UNIVERSITAT DE BARCELONA

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IMPACT ON GENE EXPRESSION AND METABOLIC HOMEOSTASIS OF BIOACTIVE COMPOUNDS ENRICHED DIETS

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A Marcela y Romilio, mis padres
Que me dieron alas para volar y
Raíces para recordar...

El derecho de vivir en Paz

El derecho de vivir
Sin miedo en nuestro país
En conciencia y unidad
Con toda la humanidad

Ningún cañón borrará
El surco de la hermandad
El derecho de vivir en paz

Con respeto y libertad
Un nuevo pacto social
Dignidad y educación
Que no haya desigualdad

La lucha es una explosión
Que funde todo el clamor
El derecho de vivir en paz

Con respeto y libertad
Y un nuevo pacto social
Que no haya desigualdad

Sentimiento, un poquitito más fuerte
Los estudiantes no los dejarán dormir
Si usted no los deja soñar
Este es el cambio.

Es la paz nuestra canción
Es fuego de puro amor
Es palomo palomar
Olivo del olivar

Es el canto universal
Cadena que hará triunfar
El derecho de vivir en paz

Es el canto universal
Cadena que hará triunfar
El derecho de vivir en paz
El derecho de vivir en paz
El derecho de vivir en paz
El derecho de vivir en paz

Víctor Jara
Versión actualizada músicos chilenos, Estallido Social - octubre 2019

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ABSTRACT

The excess of fat deposits are originated by a prolonged imbalance between the energy intake and the energy expenditure. The current food pattern based on a high consumption of saturated fats, simple sugars and processed foods, together with the sedentary lifestyle of the population, favor the development of obesity, which is responsible for 3.4 million deaths per year and is the main risk factor for the development of associated comorbidities, such as type 2 diabetes mellitus, non-alcoholic fatty liver and cardiovascular diseases. Given the current pandemic scope of obesity, finding therapeutic targets and strategies for their control and treatment is of great importance. The Mediterranean Diet, characterized by a high consumption of fruits and vegetables, provides a high levels of bioactive compounds mainly carotenoids and polyphenols, which have shown antiobesogenic properties.

The main aim of this thesis is to define the molecular mechanisms involved in the metabolic impact of foods rich in bioactive compounds in animal models of obesity. We worked with sofrito and maqui to be two food products rich in bioactive compounds.
Sofrito is as a typical Mediterranean preparation with a high nutritional interest due to the high content of bioactive compounds, mainly carotenoids and because beneficial effects in the primary prevention of cardiovascular and metabolic diseases such as type 2 diabetes has been attributed to it. On the other hand, the berry known as maqui (Aristotelia chilensis), characteristic of southern Chile, with a high polyphenols contents, especially from the anthocyanin group, has shown cardioprotective and hypoglycemic effects.

The results show that both sofrito and maqui have beneficial effects on insulin sensitivity and glucose tolerance, respectively. As well, they are

also able to increase the expression and signaling of the fibroblast growth factor pathway 21 and increase the expression of uncoupling protein 1 and browning in white adipose tissue. In addition, in the liver, the supplementation with maqui improves the hepatic steatosis caused by a high-fat diet by the expression of small heterodimer partner–interacting leucine zipper protein.
The data presented allow us to point out that both dietary supplementation with sofrito and with maqui, could be good strategies in the prevention and / or treatment of obesity and its associated comorbidities.

ABREVIATURAS

ACC: Acetyl-CoA carboxylase
ACLY: ATP citrate lyase
AHA: Asociación Americana del Corazón
AMP: Adenosine Monophosphate
AMPK: AMP-activated protein kinase
ATGL: Adipose triglyceride lipase
BAT: Tejido adiposo marrón
cAMP: Cyclic Adenosine Monophosphate
CD36: Cluster of differentiation 36
C-fos: Cellular Oncogene fos
ChoRE: Carbohydrate- responsive element
ChREBP: Carbohydrate Responsive Element Binding Protein
ChREBPα: Carbohydrate Responsive Element Binding Protein Alpha
ChREBPβ: Carbohydrate Responsive Element Binding Protein beta
CIDE: Cell death-inducing DFF45-like effector
Cidea: Cell Death-Inducing DFFA-Like Effector A
Cideb: Cell Death-Inducing DFFA-Like Effector B
Cidec: Cell Death-Inducing DFFA-Like Effector C (Cidec)
COX-2: Cyclooxygenase-2
CRP: Proteína C Reactiva
CREG1: Cellular repressor of adenovirus early region 1A–stimulated genes 1
CTP1a: Carnitina palmitoiltransferasa 1a
DAG: Diglicérido
DietMed: Dieta Mediterránea
DLP: Dislipidemia
DM2: Diabetes Mellitus tipo 2
DNL: De novo lipogénesis
ECVs: Enfermedades Cardiovasculares
Egr-1: Early growth response protein 1
Ehhdah: Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase
ELOVL6: Elongation of long-chain fatty acids family member 6
eNOS: Óxido Nítrico Sintasa endotelial
Fabp1: Fatty Acid-Binding Protein 1
FAO: Oxidación de ácidos grasos
FASN: Fatty Acid Synthase
FATP1: Fatty acid transport protein 1.
FFA: Ácidos grasos libres ó ácidos grasos no esterificados
FGF21: Fibroblast Growth Factor 21
Fgfr1: FGF21 receptor 1
Fsp27: Fat-Specific Protein of 27
Fsp27α: Fat-Specific Protein of 27 alpha
Fsp27β: Fat-Specific Protein of 27 beta

G6Pase: Glucosa-6-fosfatasa
GTT: Test de tolerancia a la glucosa
HDL-c: High Density Lipoportein-Cholesterol
HFD: Dietas altas en grasas
HOMA-IR: Homeostasis Model Assessment for Insulin Resistance
HSL: Hormone sensitive-lipase
IAS: Sociedad Internacional de Aterosclerosis
IASO: Asociación Internacional para el Estudio de la Obesidad.
IDF: Federación Internacional de Diabetes
IL-6: Interleukin-6
IMC: Índice de Masa Corporal
IR: Resistencia a la Insulina
ITT: Test de tolerancia a la insulina
LDL-c: Low Density Lipoportein-Cholesterol
LDs: Gotas lipídicas
LPL: Lipoprotein lipase
LZR: Ratas zucker delgadas
MAG: Monoglicérido
MCP1: Monocyte Chemoattractant Protein-1
MGL: Monoacylglycerol lipase
Mlx: MAX- like protein X
mRNA: Ácido ribonucleico mensajero
NAFLD: Hígado graso no alcohólico
NHLBI: Instituto Nacional del Corazón, los Pulmones y la Sangre
OMS: La Organización mundial de la Salud
OZR: Ratas zucker obesas
PEPCK: Phosphoenolpyruvate carboxykinase
PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PKA: cAMP-dependent protein kinase
PPARα: Peroxisome Proliferator-activated receptor alpha
PREDIMED: Estudio de Prevención con Dieta Mediterránea
REP: Retículo endoplásmico
SCD1: Stearoyl CoA Desaturase 1
SM: Síndrome Metabólico
SMILE: Small heterodimer partner–interacting leucine zipper protein
SREBP-1c: Sterol Regulatory Element Binding Protein-1
TAG: Triglicéridos
TCA: Ciclo del ácido tricarboxílico
TNFα: Tumor Necrosis Factor Alpha
UCP1: Uncoupling Protein 1
VLDL: Very low density lipoprotein
WAT: Tejido adiposo blanco
WHF: Federación Mundial del Corazón

Introducción

INTRODUCCIÓN

-Etiopatogenia de la obesidad

Causas

La Organización mundial de la Salud (OMS), en su sistema de clasificación internacional de enfermedades, dice que la obesidad es una enfermedad con el código ICD-10: E66.0 y hace referencia a una condición marcada por una cantidad anormalmente alta y poco saludable de grasa corporal[1]. La obesidad es un trastorno multifactorial de connotación ambiental, psicológica, y genética[2–4] caracterizado por una acumulación anormal o excesiva de tejido adiposo blanco (WAT). Este aumento del WAT tiene por objetivo almacenar el exceso de energía proveniente de un balance energético positivo sostenido, en forma de triglicéridos (TAG), la cual se consigue por medio de un incremento en el tamaño de las células adiposas existentes (hipertrofia) junto con la generación de nuevos adipocitos maduros a partir de pre-adipocitos (hiperplasia)[5-6].

Clasificación

Para una clasificación general de obesidad en adultos y en ambos sexos, la OMS propone un parámetro ampliamente utilizado por su sencillez, reproducibilidad y bajo coste, el Índice de Masa Corporal (IMC). El IMC es un indicador clínico que relaciona el cociente entre el peso en kilogramos y la talla en metros al cuadrado (kg/m²) y se ha asociado que a mayor IMC mayor mortalidad[7].

IMC (Kg/m2)	Range
< 18,5	Under weight
18,5 - 24,9	Normal
25 - 29,9	Overweight
> 30	Obese
> 40	Morbidly Obese

Tabla 1.-Tabla de Clasificación según OMS para mayores de 18 años

La clasificación se realiza según el resultado del ratio y va desde bajo peso hasta obesidad, cuando se obtiene un valor igual o superior a 30 kg/m^2[8] (Tabla 1).

La limitación de esta herramienta es que no considera la composición corporal ni la distribución de la masa magra y la masa grasa, solo mide el peso neto, por esto se ha incorporado la importancia del % de grasa para un diagnóstico global de obesidad. Como herramienta rápida, económica y no invasiva para este criterio se utiliza la bioimpedancia, la cual mide la resistencia del cuerpo al paso de una corriente alterna de baja intensidad, midiendo la masa magra y obteniendo por diferencia la masa grasa[3][9]. Los límites fisiológicos, en hombres no deben superar al 20-22% y en mujeres el 33-34% del total del peso corporal. (población afro-americana y caucásica)[10]. Mayor a estos porcentajes, ya se considera un contenido de tejido adiposo patológico el cual está asociado a las comorbilidades de la obesidad.

Prevalencia

la OMS estima que más de 1.9 mil millones de adultos en todo el mundo tienen sobrepeso y otros 650 millones son obesos[8][11]. Entre 1975 y 2016, la prevalencia de la obesidad se ha triplicado y con tendencia al aumento. Las proyecciones al 2030 es que aparezcan 3.1 millones de nuevos casos de exceso de peso. Con una repercusión directa en el costo sanitario en unos 3.000.000.000 euros/años[12-13]. En casos extremos, la obesidad puede reducir la esperanza de vida entre 6 y 14 años y se estima que es responsable de 3.4 millones de muertes anuales[14]. Datos que posicionan a la obesidad como una pandemia mundial, lo que se conoce como "globesity"[15].

-Obesidad y lipotoxicidad

La homeostasis es la estabilidad de los sistemas biológicos para mantener la vida. En el caso de la obesidad y sus comorbilidades, esta homeostasis se ha desregulado y el tejido adiposo y el hígado juegan un papel crucial en este desequilibrio metabólico.

Acumulación ectópica de lípidos

En un balance energético positivo y prolongado, existe un engrosamiento del WAT para almacenar lípidos, conlleva la expansión de éste seguido de angiogénesis, infiltración de macrófagos y finalmente sobreproducción de la matriz extracelular que es lo que determina la capacidad de expansión del WAT[16]. Esta capacidad de expansión se encuentra limitada por factores genéticos y ambientales y ante un exceso de nutrientes y frente a la incapacidad del WAT para expandirse más, el organismo seguirá almacenando depósitos de lípidos, pero lo hará en órganos no adiposos como el hígado, músculo esquelético-cardíaco y páncreas. Esta acumulación ectópica de lípidos da lugar al fenómeno de la lipotoxicidad, que es la responsable de gran parte de las comorbilidades asociadas a la obesidad como la resistencia a la insulina (IR), la diabetes mellitus tipo 2 (DM2), el hígado graso no alcohólico (NAFLD), la dislipidemia (DLP), las enfermedades cardiovasculares (ECVs), la artritis e incluso algunos tipos de cáncer[17] (Figura 1). Además del engrosamiento en el WAT, en el fenotipo obeso existe un estado inflamatorio crónico de bajo grado, que se produce debido a una producción alterada de adipoquinas en este mismo tejido. Es así como hay un aumento de la secreción de adipoquinas pro inflamatorias tales como la *Interleukin-6* (IL-6), y *Tumor Necrosis Factor Alpha* (TNFα) o de la leptina y la resistina, mientras que las de fenotipo antiinflamatorio como la adiponectina están disminuidas[18-19].

Esta producción de adipoquinas se producen en parte por la infiltración de macrófagos en el WAT y de su polarización hacia macrófagos de tipo 1 (M1). Los macrófagos cambian su polarización según estímulos externos y existen de tipo M1 y M2. Los macrófagos de tipo M1 tienen efectos citotóxicos y proinflamatorios y los de tipo M2 están involucrados en el mantenimiento de la homeostasis del WAT, ya que inhiben la respuesta inflamatoria[20]. En el caso de la obesidad se produce la polarización de tipo M1 provocada por la hipertrofia excesiva de los adipocitos. Esta hipertrofia del WAT provoca muerte celular, daño tisular y posteriormente una fibrosis que estimula respuestas inflamatorias locales que, movilizados por *Monocyte Chemoattractant Protein-1* (MCP1), atrae a los macrófagos desde la médula ósea hasta el WAT. Todo esto, sumado al flujo exacerbado de ácidos grasos libres o no esterificados (FFA) de la lipólisis de los adipocitos hipertrofiados, contribuyen a esta infiltración y polarización de los macrófagos[21](Figura 1).

