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DIETARY STRATEGIES IN THE MANAGEMENT OF TYPE 2 DIABETES IN PATIENTS WITH CORONARY HEART DISEASE: FROM CORDIOPREV STUDY

ESTRATEGIAS DIETÉTICAS EN EL MANEJO DE LA DIABETES MELLITUS TIPO 2 EN PACIENTES CON ENFERMEDAD CORONARIA ESTABLECIDA: ESTUDIO CORDIOPREV

Tesis doctoral presentada por:

M MAGDALENA PÉREZ CARDELO

Dirigida por:

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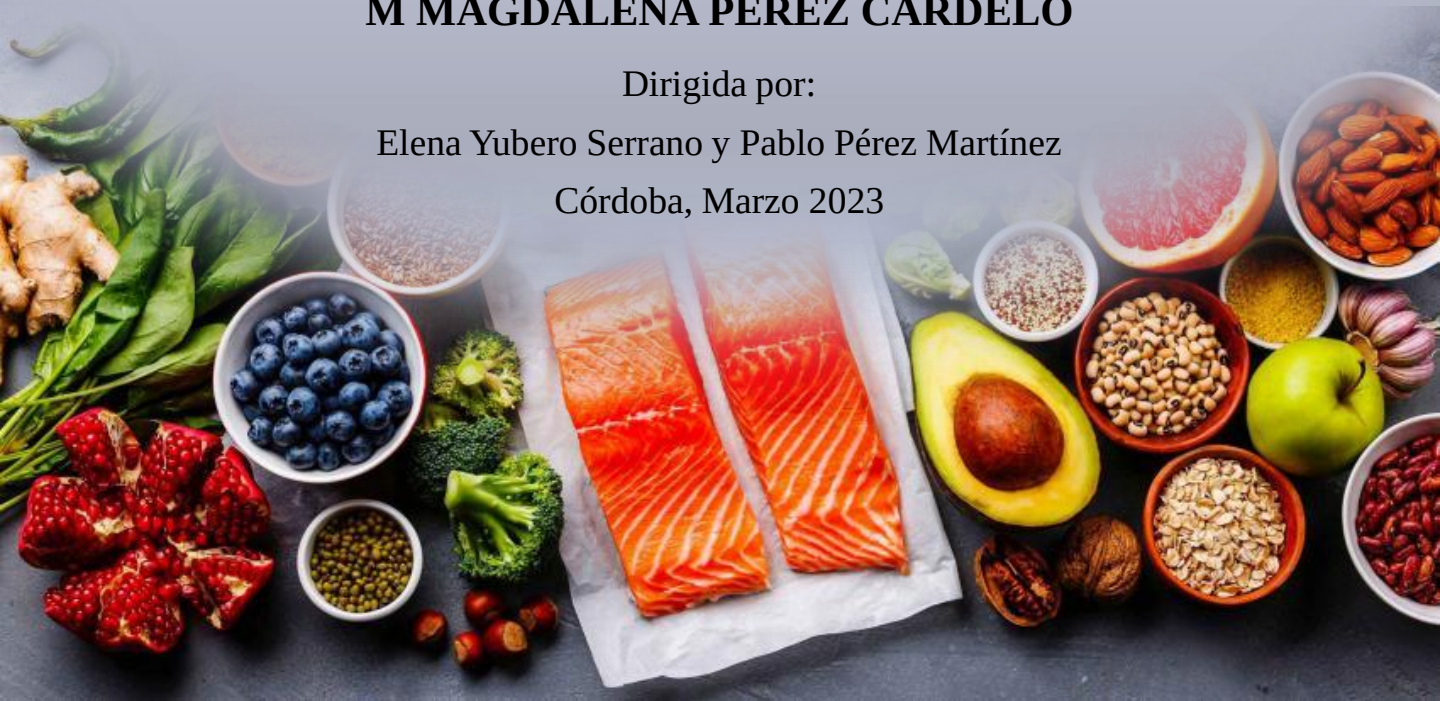
Córdoba, Marzo 2023



TITULO: *Dietary strategies in the management of Type 2 Diabetes in patients with coronary heart disease: from the CORDIOPREV study*

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ABREVIATIONS

ADA: American diabetes association

Adipo-IR: Adipose tissue insulin resistance index

AGER1: Advanced Glycation End Product Specific Receptor 1

AGEs: Advanced glycation end products

AHA: American Heart association

Akt: Protein kinase B

AUC: Area under the curve

BCAA: Branched chain amino acids

BCKD: Branched chain keto acid dehydrogenase

BMI: Body mass index

CHD: Coronary heart disease

CKD: Chronic kidney disease

CML: Carboxymethyl-lysine

CRP: C reactive protein

CVD: Cardiovascular disease

EAS: European Atherosclerosis Society

ESC: European Society of Cardiology

EVOO: extra virgin olive oil

FMD: Flow-mediated dilatation

FFA: Free fatty acids

GloxI: Glyoxalase I

GLP1: Glucagon-like peptide 1

HbA1c: Glycated hemoglobin

HIRI: Hepatic insulin resistance

HOMA-IR: homeostatic model assessment for insulin resistance

IDF: International diabetes federation

IGI: Insulinogenic index

IMT-CC: Intima-media thickness of both common carotid arteries

IR: Insulin resistance

ISI: Insulin sensitivity index

LDL-C: Low-density lipoprotein cholesterol

MG: Methylglyoxal

MISI: Muscle insulin sensitivity index

mTOR: Mammalian target of Rapamycin

MUFA: Monounsaturated fatty acids

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

OGTT: Oral glucose tolerant test

PBMCs: Peripheral blood mononuclear cells

PDH complex: Pyruvate dehydrogenase complex

PI3K: Phosphatidylinositol 3-kinase

PUFA: Polyunsaturated fatty acids

RAGE: Receptor for Advanced Glycation End Products

ROS: Reactive oxygen species

SFA: Saturated fatty acids

sICAM-1: Soluble intercellular adhesion molecule-1

SIRT-1: Sirtuin 1

sVCAM-1: Soluble vascular cell adhesion molecule-1

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

TCA: Tricarboxylic acid

TG: Triglycerides

TNF-α: Tumor necrosis factor-alpha

UCP2: Mitochondrial uncoupling protein 2

VLDL-TG: Very low-density lipoprotein-triglycerides

VOO: Virgin olive oil

WAT: White adipose tissue

WHO: World Health Organization

RESUMEN

RESUMEN

Introducción

En las últimas décadas la prevalencia de diabetes mellitus tipo 2 (T2DM) ha incrementado drásticamente, suponiendo un grave problema de salud a nivel mundial. Sin signos de recesión en un futuro cercano, la T2DM supone una gran carga al sistema de salud, la economía y la sociedad. Este hecho es especialmente relevante para pacientes con enfermedad coronaria establecida (CHD) y T2DM concomitante, pues la presencia simultánea de ambas enfermedades aumenta significativamente el riesgo de desarrollar un nuevo evento cardiovascular y, por tanto, un incremento en la mortalidad. La duración de la T2DM y el bajo control de la misma aumenta el riesgo de recurrencia cardiovascular, lo que evidencia la relevancia de la identificación precoz y el control eficaz de la enfermedad para reducir la morbimortalidad asociada, así como identificar aquellos subgrupos a los que se pueden aplicar con éxito recomendaciones dietéticas u otro tipo de tratamiento para prevenir o remitir la T2DM.

La T2DM se considera una enfermedad metabólica caracterizada principalmente por una alteración en la secreción y acción de la insulina que está fuertemente relacionada con la nutrición y otros hábitos de vida. De hecho, los estudios de intervención dietética han demostrado que los cambios en los hábitos dietéticos y el aumento de la actividad física pueden reducir la incidencia de T2DM, estableciendo una asociación entre patrones dietéticos y el riesgo de la misma. Más concretamente, las dietas bajas en carbohidratos, bajas en grasa, con restricción calórica o una dieta Mediterránea están asociadas con beneficios cardiovasculares, una mejor homeostasis de la glucosa y sensibilidad a la insulina. Sin embargo, la adaptación de estos enfoques dietéticos o el tratamiento a seguir podría variar en función de la situación basal y metabólica del paciente y del estado de la enfermedad en sí, ya que la flexibilidad metabólica podría afectar a los potenciales beneficios de los cambios en el estilo de vida o los tratamientos farmacológicos.

Recientemente se ha demostrado que la T2DM de reciente diagnostico puede revertirse a través de una pérdida intensiva de peso que puede ser conseguida mediante cirugía bariátrica o el seguimiento de una dieta hipocalórica. Sin embargo, últimos estudios sobre el tema han evidenciado la remisión de la T2DM a través de dietas saludables sin pérdida de peso asociada (una dieta baja en grasa o una dieta Mediterránea), provocando en estos pacientes una mejora de la sensibilidad a la insulina hepática, una reducción de la hemoglobina A1c (HbA1c) y una recuperación de la funcionalidad de las células β en pacientes con T2DM de reciente diagnóstico y CHD. Sin embargo, el consumo de estas dietas saludables no consiguió la remisión de la T2DM en el total de los pacientes del estudio, lo que destaca la falta de comprensión sobre los mecanismos que subyacen a la remisión de la T2DM promovida por cambios dietéticos.

Los productos finales de glicación avanzada (AGEs) son un grupo de compuestos prooxidantes y citotóxicos, generados a partir de la reacción de Maillard, que contribuyen a la aparición y progresión de ciertas enfermedades crónicas, como la T2DM y las enfermedades cardiovasculares. Los AGEs proceden principalmente de fuentes exógenas, pero una pequeña parte se genera de forma endógena como consecuencia del metabolismo normal, especialmente en pacientes con T2DM debido a la hiperglucemia crónica. La principal fuente exógena de AGEs procede de la dieta, que depende tanto de la composición como del procesado de los alimentos. Existen evidencias del papel que juegan los AGEs en la progresión de la T2DM y sus complicaciones. Sin embargo, no hay evidencia de la participación de los AGEs en la remisión de la T2DM.

Por otra parte, la incidencia de T2DM, así como con otras anomalías metabólicas como la resistencia a la insulina, la obesidad o el riesgo cardiovascular se relacionan con niveles plasmáticos elevados de aminoácidos de cadena ramificada (BCAA). En este sentido, los BCAA se consideran biomarcadores potenciales para la T2DM y la enfermedad cardiovascular. Aunque, hasta la fecha, los mecanismos moleculares no se comprenden bien, las múltiples funciones de los BCAA en el desarrollo de la resistencia a la insulina podrían conducir al desarrollo de terapias más efectivas para la T2DM.

En este contexto, diversos estudios han demostrado que existe relación entre la dieta Mediterránea y la reducción de los niveles dietéticos y circulantes de AGEs y los niveles plasmáticos de BCAA, así como el grado de estrés oxidativo y la inflamación en los que la siguieron. La dieta Mediterránea se caracteriza por su riqueza en grasas monoinsaturadas (principalmente del aceite de oliva virgen), verduras, frutas, frutos secos, legumbres y cereales integrales, que aportan fibra, antioxidantes, vitaminas, minerales y polifenoles con un bajo consumo de alimentos procesados. Las propiedades saludables de la dieta Mediterránea se deben en su mayoría al poder sinérgico de sus componentes provocando efectos antiinflamatorios y antioxidantes, que se potencia con la forma de cocinar de esta región. Múltiples estudios relacionan este patrón dietético con la reducción y prevención de múltiples enfermedades como la T2DM o la enfermedad cardiovascular. Sin embargo, son necesarios más estudios para desentrañar los efectos de esta dieta en la fisiopatología de la enfermedad y su relación la remisión de la T2DM. Ya que esto podría no solo reducir la incidencia de esta enfermedad a nivel mundial sino también la prevalencia a través de la posibilidad de remisión de la T2DM, que adquiere aún más relevancia cuando puede estar mediada por cambios en el estilo de vida y patrones dietéticos saludables y de relativamente fácil adherencia, como la dieta Mediterránea.

Hipótesis

En base a estos hallazgos, nuestra hipótesis de estudio es que los niveles iniciales de productos finales de glicación avanzada (AGEs) y/o aminoácidos de cadena ramificada (BCAAs) podrían conducir potencialmente a la identificación de pacientes con diabetes mellitus tipo 2 (T2DM) y enfermedad coronaria establecida (CHD) que se beneficiarían de intervenciones dietéticas saludables (una dieta Mediterránea o una dieta baja en grasa) para reducir las complicaciones cardiovasculares. También planteamos la hipótesis de que la reducción de los niveles circulantes de AGEs y/o BCAAs, a través del consumo de estas dietas saludables, podría estar involucrada en los mecanismos moleculares que subyacen a la remisión de la T2DM en pacientes con T2DM de reciente diagnóstico y CHD.

Objetivos

Determinar si los niveles iniciales de productos finales de glicación avanzada (AGEs) y/o aminoácidos de cadena ramificada (BCAAs) están asociados con la remisión de la diabetes mellitus tipo 2 (T2DM) en pacientes con T2DM de reciente diagnostico con enfermedad coronaria establecida (CHD) y si la reducción de sus niveles circulantes, tras el consumo de dos modelos de dietas saludables (una dieta Mediterránea y/o una dieta baja en grasas) contribuye a la remisión de la T2DM con el objetivo de establecer estrategias dietéticas terapéuticas para el manejo de estos pacientes.

Objetivos específicos

1. Comparar los niveles AGEs en pacientes con CHD y T2DM de recién diagnóstico y T2DM establecida y su asociación con marcadores ateroscleróticos subclínicos [vasodilatación mediada por flujo braquial (FMD) y grosor de la íntima media de ambas carótidas comunes (IMT-CC)] para establecer estrategias terapéuticas para prevenir o reducir los niveles AGE y retrasar la aparición de complicaciones cardiovasculares en este tipo de pacientes.
2. Analizar si la reducción de los niveles circulantes de AGEs y la modulación de la expresión génica relacionada con el metabolismo de los AGEs, tras el consumo de dos modelos de dietas saludables (una dieta Mediterránea y una dieta baja en grasas), se asocian con la remisión de la T2DM en pacientes con T2DM de reciente diagnóstico y CHD.
3. Evaluar la relación entre los niveles de BCAA circulantes (basales y cambios después del consumo de dos modelos de dietas saludables (una dieta Mediterránea y una dieta baja en grasas)) y la remisión de la T2DM en pacientes con T2DM de reciente diagnóstico y CHD.

Participantes, diseño y metodología

Esta tesis se ha desarrollado en el marco del estudio CORDIOPREV (Clinicaltrials.gov número NCT00924937). El estudio CORDIOPREV es un ensayo clínico unicéntrico, prospectivo, aleatorizado y controlado de intervención dietética que incluye 1002 pacientes con CHD, desarrollado en el Hospital Universitario Reina Sofía de Córdoba, España. Antes del reclutamiento y el inicio del protocolo de estudio, se obtuvieron los consentimientos por escrito de todos los participantes. Todos los análisis se realizaron bajo el principio de intención de tratar y todas las modificaciones siguieron la Declaración de Helsinki y las buenas prácticas clínicas, aprobadas por el Comité Ético del Hospital Reina Sofía (Córdoba, España).

Los pacientes incluyeron hombres y mujeres de 20 a 75 años con enfermedad coronaria establecida, sin ningún evento clínico relacionado con la cardiopatía coronaria en los 6 meses previos, fueron aleatorizados para seguir una intervención dietética de 5 años (dieta Mediterránea y dieta baja en grasas, con la misma intensidad de asesoramiento dietético). Los detalles sobre los métodos de estudio, los criterios de inclusión y exclusión y las recomendaciones dietéticas han sido publicados anteriormente.

1. **Publicación nº 1.** Se realizó un estudio transversal, en el marco del estudio CORDIOPREV, incluyendo a aquellos pacientes con T2DM (n = 540) que cumplían, al inicio del estudio, los criterios para el diagnóstico de diabetes propuestos por la Asociación Americana de Diabetes (ADA). Los pacientes con T2DM se clasificaron en dos grupos: (a) pacientes con T2DM establecida (n = 350), considerando aquellos con antecedentes médicos previos de T2DM antes de ingresar al estudio que estaban recibiendo tratamiento (medicamentos para controlar la glucosa o tratamiento dietético), y (b) pacientes con T2DM de reciente diagnóstico (n = 190), que no tenían antecedentes de T2DM al inicio del estudio, por lo que fueron diagnosticados durante el periodo de reclutamiento del estudio CORDIOPREV. Se midieron los niveles séricos de AGEs (metilglioxal (MG) y N-carboximetil-lisina (CML) y marcadores ateroscleróticos subclínicos (vasodilatación mediada por flujo braquial, FMD y grosor de la íntima media de ambas carótidas comunes, IMT-CC). Se consideró disfunción endotelial severa en pacientes con FMD < 2%.

2. **Publicación nº 2 y 3.** Se incluyeron en ambos estudios a aquellos pacientes con T2DM de nuevo diagnóstico que no habían estado recibiendo tratamiento hipoglucemiante al inicio del estudio (190 de 1002 pacientes). Los pacientes fueron evaluados al inicio y después de 5 años de seguimiento [dieta Mediterránea (n=80) y dieta baja en grasa (n=103)] en aquellos pacientes con muestra disponible en ambos tiempos (n=183). Los pacientes se clasificaron como Respondedores (n=73, pacientes que revirtieron de T2DM durante la intervención dietética sin el uso de medicamentos para la diabetes y No respondedores (n=110, que no lograron la remisión de la diabetes durante el período de seguimiento). La remisión de T2DM fue definida como una glucemia por debajo del rango diabético según los criterios de la ADA durante al menos dos años consecutivos. En la **publicación nº 2**, los niveles de AGEs se evaluaron al inicio y después de 5 años de seguimiento de cada intervención dietética. Los AGEs dietéticos fueron determinados mediante cuestionarios dietéticos, los niveles séricos de AGEs (MG y CML) se midieron mediante técnicas ELISA y la expresión génica de los receptores relacionados con el metabolismo de los AGEs (AGER1 y RAGE) se determinaron mediante PCR en tiempo real a partir del ARN de las células mononucleares de sangre periférica de los pacientes del estudio. En la **publicación nº 3** se realizó una prueba de tolerancia oral a la glucosa (OGTT) (75 g de dextrosa monohidratada en 250 mL de agua, con muestreo de 0, 30, 60, 90 y 120 min) al inicio del estudio y cada año hasta el 5º año, para establecer los niveles de glucosa e insulina en plasma que se basaron en el cálculo de los índices de sensibilidad a la insulina derivados de OGTT. Los niveles plasmáticos de BCAA (isoleucina, leucina y valina) se midieron mediante GC-TOF/MS en ayunas y después de 120 min de una OGTT al inicio del estudio y después de 3 años de cada intervención dietética.

Resultados

1. **Publicación nº 1**. Los niveles séricos de AGEs e IMT-CC fueron mayores en pacientes con T2DM establecida en comparación con los pacientes con T2DM de nuevo diagnóstico ($p<0,001$ y $p=0,025$, respectivamente), mientras que el FMD no difirió entre los dos grupos. Los pacientes con T2DM establecida que presentaban disfunción endotelial grave (FMD < 2 %) tenían niveles séricos de MG, IMT-CC, HOMA-IR y niveles de insulina en ayunas más elevados que aquellos con T2DM de reciente diagnóstico y disfunción endotelial no grave (FMD ≥ 2 %) (todas las $p<0,05$). Los niveles séricos de CML fueron mayores en pacientes con T2DM establecida que en pacientes con T2DM de reciente diagnóstico, independientemente de la gravedad de la disfunción endotelial.

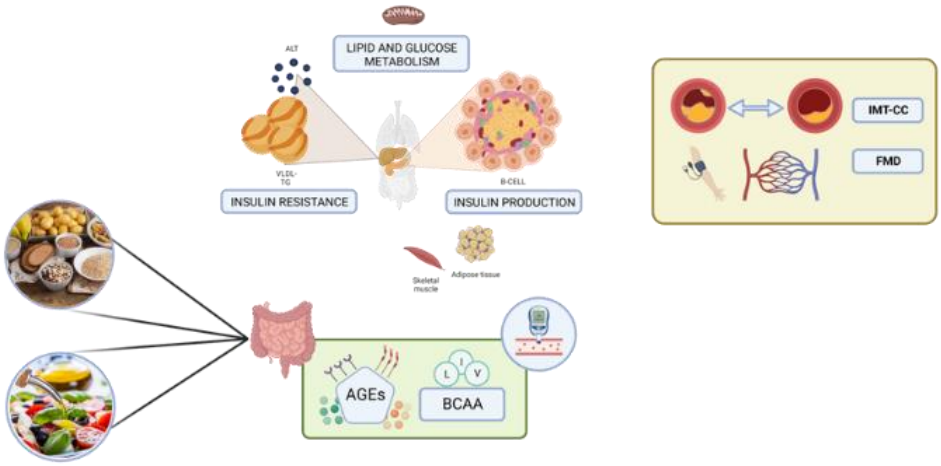
2. **Publicación nº 2**. La dieta Mediterránea disminuyó los niveles séricos de AGEs (MG y CML) y modificó la expresión génica de los principales receptores de AGEs implicados en su metabolismo, disminuyendo RAGE y aumentando AGER1 (todas las $p<0,05$). Si bien no se encontraron diferencias en las tasas de remisión de la diabetes entre las dietas (dieta Mediterránea y dieta baja en grasa), un análisis de regresión de COX mostró que cada disminución de la desviación estándar (DE) de MG, después de la dieta Mediterránea, aumentó la probabilidad de remisión de la T2DM con HR: 2,56 (1,02-6,25) y $p=0,046$ y cada aumento de la DE en el índice de disposición al inicio del estudio aumentó la probabilidad de remisión de la diabetes con HR: 1,94 (1,32-2,87) y $p=0,001$.

3. **Publicación nº 3**. Los niveles plasmáticos de isoleucina, leucina y valina tras 120 min de sobrecarga oral de glucosa al principio del estudio en pacientes que siguieron una dieta

Mediterránea se asociaron, mediante análisis COX, con una remisión de la T2DM, HR por DE (IC 95%): 0,53 (0,37-0,77), 0,75 (0,52–1,08) y 0,61 (0,45–0,82), respectivamente. En base a esto se creó un score de los niveles plasmáticos de BCAA, clasificando a los pacientes según la mediana del score. Considerando el conjunto de la población, los pacientes con puntuación más alta (niveles más bajos de BCAA) presentaron HR (IC 95%): 1,91 (1,19-3,07) mayor probabilidad de remisión de la T2DM que aquellos con puntuación más baja (niveles más altos de BCAA), HR (IC 95%): 1,82 (1,12-2,96). En cuanto a las dietas administradas, el grupo de dieta Mediterránea con un score alto (niveles más bajos de BCAA) presentó HR (IC 95%): 3,33 (1,55-7,19) mayor probabilidad de remisión de la T2DM que aquellos con puntuación baja, y una HR (IC 95%): 3,13 (1,39-7,09), mientras que no se encontró asociación en el grupo de dieta baja en grasa.

Conclusión

Los pacientes con T2DM establecida mostraron mayores niveles basales de AGEs y un mayor IMT-CC, particularmente en aquellos con disfunción endotelial, en comparación con los pacientes con T2DM de reciente diagnóstico. Por otro lado, los pacientes con T2DM de reciente diagnóstico que remitieron de la T2DM fueron aquellos que presentaron niveles basales más bajos de BCAAs o una reducción de los niveles de AGEs circulantes y una modulación del metabolismo de los AGEs tras el consumo de la dieta Mediterránea. Estos hallazgos no se observaron después del consumo de una dieta baja en grasa. Nuestros resultados sugieren que la relación diferencial encontrada entre el metabolismo de los AGEs y los niveles de BCAAs y la remisión de la T2DM, según la dieta consumida, puede potencialmente utilizarse como una herramienta para seleccionar las recomendaciones dietéticas más adecuadas para inducir la remisión de la T2DM, y también reducir las complicaciones cardiovasculares, a través de estrategias nutricionales en pacientes con T2DM de reciente diagnostico con CHD.



ABSTRACT

ABSTRACT

Introduction

Type 2 diabetes mellitus (T2DM) has currently become a global pandemic and its prevalence has increased over the past few decades, with no signs of receding in the near future, imposing a socioeconomic burden on health services, economy and society. In addition, patients with concomitant presence of coronary heart disease (CHD) and T2DM have a significantly increased risk of developing a new cardiovascular event than those without T2DM. This risk of cardiovascular recurrence seems to be increased by the duration of the T2DM, evidencing the relevance of early identification and efficiently control of the disease to reduce associated morbidities and mortalities.

T2DM is considered a metabolic disease mainly characterized by impaired insulin secretion and action that is strongly related with nutrition and other lifestyle habits. In fact, dietary intervention studies have shown that changes in dietary habits and increased physical activity can reduce T2DM incidence establishing an association between specific dietary patterns and diabetes risk. More specifically, low-carbohydrate, low-fat calorie-restricted or Mediterranean-style diets are associated with cardiovascular benefits, improved glucose homeostasis and insulin sensitivity. However, these dietary approaches appear to be disease status-dependent, as metabolic flexibility decreases over time reducing the potential benefits of lifestyle changes or pharmacological treatments.

Newly diagnosed T2DM has recently been proved to be reversible by different strategies, such as intense weight loss (either bariatric surgery or very low caloric diet). We have recently found that the consumption of a healthy dietary pattern (a low-fat diet or a Mediterranean diet) determined an improvement of hepatic insulin sensitivity, a reduction in hemoglobin A1c (HbA1c) and a recovery of β-cell functionality in newly diagnosed T2DM patients with CHD. However, consumption of these healthy diets did not promote T2DM remission in every patient, highlighting the lack of comprehension about the mechanisms underlying T2DM remission promoted by dietary changes.

Advanced glycation end products (AGEs) are a group of pro-oxidant and cytotoxic compounds, generated from the Maillard reaction, which contribute to the onset and progression of certain chronic diseases, such as T2DM and cardiovascular disease. While a small amount of AGEs is generated endogenously as normal metabolism consequence, especially in T2DM patients because of chronic hyperglycemia, the main exogenous sources of AGEs is through diet, which depends both on the composition and the food processing. In this context, we have recently showed that the Mediterranean diet could be considered a good dietary model for reducing the content of dietary and circulating AGE levels, as the degree of oxidative stress and inflammation. It has been evidenced that AGEs play an important role in the progression of T2DM and its complications. However, there is no evidence of the participation of AGEs in T2DM remission.

Previous results also support the fact that elevated plasmatic levels of branched-chain amino acids (BCAAs) have been related with T2DM incidence and metabolic abnormalities such as insulin resistance, obesity, cardiovascular risk and glucose intolerance. In this sense, BCAA are considered as potential biomarkers for T2DM and cardiovascular disease. Recent studies have shown that the Mediterranean diet is able to reduce fasting plasma BCAA levels (valine, leucine,

and isoleucine). Although, to date, the molecular mechanisms are not well understood, the multiple roles of BCAAs in the development of insulin resistance could lead to the development of more effective therapies for T2DM.

In this context, several studies support the relation between Mediterranean diet consumption and a reduction in dietary and circulating AGEs and plasma BCAAs, as a reduction in inflammation and oxidative stress. Mediterranean diet is characterized by its richness in monounsaturated fat (mainly from virgin olive oil), vegetables, fruit, nuts, legumes and whole grain cereals, which provide fiber, antioxidants, vitamins, minerals and polyphenols with a low consumption of processed foods. The healthy properties of the Mediterranean diet are mostly due to its anti-inflammatory and anti-oxidative effects and the way of cooking boost these beneficial effects. Multiple studies relate this dietary pattern to the reduction and prevention of multiple diseases such as T2DM or CVD. However, more studies are needed to determine the effects of this diet on the pathophysiology of the disease and its relation to T2DM remission. This research can reduce T2DM incidence worldwide but also reduce its prevalence through the possibility of T2DM remission, which becomes even more relevant when it can be mediated by lifestyle changes and healthy dietary patterns, that have high adherence ratio, such as the Mediterranean diet.

Hypothesis

Based on these findings, our hypothesis is that baseline levels of advanced glycation end products (AGEs) and/or branched-chain amino acids (BCAAs) could potentially lead to the identification of patients with type 2 diabetes mellitus (T2DM) and coronary heart disease (CHD) who would benefit from healthy dietary interventions (a Mediterranean diet and a low-fat diet) to reduce cardiovascular complications. We also hypothesize that reduction of circulating levels of AGEs and/or BCAAs, through consumption of these healthy diets, might be involved in the molecular mechanism underlying T2DM remission of newly diagnosed T2DM patients with CHD.

Objectives

To determine whether baseline levels of advanced glycation end products (AGEs) and/or branched-chain amino acids (BCAAs) are associated with type 2 diabetes mellitus (T2DM) remission in newly diagnosed T2DM patients with coronary heart disease (CHD) and whether reduction of their circulating levels, after the consumption of two models of healthy diets (a Mediterranean diet and/or a low-fat diet) contributes to T2DM remission with the aim to establish therapeutic dietary strategies for the management of these patients.

Specific objectives

1. To compare the levels of AGEs in both newly diagnosed and established T2DM patients with CHD and their association with subclinical atherosclerotic markers [brachial flow-mediated vasodilation (FMD) and intima-media thickness of common carotid arteries (IMT-CC)] for establishing strategies to prevent or reduce AGE production and delay the onset of cardiovascular complications in this type of patients.

