The gut microbiota pattern predictive for development of Metabolic Syndrome and dietary modulation

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THE GUT MICROBIOTA PATTERN PREDICTIVE FOR DEVELOPMENT OF METABOLIC SYNDROME AND DIETARY MODULATION

Trabajo presentado por

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Doctorado Internacional

Dirigido por

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TÍTULO DE LA TESIS: IDENTIFICACION DEL PATRON DE MICROBIOTA INTESTINAL PARA LA PREDICCION DEL DESARROLLO DE SINDROME METABÓLICO Y SU MODULACION POR LA DIETA

DOCTORANDO/A: Sonia García-Carpintero Fernández-Pacheco

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS: El trabajo de tesis realizado por Sonia García Carpintero Fernández Pacheco, bajo nuestra dirección en la Unidad de Lípidos y Arteriosclerosis del Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC/Hospital Universitario Reina Sofía de Córdoba/Universidad de Córdoba), se ha basado en la demostración de un patrón de microbiota intestinal capaz de predecir el desarrollo de Síndrome Metabólico en una subpoblación de pacientes con enfermedad coronaria del estudio CORDIOPREV. Los resultados obtenidos corresponden con los objetivos planteados inicialmente. En cuanto a la difusión de resultados de la tesis, la doctoranda ha participado 10 congresos científicos, presentando comunicaciones orales y de tipo póster. Así mismo dichos resultados ya se han publicado o están en vías de publicación en colaboración con sus tutores y otros colaboradores de nuestro grupo de investigación.

La publicación derivada de esta tesis es:

- Carmen Haro*, **Sonia García-Carpintero***, Oriol A. Rangel-Zúñiga, Juan F. Alcalá-Díaz, Blanca B. Landa, José C. Clemente, Pablo Pérez-Martínez, José López-Miranda, Francisco Pérez-Jiménez, Antonio Camargo. *Consumption of two healthy dietary patterns restored microbiota dysbiosis in obese patients with metabolic dysfunction.* Mol. Nutr. Food Res. 2017 sep. *These authors contributed equally to this work.

A nuestro juicio, el trabajo realizado por la doctoranda Sonia García Carpintero Fernández Pacheco reúne los méritos suficientes para ser defendido ante el tribunal correspondiente y poder optar al grado de Doctor. Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 3 de Noviembre de 2017

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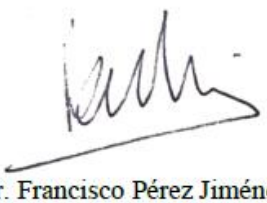

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"El conocimiento no es una vasija que se llena, sino un fuego que se enciende".
Plutarco

"Investigar es ver lo que todo el mundo ha visto, y pensar lo que nadie más ha pensado".
Albert Szent-Györgyi

"En algún lugar, algo increíble está esperando ser conocido". *Carl Sagan*

"La ciencia es el alma de la prosperidad de las naciones y la fuente de vida de todo progreso".
Louis Pasteur.

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ABBREVIATIONS

The most used abbreviations throughout the text are outlined below:

AACE: American Association of Clinical Endocrinologists

ADA: American Diabetes Association

AHA: American Heart Association

AMPK: Adenosine Monophosphate-activated Protein Kinase

ApoB: Apolipoprotein B

AT: Adipose Tissue

ATP III: Adult Treatment Panel III

BMI: Body Mass Index

CBR-1: Cannobinoid Receptor 1

CD: Crohn´s Disease

CETP: Cholesterol Ester Transport Protein

CFU: Colony Forming Units

CHD: Coronary Heart Disease

CRC: Colorectal Cancer

CVD: Cardiovascular Disease

DBP: Diastolic Blood Preassure

EGIR: European Group for the study of Insulin Resistance

FDR: False Discovery Rate

FFA: Free Fatty Acids

FIAF: Fasting –induced Adipocyte Factor

GALT: Gut –associated Lymphoid Tissue

GC-MS: Gas Chromatography –Mass Spectoscopy

GLP-1: Glucagon like Peptide 1

GPR41: G Protein coupled receptor 41

GPR43: G Protein coupled receptor 43

GIT: Gastrointestinal Tract

HDL (-c): Hight Density Lipoprotein (-cholesterol)

HPLC: High Performance Liquid Chromatography

IBD: Inflammatory Bowel Disease

IBS: Irritable Bowel Syndrome

IDF: International Diabetes Federation

IFG: Impaired Fasting Glucose

IGT: Impaired Glucose Tolerance

IL-6: Interleukin 6

IR: Insulin Resistance

ISI: Insulin Sensitivity Index

LDL (-c): Low Density Lipoprotein (-cholesterol)

LF: Low-Fat diet

LPL: Lipoprotein lipase

LPS: Lipopolysaccharide

MED: Mediterranean

MetS: Metabolic Syndrome

MGWAS: Metagenome -Wide Association Study

NAFLD: Non –Alcoholic Fatty Liver Disease

NCEP ATP III: National Cholesterol Education Programme Adult Treatment Panel III

NGS: Next Generation Sequencing

NHLBI: National Heart, Lung and Blood Institute

OGTT: Oral Glucose Tolerance Test

OTU(s): Operational Taxonomic Unit(s)

PAI-1: Plasminogen activator inhibitor 1

PCoA: Principal Coordinate Analysis

PD: Phylogenetic Diversity

PRRs: Pattern –recognition receptors

QA/QC: Quality Assurance / Quality Control

QIIME: Quantitative Insight into Microbial Ecology

qPCR: Quantitative Polymerase Chain Reaction

RAS: Renin Angiotensin System

ROS: Reactive Oxygen Species

Rx: Receiving Treatment

SBP: Systolic Blood Pressure

SCFA(s): Short Chain Fatty Acid(s)

T2DM : Type 2 Diabetes

TC : Total Cholesterol

TG: Triglycerides

TLRs: Toll like Receptor(s)

TMA: Trimethylamine

TMAO: Trimethylamine

TNF-α: Tumor Necrosis Factor Alpha

UC: Ulcerative Colitis

UPLC-MS/MS: Ultrahigh Performance Liquid Chromatography –Tandem Mass Spectroscopy

VLDL: Very Low Density Lipoprotein

WC: Waist Circumference

WHO: World Health Organization

I. ABSTRACT
RESUMEN

1. ABSTRACT

Introduction:

The metabolic syndrome (MetS) is a condition characterized by a cluster of metabolic disorders including abdominal obesity, glucose intolerance, dyslipidemia and hypertension. The MetS is related with the increasing prevalence of cardiovascular disease (CVD) and type-2 diabetes mellitus (T2DM). The gut microbiota is a complex ecosystem consisted of trillions of microbes that have co-evolved with their host for hundreds of millions of years. During the last decade, a growing body of knowledge has suggested that there is a compelling set of connections among diet, gut microbiota and human health. At present, it is known that microbial imbalance or dysbiosis of the gut microbiota is associated with metabolic disorders such as MetS, obesity, T2DM and CVD. In this context, the relationship between the composition of the intestinal microbiota and human health has led to the design of strategies to promote the prevalence of beneficial bacteria that improve health status. In this sense, studies are needed to research the possibility the develop strategies to dilute microbial patterns associated with metabolic diseases with the aim of predict and treat the course of the disease.

Hypothesis:

Our hypothesis is the existence of a gut microbiota pattern predicts the development of MetS. On the other hand, the null hypothesis is the absence of a specific pattern capable of predicting the development of MetS.

Objectives:

Main objective:

To determinate whether the composition of the intestinal microbiota and fasting and postprandial levels of Lipopolysaccharide (LPS) and Lipopolysaccharide Binding Protein (LBP) might predict the development of MetS, defined by the criteria established by National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III) for MetS, in a subpopulation of patients within the CORDIOPREV study with coronary heart disease.

Secondary objectives:

1) *T*o evaluate whether the chronic consumption of two healthy diets (Mediterranean diet and low-fat diet) may restore the gut microbiome dysbiosis in obese patients depending on the degree of metabolic dysfunction.

2) To analyze the differences in the composition of the intestinal microbiota and plasma levels of LPS after consumption of two healthy diets (Mediterranean diet and low-fat diet) for 3 years in PreDM patients who regressed from Normoglucemia (NGC) compared with patients who did not regressed during these time periods.

Participants, design and methodology:

The current Thesis was conducted in patients within the CORDIOPREV study (Clinical Trials.gov.Identifier: NCT00924937), an ongoing prospective, randomized, opened and controlled trial in patients with coronary heart disease who had their last coronary event over 6 months before enrolling in the study. In CORDIOPREV study the participants were randomized to receive two dietary models, a Mediterranean diet and a Low-Fat diet, over a period of 7 years, in addition to conventional treatment for coronary heart disease. All patients gave written informed consent to participate in the study. The trial protocol and all amendments were approved by the local ethics committees, following the Helsinki declaration and good clinical practice.

In this thesis, we performed 3 different studies according to the objectives proposed.

- *Study I from Main objective*: We selected 182 patients without MetS from CORDIOPREV study, according to the NCEP ATP III criteria for MetS, from which 34 patients MetS develop during the first 3 years of follow-up (MetS develop group) whereas the remaining 148 patients did not developed if during this period of time (control group). The biochemical determinations and the analysis of the intestinal microbiota were performed at baseline and after 3 years of follow up. The analysis of intestinal microbiota was carried out by sequencing of the variable region V3-V4 of the microbial 16S rRNA gene in the MiSeq platform of Illumina. In additions, we determinate the plasma levels of LPS and LBP in fasting and postprandial state.
- *Study II from secondary objective 1*: We selected 106 male patients, according to the severity of metabolic dysfunction: MetS-OB group, 33 obese people with MetS; NonMetS-OB group, 32 obese people without MetS (2 or less criteria for the metabolic

syndrome); NonMetS-NonOB group, 41 nonobese people without MetS. The sequencing of the variable region V4 of the microbial 16S rRNA gene in the MiSeq platform of Illumina. The biochemical determinations and the analysis of the intestinal microbiota were performed at baseline and after 2 years of follow up.

• *Study III from secondary objective 2*: We selected 182 pre-diabetic (PreDM) patients included the CORDIOPREV, according to the American Diabetes Association (ADA) diagnosis criteria, 69 from which regressed to NGC after 3 years of follow-up (PreDM regression group) and 113 did not regress to NGC (PreDM group) during same period of time. Half of each group received two healthy diets (MED and LF diets). The analysis of intestinal microbiota was carried out by sequencing of the variable region V3-V4 of the microbial 16S rRNA gene in the MiSeq platform of Illumina. In addition, we also determinated the fasting plasma levels of LPS.

Results:

- *Study I: Study I:* We observed higher abundance of *Ruminococcus* and *Holdemania* and lower *Prevotella* and *Clostridium*, in patients without MetS who developed MetS than patients who did not developed MetS at baseline. Moreover, lower richness and diversity were associated to the development of MetS. In addition, we observed an increase in unknown genera of *Erysipelotrichaceae* family in patients who developed MetS whereas the abundance of this genus did not change in the patients who did not develop MetS after 3 years follow-up. Moreover, we also found higher levels of LBP at baseline in patients who further developed MetS, and higher levels of LBP and LPS when patients develop MetS after 3 years follow-up compare with patients who did not develop MetS.

- *Study II***:** Our study showed a marked dysbiosis in people with severe metabolic disease, who had the full characteristics of MetS (5 criteria) (Met-OB), compared with obese people without MetS (NonMetS-OB) and non-obese people (NonMetS-NonOB). Interestingly, the dysbiosis pattern was reversed by the chronic consumption of both MED or LF diets, altering its microbiota composition to the pattern found in metabolically healthy people. However, in the NonMetS-NonOB and NonMetS-OB groups, no significant microbiota changes were observed after the dietary intervention.

- *Study III***:** Our study showed an increase in the diversity of bacterial genera of patients who regressed from PreDM to NGC, which agree with the notion about the

association between low diversity microbiota and metabolic disease risk. Moreover, a higher relative abundance of *Akkermansia* and lower *Haemophilus* genera associated to PreDM regression when patients consumed the LF diet, whereas a higher abundance of *Prevotella* and lower *Streptococcus* genera was associated to PreDM regression when patients consumed the MED diet. In addition, lower plasma levels of LPS were associated with PreDM regression after consumption of both diets compare with patients who did not regressed.

Conclusions:

Main conclusion:

Our study showed that early detection of MetS development is possible by plasma levels of LBP and specific gut microbiota pattern. High abundance of *Ruminococcus* and *Holdemania,* and low abundance of *Prevotella* and *Clostridum* as well as, high plasma levels of LBP are potential biomarkers to predict the develop of MetS in our population.

Secondary conclusions:

***1*)** Our results suggest that the chronic intake of two healthy dietary patterns partially restores the gut microbiome dysbiosis in obese patients with coronary heart disease depending on the degree of metabolic dysfunction. In this way, a healthy dietary intervention could be a key tool to reverse microbiota dysbiosis and, as a result, help to correct metabolic imbalance.

2) Our results showed that the consumption of two healthy diets led to different specific changes in the gut microbiota, associated to regression from PreDM to normal glucose condition. Moreover, the higher abundance of *Akkermansia* and *Prevotella*, for patients who regressed consuming LF and MED diets respectively was consistent with an improvement in the intestinal barrier integrity. In addition, plasma levels of LPS were associated with the regression of PreDM from NGC after consumption of healthy diets.

I. Resumen

Introduccion:

El síndrome metabólico (SMet) es una afección caracterizada por un grupo de alteraciones metabólicas que incluyen, entre otras, obesidad abdominal, intolerancia a la glucosa, dislipidemia e hipertensión. Este síndrome está implicado en la creciente prevalencia de enfermedades cardiovasculares y diabetes mellitus tipo 2 (DM2). La microbiota intestinal es un ecosistema complejo formado por billones de microbios que han coevolucionado con su huésped durante cientos de millones de años. En la actualidad, se sabe que el desequilibrio microbiano, disbiosis de la microbiota intestinal se asocia con trastornos metabólicos tales como el propio SMet, Obesidad, DM2 y Enfermedad Cardiovascular (CVD). En este contexto, la relación entre la composición de la microbiota intestinal y la salud humana ha llevado al diseño de estrategias para promover la prevalencia de bacterias beneficiarias que pudieran mejorar el estado de salud. En este sentido sería de interés la búsqueda de patrones microbianos asociados a enfermedades metabólicas con el objetivo de predecir y tratar la enfermedad.

Hipotesis:

Nuestra hipótesis consiste en que existe un patrón de microbiota intestinal capaz de predecir el desarrollo de SMet. Por otro lado, la hipótesis nula es la ausencia de un patrón específico capaz de predecir el desarrollo de SMet.

Objetivos:

Objetivo principal:

Establecer que la composición de la microbiota intestinal y los niveles plasmáticos de Lipopolisacárido (LPS) y Proteína de unión a lipopolisacárido (LBP) pueden predecir el desarrollo de SMet, definido a través de los criterios establecidos por NCEP ATP III para SMet, en una subpoblación de pacientes del estudio CORDIOPREV con enfermedad coronaria.

Objetivos secundarios:

1) Evaluar si el consumo crónico de dos dietas saludables (dieta mediterránea y dieta baja en grasas) puede restaurar la disbiosis de la microbiota intestinal en pacientes obesos dependiendo del grado de disfunción metabólica.

2) Analizar las diferencias en la composición de la microbiota intestinal y los niveles plasmáticos de LPS después del consumo, durante 3 años, de dos dietas saludables (dieta mediterránea y dieta baja en grasa) en pacientes PreDM que regresaron a Normoglucemia (NGC) comparado con pacientes que no regresaron.

Participantes, diseño y metodología:

La tesis actual se realizó en pacientes dentro del estudio CORDIOPREV (Clinical Trials.gov.Identifier: NCT00924937), un ensayo prospectivo, aleatorizado, abierto y controlado en curso en pacientes con enfermedad coronaria que tuvieron su último evento coronario durante 6 meses antes de inscribirse el estudio. En el estudio CORDIOPREV, los participantes fueron aleatorizados para recibir dos modelos dietéticos, una dieta mediterránea y una dieta baja en grasa, durante un período de 7 años, además del tratamiento convencional para la enfermedad coronaria. Todos los pacientes dieron consentimiento informado por escrito para participar en el estudio. El protocolo del ensayo y todas las enmiendas fueron aprobados por los comités locales de ética, siguiendo la declaración de Helsinki y la buena práctica clínica.

En esta tesis, se realizaron 3 estudios diferentes según los objetivos propuestos.

• E*studio I del Objetivo principal:* Seleccionamos 182 pacientes sin SMet del estudio CORDIOPREV, de acuerdo con los criterios NCEP ATP III para SMet, de los cuales 34 pacientes desarrollaron SMet durante los primeros 3 años de seguimiento (grupo de desarrollo SMet) mientras que los 148 pacientes restantes no desarrollaron SMet durante este período de tiempo (grupo de control). Las determinaciones bioquímicas y el análisis de la microbiota intestinal se realizaron al inicio del estudio y después de 3 años de seguimiento. El análisis de la microbiota intestinal se llevó a cabo mediante la secuenciación de la región variable V3-V4 del gen 16S rRNA microbiano en la plataforma MiSeq de Illumina. Además, determinamos los niveles plasmáticos de LPS y LBP en ayunas y estado posprandial.

• *Estudio II del objetivo secundario 1*: Seleccionamos 106 pacientes varones, de acuerdo con la gravedad de la disfunción metabólica: grupo SMet-OB, 33 personas obesas con SMet; Grupo NonSMet-OB, 32 personas obesas sin SMet (2 o menos criterios para el síndrome metabólico); Grupo NonSMet-NonOB, 41 personas no obesas sin SMet. La secuenciación de la región variable V4 del gen microbiano 16S rRNA en la plataforma MiSeq de Illumina. Las determinaciones bioquímicas y el análisis de la microbiota intestinal se realizaron al inicio del estudio y después de 2 años de seguimiento.

• *Estudio III del objetivo secundario 2:* Seleccionamos 182 pacientes pre-diabéticos (PreDM) incluidos CORDIOPREV, de acuerdo con los criterios de diagnóstico de la Asociación Americana de Diabetes (ADA), 69 de los cuales regresaron a NGC después de 3 años de seguimiento (grupo de regresión PreDM) y 113 no regresaron a NGC (grupo PreDM) durante el mismo período de tiempo. La mitad de cada grupo recibió dos tipos de dietas (Mediterránea y dieta baja en grasa). El análisis de la microbiota intestinal se llevó a cabo mediante la secuenciación de la región variable V3-V4 del gen 16S rRNA microbiano en la plataforma MiSeq de Illumina. Además, también determinamos los niveles plasmáticos de LPS en ayunas.

Resultados:

- *Estudio I:* Al inicio del estudio observamos alta abundancia de *Ruminococcus y Holdemania* y baja abundancia de *Prevotella* y *Clostridium,* en pacientes antes del desarrollo de SMet comparado con pacientes que no desarrollan SMet. Además, la riqueza y la diversidad más bajas se asociaron con el desarrollo de SMet en nuestro estudio, tanto al inicio como a los 3 años de seguimiento. Por otro lado, se observó un aumento en el género desconocido de la familia *Erysipelotrichaceae* en pacientes que desarrollan SMet después de 3 años de seguimiento, mientras que la abundancia de este género no cambió en los pacientes que no desarrollaron después de 3 años de seguimiento. También, encontramos mayores niveles de LBP en pacientes antes de desarrollar SMet y mayores niveles de LBP y LPS cuando los pacientes desarrollan SMet después de 3 años de seguimiento comparado con los pacientes que no desarrollaron SMet.

- *Estudio II:* Nuestro estudio mostró una marcada disbiosis en personas con enfermedad metabólica severa, que tenía las características completas de SMet (5 criterios) (SMet-OB), en comparación con las personas obesas sin SMet (NonSMet-OB) y las personas no obesas (NonSMet-NonOB). Curiosamente, el patrón de disbiosis se invirtió por el

consumo crónico de dietas MED o LF, alterando su composición microbiana al patrón encontrado en personas metabólicamente sanas. Sin embargo, en los grupos NonSMet-NonOB y NonSMet-OB, no se observaron cambios significativos de microbiota después de la intervención dietética.

- *Estudio III:* Nuestro estudio mostró un aumento en la diversidad de géneros bacterianos de pacientes que retrocedieron de PreDM a NGC, lo que concuerda con la noción acerca de la asociación entre microbiota de baja diversidad y riesgo de enfermedad metabólica. Por otra parte, se observó una mayor abundancia relativa de los géneros *Akkermansia* y baja abundancia de *Haemophilus* asociados a la regresión PreDM cuando los pacientes consumieron la dieta baja en grasa, mientras que una mayor abundancia de *Prevotella* y menor *Streptococcus* se asoció a la regresión PreDM cuando los pacientes consumieron la dieta Mediterránea. Además, los niveles plasmáticos más bajos de LPS se asociaron con la regresión de PreDM después del consumo de ambas dietas comparado con los pacientes que no retrocedieron.

Conclusiones:

Conclusión principal:

Según nuestro estudio, la detección temprana del desarrollo de SMet es posible, a través de los niveles plasmáticos de LBP y un patrón especifico de microbiota intestinal. La alta abundancia de *Ruminococcus* y *Holdemania* y la baja abundancia de *Prevotella y Clostridum*, así como altos niveles plasmáticos de LBP, son biomarcadores potenciales para predecir el desarrollo de MetS en nuestra población.

Conclusiones secundarias:

1) Nuestros resultados sugieren que la ingesta crónica de dos patrones dietéticos saludables restaura parcialmente la disbiosis del microbioma intestinal en pacientes obesos con enfermedad coronaria dependiendo del grado de disfunción metabólica. De esta manera, una intervención dietética saludable podría ser una herramienta clave para revertir la disbiosis microbiana y, como resultado, ayudar a corregir el desequilibrio metabólico.

2) Nuestros resultados mostraron que el consumo de dos dietas saludables condujo a diferentes cambios específicos en la microbiota intestinal, asociados a la regresión de

PreDM a condición de glucosa normal. Por otra parte, la mayor abundancia de *Akkermansia* y *Prevotella*, en los pacientes que regresaron tras consumir dieta baja en grasa y mediterránea, respectivamente, fue compatible con una mejora en la integridad de la barrera intestinal. Además, los niveles de LPS se asocian con la regresión de PreDM a NGC tras la ingesta de dietas saludables.

II. INTRODUCTION

1. METABOLIC SYNDROME

1.1 Definition and Epidemiology

The metabolic syndrome (MetS) is a condition characterized by a cluster of metabolic disorders including abdominal obesity, insulin resistance/glucose intolerance, dyslipidemia and hypertension that result from the increasing prevalence of obesity and type 2 diabetes mellitus (T2DM) [1]. In addition to reducing the patients' quality of life, MetS has a significant economic impact on public health expenditure due to the morbidity generated: the increased risk of developing T2DM raises by over 30% the probability of cardiovascular diseases (CVD), which is currently the leading cause of mortality worldwide [2, 3].

In spite of the extensive worldwide research in this field, during the 1990s there was no widely-accepted international definition to describe precisely what the syndrome includes. The different terms used to describe it, such as 'metabolic syndrome' [4], 'syndrome X' [5] or 'insulin resistance syndrome' [6] represented views of the syndrome from different perspectives and did not all include the same symptoms. In 1981, Leonhardt and Hanefeld [7] coined for the first time the term "metabolic syndrome" to include T2DM, hyperinsulinaemia, obesity, hypertension, hyperlipidaemia, gout and thrombophilia. The authors observed how these complaints developed against a background of genetic predisposition and environmental influences, such as overeating and lack of physical exercise, and led to the development of atherosclerosis. In 1985, Modan [8] detected the presence of a link between hyperinsulinemia, hypertension, T2DM, glucose intolerance and dyslipidemia. In 1988, Gerald Reaven [5], in his Banting Lecture at the American Diabetes Association's national convention, introduced the concept of what he called "Syndrome X" to describe an aggregation of independent, coronary heart disease (CHD) risk factors concentrated in one individual patient. The risk factors included in the syndrome were insulin resistance, defined as the inability of insulin to stimulate the transport of glucose efficiently into the body cells (hyperinsulinemia or impaired glucose tolerance), hypertension, hypertriglyceridemia, and low, high-density lipoprotein cholesterol (HDL). However, neither obesity nor visceral obesity were included in the definition, despite the fact that we now consider them crucial symptoms.

Diagnostic criteria have changed considerably over the last decade. In 1998, the first international effort to define the MetS was released, as part of the World Health Organization (WHO) report on the definition and classification of diabetes mellitus and

its complications [9]. Later, an important paper was published by the ATP III on abdominal obesity, which is considered one of the most important diagnostic criteria established since 2001, and is shown in **Table 1** [10]. At present, the most commonly-used criteria for definition have been described by the World Health Organization (WHO) [11], the European Group for the study of Insulin Resistance (EGIR) [12], the National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III) [10], the American Association of Clinical Endocrinologists (AACE) [10] and the International Diabetes Federation (IDF) [13]. The ATP III criteria are used in most epidemiological studies.

Table 1. Diagnostic criteria of the metabolic syndrome considering its definition, according to the World Health Organization (WHO), National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) and International Diabetes Federation (IDF).

Clinical measurements	WHO (1998)	ATPIII (2001)	IDF (2005)
Insulin resistance	Lowered insulin sensitivity[a] plus any 2 of the following	None, but any 3 of the following 5 features	None
Waist circumference	Waist-to-hip ratio: >0.90 in men, >0.85in women and/or BMI>30 kg/m2	WC: ≥102 cm in men ≥88 cm in women	Increased WC (population specific) plus any 2 of the following
Lipids	TG ≥150 mg/dL and/or HDL-C: <35 mg/dL in men <39 mg/dL in women	TG ≥150 mg/dLand /or HDL-C: <40 mg/dL in men <50 mg/dL in women	TG ≥150 mg/dL or on TGs Rx. HDL-C: <40 mg/dL in men <50 mg/dL in women or on HDL-C Rx
Blood pressure	≥140/90 mm Hg	≥130/85mm Hg	≥130 mm Hg systolic ≥85 mm Hg diastolic o
Glucose	IGT, IFG, or T2D	>110 mg/dL (includes diabetes)[b]	≥100 mg/dL (includes diabetes)[b]
Others	Microalbuminuria		

[a]Insulin sensitivity measured under hyperinsulinemic euglycemic conditions, glucose uptake below lowest quartile for background population under investigation. [b]In 2003, the American Diabetes Association (ADA) changed the criteria for IFG tolerance from >110 mg/dl to >100 mg/dl.

In 2009, a number of different organizations participated in publishing a joint statement that provided a "harmonized" definition of MetS. This statement was published in the article entitled "Harmonizing the Metabolic Syndrome" [14] and is summarized in **Table 2**. The aim of this consensus on the diagnosis of MetS was to obtain a useful,

practical tool for assessing the risk of CVD and diabetes, while taking into consideration the study population and geographical region.

Diagnostic criteria 3 of the 5 components proposed	**Values**
Waist circumference	**High Waist circumference:** Population- and country-specific definitions *(table 3)*
High blood pressure	**SBP ≥130 mmHg and/or DBP ≥85 mmHg** or antihypertensive treatment
High fasting glucose	**Fasting glucose ≥110 mg/dL** or treatment for high blood sugar
High triglycerides	**>150 mg/dL** or treatment with specific lipid-lowering drugs
Low HDL-C	**<40 mg/dL in men or <50 mg/dL in women** or treatment effect on HDL-C

Table 2: Criteria for clinical diagnosis of Metabolic Syndrome according to the currently accepted definition. Abbreviations: HDL-c, high-density lipoprotein-cholesterol.

- ***Abdominal obesity***

According to the ATP III criteria, men are considered as having abdominal obesity when the waist circumference is equal to or greater than 102 cm and in women when it is equal to or greater than 88 cm [11].The recommendation to measure waist circumference rather than body mass index (BMI) recognized the important part played by abdominal obesity in MetS. By singling out waist circumference, the NCEP-ATP III recommendation acknowledged that health professionals and clinicians are struggling with a demographic explosion: more and more patients are overweight or obese and show the related metabolic effects of an affluent, sedentary lifestyle characterized by excess consumption of highly processed, energy-dense food of poor nutritional value.

The pathophysiological consequences of abdominal obesity include the expansion of adipose tissue, and the dysfunction of adipocytes causes alterations in the secretion of adipokines and cytokines, which contributes to the development of insulin resistance (IR) and MetS [15].

- ***Atherogenic dyslipidemia***

The major components of dyslipidemia associated with the MetS are increased triglyceride (TG >150 mg/dl) and low high-density lipoprotein (HDL-c) (<50 mg/dl in women, <40 mg/dl in men). [13, 16, 17]. Dyslipidaemia is widely established as an independent risk factor for cardiovascular disease. Low HDL cholesterol and hypertriglyceridaemia have been found to be independently and significantly related to myocardial infarction/stroke in patients with MetS [17-20]. Additionally, a combination of high fasting glucose and low HDL cholesterol were shown to have primary predictive ability for coronary heart disease. These abnormalities may be the consequence of a global metabolic effect of IR [20].

- ***Hypertension***

Blood pressure is a classic feature of the MetS, and it has been reported that the MetS is found in up to one third of hypertensive patients [12, 21, 22] . For this reason, high blood pressure is included in the definition of the MetS presented by the WHO, the NCEP, the IDF and the American Heart Association/National Heart, Lung, and Blood Institutes [11, 13, 21, 23]. Blood pressure levels are closely linked to visceral obesity and IR [21, 24, 25], which are the main pathophysiologic features underlying the MetS.

Another reason why high blood pressure was included in the diagnosis criteria of MetS is because the reduction of blood pressure below 130/85 decreased the probability of a cardiovascular event in diabetic patients or in those with other cardiovascular risk factors [11].

Visceral obesity, IR, oxidative stress, endothelial dysfunction, activated renin-angiotensin system, increased inflammatory mediators and obstructive sleep apnea have been proposed as possible factors involved in the development of hypertension in the MetS [26-29].

- ***High Fasting glucose levels: Insulin Resistance***

A fasting glucose measurement of ≥100 mg/dL is usually an IR indicator, and is frequently accompanied by other MetS components. Hyperglycemia is associated with an increased risk of developing T2DM and CVD [11]. Although the ATP III panel did not consider the IR value as a criterion for the diagnosis of MetS, it is usually accepted as a feature which is commonly present in this syndrome.

Epidemiology of MetS

The cluster of cardiovascular risk factors known as the MetS greatly increases the risk of developing diabetes, kidney disease and CVD. Individuals with the MetS are also at increased risk for premature death from cardiovascular disease or all-cause mortality [30, 31]. Cross-sectional and longitudinal epidemiologic studies provide prevalence data on the syndrome based on criteria proposed by the WHO and NCEP AT-III [11, 21]. Owing to differences in the criteria, estimates of the prevalence of the syndrome vary according to the criteria used. The most recent definitions have introduced region-specific cut-off points for the level of obesity (waist circumference) to define the MetS [32], and the introduction of these cut-off points is rational from the point of view that the association between obesity and glucose intolerance [32], blood pressure [33] and dyslipidaemia [34] varies between ethnic groups. On the other hand, the use of the region-specific cut-off points may also mask some of the true regional differences in the prevalence of the syndrome **(Table 3).**

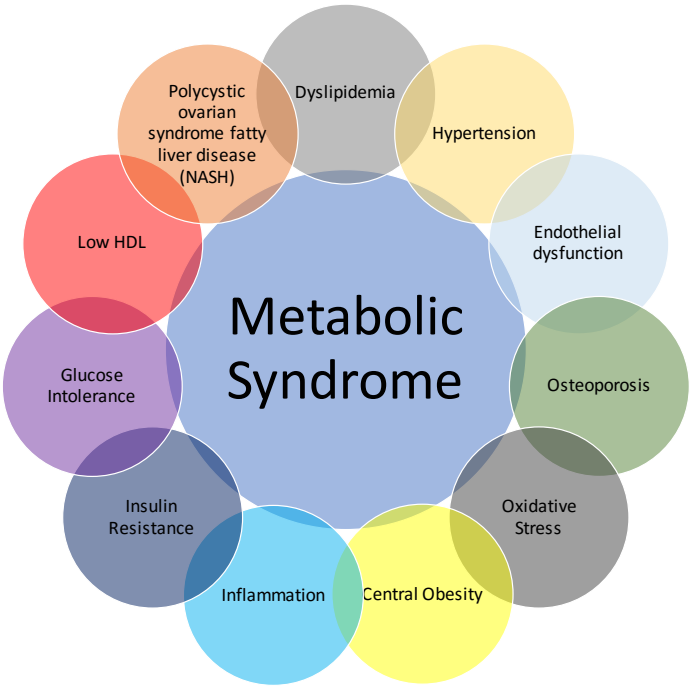

Figure 1: Diseases associated to Metabolic syndrome

Country	Age (year)	Number	Prevalence (%)	Diagnostic criteria	Study
Saudi Arabia	10–18	1,231	10 (M) 8 (F)	NCEP	[35]
Oman	20+	1,419	20 (M) 23 (F)	NCEP	[36]
Turkey	49±13	2,398	27 (M) 39 (F)	NCEP	[37]
Finland	42–60	1,005 males	14 21	NCEP WHO	[38]
India	20+	1,091	8 (M) 18 (F)	NCEP	[39]
United States	12–17	2,014	7 (M) 2 (F)	IDF	[40]
United States	30–79	Framingham offspring 3,224 San Antonio Heart S. 1,081 (white) 1,656 (Mexican Hispanic)	15 (M) 14 (F) 9 (M) 13 (F) 14 (M) 21 (F)	NCEP	[41]
China (urban)	15+	1,206	26 (M) 28 (F)	NCEP	[42]
China (rural)	18–74	13,505 females	22 17 23	IDF NCEP ATP-III modified	[43]

Table 3: Prevalence of the metabolic syndrome.

The prevalence of MetS increases with age in the world population [44]. In Europe, the prevalence of MetS in men is 10.7% for the 20-39 age range, 33% in 40-59 year olds and 39.7% in those over 60 years of age, whereas in women these figures are 18%, 30.6% and 46.1%, respectively [3, 45, 46]. In a comparative study of patients with or without ischemic heart disease, in 41.1% of the patients who had a myocardial infarction, MetS had been diagnosed before the cardiovascular event [47].

In Spain, the existing data show similar results to those found in international studies. Sholze et al. report that in Spain, 22% of the hypertensive population has sufficient criteria for the diagnosis of MetS and this percentage is likely to rise steadily [48]. Furthermore, it was concluded that all the components of the MetS are significantly more prevalent in men, except the low values of HDL-c, which is more prevalent in women [49]. In addition, data provided by the Canary Islands Nutrition Survey, which included 578 adults aged between 18 and 74 years old, showed that the prevalence of MetS (in men and women together) was 24.4% [50].

One highly important factor affecting patients with MetS is the risk of developing T2DM, and having this disease practically constitutes a pre-diabetic state, due, among other physiopathological phenomena, to the IR generated in these individuals. Specifically, in a meta-analysis carried out on 16 studies with patients MetS, the relative risk of developing T2DM varied from 3.53 to 5.17 in some populations [51]. In addition, the number of components of MetS is related linearly with an increased risk of developing diabetes [52, 53]. The WOSCOPS study [54] demonstrated that the risk of developing T2DM increased 4-fold in patients with MetS and especially in those whose plasma levels of hs-CRP were high, which indicates the role played by the proinflammatory state of these individuals in the development of T2DM. This phenomenon has also been observed in women's Health Study [41], where the values of hs-CRP and the number of MetS were associated with higher incidence of T2DM.

Further studies are needed to determine the prevalence of MetS in the Spanish population, as it is highly relevant epidemiological data which directly links the greater prevalence of MetS with increased CVD [23, 55].

1.2 Clinical and biological manifestations

Although the pathophysiology is largely unknown, clinical findings most commonly associated with MetS include IR, dyslipidemia (specifically high TG and low levels of HDL), small dense low-density lipoprotein [LDL]), central obesity,

hypertension, impaired glucose tolerance or diabetes mellitus and high rates of atherosclerotic disease. Recently, inflammation markers and accelerated hemostasis/impaired fibrinolysis have been added to this list [56].

- ***Insulin Resistance (IR)***

Many tissues, including skeletal muscle, liver and adipose tissue may exhibit IR [57]. MetS is associated with IR, but it is not a consequence of IR alone, nor is it a direct consequence of the lack of insulin action. IR and decreased insulin secretion are metabolic disorders that generate hyperglycemia, and increased plasma concentrations of non-esterified fatty acids can cause damage to β-cells and peripheral tissue function. In turn, IR is related to the state of low-grade inflammatory disease and an increase in oxidative stress, which is characteristic of MetS [58].

IR may be caused by defects in the insulin receptor or by action at the post-receptor level. Authors have observed that mutations in the receptor are infrequent, so in most of patients with IR there are defects at post-receptor levels; this derives from genetic alteration and its clinical expression is favoured by environmental factors and abdominal obesity. In addition, a sedentary lifestyle and smoking are also factors which contribute to IR [21].

Most obese subject had IR characteristic of MetS, and the altered glucose metabolism favours the development of T2DM [59]. In a 10-year follow-up study, it was observed that the weight loss in the patients produced an improvement in the IR and MetS and decreased total mortality. In addition, even in the absence of weight loss, it has been noted that physical activity, measured by cardiorespiratory capacity, prevents MetS and the onset of other diseases and reduces mortality [60, 61].

- ***Impaired glucose homeostasis***

High fasting glucose levels are a major component of MetS, but neither impaired fasting glucose (IFG) nor diabetes is an absolute criterion. IR is one factor in the development of IFG, impaired glucose tolerance (IGT) and T2DM [11, 58, 62-65]. Increased free fatty acid (FFA) levels are also associated with resistance to insulin-mediated glucose uptake and metabolism in muscle and an increase in glucose production and release by the liver. Pancreatic β-cell dysfunction is a critical component in the pathogenesis of T2DM [66]. Glucose intolerance develops when insulin secretion capacity of pancreatic β-cells declines, losing the ability to suppress hepatic glucose

uptake and to compensate for IR in target tissue [66, 67]. Whereas insulin insensitivity is an early phenomenon partly related to obesity, in genetically-susceptible individuals, pancreatic β-cell function declines gradually over time, even when the glucose level is still within the normal range, before the onset of clinical hyperglycemia [58, 67]. When IR is combined with β-cell defects in glucose-stimulated insulin secretion, IGT, IFG, or T2DM can result [68]. Peripheral IR is most characteristic of IGT, whereas insulin-mediated failure to suppress hepatic glucose output is a more prominent feature of IFG [69]. IFG is more common in men than women in virtually all ages, but it is up to eight times higher in European men aged 50 to 70 years [69]. Conversely, the prevalence of IGT is higher in women than men, except in Europeans over the age of 80 [69]. IFG and IGT are strong predictors of future diabetes [70, 71] and represent intermediate stages in the progression from MetS to T2DM [72]. The incidence of subsequent diabetes is highest in individuals with combined IGT and IFG [69], and the risk of CVD increases with the progress of glucose intolerance [73].

- ***Abdominal obesity***

Abdominal obesity is a typical characteristic of MetS and is characterized by the presence of activated adipocytes and by the infiltration of macrophages into adipose tissue (AT) [74].

Obesity and excess weight are major risk factors for the development of MetS and other chronic diseases, such as cancer and T2DM. In 2003, Park et al. studied the relationship between obesity and MetS, describing a prevalence of MetS of 5% in subjects with normal weight, 22% in those who were overweight and up to 60% of obese people according to the Third National Health and Nutrition Examination Survey (NHANES III) [75]. On the other hand, cohort studies show how the existence of obesity in an individual increases the risk of premature death with respect to those individuals whose BMI is between 20-25 kg / m^2 [76]. The existence of greater adiposity at the central-abdominal level leads to a higher risk of developing MetS and therefore an increased risk of CVD, so much so that up to 46% of individuals who develop MetS have a high waist circumference as defined in the above criteria [77].

The WHO defines overweight as a BMI equal to or greater than 25 kg / m^2, and obesity as a BMI equal to or greater than 30 kg / m^2. The Latest data from the International Association for the Study of Obesity / International Obesity Task Force (IASO / IOTF) (www.iaso.org), in 2010, gives a number of 1 trillion adults with a BMI between 25-29.9 kg / m^2 and a global prevalence of 474 Millions of obese people with a BMI> 30 kg / m^2,

adjusted for ethnic differences. In addition, the prevalence of obesity is increasing exponentially, as *Finucane et al*. [78] showed through a population study of subjects over 20 in 199 countries. The results of this study show that between the 1980s and 2008, the mean age-adjusted BMI increased in virtually all regions of the world except Africa and South Asia. In developed countries, the largest increase per decade was in the United States (1.1 Kg / m^2) followed by UK (1.0 kg / m^2). Based on these data, Stevens [79] estimated that the increased prevalence of obesity in these 199 countries since the 1980s and the prevalence of obesity in global terms, adjusted for age, rose from 6.4% in 1980 to 12% in 2008. By gender, between 1980 and 2008, the prevalence of age-adjusted obesity was still higher in women than in men (1980: 4.8% H; 7.9% M / 2008: 9.8% H, 13.8% M). The latest WHO calculations indicate that by 2015, there will be around 2.3 billion Adults (over 15 years old) with overweight, and at least 700 million obese adults [80]. A BMI> 30 kg / m^2 is one of the 8 risk factors that represent 61% of deaths due to CVD (currently the number one cause of death in the world) and 75% of ischemic heart disease [81]. In our country, the prevalence of obesity is among the highest in Europe.