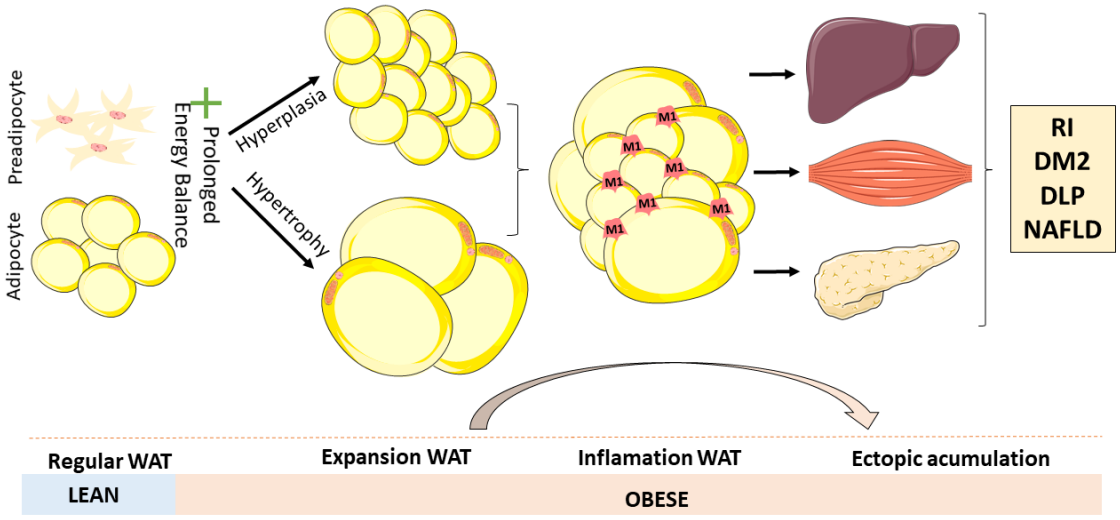

Figura 1. Diagrama de los adipocitos en estado sano y el cambio provocado por un balance energético positivo y prolongado, con consecuencia de la acumulación ectópica de depósitos de grasa y las lesiones lipotóxicas causantes de las enfermedades como IR, DM2, DLP y NAFLD.

Síndrome metabólico y obesidad central

La ubicación topográfica de la grasa ectópica también influye en tener mayores o menores riesgos metabólicos, existiendo dos principales clasificaciones: obesidad ginoide o gluteofemoral y obesidad androide o también llamada abdominal. En la obesidad ginoide ocurre mayoritariamente hiperplasia y en la clínica se identifica porque el exceso de grasa se localiza en la zona de los glúteos y muslos. Este tipo de obesidad se relaciona con problemas venosos en extremidades inferiores, incluso algunos autores le atribuyen un perfil pasivo, con bajos niveles de citoquinas inflamatorias[9][22]. En cambio, en la obesidad androide o abdominal se da una hipertrofia del adipocito, en este caso la grasa se localiza en la zona central del abdomen, rodeando los órganos[23] y es uno de los principales factores de riesgo para padecer síndrome metabólico (SM)[5][24].

El SM es un trastorno metabólico que incrementa los riesgos de padecer ECVs y DM2 [25-26]. Varios criterios de diagnóstico han sido propuestos por diferentes organizaciones durante la última década. Como los de la Federación Internacional de Diabetes (IDF), Instituto Nacional del Corazón, los Pulmones y la Sangre (NHLBI), Asociación Americana del Corazón (AHA), Federación Mundial del Corazón (WHF), Sociedad Internacional de Aterosclerosis (IAS), Asociación Internacional para el Estudio de la Obesidad. (IASO) y la principal diferencia entre todas ellas, respecta a la medida de la obesidad central. Por lo que actualmente se utiliza un criterio armonizado entre todas las organizaciones donde la obesidad central sigue siendo importante, pero se debe diferenciar por población[27]. Los otros factores de riesgo son presión arterial elevada, dislipidemia, glucosa en ayunas elevada (Tabla 2).

Metabolic Syndrome Harmonized criteria (2009) (IDF, NHLBI, AHA, WHF, IAS, IASO) Presence of at least 3 of following factor	
Waist circunference*	≥ 94 cm (men) or ≥ 80 cm (women)/European Population and country-specific definitions*
Fasting blood glucose	≥ 110 mg/dL**
Blood pressure	≥ 130/85mm Hg **
TAG	≥ 150mg/dL**
HDL	< 40mg/dL (men) y < 50mg/dL (women)

** or drug treatment

Tabla 2.-Clasificación para Síndrome Metabólico, según Harmonized Criteria 2009

Diabetes Mellitus tipo 2: hiperinsulinemia, hiperglicemia e hipertrigliceridemia

La presencia de los factores de riesgo del SM incrementa los riesgos de desarrollar DM2. La DM2 es una enfermedad crónica que se caracteriza por una hiperglicemia y se diagnostica con valores de glicemia en ayuna mayores a 126 mg/dL y a 200 mg/dL en cualquier momento, recientemente también se utiliza la hemoglobina glicosilada mayor a 6,5% como un criterio diagnóstico. Esta enfermedad exhibe una tríada de hiperinsulinemia, hiperglicemia e hipertrigliceridemia. La hiperglicemia frente a la hiperinsulinemia se atribuye a la IR, la que es una fase previa al desarrollo de DM2. La IR se caracteriza por altos niveles de insulina circulante (hiperinsulinemia) y mala señalización de ésta en órganos periféricos. Dada la incapacidad de que la insulina actué en los órganos periféricos y así disminuya los niveles de glicemia en sangre, esta resistencia tiene como resultado una sobreproducción e hipersecreción de insulina proveniente de las células beta del páncreas para intentar reducir los niveles elevados de glucosa en sangre. Además, se ha descrito que en un estado de resistencia a insulina ésta no inhibe la

gluconeogénesis, pero sí continúa activando la lipogénesis, produciendo la combinación de hiperglucemia e hipertrigliceridemia[28].

El diagnóstico de una IR en humanos conlleva el examen físico de Acantosis Nigricans, la cual es una hiperpigmentación de la piel y es una típica manifestación clínica de la IR y como así también el índice *Homeostasis Model Assessment for Insulin Resistance* (HOMA-IR) con el que se evalúa la relación entre la insulina plasmática en ayuno y la glucosa plasmática en ayuno. El HOMA-IR se calcula según la fórmula: (insulina plasmática en ayuno (μU/ ml) x glucosa plasmática en ayuno (mmol/L) /22.5) y su valor no debe ser mayor a 2.5. Otras pruebas para evaluar el metabolismo de la glucosa, es la prueba de la tolerancia a la glucosa (GTT) la cual monitorea la eliminación periférica de una carga de glucosa administrada y por ende la secreción de insulina en intervalos de tiempo determinados (30,60,90,120 minutos), por su parte la prueba de tolerancia a la insulina (ITT).) evalúa los niveles de glucosa en sangre tras una administración de insulina en intervalos de tiempo (30,60,90,120 minutos)[29]. La ITT es una prueba estándar para determinar el estado de resistencia a la insulina sobre todo en ratones, ya que en humanos los riesgos de una hipoglicemia son elevados, por lo es más aplicable el HOMA-IR[30]. Cuando el escenario no se revierte, y existe una progresiva resistencia a la insulina, al cabo de una década aproximadamente se establece la DM2

Hígado graso no alcohólico (NAFLD), dislipemia y enfermedad cardiovascular

El hígado es un órgano clave en el mantenimiento de la homeóstasis metabólica y es uno de los primeros órganos afectados por el acúmulo de lípidos ectópicos y por tanto de sufrir el fenómeno de la lipotoxicidad. La acumulación de grasas a nivel hepático da lugar inicialmente a una esteatosis hepática que puede dar lugar a patología que abarcan desde la NAFLD a una cirrosis e incluso cáncer hepático. La acumulación excesiva

de TAG en los hepatocitos a causa de una De Novo Lipogenesis (DNL) exacerbada[31] es la principal causa de esta la esteatosis hepática[23].Por su parte la DLP engloba patologías asociadas a niveles elevados de lípidos en suero; > 200 mg/dL Colesterol total, >150 mg/dL TAGs y 170 mg/dL LDL-c (*Low Density Lipoportein-Cholesterol*) y niveles < 40-50 mg/dL HDL-c (*High Density Lipoportein-Cholesterol*). Estos valores, tienen influencia directa con el desarrollo de las ECVs debido a su papel en la formación de la placa aterogénica, responsable del estrechamiento de las arterias, y en la generación de LDL-c oxidadas, característica de las enfermedades cardiovasculares, como la cardiopatía coronaria, la insuficiencia cardíaca o la hipertensión arterial. Las ECVs son la primera causa de muerte a nivel mundial[26][32].

-Tejido adiposo y metabolismo de lípidos

Se sabe que el tejido adiposo es un órgano clave en la regulación metabólica ya que desempeña un papel activo en el almacenamiento de lípidos, pero también por su función como órgano endocrino[16]. A la fecha se han descrito 3 tipos de tejidos adiposo, cada uno localizado en distintas partes del cuerpo. WAT: subcutáneo, visceral retroperitoneal, mesentérico perigonadal, tejido adiposo marrón (BAT): cervical, supraclavicular, paravertebral, perirrenal y tejido adiposo pardo o beige: subcutáneo inguinal[33].

Tejido adiposo blanco (WAT)

El WAT almacena energía como TAG en preparación para el ayuno o señales hormonales. El equilibrio entre procesos como la lipogénesis y la lipólisis suceden de manera coordinada para mantener la homeostasis lipídica (Figura 2). En condiciones patológicas, este equilibrio se ve alterado esencialmente por la incapacidad del tejido adiposo de almacenar más grasas. En este contexto, una lipólisis exacerbada en WAT aumenta la liberación de ácidos grasos, lo que conduce a una

acumulación de éstos en tejidos periféricos no adiposos, la consecuente lipotoxicidad y resistencia a la insulina[34]. Por otro lado, en esta situación, la lipogénesis en WAT está disminuida y esto también conduce a la resistencia a la insulina y DM2, ya que el ácido graso palmitoleato (C16:1n-7) que es un producto derivado de la lipogénesis también se reconoce como un insulino-sensibilizador[35].

En el WAT, la acumulación de TAG se realiza por medio de fuentes dietéticas y endógenas. Los TAG circulantes provienen de los absorbidos en el intestino o de los sintetizados en el hígado y circulan en sangre empaquetados en quilomicrones o *very low density lipoprotein* (VLDL), respectivamente. Para incorporarse al WAT, estos TAG se hidrolizan en FFA por la acción de la *lipoprotein lipase* (LPL), cuya actividad es estimulada por insulina. Los FFA liberados ingresan en los adipocitos a través de transportadores de ácidos grasos como el *Cluster of differentiation 36* (CD36) y la *Fatty acid transport protein 1* (FATP1). Por otro lado, la insulina también estimula la absorción de glucosa en los adipocitos, lo que impulsa DNL[36].

Tejido adiposo Marrón (BAT)

La principal función del BAT es la generación de calor (termogénesis) mediante el desacoplamiento de la cadena de transporte electrónico de la síntesis de ATP. Los infantes tienen depósitos de BAT interescapular y perirrenal, y hace aproximadamente una década se hizo evidente que el BAT está presente también en los humanos adultos principalmente en depósitos cervicales, supraclaviculares, paravertebrales y perirrenales[37]. El BAT genera calor mediante un proceso adaptativo llamado termogénesis sin temblores, que requiere un suministro abundante de combustible y la expresión de la *uncoupling protein 1* (UCP1). En concreto, el BAT oxida ácidos grasos y disipa en forma de calor la energía contenida en el gradiente de protones de la cadena de transporte de electrones[38]

gracias a la presencia de la UCP1, la cual se considera un marcador en el BAT y de la capacidad termogénica[39](Figura 2).

La termogénesis en BAT es activada como primera respuesta al frío, pero también por medio de la dieta. Mediante la activación del sistema nervioso simpático y la secreción de catecolaminas. La vía de transducción de señal de las catecolaminas pasa por la producción de *cyclic adenosine monophosphate* (cAMP), que activa la *protein kinase cAMP-dependant* (PKA), esta promueve la lipólisis por fosforilación directa de la HSL y de diferentes perilipinas pero también por activación indirecta de la ATGL y a la vez impulsa la transcripción de genes termogénicos (incluyendo la UCP1). Durante la termogénesis, los FFA son el combustible principal. En consecuencia, los lípidos intracelulares liberados por la lipólisis dentro de los adipocitos marrones se dirigen a las mitocondrias activas para la producción de calor[40-41].

La termogénesis juega un papel importante en la regulación del peso corporal ya que la activación del BAT aumenta el consumo de grasas y por ende el gasto energético. Esta propiedad ha hecho que hoy en día la activación del BAT se considere una estrategia terapéutica para patologías como la DM2 y la obesidad.

Recientemente se ha descrito que la termogénesis en BAT requiere de la activación simultanea de la DNL y la oxidación de ácidos grasos (FAO), dos procesos regulados de manera coordinada y excluyente en la mayoría de los tejidos y órganos[42-43].

Tejido adiposo Beige

A parte del WAT y el BAT, existe un tejido adiposo intermedio entre ambos que se conoce como tejido adiposo beige. Este tejido adiposo beige es una WAT que presenta características metabólicas y fenotípicas similares al BAT ya que también contribuye al aumento del gasto energético, además de expresar genes implicados en la DNL, la FAO y la

lipólisis. En este caso hablamos de que el tejido adiposo beige se origina por pardeamiento o browning del WAT. Es importante destacar que el BAT se desarrolla embrionariamente a partir de precursores específicos diferentes de los que derivan en WAT. Los precursores del WAT son también precursores también del músculo esquelético y por esto no es casual que sea un tipo de célula más oxidativa y menos de almacenaje que la blanca. Por su parte la grasa beige se desarrolla postnatalmente a partir de adipocitos del WAT[44-45](Figura 2).