2. To analyze whether the reduction of circulating AGE levels and the modulation of gene expression related to AGE metabolism, after the consumption of two models of healthy

diets (a Mediterranean diet and a low-fat diet), were associated with T2DM remission in newly diagnosed T2DM patients with CHD.

3. To evaluate the relationship between levels of circulating BCAA (baseline and changes after the consumption of two models of healthy diets (a Mediterranean diet and a low-fat diet) and T2DM remission in newly diagnosed T2DM patients with CHD.

Participants, design and methodology

This thesis has been performed within the framework of the CORDIOPREV study (Clinicaltrials.gov number NCT00924937). The CORDIOPREV study is a single center, prospective, randomized, single-blind and controlled dietary intervention clinical trial that includes 1002 patients with CHD, developed at Reina Sofia University Hospital in Córdoba, Spain. Prior to recruitment and initiation of the study protocol, written consent was obtained from all participants. All the amendments follow the Helsinki Declaration and good clinical practices and were approved by the Ethics Committee of the Hospital Reina Sofía (Cordoba, Spain). All analysis were done under the principle of intention to-treat.

Eligible patients included men and women aged 20–75 years who had established CHD, without any clinical events related to CHD in the previous 6 months, were randomized to follow-up a 5 year dietary intervention (a Mediterranean diet and a low-fat diet) who received the same intensive dietary counselling. Details about study methods, inclusion and exclusion criteria and dietary recommendations has been described previously.

1. **Paper nº 1.** A cross-sectional study was carried out, within the framework of the CORDIOPREV study, those T2DM patients (n = 540) who met, at baseline, the criteria for diabetes diagnosis proposed by the American Diabetes Association were included. T2DM patients were categorized in two groups: (a) patients with established T2DM (n = 350), considering those with a prior medical history of T2DM before entering the study that were receiving treatment (glucose-lowering drugs or diet), and (b) patients with newly diagnosed T2DM (n = 190), who had no previous history of T2DM at the beginning of the study, thus being diagnosed during the recruitment period of the CORDIOPREV study. Serum levels of AGEs (methylglyoxal (MG) and N-carboxymethyl-lysine (CML)) and subclinical atherosclerotic markers (brachial flow-mediated vasodilation (FMD) and IMT-CC) were measured. Severe endothelial dysfunction was considered in patients with a FMD < 2%.

2. **Paper nº 2 & 3.** Newly diagnosed T2DM patients who had not been receiving glucose-lowering treatment at the beginning of the study were included in both sub-analysis (190 out of 1002 patients). Patients were evaluated at baseline and after 5 years of follow-up [the Mediterranean diet (n=80) and a low-fat diet (n=103)] in those patients with available sample in both timepoints (n=183). Patients were classified as Responders (n=73, patients who reverted from T2DM during dietary intervention without the use of diabetes medication and Non-responders (n=110, who did not achieve diabetes remission during the follow-up period). T2DM remission was defined as glycemia below the diabetic range according to the ADA criteria for at least two consecutive years. In the **paper nº 2,** the levels of AGEs were evaluated at the baseline and after 5 years of follow-up of each dietary intervention. Dietary AGEs were determined by dietary questionnaires, serum

AGE levels (MG and CML) were measured by ELISA techniques and gene expression of receptors related to AGEs metabolism (AGER1 and RAGE) were determined by real time PCR of the RNA from peripheral blood mononuclear cells of the study patients. In the **paper nº 3,** an oral glucose tolerance test (OGTT) was performed at the beginning of the study and every year until the 5th year. OGTT (75 g dextrose monohydrate in 250 mL water) was performed with 0, 30, 60, 90 and 120 min sampling to establish plasma glucose and insulin levels which based the calculous of OGTT-derived insulin sensitivity indexes. Plasma levels of BCAA (isoleucine, leucine, and valine) were measured by GC–TOF/MS at fasting and after 120 min of an OGTT at the baseline of the study and after 3 years of each dietary intervention.

Results

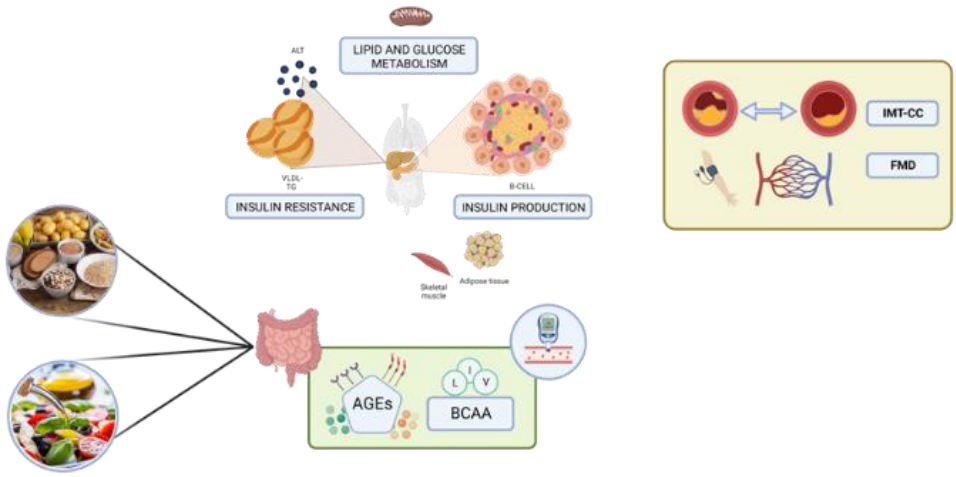
1. **Paper nº 1**. Serum AGE levels and IMT-CC were higher in patients with established T2DM compared to newly diagnosed T2DM ($p < 0.001$ and $p = 0.025$, respectively), whereas FMD did not differ between the two groups. Patients with established T2DM who exhibited severe endothelial dysfunction (FMD < 2%) had higher serum MG levels, IMT-CC, HOMA-IR and fasting insulin levels than those with newly diagnosed T2DM and non-severe endothelial dysfunction (FMD ≥ 2%) (all $p < 0.05$). Serum CML levels were greater in patients with established compared to newly diagnosed T2DM, regardless of endothelial dysfunction severity.

2. **Paper nº 2.** The Mediterranean diet decreased serum AGE levels (MG and CML) and modified the gene expression of the main AGE receptors involved in their metabolism, decreasing RAGE and increasing AGER1 (all $p < 0.05$). While no differences in diabetes remission rates were found among diets (the Mediterranean diet and a low-fat diet), a COX regression analysis shows that each SD decrease in the MG, after the Mediterranean diet, increases the probability of T2DM remission with HR: 2.56 (1.02–6.25) and $p = 0.046$ and each SD increase in disposition index at baseline increases the probability of diabetes remission with HR: 1.94 (1.32–2.87) and $p = 0.001$.

3. **Paper nº 3.** Isoleucine, leucine, and valine plasma level after 120 min of an OGTT in the Mediterranean diet were associated, by a COX analysis, with T2DM remission: HR per SD (95% CI): 0.53 (0.37–0.77), 0.75 (0.52–1.08), and 0.61 (0.45–0.82), respectively. We built a score of BCAA plasma levels and patients were classified by median scores. Considering the whole population, patients with a high score (lower BCAA levels) presented 1.91 (1.19–3.07) a higher probability of T2DM remission than those with a low score (higher BCAA levels), 1.82 (1.12–2.96). Regarding the diets administered, the Mediterranean diet group with a high score (lower BCAA levels) presented 3.33 (1.55–7.19) a higher probability of T2DM remission than those with a low score, and a HR of 3.13 (95% CI 1.39–7.09), whereas no association was found in the low-fat diet group.

Conclusion

Patients with established T2DM exhibited higher baseline levels of AGEs and increased IMT-CC, particularly in those with endothelial dysfunction, compared with newly diagnosed T2DM patients. On the other hand, newly diagnosed T2DM patients who achieved T2DM remission were those who presented lower baseline levels of BCAA or a reduction in circulating AGEs levels and a modulation of AGEs metabolism after consumption of the Mediterranean diet. These findings were not observed after consumption of a low-fat diet. Our results suggests that the differential relationship found between AGE metabolism and BCAA levels and T2DM remission, according to the dietary consumed, may potentially be used as a tool to select the most suitable dietary recommendations to induce T2DM remission, and also reduce cardiovascular complications, by nutritional strategies in newly diagnosed T2DM patients with CHD.



I. INTRODUCTION

I. INTRODUCTION

1. Diabetes: Clinical aspects and classification

Diabetes mellitus is a disease characterized by chronic exposure to hyperglycemia, as a consequence of an insufficiency insulin production and/or response. Long term exposure to hyperglycemia increases the risk of developing chronic microvascular and macrovascular complications that are associated increased morbidity and mortality affecting life quality.[1] The main symptoms of diabetes mellitus are polydipsia, polyuria, and weight lost. Other symptoms may include polyphagia, blurred vision, and fatigue.[1] There are different types of diabetes mellitus, classified according to the etiological factor: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus.[2]

T1DM is generally caused by an immune-associated destruction of insulin-producing pancreatic β-cells. Historically, T1DM was considered a disorder in children and adolescents, but over the past decade, age is no longer a restricting factor. Polydipsia, polyphagia, and polyuria along with hyperglycemia remain diagnostic hallmarks in children and adolescents, and to a lesser extent in adults. The immediate need for exogenous insulin replacement is also a hallmark of T1DM, for which lifelong treatment is required. To date, key questions regarding the epidemiology of T1DM, the efficacy of current therapies, the understanding of how the disorder develops and the prevention or cure the disease remain unclear.[3]

T2DM is characterized by a relative and progressive impairment of insulin secretion associated with co-occurring insulin resistance.[4] T2DM is the most common type representing 90-95% of diabetes cases. The risk of developing T2DM increases with age, obesity, and lack of physical activity. However, this disease is often not diagnosed in the first years because of the mild symptoms caused by its gradual progression.[5] It is estimated that 30-80% of diabetics remain undiagnosed. Therefore, early detection of this disease is crucial to control the progression and its complications, especially cardiovascular effects.[4]

On the other hand, gestational diabetes mellitus appears in the second or third trimester of pregnancy, but this does not last, as it usually returns to normoglycemia after pregnancy. Nonetheless, gestational diabetes mellitus increases the risk of developing T2DM over the years. The diagnostic criteria of gestational diabetes mellitus is still controversial, which complicates the comparison of data between studies.[6]

A recent report published by the American Diabetes Association (ADA) also includes the classification "other types specific of diabetes", which are less common and with diverse origins, including diabetes caused by a single gene disorder, such as MODY (mature-onset juvenile diabetes), diabetes caused by diseases of the exocrine pancreas and diabetes induced by drugs or chemicals that can stop insulin secretion or action.[5]

This project focuses mainly on T2DM.

2. *Type 2 Diabetes Mellitus*

The World Health Organization (WHO) defines T2DM as a "*metabolic disorder of multiple etiology characterized by chronic hyperglycemia with impaired carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both*".[7] T2DM is largely caused by impaired insulin production and secretion by pancreatic β-cells, as well as insulin resistance of peripheral tissue.[8,9] The feedback between β-cell and insulin-sensitive tissues promotes an increase in insulin secretion due to the lack of insulin response from these tissues.[10] Nevertheless, β-cell cannot chronically maintain this situation, resulting in a hyperglycemia and leading to the progression of T2DM.[8] Incretins, oxidative stress, advanced glycation end products, amino acid metabolism, the microbiome, immunology dysregulation and inflammation have emerged as possible factors involved in the pathophysiological mechanisms of the disease. In addition, around 90% of T2DM patients are obese or overweight at the time of diagnosis. The etiology of T2DM is thought to be largely linked to hypercaloric diets combined with insufficient energy expenditure and sedentary lifestyle.[4] There are a range of effective treatments that reduce hyperglycemia in T2DM patients, which mediate their effects by improving insulin secretion or decreasing insulin resistance in peripheral tissue.[11] The adoption of healthy lifestyles in combination, in some cases, with hypoglycemic medication are necessary when the disease progresses, to prevent the onset and/or progression of diabetes-associated complications. Despite this, post-diagnosis complications, especially long-term complications, are prevalent globally. As a consequence, T2DM remains a leading cause of blindness, end-stage renal disease, lower limb amputation and cardiovascular disease.[7]

2.1.Epidemiology of T2DM

T2DM is the most common and clinically important metabolic disorder that has become a global pandemic in recent decades and a major healthcare burden worldwide. According to International Diabetes Federation (IDF), the estimation of patients with T2DM was 151 million in 2000 and has doubled in the recent decades with 382 million in 2013 and around 463 in 2019.[12] The incidence of T2DM continues to increase, and it is projected that there will be greater than 590 million T2DM patients by 2035 **(Figure 1).**[1,12] When related to the global population, this disease is affecting to 9.3% of which 90-95% of patients with diabetes are type 2. Specifically, the prevalence of diabetes in Europe is estimated to be 61 million people according to diabetes atlas, with an incidence of 3 and 6 cases/1000 person-years, however a high heterogeneity were found among the studies.[13,14] In Spain, the first part of the di@bet.es study reported the prevalence of the country in 2012 that covered the 13.8% of the population, around 4.5 million people.[15] The second part was published in 2020 that estimated the incidence of T2DM in a cohort of 5072 adults, evaluated during 7.5 years of the study. The incidence of T2DM was 11.6 cases/1000 person-years (IC 95% 11.1-12.1), 3.7 cases/1000 person-years of known diabetes and, therefore, the incidence of the unknown was 7.9 cases/1000 person-years.[14]

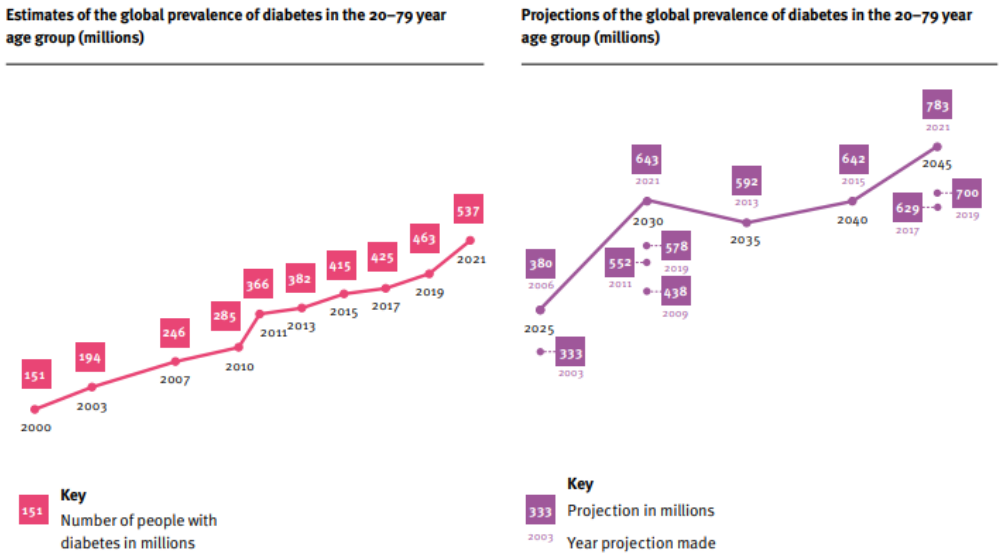


Figure 1. T2DM prevalence evolution and projection over the years 2000-2035 according to IDF Diabetes Atlas (2021).[1,16]

The epidemiology of T2DM is affected by genetic and environmental factors. Its incidence has increased due to longer life expectancy as well as obesity, sedentary lifestyles and the consumption of unhealthy and hypercaloric diets rich in processed foods, refined grains, sugar and saturated fats. For this reason, despite genetic predisposition, T2DM can be prevented with healthy and adequate lifestyle.[17] However, the distribution of this disease is no homogeneous across the globe. It has been established that region is an important variation factor in the prevalence and incidence of T2DM, but it is still considered a global disease.[18] Regarding the distribution, the increase in the prevalence of T2DM is estimated to be considerably higher in developing countries than in developed countries.[12,19] According to IDF in 2020, low- and middle-income countries represent approximately 80% of diabetic population, located mainly in East and Southeast Asia, Latin America, the Middle East and North Africa (**Figure 2**).[1] This circumstance could be attributed to rapid economic development that increases obesity and overweight rates, interposed by an adoption of "Western lifestyles", led by increased urbanization, rapid nutritional changes and sedentarism.[12,18,20] Moreover, in these countries, the capability of health services to manage the increase in diabetes are limited and prevention policies are lacking.[21] Many developing countries do not have adequate infrastructure to treat this pandemic, meaning that diabetes is a serious concern for the future.[18,21] Globally, it was estimated that diabetes accounted for 12% of health expenditures in 2010 ($376 billion), and the healthcare cost will continue to rise to $490 billion in 2030.[18] In addition, there is continuing emerging evidence that the prevalence and incidence of T2DM is likely to be higher in developing countries than currently documented due to undiagnosed diabetes.

In terms of mortality, T2DM was the 9th leading cause of death worldwide and the 6th leading cause of death in upper-middle-income countries in 2019 according to the WHO (**Figure 3**).[22] There is an increase in mortality that is consistent with the increase in T2DM prevalence. Patients with T2DM have a 15% increased risk of all-cause mortality two-fold increased risk of cardiovascular mortality.[23] In fact, according to the IDF, T2DM is associated with 11.3% of total global mortality in ages 20-79 years. The highest number of diabetes-related deaths in 2019 was

located in the Western Pacific region. [1] However, in developed countries, a reduction in diabetic complications and mortality from vascular causes in T2DM patients has been observed, which are associated with improved treatments and control of the disease.[24]

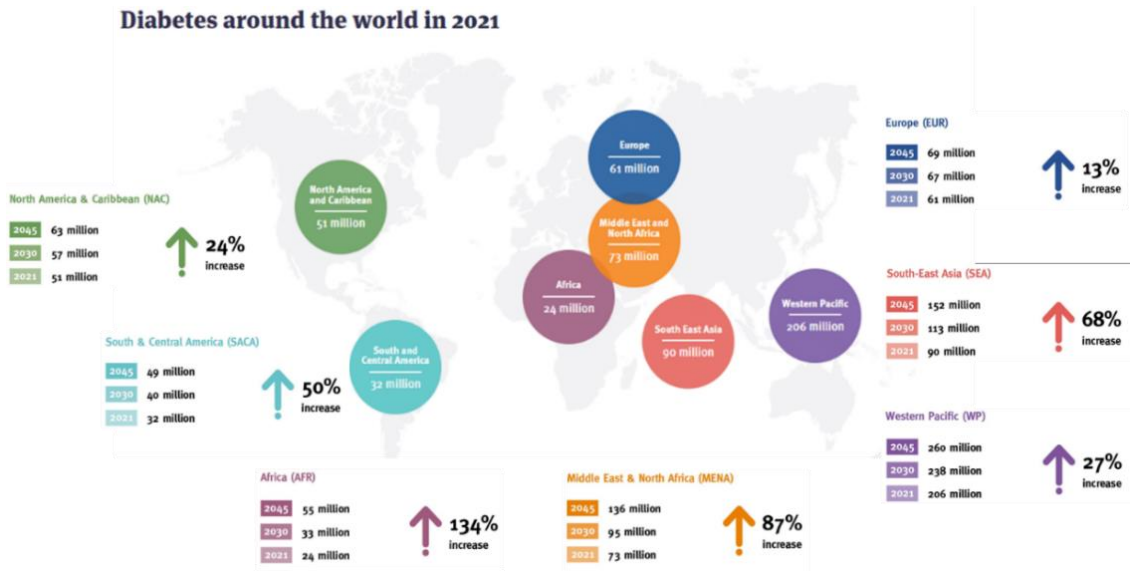


Figure 2. Geographic profile of T2DM prevalence in adults according to IDF Diabetes Atlas (2021).[1]

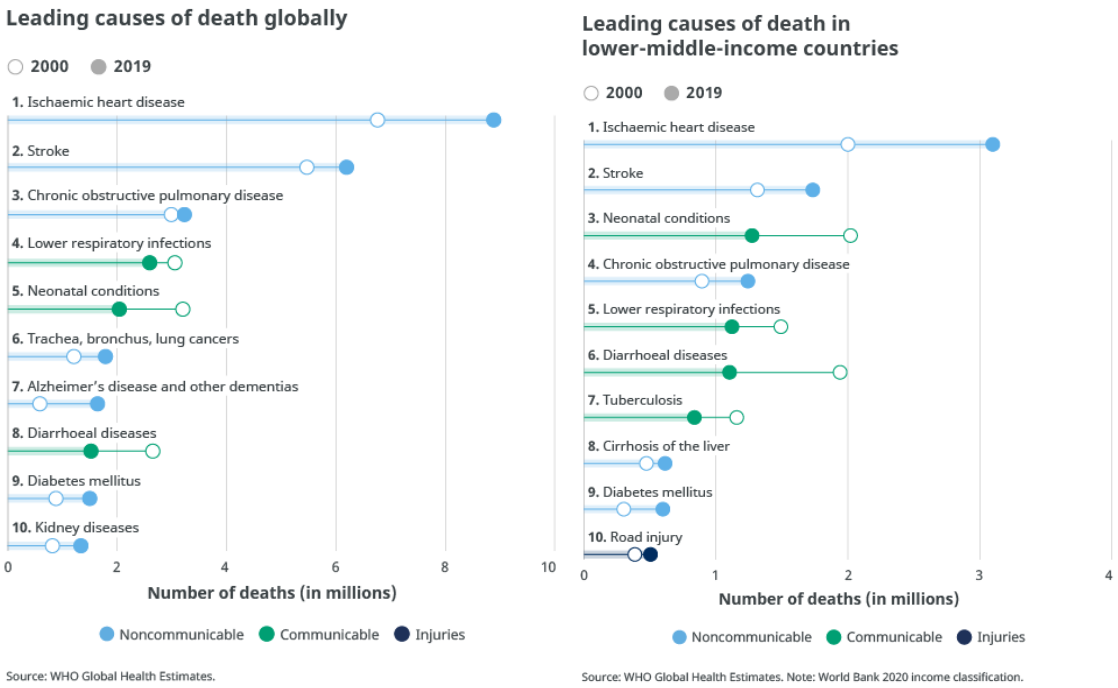


Figure 3. Global leading cause of death according to WHO (2019).[22]

2.2.Clinical complications of T2DM

Diabetic complications are the leading cause of premature death and poor quality of life in T2DM patients.[25] In addition, more than 50% direct cost of T2DM is accounted for by T2DM complications.[1,26] The complications of T2DM can be classified into macrovascular (CHD, myocardial infarction, stroke, and peripheral arterial disease) and microvascular complications that includes nephropathy, retinopathy, neuropathy, diabetic foot, among others (**Figure 4**).

Hyperglycemia, insulin resistance, excess fatty acids, dyslipidemia with elevated levels of circulating triglycerides and low-density lipoprotein cholesterol (LDL-C), increased oxidative stress, disrupted protein kinase C signaling and increased advanced glycation end products (AGEs) result in vascular inflammation, vasoconstriction, thrombosis and atherogenesis.[10,27] Therefore, adequate and sustained control of blood pressure and blood lipid and glucose levels can prevent or delay the onset of diabetes-related complications in people with T2DM. As demonstrated, intensive T2DM treatment promotes a ≥10% risk reduction in major macrovascular and microvascular events. [26,28]

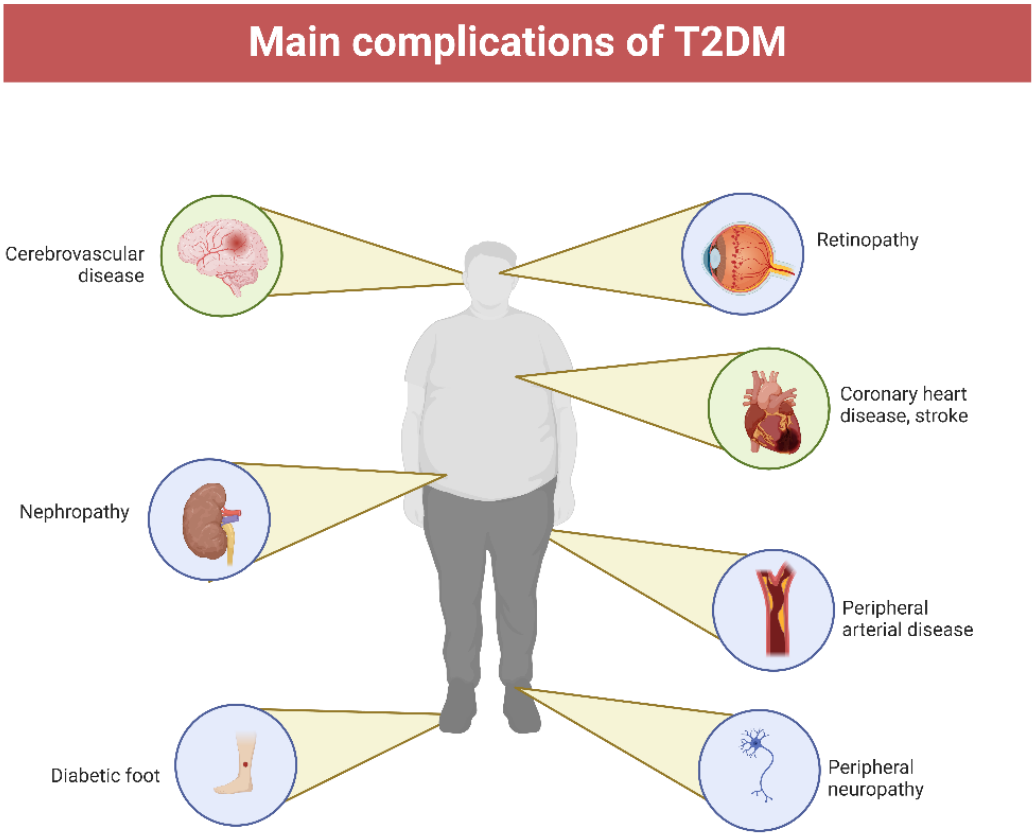


Figure 4. T2DM cardiovascular complications scheme created with BioRender.com.

2.2.1. Macrovascular complications

Diabetes increases the risk of macrovascular complications which mainly comprises coronary heart disease (CHD), myocardial infarction, cerebrovascular disease, peripheral arterial disease and stroke. This is a progressive process that start in early stages and is sensitive to risk factors as T2DM. Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in T2DM patients and the risk increases with worsening glucose status because of a higher risk of accelerated atherosclerosis and other more direct lipotoxic and glucotoxic effects.[13] Acute ischemic stroke and transient ischemic attacks are three to four times more common in T2DM.[17] Concomitant risk factors such as smoking, arterial hypertension, obesity, and dyslipidemia further increase the likelihood of these complications.[26] In fact, CVD represents more than half of the mortality in diabetic population and it is also the largest contributor to diabetes-associated healthcare spending, estimated at $37.3 billion per year in United States. [29]

2.2.2. ***Microvascular complications***

Diabetic nephropathy

Chronic kidney disease (CKD) attributed to diabetes (diabetic nephropathy) occurs in 20-40% of patients with T2DM. It is characterized by albuminuria, a low estimated glomerular filtration rate and manifestations of renal damage.[30,31] The most effective strategy to reduce the impact of diabetic nephropathy is to prevent T2DM, as it may be present at the time of T2DM diagnosis. Also, CKD can progress to end-stage renal disease. Diabetic nephropathy is becoming the most common cause of end-stage renal failure. In Europe, more than 10% of patients requiring dialysis or transplantation have T2DM and this figure is twice as high in the USA[1].

Diabetic retinopathy

Diabetic retinopathy is a highly specific vascular complication and one of the most frequent causes of blindness among adults 20 to 74 years of age, with strong personal and socioeconomic repercussions.[1] Diabetic retinopathy is characterized by a spectrum of lesions resulting from cumulative damage from long-term elevated glucose levels to the small blood vessels of the retina. These lesions include changes in vascular permeability, capillary microaneurysms, capillary degeneration and excess new blood vessels (neovascularization). Consequently, glaucoma, cataracts, and other eye disorders appear earlier and more frequently in people with T2DM. The neural retina is also dysfunctional with the death of some cells, which alters the electrophysiology of the retina and results in an inability to discriminate colors [36]. In addition to the duration of diabetes, factors that increase the risk of retinopathy include chronic hyperglycemia, renal disease, hypertension, and dyslipidemia.[32] Correct management of T2DM with the objective of maintaining optimal glycemia, together with the treatment of blood pressure when required, has been shown in large prospective randomized studies to prevent and/or delay the onset and progression of diabetic retinopathy.[4]

Diabetic neuropathy

More than half of people with T2DM end up developing neuropathy. Traditionally, the progression of the disease is clinically characterized by the development of vascular anomalies, such as capillary basement membrane thickening and endothelial hyperplasia, with consequent decrease in oxygen tension and hypoxia.[19] Peripheral neuropathy is the most common form of diabetes-associated neuropathy, which includes a heterogeneous group of disorders. It takes place

in the distal nerves of the extremities, in particular, numbness of the feet (diabetic foot) is very common.[33]

Diabetic foot

Diabetic foot and lower extremity complications are characterized by triggering chronic ulcers and abnormal distribution of internal bone pressure, sometimes leading to gangrene and amputations that significantly reduce quality of life and increase the risk of premature death.[1] Amputation rates in populations with diagnosed T2DM are typically 10 to 20 times higher than in non-diabetics.[25] Early recognition and treatment of patients with diabetes and feet at risk for ulcers and amputations may delay or prevent adverse outcomes.[32] In fact, an example of this is that several studies have shown a reduction of between 40% and 60% in amputation rates among adults with diabetes during the last 10-15 years in several European countries, including Spain, USA and Australia.[25]

2.3.Diagnostic criteria for T2DM

Most guidelines use the standard diagnostic criteria proposed by IDF, World Health Organization (WHO) or American Diabetes Association (ADA). According to the 2021 ADA report, diabetes mellitus can be diagnosed through the following criteria: fasting plasma glucose (FPG) levels ≥ 126 mg/dL (7.0 mmol/L), 2-hour glucose plasma levels (2h PG) during 75 g oral glucose tolerance test (OGTT) ≥ 200 mg/dL (11.1 mmol/L) or HbA1c percentage ≥ 6.5% (48 mmol/mol) (**Table 1**).

