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NEW CLINICAL STRATEGIES TO DETECT CARDIOVASCULAR DISEASE IN SUBJECTS WITH TYPE 2 DIABETES

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DOCTORAL THESIS PRESENTED BY

ALEJANDRA PLANAS VILASECA

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Directors:

Cristina Hernández Pascual PhD

Olga Simó Servat PhD

Tutor:

Rafael Simó Canonge PhD

Programa de doctorat en Medicina, Departament de Medicina
Universitat Autònoma de Barcelona.

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Abbreviations

AACE/ACE 2020: Association of Clinical Endocrinologists and American College of Endocrinology on the management of dyslipidemia and prevention of cardiovascular disease algorithm

AGE: advanced glycation end-products

ALT: alanine transaminase

ASCVD: atherosclerotic cardiovascular disease

AST: aspartate aminotransferase

AU: Agatston units

AUROCs: area under the receiver operating characteristic curves

AUs: arbitrary units

BMI: body mass index

BMs: basement membranes

CACs: Coronary Artery Calcification Score

CAD: coronary artery disease

CHD: coronary heart disease

CIMT: carotid intima-media thickness

CKD: chronic kidney disease

COX 2: cyclooxygenase 2

CT: computed tomography

CV: cardiovascular

CVD: cardiovascular disease

CVE: cardiovascular events

DAG-PKC: diacylglycerol-proteinkinase C

DN: diabetic neuropathy

DR: diabetic retinopathy

DRS: Diabetic Retinopathy Study

ECM: extracellular matrix

ERM: ezrin, radixin, and moesin

ETDRS: Early treatment diabetic retinopathy Study

GFR: glomerular filtration rate

GGT: gamma-glutamyl transferase

HDL: high-density lipoprotein

HR: hazard ratio

HUVEC: human umbilical vein cell

LDL: low-density lipoproteins

NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells

NO: nitric oxide

NOS: nitric oxide synthase

NPDR: non-proliferative diabetic retinopathy

O-GlcNAc: O-linked N-acetylglucosamine

OR: odds ratio

PDR: proliferative diabetic retinopathy

PG: proteoglycans

PGI synthase: prostacyclin synthase

RAGEs: receptors for AGEs

RCTs: Randomized clinical trials

ROS: reactive oxygen species

SAF: skin autofluorescence

SMC: smooth muscle cells

T2D: type 2 diabetes

TG: Triglyceride

UKPDS: UK Prospective Diabetes Study

VEGF: vascular endothelial growth factor

VSMC: Vascular smooth muscle cells

Resumen

Los pacientes con diabetes tipo 2 presentan un mayor riesgo de enfermedad macrovascular, siendo éste de dos a cuatro veces mayor en comparación con los pacientes no diabéticos. A pesar que la diabetes tipo 2 es un factor de riesgo independiente de enfermedad cardiovascular (ECV), no todos los pacientes con diabetes parecen tener el mismo riesgo. De hecho, un alto porcentaje de estos pacientes nunca experimentarán complicaciones macrovasculares. Por lo tanto, la identificación precoz de pacientes diabéticos con riesgo de desarrollar ECV sigue siendo un desafío.

Es conocido que la hiperglucemia crónica está relacionada con las complicaciones cardiovasculares de la diabetes. Sin embargo, el riesgo exagerado de ECV en esta población no se explica completamente por los factores de riesgo clásicos como la obesidad, la hiperglucemia, la dislipidemia y la hipertensión; de hecho, una proporción sustancial de este riesgo permanece sin explicación. Por lo tanto, se cree que condiciones específicas relacionadas con la diabetes deberían estar involucradas en el exceso de riesgo de ECV en esta población, y la acumulación tisular de productos finales de glicación (AGEs) podría ser uno de ellos. Los AGEs se acumulan en el cuerpo durante el envejecimiento, y este proceso se acelera por la hiperglucemia crónica y el estrés oxidativo, dos condiciones presentes en la diabetes tipo 2. Por tanto, la formación y acumulación de AGEs están aceleradas en la diabetes mellitus y podrían contribuir a la disfunción vascular y al desarrollo acelerado del proceso aterosclerótico. En los últimos años se ha validado un método simple y no invasivo para la evaluación de AGEs a través de la autofluorescencia de la piel (SAF) y se ha relacionado con la presencia de micro y macroangiopatía en sujetos con diabetes tipo 2.

Múltiples estudios indican que la presencia de complicaciones microvasculares de la diabetes aumenta el riesgo de ECV. En particular, la retinopatía diabética (RD) se ha relacionado con un aumento en el riesgo de mortalidad cardiovascular y de muerte por cualquier causa en pacientes con diabetes. En este sentido,

cabe señalar que las alteraciones microvasculares inducidas por la diabetes que se producen en la retina también pueden surgir en otros lechos vasculares, como la microcirculación miocárdica. Sin embargo, la RD a menudo no se considera un factor de riesgo en los estudios dirigidos a evaluar las ECV.

En base a ello, el principal objetivo de esta tesis doctoral es desarrollar nuevas estrategias clínicas para identificar mejor a aquellos pacientes con DM2 con alto riesgo de desarrollar un evento CV.

En la primera parte de esta tesis doctoral, se plantean dos objetivos específicos. En primer lugar, examinar si la carga y el grado de microangiopatía es un factor de riesgo independiente para determinar la presencia de ECV subclínica; en segundo lugar, examinar la utilidad de la medición no invasiva de AGEs en la piel y su relación con las complicaciones diabéticas y la ECV subclínica, en sujetos diabéticos sin antecedentes de ECV. Para evaluar estos objeticos, se realizó un estudio prospectivo de casos y controles que incluyó 200 sujetos diabéticos tipo 2 sin antecedentes de ECV y 60 sujetos no diabéticos pareados por edad. La presencia de ECV subclínica se examinó utilizando dos parámetros: el score de calcio arterial coronario (CACs) y una combinación de CACs> 400 UA, placa carotidea ≥ 3 mm, grosor de íntima-media carotidea> 1, o la presencia de cambios en el ECG que sugieran un infarto de miocardio previo. En este estudio evidenciamos que la RD es un predictor independiente de ECV subclínica y que SAF es un buen predictor de CAC> 400 AU (un marcador fiable de aterosclerosis coronaria). Por lo tanto, la evaluación de la RD y de SAF puede plantearse como una buena estrategia que nos ayude a seleccionar una población diabética de alto riesgo CV en la que se deben centrar las estrategias encaminadas a reducir los factores de riesgo CV y optimizar el control metabólico.

En la segunda parte de esta tesis doctoral, el objetivo fue evaluar si la presencia de retinopatía diabética y la acumulación en el tejido subcutáneo de AGEs pueden ayudar a identificar pacientes con diabetes tipo 2 con alto riesgo de desarrollar un evento CV. Para ello, hemos realizado un seguimiento de la misma cohorte hasta presentar un evento CV. Hemos definido evento CV como un compuesto de infarto de miocardio, revascularización coronaria, ictus,

amputación de miembros inferiores o muerte CV. Tras un seguimiento de 4,35 años, se registraron un total de 24 eventos CV, 23 eventos CV en el grupo de diabetes y 1 en el grupo control de no diabéticos. Consecuentemente, confirmamos que los pacientes con diabetes tipo 2 tiene significativamente más riesgo de sufrir un evento CV que los sujetos no diabéticos. Además, evidenciamos que la RD y SAF (como medida de la acumulación de AGEs en la piel) son eficaces predictores de eventos cardiovasculares en sujetos con diabetes tipo 2. En conclusión, reconfirmamos que los pacientes con diabetes tipo 2 tienen significativamente más eventos cardiovasculares que los sujetos no diabéticos. Asimismo, RD y valores altos de SAF son importantes predictores de eventos CV en sujetos con diabetes tipo 2 y, por lo tanto, podrían incluirse como variables significativas en la estratificación del riesgo CV. Además, RD y valores altos de SAF podrían considerarse como biomarcadores útiles en la selección de pacientes diabéticos tipo 2 en los que se debe priorizar el cribado de enfermedad cardiovascular.

Este trabajo contribuye a la identificación de nuevos marcadores de riesgo cardiovascular que pueden ayudarnos a seleccionar aquellos pacientes con mayor riesgo cardiovascular y en los que el cribado de ECV puede ser rentable. Además, nuestros hallazgos podrían tener implicaciones tanto terapéuticas como de investigación. De hecho, la detección y clasificación de la RD y la evaluación de SAF nos permiten identificar pacientes con alto riesgo CV que, según las guías actuales, se beneficiarían más de una reducción más agresiva del perfil lipídico, y del control metabólico. Además, este hallazgo podría usarse para enriquecer las cohortes para futuros ensayos clínicos de intervención con pacientes más propensos a desarrollar eventos cardiovasculares, reduciendo así el tamaño de la muestra, la duración y el coste de los estudios. Por otra parte, un enfoque holístico e integrador que comprendiera los factores de riesgo CV clásicos, las variables clínicas, SAF y DR, nos podría ayudar a crear un score que permitiría identificar mejor a aquellos pacientes con DM2 con riesgo CV muy alto.

Abstract

Type 2 diabetes confers a substantial burden of macrovascular disease, with two to four-fold increased risk of any cardiovascular event in comparison with non-diabetic patients. Although type 2 diabetes is an independent risk factor for cardiovascular disease (CVD), not all patients with diabetes appear to be at equal risk. In fact, a high percentage of these patients will never experience vascular complications. Therefore, early identification of diabetic patients at risk of developing CVD remains a challenge to be met.

It is well known that chronic hyperglycemia is related to cardiovascular (CV) complications of diabetes. However, the exaggerated risk for CVD in this population is not explained fully by conventional risk factors such obesity, hyperglycemia, dyslipidemia and hypertension, and, in fact, a substantial proportion of this risk remains unexplained. Therefore, specific diabetes-related risk factors should be involved in the excess risk for CVD, and the tissue accumulation of advanced glycation end-products (AGEs) could be one of them. AGEs accumulate in the body during aging, and this process is accelerated by chronic hyperglycemia and oxidative stress, two conditions commonly present in type 2 diabetes. Therefore, the formation and accumulation of AGEs are accelerated by the diabetic milieu and contribute to vascular dysfunction and the accelerated development of atherosclerotic processes. In recent years, a simple and non-invasive method for AGEs assessment through skin autofluorescence (SAF) has been validated and related to the presence of micro- and macroangiopathy in individuals with type 2 diabetes.

Emerging data indicate that the presence of diabetic microvascular complications increases the risk of CVD. In particular, diabetic retinopathy (DR) has been linked with an increase in risk for all-cause and cardiovascular mortality in patients with diabetes. In this regard, it should be noted that diabetic-induced microvascular abnormalities that occur in the retina may also arise in other vascular beds, such as myocardial microcirculation. However, DR is often missing as a risk factor in studies addressed to evaluate CVD.

On this basis, the main objective of this doctoral thesis is to develop new clinical strategies to better identify those patients with T2D at high risk of developing a CV event.

In the first part of this doctoral thesis, there are two specific objectives. First, to examine whether the burden and degree of microangiopathy is an independent risk factor for subclinical CVD; second to examine the usefulness of non-invasive measurement to determine skin AGEs and their relationship to diabetic complications and subclinical CVD, in diabetic subjects with no history of clinical CVD. For this purpose, a prospective case-control study comprising 200 type 2 diabetic subjects without history of clinical CVD and 60 age-matched non-diabetic subjects. The presence of subclinical CVD was examined using two parameters: calcium coronary score (CACs) and a composite of CACs >400 UA, carotid plaque ≥3 mm, carotid intima media thickness ratio >1, or the presence of ECG changes suggestive of previous asymptomatic myocardial infarction. We provided evidence that DR is an independent predictor of subclinical CVD and that SAF is a good predictor of a CACs > 400 AU (a reliable marker of coronary atherosclerosis). Therefore, assessment of DR and SAF measurement can be envisaged as a method to help us to select a high CV risk diabetic population in whom the strategies aimed at reducing CV risk factors and optimizing metabolic should be focused.

In the second part of this doctoral thesis, the aim was to evaluate whether the presence of diabetic retinopathy and the accumulation of advanced glycation end-products (AGEs) in subcutaneous tissue can help to identify patients with type 2 diabetes at high risk of developing CV events. For this purpose, we conducted a follow-up of the same cohort. The primary outcome was the time to the first CV event. We defined a CV event as a composite of myocardial infarction, coronary revascularization, stroke, lower limb amputation or CV death. After a follow-up of 4.35 years, a total of 24 CV events were registered, 23 CV events in type 2 diabetes group, and 1 in the non-diabetic control group. We confirmed that patients with type 2 diabetes had significantly more risk of suffering a CV event than non-diabetic subjects. Furthermore, we provide evidence that DR and SAF (as a measure of tissue AGE accumulation) are powerful predictors of CV events

in subjects with type 2 diabetes. In conclusion, we reconfirmed that patients with type 2 diabetes have significantly more CV events than non-diabetic subjects. In addition, DR and higher values of SAF are powerful predictors of CV events in subjects with type 2 diabetes and, therefore, might be included as meaningful variables in CV-risk stratification. Furthermore, DR and higher values of SAF could be useful biomarkers in selecting type 2 diabetic patients in whom the screening for cardiovascular disease should be prioritized, thereby generating more personalized and cost-effective medicine.

Overall, this work contributes to the identification of new cardiovascular risk markers that can help us to select those patients with the highest cardiovascular risk in whom CVD screening can be cost effective. Moreover, our findings could have both therapeutic and investigational implications. In fact, the detection and grading of DR and assessment of SAF permits us to identify patients at high CV risk who might, under current guidelines, benefit most from further lipid and blood glucose lowering. In addition, it could be used to enrich the cohorts for future intervention trials with patients more prone to develop clinical outcomes, thus reducing sample size, duration, and the cost of studies. Moreover, a holistic and integrative approach taking into account classical CV risk factors, clinical variables and SAF and DR, will allow us to create a score that will permit better identify those T2D patients with very high CV risk.

1. Introduction

1.1. Cardiovascular risk in type 2 diabetes.

1.1.1. Epidemiology

Diabetes mellitus affects more than 500 million people worldwide, and its prevalence is increasing. Most of the burden of this chronic disease comes from its complications, and given that diabetes is expected to increase from 537 million in 2021 to 783 million by 2030, the complications derived from diabetes will become even a more serious problem in the future (1).

Diabetes mellitus is one of the leading causes of mortality and major morbidities, including cardiovascular disease (CVD), chronic kidney disease, and blindness in the working age population (1). In addition, the high frequency of micro and macrovascular complications have a devastating effect in the quality of life for people with diabetes (2).

Healthcare costs for diabetic subjects are more than the double of the costs for those without diabetes (3), and the occurrence of major diabetes related complications in type 2 diabetic patients is associated with increased average medical costs (4). In this regard, it has been reported that the consumption of health care resources was almost the double in type 2 diabetic patients with macrovascular complications compared to patients without them (5).

Fortunately, in the last two decades we have seen reductions in the mortality and the incidence of cardiovascular (CV) complications among adults with diabetes (6). Probably, the increasing emphasis on integrated care of patients with chronic disease, improved patient education in disease management, and advancements in clinical decision-making support have probably reduced the rates of cardiovascular complications among patients with diabetes (7). Advances in revascularization and increased use of glucose-monitoring systems may have also played a role (8). Moreover, and probably most importantly, the improved management of risk factors such as hypertension, high levels of LDL cholesterol and glycated hemoglobin and the increasing use of statins and antihypertensive medications had led to a reduction in cardiovascular risk (9–12).

Despite the improvement in cardiovascular risk prevention and the tendency to decrease the incidence of cardiovascular disease in patients with diabetes, CVD continue to be the leading cause of death among subjects with diabetes, in whom adverse cardiovascular outcomes occur, which on average is 14.6 years earlier and with increased severity compared to individuals without diabetes (13,14).

People with type 2 diabetes (T2D) have two-fold increased risk of developing CVD (13,14), and two to four times more risk of dying from CVD. Atherosclerotic disease is the main cause of morbi-mortality in people with diabetes: up to 80% will die for this reason, being coronary heart disease the most frequent cause (15). In addition, cardiovascular complications are the most common cause of hospitalization in diabetes. It is estimated that T2D diagnosis reduces 7 years life expectancy (15).

Subjects with T2D have a high prevalence of hypertension (77-87%), dyslipidemia (74-81%) and obesity (16). It is known that CV risk in diabetes is associated to sex, age, duration of diabetes and the rest of classical risk factors (17). However, the exaggerated risk for CVD in this population is not fully explained by conventional risk factors such as obesity, hyperglycemia, dyslipidemia, and hypertension, and in fact a substantial proportion of this risk remains unexplained (18,19).

1.1.2. Concept and pathogenesis of atherosclerosis

CVD is defined as a group of disorders of the heart and blood vessels including coronary heart disease, cerebrovascular disease, heart failure and peripheral artery disease. The main cause of CVD is the early development of atherosclerotic lesions.

Atherosclerosis is a chronic disease that affects the arterial wall, causing the uncontrolled accumulation of lipids. This accumulation causes a narrowing of the blood vessel lumen and can lead to the occlusion of the vessel and trigger an ischemic process in the surrounding tissue. The histological course of atherosclerosis in patients with diabetes follows almost the same course as in people without diabetes.

Atherosclerosis is a complex process that develops silently for several decades and has inflammatory, immunological, proliferative, and thrombotic components. Below we will explain in more detail this complex process (20).

The Stary classification (**Figure 2**) defines the different evolutionary phases of the atherosclerotic lesion. The incipient lesion is usually the fatty streak (type 2 lesion) and the fully formed lesion is the atherosclerotic plaque (type 4). Generally, two phases are distinguished in the progression of the atherosclerotic lesion; an initial phase in which the arterial wall progresses to the formation of an atherosclerotic plaque; and an advanced phase where the atherosclerotic plaque grows, causing stenosis of the blood vessel and the appearance of clinical complications (20).

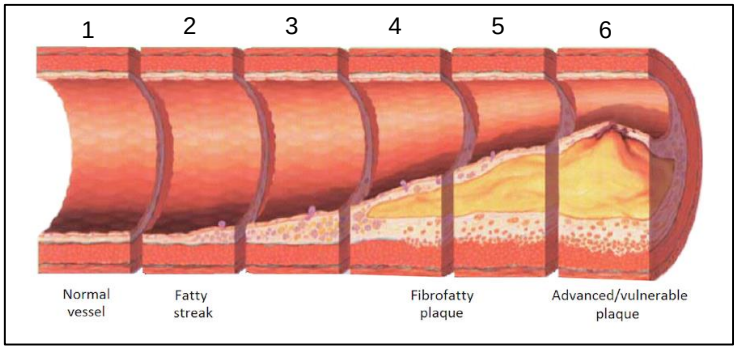


Figure 2. Stary classification: evolutionary phases of the development of the atherosclerotic lesion.

<u>Early stages of atherosclerotic lesion</u>:

Russel Ross, in the 1990s, postulated that endothelial dysfunction was the initial mechanism in the development of atherosclerosis (21). The atherosclerotic lesion begins as a response of the endothelium to an external aggression, such as a high concentration of cholesterol, hypertension, oxidative stress, hyperglycemia, etc. The result of this aggression is a dysfunction in the gene expression of endothelial cells, causing a decrease in the synthesis of nitric oxide synthase (NOS) and prostacyclin synthase (PGI synthase), two key enzymes for maintaining arterial homeostasis. Moreover, it increases the activity of cyclooxygenase 2 (COX 2), which generates the powerful vasoconstrictor

thromboxane A2. It also increases the expression of inflammatory cytokines, which initiates the inflammatory process which is characteristic of the atherosclerosis process.

