processed using the IBM SPSS for Windows v22.0.

We observed significantly higher concentrations of CRP, Hcys, MDA and GPx and a significantly lower TAS in the DM2-DR and DM2+DR groups compared to the control group (CTRL) ($p<0.05$). Considering gene expression, we observed a lower expression of SLC23A2 and a greater expression of MMP9 in the DM2-DR and DM2+DR groups compared to the CTRL ($p <0.05$). The TP53 gene expression was only significantly higher between DM2+DR and the CTRL ($p<0.05$).

Consequently, genes related to apoptosis (TP53) and extracellular matrix integrity (MMP9) could be considered as markers of susceptibility to the development and progression of DR. As well as the SLC232A2 gene (ascorbic acid transporter) may be considered as a protector of the risk of suffering or progressing the retinopathy in DM2 patients.

Key words: diabetes mellitus, diabetic retinopathy, genetics, gene expression, oxidative stress, vitamin B12, homocysteine.

Resumen

La Diabetes Mellitus (DM) constituye una de las causas más importantes de pérdida de visión y de la calidad de vida relacionada con la visión en todo el mundo, puesto que presenta un gran número de complicaciones en su desarrollo, siendo una de las más importantes la retinopatía diabética (RD). En la actualidad la DM se considera una pandemia, puesto que su prevalencia no deja de aumentar.

La RD es una patología ocular en auge, causa de ceguera en todo el mundo con una prevalencia del 34,6%. Las personas con RD a menudo no se dan cuenta de los cambios en su visión durante las primeras etapas de la enfermedad pero, a medida que avanza, causa una pérdida importante de visión e incluso ceguera. El diagnóstico precoz es esencial para favorecer el buen pronóstico visual y poder prevenir la ceguera producida por esta enfermedad.

En este contexto, planteamos un estudio con el objetivo de establecer la relación entre las concentraciones plasmáticas de ácido fólico, vitamina B12, homocisteína (Hcys), proteína total, proteína C reativa (PCR), marcadores de estrés oxidativo (malonildialdehído – MDA, glutation peroxidasa – GPx, estado antioxidante total – EAT) y la expresión de ciertos genes relacionados con dichas moléculas, en pacientes diabéticos tipo 2 (DM2), y analizar su correlación con la presentación y progresión de la RD en una población mediterránea.

Para ello, llevamos a cabo un estudio epidemiológico multicéntrico de casos y controles que incluyó 81 sujetos, 49 con DM2 (14 +RD, 35 –RD) y 32

controles. Se determinaron las concentraciones de las moléculas antes mencionadas y se analizó la expresión de los genes. Los datos obtenidos fueron procesados mediante el programa IBM SPSS para Windows v22.0.
Observamos una concentración significativamente superior de PCR, Hcys, MDA y GPx y un EAT significativamente inferior en los grupos DM2-RD y DM2+RD respecto al grupo control ($p<0.05$). En cuanto a la expresión de genes, observamos una menor expresión de SLC23A2 y una mayor expresión de MMP9 en los grupos DM2-RD y DM2+RD respecto al grupo control ($p<0.05$). La expresión del gen TP53 sólo fue significativamente superior entre DM2+DR y el grupo control ($p<0.05$).

Por tanto, los genes relacionados con apoptosis (TP53) e integridad de la matriz extracelular (MMP9) podrían ser considerados como marcadores de susceptibilidad al desarrollo y progresión de la RD. Así como el gen SLC232A2 (transportador del ácido ascórbico) puede considerarse como protector del riesgo de padecer o progresar en la retinopatía en pacientes DM2.

Palabras clave: diabetes mellitus, retinopatía diabética, genética, expresión genética, estrés oxidativo, vitamina B12, homocisteina.

INTRODUCTION

1. About diabetes

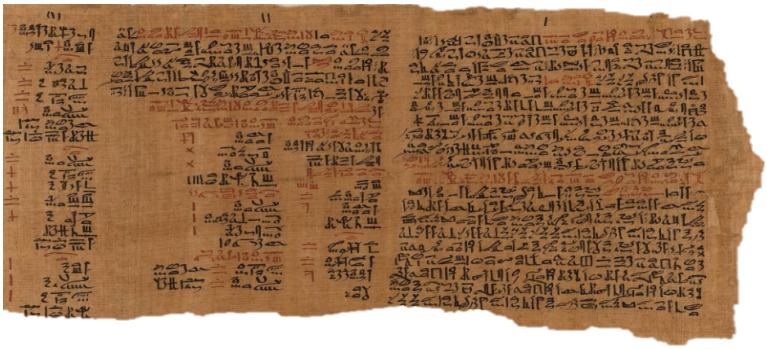
Diabetes is a collection of names for different diseases. Diabetes Mellitus (DM) indicates higher levels of glucose in the blood system. The root of the pathogenic mechanisms is different due to which different type of diabetes we refer too. There are many different types of DM, which have totally different causes, however type 2 (DM2) is the most common type of the disease. Therefore, finding proper diagnosis of the disease requires adequate type classification and thereafter careful treatment.

The DM is increasing continuously in the world, specifically DM2, which shows pertinence in our modern living due to lower physical activity, and higher body weights, both in adults and in children (1). Increasing prevalence worldwide demands more action on DM prevention and management, with careful planning within the healthcare and the organizations behind it. Although it is not only a matter of a good healthcare system, yet also the modern western lifestyle must act for a more preventive lifestyle.

Despite all modernized and refined drugs and treatments of the disease, complications in lingering patients are a wide problem. As an example, it is worth mentioning that, DM is the most common reason for severe poor vision in working adults in the western world and diabetes or pre-diabetes occur in most cases of myocardial infarctions (2). Notwithstanding intensive research behind the risk factors and the pathogenic mechanisms of this disease, there is still no exclusive medication or treatments that could specifically prevent it.

2. History

The disease is first time described in an Egyptian papyrus roll (Figure 1) from 1550 b.c., in the so called “Papyrus Ebers” describing a polyuria condition (3).



(Figure 1) Papyrus and Ostraca Collection of the University Library Leipzig.
Source:http://www.organapapyrologica.net/content/members_leipzig.xml;jsessionid=7839B01E6F247940F893D43993AEB892;jsessionid=7839B01E6F247940F893D43993AEB892?XSL.lastPage.SESSION=/content/members_leipzig.xml&lang=en

Around 500 b.c. an Indian physician called Sushruta was the first who described the two types of DM, differentiating between those cases in young people, which caused death, and others in adults (4).

(Figure 2) Sushruta statue.
Source: http://www.newsgram.com/father-of-surgery-was-sushruta-the-first-plastic-surgeon-in-600-bc/

Aretaios from Cappadocia (5), (Figure 3) is known to be the first to describe and used the word “Diabetes” which is Greek, meaning "a siphon”, because people with diabetes "passed water like a siphon”.

(Figure 3) Aretaios from Cappadocia.
Source: https://en.wikipedia.org/wiki/Aretaeus_of_Cappadocia

John Rollo (6), a Scottish surgeon, deceased 1809, was the first person using the term “diabetes mellitus”, where the word “mellitus” means “honey” or “sweet” which was described earlier by Thomas Willis year 1674 who tasted the urine and described its flavour by adding the term “mellitus”. He also described the first functional diet that could help those with the disease. He thought glucose was made in the stomach from vegetables and his “diabetic diet” also called the “animalistic diet” consisted mainly of fat and meat. With some modifications, this was actually the only treatment of the disease until beginning of the 1920s. A famous variant of this diet was created by a Swedish doctor, Karl Petrén (1868-1927) consisting mainly of cabbage and killer in different forms rinsed with wine (7). French chemist, Michel Chevreull (1786-1889) showed that the sugar in human urine was glucose (8).

Claude Bernard (1813-1878) has a profound role in the history of DM research, since he discovered and mapped the metabolism of glucose and that it could be find in the bloodstream, also during fasting and not only during meals as one has though before. He also found that the liver contained a starch-like substance that he called “glycogen” meaning “sugar-binding”(9). Paul Langerhans (1847-1888) discovered that the pancreas was designed as accretions of cells looking like islets (10). Some years later, Gustav Laguesse (1861-1927) suggested that these islets could be involved in secretion of a substance that could lower the glucose levels in the bloodstream. He called them the “islets of Langerhans” and later this hypothetical substance was called insulin, coming from the Latin, meaning: island (11). Another Scottish doctor, George Harley (1829-1896) noticed that there are at least 2 different types of diabetes that needs totally different treatment (12). Etienne Lancreaux (1829-1910) set a difference between fat and thin diabetes: diabéte gras and diabéte maigre. Before the discovery of the insulin these terms had a dramatic role seeing that the latter often died within a few months. After the discovery of insulin the dying group could survive and the other group could often live with a dietary treatment (13).

The terms DM types 1 and 2 are actually given from anthropometry. In the 1940s an American anthropologist named Dupertius measured body constitution in diabetic individuals (14). As an anthropologist, he had vague knowledge about diabetes and though it is one uniform disease. To his surprise, he realized that people with DM had totally different body constitution, which he classified as IA and IB. These where later named group I and II, and the latest classification as “type 1” and “type 2” diabetes was made by Andrew Cudworth in the 1970s (15).

Before 1923, the chance was small for individuals to survive long enough in order to develop diabetes complications. However, the most common

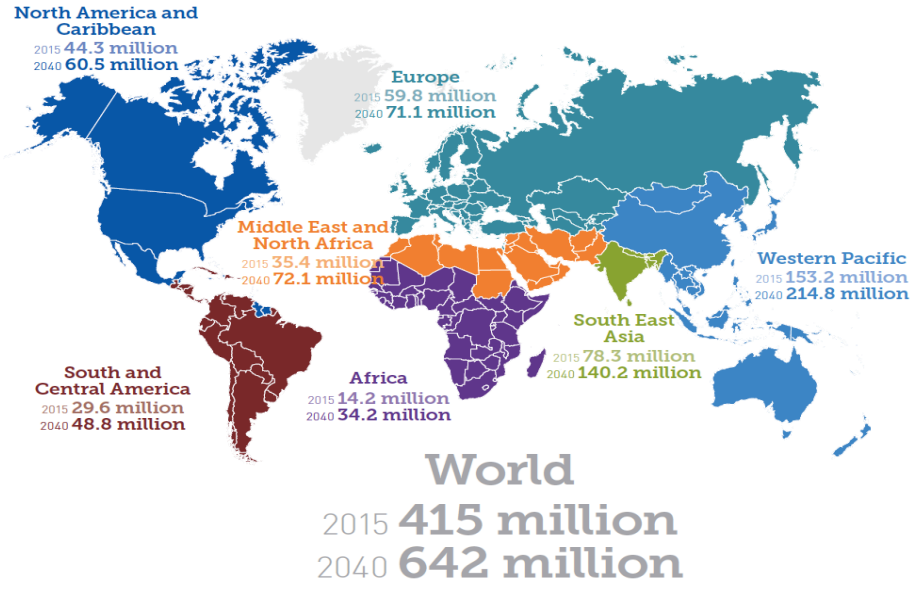
complications were known as early as the beginning of 1800s. Domenico Cotugo (1736-1822) wrote in 1770 the first description of proteinuria with DM (16), while Wilhelm Griesinger (1817-1868) described albuminuria for the first time (17).

In 1855, Eduard von Jaeger (1818-1884), draw the first picture of retinopathy in the ocular fundus on a diabetic 22-year-old patient with the new discovered ophthalmoscope (18). Julius Hirshbergh (1843-1925) was the first claiming that retinopathy was typical in DM patients and he classified these retinal findings to 4 different forms: retinitis centralis punctuate, haemorrhagic form, retinal infarction, and haemorrhagic glaucoma (19). Although these expansive complications of DM disease were most clear after the discovery of insulin by Banting and McLeod (Nobel Prize 1923). Less than 10 years after this significant discovery, an American specialized in DM, Elliot P. Joslin (10) stated that, due to the discovery of insulin, we now move from the insulin-coma ages to the diabetes-complication age (6). Even if modern and improved measurements with portable blood glucose meters, more specific drugs, better blood pressure measurements and treatments are available, among ocular fundus screenings and laser treatments, we face the biggest global challenge ever due to increased prevalence of DM2.

3. Ethnicity

It is very often said that DM, particularly DM2, is a disease from the western world and habits. It is usually associated with the American lifestyle and many European countries, where the fast-food culture and sedentary work have a significant role. However, this is not far away from the truth, and we can clearly see that the prevalence of the disease is rapidly increasing all over the world (Fig. 4). Regardless of this association, the IDF (International Diabetes Foundation) reveals the fact, showing most of the increase happen

in low or middle income countries, and the trend appears to continue in years ahead. Lifestyles changes along with urbanization, for having a more westernized lifestyle has often been discussed and has been a subject for research, with proven facts that the prevalence of diabetes, for the same ethnical group, living in different areas, has been higher due to its outcome. At the same time the environment of these ethnical groups have varied and have shown different consequences. However, the most significant factor seems to be genetic factors regarding prevalence, progression and occurrence of sequelae between ethnical groups.



(Figure 4) Estimated number of people with diabetes worldwide and per region in 2015 and 2040 (20-79 years). Source: IDF Diabetes Atlas, Seventh Edition, 2015

4. Clinical types

As mentioned earlier, there are different ethnological classifications of DM. There are older sayings asserting that some main differences in classification of DM types 1 and 2 is the propensity of producing acids, so called ketones.

In older days scientist thought the production of ketones only seemed to appear in type 1 diabetes (DM1), due to destroyed islet cells caused of an autoimmune process, but also non-autoimmune DM1 was described which seemed to debut as dramatically as the classical forms with the difference that this form arise secondary after a chemical destruction of beta cells after acute form of pancreatitis, non-alcohol induced (20). However several cases of ketoacidosis have been noticed also in DM2 patients (21).

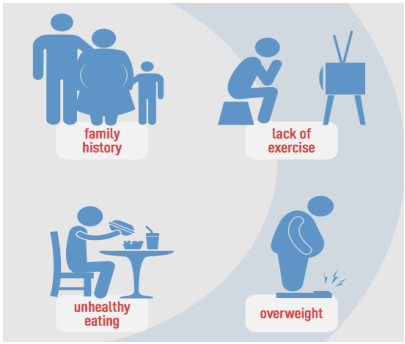
Type 1: This DM type is characterized of a progressive betel destructivity which sooner or later will cause insulin deficiency, which means that the person suffering from this type of the disease need external injections of insulin in order to survive. Usually this is caused by an autoimmune response where antibodies attack islet tissue in the pancreas. The autoimmunity itself can be divided in two groups where one is referred to the fast progressive form and the other one, which is most common is called the slow progressive form or LADA (Latent Autoimmune Diabetes in Adults).

Type 2: The DM2 covers the majority of all diabetic types. Variation in pervasiveness is usually seen and the disease progress undetected for several years before a diagnosis is set and the individual has by then often developed symptoms with complications. There are usually several causes, however the diagnosis presumes no signs of autoimmunity with destructiveness of the pancreas, and the fact that the individual has no other known reasons for having diabetes.

It is said that there is a reduction in the amount of beta cells in DM2, but most of them exists and are intact. A small increase of A-cells leads to higher levels of the hormone, glucagon. Due to prolonged duration of the disease, the appearance of amyloid inclusion is seen in the islets of

Langerhans. With this said, scientists conclude that the quality and mechanisms and capacity of secretion is more important than the amount of functional cells in DM2 (22). More recently it has been said that DM2 is actually a slow progressive form of DM1 (13). Those can be identified with attribution of so called GAD-antibodies. This "new" form is seen mostly in normal or slightly obese individuals, referred to a group called LADA (23). However, old terms such as latent or chemical diabetes is more seldom used nowadays and new subtypes of DM 1 and 2 have been described and most diabetics adopts having type 1 or mixed forms of types 1 and 2.

With this said, we could affirm that the prevalence of classical DM2 has reduced, not assert the amount of cases has been reduced, but more likely, those we used to call DM2 is discover having type 1 or type 1-alike diabetes. Individuals with DM2 suffer usually from an insulin resistance and some grade of insulin deficiency. Furthermore, no insulin injection is necessary, at least during the first year, and changing lifestyle, diet and per oral medication might reduce the risk of injections. Let's bear in mind that this type has a strong hereditary component which means that the disease results in interaction with several different genes, environmental factors, obesity, higher age and physical inactivity.



(Figure 5) Diabetes Risk Factors.

Source: International diabetes federation.http://www.idf.org/worlddiabetesday/toolkit/gp/risk-factors

5. Prevalence and incidence of diabetes mellitus type 2

There is no ideal screening system to evaluate incidence of DM2. Preferably one with a yearly examination, with yearly-added new diagnosis of the whole population in each specific country. A study made in Laxå, Sweden, with a follow-up over 33 years showing an age standardized yearly incidence of about 3 new cases per 1.000 inhabitants (Jansson, 2010, Mortality trends in subjects with and without diabetes during 33 years of follow-up) and a retro-perspective study from Uppsala, also in Sweden, showing 3,8 cases per 1.000 inhabitants (22, 24). Another study from Gothenburg was the yearly incidence 4,3 per 1.000 inhabitants between ages 51-60 and 4,9 per 1.000 inhabitants between ages 61-67. The cumulative risk of developing DM2 between age 61-67 was close to 8% (24). This is not far away from fact that the WHO represents for the whole world presented in their "key facts" below:

Key facts from the World Health Organization (WHO)

- The number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014 (25).
- The global prevalence of diabetes* among adults over 18 years of age has risen from 4.7% in 1980 to 8.5% in 2014 (25).
- Diabetes prevalence has been rising more rapidly in middle- and low-income countries (25).
- Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation (25).
- In 2012, an estimated 1.5 million deaths were directly caused by diabetes and another 2.2 million deaths were attributable to high blood glucose**(25).

- Almost half of all deaths attributable to high blood glucose occur before the age of 70 years (25). WHO projects that diabetes will be the 7th leading cause of death in 2030 (26).
- Healthy diet, regular physical activity, maintaining a normal body weight and avoiding tobacco use are ways to prevent or delay the onset of DM2 (25).
- Diabetes can be treated and its consequences avoided or delayed with diet, physical activity, medication and regular screening and treatment for complications (25).

** Defined as fasting blood glucose equal to or higher than mmol/L, or on medication for raised blood glucose, or with a history of diagnosis of diabetes.*

*** High blood glucose is defined as a distribution of fasting plasma glucose in a population that is higher than the theoretical distribution that would minimize risks to health (derived from epidemiological studies). High blood glucose is a statistical concept, not a clinical or diagnostic category.*

In Sweden, there are also gender related differences in incident risks and the prevalence of DM2. Apparently the disease is more common in women but this gender difference disappears when the whole population in all ages is considered. In higher ages, it's more common in women, which could be related to higher diabetes incidence and lower mortality in women (24).

6. The metabolic syndrome

Gerald Reaven was the first one introducing this syndrome in 1988, described as "syndrome X" (27). The metabolic syndrome is referred to a group of risk factors related to cardiovascular disease, which all are related to insulin resistance (28). Suffering from insulin resistance among obesity,

particularly abdominal obesity, along with dyslipidaemia, hypertension, DM2 or decreased glucose intolerance connote a significant higher risk of diseasing or even death in cardiovascular diseases.

6.1. Definition

Due to its complicity, there is no single definition of the metabolic syndrome. However, all different definitions share the same major components as you can see in the table 1.

The differences in these definitions may cause different prevalence figures and the cardiovascular disorder risks may differ, due to which definition is used in different population studies.

	WHO (1999)	EGIR (1999)	NCEP ATP III (2001 revised 2005)	AACE (2003)	IDF (2005)	AHA/NHLBI (2005)
Insulin Resistance	IGT, IFG, DM2 or decreased insulin sensitivity & 2 of the following characteristics	Plasma insulin >75% & 2 of the following characteristics	No definition: 3 of the following characteristics	IGT or IFG & any of the following characteristics	No definition	No definition: 3 of the following characteristics
Obesity	Waist size: men ≥0,9 women ≥0,85 &/or BMI >30 kg/m2	Waist size: men ≥94 cm women ≥0,80 cm	Waist size: men ≥ 102 cm women ≥88 cm	BMI ≥ 25 kg/m2	Increased wist size (population specific) & 2 of the following characteristics	Waist size: men ≥ 102 cm women ≥88 cm
Dyslipidemia (mmol/l)	TG ≥1,7&/or HDL: men< 0,9 women ≥1,0	TG ≥1,7&/or HDL: men& women < 1	TG ≥1,7 HDL: men ≤ 1,0 & women ≤ 1,3	TG ≥1,7 HDL: men< 1,0 & women< 1,3	TG ≥ 1,7 or treatment against high TG HDL: men ≤ 1,0 women ≤1,3 or with treatment	TG ≥ 1,7 or treatment against high TG HDL: men ≤ 1,0 women ≤1,3 or with treatment
Hypertension (mmHg)	≥ 140/90	≥ 140/90 or hypertension treatment	≥ 130/85	≥ 130/85	SBP ≥ 140 or DBP ≥ 85 or with hypertension treatment	SBP ≥ 140 or DBP ≥ 85 or with hypertension treatment
Glucose (mmol/l)	IGF, IFG or DM2	IGT or IFG (not diabetes)	> 6,1	IGT or IFG (not diabetes)	≥ 5,6	≥ 5,6 or diabetes treatment
Other	Micro-albuminurea			Other insulin resisters characteristics (family history of DM2, polycystic ovarial syndrome, inactive life-style, age & some ethnical relation to a high risk group of DM2)		

(Table 1) Different definitions of the Metabolic Syndrome. Source: Carl-David Agardh and Cristian Berne. Diabetes. 4th rev. Stockholm: Författarna och Liber AB; 2009. Part 1, Definition, diagnostik och klassificiering: p.28

Abbreviations: WHO (World Health Organisation), EGIR (European Group for the Study of Insulin Resistance), NCEP ATP III (National Cholesterol Education Program Adult Treatment Panel III), AACE (The American Association of Clinical Endocrinologists), IDF (International Diabetes Federation), AHA/NHLBI (American Heart Association and the National Heart, Lung, and Blood Institute), IGF (Insulin Like Growth Factor), IFG (Interferon gamma), DM2 (Type 2 Diabetes Mellitus), BMI (Body Mass Index), TG (Transglutamase), HDL (High Density Lipoprotein), SBP (Systolic Blood Pressure), DBP (Diastolic Blood Pressure), mmol/l (milimole per liter), mm Hg (millimeter Quicksilver)

6.2. Cause

There is a multifactorial background to the metabolic syndrome where life-style, metabolism and genetic factors all play its role somehow. Age, ethnicity, gender and social-economic factors influence the prevalence. Figures about 15-40% have been reported for adults' population in a westernized population. The prevalence increases with age in young adults and starts to decrease about their 70s.

Unfortunately, the increase is major in the USA, Europe and Asia, due to its similarity in life-style changes and the occurrence of obesity, waist-obesity and DM2. It is said that about half of the factors causing the metabolic syndrome is due to heredity and the other half due to the life-style, which indicates higher energy intake, stress and less physical activity. In genetic studies with help of singe nucleotide polymorphism (29), in both DM2 and insulin resistance, indications that the "old" gene (ancestral gene) is associated with increased risk, while mutations occurring in later generations are protected from metabolic deviations. This leads to the fact that recent life-style and habitat is the dominating cause to this powerful increase of the metabolic syndrome, in many populations. There is also known that stress, anxiety, depression, lack of sleep, using tobacco and alcohol all contributes in development of the metabolic syndrome. In women with insulin resistance, with heightened level of testosterone levels among low levels of the sexual hormone binding globulins (SHBG), results in higher free testosterone levels, which is related to the metabolic syndrome.