Table 1. T2DM diagnostic criteria according to American Diabetes Association.

	DIAGNOSTIC VALUES		
ANALITICAL TEST	**NORMAL**	**PREDIABETES**	**DIABETES**
HbA1c	<5.7% (<39 mmol/mol)	5.7-6.4% (39-47 mmol/mol)	≥ 6,5% (≥48 mmol/mol)
Fasting plasma glucose (FPG)	<100 md/dL	100-125mg/dL (56-6.9 mmol/L)	≥126 mg/dL (7.0 mmol/L)
2-hour plasma glucose during an OGTT	<140 mg/dL	140-199 mg/dL	≥ 200 mg/dL (11.1mmol/L)
In patients with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose	–	–	≥ 200 mg/dL (11.1 mmol/L)

T2DM: type 2 diabetes mellitus; HbA1c: glycated hemoglobin (The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay); NGSP: National Glycohemoglobin Standardization Program; DCCT: Diabetes Control and Complications Trial; FPG: fasting plasma glucose (Fasting is defined as no caloric intake for at least 8 hours*); OGTT: oral glucose tolerance test (The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75g anhydrous glucose dissolved in water*). In the absence of symptoms of hyperglycemia, two abnormal tests are required for the diagnosis of diabetes mellitus.*

In general, these three criteria are accepted for diagnosis of T2DM. HbA1c is an indirect measure of average blood glucose levels.[34] Its advantage is the lack of fasting requirement and the lower preanalytical variation. Nevertheless, it has lower sensitivity and higher cost, conditioning its accessibility in some regions. Hemoglobin variants or abnormal red blood cell turnover should be

considering before select this diagnostic technique because of possible alteration in the results and the diagnosis. Those conditions may interfere with HbA1c results, forcing to use glycemic tests as FPG or OGTT for a more reliable diagnosis.[34,35]

For T2DM in the presence of symptoms (e.g. polyuria, polydipsia or unexplained weight loss) the diagnosis can be based on: a random FPG ≥ 200 mg/dL or 11.1 mmol/L. For the diagnosis of diabetes mellitus in individuals with elevated levels of any of the criteria in absence of any symptoms, two abnormal tests are required.[36]

2.4.Etiology/Risk factors for T2DM

Many risk factors for T2DM have been identified,[17] one of the most important ones are body mass index (BMI), specifically over 30, stablished as obesity. The incidence of T2DM and obesity has grown in parallel in the last decades. Several studies reported that obese people have 90-fold increased risk of developing T2DM than those with normal weight.[37] In Western countries, it is estimated that about 50% of T2DM patients are obese and 30–40% were overweight.[38] Increased deposition of fat in the ectopic regions of the body (mostly visceral fat) also increases T2DM risk by more than double.[39,40] This can be attributed to a number of metabolic abnormalities that lead to insulin resistance. In addition, waist circumference or waist-hip ratio predicts T2DM risk regardless of BMI.[41] This is advisable in some populations (such as Asian Indians), where the onset of diabetes occurs with a lower BMI although the percentage of visceral fat is higher.[42]

Genetics are known to play an important role in T1DM but, recently, there is increasing recent research on the genetic factor in T2DM.[43] A study of monozygotic twins showed a concordance of 70% for T2DM and 30-50% for T1DM, and the association with parents is also strong.[44,45] Family history of T2DM is not only associated with increased diabetic risk but is also inversely related to the age of onset of T2DM.[46] However, increased susceptibility to T2DM is due to more variants that have not yet been analyzed, as the highest odds ratio reported for a risk locus is 1.57.[43] This suggest that the increased risk of developing T2DM in relatives of T2DM patients may be due to sharing similar lifestyles as well as genetics.[44]

In addition, risk factors for T2DM include sex (men are at higher risk than women[47]) and race/ethnicity, (i.e., African American, Latino, Native American, Asian American, Pacific Islander origin confer increased risk).[42] Previous gestational diabetes also confers a markedly increased risk of T2DM because of underlying β-cell dysfunction.[5,48] Genetic factors, advanced age, metabolic syndrome criteria, such as hypertension or dyslipidemia, and polycystic ovarian syndrome also contribute to increased T2DM risk.[17,49]

Regarding non-genetic factors, lifestyle plays a crucial role in the development and the progression of T2DM. Alcohol and smoking[50] increase T2DM risk and prognosis as well as stress or depression.[17,51] Similarly, diet and sedentary lifestyle clearly predispose to increase in body fat leading to overweight and obesity, increasing the risk of T2DM. Public health guidelines recommend at least 150 min of moderate-vigorous physical activity per week, which has been associated with a 26% reduction in T2DM risk,[52] by improving insulin sensitivity.[17] Diet is also considered a critical risk factor in the development and progression of T2DM. Typically Western diets, characterized by high total caloric intake and rich in saturated fat, low fiber consumption and regular intake of soft drinks, juices, red meat and processed food are associated with increased risk of T2DM.[53]

Recent advances in genomics, metabolomics and gut microbiome technologies have offered opportunities and challenges for current research for their potential use for more effective prevention and management of T2DM.[54]

2.5.T2DM remission

T2DM has long been regarded as a chronic and inevitably progressive disease that requires increasing numbers of oral hypoglycemic agents and eventually insulin, caused by an inexorable decline in β-cell function and insulin resistance. However, it is now certain that the disease process can be halted with restoration of normal carbohydrate and fat metabolism, in newly diagnosed diabetic patients.[53] Most of these studies have been conducted in patients undergoing bariatric surgery, who normalized their plasma glucose levels in a sustained manner after surgery, providing the first evidence of T2DM remission.[55-57] T2DM remission is defined as glucose levels below the diagnostic level of diabetes or HbA1c <6.5% maintained over time for at least one year, in the absence of hypoglycemic medication.[53] Clinical factors such as younger age, initial hyperglycemia, substantial weight loss and the duration of the disease, and the absence of insulin treatment have been related to a greater probability of remission.[58-61] Weight loss after surgery is mainly promoted by caloric restriction and/or malabsorption from the surgery.[58]

Similarly, several studies have shown that very caloric restricted diets also induce a rapid metabolic improvement and T2DM remission through significant weight loss, without the need for surgery.[62,63] This is exposed in the DIRECT study where a weight-loss maintenance program through very-low-calorie diets induced a 36% rate of maintained T2DM remission after two years post-intervention.[64] However, the main concern of this strategy is that very low-calorie diets may not be achievable or sustainable for a long time in most of the patients.[53,65] Nevertheless, weight loss may not be the main or only cause of T2DM after bariatric surgery because it has been observed that normalization of plasma glucose levels can occur in some patients only hours or days after bariatric surgery, before achieving significant weight loss.[53,65] Therefore, caloric restriction could also orchestrate independent mechanisms,[58,63] pointing out the role of glucose regulation by the gastrointestinal tract and its involvement in T2DM remission. However, despite years of research, the exact mechanisms underlying the beneficial glycemic effects after bariatric surgery and/or the mechanisms of T2DM remission are still not fully understood.[66,67]

Bariatric surgery or caloric restriction are not the only strategies that promotes T2DM remission. Certain long-term clinical trials where the quality of the consumed diet, such as its macronutrient composition or antioxidants content should be considered. Some studies have shown that isocaloric diets without significant weight loss[68-70] such as a low-carbohydrate diet,[62] a low-fat diet or a Mediterranean diet rich in extra virgin olive oil (EVOO) [71,72] have also successfully reverse T2DM, with significant reductions in HbA1c.[69,70] In order to explain this situation, a series of potential mechanisms have been proposed, without being exclusive like changes in the metabolism of bile acids, the detection of nutrients from the gastrointestinal tract and the use of glucose, intestinal hormones and the intestinal microbiota.[58,64,66,67,73-75]

On the other hand, the pathophysiological changes in relation to the two main etiological components of T2DM are better described, both in remission through bariatric surgery and by consumption of very low-calorie diets.[58,76] One of the most important mechanisms endorsed to date through these strategies is the one that acts through the existing relationship between hepatic insulin resistance and β-cell functionality through the twin cycle.[77] The twin cycle hypothesize

that T2DM etiology and physiopathology occur due to an accumulation of fat in the liver, which induces insulin resistance, and hyperinsulinemia; this hyperinsulinemia leads into a self-reinforcing cycle, stimulating fat production, which spills into the pancreas causing T2DM.[78] Various studies have supported that obtaining remission of diabetes through these previously exposed strategies resulted, on the one hand, a reduction in the production and plasmatic levels of low density lipoprotein-triglyceride (VLDL-TG) together with a reduction in de novo lipogenesis.[79] This reduces alanine aminotransferase (ALT) levels and liver fat, also reducing β-cell exposure to lipid metabolites such as VLDL-TG, chylomicrons or any amount of saturated fatty acids.[79] As for the pancreas, the fat content is reduced and the morphology is modified by an increase in volume and a reduction in the irregular morphology.[80] All this causes a redifferentiation of the β-cells of the pancreas, improving the functionality and increasing the secretion of insulin, regulating HbA1c levels and increasing hepatic insulin sensitivity, favoring oxidative lipid and glucose metabolism, but skeletal muscle insulin resistance remains essentially unchanged.[81,82] The relevance of this studies are crucial in clinical practice to avoid disease burden, as T2DM remission has been described to lead an improvement in cardiovascular risk, hypertension and lipid profile.[53,83] However, patients who underwent the same dietary strategies but did not experience diabetes remission showed no signs of improvement in insulin, HbA1c, ALT or pancreatic morphology, as well as in both oxidative lipid and glucose metabolism. Furthermore, even though there was a reduction in the plasmatic levels of VLDL-TG, their hepatic production increased.[79,80]

This raises the hypothesis that patients with the same strategy for diabetes remission, either by bariatric surgery or substantial weight loss, may or may not remit depending on whether their diabetes status is more advanced and irreversible (with greater pancreatic β-cells dedifferentiation) and/or have different etiology of the disease (not just lipid-promoted effect on pancreatic β-cells). However, the mechanisms underlying diabetes remission through other dietary strategies remain to be explored.

3. Parameters for evaluation of the progression of diabetes and CVD

3.1.Insulin resistance and β-cell function

Two main abnormalities are known to underlie most cases of T2DM: insulin resistance (IR) and defects in pancreatic β-cell function, which has been expanded to what was subsequently termed the twin cycle hypothesis. An excess of fat accumulation in the liver induces hepatic IR, causing rises in fasting plasma glucose and triggering an increase in insulin production, leading into impaired pancreatic β-cell function and resulting in the development of T2DM. For this reason, the measurement of IR is an important point to determine the progression on T2DM.

There are different ways to measure IR, but the most common is by fasting methods and the OGTT. The OGTT is a technique that uses a 75- or 100-g glucose load and measures glucose and insulin at various intervals, it does not require intravenous access but does involve several venipunctures between 2 to 4 hours, being the most common timepoints used in clinical studies 0, 30, 60, 90 and 120 min after glucose load. This technique provides information on β-cell secretion and peripheral insulin action that can be determined through various mathematical equations. Insulin sensitivity has been assessed by calculating insulin area under the curve (AUC insulin), AUC glucose/AUC insulin, and by an insulin sensitivity index (ISI) which applies only the 0 and 120 min glucose and insulin values in a complex mathematical formula.[84]

The most commonly used equations are homeostatic model assessment (HOMA-IR) and hepatic, adipose and muscular IR. HOMA-IR has been widely employed in clinical research to assess insulin sensitivity. Instead of using fasting insulin or a glucose/insulin ratio, the product of the fasting values of glucose (expressed as mg/dL) and insulin (expressed as μU/mL) is divided by a constant: baseline insulin x baseline glucose/405 or 22.5 if glucose is expressed in S.I. units.[85] This IR can be classified as hepatic, muscular or adipose which have their specific indexes: Hepatic insulin resistance index derived from fasting values (Hepatic-IR fasting or HIRI) that primarily reflects hepatic insulin resistance.[86] Muscle insulin sensitivity index (MISI) was measured according to the Abdul-Ghani et al method using values in the OGTT.[87] Adipose tissue insulin resistance index (Adipo-IR) was calculated as the product of fasting free fatty acids (FFA) x fasting plasma insulin.[88] Other common indexes are insulin sensitivity index (ISI),[84] insulinogenic index (IGI), which measured insulin secretion[89] and disposition index (DI) that estimate β-cell functionality.[90]

The relationship between glucose and insulin is quite complex and involves the interaction of many metabolic and regulatory factors. Normal values of insulin sensitivity varies widely and is influenced by age, ethnicity, and obesity, so it is important to evaluate these measures cautiously. However, they are very good techniques to determine the evolution of the patients over time in clinical trials.

*3.2.*Evaluation of cardiovascular disease risk.

Cardiovascular risk assessment is a decisive tool for clinical decision-making to assess cardiovascular risk and monitor those patients with established CVD. For this reason, different risk scales have been developed over the years, in order to adopt the best strategy to prevent the onset of cardiovascular events. In Europe, SCORE system is used in primary prevention for prediction of cardiovascular events[91] while, in the United States,[92] specific risk equations derived

from different cohorts are used, like Framingham Heart Study. From those studies, several risk factors have been identified such as gender, smoking status, total cholesterol or blood pressure, to determine vascular risk of certain population.[93,94] However, these scores do not fully adjust to the actual incidence of CVD.[95] For this reason, several clinical parameters, such as C-reactive protein (CRP) levels, were added to these scales to improve the prediction of cardiovascular risk.

In secondary prevention, all patients are considered as high or very high cardiovascular risk due to the accumulation of different risk factors, such as T2DM. The evaluation of those factors has been shown to help to improve the prediction of new cardiovascular events. To identify those patients who may benefit from more intensive therapy, established risk factors such as LDL-C and CRP, are monitored as therapeutic targets. [96]

The study of the etiopathogenesis of atherosclerosis, added to the need to find new diagnostic and predictive biomarkers of disease that allow better stratification of cardiovascular risk, constitutes primary aims of cardiovascular research. [97] In fact, new advances in atherosclerotic etiopathogenesis allows the discovery of new biomarkers, such as miRNAs, myeloperoxidase, or vascular morphological evaluation through imaging tests.

Intima-media thickness of both common carotid arteries (IMT-CC)

Nowadays there are some non-invasive techniques to evaluate subclinical atherosclerosis such as high-resolution carotid ultrasound, which measures the evaluation of carotid intima-media thickness, specifically the intima-media thickness of both common carotid arteries (IMT-CC) and the detection of presence of the presence of carotid plaques, coronary calcium quantification by cardiac computed axial tomography and flow-dependent endothelial function.[97,98] The assessment of the presence of subclinical CVD through non-invasive imaging tests is increasingly widespread in clinical practice. The latest consensus and guidelines for the management of dyslipidemia[99] recommend the evaluation of the presence of plaques, in order to modify the intensity of lipid-lowering treatment in patients without known CVD. These advances allow specialized vascular units to standardize the evaluation of subclinical CVD, through ultrasound equipment, used in both clinical practice and research.[100,101] In fact, high-resolution carotid ultrasound is a technique that allows the evaluation of morphology and the determination of the presence of atherosclerotic plaques or measurements of the carotid wall such as IMT-CC. This technique correlates small changes in the vascular wall with early atherosclerotic stage, correlating with the incidence of carotid plaques[102] and making it possible to anticipate the diagnosis of a clinical events.[103] This is because precursor lesions of atherosclerosis occur in the intima layer and subsequently progress to the rest of the wall, initially respecting the vascular lumen. The accumulation of damage caused to the wall gradually causes distortion and thickening of the intima layer of the blood vessel, sometimes without clinical symptoms until complete vessel obstruction. Hence the importance of evaluating these morphological changes through vascular imaging tests in order to prevent future events through more invasive treatments.[104]

Regarding the opinion of the main scientific societies, the guidelines published by the American Heart Association (AHA) in 2013, defended the use of the IMT-CC as a clinic biomarker. Recommendations were limited to the evaluation of cardiovascular risk in those patients who remained asymptomatic with an intermediate level of risk.[92] However, the European Society of Cardiology and European Atherosclerosis Society (ESC/EAS) guidelines published in 2021, recommend the use of such techniques in those patients with a low or moderate risk. The reason

for these differences has been justified by the lack of standardization in the methods of the technique. Consequently, the possibility of evaluating the presence of carotid plaques and IMT-CC as a modifying factor of cardiovascular risk in intermediate-risk patients is displaced because of the impossibility of performing coronary calcium quantification, that are the more reliable measure for cardiovascular risk in those patients.[105]

The procedure to determine IMT-CC are described in the guidelines published by the American Society of Echocardiography Carotid Intima-media thick Task Force.[106] Likewise, some of the recommendations included in the Mannheim Carotid Intima-Media Thickness and Plaque Consensus in its 2012 update are included.[103]

Flow-mediated dilatation (FMD)

Vascular endothelium plays an essential role in various pathological and physiological processes, for instance, regulating vascular homeostasis, vasoconstriction and dilation, thrombosis, fibrinolysis, inhibition of inflammation and smooth muscle cell function that are crucial in the onset and progression of microvascular diseases.[107] Endothelial cells control vascular function by responding to various hormones, neurotransmitters, and vasoactive factors which affect vasomotion, thrombosis, platelet aggregation, and inflammation.[108] In response to various chemical (acetylcholine, ACh) or physical (shear stress) stimuli, the endothelium modulates vasomotor tone, by synthesizing and releasing different vasodilators and vasoconstrictors.[108,109] Endothelium occupies a unique position in that it is able to secrete a variety of vasoactive molecules and is also exposed to direct vascular injury. Therefore, it is an important mediator of atherosclerosis formation and is considered an indicator of vascular risk. It also prevents platelet adhesion and aggregation, as well as leukocyte adhesion and migration into the arterial wall and inhibits smooth muscle cell proliferation and intimal migration, oxidation of LDL-C, apoptosis of smooth muscle cells, all key events in the development of atherosclerosis.[110] Previous studies have demonstrated a correlation between measures of coronary vasodilator function and FMD.[111] Early studies established that attenuated vascular responses occur prior to the development of atherosclerosis in response to a risk factor milieu, making the measurements attractive as a screening tool for cardiovascular risk.[112,113] It is important to note that changes in FMD over time during a treatment intervention have been found to be a prognostic marker of CVD greater than a single FMD measurement.[114]

An imbalance between the magnitude of injury and the repair capacity of the endothelium is a key factor in the occurrence and development of various cardiovascular diseases, such as myocardial infarction, stroke, and hypertension.[115] Accumulating evidence has confirmed that endothelial dysfunction is considered an important pathological feature of early atherosclerosis.[116] Endothelial function is suggested as a potential biomarker because of it deteriorates during the natural progression of CVD.[117] Therefore, early detection of vascular endothelial dysfunction, timely intervention, and guided treatment are significant for maintaining cardiovascular health and reducing morbidity and mortality associated with cardiovascular diseases and medical costs.[118,119]

Vascular function can be measure by invasive and noninvasive methods. Invasive tests require the injection of acetylcholine into human coronary arteries, but their application is very limited because they are invasive, time-consuming, and expensive, and they are not recommended for healthy or asymptomatic patients.[118,119] A noninvasive test is brachial artery FMD is the most

popular method for evaluating vascular endothelial function.[120] This test is noninvasive, low cost, good repeatability, and wide clinical and research application, especially for clinical trials.[120] Many large prospective cohort studies have accepted FMD as an adjunctive marker of CHD. Several studies found that a decreased FMD could increase the risk of CHD.[121,122] The procedure to determine FMD are described the guidelines established by the International Brachial Artery Reactivity Task Force.[123] Likewise, some of the recommendations including cutoffs values (FMD < 2% as severe endothelial dysfunction) which serves as a benchmark to predict cardiovascular events in high-risk populations were described previously.[124]

4. Advanced Glycation End Products AGEs

4.1.*AGEs: Introduction, Chemistry, Classification*

Advanced Glycation End Products (AGEs) belong to a heterogeneous, complex group of compounds formed either exogenously or endogenously by different mechanisms and from a variety of precursors. In general, AGEs are created by means of non-enzymatic condensation (without any biological catalyst involved) between carbonyl groups of reducing sugars and free amine groups of nucleic acids, proteins or lipids, followed by further rearrangements yielding stable and irreversible end-products. Reactions leading to the formation of AGEs have been known for more than a hundred years, as Maillard reaction.[125] This reaction has been studied for years in the food industry, since its products provide a beautiful brown color and pleasant and addictive taste, aroma, savor and flavor in thermally processed foods with high sugar and protein content. Moreover, the developing western diet and our stressful lifestyle has increased the consumption of highly processed foods, which can also undergo reactions leading to AGEs formation upon thermal processing. Coextensively, a decreased in physical activity and an instauration of western lifestyle has led to civilization diseases and has brought the attention to research on the etiology of pathologic states, such as insulin resistance, diabetes, CVD, cancer and aging and the relationship with AGEs formation.[126-129]

4.2.Formation and Chemistry of AGEs

The diverse nature of the structures of AGEs suggests a great variety in the mechanisms of their formation. Indeed, both endogenous and exogenous AGEs can be generated in several pathways from a number of precursors, however as the most common formation is Maillard reaction. This reaction can be summarized in three main phases **(Figure 5).**

The first step of the Maillard pathway consists of the condensation of a carbonyl group, e.g., from a reducing sugar and an amine group from moieties such as the side chains of lysine or arginine[125,130] in a non-enzymatic way to form a Schiff base.[131] A Schiff base is a compound that has a carbon to nitrogen double bond in which the nitrogen is not bond to any hydrogen. The initiation of this first step depends on the glucose concentration and takes place in a couple of hours. If the glucose concentration decreases, this reaction is reversible.[132] During the second phase, the Schiff base undergoes a chemical rearrangement over a period of days and forms the Amadori products (also known as early glycation products). Amadori products are more stable compounds (HbA1c is the best known), but the reaction is still reversible.[133] If there is accumulation of Amadori products, they will undergo a complicated chemical rearrangement (oxidations, reductions and hydrations) and form cross-linked proteins. This process takes place over weeks or months and is irreversible. The brown colored end products are called AGEs and

some of them have fluorescent properties. In addition to the Maillard reaction, other pathways can also form AGEs. For example, glucose autoxidation and lipid peroxidation into dicarbonyl derivatives due to an increase in oxidative stress is another pathway described for the formation of AGEs.[134] These compounds can interact with monoacids and form AGEs. The other well-studied mechanism for the formation of AGEs is the polyol pathway, where glucose is converted to sorbitol by the enzyme aldose reductase and then to fructose by the action of sorbitol dehydrogenase.[135]

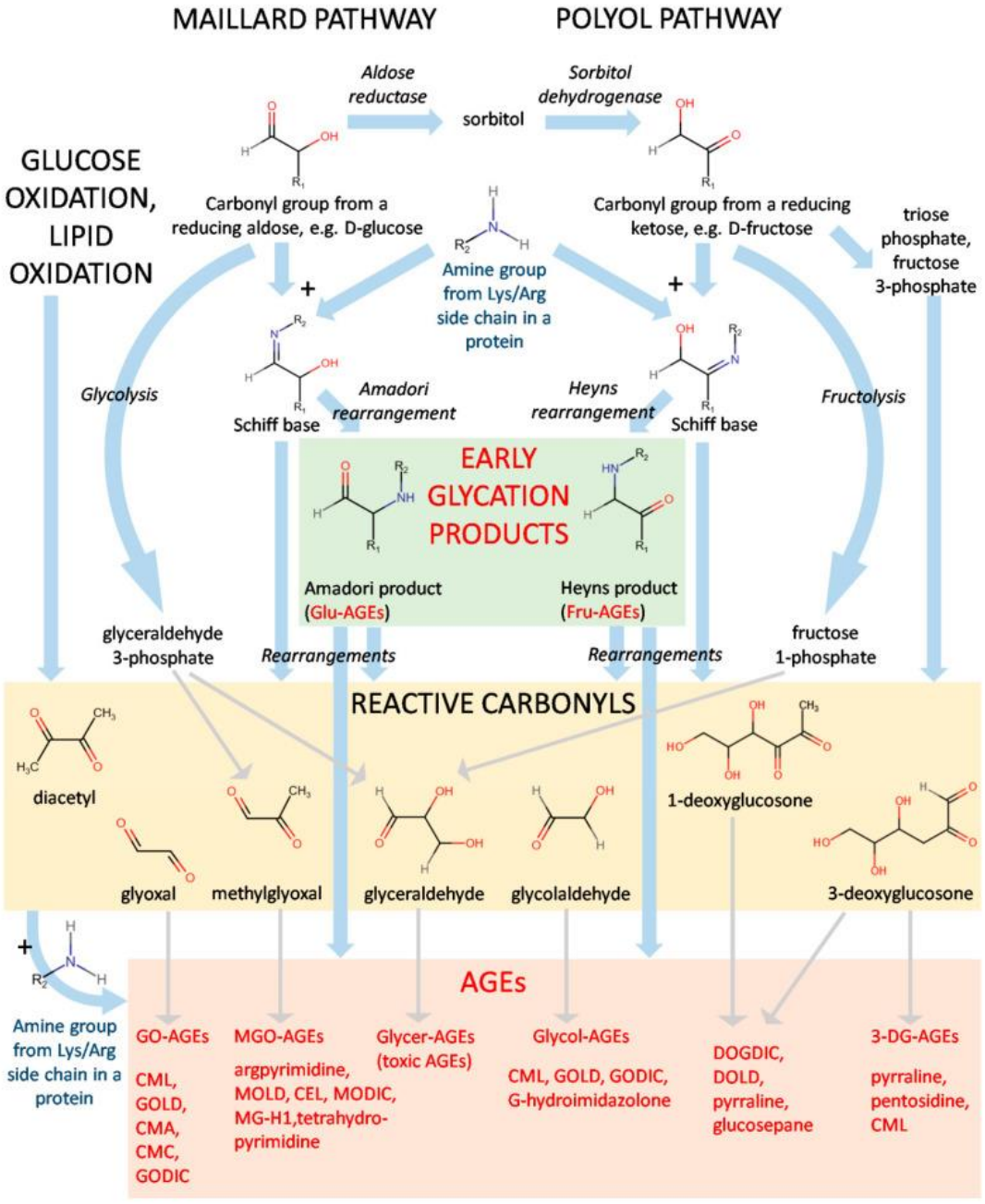


Figure 5. A scheme of formation for AGEs, adapted from Twarda-Clapa, A. in Cells 2022.[125] Abbreviations: glucose-derived (Glu-AGEs), fructose-derived (Fru-AGEs), glyoxal-derived (GO-AGEs), methylglyoxal-derived (MGO-AGEs), glyceraldehyde-derived (Glycer-AGEs), glycolaldehyde-derived (Glycol-AGEs), and 3-deoxyglucosone-derived (3-DG-AGEs).

4.3.Defense systems, receptors and elimination of AGEs

Endogenous AGE production, exogenous AGE intake (mainly through diet) and renal and enzymatic clearance are the cause of AGEs fluctuation levels in an organism which together produce transient increases and decreases in serum or plasma levels of AGEs. Several enzymes and receptors for these glycotoxins have been shown to be part of a detoxification system and counterregulation against the prooxidant effects of glycation.[136] Defense systems for maintaining AGE homeostasis include innate defenses, enzymatic degradation, renal clearance, and receptor-mediated cellular uptake and degradation. The innate defense against AGEs includes skin pigmentation, chelation of redox metals, and the structural conformation of enzymes that protect reactive sites.[137,138] Exogenous AGEs have also been suggested to be metabolized by the gut microbiome and the microbiome to be modified by the amount of AGEs consumed.[139-142]

A receptor system for AGEs has been described that generally participates in detoxification of AGEs, like Advanced Glycation End Product Specific Receptor 1 (AGER1), while Receptor for Advanced Glycation End Products (RAGE) and triggers the inflammatory response, promoting oxidative stress.[143] Enzymes that degrade AGEs include the glyoxalase system, mainly glyoxalase I (GloxI) and glyoxalase II, aldoreductases, and aldehyde dehydrogenases. Particularly, GloxI catalyzes the metabolism of dicarbonyls and prevents their binding with proteins to form AGEs.[136,138] Renal clearance includes the mechanisms of filtration, reabsorption, and tubular secretion to carry out the elimination of AGEs.[144]

AGEs can promote the generation of reactive oxygen species (ROS) and new AGEs, deplete antioxidant systems, and trigger the secretion of inflammatory mediators.[145] The main form of evaluation and quantification of AGEs is based on sensitive immunoassays characterized by high-pressure liquid chromatography, gases and mass spectrometry for certain AGEs, such as carboxymethyl-lysine (CML) or methylglyoxal (MG).[145] Despite the fact that these products are not the most prevalent ones, they are very common in vivo and are positively correlated with native oxidants and their activities.[138,145,146] CML is considered one of the most important forms of AGEs,[147] because it can lead to the formation of highly reactive dicarbonyl compounds that react with proteins and propagate the formation of intra- or intermolecular cross-links.[148] However, MG is an intermediate metabolite in AGEs metabolism, formed spontaneously during glycolysis as a result of the transformation of triose phosphates or during other metabolic processes like lipolysis, but it is highly reactive.[149] It can leading to cross-linking and denaturation of the protein, producing new AGEs.[150]

AGEs are eliminated by the kidneys by filtration (CML) and by absorption and active secretion (MG), two crucial processes to the excretion of AGEs in the urine.[151,152] As a consequence of their large blood supply, the kidneys are directly exposed to a greater amount of circulating AGEs, making them vulnerable to injury by the constant circulation of reactive carbonyls and ROS[153], especially in T2DM, which amounts are even higher and there's a high risk of diabetic nephropathy. Moreover there is an early reduction in AGE clearance documented in T2DM patients, before recognizable alterations in renal function occur[154] that can cause an accumulation of AGEs in the organism, which leads to an increase in the new AGEs formation and increase oxidative stress.