Some years later, William et al, proposed a new hypothesis about atherosclerosis pathogenesis, called the response-to-retention hypothesis and it is complementary to endothelial dysfunction theory (22). They suggested that the initial event is the retention of lipoproteins, mainly low-density lipoproteins (LDL), in the subendothelial space. Typically, lipoproteins can cross the endothelium by a natural phenomenon called endothelial transmigration. Any factor that alters the balance of input and output of lipoproteins can cause the accumulation of cholesterol in the tunica intima. Thus, hypercholesterolemia will favor the entry of LDL into the tunica intima, while the alterations and physical-chemical modifications both in the LDL particles and in the proteins of the extracellular matrix mediated by oxidative stress, cause the retention of these particles.

It is difficult to know which of the two events, endothelial dysfunction or LDL retention, occurs first, although it is likely that they occur at the same time and potentiate each other. In any case, the result is that a localized inflammatory focus is created, and so the atherosclerotic plaque is formed, characterized by a large lipid-rich necrotic core surrounded by a fibrous layer of PG and collagen, that provides momentary stability to the atherosclerotic lesion, known as an advanced stable atherosclerotic lesion (23).

Advanced stages of atherosclerotic lesion:

Normally the presence of an atherosclerotic plaque does not cause symptoms. However, this lesion will continue to evolve, getting bigger, increasing inflammation and favoring cell proliferation. A proliferation of SMCs in the tunica media occurs, causing different alterations. Not only, there is a loss of contractile function and an alteration of the ability to regulate blood pressure; but also, SMC also acquire the ability to capture lipoproteins, transforming into foam cells and increasing the size of the lesion. In addition, there is a loss of consistency of the fibrous layer of the atherosclerotic lesion, generating a weak plaque, which is easily broken by the force of the bloodstream. Atherosclerotic plaque rupture is

the key event that will cause platelet aggregation and thrombus formation, and therefore, clinical events will appear (20).

1.1.3. Atherosclerosis and diabetes: specific conditions.

The evolution of atherosclerotic lesions in diabetes is similar to other non-diabetic patients with atherosclerosis. However, diabetes is characterized by specific alterations that are involved in most phases of atherosclerotic plaque development, accelerating the process. These conditions are mainly hyperglycemia and insulin resistance; both present in subjects with type 2 diabetes. It is known that patients with type 2 diabetes mellitus have greater atherosclerotic plaque burden, higher atheroma volume, more calcified lesions and smaller coronary artery lumen diameter than persons without diabetes mellitus (24).

Role of hyperglycemia:

Diabetes mellitus is a heterogeneous disorder defined by the presence of hyperglycemia, and despite markedly different genetic and mechanistic causes, both type 1 and type 2 diabetes mellitus are associated with higher prevalence of atherosclerotic cardiovascular disease (ASCVD). Therefore, it is natural to consider hyperglycemia among the causes for accelerated ASCVD observed in patients with diabetes mellitus. There is strong evidence demonstrating greater risk for ASCVD with increasing glycemia, with an estimated 11% to 16% increase in cardiovascular events for every 1% increase in HbA_{1c} (9).

Hyperglycemia causes the exacerbation of two highly pathological molecular processes, non-enzymatic glycation and oxidative stress. Both processes are closely interrelated. The glycation of certain proteins alters their function, promoting oxidative stress and, on the other hand, oxidative stress stimulates chemical reactions that promote the formation of advanced glycation end products (later, we will discuss in detail the role of advanced glycation end products in CVD). Both processes are also responsible for the inflammatory response that occurs in atherosclerotic plaques.

Role of insulin resistance:

Insulin resistance (IR) has been considered a strong predictor of ASCVD. IR is defined as an inadequate response of insulin-sensitive target tissue to insulin-mediated cellular action, accompanied by hyperinsulinemia as a compensatory response. IR is closely related to visceral adiposity and obesity, and it results in a wide range of deleterious metabolic derangements, including hyperglycemia, hypertension, and dyslipidemia. Persistent exposure to IR can lead to an increased risk of metabolic syndrome, T2DM, and CVD (25).

Impairment of insulin signaling at multiple points in the insulin signaling pathway in endothelial cells, VSMC, and macrophages promotes the development and progression of atherosclerosis. Moreover, insulin resistance promotes a proinflammatory state further contributing in atherosclerosis (26).

Therefore, both insulin resistance and hyperglycemia trigger the activation of the same proinflammatory pathways, which contribute to the development of atherosclerosis. Insulin resistance is very important at the beginning of the atherosclerotic process, contributing significantly to the initial development of atherosclerosis. In Figure 3 we can see the distinct and overlapping mechanisms by which hyperglycemia and IR promotes atherogenesis and accelerates the progression of atherosclerosis **(Figure 3)**.

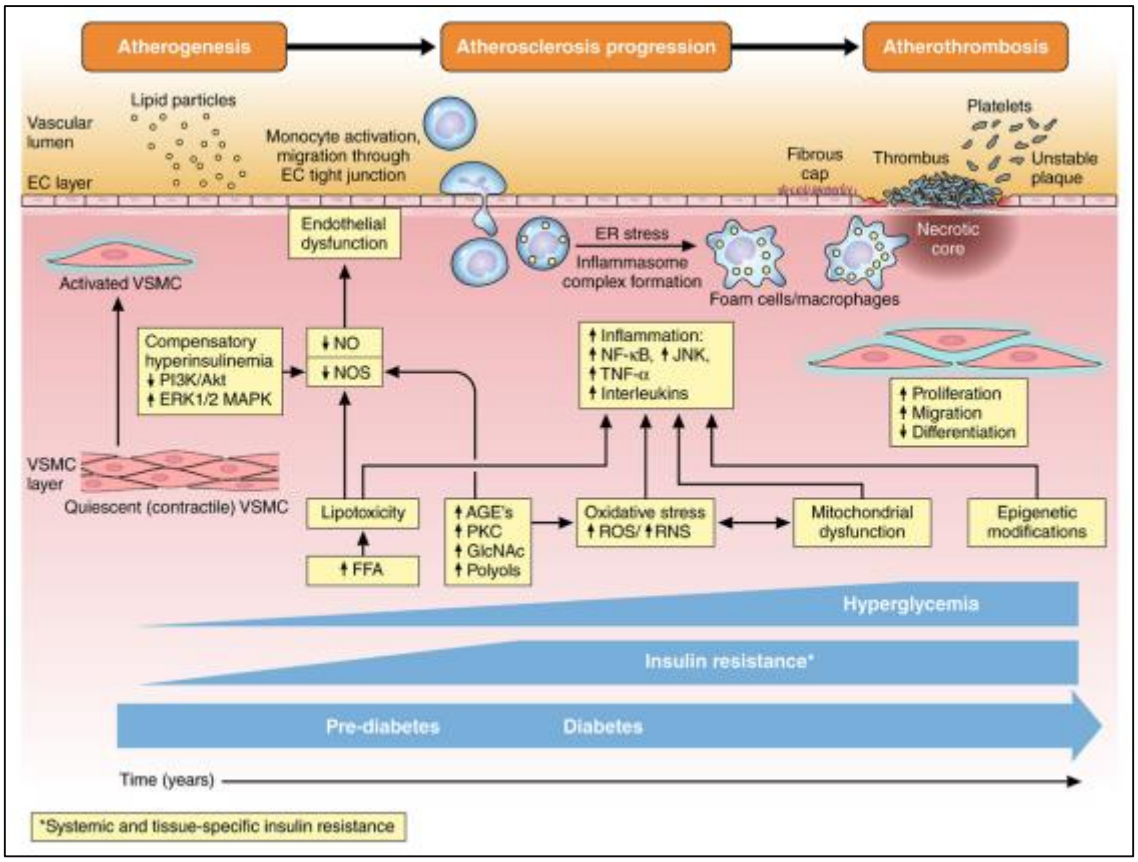


Figure 3. Development and progression of atherosclerosis in diabetes mellitus (19).

<u>Role of vascular calcification</u>:

Individuals with diabetes mellitus are more likely to have calcified atherosclerotic lesions. Underlying mechanisms for this may be related to the role of hyperglycemia with development of AGEs which accelerate vascular calcification. Hyperglycemia also leads to increased post-translational protein modification, including modification by O-linked N-acetylglucosamine (O-GlcNAc). O-Glc-N-acylation starts a cascade of proatherogenic pathways which potentiates vascular calcification. In addition, altered regulation of osteoprotegerin and osteocalcin may promote arterial calcification in diabetes mellitus. Lastly, as noted above, diabetes mellitus is associated with arterial inflammation with increased levels of tumor necrosis factor-alpha, which is a mediator of arterial calcification (27).

1.1.4. CV risk assessment in diabetes

The relationship between diabetes and cardiovascular risk has been a matter of debate during the past two decades. Several studies confirmed the importance of diabetes mellitus as a CV risk factor in diverse populations and suggested diabetes mellitus as a risk equivalent for established coronary heart disease. Persons with diabetes mellitus but without known cardiovascular disease have high risk of myocardial infarction (20.2% incidence over 7 years), similar to individuals with a previous myocardial infarction but no diabetes mellitus as shown in the East–West study conducted within the Finnish population (28). Likewise, comparable hazard ratios for cardiovascular death were found in people aged ≥30 years with diabetes mellitus requiring glucose-lowering medications but without previous myocardial infarction in comparison with people with previous myocardial infarction, among 3.3 million individuals from Denmark, including 71801 with diabetes mellitus and 79575 with previous myocardial infarction but without diabetes mellitus (29). These studies led to the National Cholesterol Education Program- Adult treatment Panel III that defined T2D as a risk equivalent for established coronary heart diseases (CHD) (30).

Although T2D is recognized as an independent risk factor for CVD, and sometimes is considered as a risk equivalent of CHD not all patients with diabetes appear to be at equal risk. In fact, a significant number of these patients will never experience cardiovascular complications. Actually, the early identification of diabetic patients at risk of developing CVD remains a challenge, and the high prevalence of T2D precludes implementing a generalized screening for CVD in asymptomatic patients (31,32). It is well known that chronic hyperglycemia is related with chronic complications of diabetes. However, two large studies revealed that tight glucose control slightly but not significantly reduced the risk of cardiovascular disease in either type 1 (33) or type 2 diabetes patients (34). Moreover, the traditional risk factors associated with diabetes (ie, hypertension, dyslipidemia, obesity) are estimated to contribute in less than 40% of CV events. In fact, these factors are not useful for identifying diabetic subjects with subclinical CVD patients in which the screening could be cost-effective (18,19). Therefore, cheap and routinely available measures that identify those patients at high risk of developing CVD are needed. Accurate CVD and CVD risk prediction is important

to identify diabetic patients who may benefit from primary preventive pharmacological therapies while avoiding unnecessary therapies in those who are at low risk.

Cardiovascular risk assessment scores for people with diabetes

Multiple guidelines recommend the calculation of individual cardiovascular risk using risk calculators. Multivariate risk scores have, therefore, been used to predict CVD risk in individuals with diabetes. There are a large number of scores for the general population, but few that are specific to people with diabetes. Whether general population scores can accurately be used in individuals with diabetes is unclear (32). For diabetic population, the older and most commonly used prediction model is the UK Prospective Diabetes Study (UKPDS) risk engine (35). It has been externally validated, and only showed a moderate ability to discriminate between patients who will and will not get an event. Further, there was poor agreement between predicted and actual cardiovascular risk (36). Nevertheless, this risk score was included in the influential National Institute of Health and Care Excellence guideline for the management of diabetes (37). In recent years, diabetes management has changed considerably (e.g. wider use of lipid and blood pressure-lowering agents), which questions the use of such an antiquated model in current clinical practice. Currently, cardiovascular risk equations in the diabetic population are not used in routine clinical practice and their use is not recommended by the main clinical guidelines for diabetes and CVD.

Stratification of cardiovascular risk in individuals with diabetes according to the main clinical guidelines for diabetes and CVD.

As we have seen, risk scores are not useful in cardiovascular risk stratification in subjects with diabetes. For this reason, some clinical guidelines propose cardiovascular risk stratification based on comorbidities and risk factors, such as the following two guidelines: a) Consensus statement by the American Association of Clinical Endocrinologists and American College of Endocrinology on the management of dyslipidemia and prevention of cardiovascular disease algorithm (AACE/ACE 2020); b) 2019 ESC Guidelines on diabetes, pre-diabetes,

and cardiovascular diseases developed in collaboration with the EASD. The first is designed for the management of dyslipidemia and prevention of CVD; and the second is specific for diabetes and CVD.

a) Consensus statement by the American Association of Clinical Endocrinologists and American College of Endocrinology on the management of dyslipidemia and prevention of cardiovascular disease algorithm (AACE/ACE 2020)

AACE has defined 5 risk categories based on the number and severity of major risk factors, each category has goals for LDL-C, non-HDL-C, and apo B levels, proportional to the degree of risk **(Figure 5).** Subjects with diabetes plus established CVD are considered as at extreme risk for ASCVD events; Very-high risk include individuals with diabetes who have 1 or more major risk factor (advanced age, high total serum cholesterol, high non–HDL-Cholesterol, high LDL-cholesterol, low HDL Cholesterol, hypertension, chronic kidney disease, cigarette smoking or family history of ASCVD). Most diabetic patients are included in this category. Finally, they considered that subjects with diabetes with no other risk factor are at high risk (38).

This guideline is interesting because it creates a category of extreme risk that was not previously contemplated in other guidelines. On the other hand, it does not take into account important variables in the diabetic patient which are the presence of microangiopathic complications, metabolic control or the duration of the disease.

ASCVD Risk Categories and Treatment Goals					
Risk category	Risk factors and 10-year risk	Treatment goals (mg/dL)			
		LDL-C	Non-HDL-C	Apo B	TG
Extreme risk	• Progressive ASCVD including unstable angina • Established clinical ASCVD plus diabetes or CKD ≥3 or HeFH • History of premature ASCVD (<55 y, male; <65 y, female)	<55	<80	<70	<150
Very high risk	• Established clinical ASCVD or recent hospitalization for ACS, carotid, or peripheral vascular disease, or 10-year risk >20% • Diabetes with ≥1 risk factor(s) • CKD ≥3 with albuminuria • HeFH	<70	<100	<80	<150
High risk	• ≥2 risk factors and 10-year risk 10-20% • Diabetes or CKD ≥3 with no other risk factors	<100	<130	<90	<150
Moderate risk	• <2 risk factors and 10-year risk <10%	<100	<130	<90	<150
Low risk	• No risk factors	<130	<160	NR	<150

Figure 5. ASCVD risk categories and treatment goals in AACE/ACE -

b) <u>2019 ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD.</u>

This guideline is specific for diabetes, and within the stratification criteria it includes parameters such as the duration of diabetes or the presence of chronic kidney disease or diabetic retinopathy. On the other hand, it is less aggressive in terms of risk categories (since it does not include the extreme risk category) **(Figure 6**).

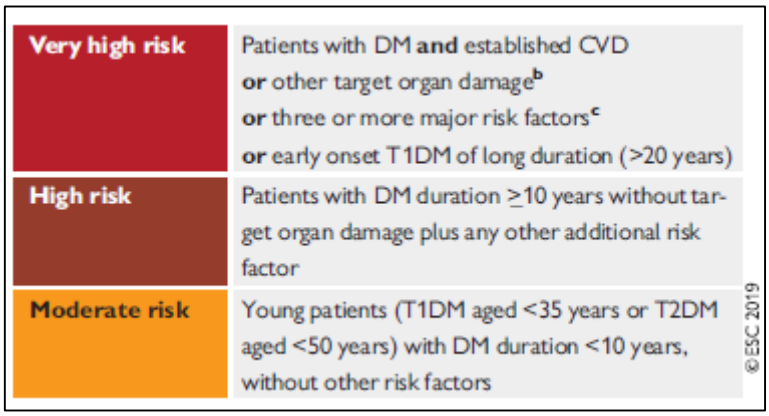


Figure 6. Cardiovascular risk categories in subjects with diabetes according ESC/EASD 2019.

Individuals with DM and CVD or DM with target organ damage (retinopathy, proteinuria, renal impairment or left ventricular hypertrophy), are at very high risk. Patients with DM with three or more major risk factors (hypertension, dyslipidemia, obesity or cigarette smoking), or with a DM duration of >20 years, are also at very high risk. Furthermore, T1DM at the age of 40 years with early onset (i.e. 1 - 10 years of age) and particularly in female individuals is associated with very high CV risk. Most others subjects with DM are high risk, with the exception of young patients (aged <35 years) with T1DM of short duration (<10 years), and patients with T2DM aged <50 years with a DM duration of <10 years and without major risk factors, who are at moderate risk (39).

It should be noted that the main objective of these CV risk classifications in both guidelines is to establish treatment objectives, especially for LDL cholesterol. None of them help us decide whether to perform an imaging test to detect silent cardiovascular disease or to be more aggressive in screening for cardiovascular disease.

Screening for CVD in asymptomatic subjects with diabetes

The strategy of screening patients with diabetes to detect advanced asymptomatic CVD is motivated by the goal of identifying patients with high cardiac risk whose outcomes might be improved through more aggressive risk factor modification, medical surveillance, or revascularization of their coronary artery disease. However, clinical factors that confer risk for adverse cardiac outcomes do not always predict which patients will have abnormal screening tests (40), and negative screening tests in patients with diabetes do not uniformly confer a benign prognosis (41). Similarly, diagnostic tests to detect inducible ischemia or assess atherosclerotic burden such as carotid or femoral ultrasonography do not always identify those patients at risk for plaque rupture and thrombosis, which typically leads to acute coronary events.

Screening for asymptomatic CHD in patients with DM remains controversial. Randomized clinical trials (RCTs) evaluating the impact of routine screening for CHD in asymptomatic DM and no history of CHD have shown no differences in cardiac death and unstable angina at follow-up in those who underwent stress testing, or CTCA, compared with current recommendations (42–44). The low event rates in RCTs and the disparities in the management of screening results (invasive coronary angiography and revascularization were not performed systematically) may explain the lack of benefit of the screening strategy. Further large and appropriately powered trials (with more targeted population) are required to allow a more precise analysis of the magnitude of benefit and to assess pre-specified subgroups in which screening strategies may offer larger benefits. Then, cost-effectiveness studies should assess the financial impact and economic benefits of a CHD screening program in diabetic patients.

Examinations to assessment subclinical atherosclerosis disease

It is important to remark that a diabetic patient with clinically evident atherosclerotic disease involving lower-extremity, cerebral, renal, or mesenteric arteries, is at increased risk for adverse cardiovascular outcomes and might have advanced coronary atherosclerosis (45). Clinical history is important to determine the presence of vascular symptoms (transient ischemic attack, mesenteric ischemia, or claudication), as well as it is the physical exam for bruits and peripheral pulses.

Classic tests for the detection of subclinical CV disease include: a) a diminished ankle-brachial index is a sensitive indicator of increased risk for future CVD events (46), b) a resting ECG, it is a simple, non-invasive and inexpensive technique and may detect evidence of prior myocardial injury or ischemia (47), c) an echocardiography is the first choice test to evaluate structural and functional abnormalities, however has the limitation of being unable to detect small myocardial infarctions that do not cause significant wall motion abnormalities (48), d) carotid or femoral ultrasonography is useful for detecting underlying atherosclerotic disease. The presence of atheromatous plaques in the carotids or in the lower extremities is highly suggestive of coronary atherosclerotic disease. In addition, using carotid ultrasound we can measure the carotid intima-

media thickness (CIMT). CIMT has been widely used as a marker to identify subclinical atherosclerosis, as increased CIMT has been associated with a high prevalence of CHD and future CV events (49).

In recent years, new imaging tests have been developed to assess subclinical CVD. CT coronary angiography (CTCA) makes possible to determine both the degree of luminal stenosis and the morphology of the plaque and can help to stratify asymptomatic subjects with diabetes. Stress testing (exercise or pharmacological) can detect silent ischemia in asymptomatic individual with diabetes, and the most used stress test is the SPECT. However, due to the cost of these examinations, it is not feasible to perform them in all patients with diabetes. Finally, the measurement of coronary artery calcium by means of a computed tomography (CT) is postulated as one of the most important and valuable technique for the detection of subclinical CV disease, which I will discuss in more detail below.