In this syndrome, the risk of cardio-vascular diseases is about doubled in all age categories. The risk is also increased if more factors behind it are presence. If one has DM2, the risk of having the metabolic syndrome is about four to 6 times higher compared with those not having DM2.

About 90% of people suffering from DM2, teenagers up to 70-80 year old individuals, all fulfil the criteria for metabolic syndrome. It should be mentioned that the metabolic syndrome is not very rare in DM1, since about 22% of the patients in the Diabetes Control and Complication Trial (DCCT), fulfilled the criteria, following IDFs criteria, at start of the study. It has been shown that individuals with DM1 fulfilling the criteria for the metabolic syndrome have higher risk developing cardiovascular diseases and microvascular complications.

7. Other diabetic types

7.1. Genetic defects in beta-cell-functionality

7.1.1. MODY (maturity-onset diabetes of the young) is a variant where mutation in genes causes the disease. Less than 5% of this type causes DM2. So far, this type of mutations has been found in several genes with different symptoms. Therefore giving name to MODY 1, MODY 2 up to MODY 6.

7.1.2. Mitochondrial diabetes, also called MIDD (30). As the name indicates this type is inherited from the mother and associated with deafness. There are so far 40 types of mitochondrial mutations and these mutations stands for about 1% of all cases of diabetes (30).

7.1.3. Genetic defects that influences the effect of insulin

These types are very rare types, except in lipoatrofic diabetes, which is caused by a mutation in an insulin-receptor gene, called A-insulin resistance. This mutation causes many different types of symptoms and syndromes, such as Donahue-syndrome and Rabson Mendenhall

in children. Lipoatrophy, hyperinsulinemia, DM, hypertriglyceridemia, acanthosis nigricans and liver steatosis might be other difficulties these mutations might cause.

Also worth mentioning in this category one might suffer from:

- Diseases in the exocrine pancreas
- Endocrine diseases
- Medical or chemical induces diabetes
- Inflectional diseases
- Specific forms of immune-mediated diabetes
- Other genetic syndromes associated with diabetes
- Pregnancy diabetes

7.2. Diabetes mellitus diagnosis

The debut of DM classical symptoms is sometimes very sudden, with: frequent and lots of urination, thirst, unplanned weight-loss, infections and poor vision. In worst-case scenarios, ketoacidosis might happen and if not rapidly treated, lead to ones death. Usually the symptoms are less complicated and severe, with hyperglycaemia during long periods, before the prognosis is obvious. However, when a diagnosis is determined the individual might have developed complications.

7.2.1. Diagnostic criteria

In order to determine the diagnosis of DM, it needs to be evidence of high plasma glucose levels in the bloodstream. Generally, DM represents a group of metabolic diseases, which are characterised by hyperglycaemia, which is usually a cause of shortage in insulin secretion or insulin defects, or both together. Temporary or occasional rise of the plasma glucose that might have been caused due to extreme stress, difficult infections or trauma is therefore not a criteria for putting the diagnosis. Through the years, the criteria have differed and the first general criteria were introduced by the National Diabetes Data Group in the USA and by the World Health Organization (WHO) in 1980. After nearly two decades, in 1999, further changed was introduced by the (WHO) and used by medical professionals until more recent years. Most recent changes have been presented by the American Diabetes Association.

	WHO 1999	ADA 2003
Diabetes Fasting glucose 2–h glucose*	≥7.0mmol/l **or** ≥11.1mmol/l	≥7.0mmol/l **or** ≥11.1mmol/l
IGT Fasting glucose 2–h glucose	<7.0mmol/l (if measured) **and** ≥7.8 and <11.1mmol/l	Not required ≥7.8 and <11.1mmol/l
IFG Fasting glucose 2–h glucose	6.1 to 6.9mmol/l **and (if measured)** (measurement recommended)	5.6 to 6.9mmol/l Measurement not recommended (but if measured should be <11.1 mmol/l)

(Table 2) Venous plasma glucose 2 hours after ingestion of 75 grams oral glucose. (2)
Source: Författarna och Liber AB; 2009. Part 1, Definition, diagnostik och klassificiering: p.28
Abbreviations: WHO (World Health Organization), ADA (American Diabetes Association), IGT (Impaired Glucose Intolerance), IFG (Impaired Fasting Glucose)

7.3. Managing diabetes mellitus prevention and therapy

In all DM types, the aim of treatment is always to achieve normal plasma glucose levels in hope to reduce the risk of hypoglycaemia. The main pillars of the treatment are an adapted diet and physical activity for the individual with the self-care as a key point. Except diet control and physical activity, there are several other methods of prevention and treatment of DM. Among these, oral medications such as metformin, which is among the bi-guanides that helps, decrease the production of glucose in the liver. They also decrease how much glucose the intestines absorb and makes the body more sensitive to insulin, and help muscles absorb glucose.

Glitazones, which are among the sulfonylureas, which are among the oldest diabetes drugs, still used today work by stimulating the pancreas with the help of beta cells. This causes the pancreas to make more insulin.

Other medications such as meglitinides help release insulin from the pancreas. Thiazolidinediones work by decreasing glucose in the liver. They also help fat cells use insulin better.

Glucagon-like peptides are similar to the natural hormone called incretin. They increase B-cell growth and how much insulin the body uses. They decrease appetite and how much glucagon the body uses. They also slow stomach-emptying which all are important actions for patients with DM.

If diet control, physical activity and oral medications are not given satisfactory results in the DM patient, combined with insulin medication

which most frequently used as injections in fat tissues can be used. There are short-acting, rapid-acting, intermediate-acting, long-acting and combination insulin that either individually or in combination are used to obtain maximum balance of blood glucose to reduce the risk of hypo or hyper glycaemia.

7.3.1. The importance of prevention of diabetes mellitus type 2

Some suggestions have been made on big groups of people in the USA during a long period of life, regarding the effect of regular physical activity and development of DM2. A follow-up of 87.000 women and 21.000 men in their middle ages, during 8 years showed that those individuals with at least 30 minutes of physical activity at least once a week, reduced their risks getting affected over 30%. There are of course many arguments why DM2 should be prevented. It is often said that the disease increases epidemically and that costs and maintenance of the disease is a big effort for every countries health care system. However, studies have shown that changing life-styles in individuals with high risk of developing the disease and/or with some oral medicament treatments might have preventive outcomes (31, 32). Also, obesity and bariatric surgery, more known as gastric banding or gastric bypass and gastric-plastic surgeries, have led to decrease in medication for DM2 patients or even ending treatment due to loose of with and changing lifestyle. A study made on 22.000 individuals with high body mass index, undergone some type of surgery due to obesity, showed results of 75% of the participants could terminate usage of oral medication. Since today's ability of affecting the beta-cells in pancreas is limited, we are suggested to focus on the insulin effect. This goal is most effectively achieved if focus is on change of lifestyles, possibly with types of pharmaceuticals designed to prevent insulin resistance, mainly in

people with higher risk factors developing the disease, such as: obesity, physical inactivity, use of tobacco and stress.

8. The diet

Understanding the importance of balanced diet and the ability to control, prevent and treat individuals with dietary treatments is fundamental in prevention of insulin intolerance, especially for people with DM2. The aim of dietary treatments or dietary awareness is to prevent vigorously symptomatic plasma glucose turns, which could cause complications.

In this effort, the target is not only the plasma glucose control, but also reducing the risk of hypertension and development of disorders in the lipid metabolism among coagulation disorders and fibrinolysis. These mentioned risks are all linked to possible development of insulin-resistance and all these risk factors are included in the so-called “metabolic syndrome” that we will be more closely described further below.

Studies has provided evidence supporting a beneficial effect from the traditional Mediterranean diet on the risk of DM2 and metabolic syndrome. A recent meta-analysis of prospective cohort studies showed that greater adherence to the Mediterranean diet was associated with a significant reduction in the risk of diabetes.

The Mediterranean diet has also been found to be beneficial in the prevention of gestational diabetes. Results have show that participants assigned to the Mediterranean diet had a significant 30% reduction in the risk of DM2 and that it also promoted the reversion of metabolic syndrome and its components, hyperglycaemia and central obesity (33).

9. Physical activity

In DM1 patients, without any complications, physical exercise shows improving effects in the cardiovascular functionality, it might even reduce any hyperlipidaemia and increase the general health quality. However, it does not control of glucose improve with physical exercise.

It is recommended and sometimes vital, for a person with DM1, to decrease the amount of insulin or intake of extra carbohydrates, or often both, prior to any extreme physical activity. In DM2, which are the most common type, is usually followed by obesity, hypertension, dyslipidaemia, and severe insulin deficiency.

All of these factors, individually might be a profound cause of cardiovascular complications. This leads to the fact that necessary treatment for this type is to normalize glucose and lipid levels in the bloodstream as much as possible.

Traditionally a combination of changing lifestyle and diet habits with oral medications are used to attain set treatment goals. Lower energy intake and higher energy consumption is the simple rule for this treatment. If/when this treatment is insufficient in order to obtain normal glucose balance, guidance from the USA (34) and Europe (EASD) stated 2009, suggests pharmacological treatment which will be more closely explained below.

10. Oral medication

As mentioned earlier, if or when physical activity and proper diet treatment are not sufficient in order to obtain normal glucose balance, it is suggested to use pharmacological treatments with metformin, which is a bi-guanid with antihyperglycaemic effects which both decreases the basal and post gradual levels of plasma blood glucose (35). Notwithstanding stimulation of insulin

secretion and therefore not cause hypoglycaemia. It has been suggested that treatment with metformin should begin as soon as the diagnosis of DM2 is determined (36) which some healthcare professionals plainly do not agree upon.

11. Use of insulin

Before 1922, everyone suffering of DM1 deceased shortly after onset of the disease. The discovery of insulin has been of such importance in our world and has been priced with the Nobel Price the year after its discovery. First person treated was Leonard Thompson, a 13 year old boy from Toronto, Canada (37).

The drug has developed significantly during the years and today we use such perfect combinations of amino-acid sequences and high degree of purification, which makes the insulin identical to human insulin. We are also able to modificate its properties for selective uses and these types are called insulin analogues.

The injection supply has reached such perfection that insulin pens can be used and many other different researches and inventions are being tested to create even better injection methods, among these also insulin pumps are other inoperable options, which gives more precise and customized treatment with better glucose control in relation to meal related requisites. In spite of improvements and the technology, usage of insulin demands great knowledge from the user.

Many often users will experience hypo- or hyperglycaemia episodes due to miscalculations or not fair predictions of ones digestion and physiology. Great patient-healthcare contact is required in order to tailor decorous usage of the drug. Many times, it’s advised to the patients, to connect with other

individuals suffering from the same disease, in order to apprehend and utilize the drug fairly in their daily life.

12. Transplantation

Despite great technology and methods of treatment with insulin, whether with insulin pens or pumps, in DM1 patients, sometimes the desired treatment isn't fulfilled. Which leads to daily life complications effecting the individuals' private life, family life, education possibilities and work life. It's worth mentioning that even today, sudden death in young individuals, treated for DM1, is surprisingly high. It is thought that about 6% of all deaths in people below 40 years old, suffering from DM1, high be caused from a hypoglycaemia leading to a ketoacidosis, more commonly described as "dead in bed syndrome" (38). Patients with this type of unstable disease with frequent hypoglycaemia tend to under-treat themselves resulting worsening blood glucose controls and higher risk for complications. Scientists agree upon the fact that antipathy in DM1 is caused by the metabolic disorders which itself is a result of malfunctioning of the beta-cells in the islets of Langerhans. This fact has led scientist to believe of a cure for the disease through transmitting of functional beta cells. There are two different ways of transplanting functional cells to such patients. One of them is by transplanting a whole new pancreas (39). Although this sounds promising, there are too great dangers for someone planned undergoing transplantation. Specially if the individual haven't already or is not about to remove or transplant a kidney, both because of the operation itself and because of the immunosuppressive treatment that follows. Therefore, only a pancreas transplant solely is seldom advised and must be predicted to result to great advantages before it's performed.

The other method used is to just take isolated islets of Langerhans so called "islet transplantation" (40). The latter has been more enticing since only the

islets needed is transplanted. The result of transplantation has been very promising and developed rapidly since it first was first performed in the USA, Minneapolis, year 1966.

13. Self-care

The concept of "self-care" was originally introduced for easier disease conditions, which could be managed self-handed. Great responsibility and knowledge about the disease, condition that might betide and treatments is requested from the patient, in order to minimize risks and complications. There are times when a patient, suffering from diabetes should be more observant. For example, before trips abroad or if getting acute diseases and infections. They should always think about suitable diet, physical activity. Always be careful in use of tobacco, use of alcohol and in traffic. They should pay attention to signs of neuropathy, signs of undesirable sudden or prolonged aces in the musculoskeletal system. There should be awareness for cardiovascular signs, pregnancy and menstruations disorders and preferably also care extra about oral health and sleeping habits or disorders. Modern methods of treatment for DM patients are based on good collaboration between the patient and the health-care team involved (41). This chronic and sometimes life threatening disease calls for a considerable contribution of the patient with enough knowledge about the disease and its complications. One of the hardest tasks for the health-care providers are to educate these individuals about the assignations that need to be carried out by the patients alone and often single-handed. Mainly, the actions and precautions required of the patient is to understand the mechanisms of their disease, how to measure their blood glucose levels, learn how to use the treatment suggested, either if it is tablets or with insulin. Furthermore, the patient needs to decide when contact with the healthcare should be made in order to avoid complications. However, the healthcare providers need to have trained doctors and nurses, ready to guide the patient whenever needed

with regular instructions and education about the disease and if new guidance and better suitable drugs are introduces in the market. Regular foot care or chiropody is always recommended (43, 44). Individual physical exercise programs and other screenings aimed for specific complications are essential which will be more closely discussed in next chapter.

14. Complications

During prolonged diagnosis with diabetes, one usually finds complications from smaller and bigger blood vessels in the human body. The problem is seen in many parts of the body and organs, such as the vessels in the eye, kidneys and the nervous system. Also, a higher risk for developing atherosclerosis in coronary vessels and those vessels in the brain. Not to forget risks for diabetic foot ulcers.

I will briefly mention these complications as follows;

14.1. Microangiopathy

This term is a collective term referring to the biochemical, structural and haemodynamic changes occurring, in smaller vessels, in different organs due to conical hyperglycaemia such in diabetes. Body parts usually affected by microangiopathy are in the retina in the eye (retinopathy), the glomeruli in the kidneys (nephropathy) and the vasa nervorum in the nervous system (neuropathy) (42). The most usual complication is retinopathy in the eyes and the most usual cause of blindness below age 65 in the western world (43). Nephropathy is one of the most usual causes to uraemia, which means higher level of urine in the bloodstream, indicating kidney failure. Peripheral neuropathy is a strong contributor to diabetic foot ulcers risking amputations in extremities.

14.2. Macroangiopathy

The changes occurring in bigger or middle-sized vessels are quite identical in their evolution such as atherosclerosis. Although the outcome and development of plaques are similar, the process is much higher and usually more aggressive in diabetic patients, which leads to more complications (5). The cause of this rapidness and aggressively is not yet discovered but, so far, it's been shown that there is a high oxidative stress in the endothelium cells which seems to have a profound role in the process. Also, increased glycation and lipid oxidation might be involved in the damage and inflammations caused in the vessel walls and mechanisms directly connected to the development of plaques (44). Let's have in mind that presence of hypertension, hyperlipidaemia and low HDL-cholesterol among coagulation disturbances are important factors to cardiovascular diseases in diabetes patients. Microangiopathy, which has been discussed earlier might bind links to the increased complications in diabetic patients, suffering from cardiovascular diseases.

14.3. Hypertension

High blood pressure is an important risk factor in development of macro and microangiopathies (45). There is strong scientific support, showing that development of nephropathy is decreased when treatment of hypertension is initiated in DM1 patients (46). Hypertension is usually followed by nephropathy in these patients. There are several causes, developing hypertension in DM2, notwithstanding that hypertension most likely precede DM2 diagnosis than being a cause of it. Most of the causes are linked to chosen lifestyle habits and It seems to be very important to start treating for hypertension early in the disease, if indicated, since evidence has shown that cardiovascular diseases and its complications are decreased and lower mortality is observed,

specifically in the development of stroke. Even though reduced risk in nephropathy and retinopathy is also benefits gaining from hypertension treatment in these groups of patients.

14.4. The diabetic foot

This term is used as a collection of complications such as: ulcers, infections, destruction of deeper tissue associated with neuropathy and periphery vascular diseases in lower extremities. In the western world, the diabetic foot is the main cause of hospitalization (47) of these groups of patients. The treatment is very costly, especially in situations when amputation is necessary, where length of stay, rehabilitation and increased need of home nursing is required. It is calculated that of all amputation, about 40-50% (excluding trauma or malignity) is due to diabetes. In some countries up to 70-90%. Population based studies regarding amputation in lower extremities are limited. The incident (48) is considered to be about 7 and 206 per 100.000 inhabitants per year. The highest reported incidents are from American-Indian sanctuaries and the lowest reported are from Denmark. The differences are due to demographical factors, diagnostic and treatment of diabetes among different reporting systems. Most of the amputations are made below the ankle and 80% of them were previously caused by an ulcer. The most usual cause of amputation is gangrenes and infections. Slow healing or even not healing ulcers is not an indication for amputation. These foot complications in diabetes patients represent a serious threat, both towards the patients' extremities and for their survival.

14.5. The diabetic eye

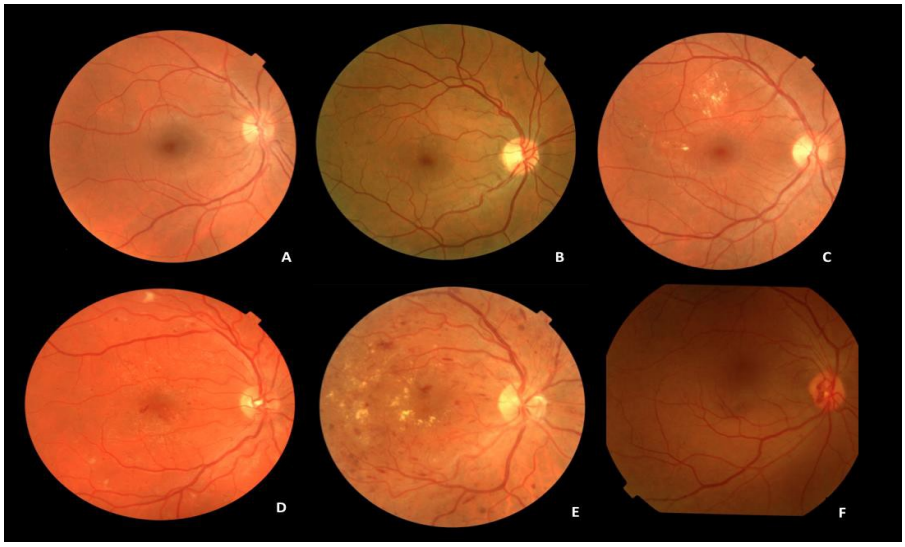
According to the National Eye Institute (NEI, USA), diabetic eye disease includes a variety of ocular pathologies that affect to the diabetic patients. Mainly the DR, the diabetic macular edema (DME), cataracts

and glaucoma. All of these DM-related disorders potentially cause visual impairment and blindness. In fact, DR is the main responsible of vision loss and blindness among working age adults worldwide. Early detection and accurate therapy and follow-up of the affected patients are major protecting strategies for fighting against diabetic blindness.

The DR is a microvasculopathy affecting the retina as well as the vitreous body that can be classified according to the Early Treatment Diabetic Retinopathy Study (ETDRS) criteria (X). Main ocular fundus signs and symptoms of DR are: microaneurisms, haemorrhages, hard exudates (spots corresponding to lipidic storage), soft exudates (spots corresponding to accumulation of axoplasmic detritus inside the axons of the ganglion cells), dilation and tortuosity of the veins, and intraretinal microvascular abnormalities (IRMA).

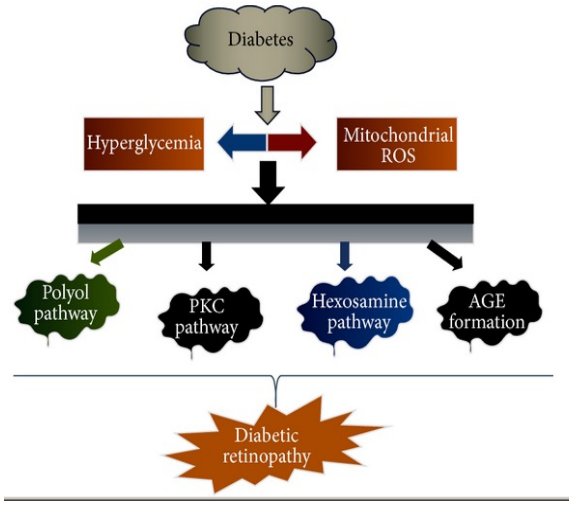
Figure 6 shows the ocular fundus appearance according to the DR stages (ETDRS classification). According to the epidemiological studies, the risk of initiation and progression of DR is closely related to the type and duration of diabetes, blood glucose, blood pressure and lipidic profile. It has also to be considered that long term lesions of DR are characterized by a complex array of vascular and neurodegenerative changes leading to the apparition of specific areas of retinal ischemia.

This, in turns, frequently triggers the onset of macular edema and/or the proliferative stages of DR with risk of visual impairment and blindness. Inflammation and neurodegeneration has also been reported in the diabetic retina in human and experimental diabetic models of retinopathy.



(Figure 6) Retinopathy Stages. A: Initial DR, B: Mild DR, C: Moderate DR, D: Severe DR, E: Very severe DR, F: Proliferative DR. Source: Extracted from doctoral thesis of Dr. MARÍA JOSÉ ROIG REVERT (49)

Different cellular pathways and a variety of molecular mechanisms have been implicated in the DR, such as the increased polyol pathway flux, augmented advanced glycation end-products (AGE) formation, alterations in the activation of signalling cascades (among them the protein kinase C, (PKC)). Pathway, elevated oxidative stress, increased hexosamine pathway flux, and others. Interestingly, the above signalling pathways are in relation to oxidative stress, inflammation, and ischemia, therefore inducing up-regulation of a wide spectrum of factors such as insulin-like growth factor (IGF), stromal derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), angiopoietins (Ang-2), tumour necrosis factor (TNF), and basic fibroblast growth factor-2 (bFGF) contributing themselves to the pathogenesis of DR, as can be seen in the figure 7.



(Figure 7) Major mechanisms involved in diabetes retinopathy (DR) (elevated polyol pathway flux, augmentation of AGE formation, activation of PKC and increased polyol pathways).

After an intensive search of the scientific literature, it became evident that the mechanisms involved in the pathogenesis of DR, reflects a single hyperglycaemia-induced process. However, it is presumable that the above-mentioned pathogenic mechanisms involved in DR are interactive and interdependent. As a consequence of this, the contributions of large trials and preclinical research into the underlying mechanisms of action of the processes involving the onset and progression of DR could lead to new treatments of the disease to avoid the diabetic visual impairment and blindness.

However, further research is needed to achieve full knowledge of the risk factors and pathogenic mechanisms occurring in the diabetic retina to achieve better eye care in diabetics.