Most obese patients have IR, which is characteristic of MetS, as well as having an altered glucose metabolism, which favours the development of T2DM, hypertension, endothelium dysfunction, inflammation (increased IL-6, IL-18, TNF-a or leptin and decreased adiponectin) and dyslipidemia (TG increase and HDL decrease) [82-86]. Overweight and obesity both have serious health consequences. Obesity is an important risk factor for chronic diseases (also called 'non-communicable diseases' by the WHO) related to dietary and nutritional habits, such as:

- CVD (especially ischemic and cerebrovascular heart disease).
- Diabetes, which has rapidly transformed into a global epidemic.
- Diseases of the locomotor system, and in particular osteoarthritis.
- Some types of cancer, such as endometrial, breast and colon.

AT is composed of adipocytes, stromal preadipocytes, immune cells and the vascular endothelium. This tissue plays a major role in the energy homeostasis of the whole body, since it is able to respond rapidly and dynamically to the excess of nutrients through hypertrophy and hyperplasia of the adipocytes to store more fat, a phenomenon which is known as expansion of AT [87]. The AT secretes adipokines, such as TNF-α

and IL-6, with a pro-inflammatory function, and in obese populations high levels have been found [81].

A recent study [88] concluded that there was a link, at the epidemiological level, between obesity and T2DM and that the attachment mechanism of these diseases was the capacity for expansion of adipose tissue. According to these authors, the AT of each individual has a capacity of limited expansion determined by genetic and environmental factors. Once the AT reaches its limit of expansion, it loses the capacity to store fat efficiently and this tends to accumulate in other organs where the phenomena of IR, apoptosis, inflammation and cardiovascular complications are generated **(Fig 2).**

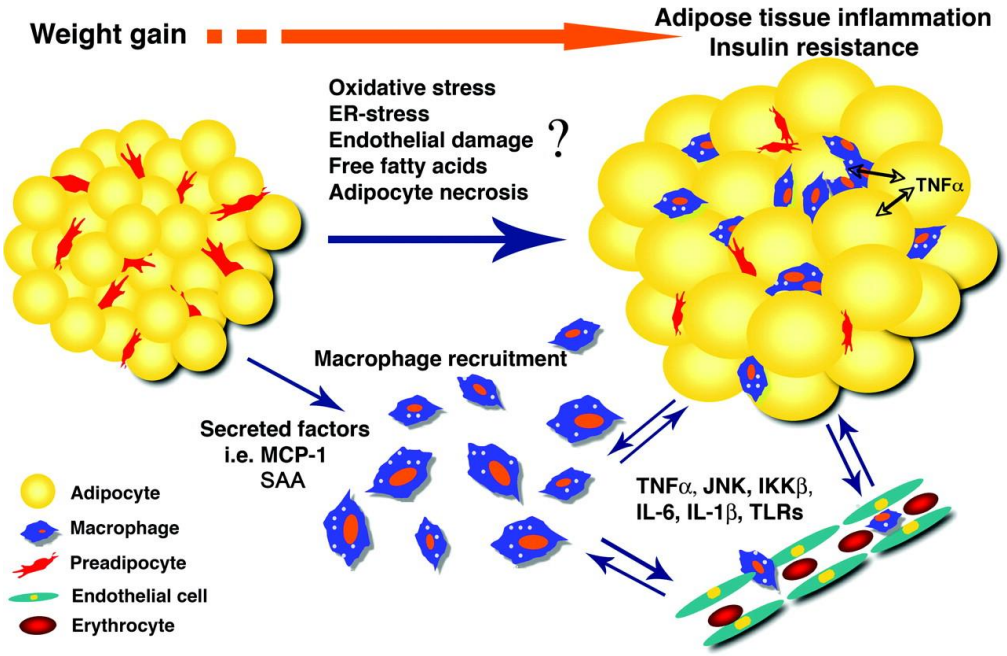

Fig. 2 Adipose tissue expansion. Modified from [89]

The MetS is associated to chronic low grade inflammation, and in this step, the AT is infiltrated by macrophages and the production of pro-inflammatory adipokines increases, contributing to the dysfunction of AT. The MetS and its clinical consequences are therefore related to the abnormal secretion of adipokines, hypertrophy of the adipocyte, excessive release of fatty acids, low grade inflammation and oxidative stress (OS) as a result of AT dysfunction [90].

However, this infiltration capacity of AT is reversible, as has been observed in weight loss regimes that have resulted in a reduction in inflammatory markers [91].

Recent evidence suggests that certain "metabolic phenotypes" exist in which not all obese subjects show alterations in their metabolic parameters: IR, central adiposity, low HDL-c and high TG levels, as well as HT. In the same way, not all subjects with a normal BMI have a healthy metabolism [92-94]. It is also interesting to note that individuals with a normal BMI (<30 kg / m^2), but who are metabolically obese, are characterized by hyperinsulinism and IR, which gives them an increased risk of developing T2DM and CVD [95, 96].

- ***Hypertension***

Hypertension plays a pivotal role in MetS. Indeed, risk estimates according to the Framingham study show that roughly 80% of essential hypertension in men and 65% in women can be directly attributed to obesity [97]. There is a clear association between body mass index and arterial pressure even in non-obese, lean people [98-100]. Hyperinsulinemia, hyperleptinemia and hyperlipidemia are related with sympathetic tone. In addition to insulin, leptin can also be a link between obesity and increased sympathetic activity. Besides its effect on appetite and metabolism, leptin acts in the hypothalamus to increase blood pressure through activation of the sympathetic nervous system [101, 102]. Moreover, high circulating levels of FFA in visceral obese individuals may participate in the activation of the sympathetic nervous system. The increased release of FFA into the portal vein from lipolysis in visceral fat depots could explain the strong association between visceral obesity and increased sympathetic nerve outflow [1].

- ***Dislypidemia***

Dyslipidemia is characterized by increased levels of LDL, decreased HDL and increased blood TG levels [98, 103-105].

Among several causes of IR, it has been speculated that it may be mediated partly by an increase in FFAs that inhibits post-insulin receptor signaling and thus contributes to IR. FFAs may also be an important determinant of the MetS as their level is generally high in this condition. As resistance to insulin action or insulin deprivation is associated with increased lipolysis, intra-abdominal fat, which is metabolically very active, releases FFAs into portal circulation. The liver converts the FFAs into TG and this may explain the link between hypertriglyceridemia and the MetS. An increased supply of glucose and overproduction of VLDL raises the concentration of triglyceride-enriched particles, leading to a reciprocal exchange of fatty acids: cholesterol-esters are transferred to VLDL

and chylomicron remnants, while tTG are transferred to LDL and HDL particles to form small-dense LDL and HDL. These dense particles are well known for their high atherogenic potential [21, 106, 107].

In addition, the factors contributing to hypertriglyceridemia in the general population include obesity, overweight, physical inactivity, excess alcohol intake, high-carbohydrate diet, T2DM and other diseases (e.g. chronic renal failure, nephrotic syndrome), certain drugs (e.g. corticosteroids, estrogens, retinoids, high doses of adrenergic blocking agents), and genetic disorders (familial combined hyperlipidemia, familial hypertriglyceridemia and familial dysbetalipoproteinemia) [108]. In daily practice, high serum TG is mainly found in patients with MetS [109, 110]. Moreover, low HDL-c is the strongest predictor of future cardiovascular events in patients with angiographically-confirmed events and levels of total cholesterol within the normal range. According to the current guidelines, the presence of low HDL-c should be considered a major cardiovascular risk factor that modifies the goal for LDL-lowering therapy and is used as a risk factor to estimate the 10-year risk for CHD. A low HDL-c level has several causes, some of which are linked to IR, e.g. high TG, overweight and obesity, physical inactivity and T2DM. The combination of a low HDL-c with a high plasma triglyceride level has therefore been considered an IR state [17, 98].

- ***Inflammation***

Inflammation is a physiological response of the organism to harmful stimuli, be they physical, chemical or biological. The response provided usually leads to the reestablishment of homeostasis. It involves the coordinated action of many cell types and mediators, whose intervention depends on the nature of the initial stimulus and the ensuing responses [111]. Our innate immune system is made up og epithelial barriers which, together with different types of cells and circulating proteins, defend us from aggressive external factors. Some of these cells, especially macrophages and lymphocytes, trigger the inflammatory reaction and involve a large number of physiological mechanisms which, sustained over time, cause injury to the affected tissue.

The inflammatory response is the first response mechanism which combats exogenous agents such as micro-organisms, or substances generated by them, and is able to eradicate them from the organism. In this way, on a local level where the contact of the pathogen with the organism generates a local reaction which favours the recurrent acute phase (RFA), of which CRP stands out. These phase reactants can be present in acute and

chronic inflammatory processes and their levels can be high in many situations like infections, trauma, autoimmune diseases and even neoplastic processes. This local response generates a series of signals that will attract the different cells of the immune system mainly to the leukocytes, which are the cells that are ultimately responsible for eradicating the external agent [112].

Changes in FFA levels are induced by a number of molecules in anti-inflammatory drugs called cytokines. Cytokines are secreted by macrophages, monocytes and other immune cells involved in the early stages of the inflammatory response. The performance of these cytokines is limited on a local level with the exception of interleukin (IL) -6 and IL-1 and TNF-α, which are the main proinflammatory cytokines of our immune system and constitute authentic hormones which participate in multiple processes of the body. These cytokines favour a systemic reaction that entails a large number of physiological and biochemical alterations such as increased body temperature (fever) or neuroendocrine changes. [112, 113].

The inflammatory response can be divided into 3 steps [114]: 1) proinflammatory production of cytokines that act as markers for the chemotaxis of cells in the immune system called macrophages, as well as favouring prothrombotic and repair phenomena at this level. 2) Systemic inflammatory reaction that amplifies or attenuates the inflammatory process through a clearly endocrine function. 3) Massive release into the bloodstream of inflammatory mediators that may even cause tissue damage if they persist in time.

- ***Oxidative stress***

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and their disposal defense mechanisms, known as antioxidants [115]. Oxidative stress is a well-recognized mechanism which plays an important role in many pathological conditions [116], and several human diseases have been closely linked to oxidative stress [117]. The protein redox state is implicated in the regulation of several cellular activities, including cell differentiation and the activation of specific metabolic pathways [118, 119].

ROS are ubiquitous, highly reactive, short-lived derivatives of oxygen metabolism produced in all biological systems that react with the surrounding molecules wherever they are formed. These species, which include the superoxide radical ($O_2^{\bullet -}$), the hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), along with reactive nitrogen species, such as nitric oxide (NO) and the peroxynitrite radical ($ONOO^{\bullet}$), are oxygen derivatives that

play important roles in vascular biology. Additionally, low levels of ROS are indispensable in several biochemical processes, including intracellular messaging, cellular differentiation, growth arrestment, apoptosis [120], immunity [121] and defense against microorganisms [122].

Oxidative stress, also referred to as an ROS-antioxidant imbalance, occurs when the net amount of ROS exceeds the antioxidant capacity. Thus, oxidative stress can occur as a consequence of a general increase in ROS generation, a depression of the antioxidant systems, or both [123, 124].

The natural antioxidant system consists of a series of antioxidant enzymes and numerous endogenous and dietary antioxidant compounds that react with and inactivate ROS. The primary antioxidant enzymes include, but are not limited to, superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPX). Cells must maintain their levels of antioxidants, often defined as their antioxidant potential, through dietary intake and/or *de novo* synthesis [125].

Reported results support the concept that increased oxidative stress may play an important role in MetS-related manifestations, including atherosclerosis, hypertension and T2DM [115, 126]. Oxidative stress is also associated with adiposity and IR in men [127] and those with MetS, which suggests that oxidative stress could be an early event in the pathology of these chronic diseases, rather than merely a consequence or an innocent bystander [128]. In models of obese-diabetic and obese non-diabetic mice, antioxidant activity was low, possibly due to the obesity per se [128]. More recently, the Coronary Artery Risk Development in Young Adults (CARDIA) noted that a higher concentration of oxLDL was associated with an increased incidence of MetS overall, as well as its components of abdominal obesity, hyperglycemia and hypertriglyceridemia [129]. Furthermore, the odds ratio for the highest oxLDL quintile was 3 times higher than the lowest quintile and oxLDL was associated with high C reactive protein (CRP) and insulin and inversely linked to adiponectin and HDL levels [129].

Moreover, there are multiple sources for oxidative stress in relation to obesity. Some of them are inherently related to increased adiposity and fat distribution, whereas others are the result of comorbidities or behavioral changes associated with being obese. Increased adipose tissue and, in particular, visceral adiposity are significantly correlated with systemic levels of oxidative stress biomarkers [130] .

- ***Prediabetes***

Prediabetes (PreDM), which is a combination of excess body fat and IR, is considered to be an underlying etiology of MetS. PreDM manifests as impaired fasting glucose and/or impaired glucose tolerance. Impaired fasting glucose (IFG) is defined as a fasting blood glucose level of 100 to 125 mg/dL; impaired glucose tolerance (IGT) requires a blood glucose level of 140 to 199 mg/dL 2 hours after a 75-g oral intake of glucose, while the ADA defines PreDM by including 5.7% to 6.4% additional hemoglobin A1c (HbA1c)-based criteria. Glucose intolerance, one of the 5 MetS components, is an established CVD risk factor. Subjects with PreDM may therefore be a high risk subgroup among the population with MetS [131, 132].

There is certainly an overlap in the presence of PreDM and MetS in the general population, and the two seem to be closely interrelated. One study found that among non-diabetic patients above the age of 50, about twice as many have IFG plus MetS as those with IFG alone [133]. Another study compared patients with MetS and PreDM in terms of cardiovascular risk factors, target-organ dysfunction and IR [134]. Individuals with MetS have about a five times higher risk of diabetes [135, 136], and epidemiologic studies have shown that the adverse effects of MetS begin in childhood [137]. The researchers also found that individuals with PreDM (compared to those with normoglycemia) had higher LDL cholesterol, insulin and the homeostasis model assessment of insulin resistance (HOMA-IR) beginning in adolescence. Starting from childhood, diabetic subjects had significantly higher levels of BMI, TG, glucose, insulin and HOMA-IR, and lower levels of HDL cholesterol. There has been less research into whether MetS in those with PreDM predicts the future development [138]. However, one group of researchers recently published a study looking into this very question. About 3,234 participants with IGT were assessed for the presence of MetS and followed over an average of 3.2 years [139]. The development of diabetes was measured by an annual OGTT and twice-yearly fasting PG tests. The presence of the metabolic syndrome at baseline in PreDM individuals was associated with a 70–100 % increased risk of diabetes. Baseline fasting glucose was the strongest component linking MetS with incident diabetes, followed by baseline hypertriglyceridemia and changes in waist circumference. They concluded that the baseline presence of MetS in patients with IGT was indeed linked with an increased risk of developing diabetes.

1.3 Evolution and complications

The clinical identification and management of patients with the MetS is an essential step in the effort to implement suitable treatments to reduce their risk of subsequent diseases [140]. Effective preventive approaches include lifestyle changes, primarily weight loss, diet, and exercise, and the treatment includes the careful use of pharmacological agents to reduce the specific risk factors. Pharmacological treatment should be considered for those whose risk factors have not been reduced sufficiently with preventive measures and lifestyle changes [141]. MetS is difficult to manage clinically since there is no recognized method to prevent or improve the whole syndrome, the background of which is essentially IR [5]. Most physicians therefore treat each component of MetS separately, with particular emphasis on those components that are most easily treated with drugs. In fact, it is easier to prescribe drugs to lower blood pressure, blood glucose or TG than to initiate a long-term strategy to change people's lifestyle (exercise more and follow a healthier diet) in the hope that they will ultimately lose weight and tend to have a lower blood pressure, blood glucose and TG.

- Lifestyle

The prevalence of central obesity in the western world and developing countries is on the rise and, given its association with IR, it is a key factor in the relevance of MetS as a public health problem [48, 142]. Individual lifestyle, in terms of dietary habits and physical activity, strongly influences the pathogenesis of obesity and consequent cardiovascular and metabolic risk. MetS mostly affects people with excess weight who lead a sedentary lifestyle. In the expression of disease, the balance between genetic predisposition and environment is always variable, but in this case, maintaining a normal weight and doing regular physical exercise are still the primary and most effective prevention strategy [143]. Similarly, any treatment should be based on promoting effective weight loss and physical exercise, although attempts at encouraging patients to lead healthy lifestyles to achieve long-term results require specific strategies. Randomized controlled trials indicate that doing regular exercise has positive effects on the individual components of metabolic and cardiovascular risk factors related to the MetS such as body weight and fat [144], insulin sensitivity [145], lipid profile [146], blood pressure [147, 148] and low-grade inflammation [149].

-Weight loss

Weight loss is the main objective of most intervention studies into MetS. There is general agreement that weight loss is linked to significant improvements in the clinical abnormalities of MetS, including blood glucose, lipid profile and blood pressure [150-152], and even a moderate weight loss (7% reduction over 4 weeks) can improve the metabolic profile, even if the high body mass index (BMI) does not change [152]. However, the greater the reduction in BMI, the more significant the metabolic improvements. Lifestyle modifications are also desirable in subjects who have only one or two MetS criteria, not the full disorder. In the large US Diabetes Prevention Program (DPP), the effects of lifestyle intervention have been investigated in more than 3000 participants with IGT [153]. Metformin is an oral diabetes medicine that helps control blood sugar levels. The patients in the intervention group were allocated to an intensive lifestyle intervention (including a low-calorie, low-fat diet and weight loss) or to the metformin therapy (850 mg three times daily.). MetS incidence was reduced by 41% in the lifestyle group and by 17% in the metformin group compared with the placebo arm [153].

-Diet

Most lifestyle modification programs recommend a low-calorie diet. In the last decade there has been a growing interest in following a healthy diet model like the Mediterranean Diet (MED). The MED is recognized as one of the healthiest dietary patterns. It has shown benefits in patients with CVD [154] and in the prevention and treatment of related conditions, such as diabetes [155], hypertension [156] and MetS [157]. However, the beneficial effects of traditional MED are being lost, and in fact, the Eurodiet [158] project showed that in the southern countries of Europe in which a MED rich in olive oil was traditionally consumed, the percentage of fat ingested was higher than that of the Nordic countries. For this reason, in countries are making dietary recommendations to reduce fat consumption, especially saturated fatty acids (SFA) [159].

MED is characterized by high consumption of fruit, vegetables, legumes and grain, a moderate alcohol intake, a moderate-to-low consumption of dairy products and meats/meat products and a high monounsaturated- to-saturated fat ratio [160], and this diet has been inversely associated with the cumulative incidence of MetS [157]. The consumption of Monounsaturated Fatty Acids (MUFA), namely oleic acid in olive oil, is

considered the key component of MED which is responsible for its protective effect. ATP III dietary recommendations for patients with MetS are in line with the general recommendations. According to the ATP III [11], a healthy eating model should have a fat intake of 25-35% of the total calories, with a proportion of SFA <10%, PUFA <7% and a maximum percentage of 20% of MUFA. In this model, the intake of SFA and cholesterol is low and most of the calories come from foods of plant origin. Among the MUFA present in the diet, oleic acid (cis C18: 1 n-9) is the most consumed and 85% of the intake of this fat comes from olive oil [11, 161].

- Physical Activity

Physical activity plays a pivotal role in the treatment of MetS. A number of studies have described how cardiorespiratory exercise is able to modulate the relationship between MetS and cardiovascular events, providing a strong protective effect against cardiovascular mortality [162]. The amount and intensity of physical exercise regulate the circulating levels of lipids and other metabolic abnormalities responsible for cardiovascular risk [163]. In addition, when physical activity is combined with diet, the healthy effect is greater, compared with studies in which either approach is used alone [144, 164].

- Pharmacological Approach

The National Institute of Health's current guidelines for the treatment of obesity recommend pharmaceutical therapy for weight loss for individuals with a BMI of 30 kg/m^2 or over and in the case of patients with a BMI of 27 kg/m^2 or more with comorbidities linked to their excess weight. There are two main types of pharmacological approach to weight loss: appetite suppressants and antinutrient inhibitors. One single drug is generally recommended and average weight losses range widely, from 5% to 10% of the initial weight [165]. Appetite suppressants include phentermine derivatives and sibutramine, which are usually taken in the late morning and reduce appetite in the late afternoon and evening. In randomized clinical trials, treatment with Orlistat in obese persons with T2DM at baseline led to an improved glycemic control and a weight reduction of 6% over 1 year, versus 4% weight loss with the placebo [166]. However, the main problem with these currently available anti-obesity drugs is a relatively high rate of adverse side effects which lead to poor tolerance and compliance in long-term use.

In conclusion, persons with overweight or obesity need to reduce their body weight by 7%-10% over a period of 6 to 12 months. Weight reduction should be combined with a daily minimum of 30 minutes of moderate-intensity physical activity. Nutritional therapy calls for low intake of saturated fat and low total fat intake, reduced consumption of simple sugars and high glycemic index foods, and increased intakes of fruit, vegetables, legumes and whole grains. Metformin, thiazolidinediones and acarbose will lower the risk of T2DM in subjects with IFG or IGT.

2. INSTESTINAL MICROBIOTA

2.1 The human gastrointestinal tract and its interaction with the host

The complex microbial ecosystem in the gastrointestinal tract (GIT) is known as the gut microbiota. The gut microbiota consists of the entire microbial community in the human gut including bacteria, yeast, fungi, Archaea and viruses, comprising a total of 1013-1014 microorganisms [167]. The human intestinal microflora is estimated to contain 500 to 1000 species and the size of the population is ~10 times greater than the total number of our somatic and germ cells [168]. However, it is highly probable that 99% of the bacteria come from about 30 or 40 species.

Humans consume food and drink daily, most of which the body utilises for energy, and a number of different microorganisms reside on these foods, which are passed down the GIT with the food. The food is consumed through the mouth, where it is chewed and swallowed, before passing through the esophagus down to the stomach, where digestive enzymes such as pepsin are released, as well as hydrochloride acid, which causes the pH to drop. The food content passes through the different sections of the small intestine, the duodenum, the jejunum, and continues on to the ileum, where the pH rises as bicarbonate is released. During this passage, more digestive enzymes are released, resulting in the cleavage and absorption of carbohydrates, proteins and lipids. Finally, the luminal content is passed to the colon, which is divided into ascending, transcending, descending and sigmonal sections [169].

On the other hand, the diversity of microorganisms varies along the GIT [170] and depends on the morphological and physiological characteristics of each part of the digestive system. The further we move along the GIT, the greater the quantity and diversity of the microorganisms [171] **(Figure 3).**

The environment in the GIT is generally anaerobic, but environmental conditions such as pH, transit times and the availability of nutrients or substrates change throughout the GIT. This has an effect on the microbial ecosystem in the gut, leading to varying numbers of microorganisms which accumulate at the colon [172]. Besides being the place where food is processed and nutrients absorbed, the GIT is also the largest immune organ of the body and, positioned at the interface with the external environment, it constitutes an important barrier against ingested pathogens and toxins. In addition, dysregulation of intestinal epithelial cell performance is linked to an array of different pathologies [173].

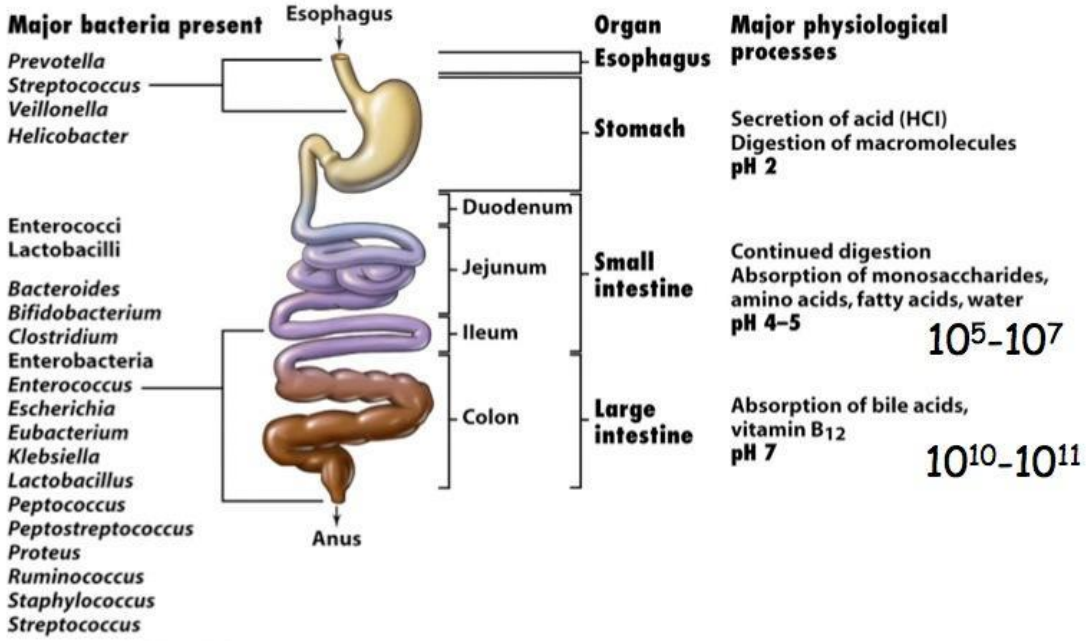

Figure 3. Variations in number of bacteria and their composition along the length of the gut.
Modified from [174]

The bacteria in the gut reside there due and feed on the available substrates, but their presence is also based on natural selection by the host [175]. This has led to a symbiotic relationship between host and the gut microbiota which brings mutual benefits [173]. The host benefits from the bacterial community in the GIT, as it, among other tasks, degrades otherwise indigestible substrates for the host. The host may subsequently use the produced metabolites. In addition, the gut microbiota protects the host from pathogenic bacteria by reducing the space and the availability of substrates [176].

The gut microbiota is mainly represented by a limited number of bacterial phyla, as *Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia* and *Actinobacteria*, with *Firmicutes* and *Bacteroidetes* as the dominant phyla, [168, 177]. Within the *Firmicutes* phyla, the main genera found are *Faecalibacterium*, and *Roseburia* [178]. These genera include species that are able to produce butyrate, as they belong to specific *Clostridium* clusters [168]. Besides the butyrate-producing bacteria, the *Firmicutes* also include the *Lactobacillus* genus, of which specific strains, as well as *Bifidobacterium* (phylum *Bacterioidetes*), are used as probiotics [179].

In a healthy colonic microbiota, the most predominant phyla are *Firmicutes* and *Bacteroidetes*, whereas the phylum *Proteobacteria* is less abundant. At the genera level, *Bacteroides, Prevotella* (phylum *Bacteroidetes)* and *Ruminococcus* (phylum *Firmicutes)*

are the most abundant, followed by the genera *Bifidobacterium* (phylum *Actinobacteria),* *Eubacterium* and *Peptostreptococcus* (phylum *Firmicutes) [178]*. In addition, the genus *Clostridium* is well-represented, and in lesser proportions, there are populations of facultative anaerobic or aerotolerant bacteria such as *Enterococcus, Lactobacillus* and *Streptococcus,* which are essential for microbial homeostasis in the large intestine, all of which belong to the phylum *Firmicutes [180-182]*.

The collective genome of these microorganisms (the "microbiome") exceeds the size of the human nuclear genome by two orders of magnitude, contributing a broad range of biochemical and metabolic functions that the host could not otherwise perform [183]. Furthermore, unlike its host's genome, the collective genome of the microorganisms can dynamically change the configuration of its components to adapt to the needs of its individual constituents, of the community as a whole and of the host, whose environment varies widely in response to factors such as dietary nutrients, illness and antibiotic use.

The gut microbiota is essential for intestinal development, homeostasis and protection against pathogenic challenges, to such an extent that some researchers refer to it as an "extra organ" of the host [184]. In particular, gut microbes are involved in metabolic reactions, such as the fermentation of non-digestible dietary fiber (resistant starch, some oligosaccharides), the harvest of energy ingested but not digested by the host, the biotransformation of conjugated bile acids, the degradation of oxalate-based complexes and the synthesis of certain vitamins (such as B12 and K); they also have trophic effects on the intestinal epithelium, by favouring the development of intestinal microvilli and play a fundamental role in the maturation of the host's innate and adaptive immune responses [167, 185].

The metabolic function of the intestinal microbiota is essential for the host, and one of the main functions of gut microbiota is controlling the host's metabolism.

In the colon, the microbiota ferments carbohydrates (CHO) of vegetable origin which cannot be digested by the digestive enzymes of mammals and escape proximal digestion. The main end products resulting from the CHO catabolism of intestinal microbes are acetate, propionate and butyrate. Lactate, despite not being a short chain fatty acid (SCFA), is also produced by certain members of the microbiota, such as lactic acid bacteria, *Bifidobacteria* and *Proteobacteria*, but under normal physiological conditions it does not accumulate in the colon due to the presence of species like *Eubacterium hallii*, which can convert lactate into different SCFAs [186].

Acetate is the most abundant SCFA in the colon and makes up more than half of the total SCFA detected in feces [187]. Two main metabolic routes have been described for acetate production by the gut microbiota **(Figure 4).** Most acetate is produced by most enteric bacteria as a result of CHO fermentation, but approximately one-third of colonic acetate comes from acetogenic bacteria, which are able to synthesize it from hydrogen and carbon dioxide or formic acid through the Wood–Ljungdahl pathway [188, 189].

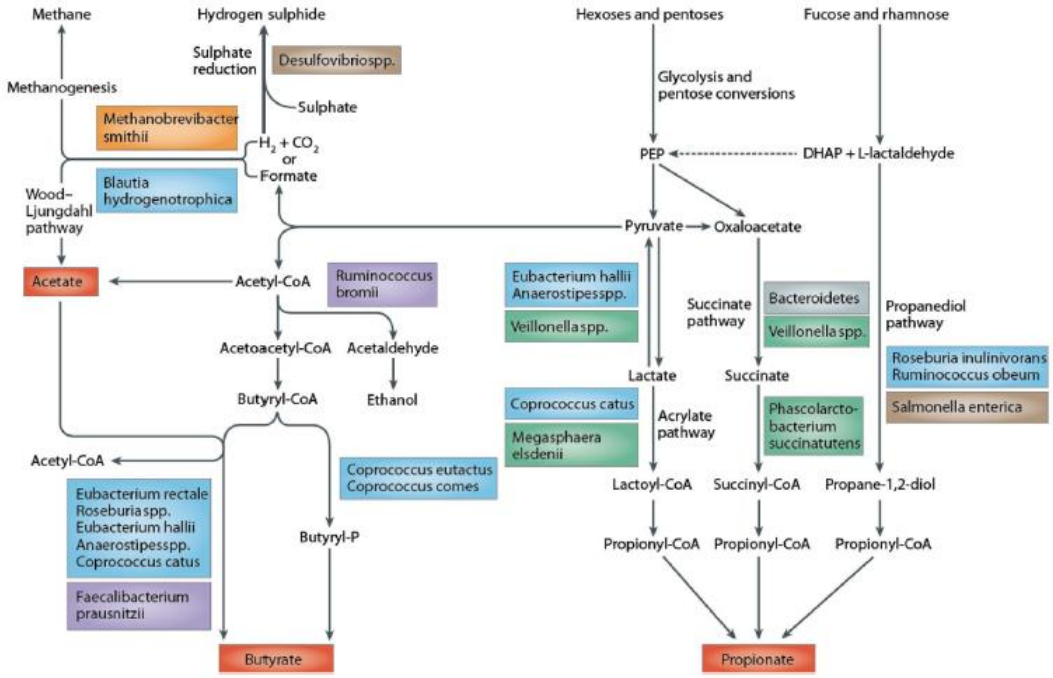

Figure 4. Schematic representation of microbial metabolic pathways and cross-feeding mechanisms, contributing to SCFA formation in the human gut. Modified from [190]

Propionate and butyrate metabolism have received much attention over the last few years, mainly due to the connection between low levels of butyrate, propionate bacterial producers and certain diseases in which inflammatory processes are involved. For instance, butyrate producers are normally low in ulcerative colitis [191] and lower levels of propionate producers have been detected in children at risk of asthma [192].

One of the health effects attributed to the production of SCFA is the concomitant reduction of the luminal pH, which inhibits pathogenic microorganisms and increases the absorption of some nutrients by itself [193]. Acetate has been found to be a key player in the ability of *Bifidobacteria* to inhibit enteropathogens [194]. Moreover, butyrate fuels the intestinal epithelial cells and increases mucin production, which may result in changes on bacterial adhesion [195] and improved tight-junction integrity [196]. Thus, the

production of SCFA seems to play an important role in the maintenance of the gut barrier function.

After their production, SCFA will be absorbed and used in different biosynthetic routes by the host [197]. During the process of intestinal absorption, part of the SCFA, mainly butyrate, will be metabolized by the colonocytes [179] while the rest will be transported by the hepatic vein to the liver, where they will be metabolized [186]. These SCFA will enter diverse CHO and lipid metabolic routes; propionate will be mainly incorporated into gluconeogenesis, while acetate and butyrate will be mostly passed into lipid biosynthesis.

SCFAs also control gene expression through the inhibition of histone deacetylase enzymes by butyric acid and metabolic regulation through the G protein-coupled receptors GPR43 and GPR41 (also known as FFA2 and FFA3, respectively). Acetate and propionate are the most potent activators of GPR43, while GPR41 is activated in the following order of affinity: propionate> butyrate> acetate. The activation of GPR43 by SCFAs in neutrophils leads to the suppression of inflammation, while its activation in the L cells of the small intestine and colon stimulates the secretion of glucagon-like peptide 1 (GLP-1). In addition, the gut microbiota also activates GPR41 through the production of SCFA. When this activation occurs in L cells of the intestine, it induces the secretion of peptide YY. Both GLP-1 and peptide YY decrease appetite and energy intake. Additionally, butyrate and propionate induce the expression of leptin in the AT, and so also regulate appetite [198].

The intestinal microbiota also plays a crucial role in the metabolism of bile acids (BA). The BA pool size and composition are inherently linked with the presence, and presumably, composition of the microbial community [199]. BA are amphipathic biological detergents that function mainly in lipid metabolism but also serve a wide range of regulatory functions throughout the body [200]. The primary BA, chenodeoxycholic acid (CDCA) and cholic acid (CA) are synthesized from cholesterol in the liver, in a process involving at least 14 enzymes[201].

In the liver, primary BA are conjugated into either glycine (predominantly in humans) or taurine. Conjugated BA subsequently accumulates in the gall-bladder [200]. Following food intake, the gall-bladder releases BA into the duodenum, where they contribute to the solubilization of ingested lipids, as the bile acids proceed through the small intestine and colon [201, 202]. BA are then reabsorbed in the distal ileum, primarily through active

transport by the apical sodium-dependent bile salt transporter or the ileal bile acid transporter. Typically, this results in a ∼50 to 90% recovery of conjugated BA.

BA that are not reabsorbed can serve as substrates for microbial metabolism and undergo biotransformation to secondary BA [202]. The predominant secondary BA, formed via 7α-dehydroxylation by gut bacteria, are deoxycholic acid (DCA), formed from CA, and lithocholic acid (LCA), formed from CDCA. The secondary BA bind to the G protein-coupled receptor, the TGR5 receptor, in intestinal L-cells and induces the secretion of glucagon-like peptide 1, which increases insulin. Meanwhile, TGR5 receptor activation in muscle and adipose tissue increases energy expenditure [201, 203].

On the other hand, an early study has already contributed to the hypothesis that gut bacteria may play an important role in host amino acid homeostasis and health by showing that germ-free mice had an altered distribution of free amino acids along the GIT compared with normal mice [204]. The most abundant amino acid-fermenting bacteria in the human small intestine are bacteria belonging to the *Clostridium* clusters, the *Bacillus-Lactobacillus-Streptococcus* groups and *Proteobacteria* [205]. In the large intestine of healthy humans, bacteria belonging to the *Clostridia* and *Peptostreptococci* appear to be the most prevalent species involved in amino acid fermentation [205, 206]. Moreover, the diet includes protein and peptides, which can be hydrolyzed to amino acids by the proteases and luminous peptidases, and the amino acids derived from dietary proteins can serve as substrates for luminal bioconversion by the intestinal microbiota. Interestingly, amino acids can also serve as precursors for the synthesis of SCFA by bacteria [206], suggesting an interplay between microbial activity, host amino acid and SCFA homeostasis.

In addition, the gut microbiota has been shown to be an important factor in supplying aromatic amino acids and branched-chain amino acids (BCAAs) including leucine, phenylalanine, isoleucine, and valine to mammalian hosts [207]. BCAAs are, in fact, related to IR, as has been confirmed in a cross-sectional study of sedentary MetS subjects, using the frequently sampled glucose tolerance test to measure insulin sensitivity [208], and in cohorts of Chinese and Asian-Indian men in Singapore, in which BMI was matched at around 24 [209].

Another mechanism for immunomodulation by microbiota is the capacity to regulate the production of mucins from intestinal goblet cells. Mucins may directly limit intestinal infections by adhering to pathogens and protect against acid gastric and duodenal secretions. The mucus also provides a medium in which bacterial-derived metabolites

with signaling functions are secreted and concentrated. Thus, the mucus layer may promote mutualism by keeping bacteria at bay and restricting overt immune stimulation while facilitating host-commensal or commensal-commensal cross-talk through the diffusion of bacterial products [210].

In addition, the gut microbiota also contributes to maintaining the proliferation and differentiation of intestinal cells, which are required to preserve the structure of the intestine. Moreover, the gut microbiota plays an important role in the defence barrier of the intestinal mucosa, thus avoiding the colonization of other pathogenic microorganisms [211] and acting like a protective organ against invasive pathogens.

2.2 Endotoxemia

The gut microbiota is in close contact with the mucosa, and so, together with its products, it necessarily affects the host, and vice-versa. The mucus associated bacteria must of course be in closer contact with intestinal epithelial cells (IEC), than the bacteria which simply resides in the lumen mucus-associated bacteria may therefore be very important for stimulation of the host immune system [212], as well as intestinal integrity. However, bacteria residing in the lumen may also have a part to play. Interaction between gut microbiota and IEC is therefore important for intestinal integrity [213], this interaction is based on a balanced system, in which the host must not induce inflammation upon contact with commensal bacteria, as that would result in a continual inflammatory state, leading to impaired intestinal integrity. However, the host must induce an immune response when faced with bacterial invasion, and the host cells therefore need to differentiate between bacteria in the gut microbiota.

The epithelial layer is the final barrier between the luminal content and the host. It is therefore absolutely essential to maintain this barrier, and an imbalance in barrier function has been linked to diseases such as irritable bowel syndrome (IBD) or Celiac disease [214, 215].

The term ‘intestinal integrity’ is quite often used in connection with intestinal permeability. Although these two terms are closely related, they are not the same thing. Intestinal integrity is defined as ‘maintaining the intestinal barrier whole and assembled’, which occurs through the mucus layer, the epithelial cells and the connection between the Tight junction proteins (TJs) [216]. The interaction with IEC is important for maintaining intestinal barrier integrity. IEC interacts through TJs, desmosomes, adhesion junctions and gap junctions **(Figure 5**). On the other hand, intestinal permeability is considered to

reflect the passage of molecules across the IEC layer, which can be affected both by the epithelial cells forming the barrier, as well as interactions by TJs.

Bacterial translocations are believed by some researchers to indicate impairment of intestinal integrity [198], which is connected to inflammation and gastrointestinal disorders and can cause adverse effects. Impaired intestinal integrity or altered intestinal permeability has also been connected to conditions like IBD, MetS, T2DM and obesity [217].

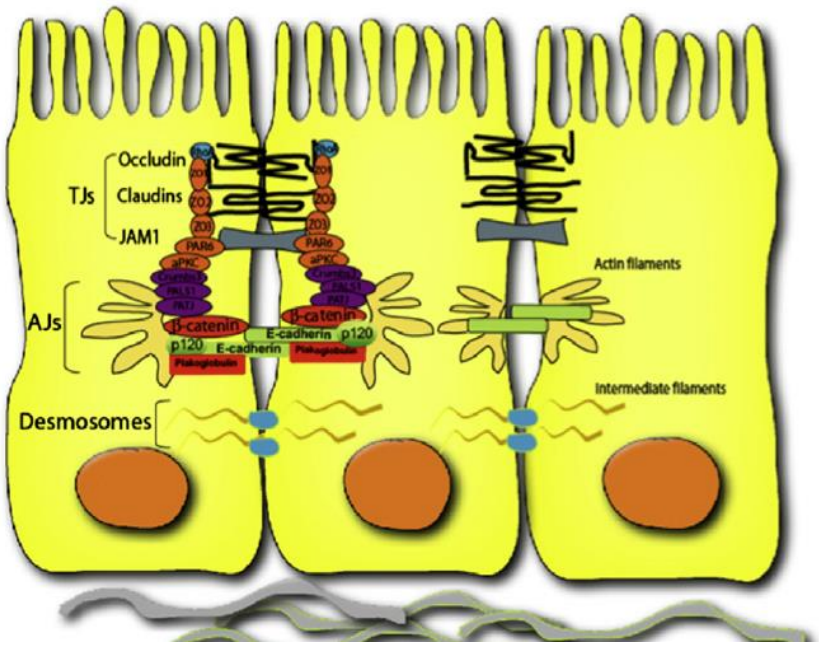

Fig. 5. Interaction between epithelial cells through tight junction (TJ), adherens junction, and desmosome. Modified from [218]

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. It is considered an endotoxin, which contributes to the structural integrity and protection of that membrane from chemical attack. Once it reaches the blood stream, it causes metabolic endotoxemia (ME), which can trigger an immune response leading to inflammation. It can also inhibit insulin signalling, contributing to IR [219]. Endotoxemia may therefore favour obesity and the onset of T2DM [220, 221]. Importantly, the gut is the main source of LPS, and it is estimated that the intestinal lumen has over 1g of LPS. Just a small dose of endotoxin in the circulatory system can cause inflammatory reactions [222].