Coronary Artery Calcification Score (CACs):

Quantifying CAC using a non-contrast cardiac CT is an accurate and non-invasive method for assessing burden of subclinical atherosclerosis and can help to identify asymptomatic individuals at higher risk for inducible ischemia.

Extensive data indicate a close relationship between the coronary artery calcium score and clinical coronary events among individuals with and without diabetes (50,51). In addition, it has been shown that the prognostic significance of elevated CACs in predicting coronary events appears to be greater in patients with diabetes than in those without diabetes, and it can further enhance the predictions provided by established risk models (51,52). Moreover, the CACs has been related to moderate to large perfusion defects assessed by scintigraphy (53). For all these reasons, the Imaging Council of the American College of Cardiology concluded that CAC screening is the most sensitive risk stratification tool among asymptomatic persons with diabetes (54). Patients are typically stratified by Agatston units (AU), yielding a CACs <100 (low risk), 100 to 400 (moderate risk), and >400 (high risk). Notably, the finding of a CACs of <10 AU may facilitate risk stratification by enabling the identification of people at very low risk within the

overall high-risk population of diabetic patients (55). In this regard, it should be noted that the absence of coronary calcium confers a remarkably favorable prognosis despite the presence of DM, with no patients experiencing adverse cardiac events during 5 years of follow-up (51). Moreover, Anand et al. (53) studied asymptomatic patients with diabetes and confirmed the higher incidence of inducible ischemia in patients with higher calcium scores. Nearly one-third of those patients had a calcium score >400, and 28% of which had large ischemic defects.

Nevertheless, widespread screening for silent CHD in diabetes cannot be recommended at this time, since RCTs evaluating the impact of routine screening for CHD in asymptomatic have not proved benefit of the screening strategy in diabetic subjects with subclinical cardiovascular disease, and are not cost-effective. Consequently, the identification of a more targeted population in which the CACs would be more cost-efficient seems warranted. In fact, it is currently recommended, by the main clinical guidelines for diabetes and CVD, to perform a CACs in asymptomatic diabetic patients with moderate CV risk.

1.2. Advanced glycation end products in type 2 diabetes

1.2.1. Formation

Advanced glycation end Products (AGEs) are formed by the Maillard process, which is a non-enzymatic glycation of proteins, lipids, or nucleic acids. Protein glycation is mainly started when the carbonyl groups of reducing sugars, such as glucose, interact non-enzymatically with the reactive amino group of proteins, such as lysine or arginine residues. This interaction results in the formation of an unstable aldimine compound, the Schiff base. The Schiff base can be rearranged to produce a stable Amadori product (for example HbA1c), which accumulates on proteins over a period of several weeks. The Amadori product undergoes oxidative degradation to generate highly reactive intermediate dicarbonyl compounds that interact again with free amino groups of proteins. Following complex chemical reactions, a highly heterogeneous, often fluorescent, insoluble, and irreversible group of AGEs is formed, which accumulates and damages long-lived proteins such as extracellular matrix collagen. In summary, in the Maillard process, there are early stage reactions that lead to the formation of early

glycation adducts (such as HbA1c), and later-stage reactions that subsequently form AGEs (56).

1.2.2. Pathogenesis

AGEs accumulate in the tissues with aging. The degree of accumulation of AGEs is associated with increased production and decreased degradation and renal clearance. In patients with diabetes, chronic hyperglycemia accompanied by hyperlipidemia, oxidative/carbonyl stress, and, sometimes, decreased renal function leads to the accumulation of AGEs (57). Accumulation of AGEs could be considered as one of the major pathogenic mechanisms resulting in end-organ damage in subjects with diabetes (58).

The formation and accumulation of AGEs can contribute to diabetic complications mainly by two pathways. First, cross-links can be formed with long-lived proteins in the body such as those constituting the extracellular matrix (ECM) and vascular basement membranes (BMs). These proteins are highly susceptible to AGE modification. Functionally, AGE-mediated crosslinks in BM are known to cause reduced solubility and decreased enzymatic digestion (59). Moreover, AGE formation has shown to affect the three-dimensional nature of BM proteins, thereby causing structural and functional abnormalities. Thus, the presence of AGE on vascular BMs may have direct pathological consequences, particularly in diabetes, who have accelerated formation and accumulation of AGEs.

Second, AGEs can cause deleterious effects by the activation of receptors for AGEs (RAGEs). RAGE is a member of the immunoglobulin superfamily of receptors. AGEs, by interacting with RAGE, trigger the activation of secondary messenger pathways such as protein kinase C. A crucial target of RAGE signaling is nuclear factor (NF)-KB, which is translocated to the nucleus where it increases transcription of a number of proteins, including endothelin-1, intercellular adhesion molecule-1, tissue factor, E-selectin, vascular endothelial growth factor (VEGF), and proinflammatory cytokines and mediators of oxidative stress (60,61). All these molecular mediators are involved in the development of diabetic complications. The main mechanisms by which AGE accumulation

participates in the development of complications in T2D are summarized in **Figure 5.**

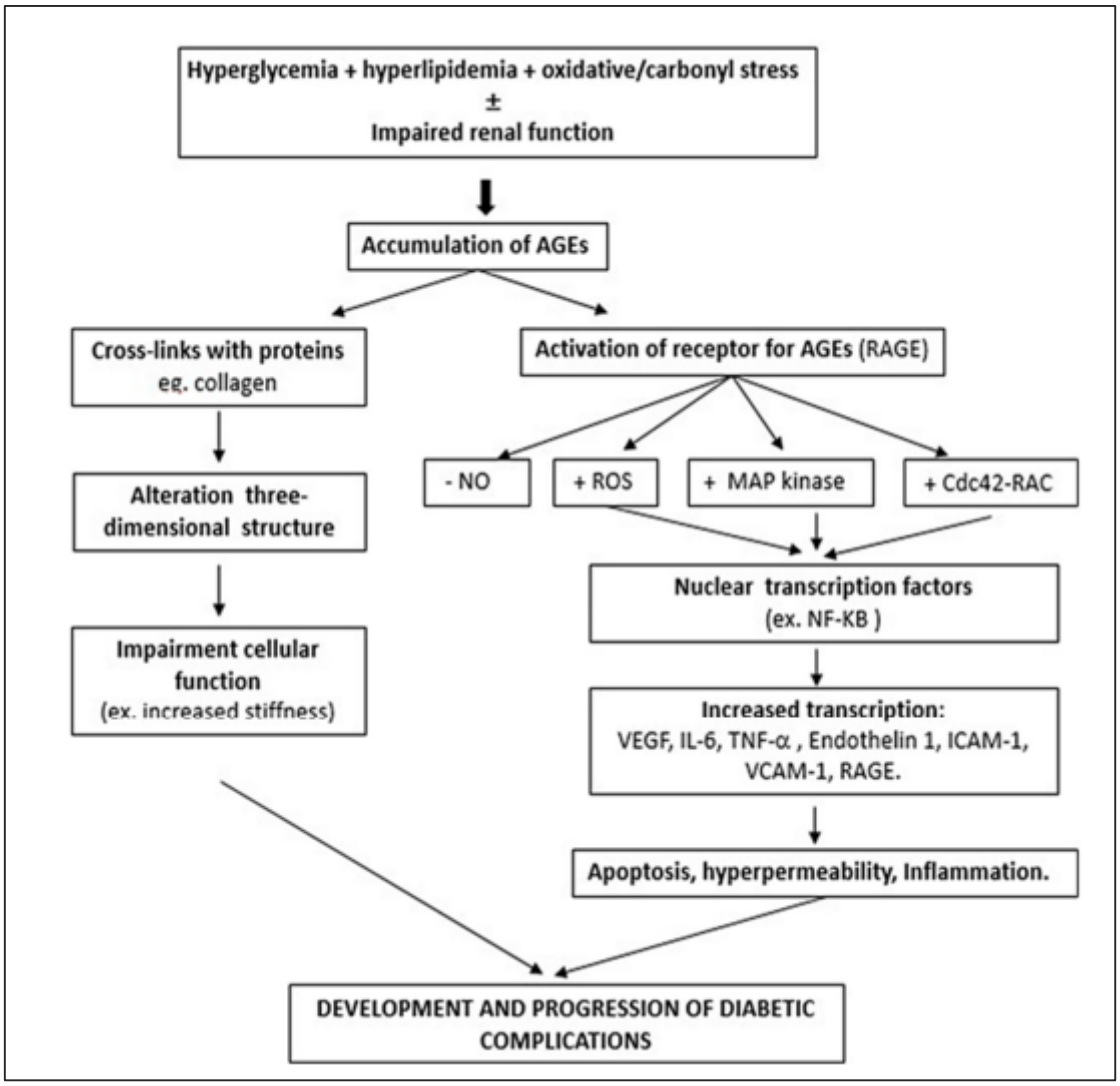


Figure 5. Multi-pathway contribution of AGEs to diabetic complications. NO, nitric oxide; ROS, reactive oxygen species; MAP, mitogen-activated protein; Cdc42, cell division cycle 42 protein; NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor α; ICAM-1, intercellular adhesion molecule-1, VCAM-1 Vascular cell adhesion protein 1.

1.2.3. AGE assessment

The plasmatic determination of AGEs, such us N-ε-carboxymethyl lysine (N-ε-CML) or pentosidine, have been proposed as biomarkers for diabetic complications. Several papers have shown that circulating levels of AGEs in patients with diabetes are associated with the progression of atherosclerosis (62),

renal failure (63), or diabetic retinopathy (DR) (64). However, there are also other studies that did not show the same association (65,66). Circulating AGEs are rapidly broken down to AGE peptides or free AGEs, which are excreted by the kidney, thus having a fast turnover (67). Moreover, biochemical and immunochemical assays for circulating AGE determinations are complex, time consuming, expensive, and of low reproducibility (68). In addition, there is a significant variation with renal function. All these reasons limit their use in current clinical practice. Serum AGEs do not necessary reflect tissue AGE levels.

Since AGEs accumulate in long-lived proteins, it seems reasonable to assess AGEs in accessible tissues such as the skin, where long-lived proteins are present. Skin AGEs are mainly accumulated in collagen, which has a low turnover and represents the diabetic milieu influence over a longer time period than HbA1c; thus, skin AGEs may reflect the impact of both oxidative stress and a history of sustained hyperglycemic episodes (69).

The measurement of HbA1c has been the gold standard for metabolic control. However, there is growing evidence to suggest that ‘‘metabolic memory’’ plays an important role in the development of long-term metabolic complications in diabetic patients (70), and HbA1c only represents the mean level of glycemia over the previous 3 months, but not the long-term exposition to hyperglycemia. Therefore, the use of new biomarkers that reflect the accumulated exposition to hyperglycemia could be useful to predict diabetes outcomes, and skin Ages could be one of them. The first evidence that accumulation of AGEs in skin tissue was related to the presence of micro and macrovascular complications in type 1 diabetes was in 1986 (69). Some years later, the DCCT-EDIC sub study showed that skin AGEs levels measured in biopsy specimens were associated with the development and progression of diabetic complications in type 1 diabetes, even after adjustment for HbA1c (71). Similar results were also reported in type 2 diabetes in the UKPDS (34). Nevertheless, the assessment of AGEs in skin biopsy is not feasible in daily clinical practice.

Based on specific fluorescence of some AGEs, a simple and non-invasive method for skin AGEs assessment has recently been developed through skin autofluorescence (SAF). Skin autofluorescence is measured using an

autofluorescence reader (AGE Reader™ device **(Figure 6)** (DiagnOptics TechnologiesBV, Groningen, the Netherlands)), which illuminates 4 cm2 of the skin surface on the volar side of the forearm, guarded against surrounding light, and uses an excitation light source with a peak excitation of 370. Subsequently, the emitted fluorescence light (within the wavelength range of 420–600 nm) and the reflected excitation light (within the wavelength range of 300–420 nm) from the skin are measured with a spectrometer. SAF is calculated in arbitrary units (AUs) as the ratio between the emitted light and the reflected light, multiplied by 100. A series of three consecutive measurements are carried out, taking less than a minute (72). Notably, it has been demonstrated that SAF has a strong correlation with the specific AGEs, such pentosidine, carboxymethyl-lysine, or carboxyethyl lysine content in skin biopsies (73).

Figure 6. AGE Reader device ™

1.2.4. Skin advanced glycation end products and diabetic complications.

SAF and Diabetic Microvascular Complications

It is well known that SAF values are related with the development of diabetic micro and macrovascular complications, and this is supported by multiple evidence, not only in cross-sectional studies (74–80) but also in prospective trials (81,82). Wang et al. (83) recently published a large cross-sectional study comprising 825 subjects with type 2 diabetes showing that SAF is an independent predictor of T2D complications, including DR, diabetic kidney disease, diabetic cardiovascular disease, and diabetic peripheral neuropathy. Additionally, as the number of complications increases, the SAF value also increases. Hosseini et al. (84), in a systematic review and meta-analysis, suggested that SAF levels could

be a predictor of chronic micro and macrovascular complications in DM. Regarding diabetic nephropathy, the majority of studies has reported a positive association with SAF (66,79), but some of them did not find this association (75,85). It seems that in the kidney, activation of RAGE with AGEs may induce podocyte apoptosis and generation of monocyte chemoattractant peptide-1 and transforming growth factor-β, leading to albuminuria and glomerular sclerosis (86). Moreover, the association between SAF and cardiovascular events has been explored in patients with chronic kidney disease stage 3 (87) and showed a progressive increase in CVE across tertiles of baseline SAF. These findings have not only been seen in subjects with early stages of CKD, but also in patients with end-stage kidney disease(88).

In the case of DR, evidence is controversial. Some studies reported a lack of association between DR and skin AGEs (75,89). However, most recent studies have found a clear independent correlation with development of retinopathy and its severity (77,85,90,91). Interestingly, Takayanagi et al. (91) demonstrated that skin AGEs are not only related with the presence and severity of DR but also with the progression of DR. It is believed that the association between skin AGEs and DR is due to the important role of AGEs in the oxidative stress-induced apoptosis of the retinal pericytes (92). It is known that AGEs can induce intrinsic signaling pathways mediated mainly through RAGEs expressed on the membrane of pericytes, leading to apoptosis (93). Since pericyte function is the main regulator of the basement membrane at the blood retinal barrier, selective pericyte loss leads to disruption of the blood retinal barrier and the development of DR (94). In addition, AGE accumulation upregulates VEGF, a major mediator of diabetic macular edema and proliferative DR (95).

The association between diabetic neuropathy (DN) and SAF has been reviewed recently by Papachristou et al. (96), and the association is quite unanimously. Most evidence shows that increasing SAF levels predicts the development of DN. It is believed that the accumulation of AGEs in the peripheral nerves leads to the enhancement of reactive oxygen species, which promotes neural inflammation and impairs axonal transport. These perturbations, along with direct neuronal toxicity from intracellular sorbitol accumulation (due to hyperglycemia), culminate

in DN (96). Nevertheless, it should be noted that published studies are heterogeneous, including populations with different diabetes type, different SAF cut-off values, and different methods of DN assessment, so this evidence must be taken with caution.

SAF and Diabetic Macrovascular Complications

As previously mentioned, subjects with diabetes presented an increased risk for myocardial infarction and stroke caused by vascular occlusion and are more likely to develop serious cardiovascular and cerebrovascular disease than non-diabetic subjects (97,98). Atheromatous plaque formation in subjects with diabetes is practically the same form that occurring in non-diabetic subjects, although the distribution of plaques may be different, and diabetic lesions characteristically show a higher tendency for focal medial calcification (99). AGEs have been accepted as having a key role in the formation and acceleration of atherosclerotic lesions, even in normoglycemic patients, but especially in diabetics (58).

The assessment and stratification of cardiovascular risk in subjects with T2D is a challenge. The UKPDS risk score is still one of the most used tools to give cardiovascular risk estimates in people type 2 diabetes. Lutgers et al. demonstrated that SAF provides additional information to the UKPDS risk score for the estimation of cardiovascular prognosis in T2D (67). In addition, there is emerging evidence indicating that SAF is an important biomarker not only of the presence of cardiovascular disease but also of their outcomes (100).

AGEs may contribute to cardiovascular events and cardiovascular mortality by three well-established pathophysiological mechanisms: (a) AGEs can affect the physiological properties of cardiac proteins in the extracellular matrix by creating cross-links, which provoke decreased flexibility of the matrix proteins and produce stiffness in vascular walls; (b) AGEs induce endothelin-1 production and reduce nitric oxide at the vascular level, thus resulting in vasoconstriction and the loss of vascular compliance; and (c) AGEs can cause multiple vascular and myocardial changes through the interaction with RAGEs, leading to atherosclerosis, thrombosis, and vasoconstriction (101). It should be noted that

RAGEs mediate the induction of fibrosis through the increase of TGF-β and influence calcium metabolism in cardiac myocytes (102).

Data regarding the important role of oxidative stress on endothelial dysfunction and coronary artery disease are extensive (103). However, most markers for oxidative stress are n ot readily available for clinical practice. It is well known that AGEs, by interacting with their own receptor RAGE, can induce intracellular signaling that leads to enhanced oxidative stress (57). Moreover, skin AGEs are stable and could be non-invasively assessed, thus serving as a reliable biomarker of cardiovascular disease.

SAF and subclinical cardiovascular disease

It is well established that SAF is a good predictor of subclinical cardiovascular disease in patients with and without diabetes.

Arterial stiffness is associated with the prevalence of CVD and predicts future cardiovascular events in healthy and high-risk patients. The main components of the extracellular matrix within the arterial wall are type I collagen, type III collagen, and elastin. AGE accumulation leads to quantitative and qualitative alterations of collagens and elastin, which could contribute to the decreased elastic properties of the vessels, thereby playing a role in arterial stiffness (104). SAF is strongly correlated with pulse wave velocity, brachial and aortic augmentation indices, and ankle-brachial index, all of them markers of arterial stiffness (105). Birukov et al. (106) recently investigated the relationships between SAF and vascular stiffness in a large study performed in diabetic and non-diabetic populations. These authors concluded that SAF might be involved in vascular stiffening independently of cardiometabolic risk factors, and it could be a rapid and non-invasive method for the assessment of macrovascular disease progression across all glycemic strata (106).

CIMT is a useful marker of the progression of atherosclerosis and is an excellent predictor of cardiovascular events. SAF was an independent determinant of max-IMT ($R = 0.45$, $\beta = 0.425$, $p < 0.01$) in a small study with T2D subjects (76).

SAF and cardiovascular events and death

There is increasing evidence that SAF is a robust predictor of cardiovascular events and cardiovascular death in subjects with T2D.

In a multicenter cross-sectional study comprising more than 500 T2D subjects, Noordzij et al. (75) showed that SAF values were higher when a greater number of diabetic complications was present. In addition, these authors observed that SAF was associated with the presence of macrovascular complications in patients with diabetes, independently of classical risk factors.

Mulder et al. (107) showed that SAF is elevated in acute ST-elevation myocardial infarction compared with healthy controls. In addition, higher values of SAF were related with more risk to die or to present a new myocardial infarction or heart failure in the following year.

Skin AGEs are not only associated with CVD and are useful as predictors of cardiac events but are also associated with peripheral artery disease and can be considered as a useful biomarker to predict amputations in these patients

These findings support the concept that AGEs and their receptor system play an important role in the impairment of vascular function. Thus, AGEs are not only markers of “metabolic memory” in diabetic subjects but also have an important pathogenic role both in endothelial dysfunction and in the atherosclerotic process.

1.3. Diabetic retinopathy

1.3.1. Concept and epidemiology

Diabetic retinopathy is a cardinal manifestation of diabetes and, classically, it has been considered to be due to microangiopathic involvement of the retina. The alteration of the small retinal vessels is characterized by vascular occlusion and increased vascular leakage. DR has long been considered a microvascular complication of diabetes, however, growing evidence suggests that neurodegeneration is an early event in its pathogenesis. Recently, the American association of Diabetes (ADA) has defined DR as a highly specific neurovascular

complication of both type 1 and type 2 diabetes, the prevalence of which strongly correlates to both the duration of diabetes and level of glycemic control (108).