15. Mortality in diabetes mellitus type 2

Mortality in age standardizing DM2 patients is approximately twice as high than for those without the diagnosis (50). For individuals in their middle ages, the expected reduction of living years is about 5-10 years (24). However, several population studies from Denmark, Holland and Sweden has shown that mortality in DM2 has decreased around 4% yearly over the past decade (24). Cardiovascular diseases, in particular myocardial infarctions and stroke have been the main cause of death in DM2 patients. Occurrence of these causes has been reported to be twice to four times higher in DM2 patients compared with those not suffering from the disease. Risk factors to increased risk of mortality in DM2 is age, male gender, prolonged disease duration with poor glucose control among cardiovascular factors such as usage of tobacco, hypertension, lipid disorders, abdominal obesity and indigent physical activity. During 2006, 47.039 Swedish women and 44.232 Swedish men, which is equivalent to 1.028 per 100.000 women and 982 per 100.000 men from the means population, died (48). The amount of population dying from diabetes as the main cause was 972 women and 1.025 men whence 941 women and 984 men were diagnosed with DM2 or non-specific type of diabetes. This gives a mortality rate of 16,8 for women and 26,9 for men in each 100.000 of the mean population. These figures have been pretty much alike for women since the 1980s while it has been increasing slightly for men since 1998. The mortality rate increases with age, which gives us the fact, that 6,7 of each 100.000 women in ages between 45-64 years old, 27 in ages between 65-74 and 160 in ages above 74. For men, the similar mortality rate showed 12 of each 100.000 in ages between 45-64, 54 in ages between 65-74 respectively 213 for those above 74 (2).

16. Molecules involved in diabetes risk and progression

There are several studies searching for biomarkers of DM risk and DM complications. Among the outstanding molecules: vitamins, minerals, homocysteine (Hcys) and oxidative stress metabolic by products, have extensively been reviewed in relation to DM and the diabetic course (51-53). In upcoming paragraphs we will study different vitamins of importance to the project carried out.

16.1. Vitamins

There are well-documented complications caused by DM2, which is affecting a remarkable proportion of the world's population. DM2 is considered to be one of world's most chronic diseases (54).

16.1.1. Vitamin B12

This vitamin, also known as cobalamin, is a water-soluble vitamin with a key role in the normal functioning of the brain and nervous system, and for the formation of blood. It is one of the eight B vitamins. It is normally involved in the metabolism of every cell of the human body, especially affecting DNA synthesis and regulation, but also fatty acid metabolism and amino acid metabolism (55). The effective use of vitamin B supplements, in general, has been described for prevention of cardiovascular disease in individuals with kidney disorders (56). Vitamin B12 is known to be essential in maintaining the general integrity of the vascular system, among others B vitamins. It is known that individuals using the drug metformin, may reduce their serum FA and vitamin B12 levels which substantially increases the risk of B12 deficiency, which in turn might be an independent risk factor for cardiovascular disease, especially among individuals with DM2 (57).

16.1.2. Vitamin B9 (Folate, folic acid)

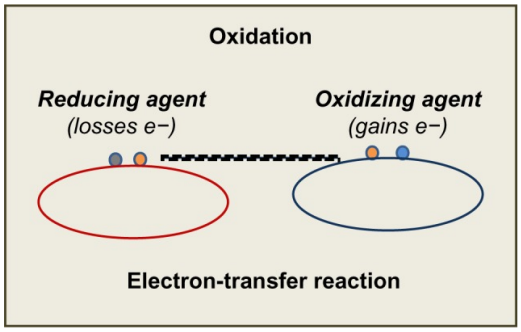
Folate, also known as folic acid, vitamin B9 and vitamin M (58), is as important as vitamin B12 in the maintenance of the overall integrity of the vascular system. The human body needs folate to synthesize DNA, repair DNA, and methylate DNA as well as to act as a cofactor in certain biological reactions (59) We all require folate to produce healthy red blood cells and prevent anaemia (60). Folate, together with B12, contributes requisite absorption of essential nutrients. It has also been shown that individuals suffering from diabetic peripheral neuropathy have had significant improvements from their symptoms (61).

16.2. Homocysteine

Hcys is a non-protein amino acid. It is a homologue of the amino acid cysteine, differing by an additional methylene bridge. It is biosynthesized from methionine by the removal of its terminal Cε methyl group. Hcys can be recycled into methionine or converted into cysteine with the aid of certain B-vitamins. A high level of Hcys in the blood (hyperhomocysteinemia) makes a person more prone to endothelial cell injury, which leads to inflammation in the blood vessels, which in turn may lead to atherogenesis, which can result in ischemic injury (62). Hyperhomocysteinemia is strongly associated with increased risk of cardiovascular disease (53). Coronary artery disease occurs when an atherosclerotic plaque blocks blood flow to the coronary arteries, which supply the heart with oxygenated blood. Hcys has been associated to cardiovascular diseases and atherovascular plaques in the arteries such as the complications mentioned earlier in DM2 patients affected by macroangiopathies.

16.3. Oxidative stress biomarkers

In a cell, different stress factors might cause a chemical reaction involving the loss of electrons or an increase in the oxidation state. This is more commonly known as oxidation. Although oxidation reactions are crucial for life, they can also be damaging if it exceeds from the physiological levels. The oxidation reactions can produce free radicals, which in turn could start a chain reaction that could be hazardous to the cell and could cause damage/death to the cell.



(Figure 8) Oxidation process (Extracted from Pinazo-Durán et al., Clin Inter Aging 2014) (63)

Furthermore, the chain reaction of lipid peroxidation was initiated by the superoxide anion ($\mathbf{O_2^{\cdot-}}$) that in the presence of hydrogen peroxide triggers the formation of hydroxyl radical ($OH^{\cdot}$) the most damaging to cells and tissues. The following reactions were set up by Haber-Weiss and Fenton to explain the generation of free radicals in the presence of oxygen, hydrogen peroxide and iron.

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

Antioxidant defences stop terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves. An insufficient level of antioxidants, or inhibition of the antioxidant enzyme, causes oxidative stress. Oxidative stress is the result of the imbalance between the pro-oxidants and antioxidants leading to damage to cell structure and cell function by overly reactive oxygen species (ROS) and chronic excessive inflammation. Oxidative stress seems to play a significant role in many human diseases, such as diabetes (64, 65). In many of these cases, it is unclear if oxidants trigger the disease, or if they are produced as a secondary consequence of the disease and from general tissue damage (66). Antioxidants are molecules that inhibit the oxidation of other molecules. There is increased evidence that these complications induce metabolic derangements leading to oxidative complications and stress. Vast investigation has been done the past decades in this field, in the development of diabetes complications (67, 68). Stimulation and imbalance between production and elimination of ROS and reactive nitrogen species (RNS) (66) is a common cause of diabetic hyperglycaemia. Oxidative stress is associated with increased production of ROS or with a significant decrease in the effectiveness of antioxidant defences (69). As mentioned before, complications from micro- and macroangiopathies, such as DR, nephropathy, neuropathy, atherosclerosis and cardiovascular disease, all have a significant role in the oxidative and nitrosative stress processes. Hyperglycaemia can stimulate an imbalance between production and elimination of reactive oxygen species (ROS) and reactive nitrogen species (66, 70, 71).

Up today there is general agreement that ROS are involved in the physical, biochemical, molecular, and pathological changes associated with aging. Oxidative damage to lipids, proteins, and nucleic acids

accumulates and increases with age, whereas the antioxidant mechanisms decrease in parallel. These facts are closely related to age-related diseases as glaucoma, age macular degeneration or DR.

17. Genes involved in diabetes risk and progression

17.1. Genetic analysis and expressions

Genetic analysis is the overall process of studying and researching in fields of science that involve genetics and molecular biology. There are a number of applications that are developed from this research and these are also considered parts of the process. The base system of analysis revolves around general genetics. Basic studies include identification of genes and inherited disorders. This research has been conducted for centuries on both a large-scale physical observation basis and on a more microscopic scale. Genetic analysis can be used generally to describe methods both used in and resulting from the sciences of genetics and molecular biology, or to applications resulting from this research. Genetic analysis may be done to identify inherited disorders, to make a differential diagnosis in certain somatic diseases, and to identify subjects in high risk for developing diseases. Genetic expression is the process by which genes are transcribed to messengers RNAs (mRNAs). Changes in the nucleotide sequence of the genome could lead to mutations, which result from errors during DNA replication or other types of DNA damage. When the genetic alteration affects to only one nucleotide, this definition could be also used for single nucleotide polymorphism (SNP) (29). The difference between mutation and polymorphism is based on allelic frequencies. It is a polymorphism when the allelic frequency for the minor allele is more than 1%. A polymorphism is a population attribute. Genetic polymorphism promotes diversity within a population. A gene is said to be polymorphic if more than one allele occupies that

gene's locus within a population (13). Polymorphisms of DNA repair genes might be linked to distinct mutation rate, which is a genomic trait. Mutation can be neutral, deleterious, or beneficial, in terms of the fitness of the corresponding phenotype, yet such a classification is only meaningful in an evolving population, i.e., invoking the concept of polymorphism, which itself does not have any functional implications. The genomic distribution of SNPs is not homogenous; SNPs occur in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and "fixing" the allele (eliminating other variants) of the SNP that constitutes the most favourable genetic adaptation (72). Other factors, like genetic recombination and mutation rate, can also determine SNP density (73). Within a population, SNPs can be assigned a minor allele frequency. This is simply the lesser of the two-allele frequencies for SNPs. There are variations between human populations, so a SNP allele that is common in one geographical or ethnic group may be much rare in another. These genetic variations between individuals (particularly in non-coding parts of the genome) are sometimes exploited in DNA fingerprinting, which is used in forensic science. Also, these genetic variations underlie differences in our susceptibility to disease. The severity of illness and the way our body responds to treatments are also manifestations of genetic variations. There are several rare types of diabetes caused by a mutation in one gene. In this sense, Garin et al. reported that recessive mutations in the human insulin gene (74) result in permanent neonatal diabetes (74). However, diabetes is a complex disease with many genes related to the risk of its onset and progression.

17.2. Genes

Some candidate genes have been linked to DM and DR. A selection of the most relevant genes is enclosed below:

17.2.1. TP53

Tumour proteins p53, also known as transformation-related protein 53 (TRP53), are encoded by homologous genes in various organisms such as TP53. This homolog is crucial in multicellular organisms, where it prevents cancer formation, thus, functions as a tumour suppressor (75). As such, p53 has been described as "the guardian of the genome" because of its role in conserving stability by preventing genome mutation (76). Hence TP53 is classified as a tumour suppressor gene (76-81). The name p53 was given in 1979 describing a protein mass of 53 kDa. However recent techniques and sequencing of the human genome enabled to establish that the human TP53 gene encodes not only one, but at least 12 proteins, which are ranging from 28 to 53 kDa. All these p53 proteins are called the p53 isoforms (76). The International Cancer Genome Consortium has established that the TP53 gene is the most frequently mutated gene (>50%) in human cancer, indicating that the TP53 gene plays a crucial role in preventing cancer formation (76). TP53 gene encodes proteins that bind to DNA and regulate gene expression to prevent mutations of the genome (82).

17.2.1.1. TP53 in diabetes mellitus type 2

In the context of tumour suppression, p53 is an undisputedly critical protein. Functioning primarily as a transcription factor, p53 helps fend off the initiation and progression of tumours by inducing cell cycle arrest, senescence or programmed cell death (apoptosis) in cells at the earliest stages of precancerous

development. Compelling evidence, however, suggests that p53 is involved in other aspects of human physiology, including metabolism. Indeed, recent studies suggest that p53 plays a significant role in the development of metabolic diseases, including diabetes, and further that p53's role in metabolism may also be consequential to tumour suppression (83). Kung et al. (84), studied genetic variants in the TP53 gene of a humanized knock-in mouse model and showed that the R72P polymorphism of this gene had a significant impact on the metabolic response to a high-fat diet, demonstrating an association between TP53 polymorphisms and metabolism (84). This research group carried out another study using the same mouse model and found that R72 mice developed insulin resistance, islet hypertrophy, increased infiltration of immune cells, and fatty liver disease. Also, they observed more-severe obesity and glucose intolerance in R72 mice compared to P72 mice, suggesting that this SNP predisposes to obesity and metabolic dysfunction (85). Scientists have found an association between Arg72Pro (R72P) polymorphism in the TP53 gene and DM1 (86) but the association between this gene and DM2 is still not clear. Gloria-Bottini et al. did not found any relation between TP53 gene and DM2 (87) whereas Gaulton et al. reported a significant association between the gene and this disease (29).

17.2.2. TMCO1

Transmembrane and coiled-coil domain-containing protein 1 is a protein that in humans is encoded by the TMCO1 gene. This locus encodes a transmembrane protein. Mutations at this locus have been associated with craniofacial dysmorphism, skeletal anomalies, and

mental retardation. Mutations at this locus have also been associated with open angle glaucoma blindness. Alternatively, spliced transcript variants have been described (88).

17.2.2.1. TMCO1 in diabetes mellitus type 2

This gene has been involved in ocular diseases. However, its role in DM and the diabetic eye needs further research in human and experimental animals.

17.2.3. SLC23A2

Solute carrier family 23 member 2 is a protein that in humans is encoded by the SLC23A2 gene (89, 90). The absorption of vitamin C into the body and its distribution to organs requires two sodium-dependent vitamin C transporters. This gene encodes one of the two required transporters and the encoded protein accounts for tissue-specific uptake of vitamin C. Previously, this gene had an official symbol of SLC23A1. The solute carrier (91) group of membrane transport proteins include over 300 members organized into 52 families (91). Most members of the SLC group are located in the cell membrane. The SLC gene nomenclature system was originally proposed by the HUGO Gene Nomenclature Committee (HGNC) and is the basis for the official HGNC names of the genes that encode these transporters. A more general transmembrane transporter classification can be found in TCDB database. Solutes that are transported by the various SLC group members are extraordinarily diverse and include both charged and uncharged organic molecules as well as inorganic ions and the gas ammonia. As is typical of integral membrane proteins, SLCs contain a number of hydrophobic transmembrane alpha helices connected to each other by hydrophilic intra- and extra-cellular loops. Depending on

the SLC, these transporters are functional as either monomers or obligate homo- or hetero-oligomers.

17.2.3.1. SLC23A2 function in diabetes mellitus type 2

Analyses have shown that there were significant differences in the vitamin C consumption in energy intake, activity level, dietary fiber intake, nutritional supplementation status, drinking or not drinking, education level among the different vitamin C intake groups. There were also significant differences in age, sex, body mass index (BMI), smoking status and vitamin C intake between the DM2 group and the non-diabetes group, suggesting that there is a significant negative correlation between the dietary vitamin C intake and the risk of DM2 (92).

17.2.4. MMACHC

The exact function of the protein encoded by this gene is not known, however, its C-terminal region shows similarity to TonB, a bacterial protein involved in energy transduction for cobalamin (vitamin B12) uptake. It has been shown that this gene may be involved in in the binding and intracellular trafficking of cobalamin, and it is demonstrated that metformin treated diabetic patients have a higher prevalence of cobalamin deficiency (93). Hence, it is postulated that this protein may have a role in the binding and intracellular trafficking of cobalamin. Mutations in this gene are associated with methylmalonicaciduria and homocystinuria type cblC. Methylmalonicaciduria and homocystinuria type C protein also known as MMACHC is a protein that in humans is encoded by the MMACHC gene (94). The C-terminal region of the product of the MMACHC gene is similar to TonB, a bacterial protein involved in energy transduction for cobalamin uptake (94).

17.2.5. MMADHC

Methylmalonicaciduria and homocystinuria type D protein, mitochondrial also known as MMADHC is a protein that in humans is encoded by the MMADHC gene (95). This gene encodes a mitochondrial protein that is involved in an early step of vitamin B12 metabolism. Vitamin B12 (cobalamin) is essential for normal development and survival in humans (96). Mutations in this gene cause methylmalonicaciduria and homocystinuria type cblD (MMADHC), a disorder of cobalamin metabolism that is characterized by decreased levels of the coenzymes adenosylcobalamin and methylcobalamin (95).

17.2.5.1. MMADHC and MMACHC (vitamin B12) function in diabetes mellitus type 2

Several cross sectional studies (97-99) and case reports (34, 100, 101) have documented an increased frequency of vitamin B12 deficiency among DM2 patients.

17.2.6. MMP9

Proteases of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, angiogenesis, bone development, wound healing, cell migration, learning and memory, as well as in pathological processes, such as arthritis, intracerebral haemorrhage (102) and metastasis (103). Most MMPs are secreted as inactive proproteins, which are activated when cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type IV and V collagens and other extracellular matrix proteins (102). MMP9 is greatly up regulated during human respiratory epithelial healing (104). Using a

MMP9 deficient mouse model, it was seen that MMP9 coordinated epithelial wound repair and deficient mice were unable to remove the fibrinogen matrix during wound healing (105). When interacting with TGF-ß1, Gelatinase B also stimulates collagen contraction, aiding in wound closure (106).

17.2.6.1. MMP9 function in diabetes mellitus type 2

Coordinated extracellular matrix deposition is a prerequisite for proper wound healing, which is mainly orchestrated by MMPs. Diabetic wounds generally show compromised, wound healing cascade and abnormal MMP9 concentration is one of the cause. MMP9 gene promoter may result in altered expression of MMP9 in wounds of DM2 cases resulting into non-healing chronic ulcers in them (107). In addition, several studies show an association between MMP9 polymorphisms and diabetes. In example, Feng et al. found an interesting association between the MMP9 -1562C/T polymorphism and diabetic nephropathy in a Chinese population (108) being supported these results by other recent study carried out by Zhang et al. (109).

HYPOTHESIS AND OBJECTIVES

1. **Hypothesis**

DR is one of the most important causes of vision loss and quality of life related to vision worldwide. Early diagnosis is essential to promote visual prognosis. It consists not only in detecting loss of visual function, but also in defining the extent and depth of the lesion and its etiopathogenic characteristics, for a better diagnostic and therapeutic approach. However, practice indicates that subjective and objective ophthalmological manifestations appear on many occasions when the lesion is already at an advanced stage. The new advances in biomedicine and biotechnology and the development of computer science have favoured the emergence of new exploratory techniques, many of which have been a great help to the current ones, especially in relation to retinal diseases. Among these techniques, the analysis of the genetic expression, in relation to the oxidative stress and antioxidant defences stand out. In addition, the new disciplines, nutrigenetics and nutrigenomics, are subject to continuous advances reason why they are more and more useful in ophthalmology and sciences of the vision. Although DR and diabetic macular edema currently only have a palliative treatment that tries to prevent or slow vision loss, different diagnostic and therapeutic approaches are needed to combat diabetic blindness and its consequences for patients, families and the elderly society. We propose a multicentral study (Valencia Study) to analyse several clinical, biochemical, molecular and genetic aspects about DR, among them this subproject that aims to analyse the role of protein transport proteins of vitamins and molecules related to vascular risk with the availability of antioxidants and their implications for the presentation or progression of DR.

2. Objectives

2.1. General:

To study the relationship between plasma concentrations of folic acid, vitamin B12, Hcys, MDA, total TAS, GPx and the expression of genes related to these molecules in DM2 patients, and to analyse their correlation with the presentation and progression of DR in a Mediterranean population.

2.2. Specifics:

1. To explore the visual acuity and ocular fundus of the diabetic patients to characterize their DR.

2. To analyse plasma concentrations of folic acid, vitamin B12 and Hcys in DM2 patients.

3. To determine the expression of the following genes: SLC23A2, TP53, RBP1, MMP9, MMACHC, MMADHC, TMCO1 and THRA.

4. To integrate the results of the clinic ophthalmology, biochemistry and genetics to establish a relationship between the ingestion and availability of these molecules. Including the expression of genes and the possible variations of these in relation to DM2. In order to clarify, if there is a need or not, for antioxidant supplementation.

5. To establish a new protocol and evaluate the need for micronutritional and antioxidant supplementation in DM2 patients, and to curb the presentation or progression of DR and loss of vision.

MATERIALS AND METHODS

1. Study design and participants

We carried out a case-control study matched by age and gender, involving 81 participants recruited from June to December 2012 at the University and Polytechnic Hospital La Fe, Dr. Peset University Hospital and Rahhal Clinic, according with the inclusion/exclusion criteria (Table 3).

The sample size was calculated using the eNe 2.0 statistical program (GlaxoSmithKline S.A.) to get a statistical power of 80% and detect differences in the hypothesis contrast (Ho: p1=p2) using a bilateral $\chi 2$ test for two independent samples, considering that the level of significance would be 5%.

All tests and study procedures were carried out in accordance with the Helsinki Declaration on Human Experimentation (Helsinki 1964, updated version 2004) and are in line with the current regulations for this type of studies in the European Community.

The participants were informed about the details of the study and researchers explained to them the details of the study, giving them all details in a written document. Once they decided to participate, the informed consent was signed. Likewise, approval was obtained from the Ethics and Research Committees of the Dr. Peset University Hospital.

The following table shows the inclusion/exclusion criteria for the selection of participants:

INCLUSION CRITERIA	
DM2 PATIENTS	**CONTROLS**
Age from 25 to 85 years	Age from 25 to 85 years
Suffer from DM2 at least 5 years ago	Not having diabetes (type 1 or 2)
Not suffering from systemic disease	Not suffering from systemic disease
EXCLUSION CRITERIA	
DM2 PATIENTS	**CONTROLS**
Age <25 or >85 years	Age <25 or >85 years
To have DM type 1	To have diabetes (type 1 or 2)
Having any other eye disease that may interfere with the study	Having any other eye disease that may interfere with the study

(Table 3) Criteria for selecting the study participants

The participants selected were classified into 2 groups:

1. Patients with DM2 (n=49)
2. Healthy controls (CTRL, n=32)

Similarly, the DM2 group was subdivided into 2 other groups:

1. Patients without DR (DM2-DR, n=35)
2. Patients with DR (DM2+DR, n=14)

Once an individual accepted to participate in the study, he/she was programmed for the ophthalmic examination and blood sample extraction.

From each participant, 3 blood samples were extracted. One for the gene expression analysis, another one for several biochemical parameters and oxidative stress markers analysis and the last one for a simple blood analysis. This blood analysis included haematocrit, glycosylated haemoglobin, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and folic acid, and was carried out at the department of Clinical Analysis of the centres participating in the study.

The biochemical parameters and oxidative stress markers were analysed in plasma. For doing this, the blood sample was centrifuged at 2000 rpm during 10 minutes at 4ºC. Then, the plasma was collected and aliquoted into cryotubes and stored at -80ºC.

2. Ophthalmological exploration

All patients underwent an ophthalmic examination of both eyes, which included:

- Best corrected visual acuity (BCVA), with Snellen optotypes and expressed according to the decimal logarithm of angular size in minutes of arc (logMAR).

- Complete biomicroscopy in slit lamp discarding ocular associated pathology and verifying that there was no contraindication for pupillary dilatation.

- Goldmann applanation tonometer for intraocular pressure (IOP), estimated in millimetres of mercury (mmHg).

- Funduscopy of the seven fields of DIABETIC. The assessment of the eye fund was carried out with slit lamp and lens of 78 Diopters. In addition, retinographies were taken from each patient with TOPCON ImageNet TRC-50JA.

- The macular morphometric study was performed using spectral domain optical coherence tomography (HD-Cirrus, Carl Zeiss Meditec, Dublin, CA)

The absence or presence of DR according to the characteristic funduscopic lesions was defined based on the DIABETIC study group (110): microaneurysms, haemorrhages, hard exudates, soft or cottony exudates, dilatation or venous tortuosity, intraretinal microvascular abnormalities (IRMAS) and/or neovascularization. Depending on these signs, we classify DR patients according to the early treatment diabetic retinopathy study (ETDRS).