4.4.AGES, diet and diseases

The stressful rhythm that characterized our lifestyle in developed countries have promoted an increase of processed foods offer by food industry and demand. It generates changes in our dietary patterns to a more hypercaloric, high-sugared and ultra-processed diet that, in this context, is translated as an increase in dietary AGEs consumed.[155] Those AGEs, which are created in the non-enzymatic multi-stage glycation process, proceed mainly from the way of cooking, at high temperatures and low moisture, including grilling, roasting, and frying.[126,156,157] The combination of the compounds that protect form excessive AGE accumulation such as antioxidants, products with anti-glycation properties (mainly in fruits, vegetables, herbs, and spices)[158] and a low of moderate amount of sugar and processed integrated with a slow and low temperature way of cooking,[159] could characterize the perfect diet to avoid exogenous AGEs. That could be the case of a Mediterranean diet, considered a low-AGE diet, because its properties and compounds, rich in vegetables and fruits and reduced in the processed foods, between others.[75] However, more studies must be done to determine the effect on this diet on the circulating AGEs levels in different conditions.

Accumulation of AGE increases oxidative stress and initiates various disorders, leading to the progression of atherosclerosis, cardiovascular disease, diabetes and their complications. Dietary AGEs have been associated with multiples diseases such as T2DM, metabolic syndrome, neurodegenerative diseases and CVD. However, inborn defensive mechanisms, recovery systems, renal clearance and exogenous antioxidants protect from excessive AGE accumulation. Nowadays, the concentration of dietary AGEs can be determined by a validated semiquantitative food frequency questionary,[160] based on CML database for 549 commonly consumed food items for the Northeastern American multiethnic urban population, which was measured using a validated immunoassay method by Goldberg et al. and Uribarri et al.[156,157]

4.4.1. T2DM and AGEs

AGE production is enhanced by chronic hyperglycemia common in diabetes and, at the same time, their clearance seems to be reduced, triggering oxidative stress, inflammation and apoptosis. This high concentration of AGEs in T2DM, a sign of bad glycemic control, can develop into a number of complications, such as retinopathy[161], nephropathy, bone strength weakness,[162] neuropathy,[163] atherosclerosis,[164] and CVD.[165] Moreover, AGEs are a possible factor for causing diabetes states and are believed to be a good biomarker of this disease.

Chronic oxidative stress and an overload of AGEs in serum, despite acting on many molecular pathways[166], can inhibit two receptors, AGER1 and sirtuin 1 (SIRT-1), which are responsible for recognizing and repressing AGEs,[167] and in-vivo decrease insulin efficiency[137] as has been proved on 3T3-L1 cell line and mice with a high intake of MG. However both AGER1 and SIRT1 are restored after lowering the external oxidant burden by AGE restriction.[137,168] High concentrations of AGEs during diabetes has been proved to increase of tumor necrosis factor-alpha (TNF-α) and CRP, highly related with inflammation.[169] Dietary AGEs can also impair insulin secretion in pancreatic islet β-cells, by suppressing the deacetylase SIRT1 which regulates mitochondrial uncoupling protein 2 (UCP2), thus impairing membrane depolarization and β-cell secretory function.[170] More studies and randomized controlled clinical trials are needed to confirm and expand on the notion that AGE reduction is a promising prevention and treatment to T2DM, especially nowadays. As the current diabetes epidemic is undeniable related to environmental

changes enhanced by lifestyle changes and an increase in the enrichment of processed foods rich in AGEs, highly palatable and appetite-enhancing. These pro-oxidants molecules that simultaneously drive overnutrition and oxidant overload, may overwhelm host defenses and raise oxidative stress, presaging chronic inflammation. These states result in production and sensitivity insulin impairment that conclude in T2DM. Efforts to curtail the current diabetes epidemic addressed by circulating AGEs decrease may reduce the pre-existing elevated basal oxidative stress and inflammation in order to prevent, improve or reverse T2DM in a better cost-effective manner.[171]

4.4.2. CVD and AGEs

As we described before, CVDs, is often listed as complications during T2DM, where AGEs can play a key role in the appearance and progression of this complications by multiple ways. AGEs has been described as take part on the crosslinking of elastin and collagen, which leads to myocardial stiffening of the blood vessels and cardiac fibrosis[172]; the interaction between AGEs and RAGE resulting in ROS release, by the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inducing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway[173]; the inhibition of nitric oxide activity and the reduction of endothelial nitric oxide synthase activity; and an increase in vascular permeability.[174-176] Moreover, the amount of serum AGEs affect the levels of soluble intercellular adhesion molecule-1 (sICAM-1), and soluble vascular cell adhesion molecule-1 (sVCAM-1) highly related with vascular injury and CVD.[177] And several AGEs types, especially MG, have been described to present high levels in CVD patients with a pre-existing with diabetes mellitus.[178]

These data, combined with the findings that AGEs are increased in human atherosclerotic tissues, support the idea that AGEs contribute to cardiovascular diabetic complication through endothelial dysfunction and atherosclerosis.[179-181] It points out the need of study the implication of dietary AGEs as a powerful tool to manage the progression on this disease especially when is combined to a T2DM.

5. Branched chain amino acids (BCAA)

Branched-chain amino acids (BCAA) (isoleucine, leucine and valine) are a group of aminoacids that share a structural feature with a branched-side chain and common steps in their catabolism.[182] In addition to being substrates for the synthesis of proteins, they play several roles in metabolim and physiological processes. BCAAs have been described to act as signaling molecules regulating metabolism of glucose, lipid and protein, playing an important role in interorgan metabolic crosstalk.[183] A dysregulation of their catabolism seems to have a significant contribution in several metabolic diseases such as T2DM or CVD.[184-186]

*5.1.*BCAA and T2DM

Over the last decade, BCAA catabolism has been increasingly considered to play an emerging role in the development of insulin resistance, especially linked to obesity and T2DM. Several studies reported an elevated plasma[187,188] and tissue specific (especially white adipose tissue (WAT), liver and muscle)[189,190] BCAA levels in T2DM patients.[191,192] A strong relationship between elevated plasma BCAA levels and insulin resistance has been widely studied,[185,187,193]

suggesting that BCAA levels in plasma may predict future T2DM as it was seen in several clinical studies.[74,192] To date, there are no reports investigating whether, particularly, increased plasma BCAA levels in humans induce insulin resistance.The underlying mechanisms regarding to the role of elevated plasma BCAA levels on insulin-stimulated glucose uptake in humans remain largely unknown.

BCAA homeostasis is a complex process that is regulated by multiple factors where there is an increase in BCAA levels, by protein breakdown (inhibited by insulin), food intake and gut microbial syntesis and a reduction in BCAA circulating levels due to an increase in protein synthesis, excretion or BCAA catabolism. This homeostasis has been proved to be regulated for different hormones such as adiponectin which decreases BCAA plasma levels by activation of liver catabolism, or ghrelin which rise BCAA plasma leves by reducing the catabolism and stimulating AgRP neurons that promotes overfeeding and insulin resistance. However, the hormone that playes mayor role in BCAA homeostasis seems to be insulin. Insulin act as a regulator of BCAA levels, through the activation of protein kinase B (Akt) [194-196] that reduces protein breakdown in muscle tissue[197,198] resulting in low levels of BCAA in plasma.[199,200] However, in patients with insulin resistance, insulin effect that reduce protein breakdown is blunted,[194,201] causing an increase in muscle breakdown that reducemuscle potential capacity to catabolize BCAA[202,203] with an increase in circulating BCAA levels.[204-206] To this, it should be noted that T2DM patients also showed lower mitochondrial oxidative capacity [207] that result in disturbed glucose and fat oxidation. This fact could be caused by defects in BCAA-catabolic enzymes especially in via branched chain keto acid dehydrogenase (BCKD) complex [208-215] that results in anaplerotic stress[212,216,217] and increasing BCAA-derived carbon flux to tricarboxylic acid (TCA) cycle intermediates,[218,219] which can have toxic effects on cellular function, mitochondrial dysfunction lipotoxicity and insulin resistance.[220-224] **(Figure 6)**

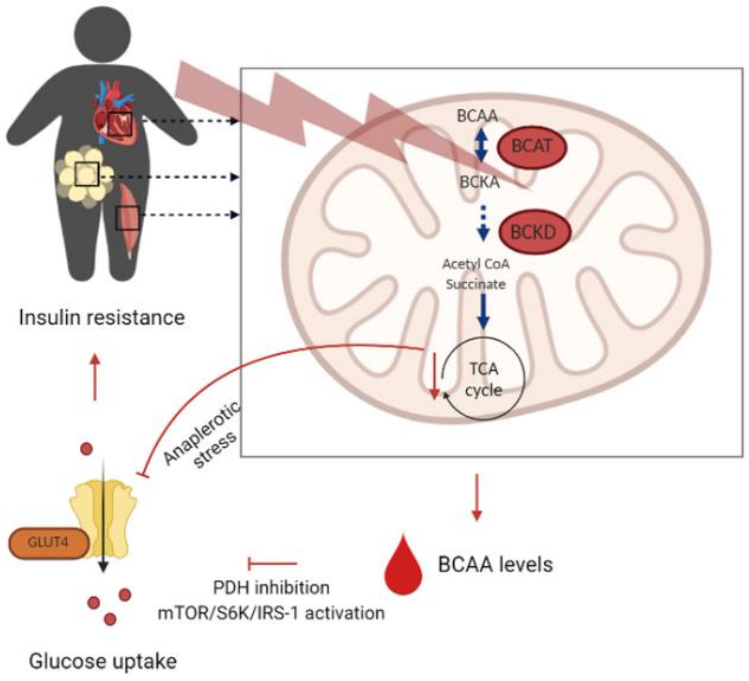


Figure 6. Schematic overview of mechanisms linking BCAA catabolism with insulin resistance. Adapted form Vanweert, F. et al. Nutr. Diabetes (2022).Abreviations: BCAA branched-chain amino acids, mTOR mammalian target of rapamycin complex, S6K ribosomal S6 kinase, IRS-1

insulin receptor substrate-1, PDH pyruvate dehydrogenase complex, GLUT4 glucose transporter type 4.

Besides anaplerotic stress in the mitocondria its consequences, there is evidence that elevated BCAA levels hamper insulin signaling pathways, such as mTOR (mammalian target of Rapamycin) acitvation or PDH (pyruvate dehydrogenase) complex inhibition. In this sense, elevated BCAA plasma levels specially under high fat conditions increase insulin resistance, mediated by downregulation of PI3K-Akt signaling pathway and hyperactivation of the mTOR/p70S6K pathway.[225-227] PDH complex is also inhibit by the high presence of BCAA in plasma, promoting an impairment of glucose uptake and oxidation, booting insulin resistance. This strong relation suggests a key point in the prevention and treatment of T2DM that can even be related to T2DM remission. Interestingly, bariatric surgery has been proved to be able to promote T2DM remission in some patients and also has been described that, after this intervention, there was a reduction in circulating BCAA levels and an special increase in BCKD (catabolic enzyme of BCAA) [228] in WAT [210,229]. However, WAT are suggested to be responsible for around 5% of whole-body BCAA oxidation,[214] therefore modulation of BCAA levels must have additional origins, and so T2DM remission.[202]

*5.2.*BCAA and CVD

There are also evidence that changes in BCAA metabolism are related to CVD. BCAAs are essential for normal growth and function at the cell and organism levels. However, there are different studies that suggest that high levels of BCAA could increase cardiovascular risk. The PREDIMED study concluded, in a case-cohort analysis with 226 incident CVD cases and 781 non-incident CVD cases, that high concentrations of baseline BCAAs were associated with increased risk of CVD, especially stroke,[74] which may be explained by an obesity-related decline in their catabolism.[203] Nevertheles the relevance of the metabolism of BCAA in CVD remains poorly understood.

Patientes with obesity or insulin resistance tend to present metabolism inflexibility, characterized by a resistance to switch from fatty acid oxidation to glucose oxidation, which increases triglycerides levels, especially in heart tissue.[230] Aditionally, these patients have been described to have higher levels of BCAA in heart tissue[231-233] and also in liver,[216] that could be caused by a disfunctional BCAA catabolism and a lack of their enzymes.[234,235] Moreover sustained elevated levels of BCAA in heart tissue can activate mTOR-C1 that contributes to unhealthy cardiac hypertrophy by chronic activation of protein synthesis, cardiac phosphoproteomic remodeling and diabetes-related cardiomyopathy.[236,237] This highlight the relevance of the study of BCAA in CVD and the mechanisms involved in the pathogenesis of the disease that may shed light on new strategies in the management of CVD, especially when concomitant with T2DM.

*5.3.*BCAA and DIET

BCAA are a group of essential aminoacids that cannot be synthesized by humans, so they must be ingested through diet; approximately 20% of dietary protein are BCAAs.[238] BCAA major sources come from animal products such as dairy products, red meat and poultry.[239,240] Several studies associate a high consumption of animal products, characteristic of the Western diet, with an increased risk of T2DM and insulin resistance.[240,241] In addition, several studies have

investigated the effect of dietary BCAA restriction on physiological phenotypes and have shown improved insulin- sensitivity, insulin-stimulated glucose uptake, glycogen storage in skeletal muscle,propensity to fat oxidation relative to glucose for energy production [242-245] and cardiac metabolism, including reduced myocardial triglyceride levels and increased use of fatty acids relative to glucose for oxidation and energy production.[230]

Despite the fact that BCAA are essential aminoacids, an elevated protein intake should increase plasma BCAA levels. However, most of the studies showed a weak correlation between BCAA consumption and plasma BCAA.[239,246] Interestingly, a recently published a case-control that in patients with incident T2DM, the regular consumption of a Mediterranean diet with EVOO decrease the levels of plasma BCAA.[74] Nevertheless, the mechanisms that underline this link between Mediterranean diet and circulating BCAA levels are still unknown.

Taken together, plasma BCAA levels and its relationship with T2DM and CVD may be influenced exogenously by food intake, and, especially, a dietary pattern such as a Mediterranean diet and endogenously by dysregulation of BCAA catabolism. However, further studies are needed to understand knowledge gap between diet, BCAA catabolim and T2DM and to explore their mechanisms in order to establish potential strategies in the treatment of metabolic diseases such as T2DM or CVD.

6. Dietary strategies

Lifestyle is considered a pivot factor for the development of chronic diseases such as T2DM and CVD where dietary factors are of paramount importance in the management and prevention of several of them. The relationship between not only isolated nutrients and foods but specific dietary patterns and these diseases has been extensively investigated over the years. Starting with the critical connection between CVD and diet, specifically the fat type consumption, the relevance of nutrition has been growing in the interest of many scientists that claims for a less invasive and populated way of prevention and treatment of the most common diseases in the development world. Nonetheless, the connection between dietary patterns and T2DM is undeniable and considering that both chronic conditions (T2DM and CVD) and their risk factors are interred, targeting dietary factors for T2DM and the mechanisms may also help to prevent or manage CVD in those patients.

Consequently, clinical organizations recommend following specific dietary guidelines in order to prevent or help the management of those diseases. Most of them, like the National lipid association, American Heart Association or American Diabetes Association, concur on most of the guidelines, like the reduction of saturated fatty acids (SFA), sugars, high amounts salt or high processed foods and the increase of vegetables, fruits, whole grains and legumes. They also pay attention to the composition of the diet, regarding macronutrients they mainly agree on the proportion of SFA that should not increase the 10% of the total energy intake and carbohydrates which 50-55% of the total energy intake has been described as the better proportion for a reduction of the mortality.

Despite the similarities of the clinical organization guidelines can be recruited by many different diets, the complexity of the dietary patterns allows them to differentiate not only in the composition and palatability but in their benefits on the patients and their potential adherence, taking special relevance, diets like low-fat diet, DASH, healthy vegetarian or the Mediterranean for their evidence on T2DM and CVD prevention and management. Curiously, despite the reticence of this guidelines to the fat content on their dietary recommendations, the Mediterranean diet highlights for having a 35-45% of fat (mainly monounsaturated fat -MUFA) that increase the palatability ratio compared with the rest of the recommended diets and consequently the adherence over the years.

6.1.Mediterranean diet

The Mediterranean diet is the legacy of millennia of exchange of cultures, food and people around Mediterranean basin, which has currently been considered an Intangible Cultural Heritage by UNESCO (2010).[247] The term was coined in 1960 and today it is one of the most studied dietary patterns. Within this lifestyle, the traditional Mediterranean dietary pattern is compound for a wide variety of nutrients which has been proven for cardiovascular and metabolic health. The Mediterranean diet is most closely tied to traditional areas of olive cultivation in the Mediterranean area, and it has historically been associated with low chronic diseases rates and high longevity, however, shifts in lifestyles over recent years have bleared these relationships.[248] Scales to measure adherence as well as studies of food components and the synergy between them have consolidated a body of knowledge that is of great interest to institutions and governmental agencies. The recognition of its benefits for health has made the widespread introduction of the Mediterranean diet an urgent challenge.

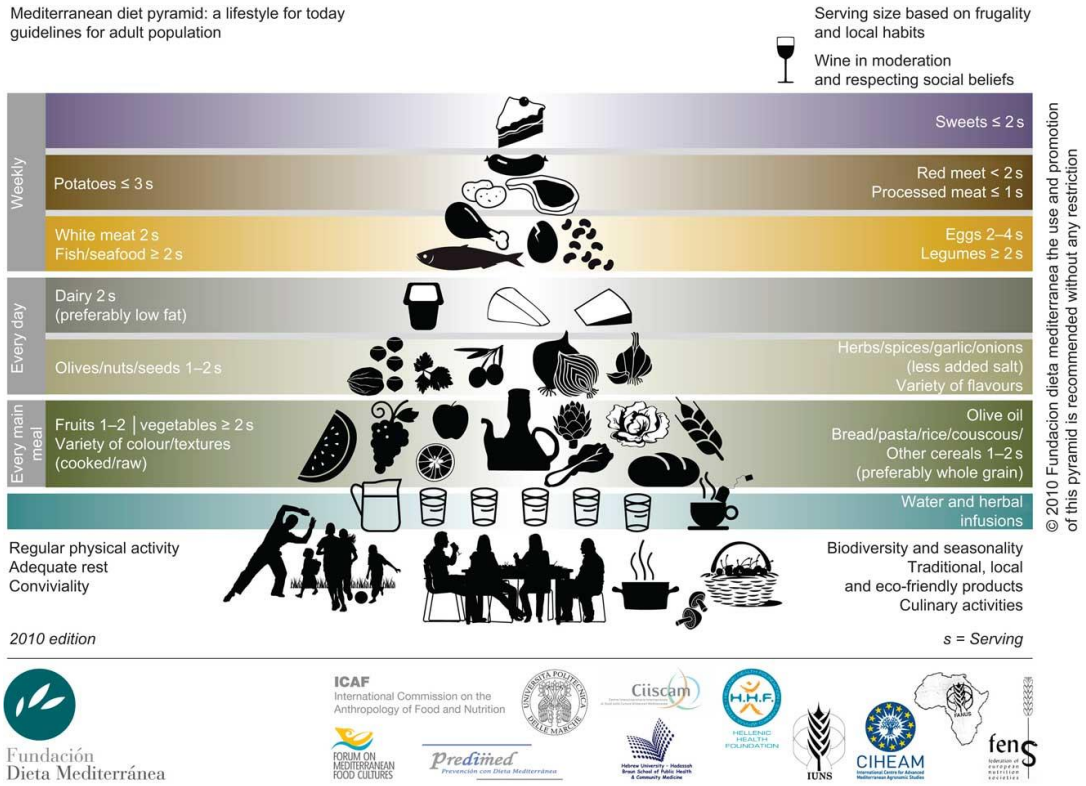


Figure 7. Mediterranean diet pyramid: a lifestyle for today adapted from "Fundación Dieta Mediterránea (2010)".[249]

The main characteristic of this diet seems to be the amount and composition of fat content. Fat consumption constitutes 35-45% of total calorie intake, being 50% of this fat MUFA, from virgin olive oil and extra virgin olive oil (VOO and EVOO, respectively), also considering minority compounds found in EVOO that has been widely studied for their health benefits. Moreover, the amount of SFA and cholesterol, is ~7%, due to a very low consumption of red and processed meat and dairy products. On the other hand, the Mediterranean diet is also characterized by its low animal origin protein content and a high consumption of complex carbohydrates (approximately 50% of the total caloric intake).[250] Plant-based foods (fruits, vegetables, minimally refined cereals, legumes, nuts and seeds) (**Figure 7**), with minimal processing and emphasizing seasonal and field-grown fresh vegetables, are important characteristics of the Mediterranean diet that increase the intake of dietary fiber, linoleic and linolenic acid, antioxidants and bioactive compounds (such as vitamins C and E, β-carotene, flavonoids, isoflavonoids, phenols, phytosterols).[249] Everyday consumption of row and cooked vegetables is highlighted. Furthermore, the base of most of the dishes in the Mediterranean cuisine are the "sofrito" that is a slow-cooked homemade sauce with tomato, garlic, onion, aromatic herbs, and olive oil, that a lipidic medium while slow cook changes increase absorption and bioavailability boosting the beneficial effects of some molecules like lycopene,[251] although some other molecules as vitamin C reduces their nutritional qualities when they are cooked. [252] Also, a moderate intake of dairy products (mostly cheese and yogurt), zero to four eggs a week, fish and poultry consumed in low to moderate amounts, red meat consumed in very low amounts and wine in moderation, consumed with meals, are also the Mediterranean diet recommendations. The Mediterranean diet is considered as a plant-based diet which a relatively high intake of nuts and EVOO and a particular way of cooking, that makes this diet unique and

different from the other healthy diet patterns (**Figure 7**).[249] Individual foods and components of the Mediterranean diet (e.g. EVOO) have well-documented health benefits,[253,254] but in recent years special attention has been given to the overall combination of foods, expressed as a dietary pattern, which may be more strongly related to health due to the additive or synergistic effects of the components. This scientific statement emphasizes the importance of dietary patterns beyond individual foods or nutrients. For all these reasons, it is a diet that has been recommended by nutritionists for decades and has been related to greater longevity, lower incidence of CVD and T2DM and less development of cognitive impairment.[255,256] (**Figure 8**)

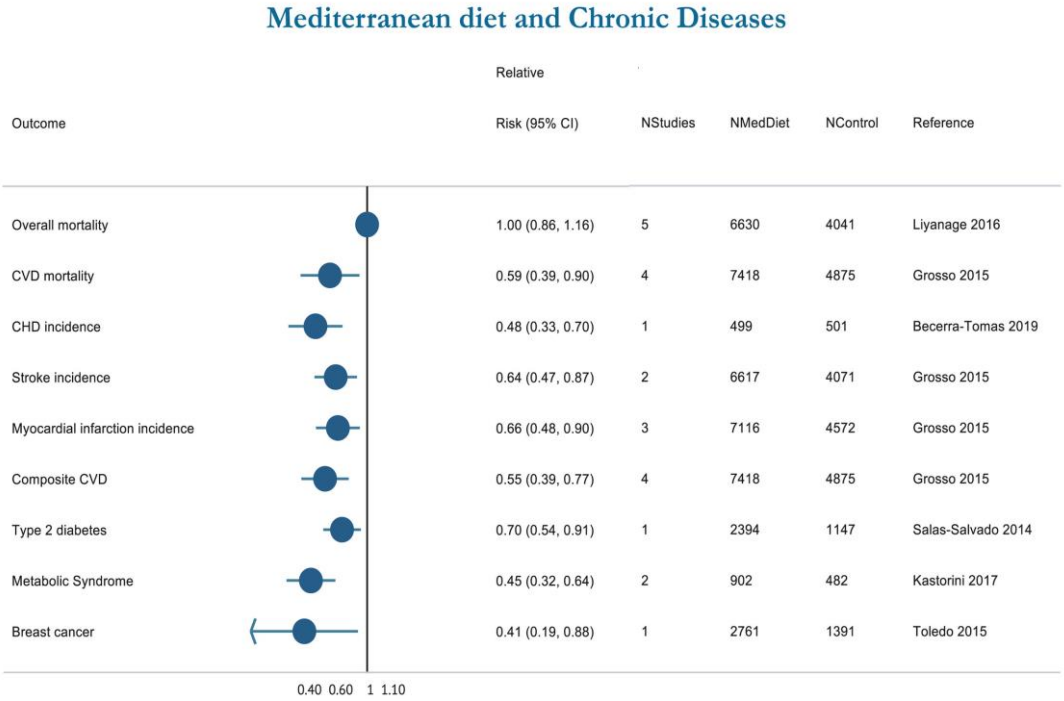


Figure 8. Relative risks (blue circles), and horizontal bars represent 95% confidence intervals for Mediterranean diet intervention versus control groups in Meta-Analyses of Randomized Controlled Trials on Mediterranean Diet and Chronic Diseases adapted from Guasch-Ferré M. et al. in JIM (2021).[249]

6.1.1. The Mediterranean diet and CVD

The association between the Mediterranean diet, low rates of cardiometabolic diseases and longevity prompted the Seven Countries Study, which provided epidemiological evidence on the effects of diet on health. From this study, several epidemiological and interventional studies have emerged that seek to study this relationship and explain the underlying mechanisms. The Seven Country Study was one of the first to evaluate CVD and one of the main characteristics of the Mediterranean diet, the type of fat.[257] In this study, the type and amount of fat in the diet were found to be highly relevant in CVD, with increased cardiovascular mortality observed after the consumption of diets rich in SFA, compared to diets rich in MUFA.[248] Since then, a large number of observational studies,[258-260] intervention studies, reviews and meta-analyses have been published, and all of them conclude a strong association between the consumption of a Mediterranean diet and a decrease in cardiovascular risk,[261-263] as well as an improvement in the control and appearance of some of the classic cardiovascular risk factors.[10,264,265]

The Lyon study was the first dietary intervention clinical trial that showed a decrease in the composite outcome of mortality and development of cardiovascular events, from 20 to 8%, after a Mediterranean diet consumption for 46 months compared with a low-fat control diet, similar to AHA recommendation.[266] This secondary prevention study was performed in 605 patients which had previously suffer an acute myocardial infarction. However, the main source of fat in the recommended Mediterranean diet in this study was canola oil, a rich source of linoleic acid, PUFA (polyunsaturated fatty acids), but not common in the Mediterranean countries that use EVOO or nuts, rich in MUFA.

Recently, another big interventional study was published the PREDIMED study (PREvención con DIeta MEDiterránea). This study was carried out in 7447 patients at high cardiovascular risk who had not presented, until their inclusion, any cardiovascular event, who were randomized into three arms: a Mediterranean diet supplemented with EVOO, a Mediterranean diet supplemented with walnuts and a control diet. This study, in primary cardiovascular prevention, showed that the consumption of a Mediterranean diet, supplemented with EVOO or nuts, reduces the incidence of cardiovascular events (31% reduction after the Mediterranean diet supplemented with EVOO and 28% in those who followed the same diet but supplemented with walnuts, compared to the control diet).[267] Likewise, different sub-analyses of this study showed an improvement over the classic risk factors,[268] T2DM or lipid parameters, as well as emerging risk factors, endothelial dysfunction,[269] inflammation, oxidative stress and carotid atherosclerosis.[270]

Finally, a secondary cardiovascular prevention and dietary interventional study with the Mediterranean diet has been recently published, the CORDIOPREV study (CORonary Diet Intervention with Olive oil and cardiovascular PREVention study). This study was performed in 1002 patients with stablished coronary heart disease that were randomly assigned to a Mediterranean diet rich in EVOO and a low-fat and high complex carbohydrate diet, as recommended by the National Cholesterol Education Program, and followed up for 7 years with not weight loss.[271] The incidence of a major cardiovascular event during the study was 28,1% [95% CI 27,9-28,3] in the Mediterranean diet group and 37,7% [37,5-37,9] in the low-fat group especially visualized in men where those 16.2% was occurred in the Mediterranean diet and 22.8% in the low-fat diet. This study highlights the benefits in clinical practice of a Mediterranean diet vs a low-fat diet in long term secondary cardiovascular prevention that is increased by the high levels of adherence achieved by the Mediterranean diet in both, short and long term.[272] Furthermore, different sub-analyses of this study showed an improvement on atherosclerosis progression measured by IMT-CC and carotid plaques height[273] and other risk factors as endothelial function, T2DM, lipid parameters, inflammation, oxidative stress, miRNAs or microbiome.[274-278]

6.1.2. The Mediterranean diet and T2DM

As a result of the studies on the Mediterranean diet and CVD, the study of how the Mediterranean diet could prevent or help in the management of other cardiometabolic diseases such as T2DM became of relevance for the scientific community.