LPS consists of covalently-bound lipid and polysaccharide. Three structurally and genetically distinct regions form this glycoconjugate: a portion called lipid A (responsible

for endotoxic activity), core oligosaccharide and the external region or *O*-antigen [223]. **See Figure 6**.

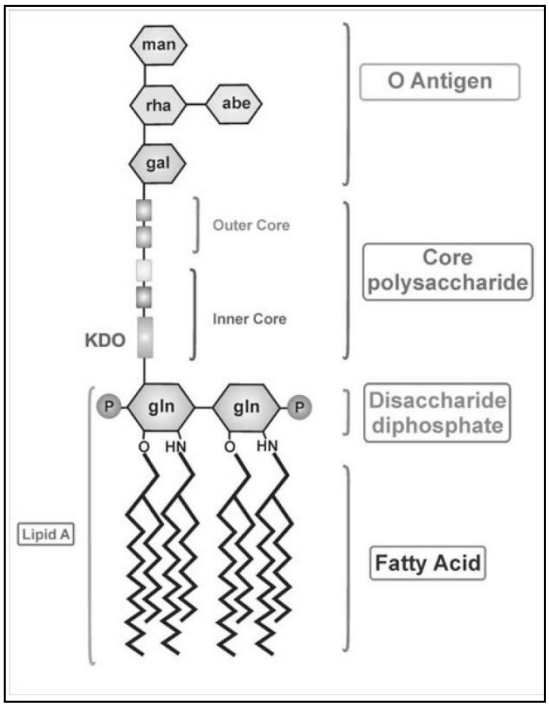

Figure 6. Schematic representation of the structure of lipopolysaccharide (LPS). Modified from [224]

Weight gain has been associated with greater gut permeability, and subsequent systemic exposure to mildly-increased LPS circulating levels [225] demonstrated that a high-fat diet promotes LPS absorption across the intestinal barrier, increasing its plasma levels two- or three-fold, a threshold defined as ME. These data are supported by previous studies which also found that higher concentrations of fatty acids impaired intestinal barrier integrity [226, 227].

It has been proposed that two mechanisms of LPS absorption [222] shown in an in vitro model of human epithelial adenocarcinoma cells demonstrate that the formation of quilomicron promotes LPS absorption. Other suggested mechanisms include LPS absorption through internalization by intestinal microfold cells [228] and enterocytes, with the involvement of TLR4 and myeloid differentiation protein-2 (MD-2) [229]. Moreover, some bacteria can induce and/or modulate the expression of genes involved in the barrier function in host epithelial cells [230]. It has been shown that the introduction of a high-fat diet in mouse models resulted in a decreased expression of genes involved in the barrier function, namely zonula occludens 1 (ZO-1) and occludin genes.

The starting point for innate immunity activation is the recognition of the conserved structures of bacteria, viruses and fungal components through pattern-recognition receptors (PRRs) [231]. TLRs are PRRs that recognize microbe-associated molecular patterns (MAMPs) [232], such as several bacterial structures of gram-negative outer membrane (e.g., LPS) and components of the gram-positive cell wall such as lipoteichoic acid or peptidoglycan [231]. TLRs are transmembrane proteins containing extracellular domains rich in leucine repeat sequences and a cytosolic domain homologous to the IL1 receptor intracellular domain (TIR domain) [233].

In mammals, TLRs are present in macrophages, neutrophils, dendritic cells (DCs), IEC and other cells belonging to innate immune system. Over the past few years, researchers have carried out various studies demonstrating that microbiota can regulate the intestinal innate immune system by modulating TLRs expression on the immunosensory cell surface through MAMPs; the recognition of microbes leads to the activation of nuclear factor-kappa B (NF-κB) signaling pathway and consequently triggers cytokine production, and up-regulation of co-stimulatory molecules on antigen presenting cells, leading to the activation of T cells [234].

The target tissues for LPS are the adipose tissue, liver and endothelium [235]. Thus, the portion responsible for endotoxic activity of LPS (lipid A) binds to the TLR-4 present in the plasma membrane. The recognition of LPS by this receptor is mediated by LBP protein, the CD14 co-receptor of TLR-4 and by the MD-2 [236]. The TLR-4 is found on the surface of both immune cells (monocytes, macrophages, Kupffer cells andpreadipocytes) and non-immune cells (adipocytes, hepatocytes and endothelial cells). **See Figure 7.**

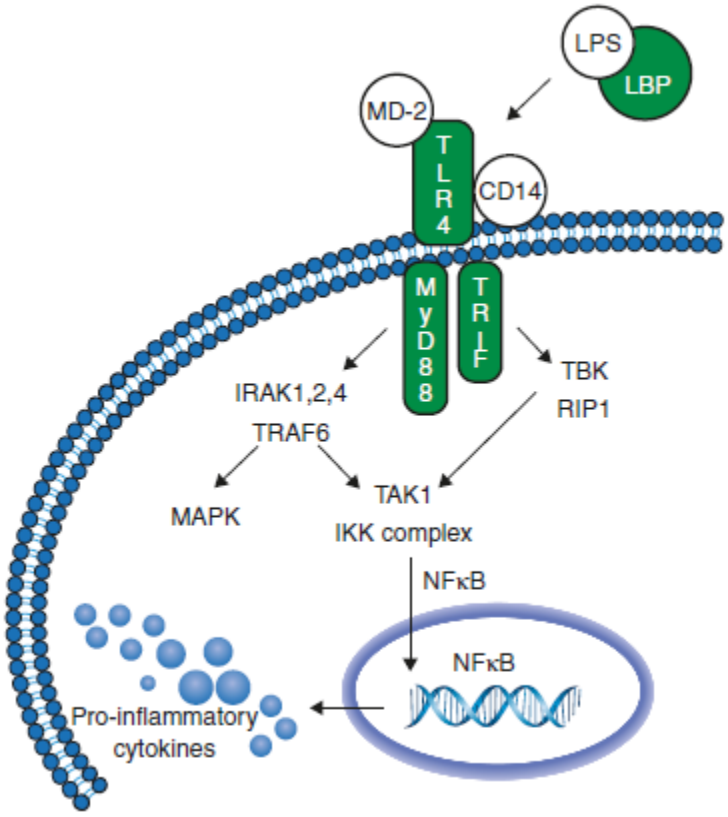

Figure 7. LPS-TLR4 signalling pathway. Modified from [237]

LPS can also be found in the plasma of healthy individuals [223, 238], since besides colonizing the gut, gram-negative bacteria are also present in the oral cavity and the respiratory and genito-urinary systems. However, clinical studies have shown that the concentrations of circulating LPS and LBP (an endotoxemia marker) are higher in type 1 or 2 diabetic individuals, and in obese subjects [239-242], thus strengthening their link with IR and metabolic diseases.

Blood samples from obese, overweight and normal weight Afro-American women were collected and stimulated in vitro with varying doses of LPS. The obese class III (BMI > 40 kg/m^2) expressed 365% more TNF-α than normal weight (BMI between 20-25 kg/m^2) women when intermediate concentrations of LPS (20 μg/mL) were tested. When the maximum expression of TNF-α was assessed independently of the LPS dose, obese class III produced 230% more than normal-weight women. The obese class I (BMI between 30-35 kg/m^2) produced 190% more TNF-α than the normal-weight group. The results of this study reinforce the effect of excess body weight on the expression of inflammation markers such as TNF-α [243].

In a study conducted by Creeley et al. [244], individuals with T2DM presented mean LPS values 76% higher than the individuals without the disease. The diabetic patients were treated with rosiglitazone and showed a reduction of 51% in insulin and 35% in LPS levels. Rosiglitazone is an agonist of the peroxisome proliferator-activated receptor (PPAR-γ), which presents anti-inflammatory properties [245]. This antidiabetic agent may attenuate inflammation, favouring the clearance of LPS by reducing insulinemia and increasing HDL bioavailability. Reductions in plasma insulin concentrations favour the function of Kupffer cells, which are partly responsible for LPS clearance [244]. When LPS interacts with HDL, the activation of TLRs is impaired [245], and individuals with low HDL levels showed a greater inflammatory response (TNF-α, IL-1β, IL-6, IL-8) after the infusion of low doses of LPS [238]. In addition, Pajkrt et al. verified that the infusion of HDL can reduce the deleterious effects of LPS [246].

2.3 Factors modifying the intestinal microbiota

The composition of microbial communities is generally considered stable in each individual [247], but different factors can modify the intestinal microbiota, such as antibiotics, diet, genus and age, among others.

- ***Antibiotics***

Numerous studies have confirmed that antibiotics have an extremely powerful impact on the composition and functionality of the human microbiota. One study documented that healthy volunteers treated for 1 week or less with antibiotics reported effects on their bacterial flora that persisted 6 months to 2 years after treatment, including a dramatic loss in diversity as well as in representation of specific taxa, the insurgence of antibiotic-resistant strains and the upregulation of antibiotic-resistant genes (ARGs) [248]. Antibiotic treatment in mice mimics the impact on and long-term shifts in human gut communities. For example, a single dose of clindamycin has been shown to induce profound changes in the composition of mouse microbiota and, consequently, to confer long-lasting susceptibility to infection by *C. difficile* [249].

Surveys on thousands of children have highlighted the link between the use of antibiotics during the first year of life and the development of asthma by the age of 6–7 [250, 251]. The early use of macrolides in Finnish children was found to generate a distinct microbial profile characterized by a loss of *Actinobacteriaceae* and an increase in *Bacteroidetes* and *Proteobacteria*, an induction of ARGs and a decrease in bile salt

hydrolases. This profile positively correlated with either a later development of asthma or an increase in body mass index [252].

Therefore, exposure to antibiotics, even for short periods of time and especially during infancy, has long-lasting effects on the microbiota, and this can predispose the host to a variety of diseases, some of which are yet to be identified. This is evidently a matter of crucial importance for public health.

- ***Aging***

The human gut microbiota alters with the aging process. In the first 2-3 years of life, the gut microbiota varies extensively in composition and metabolic functions. After this period, the gut microbiota changes to the more stable and diverse microbial species, as found in adults. However, in old age, the deterioration of physiological functions of the human body leads to an inevitable decrease in beneficial species (e.g. *Bifidobacteria*) in the gut microbiota, which, in turn, leads to a number of various gut-related diseases [253]. Moreover, another major negative consequence of aging is immunosenescence, which can be defined as a decline in the functionality of the immune system, which can cause a chronic low-grade inflammatory status in the gut. Immunosenescence can therefore cause unfavourable changes in the composition and structure of the gut microbiota in older people [254].

Studies in aging gut microbiota have revealed that the composition of microbiota in the elderly is considerably different from that of younger adults. To be exact, a very low abundance of the phylum *Firmicutes* and low general diversity were detected in elderly subjects [167, 255, 256], and this low diversity has been linked with increased health risks [257]. Another study has reported a significantly higher abundance of *Bacteroidetes*, and a lower abundance of *Clostridium* cluster IV in elderly patients compared to younger subjects [258].

At the genus level, the available studies are in general agreement, reporting an age- related increase in facultative anaerobes, including *Streptococcus, Staphylococcus*, *Enterococcus* and *Enterobacteria [259, 260]*. These gram-negative bacteria have the ability to secrete LPS, which may act as an endotoxin [261]. The enterobacteria group includes potentially pathogenic species (pathobionts), and could be the cause of infections when the host resistance mechanisms fail as a result of the ageing process. Antibiotic treatment and hospitalization are known to favor the increase of enterobacteria in the microbiota of elderly people [262].

Changes in microbial composition occur with a high degree of variability at the two extremes of life, childhood and old age, while they are characterized by relative stability in adulthood. It is believed that during birth, the infant gut contains no microbial community at all, or at least very few species. Subsequently, the gut microbiota alters drastically in the first two years of life, during the different stages such as colonization, development and maturation (**See Figure 8).** Along with the factors described above, there are more factors such as mode of delivery (vaginal or cesarean), exposure to antibiotics, feeding method, hospital environment and use of prebiotic/probiotic, which play significant roles in the variability of microbial community in the first few years of life [253].

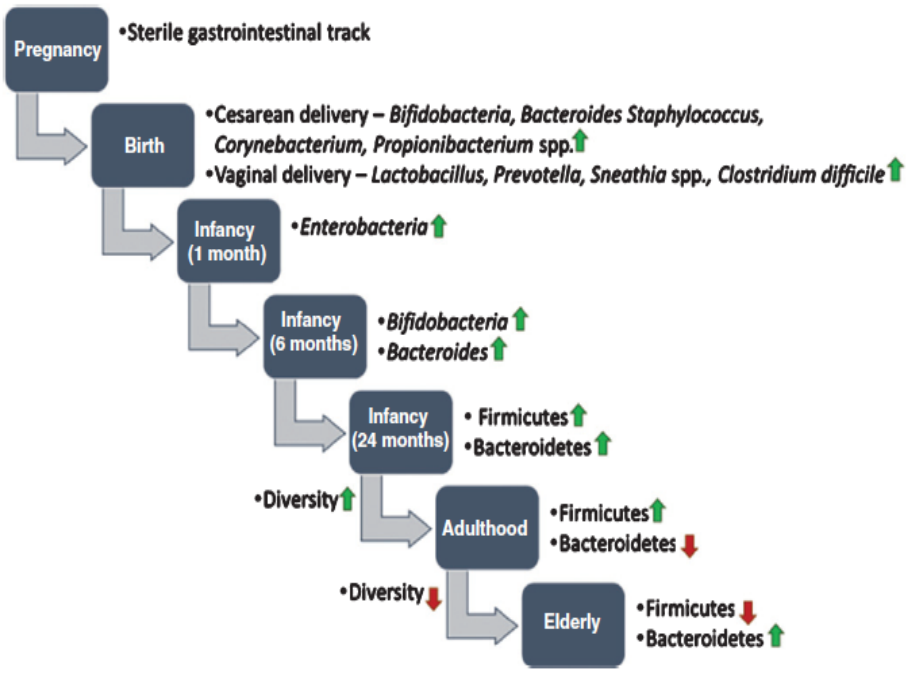

Figure 8. Development of human gut microbiota from pre-birth to old age. Modified from [253]

- ***Gender***

There is increasing evidence that sex steroid hormone levels are associated with the human gut microbiome [263]; however, there have been no comprehensive studies into how the factors associated with these sex steroids are linked to the human gut microbiome. A recent animal study reported sex-specific differentials in gut microbiome composition [264]. Adiposity, a source of sex steroids [265], may play an additional role in forming a sex-specific microbiome composition, as suggested by animal models and in human weight-loss trials [266, 267].

In an American study, it was found that *Bacteroidetes* showed a greater abundance in men than women [268]. A cross-sectional study in European countries also showed that the *Bacteroides-Prevotella* group was more prominent in males than in females [269]. *Bacteroides thetaiotaomicron* was also identified as being higher in men than women in a Chinese population [255]. Moreover, male mice had lower bacterial diversity [270], and higher *Parabacteroide*s belonging to *Bacteroidetes* than females, which is consistent with the above results of our clinical trial [264].

In another study, sex differences were identified in the gut microbiota of obese people with different BMIs [271]. They observed that the abundance of the *Bacteroides* genus was lower in men than in women when the BMI was < 33. In fact, the abundance of this genus decreased in men, the higher the BMI, whereas in women it remained unchanged in the different ranges of BMI. They also observed that the abundance of *Bilophila* was lower in men than in women, regardless of BMI [271].

- ***Diet***

Diet is a major factor in the composition and metabolism of the colonic microbiota. The amount, type and balance of the main dietary macronutrients (carbohydrates, proteins and fats) have a great impact on the large intestinal microbiota [272].

In general, it has been shown that diets rich in fats and proteins increase the abundance of species of the genus *Bacteroides,* while diets high in carbohydrates increase the abundance of the genus *Prevotella*. Furthermore, it has been observed that the consumption of diets rich in vegetables, fruit and fiber increases the microbial richness [178, 273, 274]. In this thesis, we have included a special section to address this issue in depth (*Section 4: Modulation of the intestinal microbiota by diet*).

- ***Others***

Other microbial factors include the metabolic activity of the commensal microbiota itself, which can alter the conditions of the ecosystem in terms of pH, oxygen, and so on, as well as the generation of metabolites and other substances by the gut microbiota, which may promote or prevent the growth of other species (nutrients, growth factors, etc.) [182, 230, 275].

They also include the production of stomach acid, pancreatic secretions and bile salts, which will ultimately determine the pH level of the mucose, the production of hydrolytic

enzymes such as lysozyme and trypsin, the production of antimicrobial substances such as defensins, intestinal motility and mucus production, which will determine the conditions of the habitat to be colonized [230].

3. INTESTINAL MICROBIOTA AND CARDIOMETABOLIC DISEASE

The normal intestinal microbiota plays a major role in staying healthy and preventing disease. In fact, the alteration of the intestinal microbiota has been shown to contribute to the pathogenesis of several pathological conditions, including obesity, MetS, T2DM and insulin resistance, among others.

3.1 Obesity

A recent body of evidence reports that, in obesity and metabolic disorders, various phylogenetic signatures exist in gut microbiota and genes within the gut microbiome. In addition, the metabolic activity of the so-called obese, metabolically-impaired microbiome is seen to vary significantly from its lean counterpart [185, 276]. Thus, gut microbiota can influence the functions in the host relating to the energy harvest and expenditure, fat storage and eating behaviour. Interestingly, germ-free (GF) animals are shown to be resistant to high fat diet (HFD)-induced obesity [277].

The earliest finding was published by Gordon et al, who put forward the role of gut microbiota in obesity disorder [278-280]. They showed, in a series of experiments on mice, that animals with gut microbiota gained more weight and expressed the increased accumulation of TG into adipocytes, enhanced monosaccharide uptake from the gut and enhanced fatty acid (FA) oxidation in muscle and liver compared to the GF mice after an eight-week HFD intervention [278]. In addition, GF mice showed a permanent hepatic and skeletal expression of the adenosine monophosphate (AMP)-activated protein kinase (AMPK), which stimulates the catabolic (ATP-producing) pathways under stressful conditions, thus monitoring the energy status of the cell. The absence of AMPK in GF mice may at least partly explain their resistance to HFD-induced obesity and IR [277, 278]. Furthermore, the presence of the gut microbiota suppressed intestinal expression of fasting-induced adipose factor (Fiaf), which is a glycosylated protein produced by the enterocytes, adipocytes and hepatocytes [281]. The gut microbiota regulates the expression of Fiaf inhibition, which, in turn, triggers lipoprotein lipase (LPL) in the adipose tissue, which further leads to reduced triglyceride and FA accumulation [281].

Other studies have observed, in genetically obese mice (ob/ob mice), compared to their lean counterparts, a 50% reduction in the abundance of the phylum *Bacteroidetes*

and a proportional increase in the phylum *Firmicutes* [279]. These specific changes in ob/ob mice suggest that these mice present an intestinal microbiota with a greater ability to extract energy from dietary nutrients. This fact was confirmed by transplanting stools from ob/ob mice to normal weight mice: the latter developed obesity in two weeks [279].

Data from human studies were generally consistent with the results from animal models, e.g., 12 obese subjects had lower *Bacteroidetes* and more *Firmicutes* in their distal gut than the lean control subjects. After randomization to either carbohydrate-restricted or fat-restricted diets for 52 weeks, the proportion of *Bacteroidetes* increased over time, mirroring reductions in the host's weight, but not dietary changes [280].

A subsequent metagenomic study [276] with 154 individuals including monozygotic and dizygotic twins, concordant for leanness or obesity, and their mothers, also showed that obesity was associated with a markedly reduced bacterial diversity, a relative depletion of *Bacteroidetes*, and a higher proportion of *Actinobacteria* compared with leanness.

Another example of the role of microbiota in obesity has been seen in patients undergoing a Rouex-en-Y gastric bypass. After surgery, the patients observe a dramatic metabolic improvement that cannot be explained by the caloric restriction and the weight loss alone. Changes in the gut microbiota have been shown to play a role in this improvement, as a shift in bacterial population has been observed in a number of studies [282-285]. In order to demonstrate the role of bariatric surgery in the changes of the gut microbiota, Liou et al. showed that fecal transplantation from RYGB-treated mice into germ-free mice led to weight loss and decreased fat mass in mice [286].

Other relatively minor studies examined gut microbiota composition in human obesity and T2DM and the impact of weight reduction on microbial flora. Although generally confirming the above findings, the results were more heterogeneous, due to different methodologies and the complexity of our modern human lifestyle as compared with experimental animal models, where all potential confounding factors, including the frequency and composition of meals, can be precisely controlled [172, 198, 256, 281, 282].

One of the mechanisms through which gut microbes contribute to increased energy absorption seems to be the production of SCFAs, resulting from the hydrolysis and the fermentation of dietary polysaccharides. SCFAs, such as propionate, butyrate and acetate, could be absorbed and used as source of energy, but seem to exert more complex metabolic functions influencing the host's appetite [287], intestinal transit time [288],

energy absorption and energy harvest [289]. For example, SCFAs increase intestinal absorption of monosaccharides stimulating the expression of sodium/glucose transporter-1 [288, 290].

SCFAs also contribute towards modulating the host's appetite and food intake, interacting with G-coupled proteins expressed by enteroendocrine cells and promoting the release of glucagon-like peptide-1 (GLP-1) and peptide YY, which directly influence the host's satiety [290]. Moreover, SCFAs influence lipid metabolism by increasing lipogenesis [291] and inhibiting fatty acid oxidation [292], as previously reported.

Other possible mechanisms have also been proposed. For example, a high-fat diet has shown to increase the proportion of gram-negative species in the gut microbiota, thus contributing to an increased intestinal absorption of bacterial fragments, such as LPS. As a consequence, the increased levels of circulating LPS lead to a condition defined as "metabolic endotoxemia" [293], in which blood LPS levels are lower than those observed in septic shock. The experimentally induced endotoxemia in mice leads to body weight gain, fasted hyperglycemia and hyperinsulinemia, similar to that observed in high-fat-fed mice [293]. Increasing evidence suggests that a high-fat diet promotes changes in gut microbiota composition, but the subsequent development of the obese phenotype occurs only in the presence of metabolic endotoxemia [294].

Another mechanism involved in the regulation of gut ecosystem homeostasis is the endocannabinoid system. In fact, endocannabinoid receptors expressed in the gut (eCB1) interact with bacterial LPS, modulating gut permeability, LPS translocation and inducing metabolic endotoxemia [295].

In conclusion, evidence emerging from studies conducted on animal models has confirmed the pathogenic role played by the gut microbiota in the development of obesity.

3.2 Metabolic syndrome (MetS)

The MetS is normally associated with obesity. The onset of obesity is a complex process that involves genetic and environmental factors and is often associated with the development of a number of chronic complications[1]. The gut microbiota plays a significant role in obesity, T2DM, plasma lipids metabolism and hypertension, and, therefore, in the pathogenesis of the MetS [282, 284, 286, 296, 297].

A recent study with the CORDIOPREV cohort [298] found lower *Bacteroides*, *Eubacterium* and *Lactobacillus* in patients with MetS, compared with the control group without MetS. In addition, *Bacteroides fragilis*, *Parabacteroides diastonis*, *Bacteroides*

thetaiotaomicron, Faecalibacterium prausnitzii, Fusobacterium nucleatum, Bifidobacterium longum, Bifidobacterium adolescentis, Ruminococcus flavefaciens subgroup and *Eubacterium rectale* were depleted in MetS patients [298].

The most striking evidence for the involvement of the gut in the MetS comes from the effects of weight-reducing (bariatric) gastrointestinal surgery [299, 300]. Bariatric surgery was originally based on two general principles: restricting the capability for food intake (e.g. gastric bands creating a resistance for luminal flow) and creating malabsorption by bypassing a portion of the intestine (gastric bypass). Today, the commonest procedures are the Roux-en-Y gastric bypass and vertical sleeve gastrectomy [301]. The Roux-en-Y gastric bypass (RYGB) results in rapid weight loss, reduced adiposity and improved glucose metabolism. These effects are not simply attributable to decreased caloric intake or absorption, and the mechanisms linking rearrangement of the gastrointestinal tract to these metabolic outcomes are largely unknown. Studies have revealed that alterations to the gut microbiota after RYGB are conserved among humans, rats, and mice, resulting in a rapid and sustained increase in the relative abundance of *Gammaproteobacteria* (*Escherichia*) and *Verrucomicrobia* (*Akkermansia*). These changes were independent of weight change and caloric restriction, were detectable throughout the length of the gastrointestinal tract and were most evident in the distal gut, downstream of the area where the surgery was carried out [286].

Zhang et al. [284] found that the reduction of gastric acid and the modification of the total length of the small bowel contributed to the growth of facultative anaerobes, with a significant increase in *Gammaproteobacteria*. On the other hand, *Firmicutes,* and in particular, methanogens bacteria, which seem to contribute to increased energy extraction from fermentation of polysaccharides in obese subjects, decrease dramatically after RYGB [284].

The direct transit of carbohydrates to the small intestine, without prior exposure to gastric acids, promotes the growth of *Proteobacteria* and *Enterobacteria,* which ferment complex carbohydrates [302]. It is well-known that the increased production of metabolites deriving from oligosaccharide fermentation contributes to increased GLP-1 and peptide YY production, which, in turn, contributes to reducing appetite and improving beta-pancreatic cell function and insulin secretion [303].

The increase in the *Bacteroides-Prevotella* group was also observed after weight loss promoted by RYGB, together with an increase in the *Faecalibacterium prausnitzii* species, which is directly linked to the reduction in low-grade inflammation [304].

One characteristic of MetS is low-grade inflammation, which manifests itself in high pro-inflammatory gene expression. The gut microbiota controls the inflammation through the TLRs and Nod-like receptors (NLRs). Both TLRs and NLRs recognize a wide variety of microbial components and allow the innate immune system to tolerate a wide variety of bacteria, viruses, fungi and parasites [234]. This concept was first suggested by Cani et al. (2007), who showed that obesity can result in the loss of epithelial barrier function, leading to receptor TLR4 activation by the bacterial product LPS, which can, in turn, result in the release of cytokines that promote IR. As outlined below, our results show that alterations in the gut microbiota can, in fact, be a primary cause of obesity and other aspects of the MetS [293].

3.3 Prediabetes

As was explained in *Section 1,* the PreDM is an intermediate step between a subject with normal levels of glucose and a subject with T2DM. Subjects with PreDM may therefore constitute a high-risk subgroup in the population with MetS. Recent evidence suggests that gut microbiota is involved in PreDM, although its role is as yet unknown. For now, only a few studies have revealed the role of the microbiota in the pathogenesis of PreDM [11, 305, 306].

A study with Xylooligosaccharide supplementation in 40 subjects with PreDM identified associated bacterial taxa and PreDM. Among them, the abundance of the genera *Enterorhabdus, Howardella,* and *Slackia* was greater in Pre-DM. In addition, the abundance of the species *Blautia hydrogenotrophica* was lower in pre-DM subjects [307].

Another study involving 52 Chinese patients with PreDM given treatment with acarbose or placebo, demonstrated that the diversity and composition of the gut microbiota changed significantly during acarbose treatment in PreDM individuals. After consumption of acarbose, the abundance of *Lactobacillus* and *Dialister* increased in the subject, while *Butyricicoccus, Phascolarctobacterium,* and *Ruminococcus* were inhibited [306].

In addition, a recent study suggests that changes in human gut microbiota may be involved in the progression of glucose intolerance and insulin resistance [11]. In a Chinese population, it was observed that *Bacteroides* and *Clostridium* undergo marked modulations in the progression of glucose tolerance and insulin resistance [308]. In line with this, it was seen that *Akkermansia muciphila* and *Faecalibacterium prausnitzii* had

a greater abundance in the Normoglucemia group than in the PreDM group. At genus level, the abundance of *Bacteroides* in the T2DM group was only half that of the NGT and Pre-DM groups.

Moreover, another study in a Russian population showed low levels of *Verrucomicrobia* was associated with glucose intolerance [305]. In addition, the presence of *Blautia* and *Serratia* was lower in PreDM than in T2DM patients, and even lower in those with normal glucose tolerance.

In male African-American veterans with differences stages of glucose tolerance, it was detected that the relative abundance of *Bacteroidetes* was lower and *Firmicutes* greater in PreDM vs normal glucose tolerance. In addition, the *Bacteroidia/Clostridia* ratio was twice as high in subjects who maintained normoglycemia, compared with those who remained PreDM, suggesting the beneficial profile of a higher ratio. At the family level, the relative abundance of *Ruminococcaceae* and *Veillonellaceae* (both of the Clostridia class) was higher in PreDM compared with normoglycemia groups. This study showed that *Veillonellaceae* was more abundant in PreDM vs normoglycemia, and also found that *Streptococcaceae* was more abundant in high vs low HbA1c quartiles [309].

In conclusion, human studies have confirmed the pathogenic role of gut microbiota in the PreDM stage. Further controlled studies, in which finer taxonomic resolution of the gut microbiome is assessed, will be necessary to understand the complex interconnections between the gut microbiome, the environment and the development of PreDM and T2DM.

3.4 Type 2 Diabetes

Type 2 diabetes mellitus (T2DM) is characterized by IR and sometimes by reduced insulin production, resulting in a poor cellular uptake of glucose and high levels of blood glucose. Obesity is a major risk factor for T2DM, and the two are closely linked. Recent studies have shown that the gut microbiota plays a critical role in the regulation of development of T2DM.

Recent evidence suggests that the composition of the gut microbiota seems to be impaired (dysbiosis) in patients with T2DM compared to non-T2DM patients [310, 311], and T2DM subjects present a reduction in butyrate bacteria-producing, especially *Roseburia intestinalis* and *Faecalibacterium prausnitzii*, which are an important group of bacteria known for anti-inflammatory properties and an increase in various opportunistic pathogens, such, *Lactobacillus gasseri*, *Streptococcus mutan* and others from the

Clostridium class. On the other hand, there is a high proportion of *Proteobacterias* and an increase in the gene expression of microbiota which play an important role in oxidative stress and inflammation.

In 2010, Larsen et al.[311] analyzed the intestinal microbiota of 18 subjects with T2DM and 18 non-diabetic controls, all males between 31 and 73 years old with body mass indices ranging from 23 to 48. In this research, it was demonstrated that T2DM is associated with compositional changes in the intestinal microbiota which are mostly apparent at phylum and class levels. The relative abundance of *Firmicutes* was significantly lower, while the proportion of *Bacteroidetes* and *Proteobacteria* was somewhat higher in diabetic subjects compared to their non-diabetic counterparts. Accordingly, the ratios of *Bacteroidetes* to *Firmicutes* correlated significantly and positively with reduced glucose tolerance [311]. In addition, the intestinal microbiota among subjects with T2DM was relatively enriched with gram-negative bacteria belonging to the phyla *Bacteroidetes* and *Proteobacteria.* In addition, the main compounds of the outer membranes in gram-negative bacteria, LPS, known as potent stimulators of inflammation, can trigger an inflammatory response and play a role in the development of diabetes [312].

Akkermansia muciniphila (*A. muciniphila*) was somewhat more abundant in Chinese patients with diabetes [313]. In contrast, the abundance of *A. muciniphila* was 3,300 times lower in leptin-deficient obese mice than in their lean littermates [314]. Zhang et al [313] found lower levels of *A. municiphila* in a small number of patients with newly diagnosed T2DM (n=13).

In a study from Europe, the authors applied shotgun sequencing to study only postmenopausal female patients with T2DM. The subjects showed increases in the abundance of four *Lactobacillus* species, including *Lactobacillus gasseri, Streptococcus mutans* and certain *Clostridiales* such as *Clostridium clostridoforme,* and also showed decreases in at least five other *Clostridium* species. *R intestinalis* and *F prausnitzii*, both prototypical butyrate producers, were highly discriminate for T2DM [315]. Interestingly, when comparing patients with T2DM with a group of women with impaired glucose tolerance, an increase in energy metabolism/harvest and fatty acid metabolism could be observed in T2DM. The Chinese cohort and a Scandinavian cohort reported somewhat discrepant results; however, both cohorts were considerably different in various ways. In both cohorts, *C clostridoforme* and *Lactobacillus* species increased, whereas *Roseburia*, a major butyrate producer, decreased. These two studies are an exciting, important start

in the field of T2DM and clearly suggest that a 'gut signature' might exist and a more importantly functional analysis also revealed that a proinflammatory tone might be initiated in the intestine which could reflect the starting point of low-grade systemic inflammation, as commonly observed in T2DM [313, 315]

In conclusion, human studies confirmed the pathogenic role of metabolic endotoxemia in the development of insulin resistance and T2DM. The progressive development of glucose intolerance and diabetes is accompanied by a corresponding decrease in anti-inflammatory and butyrate-producing bacteria, as well as an increase in pathogens. Indeed, the experimental enrichment of butyrate-producing bacteria is associated with an improvement in insulin sensitivity.

3.5 Atherosclerosis and cardiovascular disease

Cardiovascular disease (CVD), with manifestations such as heart attack and stroke, is the most common cause of death and represents about 30% of deaths worldwide. Diabetes and obesity are major risk factors for cardiovascular disease. With the prevalence of cardiometabolic disorders reaching pandemic proportions, the search for modifiable causative factors has intensified. One such potential factor is the gut microbiota. In recent years, evidence has accumulated showing the association of distinct changes in gut microbiota composition with cardiovascular disease. The gut microbiota metabolizes certain dietary nutrients such as phosphatidylcholine (PC) and L-carnitine into proatherosclerotic trimethyl metabolites [316]. This may further lead to the development of atherosclerosis and CVD in obese rodents and humans. PC and L--carnitine, via the formation of TMA--N--oxide that possess trimethylamine (TMA)--moiety, take part in the development of CVD [317]. Thus, gut microbiota possesses many endocrinological functions, and may serve as an active participant in the development of atherosclerosis and CVD [318]**.**

TMAO had previously been suspected to arise from the bacterial metabolism of choline via an intermediate, trimethylamine (TMA), and subsequent hepatic oxidation via flavin monooxygenase 3 (FMO3), thus forming TMAO [319, 320]. Betaine is a known direct oxidation product of choline. PC is the major dietary source of choline in omnivores [316, 318-320], and direct ingestion of PC was shown to result in rises in choline, betaine and TMAO levels [316]. Furthermore, our studies suggest that plasma levels of TMAO showed the strongest positive correlation with CVD risk [316]. Thus, our initial

metabolomic studies suggest that the plasma levels of 3 metabolites of dietary PC and gut microbiota might be linked to CVD in humans [316].

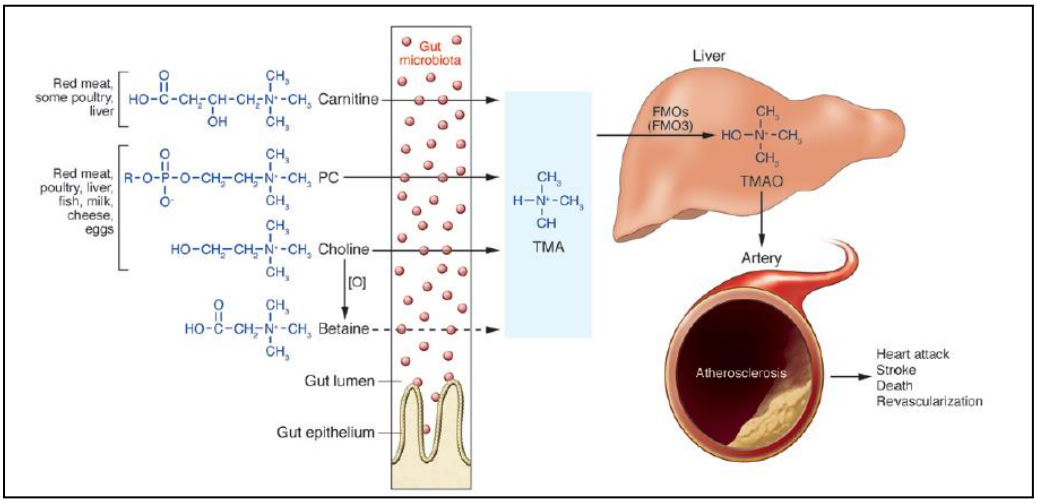

Figure 9. Nutrient/meta-organismal pathway associated with atherosclerosis and major adverse cardiovascular events. *Modified from [321]*.

Recent studies have revealed that the potential pathogenic contribution of the gut microbiota–dependent generation of TMAO may extend beyond the development and progression of atherosclerosis and its adverse complications **(Figure 9).** For example, it was recently observed that circulating TMAO levels are higher in patients with heart failure compared with age- and gender-matched subjects without heart failure [322]. Moreover, a remarkably strong adverse prognostic value was seen, associated with high plasma TMAO levels among a cohort of stable patients with heart failure (n = 720) that was incremental to traditional risk factors, cardio-renal indices (B-type natriuretic peptide and glomerular filtration rate) and markers of systemic inflammation (C-reactive protein) [322].

Studies in germ-free mice have shown that TMAO was undetectable following a carnitine or choline challenge, but following colonization of the animals with microbiota from conventional mice, germ-free mice were able to produce TMA. In addition, when conventional mice were treated with antibiotics, a reduction in TMAO production was observed [316, 323]. Similar results were reported in human studies [318, 323, 324]. It is thus well-established that the gut microbiota is intrinsically involved in TMAO production and that TMAO, and not choline and carnitine, is the pro-atherogenic compound. Interestingly, high TMAO levels have also been reported in patients with T2D [325], chronic kidney disease [324] and heart failure [326], which are all disorders associated with an increased risk of CVD. Although the way in which TMAO promotes

the formation of atherosclerotic plaques is not entirely clear, several contributing mechanisms have been identified. TMAO has been show to inhibit reverse cholesterol transport by reducing the expression of the hepatic enzymes Cyp7a1 and Cyp27a1, as well as multiple bile acid transporters, while simultaneously inhibiting the expression of intestinal bile acid transporters Npc1L1 and Abcg5/8, thus reducing bile acid synthesis and excretion by the liver and bile acid uptake in the intestine [323]. In macrophages, TMAO increases the efflux of cholesterol through increased expression of the cholesterol transporters Abca1 and Abcg1, while simultaneously increasing expression of the proatherogenic scavenger receptors, CD36 and scavenger receptor A (SRA) [316, 323].

In summary, it is clear that a link exists between TMAO levels and atherosclerosis. In the human body, atherosclerosis can lead to a number of complications, including cardiovascular disease. To understand the clinical applications of research concerning the gut microbiome, in this case microbiota-dependent metabolism of quaternary amines, it is important to explore the relationship between TMAO and cardiovascular disease.

4. MODULATION OF THE INTESTINAL MICROBIOTA BY DIET

Diet is probably the most important singular factor that shapes the gut microbiota composition, since food consumed by the host simultaneously feeds the hundreds of trillions of bacteria in the GIT [272, 273]. For example, in a study conducted with humans and 59 other mammalian species, the gut metagenomes of carnivores, omnivores and herbivores each clustered separately [327]. In addition, diet-microbiota interactions are likely to play a crucial role in the link between gut microbiota and health, since different components of the diet are known to activate different bacterial genes in the microbioma [273, 328]. It estimated that the effect of diet outweighs the effect of genetics on the gut microbioma. Almost a decade ago now, Mueller et al. studied country-related differences in fecal microbiota composition [269]. The levels of *F. prausnitzii* were higher in the Swedish study group compared to the Italian, German and French group [269], and these differences were explained by the differences in dietary habits.

Recently, the analysis of gut microbial populations has shown three main variants or "enterotypes" in adults, represented by *Bacteroides, Prevotella* and *Ruminococcus* [273]. Wu *et al.* [273] investigated the relationship between dietary and environmental variables and gut microbiota in 98 healthy subjects, in a cross-sectional study approached by 16S rDNA pyrosequencing. The study showed that *Bacteroides* "enterotype" was closely linked to animal protein and saturated fat consumption, which implies that meat intake (as in the "Western" diet for instance) actually characterized this "enterotype". In contrast, the *Prevotella* "enterotype" was linked to high values of carbohydrates and simple sugars, indicating a relationship with a carbohydrate-based diet, typical of agrarian societies, and indeed, vegetarians and vegans showed enrichment in the *Prevotella* "enterotype" too. Furthermore, the authors performed a controlled-feeding trial based on a small subject cohort (10 subjects), which was randomized, subjected to high-fat/low-fiber or low-fat/high-fiber diets and sampled over 10 days. The results showed that microbiome profiles clearly changed within 24 h of diet, while the "enterotype" identity remained stable, indicating that long-term diet is strongly related with specific "enterotypes" [273].