3. Study of classic biomechanical parameters

The biochemical parameters assayed were: total protein concentration, CRP, vitamin B12 and Hcys.

All these parameters were assayed by duplicate.

3.1. Total protein concentration

Total protein determination was performed using the bicinchoninic acid (BCA) technique, using the Bicinchoninic Acid Protein Assay Kit (BCA1 and B9643, Sigma-Aldrich). It is a method similar to that of Lowry (111), and is based on the formation, under alkalinity conditions, of a Cu^{2+}-protein complex, followed by a reduction of Cu^{2+} to Cu^{1+}. Then, the BCA reacts with the reduced copper to form a compound that absorbs light at 560 nm, which can be measured by spectrophotometry (112-117).

The kit includes the following reagents:

- Reagent A: contains BCA, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1N sodium hydroxide (pH = 11.25)
- Reagent B: contains 4% (w/v) copper (II) sulphate pentahydrate.
- Protein Standard: 1.0 mg/mL BSA (bovine serum albumin) in 0.15M sodium chloride, with 0.05% sodium acid as preservative.

From the protein standard, we must prepare serial dilutions of different concentrations of BSA. The way to prepare these standards is shown in the following table.

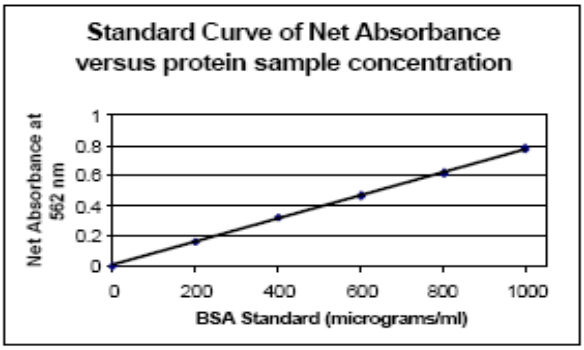
#	(BSA) µg/mL	BSA Volume (mL)	H_2O Volume (mL)
1	1000	75	0
2	800	60	15
3	600	45	30
4	400	30	45
5	200	15	60
6	0	0	75

(Table 4) Preparation of BSA standard.

In the same way, we must prepare a solution with reagents A and B, mixing 50 parts of reagent A with 1 part of reagent B. Once the solution A + B and the standards are prepared, we carry out the analysis, on a 96-well plate, as follows:

- Add 25 µL standard/sample to the corresponding wells.
- Add 200 µL of solution A+B to each well. The relationship between this volume and the volume of the previous step must be 8:1.
- Incubate 1 hour at 60°C.
- Measure the absorbance at 562 nm (545-590 nm filters can be used).

We draw the standard curve (absorbance vs. concentration of BSA, figure 9) and, from this, we will calculate the total protein concentration in each of the samples, considering the dilution factor (in case we have had to dilute the sample).



(Figure 9) Standard curve example of the bicinchoninic acid assay for protein analysis. Taken from the booklet included in the SIGMA kit, code BCA1 and B9643.

3.2. C reactive protein

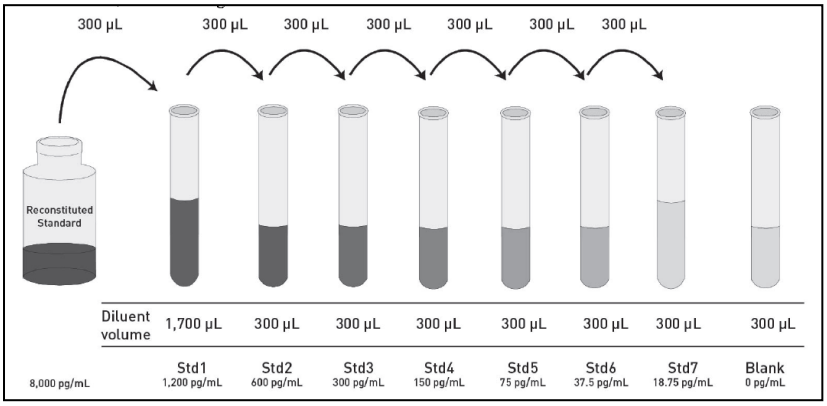
The determination of plasma levels of CRP was performed using the CRP Human ELISA Kit (KHA0031, Thermo Fisher).

The reagents included in the kit were:

- Hu CRP Standard
- Standard Diluent Buffer
- Antibody Coated Wells
- Hu CRP Biotin Conjugate
- Streptavidin-HRP (100X)
- Streptavidin HRP Diluent
- Wash Buffer Concentrate (25X)
- Stabilized Chromogen (tetramethylbenzidine – TMB)
- Stop Solution

To prepare the standard curve, we followed the manual as follows:

- Reconstitute Hu CRP Standard to 8000 pg/mL with Standard Diluent Buffer.
- Add 300µL of reconstituted standard to a tube containing 1700µL of Standard Diluent Buffer, mix and label as 1200 pg/mL (STD1).
- Follow the serial dilutions as is showed in the next figure:



(Figure 10) Preparation of the standard curve, obtained from the manual of the kit.

Before starting with the protocol, we also have to prepare the samples. Human plasma requires, at least, a 3000–fold dilution in the Standard Diluent Buffer.

Once the standard and samples are prepared, we proceed with the assay:

- Add 100µL of Standard Diluent Buffer to the zero standard wells. The wells reserved for the chromogen blank should be left empty.
- Add 100µL of standards and diluted samples to the corresponding wells.
- Cover the plate and incubate for 2 hours at 37ªC.
- Remove the solution and wash the wells 4 times with diluted Wash Buffer.
- Add 100µL Hu CRP Biotin Conjugate to all wells, except those for the chromogen blanks.

- Cover the plate and incubate for 1 hour at RT.
- Remove the solution and wash the wells 4 times with diluted Wash Buffer.
- Add 100µL of 1X Streptavidin-HRP into each well, except chromogen blanks.
- Cover the plate and incubate for 30 minutes at RT.
- Remove the solution and wash the wells 4 times with diluted Wash Buffer.
- Add 100µL of Stabilized Chromogen to each well (the substrate solution will begin to turn blue).
- Incubate 30 minutes at RT in dark.
- Add 100µL of Stop Solution to each well (the colour will change from blue to yellow)
- Read the absorbance at 450nm within 30 minutes after adding the stop solution.

3.3. Vitamin B12

The plasma determination of this vitamin was carried out by means of the Vitamin B12 ELISA Kit (KA1170, Abnova).

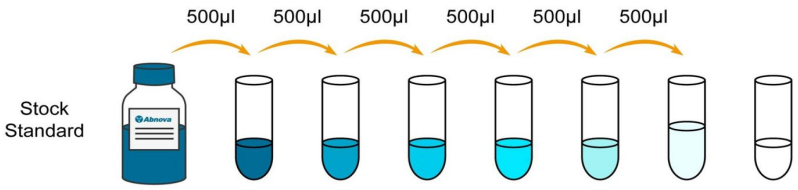
The microtiter plate provided in this kit has been pre-coated with an antibody specific to Vitamin B12. During the reaction, Vitamin B12 in the sample or standard competes with a fixed amount of biotin-labelled Vitamin B12 for sites on a pre-coated Monoclonal antibody specific to Vitamin B12. Excess conjugate and unbound sample or standard are washed from the plate. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and

the colour change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of Vitamin B12 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

To make the experiments, the reagents used were:

1. Stock Standard
2. Sample Diluent
3. Assay Diluent (A and B)
4. Detection Reagent (A and B)
5. Wash Buffer
6. Substrate Solution
7. Stop Solution

The first step was to prepare the standard curve. The Stock Standard was diluted with Sample Diluent in order to produce a solution of 1000 pmol/L. Then, this solution was used for making serial dilutions with Sample Diluent, which serves as the zero standard (0 pmol/L).



Standard	A	B	C	D	E	F	G	H
pmol/L	1000	500	250	125	62.5	31.2	1.56	0

The assay procedure was as follows:

- Add 50 µl of Standard, Blank, or Sample per well.
- Immediately add 50 µl of Detection Reagent A working solution to each well. Cover with the Plate sealer. Tap the plate and incubate for 1 hour at 37°C.
- Aspirate each well and wash (total 3 washes) by filling each well with 400µL of Wash Buffer. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
- Add 100 µL of Detection Reagent B working solution to each well. Cover with a new plate sealer and incubate for 45 minutes at 37°C.
- Repeat the aspiration/wash process for five times as conducted in step 3.
- Add 90 µL of Substrate Solution to each well. Cover with a new plate sealer and incubate within 15-30 minutes at 37°C. Important: protect from light.
- Add 50 µL of Stop Solution to each well.

Measure the optical density of each well at once, using a microplate reader set to 450 nm.

In order to calculate the vitamin B12 concentration in samples, we had to create a standard curve using computer software capable of generating a four-parameter logistic (4-PL) curve-fit.

3.4. Homocysteine

The determination of plasma levels of Hcys was performed using the Hcys ELISA Kit (STA-670, Cell Biolabs) and the reagents included in the kit were:

- Anti-Hcys Antibody.
- Secondary Antibody, HRP Conjugate.
- Assay Diluent (Part No. 310804): One 50 mL bottle.
- 10X Wash Buffer.
- Hcys Conjugate.
- Hcys -BSA Standard.
- Substrate Solution.
- Stop Solution.

To prepare the standard curve we followed the manual from the kit. See table 5

Standard Tubes	4 mg/mL Homocysteine-BSA Standard (µL)	Assay Diluent (µL)	Homocysteine-BSA (µg/mL)
1	4	396	40
2	100 of Tube #1	300	10
3	100 of Tube #2	300	2.5
4	100 of Tube #3	300	0.625
5	100 of Tube #4	300	0.156
6	100 of Tube #5	300	0.039
7	100 of Tube #6	300	0.010
8	0	300	0

(Table 5) Standard curve preparation for homocysteine assay

After the standard preparation, we proceeded with the assay as follows:

- Remove the Assay Diluent from the plate and add 50 µL of unknown sample or standard to the Hcys Conjugate Coated Plate. Incubate at RT for 10 minutes on an orbital shaker.
- Add 50 µL of diluted Anti- Hcys Antibody to each well. Incubate at RT for 1 hour on an orbital shaker.
- Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After each wash, empty wells and tap microwell strips on absorbent paper towel to remove excess 1X Wash Buffer.
- Add 100 µL of the diluted Secondary Antibody, HRP Conjugate to each well. Incubate at RT for 1 hour on an orbital shaker.

- During this incubation, warm Substrate Solution to RT.
- Wash the strip wells 3 times according to step 5 above. Proceed immediately to the next step.
- Add 100 µL of Substrate Solution to each well, including the blank wells. Incubate at RT on an orbital shaker.
- Stop the enzyme reaction by adding 100 µL of Stop Solution into each well, including the blank wells. Results should be read immediately (colour will fade over time).
- Read absorbance on a spectrophotometer using 450 nm as the primary wave length.

4. Oxidative stress

We analysed the plasma concentration of MDA, marker of oxidative damage), plasma levels of GPx (GSH, an antioxidant enzyme) and the plasma Total TAS.

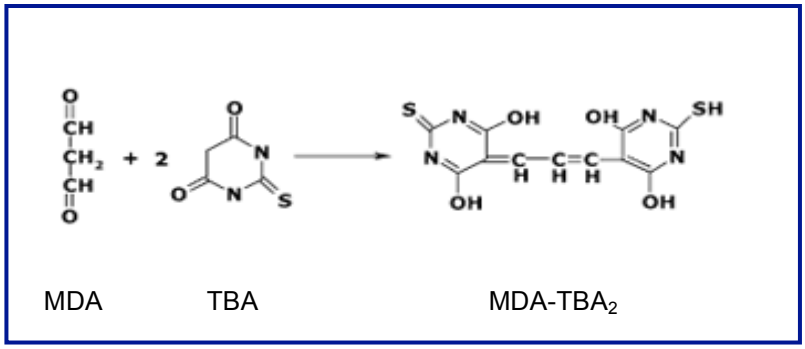
All samples were assayed by duplicate.

4.1. Determination of lipoperoxidative activity (MDA/TBARS)

The measurement of MDA as a product of lipid peroxidation is carried out following the technique of thiobarbituric acid and its reactive species (TBARS) (118-120).

The aldehyde group of MDA reacts with two molecules of thiobarbituric acid (TBA) at acidic pH and high temperature (approximately 100°C) to form a pink complex, which is extracted with butanol. The fluorescence

of this complex is measured and compared with the standard ones (standard curve) to calculate the MDA concentration in samples.



(Figure 11) Reaction between malondialdehyde and thiobarbituric acid. Source: Northwest Life Science Specialties L.L.C

The reagents used were:

- MDA (1,1,3,3-Tetraethoxypropane)
- Phosphate buffered saline (PBS)
- Sodium Dodecil Sulfate (SDS)
- Chlorhydric acid (HCl)
- Phosphotungstic acid
- Thiobarbituric acid (TBA)
- N-butanol

First, we had to prepare the standards, following the indications showed in the next table:

	MDA (µM)	**V_{MDA} (µL)**	**V_{PBS} (µL)***
P1	**0**	**0**	**60**
P2	1	30	30
P3	3	9	51
P4	5	15	45
P5	10	30	30

* 0.01M PBS

(Table 6) Preparation of standard curve

After this, we proceed as follows:

- Add 250µL of standard / diluted sample into the appropriate tubes.
- Add 125 µL of PBS buffer.
- Add 100µL of 7% SDS.
- Add 1mL of 0.1N HCl.
- Add 150µL of 1% phosphotungstic acid.
- Add 500µL of 0.67% TBA.
- Shake the tubes and boil in the bath for 1 hour at 98°C (protect from light).

- Cool the tubes with ice for about 10 minutes.
- Add 1mL of n-butanol.
- Centrifuge at 2800 rpm for 10 min at room temperature (RT).
- Add 200µL of the supernatant to the appropriate well of the plate.
- Measure the fluorescence at 544nm excitation and 590nm emission, using the Fluoroskan Ascent FL (Termo Electron Corp., Waltham, MA, USA).

4.2. Determination of total antioxidant status (TAS)

The plasma levels of TAS was determined using the Antioxidant Assay Kit (709001, Cayman Chemical), which relies on the ability of antioxidants to inhibit the oxidation of 2,2'-Azino-di-3-ethylbenzthiazoline sulphonate (ABTS) to $ABTS^{\cdot+}$ by metmyoglobin. The amount produced of this cation is inversely proportional to the antioxidants present in samples, and it can be monitored by reading the absorbance at 750 nm.

The reagents needed for the determination of TAS are:

- Phosphate buffered saline (PBS).
- ABTS® chromogen (2,2 azino-bis-(3-etilbenzotiazolina-6-sulfonato))
- Metmyoglobin
- Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid).
- Substrate (hydrogen peroxyde - H_20_2)

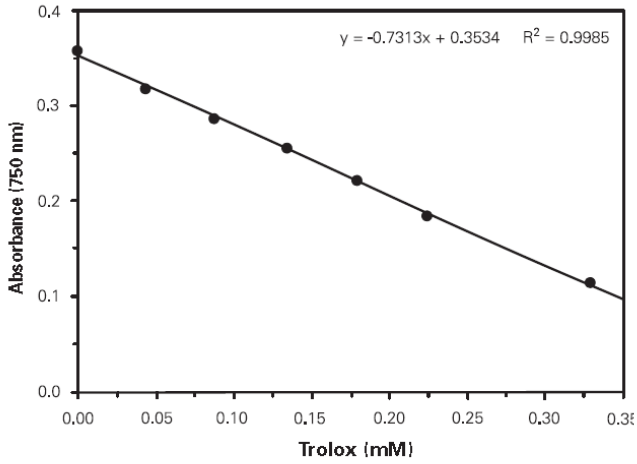
First, we had to prepare the standard curve, following the instructions of the manual.

Standard (STD)	Trolox (µL)	Assay Buffer (µL)	Final conc. (mM Trolox)
A	0	1000	0
B	30	970	0.044
C	60	940	0.088
D	90	910	0.135
E	120	880	0.180
F	150	850	0.225
G	220	780	0.330

(Table 7) Standard preparation for determining the total antioxidant status

Once standards and samples are ready to be processes, the procedure is as follows:

1. Add 10µL of standard / sample to the corresponding well
2. Add 10µL of metmyoglobin and 150µL of chromogen per well.
3. Initiate the reactions by adding 40µL of H_2O_2 working solution to the wells, as quickly as possible.
4. Cover the plate and incubate on a shaker for 5 minutes at RT.
5. Remove cover and read absorbance at 750nm.



(Figure 12) Example of standard curve for total antioxidant status assay. Source: manual of Antioxidant Assay Kit (709001, Cayman Chemical)

4.3. Determination of glutathione peroxidase activity (GPx)

The determination of the GPx activity was performed using the GPx Assay Kit (703102, Cayman Chemical), which measures the GPx activity indirectly by a coupled reaction with glutathione reductase (GR):

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

This process is accompanied by a decreasing in absorbance at 340nm.

The reagents used in this protocol were:

1. GPx Assay Buffer (10X)
2. GPx Sample Buffer (10X)
3. Glutathione Peroxidase (positive control)
4. GPx Co-Substrate Mixture
5. GPx Cumene Hydroperoxide

The next steps to determine the GPx activity were:

1. Add 120µL of Assay Buffer and 50µL of Co-Sustrate Mixture to the background wells.
2. Add 100µL of Assay Buffer, 50µL of Co-Sustrate Mixture and 20µL of diluted GPx (control) to the positive control wells.
3. Add 100µL of Assay Buffer, 50µL of Co-Sustrate Mixture and 20µL of diluted GPx (control) to the sample wells.
4. Add 20µL of Cumene Hydroperoxide to all the wells and shake the plate carefully during a few seconds.
5. Read the absorbance on every minute at 340nm to obtain at least 5 time points.

After reading the absorbance, use the next equation to calculate the change in absorbance per minute:

$$\textbf{GPx activity} = \frac{\Delta A_{340}/\text{min.}}{0{,}00373\ \mu M^{-1}} \times \frac{0{,}19\ \text{ml}}{0{,}02\ \text{ml}} \times \textbf{sample dilution} = \text{nmol/min/ml}$$

Determine the rate ΔA_{340}/min for the background, subtract this rate from that of the sample wells and use the following formula to calculate the GPx activity:

$$\Delta A_{340}/\text{min.} = \frac{* \left| A340\ (\text{Time 2}) - A340\ (\text{Time 1}) \right|}{\text{Time 2 (min.)} - \text{Time 1 (min.)}}$$

5. Gene expression analysis

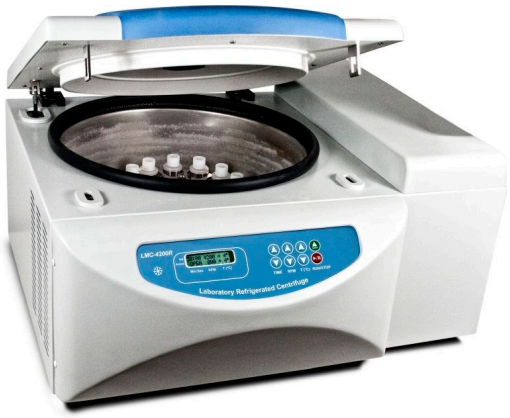
We studied the expression of 8 genes: SLC23A2, TP53, RBP1, MMP9, MMACHC, MMADHC, TMCO1, THRA.

The gene expression analysis was carried out in the laboratory of the department of Preventive Medicine & Public Health of the School of Medicine (University of Valencia).

5.1. Extraction and quantification of RNA

1. Let the blood tubes rest between 60-120 minutes, so that the serum is separated by sedimentation.
2. Transfer the serum to a 15 mL sterile plastic tube, avoiding to take anything from the interface.
3. Centrifuge at 1500-2000 rpm for 15 minutes.

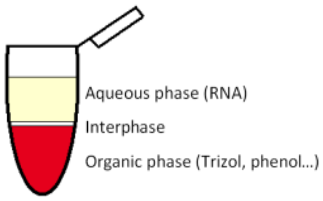
4. Remove the supernatant by decantation; fill the tube with PBS and vortex to resuspend the pellet.
5. Centrifuge at 1500-2000 rpm for 15 minutes.
6. Remove the supernatant by decantation and add 500-1000 µL of Trizol, depending on the size of the pellet.
7. Mix well with the vortex until the pellet dissolves completely in the Trizol.
8. Transfer by decantation to a 1.5 mL eppendorf tube.
9. Freeze the tubes at -80 °C and continue the protocol the next day.



(Figure 13) Centrifuge used for RNA extraction.

1. Homogenize the samples, once thawed, to ensure the rupture of the cells.
2. Transfer to a 2 mL tube.
3. Add 200 µl of chloroform if we started with 1000 µL of trizol (100 µL if we did with 500 µL of trizol).

4. Mix by inversion.
5. Let stand for 2-3 minutes.
6. Centrifuge at 13000 rpm for 15 minutes. When the centrifuge is finished, we can observe three phases in the tubes:



(Figure 14) Separation of the RNA after centrifugation

1. While centrifuging, prepare and label one eppendorf per sample.
2. Pipette as much of the aqueous phase as possible, without taking any of the interface or the organic phase.
3. Add 500 µL of isopropanol.
4. Add 2 µL of glycogen.
5. Shake the tubes manually and leave them at -20^{o}C overnight.
6. Remove from the freezer and centrifuge at 13000 rpm for 15 minutes.
7. Remove the supernatant completely (by decantation and with pipette).
8. Add 500 µL of cold 75% EtOH.
9. Vortex for 3 seconds.
10. Centrifuge at 13000 rpm and 4^{o}C for 15 minutes.
11. Remove EtOH with pipette and remaining, by stoving at 37^{o}C for a couple of minutes.

12. Resuspend the pellet in 20 µL of H_2O PCR quality.

13. Quantify the RNA by spectrophotometry (NanoPhotometer, Implen GmbH, Schatzbogen 52, D-81829, Germany).

14. Store RNA into the freezer at -70^{o}C

5.2. Obtaining and quantifying complementary DNA (cDNA)

To perform the conversion of RNA to cDNA we used the High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA, USA), whose protocol is as follows:

A. We used 300ng of RNA from each sample, and we followed the indications of the manufacturer to continue the protocol.

Product	**Volume per sample**	**Final concentration**
Master mix	4 µL	1X
RNA	Max - 16 µL	1 pg – 1 µg
H_2O (nuclease-free)	Until 20 µL	
Total	20 µL	

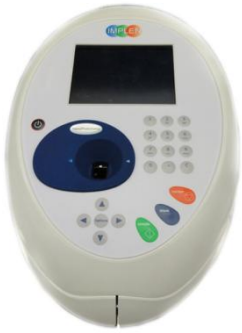
(Table 8) Products, volume and concentration in High Capacity RNA to cDNA kit

B. PCR:

	Phase 1	**Phase 2**	**Phase 3**	**Phase 4**
Temperature (^{o}C)	25	42	85	4
Time	5 min	30 min	5 min	∞

(Table 9) PCR protocol for the High Capacity RNA to cDNA kit

At the end of this protocol, the quantification of the samples was performed by spectrophotometry (NanoPhotometer, Implen GmbH, Schatzbogen 52, D-81829, Germany)



(Figure 15) Spectrophotometer

5.3. Gene expression analysis

Using Taqman probes and a real-time thermalcycler (7900HT Fast Real-Time Thermalcycler, Applied Biosystems, Foster City, CA, USA).