Several studies, reviews and meta-analyses showed an association between high Mediterranean diet adherence and approximately 20% decrease in T2DM incidence compared with low Mediterranean diet adherence.[265,279,280] These data were also supported by several clinical trials, such as the PREDIMED study, that reported a 40% and 30% reduction of T2DM incidence in

those patients (with a high cardiovascular risk) who consumed a Mediterranean diet enriched with EVOO and nuts respectively, compared to a control diet. This beneficial effect was mainly attributed to the overall composition of the Mediterranean dietary pattern and not to caloric restriction, increased physical activity or weight loss.[265]

Comparing different dietary patterns in some systematic review and meta-analysis,[281,282] there are some specific common components on the diets that prevent the development of T2DM, based specifically on increasing plant based foots, such as olive oil, vegetables, legumes, whole grains, fruits, fish and poultry and reducing red or processed meat, refined grains, sugars, high-fat dairy, fried products and soft drinks. These components and guidelines are followed by many different diets, as the DASH or Mediterranean diet that has been proved to reduce the risk of T2DM by 20%, although other diets, such as low-fat, low-carbohydrate or vegetarian/vegan also showed positive results.[283-289] However, the complexity of dietary patterns, cultural habits and the genetic variants between patients creates substantial differences between them in order to prevent or manage T2DM and to implement the diets in patients' lives. There must be multiple mechanisms that underline the role of the Mediterranean diet in T2DM, because of the multifactorial nature of T2DM and the complex synergy between the nutrients in this diet.[290] Antioxidant, anti-inflammatory effect, glucagon-like peptide (GLP1) agonist, BCAA or the microbiome are some of the most studied mechanisms.[291]

Regarding the pathogenesis of the disease, an important factor is the reduced antioxidant capacity of these patients, such as ascorbic acid, β-carotene, and the α-tocopherol/cholesterol ratio, consequently a well-studied effect is the anti-inflammatory and antioxidant compounds in the Mediterranean diet.[292] The Mediterranean diet has been described to increase plasma levels of diet-derived plasma antioxidants,[293] plasma ferric reducing antioxidant potential, total radical-trapping antioxidant parameter [294] and nitric oxide bioavailability (which enhances endothelium-dependent vasodilator response),[295] and reduce high sensitivity CRP levels[296] an important marker of inflammation. The Mediterranean diet also affects GLP-1 levels, an incretin that promotes insulin generation and secretion by β-cell,[297]inhibits gastric emptying and glucagon secretion, improving T2DM and endothelial function[297-299] and reducing postprandial hyperglycaemia.[300] Moreover, the Mediterranean diet and some of its compounds, such as vitamins, phenolic compounds, PUFA and MUFA[253,264,301,302] modify the release of cytokines and chemokines in the adipose tissue and improve insulin sensitivity[303,304] and β-cell function.[305,306] Other mechanisms involved in the relationship between T2DM and Mediterranean diet could be related with the regulation of energy homeostasis, gut health, immunity, AGEs[307-309],BCAA[74,183,310-312] or the microbiome.[313-319]

Despite the fact that Mediterranean diet has consistently been associated with lower incidence of T2DM and CVD outcomes across and within populations, the mechanisms underlying the disease are still not elucidated. Further studies are needed in the disentangle of the unexplored and still unknown causes and to address T2DM remission by this complicated and synergistic diet. While globally the reduction of the incidence and prevalence of T2DM seems to be critical it takes on even more relevance when it can be mediated by lifestyle changes and healthy and easily adherent dietary patterns such as the Mediterranean diet.

II. HYPOTHESIS

II. HYPOTHESIS

Type 2 diabetes mellitus (T2DM) has currently become a global pandemic and its prevalence has increased over the past few decades, with no signs of receding in the near future, imposing a socioeconomic burden on health services, economy and society.[1,12] T2DM is considered a complex metabolic disease where genetic, metabolic and environmental factors interact resulting in impaired insulin secretion and action.[17] T2DM patients are at increased risk of developing cardiovascular complications. Those patients with concomitant presence of coronary heart disease (CHD) and T2DM have a significantly higher risk of recurrence of a cardiovascular event than those without T2DM.[17] Moreover, these patients have been shown to present higher levels of circulating advanced glycation end products (AGEs)[165,171] and branched chain amino acids (BCAAs),[184-186] metabolites related to both diseases, which are suggested to be involved in pathophysiology of T2DM. AGEs and BCAAs are closely related to lifestyle, especially to type of diet. Consumption of a Mediterranean diet has been proved to reduce T2DM risk and may be related to modulate AGEs and BCAA levels. We hypothesize that the counteraction between an specific dietary pattern, AGEs, BCAAs and T2DM progression could benefit especially to newly-diagnosed T2DM patients because of their remain metabolic flexibility that allowed them to be able to reverse T2DM.[64,66,67] Previously T2DM remission has been described after bariatric surgery[56,57] or intense weight loss but recently, healthy dietary models consumption such as a Mediterranean diet have been proved to reverse T2DM even without a calorie restriction,[68-70] but not in every patient.[74,75]

Based on these findings, our hypothesis is that baseline levels of AGEs and/or BCAAs could potentially lead to the identification of patients with T2DM and CHD who would benefit from healthy dietary interventions (a Mediterranean diet and/or a low-fat diet) to reduce cardiovascular complications. We also hypothesize that reduction of circulating levels of AGEs and/or BCAAs, through consumption of these healthy diets, might be involved in the molecular mechanism underlying T2DM remission of newly diagnosed T2DM patients with CHD.

III. OBJECTIVES

III. OBJECTIVES

Main objective

To determine whether baseline levels of advanced glycation end products (AGEs) and/or branched-chain amino acids (BCAAs) are associated with type 2 diabetes mellitus (T2DM) remission in newly diagnosed T2DM patients with coronary heart disease (CHD) and whether reduction of their circulating levels, after the consumption of two models of healthy diets (a Mediterranean diet and/or a low-fat diet) contributes to T2DM remission with the aim to establish therapeutic dietary strategies for the management of these patients.

Specific objectives

1. **Paper nº1** *"Endothelial Dysfunction and Advanced Glycation End Products in Patients with Newly Diagnosed Versus Established Diabetes: From the CORDIOPREV Study."*
   To compare the levels of advanced glycation end products (AGEs) in both newly diagnosed and established type 2 diabetes mellitus (T2DM) patients with CHD and their association with subclinical atherosclerotic markers [brachial flow-mediated vasodilation (FMD) and intima-media thickness of common carotid arteries (IMT-CC)] for establishing strategies to prevent or reduce AGE production and delay the onset of cardiovascular complications in this type of patients.

2. **Paper nº2** *"Reduction in Circulating Advanced Glycation End Products by Mediterranean Diet Is Associated with Increased Likelihood of Type 2 Diabetes Remission in Patients with Coronary Heart Disease: From the Cordioprev Study."*
   To analyze whether the reduction of circulating AGE levels and the modulation of gene expression related to AGE metabolism, after the consumption of two models of healthy diets (a Mediterranean diet and a low-fat diet), were associated with type 2 diabetes mellitus (T2DM) remission in newly diagnosed T2DM patients with CHD.

3. **Paper nº3** *"Diabetes Remission Is Modulated by Branched Chain Amino Acids According to the Diet Consumed: From the CORDIOPREV Study.*
   To evaluate the relationship between levels of circulating BCAA (baseline and changes after the consumption of two models of healthy diets -a Mediterranean diet and a low-fat diet) and type 2 diabetes mellitus (T2DM) remission in newly diagnosed T2DM patients with CHD.

IV. PUBLICATIONS

1. Endothelial Dysfunction and Advanced Glycation End Products in Patients with Newly Diagnosed Versus Established Diabetes: From the CORDIOPREV Study

nutrients

MDPI

Article

Endothelial Dysfunction and Advanced Glycation End Products in Patients with Newly Diagnosed Versus Established Diabetes: From the CORDIOPREV Study

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Abstract: Endothelial dysfunction and intima-media thickness of common carotid arteries (IMT-CC) are considered subclinical markers of atherosclerotic cardiovascular disease (ASCVD). Advanced glycation end products (AGEs) are increased in type 2 diabetes mellitus (T2DM) patients, compared with non-diabetics, being implicated in micro- and macrovascular complications. Our aim was to compare serum AGEs levels and subclinical atherosclerotic markers between patients with established and newly diagnosed T2DM. Among 540 patients with T2DM and coronary heart disease from the CORDIOPREV study, 350 patients had established T2DM and 190 patients had newly diagnosed T2DM. Serum levels of AGEs (methylglyoxal (MG) and N-carboxymethyl lysine (CML)) and subclinical atherosclerotic markers (brachial flow-mediated vasodilation (FMD) and IMT-CC) were measured. AGEs levels (all $p < 0.001$) and IMT-CC ($p = 0.025$) were higher in patients with established vs. newly diagnosed T2DM, whereas FMD did not differ between the two groups. Patients with established T2DM and severe endothelial dysfunction (i.e., FMD < 2%) had higher serum MG levels, IMT-CC, HOMA-IR and fasting insulin levels than those with newly diagnosed T2DM and non-severe endothelial dysfunction (i.e., FMD ≥ 2%) (all $p < 0.05$). Serum CML levels were greater in patients with established vs. newly diagnosed T2DM, regardless of endothelial dysfunction severity. Serum AGEs levels and IMT-CC were significantly higher in patients with established vs. newly diagnosed T2DM, highlighting the progressively increased risk of ASCVD in the course of T2DM. Establishing therapeutic strategies to reduce AGEs production and delay the onset of cardiovascular complications in newly diagnosed T2DM patients or minimize ASCVD risk in established T2DM patients is needed.

Keywords: CORDIOPREV; type 2 diabetes mellitus; endothelial dysfunction; advanced glycation end products; methylglyoxal; N-carboxymethyl lysine; flow-mediated vasodilation; intima-media thickness of common carotid arteries

1. Introduction

Type 2 diabetes mellitus (T2DM) is associated with an increased risk of atherosclerotic cardiovascular disease (ASCVD) which is fostered by chronic exposure to hyperglycemia, as well as other factors such as hypertension, dyslipidemia or genetic predisposition [1]. Indeed, T2DM patients are up to four times more likely to suffer cardiovascular (CV) events than patients without the disease [2].

Endothelial dysfunction, a condition that contributes to the pathogenesis of vascular disease in T2DM, is considered a reliable marker of subclinical ASCVD, as it appears before the development of atherosclerotic lesions or the occurrence of clinical events [3,4]. Flow-mediated vasodilation (FMD) of the brachial artery is the most widely used non-invasive marker of endothelial dysfunction in the clinical setting [5]. However, in a more advanced stage of the disease, changes in the intima-media thickness of common carotid arteries (IMT-CC) can be seen as structural modifications within the vessel wall occur. In this context, increased IMT-CC is used as a surrogate marker of elevated CV risk [6]. As endothelial dysfunction represents a key early step in the development of atherosclerosis, establishing strategies to prevent or improve endothelial dysfunction may minimize the risk of developing atherosclerotic diseases.

Advanced glycation end products (AGEs), a complex group of oxidant compounds synthesized from slowly occurring non-enzymatic glycation between reducing sugars, such as glucose or fructose, and proteins or lipids, play an important role in the pathogenesis of diabetic vascular disease [7–9]. Methylglyoxal (MG), a highly reactive carbonyl species formed as a by-product of glycolysis, is a strong precursor of AGEs [10], whereas N-carboxymethyl lysine (CML) is a well-characterized AGE present in long-lived proteins that has been used as an indicator of oxidative stress in biological systems [11]. Small amounts of AGEs are generated in vivo as a normal consequence of metabolism, and they gradually accumulate during aging, especially in the context of associated chronic diseases [12]. In T2DM, AGEs production is enhanced by chronic hyperglycemia and, at the same time, their clearance is reduced. Their accumulation triggers oxidative stress, inflammation and apoptosis [13–16]. It is well established that AGEs can reduce nitric oxide (NO) production and endothelial NO synthase activity under high glucose concentrations [17]. These data, combined with the findings that AGEs are increased in human atherosclerotic tissues, support the idea that AGEs may contribute to vascular diabetic disease through endothelial dysfunction [18–20]. To date, no studies have evaluated AGEs levels in patients with established T2DM compared with those with newly diagnosed T2DM, thus consequently discriminating diabetic subpopulations at increased CV risk.

Considering all the above, the aim of the present study was to compare serum levels of AGEs and subclinical atherosclerotic markers (i.e., endothelial dysfunction and IMT-CC) between patients with established T2DM and those with newly diagnosed T2DM from the CORonary Diet Intervention with Olive oil and cardiovascular PREVention (CORDIOPREV) study.

2. Patients and Methods

2.1. Patient Population

This work was carried out within the framework of the CORDIOPREV study (Clinicaltrials.gov number NCT00924937), which is a randomized, single-blind, controlled trial that includes 1002 patients with coronary heart disease (CHD). The rationale, methodology and baseline characteristics of the participants in the CORDIOPREV study have been published elsewhere [21]. Prior to recruitment and

initiation of the study protocol, written consent was obtained from all participants. All the amendments follow the Helsinki Declaration and good clinical practices and were approved by the Ethics Committee of the Hospital Reina Sofía (Cordoba, Spain).

In the present cross-sectional study, we included those T2DM patients (n = 540) who met, at baseline, the criteria for diabetes diagnosis proposed by the American Diabetes Association [22]. T2DM patients were then categorized in 2 groups: (a) patients with established T2DM (n = 350), i.e., those with a prior medical history of T2DM before entering the study that were receiving treatment (medication or diet), and (b) patients with newly diagnosed T2DM (n = 190), who had no previous history of T2DM, thus being diagnosed during the recruitment period of the CORDIOPREV study.

2.2. Anthropometric Measurements and Laboratory Tests

Patients were given an appointment at 8.00 a.m., following a 12 h fast, and were admitted to the laboratory for anthropometric and biochemical tests. Anthropometric parameters were measured by trained dietitians using calibrated scales (BF511 Body Composition Analyzer/Scale, OMROM, Kyoto, Japan) and a wall-mounted stadiometer (Seca 242, HealthCheck Systems, Brooklyn, NY, USA). Waist circumference was measured midway between the lowest rib and the iliac crest. Body mass index (BMI) was then calculated as weight per square meter (kg/m^2). Smoking status, alcohol intake and drug therapy were also recorded for each participant. Systolic and diastolic blood pressure were measured with a validated digital automated blood pressure monitor.

Venous blood samples were collected from the antecubital vein in Vacutainer™ tubes containing EDTA or no anticoagulant. Serum glucose, HbA1c, total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels were measured by spectrophotometry using an Architect c-16000 analyzer (Abbot®, Chicago, IL, USA). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula (provided serum TG levels were <400 mg/dL) [23]. Apolipoprotein (ApoA1 and ApoB) concentrations were determined by immunoturbidimetry and plasma insulin by chemiluminescent microparticle immunoassay using an Architect i-2000 analyzer (Abbott®). Insulin resistance was defined by the homeostasis model assessment of insulin resistance (HOMA-IR) index, calculated as fasting insulin (mU/L) × fasting plasma glucose (mmoL/L)/22.5 [24].

2.3. Evaluation of Endothelial Function

Ultrasonography of the brachial artery was performed to measure endothelial-dependent FMD following the guidelines established by the International Brachial Artery Reactivity Task Force [25]. Briefly, tests were performed during the morning, after overnight fast, under stable temperature conditions, refraining patients from doing any physical activity in the previous 48 h, and without stopping medication. Patients remained in supine position for at least 10 min before the test, and until the last image was recorded. The patients' right arm was immobilized with a stereotactic device, and a high resolution 12 mHz transducer connected to an ultrasound scanner (EnVisor HD, Philips®, Bothell, WA, USA) was placed on the antecubital fossa, in a location where anterior and posterior intimal layers of a non-tortuous longitudinal segment of the brachial artery could be located, to acquire a baseline rest image. Subsequently, the pressure cuff was inflated to 300 mmHg and maintained for 4.5 min. Next, the pressure cuff was deflated, reactive hyperemia was measured and, 1 min after cuff deflation, the new artery diameter and blood-flow velocity were recorded. The whole procedure (from 30 s before deflating the cuff until 2 min after its deflation) was recorded on video.

FMD was defined as the percentage of change between the diameter of the brachial artery after cuff deflation (D_2) and the basal diameter pre-occlusion (D_1), calculated as $[(D_2 - D_1)/D_1] \times 100$. Participants were electrocardiogram-monitored throughout the whole procedure, and arterial diameter was measured coinciding with the R wave of the electrocardiogram. Intra-observer and inter-observer variabilities were 8.85% and 8.70%, respectively.

2.4. Estimation of Severity of Endothelial Dysfunction

Patients with available FMD measurements were classified according to the FMD cutoff value of 2%, which serves as a benchmark to predict cardiovascular events in high risk populations [26]. Among patients with severe endothelial dysfunction (i.e., FMD < 2%) (n = 172), 116 had established T2DM and 56 had newly diagnosed T2DM. Among patients with non-severe endothelial dysfunction (i.e., FMD ≥ 2%) (n = 320), 202 had established T2DM and 118 had newly diagnosed T2DM.

2.5. Ultrasound Measurement of IMT-CC

Carotid arteries were examined using a Doppler ultrasound high-resolution B-mode (Envisor C Ultrasound System, Philips, Eindhoven, The Netherlands), following the recommendations of the American Society of Echocardiography Carotid Intima-Media Thickness Task Force [27]. Measurements were registered using semi-automatic software (QLAB Advance Ultrasound Quantification Software, v5.0, Phillips, Eindhoven, The Netherlands). Measures were performed in triplicate, obtaining the general mean of the IMT of both common carotid arteries (IMT-CC) for each patient.

2.6. Determination of Serum Levels of AGEs

Methylglyoxal (MG) and N-carboxymethyl lysine (CML) were measured in the serum using competitive ELISA kits (OxiSelect™ Methylglyoxal Competitive ELISA Kit and OxiSelect™ N-epsilon-(Carboxymethyl) Lysine Competitive ELISA Kit, Cell Biolabs, Inc., San Diego, CA, USA), following the manufacturer's instructions.

2.7. Statistical Analysis

Statistical analyses were carried out using SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov normality test was performed for the evaluation of the distribution of the quantitative variables, and continuous variables that deviated significantly from the assumption of normality were transformed. Continuous variables were compared using t-test when comparing between two groups, or one-way analysis of variance (ANOVA) and post-hoc multiple comparisons analysis using the LSD test (homogeneous variances) or Tamhane's statistic (non-homogeneous variances) when comparing more than two groups. Pearson chi-squared test was employed to compare categorical characteristics. One-way ANCOVA was conducted to compare MG and CML serum levels to control for age, sex and body mass index as covariates.

3. Results

Anthropometric and biochemical characteristics for the patients with established T2DM (n = 350) and those with newly diagnosed T2DM (n = 190) are shown in Table 1. Patients with established T2DM were older, had higher systolic blood pressure, fasting glucose, fasting insulin, HbAc1 and HOMA-IR values, as well as lower levels of HDL-C, LDL-C and ApoB than those with newly diagnosed T2DM ($p < 0.05$ for all comparisons). Furthermore, IMT-CC was higher in patients with established vs. newly diagnosed T2DM ($p = 0.025$), with all average values higher than 0.7 mm, which is the suggested cutoff value to determine the presence or absence of carotid atherosclerotic disease [28]. In contrast, FMD did not differ among the two study groups.

Table 1. Characteristics of the study groups [1].

	Patients with Newly Diagnosed Diabetes (n = 190)	**Patients with Established Diabetes (n = 350)**	**p Value**
Age (years)	**60.0 ± 0.6**	**61.9 ± 0.4**	**0.015**
Sex (men/women)	158/32	285/64	0.724
Weight (kg)	86.7 ± 1.0	85.0 ± 0.8	0.191
BMI (kg/m^2)	31.4 ± 0.3	31.1 ± 0.3	0.431
Waist circumference (cm)	106 ± 0.8	105 ± 0.6	0.294
DBP (mmHg)	76.9 ± 0.9	76.1 ± 0.6	0.448
SBP (mmHg)	**137 ± 1.5**	**143 ± 1.1**	**0.001**
HDL-cholesterol (mg/dL)	**41.5 ± 0.7**	**39.6 ± 0.5**	**0.023**
LDL-cholesterol (mg/dL)	**92.4 ± 1.9**	**82.5 ± 1.4**	**<0.001**
Triglycerides (mg/dL)	146 ± 6.0	150 ± 4.1	0.604
ApoA-1 (mg/dL)	127± 1.4	125 ± 1.2	0.294
ApoB (mg/dL)	**77.3 ± 1.4**	**72.4 ± 1.0**	**0.005**
Fasting glucose (mg/dL)	**120 ± 1.7**	**157 ± 3.0**	**<0.001**
Fasting insulin (mU/L)	**10.8 ± 0.5**	**13.6 ± 0.9**	**0.024**
HOMA-IR	**4.27 ± 0.25**	**6.50 ± 0.43**	**< 0.001**
HbAc1 (%)	**6.67 ± 0.06**	**7.66 ± 0.07**	**<0.001**
FMD (%)	4.11 ± 0.45	3.46 ± 0.35	0.256
IMT-CC (mm)	**0.72 ± 0.01**	**0.75 ± 0.01**	**0.025**
Alcohol intake (>16 g/day) (%)	19.9	23.1	0.865
Current tobacco use (%)	12.2	9.8	0.380
Antihypertensive drugs (%)			
Angiotensin converting enzyme inhibitors or angiotensin II receptor blockers	**38.9**	**55.6**	**<0.001**
Calcium channel blockers	**16.8**	**24.9**	**0.039**
Beta-blockers	62.6	63.6	0.852
Nitrates	10	10	1.000
Diuretics	42.1	46.7	0.320
Lipid lowering drugs (%)			
Statins	86.8	85.7	0.795
Fibrates	**0**	**2.9**	**0.017**
Oral hypoglycemic agents (%)	**0**	**72.8**	**<0.001**
Insulin (%)	**0**	**25.8**	**<0.001**

[1] Values are presented as mean ± SEM (standard error of the mean). Numerical variables were analysed using independent t-test (95% confidence interval) for mean difference, whereas categorical variables were analysed using χ^2 test. Values in bold were significantly different ($p < 0.05$). n, sample size; BMI, body mass index; DBP, diastolic blood pressure; systolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo, apolipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; FMD, flow-mediated dilation; IMT-CC, intima-media thickness of both common carotid arteries; HbAc1, glycated haemoglobin.

Significantly more patients with established T2DM were taking calcium channel blockers, angiotensin converting enzyme inhibitors or angiotensin II receptor blockers and fibrates than those with newly diagnosed T2DM ($p < 0.05$ for all comparisons) (Table 1). Obviously, patients with established T2DM were on antidiabetic therapy, whereas those with newly diagnosed T2DM were not.

Serum levels of both MG and CML were significantly higher in patients with established T2DM vs. those with newly diagnosed T2DM (4.00 ± 0.09 vs. 3.09 ± 0.13 for MG and 0.66 ± 0.03 vs. 0.35 ± 0.05 for CML (all $p < 0.001$) (Figure 1A,B, respectively).

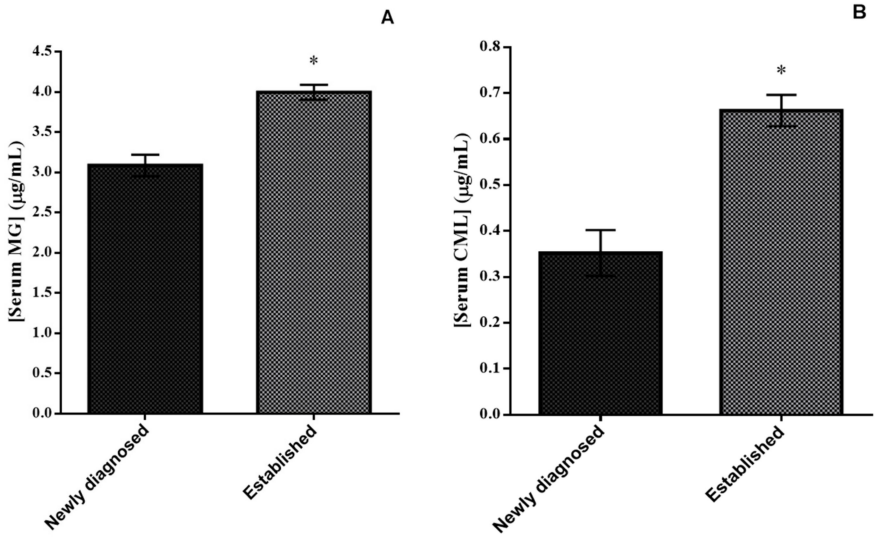

Figure 1. Serum levels of methylglyoxal (μg/mL) (**A**) and carboxy-methyl-lysine (μg/mL) (**B**) in T2DM patients studied. One-way ANCOVA results (adjusted mean ± SEM) controlling for age, sex and BMI. * $p < 0.001$ indicates significant differences.

Patients with established T2DM and severe endothelial dysfunction had the highest serum MG levels (4.37 ± 0.16) (Figure 2A). These patients also had higher IMT-CC, HOMA-IR, and fasting insulin levels than those with newly diagnosed T2DM and non-severe endothelial dysfunction (Table 2) ($p < 0.05$ for all comparisons). Serum CML levels were higher in patients with established vs. newly diagnosed T2DM, regardless of the severity of endothelial dysfunction (Figure 2B). Moreover, patients with established T2DM, with severe or non-severe endothelial dysfunction, had higher systolic blood pressure compared with patients with newly diagnosed T2DM with non-severe endothelial dysfunction (Table 2) ($p < 0.05$ for all comparisons).

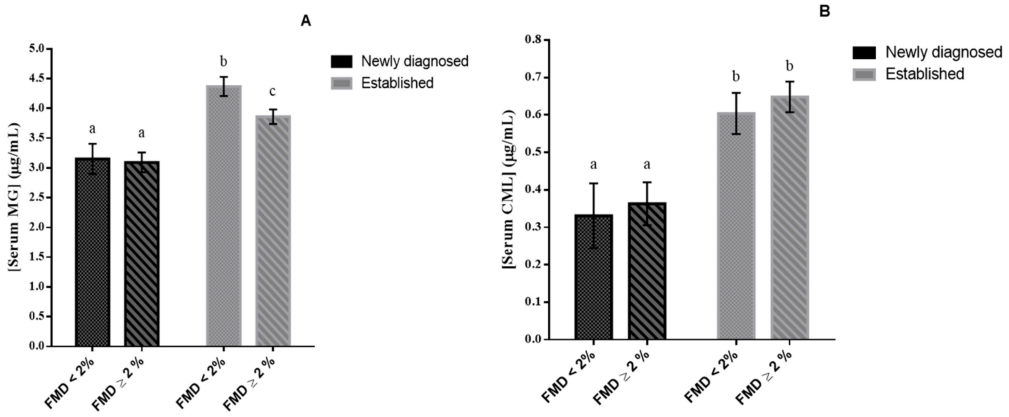

Figure 2. (**A**) Serum levels of methylglyoxal (μg/mL) and (**B**) carboxy-methyl-lysine (μg/mL) according to FMD classification (2% cutoff) in T2DM patients studied. One-way ANCOVA results (adjusted mean ± SEM) controlling for age, sex and BMI. Bars with different superscript letters (a, b, c) are significantly different ($p < 0.05$).

Table 2. Characteristics of the study groups considering the severity of endothelial dysfunction [1].