El browning del WAT se considera una buena aproximación terapéutica contra la obesidad y patologías asociadas. Ya que en obesidad el BAT está inactivo y el pardeamiento de los adipocitos del WAT, contrarrestaría esta situación[46], además la inducción de los adipocitos beige, es altamente inducible por la dieta[47].

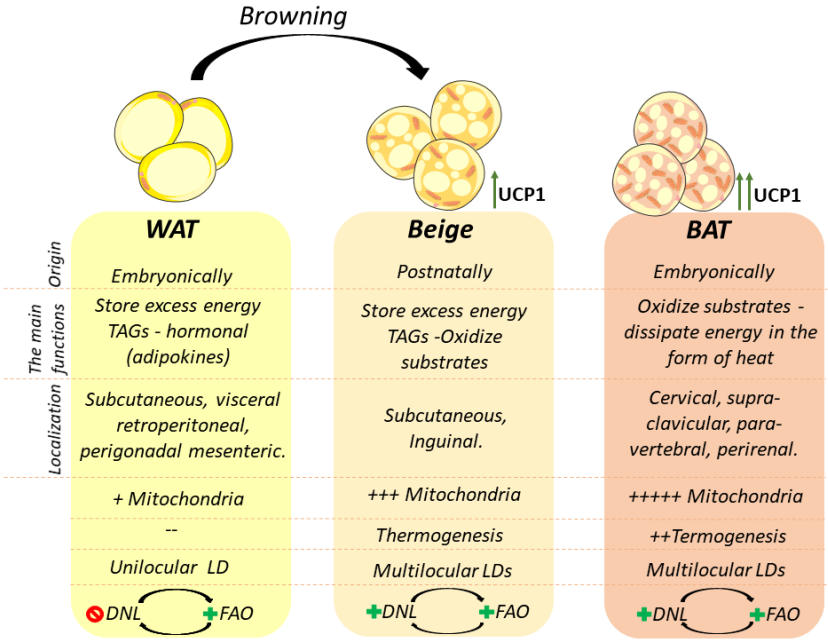

Figura 2. El origen, función, localización y características de los diferentes adipocitos blancos, adipocitos beige y marrón.

-Vías metabólicas

De novo lipogénesis (DNL)

Es una vía de síntesis de ácidos grasos a partir de precursores no lipídicos, esencialmente carbohidratos simples de la dieta como la glucosa y la fructosa, teniendo ésta última un mayor efecto lipogénico. Ocurre mayoritariamente en el hígado donde se relaciona con obesidad, NAFLD, IR y DM2 y en el tejido adiposo donde se relaciona con efectos insulino sensibilizadores[35].

La DNL está altamente regulada por las hormonas y el estado nutricional. Durante el ayuno, la DNL es baja, principalmente debido al aumento de los niveles de glucagón en sangre que causa un aumento de los niveles de cAMP intracelular y PKA que inhibe la DNL. Por otro lado, en ayuno hay también un aumento de los niveles de AMP que activan a la *AMP-activated protein kinase* (AMPK).

Por el contrario, después de una comida alta en carbohidratos, los niveles de glucosa e insulina en sangre aumentan, lo que estimula la DNL al aumentar la disponibilidad de sustratos (acetil-CoA y malonil-CoA), la expresión de genes lipogénicos y la actividad de las enzimas lipogénicas.

El proceso de síntesis de ácidos grasos puede dividirse en tres etapas secuenciales: síntesis de palmitato, elongación/desaturación de ácidos grasos y ensamblaje en triglicéridos.

Para la DNL se requiere acetil-CoA, que proveniente de sustratos no lipídicos se incorpora en el ciclo del ácido tricarboxílico (TCA) combinándose con el oxalacetato para producir citrato. El citrato en exceso puede salir del TCA y de la mitocondria para volverse a convertir en acetil-CoA por la acción de la *ATP citrate lyase* (ACLY), que es el primer paso de la DNL. Además, el citrato es un activador alostérico, de la *Acetyl-CoA carboxylase* (ACC), cuya acción es convertir acetil-CoA en malonil-

CoA, iniciando así la síntesis de palmitato. La enzima *fatty acid synthase* (FASN) es, junto la ACC, enzima limitante de la velocidad de la DNL. La FASN es el enzima clave en la síntesis de ácidos grasos y responsable de agregar secuencialmente dos unidades de carbono a una cadena de ácido graso en crecimiento hasta formar palmitato, un ácido graso saturado de 16 carbonos[36]. Una vez sintetizado el palmitato la enzima *elongation of long-chain fatty acids family member 6* (ELOVL6) alarga las cadenas carbonadas de los ácidos grasos y mediante la *stearoyl CoA desaturase 1* (SCD1) se introducen dobles enlaces convirtiendo los ácidos grasos saturados en ácidos grasos monoinsaturados o poliinsaturados. La síntesis de palmitato tiene lugar en el citosol de las células mientras que la elongación y saturación suceden en el retículo endoplásmico rugoso[48] (Figura 3).

Una vez que los ácidos grasos se sintetizan, se alargan y se desaturan, se pueden esterificar en el esqueleto de glicerofosfato que también es suministrado por el metabolismo de los carbohidratos para formar lípidos más complejos, como los triglicéridos.

Cuando la DNL esta activada, la acumulación de malonil-CoA en el citoplasma inhibe la enzima *carnitine palmitoyltransferase 1a* (CTP1a), la enzima limitante de la FAO[49]. Esto asegura que la síntesis y degradación de ácidos grasos no ocurran de manera simultánea. Además, el aumento en las concentraciones citoplasmáticas de palmitato inhiben alostéricamente la actividad ACC y reduce las tasas de DNL[50-51].

La DNL es principalmente regulada por los factores de transcripción *Carbohydrate Responsive Element Binding Protein (ChREBP)* y *Sterol Regulatory Element Binding Protein-1* (SREBP-1c), los cuales regulan la expresión de las enzimas involucradas en la síntesis de TAG.

Carbohydrate Responsive Element Binding Protein (ChREBP)

En respuesta a la glucosa y la fructosa, ChREBP y la *MAX-like protein X* (Mlx) se heterodimerizan y se activan como factores de transcripción. El heterodímero se une directamente a sus genes diana a través del Carbohydrate- responsive element (ChoRE)[52]. Este mecanismo de regulación transcripcional desempeña un papel fundamental en la activación de la DNL inducida por azúcares y en la homeostasis global del metabolismo de la glucosa a través de la coordinación del metabolismo intermediario hepático, la digestión de carbohidratos y el transporte.

Existen dos isoformas de ChREBP: ChREBPα y ChREBPβ. Ambas isoformas, en el tejido adiposo, están implicadas en la señalización de la insulina y en la activación de la DNL, bajo la captación de glucosa dependiente de transportador de glucosa 4 (GLUT4) [43][53]. En concreto, ChREBPβ está bajo control de ChREBPα y tiene una mayor actividad transcripcional siendo, según datos publicados, el principal regulador de la lipogénesis en respuesta a los carbohidratos de la dieta y un marcador de sensibilidad a la insulina en el tejido adiposo humano[54].

Sterol Regulatory Element Binding Protein-1 (SREBP-1c)

SREBP1c es un factor de transcripción que regula la expresión de genes que codifican para enzimas lipogénicas. La forma inmadura e inactiva de SREBP1c está anclado en la membrana del retículo endoplásmico hasta que llegan señales como la insulina que promueven su maduración. La maduración de SREBP1c depende de dos reacciones de proteólisis, una en el propio retículo y otra en el aparato de Golgi que generan su conformación como factor de transcripción maduro y activo. Este proceso de maduración, así como la expresión de SREP1c son activados ante la presencia de insulina tanto en células hepáticas como en adipocitos. Si bien SREBP1c no se considera tan transcendental para la DNL en WAT, sí lo es para el hígado.

Lipolisis

Los TAG que llegan al tejido adiposo son almacenados en gotas lipídicas y son liberados y utilizados según requerimientos de energía en el organismo. Así, situaciones como el ayuno o el ejercicio activan señales hormonales como el glucagón o las catecolaminas que activan la lipólisis y por lo tanto la liberación de ácidos grasos, principalmente en el tejido adiposo.

Este proceso de liberación de ácidos grasos consiste en hidrolizar los TAG en 3 moléculas FFA y glicerol. El primer paso de la hidrólisis de TAG está catalizado por la *adipose triglyceride lipase* (ATGL) que convierte un TAG en un diacilglicérido (DAG) liberando un ácido graso. Posteriormente, la *hormone sensitive-lipase* (HSL) libera un segundo ácido graso produciendo un monoacilglicérido (MAG) que mediante la acción de la *monoacylglycerol lipase* (MGL), libera el tercer ácido graso y una molécula de glicerol. Los ácidos grasos liberados pueden ser oxidados en el músculo esquelético y tejido adiposo. El glicerol por su parte puede ser utilizado para la gluconeogénesis en el hígado o reutilizado para producir nuevos TAG[42](Figura 3).

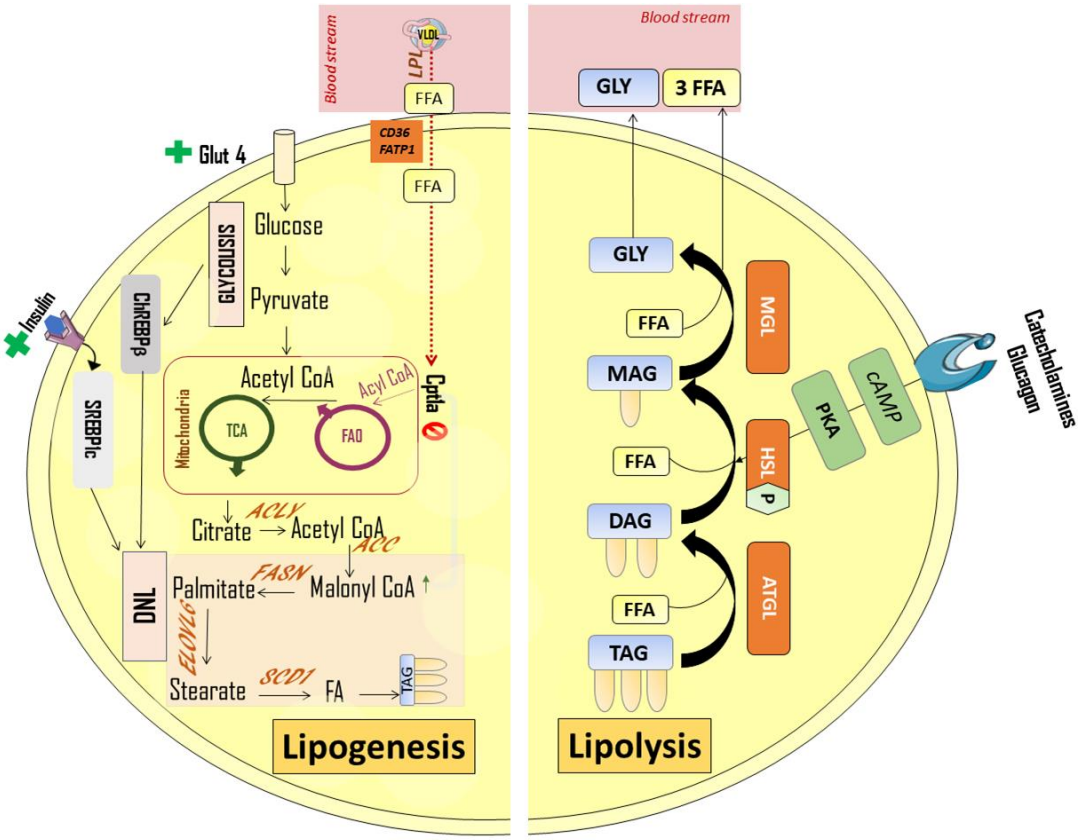

Figura 3. Lipogénesis y Lipolisis en el WAT.

Formación de gotas lipídicas (LDs)

Tanto en la lipólisis como en la DNL, la estructura de las LDs es crucial para poder almacenar o liberar los ácidos grasos correctamente.

La familia de proteínas *Cell death-inducing DFF45-like effector* (CIDE) incluye *Cell Death-Inducing DFFA-Like Effector A* (Cidea), *Cell Death-Inducing DFFA-Like Effector B* (Cideb) y *Cell Death-Inducing DFFA-Like Effector C* (Cidec) / Fat-Specific Protein of 27 (Fsp27), humanos/ratones respectivamente[55]. Son proteínas reguladoras cruciales de muchas vías metabólicas lipídicas, incluidas la fusión y el crecimiento de LDs. Además, están estrechamente relacionadas con el desarrollo de muchas enfermedades metabólicas, incluidas la obesidad, DM2 y NAFLD. Donde Cidea es expresada en tejido adiposo marrón y en la glándula mamaria, Cideb es expresada principalmente en el hígado, y en pequeñas cantidades en riñón, intestino delgado y colon[56]. Por su parte

Cidec/FSP27 se expresa en los tejidos adiposos principalmente en humanos y ratones[57]. FSP27 se localiza en la membrana de las LDs y se ha demostrado que existen dos isoformas: FSP27α y FSP27β. La isoforma β contiene 10 aminoácidos adicionales en el extremo N-terminal de la proteína. A nivel funcional, FSP27α es la encargada de la formación de LDs grandes y del fenotipo unilocular del WAT favoreciendo la fusión de las gotas más pequeñas. Por el contrario, FSP27β bloquea la fusión de las gotas lipídicas, siendo el responsable en parte del fenotipo multilocular con LDs pequeñas que se observa en hígado y BAT. Estas LDs más pequeñas facilitan la liberación de los ácidos grasos y su transporte a las mitocondrias adyacentes para su oxidación. Así, mientras FSP27α se expresa en WAT, FSP27β se expresa en hígado y adipocitos del BAT[58].