We used 2 probes for each assay: the probe of the gene from which we want to analyze the expression (target gene), and a probe of a normalizing gene or housekeeping (specifically, the GAPDH gene was used as normalizer).

The protocol carried out wasas follows:

1. Prepare 2 reaction mixtures (one with the target gene probe and one with the normalizing gene probe) with the following volumes per sample: 5 µL of master mix, 0.5 µL of probe and 3.5 µL of H_2O (total volume = 9 µL).

2. Prepare 2 reaction mixtures (one with the target gene probe and one with the normalizing gene probe) with the following volumes per sample: 5 µL of master mix, 0.5 µL of probe and 3.5 µL of H2O (total volume 9 µL) .

3. We perform the test by duplicate.

4. Add 9 µL of the reaction mixture to the target/housekeeping wells of the 96-well plate, spin the plate for 5 seconds and add 1 µL of cDNA into each well.

5. Seal the plate with an adhesive and spin for 5 seconds.

6. PCR Protocol::

    - 2 minutes at 50ºC
    - 10 minutes at 95ºC
    - 15 seconds at 95ºC
    - 1 minute and 25 seconds at 60ºC

7. Repeat the last two steps in 50 cycles.

To calculate the relative expression, the following equation was used:

$$RQ = 2^{-\Delta\Delta Ct}$$

The first ΔCt was: Ct_{target} - $Ct_{housekeeping}$

The second ΔCt was: 1st ΔCt – (average of $Ct_{housekeeping}$ from control samples)

To represent the fold change of gene expression, the mean of gene expression in all the study groups (controls, DM2, DM-DR, DM+DR) was divided by the mean of gene expression of CTRL.

6. Statistical analysis

The data were processed to descriptive statistical analysis with the IBM SPSS Statistics for Windows 22.0 program (IBM Corp., Armonk, NY, USA).

The normality of the quantitative variables was checked by the Kolmogorov-Smirnov test. The categorical variables were compared using the Pearson Chi-square test.

The comparison of 2 means was performed using Student's t-test for normal variablles or the Mann-Whitney U test for non-normal variables. The comparison of more than two means was performed by analysis of variance (ANOVA) for normal variables or by the Kruskal-Wallis test for non-normal variables.

The Pearson correlation coefficient (normal variables) and Spearman's Rho coefficient (non-normal variables) were used to determine the magnitude of association of 2 quantitative variables.

All statistical analysis were performed assigning a significance level of 0.05.

RESULTS

1. Basic characteristics of groups and subgroups

81 participants were included in this study from both sexes, between ages 26-82 years old. These were divided in two groups consisting of a control group (CTRL) (n = 32) and a DM2 group (DM2-G) (n = 49).

The DM2 group has two subgroups consisting of participating with DR (DM2+DR) (n = 14, 28,6%) and without DR (DM2-DR) (n = 35, 71,4%).

Participants have also been divided into groups consisting of a younger group (age from 25-55) and an older group (age from 56-85).

2. Ophthalmic results

The results of the quantitative variables best corrected visual acuity (BCVA), intraocular pressure (IOP) and optical coherence tomography (OCT) are expressed as average ± standard deviation of right eye (RO) and left eye (LO). The results of the qualitative variables are expressed in percentage and frequency of RO and LO. All these data are reflected in following tables.

Ophthalmic parameter		CTRL	DM2	p
BCVA	**RO**	1,000	0,780	0,000*
	LO	1,036	0,730	0,000*
IOP (mmHg)	**RO**	14,0	15,2	0,786
	LO	15,0	15,3	0,950
OCT (µm)	**RO**	248,1	259,8	0,299
	LO	248,6	272,7	0,393

(Table 10) Differences in ophthalmic parameters between control and DM2 groups

CTRL: control group; DM2: diabetes mellitus type 2 group; BCVA: best corrected visual acuity; IOP: intraocular pressure; OCT: optical coherence tomography; RO: Right orbit; LO: Left orbit

* statistically significant ($p<0,05$)

Ophthalmic parameter		CTRL	DM-DR	DM+DR	p
BCVA	**RO**	1,000	0,843	0,663	0,000*
	LO	1,036	0,798	0,598	0,000*
IOP (mmHg)	**RO**	14,0	14,2	16,1	0,610
	LO	15,0	14,8	15,8	0,886
OCT (µm)	**RO**	248,1	247,4	290,3	0,014*
	LO	248,6	262,5	299,3	0,280

(Table 11) Differences in ophthalmic parameters between control and DM2 without/with diabetic retinopathy

CTRL: control group; DM2-DR: diabetes mellitus type 2 without diabetic retinopathy; DM2+DR: diabetes mellitus type 2 with diabetic retinopathy; BCVA: best corrected visual acuity; IOP: intraocular pressure; OCT: optical coherence tomography; RO: Right orbit; LO: Left orbit

* Statistically significant ($p<0,05$)

3. Classical biochemistry

3.1. Different molecules

Molecule	CTRL	DM2	p
Glu (mg/dL)	83,3	127,6	7,18E-7*
Hct (%)	43,6	41,7	0,179
HbA1c (%)	5,5	7,0	6,43E-12*
FA (ng/mL)	8,2	10,3	0,135

(Table 12) Difference in plasma levels of different molecules between control and DM2 groups

CTRL: control group; DM2: diabetes mellitus type 2 group; Glu: glucose; Hct: haematocrit; HbA1c: glycosylated haemoglobin; FA: folic acid

* Statistically significant (p<0,05)

Molecule	MALE		p	FEMALE		p
	CTRL	DM2		CTRL	DM2	
Glu (mg/dL)	85,13 ± 4.09	119,61 (5,02)	0,009*	81,6	138,2	2,21E-7*
Hct (%)	44,9	43,0	0,070	41,0	40,2	0,762
HbA1c (%)	5,6	7,2	0,000	5,5	6,9	2,07E-6*
FA (ng/mL)	7,8	9,3	0,259	8,6	11,4	0,345

(Table 13) Difference in plasma levels of different molecules between control and DM2 groups by age

CTRL: control group; DM2: diabetes mellitus type 2 group; Glu: glucose; Hct: haematocrit; HbA1c: glycosylated haemoglobin; FA: folic acid

* Statistically significant (p<0,05)

The results are expressed as mean (standard deviation)

Molecule	MALE			p	FEMALE			p
	CTRL	DM-DR	DM+DR		CTRL	DM-DR	DM+DR	
Glu (mg/dL)	85,1[Þ]	134,7	82,0[Þ]	0,006*	81,6	138,8[§]	136,8[§]	1,81E-6*
Hct (%)	44,9	43,1	42,8	0,402	41,0	40,9	39,1	0,692
HbA1c (%)	5,6	7,2[§]	6,9[§]	0,0002*	5,5	6,9[§]	6,8[§]	0,00001*
FA (ng/mL)	7,8	8,9	10,9	0,249	8,6	9,8	15,2	0,223

(Table 14) Difference in plasma levels of different molecules between controls and DM2 without/with diabetic retinopathy by gender

CTRL: control group; DM-DR: diabetes mellitus type 2 without diabetic retinopathy; DM+DR: diabetes mellitus type 2 with diabetic retinopathy; Glu: glucose; Hct: haematocrit; HbA1c: glycosylated haemoglobin; FA: folic acid

* Statistically significant ($p<0,05$)

Þ Significant differences respect to the DM-DR group

§ Significant differences respect to the control group

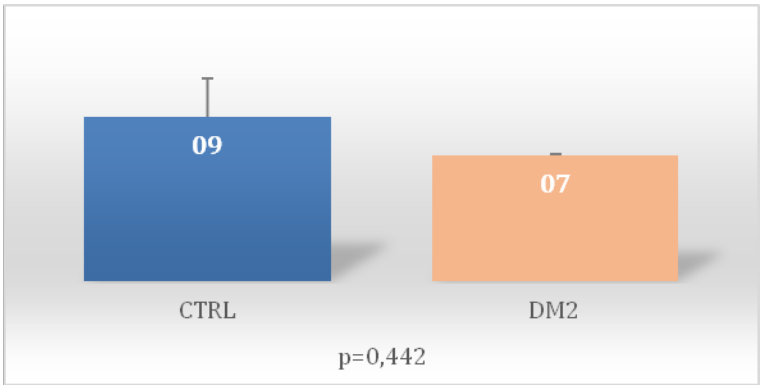
Molecule	YOUNG GROUP		p	OLD GROUP		p
	CTRL	DM2		CTRL	DM2	
Glu (mg/dL)	79,2	137,5	0,055	88,6	125,7	0,00003*
Hct (%)	44,1	45,0	0,540	42,5	41,0	0,509
HbA1c (%)	5,5	7,2	0,014*	5,6	7,0	5,62E-9*
FA (ng/mL)	6,9	8,9	0,156	8,9	10,6	0,933

(Table 15) Difference in plasma levels of different molecules between control and DM2 groups by age

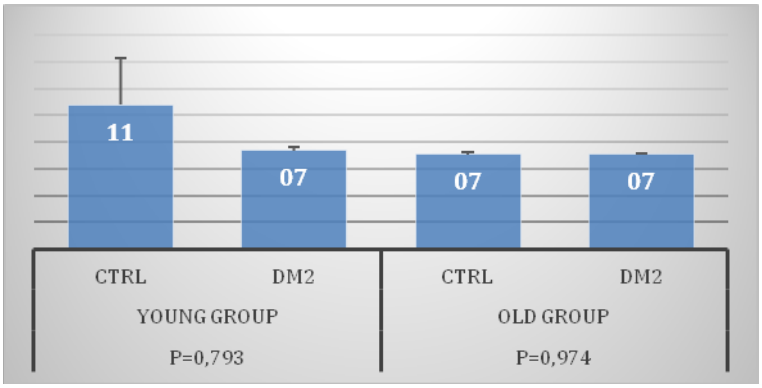
CTRL: control group; DM2: diabetes mellitus type 2 group; Glu: glucose; Hct: haematocrit; HbA1c: glycosylated haemoglobin; FA: folic acid

* Statistically significant ($p<0,05$)

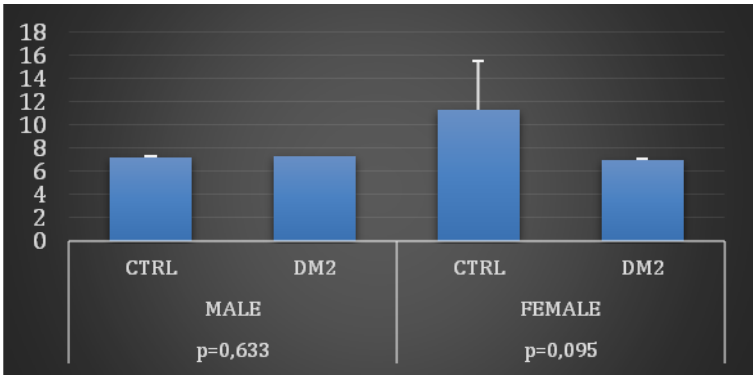
3.2. Total protein concentration



(Chart 1) Total protein concentration (g/dL)

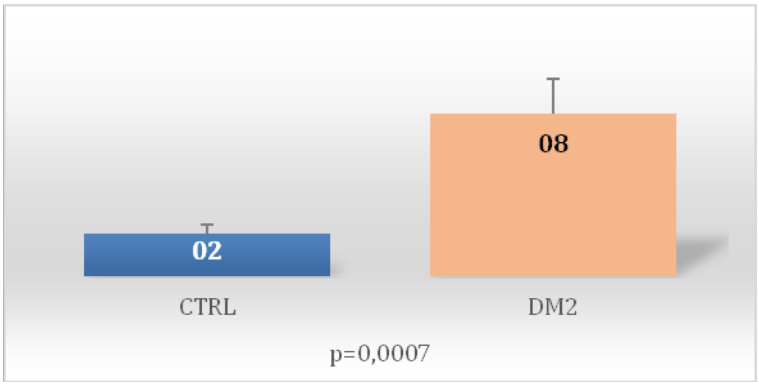


(Chart 2) Total protein concentration (g/dL) by age

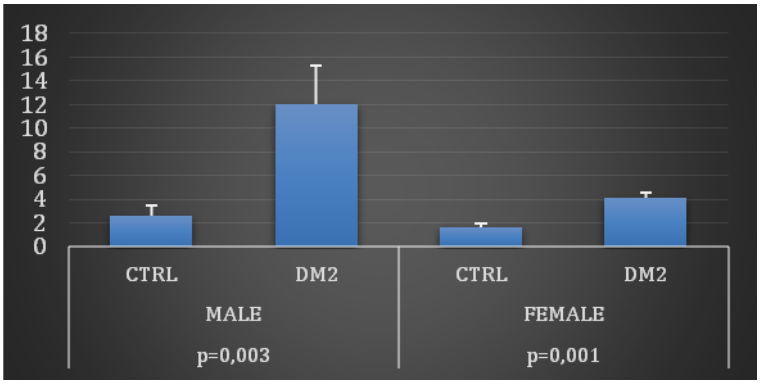


(Chart 3) Total protein concentration (g/dL) by gender

3.3. C reactive protein

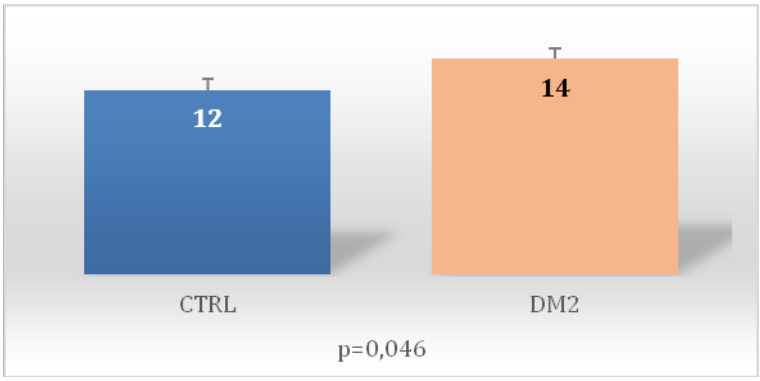


(Chart 4) C Reactive Protein (mg/L)

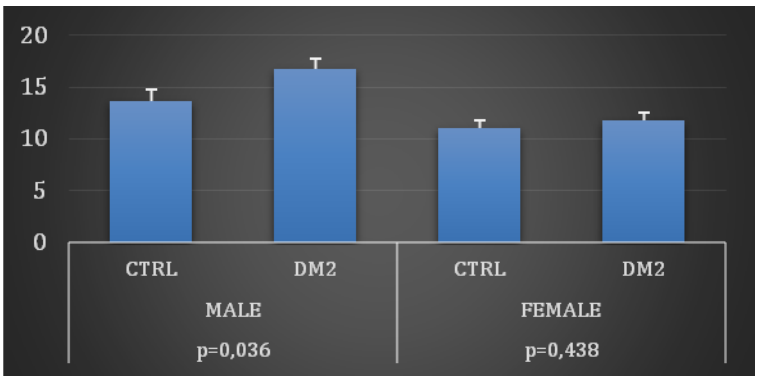


(Chart 5) C Reactive Protein (mg/L) by gender

3.4. Homocysteine

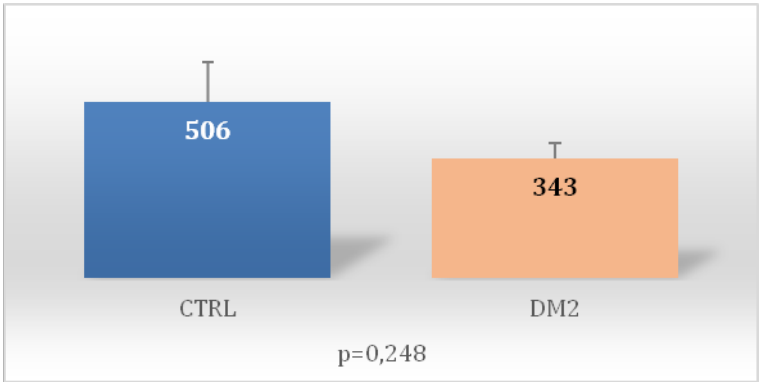


(Chart 6) Homocysteine (μmol/L)

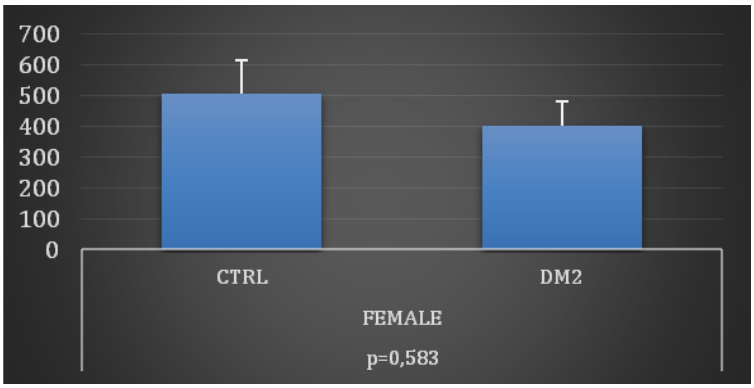


(Chart 7) Homocysteine (μmol/L) by gender

3.5. Vitamin B12



(Chart 8) Vitamin B12 (pg/mL)



(Chart 9) Vitamin B12 (pg/mL) in women

3.6. Lipids

Lipid	CTRL	DM2	p
T-Ch (mg/dL)	209,4	183,6	0,004*
HDL-Ch (mg/dL)	66,2	49,4	0,000*
LDL-Ch (mg/dL)	129,1	104,5	0,001*
TG (mg/dL)	87,6	153,8	0,000*

(Table 16) Difference in plasma lipid levels between control and DM2 groups

CTRL: control group; DM2: diabetes mellitus type 2 group; T-Ch: total cholesterol; HDL-Ch: HDL cholesterol; LDL-Ch: LDL cholesterol; TG: triglycerides

* Statistically significant (p<0,05)

Lipid	MALE		p	FEMALE		p
	CTRL	DM2		CTRL	DM2	
T-Ch (mg/dL)	218,8	182,1	0,010*	200,6	185,3	0,162
HDL-Ch (mg/dL)	57,5	44,3	0,015*	74,9	55,2	0,004*
LDL-Ch (mg/dL)	141,9	106,0	0,001*	116,4	102,9	0,188
TG (mg/dL)	100,5	169,6	0,006*	75,6	134,9	0,001*

(Table 17) Differences in plasma lipid levels between control and DM2 groups by gender

CTRL: control group; DM2: diabetes mellitus type 2 group; T-Ch: total cholesterol; HDL-Ch: HDL cholesterol; LDL-Ch: LDL cholesterol; TG: triglycerides

* Statistically significant (p<0,05)

Lipid	YOUNG GROUP		p	OLD GROUP		p
	CTRL	DM2		CTRL	DM2	
T-Ch (mg/dL)	214,5	212,9	0,940	202,2	178,2	0,017*
HDL-Ch (mg/dL)	60,3	50,3	0,495	75,6	49,2	0,003*
LDL-Ch (mg/dL)	138,0	121,1	0,350	115,2	101,2	0,093
TG (mg/dL)	89,1	211,7	0,041*	89,6	142,9	0,004*

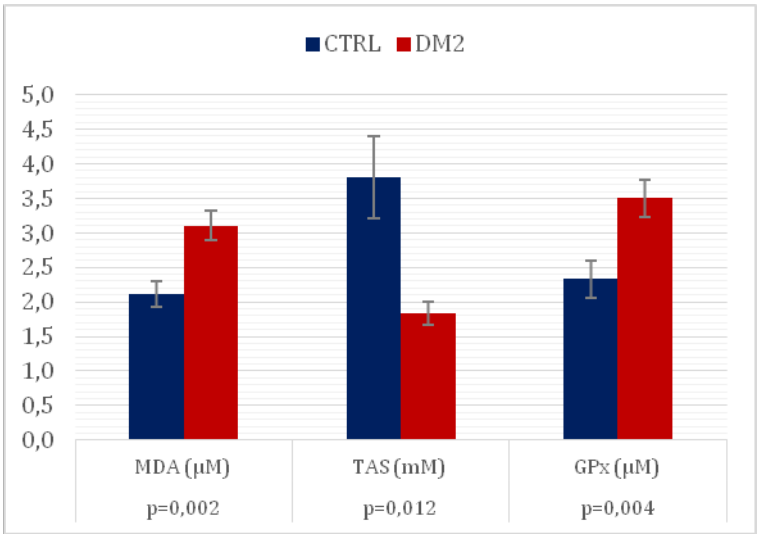
(Table 18) Differences in plasma lipid levels between control and DM2 groups by age

CTRL: control group; DM2: diabetes mellitus type 2 group; T-Ch: total cholesterol; HDL-Ch: HDL cholesterol; LDL-Ch: LDL cholesterol; TG: triglycerides

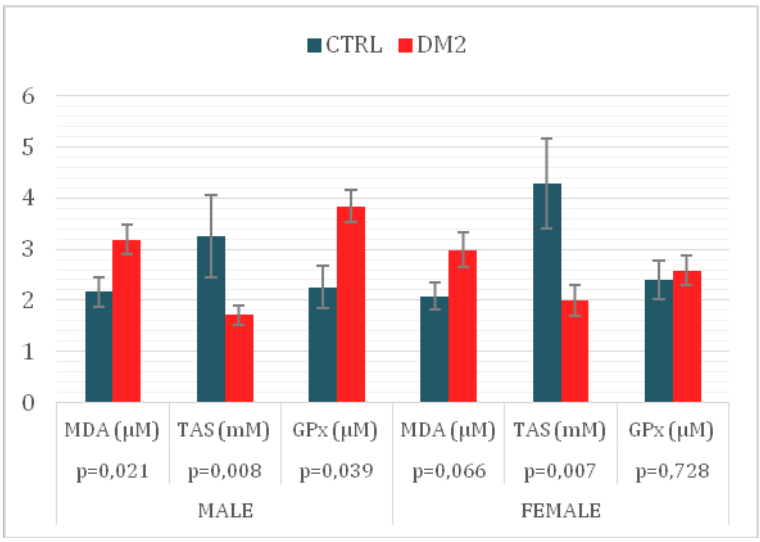
* Statistically significant

(p<0,05)