Retinopathy is the most common "microangiopathic" complication of DM and the world's leading cause of blindness in the working-age population (1). It is estimated that the prevalence of DR in the population with diabetes can reach up to 30%, in addition this prevalence increases with the time of evolution of the DM and the age of the patient. It is believed that at the time of diagnosis of T2D, one third of patients already have diabetic retinopathy, and the prevalence increases to 60% with more than 20 years of evolution (109).

The main factors for the development of DR are hyperglycemia and chronic hypertension. Other factors may be microalbuminuria, pregnancy, hyperlipidemia, smoking habit …(110) Traditionally it has been considered that a glucose greater than 126mg/dL and $HbA_{1c} > 6.5\%$ are the cut-off point from which patients with DM can develop DR, although in clinical practice there are patients with DR and lower values (110). However, it has been shown that an intensive reduction of HbA1c concentrations reduces the risk of presenting DR in both TD1 (33) and T2D (2).

DR is classified in different stages according with the findings in the fundus exam. These classifications are very useful for establishing the prognosis, therapeutic and follow-up. DR is classified as non-proliferative (NPDR) and proliferative (PDR), depending on the presence of neovessels and/or fibrovascular proliferation. Currently, we classify diabetic retinopathy based on the results of two large studies: Diabetic Retinopathy Study (DRS) (111) and Early treatment diabetic retinopathy Study (ETDRS) (112) **(Figure 7).**

Measure	Score	Observable Findings
ICDR severity level		
No apparent retinopathy	0	No abnormalities (Level 10 ETDRS)
Mild non-proliferative diabetic retinopathy	1	Microaneurysm(s) only (Level 20 ETDRS)
Moderate non-proliferative diabetic retinopathy	2	More than just microaneurysm(s) but less than severe non-proliferative diabetic retinopathy (Level 35, 43, 47 ETDRS)
Severe non-proliferative diabetic retinopathy	3	Any of the following: > 20 intra-retinal haemorrhages in each of 4 quadrants, definite venous beading in ≥2 quadrants, prominent intra-retinal microvascular abnormalities in ≥1 quadrant, or no signs of proliferative retinopathy. (Level 53 ETDRS: 4-2-1 rule)
Proliferative diabetic retinopathy	4	One or more of the following: neovascularization and/or vitreous or preretinal haemorrhages. (Levels 61, 65, 71, 75, 81, 85 ETDRS)
Macular oedema severity level		
No macular oedema	0	No exudates and no apparent thickening within 1 disc diameter from fovea
Macular oedema	1	Exudates or apparent thickening within 1 disc diameter from fovea

Abbreviations: ETDRS, Early Treatment Diabetic Retinopathy study; ICDR, International Clinical Diabetic Retinopathy

doi:10.1371/journal.pone.0139148.t001

Figure 7. Grading protocol ICDR (International clinic diabetic retinopathy) and ETDRS Severity Levels.

Another form of presentation of diabetic retinopathy is macular edema, which has an independent course to DR. Macular edema is characterized by an increased thickness in central retina, which is usually accompanied of alterations in the fundus exam at the central area of the retina (macula) such as microaneurysms, lipid exudates, and retinal thickening.

1.3.2. Pathogenesis

One important pathogenic event of DR is the endothelial damage caused by hyperglycemia, which in turn causes the two basic phenomena in DR: capillary occlusion and increased vascular leakage. The occlusion of the retinal capillaries causes ischemia of the retina, which translates into increased ocular production of vascular growth factor by hypoxic retinal cells (pericytes, Muller cells, and pigment epithelium), whose objective is to favor the development of new vessels to respond the lack of initial perfusion. However, VEGF production turns out to be an abnormal response that causes growth of new vessels (proliferative retinopathy). Likewise, the increase in vascular permeability (favored by VEGF

and other cytokines) causes fluid accumulation and exudation in the retinal parenchyma, causing its dysfunction.

The pathways involved in DR development and that are triggered under hyperglycemic conditions are the following: polyol and the hexosamine pathways, the synthesis de novo of diacylglycerol-proteinkinase C (DAG-PKC), the formation of advanced glycation end-products (AGEs) and free radical production. All these pathways are crucial to the development of DR and other diabetic complications and are summarized in **Figure 8** (113). Moreover, the activation of all these pathways induces an imbalance between angiogenic and antiangiogenic factors (angiogenic >> antiangiogenic).

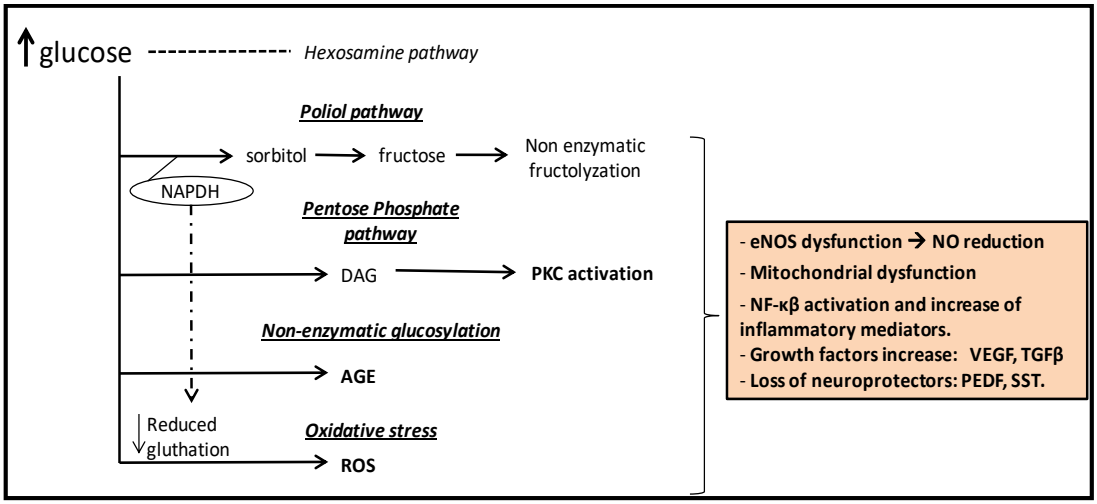


Figure 8. Pathogenic pathways involved in diabetic complications

1.3.3. Assessment of DR.

Currently, retinopathy screening is recommended in diabetic population. An initial ophthalmological assessment is recommended in patients with T2D at the time of diagnosis, and subsequently every 1-2 years, while in patients with T1D it is recommended 5 years after diagnosis and annually thereafter (109).

Usually, the ophthalmological examination is based on taking visual acuity, intraocular pressure, and examination of the fundus through pupillary dilation (to see the retinal periphery). Due to the high care burden, telemedicine programs have been implemented, and retinographies are performed using non-mydriatic

cameras, which are later evaluated by trained personnel. It seems that the implementation of these programs are cost-effective (114).

The generalized screening of the DR has allowed an early diagnosis. The early diagnosis of DR lead to physicians and patients to reinforce the diabetes care and the treatment of other risk factors and occasionally, when it reaches more advanced stages, may lead to the implementation of therapeutic procedures to prevent its progression (108). In addition, the presence of DR is an independent indicator of other diabetic complications such as diabetic nephropathy (115), cardiovascular disease (116) and stroke (117), thus increasing the risk of morbi-mortality of T2D patients.

1.3.4. Diabetic retinopathy and CVD.

It is known that the burden of microvascular disease is determinant of future cardiovascular risk (117). Brownrigg et al (117), in a large population cohort (with 259686 person-years of exposure and 2822 first cardiovascular events), found that the burden of microvascular disease is a determinant of future cardiovascular risk. The risk of a first cardiovascular event increased linearly with the number of manifestations of microvascular disease present. Furthermore, the presence of isolated retinopathy, peripheral neuropathy, or nephropathy confer at least a similar risk of cardiovascular events as factors contained in contemporary risk equations such as blood pressure, low-density cholesterol and hemoglobin A1c. Despite significant differences in baseline values of glycated hemoglobin, low-density cholesterol and blood pressure among individuals with increasing burden of microvascular disease, these factors did not modify associations between microvascular disease and cardiovascular outcomes, as we can see in **figure 9**.

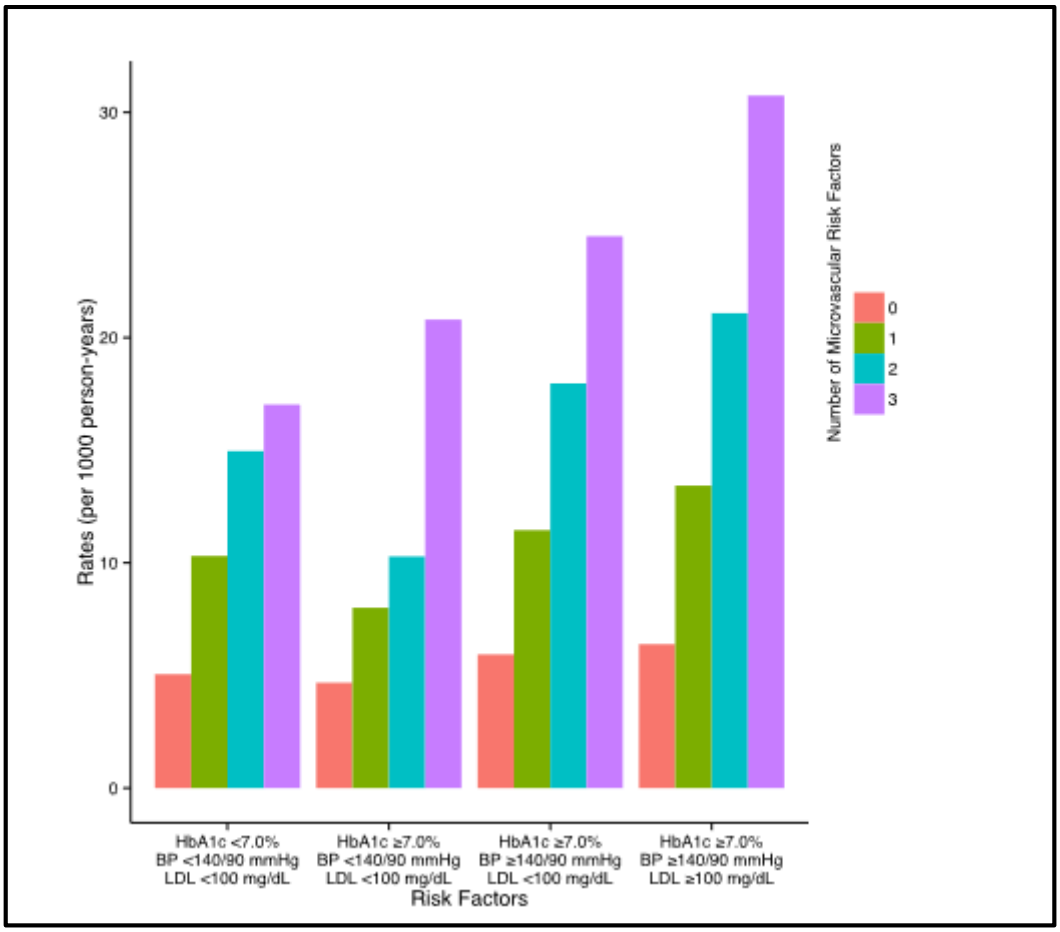


Figure 9. Adjusted cardiovascular event rates by cumulative burden of microvascular disease and established risk factor goals(117).

As mentioned, DR is an independent indicator of other diabetic complications. Several large prospective studies have shown that the presence and severity of diabetic retinopathy are independent determinants of future CV events in people with diabetes (118–120). In a recent systematic review of 17 studies comprising 14,896 people with type 2 diabetes (of mean age 58 years and mean follow-up 9 years), people with any kind of retinopathy were more than twice as likely to die or suffer a fatal or nonfatal CV event than people without retinopathy, and a fourfold higher risk was noted for people with advanced retinopathy (121).

Several explanations may account for the relationship between retinopathy and CV outcomes. First, both retinopathy and incident CV outcomes are recognized consequences of diabetes and their association with each other may therefore be due to their mutual link to diabetes. Second, the degree of retinopathy is progressively related to the degree of several independent factors of CV

outcomes, including hyperglycemia, blood pressure, albuminuria, renal insufficiency, dyslipidemia, and other abnormalities. People with higher levels of these factors would therefore have both more severe retinopathy and a higher incidence of CV outcomes. Third, the microvascular abnormalities present in the retina may also be occurring in many other vascular beds, and CV outcomes may be due in part to accumulated microvascular abnormalities in the myocardial microcirculation, arterial wall (i.e., vasa vasorum), and elsewhere. Thus, pathologic changes in vasa vasorum of conductance vessels or in the myocardial capillaries are similar to the changes seen in the retina. Recent evidence indicates that in subjects with type 2 diabetes, the vasa vasorum presents evolutionary changes similar to those observed in the retina: an initial stage in which endothelium dysfunction and loss of capillaries predominates (122), and more advanced stages in which ischemia plays a key role, leading to angiogenesis and plaque neovascularization (122–124). This change in plaque phenotype results in a more inflamed and unstable plaque, favoring plaque rupture and a poor outcome of CV events. Thus, microcirculation represents a “common soil” between DR and CHD, and it may no longer make sense to consider microangiopathy and macroangiopathy as entirely separated entities in the setting of DM.

Therefore, changes in retinal pathology may closely reflect changes in microvascular pathology in these other vascular beds, and people with the most rapid progression in retinal pathology may be the ones most likely to suffer incident CV outcomes. Moreover, changes in the retina (which are readily accessible for measurement) may reflect changes in an individual’s CV risk and may therefore identify those individuals whose CV risk is rising and who may benefit from particularly aggressive CV risk reduction therapies.

2.Hypothesis

a) AGEs are postulated as a major pathogenic mechanism in the development of diabetes complications. Our hypothesis is that the AGEs determined in the skin in a non-invasive way are related to the development of diabetic complications, and they could explain the residual cardiovascular risk which is currently not explained by traditional risk factors.

b) Since microangiopathy is a strong and independent risk factor for CVD, our second hypothesis is that the greater the extent of microangiopathy, the greater the risk of CV events.

3.Objectives

Main Objective:

To develop new clinical strategies to better identify those patients with T2D at high risk of developing a CV event.

Secondary objectives:

1. To examine whether the burden and degree of microangiopathy is an independent risk factor for subclinical CVD.
2. To examine the usefulness of non-invasive measurement to determine skin AGEs and their relationship to diabetic complications and subclinical CVD.
3. To evaluate whether the presence of diabetic retinopathy and accumulation of advanced glycation end-products in subcutaneous tissue can help to identify patients with type 2 diabetes at high risk of developing CV events and allow us to develop more personalized and cost-effective medicine.

4.Methods

The methodology followed is divided into two clinical studies:

(i) **Cross-Sectional Study:** Case-control study including patients with T2D without known cardiovascular disease matched by age and sex with non-diabetic patients, who were assessed for the degree and extent of microangiopathy, non-invasive AGEs measurement in the skin, and the presence of subclinical CVD.

(ii) **Prospective study**: Follow-up of the same cohort, where the relationship of microangiopathy and AGEs in the skin with the development of cardiovascular events is evaluated.

Study design, data source, and patient enrollment:

This was a prospective case–control study comprising a total of 200 type 2 diabetic subjects with no history of clinical CVD and 60 non-diabetic subjects matched by age (control group) (PRECISED study: ClinalTrial.gov NCT02248311).

The inclusion criteria were:

1. Age from 50 to 79 years and
2. A history of type 2 diabetes diagnosed at least 1 year prior to the day of screening.

The exclusion criteria were:

1. A medical history of a CV event.
2. Type 1 diabetes.
3. Any contraindication for the performance of PET/CT or MRI.
4. Any concomitant disease associated with a short life expectancy.

The subjects were recruited from the Outpatient Diabetic Clinic of Vall d'Hebron Hospital and the Primary Healthcare centers within its catchment area (North Barcelona). A total of 2631 clinical records of subjects with type 2 diabetes were

reviewed: 1912 did not meet the inclusion criteria, leaving 719 eligible for screening; out of these, 200 subjects with type 2 diabetes agreed to participate in the study. A total of 60 age-matched non- diabetic subjects without a history of CVD served as a control group. These individuals were recruited from the same Primary Healthcare centers and most were relatives of the diabetic subjects.

The study was conducted according to the declaration of Helsinki and was approved by the local ethics committee. All subjects provided written informed consent before study entry.

4.1. Cross-sectional study

Assessment of CV risk factors.

Anthropometric measurements and classical risk factors

Clinical data were obtained on the first visit by an endocrinologist. Anthropometric data were obtained by standardized protocols at the same visit. Weight and height were measured with a balance with a fixed stadiometer to determine the body mass index (BMI). Hypertension was defined as systolic blood pressure ≥140mmHg or diastolic blood pressure ≥90mmHg, or when patients were under treatment with antihypertensive agents.

A history of smoking habits (non-smoker/current smoker/ex-smoker) was recorded. Smokers who stopped smoking ≥1year prior to recruitment were considered ex-smokers.

Dyslipidemia was defined by the use of lipid-lowering drugs, decreased values of high-density lipoprotein (HDL) cholesterol (men 5.2mmol/L), low-density lipoprotein (LDL) cholesterol (>4.3mmol/L), or triglycerides (>1.7mmol/L). Framingham and United Kingdom Prospective Diabetes Study-coronary heart disease (UKPDS-CHD) were calculated as described elsewhere (125).

Fundus examination

DR was evaluated by experienced ophthalmologists in mydriasis using slit-lamp biomicroscopy and retinography with the same camera (Topcon-DRI-

OCTTRITON). The examiners classified DR according to the International Clinical Diabetic Retinopathy Disease Severity Scale (112): (1) no apparent retinopathy, (2) mild non-proliferative retinopathy (NPDR), (3) moderate NPDR, (4) severe NPDR, and (5) proliferative diabetic retinopathy (PDR).

Laboratory tests

A venous blood sample was collected from the antecubital vein, separated by centrifugation (2000×g at 4°C for 20min) and frozen at −80°C for batched storage and analysis. Glycosylated hemoglobin was determined by the Cobas B 101 (Roche) system. The remaining biochemical parameters were measured using an Olympus AU5400 automatic biochemistry analyzer (Olympus, Tokyo, Japan).

Measurement of skin autofuorescence

SAF was measured using the AGE ReaderTM™ (DiagnOptics TechnologiesBV, Groningen, the Netherlands), a noninvasive desktop device. This device detects the characteristic fuorescence of some AGEs and was used to estimate the level of AGEs in the skin. Technical and optical details of this non-invasive method have been described more extensively elsewhere (72). In short, the AGE ReaderTM uses an excitation light source with a peak excitation of 370 nm to illuminate a skin surface area of 4 cm2. Subsequently, the emitted fluorescence light (within the wavelength range of 420–600 nm) and the reflected excitation light (within the wavelength range of 300–420 nm) from the skin are measured with a spectrometer. SAF is calculated in arbitrary units (AU) as the ratio between the emitted light and the reflected light. SAF was measured three times in series at a site on the ventral side of the forearm by the same operator and the mean of those assessments was used as the representative value.

Assessment of subclinical CVD

Subclinical CVD was defined as the composite of a CACs >400, carotid plaque ≥3mm, or CIMT>1. The presence of ECG changes suggestive of previous asymptomatic MI (Minnesota codes 1.1. and 1.2) was also considered subclinical CVD.

Electrocardiogram

A 12-lead ECG was taken after the patient had been lying down for at least 5 min. The ECG was evaluated by a cardiologist.

Carotid ultrasonography

All study participants underwent a standard echo-color Doppler examination of the extracranial carotid arteries (common carotid artery, internal and external carotid artery) by means of a high-frequency linear probe (vivid 7-GE and vivid 9, Medical Systems, GE Healthcare, with linear probe 7 MHz).