-Estrategias terapéuticas para el control y tratamiento de la obesidad

El balance energético positivo, característico de la obesidad, está directamente relacionado con los patrones alimentarios actuales, donde destaca la occidentalización de la dieta, también conocida como "Western Diet", la cual se considera una dieta obesogénica, que se define por una alta ingesta de grasas saturadas, sacarosa y bajo consumo de fibra, todo esto sumado al sedentarismo representan un elevado riesgo para el desarrollo de la obesidad[59-60]. Se conoce que la principal herramienta para combatir la aparición y desarrollo de la obesidad es seguir un estilo de vida sano, que incluya actividad física y alimentación saludable, como estrategias nutricionales contra la obesidad, se han utilizado dietas hipocalóricas, hiperproteicas, cetogénicas, pero todas ellas tienen una baja/moderada adherencia en la población y estas dos últimas además presentan problemas asociados a su uso prologando, como la sobrecarga renal y hepática, respectivamente[24][61-62].

En este escenario, la Dieta Mediterránea (DietMed) que es una dieta de fácil adherencia y seguimiento ha mostrado múltiples beneficios en la prevención y tratamiento de la obesidad e incluso revirtiendo sus comorbilidades ya que reduce la inflamación crónica de bajo grado y mejora la función endotelial, ofreciendo de esta manera efectos cardioprotectores[63]. El estudio de Prevención con Dieta Mediterránea (PREDIMED) muestra que esta dieta es efectiva como prevención primaria y secundaria de ECVs y se ha asociado con una disminución de riesgo de mortalidad, incidencia de cáncer y las enfermedades de Parkinson y Alzheimer[64–67].

La DietMed se caracteriza por un alto consumo de aceite de oliva extra virgen, frutas, verduras, frutos oleaginosos, legumbres y cereales integrales (Figura 4). Por lo que es una dieta que favorece el consumo de alimentos altos en compuestos bioactivos.

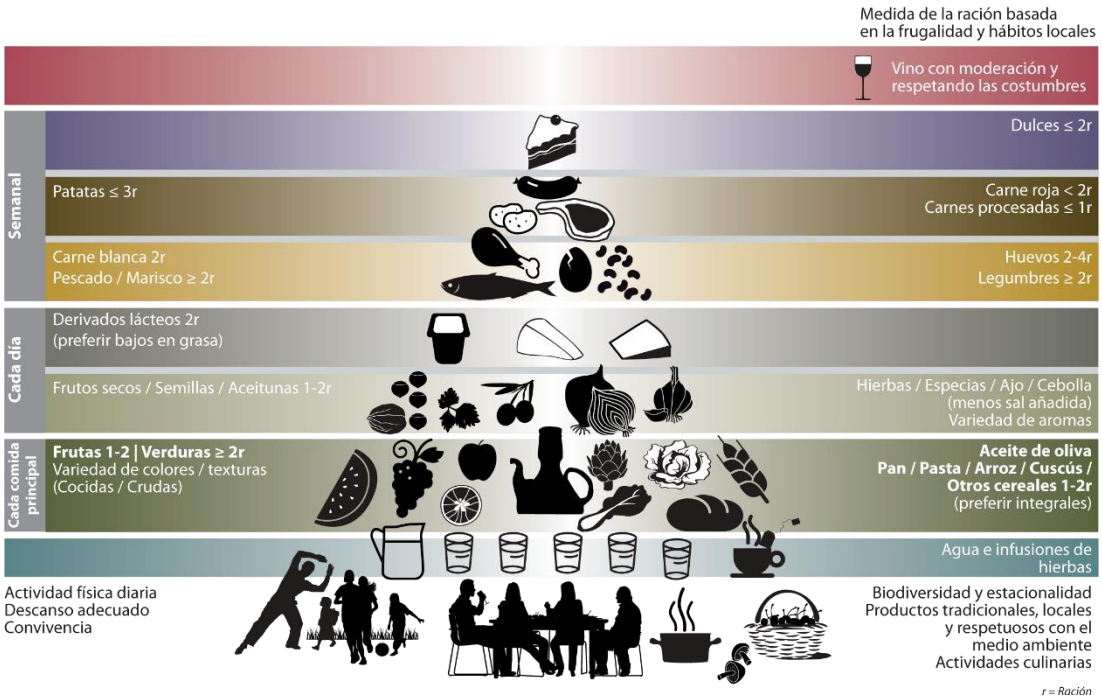

Figura 4. Distribución Pirámide de la Dieta Mediterránea (Fundación Dieta Mediterránea)

Compuestos bioactivos

Los compuestos bioactivos presentes en las frutas y vegetales son principalmente los carotenoides y polifenoles a los cuales se les han atribuido beneficios contra el cáncer enfermedades cardiovasculares, metabólicas y en el control del peso, por ende la prevención o tratamiento de la obesidad[66][68-69]. Los efectos de los compuestos bioactivos no solo dependen de la cantidad consumida, sino también de la bioaccesibilidad y biodisponibilidad, y no necesariamente el polifenol más abundante es el más biodisponible. La bioaccesibilidad se define como la fracción de un compuesto ingerido que se libera de la matriz alimentaria en el tracto gastrointestinal y una vez allí, estos compuestos o sus metabolitos alcanzan la circulación sistémica, lo que se conoce como biodisponibilidad y posterior bioactividad en el organismo[70-71].

Los carotenoides

Los carotenoides son pigmentos vegetales responsables de los tonos rojos, amarillos y naranjas en muchas frutas y verduras. Juegan un papel importante en la salud de las plantas e indirectamente en la salud humana.

Estos pigmentos naturales son sintetizados principalmente por plantas y muchos microorganismos, responsables de los colores amarillo, naranja y rojo en varias frutas y verduras. La mayoría de los carotenoides son terpenoides de 40 carbonos que tienen el isopreno como su unidad estructural básica. Los carotenoides se pueden clasificar en dos grupos en base a sus grupos funcionales: xantofilas que contienen oxígeno como grupo funcional y son luteína, zeaxantina, β-criptoxantina y carotenos, que contienen solo la cadena de hidrocarburos madre, sin ningún grupo funcional, y donde se incluyen el licopeno, β-caroteno y α-caroteno[72] (Figura 5).Varios procesos son necesarios para una óptima absorción de los carotenoides; la bioaccesibilidad, formación de micelas lipídicas en el

intestino delgado, captación de carotenoides por las células de la mucosa intestinal, y transporte de carotenoides o sus productos metabólicos a la circulación linfática o portal. Después de la absorción por difusión pasiva, los carotenoides siguen el metabolismo de los quilomicrones, son absorbidos por el hígado y liberados en el torrente sanguíneo en lipoproteínas.

Los carotenoides tienen un papel muy importante en la dieta y a menudo se describen como provitaminas A, ya que la vitamina A es un producto del metabolismo de los carotenoides. Otro rol potente es su actividad antioxidante y antiinflamatoria. Estudios epidemiológicos han demostrado que el consumo de dietas ricas en carotenoides está asociado con un menor incidencia de cáncer y ECVs[70][72].

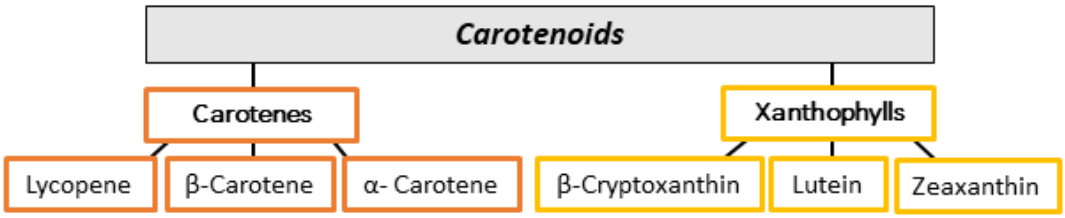

Figura 5. Clasificación de carotenoides.

Los Polifenoles

Los polifenoles son la principal clase de fitoquímicos de las plantas. Se sintetizan como metabolitos secundarios en respuesta a agresiones ambientales y de protección contra herbívoros, infecciones microbianas y radiación ultravioleta[73]. Los polifenoles ejercen un papel protector dado que disminuyen el estrés oxidativo, tienen efectos antiinflamatorios, vasodilatadores, vasoprotectores, antitrombóticos, hipolipeminantes y antiateroescleróticos y recientemente se ha visto que activan vías de señalización celular involucradas en la regulación metabólica[74–76].

Los polifenoles se pueden clasificar en dos grandes familias: flavonoides y no flavonoides, y cada uno de ellos se subcategoriza en varios grupos. Los flavonoides pueden subdividirse en antocianinas, chalconas, flavanoles, flavanonas, flavonas, isoflavonoides de flavonoles, mientras que los no flavonoides incluyen estilbenos, lignanos, ácidos fenólicos y otros polifenoles (Figura 6). Se han reportado aproximadamente 8000 estructuras químicas cada una con diferente impacto fisiológico en el organismo, aunque todas ellas tienen en común uno o más grupos fenólico[71][77-78]. Si bien el consumo de los polifenoles en la salud humana no se considera esencial, los estudios epidemiológicos, ensayos controlados aleatorizados y ensayos in vivo e in vitro con modelos animales y líneas celulares, muestran que las ingestas aguda y a largo plazo pueden tener efectos beneficiosos sobre la incidencia de enfermedades crónicas, incluidas las ECVs, los accidentes cerebrovasculares, la obesidad, la DM2, algunos tipos de cáncer y el deterioro de la función cognitiva[79–81].

El efecto de los polifenoles, van directamente relacionado a la biodisponibilidad de estos, y entre los factores principales involucrados en la biodisponibilidad se encuentran; la bioaccesibilidad, la dosis, el tamaño del compuesto fenólico. Solo el 5% -10% de la ingesta total de polifenoles de la dieta se absorben directamente a través del estómago y/o el intestino delgado. La mayoría de los polifenoles ingeridos alcanzan el colon donde actúa directamente la microbiota y se cree que ejercen un efecto prebiótico. Después de la absorción, los polifenoles experimentan biotransformación de fase I y II (sulfatación, glucuronidación, metilación y conjugación de glicina) por parte de los enterocitos en el hígado para aumentar la hidrofilia favoreciendo la secreción urinaria. Los metabolitos de polifenoles derivados del metabolismo hepático interactúan con el tejido adiposo, el páncreas, los músculos y el hígado, donde ejercen bioactividad[71][82-83].

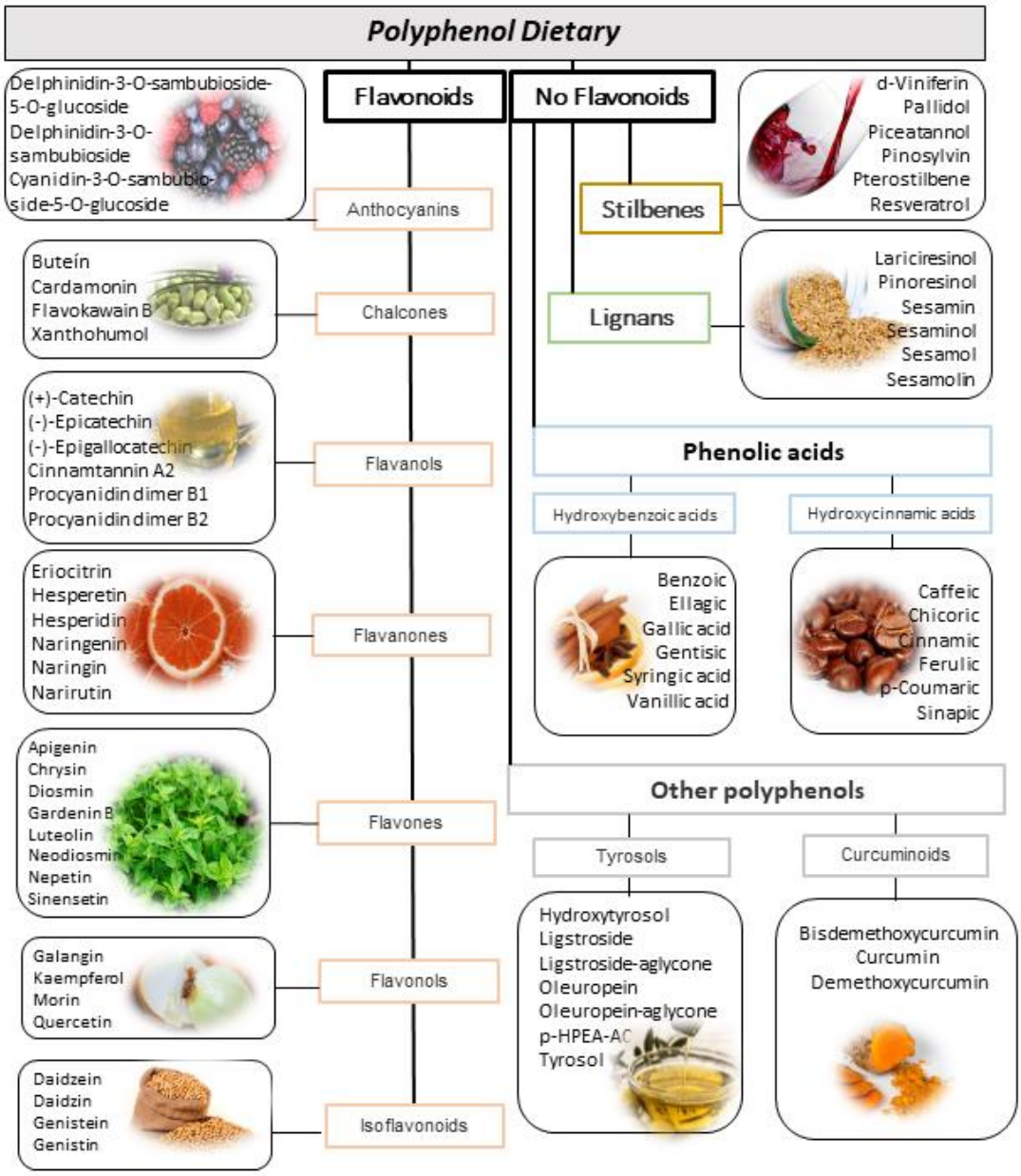

Figura 6: Distribución y clasificación de los Polifenoles.