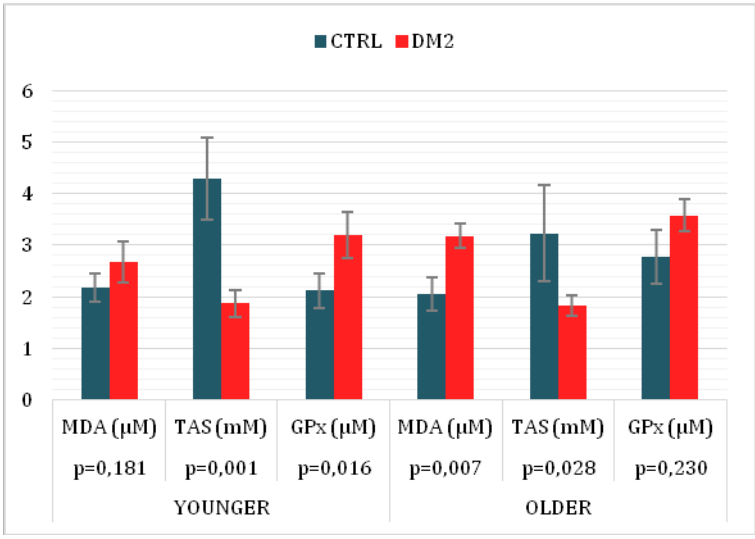
3.7. Oxidative stress



(Chart 10) Oxidative stress markers



(Chart 11) Oxidative stress by gender



(Chart 12) Oxidative stress by age

4. Molecular biochemistry of diabetic retinopathy

4.1. Different molecules

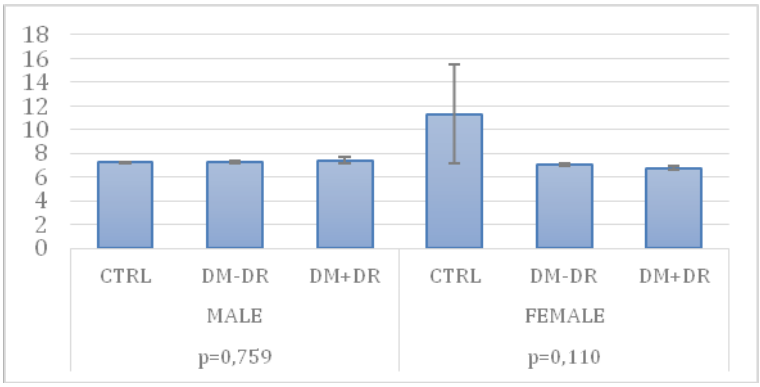
Molecule	CTRL	DM-DR	DM+DR	p
Glu (mg/dL)	83,3	136,4§	105,5	7,22E-6*
Hct (%)	43,6	42,2	40,7	0,243
HbA1c (%)	5,5	7,1§	6,9§	3,36E-9*
FA (ng/mL)	8,2	9,3	13,3	0,059

(Table 19) Differences in plasma levels of different molecules between controls and DM2 without/with diabetic retinopathy

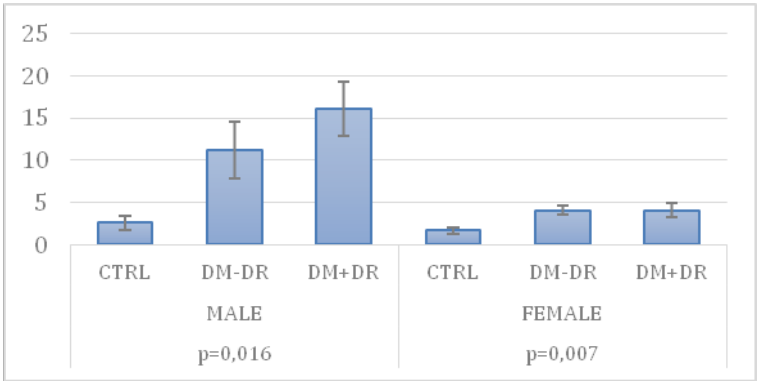
CTRL: control group; DM-DR: diabetes mellitus type 2 without diabetic retinopathy; DM+DR: diabetes mellitus type 2 with diabetic retinopathy; Glu: glucose; Hct: haematocrit; HbA1c: glycosylated haemoglobin; FA: folic acid

* Statistically significant ($p<0,05$)

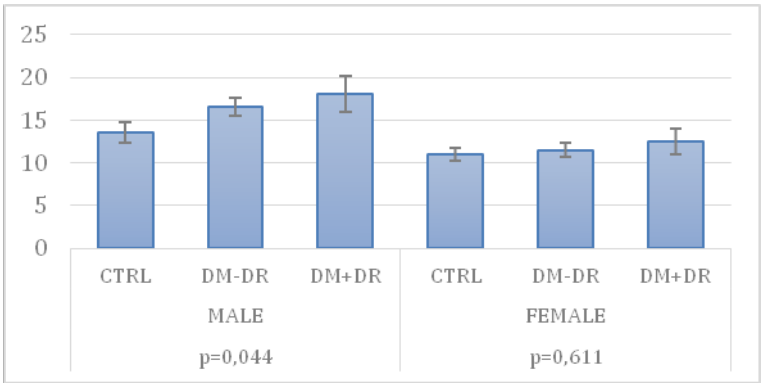
§ Significant differences respect to the control group



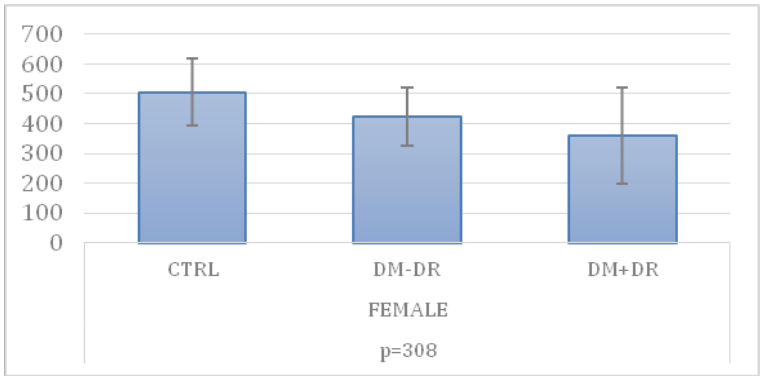
(Chart 13) Total Protein Concentration (g/dL) by gender



(Chart 14) C Reactive Protein (mg/L) by gender



(Chart 15) Homocysteine (μmol/L) by gender



(Chart 16) Vitamin B12 (pg/mL) in women

4.2. Lipids

Lipid	CTRL	DM-DR	DM+DR	p
T-Ch (mg/dL)	209,4	184,4	181,0	0,015*
HDL-Ch (mg/dL)	66,2	48,8	51,4	0,001*
LDL-Ch (mg/dL)	129,1	103,3	108,4	0,005*
TG (mg/dL)	87,6	162,0	129,4	0,000*

(Table 20) Differences in plasma lipid levels between controls and DM2 without/with diabetic retinopathy

CTRL: control group; DM-DR: diabetes mellitus type 2 without diabetic retinopathy; DM+DR: diabetes mellitus type 2 with diabetic retinopathy; T-Ch: total cholesterol; HDL-Ch: HDL cholesterol; LDL-Ch: LDL cholesterol; TG: triglycerides

* Statistically significant ($p<0,05$)

Lipid	MALE			p	MALE			p
	CTRL	DM-DR	DM+DR		CTRL	DM-DR	DM+DR	
T-Ch (mg/dL)	218,8	181,2§	185,6	0,037*	200,6	188,5	177,2	0,295
HDL-Ch (mg/dL)	57,5	43,5§	47,4	0,028*	74,9	55,4§	54,7§	0,017*
LDL-Ch (mg/dL)	141,9	102,8§	120,3	0,003*	116,4	103,9	100,5	0,414
TG (mg/dL)	100,5	173,6§	154,4§	0,025*	75,6	146,2§	108,5	0,002*

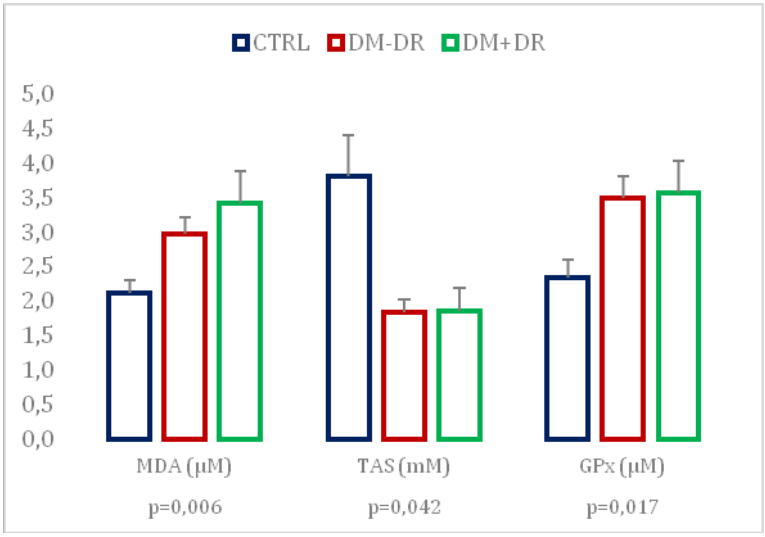
(Table 21) Differences in plasma lipid levels between controls and DM2 without/with diabetic retinopathy by gender

CTRL: control group; DM-DR: diabetes mellitus type 2 without diabetic retinopathy; DM+DR: diabetes mellitus type 2 with diabetic retinopathy; T-Ch: total cholesterol; HDL-Ch: HDL cholesterol; LDL-Ch: LDL cholesterol; TG: triglycerides

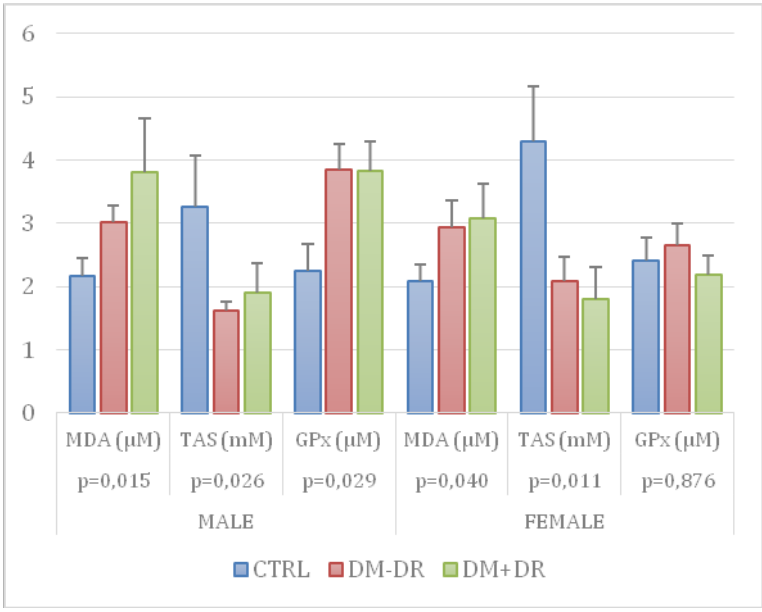
* Statistically significant ($p<0,05$)

§ Significant differences respect to the control group

4.3. Oxidative stress



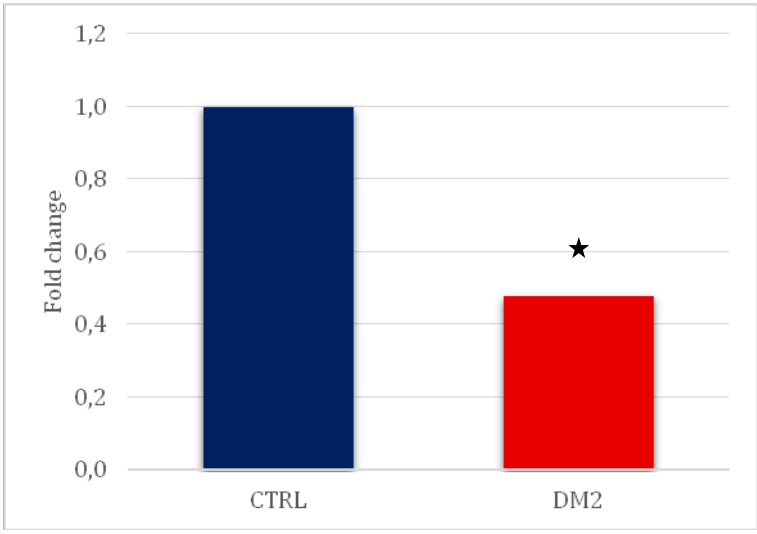
(Chart 17) Oxidative stress markers



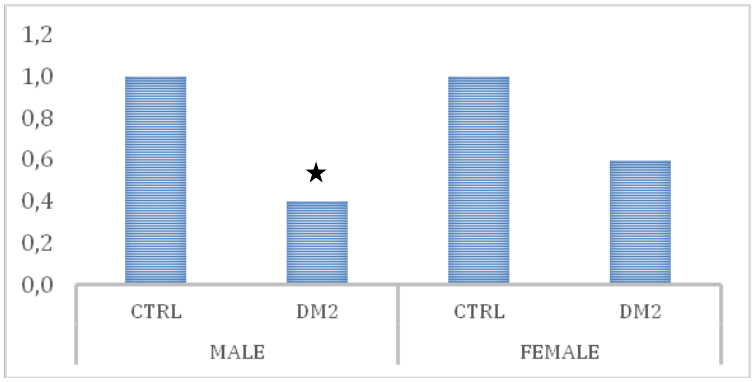
(Chart 18) Oxidative stress by gender

5. Genetics of diabetic retinopathy

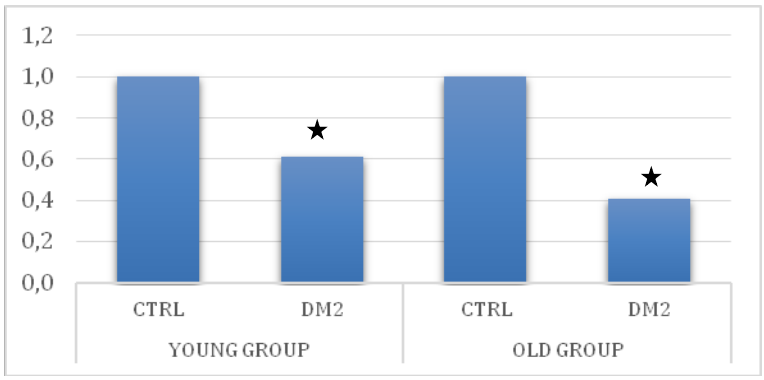
5.1. Relative expression of SLC23A2 gene



(Chart 19) Relative expression of SLC23A2 gene (p=0.026)

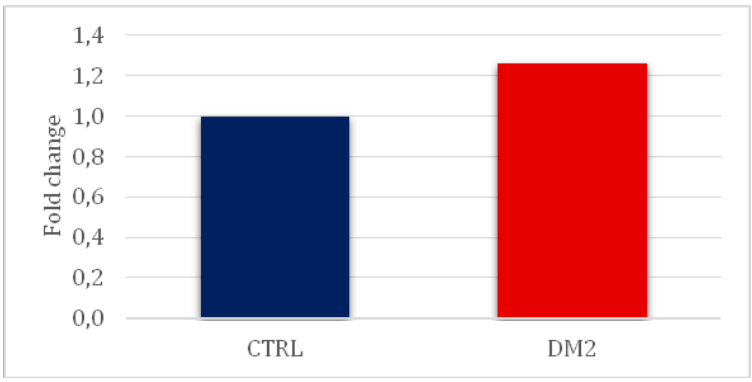


(Chart 20) Relative expression of SLC23A2 gene by gender (male: p=0.019; female: p=0.061)

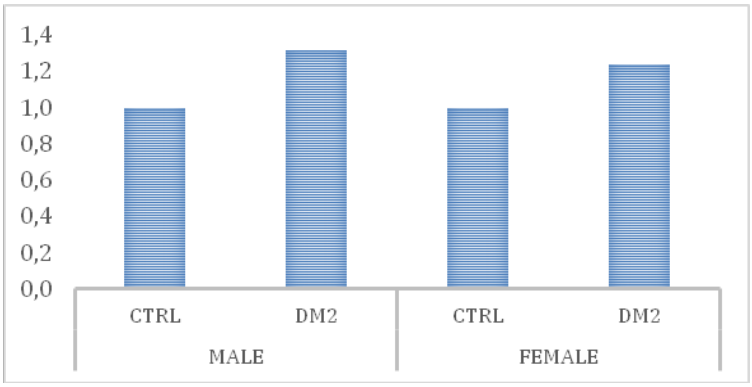


(Chart 21) Relative expression of SLC23A2 gene by age (young: p=0.047; old: p=0.027)

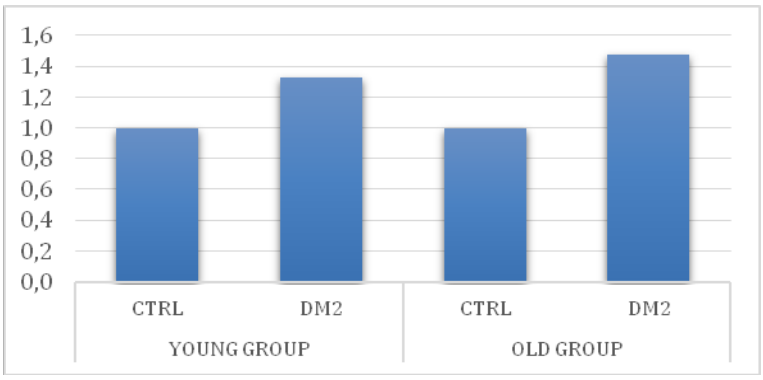
5.2. Relative expression of TP53 gene



(Chart 22) Relative expression of TP53 gene (p=0.084)

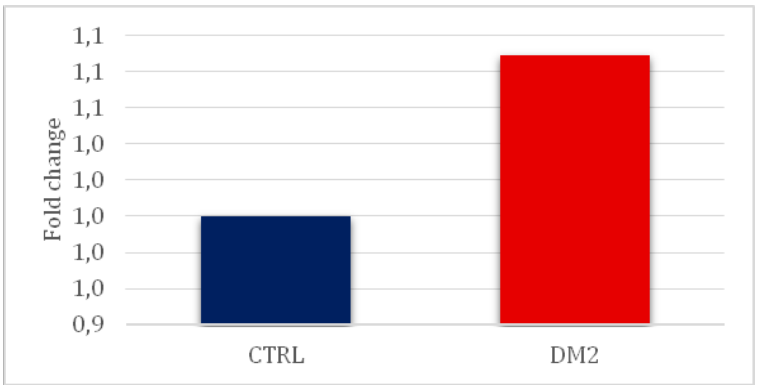


(Chart 23) Relative expression of TP53 gene by gender (male: p=0.350; female: p=0.456)

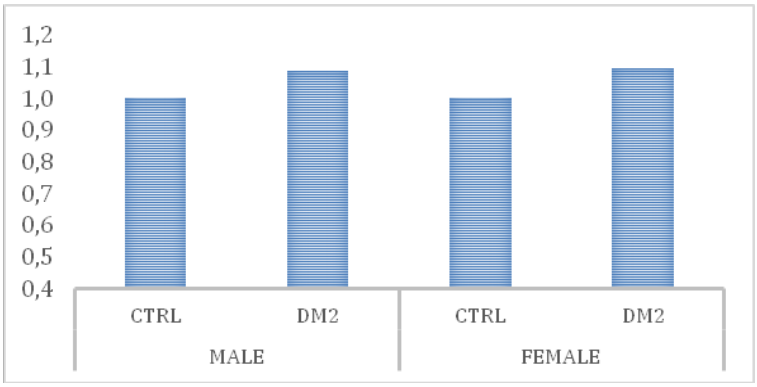


(Chart 24) Relative expression of TP53 gene by age (young: p=0.442; old: p=0.234)

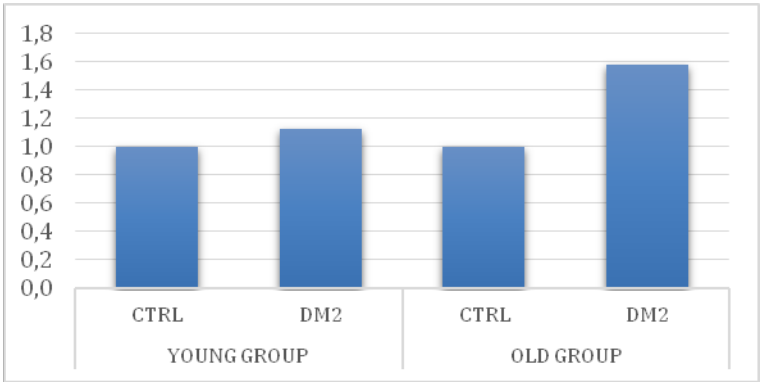
5.3. Relative expression of RBP1 gene



(Chart 25) Relative expression of RBP1 gene (p=0.612)

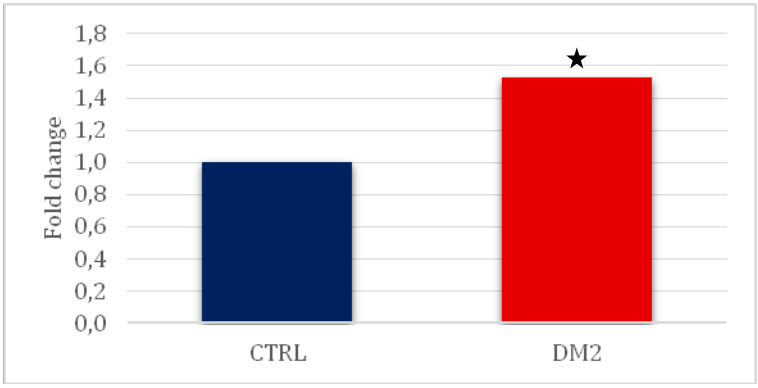


(Chart 26) Relative expression of RBP1 gene by gender (male: p=0.748; female: p=0.682)

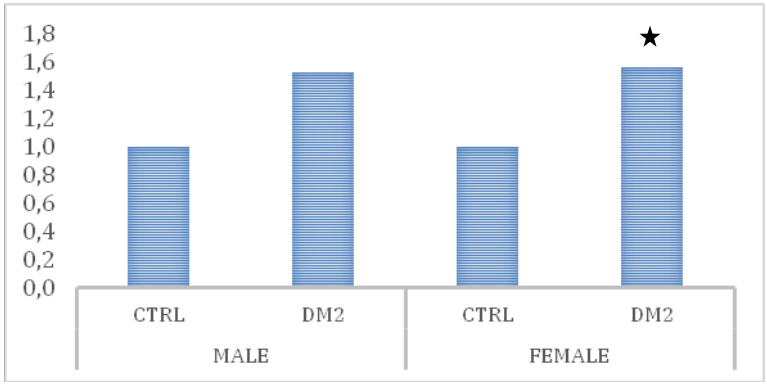


(Chart 27) Relative expression of RBP1 gene by age (young: p=0.694; old: p=0.117)

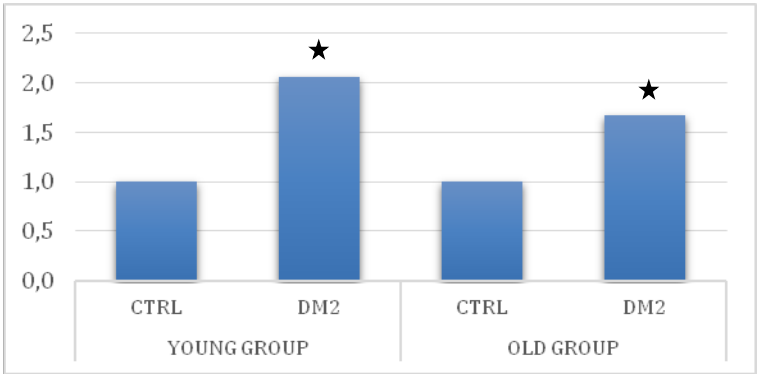
5.4. Relative expression of MMP9 gene



(Chart 28) Relative expression of MMP9 gene (p=0.036)