Conventional ultrasound (B-mode and color Doppler) was used to measure common intimia media thickness (IMT) and to identify the presence of carotid plaques according to the Mannheim consensus (126). Extracranial carotid arteries were explored axially and longitudinally. We measured the IMT of the far wall of the common carotid artery at the level of 1cm proximal to the bifurcation bilaterally. We used the mean value obtained from three measurements for our analysis. Frequency of carotid plaques was defined as the presence of plaques in any of the explored territories. The assessment of all the measurements and the performance of the ultrasound studies were performed by two researchers, who were blinded to the conditions of the participants.

CT-CAC scanning

An ECG synchronized prospective contrast-enhanced coronary CT was performed with Siemens Biograph mCT 64 s equipment. The calcium score was analyzed using semiautomatic methodology with “Syngo.Via” cardiac CT software, the global and individual Agatston score for coronary vessels being calculated. Patients were classified by AU as low risk (<100AU), moderate risk (100-400AU), or high risk (>400AU).

Coronary angio-CT

After patient preparation with beta blockers for decreased heart rate, and nitroglycerin for vasodilatation if required, an ECG synchronized prospective

contrast enhanced coronary CT was performed with Siemens Biograph mCT 64s equipment. Automatic coronary vessel extraction of all coronary vessels with visual analysis of coronary stenosis was performed by researchers blind to the patient's condition with "Syngo.Via" cardiac CT software as described elsewhere (127).

Statistical analysis

The categorical variables are presented as percentages. For the quantitative variables, the mean and SD are displayed, except for triglycerides, lipoprotein (a), and homocysteine in which median and range were used. Differences among groups were assessed using the $\chi 2$ test for qualitative variables, while t-test quantitative variables with a normal distribution, and non-parametric tests were used for those quantitative variables without a normal distribution.

Diabetic subjects were divided into two groups: those with calcium scores higher and lower than 400 AU. Logistic regression analysis to predict a CACs higher than 400 AU was performed using the variables that were significant at the univariant analysis. The complete logistic model being compared with a logarithmic transformation of the microalbuminuria: creatinine ratio. ROC curves were calculated and the $\chi 2$ test for ROC area comparison was performed.

In view of the skewed distribution of CACs values, they were logarithmically converted to use parametric tests. For the bivariate correlation, Pearson correlation coefficient was calculated using Bonferroni-adjusted signifcance.

We created a variable (higher or lower SAF) taking into account the SAF tertile in diabetic subject (the first and second tertile or lower SAF measurement subjects were compared with those within the third tertile or higher SAF measurement).

The differences between diabetic subjects with the combined endpoint defined above and those without it were assessed. Selected variables that were significant at the univariate analyses were used to predict the combined endpoint by forward stepwise selection method in a binary logistic regression analysis. ROC curves were calculated. The area under the receiver operating characteristic curves (AUROCs) for the UKPDS-CHD and Framingham risk

scores for predicting the presence of the combined endpoint and the effect of the addition of DR and/or microalbuminuria were calculated.

Significance was accepted at the level of p value <0.05 for all the analyses. Statistical analyses were performed with the Stata statistical package.

4.2. Prospective Study

Outcome:

The primary outcome was the time to the first CV event. We defined a CV event as a composite of myocardial infarction, coronary revascularization, stroke, lower limb amputation or CV death.

Statistical analysis

Differences among groups were analyzed using Student's t-test for quantitative variables with a normal distribution and Pearson's chi-squared test for categorical variables. We calculated event-free survival according to the Kaplan–Meier method.

The Cox proportional hazard multiple regression analysis was used to determine independent predictors of CV events. Statistical analyses were performed with Stata statistical package 15. Significance was accepted at the level of p< 0.05 for all analyses.

5.Results

5.1. Clinical Characteristics.

The general characteristics as well as the main laboratory findings in subjects with type 2 diabetes and non-diabetic controls are shown in **table 1**. We did not find any significant difference between groups regarding age, gender, ethnicity, smoking habit, family history of CVD, or in laboratory parameters (creatinine and glomerular filtration rate (GFR), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT). Subjects with type 2 diabetes presented a significantly higher BMI and waist circumference, as well as a higher prevalence of hypertension and dyslipidemia. Therefore, as expected, significantly more subjects were treated with angiotensin receptor blockers, ACE inhibitors, and statins in the diabetic group in comparison with non-diabetic controls. As expected, HbA1c was significantly higher in the diabetic population than in control subjects. We also found significantly higher levels of alanine transaminase (ALT) in diabetic subjects in comparison with controls.

Table 1. Characteristics of type 2 diabetic patients and non-diabetic control subjects.

	Type 2 diabetes (n=186)	**Control group (n=57)**	**P**
Sex (woman) (n,%)	107 (57.53%)	37 (64.91%)	0.32
Ethnicity (Caucasian n,%)	178 (95.7%)	56 (98.25%)	0.64
Age (years)	65.7 ±6.47	66.02 ±6.63	0.75
BMI (kg/m^2)	30.25 ± 4.9	26.83 ±4.77	**<0.0001**
Waist circumference (cm)	103.9±13.53	91.2±13.92	**<0.0001**
Smoking No (n,%) Current Smoker (n, %) Ex-smoker (n, %)	 99 (53.23%) 25 (13.44%) 62 (33.33%)	 34 (59.65%) 7 (12.3%) 15 (26.32%)	0.59
CV family history (n, %)	22 (11.83%)	8 (14.04%)	0.65
Hypertension (n, %)	134 (72.04%)	28 (49.12%)	**0.001**
Dyslipidemia (n, %)	148 (78.57%)	25 (43.86%)	**<0.0001**

HbA1c (%)	7.43 ±1.18	5.55±0.31	**<0.0001**
Creatinine (mg/dl)	0.82 ±0.24	0.76 ±0.19	0.76
GFR ml/min	81.76 ±16	85.58 ±10.88	0.09
AST (UI/L)	25.51 ±15.71	23.48 ±5.73	0.34
ALT (UI/L)	25.94 ±16.88	21.12 ±10.55	**0.043**
GGT (UI/L)	44.46 ±71.82	31.04 ±29.77	0.17

Data are expressed as % or mean ± SD

The specific characteristics of subjects with diabetes are displayed in **table 2**. In summary, the type 2 diabetic patients included in the study exhibited a long-term duration of diabetes with relatively good metabolic control (HbA1c: 57.85±12.56mmol/mol (7.43%±1.18%)). Around 60% were under combined treatment with metformin plus insulin or insulin alone, and up to 30% presented at least one microangiopathic complication.

Table 2. Characteristics of type 2 diabetic patients

	N=186
Diabetes duration (years)	12 ±9.4
HbA1c (%)	7.4 ±1.18
Microvascular complications	
Retinopathy (n, %)	50 (26.88%)
Non-proliferative	44 (23.66%)
Mild	23 (12.37%)
Moderate	16 (8.6%)
Severe	5 (2.69%)
Proliferative	6 (3.23%)
Urine albumin/creatinine ratio (mg/g)	
<30mg/g (n, %)	120 (64.52%)
30-300mg/g (n, %)	54 (29.03%)
>300mg/g (n, %)	9 (4.84%)
Neuropathy	35 (18.82%)
Diabetes treatment	

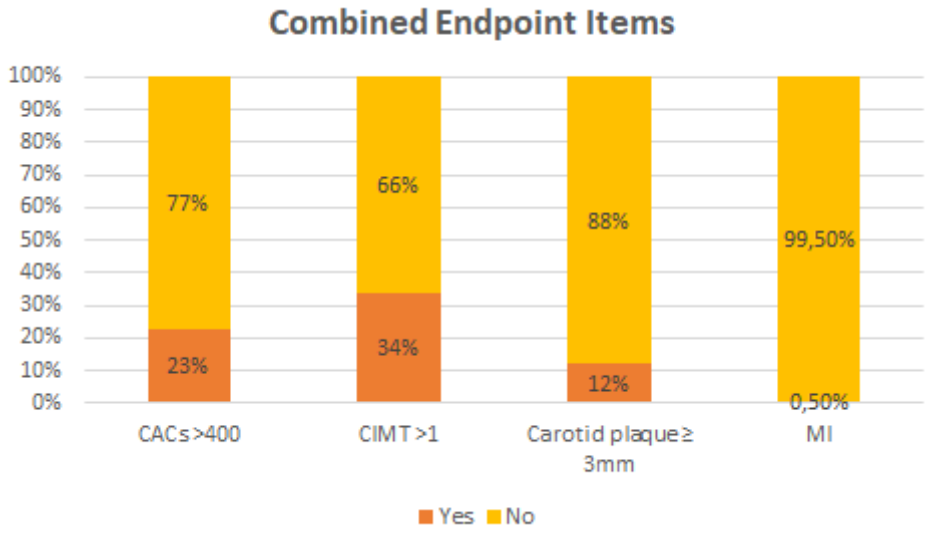
Oral agents	42.47%
Insulin	4.84%
Oral agents + insulin	53.76%

Data are expressed as % or mean ± SD

The presence of subclinical CVD assessed by the composite of a CACs >400, carotid plaque ≥3mm, or CIMT>1 and ECG signs of a previous MI was higher in type 2 diabetic patients than in the control group (65.7% vs 44%; p=0.05).

In the diabetic group 41 subjects (23%) presented CACs>400, 57 subjects (34%) presented a carotid plaque ≥3mm and 20 subjects (12%) presented a CIMT>1; 10.5% present altered 2 of the three items, and 3% all 3. One out of 200 included patients (0.5%) presented ECG signs of a previous MI **(Figure 10)**.

Figure 10. Percentage of patients with diabetes presenting each item of the combined endpoint.



5.2. Relationship between the burden and the degree of microangipathy with subclinical CVD.

Relationship between DR and subclinical CVD.

The main clinical characteristics of type 2 diabetic patients according to the presence or absence of the combined endpoint is displayed in **table 3.**

Table 3. Comparison of type 2 diabetic patients with and without the composite end-point.

	Endpoint NO N=57	Endpoint YES N=106	P
Sex (woman) (n, %)	38 (66.67%)	58 (54.72%)	0.14
Age (years)	64.035 ±7.19	66.98 ±5.71	**0.004**
BMI (kg/m^2)			
Diabetes duration (years)	11.75 ±8.98	15.96 ±9.52	**0.007**
Waist circumference	103.64 ±13.65	104.3 ±13.31	0.76
Smoking • No (n, %) • Current smoker (n, %) • Ex-smoker (n, %)	 33 (57.89%) 7 (12.3%) 17 (29.82%)	 52 (49.06%) 17 (16.04%) 37 (34.91%)	0.54
Hypertension (n, %)	36 (63.16%)	81 (76.42%)	0.07
Dyslipidemia (n, %)	46 (80.7%)	84 (79.25%)	0.82
HBA1c (%)	7.27 ±1.25	7.41 ±1.07	0.45
Total cholesterol (mg/dl)	191.96 ±40.68	182.77 ±34.1	0.12
HDL cholesterol (mg/dl)	50.63 ±11.05	48.9 ±12.55	0.38
LDL cholesterol (mg/dl)	109.3 ±34.26	104.43 ±29.5	0.34
Triglycerides (mg/dl)	142 [57-503]	130[45-418]	0.45
Homocysteine	10.5 [5.8-37.6]	12.4 [6.3-127]	**0.02**
Lipoprotein (a) (mg/dl)	9.35 [1-99.9]	8.46 [1-162.9]	0.35
Creatinine (mg/dl)	0.765 ±0.19	0.86 ±0.26	**0.02**
GFR (ml/min)	85.63 ±13.05	79.3 ±16.85	**0.01**
albumin/creatinine ratio (mg/g) • <30 mg/g (n, %) • 30-300 mg/g (n, %) • >300 mg/g (n, %)	 42 (73.68%) 8 (14.04%) 2 (3.51%)	 65 (61.32%) 34 (32.08%) 7 (6.6%)	0.27
Log albumin/creatinine ratio	2.41 ±1.75	3.1 ±1.49	**0.009**
Retinopathy (n, %)	10 (17.54%)	35 (30.19%)	**0.04***
• NPDR - Mild - Moderate - Severe • PDR	 10 8 2 0 0	 27 14 13 3 5	**0.005****
Neuropathy (n, %)	8 (14.04%)	21 (19.81%)	0.35

Data are expressed in median ± SD or in median [range].*absence vs presence of DR. ** absence/mild DR vs. moderate/severe DR

Type 2 diabetic patients with the combined endpoint were older, presented longer diabetes duration, more frequent hypertension, higher homocysteine levels, higher levels of creatinine, a lower GFR, higher microalbuminuria levels, and a higher frequency of DR. In addition, those patients with moderate–severe DR presented a higher prevalence of subclinical CVD assessed by the combined endpoint in comparison with patients with mild DR or the absence of DR (91.3% vs 60.8%; p=0.004).

The logistic regression model in which DR was introduced as a categorical variable (yes/no) showed that DR was not an independent variable accounting for the combined endpoint **(table 4A)**. However, when DR was introduced as absence–mild DR versus moderate–severe, it was strongly associated with the combined endpoint (OR 8.53 (95% CI 1.05 to 69.12)) **(table 4B).**

Table 4. (A) Results of the logistic regression analysis for predicting the presence of subclinical cardiovascular disease in subjects with type 2 diabetes assessed by the combined endpoint (CACs ≥400AU, carotid plaque ≥3mm, carotid intima–media thickness >1, or ECG changes suggestive of previous asymptomatic myocardial infarction). Variables that were significant at a p value <0.05 in the univariate analysis of **table 3** (comparison between the presence or not of the combined endpoint) were entered in the model as independent variables (age, diabetes duration, hypertension, homocysteine, GFR, the presence of microalbuminuria and/ or DR), adjusting for gender and dyslipidemia. A forward stepwise selection method was used with a significance level of 0.05. **(B)** The same analyses have been performed but DR was classified into no-DR or mild and moderate to severe DR/proliferative DR.

A				
	Coefficient	SE	OR (95%CI)	P value
Age (years)	0.077	0.027	1.06 (1.02 to 1.11)	**0.005**
DR and/or microalbuminuria				**0.011**
Microalbuminuria (no/yes)	0.944	0.458	2.70 (1.05 to 6.30)	
DR (no/yes)	0.932	0.523	2.54 (0.91 to 7.08)	
Microalbuminuria and DR	1.199	0.621	3.32 (0.98 to 11.2)	
Constant	4.854	1.81		
Dependent variable: combined endpoint. Independent variables included in the model: age, gender, diabetes duration, GFR, homocysteine, hypertension (no/yes), dyslipidemia (no/yes), and the presence of microalbuminuria and/or DR (no/yes).				

B				
Age (years)	0.077	0.028	1.06 (1.02 to 1.11)	**0.006**
DR and/or microalbuminuria				**0.001**
Microalbuminuria (no/yes)	0.816	0.407	2.26 (1.02 to 5.02)	
DR (no–mild/moderate–severe)	2.144	1.096	8.53 (1.05 to 69.1)	
Microalbuminuria and DR	2.202	1.096	9.04 (1.05 to 77.6)	
Constant	−4.873	1.842		
Dependent variable: combined endpoint. Independent variables included in the model: age, gender, diabetes duration, GFR, homocysteine, hypertension (no/yes), dyslipidemia (no/yes), and the presence of microalbuminuria and/or DR (no or mild /moderate to severe).				

The AUROC of this model for identifying subclinical CVD using the combined endpoint was 0.69 (95% CI 0.61 to 0.76; p<0.05). When, in the same model, microalbuminuria was added to the DR categorized as moderate–severe versus no or mild DR, the strength of the association increased (OR 9.04 (95% CI 1.05 to 77)) as well as the AUROC (0.72 (95% CI 0.64 to 0.78; p<0.05)).

The probability of having a CACs ≥400AU, carotid plaque ≥3mm, CIMT >1, and the composite endpoint according to the presence and degree of DR is represented in **table 5**. The presence of DR significantly increases the risk of CACs ≥400AU and the composite endpoint.

Table 5. Probability of having carotid plaque ≥3mm, carotid-media thickness >1, CACs ≥400 AU, and the composited endpoint according to the presence and degree of DR.

	No DR	DR	p	No DR-Mild DR	NPDR moderate-severe / PDR	p
Carotid plaques ≥3cm	32.2%	40.0%	0.38	33.1%	43.4%	0.33
CIMT > 1	12.3%	12.5%	0.82	13.3%	4.3%	0.21
CACs ≥ 400 AU	19.2%	36.2%	**0.019**	22.8%	40.9%	0.07
Composite End Point	53.3%	70.6%	**0.029**	60.8%	91.3%	**0.004**

Finally, the discrimination abilities of the United Kingdom Prospective Diabetes Study (UKPDS) and Framingham cardiovascular risk scores to predict the combined endpoint of subclinical cardiovascular disease, adding DR (categorized

as either “yes or no” or “moderate–severe vs no or mild DR”) alone or in combination with microalbuminuria, were calculated and compared employing the AUROC. We found that it was only when DR was categorized as moderate to severe versus no or mild DR that the AUROC increased significantly **(table 6).**

Table 6. Comparisons between UKPDS-CHD (CHD=coronary heart disease) (A) and Framingham risk scores (B) with and without the addition of microalbuminuria (no/yes), diabetic retinopathy (DR) (no/yes), DR+microalbuminuria, moderate–severe DR, and moderate–severe DR+microalbuminuria in assessing the presence of the combined endpoint

A				
	ROC area	SE	95% CI	P value
UKPDS-CHD	0.658	0.044	0.58 to 0.75	
UKPDS-CHD+microalbuminuria	0.683	0.044	0.60 to 0.77	0.191
UKPDS-CHD+DR	0.687	0.045	0.60 to 0.77	0.198
UKPDS-CHD+microalbuminuria and DR	0.701	0.044	0.62 to 0.79	0.074
UKPDS-CHD+moderate–severe DR	0.729	0.041	0.65 to 0.818	**0.001**
UKPDS-CHD+microalbuminuria and moderate–severe DR	0.733	0.042	0.65 to 0.82	**0.008**
B				
Framingham	0.649	0.045	0.56 to 0.78	
Framingham+microalbuminuria	0.66	0.045	0.57 to 0.75	0.52
Framingham+DR	0.678	0.046	0.59 to 0.77	0.269
Framingham+microalbuminuria and DR	0.683	0.045	0.60 to 0.77	0.223
Framingham+moderate–severe DR	0.717	0.042	0.67 to 0.83	**0.007**
Framingham+microalbuminuria and moderate–severe DR	0.714	0.043	0.63 to 0.80	**0.034**

UKPDS, United Kingdom Prospective Diabetes Study.

Relationship between DR and CACs

The distribution of control and diabetic subjects, taking into account the CACs, is shown in **table 7**. As expected, type 2 diabetic patients presented higher CACs than non-diabetic control subjects. In fact, in the control group, no patient presented CACs≥400AU, while in the T2D group 35 patients (22%) did ($p < 0.0001$).