	Patients with Newly Diagnosed Diabetes		**Patients with Established Diabetes**		
	Severe Endothelial Dysfunction (n = 56)	**Non-Severe Endothelial Dysfunction (n = 118)**	**Severe Endothelial Dysfunction (n = 116)**	**Non-Severe Endothelial Dysfunction (n = 202)**	***p* Value**
Age (years)	60.4 ± 1.2	59.8 ± 0.8	62.3 ± 0.7	61.4 ± 0.6	0.112
Sex (men/women)	46/10 ^{ab}	98/20 ^{ab}	87/29 ^{b}	175/27 ^{a}	0.074
Weight (kg)	84.7 ± 1.5	88.1 ± 1.3	84.6 ± 1.4	85.2 ± 1.1	0.229
BMI (kg/m^2)	31.5 ± 0.5	31.6 ± 0.4	31.2 ± 0.4	31.2 ± 0.4	0.877
Waist circumference (cm)	104 ± 1.3	107 ± 1.1	104 ± 1.1	105 ± 0.8	0.204
DBP (mmHg)	77.6 ± 1.6	76.7 ± 1.0	74.2 ± 0.9	77.4 ± 0.7	0.062
SBP (mmHg)	**141 ± 2.7 ^{ab}**	**136 ± 1.8 ^{a}**	**145 ± 2.0 ^{b}**	**143 ± 1.4 ^{b}**	**0.018**
HDL-cholesterol (mg/dL)	41.0 ± 1.4	41.6 ± 0.8	40.0 ± 1.0	39.1 ± 0.6	0.128
LDL-cholesterol (mg/dL)	**93.5 ± 3.8 ^{a}**	**92.6 ± 2.5 ^{a}**	**85.8 ±2.7 ^{ab}**	**81.7 ± 1.7 ^{b}**	**0.001**
Triglycerides (mg/dL)	151 ± 12.2	142 ± 7.0	161 ± 7.2	146 ± 5.4	0.253
ApoA-1 (mg/dL)	125 ± 2.6	128 ± 1.7	127 ± 2.2	124 ± 1.4	0.221
ApoB (mg/dL)	**81.5 ± 3.0 ^{a}**	**75.8 ± 1.8 ^{ab}**	**74.9 ± 1.8 ^{ab}**	**71.8 ± 1.3 ^{b}**	**0.007**
Fasting glucose (mg/dL)	**122 ± 3.4 ^{a}**	**118 ± 2.1 ^{a}**	**155 ± 6.0 ^{b}**	**155 ± 3.4 ^{b}**	**<0.001**
Fasting insulin (mU/L)	**11.2 ± 1.0 ^{ab}**	**10.4 ± 0.7 ^{b}**	**15.2 ± 1.7 ^{a}**	**12.9 ± 1.0 ^{ab}**	**0.052**
HOMA-IR	**4.27 ± 0.52 ^{ab}**	**4.18 ± 0.31 ^{b}**	**7.06 ± 0.9 ^{a}**	**6.09 ± 0.45 ^{ab}**	**0.002**
HbAc1 (%)	**6.81 ± 0.11 ^{a}**	**6.63 ± 0.08 ^{a}**	**7.57 ± 0.13 ^{b}**	**7.72 ± 0.09 ^{b}**	**<0.001**
FMD (%)	**−1.78 ± 0.70 ^{a}**	**6.91 ± 0.34 ^{b}**	**−2.19 ± 0.45 ^{a}**	**6.70 ± 0.30 ^{b}**	**<0.001**
IMT-CC (mm)	0.74 ± 0.02 ^{ab}	0.72 ± 0.01 ^{a}	0.76 ± 0.02 ^{b}	0.75 ± 0.01 ^{ab}	0.162
Alcohol intake (>16 g/day) (%)	21.8	19.1	22.4	23.1	0.682
Current tobacco use (%)	17.9 ^{a}	7.8 ^{b}	9.9 ^{ab}	8.7 ^{ab}	0.176
Antihypertensive drugs (%)					
Angiotensin converting enzyme inhibitors or angiotensin II receptor blockers	**28.6 ^{a}**	**43.2 ^{ab}**	**61.2 ^{c}**	**51.5 ^{b,c}**	**<0.001**
Calcium channel blockers	12.5	19.5	21.6	24.8	0.236
Beta-blockers	62.5	62.7	65.5	62.4	0.950
Nitrates	8.9	11.0	6.0	11.4	0.442
Diuretics	41.1 ^{abc}	40.7 ^{c}	55.2 ^{b}	43.1 ^{ac}	0.094
Lipid lowering drugs (%)					
Statins	87.5	84.7	83.6	87.1	0.805
Fibrates	0 ^{ab}	0 ^{b}	3.4 ^{a}	3.0 ^{ab}	0.129
Oral hypoglycemic agents (%)	**0 ^{a}**	**0 ^{a}**	**73.3 ^{b}**	**72.8 ^{b}**	**<0.001**
Insulin (%)	**0 ^{a}**	**0 ^{a}**	**26.7 ^{b}**	**24.8 ^{b}**	**<0.001**

[1] Values are presented as mean ± SEM (standard error of the mean). Numerical variables were analysed using one-way ANOVA, whereas categorical variables were analysed using χ^2 test. Values in bold were significantly different ($p < 0.05$). Values in the same row with different superscript letters (a, b, c) are significantly different. BMI, body mass index; DBP, diastolic blood pressure; systolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo, apolipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; HbAc1, glycated haemoglobin; FMD, flow-mediated dilation; IMT-CC, intima-media thickness of both common carotid arteries.

4. Discussion

Most of the studies published to date have reported increased levels of AGEs in T2DM patients compared with healthy populations [14–17]. However, to the best of our knowledge, this is the first study that assessed differences in AGEs concentrations between patients with CHD and established T2DM vs. those with CHD and newly diagnosed T2DM. In the present study, patients with established T2DM had higher serum AGEs levels (both MG and CML) than those with newly diagnosed T2DM.

Although FMD (an early subclinical atherosclerosis marker) did not differ between these two study groups, IMT-CC (a subclinical atherosclerotic marker related to vascular damage) was increased in patients with established vs. newly diagnosed T2DM. Moreover, when the presence of severe endothelial dysfunction was considered, patients with established T2DM exhibited the highest serum MG levels among all groups.

Experimental and clinical studies have suggested that the increased formation of AGEs is one of the causes of endothelial dysfunction in T2DM [18]. AGEs enhance vasoconstriction (by increasing endothelin-1 levels), reduce vasodilation (by decreasing NO levels) and stimulate AGE-modification of extracellular matrix to accelerate the progression of atherosclerosis [17]. In this context, Ninomiya et al. [19] reported an inverse association between FMD and accumulated fluorescent AGEs in the skin of T2DM patients. This is in line with our findings in patients with established T2DM, who exhibited the highest MG levels when severe endothelial dysfunction was present.

The harmful effects of AGEs are mainly due to their ability to cross-link proteins, thus affecting their conformation, altering their enzymatic activity and reducing their degradation capacity and clearance [8]. In T2DM patients, AGEs accumulate as their production is enhanced by chronic hyperglycemia, thus worsening AGE-induced deleterious effects. This could explain our results as a greater dysregulation of glucose homeostasis seen in patients with established T2DM (who had higher fasting glucose, insulin and HbA1c levels compared with patients with newly diagnosed T2DM) could lead to higher serum levels of AGEs.

AGEs could also contribute to T2DM progression, not only due their oxidant properties *per se*, but also by triggering the cascade of oxidative damage and inflammatory response through the action of receptors such as nuclear factor kappa B (NFkB), nuclear factor erythroid 2-related factor 2 (Nrf2) and more specifically the receptor for AGEs (RAGE) [29]. Therefore, AGEs can lead to vascular inflammation and pathological angiogenesis, contributing to the long-term vascular complications of T2DM [30,31]. This might also explain, at least partly, our finding that patients with established T2DM and severe endothelial dysfunction had, in addition to the highest levels of serum MG, higher IMT-CC than patients with newly diagnosed T2DM and non-severe endothelial dysfunction. In this context, it has been shown that accumulation of AGEs in the vessel wall can be responsible for the formation of a rigid fibrin network and collagen stiffness that contribute to increase IMT-CC in T2DM patients [7].

Endogenous AGEs formation represents a minor component of the total body load of AGEs; dietary AGEs are one of the most important exogenous sources of AGEs that depends on nutrient composition and food processing methods applied [32,33]. The intake of meals rich in AGEs can acutely impair endothelial function in patients with and without T2DM [34]. Conversely, low-AGE diets were shown to reverse insulin resistance and chronic inflammation, inhibit the progression of atherosclerosis and prevent experimental diabetic complications [35,36]. In this context, we have recently published that, in both elderly adults and patients with the metabolic syndrome, a Mediterranean diet could be a beneficial dietary model in terms of AGEs reduction as it has a low content of dietary AGEs, thus consequently reducing their circulating levels and the degree of oxidative stress and inflammation [37,38].

The present study has some limitations. As it is a cross-sectional study, associations between serum levels of AGEs, FMD and IMT-CC values cannot be inferred. Furthermore, we could not assess the effects of drug therapy on AGEs, FMD and IMT-CC. Moreover, the results are limited to a population of T2DM patients with CHD and may not be suitable for extrapolation to healthy

populations. Finally, as this is an ancillary study, information about the time of diagnosis of T2DM in established diabetes patients was not available for all the cases.

5. Conclusions

To the best of our knowledge, this is the first study to show differences in AGEs levels between patients with established T2DM and those with newly diagnosed disease. Furthermore, although FMD did not differ between the two groups, IMT-CC was higher in patients with established vs. newly diagnosed T2DM. The presence of severe endothelial dysfunction was associated with increased IMT-CC and MG levels. As AGEs accumulation is the result of endogenous formation, oral intake and renal clearance, they can be lowered by changes in dietary habits and pharmacological treatment. The present findings support the need for establishing strategies to prevent or reduce AGEs in order to delay the onset of CV complications in newly diagnosed T2DM patients and to minimize CV risk in patients with established T2DM.

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2. *Reduction in Circulating Advanced Glycation End Products by Mediterranean Diet Is Associated with Increased Likelihood of Type 2 Diabetes Remission in Patients with Coronary Heart Disease: From the Cordioprev Study.*

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Reduction in Circulating Advanced Glycation End Products by Mediterranean Diet Is Associated with Increased Likelihood of Type 2 Diabetes Remission in Patients with Coronary Heart Disease: From the Cordioprev Study

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Scope: It is hypothesized that decreased advanced glycation end products (AGEs) levels could affect type 2 diabetes mellitus (T2DM) remission in newly diagnosed patients through the consumption of two healthy diets.
Methods and Results: Patients from CORDIOPREV study, all with previous cardiovascular events, with T2DM at the beginning of the study are included. Patients are randomized to a Mediterranean or a low-fat diet for five years. No different diabetes remission rates are found among diets. Serum methylglioxal (MG) and carboximethyllysine (CML), levels dietary AGE, as well as gene expression of AGER1 and RAGE are measured. Serum MG decreases only after the consumption of the Mediterranean diet. Moreover, a COX regression analysis shows that each SD decrease in the MG, occurring after the Mediterranean diet, increases the probability of T2DM remission with HR:2.56(1.02–6.25) and $p = 0.046$ and each SD increase in disposition index at baseline increases the probability of remission with HR:1.94(1.32–2.87) and $p = 0.001$.
Conclusions: It is demonstrated that the reduction of serum AGEs levels and the modulation of its metabolism, occurring after the consumption of a Mediterranean diet, might be involved in the molecular mechanism underlying the T2DM remission of newly diagnosed patients with coronary heart disease.

1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) is steadily increasing worldwide, imposing a heavy socioeconomic burden on health services, the economy, and society in general.[1,2] T2DM incidence is strongly linked to environmental and lifestyle factors (limited physical activity and consumption of high-energy diets rich in processed foods, refined sugar, or saturated fats).[3] Dietary intervention studies have shown that changes in diet and physical activity can protect against T2DM,[4,5] establishing an association between specific dietary patterns and diabetes risk.[6] In this context, low-carbohydrate, low-fat calorie-restricted, or Mediterranean-style diets have been shown to be effective for T2DM patients, due to emerging evidence on cardiovascular benefits, glucose homeostasis, and insulin sensitivity.[7,8, 9]

Early T2DM has now been shown to be reversed by different dietary strategies, such as caloric restriction[10,11] or consumption of a low-carbohydrate

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Mediterranean diet model,[12] which was mainly responsible for the substantial weight loss.

The Mediterranean diet is characterized by its richness in monounsaturated fat (mainly from virgin olive oil), vegetables, fruit, nuts, legumes, and whole grain cereals, which provide fiber, antioxidants, vitamins, minerals, and polyphenols, and a low consumption of processed foods. The healthy properties of a Mediterranean diet are mostly due to its anti-inflammatory and anti-oxidative effects.[13] Advanced glycation end products (AGEs) are a group of prooxidant and cytotoxic compounds, generated from the Maillard reaction, which contribute to the onset and progression of certain chronic diseases, such as T2DM.[14] Small amounts of AGEs are generated as a consequence of a normal metabolism, but in the context of chronic diseases, AGE production increases.[15] In T2DM patients, AGEs tend to accumulate because their production is boosted by chronic hyperglycemia, thus worsening AGE-induced deleterious effects. In fact, it has been suggested that AGEs may even play a role in the progression of T2DM and its complications.[16] Endogenous AGE formation represents a minor component of the total body load of AGEs, and diet is one of the most important exogenous sources of AGEs, which depend on the nutrient composition and the food processing methods applied.[17,18] It has been demonstrated that AGE-restricted diets produce an improvement in insulin resistance, a reduction in serum AGEs levels and markers related to inflammation and oxidative stress, after the consumption of AGE-restricted diets in T2DM patients.[19] In this context, we have recently published that a Mediterranean diet could provide a good dietary model for the reduction of AGE content, since, during its consumption, it provides a low content in dietary AGEs, with the consequent reduction of their circulating levels and oxidative stress and inflammation in both elderly adults and metabolic syndrome patients.[20,21] However, to the best of our knowledge, there is no evidence of the involvement of AGEs in T2DM remission.

Taking all the above into consideration, the main aim of this study was to analyze whether the reduction of AGE levels and the modulation of gene expression related to AGE metabolism after the consumption of two models of healthy diets (a Mediterranean diet and a low-fat diet) were associated with T2DM remission in patients with coronary heart disease (CHD).

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Table 1. Baseline anthropometric, clinical, and metabolic characteristics according to remission of T2DM.

	Responders	Non-Responders	*p*-value
n	73	110	
Mediterranean/Low-fat diets	33/40	47/63	0.741
Men/Women [n]	60/13	92/18	0.799
Age [years]	60.8 ± 1.0	59.3 ± 0.9	0.252
Weight [kg]	80.2 ± 1.3	88.4 ± 1.4	<0.001
BMI [Kg/m^2]	29.9 ± 0.4	32.1 ± 0.4	0.001
Waist circunference [cm]	101 ± 1	108 ± 1.	<0.001
SBP [mmHg]	137 ± 2.3	138 ± 2	0.717
DBP [mmHg]	76.6 ± 1.6	77.2 ± 1.0	0.755
Glucose [mg dL^{-1}]	98.9 ± 1.6	118 ± 3	<0.001
Insulin [mU L^{-1}]	9.28 ± 0.79	13.4 ± 1.1	0.007
HbA1c [%]	6.53 ± 0.08	6.79 ± 0.08	0.032
HDL-cholesterol [mg dL^{-1}]	43.0 ± 1.3	40.7 ± 0.8	0.141
LDL-cholesterol [mg dL^{-1}]	89.2 ± 2.8	93.3 ± 2.7	0.302
Triglycerides [mg dL^{-1}]	131 ± 9	150 ± 7	0.090
C-reactive protein [mg L^{-1}]	3.89 ± 0.56	3.51 ± 0.38	0.558
Disposition Index	0.68 ± 0.06	0.43 ± 0.02	<0.001
Use of Statins [%]	88.2 %	86.3%	0.821
Smokers [%]	11.9%	10.5%	0.789
Active alcohol consumer [%]	85.1%	78.9%	0.412
Serum MG [mg mL^{-1}]	3.11 ± 0.16	3.04 ± 0.13	0.727
Serum CML [mg mL^{-1}]	0.29 ± 0.05	0.32 ± 0.04	0.659
AGER1 gene expression [AU]	0.18 ± 0.03	0.18 ± 0.02	0.994
RAGE gene expression [AU]	0.18 ± 0.02	0.22 ± 0.02	0.245

Values expressed as mean ± SEM. Men/women in each of the diets frequencies; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, Hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MG, Metylglyoxal; CML, CarboxyMethylLysine. *t*-test analysis for unpaired data, *p*-values ($p < 0.05$).

2. Results

2.1. Characteristics of the Study Population

There were no significant differences in any of the anthropometrics and biochemical characteristics, at baseline, in the 183 T2DM patients assigned to each dietary intervention (Supporting information Tables S1 and S2, Supporting Information). The baseline characteristics of the patients according to T2DM remission are shown in **Table 1**. Briefly, Responders had lower weight, BMI, waist circumference, fasting glucose, fasting insulin, and HbA1c, but higher DI values than non-Responders. We observed that non-Responders patients increased fasting glucose and decreased LDL-cholesterol after the dietary intervention as compared to Responders, while Responders decreased HbA1c compared to non-Responders (Supporting information Table S3, Supporting Information). There were no significant differences in ΔChange of anthropometrics or biochemical characteristics after the dietary intervention according to the diet consumed (Table S4, Supporting Information). We evaluated the severity of coronary artery disease at baseline of the study by coronary angiography. Single-vessel lesion were found in the 40% of non-responders

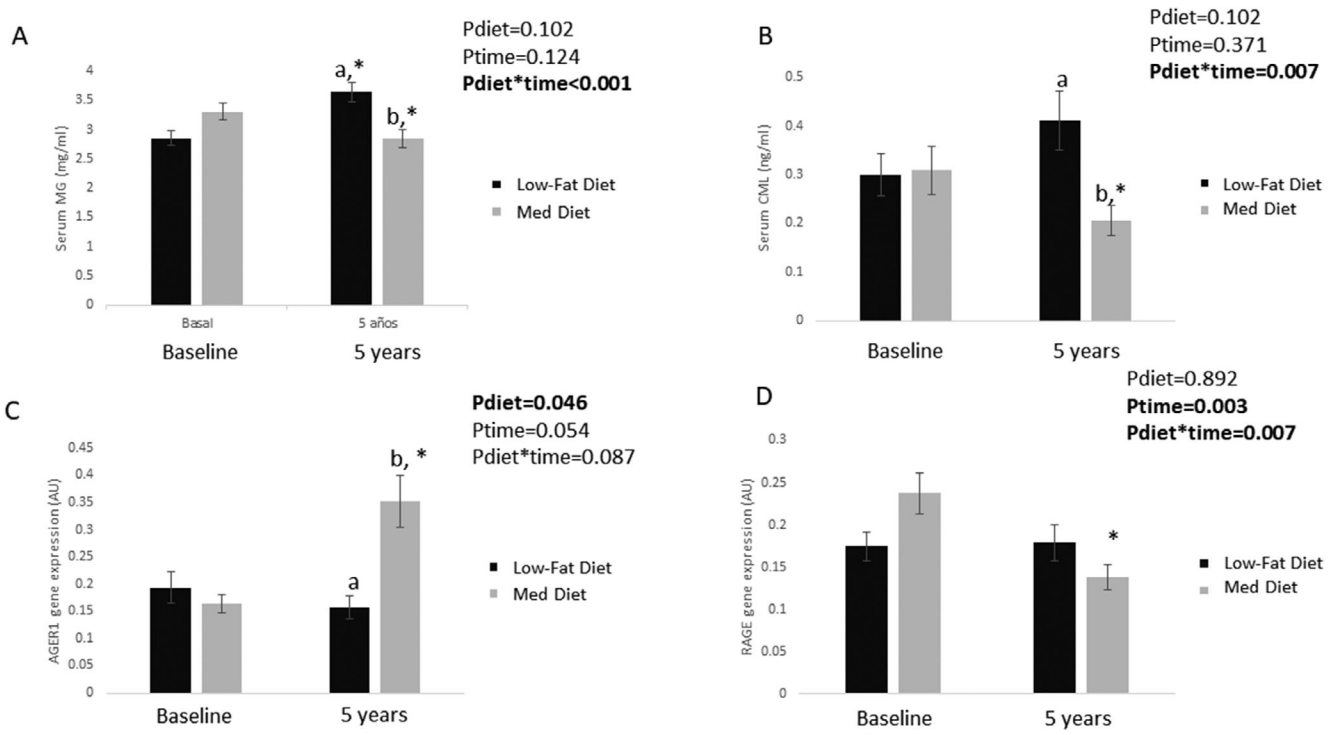

Figure 1. Influence of dietary intervention on AGE metabolism. Mean ± S.E.M. of the serum levels of A) methylglyoxal (MG), B) carboxymethyllysine (CML), C) gene expression of AGER1, and D) RAGE, at baseline and post dietary intervention for the two diets studied. ANOVA for repeated measures *p*-values adjusted by age, BMI, sex, triglycerides, HDL-cholesterol, smoking, alcohol consumption, and used of statins. Global *p*-values: *p*(diet): diet effect; *p*(time): time effect; *p*(diet*time): time by diet interaction. Different letters indicate significant differences ($p < 0.05$) between groups in the Post Hoc Bonferroni's multiple comparison tests.

and 34.2% of responders, and multi-vessel lesion (more or equal than two lesions) were found in the 53.6% of non-responders and in the 63% of responders. We did not find statistical differences in the number of affected vessels at baseline in responders patients compared to non-responder patients.

2.2. Dietary Intervention Modulates AGEs Levels and Gene Expression Related to AGE Metabolism

Dietary intervention reduced the amount of dAGEs after the consumption of both diets studied, as well as in both group of Responders and non-Responders (Supporting information Figures S1A and S1B, Supporting Information, respectively) (all $p < 0.05$). We also analyzed the effect of dietary intervention (Δchanges produced between post and pre-intervention) on serum levels of AGEs (MG and CML) and gene expression of the main AGE receptors involved in their metabolism, AGER1 and RAGE. The Mediterranean diet decreased serum levels of both MG and CML whilst the low-fat diet increased serum levels of MG but did not modify the levels of CML (Figure 1A,B, respectively). Regarding the expression of AGE receptors, Mediterranean diet increased AGER1 expression and diminished RAGE expression (all $p < 0.05$). Consumption of the low-fat diet had no effect on the expression of these genes (Figure 1C,D, respectively).

2.3. Mediterranean Diet Modulates AGEs Metabolism in Responders

The effect of each dietary intervention (Δchanges produced between post and pre-intervention) on AGE levels and gene expression related to AGE metabolism are shown in **Figure 2**. The Mediterranean diet produced a decrease in serum levels of MG in Responders but did not exert significant changes in the levels of this glycotoxin in non-Responders. However, the low-fat diet increased serum levels of MG, regardless of T2DM remission (all $p < 0.05$) (Figure 2A). In addition, Responders showed lower serum levels of CML after the Mediterranean diet compared to the low-fat diet ($p = 0.015$). We observed no changes in serum levels of CML in non-Responders (Figure 2B).

Regarding the gene expression of AGE metabolism, the Mediterranean diet increased AGER1 expression in non-Responders, but not in Responders although we observed a biological tendency ($p = 0.056$), and decreased RAGE expression in both non-Responders and Responders (Figure 2C,D, respectively). The low-fat diet did not produce any effect on AGER1 and RAGE expression as regards T2DM remission (Figure 2C,D). We observed no differences in the percentage of Responders and non-Responders after both diets, with 38.8% of Responders following the low-fat diet and 41.3% the Mediterranean diet (Chi-square value for comparison $p = 0.741$).

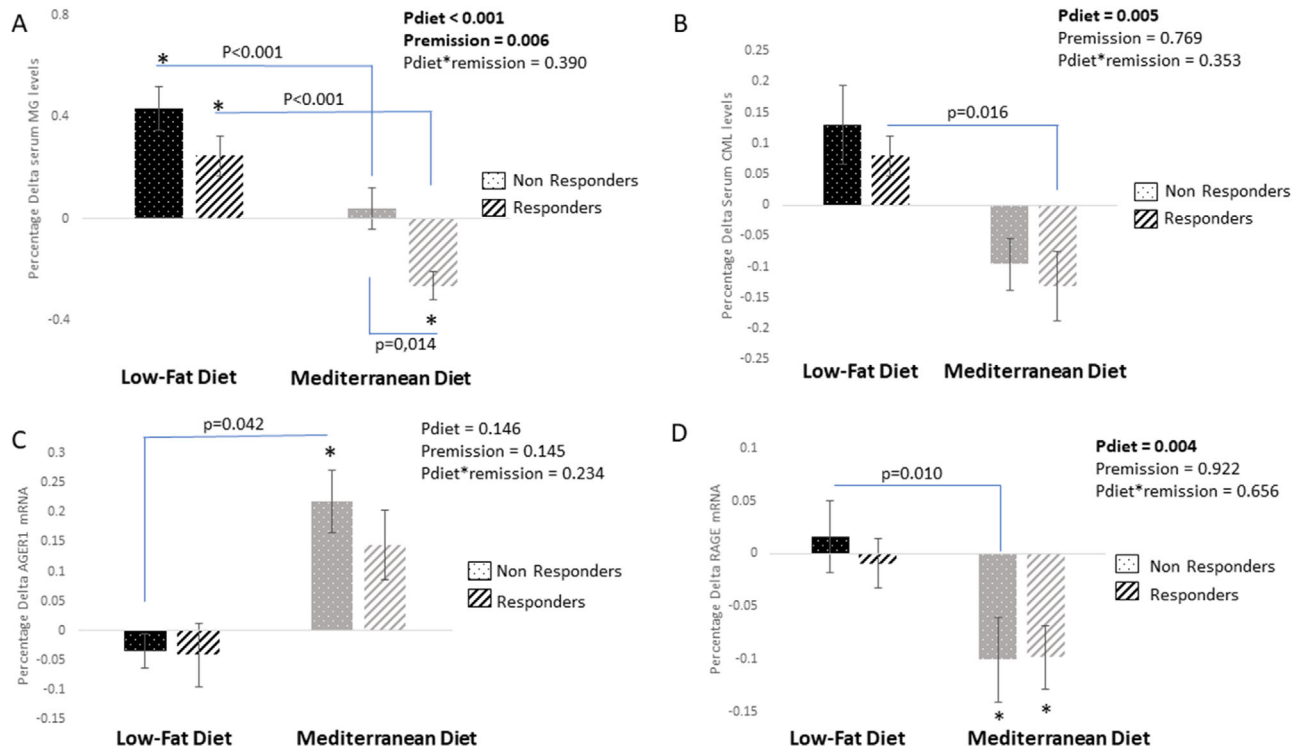

Figure 2. AGEs metabolism during dietary intervention comparing responder versus non responder patients. Data are shown as percentage Δ Change (changes produced between post and pre-intervention: as the value for five years, after dietary intervention, minus value at baseline divided by value at baseline). expressed by Mean ± S.E.M. of the serum levels of A) methylglyoxal (MG), B) carboximethyllysine (CML), C) gene expression of AGER1, and D) RAGE, in each of the diet studied. Univariate ANOVA was performed where p-values were adjusted by age, BMI, sex, triglycerides, HDL-cholesterol, smoking, alcohol consumption, and used of statins. Global *p*-values: *p*(diet): diet effect; *p*(remission): group of patient effect; *p*(diet*remission): diet by group interaction. * means $p < 0.05$ comparing post versus baseline. *p* values for comparisons between groups in the Post Hoc Bonferroni's multiple tests are included in the figures.

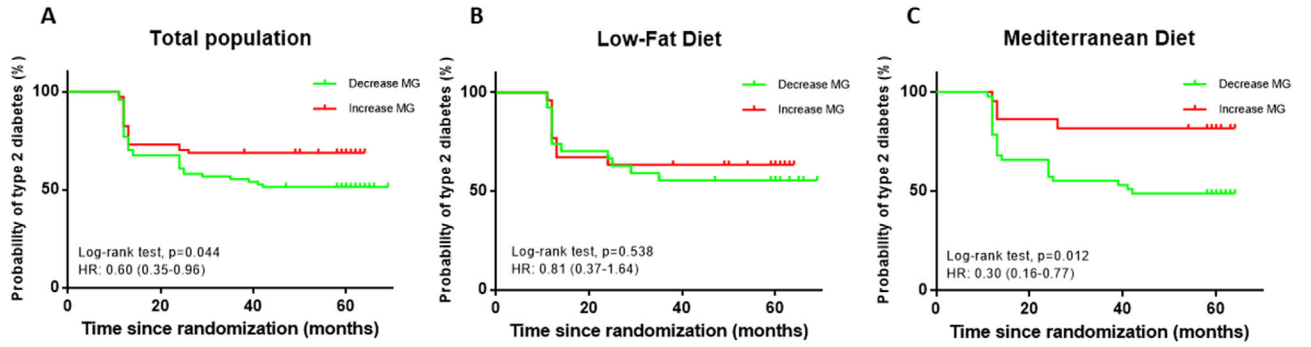

Figure 3. Probability of Type 2 diabetes. Probability of T2DM is represented for two groups of patients, those with percentage ΔMG (changes produced between post and pre-intervention: as the value for five years, after dietary intervention, minus value at baseline divided by value at baseline). A) Below the median and those above the median, for the entire population studied and B,C) separated by dietary intervention, Mediterranean diet and Low-Fat diet.

2.4. Molecular Mechanism Involved in T2DM Remission

Using a Kaplan–Meier survival curve, we estimated the probability of being T2DM depending on pΔMG during dietary intervention. To do that, patients were classified according to the median of pΔMG in our population (0.0706 mg mL^{-1}). Those patients who were under the median of pΔMG, decreased serum MG levels, whilst those who are up of the median of ΔMG, increased them. We observed that those patients who decrease their MG levels during the intervention had higher probability of T2DM remission than those who did increase them with Hazard ratio non-adjusted of HR:0.60 (0.35–0.96) ($p = 0.044$) (Figure 3A). When we performed these analyses divided by diet, we observed that those patients who followed a low-fat diet showed no differences in the probability of T2DM remission between ΔMG groups with a HR: 0.81 (0.37–1.64)

($p = 0.538$) (non-adjusted) (Figure 3B). However, only those who reduced serum MG after the consumption of Mediterranean diet had higher probability to T2DM remission than those who increased them with a HR:0.30 (0.16–0.77) ($p = 0.012$) (Figure 3C).

In order to identify significant factors associated with the time/group interaction of T2DM remission, we performed a COX proportional hazards models. The analysis showed that T2DM remission appears to be associated to changes in serum MG levels (pΔMG: changes produced between post and pre-intervention corrected by baseline values) after the consumption of a Mediterranean diet, where each standard deviation decrease in pΔMG has 2.54 more probability of disease remission compared with those who do not modify or increase their levels, with an adjusted HR:2.56 (1.02–6.25) and $*p = 0.046$. Indeed, decreasing observed in MG serum levels after the consumption of the Low-Fat diet showed an adjusted HR of 1.38 (0.67–2.86) and $*p = 0.381$ for diabetes remission. Moreover, COX proportional hazards models showed that T2DM remission was also associated to values of DI at baseline where each standard deviation increase of DI has 1.94 more probability of disease remission compare with those who do not modify or decrease its levels, with an adjusted HR:1.94 (1.32–2.87) and $p* = 0.001$.