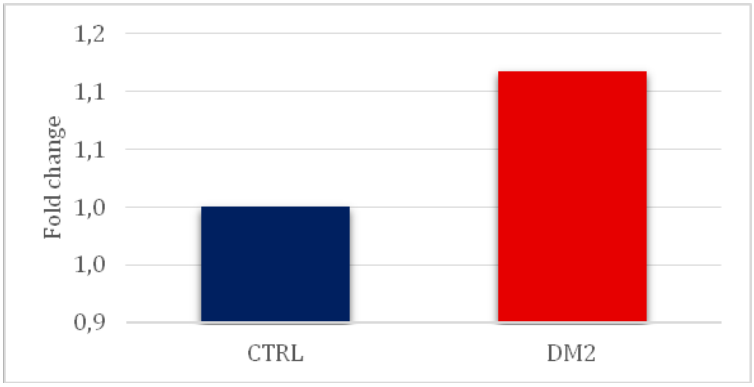


(Chart 29) Relative expression of MMP9 gene by gender (male: p=0.058; female: p=0.010)

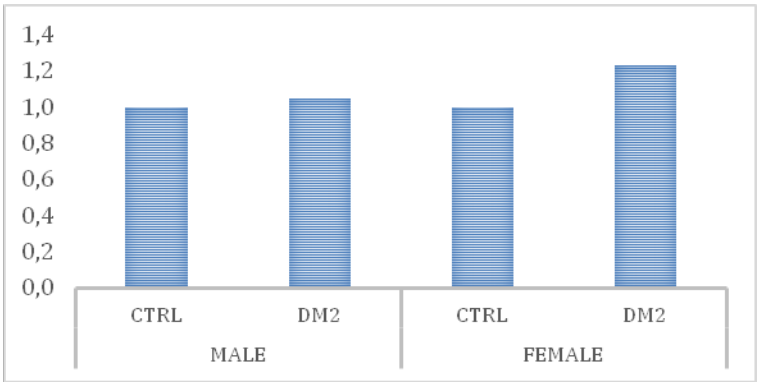


(Chart 30) Relative expression of MMP9 gene by age (young: p=0.006; old: p=0.039)

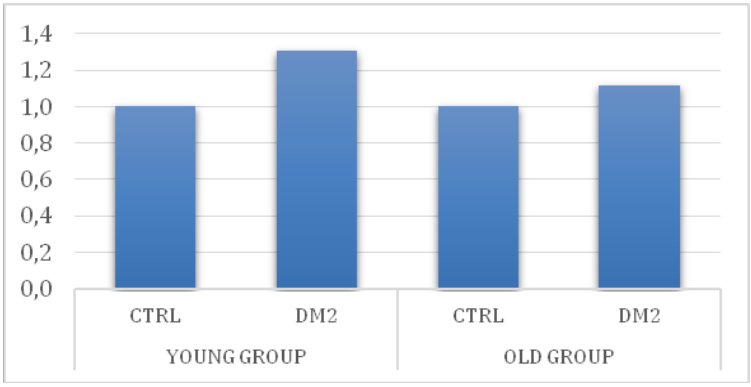
5.5. Relative expression of MMACHC gene



(Chart 31) Relative expression of MMACHC gene (p=0.517)

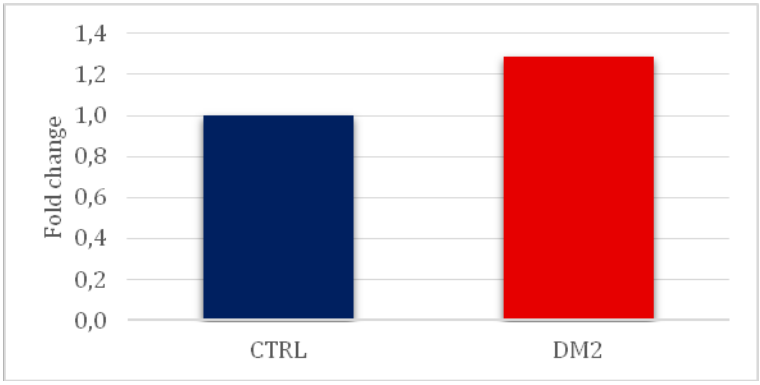


(Chart 32) Relative expression of MMACHC gene by gender (male: p=0.884; female: p=0.267)

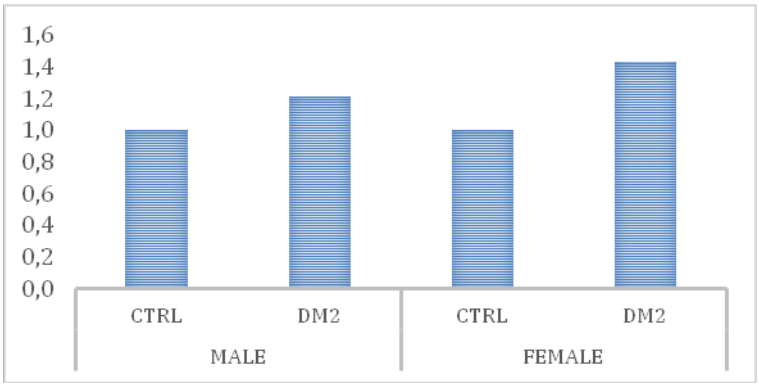


(Chart 33) Relative expression of MMACHC gene by age (young: p=0.280; old: p=0.738)

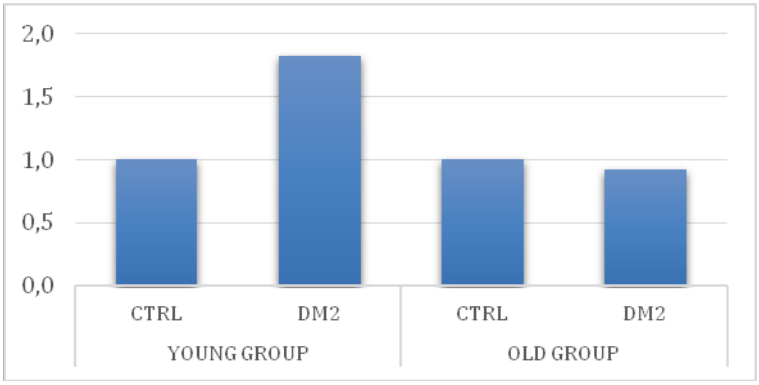
5.6. Relative expression of MMADHC gene



(Chart 34) Relative expression of MMADHC gene p=0.120)

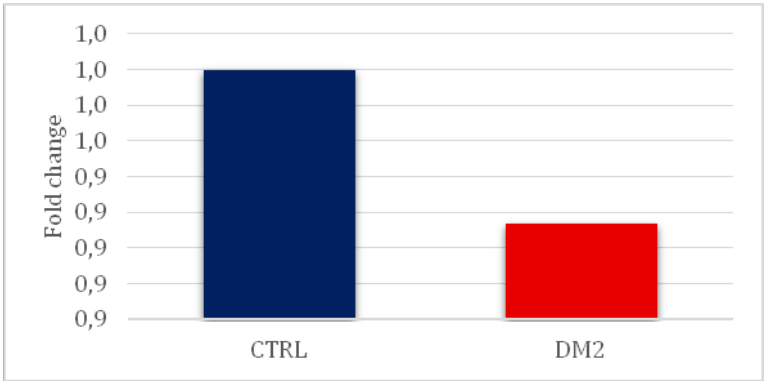


(Chart 35) Relative expression of MMADHC gene by gender (male: p=0.164; female: p=0.294)

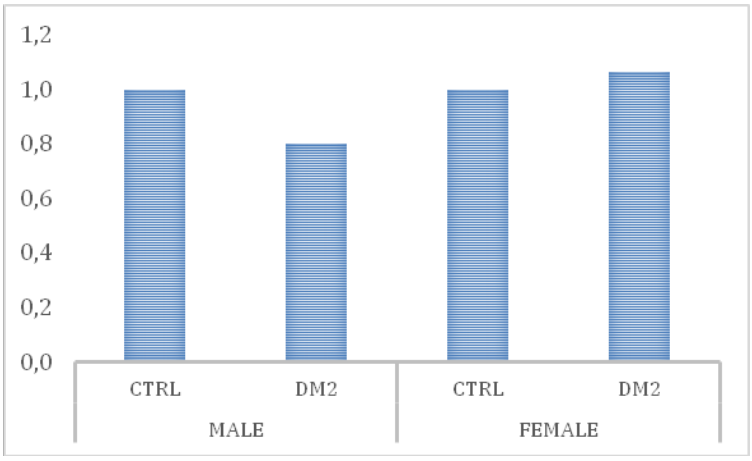


(Chart 36) Relative expression of MMADHC gene by age (young: p=0.399; old: p=0.822)

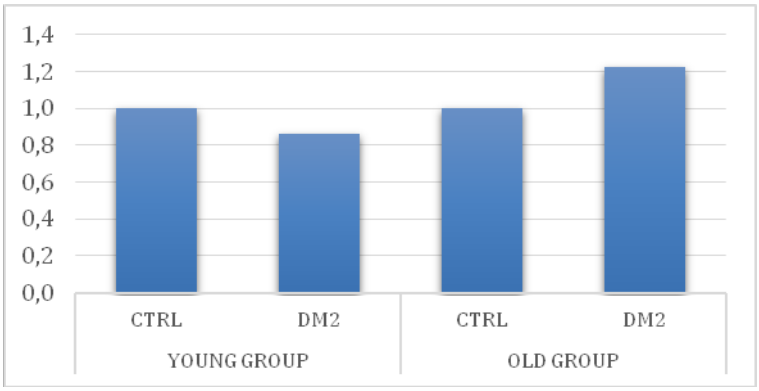
5.7. Relative expression of TMCO1 gene



(Chart 37) Relative expression of TMCO1 gene (p=0.492)

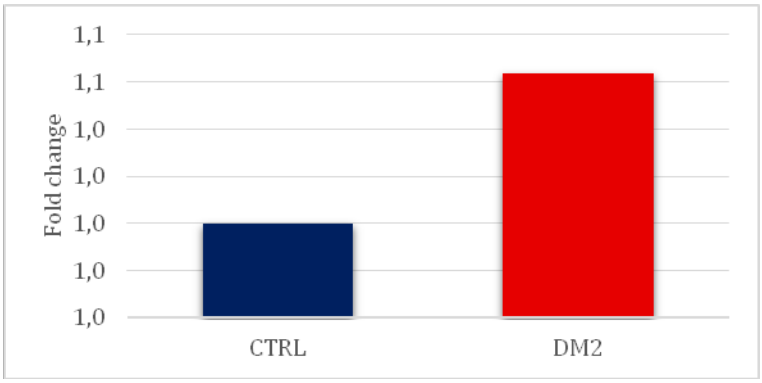


(Chart 38) Relative expression of TMCO1 gene by gender (male: p=0.232; female: p=0.745)

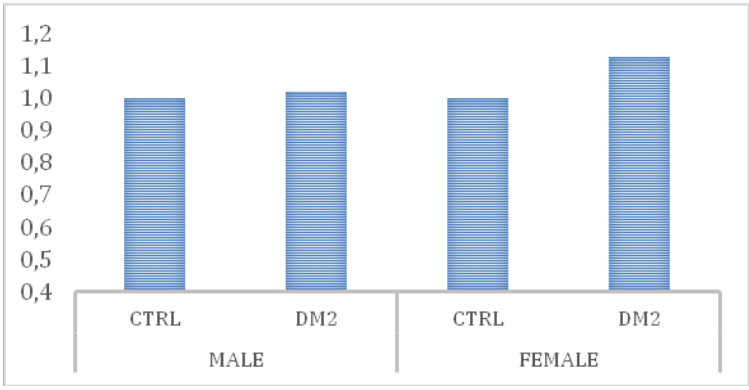


(Chart 39) Relative expression of TMCO1 gene by age (young: p=0.540; old: p=0.346)

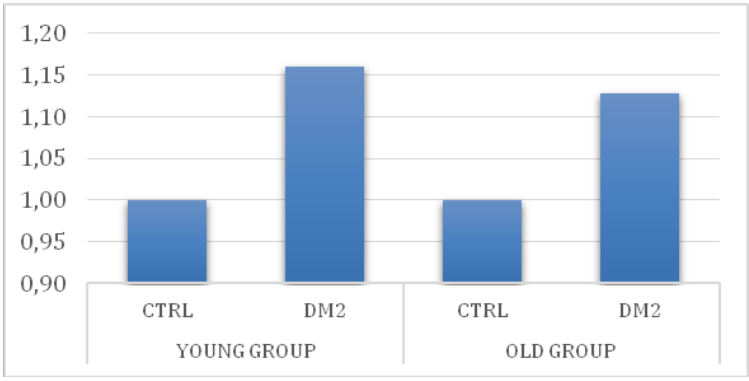
5.8. Relative expression of THRA gene



(Chart 40) Relative expression of THRA gene (p=0.455)



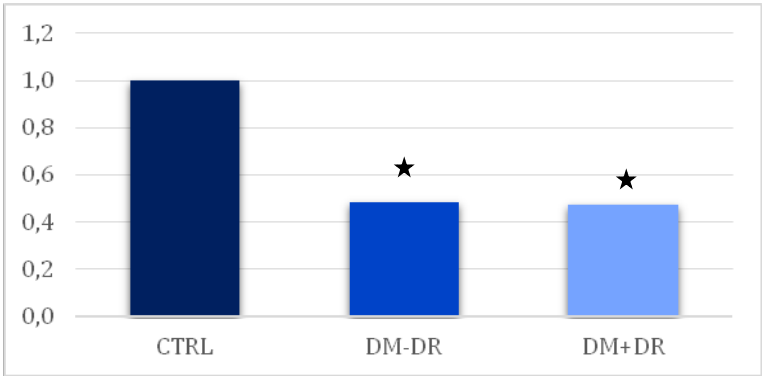
(Chart 41) Relative expression of THRA gene by gender (male: p=0.859; female: p=0.342)



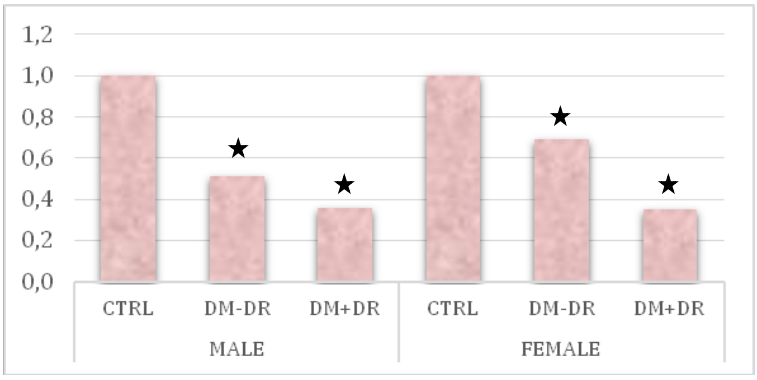
(Chart 42) Relative expression of THRA gene by age (young: p=0.281; old: p=0.363)

6. Relative gene expression with diabetes retinopathy

6.1. Relative expression of SLC23A2 gene

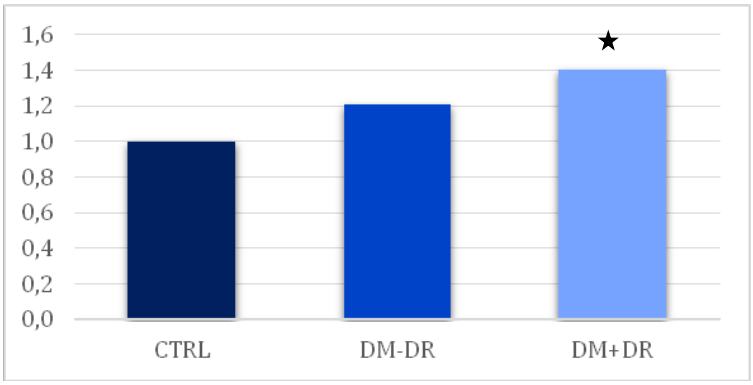


(Chart 43) Relative expression of SLC23A2 gene (p=0.018)

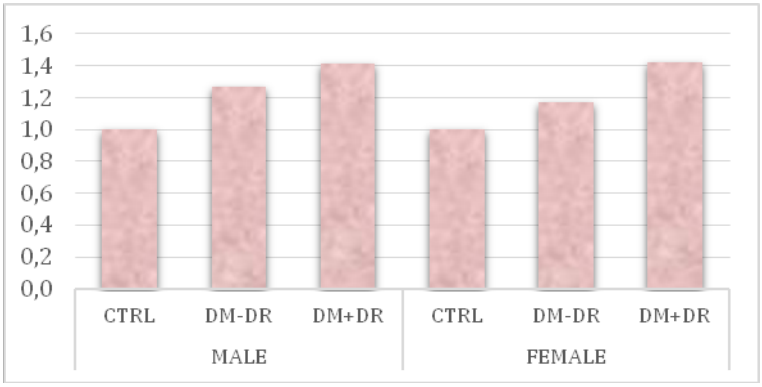


(Chart 44) Relative expression of SLC23A2 gene by gender (male: p=0.002; female: p<0.001)

6.2. Relative expression of TP53 gene

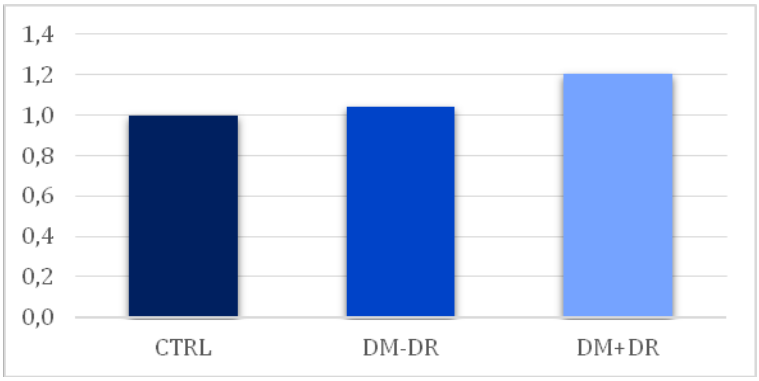


(Chart 45) Relative expression of TP53 gene (p=0.018)



(Chart 46) Relative expression of TP53 gene by gender (male: p=0.068; female: p=0.055)

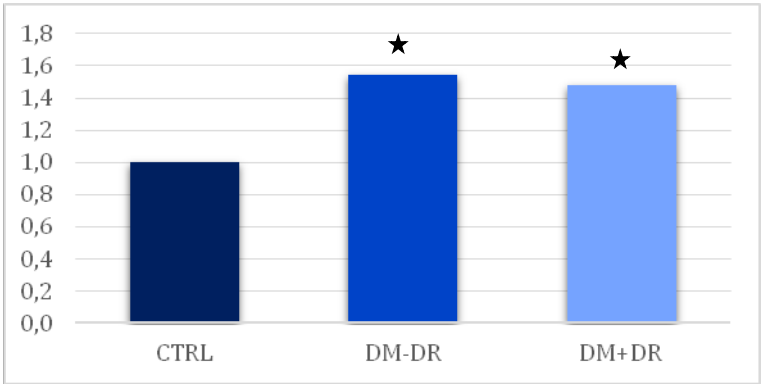
6.3. Relative expression of RBP1 gene



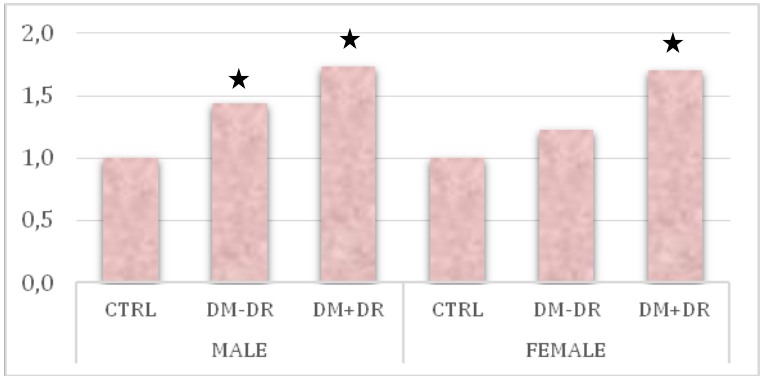
(Chart 47) Relative expression of RBP1 gene (p=0.710)

(Chart 48) Relative expression of RBP1 gene by gender (male: p=0.662; female: p=0.918)

6.4. Relative expression of MMP9 gene

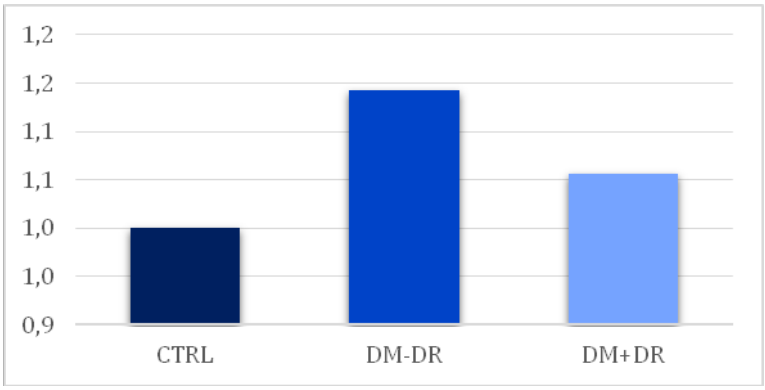


(Chart 49) Relative expression of MMP9 gene (p=0.021)

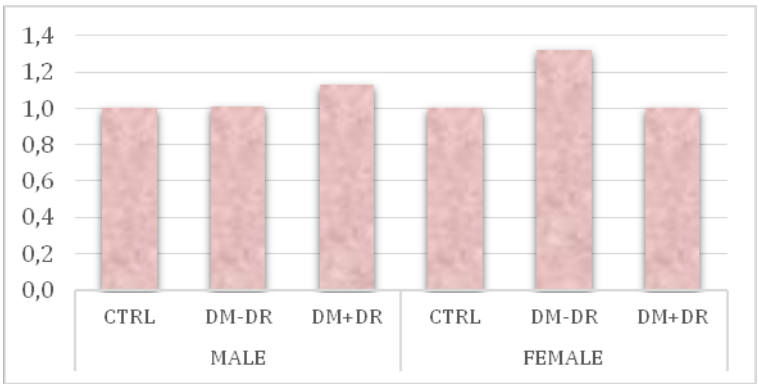


(Chart 50) Relative expression of MMP9 gene by gender (male: p=0.001; female: p=0.012)

6.5. Relative expression of MMACHC gene

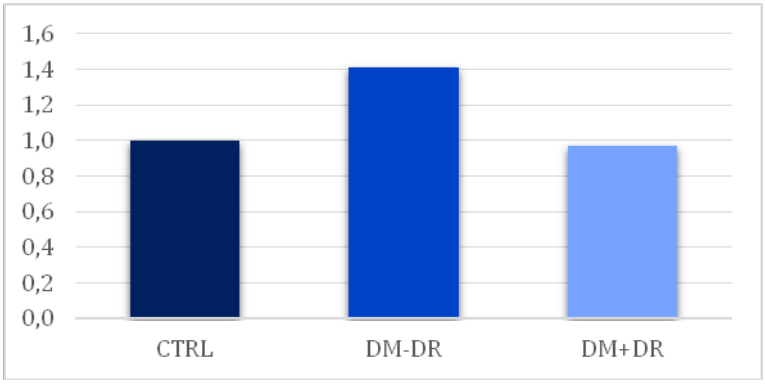


(Chart 51) Relative expression of MMACHC gene (p=0.793)