Of the numerous dietary components that modify gut microbiota composition, fiber is well known for its various beneficial health effects, such us the reduction of gut transit time and binding of fecal carcinogens, both of which protect the body from colon cancer

[329]. Fibers and other complex, dietary carbohydrates are indigestible in the upper parts of the GI tract and metabolized to SCFAs in the colon by the gut bacteria, which further affects host metabolism and health [330, 331]. Fermentation is possible since the colon microbiome possesses genes for the various carbohydrate-active enzymes [185, 332].

SCFAs are probably the most important metabolites that are produced in the colon via the gut microbiota fermentation process [330]. The most important SCFAs are acetate, propionate and butyrate that are produced in the ratio 60:25:15 [333]. They are all rapidly absorbed into the intestine [330]. Butyrate is one of the most commonly studied health-promoting metabolites. Members of the gram--positive *Clostridium* clusters IV or XIV are the main producers of butyrate which have various other functions such as serving as an energy source for the colonic epithelial cells, improving the integrity of the gut barrier and alleviating inflammation [334]. Therefore, changes in the relative production rates of the major SCFAs by the colonic microbiota are likely to have important physiological consequences.

Two butyrate-producing *Firmicutes*, *F. prausnitzii* and *Eubacterium rectale*, for example, are among the most abundant bacteria in the healthy colonic community [335]. Although these two species use similar routes for butyrate synthesis, relying on butyryl CoA:acetate CoA transferase [336], evidence indicates that they have distinct ecological niches. *E. rectale* and the closely related *Roseburia spp.* are flagellated bacteria with the ability to utilize a range of dietary polysaccharides, especially starch [337, 338]. *F. prausnitzii* is nonflagellated and fails to utilize many dietary polysaccharides, including starch [339]. Furthermore, the stimulation of *F. prausnitzii* by low concentrations of oxygen was not observed for the close *E. rectale* relative *Roseburia inulinivorans* [340]. In human volunteer trials, weight-loss diets low in total carbohydrate have been shown to decrease the percentage of butyrate among fecal SCFAs [341, 342], correlating with decreased populations of the butyrate-producing *Roseburia* plus *E. rectale* group [341, 343]. By contrast, *F. prausnitzii* showed little change in its representation among the fecal microbiota with low-carbohydrate diets [341]. Fecal butyrate concentrations have been shown to increase with total SCFA concentrations under conditions of more rapid gut transit [344, 345], an effect that might be mediated partly via the influence of pH on the gut community [346].

- ***Dietary pattern***

- ***Vegetarian diets*:** Studies on vegetarian/vegan diets often lack clear definitions regarding their dietary pattern. The results of these studies indicate that the microbiota of omnivores is enriched with bacteria of the *Clostridium* cluster XIVa, which are butyrate-producing bacteria [347, 348]. Furthermore, Kabeerdoss et al. [347] reported an increased gene level of butyryl-CoA CoA-transferase in the omnivore group. This indicates an enhanced capacity for the production of butyrate. However, this study was conducted in rural southern India and the dietary habits of Indian omnivores are very different to those of Western country omnivores. Therefore, it is questionable whether these results can be translated to the typical Western vegetarian/omnivorous diet.

In addition, Matijasic et al. [348] also reported increased proportions of *Bacteroides/Prevotella* group, *B. thetaiotaomicron*, *C. clostridioforme*, and *F. prausnitzii* in vegetarians.

Kim et al. [349] conducted a strict vegetarian diet intervention for 30 days in 6 obese patients with comorbidities such as T2DM and high blood pressure. The intestinal microbiota was analyzed using 454 pyrosequencing of 16S ribosomal RNA gene sequences. The results showed an increase in bacteria of the phylum *Bacteroidetes,* in *Bacteroides fragilis,* and in *Clostridium* spp. belonging to the clusters XIVa and IV, as well as a decrease in bacteria of the phylum *Firmicutes* and in pathobionts such as members of the family *Enterobacteriaceae,* followed by a reduction in fecal concentrations of small-chain fatty acids and lipocalin-2.

- ***Western diet:*** Western diet is enriched in total fat, animal proteins, n-6 polyunsaturated fatty acids and refined sugars, and it is considered as a major factor which triggers off the development of CVD [331, 350]. The diversity and richness of gut microbiota seems to decrease due to the long-term consumption of typical Western food, which includes excessively high amounts of fat (high-fat diet, HFD) and low amounts of complex carbohydrates [331]. A HFD does not provided the nutritional contents required for balanced gut microbiota functions, since most of the food is already absorbed in the upper part of the digestion system without reaching the lower parts of the small intestine and colon [331, 351]. The gut microbiota structure of Italian children (n= 15), for instance, had significantly less diversity compared to their healthy counterparts of the same age in Burkina Faso (n=14). De Filippo *et al.*, [274] compared the gut microbiota composition of European and rural African children, focusing on respect to

the contribution of the diet. This work demonstrated significant differences in gut microbiota between the two groups; in particular, African children showed a significant increase in *Bacteroidetes* and less *Firmicutes*, especially with the increment in the *Prevotella* and *Xylanibacter* genera, containing gene sets for cellulose and xylan hydrolysis. Furthermore, *Enterobacteriaceae* were seen to decrease in African children compared to their European counterparts. The authors hypothesized that gut microbiota co-evolves with the polysaccharide-rich diet of African individuals, allowing them to maximize energy intake from fibers and protecting them from inflammatory and gut diseases. The diets of North American and Italian urban children are much richer in animal protein and saturated fats, whereas the diets of the other two populations are plant-based and have higher levels of fiber. The *Bacteroidetes:Firmicutes* ratio was lower for children in the Western countries. The diet resembled that of early humans the and rural living conditions in Africa were thought to be the main reason to explain the differences detected [352]. In addition, HFD seems to play a role in the induction of IR and glucose intolerance [293]. In addition, enrichment in *Succinivibrio* and *Treponema* has been reported in several African populations [274, 353, 354]. These bacteria possess a high-fiber-degrading potential, which is important since the typical diet of rural African populations is high in fiber and complex carbohydrates [274, 353]. Interestingly, Schnorr et al. [354] reported that *Bifidobacteria* were detected in the Hadza samples. The authors hypothesize that the occurrence of *Bifidobacteria* in the gut of adult humans is associated with the consumption of agro-pastoral-derived foods. So far, *Bifidobacteria* have been assumed to be an important part of a healthy human gut microbiota, but this observation raises the question about whether healthy gut microbiota *per se* really exists.

- ***Mediterranean diet***: The Mediterranean diet (MED) is considered one of the healthiest dietary models. Many of the characteristic components of the MED have functional features with positive effects on health and wellness. MED are characterized by the consumption of cereals (preferably as whole grains), legumes, nuts, vegetables and fruit, very often and in large amounts; the MED also includes a lower consumption of fish or seafood, white meat and eggs, moderate to small amounts of poultry and dairy products and low ethanol intake, usually in the form of wine. The main source of dietary lipids in the MED is olive oil, and a sufficient daily intake of water should be guaranteed. In addition, the MED also includes carrying out physical activity in order to maintain a good state of physical and mental health [355]. **Figure 10.**

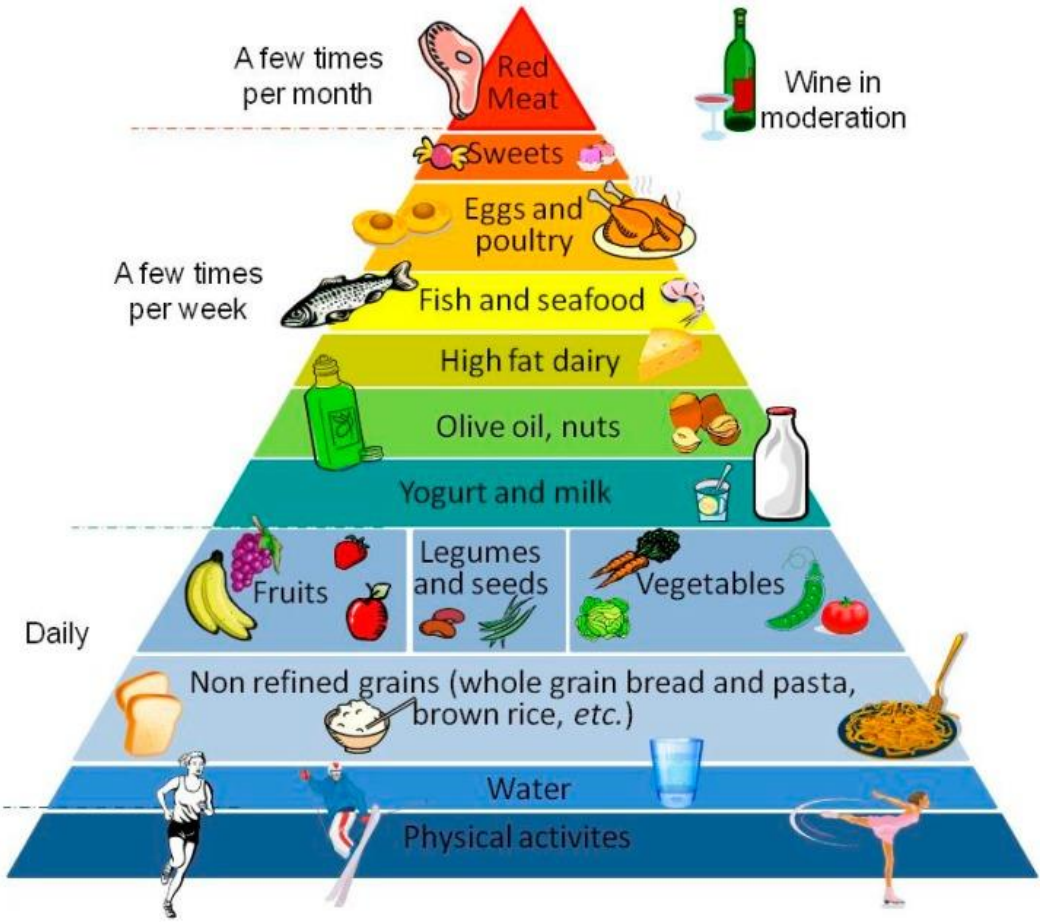

Figure 10. Pyramidal representation of Mediterranean diet and the frequency of recommended intake. Modified from [355]

A study [356] with 20 obese men within the CORDIOPREV study demonstrated that after long term-consumption of MED, *Prevotella* decreased and the *Roseburia* and *Oscillospira* genus increased. *Roseburia* is an important butyrate-producing genus that prevents the development of T2DM, so this study demonstrated the important role of MED in the prevention of T2DM [356]. Additionally, the MED increased the relative abundance of *Oscillospira*, a genus belonging to the *Ruminoccocaceae* family, associated with the feeding of fresh forage in ruminants such as cattle and sheep, which suggests that the microbiota adapt to a vegetable-rich diet such as MED [357]. In agreement with this, MED consumption has been associated with the low rate of cardiovascular mortality found in Mediterranean countries [358, 359].

In another transversal study, MED was associated with greater abundances of *Bacteroidetes*, *Prevotellacea* and *Prevotella* and lower frequency of *Firmicutes* and

Lachnospiraceae. Also, in subjects with a score MED≥ 4, higher concentrations of fecal propionate and butyrates were detected. These results confirm the complexity of the diet-microbiota relationship [360].

Filippis et al. [361] studied gut microbiota composition in 153 people following three different diets: omnivores, vegetarians and vegans. The majority of vegan and vegetarian subjects and 30% of the omnivore subjects had a high adherence to the MED. They detected significant associations between consumption of vegetable-based diets and increased levels of faecal SCFAs, *Prevotella* and some fibre-degrading *Firmicutes*, whose role in the human gut warrants further research.

- ***Probiotics and Prebiotics***

Due to our current state of knowledge of the imbalance in the intestinal microbiota and its relation with disease, treatments involving the modulation of the gut microbiota may be used in an attempt to treat these illnesses. One such treatment is the use of prebiotics and probiotics.

- ***Probiotics:*** A large number of organisms meet the criteria established by the WHO to define probiotics: "A live organism which provides a benefit to the host when provided in adequate quantities [362]." The gram-negative *Escherichia coli*, strain Nissle 1917, various lactic acid producing *Lactobacillus* strains, and a number of *Bifidobacteria* represent the primary microorganisms classified as probiotic agents. Probably the most effective strategy to select probiotic species is based on production of beneficial clinical outcomes in humans [363]. The beneficial effects of probiotics may be related to their capacity to produce vitamins, antioxidants and defensins against pathogenic competitors. Probiotics are also characterized by their production of SCFAs and the absence of toxins [364]. Probiotic bacteria may also inhibit the growth of pathogens through various mechanisms. Many beneficial probiotics such as *Bifidobacteria* and *Lactobacilli* are gram-positive bacteria, which are devoid of LPS. Such bacteria may reduce the risk of infection by competing with pathogens for dietary nutrients or receptors in the gut wall [365]. Other bacterial genera that include *Bacteroides, Enterococci, Eubacteria,* and *Streptococci* are potentially beneficial or harmful to the host, depending on the particular bacterial species being studied. Moreover, the butyrate producer *Roseburia [366]* and the mucin-degrading bacterium *Akkermansia muciniphila* have also been reported as potential probiotics [367]. The use of *Bifidobacterium longum* and *Bifidobacterium breve*

for the prevention and treatment of acute diarrhea in newborns and infants has also gained interest [368].

- ***Prebiotics*:** Nutrients that restore a healthy gut microbiota by modulating its composition are being developed as new therapeutic approaches to treat inflammatory diseases. Since the gut microbiota plays a major role in maintaining physiological reactions in the host, new dietary treatments based on the use of dietary supplements (organic selenium and *Lithothamnium muelleri* algae) and probiotics (*Saccharomyces boulardii* UFMG 905 and *Bifidobacterium*) have been developed to modulate the gut immune response and restore intestinal homeostasis [369, 370]. In addition, these changes in the host's diet could be used to modulate the gut microbiota and restore homeostasis. Prebiotics stimulate the growth or activities of specific microbial genera and species in the gut microbiota in order to confer health benefits to the host. In general, prebiotics favour the growth of *Bifidobacteria* and *Lactobacilli* over potentially harmful proteolytic and putrefactive bacteria. Prebiotics have been classified mainly into two groups, the inulin-type fructans (ITF) and the galacto-oligosaccharides (GOS), based on their chemical structures [371]. A high consumption of dietary fiber has long been recognized to provide health benefits [372], and foods rich in dietary fiber have been shown to enrich *Bacteroidetes*, especially *Prevotella and Xylanibacter*, and to reduce *Firmicutes and Enterobacteriaceae.* High-level dietary fiber supplements also increase the level of several bacteria including *Bifidobacterium*, the clostridial cluster XIVa, and *F. prausnitzii*, which are usually associated with a healthy status [373]. In addition to traditional foods, pure polyphenols and polyphenol-rich foods (such as cocoa, tea, wine, soy products, and fruit) may significantly affect the composition of the gut microbiota [374]. Based on these results, increasing the number of *Bifidobacteria* and *Lactobacilli* is currently regarded as a marker of intestinal health and as a possible screening marker for the identification of prebiotics.

Over the last few decades, a number of studies in humans have evaluated the effects of probiotics and prebiotics on gut microbiota composition [375-377]. On the other hand, other studies have demonstrated changes in gut microbiota composition in healthy adults. For example, a study in 30 adult subjects showed an increase in the abundance of *Bifidobacterium* genus and especially *Bifidobacterium adolescentis* after the consumption of the prebiotic lactulose [378]. Other studies have also observed a significant change in *Bifidobacterium* communities after the use of a probiotic fermented oat drink containing *Bifidobacterium longum* and *Bifidobacterium longum* [379, 380].

In summary, the gut microbiota has been shown to modulate the activity of a broad range of tissues and organs, with effects ranging from immunity to stimulation of brain centers responsible for appetite and food intake control. Furthermore, recent studies suggest that the gut microbiota could be manipulated using diet, prebiotics and probiotics in order to maintain health. Diets containing nutrients that are fermentable by intestinal bacteria may be used to stimulate the growth of beneficial bacteria. In fact, the gut microbiota is now considered as a separate organ of the body which produces both physiological and pathological effects. Modifying the gut microbiota using prebiotics and probiotics represents an important therapeutic strategy for the prevention and treatment of human disease.

- **Metabolic syndrome and diet:**

Numerous studies have shown that the changes in gut microbiota by diet could affect the prevalence or incidence of MetS [296, 298, 355, 356]. For example, Roopchand et al. demonstrated that concord grape polyphenols (GP) led to changes in the gut microbiota and reduction in the conditions associated with MetS arising from HFD in mice [381]. A grape polyphenol-based diet led to a decreased ratio of *Firmicutes* to *Bacteroidetes* and a significant increase in the relative abundance of *Akkermansia muciniphila* in the cecal and fecal microbiota [381]. In line with this, lifestyle interventions using a Mediterranean-type diet reported inverse associations between a good adherence to this pattern and the risk of CVD or T2DM. They also showed a reduction in the incidence of key components of MetS, including obesity, hypertension, glucose tolerance, dyslipidemia and insulin resistance [382-384]. Haro C. et al. demonstrated that a long term consumption of MED increased saccharolytic bacteria, with a healthy influence on the metabolism of the host in patients with MetS.

Larger and more experimental studies and clinical trials are needed in this field, but all the current studies suggest that diet is a good strategy for treating MetS, through changes in the intestinal microbiota.

III. HIPOTHESIS

The metabolic disorders such metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM) and Obesity, present a gut microbiota alters (dysbiosis), but the question remains whether dysbiosis of the intestinal microbiota is a direct cause for any metabolism-related disorder, or whether changes in the intestinal microbial communities in affected and obese individuals are an adaptation to a change in the host diet.

Although previous studies have pointed out that microbiota is individual-specific and shows high stability and resistance to perturbations over time [378], recent research in humans indicates that the changes in the eating habits could to explain until 57% of the variability in the composition of the intestinal microbiota, while the genetic polymorphisms of the host not explain more of 12% of the predisposition of suffer MetS [225, 246, 298, 374]. In this sense, studies are needed to research the possibility the develop strategies to dilute microbial patterns associated with metabolic diseases with the aim of predict and treat the course of the disease.

Based on these facts, our hypothesis is the existence of a gut microbiota pattern predicts the development of MetS. On the other hand, the null hypothesis is the absence of a specific pattern capable of predicting the development of MetS.

IV.OBJETIVES

IV. OBJECTIVES

Main objective

To determinate whether the composition of the intestinal microbiota and plasma levels of Lipopolysaccharide (LPS) and Lipopolysaccharide Binding Protein (LBP) might predict the development of MetS, defined by the criteria established by National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III) for MetS, in a subpopulation of patients within the CORDIOPREV study with coronary disease.

Secondary objectives:

1. To evaluate whether the chronic consumption of two healthy diets (Mediterranean diet and low-fat diet) may restore the gut microbiome dysbiosis in obese patients depending on the degree of metabolic dysfunction**.**

2. To analyze the differences in the composition of the intestinal microbiota and plasma levels of LPS after consumption of two healthy diets (Mediterranean diet and low-fat diet) for 3 years in PreDM patients who regressed from Normoglucemia (NGC) compared with patients who did not regressed during these time periods.

V. MATERIALS AND METHODS

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1. STUDY POPULATION

The current work was conducted in a subgroup of patients (n=182 for *Study I,* , n=106 Study II and n=182 for *Study III*) within the CORDIOPREV study (Clinical Trials.gov.Identifier: NCT00924937), an ongoing prospective, randomized, opened, controlled trial in patients with coronary heart disease (CHD), who had their last coronary event over six months before enrolling in two different dietary models (Mediterranean and low-fat) over a period of seven years, in addition to conventional treatment for CHD [385]. The study is being conducted at the Instituto Maimonides de Investigación Biomedica de Cordoba, a scientific institute which carries out research into biomedical areas for the Reina Sofia University Hospital, and the University of Cordoba, Spain. The lipids and atherosclerosis unit, internal medicine unit, is also a member of the CIBER Fisiopatologia de la Obesidad y Nutrición (CIBERobn), a national research organization studying obesity and nutrition and their impact on health and disease. Moreover, the leading researchers have established collaboration agreements with national and international research groups to conduct additional ancillary studies. All its amendments have been approved by the Ethics Committee of the Hospital Universitario Reina Sofía following the indications of the Helsinki Declaration and the code of good clinical practice.

1.1 Sample size calculation

The sample size calculation was conducted using the GRANMO sample size calculator (Version 7.12 April 2012).

- ***Study I from Main objective:***

The calculation was made based on the following premises:

- Primary endpoint of the study: Relative abundance of *Ruminococcus* genus
- Standard deviation of the primary endpoint: 0.025
- Difference in minimum expected: 0.014
- Alpha error = 0.05 (Confidence level: 95%)
- Beta error = 0.20 (Power: 80%)
- Estimated losses: 10%
- Hypothesis Contrast: Bilateral.

Based on these premises are required 28 participants per group.

- ***Study II from secondary objective 1:***

The calculation was made based on the following premises:

- Primary endpoint of the study: Relative abundance of the specie *Faecalibacterium prausnitzii*
- Standard deviation of the primary endpoint: 0.02
- Difference in minimum expected: 0.01
- Alpha error = 0.05 (Confidence level: 95%)
- Beta error = 0.20 (Power: 80%)
- Estimated losses: 10%
- Hypothesis Contrast: Bilateral.

Based on these premises are required 55 participants per group.

- ***Study III from secondary objective 2:***

The calculation was made based on the following premises:

- Primary endpoint of the study: Relative abundance of *Prevotella* genus
- Standard deviation of the primary endpoint: 0.02
- Difference in minimum expected: 0.01
- Alpha error = 0.05 (Confidence level: 95%)
- Beta error = 0.20 (Power: 80%)
- Estimated losses: 10%
- Hypothesis Contrast: Bilateral.

Based on these premises are required 35 participants per group.

1.2 Inclusion criteria

1. Informed consent: All participants will agree to being included in the study by signing the protocol approved by the Reina Sofia University Hospital Clinical Research Ethics Committee. In this written statement of consent, it will state that patients will be chosen for inclusion in the groups on a random basis.

2. Diagnostic criteria: The patients were selected with acute coronary syndrome (unstable angina, acute myocardial infarction) and high-risk chronic CHD according to the following criteria:

a) Acute myocardial infarction: The existence of at least 2 of the following 3 signs: angina-type chest pain (or anginal equivalents), typical ECG changes (appearance of new Q waves and/or changes in ST segments and/or T waves), and a rise in myocardial enzymes (CPK and/or CPK/MB more than twice the normal laboratory limits). The MB value criterion will prevail in case of discrepancies over the total CPK.

b) Unstable angina: Admission to hospital for angina-type chest pains lasting at least 15 min, both at rest and after exercise, which have increased in frequency and duration in recent days or weeks. The latest episode must have occurred at least 48 h before admission and must be accompanied by at least 1 of the following electrocardiographic or analytical changes:

- ST depression of at least 0.5 mm in 2 contiguous leads.
- ST elevation of at least 1 mm in 2 contiguous leads.
- T-wave inversion of at least 2 mm in 2 contiguous leads.
- Positive troponin result.

c) Chronic high-risk ischemic heart disease: patients will be included who have been hospitalized for a coronary event and/or stable angina at least once in the past 2 years and who have undergone diagnostic coronary angiography with evidence of severe coronary disease, which is defined as the existence of an epicardial vessel greater than 2.5 mm in diameter with stenosis of N50%.

1.3 Exclusion criteria

1. Patients: <20 y of age or >75 y old, or with a life expectancy < 5 year.

2. Severe heart failure, NYHA functional class III or IV, with the exception of self-limited episodes of acute heart failure at the time of the acute ischemic event.

3. Severe left ventricular systolic dysfunction (with ejection fraction ≤35%).

4. Patients with restricted capacity to follow the protocol: those unable to follow the prescribed diet for whatever reason, due to personal or family circumstances.

5. Risk factors which are severe or difficult to control: Patients with hypertension and diabetes, where there is organ involvement that limits their survival, were excluded (chronic renal failure with creatinine which is persistently N2.5 mg/dL) and disabling clinical manifestations of cerebral atherosclerosis.

6.- Chronic diseases unrelated to coronary risk: severe psychiatric illnesses, chronic conditions requiring treatment that could limit the dietary intervention (chronic renal failure, chronic liver disease, neoplasia under treatment, chronic obstructive pulmonary

disease involving respiratory pulmonary failure with home oxygen therapy, endocrine diseases susceptible to decompensation, and diseases of the digestive tract that involve episodes of diarrhea).

7. Participants in other studies: Patients taking part in other studies, at the time of selection or up to 30 d before the study begins, were excluded.

8. Antibiotic usage and T2DM: In addition to the general exclusion criteria defined in the CORDIOPREV study, antibiotic usage and patients without T2DM was included as an exclusion criterion for the current study.

2.STUDY DESIGN

In this thesis, we performed 4 different studies according to the objectives proposed.

- ***Study I***: For study if the gut microbiota might predict the development of MetS, we selected 182 patients from CORDIOPREV study according to the NCEP ATP III criteria for MetS, from a total of 182 which had not had a MetS, 34 patients MetS develop (MetS develop group) according to the NCEP ATP III criteria for MetS during the first 3 years of follow-up and 148 patients did not develop MetS (control group) during same period of time. Half of each group received a type of diet, Mediterranean diet (MED) and low-fat diet (LF).
- ***Study II:*** for evaluated whether the chronic consumption of two healthy diets may restore the gut microbiome dysbiosis in obese patients depending on the degree of metabolic dysfunction, we selected 106 male patients at baseline and after 2 years of dietary intervention (Mediterranean or Low-fat diet), which were also divided into three groups: the MetS-OB group, 33 obese people with MetS; the NonMetS-OB group, 32 obese people without MetS (2 or less criteria for the metabolic syndrome); the NonMetS-NonOB group, 41 nonobese people without MetS.
- ***Study III:*** For analyse the differences in the composition of the intestinal microbiota among PreDM patients who regressed from Normoglucemia (NGC) after consumption for 3 years of two healthy diets (Mediterranean diet, a low diet) compared to PreDM patients who did not regressed during these time periods. The study was performed in 182 pre-diabetic (PreDM) patients included the CORDIOPREV, according to the American Diabetes Association (ADA)

diagnosis criteria, 69 from which regressed to NGC after 3 years of follow-up. Half of each group received a type of diet (Mediterranean diet, low fat diet).

The study design has been previously described [386]. The cardiologist attending the patient would suggest their participation in the study, after verification that meets the criteria for inclusion and does not meet the exclusion criteria. If the patient decides to collaborate, one of the internists will open the corresponding CRF data, which will be assessed by the coordinator and then by external independent cardiologists. If the information is favourable, they will be asked to patient informed consent. Next will be made baseline studies and will be randomized and assigned to one of two study diets. The randomization will be made by the following variables:

- Male or female
- Be under 60 years old or above.
- Having or not a previous myocardial infarction.

The randomization procedure consisted in a fixed randomization, stratified into blocks blindly keeping the whole process of scrambling by researchers who will be involved in data collection and treatment of patients. This randomization process will be conducted by an international organization renowned in the field of Epidemiology and Clinical Research (Escuela Andaluza de Salud Pública) which know in detail the entire process of randomization of patients.

Participants were randomized to receive two diets: a Med diet and a LF diet. The composition was as follows: (a) LF diet: 28% fat (12% monounsaturated, 8% polyunsaturated and 8% saturated) and (b) Med diet: 35% fat (22% monounsaturated, 6% polyunsaturated and 7% saturated). Furthermore, to ensure that the main fat source of the Med diet (olive oil) was identical for all patients in this group, the olive oil was given to the participants by the research team. Food packs, including low-fat foods (cereals, biscuits, pasta, etc.) of similar cost, were provided for the patients who were randomized to the LF group. The workflow of CORDIOPREV is represented in the **Figure 11**.

For the definition of MetS on the CORDIOPREV study we used the criteria defined by the ATP III – NCEP as amended [14] and for the Prediabetes were classified according to the ADA pre-diabetes criteria classification [9]. In the analysis of our work were only included, those patients with all parameters available, biochemical

determinations, postprandial lipemia and compliance with diet during the 36-month period.

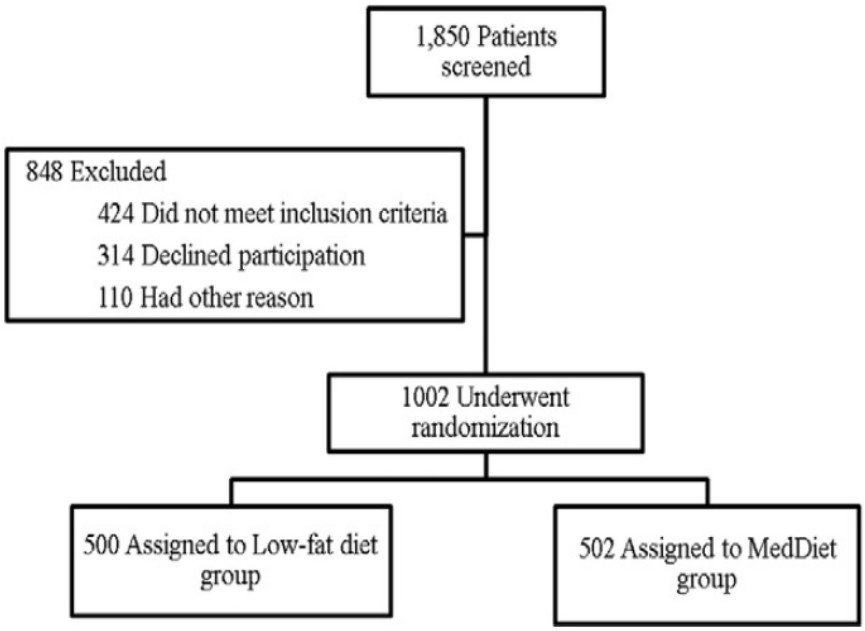


Figure 11. Screening and Randomization of CORDIOPREV study [387] .

Diet assessment was performed using a validated 14-item questionnaire to assess adherence to the Med diet [388] (see **Table 4**) and a similar 9-point score to assess adherence to LF diet [154] (see **Table 5**) before the start of the dietary intervention and yearly follow-up visits.

Questions	Criteria for 1 point
1. Do you use olive oil as main culinary fat?	Yes
2. How much olive oil do you consume in a given day (including oil used for frying, salads, out-of-house meals, etc.)?	≥4 tbsp
3. How many vegetable servings do you consume per day? (1 serving: 200 g [consider side dishes as half a serving])	≥2 (≥1 portion raw or as a salad)
4. How many fruit units (including natural fruit juices) do you consume per day?	≥3
5. How many servings of red meat, hamburger, or meat products (ham, sausage, etc.) do you consume per day? (1 serving: 100–150 g)	<1
6. How many servings of butter, margarine, or cream do you consume per day? (1 serving: 12 g)	<1
7. How many sweet or carbonated beverages do you drink per day?	<1
8. How much wine do you drink per week?	≥7 glasses
9. How many servings of legumes do you consume per week? (1 serving: 150 g)	≥3
10. How many servings of fish or shellfish do you consume per week? (1 serving 100–150 g of fish or 4–5 units or 200 g of shellfish)	≥3
11. How many times per week do you consume commercial sweets or pastries (not homemade), such as cakes, cookies, biscuits, orcustard?	<3
12. How many servings of nuts (including peanuts) do you consume per week? (1 serving 30 g)	≥3
13. Do you preferentially consume chicken, turkey, or rabbit meat instead of veal, pork, hamburger, or sausage?	Yes
14. How many times per week do you consume vegetables, pasta, rice, or other dishes seasoned with sofrito (sauce made with tomato and onion, leek, or garlic and simmered with olive oil)?	≥2

Table 4. Validated 14-item Questionnaire of Mediterranean diet adherence

2.1 Description of dietary patterns

Mediterranean Diet (MED)

The qualitative composition of this diet is based on a total caloric intake of 35% fat (22% MUFA, 6% PUFA, 7% SFA), 50% HC and 15% protein). The recommendations

of this intervention group are based the questionnaire of 14-items of adherence to MED **(Table 4).** The recommendations provided to the participants of this group are:

A) An abundant consumption of extra virgin olive oil both for cooking and for the production of salads (≥ 4 tablespoons daily, 20 g).

B) Consumption of 2 or more daily servings of vegetables (1 serving = 200 g) and Least one of the rations should be raw.

C) Consume at least 3 pieces of fruit (average size = 125 g) per day Predominating the consumption of fruits as dessert. The preference of fruit on the juice of fruits to maintain an adequate supply of fiber.

D) Include legumes in at least 3 meals a week (150 g per serving cooked).

E) Eat more than 3 servings (1 serving = 100-150 g) per week of fish Varied including fatty fish such as salmon, mackerel and sardines.

F) Consume nuts weekly in small amounts, approximately 3 handfuls (30 g).

G) Include consumption of whole wheat bread and cereals at meals.

(H) Consume low-fat dairy products, with a preference for Fermented dairy products (yogurt or cheese).

(I) Reduce meat consumption and preferably take white meat (without Skin) rather than red meat or processed meats. Eggs can be consumed several times a week (2-4 units).

J) Culinary preparations with "sofrito" (tomato, onion, garlic, spices and everything this is simmered with olive oil). The Mediterranean diet model includes a moderate amount of wine during meals. This approximate amount oscillates between no more than 1 glass of wine a day (8g alcohol) for women and no more than 2 glasses of wine (16g alcohol) per day for men. This recommendation was only carried out if the patient was a Regular consumer of wine. It was recommended to eliminate the consumption of other fats other than those mentioned from butter, margarine, seed oils, creams, etc, and food Rich in sugars or unhealthy fats (industrial pastries, snacks, foods Industrial desserts, sugary carbonated drinks, etc.).

Low fat diet (LF):

In the intervention group with the LF diet, the fat exceeded 28% (12% MUFA, 8% PUFA, 8% SFA), 57% HC and 15% protein). The recommendations of this intervention group are carried out in accordance with the recommendations of the ATP III - NCEP guide [10, 11], fundamentally in limiting the consumption of fat, both animal and vegetable, increasing the consumption of HCC to replace it. The main differences

with the MED group are also based on the Questionnaire 9-items of adherence to the LF diet **(Table 5)** and are:

A) Minimize the amount of oil used for cooking and salads (≤ 2 tablespoons per day, 10 g).

B) Remove visible fat (or skin) from chicken, duck, pork, lamb etc. Before the after cooking (soup and cooked dishes).

C) Do not eat more than 1 serving of red meat (1 serving = 150 g) per week.

D) Consume low-fat and especially fermented dairy products (cheese and yogurt). You should consume 2-3 servings per day.

(E) The consumption of lean fish (cod, hake, monkfish etc.) instead of Fat or canned fish (<1 serving per week, 50 g).

F) Restrict the consumption of nuts (<1 serving per week, 10 g).

G) Eliminate the consumption of industrial bakery.

H) Use cooking techniques without oil (boiled, baked, grill, etc.). Recommendations on the consumption of vegetables (vegetables, fruits, Legumes or cereals), eggs and white meat are the same as those included in the group of MED.

Questions	*Criteria for 1 point*
1 How much oil do you consume daily (included for frying, Salads, breakfast etc.)? (1 tablespoon = 10mL).	≤ 2
2 Does it remove visible fat (or skin) from chicken, duck, pork, lamb etc. Before or after cooking (soups and dishes cooked)?	Yes
3 How many meals rich in hamburger fat, sausage, sausage, bacon etc. Consumed per week? (Meat ration = 100g, sausage or bacon = 30g)	≥1
4 How many servings of butter, margarine, butter pork, mayonnaise, cream or ice cream consumed per week? (Serving = 12g, ice cream = 100g)	≥1
5 Do you consume low fat products exclusively?	Yes (include not consume diary)
6 How many times a week do you prepare pasta, rice, potatoes, pulses using "sofrito" (with olive oil), bacon, salami or fatty meats like pork or lamb?	≤ 2
7 How many times a week do you eat fatty fish or fish or canned fish in oil?	≥1
8 How many servings of industrial bakery, such as cake, cookies or custard consumed per week? (Cake serving = 80g; 6 Cookies = 40g)	≥1
9 How many times a week do you eat nuts (Including peanuts), chips or other snacks?	≥1

Table 5. Questionnaire 9-items of adherence to Low Fat diet.

3. POSTPRANDIAL STUDY

At the baseline and after 3 years follow up, subjects were given a fatty breakfast with the same fat composition as consumed in each of the dietary intervention period. Participants presented at the clinical centre at 8-hours following 12 hours fast (time 0), abstained from alcohol intake during the preceding 7 days.

At 8:00 a.m. the patients arrived at the laboratory, the measurements were taken (Weight, height and waist circumference) and their blood pressure was measured. For measurements, biochemical tests, a fasting blood sample was obtained and then 2 and 4h after the fat overload: 0.7 g fat / kg Body weight (12% SFA, 10% PUFA, and 43% MUFA), 10% protein and 25% HC. Breakfast was ingested in 20 min. Patients could only drink water during postprandial determinations. Blood samples for biochemical determinations were collected following the recommendations of Mihas et al. (378).

4. SAMPLING AND BIOCHEMICAL MEASUREMENTS

4.1 Blood samples

Biochemical measurements were collected from the participants after a 12-h overnight fast at the beginning of the study and after 3 years of follow-up. Venous blood was obtained from the antecubital vein, collected in EDTA collection tubes and serum separator tubes, and immediately transferred to 4ºC. To minimize proteolytic degradation, the plasma was supplemented with protease inhibitor cocktail (Roche Diagnostic, Germany) 40µL/mL of plasma.

4.2 Isolation of plasma

Immediately after the collection blood samples, we proceeded to the separation of plasma by centrifugation (1500 x g during 15 min at 4 ºC). The plasma samples were stored in aliquots at -80 ºC until their use to avoid inter-assay variations.

4.3 Biochemical measurements

The biochemical measurements were performed at the Reina Sofia University Hospital by personal that were unaware of the interventions. Lipid variables were assessed with a DDPPII Hitachi modular analyzer (Roche, Basel, CH) using specific reagents (Boehringer-Mannheim, Ingelheim am Rhein, DE). Plasma triglycerides (TG) and cholesterol concentrations were assayed by enzymatic procedures. High density lipoproteins (HDL-C) were measured following precipitation of a plasma aliquot with dextran sulphate-Mg2+. Low density lipoprotein (LDL-C) concentration was calculated by the Friedewald equation [389], using the following formula: LDLc = CT − (HDL + TG / 5). Glucose was measured by the hexokinase method and insulin was determined by chemiluminescent microparticle immunoassay (CMIA) for quantitative determination using the Architect c-16000 analyzer (Abbott®, Chicago (IL), USA). HbA1c was measured by means of high performance liquid chromatography (HPLC) with a VARIANT II Turbo BioRad® analyzer (Hercules, California, USA).

The insulin sensitivity was measured using the oral glucose tolerance test. Before the test started, the patients had fasted for 12 h 28 and were asked to refrain from smoking during the fasting period and from alcohol intake during the preceding 7 days. They were also asked to avoid strenuous physical activity the day before the test was taken. At 8:00 a.m., the patients were admitted to the laboratory and an oral glucose tolerance test (OGTT) (75g dextrose monohydrate in 250 ml water) was performed with 0, 30, 60, and

120 min sampling to establish plasma glucose and insulin levels. The insulin sensitivity index (ISI) was calculated as previously described [390, 391]. For the estimation of the insulin resistance were determined as a sensitivity parameter to the insulin and HOMA-IR by means of the mathematical evaluation of the balance Production of hepatic glucose and insulin secretion from basal levels of both. The HOMA-IR index (fasting glucose (mg / dL) x fasting insulin (mU/L)/22.5), was considered a reliable measure of insulin resistance.

5. ENDOTOXEMIA STUDY

5.1 Lipopolysaccharide (Endotoxin-LPS) concentration in plasma

Endotoxin (LPS) was measured by a limulus amebocyte lysate test (QCL-1000; *Lonza Iberica S.A. Barcelona, Spain*). All procedures were performed under nonpyrogenic conditions. Plasma samples at 0 and 4 hours after the intake were diluted 1:5, mixed with limulus amebocyte lysate and incubated at 37 ºC for 10 min. The substrate solution was then mixed with the LAL-sample and incubated at 37 ºC for 6 min. The reaction was stopped with 10% Sodium Dodecylsulfate (SDS). If the endotoxin was present in the plasma, a yellow colour developed. The absorbance of the coloured solution was read at 405 nm in a plate reader (SPECTRA Fluor, Tecan). The recovery of spiked lipopolysaccharide was between 50 and 200%. The sensitivity of the assay was sensitivity range of 0.1-1.0 endotoxin units (EU) / ml). Intra- and inter-assay coefficients of variation for LPS determinations were between 5–10%.

5.2 Lipopolysaccharide binding protein (LBP) concentration in plasma

Fasting and postprandial LBP levels were performed on EDTA plasma samples with the Hycult Biotech immunoassay kit (Frontstraat, The Netherlands). Before to the assay the plasma samples were diluted 1: 1000 using polypropylene tubes to prevent leakage in the tube walls, then added to the wells of the plate and incubated 1 h, elapsed time was washed 4 times and added The biotinylated tracer Antibody (ATB) was added and incubated for 1 h, after 4 washes streptavidin conjugated peroxidase was added and incubated in the dark again 1h then the tracer was added and incubated for 30 min and finally the stop solution was passed to reading the plate using a plate reader (SPECTRA Fluor, Tecan) at 450 nm.