Table 7. Distribution of control and diabetic subjects taking into account the CACs

	Type 2 diabetes	Control	P
CACs ≥ 100AU	77 (47.2%)	12 (24%)	**0.003**
CACs ≥ 200AU	59 (36.2%)	8 (16%)	**0.005**
CACs ≥ 300AU	51 (31.3%)	3 (6%)	**<0.001**
CACs ≥ 400AU	41 (25.2%)	0	**<0.001**

The clinical characteristics and the main laboratory findings of subjects with T2D according to CACs are shown in **Table 8.** Among type 2 diabetic patients, gender, age, waist circumference, serum levels of homocysteine, and the presence of DR were significantly different in those patients with a CACs ≥400AU in comparison with patients with <400AU

Table 8. Characteristics of type 2 diabetic patients according calcium score

	CACs < 400 N=122	**CACs ≥400** N=41	**p**
Sex (woman) (n, %)	79 (64.75%)	17 (41.46%)	**0.009**
Age (years)	65.27 ±6.74	67.98 ±4.8	**0.018**
BMI (kg/m^2)	30.07 ±4.91	31.32 ±4.72	0.15
Diabetes duration (years)	13.82 ±9.53	16.49 ±9.34	0.12
Waist circumference	102.79 ±13.36	108 ±12.86	**0.031**
Smoking • No • Current smoker • Ex-smoker (n, %)	 66 (54.1%) 19 (15.57%) 37 (30.33%)	 19 (43.34%) 5 (12.2%) 17 (41.46%)	0.41
Hypertension (n, %)	85 (69.67%)	32 (78.05%)	0.30
Dyslipidemia (n, %)	98 (80.33%)	32 (78.05%)	0.75
HbA1c (%)	7.35 ±1.17	7.38 ±1.14	0.90
Calcium (mg/dl)	9.52 ±0.37	9.61 ±0.4	0.18
Total cholesterol (mg/dl)	186.71 ±38.073	183.83 ±32.5	0.66
HDL cholesterol (mg/dl)	50.54 ±12.22	46.414 ±11.07	0.057
LDL cholesterol (mg/dl)	106.23 ±32.54	105.81 ±27.21	0.94
Triglycerides (mg/dl)	130 [57 - 503]	158 [57 – 403]	0.25
Homocysteine	10.9 [5.8 – 37.6]	14 [6.3 - 127]	**0.018**
Lipoprotein (a) (mg/dl)	9.43 [1 – 162.9]	6.89 [1 – 129]	0.46
GFR (ml/min)	82.06 ±15.75	79.9 ±16.33	0.45
Creatinine (mg/dl)	0.81 ±0.24	0.87 ±0.24	0.16

Albumin/creatinine ratio • <30 mg/g (n, %) • 30-300 mg/g (n, %) • >300 mg/g (n, %)	 80 (65.57%) 37 (30.33%) 5 (4.1%)	 27 (65.85%) 10 (24.39%) 4 (9.76%)	0.34
Log albumin/creatinine ratio	2.77 ±1.6	3.11 ±1.67	0.25
Retinopathy (n, %) • NPDR - Mild - Moderate - Severe • PDR	27 (22.13%) 23 14 6 3 4	16 (39.02%) 14 7 8 0 1	**0.034**
Neuropathy (n, %)	18 (14.75%)	11 (26.83%)	0.08

(Data are expressed as %, mean ± SD, or median and [range])

The logistic regression model, which included the variables with statistical significance in the univariate study, showed that only age, gender, and the presence of DR were independently related to a CACs >400AU **(table 9)**. The model for identifying type 2 diabetic subjects with a CACs >400 showed an AUROC of 0.77 (95% CI 0.72 to 0.83; p<0.00001).

Table 9. Results of the logistic regression analysis for predicting the presence of a calcium coronary score (CACs) ≥400AU in subjects with type 2 diabetes.

A				
	Coefficient	SE	OR (95%CI)	P value
Age (years)	0.112	0.037	1.12 (1.04 to 1.21)	**0.002**
Gender (M/F)	−1.14	0.407	0.32 (0.14 to 0.71)	**0.005**
Homocysteine (µmol/L)	0.063	0.036	1.07 (0.99 to 1.14)	0.084
DR (no/yes)	0.898	0.412	2.46 (1.11 to 5.51)	**0.029**
Constant	-9.189	2.462		
Dependent variable: CACs ≥400AU. Independent variables included in the model: age, gender, waist circumference, homocysteine, and DR (no/yes).				

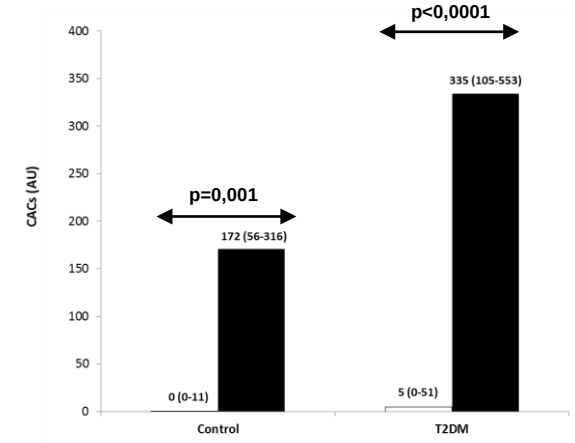
We also observed an inverse relationship between the degree of DR and a CACs <10AU. In this regard, 93.9% of patients with no or only mild DR presented a CACs <10 whereas this percentage was as low as 6.1% in those patients with moderate–severe DR.

Relationship between the CACs, and coronary stenosis.

Type 2 diabetic patients presented a significantly higher proportion of coronary stenosis (stenosis >50% in at least in one coronary artery) than non-diabetic control subjects (24.3% vs 9.1%; p=0.03).

As expected, we found a clear relationship between the CACs and the presence of coronary stenosis in both diabetic and non-diabetic control subjects (**figure 11A)**. Diabetic subjects with coronary stenosis presented a significantly higher CACs (AU) than diabetic subjects without coronary stenosis (337 (95% CI 10 to 2236) vs 5 (95% CI 0 to 293); p<0.0001). Also, a clear relationship between the presence and degree of DR and the presence of one or more coronary stenosis was found **(figure 11B).**

A)



B)

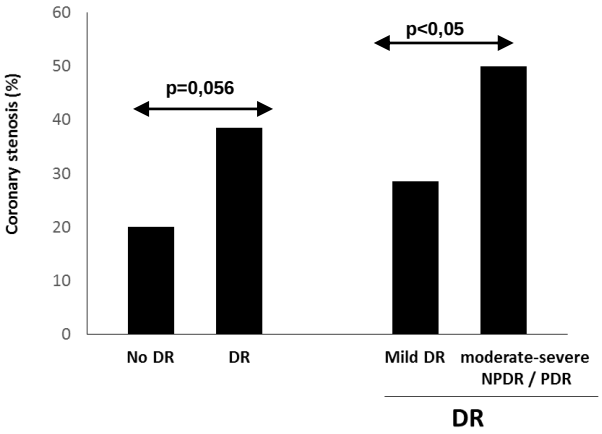


Figure 11. A) CACs (AU) and the presence of coronary stenosis in both diabetic and non-diabetic control subjects. Data are median (CI: 25%-75%). B) Association between coronary stenosis and the presence and degree of DR.

5.3. The usefulness of non-invasive measurement to determine skin AGEs and their relationship to diabetic complications and subclinical CVD

SAF was significantly higher in the diabetic group than in the control group (2.67±0.66 vs. 2.40±0.62; p=0.011). The SAF value was significantly higher in the T2D patients who had DR in comparison with patients without DR (2.89±0.79 vs. 2.58±0.59, p=0.01). We did not find correlation between SAF and HbA1c (r=−0.101, p=0.63).

When patients with a high SAF values (the superior tertile) were compared with those with low values (the two inferior tertiles), we observed that patients with a higher SAF were older, with longer diabetes duration, higher values of homocysteine and microalbuminuria, and a lower glomerular filtration **(Table 10).** We also found a significantly higher SAF value in those patients with the diagnosis of diabetic nephropathy (2.89±0.78 vs. 2.6±0.6,p=0.02).

Table 10. Characteristics of subjects with type 2 diabetes according to SAF measurement.

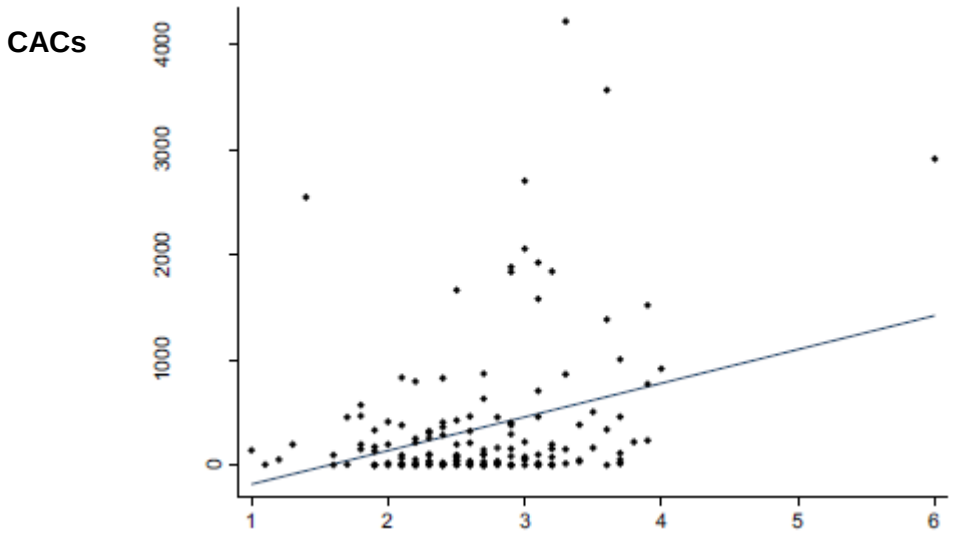
	Lower SAF (n= 109)	Higher SAF (n= 48)	P
Sex (woman) (n, %)	64 (58.7%)	22 (45.8%)	0.136
Age (years)	64.87 ±6.1	67.17 ±6.4	**0.03**
BMI (kg/m^2)	30.1±5.0	30.9±4.6	0.31
Diabetes duration (years)	13.4±8.9	17±10.3	**0.03**
Waist circumference	103.4±14.0	107.3±12	0.1
Smoking No Current smoker Ex-smoker (n, %)	 57 (52.3%) 16 (14.7 %) 36 (33,0%)	 22 (45,8%) 6 (12,5%) 20 (41,7%)	0.58
Hypertension (n, %)	74 (67.9%)	37 (77.08%)	0.24

Dyslipidemia (n, %)	93 (85.3%)	34 (70.8%)	**0.033**
HbA1c (%)	7.5 ±1.3	7.4 ± 1.0	0.58
Total cholesterol (mmol/L))	4.8±1.0	4.74±0.84	0.71
HDL cholesterol (mmol/L)	1.26±0.32	1.28±0.29	0.85
LDL cholesterol(mmol/L)	2.71±0.85	2.71±0.7	0.98
Triglycerides (mmol/L)	1.51 [0.64-5.68]	1.50 [0.7-4.55]	0.54
Homocysteine (µmol/L)	10.9 [5.8-127]	12.6 [6.3-37.6]	**0.0142**
Lipoprotein (a) (mg/dl)	7.97 [1-129]	7.7 [1-99.9]	0.3
GFR (ml/min)	83.25±15.75	76.65±18.46	**0.0234**
Creatinine (mg/dl)	0.81±0.24	0.95±0.5	**0.017**
Albumin/creatinine ratio <30 mg/g (n, %) 30-300 mg/g (n, %) >300 mg/g (n, %)	 71 (66.4%) 33 (30.8%) 3 (2.8%)	 28 (59.6%) 12 (25.5%) 7 (14.9%)	**0.019**
Log albumin/creatinine ratio	1.24±0.57	1.47±0.83	**0.0482**
Diabetic retinopathy (%) NPDR Mild Moderate Severe PDR	24 (22.02%) 12 (11%) 6 (5.5%) 3 (2.8%) 3 (2.8%)	17 (36.2%) 9 (17.02%) 5 (10.64%) 2 (4.3%) 2 (4.3%)	0.065
Log Calcium Score	1.98±0.77	2.37±0.85	**0.019**

Since not all patients could be tested for skin AGEs, we reanalyzed the clinical characteristics and the main laboratory findings of subjects with T2D according to CACS and we obtain T2D patients with CACs ≥ 400 AU were older (p= 0.0015), with higher percentage of men (p = 0.046), longer diabetes duration (p = 0.051, lower level of serum HDL cholesterol (p = 0.012) and higher values of homocysteine (p = 0.012) in comparison with those patients with CACs < 400 AU. Additionally, SAF values were significantly higher among the group with CACs ≥400AU compared to patients with CACs < 400 AU (2.96 ± 0.86 vs. 2.59 ± 0.57; p = 0.003). Similarly, patients with a higher SAF value defined by the superior tertile values presented significantly higher CACs values **(Table 10)**.

Furthermore, a direct correlation between CACs and SAF values was observed (r = 0.31; p < 0.001) **(Figure 12).**

Figure 12. Correlation between coronary artery calcium score and skin advanced glycation end products in T2D subjects. r = 0.31; p < 0.001



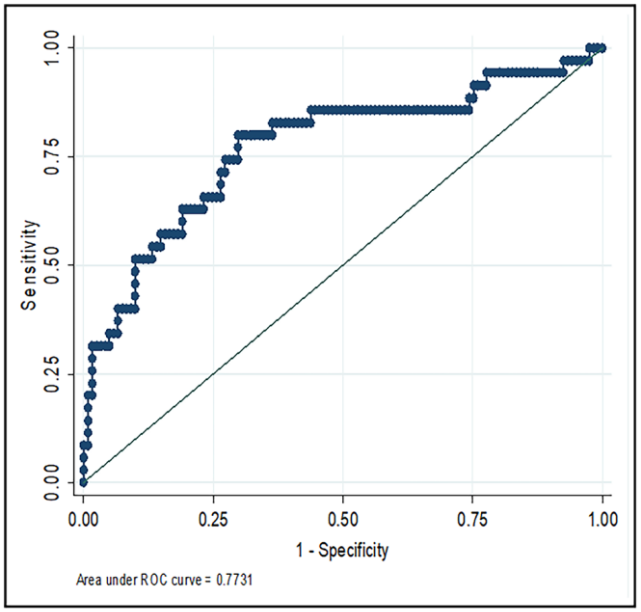
A logistic regression analysis including variables that were significant to the univariate analysis showed that age, cholesterol HDL and SAF values were independently related to CACs ≥ 400 AU **(Table 11).**

Table 11. Results of the logistic regression analysis for predicting the presence of CACs ≥ 400AU in subjects with type 2 diabetes

	Coefficient	**SE**	**OR (95% CI)**	**p value**
Age (Years)	0.127	0.040	1.13 (1.05-1.23)	**0.002**
HDL-Col (mmol/l)	-0.066	0.022	0.93 (0.89-0.98)	**0.003**
Skin AF (AU)	0.706	0.33	2.04 (1.07-3.88)	**0.033**
Constant	-8.48	2.76		

The model for identifying T2D subjects with CACs ≥ 400 AU including these variables showed an AUROC of 0.77 (95% CI 0.70–0.84). Sensibility and specificity of the model were 80% (95% CI: 64.1–90%) and 70.2% (95% CI: 61.6–77.7%), respectively **(Figure 13).**

Figure 13. Area under the curve for identifying T2D subjects with CACs ≥400AU

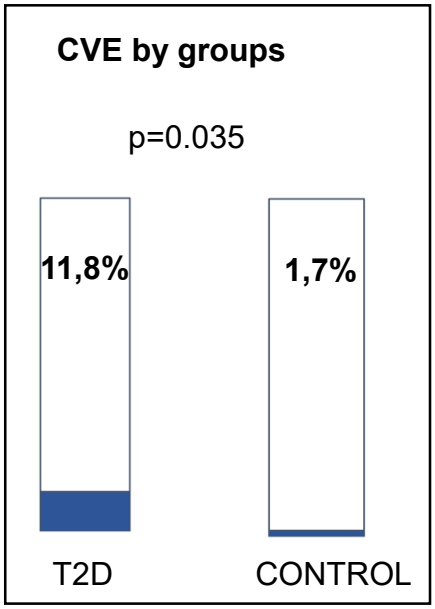


AUROC 0.77 (95% IC 0.70-0.84)

5.4. The presence of diabetic retinopathy and non-invasive skin AGEs measurement can help identify patients with type 2 diabetes at high risk of developing CV events.

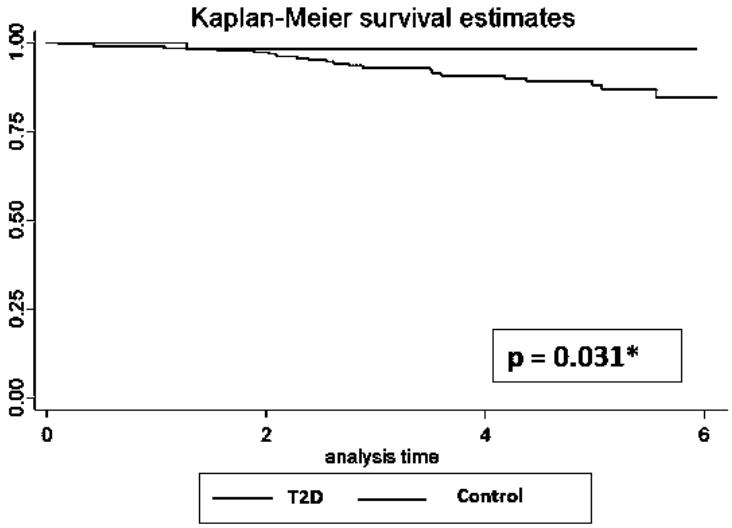
187 subjects with type 2 diabetes and 57 non-diabetic controls were followed until December 2020. After a follow up of 4.35 ± 1.43 years, a total of 24 vascular events were registered, 23 vascular events (12.3%) in type 2 diabetes group, and 1 (1.75%) in non-diabetic control group **(Figure 14)**

Figure 14. Differences in CVEs between diabetic patients and the control group.



The Kaplan-Meier analysis shows vascular event free-survival regarding groups (p=0.035), (**Figure 15**).

Figure 15. Kaplan-Meier analysis predicting cardiovascular event free-survival regarding groups.



In the type 2 diabetes cohort, we found an incidence rate of CV events of 28.2 per 1000 person years. The main basal clinical characteristics of patients with

type 2 diabetes according to the presence of the primary outcome (the first vascular event) are shown in **Table 12.**

Table 12. Clinical characteristics of patients with type 2 diabetes according the presence of primary outcome (first cardiovascular event)

	CV event + (n= 23)	**CV event - (n = 164)**	**p**
Follow up (y)	5.09 ± 1.20	5.21 ± 0.95	0.564
Sex (woman) (n, %)	8 (34.7%)	100 (60.9%)	**0.017**
Age (years)	68.61 ±6.04	65.22 ±6.49	**0.019**
BMI (kg/m^2)	30.18±4.19	30.23±4.99	0.961
Diabetes duration (years)	17.69±9.44	14.08±9.34	0.084
Waist circumference (cm)	105.6±11.89	103.69±13.7	0.552
Smoking No (n, %) Current smoker (n, %) Ex-smoker (n, %)	 11 (47.8%) 03 (13.04%) 08(34.37%)	 88 (53.65%) 22(13.41%) 55(33.53%)	0.943
Hypertension (n, %)	17 (73.9%)	118 (71.9%)	0.844
Dyslipidemia (n, %)	16 (69.76)	133 (81.1%)	0.198
Insulin treatment (n,%)	17 (73.9%)	91 (54.48%)	0.198
Fast plasma glucose (mmol/L)	7.99±2.43	8.73±2.79	0.232
HbA1c (mmol/mol)	58.45 ± 8.10	56.1 ± 9.08	0.234
HbA1c (%)	7.72 ±1.07	7.41 ± 1.20	0.234
Total cholesterol (mmol/L)	4.69±0.66	4.78±0.95	0.682
HDL cholesterol (mmol/L)	1.33±0.38	1.27±0.30	0.399
LDL cholesterol (mmol/L)	2.73±0.47	2.71±0.82	0.906
Triglycerides (mmol/L)	1.39[0.51-2.5]	1.53 [0.6-5.7]	**0.046**
Homocysteine (µmol/L)	12.5 [8.1-17.4]	11.3 [5.8-127]	0.765

Lipoprotein (a) (mg/dl)	7.21 [1-91.2]	8.45 [1-162.9]	0.745
GFR (ml/min)	86.5±11.18	81.12±16.46	0.285
Creatinine (mmol/l)	0.068±0.01	0.0734±0.02	0.278
Albumin/creatinine ratio <3.39 mg/mmol (n, %) 3.39-33.9 mg/mmol (n, %) >33.9 mg/mmol (n, %)	 9 (40.9%) 10 (47.6%) 2(9.5%)	 111 (68.5%) 44 (27.2%) 7 (11.3%)	0.06
Log albumin/creatinine ratio	1.50±0.70	1.25±0.61	0.085
Diabetic Retinopathy (n,%)	11 (47.82%)	40 (24.40%)	**0.018**
Diabetic Neuropathy (n,%)	3(13.04%)	32(19.451)	0.450
CACS>400AU (n, %)	10 (52.63%)	31(19.562)	**0.001**
Log CACs (AU)	2.55±0.84	2.05±0.78.7	**0.013**
AGEs 3rd Tertil (AU)	12 (63.15%)	39 (26.71%)	**0.001**
AAS (n,%)	6(27.27%)	54 (32,92%)	0.594
Statines (n,%)	14 (63.63%)	119 (72567%)	0.384

The multivariate Cox's regression **(Table 13),** including the selected variables that were significant to the univariate analysis and well-known risk factors of CVD, showed that only age (HR 1.09, 95% CI 1.01–1.18, p = 0.024), gender (HR 0.35, 95% CI 0.15–0.83, p = 0.0174), the presence of DR (HR 2.58, 95% CI 1.14–5.85, p = 0.023), CACS > 400 AU (HR 4.16, 95% CI 1.14–10.26, p = 0.002) and a value of SAF on the 3rd tertile (HR 4.68, 95% CI 1.83–11.96, p = 0.001) were independently associated with the presence of a CV event.