2.5. Correlation between AGE Metabolism and Insulin Sensitivity and Beta Cell Functionality Indexes

In order to establish the relation between reducing the AGEs and glucose homeostasis involved in T2DM remission, measured by insulin sensitivity and beta cell functionality indexes (HOMA-IR, HIRI, IGI, ISI, DI, and MISI), we performed a correlation analysis between values of these parameters at five years of follow up and the values of percentage Δchanges in MG. In our whole population, we found no correlation between indexes and changes in MG. However, when we analyzed them separately, by diet, we found that pΔMG correlated negatively with DI values ($R = -0.277$ and $p = 0.032$) only after the consumption of the Mediterranean diet, but not after the low-fat diet, suggesting that the reduction in the MG content improved beta cell functionality in those patients who followed the Mediterranean diet.

3. Discussion

This study presents new findings about the involvement of AGE restriction in the molecular mechanisms underlying T2DM remission in newly diagnosed diabetic patients with CHD. Our data showed that only the long-term consumption of a Mediterranean diet significantly reduced serum levels of AGEs, particularly MG, and this was associated with a higher probability of T2DM remission. Decreased MG levels can be attributed to several factors, among them AGER1 activation. However, this activation was not associated specifically with diabetes remission. Nevertheless, in those who achieved a remission from T2DM after consumption of a low-fat diet, other mechanisms must be involved, since this diet did not reduce significantly the AGEs serum levels nor modify their metabolism.

Diabetes remission is a clinical fact that has been demonstrated by several relevant publications.[22–24] Most of these studies have been conducted in diabetic patients undergoing bariatric surgery for obesity treatment or after a very low-calorie dietary intervention, in which T2DM remission was associated with a substantial weight loss.[11,23,25] However, in long-term trials studying T2DM remission, the quality of the consumed diet, such as its fatty acid composition or antioxidants content, should also be taken into account.[26,27] It is worth noting that in our study, T2DM remission took place after the consumption of two healthy dietary models, a Mediterranean diet and a low-fat diet, without significant weight loss. To the best of our knowledge, our study represents an important breakthrough, since it partially explains the mechanisms involved in the remission of T2DM by the action of a dietary intervention without caloric restriction, such as the reduction of oxidant and inflammatory status measured by AGEs (an established biomarker of these situations)[28] and its association with beta cell pancreatic functionality.

Compelling evidence shows that oxidative stress plays a pivotal role in the pathophysiological processes behind T2DM and its complications,[29,30] mainly as a causal factor for beta cell dysfunction and insulin resistance, the two hallmarks of T2DM.[31] In this context, AGEs might contribute to T2DM development not only due to their oxidant properties per se, but also during their formation, since they unleash a cascade of oxidative damage and inflammatory response through the action of receptors such as nuclear factor kappa B (NFkB), Nuclear factor erythroid 2-related factor 2 (Nrf2), and more specifically, RAGE.[32]

Moreover, AGEs could affect the normal function of beta cells in the pancreas by inhibiting the production and secretion of insulin and, finally, by inducing a dysregulation of the glucose homeostasis (glycemic control) and the development of T2DM.[14,33]

Our study also showed that both dietary interventions reduced the oral intake of AGEs. However, we only observed a decrease in serum levels of MG and CML after the consumption of the Mediterranean diet, mainly in those who achieved a regression from T2DM. We hypothesized that this might be due to a better modulation of AGE metabolism by activation of AGER1, a receptor for AGEs that mediates the uptake and degradation of AGEs from cells and tissues, since, as shown in our results, the gene expression of this receptor increased after the intervention with the Mediterranean diet compared to the low-fat diet. Previous studies carried out by our group support the action of the Mediterranean diet on the activation of AGER1, which contributes to the clearance of AGEs.[20,21] Among other factors, this MG reduction observed after Mediterranean diet could be also related with lower amount of carbohydrates intake in this diet compare to the low-fat diet. Interestingly, the reduction in MG content was observed only in responders, due to the fact that those who remain diabetic have a worse glycaemic control, which, in turn, maintains the endogenous production of AGEs. In our opinion, the reduction on circulating MG content we observed only in responders could be explaining by the synergy of two main processes: Mediterranean diet induces a reduction in MG by triggering AGER1 and RAGE, this reduction could affect beta cell functionality,[34,35] which in fact was better from the beginning in responders. A better glucose control in these responders could help with the diminishing

of serum MG content. Even though non-responders decreased the dietary AGEs intake and triggered the AGER1 expression after the Mediterranean diet consumption, this group of patients did not reduce the MG serum levels. This fact might be due to that non-responders had worst beta cell functionality at the beginning of the study and consequently worst blood glucose control, and the beneficial effect of the Mediterranean diet could not counteract the in vivo production of MG. Moreover, the gene expression of RAGE, that binds AGEs and triggers the inflammatory and oxidative stress response, decreased after the consumption of the Mediterranean diet, whilst there were no changes in the gene expression of this receptor after the ingestion of the low-fat diet. However, we hypothesize that in all this physiological process might be involved the effect of the Mediterranean diet on the AGEs metabolism, otherwise, we would observe the same phenomenon also after the consumption of the Low-fat diet. It is possible that only those patients who have better beta cell functionality could get benefits on the consumption of the Mediterranean diet and the MG reduction triggered by this diet could be behind the physiological processes associated with the remission. The molecular mechanism behind the remission observed after the consumption of the low-fat diet in our population might be related to specific diabetes phenotypes in accordance with previous results from our groups, where we demonstrated that the consumption of low-fat diet might be more beneficial to patients with liver insulin resistance, whereas patients with muscle insulin resistance and muscle + liver insulin resistance might benefit more from a Mediterranean diet.[36]

We hypothesize therefore that reducing AGEs could be involved in the improvement of some of the hallmark signs of diabetes pathology. First, we have reported that reduction in MG serum content led to a higher probability of T2DM remission in those patients who followed a Mediterranean diet. Secondly, we have demonstrated that the remission of T2DM during the period of dietary intervention depended on the reduction in MG after adjustment with changes in weight, BMI, waist circumference, statins and other possible confounders. Finally, we have shown that a reduction in MG correlated with beta cell functionality, as measured by DI.

However, the limitations of the study must be mentioned. Firstly, this research is based on a long-term, well-controlled dietary intervention, which ensures the quality of the study but may not reflect the level of compliance in a free-living population. The second limitation is that T2DM remission was not the primary endpoint of the CORDIOPREV trial, although it was a secondary objective of this study. Therefore, our results may be taken with caution, and be supported by additional studies in which it would be the primary outcome before generalization to other populations. Finally, further molecular studies are needed to offer a complete explanation which join all the results showed in our study.

In conclusion, our study suggests that the reduction of serum levels of AGEs and the modulation of AGE metabolism, which occur after the consumption of a Mediterranean diet, might be involved in the molecular mechanism underlying the T2DM remission of newly diagnosed T2DM patients with CHD. Further studies are still needed to explore the mechanisms involved in T2DM remission after the consumption of a low-fat diet.

4. Experimental Section

Population: This work was carried out in the setting of the CORDIOPREV study (Clinical Trials Registry NCT00924937). The study protocol was designed in accordance with the principles of the Declaration of Helsinki, approved by the Human Investigation Review Committee of the Reina Sofia University Hospital, and followed institutional and Good Clinical Practice guidelines. The CORDIOPREV study was a prospective, randomized, controlled trial that includes 1002 CHD patients, who had their last coronary event more than six months before joining the study. Patients were recruited from November 2009 to February 2012, mostly at the Reina Sofia University Hospital (Cordoba, Spain). All patients gave written informed consent to participate in the study.

The inclusion and exclusion criteria was published previously.[37] To summarize briefly, the patients were eligible if they were aged 20–75 years, with established CHD but without clinical events in the last six months, with the intention of following a long-term monitoring study, with no other serious illnesses and a life expectancy of at least five years.

At the beginning of the study, short-duration diabetes was diagnosed, according to American Diabetes Association,[38] in patients who had not been diagnosed previously and were not receiving glucose-lowering treatment. These patients were included in the CORDIOPREV-DIRECT study (190 out of 1002 patients). Of these, seven patients were lost due to the inability to perform the diagnostic test used in this work; diabetes remission was evaluated in the remaining 183 patients during the five-year follow-up of each participant. Moreover, three participants died during the follow-up period without achieving diabetes remission.

In these patients ($n = 183$), T2DM remission was assessed every year during the follow-up period, and the group was subdivided into Responders ($n = 73$), whose T2DM was reversed after the dietary intervention without the use of diabetes medication to lower blood glucose levels, and non-Responders ($n = 110$), who did not respond to the dietary intervention and remained diabetic at the end of the follow-up period. These non-Responders patients were prescribed glucose lowering treatment when it was needed. Pharmacological treatment was prescribed by the primary care physicians or any other specialist who was not linked to the CORDIOPREV study, according to standardized recommendations by international guidelines.[39] None of the CORDIOPREV study researchers were involved in the decision to start glucose-lowering treatment in those patients.

Patients classified as Responder must have a T2DM remission, which was defined as achieving non-diabetic levels of hemoglobin A1c (HbA1c) $\leq 6.5\%$, fasting plasma glucose (FPG) ≤ 126 mg dL^{-1} and 2 h plasma glucose (2h-PG) of 75 gr in the oral glucose test tolerance (OGTT) ≤ 200 mg dL^{-1} and maintaining these levels for at least two consecutive years.[40]

Randomization and Dietary Intervention: Randomization was performed by the Andalusian School of Public Health, as previously described.[37] The study dietitians were the only members of the intervention team to know about the dietary group of each participant. Briefly, the randomization was based on the following variables: sex (male, female), age (<60 and ≥60 years old) and previous myocardial infarction (yes, no). Each patient was randomly stratified, in addition to the conventional treatment for CHD, to one of two potentially healthy diets: (I) the Mediterranean diet, with a minimum 35% of calories from fat [22% MUFA, 6% polyunsaturated (PUFA), and <10% saturated (SFA)], 15% proteins, and a maximum of 50% carbohydrates and (II) a low-fat, high-complex carbohydrate diet, as recommended by the National Cholesterol Education Program and the American Heart Association, with <30% total fat (12–14% MUFA, 6–8% PUFA and <10% SFA), 15% protein, and a minimum 55% carbohydrates. In both diets, the cholesterol content was adjusted to < 300 mg day^{-1}. Both study diets included foods from all major food groups, but no total calorie restriction was advised. Data on dietary assessment, follow-up visits as well as dietary adherence and long-term dietary adherence maintenance have been published recently.[37,41] No intervention to increase physical activity or lose weight was included.

Dietary AGE Intake: The assessment of dietary AGE (dAGE) content was performed using 3-day weighed food diaries completed by the participants at baseline and after every year during the dietary intervention study until the five years of follow-up has elapsed, with a strong emphasis

on cooking methods in both diets. dAGE content was estimated from a database of approximately 560 foods listing their AGE values[18] and was expressed as AGE kilounits g^{-1} food.

Laboratory Tests: At 8.00 am, following a 12-h fast, the patients were admitted to the laboratory for anthropometric and biochemical tests [BMI, waist circumference, systolic blood pressure, diastolic blood pressure, HDL-cholesterol, LDL-cholesterol, triglycerides, cholesterol, high sensitive C-reactive protein (hsCRP), glucose, HbA1c, and homeostasis model assessment of insulin resistance (HOMA-IR)]. The patients had refrained from smoking during the fasting period and abstained from alcohol intake for the past 7 days. Venous blood was sampled from the antecubital vein and collected in Vacutainer tubes with no anticoagulant and then put into tubes containing EDTA, which were immediately transferred to 4 °C. The plasma and serum samples were frozen at −80 °C for further biochemical analysis. The serum parameters were measured in Architect c-16000 analyzers (Abbott, Chicago, IL, USA) using spectrophotometric techniques (enzymatic colorimetric methods): the hexokinase method for glucose and oxidation–peroxidation for cholesterol; the triglycerides, LDL-cholesterol, and HDL-cholesterol levels were estimated using the Friedewald formula, based on cholesterol, triglycerides, and HDL-cholesterol concentrations. The plasma levels of insulin were measured by chemiluminescent microparticle immunoassay using an analyzer (i-2000Abbott Architect, Chicago, IL, USA). The plasma concentrations of hsCRP were determined by high sensitivity ELISA (BioCheck, Inc., Foster City, CA, USA). HOMA-IR was derived from fasting insulin ($\mu U\ L^{-1}$) × fasting glucose (μmoles L^{-1})/22.5.

The patients underwent a standard Matsuda test every year during the follow-up period. After an overnight fast, blood was sampled from a vein before an oral glucose intake (0 min) and again after a 75 g flavored glucose load (Trutol 75; Custom Laboratories, Baltimore, MD, USA). Blood samples were taken at 30, 60, 90, and 120 min to determine glucose and insulin concentrations.[42] The following indices were calculated at baseline and during every year of follow-up: hepatic insulin resistance index (HIRI); muscular insulin sensitivity index (MISI); insulin sensitivity index (ISI); insulinogenic index (IGI), and disposition index (DI), as described in previously published.[43]

Determination of Serum AGEs Levels: Serum and plasma samples were collected at baseline and after five years of follow-up of the dietary intervention and separated from whole blood by centrifugation at 1500 × *g* for 20 min at 20 °C and 1500 × *g* for 15 min at 4 °C, respectively, within 1 h of extraction.

Methylglyoxal (MG)[44] and *N*-carboxymethyllysine (CML) were the most well-studied AGEs and served as markers of AGE accumulation in several tissues.[45] MG and CML were measured in the serum using competitive ELISA kits (OxiSelect Methylglyoxal Competitive ELISA Kit and OxiSelect *N*-epsilon-(Carboxymethyl) Lysine Competitive ELISA Kit, Cell Biolabs, Inc., San Diego, CA, USA), following the manufacturer's instructions. These well-validated competitive ELISAs based on non-cross-reactive monoclonal antibodies (mabs) for protein-bound CML (4G9 mab) and protein-bound MG derivatives [lysine-MG-H1 (3D11 mab)], characterized by HPLC, and used as immunogens.[46,47] The resulting values reflect relatively stable protein- or peptide-associated CML and MG and not the free compounds. AGEs inter-assay coefficients of variation were 2.8% and 5.2% for CML and MG, respectively, and intra-assay coefficients of variation were 2.6% and 4.1% for CML and MG, respectively.[48]

Quantification of the Gene Expression Related to AGE Metabolism by Real-Time PCR: The blood was processed and peripheral blood mononuclear cells (PBMCs) were isolated. The PBMCs were isolated at baseline and after five years of follow-up of the dietary intervention. The total PBMC RNA was extracted using the Trizol method and quantified using a Nanodrop ND-1000 v3.5.2 spectrophotometer (Nanodrop Technology, Cambridge, UK). RNA integrity was verified on agarose gel electrophoresis and stored at −80 °C. The samples were digested with DNAse I (AMPD-1 KT, Sigma) before Real Time-PCR. The real-time PCR reactions were carried out using the Bio-Rad PCR platform, following the manufacturer's instructions. Each reaction was performed with 5 µL of a 1:5 (v/v) dilution of the first cDNA synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems) commercial kit, following the manufacturer's instructions. The relative expression for each analyzed gene was calculated, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GAPDH, Hs99999905_m1) as a housekeeping gene.

Statistical Analysis: The statistical analyses were carried out using SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA) and R software (version 3.5.0.; The R Foundation, Vienna, Austria) . Continuous data were compared by analysis of variance (ANOVA) for repeated measurements in which age, BMI, sex, triglycerides, HDL-cholesterol, smoking, alcohol consumption, and used of statins were included as covariates. Categorical variables were compared using Chi Square tests. Data are shown as mean ± standard errors. To evaluate the changes in time the percentage Δchanges (changes produced between post and pre-intervention: as the value after five years of dietary intervention, minus value at baseline divided by value at baseline) was calculated. The probability of T2DM remission was calculated using the Kaplan–Meier method of estimating the cumulative probability of an event in the group of those who decrease and increase serum MG after five years follow-up (defined to be below and upper the median of pΔMG, median = 0.0706 mg mL^{-1}). Cox proportional hazards model was specified to identify significant factors associated with the time of remission, the full model was implemented with the following variables: sex, age, pΔBMI, estatins, HDL-cholesterol, triglycerides, smoking, alcohol consumption, DI, diet/pΔMG interaction, and pΔCML and furthermore a variable selection is done in order to obtain the final model.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

F.M.G.-M., M.P.C., E.M.Y.-S., and J.L.-M. contributed equally to this work. F.M.G.-M. collected data, performed analysis, and wrote the manuscript. M.P.C. performed experimental procedure, collected data, and participated in the manuscript writing. S.d.l.C. performed experimental procedure, collected data and participated in the manuscript writing. J.F.A.-D. performed the clinical data from the patients. I.R.-R. collected

data. I.G. supervised and performed statistical analysis. C.V.-D. collected data. A.L.-M. collected data. R.M.L. reviewed the manuscript. J.D.-L. supervised the collected data collection and clinical parameters. P.P.-M. reviewed the manuscript and supervised the clinical study of the patients. E.M.Y.-S. supervised the collected data, reviewed the manuscript, and designed the study. J.L.-M. designed the study, supervised the clinical study and reviewed the manuscript.

Keywords

advanced glycation end products, Mediterranean diet, type 2 diabetes mellitus

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3. Remission Is Modulated by Branched Chain Amino Acids According to the Diet Consumed: From the CORDIOPREV Study.

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Diabetes Remission Is Modulated by Branched Chain Amino Acids According to the Diet Consumed: From the CORDIOPREV Study

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Scope: Branched Chain Amino Acids (BCAA) plasma levels may be differentially associated with type 2 diabetes mellitus (T2DM) remission through the consumption of the Mediterranean diet (Med) and a low-fat (LF) diet.

Methods: One hundred eighty-three newly diagnosed T2DM patients within the CORDIOPREV study are randomized to consume the Med or a LF diet. BCAA plasma levels (isoleucine, leucine, and valine) are measured at fasting and after 120 min of an oral glucose tolerance test (OGTT) at the baseline of the study and after 5 years of the dietary intervention.

Results: Isoleucine, leucine, and valine plasma levels after 120 min of an OGTT in the Med diet ($N = 80$) are associated by COX analysis with T2DM remission: HR per SD (95% CI): 0.53 (0.37–0.77), 0.75 (0.52–1.08), and 0.61 (0.45–0.82), respectively; no association is found in patients who consumed a LF diet ($N = 103$). BCAA plasma levels combined in a score show a HR of 3.33 (1.55–7.19) of T2DM remission for patients with a high score values in the Med diet, while in those with a LF diet, no association is found.

Conclusion: The study suggests that BCAA measurements potentially be used as a tool to select the most suitable diet to induce T2DM remission by nutritional strategies.

1. Introduction

Type 2 diabetes mellitus (T2DM) represents a serious health problem worldwide, with grave social and economic repercussions. Its mechanisms are not fully understood, but insulin resistance and beta-cell dysfunction are the two major pathophysiologic abnormalities, which underlie most cases of T2DM.[1]

Despite the clinical relevance, the current clinical goals for T2DM only include prevention or delay of complications,[2] rather than diabetes remission. Early T2DM has now been proved to be reversible,[3–5] using different strategies such as an intense weight loss by bariatric surgery,[6,7] very low-calorie[6]/low-carbohydrate diets,[8,9] or the Mediterranean (Med) dietary model[10] even without a calorie restriction.[11,12] These patients improved their hepatic insulin sensitivity and presented a recovery of beta-cell functionality[13] and significant HbA1c reductions.[8,14]

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Several studies have shown the relationship between the plasma levels of branched chain amino acids (BCAAs) and the incidence of diabetes.[15–17] In fact, high BCAA plasma levels have been associated with metabolic abnormalities such insulin resistance[18] and are currently considered as potential biomarkers for T2DM risk.[19,20] Moreover, high levels of plasma BCAAs have been associated with obesity, insulin resistance, impaired glucose tolerance and type 2 diabetes.[17,18,21,22]

Previous studies have shown that consumption of the Med diet reduced fasting plasma BCAA levels (valine, leucine, and isoleucine), which was negatively associated to cardiovascular disease (CVD)[23] and T2DM risk.[24] In fact, these studies found an association between high BCAA plasma levels and the development of these diseases when an olive oil-enriched Med diet was consumed, whereas no association was found when the participants either consumed the Med diet supplemented with nuts, or a control diet in which the participants were advised to reduce their intake of all types of fat.

Based on this, we hypothesized that BCAA plasma levels may be associated with T2DM remission in patients with coronary heart disease, and set out to discover whether this potential relationship was influenced by a specific dietary pattern. We therefore aimed to evaluate the relationship between BCAA plasma levels and the remission of T2DM in newly diagnosed T2DM patients after five years of the consumption of two healthy dietary patterns, a low-fat (LF) diet and the Med diet. Moreover, we tested the relationship between BCAA plasma levels at fasting state and after the performance of a dynamic test, an oral glucose tolerance test (OGTT), with the T2DM remission. To conclude, the potential translational value of this study lies in the fact that patients with acute myocardial infarction and T2DM have a higher risk of developing a new cardiovascular event than those without T2DM.[25]

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2. Experimental Section

2.1. Study Patients

The Coronary Diet Intervention with Olive Oil and Cardiovascular Prevention study (CORDIOPREV) is an ongoing prospective, randomized, open, controlled trial of 1002 patients receiving conventional treatment for coronary heart disease (CHD) who had their last coronary event over 6 months before enrolment in one of two different dietary models [a Med diet and a LF diet] over a period of 7 years. This clinical trial has been registered as legislation requires (Clinical Trials.gov.Identifier: NCT00924937). The eligibility criteria, design, and methods of the CORDIOPREV clinical trial have been reported elsewhere.[26] All patients gave their written informed consent to participate in the study and to the inclusion of material pertaining to themselves, that they acknowledge that they cannot be identified via the paper. The trial protocol and all amendments were approved by the Reina Sofia University Hospital Ethics Committee of Human Experimentation, following the Helsinki's Declaration and Rules of Good Clinical Practices. The experimental and clinical work conducted in this study has complied all mandatory health and safety procedures.

Newly diagnosed type 2 diabetes patients who had not been receiving glucose-lowering treatment at the beginning of the study were included in the CORDIOPREV-DIRECT study (190 out of 1002 patients). Of these, seven patients were lost due to the inability to perform the diagnostic test used in this work; diabetes remission was evaluated in the remaining 183 patients during the 5-year follow-up for each participant. Moreover, three participants died during the follow-up period without achieving diabetes remission. Thus, the remaining 183 newly diagnosed patients were classified as Responders, patients who reverted from type 2 diabetes during the 5 years of dietary intervention without the use of diabetes medication (n = 73); or Non-Responders, who did not achieve diabetes remission during the follow-up period (n = 110). Remission was defined as glycemia below the diabetic range for at least two consecutive years (HbA1c <6.5%, fasting plasma glucose <126 mg dL^{-1}, and 2 h plasma glucose in the OGTT <200 mg dL^{-1}) in absence of active pharmacological or surgical therapy according to the American Diabetes Association.[27] The baseline characteristics of the CORDIOPREV-DIRECT subjects are shown in **Table 1**.

2.2. Study Design

The study design has been previously described.[26] Briefly, participants were randomized to receive two diets: a Med diet or a LF diet. The LF diet consisted of <30% total fat [12–14% monounsaturated fatty-acid (MUFA), 6–8% polyunsaturated fatty-acid (PUFA), and <10% saturated fatty-acid (SFA)], 15% protein, and a minimum of 55% carbohydrates. The Med diet comprised a minimum 35% of calories as fat (22% MUFA, 6% PUFA, and <10% SFA), 15% proteins, and a maximum of 50% carbohydrates. In both diets, the cholesterol content was adjusted to <300 mg day^{-1}.

Table 1. Baseline anthropometric, clinical and metabolic characteristics according to T2DM remission and the diet consumed.

	LOW-FAT DIET			MEDITERRANEAN DIET		
	Non-Responders ($n = 63$)	Responders ($n = 40$)	p value	Non-Responders ($n = 47$)	Responders ($n = 33$)	p value
Male, n (%)	54 (85.7)	30 (75)	0.172	38 (80.9)	30 (90.9)	0.215
Age [years]	59.6 ± 1.2	60.0 ± 1.5	0.850	58.8 ± 1.4	61.9 ± 1.4	0.123
Weight [kg]	89.9 ± 1.7	78.7 ± 1.7	**<0.001**	87.6 ± 2.4	82.1 ± 2.0	0.103
Body mass index [kg m^{-2}]	31.9 ± 0.6	29.5 ± 0.6	**0.005**	32.3 ± 0.7	30.3 ± 0.7	0.057
Waist circumference [cm]	109 ± 1	100 ± 1	**<0.001**	107 ± 2	103 ± 2	0.086
Systolic blood pressure [mmHg]	141 ± 2	133 ± 3	**0.040**	134 ± 3	142 ± 4	0.080
Diastolic blood pressure [mmHg]	77.1 ± 1.4	75.1 ± 2.1	0.413	77.2 ± 1.4	78.4 ± 2.3	0.766
Glucose [mg dL^{-1}]	121 ± 4	99 ± 2	**<0.001**	115 ± 3	99 ± 3	**<0.001**
Insulin [mU L^{-1}]	12.4 ± 0.9	10.2 ± 1.1	0.131	14.8 ± 2.3	8.2 ± 1.2	**0.026**
HbA1c [%]	6.86 ± 0.13	6.43 ± 0.11	**0.023**	6.70 ± 0.08	6.65 ± 0.13	0.704
HDL-cholesterol [mg dL^{-1}]	40.5 ± 1.1	43.7 ± 2.1	0.147	41.1 ± 1.3	42 ± 1.5	0.627
LDL-cholesterol [mg dL^{-1}]	93.1 ± 3.3	87.9 ± 4.2	0.334	93.5 ± 4.4	90.6 ± 3.5	0.634
Triglycerides [mg dL^{-1}]	150 ± 9	147 ± 14	0.862	149 ± 9	112 ± 8	**0.005**
C-reactive protein [mg L^{-1}]	3.32 ± 0.48	4.64 ± 0.88	0.158	3.77 ± 0.60	2.99 ± 0.60	0.383

One-way ANOVA p-values ($p < 0.05$). Abbreviations: HDL-c, high-density lipoprotein; LDL-c, low-density lipoprotein. Differences in gender were tested by chi-square method. Statistically significant differences are in bold.

2.3. Dietary Assessment

Participants in both intervention groups received the same intensive and sustained dietary counseling during the whole period of the trial. At the beginning of the study and every 6 months, each patient had a face-to-face interview with a nutritionist to fill in a 137-item semi-quantitative food frequency questionnaire, validated in Spain.[28] The dietary evaluation was calculated by the validated 14-item Med Diet Adherence Screener (MEDAS), which was used for measuring adherence to the Med diet.[29] Moreover, a 9-item dietary adherence screener was used to measure adherence to the LF diet guidelines. A more detailed report on the dietary adherence has been published recently by the research group.[30] The Med and LF diet were designed to provide a wide variety of foods, including vegetables, fruit, cereals, potatoes, legumes, dairy products, meat, and fish. The participants in both intervention groups received the same intensive dietary counseling. The nutritionists administered personalized individual interviews at inclusion and every 6 months, and quarterly group education sessions were held with up to 20 participants per session and separate sessions for each group.

2.4. Clinical Plasma Parameters

Venous blood from the participants was collected in tubes containing EDTA after a 12-h overnight fast. Lipid variables, serum insulin, and plasma glucose were determined as previously reported.[31]

2.5. Methodology of the Two Metabolic Challenges

An OGTT was performed at the beginning of the study and every year. OGTT (75 g dextrose monohydrate in 250 mL water) was performed with 0, 30, 60, 90, and 120 min sampling to establish plasma glucose and insulin levels. OGTT-derived insulin sensitivity indexes (HOMA-IR, ISI, IGI, HIRI, MISI, and DI) were all calculated as previously described.[31] An oral fat tolerance test was performed at the beginning using a weight-adjusted meal (0.7 g fat and 5 mg cholesterol per kg body weight) with 12% SFA, 10% PUFA, 43% MUFA, 10% protein, and 25% carbohydrates. The meal composition was designed by a group of nutritionists with olive oil, skimmed milk, white bread, cooked egg yolks, and tomatoes. After the meal, the volunteers rested and did not consume food for 5 h but were allowed to drink water. Blood samples for biochemical testing were collected before the meal and every hour during the next 4 h, following recommendations for an oral fat tolerance test proposed by Mihas et al.[32]

2.6. Methodology of the BCAA Determination

2.6.1. Sample Preparation

Plasma samples (60 µL) were randomly deproteinized with 200 µL of 3:1 MeOH:ACN (*v/v*). The mixture was vortexed for 1 min and subsequently cooled at −20°C for 3 min. The resulting precipitate was separated by centrifugation at 14 000 × *g* for 15 min at 4°C and the supernatant phase was isolated. This phase was dried by evaporation. The residue was reconstituted with 20 µL of methoxyamine in pyridine (20 mg mL^{-1}) and maintained at 30°C for 90 min. Then, 180 µL of a 98:2 (*v/v*) BSTFA–TMCS mixture were added to the reconstituted analytical sample, which was shaken for 30 s and maintained at 37°C for 60 min. All samples were analyzed in triplicate.