6. GUT MICROBIOTA COMPOSITION

6.1 Collection and storage of fecal samples

To collect the fecal samples, we gave the patients a box with carbonic snow and a sterile plastic bottle with a screw cap to keep the frozen sample. Once delivered to the laboratory staff, the sample was stored at -80ºC until microbial DNA was extracted.

6.2 Extraction of bacterial DNA from fecal samples.

For the study of the intestinal microbiota, stool specimens collected and duly stored – 80ºC until the moment of being processed were obtained. DNA extraction was performed according to the protocol for the isolation of feces DNA using the QIAamp® DNA Stool Mini Kit Handbook Kit. The protocol was optimized for 180-220 mg of sample and it is followed in the following steps:

Add approximately 200 mg stool sample in a 2ml vial and place immediately in an ice bath; Add 1.4 ml Buffer ASL to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized, and then heat to 95 ° C for 5 minutes and centrifuge 1 minute samples at 14000 rpm. Transfer 1.2 mL of the supernatant and add to a new tube, discarding the pellet. Add 1 InhibitEX Tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended.

Following this step, incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. This solution was centrifuged at 14000 rpm for 3 minutes, so that the sample and the inhibitors were bound to the inhibitex matrix, this step was repeated twice. The supernatant was transferred completely to a new 1.5 mL tube and the pellet was discarded, again centrifuged at 14000rpm for 3 minutes. 15 µL of proteinase K was added, and then 200 µL of the centrifuge supernatant was added to each tube with the proteinase content (important not to directly add proteinase K to the sample), and added 200 µl of AL buffer (containing guanidine hydrochloride). Homogenize solution with vortex for 15 seconds and incubate at 95 ° C, 10 minutes; after the incubation time add 200 µL of ethanol (96-100%) to the lysate and vortex.

Carefully apply the complete lysate to the column of a new vial, close the cap and centrifuge at 14000 rpm for one minute and discard the vial that had the filtrate, then carefully remove the column and place it in a new vial. Carefully open the QIAamp column and add 500 µL of buffer AW1 (wash buffer), close the lid and centrifuge at 14000 rpm one minute and discard the manifold.

The column was placed in a new 2 mL tube and 500 µL of buffer AW2 (wash buffer) was added, then centrifuged 3 minutes and discarded the collection tube, then

transferred the column to a new 1.5 mL tube and 200 µL of the AE buffer was added directly to the QIAamp membrane, closing the cap and incubating for 1 minute at room temperature, finally recovering the DNA and centrifuging at full speed for 1 minute. Once isolated the bacterial DNA was quantified by a nanodrop spectrophotometer and the samples were stored at -20 ° C.

We sequenced the V4 16S microbial rRNA by Ilumina Miseq for the study II and V3-V4 for the study I and III.

6.3 Sequencing the V4 16S microbial rRNA on the Illumina MiSeq and upstream informatics analysis.

The preparation of samples was performed similarly to that described by Costello et al. [392]. To sum up, the 106 samples taken on the two dates in the study (baseline and 2 years) were amplified in triplicate by PCR to generate an amplification library (modified from Sarah Owens, Argonne National Labs), with each sample amplified in 25 µL PCR reactions. The PCR experimental conditions for the 515-806 bp region of the 16S rRNA gene and the sequencing procedures with the Illumina platform have both been described by Caporaso et al. [393].

The obtained 16S rRNA sequences were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) with default parameters unless indicated otherwise [393]. Briefly, raw sequencing data was de-multiplexed and low-quality reads were discarded. Reads were clustered using a closed-reference OTU picking protocol that assigned reads to reference sequences from Greengenes v13-8 [394]. Taxonomy was assigned to the OTUs against the Greengenes v13-8 preclustered at 97% identity. Differences between bacterial communities were calculated in QIIME using rarefaction curves of alpha-diversity indexes including estimates of community richness (such as the Chao1 estimator, Good´s coverage and the observed number of OTUs present in each sample and Phylogenetic diversity (PD) or the length of the phylogenetic branch observed in each sample). Due to the unequal size of our library per sample and with the purpose to retain all samples each library was subsampled to an even sequencing depth of exactly 2000 sequences per sample (the lowest number of reads obtained in the sequencing) to mitigate biases arising from different depths of sequence across samples. Beta diversity was estimated using weighted and unweighted UniFrac distance [395]. Beta-diversity distance matrices were built after sub-sampling all the samples to an even depth of 2000 sequences per sample, which is the same with the depth provided to script alpha_rarefaction.py.

Relative taxonomic abundance was measured as the proportion of reads over the total in each sample assigned to a given taxonomy.

6.4 Sequencing the V3-V4 16S microbial rRNA on the illumina MiSeq and upstream informatics analysis.

The DNA was quantified using a Qubit dsDNA HS Assay kit (Life technologies), as this is a high specific assay for detecting dsDNA in 10 pg/ml-100pg/ml. Working solutions were prepared according to manufactures´ recommendation where 2 µL DNA was added to a mix of 1:200 Quant-iT™ reagent and Quant-iT™ buffer respectively, to a total volume of 200 µL. Measurements were executed in a Qubit™ fluorometer. For calibration of the instrument, 10 µL of two standards were added to 1:200 working solution. The DNA was diluted to final concentration 5 ng / µl.

The V3–V4 region of the bacterial 16S rRNA gene was amplified with the universal primers reported by Klindworth et al. [396], fused with Illumina adapter overhang nucleotide sequences. Primer sequences were: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3'and 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'. The workflow is represented in **Figure 12.**

Two independent PCR reactions were performed for each sample. The products were pooled and indexed using Illumina's 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego CA) (https://www.illumina.com). Libraries were deep sequenced with the Illumina MiSeq sequencer using a reagent v3 cartridge and 600

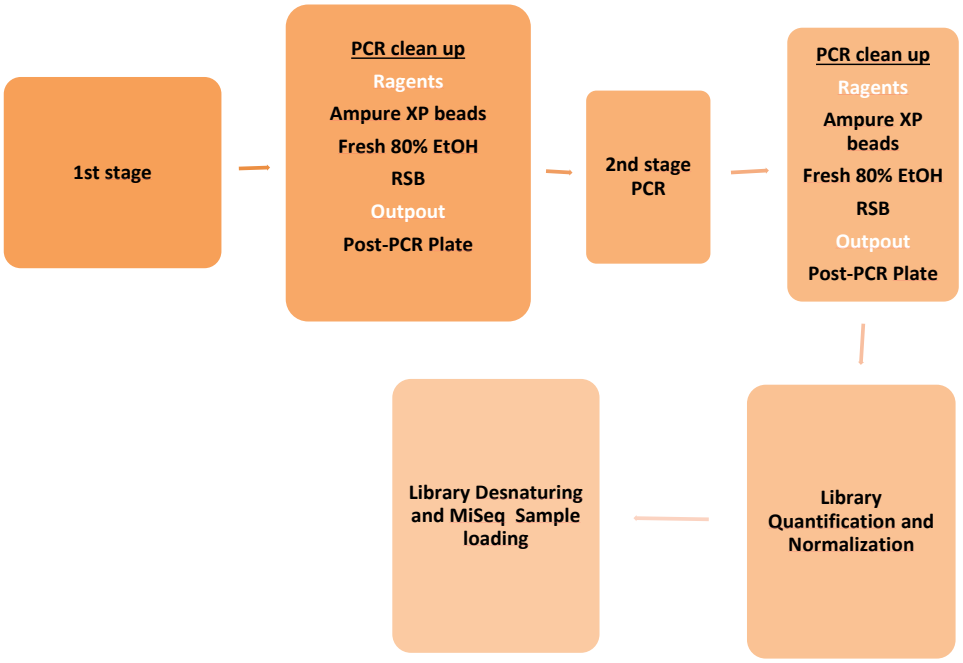


Figure 12. 16S Library Preparation Workflow

cycles. Output data included demultiplexed fastq files, which had the primer and index sequences removed.

The merged paired-end reads were analyzed using the QIIME [393] pipeline. To perform detection and clustering of 16S rRNAs, an open-reference Operational Taxonomic Units (OTUs) picking approach was used. pick_open_reference_otus.py is the primary interface for open-reference OTU picking in QIIME, and includes taxonomy assignment, sequence alignment, and tree-building steps. In this open-reference OTU picking process, reads were firstly clustered against a Greengenes 13_8 reference sequence collection [394] (available at http:// qiime.org/home_static/dataFiles.html) through closed-reference OTUs picking. Subsequently, 0.1% of the reads which failed to hit the reference sequence collection were randomly subsampled and clustered de novo using UCLUST [397], with an OTU cluster defined at a sequence similarity of 97%. Each cluster centroid was then chosen as a "new reference sequence" for another round of closed-reference OTU picking. OTU assignments for read that failed to hit the reference database were picked by an additional round of de novo clustering. The PyNAST

alignment algorithm [398] was used to align the OTU representative sequences against the Greengenes core reference alignment [394]. We generated a OTU table (biom summarize table) for downstream diversity analysis by excluding the sequences that had failed to align by PyNAST. We also used the generated OTU table to summarize microbiome communities by taxonomic levels (by default: phylum, class, order, family, genus) based on different timepoints (summarize_taxa_through_plots.py).

7. STATISTICAL ANALYSIS

We used PASW Statistics, Version 18 (Chicago, IL, USA) and version 20.0 (IBM Inc., Chicago, IL, USA) and R software, version 3.0.2 (R Foundation for Statistical Computing, http://www.R-project.org/) were used for statistical analyses of data. The normal distribution of variables was assessed using the Kolmogorov– Smirnov test. When variables followed a normal distribution (metabolic variables), One-way ANOVA in each time of study was used to compare the baseline metabolic variables between groups and ANOVA for repeated measures to assess the statistical differences with time within of each group and between groups. When variables did not follow a normal distribution, we used nonparametric methods. The Krustall Wallis and Mann–Whitney U test analysis was used to compare the statistically significant differences in the relative abundance of the bacterial between groups. Post hoc statistical analysis was completed by using the Bonferroni's multiple comparison tests. The statistically significant microbiota changes by diet were assessed by the Wilcoxon signed-rank test. In order to assess whether specific differences occurred in some bacterial taxa between groups and bacterial taxa, we compared the abundance of taxa present at least in the 75% of the human fecal DNA samples. We analyzed the changes in the abundance of these taxa per group and diet.

We also analyzed the frequency of occurrence of taxa identified at least in 25% of the total human fecal DNA samples. The χ^2 test was applied to establish differences in bacterial prevalence between the studied groups. Results were adjusted by False Discovery Rate (FDR) using Benjamini and Hochberg method [399]. A *P-value* and *Q-value* <0.05 was considered statistically significant.

A study of the relationship among parameters was also carried out using Pearson's linear correlation coefficient. All data presented are expressed as mean±S.E.M. A P value <0.05 was considered significant.

The statistical significance of differences in alpha and beta-diversity was performed with QIIME and PAST software, using a nonparametric two sample t-test with 999 Monte Carlo permutations on a number of observations, Chao1 and PD and nonparametric ANOSIM tests on unweighted and weighted UniFrac distance matrices.

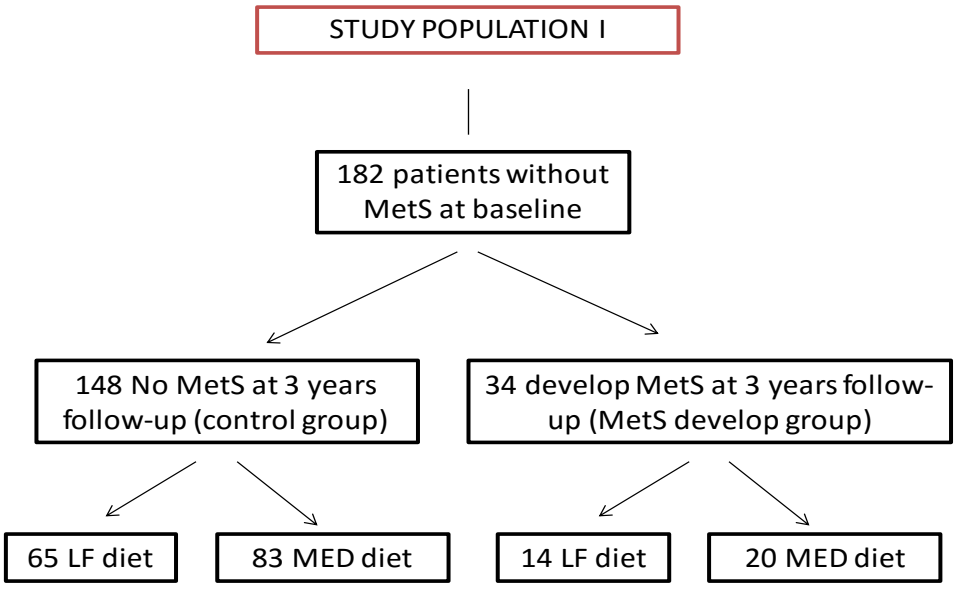
V. RESULTS

V. RESULTS

1. *CHAPTER 1: Results from Main objective*

Gut microbiota composition as predictive biomarker of the development of MetS

We included in this study to 182 participants from the CORDIOPREV study, which were divided in 2 groups according to the progression of MetS. From a total of 182 patients without MetS at baseline, 34 patients developed MetS according to the NCEP ATP III criteria for MetS during the first 3 years of follow-up whereas 148 did not develop MetS during this period of time. In order to identify a microbiota pattern predictive for the MetS development, we analyzed the differences in gut microbiota composition at baseline between patients who developed MetS as compared with patients who did not developed it.



1.1 Baseline characteristics

We did not observe differences in most of the NCEP ATP III criteria for MetS at baseline between patients who development MetS (MetS develop group) and patients without MetS (control group), but we observed that the values of waist circumference and TG was higher in the MetS develop group than in the control group without MetS (P=0.008 and P=0.009, respectively) **(Table 6).** The most of patients at baseline had waist circumference and high blood pressure criteria **(Table 7).**

Table 6: Baseline characteristics of the participant in the study according to NCEP ATP III criteria for MetS.

	Control group	*MetS develop*	*P-Value*
N (Men/Woman)	148 (127/21)	34 (23/11)	
Age (years)	58.44±0.79	61.29±1.27	0.113
Waist circumference	98.52±0.86	104.01±2.10	***0.008***
SBP (mmHg)	132.16±1.67	138.38±3.13	0.103
DBP (mmHg)	75.44±0.99	77.55±1.70	0.290
TG total (mg/dL)	95.30±3.09	115.33±7.99	***0.009***
HDL-c (mg/dL)	47.19±0.81	44.42±1.60	0.141
Glucose (mg/dL)	90.16±0.78	91.24±1.27	0.544

Values presented are the mean ± SEM of each group. Abbreviations: TG, triglycerides; HDL-c, high-density lipoprotein-cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure. One-way ANOVA statistical analysis *p*-value <0.05

Table 7. Criteria of MetS diagnosis at baseline.

Criteria of diagnosis MetS	Total (N=182)	Control group (N=148)	MetS develop group (N=34)
Waist circumference	74	53 (35.81%)	21 (61.76%)
High Blood Pressure	81	63 (42.56%)	18 (52.94%)
TG	18	10 (6.75%)	8 (23.54%)
Fasting glucose	25	22 (14.86%)	3 (8.82%)
HDL-c	46	36 (24.32%)	10 (29.41%)

The value represents the number of patients with the criteria of MetS at baseline. Abbreviations: TG, triglycerides; HDL-c, high-density lipoprotein-cholesterol.

1.2 Metabolic Syndrome development

The group who developed MetS increased significantly TG (P<0.001) and glucose (P<0.001) and decreased HDL (P<0.001) at 3 years follow-up. By contrast, we observed a decrease in TG (P=0.026) and HDL (P=0.003), and an increase in Glucose (P=0.001) in the control group who did not developed MetS at 3 years of follow-up (***Table 8*****).**

Table 8. Effect of the dietary intervention on the main metabolic variables in the population of study.

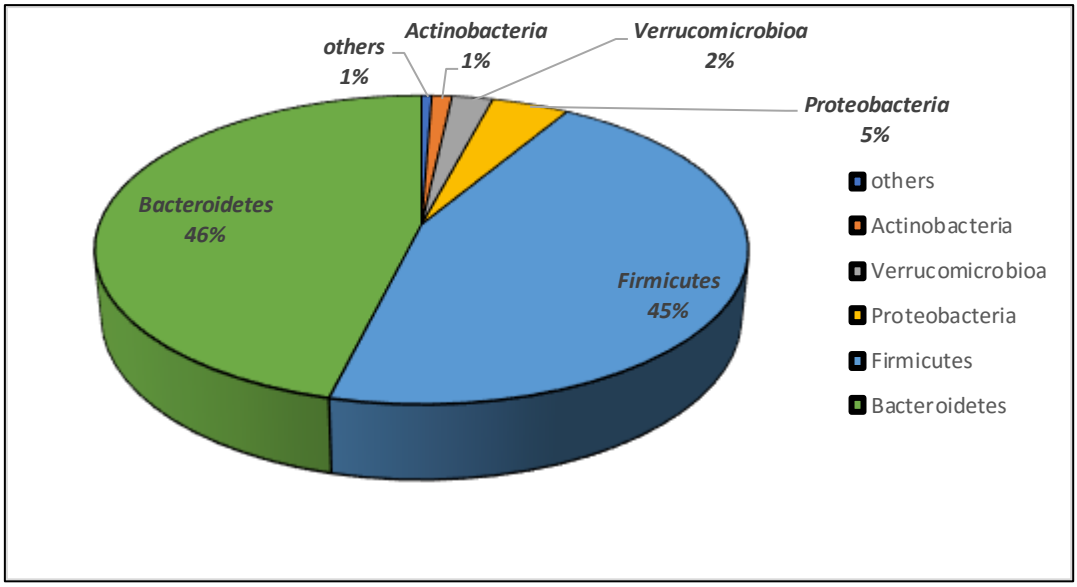
	Control group (N=148)	*MetS develop (N=34)*	*P time*	*Pgroup*	*P interaction*
Waist circumference					
basal	98.52±0.86	104.01±2.10	0.368	<0.001	0.033
3 years	97.51±0.91	105.95±1.89			
SBP (mmHg)					
basal	132.16±1.67	138.38±3.13	0.846	0.004	0.262
3 years	129.48±1.48	140.31±3.42			
DBP (mmHg)					
basal	75.44±0.99	77.55±1.70	0.590	0.049	0.218
3 years	73.75±0.85	78.22±1.59			
TG total (mg/dL)					
basal	95.30±3.09	115.33±7.99	0.031	<0.001	<0.001
3 years	85.77±2.81*	141.79±9.92*			
HDL-c (mg/dL)					
basal	47.19±0.81	44.42±1.60	<0.001	0.035	0.048
3 years	45.11±0.90*	39.55±1.39*			
Glucose(mg/dL)					
basal	90.16±0.78	91.24±1.27	<0.001	0.010	0.012
3 years	92.40±0.68*	97.85±1.34*			

ANOVA for repeated measures with time as intra-subject factor and group as the inter-subject factor was used to determine the statistical significant changes through the period of study. We considered significative when *P-value*<0.005. Abbreviations: BMI, body mass index, HDL-c, high-density lipoprotein-cholesterol; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure.

1.3 Global pattern of microbiota of the study population

We analyzed the differences in gut microbiota composition at baseline and after 3 years of follow-up between the group who developed and the group who did not develop MetS by 16S sequencing with the Illumine platform. Globally, the phylogenetic characterization of the gut microbiota of the study population at baseline showed the abundance 5 main bacterial phyla ranged about the 99.03% of total phyla: *Bacteroidetes* (45.90%), *Firmicutes* (45.06%), *Proteobacteria* (4.46%), *Verrucomicrobia* (2.39%) and *Actinobacteria* (1.1%) (**Figure 13**). Moreover, we observed that *Bacteoidia* and *Clostridia* were the most predominant class. *Bacteroidaceae* and *Ruminococcaceae* were the most abundantly detected families. The most prevalent genera in our analyzed samples were *Bacteroides* and a group of non-identified genus from *Ruminococcaceae* family.

Figure 13. Composition of the intestinal microbiota of the study population at phyla level

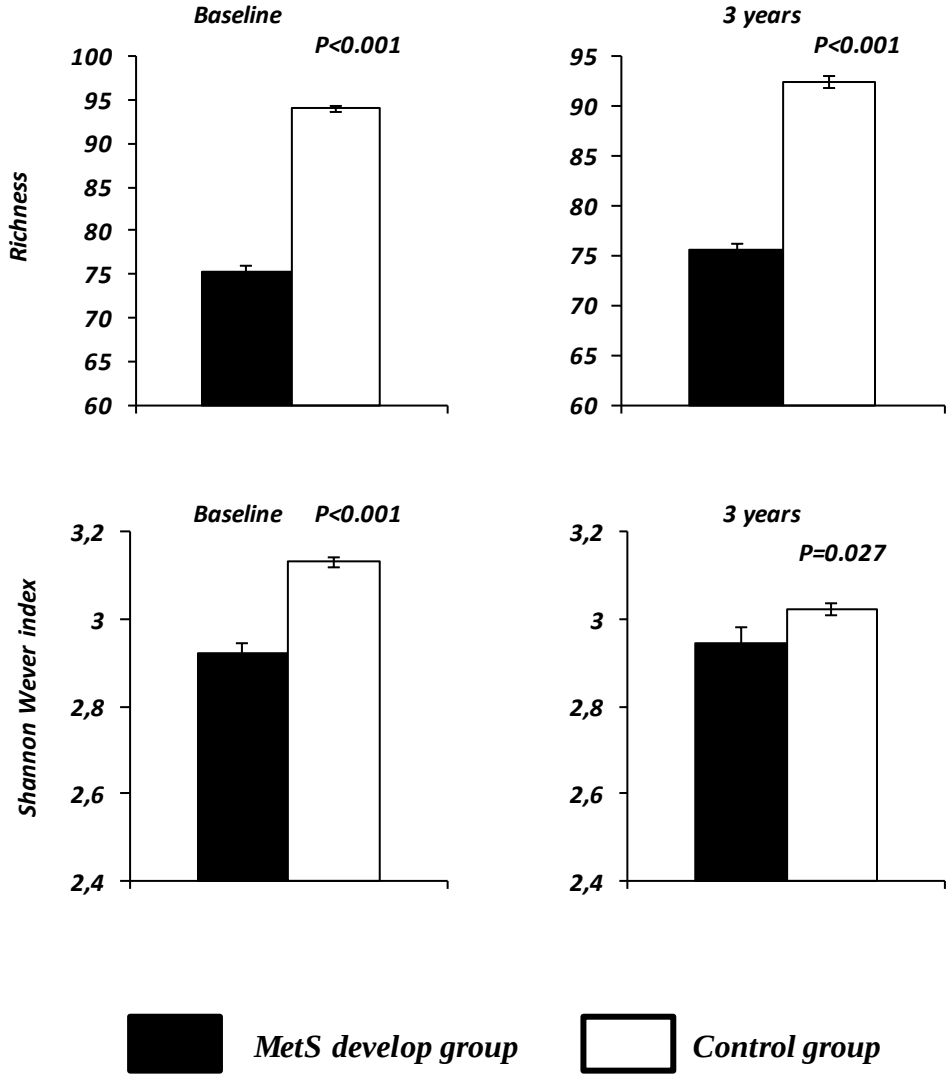


The values represent percent of relative abundance at phyla level in the study population at baseline.

1.4 Diversity and Richness determination

We analyzed the differences in gut microbiota at baseline and after 3 years follow-up between groups and we found higher richness and diversity of bacterial genera as assessed by Shannon index in control group than MetS develop group at baseline ($P<0.001$ and $P<0.001$, respectively). In addition, we found higher diversity and richness bacteria after 3 years follow-up in control group compare with MetS develop group ($P<0.001$ and $P=0.027$, respectively). **Figure 14**

Figure 14. Diversity and Richness bacteria at baseline and after 3 years follow-up.



The values represent means ± SEM of Richness and Shannon Weaver index in baseline and after 3 years follow up. The statistically significant differences between group at baseline and after 3 years follow up were tested by PERMANOVA (*p<0.05).

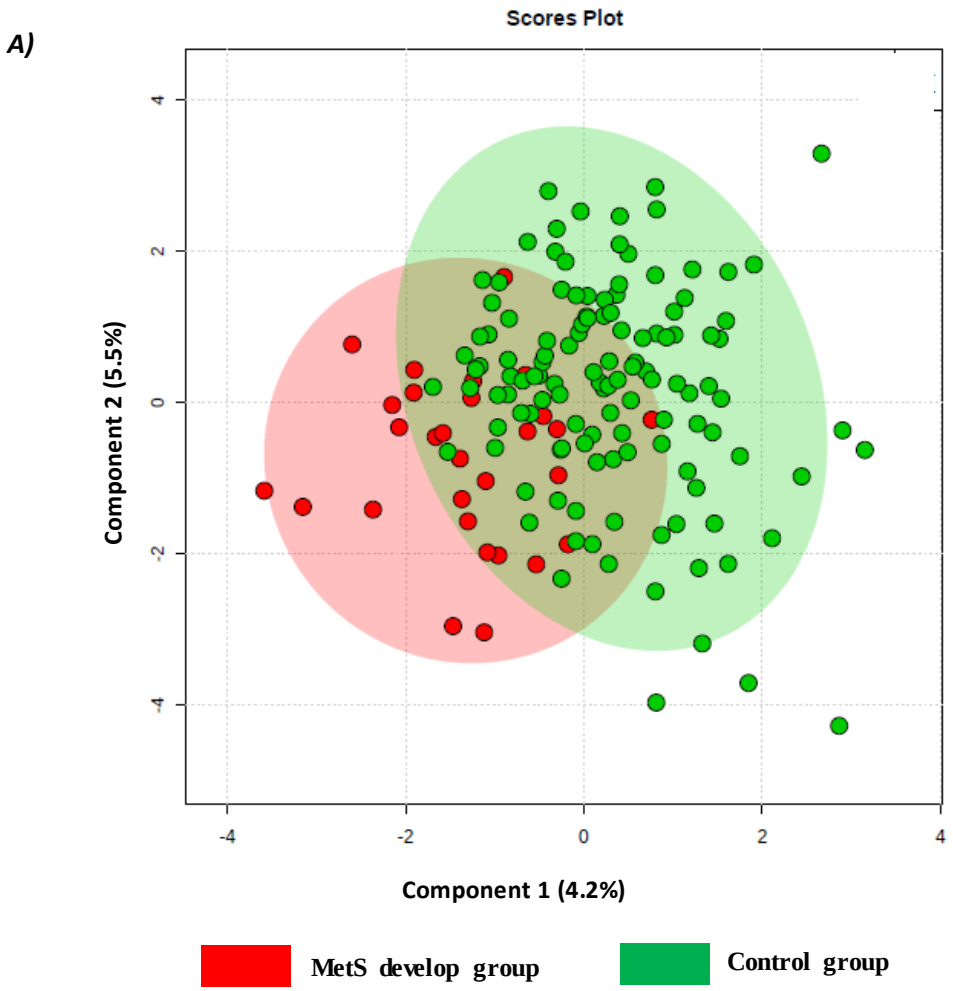
1.5 A gut microbiota pattern predictive of MetS development

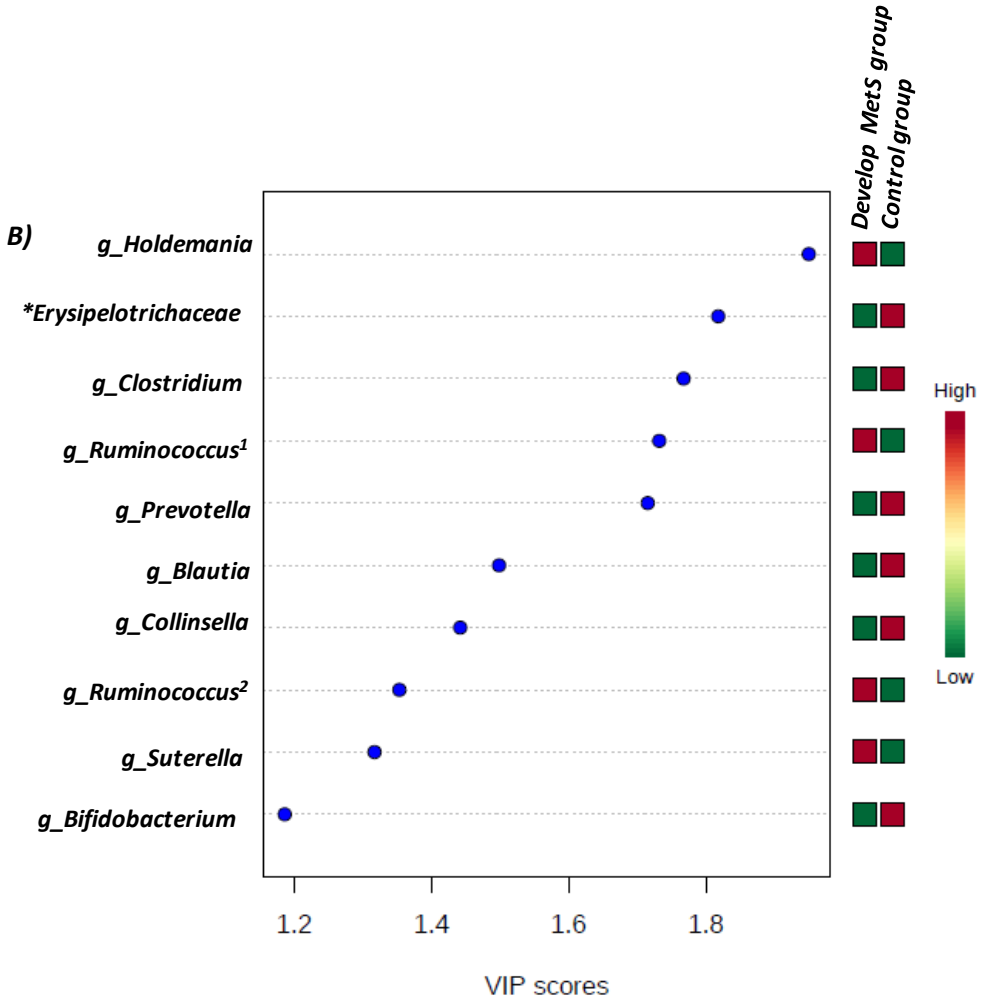
In order to identify a specific microbiota pattern that may predict the development of MetS, we study the differences in the gut microbiota at baseline between patients who developed and patients who did not develop MetS after 3 years of follow-up.

We used multivariate analysis to further investigate whether the relative abundance of specific taxa might differ between groups at baseline,before development of MetS. A Partial Least Square Discriminant Analysis (PLS-DA) revealed that model with high accuracy (two component model accuracy 0.810, R^2 0.38 and Q^2 0.083) could distinguish between patients who developed MetS from those who did not develop it. Based on the

variable importance in the projection (VIP) cut-off of 1.5, the following pattern was identified: higher abundance of *Holdemania* (1.9) and *Ruminococcus* genera (1.7) and lower, family *Erysipelotrichaceae* (1.8), *Clostridium* (1.7) and *Prevotella* (1.7) for discrimination between groups **(Figure 15).** These results were confirmed for the univariate analysis, Mann-Whitney U test adjusted by False Discovery rate (Benjamini and Hochberg method). We observed higher abundance of *Holdemania* (P=0.007; Q=0.048) and lower *Clostridium* genus (P=0.003; Q=0.014) in patients who developed MetS than in patients who did not developed MetS at baseline. Nevertheless *Ruminococcus* (P=0.024; Q=0.583), family *Erysipelotrichaceae (*P=0.015; Q=0.583) and *Prevotella* (P=0.024; Q=0.583) did not pass the FDR test.

Figure 15. Partial Least Square Discriminant Analysis (PLSDA) at genus level in baseline.





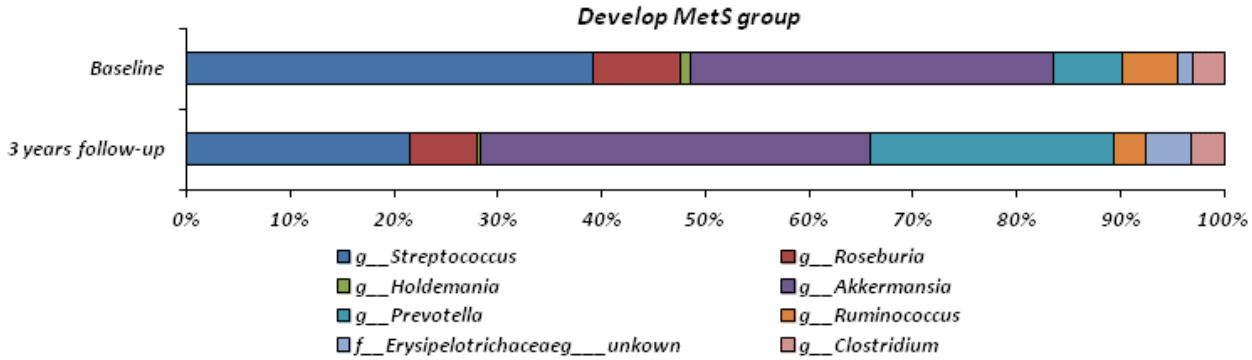
A) PCA between groups at basal time at genus level. **B)** Variable importance in projection (VIP) plot: important features (analyzed genus level) identified by PLS-DA in a descending order of importance. The graph represents relative contribution of bacteria to the variance between individuals. High value of VIP score indicates great contribution of the genus to the group separation. The black and white boxes on the right indicate whether the abundance of bacteria is increased (black) or decreased (white) in each group, develop MetS group and Control group. *Genera unknow; 1; Family *Lachnospiraceae*. 2; Family *Ruminococcaceae*

1.6 Gut microbiota changes associated to MetS development

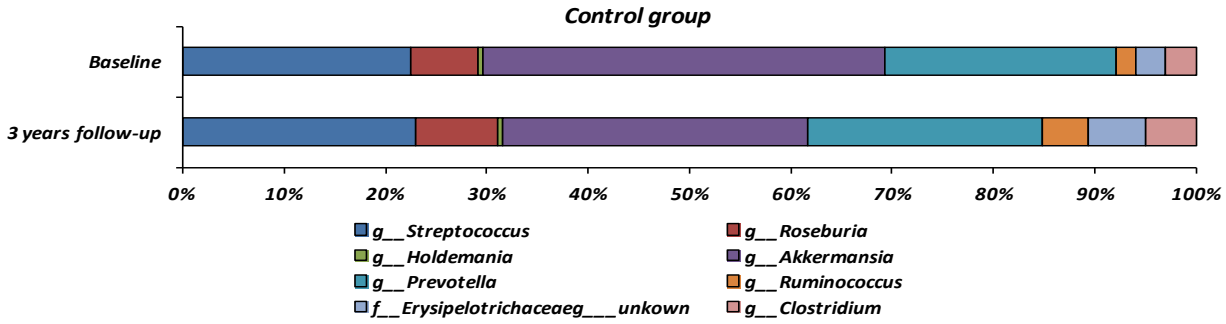
In order to deep in the role of gut microbiota in the development of MetS, we studied the changes undergone in the gut microbiota after 3 years of follow-up in the patients who develop MetS as compared with baseline. When we analyzed the changes in specific bacterial taxa, we observed an increased in the abundance of unknown genera of *Erysipelotrichaceae* family (P<0.001; Q=0.015) in the group of patients who developed MetS at 3 years of follow-up as compared with baseline. By contrast, we

observed a decrease in the abundance of *Streptococcus* (P=0.002; Q=0.039), *Roseburia* (P=0.001; Q=0.039), *Holdemania* (P<0.001; Q=0.036) and *Akkermansia* (P=0.002; Q=0.039), whereas *Erysipelotrichaceae* remained unchanged, in the control group who did not developed MetS at 3 years follow-up as compared with baseline **(Figure 16).**

Figura 16. Changes in gut microbiota composition after 3 years follow-up.



Bacteria	*P-value*	*Q-value*
f_Erysipelotrichaceae_g_unkown	**<0.001**	**0.015**
Roseburia	**0.311**	**0.670**
Akkermansia	**0.740**	**0.888**
Holdemania	**0.030**	**0.247**



Bacteria	*P-value*	*Q-value*
f_Erysipelotrichaceae_g_unkown	**0.542**	**0.747**
Roseburia	**0.001**	**0.039**
Akkermansia	**0.002**	**0.039**
Holdemania	**<0.001**	**0.036**

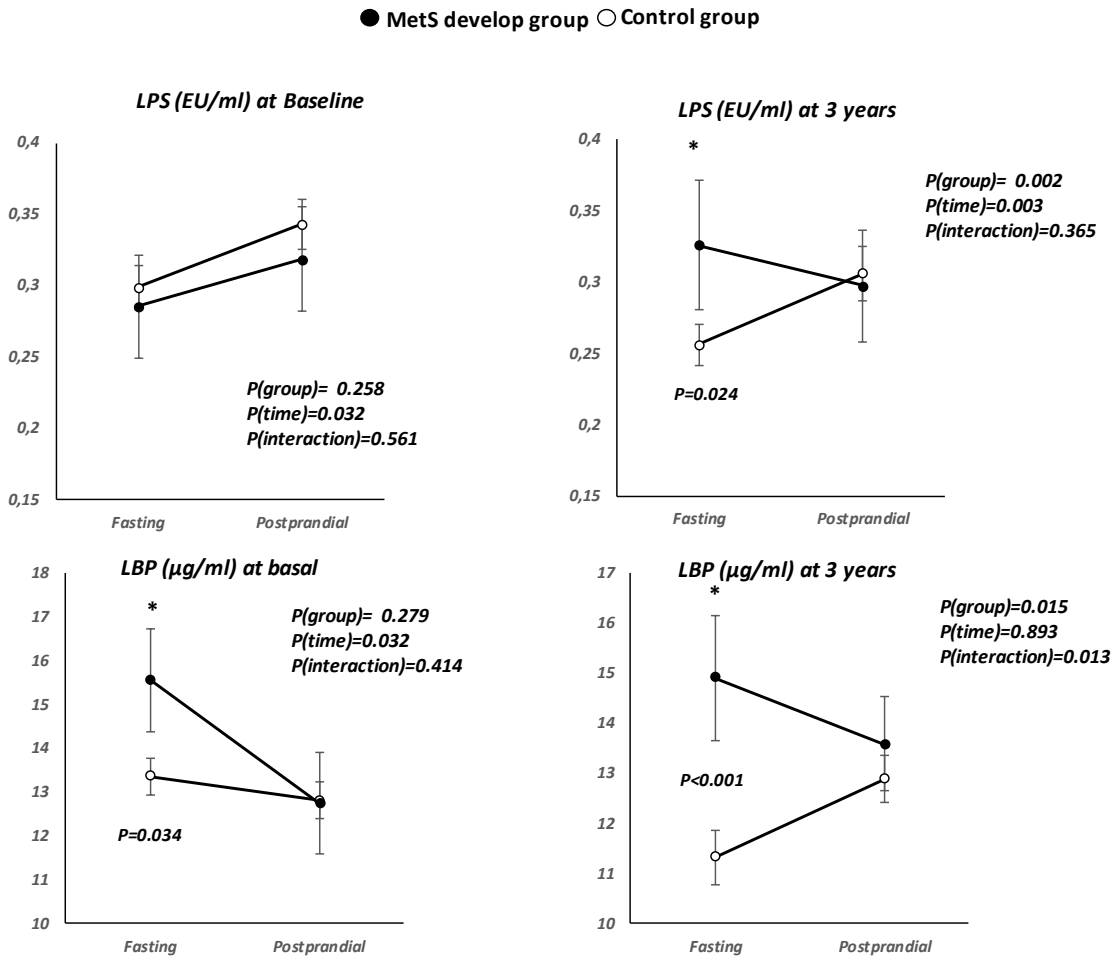
Changes in the relative abundance of genera levels in Develop MetS group and Control group compare baseline and after 3 years follow up. The different colour represents each relative abundance. The statistically significant differences between groups were tested by Krustall Wallis statistical analysis. P-values: Krustall Wallis statistical analysis. *P<0.05. Q-values: Krustall Wallis P-values adjusted by False Discovery Rate using Benjamini and Hochberg method. *Q<0.05.

1.7 Endotoxemia and MetS.

In order to study the relationship between LPS plasma levels in the development of MetS, we measured the LPS plasma levels in fasting state and 4h after the intake of a mixed meal administered at the beginning of the study and after 3 years of follow-up. We did no observe any significant differences between groups in baseline. By contract, we found higher fasting plasma level of LPS in subject who develop MetS as compared with control groups after 3 years follow-up (P=0.024).

We also studied the implication of LBP on the progression of MetS. We observed higher fasting plasma levels of LBP in patients who developed MetS compare with control group without MetS at baseline and after 3 years follow-up (P=0.034 and P<0.001, respectively) **(Figure 17).**

Figure 17. Fasting and postprandial plasma levels of LPS and LBP in the progression of MetS.