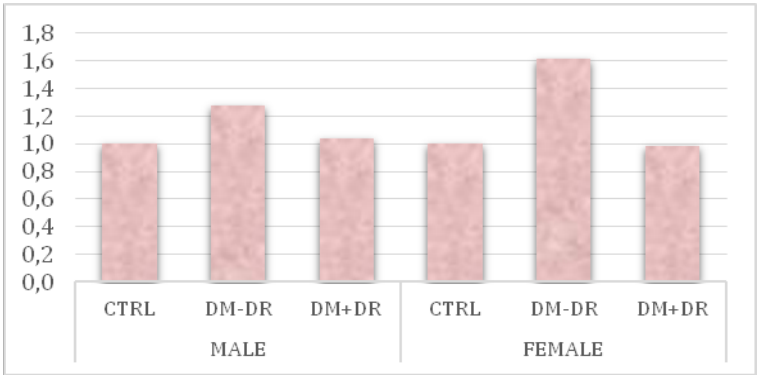


(Chart 52) Relative expression of MMACHC gene by gender (male: p=0.619; female: p=0.400)

6.6. Relative expression of MMADHC gene

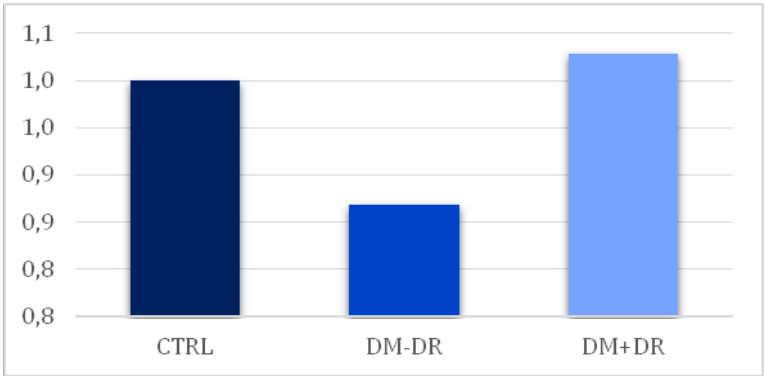


(Chart 53) Relative expression of MMADHC gene (p=0.298)

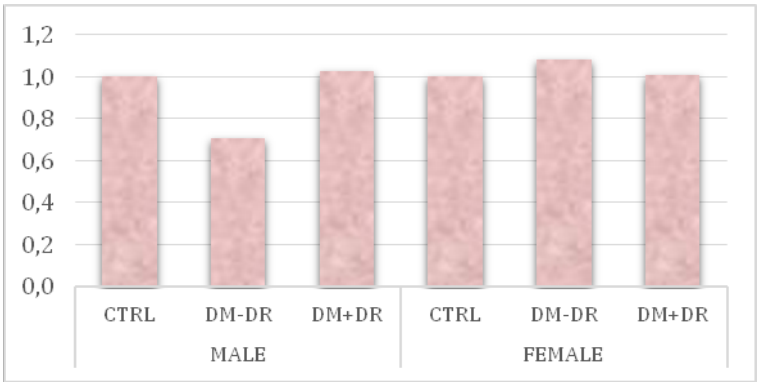


(Chart 54) Relative expression of MMADHC gene by gender (male: p=0.368; female: p=0.536)

6.7. Relative expression of TMCO1 gene

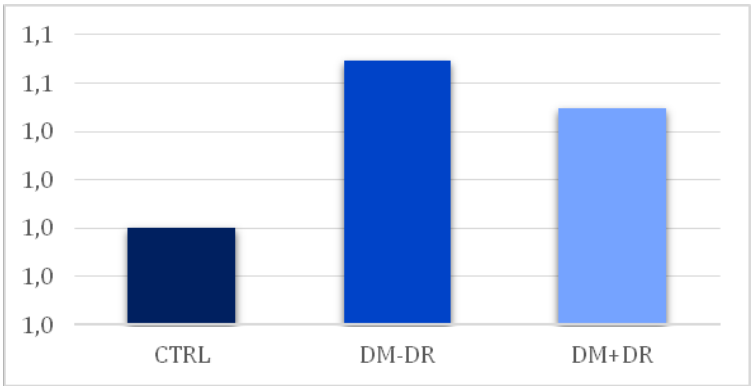


(Chart 55) Relative expression of TMCO1 gene (p=0.516)

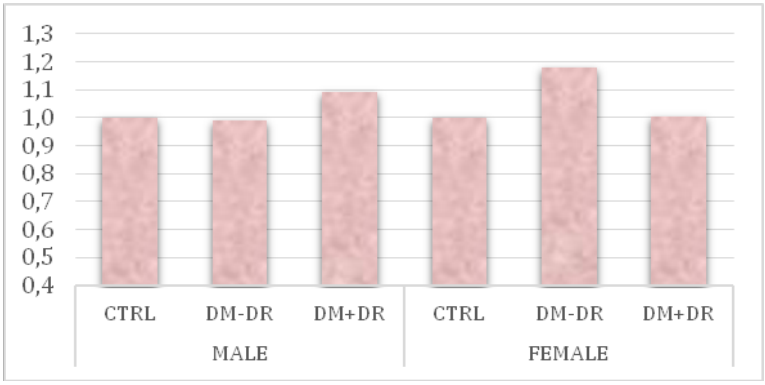


(Chart 56) Relative expression of TMCO1 gene by gender (male: p=0.170; female: p=0.917)

6.8. Relative expression of THRA gene



(Chart 57) Relative expression of THRA gene (p=0.747)

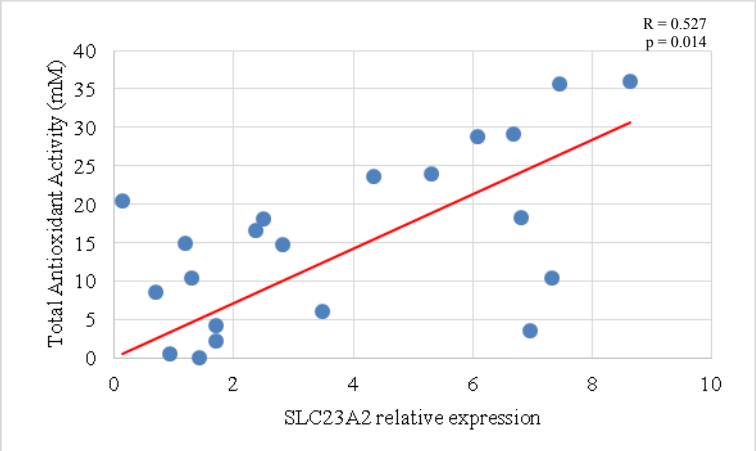


(Chart 58) Relative expression of THRA gene by gender (male: p=0.750; female: p=0.432)

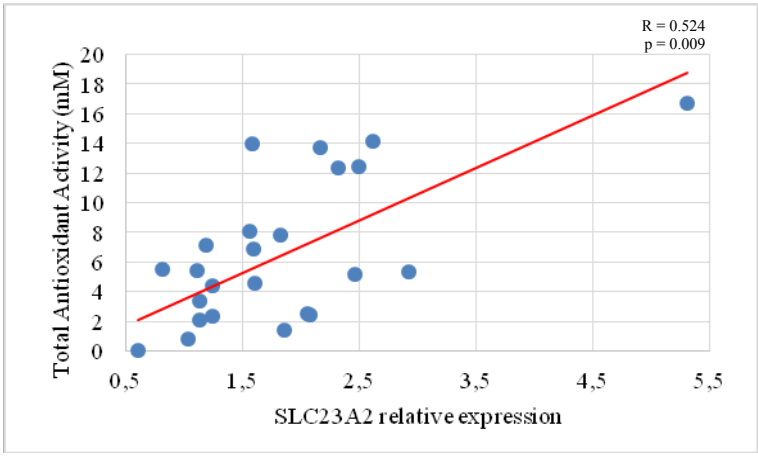
7. Correlation between oxidative stress and gene expressions

We analysed the correlation between oxidative stress markers and gene expression, and only the correlation between the expression of SLC23A2 gene and TAS or MDA was statistically significant (see graphics below).

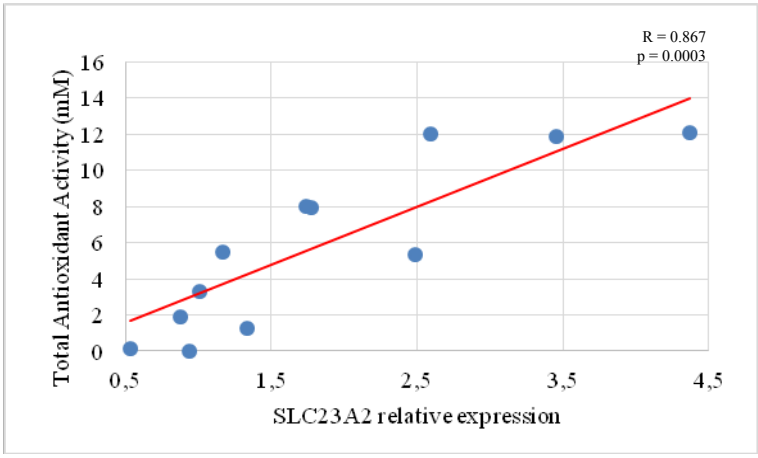
7.1. Relative expression of SLC23A2 gene and total antioxidant activity



(Chart 59) Relative expression of SLC23A2 gene and total antioxidant activity – CONTROL GROUP

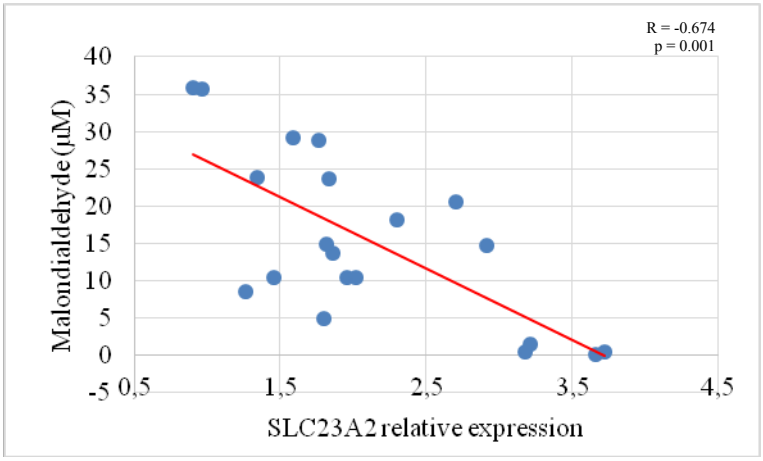


(Chart 60) Relative expression of SLC23A2 gene and total antioxidant activity – DM-DR GROUP

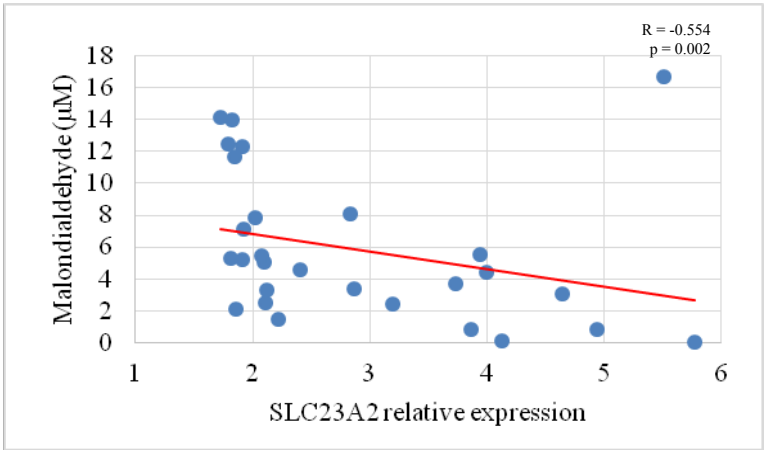


(Chart 61) Relative expression of SLC23A2 gene and total antioxidant activity – DM+DR GROUP

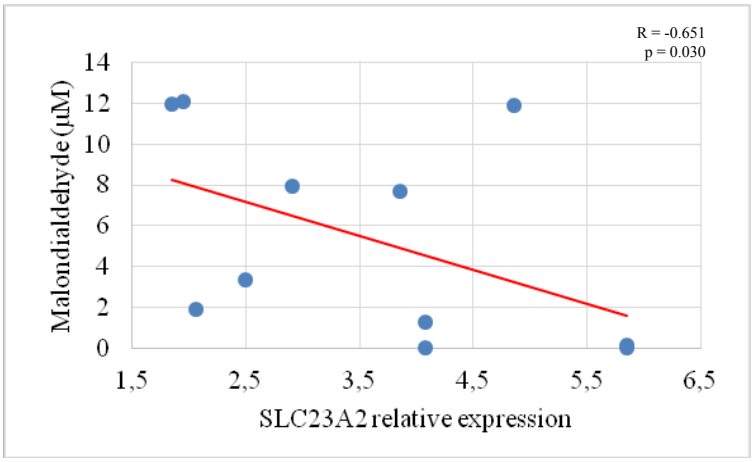
7.2. Relative expression of SLC23A2 gene and malondialdehyde concentration



(Chart 62) Relative expression of SLC23A2 gene and malondialdehyde concentration - CONTROL GROUP



(Chart 63) Relative expression of SLC23A2 gene and malondialdehyde concentration – DM-DR GROUP



(Chart 64) Relative expression of SLC23A2 gene and malondialdehyde concentration – DM+DR GROUP

DISCUSSION

1. About Diabetes Mellitus and the design of this work

Diabetes mellitus (DM) is a chronic metabolic disorder with devastating complications affecting millions of persons worldwide. In fact, DM is now considered a global epidemic, affecting more than 400 million people. The World Health Organization point to DM as one of the first leading causes of death. Moreover, it is a fact that the incidence of DM continues to rise steadily (1-6, 120, 121).

In this scenario, the identification of main risk factors accompanying a hyperglycemic background, as well as the causative environmental factors and lifestyle, will theoretically explain this phenomenon (122). Nonetheless, new preventive strategies have to be developed to counteract this pandemic disease. The Early Activity in Diabetes trial (ACTID) reported that dietary advice could reinforce appropriate glycemic control by preventing a decline in self-care in DM2 adult patients. Because of this, the benefits of this simple intervention draw attention to the pivotal role of diabetic patient education.

A major complication of DM is DR. The diabetic retina is the main responsible of the visual loss in the age frame from 20 to 65 years. As a consequence of this, the social and economic burden of the disease is enormous (123). In fact, proliferative DR and diabetic macular edema are the most important ocular complications related to DM.

There is a high prevalence for the development of DM2 in Spain (124-127). Also in the Spanish Valencian Community, the incidence of DM2 is high.

Because of this, it is critical to address whether or not there can be any genetic susceptibility/resistance for the development of these DM complications in eyes.

The main goal of present work was to illustrate the molecular and genetic aspects of the DR regarding the role of various molecules and candidate genes involved in major DR pathogenic mechanisms. Such as apoptosis, vascular/neural damage, inflammation, oxidative stress and alterations of the extracellular matrix. Also if these can be differentially expressed in DM2 with (+DR) and without (-DR) retinal affectation, to report gender and age distribution of DR incidence and ophthalmic data, which may help in the identification of patient at risk, of the disease and visual loss.

In the context of the Valencia Study on Diabetic Retinopathy (VSDR) (51), a final sample of 81 participants was selected from the databases to be included in this work. Major operative procedures were: 1) interview (*to register personal and familial data*), 2) ophthalmological examination (*best corrected visual acuity, intraocular pressure, ocular fundus examination, and the OCT examination*, 3) blood collection in fasting conditions, that were centrifuged to obtain three separated aliquots for: a) basic biochemistry, b) oxidative stress and a set of molecules (vitamin B12, CRP, total protein content and Hcys) c) candidate genes.

The database of the VSDR has been useful to achieve the patient´s information as well as to schedule the appointments to better prepare the sampling collection, including the analytical procedures for our purposes.

Genes that have been investigated in the present work have been: the apoptotic regulator TP53, the vitamin C transporter SLC23A2, the regulator of the metalloproteinase 9 (MMP9), the mitochondrial protein involved in

vitamin B12 metabolism (MMACHC & MMADHC), the RBP1 gene and the TMCO1 gene.

A question that arises was: why these genes and not others? The response is that these genes have been chosen after an extensive search of scientific literature and previous personal experience of my directors in this work.

As designed, such molecules and genes seem to be ultimately linked with DM, as well as DR. Nevertheless, all these, alone or in collaboration with other factors might prove to be useful in the development of future diagnostic and therapeutic strategies and for better eye care in diabetics. Consequently to prevent visual loss, and to contribute to an improvement of the patient's life quality.

In addition to this preface, in this section the results obtained through the project will be discussed in a stepwise fashion.

2. Socio-demographic Data
3. Ophthalmic Results
4. Classical Biochemistry
5. Molecular Biochemistry of DR
7. Genetics of DR

2. Socio-demographic data

Mean age of the total participants in the study was 50 ± 15 years. According to the main groups, mean age in the DM2 was 52 ± 6 years versus 47 ± 14 years in the controls. Attending to gender, 54.3% of total participants were men versus 45.7% women. In relation to the gender percentages in the main groups: 57.1% men and 42.9% women in the DM2 as well as 50% men and 50% women in the controls. These data regarding age and gender are similar to descriptions from a recent study on DM in a Spanish population (124)

Regarding the DR, data from this study are in agreement, in general, with those reported by Salinero-Fort et al., which were also obtained in a Spanish population (125).

3. Ophthalmic Results

According to The American Academy of Ophthalmology screening for DR includes the eye dilation and examination of the ocular fundus, as well as using validated digital imaging involving 3D retinographies and OCT macular data. These approaches have been used herein to address the diagnosis and classification stage of DR in the DM2 patients.

Our data showed significantly lower values for the BCVA in each eye in the DM2 patients versus the controls, as expected. No statistical differences were obtained when data from the IOP and the OCT examination were processed. In addition, our results demonstrated significantly reduced BCVA and OCT results in macular thickness values in the diabetics' +DR eyes as compared to the control group. This strongly indicates that the retinal images are useful enough to manage the diagnosis and subsequent therapy for the diabetic eyes, as widely demonstrated (126, 127).

The eye care in diabetics includes a medical interview, a standardized ophthalmic examination as well as screening of high quality retinal photographs of patients who have not had previous treatment for DR, and a strict follow-up of these patients. An effective screening program can determine who needs a possible treatment, and who simply requires annual screening, as recommended (128).

4. Classical Biochemistry

Previous works have widely described that intensive control of major risk factors such as hyperglycemia and hypertension are helpful in reducing the onset and progression of DR (125-128).

Higher lipidic profile has been proposed as a risk factor for DR because of its relation to endothelial dysfunction playing a role in retinal exudate formation in the course of DR (125, 126). Large epidemiological studies reached controversial results regarding the association of serum lipids with the severity of DR. In the ETDRS report (125), high total cholesterol and LDL levels were associated with retinal hard exudates. However, other studies failed in demonstrate these conclusions, such as in the Multi-Ethnic Study of Atherosclerosis and the Australian Diabetes, Obesity, and Lifestyle Study (126). Because of this, in this study, we aimed to investigate whether serum lipids have an effect on the development of DR.

Our results confirmed that mean HbA1c values were significantly higher in DM2 vs. the control group of participants. Moreover, these values were also higher in the DM2 +DR group than in the DM2 -DR and the healthy controls. Furthermore, mean total cholesterol, triglyceride, LDL, HDL levels were significantly different between specific subgroups (+DR vs. –DR), as can be seen in the corresponding tables of the results section.

Regarding other parameters, it has been shown that the vitamins B9 and B12 as well as the Hcys displayed different levels in the diabetics with or without retinopathy as compared to the healthy controls. All together these data suggest that the above molecules may be presumptive candidate biomarkers for the risk of developing and monitoring DR in DM2 patients, in agreement with previous reports (51).

Also, our data pointed to HDL and TG significant differences between men and women diabetics vs. the controls, which is an interesting point to consider for future research regarding gender disparities in the development of DR.

In spite of the interesting data from the present work regarding the lipidic profile and DR, and the reports of other authors (127, 128) large multi-centric prospective studies are needed to clarify the reasons of the discrepancies reported in distinct studies.

5. Molecular Biochemistry of Diabetic Retinopathy

DR is a multifactorial disease involving environmental and genetic factors (19, 42, 43). This microangiopathy is characterized by abnormal growth and leakage of small blood vessels that may result in local edema and functional impairment of the retina and choroid. Several mechanisms leading to the impairment of microcirculation in diabetes have been implicated, and the majority still remains unclear (51-56, 63-67, 125-127).

However, a dysregulated vascular regeneration appears to play a key role. In addition, oxidative and hyperosmolar stress, as well as the activation of inflammatory pathways triggered by advanced glycation end-products and toll-like receptors, increased polyol pathway flux, activation of PKC, etc., have been recognized as key underlying events. Therefore, searching for

biomarkers of the risk of developing DR is a challenge for researchers worldwide.

General strategies for the prevention of DR should be aimed at the identification of biomarkers for the risk factors and the main pathological mechanisms for better eye care in DM2.

Our work demonstrated the importance of oxidative stress biomarkers for DR, in agreement with previous works (51, 63-71). As a consequence of all data gathered during the present work, we may suggest that MDA and TAC can be useful as biomarkers for the risk of DR in DM2.

6. Genetics of Diabetic Retinopathy

On the role of genetics of DR, studies reported contradictory findings. Genetic susceptibility to DR has also been suggested by some authors. In the Veterans Affairs Diabetes Trial, it was described that the prevalence of moderate to severe DR was higher for Hispanics and African-Americans than for non-Hispanic whites (128). However, ethnicity could not explain by itself the differences in the DR prevalence in another population study (127).

Furthermore, no genetic variants meet criteria of useful diagnostic marker; neither significantly elucidates the basis of DR development/progression.

Current knowledge of the genetics of DR is quite limited. In spite of the strong scientific evidence suggesting that DR is a heritable trait, linkage studies, candidate gene association studies and the genome wide association study (GWAS) have failed to reveal any reproducible loci for DR (127).

Among the most relevant genes involved in DR are: polyol genes, growth factors genes, advanced glycation end products, cytokines genes,

transcription factors, etc. Based on these concepts it is necessary to go ahead with specific genetics research in DR. In this context, new possibilities and approaches associated with utilization of outstanding technologies such as the next-generation sequencing or comprehensive read analysis for identification of single nucleotide polymorphisms from pooled sequencing (CRISP) assays are now available.

Our work is conclusive in respect to the analyzed genes.

TP53, MMP9 and MMACHC/MMADHC genes showed higher expression values whereas the SLC23A2 gene exhibited significantly lower values in the DM2 patients vs. the controls. In addition, TP53, SLC23A2 and MMP9 genes showed a differential expression profile in DM2 +DR vs. DM2 –DR.

As previously reported, it can be suggested that genes related to the regulation of apoptosis and the integrity of the extracellular matrix, altogether with genes involved in the metabolism of antioxidant vitamins and cardioprotective/neuroprotective molecules can be involved in the risk of developing DR (125).

CONCLUSION

1. The present study confirms the importance of controlling the lipid profile in DM2 patients for outstanding diabetic eye care.

2. From a molecular viewpoint regarding DR, the vitamins B9, B12 and Hcys are presumptive candidate biomarkers for the risk of developing and monitoring DR in DM2 patients.

3. Searching for molecular biomarkers of DR, our data strongly suggest that oxidative stress is a relevant pathogenic mechanism. Both the MDA and TAC can be utilized as biomarkers of oxidative stress in DM2 patients at risk of developing DR.

4. Among the eight studied genes, the MMP9 gene may be consider an outstanding genetic biomarker of susceptibility to DR.

5. Among the eight studied genes the SLC232A2 can be highly considered as a protective factor for the DR development and progression.

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18 MESESEVALUACIÓN

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