-Alimentos con alto contenido en compuestos bioactivos

Sofrito

El sofrito es una preparación típica mediterránea y es una salsa hecha a base de tomate y cebolla cocidos a fuego lento con aceite de oliva; en algunos casos contiene puerro o ajo como así también algunas especias. El consumo de sofrito se incluye como uno de los 14 puntos para evaluar una buena adherencia a la DietMed y se recomienda su consumo mayor o igual a dos veces por semana. El sofrito se considera beneficioso para la prevención primaria de ECVs[64-65][68] e incluso metabólicas como DM2[84].

El sofrito tiene un alto interés nutricional debido al gran contenido en compuestos bioactivos; carotenoides y polifenoles, aportados por sus ingredientes principales, el tomate, la cebolla y el aceite de oliva. Se han identificado hasta 40 tipos de polifenoles en el sofrito, destacando los ácidos hidroxicinamoilquínicos y derivados de flavona, flavonoles y dihidrocalconas[85].

Aparte del contenido en compuestos bioactivos del sofrito se ha descrito también que el uso de determinadas técnicas culinarias pueden aumentar la biodisponibilidad de estos compuestos bioactivos y aumentar el impacto beneficioso en el organismo.
La matriz alimentaria del sofrito es compleja, ya que es un preparado con varios alimentos y que cada uno aporta componentes distintos, como los carotenoides del tomate, los polifenoles de la cebolla /ajo y los ácidos grasos del aceite de oliva, esta última actúa como excipiente del preparado, ya que se sabe que la presencia de aceite de oliva extra virgen mejora la bioaccesibilidad y biodisponibilidad de los carotenoides y polifenoles promoviendo su liberación desde la matriz alimentaria[86-87].
La preparación del sofrito considerando el tiempo de cocción y la adición de cebolla, modifica los compuestos bioactivos de los ingredientes a

formas más biodisponibles, promoviendo la formación de *cis*-licopeno y la bioaccesibilidad de polifenoles ácido clorogénico, ácido ferúlico y naringenina en comparación a un tomate crudo[88]. Es así como encontramos que en porcinas, el sofrito ejerce efectos protectores de la disfunción endotelial coronaria inducida por LDL-c al reducir el daño oxidativo, mejorar la expresión y actividad de la *endothelial nitric oxide synthase* (eNOS) y mejora la funcionalidad de las HDL[89]. En el mismo sentido, un estudio en humanos demostró mejoras sobre los biomarcadores inflamatorios plasmáticos como la proteína C Reactiva (CRP) y TNF-α, en hombres sanos con una ingesta de una única dosis de sofrito (240 g/70 kg)[90]. Finalmente, en un estudio con ratas Zucker alimentadas con una dieta suplementada con sofrito al 2% (w/w) durante 8 semanas, se observó que el sofrito tiene efectos beneficiosos sobre las alteraciones vasculares de la aorta relacionadas con la obesidad. En este mismo trabajo también se describió que las ratas obesas suplementadas con sofrito tenían mejores niveles de colesterol total, triglicéridos y glucosa que las obesas sin sofrito. Aunque no hubo variaciones en cuanto al peso entre ambos grupos sí que se detalla que las ratas obesas con sofrito eran hiperfágicas[91]. Este estudio y sus datos de hiperfagia, peso corporal y metabolismo de la glucosa fueron el punto de partida del objetivo 1 de esta tesis.

Maqui (*Aristotelia chilensis* (Mol.) Stuntz)

La *Aristotelia chilensis* (Mol.) Stuntz, denominada también maqui, es una baya comestible perteneciente a la familia Elaeocarpaceae, de alrededor de 5 mm de diámetro de color azul-negro intenso. Es una especie nativa de Chile que se considera un gran antioxidante natural[92-93] y es ampliamente utilizado por los indígenas de esta zona con fines medicinales en el tratamiento de enfermedades respiratorias, úlceras, diarrea, hemorroides, fiebre y como cicatrizante[94]. También se le han atribuido propiedades anti-aterogénicas e hipoglucemiantes[95-96].

El zumo de maqui puede inhibir la oxidación de las LDL y proteger a las células endoteliales contra el estrés oxidativo intracelular. Además, tiene actividad antiinflamatoria en las células de cáncer de colon a través de la reducción de la expresión de la cyclooxygenase-2 (COX-2)[97-98]. Los extractos de maqui han mostrado un efecto protector preventivo en estudios de isquemia/reperfusión en el corazón de ratas[99], como así también efectos antidepresivos tras sufrir un accidente cerebrovascular[100].

La baya del maqui tiene el color característico de los frutos ricos en antocianinas principalmente del grupo de delfininas[82][101–103]. Las antocianinas son una de las clases principales de flavonoides, tienen un esqueleto básico de C6-C3-C6[104] y están ampliamente distribuidas en diferentes concentraciones y con diversos grados de hidroxilación, metilación, glicosilación y acetilación en frutas, verduras, algunas bebidas y granos de cereales (incluidos maíz, arroz, trigo, cebada, sorgo, mijo y centeno). Son pigmentos naturales responsables del negro, púrpura, azul, rosa, rojo y marrón de muchas plantas, especialmente de las bayas[105].

Existe cierta controversia sobre los efectos de las antocianinas debido a su escasa presencia en suero después de su ingesta oral, alrededor del 1% [106]. La mayoría de las antocianinas de la dieta no se absorben a nivel del tracto gastrointestinal superior y alcanzan el intestino grueso, donde la microbiota intestinal las biotransforma en metabolitos fenólicos. Las formas glicosilada y acetilada reducen la bioactividad de las antocianinas, pero aumentan su estabilidad en el tracto intestinal, lo que aumenta su absorción[71][104][107]. Se ha demostrado que la suplementación dietética con alimentos que contienen antocianinas muestra potentes efectos antioxidantes, antiinflamatorios, mejora los niveles de glucosa en ayunas, la tolerancia a la glucosa, la sensibilidad a la insulina y puede ejercer control sobre el peso corporal[96][108–110]. Todos estos potentes

efectos se consiguen a pesar de la baja biodisponibilidad de las antocianinas, por lo que se cree que lo que potencia sus efectos es la sinergia entre tipos de alimentos con mezcla de antocianinas, como así también los metabolitos, ya que tienen una biodisponibilidad 42 veces mayor que la de las antocianinas originales[111].

-Revisión FGf21

Nutritional regulation of fibroblast growth factor 21: From macronutrients to bioactive dietary compounds

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Resumen Revisión

El *fibroblast growth factor 21* (FGF21) es una hormona peptídica involucrada en el mantenimiento de la homeostasis metabólica en individuos sanos y es considerado un buen candidato terapéutico para el tratamiento de la obesidad. FGF21 es producido principalmente por el hígado, pero también por otros tejidos, como WAT, BAT, el músculo esquelético y el páncreas en respuesta a diferentes estímulos, como el

frío y diferentes situaciones nutricionales, que incluyen el ayuno, dietas altas en grasas (HFD), dietas cetogénicas, algunas dietas deficientes en aminoácidos, dietas bajas en proteínas, dietas altas en carbohidratos y recientemente se han descrito los compuestos bioactivos de la dieta, como capaces de inducir la producción y señalización de FGF21.

Los tejidos diana de FGF21 son esencialmente el WAT, el BAT, el músculo esquelético, el corazón y el cerebro. Los efectos de FGF21 se producen a través del dímero de receptores formado por un miembro de la familia de los receptores del factor de crecimiento de fibroblastos (*FGF receptor*, FGFR) junto con el correceptor *β-klotho* (KLB). En concreto, FGF21 interactúa interacciona directamente con el dominio extracelular del complejo receptor FGF21-KLB-FGF (FGFR) para activar la vía de transducción de la señal de FGF21 que incluye la fosforilación del sustrato del receptor FGFR (FGF2α y la fosforilación de ERK1 / 2). Los ratones que carecen de KLB son resistentes a los efectos agudos y crónicos del FGF21. Además, los efectos de sensibilización aguda a la insulina de FGF21 también están ausentes en ratones con deleción específica de KLB adiposo o FGFR1. La mayoría de los datos muestran que la administración farmacológica de FGF21 tiene efectos metabólicos beneficiosos. El objetivo de esta revisión es recopilar información existente sobre los mecanismos que podrían permitir el control de los niveles endógenos de FGF21 para obtener los efectos metabólicos beneficiosos de FGF21 induciendo su producción en lugar de hacerlo mediante administración farmacológica.

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Nutritional regulation of fibroblast growth factor 21: from macronutrients to bioactive dietary compounds

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Abstract: Obesity is a worldwide health problem mainly due to its associated comorbidities. Fibroblast growth factor 21 (FGF21) is a peptide hormone involved in metabolic homeostasis in healthy individuals and considered a promising therapeutic candidate for the treatment of obesity. FGF21 is predominantly produced by the liver but also by other tissues, such as white adipose tissue (WAT), brown adipose tissue (BAT), skeletal muscle, and pancreas in response to different stimuli such as cold and different nutritional challenges that include fasting, high-fat diets (HFDs), ketogenic diets, some amino acid-deficient diets, low protein diets, high carbohydrate diets or specific dietary bioactive compounds. Its target tissues are essentially WAT, BAT, skeletal muscle, heart and brain. The effects of FGF21 in extra hepatic tissues occur through the fibroblast growth factor receptor (FGFR)-1c together with the co-receptor β-klotho (KLB). Mechanistically, FGF21 interacts directly with the extracellular domain of the membrane bound cofactor KLB in the FGF21- KLB-FGFR complex to activate FGFR substrate 2α and ERK1/2 phosphorylation. Mice lacking KLB are resistant to both acute and chronic effects of FGF21. Moreover, the acute insulin sensitizing effects of FGF21 are also absent in mice with specific deletion of adipose KLB or FGFR1. Most of the data show that pharmacological administration of FGF21 has metabolic beneficial effects. The objective of this review is to compile existing information about the mechanisms that could allow the control of endogenous FGF21 levels in order to obtain the beneficial metabolic effects of FGF21 by inducing its production instead of doing it by pharmacological administration.

Keywords: beta-klotho; diet; energy metabolism; fibroblast growth factor 21; obesity.

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Introduction

Fibroblast growth factor 21 (FGF21) increases energy expenditure. It thus has beneficial effects on glucose/lipid homeostasis and on body weight control and emerges as a novel therapeutic agent for the treatment of metabolic diseases such as obesity, type 2 diabetes and metabolic syndrome. In rodent and primate models of the aforementioned conditions, FGF21 has the capacity to restore glycemia and lipid profile, and to improve insulin resistance [1, 2].

It is widely accepted that FGF21 participates in metabolic homeostasis in health but its action takes on greater relevance in diseases.

To date, the pharmacological use of FGF21 is limited due to its half-life of around 1–2 h. In order to improve the pharmacokinetics, selectivity, and potency of FGF21, several laboratories have focused on designing FGF21 analogs. Two such analogs (LY2405319 and PF05231023) are currently being tested in clinical trials and have yielded similar results: benign toxicology, decreased plasma TGs and low-density lipoprotein cholesterol, increased high-density lipoprotein cholesterol, modest weight loss, elevated adiponectin, reduced insulin levels, and elevated plasma ketones. Surprisingly, no glucose-lowering effect was registered in any trial. This is a major setback as both compounds were assessed mainly as anti-diabetic drugs. However, the results of these two trials highlight the capacity of FGF21 to ameliorate lipid and cholesterol metabolism [3–5].

Nutritional signals play an important role in controlling gene expression in mammals. Macronutrients

[carbohydrates, fatty acids (FAs), proteins], micronutrients (minerals and vitamins), and some bioactive dietary compounds have the capacity to regulate gene expression and thus metabolic homeostasis. In this context, it has been described that endogenous FGF21 levels are regulated by various nutritional challenges such as high-fat diets (HFDs), low-protein diets (LPDs), amino acid-deficient diets, fasting, and polyphenols [6, 7] (Figure 1). However, the levels of this molecule are also determined by metabolic stress, including obesity, type 2 diabetes (T2DM), and non-alcoholic fatty liver disease (NAFLD) [8]. In this regard, this review summarizes how various nutrient stimuli and diet components regulate the expression of FGF21, and it also seeks to shed light on the molecular mechanisms and the clinical implications of the crosstalk between diet composition FGF21 levels and signaling.

FGF21 signal transduction

FGF21, together with FGF15/19 and FGF23, is an atypical member of the fibroblast growth factor (FGF) family. With endocrine, paracrine and autocrine properties, FGF21 lacks the heparin domain present in the rest of the family members, thus allowing it to be secreted. Defined as a hepatokine, myokine, and adipokine, FGF21 is expressed in several tissues, including the liver, pancreas, thymus, heart, testis, skeletal muscle, white adipose tissue (WAT), brown adipose tissue (BAT), heart, and brain. It also exerts action on multiple target tissues, ranging from peripheral to central [9].