Table 13. Results of the multivariate Cox's regression for predicting a vascular event.

	HR	**CI95%**	**p**
Sex (female)	0.35	0.15-0.83	**0.017**
Age (y)	1.09	1.01-1.18	**0.024**
BMI (kg/m2)	0.99	0.91-1.08	0.820
Diabetes duration (y)	1.04	0.99-1.08	0.093

Waist (cm)	1.01	0.98-1.04	0.526
Hypertension (yes)	1.13	0.45-2.88	0.792
Dyslipedemia (yes)	0.59	0.24-1.44	0.244
Insulin treatment (yes)	2.11	0.83-5.36	0.116
HbA1c (mmol/mol)	1.20	0.88-1.66	0.255
GFR (ml/min)	1.02	0.99-1.05	0.170
Creatinine (mg/dl)	0.33	0.04-2.44	0.275
Diabetic Retinopathy (yes)	2.58	1.14-5.85	**0.023**
CACS>400 AU (yes)	4.16	1.69-10.26	**0.002**
AGEs 3rd Tertil (yes)	4.68	1.83-11.96	**0.001**

HR: hazard ratio; CI: confidence interval.

6.Discussion

6.1. Relationship between DR and SAF and subclinical CVD: Results from cross-sectional study.

6.1.1. Diabetic retinopathy is a useful tool for identifying subclinical cardiovascular disease in T2D subjects.

This study provides evidence that DR is a powerful and independent risk factor for identifying diabetic subjects with subclinical CVD. In fact, we have found that the assessment of DR is a good and independent predictor of both a CACs >400 AU and the composite of a CACs >400 AU, carotid plaque ≥3 mm, CIMT >1 or ECG changes suggestive of previous asymptomatic MI. In addition, a clear relationship between the presence and degree of DR and coronary stenosis was found. These findings suggest that type 2 diabetic subjects with DR represent a subset of patients at very high risk of CVD who need a specific program aimed at reducing CV risk factors, optimizing metabolic control, and providing a periodic assessment of CVD. It should be noted that the presence of DR confers a higher risk of subclinical CVD than factors contained in contemporary risk equations, such as blood pressure, LDL cholesterol, and HbA1c. In this regard, we have found that moderate–severe DR significantly increases the AUROC of the UKDPS and Framingham risk scores CHD in predicting the presence of the combined endpoint of subclinical CVD.

Consistent with our findings, previous reports have documented an increase in CV risk in patients with DR, particularly in those with advanced DR (116,119,120,128). For instance, de Kreutzenberg et al (129), in a large cohort of type 2 diabetic subjects found that DR alone or in combination with nephropathy was independently associated with the presence of carotid plaques, and that severity of microangiopathy correlates with severity of carotid atherosclerosis. In addition, coronary atherosclerosis and plaque vulnerability are more severe in patients with DR (130). Alonso et al. (128) identified that T2D patients with DR had more atherosclerosis in their carotid arteries. Another study reported a significant association of increased CIMT with DR and peripheral vascular disease (PVD). Moreover, Gerstein et al. (116) demonstrated a clear relationship between the DR and the risk of serious CV outcomes in people with type 2

diabetes; additionally, the rise in adjusted risk from 1.5 in people with mild NPDR (vs. no retinopathy) to 2.4 in people with severe DR demonstrates than more advanced retinopathy at baseline predicts a higher risk of serious outcomes. Also they had demonstrated consistent significant relationship between progression of retinopathy over 4 years and incident of CV outcomes.

The combination of this observation with the novel finding that the relationship is dynamic and that progression of retinopathy is related to a higher CV risk has several implications. Overall, this information suggests that CHD is mediated by microvessel damage within the arterial wall (the vasa vasorum) induced by diabetes (131), suggesting a similar pathologic process may underlie both diabetic retinopathy and CV disease (132).

The screening of CVD in the diabetic population continues to be a challenge. In terms of CVD risk stratification, none of the routine cardiovascular screening approaches provide conclusive or definitive results for patients with T2D. Furthermore, the use of conventional screening tools to assess the CVD risk in diabetic patients does not account for their disproportionate cardiovascular morbidity and mortality (133).

The CAC score facilitates medical decisions for asymptomatic patients aged 40-75 who do not have a confirmed diagnosis of ASCVD (134). The CAC score reinforces angiographic findings that determine the severity of coronary calcification in high-risk patients with type 2 diabetes. Additionally, it redirects the reclassification of patients with T2D (with intermediate CVD risk) into low- and high-risk groups. The risk of cardiovascular mortality is 7-20% in patients with T2D who do not undergo CAC scoring, while CAC scores within the range of 0-10, 10-99, 100-299, 300-999, and 1000 (or above) present a risk of cardiovascular death by 2%, 9%, 6%, 12%, and 63%, respectively. CAC scans are effective screening tools for determining the amount and degree of coronary calcium deposition (133). An analysis of pooled population-based studies showed that in adults older than 60 years without known atherosclerotic CVD at baseline, the CACs has a greater association with incident CHD (follow-up 11 years) and a modestly improved prediction of incident stroke (135). Budoff et al. (136) analyzed data from 1156 diabetic subjects in the Diabetes Control and

Complications Trial (DCCT) and Epidemiology of Diabetes Interventions and Complications (EDIC) study to reveal the high incidence of CVD and major adverse cardiac events (MACE) in patients with a CAC score of 100 Agatston units or higher. These results confirm the use of CAC scores for risk stratification and prognostication of patients with T2DM. According to the literature, CAC scoring has a 68% positive predictive value, a 93% negative predictive value, a 40% specificity, and a 98% sensitivity in tracking the predisposition of T2DM patients for cardiovascular events.

Moreover, in T2D population, CACs appeared to be a more important clinical prognostic indicator than typical variables that confers poor prognosis to T2D individuals such as insulin use, bad glycemic control or diabetes duration (137). In addition, CACs has been established as a reliable marker for detecting subclinical atherosclerosis and predicting CVD events. According to the results obtained from the PROCEED study (259 diabetic patients with a mean age of 61.6 years and 57.6% South Asian ethnicity), 24% of subjects had a CACs of zero despite a mean duration of diabetes of 13.7 years (138). The CACs was an independent predictor of significant plaques on coronary angiography. According to multivariate analysis, only the duration of T2DM, CACs, number of plaques, and the presence of substantial plaque were associated with an increased likelihood of having adverse cardiovascular events, whereas blood pressure had only borderline significance in predicting these events (138). Higher CACs were associated with older age, male sex, longer diabetes history, and multiple traditional risk factors. CACs were associated with higher long-term mortality and major CVE, regardless of the risk factor burden, while those without CAC had very low event rates. The authors emphasize the importance of CAC scoring in preventing CVD in patients with diabetes. The report concludes that a CACs of 400 or above carries an 8.67 hazard rate for MACE and cardiac death in patients with T2DM and indicates the need for prophylactic cardiovascular management (138).

Our findings further support this concept and, in addition, show a clear relationship between a CACs >400 AU and coronary artery stenosis in T2D subjects. However, given the high prevalence of diabetes, it is desirable to identify

a more targeted population in which screening by using the CACs could be recommended. This would increase the cost-effectiveness of screening and avoid unnecessary irradiation. We found that by using simple clinical parameters such as age, gender, and the assessment of DR, the AUROC for predicting a CACs >400 was 0.77. In addition, CACs <10 was also associated with the absence of, or just mild DR.

Overall, our results point to T2D patients with DR as a subset of the diabetic population at a high risk of having silent CVD and, in consequence, more prone to develop a cardiovascular outcome. However, given that the prevalence of DR in T2D subjects is around 30%, it still remains a huge population which makes unfeasible to recommend a screening for silent CVD. The higher risk observed in those patients with moderate–severe DR to exhibit subclinical CVD points to this specific subset of patients as the most cost-effective candidates for screening. Therefore, healthcare professionals involved in the treatment of patients with diabetes should contemplate those individuals with moderate–severe DR as a high-risk population independently of the association of other traditional CV risk factors.

CT angiography images of coronary artery stenosis provide a better prediction risk in asymptomatic type 2 diabetics than traditional risk factors and the CACs (139,140). Notably, we have found for the first time a relationship between DR and the presence of coronary stenosis assessed by coronary CT angiography. Patients with DR presented a higher proportion of having one or more coronary stenosis ≥50% than those patients without DR (38.5% vs 20.2%). In addition, this proportion was even higher (50%) in those patients with moderate–severe NPDR and PDR.

Apart from DR, we found that microalbuminuria was associated with subclinical CVD assessed by the combined endpoint, but it was unrelated to the CACs or the presence of coronary stenosis. This finding suggests that microalbuminuria reflects a more widespread atherosclerotic process, whereas the presence and degree of DR might be more directly related to CHD. It should be noted that there is extensive evidence indicating that microalbuminuria is an independent CV risk factor and it is generally considered in the design and the analysis of the results

of clinical trials on CVD performed in the diabetic population. However, this is not the case for DR. This is a significant drawback that could lead to a serious bias on the results obtained in both epidemiologic and interventional studies.

Our results underline the importance of screening for DR, which should be envisaged as a method for studying the microvasculature of the retina and its potential implications in vision loss, and also as a herald of other systemic complications of diabetes, including CVD. Retinal imaging has the significant advantage of not using ionizing irradiation and it is performed as routine screening in the diabetic population. Given the alarming increase in the number of people with diabetes and the shortage of trained retinal specialists and graders of retinal photographs, an automated approach involving a computer-based analysis of the fundus image would reduce the burden of health systems in screening for DR (141,142). There is hence an increasing interest in the development of automated analysis software using artificial intelligence/deep neural learning for the analysis of retinal images in people with diabetes (142), and it is foreseeable that specific software will be developed to better define the cardiovascular risk of diabetic individuals based on the retinal structural and functional findings of microvessels.

The low percentage of silent MI detected by ECG in our cohort, 1 out 200 (0.5%), is quite surprising. In the UKPDS, 1 in 6 (16%) patients with newly diagnosed type 2 diabetes had evidence of silent MI on the baseline surface ECG (143). In older studies, the prevalence of ECG abnormalities in patients with type 2 diabetes and no known CHD was even higher, approaching 20% (144). The low rate of silent MI in our diabetic population could not be attributed to low diabetes duration or a selected group with low diabetes-related systemic complications. In fact, the mean of known diabetic duration was 12 years, around 30% of the subjects already presented microangiopathic complications, and more than half required treatment with insulin. It should be noted that this low rate of ECG signs of asymptomatic ischemic heart disease did not mean the presence of low myocardial microangiopathy. In this regard, we have previously reported that DR was independently associated with myocardial perfusion defects (145). Altogether, these findings suggest that our population could have a high degree

of protection against CV outcomes despite having a high rate of cardiovascular risk factors including microangiopathy. A Mediterranean diet and the high proportion of patients under treatment with statins might help explain why we detected a very low rate of silent MI; however, specific studies aimed at examining this issue are needed.

In summary, the presence of DR is an independent predictor of CACs >400 AU, and moderate–severe DR is independently associated with subclinical CVD. Therefore, the presence and degree of DR should be considered a better tool for identifying type 2 diabetic patients at risk of CVD than the conventional CV risk factors used in current risk equations

6.1.2. Skin advanced glycation end products are a useful tool to predict coronary artery calcium score in patients with T2D.

We confirmed that SAF (a non-invasive and simple marker of the accumulation of AGEs in the skin) and CACs (a reliable marker of coronary atherosclerosis) were significantly elevated in subjects with T2D in comparison with healthy controls. Furthermore, the present study showed that SAF is an independent predictor of CACs ≥ 400 AU. This finding suggests that SAF may be a useful tool for identifying patients with T2D at high risk of developing CHD.

Our findings support existing data showing that SAF is significantly higher in patients with T2D than in healthy subjects, and in particular in those patients with micro- or macrovascular complications (76,82,146,147). A large cross-sectional study comprising 825 subjects with type 2 diabetes showing that SAF is an independent predictor of T2D complications, including DR, diabetic kidney disease, diabetic cardiovascular disease, and diabetic peripheral neuropathy. Additionally, as the number of complications increases, the SAF value also increases (83). Hosseini et al. (84), in a systematic review and meta-analysis, suggested that SAF levels could be a predictor of chronic micro and macrovascular complications in DM.

We have also found a direct relationship between SAF and age and T2D duration as previously reported (73). However, we did not find any correlation between SAF and HbA1c. Since skin AGEs are mainly accumulated in collagen, which has

a low turnover and represents the diabetic milieu influence over a longer time period than HbA1c (10–15 years vs. 3 months, approximately) (60); thus, skin AGEs may reflect the impact of both oxidative stress and a history of sustained hyperglycemic episodes (69). In fact, a DCCT sub-study demonstrated that AGEs accumulation in skin collagen had a better predicting value for diabetic vascular complications compared with HbA1c in type 1 diabetes (71) and several studies reported SAF as independent and strong predictor for microvascular and macrovascular complications in T2D (75,147).

This is the first study showing an association between CACs and SAF in subjects with T2D in Caucasian population. To the best of our knowledge, there is only another study evaluating the relationship between SAF and CACs with similar results. This was a cross-sectional uncontrolled study performed in 122 Japanese with T2D (66). Altogether, these findings suggest that SAF assessment (a non-invasive, quick and simple method) is a good and independent predictor of CACs ≥ 400AU.

Little is known regarding the relationship between SAF values and subclinical cardiovascular disease in both general and T2D population. Dekker et al. (147) reported that SAF values were correlated with the degree of atherosclerosis but not with CACs in general population with subclinical and clinical atherosclerosis. However, it should be noted that in this study a lower CACs cutoff (100AU) was used and only 18 out of the 223 included subjects had diabetes. Recently, Pan et al. (148) had explored the relation between SAF and subclinical atherosclerosis in coronary and carotid arteries. They included 4416 subjects (aged 50–64 years), SAF was measured and subclinical atherosclerosis was assessed by ultrasonography of carotid arteries for evaluation of carotid plaques and CACS for coronary atherosclerosis. A total of 615 (13.9%) individuals had CACS >100 and 1340 (30.3%) subjects had bilateral carotid plaques, after controlling for confounding factors, there were significant associations between SAF and CACs >100 and carotid plaque (total carotid plaque area and also bilateral carotid plaque), concluding that elevated skin AF was significantly associated with subclinical atherosclerosis in coronary and carotid arteries independently of conventional risk factors.

In T2D population, Temma et al. (76) showed that SAF was well correlated with the degree of maximum intima-media thickness in the carotid artery, concluding than SAF might be a good surrogate marker of atherosclerosis. Ninomiya et al. (149) demonstrated that SAF was an independent determinant of brachial flow mediated dilation (an indicator of endothelial dysfunction: an early stage of atherosclerosis); and also confirmed that SAF was associated with arterial thickening assessed by maximum carotid intima-media thickness.

All these findings support the concept that AGEs and their receptor system (RAGE) play an important role in the impairment of vascular function. AGEs may contribute to CVD by three well-established pathophysiological mechanisms: (1) AGEs can affect the physiological properties of cardiac proteins in the extracellular matrix by creating cross-links, which provoke decreased flexibility of the matrix proteins and produce stiffness in vascular walls (150); (2) AGEs induce endothelin-1 production (151) and reduce nitric oxide (152) at the vascular level, thus resulting in vasoconstriction and the loss of vascular compliance; and (3) AGEs/RAGE binding induce inflammatory pathways, apoptosis of SMCs and increase vascular adhesion molecules, which in turn could promote atherosclerosis. In addition, oxidative stress generated via AGEs/RAGE pathway could promote SMCs switch to osteoblast-like cells and enhanced vascular calcification, therefore AGEs can cause multiple vascular and myocardial changes through the interaction with RAGEs, leading to atherosclerosis, thrombosis, and vasoconstriction (101). Thus, AGEs are not only markers of “metabolic memory” in diabetic subjects, but also have important pathogenic role both in endothelial dysfunction and in the atherosclerotic process (153). In fact, in the present study, the higher SAF values were related to recognized CV risk factors such as age, longer diabetes duration, presence of DR and microalbuminuria, serum levels of homocysteine and low glomerular filtration.

The assessment and stratification of cardiovascular risk in subjects with T2D and the consequent screening of CVD is a challenge. The UKPDS risk score is still one of the most used tools to give cardiovascular risk estimates in people type 2 diabetes (35). Lutgers et al. demonstrated that SAF provides additional information to the UKPDS risk score for the estimation of cardiovascular

prognosis in T2D (154). In addition, there is emerging evidence indicating that SAF is an important biomarker not only of the presence of cardiovascular disease but also of their outcomes (100,105).

As we said previously, in T2D population, CACs appeared to be a more important clinical prognostic indicator than typical variables that confers poor prognosis to T2D individuals such as insulin use, bad glycemic control or diabetes duration (137) and has been established as a reliable marker for detecting subclinical atherosclerosis and predicting CVD events. However, CT examinations can be inconvenient and rather expensive for routine practice in subjects with T2D. As we said previously, given the high prevalence of diabetes, it is desirable to identify a more targeted population in which screening by using the CACs could be recommended. This would increase the cost-effectiveness of screening and avoid unnecessary irradiation. In this regard, we provide evidence that by using simple clinical parameters such as age, cholesterol HDL and assessment of SAF values, the AUROC for predicting a CACs ≥ 400AU was 0.77, with a sensibility and specificity of 80% and 70.2%, respectively.

In conclusion, SAF is a good and independent predictor of CACs ≥ 400 in T2D population. This finding suggests that SAF could be a useful tool to identify those patients in whom CT scan for assessing CACs should be prioritized. In addition, SAF measurement can be envisaged as a method to help us to select a high CV risk population in whom the strategies aimed at reducing CV risk factors and optimizing metabolic should be focused.

6.2. Relationship between DR and SAF and CVE: results form prospective study.

After a follow up of 4.35 years, we confirmed that individuals with type 2 diabetes had significantly more risk of having a CV event than non-diabetic subjects. Furthermore, we provide evidence that DR and SAF (as a measure of tissue AGE accumulation) are powerful predictors of CV events in subjects with type 2 diabetes.

We found that patients with type 2 diabetes had significantly more risk of suffering a CV event than non-diabetic subjects (12.29% VS 1.75%). Consistent with our findings, previous reports have documented that subjects with type 2 diabetes have a higher risk of developing a CV event and with a worse outcome in comparison with non-diabetic subjects (13,155).