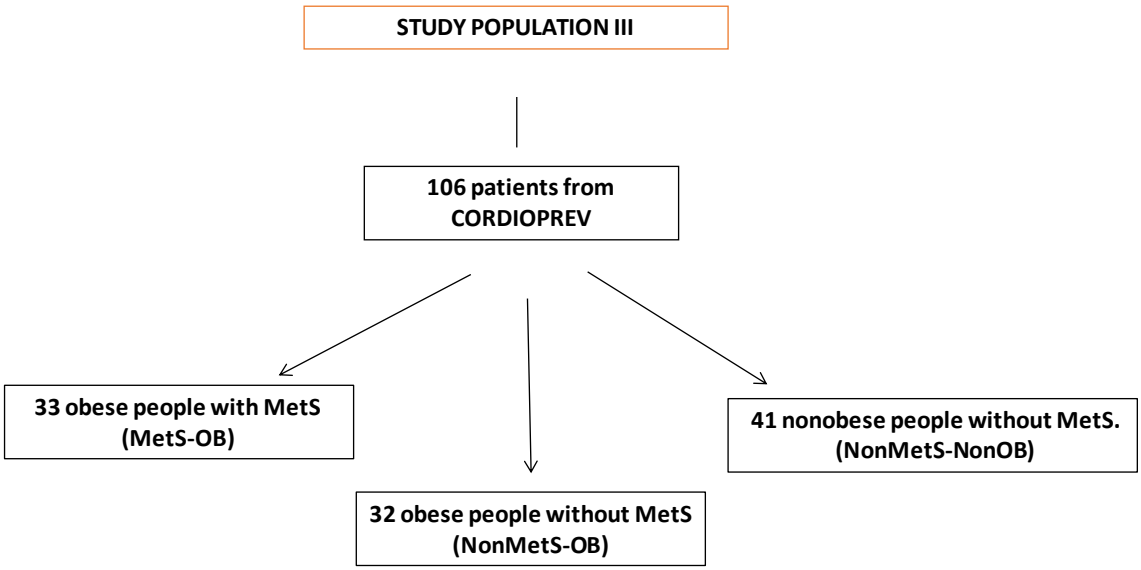


Mean (±S.E.M.) of LPS (EU/ml) and LBP (µg/ml) of the plasma levels at fasting and after the administration of the mixed meal at baseline and at 3 years. ANOVA for repeated measures p-values.
* $p<0.05$ between groups in the Post hoc Bonferroni's multiple comparison tests.

2. CHAPTER 2: Results from Objective secondary 1.

Consumption of two healthy dietary patterns restored microbiota dysbiosis in obese patients with metabolic dysfunction.

In order to carry out the study 106 patients were divided into three groups: the MetS-OB group, 33 obese people with MetS; the NonMetS-OB group, 32 obese people without MetS (2 or less criteria for the metabolic syndrome); the NonMetS-NonOB group, 41 nonobese people without MetS. We studied the microbiota composition at baseline and after 2 years of dietary intervention.



2.1 Baseline characteristic of the study population

The differences in the main anthropometric and metabolic variables between the MetS-OB, NonMetS-OB and NonMetS-NonOB groups are shown in (**Table 9**).

Table 9. Baseline characteristic of the participant in this study.

Group (N)	*MetS-OB (33)*	*NonMetS-OB (32)*	*NonMetS-NonOB (41)*	*P-value*
Age (years)	59.03±1.83	63.72±1.70	61.73±1.39	0.149
BMI (kg/m^2)	32.42±0.76^a	32.88±0.56^a	27.05±0.27^b	<0.001
WC (cm)	114.48±1.43^a	109.11±1.61^b	97.22±0.75^c	<0.001
HOMA-IR	6.50±0.95^a	3.93±0.48^b	2.31±0.21^b	<0.001
HDL-c (mg/dL)	36.79±1.54^a	45.06±2.16^b	45.38±2.14^b	0.005
LDL-c (mg/dL)	80.62±4.73	85.92±3.23	80.30±3.51	0.532
TC (mg/dL)	157.29±5.28	147.70±4.05	144.97±2.73	0.155
TG (mg/dL)	180.07±13.82^a	131.19±12.26^b	124.48±8.82^b	0.001
Glucose (mg/dL)	133.70±14.08^a	105.94±7.94^{a,b}	100.18±3.68^b	0.022
Systolic BP (mm Hg)	130.82±3.22	132.28±3.84	136.68±3.12	0.423
Diastolic BP (mm Hg)	77.22±2.01	74.81±2.05	75.71±1.76	0.681

Means values ± S.E.M. BMI: body mass index. WC: waist circumference. TC: total cholesterol. TG: triacylglycerides. BP: Blood Pressure. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. One-way ANOVA P-values. Values in the same row with different letters differ significantly between groups in the post hoc analysis using Bonferroni's multiple comparison tests.

2.2 *Global analysis of the intestinal microbiota between groups*

We did not find any significant differences in bacterial diversity at baseline nor after 2 years of dietary intervention between the 3 groups with any of the alpha diversity estimators used and at a rarefaction level of 2,000 sequences per sample. Similarly, Principal Coordinate Analysis (PCoA) or UPGMA clustering based on unweighted and weighted UniFrac distances did not show significant differences in the microbiota composition between groups at baseline nor after 2 years of dietary intervention.

2.3 Univariate analysis of the intestinal microbiota between groups at baseline

We investigated whether the relative abundance of specific taxa might differ between groups using One-way ANOVA and the results adjusted by the False Discovery Rate (FDR) using the Benjamini and Hochberg method.

2.3.1 Differences between groups at phylum level.

We observed a lower abundance of *Actinobacteria* and *Bacteroidetes* phyla in the MetS-OB group compared with the NonMetS-NonOB group (*P=0.008 Q=0.032* and *P=0.018 Q=0.032*, respectively). In addition, we observed a higher *Firmicutes/Bacteroidetes* ratio in the MetS-OB group than in the NonMetS-NonOB group (*P=0.019*), whereas no differences were observed between the NonMetS-OB and NonMetS-NonOB groups (**Figure 18**).

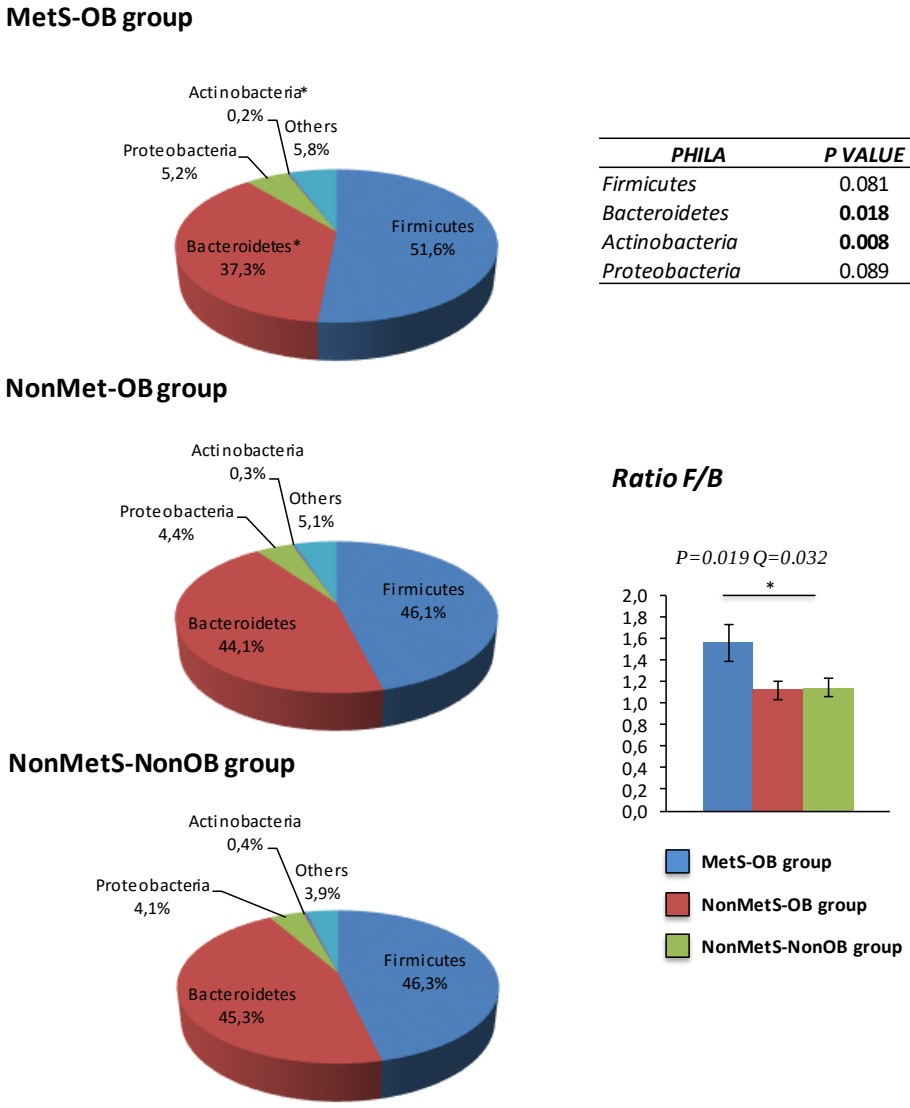
2.3.2 Differences between groups at genus level.

Moreover, we observed a lower abundance of *Bacteroides*, *Prevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcus* in the MetS-OB than in the NonMetS-NonOB group (*P=0.002 Q=0.015, P=0.007 Q=0.021, P=0.006 Q=0.021, P=0.002 Q=0.015* and *P=0.019 Q=0.040,* respectively). On the other hand, we observed a higher abundance of *Streptococcus* and *Clostridium* genera in the MetS-OB group than in the NonMetS-NonOB group (*P=0.018 Q=0.40* and *P=0.006 Q=0.021*, respectively) (**Figure 19**).

2.3.3 Differences between groups at bacterial species level.

We observed that MetS-OB patients had a lower abundance of *P. distasonis* and *F. prausnitzii* (*P=0.008 Q=0.036* and *P=0.012 Q=0.036*, respectively) than the NonMetS-OB and NonMetS-NonOB groups respectively (**Table 10**).

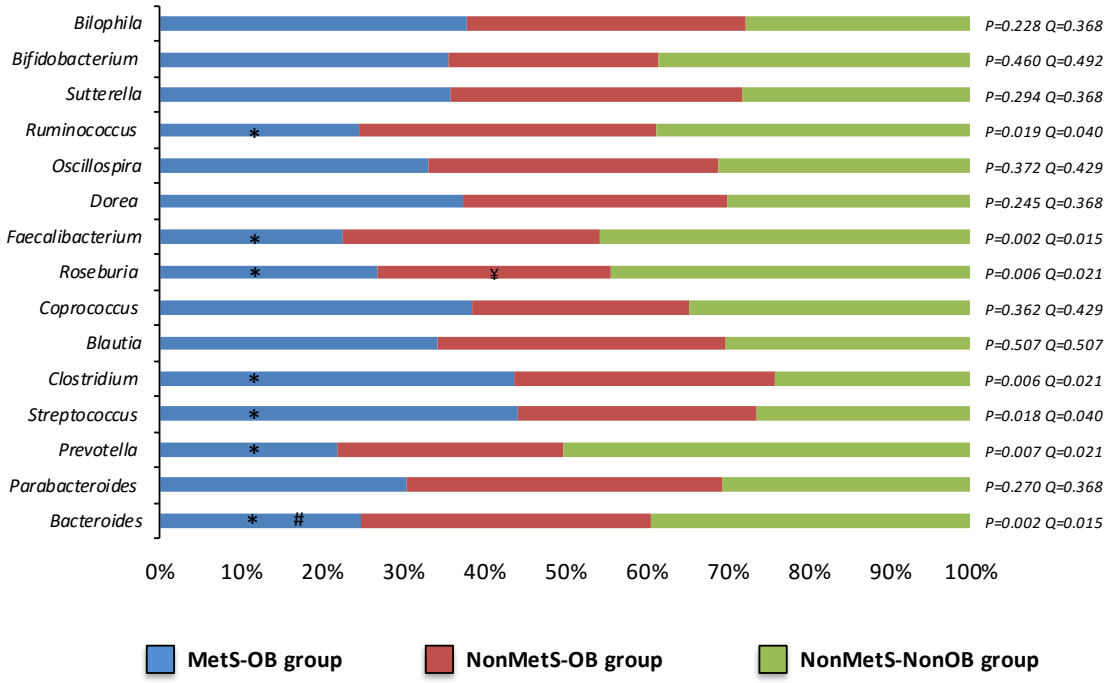
Figure 18. Composition of the intestinal microbiota of the study population at phylum level.



PHILA	P VALUE	Q VALUE
Firmicutes	0.081	0.089
Bacteroidetes	**0.018**	**0.032**
Actinobacteria	**0.008**	**0.032**
Proteobacteria	0.089	0.089

One-way ANOVA statistical analysis. Q-values: ANOVA P-values adjusted by the False Discovery Rate using the Benjamini and Hochberg method. Ratio F/B: ratio *Firmicutes*/*Bacteriodetes*. $^{*}P<0.05$ in the Post-Hoc Bonferroni's multiple comparison tests. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects.

Figure 19. Composition of the intestinal microbiota of the study population at genera level.



MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. P-values: One-way ANOVA statistical analysis. Q-values: ANOVA P-values adjusted by the False Discovery Rate using the Benjamini and Hochberg method. *Statistical significant differences between MetS-OB and NonMetS-NonOB groups. #Statistical significant differences between MetS-OB and NonMetS-OB groups. ¥Statistical significant differences between NonMetS-OB and NonMetS-NonOB groups.

Table 10. Composition of the intestinal microbiota of the study population at bacterial species level.

Bacterial Species	MetS-OB	NonMetS-OB	NonMetS-LO	P-value	Q-value
Bacteroides caccae	0.0062 ± 0.0009	0.0080 ± 0.0013	0.0093 ± 0.0014	*0.208*	*0.250*
Bacteroides ovatus	0.0026 ± 0.0004	0.0035 ± 0.0006	0.0032 ± 0.0004	*0.198*	*0.250*
Bacteroides uniformis	0.0137 ± 0.0018	0.0206 ± 0.0026	0.0239 ± 0.0040	*0.071*	*0.142*
Parabacteroides distasonis	0.0040 ± 0.0005 [a]	0.0069 ± 0.0007 [b]	0.0055 ± 0.0007 [a,b]	***0.008***	***0.036***
Prevotella copri	0.0637 ± 0.0136	0.0685 ± 0.0164	0.0846 ± 0.0180	*0.628*	*0.628*
Faecalibacterium prausnitzii	0.0155 ± 0.0018 [a]	0.0209 ± 0.0032 [a,b]	0.0297 ± 0.0039 [b]	***0.012***	***0.036***

Represented values are mean ± SEM of the relative abundance of each bacterial taxon. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic síndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. ANOVA statistical analysis P-value and Q-value. The different letters denote significant differences between groups in the post hoc comparisons.

2.4 Effect of the dietary intervention on intestinal microbiota composition

In the next step, we studied the changes in the intestinal microbiota after 2 years of the dietary intervention in comparison to baseline time.

2.4.1 Diets alter gut microbiota composition when there was dysbiosis: MetS-OB groups

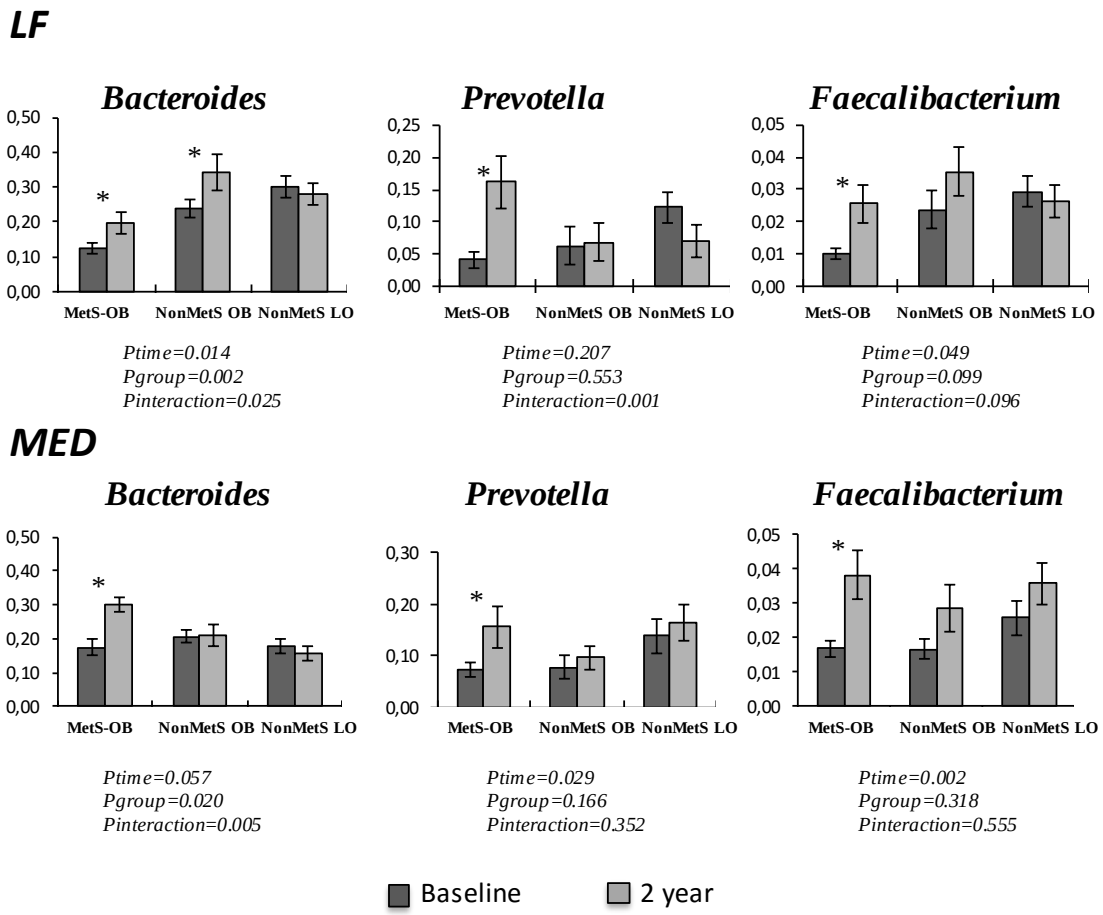
We found that the abundance of *Bacteroides*, *Prevotella* and *Faecalibacterium* genera increased in the MetS-OB group after 2 years of consumption of either the MED or LF diets as compared with the baseline (*P<0.001*, *P<0.001* and *P=0.001*, respectively). Thus, the differences between the MetS-OB group and the NonMetS-OB and NonMetS-NonOB groups found at baseline disappeared after the consumption of these diets (**Figure 20**).

Moreover, the *Bacteroidetes* phylum increased in the MetS group after 2 years of dietary intervention. However, whereas we observed a statistically significant increase in its abundance after the consumption of the LF diet (*P<0.001*), we only observed a trend after the consumption of the MED diet. Consequently, we observed a statistically significant decrease in the *Firmicutes/Bacteroidetes* ratio after the consumption of the LF (*P<0.001*) diet, whereas only a trend was noted after the consumption of the MED diet. In addition, the consumption of the LF diet for 2 years decreased the abundance of *Streptococcus* and *Clostridium* genera (*P=0.010* and

P<0.001, respectively), whereas the consumption of the MED diet did not affect the abundance of these genera (**Figure 21**).

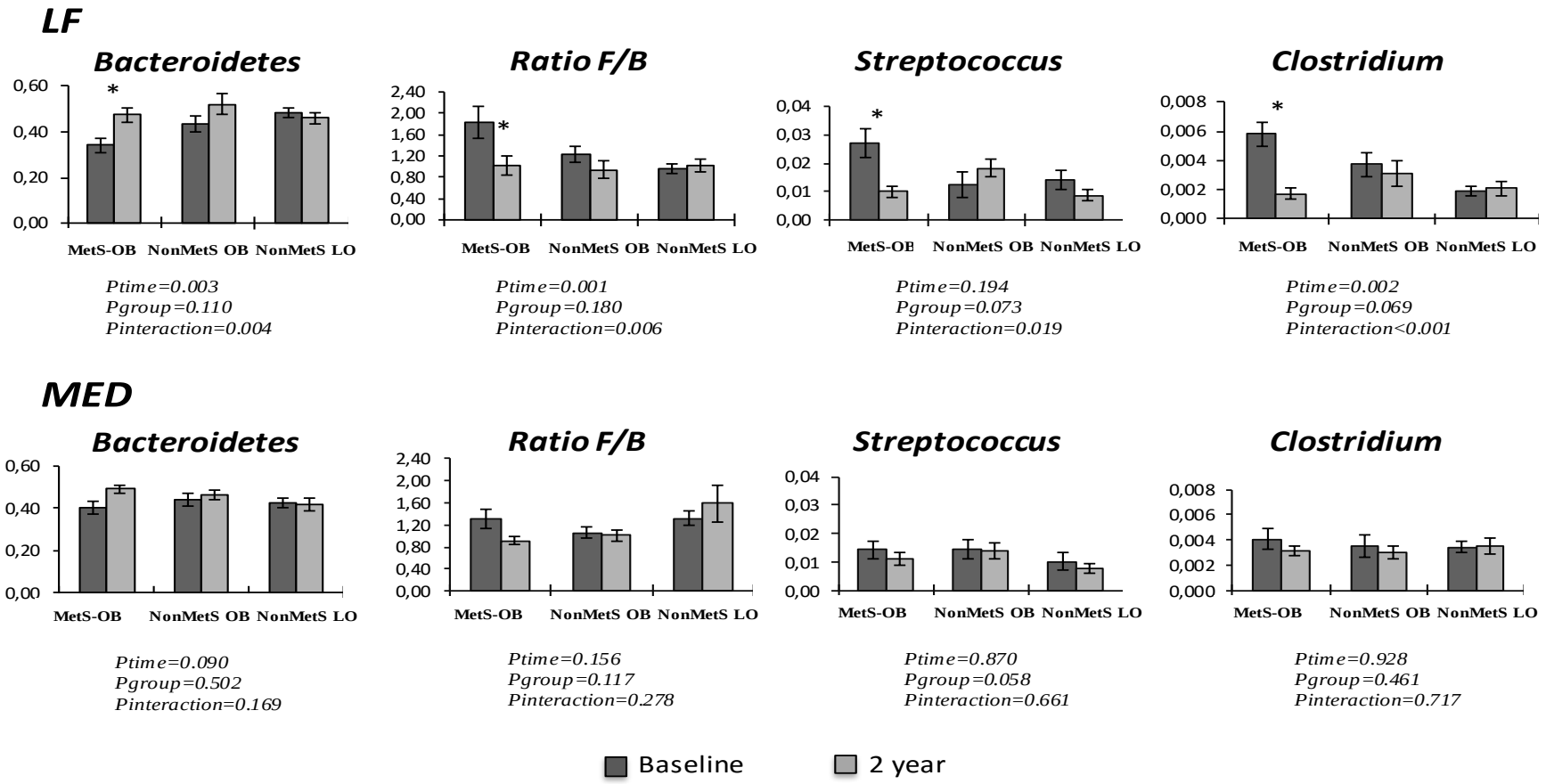
Moreover, the closest species as determined by Greengenes taxonomy were also analyzed. We observed that the consumption of the MED diet for 2 years increased the abundance of *Roseburia* and *Ruminococcus* genera and *P. distasonis* and *F. prausnitzii* bacterial species (*P=0.004*, *P=0.009*, *P=0.014* and *P=0.043*, respectively), whereas the consumption of the LF did not affect the abundance of these genera (**Figure 22**).

Figure 20. Restoration of dysbiosis by the consumption of LF and MED diets.



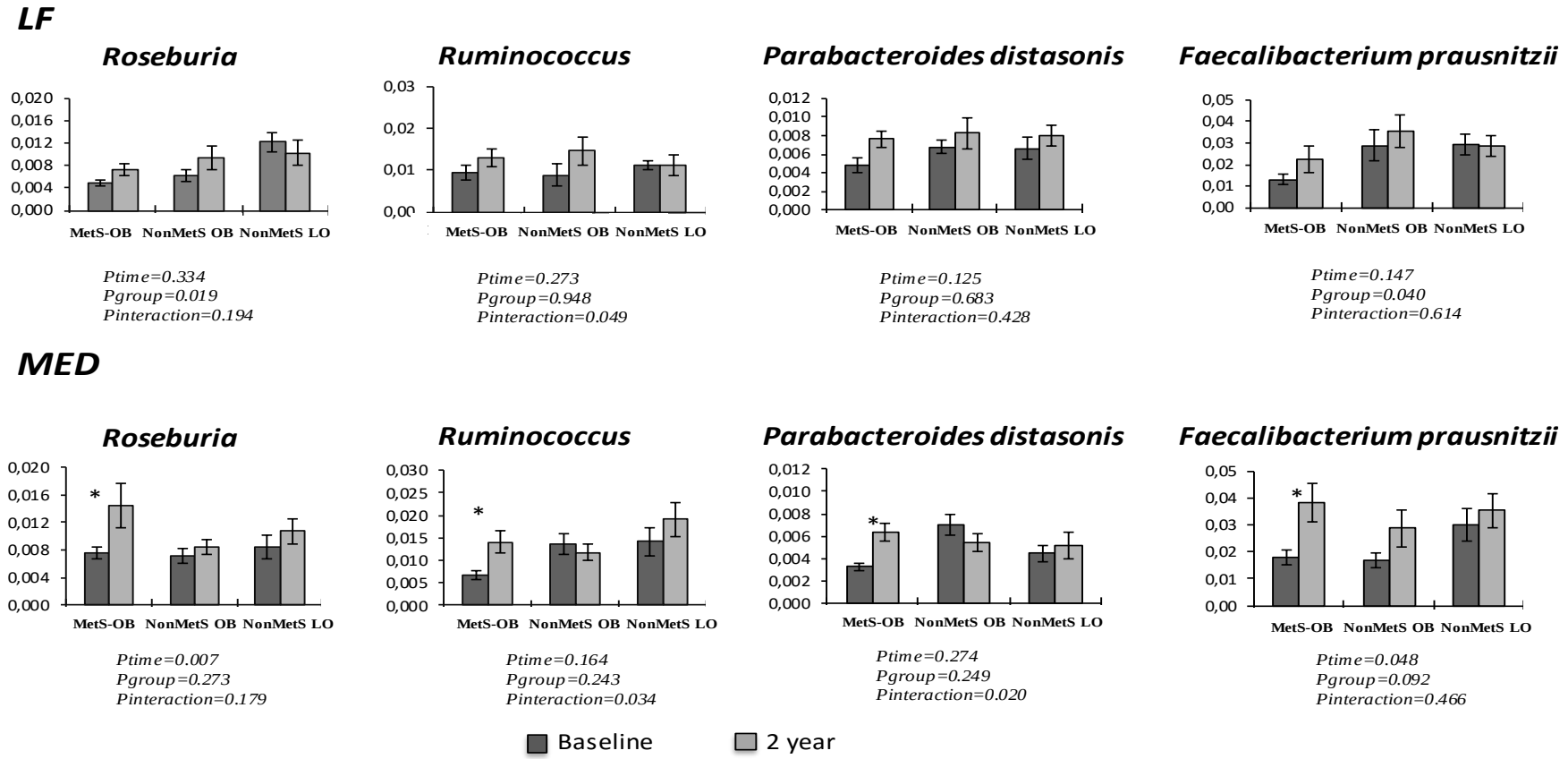
Represented values are mean ± SEM of the relative abundance of each bacterial taxon. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. LF: low-fat diet; MED: Mediterranean diet. The statistically significant differences between groups, time and sampling time by group interaction were analyzed by ANOVA for repeated measures $^{*}P<0.05$ in the Post-Hoc Bonferroni's multiple comparison tests.

Figure 21. Restoration of dysbiosis by the consumption of LF.



Represented values are mean ± SEM of the relative abundance of each bacterial taxon. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. LF: low-fat diet; MED: Mediterranean diet. The statistically significant differences between groups, time and sampling time by group interaction were analyzed by ANOVA for repeated measures $^{*}P<0.05$ in the Post-Hoc Bonferroni's multiple comparison tests.

Figure 22. Restoration of dysbiosis by the consumption of MED diet.



Represented values are mean ± SEM of the relative abundance of each bacterial taxon. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. LF: low-fat diet; MED: Mediterranean diet. The statistically significant differences between groups, time and sampling time by group interaction were analyzed by ANOVA for repeated measures $^{*}P<0.05$ in the Post-Hoc Bonferroni's multiple comparison tests.

2.4.2 Diets did not alter gut microbiota composition when there was no dysbiosys: NonMetS-OB and NonMetS-NonOB groups

No significant microbiota changes were observed after 2 years of dietary intervention in NonMetS-NonOB and NonMetS-OB groups as compared with the baseline.

2.5 Effect of the dietary intervention on the main metabolic variables

In addition, in terms of the effect of dietary intervention on the metabolic variables, we observed a decreased in the TG levels after 2 years of follow-up in the MetS-OB group ($P<0.001$) after the consumption of both diets whereas TG levels remained unchanged in NonMetS-Ob and NonMetS-NonOB groups (**Table 11**). When we analyzed the potential differences between diet in TG levels, we observed that the consumption both LF and MED diets decreased the TG levels in the MetS-OB group.

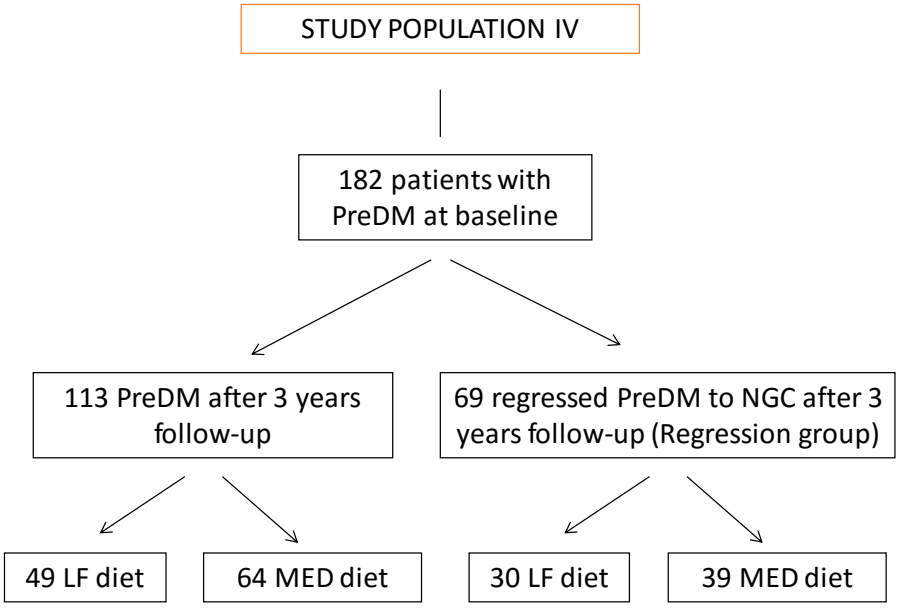
Parameter	*Time point*	*MetS-OB (33)*	*NonMetS-OB (32)*	*NonMetS-NonOB (41)*	*P-time*	*P-group*	*P-interaction*
BMI (kg/m²)	Y_0	32.42±0.76	32.88±0.56	27.05±0.27	*0.337*	*<0.001*	*0.798*
	Y_2	32.12±0.65	32.38±0.74	27.02±0.36			
WC (cm)	Y_0	114.48±1.43	109.11±1.61	97.22±0.75	*0.267*	*<0.001*	*0.593*
	Y_2	112.36±1.55	108.61±1.75	97.05±0.96			
C-reactive protein (mg/L)	Y_0	3.75±0.79	3.21±0.67	2.60±0.76	0.871	0.622	0.988
	Y_2	3.73±1.02	3.30±0.87	2.78±0.98			
HDL-c (mg/dL)	Y_0	36.79±1.54	45.06±2.16	45.38±2.14	*0.195*	*<0.001*	*0.616*
	Y_2	37.42±1.26	43.67±2.14	43.47±1.46			
LDL-c (mg/dL)	Y_0	80.62±4.73	85.92±3.23	80.30±3.51	*0.125*	*0.628*	*0.737*
	Y_2	84.00±4.37	89.13±3.4.07	85.75±3.15			
TC (mg/dL)	Y_0	157.29±5.28	147.70±4.05	144.97±2.73	*0.303*	*0.372*	*0.358*
	Y_2	154.28±5.51	153.13±4.89	151.36±4.13			
TG (mg/dL)	Y_0	188.23±11.91	131.19±12.26	124.48±8.82	*0.006*	*<0.001*	*0.842*
	Y_2	156.14±12.67*	113.50±9.35	105.10±5.34			
Glucose (mg/dL)	Y_0	133.70±14.08	105.94±7.94	100.18±3.68	*0.436*	*0.021*	*0.125*
	Y_2	112.86±4.64	107.87±6.58	105.79±3.73			
Systolic BP (mm Hg)	Y_0	130.82±3.22	132.28±3.84	136.68±3.12	*0.157*	*0.602*	*0.255*
	Y_2	138.91±3.01	133.91±2.92	136.31±2.23			
Diastolic BP (mm Hg)	Y_0	77.22±2.01	74.81±2.05	75.71±1.76	*0.940*	*0.475*	*1.000*
	Y_2	77.26±1.98	74.95±1.90	75.85±1.59			

Table 11. Effect of the dietary intervention on the main metabolic variables. Means values ± S.E.M. corresponding to fasting state at baseline and at 2 years of dietary intervention. BMI: body mass index. WC: waist circumference. TC: total cholesterol. TG: triacylglycerides. BP: Blood Pressure. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. Y_0: baseline. Y_2: 2 years of dietary intervention. ANOVA for repeated measured p-values. * *P<0.05* in the Post Hoc Bonferroni's multiple comparison tests between post-intervention and baseline.

3. CHAPTER 3: Results from Objective secondary 2.

Two healthy diets induced pre-diabetes regression through changes on gut microbiota

This study was performed in 182 pre-diabetic (PreDM) patients included in the CORDIOPREV study **(Table 12),** according to the American Diabetes Association (ADA) diagnosis criteria, 69 from which regressed to normoglycemia (NGC) after 3 years of dietary intervention during which patients consumed a MED or a LF diet



3.1 Characteristic of the study participants

No difference in the rate of regression was observed between diets (39 out of 103 patients consuming MED diet regressed to NGC; 30 out of 79 patients consuming LF diet regressed to NGC; $\chi 2$ test p=0.988). Moreover, PreDM regression was not accompanied by changes in weight, BMI, insulin and lipid parameters (**Table 13**).

Table 12. Baseline demographics and anthropometrics characteristics of the study subjects by glycemic status.

	PreDM population (N=182)
Age	*59.82±0.66*
BMI	*30.37±0.29*
Weight	*82.53±0.97*
Fasting Glucose	*93.69±0.74*
2h (OGGT) Glucose	*126.2±2.55*
HB1c	*5.95±0.02*
TG	*116.01±3.95*
HDL	*44.91±0.74*
LDL	*92.87±1.9*
Insulin	*10.03±0.41*

Abbreviation: BMI, body mass index; LDL-c, low-density lipoprotein-cholesterol, HDL-c, high-density lipoprotein-cholesterol; HB1c, glycated hemoglobin A1; TG, triglycerides.

Table 13. Effect of the dietary intervention on the Main Metabolic Variables in the population of Study at 3 years follow-up.

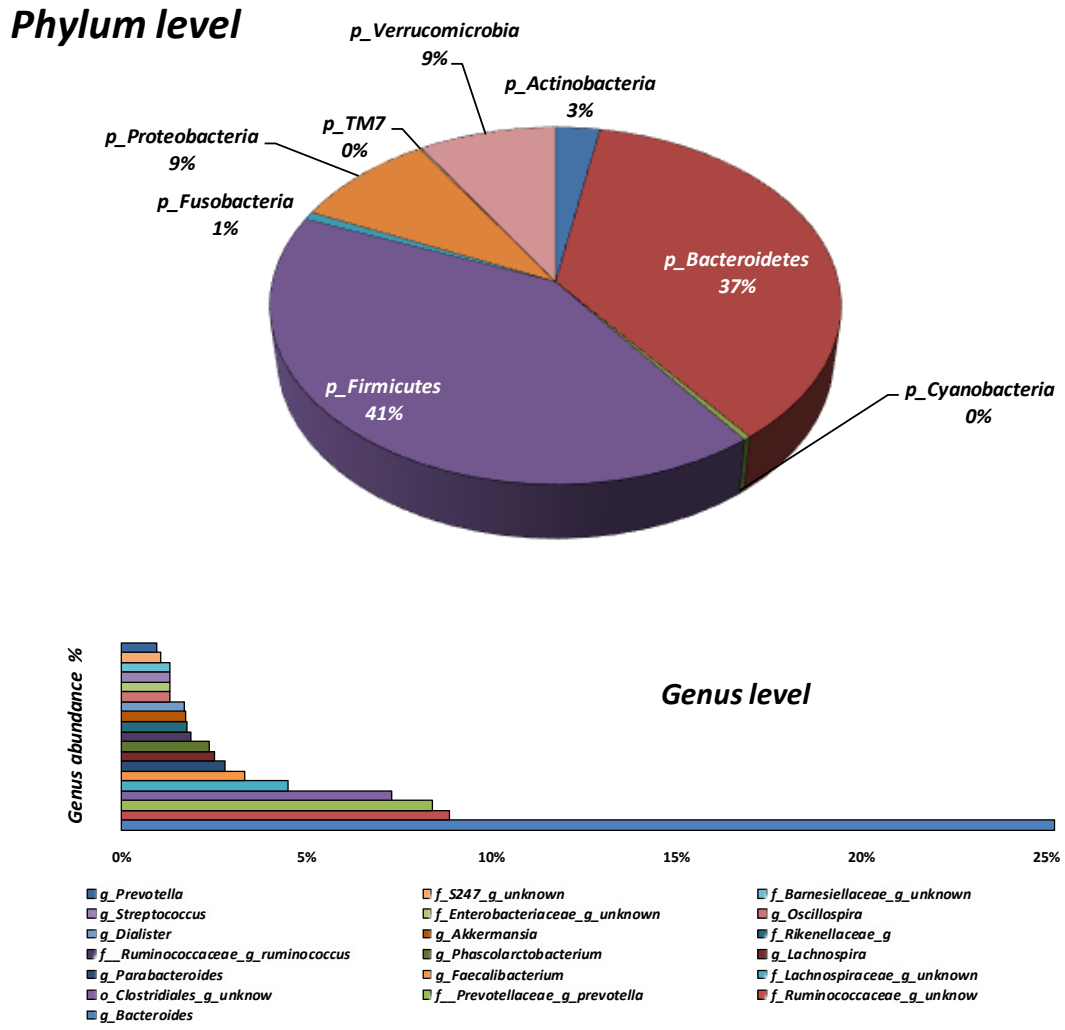
Parameter	*PreDM regression (69)*	*PreDM (113)*	*P- value*
Weight (Kg)	82.79±1.53	81.75±1.06	0.763
BMI (kg/m²)	29.99±0.54	30.37±0.41	0.508
TG total (mg/dL)	110.21±7.08	114.46±5.72	0.543
HDL-c (mg/dL)	43.51±1.2	41.33±0.8	0.116
LDL-c (mg/dL)	98.58±3.16	93.62±2.46	0.216
Insulin	7.33±0.74	8.46±0.64	0.128

One-way ANOVA statistical analysis *p*-value. We determinate significant different between groups lower $p<0.05$. Abbreviations: BMI, body mass index; LDL-c, low-density lipoprotein-cholesterol, HDL-c, high-density lipoprotein-cholesterol; TG, triglycerides.

3.2 Gut microbiota composition at baseline

Globally, the phylogenetic characterization of the gut microbiota of the study population at baseline showed the abundance 5 main bacterial phyla ranging about the 97.5% of the total bacterial population: *Bacteroidetes* (37%), *Firmicutes* (41%), *Proteobacteria* (9%), *Verrucomicrobia* (9%) and *Actinobacteria* (3%). The most prevalent genera in our population were *Bacteroides*, *Prevotella* and a group of non-identified genera from *Ruminococcaceae* family (**Figure 23).**

Figure 23. Global microbiota-pattern of the population of study



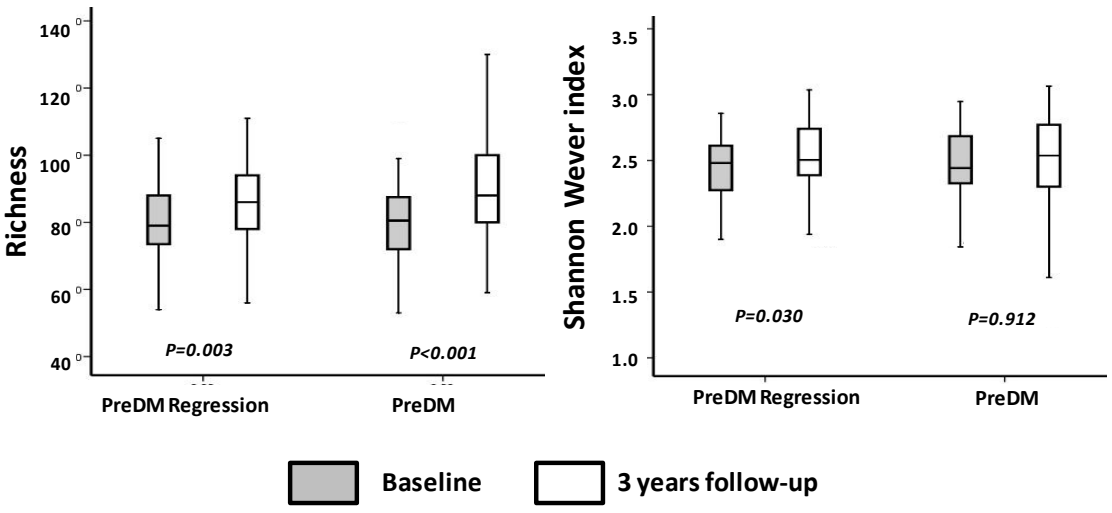
Represented the percent of abundance of each phylum and genus in the study population at baseline.