FGF21 acts as a hormone-like peptide and its signaling pathway requires FGF21 binding to a fibroblast growth factor receptor (FGFR). FGFRs are tyrosine kinase receptors, and seven isoforms have been described (1b, 1c, 2b, 2c, 3b, 3c and 4). FGFR1c has been defined as the main mediator of FGF21 response in vivo [10] through an obligate dimerization with the co-receptor β-klotho (KLB) [11]. The co-expression of these two receptors determines the sensitivity of a tissue or organ to FGF21 signaling.

Regarding the signal transduction pathway, the binding of FGF21 to the FGFR-KLB dimer stimulates the phosphorylation of FGFR substrate 2α (FRS2α) and the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt [12].

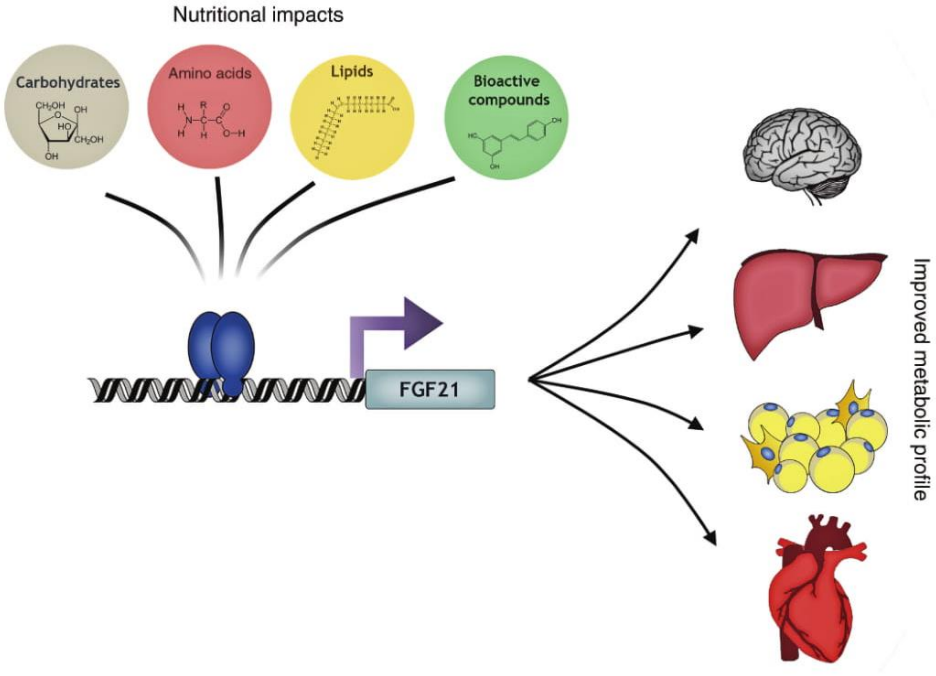

Figure 1: FGF21 expression is regulated by diet and its effects are widely distributed.
Endogenous levels of FGF21 are regulated by different macronutrients and bioactive dietary compounds. Acting as a hormone, FGF21 impacts on several tissues where regulates mainly lipid and glucose metabolism.

FGF21 as a hepatokine, myokine, adipokine and other kines

Liver production and secretion

The main source of FGF21 is the liver, where its expression is induced in response to stress. FGF21 was initially described as a fasting-adaptation hormone, as its hepatic production coupled to plasma levels is dramatically increased during prolonged fasting [13, 14]. It is now known that hepatic FGF21 expression is also induced in other liver-stress circumstances such as in obesity, specific nutritional conditions, liver injury, viral infection, chemical insult, hepatosteatosis, steatohepatitis, NAFLD, cirrhosis, and liver cancer [8, 15–18].

Hepatic overexpression of FGF21 triggers ketogenesis, gluconeogenesis, and FA oxidation (FAO) and suppresses lipogenesis in the liver [15, 19]. However, the autocrine effects of FGF21 on this organ are still under debate. FGFR4 is the predominant isoform in the liver, but the FGFR4-KLB complex cannot activate the FGF21 transduction pathway [10]. In contrast, hepatic FGFR1 levels are low, and it is unclear whether they are enough to ensure FGF21 signaling. Later studies reported contradictory results regarding the role of FGF21 in ketogenesis, thereby suggesting that the physiological effects of this molecule may differ from the pharmacological ones.

As a hepatokine, FGF21 affects WAT and BAT, tissues in which it regulates lipid metabolism – mainly by inducing lipolysis and browning and by increasing thermogenic capacity [20, 21]. It also exerts action in the brain, where it is able to reduce physical activity, induce torpor, and regulate circadian behavior [22, 23]. In conclusion, all data indicate that FGF21 is produced and secreted by the liver when the function of this organ is compromised by stress and that it is responsible for restoring and maintaining metabolic homeostasis. Hepatic FGF21 expression is highly sensitive to nutritional status, and the molecular mechanisms that modulate its expression in the liver will be reviewed in the following chapter.

White adipose tissue: autocrine and paracrine effects

Adipose tissue is the main target tissue of FGF21 and the major mediator of its beneficial effects. While the physiological effects of FGF21 during fasting remain elusive, most data on its signaling in WAT derive from studies in which it was pharmacologically administered or overexpressed in obese mice. Nevertheless, FGF21 shows paradoxical actions on WAT depending on its source. *Fgf21*-overexpressing mice show induced lipolysis [13, 24]. In contrast, *Fgf21*-knockout mice present enhanced lipolysis in late fasting [21]. In addition, while FGF21 suppresses lipolysis in mouse and human adipocytes [25], it is induced by peroxisome proliferator activating receptor g (PPARg) in WAT upon feeding, thus stimulating adipogenesis [26, 27].

Regarding glucose metabolism, FGF21 induces glucose uptake in 3T3L1 adipocytes by increasing glucose transporter 1 (GLUT1) independently of insulin action [2, 28]. Moreover, later studies showed increased glucose uptake in both WAT and BAT of lean mice infused with FGF21 and fed a chow diet [29].

In summary, in WAT, FGF21 induces genes involved in glucose uptake, lipogenesis and lipolysis, depending on the metabolic state of the adipocytes. These apparently contradictory effects may be due to compensatory effects of genetic modifications in mice, different nutritional status and different FGF21 concentrations reached between pharmacological administration and physiological secretion.

WAT is not only a FGF21 target tissue but also a mediator of the effects of this growth factor. In this regard, the glucose- and insulin-sensitizing effects of FGF21 require the production and secretion of adiponectin from WAT. Accordingly, FGF21 stimulates this mechanism in rodents, and adiponectin-knockout mice fail to reproduce the sensitizing effects of FGF21 [30]. Similarly, FGF21 also reduces the levels of the sphingolipid ceramide. Sphingolipid ceramides have been associated with insulin resistance caused by lipotoxicity. By inducing adiponectin secretion, FGF21 diminishes the accumulation of ceramides in obese animals [31]. Overall, despite some contradictory effects of FGF21 in adipose fat depots, adipose tissue is considered indispensable for the physiological and pharmacological effects of FGF21.

Finally, FGF21 induces the expression of uncoupling protein 1 (Ucp1), thus producing the so-called browning process of WAT in an autocrine, paracrine or endocrine fashion [20, 32]. Browning occurs in multilocular beige adipocytes in specific susceptible WAT depots, such as inguinal and perirenal tissue, through an increase in the expression of genes involved in thermogenesis and confers a brown fat-like phenotype to white adipocytes.

Brown adipose tissue: FGF21 induces thermogenic capacity

BAT is a FGF21 target tissue since it expresses FGFR1 and KLB; however, it is also a source of FGF21. In BAT, FGF21

stimulates glucose uptake and thermogenesis through the induction of UCP1 in the interscapular depot in an autocrine and paracrine fashion [33]. Upon cold exposure, FGF21 expression is increased in BAT and other cold-sensitive fat depots in the β-adrenergic/ATF2-dependent pathway [32–34]. In this regard, Fgf21-deficient mice respond poorly to cold exposure and show greater shivering [32]. The mechanisms underlying the action of FGF21 on BAT/WAT are still not well understood. Part of the FGF21-induced activation of the thermogenic program is driven by PGC1a, as FGF21 increases the protein levels of this molecule. Similarly, Pgc1a-knockout mice show an impaired response to FGF21 [32].

Furthermore, hepatic FGF21-mediated thermogenesis has also been described in response to maternal milk consumption in neonatal pups [35], and also in situations of metabolic stress, for example upon amino acid restriction [20]. These observations suggest that the hepatic FGF21-mediated increase in thermogenic capacity is an adaptive response to metabolic stress.

It has been proposed that the effect of FGF21 on energy expenditure and weight loss may be due to an increased thermogenic capacity of BAT and WAT (browning). However, recent experiments in Ucp1-null mice and interscapular BAT-excised mice show that when FGF21 is administered pharmacologically, UCP1 is not required for the improvement of the glucose, cholesterol, and free FA profile. Nonetheless, the increment in metabolic rate associated with the administration of FGF21 is diminished in these mice [36–38]. These data suggest that the metabolic benefits of FGF21 are partly UCP1-independent.

Skeletal muscle: FGF21 is produced in response to mitochondrial dysfunction

FGF21 expression in muscle was first described in Akt1 transgenic mice in which the mRNA and serum levels of FGF21 were induced [39]. In normal conditions, basal expression of FGF21 in skeletal muscle is low but its expression is increased by insulin [39, 40], exercise [41], mitochondrial myopathies [42], impaired mitochondrial FAO [43], muscle-specific autophagy deficiency [44] and transgenic overexpression of Akt1, perilipin-5 [45] or Ucp1 [46] in skeletal muscle. The induction of FGF21 in muscle due to a metabolic dysfunction is driven by the transcription factor ATF4, which is activated by endoplasmic reticulum (ER) stress. The AMPK and PI3K/Akt1 signaling pathways are also able to increase FGF21 expression [43].

The impact of FGF21 on skeletal muscle is not clear, as the expression levels of KLB do not seem to be sufficient to respond to FGF21. Several studies suggest that FGF21 administration can improve glucose uptake in vitro [47, 48], and a model of impaired mitochondrial FAO has recently shown the same in vivo [43].

In contrast, the role of FGF21 as a myokine is more evident. In the abovementioned conditions where FGF21 is overexpressed in muscle, the plasma levels of this growth factor also increase. The metabolic effects of this increase include the reduction of fat content in liver, increased FAO, resistance to a HFD and browning of WAT. These results show that FGF21 can act as a myokine when secreted in response to muscle stress and that it exerts its effects on metabolism in an endocrine fashion.

Heart: FGF21 exerts cardioprotective actions

Initially, the heart was discarded as a target tissue or source of FGF21 due to the low levels of FGF21 and KLB mRNA detected in this organ. However, later studies showed that FGF21 is expressed and secreted by cardiac cells in response to various stress conditions, including obesity, type 1 diabetes, fasting, ER stress, inflammation, infarct or hypertrophy, and some cardiovascular diseases [49, 50]. In the heart, FGFR1 and KLB have been detected in cardiac cells, where FGF21 exerts protective effects in an autocrine and endocrine fashion. The mRNA expression of FGF21 in these cells is driven by the transcriptional activation of Sirtuin1 – peroxisome proliferator-activated receptor a (Sirt1-PPARa) [50]; however, it can also be regulated by ATF4, especially when FGF21 induction is caused by ER or oxidative stress [49, 51].

It is now well established that FGF21 plays a key role in cardiac remodeling and pathophysiology. FGF21 protects cardiomyocytes from hypertrophy through a mechanism that involves the activation of the cAMP responsive element binding protein (CREB), the induction of PGC1a expression, and the reduction of the NF-kB pro-inflammatory pathway [50]. Cardiac FGF21 also induces the expression of anti-oxidant genes such as Ucp3 and superoxide dismutase (Sod2), thus preventing the production of reactive oxygen species (ROS) in cardiac cells and oxidative stress in the heart [52]. In an autocrine fashion, FGF21 modulates cardiac lipid homeostasis [49] and protects against diabetes-induced cardiomyopathy by activating the ERK-p38MAPK-AMPK pathway [50]. Finally, as an endocrine peptide, FGF21 can also inhibit cardiomyocyte apoptosis, thus reducing damage to the heart [53, 54].

Central nervous system: target tissue of FGF21

FGF21 has the potential to act in the central nervous system (CNS) since FGFRs are widely expressed in this tissue and KLB is specifically expressed in the suprachiasmatic nucleus, the dorsal vagal complex of the hindbrain, the area postrema, the nucleus tractus solitari, the nodose ganglia, and the paraventricular nucleus [22, 55, 56]. Immunoblotting experiments have revealed that FGF21 is expressed in several regions of the brain, such as the substantia nigra, striatum, hippocampus, and cortex [57]; however, this growth factor also crosses the blood brain barrier and is present in the cerebrospinal fluid in a linear relationship with serum levels [58]. FGF21 modulates circadian rhythm and fertility [22, 59], but current data point to the CNS as a mediator of the effects of FGF21 on energy expenditure and browning [60]. However, in all cases, the presence of KLB appears to be required for FGF21 to exert its effects. This observation thus indicates that this growth factor is an endocrine signal. Regarding the effects of FGF21 on energy expenditure and browning, experiments with diet-induced obese (DIO) mice show that the lack of KLB in the CNS abrogates all the effects of FGF21 on body weight, insulin sensitivity, metabolic regulation in liver, WAT, and BAT. These data suggest that direct signaling in the CNS causes an increase in the sympathetic outflow.

In the hypothalamus, FGF21 affects the expression of corticotropin-releasing factor. Intracerebroventricular injection of FGF21 in Fgf21-KO mice restores the metabolic effects of FGF21 essentially through the corticotropin-releasing hormone (CRH) and the activation of CREB, which finally enhances hepatic gluconeogenesis and sympathetic nerve activity in BAT [56, 61, 62]. According to that, lack of KLB in the brain blunts these effects [56, 60, 61]. Finally, treatment with the b-blocker propranolol diminishes the effects of FGF21 when it is centrally administered but not when delivered peripherally [60].