Previously, in the cross-sectional study we have already provided evidence that DR is an independent predictor of subclinical CVD, and SAF was good predictor of a CACs >400AU (a reliable marker of coronary atherosclerosis). In the follow up of the same cohort, after a mean of 4.35 years, we confirmed that both, DR and SAF, are not only related to subclinical cardiovascular disease but also are capable of predicting cardiovascular events in type 2 diabetes population.

6.2.1. DR as a biomarker of cardiovascular events in T2D subjects.

Several studies had suggested that the burden of microvascular disease is determinant of future cardiovascular risk (117,156,157). Brownrigg et al. (117) found that the burden of microvascular disease is a determinant of future cardiovascular risk: and the risk of a first cardiovascular event increased linearly with the number of manifestations of microvascular disease present. In our study, only DR is a powerful predictor of cardiovascular events in subjects with type 2 diabetes. According with our findings, previous reports have documented an increase in CV risk in patients with DR, mostly in those with advanced DR (116,119,120,128).

Another similar prospective study has recently been published, which included 374 diabetic patients without known CV disease, with a follow-up of 7.1 years, and it reported that the presence of diabetic retinopathy at the baseline of the study was a predictor of cardiovascular events, concluding that DR is a strong predictor of cardiovascular events in T2D individuals at primary CVD prevention (158).

Furthermore, in Catalonia, it has been published a retrospective cohort study with routinely collected health data from SIDIAP (primary care data base) between 2008 and 2016. 22,402 T2DM subjects with DR were identified in the database and 196,983 T2D subjects without DR. The aim of the study was to evaluate the

predictive value of diabetic retinopathy (DR) and its stages with the incidence of major cardiovascular events and all-cause mortality in type 2 diabetes mellitus. During the follow-up period among the subjects with DR, they observed the highest incidence of all-cause mortally, and in second place were the macrovascular events among the subjects with DR. After multivariable analysis, they observed that subjects with any stage of DR had higher risks for all of the study events, except for stroke. They concluded, that DR is related to CHD, macrovascular events, and all-cause mortality among persons with T2DM (159).

6.2.2. DR and Cardiovascular disease: a common soil

Although the underlying molecular mechanisms linking DR and cardiovascular disease are still a matter of debate, there are notable similarities in their pathophysiology. The presence of DR means that the microcirculation has already been damaged by the diabetic milieu and, therefore, it can be considered a reliable biomarker of the deleterious effects of diabetes in a specific individual. In this regard, recent evidence indicates that, in individuals with type 2 diabetes, the *vasa vasorum* (a network of small blood vessels that supply the walls of large blood vessels) present evolutionary changes similar to those observed in the retina: an initial stage in which endothelial dysfunction and loss of capillaries predominate (122), and more advanced stages in which ischemia plays a key role, leading to angiogenesis and inflammation in response to the progressive enlargement of the necrotic core within the plaque (124). This change in plaque phenotype results in a more inflamed and unstable plaque, favoring plaque rupture and a poor outcome of cardiovascular events. Thus, microcirculation represents a “common soil” between DR and cardiovascular event, and would explain why DR is a good predictor of CV events as we reported.

Therefore, in CV outcomes participate the pathologic changes that occur in the vasa vasorum of conductance vessels (160) or in the myocardial capillaries that are similar to the changes seen in the retina. Alternatively, the retinal microvascular abnormalities may be a consequence of macrovascular disease and reduced vascular flow. The observed link between coronary calcification and retinal abnormalities in people at high risk for CV disease supports both possibilities (161). Our findings also suggest that both processes may be

promoted by common underlying metabolic abnormalities that contribute to endothelial dysfunction such as hyperglycemia or dyslipidemia.

In the retina, the microvascular dysfunction causes a leakage in the arteriolar and capillary bed, which is characteristic of DR. In the large arterial wall, vascular leakage causes lipid accumulation, consequently leading to a pathogenic cascade of atherosclerosis (162). The endothelial inflammation may promote plaque formation and even the rupture in these large vessels and/or promote myocardial dysfunction. Regarding the underlying mechanisms, hyperglycemia causes inflammation by releasing reactive oxygen species, advanced glycation end products, cytokines, and chemokines. These collectively cause oxidative stress and endothelial dysfunction that facilitates the entry of monocytes and macrophages. Sequentially, endothelial dysfunction also helps low-density lipoprotein (LDL) particles penetrate the intimal wall of the vessel in a process called transcytosis. Further, the LDL particles get oxidized and form OxLDL, due to the inflammatory markers process. These results an intracellular uptake of oxLDL by macrophages in the arterial intima and help the formation of foam cells. Over time, the foam cells die, contributing to the production of interstitial collagen and elastin inside the foam cells; causing the formation of the necrotic core. Collectively, these overall sequential steps initiate the platelet aggregation and adhesion favors the atherosclerotic plaque formation causing micro and macrovascular complications (162)159).

Our findings support the idea that changes in the retina (which are readily accessible for measurement) may reflect changes in an individual’s CV risk and may therefore identify those individuals whose CV risk is rising and who may benefit from particularly aggressive CV risk reduction therapies. Therefore, it is important to remark that DR is not only associated with other diabetic microangiopathic diseases such nephropathy or neuropathy but also with cardiovascular disease. This is a well-established concept that should be taken into account not only in clinical practice but also in the design of any study addressed to evaluate the cardiovascular outcome in the diabetic population.

6.2.3. SAF as biomarker of cardiovascular events in T2D subjects

SAF was also a good predictor of cardiovascular events in subjects with type 2 diabetes. There are multiples studies that reported significant associations between SAF and the development of late diabetic complications (both micro and macrovascular), most of them being cross-sectional studies (66,75,77,79,80,154,163). Few prospective studies have examined the usefulness of SAF as a predictor of CVD (81,154). All of them, supports our data and concluded that SAF is a measure of metabolic burden but it is also strongly associated with the presence of CVD and cardiac mortality, as well as a biomarker of vascular damage before it becomes clinically apparent.

Cavero-Redondo et al. (100) published some years ago a systematic review and metaanalysis about SAF as a predictor of cardiovascular and all-cause of mortality in high risk subjects with renal or cardiovascular disease. Ten studies were included, but only two with diabetic populations. They concluded that higher SAF levels were significantly associated with higher pooled risk estimates for cardiovascular mortality (HR: 2.06; 95% CI, 1.58–2.67) and all cause of mortality (HR: 1.91; 95% CI, 1.42–2.56). Therefore, SAF level could be considered a predictor of all-cause mortality and cardiovascular mortality in subjects with high risk with previous cardiovascular and kidney disease.

A recent article by Boersma et al. (164) explored the relation between SAF levels and the development of type 2 diabetes, cardiovascular disease, and mortality, and it was evaluated if elevated SAF values may predict the development of CVD and mortality in individuals with T2D. A total of 2349 subjects with T2D was included; 1318 reported a previous diagnosis of T2D (median duration of the disease of 5 years), while the rest of the included subjects were “new” cases of diabetes since the diagnosis was performed at baseline due to altered fast glycaemia or an HbA1c out of range. These patients were followed a mean of 3.7 years and new CV events were collected. They observed that individuals with “new” T2D had lower SAF values than those with known type 2 diabetes, reflecting the longer period of exposure to elevated glucose levels. In addition, SAF was significantly and independently associated with the combined outcome

of new CV events and mortality in T2D subjects (OR 2.59, 95% CI 2.10–3.20, p < 0.001).

More recently, Chen et al. (165) published a meta-analysis evaluating the prospective association between AGEs and MACE where fourteen articles were included, involving almost 80000 participants. They found a significant association between AGEs and MACE. Moreover, skin AGEs were associated with a significant increase in fatal CVD. The association between AGEs and MACE was also significant in patients with diabetes. This meta-analysis indicates that higher levels of AGEs measured by skin autofluorescence are significantly correlated with a higher pooled risk of MACE, and AGEs are closely related to both nonfatal and fatal cardiovascular events; for this reason, skin AGEs are a valuable biomarker for predicting the occurrence of MACE.

Therefore, SAF could be a useful clinical tool to identify diabetic individuals with preclinical vascular damage who have a particularly high risk of developing cardiovascular events. It is important to remark, that our study is the only one that includes exclusively subjects with type 2 diabetes and no history of clinical cardiovascular disease, apparently those with less cardiovascular risk, and yet we have obtained similar results.

Mulder et al. (107) showed that SAF is elevated in acute ST-elevation myocardial infarction compared with healthy controls, and higher values of SAF were related with more risk to die or a new myocardial infarction or heart failure in the following one year. This finding suggests that SAF may play an important role in the progression of atherosclerosis. Basic research has shown that in atherosclerotic plaques AGEs interact with RAGE, resulting in increased production of inflammatory mediators, causing the plaques more vulnerable to rupture (166). Data on the important role of oxidative stress markers in endothelial dysfunction and clinically over coronary artery disease are extensive (103). However, most markers for oxidative stress are not readily available for clinical practice. By contrast, skin AGES are stable and could be non-invasively assessed, thus serving as a reliable biomarker of cardiovascular disease.

In our study, we show that higher values of SAF were independently associated with the presence of macrovascular complications. Most of the classical cardiovascular risk factors such as hypertension, dyslipidemia and HbA1c were not significantly associated with the occurrence of a cardiovascular event. However, this does not mean that they are not influencing the development of CV events, but just that are currently under control. In fact, we would need biomarkers that inform us regarding long-term deleterious effect than those reflecting a short-term impairment. In this regard, skin AGEs are mainly accumulated in collagen, which has a low turnover and represents hyperglycaemia over a longer time period than HbA1c, so SAF may reflect the impact of oxidative stress and history of hyperglycemic episodes better than classical risk factors. In fact, SAF is considered as a measure of metabolic memory in subjects with type 2 diabetes.

In addition to DR and SAF, we found that other classical factors such age, male sex and CACs >400AU also were related with the presence of a vascular event. Age is an important determinant of cardiovascular risk, and it is known that the prevalence of inducible ischemia is significantly higher in type 2 diabetes patients over 65 years old (167). Furthermore, it is well documented that the absolute risk of cardiovascular events is higher in men than women (168). CACs is a well-recognized biomarker myocardial ischemia and a good predictor of cardiovascular events (52,53). In fact, guideliness recommend that assessment of CACs could be considered in asymptomatic patients with diabetes mellitus who are over the age of 40 (18). However, CACs assessment needs of a CT scan examination, which can be inconvenient and rather expensive for routine practice in subjects with type 2 diabetes.

In conclusion, the prospective study confirms that patients with type 2 diabetes have significantly more CV events than non-diabetic subjects. In addition, DR and higher values of SAF are powerful predictors of CV events in subjects with type 2 diabetes and, therefore, might be included as meaningful variables in CV-risk stratification. Furthermore, DR and higher values of SAF could be useful biomarkers in selecting type 2 diabetic patients in whom the screening for

cardiovascular disease should be prioritized, thereby generating more personalized and cost-effective medicine.

6.3. Limitations of cross-sectional study

This study has several limiting factors. First, the analyses were restricted to individuals for whom complete information was available, and this may have resulted in selection bias. Second, the results could have been influenced by variables such as diet or ambient factors not considered in this analysis. Third, the study population was relatively small and the data presented are observational in nature. Moreover, the device used in this study, AGE reader, cannot be applied to subjects with dark skin because of the high absorption grade of the excited light. To address this problem, we did not include patients with skin reflectance below 6%, although the majority of the population in our environment is Caucasian. Further, Lutger et al. (74), previously described the other limitations of the autofluorescence reader as a marker of tissue AGES accumulation: non-fluorescence AGES will not be measured and other tissue components that fluoresce in the same range wavelength might be confounders. Finally, although our results suggest that the assessment of DR and SAF may offer a simple tool to identify very high-risk individuals with type 2 diabetes who are currently perceived to be at low absolute risk using contemporary risk models, all the risk algorithms (eg, QRISK, Framingham, Reynolds, UKDPS) are for calculating risk estimates and not for the detection of subclinical atherosclerosis. Therefore, further studies with a larger sample size are required to evaluate whether the addition of DR grading to conventional risk algorithms will confer any additional benefits.

6.4. Limitations of prospective study

First our sample was relatively small and the results could have been impacted by variables such as ambient factors or diet not considered in this analysis. Second, and probably the major limitation, was the low rate of cardiovascular events in our population. The low rate of CV events in our diabetic population could not be attributed to low diabetes duration or a selected group with low diabetes-related systemic complications. In fact, the mean of known diabetic

duration was 12 years, around 30% of the subjects already presented microangiopathic complications, and more than half required treatment with insulin. This suggest that our population could have a high degree of protection against CV outcomes despite having a high rate of cardiovascular risk factors including microangiopathy. A Mediterranean diet and the high proportion of patients under treatment with statins might help explain why we detected a very low rate CV event. Moreover, it should be noted that there is a clear trend toward a decrease in events in diabetic subjects in the last 20 years, as reported Rawshani et al. (6). This is probably due to the better management of the chronic patient with diabetes, associated with better comprehensive control of the rest of the cardiovascular risk factors, with greater use of statins and antihypertensive drugs.

6.5. Impact of our findings in clinical practice

At present, the early identification of diabetic patients at risk of developing CVD remains a challenge, and the high prevalence of T2D precludes implementing a generalized screening for CVD in asymptomatic patients. The identification of new cardiovascular risk markers can help us to select those patients with the highest cardiovascular risk in whom CVD screening can be cost effective.

Moreover, our findings could have both therapeutic and investigational implications. In fact, the detection and grading of DR and assessment of SAF permits us to identify patients at high CV risk who might, under current guidelines, benefit most from further lipid and blood glucose lowering. In addition, it could be used to enrich the cohorts for future intervention trials with patients more prone to develop clinical outcomes, thus reducing sample size, duration, and the cost of studies.

Furthermore, DR and higher values of SAF could be useful biomarkers in selecting type 2 diabetic patients in whom the screening for cardiovascular disease should be prioritized, thereby generating more personalized and cost-effective medicine.

A holistic and integrative approach taking into account classical CV risk factors, clinical variables and SAF and DR, will permit us to create a score that will permit better identify those T2D patients with very high CV risk.

7. Conclusions

1. The presence of diabetic retinopathy is an independent predictor of CACs >400 AU, and moderate–severe DR is independently associated with subclinical CVD.

2. The presence and degree of diabetic retinopathy should be considered a better tool for identifying type 2 diabetic patients at risk of CVD than the conventional CV risk factors used in current risk equations.

3. Skin AGEs assessed by autoflurescence (SAF) are a good and independent predictor of CACs ≥ 400 in T2D population.

4. SAF could be a useful tool to identify type 2 diabetic patients at risk of CVD in whom CT scan for assessing CACs should be prioritized.

5. DR and higher values of SAF are powerful predictors of cardiovascular events in subjects with type 2 diabetes

8. New Perspectives based on the provided results.

The results of the present work support the concept that the burden on microangiopathic complications, and specifically DR and skin AGEs are related with subclinical CVD and can help to predict CV events in T2D subjects.

On this basis, an automated approach involving a computer-based analysis of the fundus image would reduce the burden of health systems in screening for DR.

Several of the existing and most recent guidelines recommend the use of some algorithms to perform CVD risk assessment. Above all, they have been developed to decide the start of statin treatment. Because they were developed on specific ethnic cohorts, when applied to diverse ethnic populations, they can either underestimate or inflate the risk of cardiovascular disease. These calculators were built using regressions-based techniques that can handle a limited set of risk predictors. Due to these issues, a more reliable and precise CVD risk prediction model is required.

Some Artificial Intelligence (AI) based algorithms have proved themselves to be superior to the existing CVD risk calculators (169,170). This is the reason for the growing interest of clinicians in exploring the potential of AI in dealing with several healthcare problems, including the CVD risk assessment. AI is primarily categorized into two types of algorithms: machine learning and deep learning algorithms. Both of these algorithms require large datasets under a big data framework to build their internal models and provide accurate risk assessment. Machine learning algorithms require a series of pre-processing steps that involve data cleaning, noise reduction, feature extraction, and feature selection.

There is hence an increasing interest in the development of automated analysis software using artificial intelligence/deep neural learning for the analysis of retinal images in people with diabetes and it is foreseeable that specific software will be

developed to better define the cardiovascular risk of diabetic individuals based on the retinal structural and functional findings of microvessels.

Our group is exploring the use Deep Learning architectures on retinal fundus imaging as a tool for predicting CV risk in subjects with T2D and no history of CVD. Particularly, we use the coronary artery calcium (CAC) score as a marker, and train a convolutional neural network (CNN) to predict whether it surpasses a certain threshold defined by experts (CACs >400UI). The preliminary experiments on a reduced set of clinically verified patients show promising accuracies. In addition, we observed that elementary clinical data is positively correlated with the risk of suffering from a CV disease. We found that the results from both informational cues are complementary, and we propose two applications that can benefit from the combination of image analysis and clinical data. Particularly, we are using the coronary artery calcium (CAC) score as a marker, and train a convolutional neural network (CNN) to predict whether it surpasses a certain threshold defined by experts (>400AU) (171), This is a preliminary work that proves that there exists discriminative information in the retinal images. Results can be significantly improved gathering more clinical data (increasing the number of relevant variables) or the number of images (more patients).

The objective of this new line of research is that by means of the image of the retinal fundus together with the assessment of SAF and clinical variables we could detect those patients with imminent risk of presenting a cardiovascular event and act accordingly.

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10. Annexes

10.1. List of publications related to this doctoral thesis.

10.1.1. Diabetic retinopathy as an independent predictor of subclinical cardiovascular disease: baseline results of the PRECISED study.

Rafael Simó, Jordi BañeraS, Cristina Hernández, José Rodríguez-Palomares, Filipa Valente, Laura Gutierrez, Teresa González-Alujas, Ignacio Ferreira, Santiago Aguadé-Bruix, Joan Montaner, Daniel Seron, Joan Genescà, Anna Boixadera, José García-Arumí, **Alejandra Planas**, Olga Simó-Servat, David García-Dorado. **Diabetic retinopathy as an independent predictor of subclinical cardiovascular disease: baseline results of the PRECISED study.** BMJ Open Diabetes Res Care. 2019 Dec 29;7(1):e000845. doi: 10.1136/bmjdrc-2019-000845. eCollection 2019.

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10.1.2. Usefulness of skin advanced glycation end products to predict coronary artery calcium score in patients with type 2 diabetes.

Alejandra Planas, Olga Simó-Servat, Jordi Bañeras, Mónica Sánchez, Esther García, Ángel M Ortiz, Marisol Ruiz-Meana, Cristina Hernández, Ignacio Ferreira-González, Rafael Simó. **Usefulness of skin advanced glycation end products to predict coronary artery calcium score in patients with type 2 diabetes.** Acta Diabetol. 2021 Oct;58(10):1403-1412. doi: 10.1007/s00592-021-01735-5. Epub 2021 May 25.

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Doi: 10.1007/s00592-021-01735-5.

10.1.3. Diabetic Retinopathy and Skin Tissue Advanced Glycation End Products Are Biomarkers of Cardiovascular Events in Type 2 Diabetic Patients.

Alejandra Planas, Olga Simó-Servat, Cristina Hernández, Ángel Ortiz-Zúñiga, Joan Ramón Marsal, José R. Herance, Ignacio Ferreira-González and Rafael Simó. **Diabetic Retinopathy and Skin Tissue Advanced Glycation End Products Are Biomarkers of Cardiovascular Events in Type 2 Diabetic Patients.** J Pers Med. 2021 Dec 10;11(12):1344. doi: 10.3390/jpm11121344.

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10.1.4. Advanced Glycations End Products in the Skin as Biomarkers of Cardiovascular Risk in Type 2 Diabetes.

Alejandra Planas, Olga Simó-Servat, Cristina Hernández, Rafael Simó. **Advanced Glycations End Products in the Skin as Biomarkers of Cardiovascular Risk in Type 2 Diabetes.** Int J Mol Sci. 2022 Jun 2;23(11):6234. doi: 10.3390/ijms23116234.

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