3.3 Microbiota diversity and PreDM regression

We observed an increase in richness at genus level in both patients who regressed from PreDM to NGC and patients who did not regressed (P=0.003; P=0.001 respectively). When we analyzed the richness by diet, we observed that richness at genus level increased in patients who regressed from PreDM to NGC after the consumption of both MED and LF diets (P=0.045 and P=0.029, respectively). In addition, the richness at genus level also increased in patients who did not regress from PreDM to NGC after the consumption of LF and MED diet (P>0.001; P=0.001, respectively).

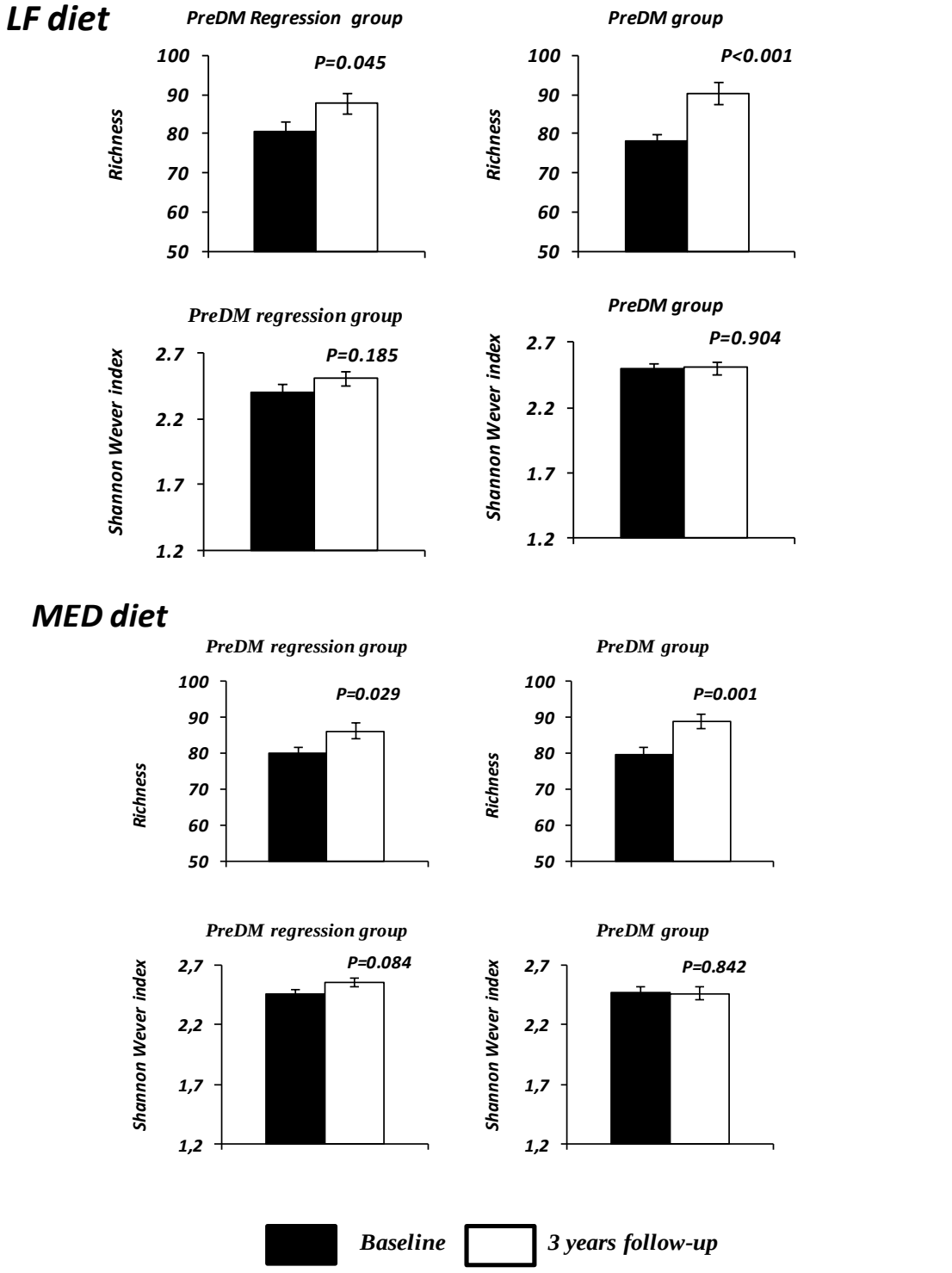
However, when we amalyzed the the diversity of bacterial genera as assessed by Shannon index, we observed an increase in the patients who regressed from PreDM (P=0.030) as compared with baseline whereas no changes were observed in the group of patients who maintained PreDM **(Figure 24).** When we analyzed the Shannon diversity index by diets separately, we observed a trend for an increase in the patients who regressed from PreDM, but it did not reach the statistical significance **(Figure 25).**

Figure 24. Evaluation of microbial diversity in the population of study



We performed Richness and Shannon Weaver index at Baseline vs. 3 years follow-up. The statistically significant differences between group at baseline and after 3 years follow up were tested by PERMANOVA (*p<0.05).

Figure 25. Richness and diversity according to the consumption of Mediterranean diet (MED) and Low-fat diet (LF).

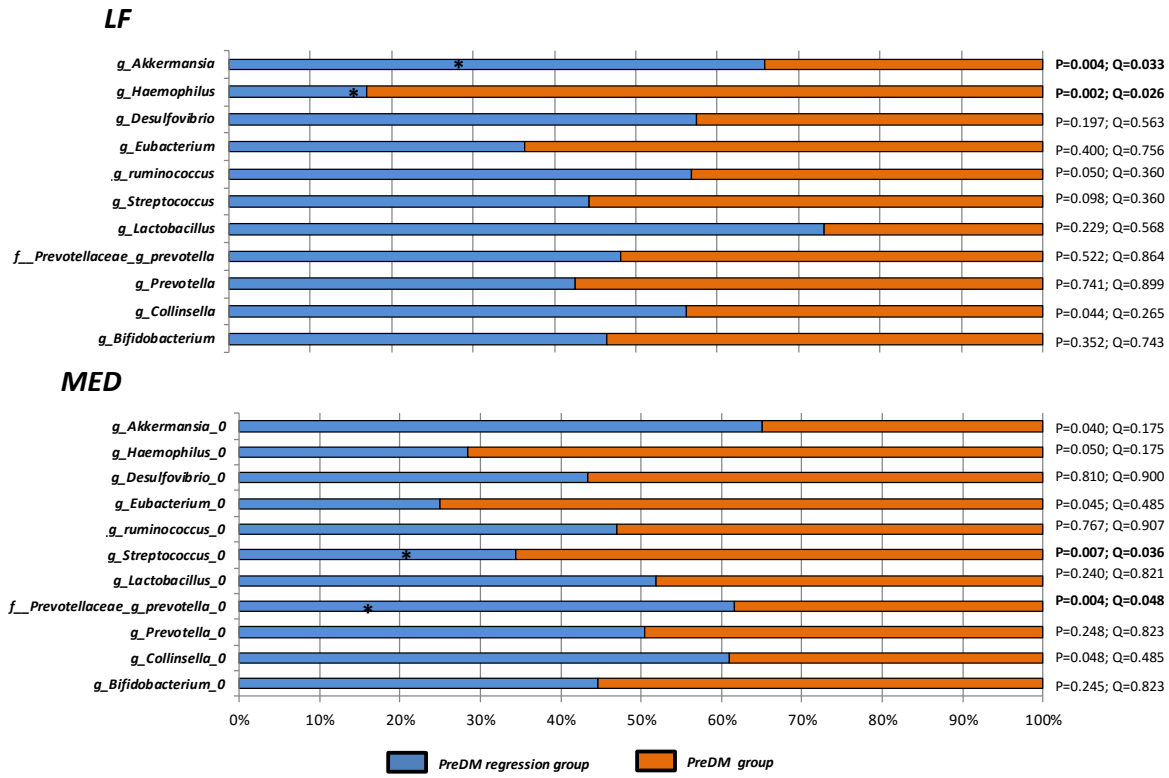


Values represent means ± SEM of Shannon diversity index and richness at baseline and after 3 years follow-up. The statistically significant differences between group at baseline were tested by PERMANOVA (*p<0.05).

3.4 Microbiota pattern associated to PreDM regression according to diets

When LF diet was consumed for 3 years, the patients who regressed from PreDM to NGC after had higher abundance of *Akkermansia* (P=0.004; Q=0.033) and lower *Haemophilus* (P=0.002; Q=0.026) than patients who did not regress. By contrast, when MED diet was consumed, the patients who regressed from PreDM have higher abundance of *Prevotella* (P=0.004; Q=0.048) and lower of *Streptococcus* genus (P=0.007; Q=0.036) than patients who did not regress **(Figure 26).**

Figure 26. Changes in gut microbiota according to the consumption of Mediterranean diet (MED) and low-fat diet (LF) at 3 years.



P-values: Krustall Wallis statistical analysis. Q-values: Krustall Wallis P-values adjusted by False Discovery Rate using Benjamini and Hochberg method. *Significant statistical differences between PreDM regression group and PreDM group after consumption LF, low-fat diet; and MED, Mediterranean diet. P<0.05.

No differences in the abundance of *Akkermansia*, *Haemophilus*, *Prevotella* and *Streptococcus* were observed between groups at baseline **(Table 14).**

Table 14. Differences in gut microbiota composition between groups at baseline before dietary intervention (MED and LF).

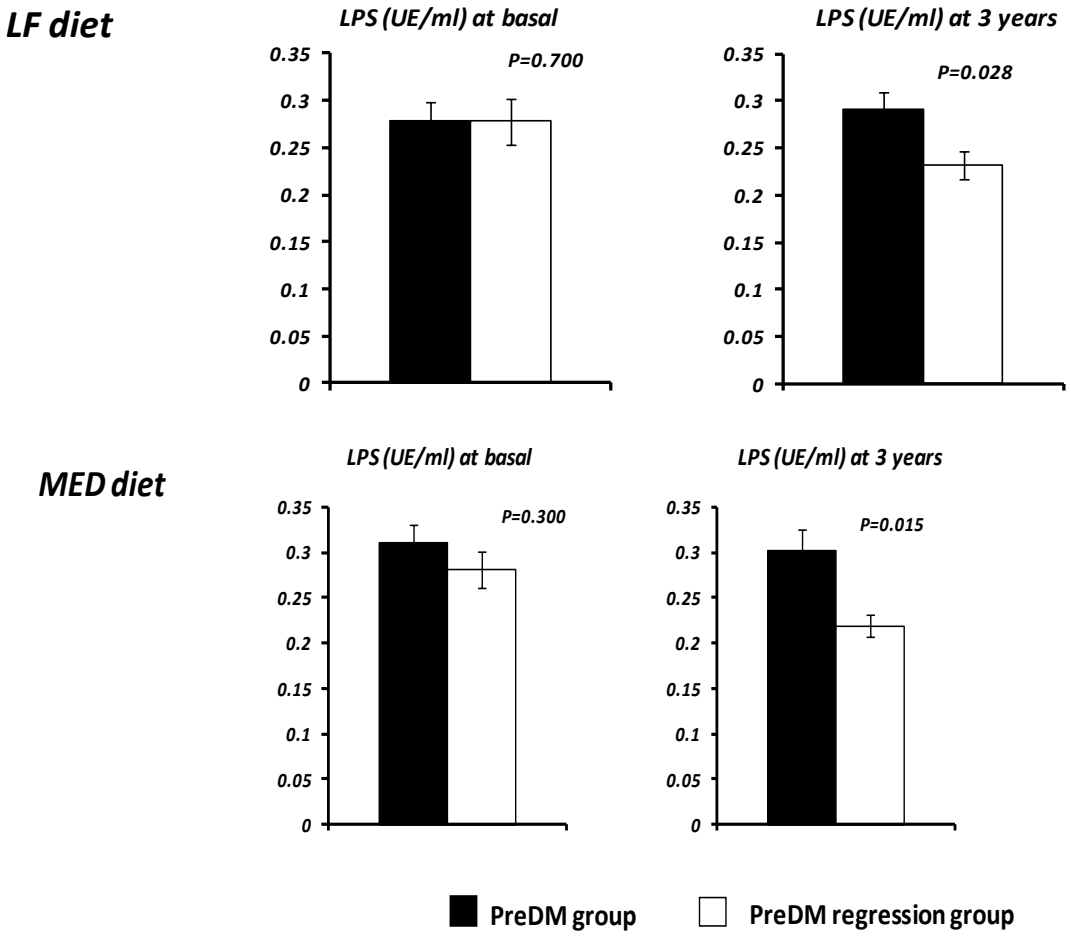
	Mediterranean diet				Low fat diet			
TAXA	***PreDM regression group***	***PreDM group***	***P-value***	***Q-value***	***PreDM regression group***	***PreDM group***	***P-value***	***Q-value***
g_Bifidobacterium	0.006±0.001*	0.003±0.001*	*0.003**	*0.041**	0.005±0.002	0.003±0.001	*0.578*	*0.995*
g_Collinsella	0.002±0.000	0.003±0.001	*0.950*	*0.950*	0.002±0.001	0.003±0.001	*0.827*	*0.995*
g_Prevotella	0.012±0.004	0.009±0.003	*0.410*	*0.842*	0.008±0.005	0.009±0.003	*0.706*	*0.995*
f__Prevotellaceae_g_prevotella	0.085±0.019	0.078±0.015	*0.845*	*0.922*	0.087±0.024	0.09±0.017	*0.953*	*0.995*
g_Lactobacillus	0.001±0.001	0.001±0.000	*0.163*	*0.652*	0.001±0.000	0.003±0.001	*0.861*	*0.995*
g_Streptococcus	0.014±0.003	0.015±0.002	*0.421*	*0.842*	0.009±0.002	0.011±0.002	*0.483*	*0.995*
g_Ruminococcus	0.001±0.000	0.001±0.000	*0.550*	*0.922*	0.001±0.000	0.002±0.000	*0.995*	*0.995*
f__Ruminococcaceae_g_ruminococcus	0.016±0.003	0.021±0.003	*0.050*	*0.299*	0.022±0.003	0.017±0.002	*0.113*	*0.995*
g_Eubacterium	0.001±0.001	0.002±0.001	*0.808*	*0.922*	0.002±0.001	0.003±0.001	*0.247*	*0.995*
g_Desulfovibrio	0.002±0.001	0.002±0.001	*0.802*	*0.922*	0.004±0.001	0.003±0.001	*0.849*	*0.995*
g_Haemophilus	0.003±0.001	0.005±0.001	*0.232*	*0.697*	0.002±0.001	0.004±0.001	*0.196*	*0.995*
g_Akkermansia	0.018±0.006	0.023±0.006	*0.701*	*0.922*	0.013±0.004	0.013±0.004	*0.591*	*0.995*

The value represent MEAN±SEM P-values: Krustall Wallis statistical analysis. Q-values: Krustall Wallis P-values adjusted by False Discovery Rate using Benjamini and Hochberg method. *Significant statistical differences between PreDM regression group and PreDM group before consumption LF, low-fat diet; and MED, Mediterranean diet. $P<0.05$

3.5 *Diet, endotoxemia and PreDM regression*

When LF diet was consumed for 3 years, patients who regressed from PreDM to NGC had lower fasting LPS plasma levels (P=0.028) than patients who did not regress. Likewise, when MED diet was consumed, patients who regressed from PreDM had lower fasting levels of LPS (P=0.015) than patients who did not regress. We did not observe differences in plasma levels of LPS at baseline between groups of patients assigned to each diet (MED and LF diets) **(Figure 27).**

Figure 37. Fasting plasma levels of LPS before and after long-term consumption of LF and MED.



Values represent means ± SEM of the fasting and postprandial plasma levels in baseline and after 3 years follow-up. The statistically significant differences between group at baseline were tested by one-way ANOVA (*p<0.05).

VII. DISCUSSION

1. CHAPTER 1: Gut microbiota composition as predictive biomarker of the development of MetS

We analyzed the gut microbiota from two groups of patients without MetS, one of which developed MetS after 3 years of follow-up, and we observed that the gut microbiota composition at baseline was different between patients who further developed MetS and patients who did not developed MetS. Moreover, we observed lower richness and increase in unknown genera of *Erysipelotrichaceae* family associated to MetS development whereas the abundance of this genus did not change in the patients who did not developed it.

Although the molecular mechanisms underlying MetS are not fully understood, the presence of MetS is commonly associated with inflammation, insulin resistance and endothelial dysfunction [400]. Current evidence indicates that the composition of gut microbes plays an important role in maintaining human health, and microbial community imbalances can predispose to certain diseases [401]. Numerous studies show the profound impact of diet on the composition of the intestinal microbiome and it has been proposed diet-based therapy to prevent the progression of MetS [402]. However, not all the patients respond similarly to diet-based strategies and a specific gut microbiota profile may determine whether a patient respond or not to diet based therapeutic treatment. In fact, the differences in the composition of gut microbiota found in baseline between groups suggest that certain dysbiosis take place in patients before MetS development. This dysbiosis or specific pattern might be considered a reason of non-responding to diets, taking into account that patients were consuming two healthy diets (MED or low-fat), and despite, 34 of them developed MetS.

Bacteria in the gut constitute a complex ecosystem in which different species exhibit specialized functions and interact as a community [168, 403, 404]. Consistent with previous findings in both mice and humans, we observed relations between the relative abundance of the *Ruminococcus* and *Holdemania* genera with MetS develop. The higher abundance of *Ruminococcus* found at baseline in the group of patients who developed MetS are in line with a study in which *Ruminococcus* abundance directly correlated with the reduction of markers of MetS, in an animal model of fructose-driven MetS [405]. In addition, a previous study found a relationship between *Holdemania* with clinical indicators of an impaired lipid and glucose metabolism in MetS patients [406]. These

finding could suggest an impaired in glucose metabolism before develop MetS in our population.

Our study also showed a reduction in of the genus with saccharolytic activity, *Prevotella,* in subjects before develop MetS. *Prevotella* is responsible for degrading many complex glycans, that constitute the mucosal layer surrounding the wall of the tract digestive, these finding suggest an impaired in gut barrier permeability before develop MetS [407] that could resulting in translocation of bacterial products across the intestinal barrier to bloodstream [408]. Our results suggest an impaired intestinal barrier by the MetS patients who will develop, which would also lead to the worst intestinal permeability compare with patients who will not develop MetS. Also, our finding suggests a loss of the capacity to degrade carbohydrates to SCFA, or the lack of further SCFA signaling, may be related with the metabolic dysfunction of the host organism.

On the other hand, *Clostridium* has been associated with obesity and other metabolic disorders in humans [409, 410], Nevertheless, a few species from *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* species, have been shown to have inhibitory activities against *Clostridium* [411, 412] who explaining the lower abundance of *Clostridium* at baseline in patients who develop MetS.

Moreover, although we did not observe differences in fasting nor postprandial LPS levels, we found higher fasting LBP levels at baseline in patients who developed MetS. Recent studies have shown that the levels of LBP, an acute phase protein which serum concentration has been shown to increase as response to invasive bacterial infection, were increased in obesity and MetS, and it has also been linked to adipocyte inflammation and insulin resistance [413-415]. This finding supports the idea of a dysbiosis or specific microbiota pattern precedes the development of MetS. Here we report a potential use of LBP, together with specific bacterial taxa as biomarkers to predict the development of MetS.

A major strength of this study was our ability to test the association between genus unknown of *Erysipelotrichacea* family and MetS development, an increase in this bacteria are in line with previous studies that have shown to be enriched in fecal samples from obese humans, genetically obese mice and high fat diet-associated mice, and to be closely linked to energy homeostasis and adiposity [284, 416, 417]. In addition, the importance of *Erysipelotrichaceae* in inflammation-related disorders of the gastrointestinal tract is highlighted [418]. Our finding suggests that deterioration of gut intestinal barrier and losing of the capacity to degrade carbohydrates to SCFA, produced

an increase of genus unknown of *Erysipelotrichacea* family causing MetS development in years.

By other hand, recent evidence has proposed that higher endotoxemia was dependent on a disruption of the key tight-junction proteins, ZO-1 and occcludin in colon [419, 420]. These changes in the intestinal barrier integrity may be associated with the changes in the gut microbiota, proposing a related with the pathogenic mechanisms to promote the development of obesity, T2DM, and MetS [421]. The composition of intestinal microbiota, gut permeability, and subsequent bacterial translocation are critical determinants of LPS exposure in health and disease [422]. Our results showed higher levels of LPS 4h after the intake of a mixed meal in the postprandial study performed after 3 years follow up suggesting that an impairment of the intestinal barrier integrity may be caused by the MetS development as a consequence of systemic inflammation in these patients, which would produce the increase in the intestinal absorption of LPS.

All together, the endotoxemia and microbiota changes observed in parallel to development of MetS, suggest a deterioration of membrane permeability which in turn could induce inflammation and therefore insulin resistance. Clarifying the precise mechanism by which microbiota affect the pathogenesis of MetS is likely to be a complicated but an important issue.

In conclusion, we identified a microbiota-based fingerprinting based in the abundance of *Ruminococcus*, *Holdemania*, *Prevotella* and *Clostridum*, as well as, fasting plasma levels of LBP, as potential biomarkers to predict the development of MetS. In addition, our result showed that the intestinal endotoxemia may play an important role in the pathogenesis of MetS, in term of, higher endotoxemia are associated with impaired intestinal barrier and it could promote develop of MetS.

2. CHAPTER 2: Consumption of two healthy dietary patterns restored microbiota dysbiosis in obese patients with metabolic dysfunction.

Our study showed a marked dysbiosis in obese people with severe metabolic disease, who had the full characteristics of MetS (5 criteria) (MetS-OB), compared with obese people without MetS (NonMetS-OB) and non-obese people (NonMetS-NonOB). Interestingly, the disbiotic pattern was reversed by the chronic consumption of both MED or LF diets, altering its microbiota composition to the pattern found in metabolically healthy people. However, in the NonMetS-NonOB and NonMetS-OB groups, no significant microbiota changes were observed after the dietary intervention.

The obese microbiota pattern, described in animal models, is characterized by a higher *Firmicutes/Bacteroidetes* ratio [279]. However, studies in obese humans do not always correspond with what is expected in animal studies [256, 280, 284, 423]. As previously commented, these different results might be explained by differences in the features of the different cohorts [424, 425]. To avoid some of these confusion factors, we selected age-matched groups, with only male participants, and our data suggested that metabolic dysfunction could be another circumstance, which may explain the different results found in the human studies. Thus, our study showed that the *Firmicutes*/*Bacteroidetes* ratio, which is considered of great importance in the development of obesity [426], is in fact related to the presence or absence of metabolic traits in humans but not with obesity itself. Moreover, the fact that our patients have CHD may also affect the gut microbiota composition, in addition to the degree of metabolic dysfunction.

We have previously reported the dysbiosis of MetS patients [427], characterized by a reduction in the abundance of genera with saccharolytic activity, which leads to a reduction in carbohydrate degradation capacity in MetS patients as compared with lean control individuals. In the present study, we have found in the subgroup of patients with MetS-OB a similar reduction of the genera with saccharolytic activity, especially *Bacteroides* and *Prevotella* (both forming the *Bacteroidetes* phylum) and *Roseburia*, *Ruminococcus*, and *Faecalibacterium* [428-430]. Our data suggest that the loss of certain functions or features of the gut microbiota, such as the loss of the capacity to degrade carbohydrates to short-chain fatty acid (SCFA) may be related with the metabolic dysfunction of the host organism [274, 431]. Taking into account that amino acids can also serve as precursors for the synthesis of SCFA by bacteria [432], the loss of the capacity to degrade carbohydrates to SCFA may imbalance the interplay between gut

microbiota and amino acid and SCFA homeostasis shifting the bacterial activity toward SCFA production from amino acids. This is consistent with the reduced insulin sensitivity observed in MetS-OB, and may be related with the high branched-chain amino acids concentration found in insulin resistance states [433] and a dysregulation in the production of incretins, which are involved in the regulation of feeding and energy balance, presumably through SCFA as signal molecules [434, 435], which, in turn, may contribute to the development of type 2 diabetes mellitus (T2DM) and MetS [436].

It is well known that individuals with obesity present a low-grade inflammation [437], which has also been proposed to promote the development of the MetS [438]. However, our study did not provide evidence of a direct relationship between gut microbiota composition and inflammatory status as assessed by C-reactive protein levels and its potential modulation by diet. Nevertheless, C-reactive measurement may not be accurately enough to evaluate the inflammatory status in the CHD population analyzed in this study and further studies focusing this issue are needed.

Dietary strategies have been shown to be of great importance in the prevention and treatment of metabolic diseases [439], which presumably work by shaping the gut microbiota, as has been suggested [440]. In fact, our study showed that the consumption of two healthy diets (the MED and LF diets) restored the microbiota composition in the population with severe metabolic dysfunction in parallel with a reduction of TG and a trend for glucose depletion (MetS-OB group), which supports this idea.

Certainly, the consumption of the two healthy diets administered in our study increased the abundance of the two genera which make up the *Bacteriodetes* phylum (*Bacteroides* and *Prevotella*), which, in turn, reduced the B/F ratio. As summary, all these changes contribute to restoring the obesity-associated microbiota pattern characterized by a high F/B ratio observed in the population with severe metabolic dysfunction (MetS-OB). In addition to the *Bacteroides* and *Prevotella* genera, the abundance of other genera with saccharolytic activity such as *Faecalibacterium* [428-430] also increased after the consumption of the two healthy diets administered, which, in turn, may restore, at least partially, the fiber-derived SCFA synthesis by gut microbiota. However, the consumption of the MED diet, rich in antioxidant phenolic compounds from foods such as fresh fruit, vegetables, red wine and olive oil also increased the abundance of *Roseburia* and *Ruminococcus* (also with saccharolytic activity [428-430], suggesting a greater potential of the MED diet for restoring gut microbiota functionality than the LF diet, which was

more abundant in whole grains, significantly lower in sources of phenolic compounds and lower in fiber than the MED diet.

In line with this finding, we have reported in a previous study, testing for a relatively reduced number of taxa by quantitative PCR, that the long-term consumption of a MED diet, but not a LF diet, restored the abundance of 5 bacterial species which had previously been reduced in patients with 3, 4 or 5 MetS criteria [427]. Here, using NGS-methodology to explore the full microbiome, we found that the LF diet was also beneficial but to a lesser extent than the MED diet.

Our study has a limitation; the 16S rRNA sequencing is suitable for microbiota analysis from phylum to genus levels, but is limited in its ability to identify bacterial species. Thus, in our results we therefore indicate that species names are only a first approximation.

In conclusion, our results suggest that the chronic intake of two healthy dietary patterns partially restores the gut microbiome dysbiosis in obese patients with coronary heart disease depending on the degree of metabolic dysfunction. In this way, a healthy dietary intervention could be a key tool to reverse microbiota dysbiosis and, as a result, help to correct metabolic imbalance.

3. CHAPTER 3: Two healthy diets induce pre-diabetes regression through changes on gut microbiota

Our study showed an increase in the diversity of bacterial genera of patients who regressed from PreDM to NGC, which agree with the notion about the association between low diversity microbiota and metabolic disease risk [441]. Moreover, a high relative abundance of *Akkermansia* and low *Haemophilus* genera was associated to PreDM regression when patients consumed the LF diet, whereas a high abundance of *Prevotella* and low *Streptococcus* genera was associated to PreDM regression when patients consumed the MED diet. In addition, lower plasma levels of LPS were associated with PreDM regression after consumption of both diets as compared with patients who did not regressed.

PreDM patients are in risk of T2DM development [442]. However, despite PreDM is associated to important metabolic disorders such as obesity, hypertension, and hypercholesterolemia [443], single changes in lifestyle such as dietary habit may reduce the risk of T2DM development by regression from PreDM to NGC [444, 445]. In fact, our study showed that 69 out from 182 PreDM patients regressed to NGC only after the consumption of healthy diets (Med or LF).

In the last few years the human gut microbiota has been shown to be an important bridge connecting diet to host metabolism. In fact, emerging evidence points to the metabolic mediation by the microbiota of both harmful and beneficial effects of dietary nutrients on health [360, 446].

The metabolic activity of the gut microbiota involves the anaerobic breakdown of dietary fiber, carbohydrates, protein and peptides, producing SCFAs, which play an important role in maintaining intestinal health [180]. The SCFAs, especially butyrate, might also enhance intestinal barrier function, which reduces the intestinal absorption of pro-inflammatory bacterial component such as lipopolysaccharide (LPS), a component of the Gram (-) bacterial cell wall, found in the gut lumen [403] and highly pro-inflammatory [293, 447, 448]. This, in turn may reduce the inflammation-induced insulin resistance [447, 448]. The fact that, when MED diet was consumed, the abundance of the SCFA-producing *Prevotella* genus was higher in patients who regressed from PreDM than in patients who did not regress suggests that the consumption of MED diet might improve the intestinal barrier by increasing of the *Prevotella* genus abundance in the PreDM patients who regressed, which in turn may reduce inflammation-induced insulin

resistance forward to NGC condition. This idea is supported by the fact that the abundance of *Prevotella*, has been shown to be favored by with vegetable-rich diet such as MED diet [352].

Moreover, *Prevotella* has been related with the degradation of the mucins and glycoproteins, both constitute the mucosal layer surrounding the wall of the tract digestive [449, 450], which decreases the bacterial translocation [451], and improves gut integrity by increasing tight junction assembly [407]. In fact, lower fasting and postprandial levels of LPS found when MED diet was consumed in patients who regressed from PreDM than in patients who did not regress, confirm the lower bacterial translocation and the improvement of the barrier.

It is well known that individuals with metabolic disease, such as MetS and T2DM, present a pro-inflammatory profile [437], which is in line with the lower abundance of specific bacterial genera such as *Streptococcus* (pro-inflammatory bacterium) observed in our patients when regressed PreDM from NGC. Moreover, the lower *Streptococcus* genus abundance in PreDM patients who regressed is consistent with a lowering in Hb1Ac as previously shown in an African American male population [452].

By other hand, when the LF was consumed, we observed higher abundance of *Akkermansia* and lower *Haemophilus* genera in patients who regressed from PreDM than in patients who did not regress. However, although the microbiota changes were different between patients who regressed from PreDM consuming Med or LF diet, functionally, both diets may improve the intestinal barrier integrity, by lower endotoxemia and improvement of insulin resistance. In fact, high levels of *Akkermansia* in patients who regressed from PreDM to NGC are in line with a previously study which showed low levels of this bacterial genera in PreDM people as compared with non-PreDM patients [308]. Overall, our results suggest that a reduced abundance of *Akkermansia* could reflect a thin mucus layer and thus an impaired gut barrier function with increased translocation of pro-inflammatory bacterial toxins potentially leading to metabolic disturbances [453].

Overall, our results suggest an improvement of the intestinal barrier integrity by different changes in gut microbiota composition as mechanism by which 69 out of 182 PreDM patients regressed from PreDM to NGC. This idea is supported by the lower plasma levels of LPS found in patients who regressed, after consumption of both Med and LF diets as compared with those who did not regress. As summary, the specific gut microbiota changes associated to PreDM regression by each diet (Med, *Prevotella*; LF,

Akkermansia), suggest an improvement in gut health and intestinal barrier integrity, which in turn may reduce inflammation and therefore, the insulin resistance [454].

In conclusion, our results suggest that the consumption of two healthy diets led to different specific changes in the gut microbiota, associated to regression from PreDM to normal glucose condition. Moreover, the higher abundance of *Akkermansia* and *Prevotella* respectively for patients who regressed consuming LF and Med diets respectively was consistent with an improvement in the intestinal barrier integrity. However, further studies are required to fully understand modulate host–microbe interactions and to establish effective pathways to prevent metabolic disease.

4. DISCUSSION GENERAL

The human gut harbors more than 100 trillion microbial cells, which have an essential role in human metabolic regulation via their symbiotic interactions with the host. An undesirable impaired of the intestinal microbiota resulting in an imbalance between bacteria protective and harmful is called dysbiosis, and has been associated with increased metabolic and immune disorders in animals and humans [410]. However, the exact contribution of gut microbiota to the development of obesity and diabetes is not very clear due to many reasons including the complexity and diversity of gut microbes, ethnic variation in studied populations and large variations between individuals studied. Scientific efforts have been focused on understanding the mechanistic basis of the crosstalk between gut microbes and host metabolism in the development and maintenance of host diseases. In this sense, the restoration and maintenance of in a healthy gut microbiota may be a remedy for diseases associated with dysbiosis. In this scenario, this thesis provides new evidence regarding the relationship between intestinal dysbiosis and MetS and PreDM. We elucidate a gut microbiota-pattern to predict the MetS development, as well as we show the potential therapeutic use of long-term consumption of a healthy diet in the restoration of a healthy microbiota, which leads ultimately to an improvement of human health. Also, as well as, two healthy diet promoted the PreDM regression from NGC accompanied by changes in gut microbiota composition. The characterization of intestinal microbiota has been conducted for massive sequencing in a population with high risk of CVD.

Composition of gut microbiota is affected by many factors such as diet, gender, age, disease state, medications as well as host genetics [190, 264, 424]. Human studies and animal models have shown that the gut microbiota is altered in obesity [455], but it is still unknown whether a gut microbiota pattern precedes the obesity or the obesity is accompanied by gut microbiota profile. In line with this, in this thesis we identified a differential gut microbiota profile associated to obese people with MetS not present in obese people as compared with non-obese people, and we identified a microbiota pattern that precedes the development of MetS.

When we compared the intestinal microbiota composition between patients who developed MetS after 3 years follow-up and patients who did not developed MetS revealed significant lower relative abundance of *Prevotella* and *Clostridium* and higher *Ruminococcus* and *Holdemania*. The differences in the composition of gut microbiota

found in baseline between groups suggest that certain dysbiosis take place in patients before MetS development. In fact, higher plasma levels of LBP support the idea of a dysbiosis or specific microbiota pattern precedes the development of MetS. Here we report a potential use of LBP, together with specific bacterial taxa as biomarkers to predict the development of MetS.

Moreover, we propose that a reduction in carbohydrate degradation capacity and deterioration of intestinal barrier precedes to MetS development. In fact, the lower levels of *Prevotella,* a saccharolytic activity bacteria, suggest a reduction in the production of SCFAs, which may be related with the metabolic dysfunction of the host organism, in patients who developed MetS. The SCFAs, especially butyrate, is essential for a well-functioning colon [291], indeed already it associated lower abundance of butyrate producing bacteria with T2DM and obesity [179, 195]. In line with this, *Prevotella* has been shown to improve permeability of intestinal barrier for degrading many complex glycans, that constitute the mucosal layer surrounding the wall of the tract digestive. In fact, a lower *Prevotella* levels, suggest a deterioration of intestinal barrier in patients who developed MetS. This idea is supported by the increase in the intestinal absorption of LPS, which may be involved in the triggering of MetS as a consequence of LPS-induced systemic inflammation.

Our results open a door to suggest that gut microbiota play an important role in the development metabolic diseases such as MetS by a specific gut microbiota dysbiosis already present in early stages of disease, and before the development or clinical diagnosis.

The main contributor to the diversity of the gut microbiota is diet [404]. It has been suggested that changes in the diet can account for 57 % of the variations in microbiota compared to genetic variations in host that can only account for 12 % [456]. In this thesis, we have shown that the chronic intake of two healthy dietary patterns partially restores the gut microbiome dysbiosis in obese patients depending on the degree of metabolic dysfunction and secondly, we also showed that the consumption of these diets induces PreDM regression from NGC, suggesting that the long-term consumption of healthy diet can be used for therapy for prevention of metabolic diseases.

Our study showed that the long-term consumption of MED diet increases the relative abundance of *Roseburia, Ruminococcus* in patients obese with MetS (OB-MetS), and *Prevotella* in patients NGC who regressed from PreDM, these several bacteria with beneficial effect in human health. These organisms possess the capability to form SCFAs,

for example *Prevotella* is one of major producers of propionate and *Roseburia* and *Ruminococcus,* butyrate [191, 337]. For this reason, the increase of these bacteria observed after the consumption of MED diet could explain the anti-inflammatory effects associated with the consumption of this diet [156]. In line with this, LF diet increases the relative abundance of *Akkermansia* (implicated of improvement of gut barrier) in PreDM who regressed from NGC and *Bacteroidetes* phylum in patients OB-MetS. The fact, these bacteria are very important in maintaining colonic epithelial homeostasis via their respective effects on mucus and a disruption of this protective layer may lead to inflammation. Therefore, the long-term consumption of both MED and LF diet generate an improvement in gut barrier which decreases the bacterial translocation [336], we found higher LPS in patients who developed MetS than control group without MetS and lower LPS in patients who regressed PreDM than PreDM patients.

All together, our results suggest that the MED and LF diets could be used as therapeutic and preventive tool for metabolic diseases, and this could open a new hypothesis to be tested in the future in bigger populations about whether the consumption of healthy diets reduces the risk of MetS and T2DM by influencing the microbiota profile.

Finally, in this thesis we have identified differential gut microbiota signatures associated to MetS development. Both, a reduced saccharolic activity and an impairment of the intestinal barrier were the signature of MetS and PreDM. Moreover, the examinations of the microbiota may this help to identify individuals at an early stage who are at risk to develop metabolic diseases such obesity, MetS and T2DM. Distinguishing different types of microbial composition and connecting these with classical clinical biomarkers may provide diagnostic patterns that allow to select the kind of prevention or treatment which is best suited for the individual patient. Moreover, we also showed that the consumption of two healthy diets such as MED and LF diets could at least partially restore the reduction and impairment of saccharolic activity and the intestinal barrier, respectively.

In conclusion, we identified a specific gut microbiota pattern associated to the development of MetS. Moreover, both a reduced saccharolic activity and an impairment of the intestinal barrier were the major signature of metabolic syndrome and pre-diabetes, but the consumption of two healthy diets such as MED and LF diets could at least partially restore these alterations in gut microbiota, suggesting that the consumption of these diets may be used as therapeutic tool for treatment of these pathologies but it also suggest their consumption as preventing strategies to avoid the development of metabolic diseases.

VIII.CONCLUSIONS

Main conclusion

Our study showed that early detection of MetS development is possible by plasma levels of LBP and specific gut microbiota pattern. High abundance of *Ruminococcus* and *Holdemania,* and low abundance of *Prevotella* and *Clostridum* as well as, high plasma levels of LBP are potential biomarkers to predict the develop of MetS in our population.

Secondary conclusions

1. Our results suggest that the chronic intake of two healthy dietary patterns partially restores the gut microbiome dysbiosis in obese patients with coronary heart disease depending on the degree of metabolic dysfunction. In this way, a healthy dietary intervention could be a key tool to reverse microbiota dysbiosis and, as a result, help to correct metabolic imbalance.

2. Our results showed that the consumption of two healthy diets led to different specific changes in the gut microbiota, associated to regression from PreDM to normal glucose condition. Moreover, the higher abundance of *Akkermansia* and *Prevotella* respectively for patients who regressed consuming LF and MED diets respectively was consistent with an improvement in the intestinal barrier integrity. In addition, plasma levels of LPS were associated with the regression of PreDM from NGC after consumption of healthy diets.

IX. REFERENCES

IX. REFERENCES

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Publications derived from the thesis:

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Consumption of Two Healthy Dietary Patterns Restored Microbiota Dysbiosis in Obese Patients with Metabolic Dysfunction

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Scope: The consumption of two healthy diets (Mediterranean (MED) and low-fat (LF) diets) may restore the gut microbiome dysbiosis in obese patients depending on the degree of metabolic dysfunction.

Methods and results: The differences in bacterial community at baseline and after 2 years of dietary intervention of 106 subjects from the CORDIOPREV study were analyzed, 33 of whom were obese patients with severe metabolic disease (5 criteria for metabolic syndrome) (MetS-OB), 32 obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome) (NonMetS-OB) and 41 non-obese subjects (NonMetS-NonOB). Our study showed a marked dysbiosis in people with severe metabolic disease (Met-OB), compared with obese people without MetS (NonMetS-OB) and non-obese people (NonMetS-NonOB). This disbiotic pattern was reversed by consumption of both MED (35% of calories as fat (22% MUFA fat, 6% PUFA fat and <10% saturated fat) or LF (<30% total fat (<10% saturated fat, 12%–14% MUFA fat and 6–8% PUFA fat) diets, whereas no significant microbiota changes were observed in NonMetS-NonOB and NonMetS-OB groups.

Conclusion: Our results suggest that the chronic intake of two healthy dietary patterns partially restores the gut microbiome dysbiosis in obese patients with coronary heart disease, depending on the degree of metabolic dysfunction.