Given the wide range of biological processes regulated by the CNS and the capacity of FGF21 to act in the brain, it is likely that future studies will reveal new actions of FGF21 signaling through the CNS.

Pancreas: FGF21 preserves b-cell function

FGF21 is highly expressed in the pancreas and has protective effects against cerulein-induced pancreatitis [63]. Supporting data show that FGF21-deficient mice are more susceptible to damage, and FGF21-overexpressing mice are partly protected. In addition, FGF21 may be involved in enhancing islet engraftment [64] and in the preservation of b-cell function and survival [65]. Another point is that KLB expression is critical for these beneficial effects, but the expression of KLB is reduced when islets are treated with high glucose concentrations [66]. In the pancreas, FGF21 increases insulin content and glucose-dependent secretion and inhibits glucagon release in isolated islets [65].

Other effects of FGF21

In addition, and consistent with its metabolic benefits, FGF21 transgenic overexpression extends lifespan in mice. The authors of that study proposed that inhibition of the GH/IGF-1 signaling pathway would explain life extension in Fgf21 transgenic mice. Furthermore, microarray analysis showed that FGF21 modulates gene expression in the liver in a similar manner to caloric restriction (which is known to extend lifespan in mammals). These data suggest that FGF21 extends lifespan by acting as a selective caloric restriction mimetic in the liver [67].

Interestingly, FGF21 appears to be not only a metabolic regulator but also a nutrient intake and taste regulator. Both pharmacologic administration and hepatic secretion of FGF21 produce a satiety signal that suppresses the intake of “sweets” [68, 69]. In addition, two genome-wide meta-analyses associated genetic variations in a locus including Fgf21 with significant differences in macronutrient intake [6, 70].

While FGF21 boasts numerous beneficial effects, a major adverse effect is a decrease in bone mass. Both genetic overexpression of *Fgf21* and pharmacological administration of this molecule lead to the inhibition of osteoblastogenesis and the stimulation of adipogenesis. In contrast, the absence of *Fgf21* leads to a high bone mass phenotype. The mechanism underlying bone mass loss is the potentiation of PPARg activity [71]. Although this non-desirable effect has been described only in rodents, it has to be taken into account when considering FGF21 as a potential drug candidate.

FGF21 expression is regulated by nutrition

Fasting and feeding

FGF21 is expressed and produced by multiple tissues. However, under normal physiological conditions, all circulating protein appears to derive from the liver [72]. FGF21 was initially described as a protein induced in the liver to control metabolic adaptation to long periods of

starvation. This mRNA induction goes through the PPARa [13, 73, 74] and CREBH [75, 76]. Later, several studies showed that FGF21 expression is regulated not only by fasting but also by other nutritional states [77]. In addition to PPARa and CREBH FGF21 expression responds to the retinoic acid (RA) receptor b (RARb) [78], the RA receptor-related orphan receptor a (RORa) [79], the thyroid hormone receptor b (TRb) [80], the activating transcription factor-4 (ATF4) [81], the farnesoid X receptor (FXR) [82], and the carbohydrate responsive element binding protein (ChREBP) [83]. The following chapter will summarize the effects of various macronutrients and bioactive dietary components on FGF21 expression, the metabolic consequences and the putative signaling transduction pathways involved.

Carbohydrates upregulate FGF21 expression

Hepatic FGF21 is induced by prolonged fasting and also by refeeding with a high carbohydrate diet (HCD) (mixed sugar and starch) [84]. Rats starved for 24 h and refed with a HCD for 12 h show an increase in hepatic mRNA and serum levels of FGF21 and metabolic adaptations in the liver and WAT. Specifically in liver, there is an induction of lipogenesis, glucose uptake and metabolism, and a reduction of FA uptake and FAO. Similarly, in WAT, refeeding with a HCD induces lipogenesis, glucose uptake and metabolism, and lipolysis [84]. These results support the hypothesis that FGF21 is produced to compensate any imbalanced nutritional states.

Hepatic FGF21 mRNA expression and plasma levels are also induced in male C57BL/6 mice fed a HC diet containing 77% of energy as dextrose, 0.5% as fat and 22.5% as protein [85]. These results agree with previous studies performed in isolated rat hepatocytes cultured with high glucose [86]. Under this dextrose-rich diet, the liver induction of FGF21 increases de novo lipogenesis, probably as a result of excess carbohydrate intake. In contrast, the same diet supplemented with exogenous lipids [a soybean oil-based emulsion that is rich in C-18:1 and C-18:2 (n−6) unsaturated FAs] reduces FGF21 mRNA levels and de novo lipogenesis.

In rodents and humans, glucose is not the only molecule able to induce FGF21 expression in vitro. The human promoter of FGF21 responds to xylitol [77] and fructose [87] through the transcription factor ChREBP, which binds to a carbohydrate response element (ChoRE) present in human and mouse promoters [77, 83]. These data again reveal the independent mechanisms that regulate the expression of FGF21 downstream of fasting and feeding signals. In vivo, both in humans and rodents, fructose ingestion, but not glucose, leads to an increase of FGF21 serum levels, which peak 2 h after an acute load [87]. In this case, the remaining question is how and why FGF21, which is considered a metabolic positive hormone, is induced by a metabolically pernicious sugar such as fructose.

Finally, it has also been demonstrated that FGF21 expression is regulated by dietary fiber. The intake of such fiber facilitates weight loss and improves lipid and glucose profiles. Sugarcane fiber (SCF: 85% insoluble fiber, 70% particles <1 μM) administered to mice fed a HFD for 12 weeks enhances insulin sensitivity, diminishes fasting plasma glucose and TGs, and attenuates weight [88]. Without altering caloric intake, SCF regulates leptin and glucagon-like peptide 1 (GLP-1) in these mice. In the liver, SCF decreases TGs, cholesterol content, and FGF21 expression, but induces mRNA levels of KLB, FGFR1, FGFR3 and PPARa, thus enhancing FGF21 signaling and ameliorating the obesity observed in the FGF21-resistant state. In parallel SCF also increases AMPK signaling [88].

High-fat diets induce FGF21 resistance

The notion of crosstalk between HFDs and FGF21 expression is controversial probably because of the variety of FAs included in diets. For example, mice fed a corn-oil based HFD (cHFD) for 5 weeks express more FGF21 in liver than those fed a HFD in which corn-oil is replaced by a fish-derived long-chain polyunsaturated n-3 FA (PUFA) [89]. Moreover, another study reported that mice fed a HFD for 16 weeks show no differences in FGF21 mRNA levels versus those fed a low-fat diet [85].

In HepG2 cells, oleate, linoleate and trans-10, cis-12 conjugated linoleic acid (t-10, c-12-CLA) induce FGF21 expression and secretion while palmitate has no effect [90, 91]. Lipid infusion in humans increases the circulating levels of FGF21 [90]. Similarly, in neonatal mice, hepatic FGF21 expression is induced at the initiation of suckling, mainly due to the high FA content of milk [35]. In this case, the FGF21 secreted induces the thermogenic program in BAT.

In the abovementioned situations, mRNA induction occurs through the activation of PPARa and causes an increase in serum levels of FGF21.

In addition to long-chain FAs, butyrate and α-lipoic acid also modulate FGF21 expression in the liver. The former is produced mainly by bacterial fermentation of dietary fiber in the large intestine, and its effect on FGF21 expression seems to be due to its capacity to inhibit histone deacetylase-3 (HDAC3) [92]. Another short FA,

α-lipoic acid, is also involved in the regulation of FGF21. α-lipoic acid can be obtained from the diet (leafy green vegetables and red meats), and its dietary supplementation induces hepatic and plasma levels of FGF21 in vivo and in vitro [93, 94]. The effect of α-lipoic acid on FGF21 expression depends on a CREBH-dependent mechanism that includes the induction of its expression and an increase in its binding to the FGF21 promoter [95].

Finally, several models of obesity in rodents, primates and humans showed that FGF21 levels are higher than in normal weight littermates [17, 96, 97]. In this context, obesity can be defined as an "FGF21-resistant state" [98]. The hepatic induction of FGF21 mRNA in DIO mice positively correlates with an attenuated responsiveness in liver and WAT as a result of a reduction in FGFR1, FGFR4 and KLB levels.

Amino acid-deficient and low-protein diets increase energy expenditure

Several studies have described that hepatic FGF21 is regulated by protein intake. In general, LPDs or diets deficient in a specific amino acid (i.e. leucine or methionine) cause an increase in the hepatic expression and serum levels of FGF21. In this regard, diets with a relatively low content of essential amino acids, such as many vegan diets, are known to be protective against cancer, autoimmunity, obesity, and diabetes. These benefits could be partly due to increased circulating levels of FGF21 [99]. Several studies have demonstrated that FGF21 is the link between imbalanced amino acid intake and adaptive metabolic response and that it serves to restore metabolic homeostasis.

In liver – but not in WAT or BAT–, FGF21 is induced by leucine deprivation. In wild-type mice, the metabolic response to leucine deprivation includes dramatic changes in lipid metabolism. In liver, such deprivation inhibits FA synthase (FAS) activity, decreases the expression of lipogenic genes, and increases the mobilization of lipid stores. In WAT, it decreases FAS activity and the expression of lipogenic genes and increases the expression of FAO genes. Finally, there is an induction of UCP1 expression in BAT [20, 100, 101]. In contrast, in *Fgf21*-deficient mice this metabolic response to leucine is impaired, thus indicating that FGF21 is a key hormone in the regulation of lipid metabolism during leucine deprivation [20, 81].

In the same way, methionine-deprived mice show a comparable phenotype to that of leucine deprivation. The metabolic response to methionine deficiency includes resistance to diet-induced obesity, improved glucose homeostasis, increased FA activation and oxidation in liver, increased lipolysis in WAT, and increased Ucp1 expression in BAT [102–104]. All these effects are coupled to an increase in FGF21 levels.

The metabolic response to protein restriction is similar to that observed under leucine or methionine restriction [7]. Serum levels of FGF21 increase in both rodents and humans upon exposure to LPDs, regardless of overall caloric intake [105, 106]. Protein restriction is accompanied by weight loss and an increase in both food intake and energy expenditure. Remarkably, neither food intake nor energy expenditure of *Fgf21*-deficient mice are altered by the administration of LPDs [7]. Moreover, ketogenic diets (KDs), which are widely known to induce FGF21 expression, are usually low in carbohydrates and proteins but rich in fat. The protein content of KDs underlies the increased levels of circulating FGF21, since protein supplementation but not carbohydrate supplementation blunts the induction [107]. This effect could also explain the induction of FGF21 observed in HCDs, which are characterized by a low protein content.

Protein undernutrition caused by LPDs or imbalanced diets (which are common in mammals confronted with deficient sources of certain amino acids like legumes, grains or corn) strongly affects aminoacidemia. Additionally, pathological situations caused by various forms of stress, such as trauma, thermal burning, sepsis and fever, can lead to a negative nitrogen balance. In this context, several scenarios that alter aminoacidemia also lead to an increase in FGF21 expression. The absence of slc6a19 (neutral amino acid transporter) causes a lack of systemic neutral amino acids, resulting in an increase in FGF21 transcription [108]. The treatment with the antileukemic agent asparaginase depletes circulating asparagine and glutamine levels, promoting FGF21 expression [109], and the skeletal muscle-specific knockout mice for glucocorticoid receptor (GR) show reduced alanine flux from skeletal muscle during fasting, resulting in an increase in FGF21 plasma levels [110]. Hence, it is likely that many other situations that reduce amino acid availability also lead to an induction of FGF21 expression.

GCN2, a kinase that acts as a sensor of amino acid supply [111], and PPARa are indispensable for the induction of FGF21 in response to protein restriction. In this regard, the respective knockout mice present blunted induction of FGF21 when fed a LPD. Nevertheless, it is worth mentioning that in both Ppara-KO and Gcn2-KO mice, the LPD still induces FGF21 expression [7]. This observation suggests that additional signaling pathways are involved in triggering the increase in FGF21 expression in response to protein restriction.

To date, there is no evidence of PPARa activation in response to a LPD, thus suggesting that PPARa plays a role in the constitutive expression of FGF21. In contrast, the LPD increases GCN2-dependent phosphorylation of eIF2a, resulting in greater ATF4 protein levels [100, 112]. Therefore, the GCN2/eIF2a/ATF4 cascade emerges as the main signaling pathway in the induction of FGF21 by protein restriction. ATF4 directly or indirectly induces the transcription of a subset of specific target genes, including FGF21, to modulate many cellular processes to adapt to amino acid deficiency [81, 113, 114]. The 5′ regulatory region of the human FGF21 gene contains two evolutionarily conserved functional ATF4-binding sequences (AARE), which are responsible for ATF4-dependent transcriptional activation in response to ER stress or amino acid restriction [81, 115].

Hepatic mTORC1 activity is also related to FGF21 expression. The mTOR signaling pathway monitors amino acid sufficiency and promotes protein translation and cell growth, among other processes [116]. In this case, L-Tsc1 KO mice, which present mTORC1 hyperactivity in the liver, show increased expression of FGF21 and depleted levels of glutamine [117]. Moreover, when these animals are treated with rapamycin (mTORC1 inhibitor) or glutamine, the increase in FGF21 is blunted. Finally, in human hepatic tumors, mTORC1 activation also correlates with FGF21 levels [117]. It has been proposed that the mechanism underling the increase in FGF21 expression occurs through PGC1a; however, additional mechanisms could be involved and it is feasible that depleted glutamine levels trigger an amino acid response (AAR).