2.6.2. GC-MS Analysis

GC–TOF/MS analyses were performed by EI ionization mode at 70 eV. Chromatographic separation was carried out with a

fused silica DB-5MS-UI (30 m × 0.25 mm i.d., × 0.25 μm film thickness) capillary column. The gas chromatography (GC) oven temperature program started at 60°C (1 min held), followed by a temperature ramp of 10°C min^{-1} to final 300°C (2 min held). Post-run time was programmed for 4 min up to 310°C to assure complete elution of the injected sample. Pulsed splitless injections of 1 μL of sample were carried out at 250°C and ultrapure grade helium was used as carrier gas at 1.0 mL min^{-1} flow rate. The injector needle was washed five times among injections with n-hexane and acetone to avoid contamination. The interface, ion source and quadrupole temperatures were set at 300°C, 300°C, and 200°C, respectively. A solvent delay of 5 min was used to prevent damage in the ion source filament. The TOF detector was operated at 5 spectra s^{-1} in the mass range m/z 50–550 and data were acquired in centroid mode. According to the manufacturer, daily mass calibration was performed with PFTBA to ensure mass accuracy and the resolution was 8500 full width half maximum (FWHM) at m/z 501.9706.

2.6.3. Data Treatment and Identification of Metabolites

Unknown Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to unzip all data files obtained by GC–TOF/MS in full scan mode. Then, MassHunter software was used to process GC–TOF/MS data files. Treatment of raw data files started by deconvolution of chromatograms to obtain a list of MFs considered as potential compounds defined by all m/z values with a common peak profile and its RT. For this purpose, the deconvolution algorithm was applied to each sample by considering all ions exceeding 1500 counts for the absolute height parameter, the accuracy error at 50 ppm and the window size factor at 150 units. The list of MFs obtained for each analysis was exported as data files in compound exchange format (.cef files). Tentative identification of compounds was performed by searching each mass spectrum in the NIST database (version 11) and also using the RI value. The identification of branched amino acids was firstly carried out by searching MS spectra on the NIST database and confirmed with standards. A table with the peak area values of branched amino acids in the samples was obtained as a result. Once the data set was extracted from the raw data files, data normalization was performed. This normalization was based on mass spectrometry total useful signal (MSTUS) method and attempts to limit the contributions of xenobiotics and endogenous substances to the normalization factor by including only peaks present in all samples.

2.7. Statistics

SPSS statistical software (IBM SPSS Statistics version 21.0) and R software (version 3·5·0.; The R Foundation, Vienna, Austria) were used for statistical analysis of data. The normal distribution of variables was assessed using the Kolmogorov–Smirnov test. The statistical differences in the metabolic variables between groups were evaluated by one-way ANOVA. Qualitative variables were compared using the Chi-square test. A repeated-measures ANOVA test was used to determine the statistical differences between variables at baseline and during the follow-up period. A Cox proportional hazards regression analysis was performed to measure the probability of diabetes remission according to BCAA individually or combined as a score and adjusted by age, gender, diet, insulin, body mass index, triglycerides, HDL, and treatment (according to dose) with statins. The data are represented as the mean ± SEM for continuous variables and as frequencies for categorical variables. p values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Baseline Characteristics of the Participants

We found no significant differences in the anthropometrics or biochemical baseline characteristics between the patients assigned to each diet (Table 1, Supporting Information). When we compared the Responders and Non-Responders groups (Table 2, Supporting Information), we observed that the Responders group had lower BMI, waist circumference, weight and HbA1c, glucose, and insulin levels than the Non-Responders group. Similar differences were found between the Responders and Non-Responders assigned to each diet (Table 1).

3.2. BCAA Plasma Levels According to T2DM Remission is Different Between Diets

We found lower isoleucine, leucine, and valine plasma levels in the OGTT (fasting and 120 min plasma levels) performed at the beginning of the study in Responders than in Non-Responders assigned to the Med diet, while we observed no such differences in Responders versus Non-Responders assigned to the LF diet (**Figure 1**). The same profile was found after 3 years of dietary intervention (Figure 1, Supporting Information). In fact, no significant changes were observed for any of the BCAA (isoleucine, leucine, and valine) after the consumption for 3 years of the LF or Med diets in the Responders and Non-Responders groups (Figure 2A and 2B, Supporting Information).

3.3. The Probability of T2DM Remission According to BCAA Depends on Diet

We tested the potential association of each BCAA plasma level at 120 min in the OGTT (**Table 2**) with the probability of T2DM remission, using Cox proportional hazards regression analysis. We found an HR per SD (95% CI) of 0.70 (0.56–0.89), 0.81 (0.64–1.03), and 0.74 (0.59–0.91) for isoleucine, leucine, and valine, respectively, with an HR (95% CI) of 0.71 (0.56–0.91), 0.80 (0.62–1.02), and 0.77 (0.62–0.96) after adjustment by covariates (age, gender, diet intensity of statins consumption, insulin, triglycerides, HDL-c, and BMI) when we included the whole population.

In contrast, we found no associations when we included the group of patients who consumed the LF diet in the Cox analysis. However, in patients who consumed the Med diet, we found an HR per SD (95% CI) of 0.53 (0.37–0.77), 0.75 (0.52–1.08), and 0.61 (0.45–0.82) for isoleucine, leucine, and valine, respectively, with an HR of 0.49 (95% CI 0.32–0.75), 0.65 (0.44–0.97), and 0.61 (0.44–0.85) after adjustment by clinical variables (Table 2).

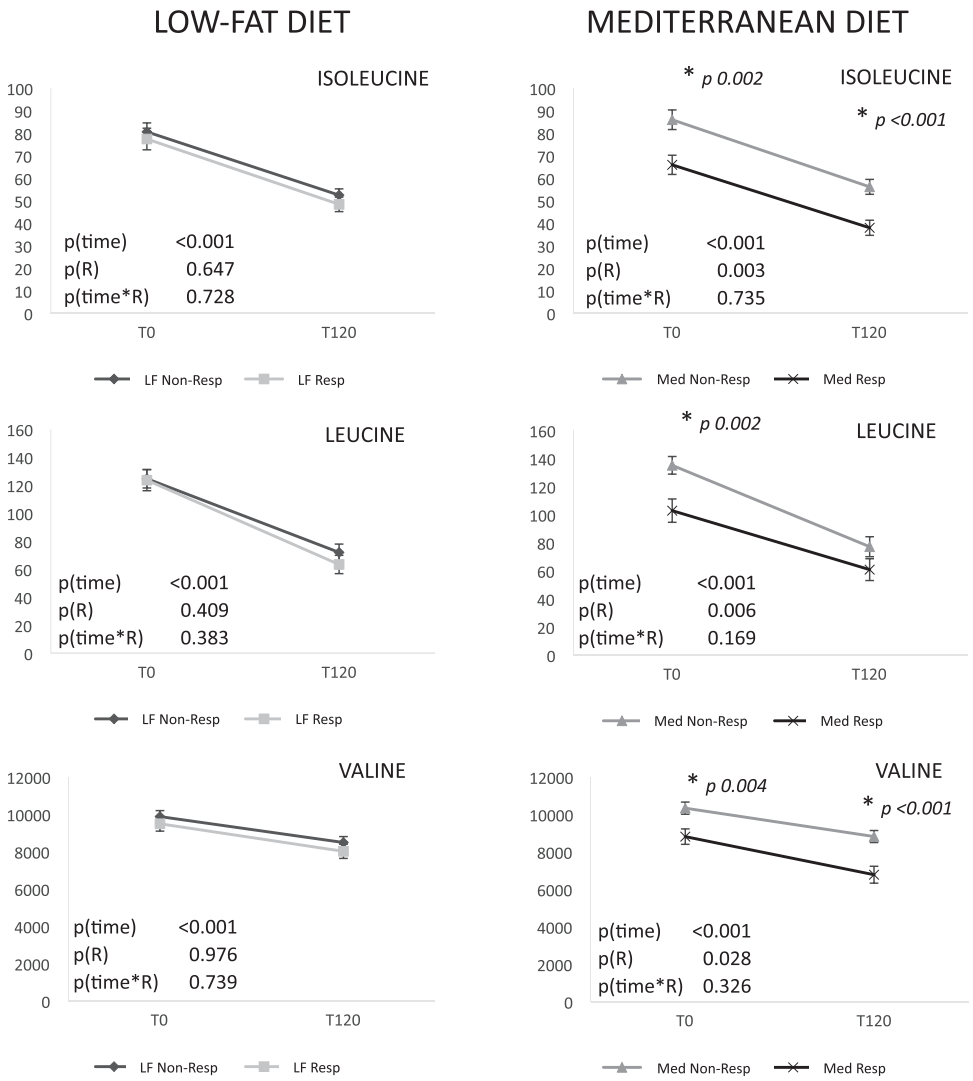

Figure 1. Baseline BCAA plasma levels in arbitrary units according to T2DM remission in the OGTT for each diet consumed. T0: fasting levels, T120: 120 min after the glucose intake. Non-Resp: Non-Responders patients. Resp: Responders patients. ANOVA for repeated measures *p*-values: p(time): OGTT time effect; p(R): remission effect; p(time*R): OGTT time by remission interaction. * $p < 0.05$ between groups in the Post Hoc Bonferroni's multiple comparison tests.

Table 2. Association of individual baseline BCAA plasma levels at 120 min in the OGTT with T2DM remission by COX regression analysis per standard deviation (SD).

		Isoleucine HR (95% CI)	Leucine HR (95% CI)	Valine HR (95% CI)
WHOLE POPULATION	*Unadjusted*	0.704 (0.555-0.894)	0.812 (0.639-1.03)	0.736 (0.594-0.913)
	*Adjusted**	0.708 (0.556-0.902)	0.796 (0.619-1.023)	0.768 (0.618-0.954)
LOW-FAT DIET	*Unadjusted*	0.880 (0.640-1.210)	0.865 (0.628-1.192)	0.887 (0.654-1.202)
	*Adjusted**	0.954 (0.686-1.327)	0.952 (0.682-1.328)	0.932 (0.678-1.281)
MEDITERRANEAN DIET	*Unadjusted*	0.531 (0.365-0.774)	0.751 (0.524-1.076)	0.611 (0.454-0.822)
	*Adjusted**	0.494 (0.326-0.748)	0.650 (0.435-0.972)	0.609 (0.436-0.851)

The analysis was adjusted by age, gender, intensity of statins treatment, insulin, HDL-c (high density lipoproteins cholesterol), and triglycerides plasma levels. HR: hazard ratio. CI: confidence interval.

Table 3. Association between diabetes remission and BCAA-based score.

			HR	(95% CI) for HR	
				Lower	Higher
WHOLE POPULATION	*Low score (ref.)*		1	1	1
	High score	*Unadjusted*	1.907	1.185	3.069
		*Adjusted**	1.822	1.121	2.963
LOW-FAT DIET	*Low score (ref.)*		1	1	1
	High score	*Unadjusted*	1.277	0.685	2.380
		*Adjusted**	1.060	0.559	2.009
MEDITERRANEAN DIET	*Low score (ref.)*		1	1	1
	High score	*Unadjusted*	3.333	1.545	7.190
		*Adjusted**	3.133	1.385	7.087

The score was built with the BCAA plasma levels in the OGTT (120 min) score in the whole population and by diet separately. A Cox regression analysis were performed with patients classified by medians of a score for each population. HR: hazard ratio. CI: confidence interval. High score values represent low BCAA levels taking into account the negative value of the COX coefficients of BCAA in both analyses, unadjusted and adjusted by age, gender, diet, intensity of statins consumption, insulin, triglycerides, HDL-c, and BMI.

3.4. A BCAA-Profile Associated to T2DM Remission by Med Diet Consumption

In order to assess the relationship between plasma BCAA levels at 120 min OGTT and T2DM remission, we built a BCAA score for each patient by multiplying the coefficients of a BCAA, obtained in the previous COX analysis and then adding the products obtained for each patient (Supplementary Materials and Methods, Supporting Information). Thus, high score values represent low BCAA levels, taking into account the negative value of the BCAA coefficients obtained in the previous COX analysis.

To assess the potential association between BCAA-based scores and the probability of T2DM remission, we performed a Cox regression analysis with patients classified by median scores (**Table 3**). Considering the whole population, patients with a high score (lower BCAA levels) presented 1.91 (1.19–3.07) a higher probability of T2DM remission than those with a low score (higher BCAA levels), 1.82 (1.12–2.96) when adjusted by covariates. Regarding the diets administered, the Med diet group with a high score (lower BCAA levels) presented 3.33 (1.55–7.19) a higher probability of T2DM remission than those with a low score, and a HR of 3.13 (95% CI 1.39–7.09) when adjusted by covariates, whereas no association was found in the LF diet group.

3.5. BCAA-Profile is Related with Insulin Resistance and Beta-Cell Functionality According to the Diet Consumed

In addition, we evaluated changes in the insulin resistance and beta-cell functionality indexes derived from OGTT according to BCAA levels and the diet consumed (**Figure 2**). We found an increase in the adipose and hepatic insulin resistance indexes in patients with high BCAA plasma levels ($p = 0.031$ and $p = 0.019$, respectively), whereas no changes were observed in patients with low BCAA levels consuming the Med diet. In contrast, we found an increase in hepatic and muscle insulin resistance indexes in patients with low BCAA levels ($p = 0.033$ and $p = 0.022$, respectively), whereas no changes were observed in patients with high BCAA plasma levels when the LF diet was consumed. In terms of beta-cell functionality, we found that both diets increased the DI in patients with low BCAA plasma levels ($p = 0.012$), while in patients with high BCAA plasma levels, the consumption of the Med diet decreased the DI compared with the consumption of the LF diet ($p = 0.032$), while no statistically significant changes were observed in the consumption of either of the diets in patients with low BCAA plasma levels.

4. Discussion

Our study showed that baseline BCAA plasma levels were negatively associated with T2DM remission in the group of patients who consumed the Med diet, whereas no association was found in the group of patients who consumed the LF diet. Moreover, the association between BCAA levels and T2DM in the Med diet group, individually or combined as a score, was stronger after the dynamic test from the BCAA plasma levels at 120 min after an OGTT than using fasting BCAA plasma levels. In addition, baseline plasma levels of BCAA were able to discern which dietary model, LF or Med diet, was more suitable for inducing T2DM remission.

Several studies have showed the relationship between BCAA plasma levels and the incidence of diabetes.[15–17] In fact, high BCAA plasma levels have been associated to metabolic abnormalities such as insulin resistance[18] by promoting an activation of the mTOR/S6K1 kinase pathway and phosphorylation of IRS1 on multiple serines, leading to incomplete fatty acid oxidation at the mitochondria[33] and the expression of several genes related to BCAA catabolism.[34] Moreover, increased BCAA catabolic flux may contribute to increased gluconeogenesis and glucose intolerance via glutamate transamination to alanine. In addition, T2DM incidence has been associated with BCAA by an overstimulation of beta cell secretion, not only by those amino acids but also by serum lipids, which contribute as secretagogues. This leads to endoplasmic reticulum stress,[35] which may ultimately contribute to beta cell dysfunction and subsequent impairment of glucose-stimulated insulin secretion (GSIS).[36]

Previous studies have shown that the consumption of Med diet reduced fasting plasma levels of BCAAs (valine, leucine, and isoleucine), which was negatively associated to CVD[23] and T2DM risk.[24] Our study showed a reduction in BCAA plasma levels after the glucose intake in the OGTT performed at baseline and after 3 years of dietary intervention, but no changes either in fasting levels or in the levels after glucose intake were observed during these years after the consumption of the Med and the LF diets, presumably due to the fact that our study included T2DM patients with CHD, whose BCAA plasma levels had been found to be abnormally high.[23]

Our study was performed in a population of newly diagnosed diabetic patients with coronary heart disease, which consumed a LF or the Med diet, without differences in the remission rate between diets. However, our results showed that baseline BCAA plasma levels, individually and combined, were associated with T2DM remission in those newly diagnosed diabetic patients who consumed the Med diet, whereas we observed no such association in those who consumed the LF diet. In fact, our results

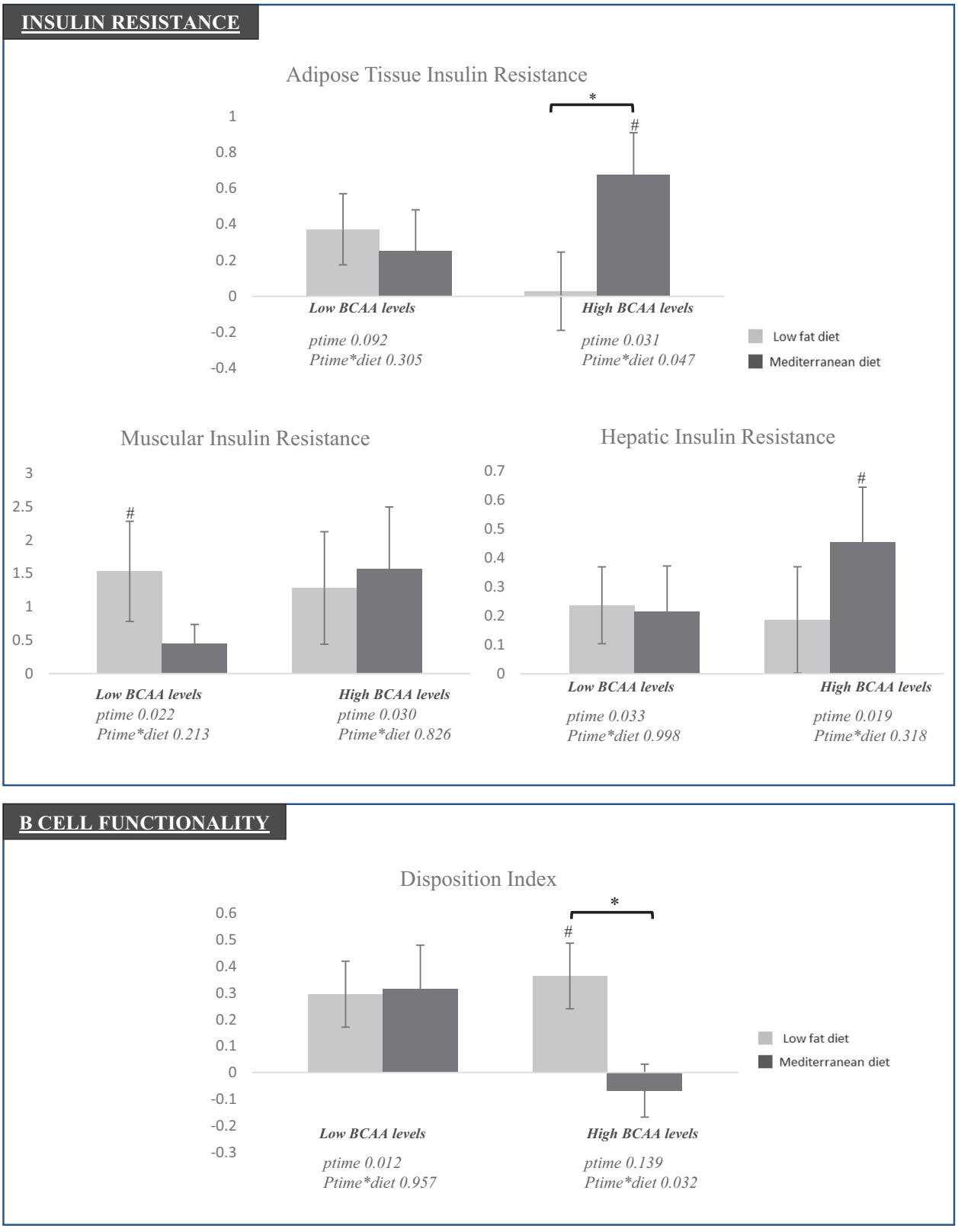

Figure 2. Percentage of change in beta-cell function and insulin resistance assessing indexes after 5 years of dietary intervention according to BCAA-based score median. Mean and standard error of adipose tissue insulin resistance index, muscular insulin resistance index, hepatic insulin resistance index and disposition index. ANOVA for repeated measures *p*-values. $^{\#}$ $p < 0.05$ compared with baseline condition in the post hoc Bonferroni's multiple comparison test. * $p < 0.05$ between diet groups in the post hoc Bonferroni's multiple comparison test.

showed that the probability of remission score, built combining the three BCAA plasma levels in addition to each BCAA individually, was negatively associated by COX regression analysis with T2DM remission in Med diet, whereas no such association was found for the LF diet. In fact, high scores, characterized by low BCAA levels, taking into account the negative value of the BCAA coefficients obtained in the previous COX analysis, were associated to a high probability of T2DM remission. Our results are consistent with previous studies in which an association was found between BCAAs, T2DM incidence[24] and cardiovascular disease[23] when an olive oil-enriched Med diet was consumed, while no association was found when participants consumed the Med diet supplemented with nuts or a control diet in which participants were advised to reduce the intake of all types of fat.

Although observational studies have shown the relationship between high BCAA levels and an impairment of glucose homeostasis,[15–17] little has been written on the potential mechanisms linking BCAA and dietary patterns. However, it seems that high circulating concentrations of BCAAs may be explained by an obesity-related catabolism in adipose tissue and a disruption in liver and skeletal muscle signaling.[37]

Some studies suggest that the deleterious effect of BCAAs on insulin sensitivity are enhanced when the fat content in the diet is high,[37] a condition in which their action as secretagogues that potentiates GSIS,[38] together with the pancreatic accumulation of fatty acid, promotes the dysfunction of pancreatic islets and hence beta-cell dysfunction.[36,39–42] Taking into account this effect, we hypothesized that the higher fat content in the Med diet compared with the LF diet may be responsible for the lower probability of remission in patients with high BCAA levels despite the beneficial effects of a Mediterranean-style diet on obesity,[43] diabetes,[44] and cardiovascular risk factors,[45,46] and, more specifically, the potential protective effect of extra-virgin olive oil.[47–49] However, low BCAA levels were associated to a higher probability of T2DM remission when a Med diet was consumed, and the consumption of the LF diet did not discriminate between high and low BCAA levels.

Taken together, our results suggest that BCAA levels are a pathophysiological key for T2DM remission and could be a crucial element in dietary pattern recommendations. BCAA levels may therefore be determinant in the design of nutritional strategies to induce T2DM remission. In fact, our study showed that patients with low BCAAs have a higher probability of T2DM remission than patients with high BCAA plasma levels who consumed the Med diet and patients with low BCAAs who consumed the LF diet. In this context, the consumption of the Med diet should be recommended to those patients with low BCAA levels, while those with higher BCAA levels would benefit more from adherence to the LF diet, which could reduce the associated insulin resistance and improve beta-cell functionality.[37] This idea is supported by the fact that consumption of the Med diet increased ATI and HIRI in patients with high BCAA levels, whereas consumption of the LF diet increased adipose tissue insulin resistance index (ATI) and muscular insulin resistance index (MISI) in patients with low BCAA levels but increased disposition index (DI) in patients with high BCAA levels.

Certain limitations of the current study must be mentioned. One limitation lies in the fact that this research is based on a long-term, well-controlled dietary intervention, which ensures the quality of the study but may not reflect the level of compliance in a free-living population. Another limitation lies in the fact that the remission of T2DM was not the primary endpoint of the CORDIOPREV trial, but was rather a secondary analysis conducted in the subgroup of newly diagnosed diabetic patients with CVD, with a sample size that did not allow us to splits data in training and test sets to validate the results. Moreover, our findings are limited to patients with CVD and precludes its generalization to healthy individuals.

In conclusion, our results imply that the dietary pattern is crucial in determining the role of BCAAs in T2DM remission. Our study also suggests that the differential relationship found between BCAA levels and T2DM remission according to the diet consumed, may potentially be used as a tool to select the most suitable dietary recommendations to induce T2DM remission by nutritional strategies. As a result, diabetes remission should definitely be the first therapeutic goal for recent-diagnosed short-duration T2DM patients, and it may be enhanced by a BCAA study at baseline, to help to provide better dietary counseling.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.P.C. and J.F.A.-D. contributed equally to this work. A.C. and J.L.-M. equally contributing senior authors. M.P.C. and J.F.A-D. wrote the draft manuscript. M.P.C., J.F.A-D., F.M.G-M., and J.L.-M. collected data and performed the classification of participants M.P.C., J.F.A-D., F.M.G-M., A.VG., A.P.A-L. performed the experiments. M.P.C., J.F.A-D., S.C-A., J.D-L., F.R-C., and R.M.L. performed the medical revisions of participants and clinical databases, and performed the statistical analysis. M.P.C. and J.F.A-D.,

J.M.O., A.C., and J.L.-M. interpreted the data and contributed to the discussion. A.C., J.M.O., and J.L.-M. contributed to the writing of the manuscript and revised it critically for important intellectual content. J.L.-M. and A.C. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

branched chain amino acids, CORDIOPREV study, Mediterranean diet, type 2 diabetes remission

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XIV. CONCLUSION

V. CONCLUSION

Patients with established T2DM exhibited higher baseline levels of AGEs and increased IMT-CC, particularly in those with endothelial dysfunction, compared with newly diagnosed T2DM patients. On the other hand, newly diagnosed T2DM patients who achieved T2DM remission were those who presented lower baseline levels of BCAA or a reduction in circulating AGEs levels and a modulation of AGEs metabolism after consumption of the Mediterranean diet. These findings were not observed after consumption of a low-fat diet. Our results suggests that the differential relationship found between AGE metabolism and BCAA levels and T2DM remission, according to the dietary consumed, may potentially be used as a tool to select the most suitable dietary recommendations to induce T2DM remission, and also reduce cardiovascular complications, by nutritional strategies in newly diagnosed T2DM patients with coronary heart disease (CHD).

This general conclusion is divided into 3 conclusions that concur with the 3 papers discussed on this doctoral thesis.

Conclusion 1. Although FMD (an early subclinical atherosclerosis marker) did not differ between both study groups, IMT-CC (a subclinical atherosclerotic marker related to vascular damage) was increased in patients with established vs. newly diagnosed T2DM. Moreover, when the presence of severe endothelial dysfunction was considered, patients with established T2DM exhibited the highest serum methylglyoxal levels (a main intermediate form of AGEs) among all groups. (**Paper 1**)

***Conclusion 2*.** The reduction of serum levels of AGEs and the modulation of AGE metabolism, which occur after the consumption of a Mediterranean diet, are associated with T2DM remission in newly diagnosed T2DM patients with CHD. Further studies are still needed to explore the mechanisms involved in T2DM remission after the consumption of a low-fat diet. (**Paper 2**)

***Conclusion 3*.** The Mediterranean diet, compared to a low-fat diet, is more suitable to benefit newly diagnosed T2DM patients with low BCAA levels in improved insulin resistance and b-cell functionality. The differential relationship found between BCAA levels and T2DM remission according to the diet consumed may potentially be used as a tool to select the most suitable dietary recommendations to induce T2DM remission by nutritional strategies. (**Paper 3**)

XV. REFERENCES

VI. REFERENCES

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TÍTULO DE LA TESIS: ESTRATEGIAS DIETÉTICAS EN EL MANEJO DE LA DIABETES MELLITUS TIPO 2 EN PACIENTES CON ENFERMEDAD CORONARIA ESTABLECIDA: ESTUDIO CORDIOPREV

DOCTORANDO/A: M MAGDALENA PEREZ CARDELO

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda M Magdalena Pérez Cardelo ha desarrollado el plan de investigación y de formación de manera adecuada. Los objetivos planteados se han desarrollado de manera íntegra, evaluando cómo distintas estrategias dietéticas a largo plazo favorecen el manejo de la diabetes mellitus tipo 2 en función de la situación metabólica de estos pacientes, evaluada mediante la determinación de nuevos marcadores metabólicos y cómo éstos pueden ser modificados a través de la dieta.

En relación a la actividad investigadora derivada del proyecto de tesis, destacar que la doctoranda ha publicado tres artículos de investigación como primera autora en revistas de alto impacto (primer cuartil –JCR de su categoría) (**1**. de la Cruz-Ares S*, **Cardelo MP***, et al., ***Nutrients***. 2020 Jan 16;12(1):238. doi: 10.3390/nu12010238; **2**. Gutierrez-Mariscal FM*, **Cardelo MP***, et al., ***Mol Nutr Food Res***. 2021 Jan;65(1):e1901290. doi: 10.1002/mnfr.201901290; **3**. **Cardelo MP**, et al., Mol Nutr Food Res. 2022 Feb;66(4):e2100652. doi: 10.1002/mnfr.202100652). Así mismo, la doctoranda ha completado satisfactoriamente las diferentes actividades propuestas en el plan de formación, incluyendo la asistencia y participación en las Jornadas de Jóvenes Investigadores organizadas por el IMIBIC; asistencia a diversos cursos de formación relacionados con su área de investigación así como a diferentes congresos regionales y nacionales e internacionales. Así mismo ha realizado una estancia predoctoral en un grupo de investigación internacional (Medicina Comparativa de la Universidad de Yale, Estados Unidos, con una duración de 10.5 meses). Todas las actividades se han justificado debidamente con documentos acreditativos. Por todo ello, se autoriza la presentación de la tesis doctoral.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 15 de Febrero de 2023

Firma del/de los director/es

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