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1. Introduction

The gut microbiota is an organ which is fully integrated in the host and plays an important role in its metabolism, physiology, nutrition and immune functions,[1] and changes in its composition and/or structure can have major repercussions on health and disease.[2] In fact, the imbalance or dysbiosis of the gut microbiota has been associated with obesity and obesity-related metabolic dysfunctions.[3] The obese microbiota pattern described in animal models is characterized by a higher *Firmicutes/Bacteroidetes* ratio,[4] and it has primarily been associated of diet-induced obesity rather than genetically induced obesity.[5,6] However, human studies have yielded inconsistent results,[7–9] including a reduced abundance of *Firmicutes* in obese subjects.[10] Previous data indicate that the variability of human population, related with factor such as age or gender, seems to affect gut microbiota composition.[11–14] We hypothesized that the microbiota profile of obese people could differ according to their metabolic status, which could also help to explain the discrepancy found in obese people, where the proportion of obese people with and without metabolic diseases may correspond more or less to the degree of dysbiosis of gut microbiota.

The shaping of the gut microbiome is currently considered a therapeutic target, since specific changes in the bacterial community might counteract the development of obesity and metabolic syndrome (MetS).[15] Although the adult human gut microbiota community is relatively stable over long periods of time,[16] diet can influence its composition and could potentially be used as a therapeutic tool to alleviate and treat conditions triggered off by microbial imbalances.[17]

Based on this previous evidence, we evaluated whether the chronic consumption of two healthy diets (MED and LF diets) may restore the gut microbiome dysbiosis in obese patients depending on the degree of metabolic dysfunction. Moreover, we explored whether the presence or absence of metabolic disturbance is associated with a different microbiota profile. For that purpose, we selected two obese populations with extreme phenotypes: a group of obese people with severe metabolic disease (MetS-OB), meaning that they met all the five defining criteria for this syndrome, and a group of obese people without metabolic syndrome (2 or less criteria for metabolic syndrome) (NonMetS-OB), in comparison with a non-MetS non-obese control group (NonMetS-NonOB).

2. Experimental Section

2.1. Study Subjects

The current work was conducted in a subgroup of 106 male patients within the CORDIOPREV study (Clinical Trials.gov.Identifier: NCT00924937), an ongoing prospective, randomized, opened, controlled trial in patients with coronary heart disease (CHD), who had their last coronary event over six months before enrolling in two different dietary models (Mediterranean and low-fat) over a period of 5 years, in addition to conventional treatment for CHD.[18] CORDIOPREV inclusion and exclusion criteria are summarized as follows: patients were eligible if they were over 20 years old, but under 75, had established CHD without clinical events in the last 6 months, were thought to follow a long-term dietary intervention and did not have severe diseases or an estimated life expectancy of less than 7 years.[18] Usage of antibiotics was included as an exclusion criteria for the current study, in addition to the general exclusion criteria defined in the CORDIOPREV study. All the subjects were receiving a standardized treatment for coronary heart disease. All patients gave their informed consent in writing to participate in the study. The trial protocol and all the amendments were approved by the local ethics committees, following the Helsinki Declaration and good clinical practice.

We analyzed the fecal samples of 106 male patients at baseline and after 2 years of dietary intervention (Mediterranean or Low-fat diet), which were also divided into three groups: the MetS-OB group, 33 obese people with Metabolic Syndrome following full MetS criteria according to the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) for MetS[19]; the NonMetS-OB group, 32 obese people without MetS (2 or less criteria for the metabolic syndrome); the NonMetS-NonOB group, 41 non-obese people without MetS. The metabolic characteristics of the subjects in the study are shown in **Table 1**.

2.2. Study Diets and Dietary Assessment

The models of healthy diet were (i) the MED diet, with a minimum 35% of calories as fat (22% MUFA fat, 6% PUFA fat and <10% saturated fat), 15% proteins and a maximum of 50% carbohydrates and (ii) the LF diet recommended by the National Cholesterol Education Program and the American Heart Association, comprising <30% total fat (<10% saturated fat, 12–14% MUFA fat and 6–8% PUFA fat), 15% protein and a minimum of 55% carbohydrates. In both diets, the cholesterol content was adjusted to <300 mg/day. Both therapeutic diets included a wide variety of foods, including vegetables, fruit, cereals, potatoes, legumes, dairy products, meat and fish. Participants in both intervention groups received the same intensive dietary counseling. Dietitians administered individual, personalized interviews at inclusion and every 6 months, and quarterly group education sessions with up to 20 participants per session and separate sessions for each group. These sessions consisted of informative talks accompanied by written information with detailed descriptions of typical foods for each dietary pattern, seasonal shopping lists, meal plans and recipes.

We performed a validated 14-item questionnaire to assess adherence to the MED Diet[20] and a similar 9-point score to assess adherence to the LF diet at baseline before starting the dietary intervention and at yearly follow-up visits. Fiber intake was calculated using the Spanish food composition tables and a validated food frequency questionnaire.[21]

2.3. Clinical Plasma Parameters

Blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) to give a final concentration of 0.1% EDTA.

Table 1. Baseline characteristic of the participant in this study

Group (N)	MetS-OB (33)	NonMetS-OB (32)	NonMetS-NonOB (41)	P-value
Age (years)	59.03 ± 1.83	63.72 ± 1.70	61.73 ± 1.39	0.149
BMI (kg m^{-2})	32.42 ± 0.76[a]	32.88 ± 0.56[a]	27.05 ± 0.27[b]	< 0.001
WC (cm)	114.48 ± 1.43[a]	109.11 ± 1.61[b]	97.22 ± 0.75[c]	< 0.001
HOMA-IR	6.50 ± 0.95[a]	3.93 ± 0.48[b]	2.31 ± 0.21[b]	< 0.001
HDL-c (mg dL^{-1})	36.79 ± 1.54[a]	45.06 ± 2.16[b]	45.38 ± 2.14[b]	0.005
LDL-c (mg dL^{-1})	80.62 ± 4.73	85.92 ± 3.23	80.30 ± 3.51	0.532
TC (mg dL^{-1})	157.29 ± 5.28	147.70 ± 4.05	144.97 ± 2.73	0.155
TG (mg dL^{-1})	180.07 ± 13.82[a]	131.19 ± 12.26[b]	124.48 ± 8.82[b]	0.001
Glucose (mg dL^{-1})	133.70 ± 14.08[a]	105.94 ± 7.94[a,b]	100.18 ± 3.68[b]	0.022
Systolic BP (mm Hg)	130.82 ± 3.22	132.28 ± 3.84	136.68 ± 3.12	0.423
Diastolic BP (mm Hg)	77.22 ± 2.01	74.81 ± 2.05	75.71 ± 1.76	0.681

Means values ± S.E.M. BMI: body mass index. WC: waist circumference. TC: total cholesterol. TG: triacylglycerides. BP: Blood Pressure. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. One-way ANOVA *p*-values. Values in the same row with different letters differ significantly between groups in the post hoc analysis using Bonferroni's multiple comparison tests.

The plasma was separated from the red cells by centrifugation at 1500× *g* for 15 min at 4 °C. Analytes determined in frozen samples were analyzed centrally by laboratory investigators of the Lipid and Atherosclerosis Unit at the Reina Sofia University Hospital, who were unaware of the interventions. Lipid variables were assessed with a DDPPII Hitachi modular analyzer (Roche) using specific reagents (Boehringer-Mannheim). Plasma TG and cholesterol concentrations were assayed by enzymatic procedures.[22,23] HDL-c was measured by the precipitation of a plasma aliquot with dextran sulphate-Mg^{2+}, as described by Warnick et al.[24] LDL-c was calculated by using the following formula; plasma cholesterol – (HDL-C + large TRL-C+ small TRL-C). Glucose determination was performed by the hexokinase method.

2.4. DNA Extraction from Fecal Samples

To collect the fecal samples, we gave the patients a box with carbonic snow and a sterile plastic bottle with a screw cap to keep the frozen sample. Once delivered to the laboratory staff, the sample was stored at −80 °C until microbial DNA was extracted, which was performed using the QIAamp DNA kit Stool Mini Kit Handbook (Qiagen, Hilden, Germany), following the manufacturer's instructions. This protocol was optimized for a 180–220 mg sample. DNA was quantified using a Nanodrop ND-1000 v3.5.2 spectrophotometer (Nanodrop Technology®, Cambridge, UK) and the DNA samples were stored at −20 °C.

2.5. Sequencing the V4 16S Microbial rRNA on the IlluminaMiSeq

The preparation of samples was performed similarly to that described by Costello et al.[25] To sum up, the 106 samples taken on the two dates in the study (baseline and 2 years) were amplified in triplicate by PCR to generate an amplification library (modified from Sarah Owens, Argonne National Labs), with each sample amplified in 25 μL PCR reactions. The PCR experimental conditions for the 515–806 bp region of the 16S rRNA gene and the sequencing procedures with the Illumina platform have both been described by Caporaso et al.[26]

2.6. Upstream Informatics Analysis of the 16S Sequences

The 16S rRNA sequences obtained were analyzed using QIIME with default parameters, unless indicated otherwise.[26] To sum up, raw sequencing data was de-multiplexed and low quality reads were discarded. The reads were clustered using a closed-reference OTU-picking protocol that assigned reads to reference sequences from Greengenes v13-8.[27] Processing consisted of the following steps: (i) demultiplexing and filtering of short (<150 nt) and low quality reads; (ii) de novo clustering of the sequences into operational taxonomic units (OTUs) with the UCLUST program using a 97% similarity threshold;[28] (iii) taxonomical assignment of each OTU by running the RDP Classifier[29] at 80% bootstrap confidence on a selected representative sequence from each OTU; (iv) alignment of representative sequences using PyNAST[30] with the Greengenes core-set alignment template. Bacterial (alpha) diversity was estimated using the total number of observed OTUs, the Chao1 estimator and Faith's phylogenetic diversity index. Beta diversity was estimated using the unweighted UniFrac distance.[31] Relative taxonomic abundance was measured as the proportion of reads over the total in each sample assigned to a given taxonomy.

2.7. Statistical Analysis

We used PASW statistical software, version 20.0 (IBM Inc., Chicago, IL, USA) to perform the statistical analysis. The normal distribution of variables was assessed using the Kolmogorov-Smirnov test. In order to assess whether specific differences occurred in some bacterial taxa between groups and bacterial species, we compared the abundance of taxa present in at least

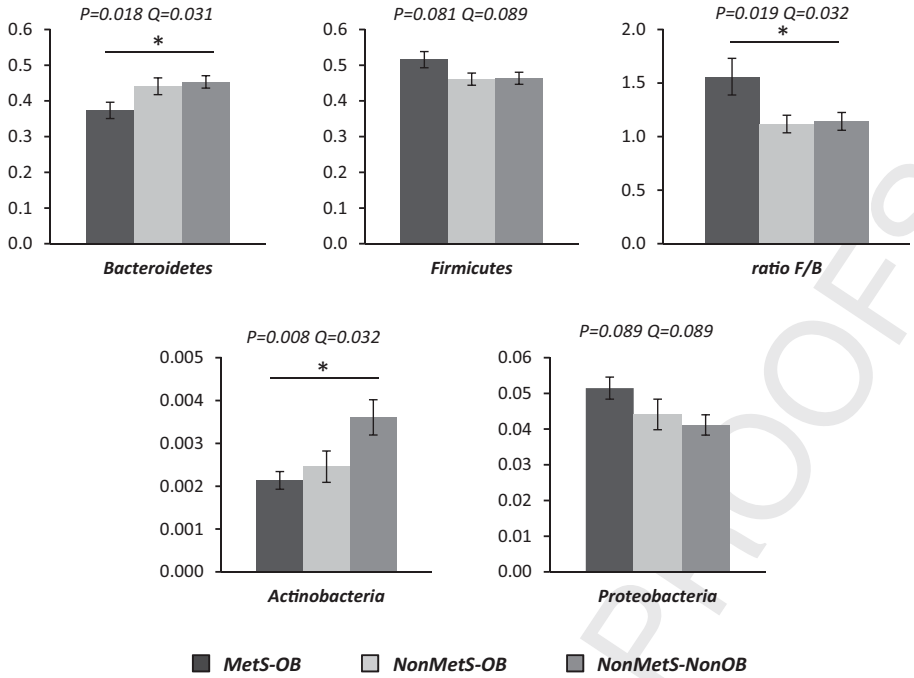

Figure 1. Composition of the intestinal microbiota of the study population at phylum level. One-way ANOVA statistical analysis. Q-values: ANOVA *p*-values adjusted by the False Discovery Rate using the Benjamini and Hochberg method. Ratio F/B: ratio *Firmicutes/Bacteriodetes*. $^{*}p < 0.05$ in the Post-Hoc Bonferroni's multiple comparison tests. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects.

75% of the human fecal DNA samples. We analyzed the changes in the abundance of these taxa per group. The differences between the groups at baseline were analyzed using One-way ANOVA and the results adjusted by the False Discovery Rate (FDR) using the Benjamini and Hochberg method. An FDR adjusted *p*-value (or Q-value) of 0.05 was considered statistically significant. We also analyzed the frequency of occurrence of taxa identified in at least 50% of one group. The χ^2 test was applied to establish differences in bacterial prevalence between the groups studied. ANOVA for repeated measures was used to evaluate the effect of diet on shaping gut microbiota with time as the intra-subject factor and group or diet as the inter-subject factor. Post hoc statistical analysis was completed by using Bonferroni's multiple comparison tests. All data presented are expressed as mean ± S.E.M.

3. Results

3.1. Baseline Characteristic of the Study Population

The differences in the main anthropometric and metabolic variables between the MetS-OB, NonMetS-OB and NonMetS-NonOB groups are shown in (Table 1).

3.2. Global Analysis of the Intestinal Microbiota Between Groups

We did not find any significant differences in bacterial diversity at baseline nor after 2 years of dietary intervention between the three groups with any of the alpha diversity estimators used and at a rarefaction level of 2000 sequences per sample (Supporting Information Figure 1). Similarly, Principal Coordinate Analysis (PCoA) or UPGMA clustering based on unweighted and weighted UniFrac distances did not show significant differences in the microbiota composition between groups at baseline nor after 2 years of dietary intervention (Supporting Information Figure 2).

3.3. Univariate Analysis of the Intestinal Microbiota Between Groups at Baseline

We investigated whether the relative abundance of specific taxa might differ between groups using One-way ANOVA and the results adjusted by the False Discovery Rate (FDR) using the Benjamini and Hochberg method.

3.3.1. Differences Between Groups at Phylum Level

We observed a lower abundance of *Actinobacteria* and *Bacteroidetes* phyla in the MetS-OB group compared with the NonMetS-NonOB group ($p = 0.008$ $Q = 0.032$ and $p = 0.018$ $Q = 0.032$, respectively). In addition, we observed a higher *Firmicutes/Bacteroidetes* ratio in the MetS-OB group than in the NonMetS-NonOB group ($p = 0.019$), whereas no differences were observed between the NonMetS-OB and NonMetS-NonOB groups (**Figure 1**).

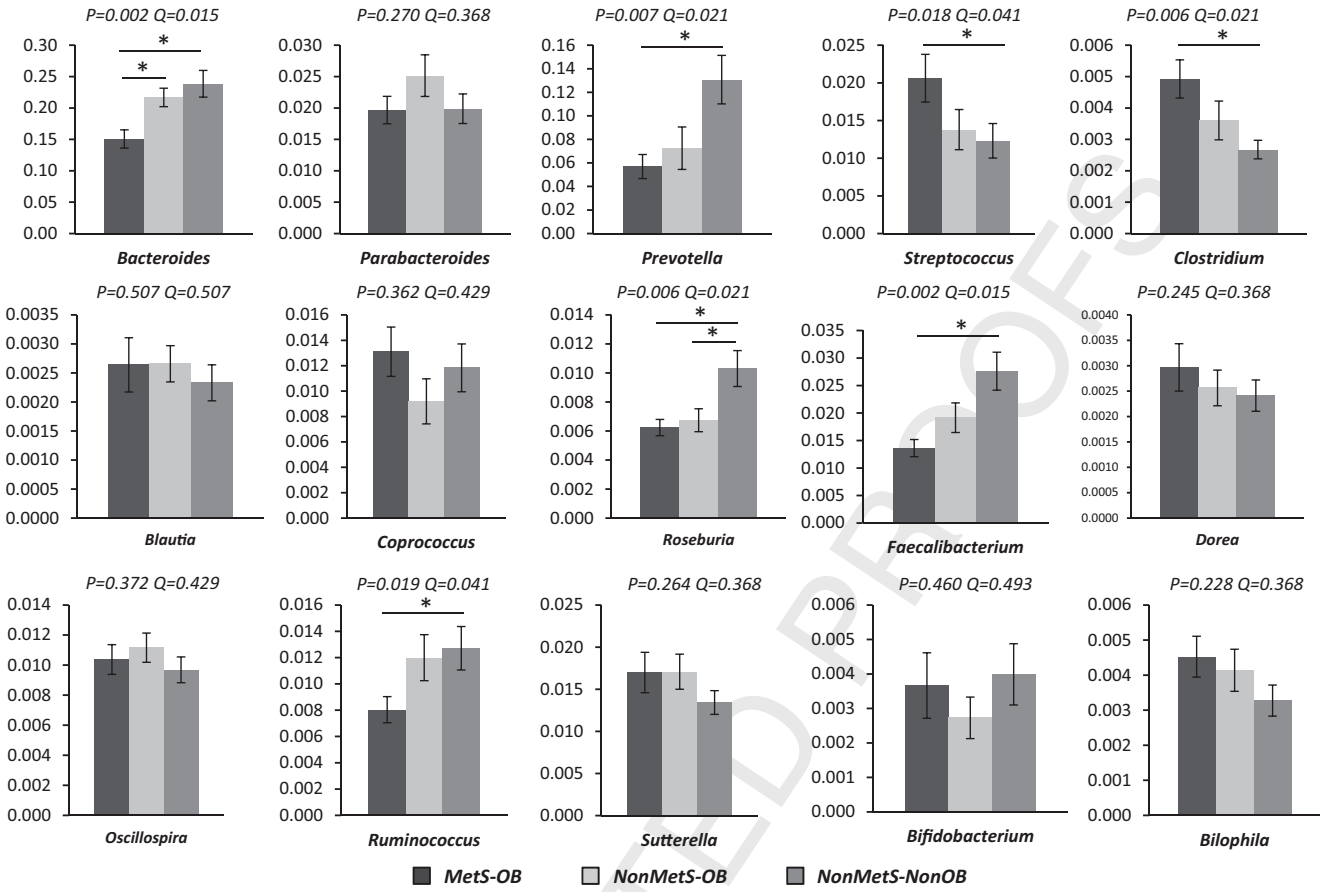

Figure 2. Composition of the intestinal microbiota of the study population at genus level. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. *p*-values: One-way ANOVA statistical analysis. Q-values: ANOVA *p*-values adjusted by the False Discovery Rate using the Benjamini and Hochberg method. $^{*}p < 0.05$ in the Post-Hoc Bonferroni's multiple comparison tests.

3.3.2. Differences Between Groups at Genus Level

Moreover, we observed a lower abundance of *Bacteroides*, *Prevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcus* in the MetS-OB than in the NonMetS-NonOB group ($p = 0.002$ $Q = 0.015$, $p = 0.007$ $Q = 0.021$, $p = 0.006$ $Q = 0.021$, $p = 0.002$ $Q = 0.015$ and $p = 0.019$ $Q = 0.040$, respectively). On the other hand, we observed a higher abundance of *Streptococcus* and *Clostridium* genera in the MetS-OB group than in the NonMetS-NonOB group ($p = 0.018$ $Q = 0.40$ and $p = 0.006$ $Q = 0.021$, respectively) (**Figure 2**).

3.3.3. Differences Between Groups at Bacterial Species Level

We observed that MetS-OB patients had a lower abundance of *P. distasonis* and *F. prausnitzii* ($p = 0.008$ $Q = 0.036$ and $p = 0.012$ $Q = 0.036$, respectively) than the NonMetS-OB and NonMetS-NonOB groups respectively (**Table 2**).

3.4. Effect of the Dietary Intervention on Intestinal Microbiota Composition

In the next step, we studied the changes in the intestinal microbiota after 2 years of the dietary intervention in comparison to baseline time.

3.5. Diets Alter Gut Microbiota Composition When there was Dysbiosys: MetS-OB Groups

We found that the abundance of *Bacteroides*, *Prevotella* and *Faecalibacterium* genera increased in the MetS-OB group after 2 years of consumption of either the MED or LF diets as compared with the baseline ($p < 0.001$, $p < 0.001$ and $p = 0.001$, respectively). Thus, the differences between the MetS-OB group and the NonMetS-OB and NonMetS-NonOB groups found at baseline disappeared after the consumption of these diets (**Figure 3**).

Moreover, the *Bacteroidetes* phylum increased in the MetS group after 2 years of dietary intervention. However, whereas

Table 2. Composition of the intestinal microbiota of the study population at bacterial species level

Bacterial species	MetS-OB	NonMetS-OB	NonMetS-LO	*p*-value	*q*-value
Bacteroides caccae	0.0062 ± 0.0009	0.0080 ± 0.0013	0.0093 ± 0.0014	*0.208*	*0.250*
Bacteroides ovatus	0.0026 ± 0.0004	0.0035 ± 0.0006	0.0032 ± 0.0004	*0.198*	*0.250*
Bacteroides uniformis	0.0137 ± 0.0018	0.0206 ± 0.0026	0.0239 ± 0.0040	*0.071*	*0.142*
Parabacteroides distasonis	0.0040 ± 0.0005[a]	0.0069 ± 0.0007[b]	0.0055 ± 0.0007[a,b]	***0.008***	***0.036***
Prevotella copri	0.0637 ± 0.0136	0.0685 ± 0.0164	0.0846 ± 0.0180	*0.628*	*0.628*
Faecalibacterium prausnitzii	0.0155 ± 0.0018[a]	0.0209 ± 0.0032[a,b]	0.0297 ± 0.0039[b]	***0.012***	***0.036***

Represented values are mean ± SEM of the relative abundance of each bacterial taxon. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic síndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. ANOVA statistical analysis *p*-value and *Q*-value. The different letters denote significant differences between groups in the post hoc comparisons.

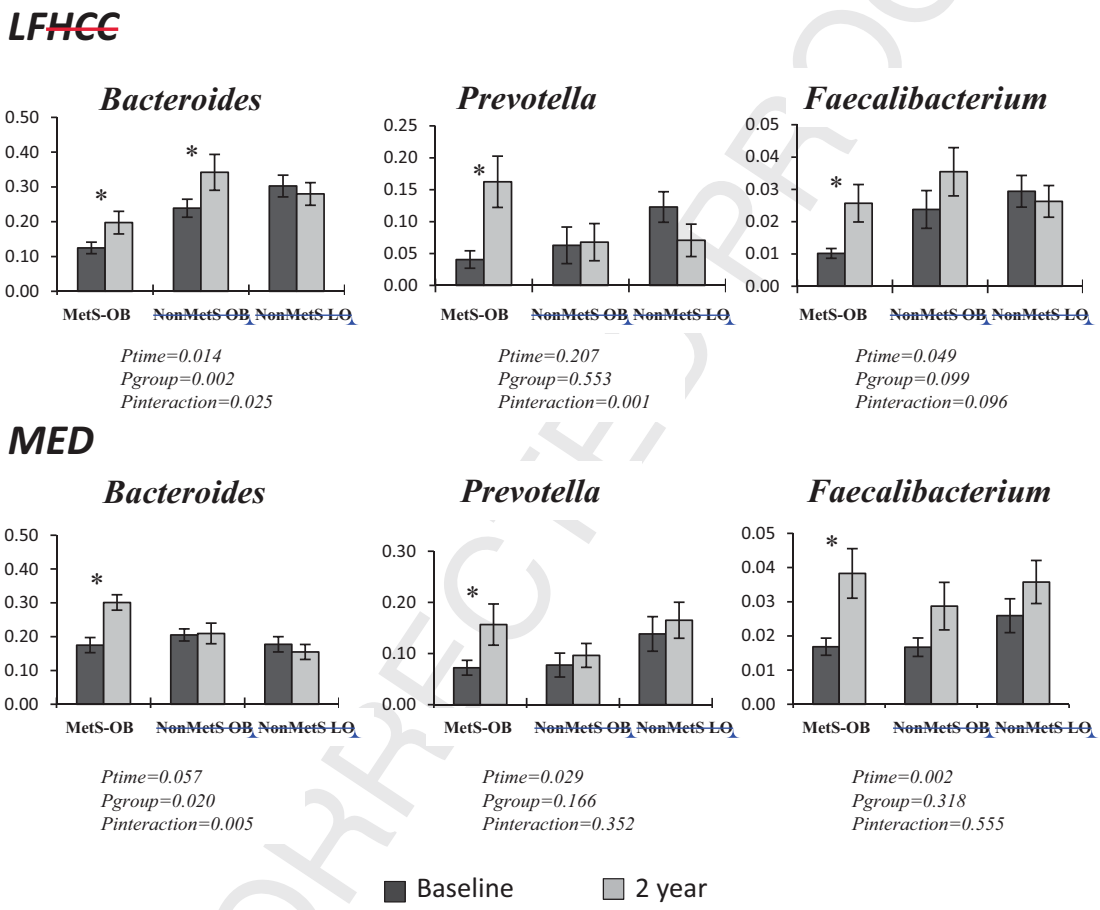

Figure 3. Restoration of dysbiosis by the consumption of LF and MED diets. Represented values are mean ± SEM of the relative abundance of each bacterial taxon. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. LF: low-fat diet; MED: Mediterranean diet. The statistically significant differences between groups, time and sampling time by group interaction were analyzed by ANOVA for repeated measures $^{*}p < 0.05$ in the Post-Hoc Bonferroni's multiple comparison tests.

we observed a statistically significant increase in its abundance after the consumption of the LF diet ($p < 0.001$), we only observed a trend after the consumption of the MED diet. Consequently, we observed a statistically significant decrease in the *Firmicutes/Bacteroidetes* ratio after the consumption of the LF ($p < 0.001$) diet, whereas only a trend was noted after the consumption of the MED diet. In addition, the consumption of the LF diet for 2 years decreased the abundance of *Streptococcus* and *Clostridium* genera ($p = 0.010$ and $p < 0.001$, respectively), whereas the consumption of the MED diet did not affect the abundance of these genera (Supporting Information Figure 7).

Moreover, the closest species as determined by Greengenes taxonomy were also analyzed. We observed that the consumption of the MED diet for 2 years increased the abundance of

Roseburia and *Ruminococcus* genera and *P. distasonis* and *F. prausnitzii* bacterial species ($p = 0.004$, $p = 0.009$, $p = 0.014$ and $p = 0.043$, respectively), whereas the consumption of the LFHCC did not affect the abundance of these genera (Supporting Information Figure 8).

3.6. Diets Did Not Alter Gut Microbiota Composition When There Was No Dysbiosys: NonMetS-OB and NonMetS-NonOB Groups

No significant microbiota changes were observed after 2 years of dietary intervention in NonMetS-NonOB and NonMetS-OB groups as compared with the baseline.

3.7. Effect of the Dietary Intervention on the Main Metabolic Variables

In addition, in terms of the effect of dietary intervention on the metabolic variables, we observed a decreased in the TG levels after 2 years of follow-up in the MetS-OB group ($p<0.001$) after the consumption of both diets whereas TG levels remained unchanged in NonMetS-Ob and NonMetS-NonOB groups (**Table 3**). When we analyzed the potential differences between diet in TG levels, we observed that the consumption both LFHCC and MED diets decreased the TG levels in the MetS-OB group.

4. Discussion

Our study showed a marked dysbiosis in obese people with severe metabolic disease, who had the full characteristics of MetS (5 criteria) (MetS-OB), compared with obese people without MetS (NonMetS-OB) and non-obese people (NonMetS-NonOB). Interestingly, the disbiotic pattern was reversed by the chronic consumption of both MED or LF diets, altering its microbiota composition to the pattern found in metabolically healthy people. However, in the NonMetS-NonOB and NonMetS-OB groups, no significant microbiota changes were observed after the dietary intervention.

The obese microbiota pattern, described in animal models, is characterized by a higher *Firmicutes/Bacteroidetes* ratio.[4] However, studies in obese humans do not always correspond with what is expected in animal studies.[7–10] As previously commented, these different results might be explained by differences in the features of the different cohorts.[11,32] To avoid some of these confusion factors, we selected age-matched groups, with only male participants, and our data suggested that metabolic dysfunction could be another circumstance, which may explain the different results found in the human studies. Thus, our study showed that the *Firmicutes/Bacteroidetes* ratio, which is considered of great importance in the development of obesity,[33] is in fact related to the presence or absence of metabolic traits in humans but not with obesity itself. Moreover, the fact that our patients have CHD may also affect the gut microbiota composition, in addition to the degree of metabolic dysfunction.

We have previously reported the dysbiosis of MetS patients,[14] characterized by a reduction in the abundance of genera with saccharolytic activity, which leads to a reduction in carbohydrate degradation capacity in MetS patients as compared with lean control individuals. In the present study, we have found in the subgroup of patients with MetS-OB a similar reduction of the genera with saccharolytic activity, especially *Bacteroides* and *Prevotella* (both forming the *Bacteroidetes* phylum) and *Roseburia*, *Ruminococcus*, and *Faecalibacterium*.[34–36] Our data suggest that the loss of certain functions or features of the gut microbiota, such as the loss of the capacity to degrade carbohydrates to short-chain fatty acid (SCFA) may be related with the metabolic dysfunction of the host organism.[37,38] Taking into account that amino acids can also serve as precursors for the synthesis of SCFA by bacteria,[39] the loss of the capacity to degrade carbohydrates to SCFA may imbalance the interplay between gut microbiota and amino acid and SCFA homeostasis shifting the bacterial activity toward SCFA production from amino acids. This is consistent with the reduced insulin sensitivity observed in MetS-OB, and may be related with the high branched-chain amino acids concentration found in insulin resistance states[40] and a dysregulation in the production of incretins, which are involved in the regulation of feeding and energy balance, presumably through SCFA as signal molecules,[41,42] which, in turn, may contribute to the development of type 2 diabetes mellitus (T2DM) and MetS.[43]

It is well known that individuals with obesity present a low-grade inflammation,[44] which has also been proposed to promote the development of the MetS.[45] However, our study did not provide evidence of a direct relationship between gut microbiota composition and inflammatory status as assessed by C-reactive protein levels and its potential modulation by diet. Nevertheless, C-reactive measurement may not be accurately enough to evaluate the inflammatory status in the CHD population analyzed in this study and further studies focusing this issue are needed.

Dietary strategies have been shown to be of great importance in the prevention and treatment of metabolic diseases,[46] which presumably work by shaping the gut microbiota, as has been suggested.[17] In fact, our study showed that the consumption of two healthy diets (the MED and LF diets) restored the microbiota composition in the population with severe metabolic dysfunction in parallel with a reduction of TG and a trend for glucose depletion (MetS-OB group), which supports this idea.

Certainly, the consumption of the two healthy diets administered in our study increased the abundance of the two genera which make up the *Bacteriodetes* phylum (*Bacteroides* and *Prevotella*), which, in turn, reduced the B/F ratio. As summary, all these changes contribute to restoring the obesity-associated microbiota pattern characterized by a high F/B ratio observed in the population with severe metabolic dysfunction (MetS-OB). In addition to the *Bacteroides* and *Prevotella* genera, the abundance of other genera with saccharolytic activity such as *Faecalibacterium*[34–36] also increased after the consumption of the two healthy diets administered, which, in turn, may restore, at least partially, the fiber-derived SCFA synthesis by gut microbiota. However, the consumption of the MED diet, rich in antioxidant phenolic compounds from foods such as fresh fruit, vegetables, red wine and olive oil also increased the abundance of *Roseburia* and *Ruminococcus* (also with saccharolytic activity[34–36]), suggesting a greater potential of the MED diet for restoring gut microbiota functionality than the LF diet, which was

Table 3. Effect of the dietary intervention on the main metabolic variables

Parameter	Time point	MetS-OB (33)	NonMetS-OB (32)	NonMetS-NonOB (41)	*p*-time	*p*-group	*p*-interaction
BMI (kg m^{-2})	Y_0	32.42 ± 0.76	32.88 ± 0.56	27.05 ± 0.27	0.337	< 0.001	0.798
	Y_2	32.12 ± 0.65	32.38 ± 0.74	27.02 ± 0.36			
WC (cm)	Y_0	114.48 ± 1.43	109.11 ± 1.61	97.22 ± 0.75	0.267	< 0.001	0.593
	Y_2	112.36 ± 1.55	108.61 ± 1.75	97.05 ± 0.96			
C-reactive protein (mg L^{-1})	Y_0	3.75 ± 0.79	3.21 ± 0.67	2.60 ± 0.76	0.871	0.622	0.988
	Y_2	3.73 ± 1.02	3.30 ± 0.87	2.78 ± 0.98			
HDL-c (mg L^{-1})	Y_0	36.79 ± 1.54	45.06 ± 2.16	45.38 ± 2.14	0.195	< 0.001	0.616
	Y_2	37.42 ± 1.26	43.67 ± 2.14	43.47 ± 1.46			
LDL-c (mg L^{-1})	Y_0	80.62 ± 4.73	85.92 ± 3.23	80.30 ± 3.51	0.125	0.628	0.737
	Y_2	84.00 ± 4.37	89.13 ± 3.4.07	85.75 ± 3.15			
TC (mg L^{-1})	Y_0	157.29 ± 5.28	147.70 ± 4.05	144.97 ± 2.73	0.303	0.372	0.358
	Y_2	154.28 ± 5.51	153.13 ± 4.89	151.36 ± 4.13			
TG (mg L^{-1})	Y_0	188.23 ± 11.91	131.19 ± 12.26	124.48 ± 8.82	0.006	< 0.001	0.842
	Y_2	156.14 ± 12.67*	113.50 ± 9.35	105.10 ± 5.34			
Glucose (mg L^{-1})	Y_0	133.70 ± 14.08	105.94 ± 7.94	100.18 ± 3.68	0.436	0.021	0.125
	Y_2	112.86 ± 4.64	107.87 ± 6.58	105.79 ± 3.73			
Systolic BP (mm Hg)	Y_0	130.82 ± 3.22	132.28 ± 3.84	136.68 ± 3.12	0.157	0.602	0.255
	Y_2	138.91 ± 3.01	133.91 ± 2.92	136.31 ± 2.23			
Diastolic BP (mm Hg)	Y_0	77.22 ± 2.01	74.81 ± 2.05	75.71 ± 1.76	0.940	0.475	1.000
	Y_2	77.26 ± 1.98	74.95 ± 1.90	75.85 ± 1.59			

Means values ± S.E.M. corresponding to fasting state at baseline and at 2 years of dietary intervention. BMI: body mass index. WC: waist circumference. TC: total cholesterol. TG: triacylglycerides. BP: Blood Pressure. MetS-OB: obese patients with severe metabolic disease (5 criteria for metabolic syndrome). NonMetS-OB: obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome). NonMetS-NonOB: non-obese subjects. Y_0: baseline. Y_2: 2 years of dietary intervention. ANOVA for repeated measured p-values.
*$p < 0.05$ in the Post Hoc Bonferroni's multiple comparison tests between post-intervention and baseline.

more abundant in whole grains, significantly lower in sources of phenolic compounds and lower in fiber than the MED diet.

In line with this finding, we have reported in a previous study, testing for a relatively reduced number of taxa by quantitative PCR, that the long-term consumption of a MED diet, but not a LF diet, restored the abundance of 5 bacterial species which had previously been reduced in patients with 3, 4 or 5 MetS criteria.[14] Here, using NGS-methodology to explore the full microbiome, we found that the LF diet was also beneficial but to a lesser extent than the MED diet.

Our study has a limitation; the 16S rRNA sequencing is suitable for microbiota analysis from phylum to genus levels, but is limited in its ability to identify bacterial species. Thus, in our results we therefore indicate that species names are only a first approximation.

In conclusion, our results suggest that the chronic intake of two healthy dietary patterns partially restores the gut microbiome dysbiosis in obese patients with coronary heart disease depending on the degree of metabolic dysfunction. In this way, a healthy dietary intervention could be a key tool to reverse microbiota dysbiosis and, as a result, help to correct metabolic imbalance.

Abbreviations

CHD, coronary heart disease; EDTA, ethylenediaminetetraacetic acid; FDR, False Discovery Rate; LF, low-fat; MED, Mediterranean; MetS, metabolic syndrome; MUFA, monounsaturated fatty acids; NCEP, National Cholesterol Education Program; NonMetS-NonOB, non-obese subjects; NonMetS-OB, obese patients without metabolic dysfunction (2 or less criteria for metabolic syndrome); MetS-OB, obese patients with severe metabolic disease (5 criteria for metabolic síndrome); PUFA, polyunsaturated fatty acids; SCFA, short-chain fatty acid; TG, triacylglycerides; T2DM, type 2 diabetes mellitus

Supporting Information

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

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Conflict of Interest

None of the authors has any conflict of interest that could affect the performance of the work or the interpretation of the data.

Keywords

CORDIOPREV study, diet, metabolic syndrome, microbiota, obesity

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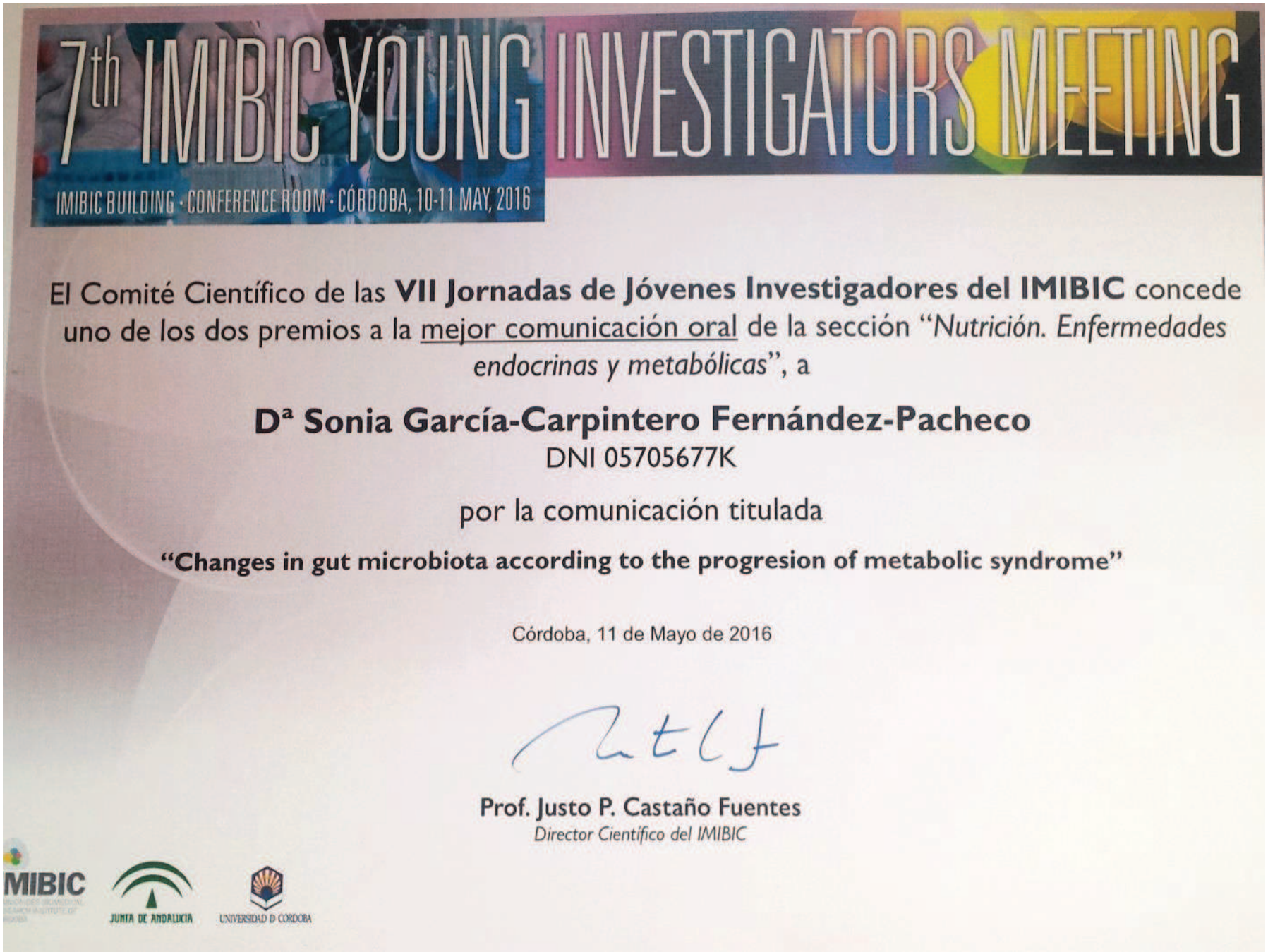

7th IMIBIC YOUNG INVESTIGATORS MEETING

IMIBIC BUILDING · CONFERENCE ROOM · CÓRDOBA, 10-11 MAY, 2016

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Dª Sonia García-Carpintero Fernández-Pacheco

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por la comunicación titulada

"Changes in gut microbiota according to the progresion of metabolic syndrome"

Córdoba, 11 de Mayo de 2016

Prof. Justo P. Castaño Fuentes

Director Científico del IMIBIC

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