In summary, amino acid-deficient diets diminish aminoacidemia and trigger AAR, thereby resulting in elevated FGF21 levels. The contribution of each single amino acid to the modulation of FGF21 and how a deficiency in specific types of dietary protein alters FGF21 expression require further study.

Bioactive dietary compounds affect FGF21 expression and signaling

Polyphenols are the most abundant phytochemicals in nature. These bioactive compounds are synthesized as secondary metabolites by plants and are thus abundant in fruits, vegetables, legumes, cocoa and some beverages, such as tea, coffee and wine. Polyphenols comprise a large heterogeneous group of chemical structures, all with a phenolic ring with one or more hydroxyl groups, and they are classified mainly into two families, namely flavonoids and non-flavonoids, and also into many subfamilies. Flavonoids are the most abundant polyphenols and consequently those most greatly ingested in human diets.

Due to the diversity of polyphenols, their absorption in the body is dose- and type-dependent and their effects are related to their bioavailability and pharmacokinetics. It is estimated that most of the polyphenols ingested go directly to the colon and only between 5 and 10% are absorbed in small intestine. The effects of polyphenols in the colon have been related to a prebiotic capacity, as they are believed to induce the growth and activity of some bacteria, such as *Bifidobacterium*, *Enterococcus* and *Prevotella*. Furthermore, once absorbed, polyphenols enter portal circulation and are metabolized in the liver. Finally, the conjugate metabolites reach the bloodstream and the target tissues [118, 119].

Various epidemiological studies have reported that the regular consumption of polyphenols has beneficial effects in obesity, insulin resistance, cardiovascular diseases, and cancer. Several lines of evidence support the notion that polyphenol-rich diets play a key role in regulating lipid and glucose metabolism and are thus pivotal in the prevention and treatment of pathologies related to energy homeostasis [120–123].

It has been described that resveratrol has a protective effect on cardiovascular risk associated to ROS overproduction but also on the prevention of hepatic steatosis and the improvement of insulin resistance [124]. Green tea polyphenols, mainly epigallocatechin-3-gallate (EGCG), reduce the LDL and increase HDL levels in humans and improve insulin sensitivity in genetic models of insulin resistance, thus exerting a beneficial effect on body weight and lipid profile [125, 126]. Isoflavones and polyphenol-rich grape extract can partially prevent hepatic steatosis associated with obesity by restoring the correct secretion of adipokines – mainly leptin and adiponectin, by up-regulating FAO, and by down-regulating lipogenesis in adipose tissue [127, 128].

For many years, the health benefits of polyphenols were attributed to their anti-oxidant capacity as free radical scavengers. It has recently been described that polyphenols activate cell-signaling pathways that are not related to ROS production but rather those involved in metabolic regulation. It is remarkable that most of these pathways are downstream of FGF21. It has been reported that, in vitro, polyphenols downregulate SREBP-1c and its main target genes in lipogenesis, namely FAS and acetyl-CoA carboxylase (ACC) [129]. Stilbens, mainly resveratrol, exert their effects by promoting the phosphorylation and activation of the AMP-activated protein kinase (AMPK) and the activity of SIRT and PGC1a, thus inducing FA catabolism through the AMPK/SIRT1/PGC1a axis [130, 131]. On

the other hand, an anthocyanin-rich juice extract up-regulates PPARa activity in mice on a HFD and down-regulates lipogenic gene expression in liver by promoting FA consumption. Coffee polyphenols and resveratrol also induce FAO in rats through PPARa and PGC1a-dependent mechanisms [132]. Chalcones and flavokawain cardamonin type B inhibit lipid accumulation and adipocyte differentiation by increasing the phosphorylation of the ERK in the early phase of adipogenesis [133].

In summary, polyphenols exert beneficial metabolic effects especially in obese and in insulin-resistant animal models, and FGF21 restores homeostasis in scenarios of metabolic stress. The crosstalk between the two signals remains unclear, but a positive correlation between the beneficial effects of polyphenol-rich fruit extracts and FGF21 activity has been described in various rodent models of diet-induced obesity. In some cases, the effects of polyphenols were due to an induction of FGF21 levels in liver [134, 135] but also to an enhancement of FGF21 signaling by increasing the expression of FGF21 receptors and KLB, [136, 137]. Moreover, flavokawain B and cardamonin also modulate the secretion of FGF21 in mature adipocytes [133].

FGF21 in humans

After the identification of rodent FGF21 as an endocrine regulator induced downstream of PPARa and its agonists by both fasting and a KD [13, 73], it was shown that fasting FGF21 levels are significantly increased in patients with T2DM without a correlation with BMI, thereby pointing to a potential role of FGF21 in the pathogenesis of insulin resistance and T2DM [138].

Later, in contrast to previously published data, it was described that serum FGF21 levels in overweight and in obese subjects are significantly higher than in lean individuals and that they correlate positively with adiposity and the metabolic syndrome [17, 139]. Also, an increase in plasma levels of FGF21 in hepatic- and muscle-insulin resistant states and a correlation with BMI have been reported [140].

Accordingly, a study performed in 2010 again showed a positive correlation between FGF21 expression and BMI; however, the authors did not find that fasting and refeeding or 12 days of a KD regulated the hormone in humans [8]. Moreover, FGF21 concentrations are reversibly increased and are related to leptin and free FAs in obese children. However, the results of that study do not support a significant relationship between FGF21, insulin resistance, and features of metabolic syndrome or NAFLD in this age group [141]. The same pattern of FGF21 expression was described in obese adolescents with T2DM compared with obese adolescents without. As in rodents, this increment in FGF21 levels in obesity and T2DM points to a FGF21-resistant state and an impaired capacity of FGF21 to improve insulin sensitivity [142]. No significant association between FGF21 and growth or IGF-1 was found in either cross-sectional or longitudinal analyses; these findings do not support a relationship between FGF21 and growth in obese children [143].

In contrast, in the pubertal transition, it was found that FGF21 concentrations do not differ by obesity status or by sex. An inverse association between FGF21 and bone mineral content (BMC) among non-obese individuals and an inverse association between FGF21 and lean mass among females were observed, which were both independent of fat mass. FGF21 was inversely associated with HOMA-IR in males but not in females. The existence of relationships between FGF21, musculoskeletal parameters, and insulin resistance raises the possibility of crosstalk between these systems. These data suggest that circulating FGF21 differs in its association with bone, lean mass and insulin resistance depending on the sex and weight of the individual [144].

Globally, several studies have proposed FGF21 as a biomarker for metabolic pathologies such as cardiovascular diseases, NAFLD, and mitochondrial disease [8, 145]. Serum FGF21 concentrations are significantly elevated in patients with mitochondrial disease. This prospective study established serum FGF21 levels as a sensitive biomarker of mitochondrial disease and demonstrated that they are the best predictor of this disorder when compared to serum levels of other classical indicators [146]. In the same way, a cross-sectional study with a large well-characterized sample (913 subjects) assessed the interaction between clinical parameters (renal function, metabolic, hepatic and vascular risk markers), as well as growth hormone (GH) status, with FGF21 levels. Those authors concluded that FGF21 serum concentrations are associated with several aspects of the metabolic syndrome, hepatocellular function, as well as with GH status [147].

In a healthy population, there is also an age-related increase in serum FGF21 levels. This observation highlights a potential age effect in response to metabolic demands during the lifespan. FGF21 levels increase with age independently of body composition. At lower levels of FGF21, bone mineral density (BMD), but not other body composition parameters, attenuates the association between FGF21 levels and age, thereby suggesting that the metabolic demands of the skeleton serve to link FGF21 and energy metabolism [148].

Regarding the nutritional regulation of FGF21 in humans, the data are less clear than in rodents. Increased levels of FGF21 in human serum after extreme fasting (7 days) and PPARa activation have been described in healthy non-diabetic individuals but with a wide inter-individual variation. The induction of ketogenesis independently of FGF21 levels suggests that the physiological role of FGF21 in humans differs from that in mice [149]. Although FGF21 is elevated in response to pharmacological activation of PPARa and PPARd, the absence of variation in human plasma during fasting and refeeding and the decrease after a 3-month KD confirm that FGF21 does not play a major role in regulating fasting response or ketosis in humans [150].

Unlike mice, which showed an increase in circulating FGF21 after only 6 h of fasting, human subjects did not have a notable surge in FGF21 until 7–10 days of fasting. Moreover, FGF21 induction was associated with decreased thermogenesis and adiponectin, an observation that contrasts with previous reports based on supraphysiological dosing. In addition, FGF21 levels increased after ketone induction, thereby demonstrating that endogenous FGF21 does not drive starvation-mediated ketogenesis in humans. Instead, a longitudinal analysis of biologically relevant variables identified serum transaminase markers of tissue breakdown as predictors of FGF21. These data establish FGF21 as a fasting-induced hormone in humans and indicate that it contributes to the late stages of adaptation to starvation, when it may regulate the utilization of fuel derived from tissue breakdown [151].

A large number of studies have shown that dietary protein content markedly influences food intake and metabolism. In this context, in humans, circulating FGF21 levels increase dramatically after 28 days on a LPD, thereby suggesting that FGF21 is a signal of protein restriction and providing an explanation of the effect of dietary protein deficiency on metabolism [7].

As mentioned earlier, FGF21 levels increase rapidly following fructose ingestion and return to baseline within 5 h. Moreover, both baseline and fructose-stimulated FGF21 levels are 2–3 fold higher in subjects with metabolic syndrome compared to those in healthy subjects. In contrast, FGF21 does not increase in the first 2 h after the ingestion of a glucose load, although a modest increase is observed after 3–4 h. These data suggest that FGF21 plays a key role in fructose metabolism in humans [87].

Other nutritional challenges that affect FGF21 levels are fish oil supplements and betaine. The inclusion of the former in a diet for 3 months markedly reduces the circulating levels of FGF21 and other biomarkers, combined with decreases in serum lipids, glucose, liver enzymes, and other NAFLD risk factors. These results suggest that fish oil might contribute to reversing FGF21 resistance [152]. Regarding betaine, it is known that plasma betaine levels are reduced in insulin-resistant humans and that they correlate closely with insulin sensitivity. In this context, in addition to the beneficial metabolic effects of betaine, supplementation with this compound robustly increases hepatic and circulating FGF21 levels in mice. On the basis of these observations, betaine supplementation merits further investigation for the treatment or prevention of T2DM in humans [153].

Circulating levels of FGF21 can also be boosted by exercise. Increased levels of the protein were observed in the serum of healthy male volunteers performing a treadmill run at 50 or 80% VO_2max. These results suggest that FGF21 is also associated with exercise-induced lipolysis [41].

Finally, in healthy human volunteers, the injection of natural glucagon increased plasma FGF21 within hours, showing for the first time that glucagon regulates glucose, energy, and lipid metabolism, at least in part via FGF21-dependent pathways [154].

Expert opinion

FGF21 is a peptide hormone involved in metabolic homeostasis and considered a promising therapeutic candidate for the treatment of obesity and associated comorbidities such insulin resistance, T2DM, and cardiovascular diseases. Although the effects of FGF21 in humans seems to be weaker than in mice and interindividual variability in the levels of this growth factor are observed, it is obvious that FGF21 exerts beneficial effects on metabolic homeostasis in both species. To date, the therapies related to FGF21 overexpression have involved pharmacological administration; however, this approach has considerable limitations due to the kinetics and bioavailability of the compound. Recently, several studies have demonstrated that specific macronutrients and bioactive dietary compounds can modulate endogenous FGF21 expression and signaling, thereby opening up the possibility to induce FGF21 production or increase activity through dietary intervention. The most feasible approaches to define a nutritional intervention able to induce FGF21 activity and reduce obesity and associated-comorbidities are probably through protein restriction and polyphenol-enriched diets. However, more research is needed in order to establish the role of FGF21 as the link between the dietary components. It is also important to highlight that all these nutritional interventions must be done in compliance

with a balanced diet in order to prevent the loss of muscle mass or BMD and essential nutrient deficiencies.

Outlook

To design therapeutic approaches based on a nutritional intervention it is essential to determine the molecular mechanisms through which macronutrients, micronutrients, and bioactive dietary compounds affect FGF21 expression and signaling. It is likely that the metabolic effects of certain diets such as vegan or the beneficial effects of established healthy diets, such as the Mediterranean diet, occur at least in part through FGF21. Further studies in humans will be required to define the effect of a nutritional profile on FGF21 levels and signaling in healthy, obese and T2DM patients. Also it will be important to define if there are any differential effects between the pharmacological administration of FGF21 and its endogenous overproduction. Moreover, obesity that is one of the possible diseases treatable with FGF21 is also a FGF21-resistant state. In this context it will be strictly necessary to study the way to overcome this resistance, thus increasing the levels of the FGF21 receptors in its target tissues. Some studies have revealed that some nutritional interventions are able to induce the expression of FGFR and KLB.

In summary, FGF21 is a promising therapeutic candidate against metabolic pathologies such as obesity or T2DM and its expression and signaling are highly regulated by different nutritional states. The remaining question is how we can increase the responsiveness to FGF21 in obese or T2DM patient just through a dietary intervention.

Highlights

In animal models has been is well described that:

- The liver is the main organ in contributing to serum levels of FGF21.
- The transcription factors network responsible for the control of FGF21 gene expression is highly complicated. Both, positive and negative factors have been described.
- The expression of hepatic FGF21 responds to several stimuli, mainly metabolic stress signals.
- The hepatic expression of FGF21 can be modified by dietary components. Mainly high fat diet, high carbohydrate diet, low protein diet or amino acid restricted diets.

In order to confirm FGF21 as a therapeutic candidate against metabolic pathologies such as obesity or T2DM:

- The role of FGF21 as the link between the dietary components and its metabolic effects has to be set precisely.
- The effect of dietary bioactive compounds on the expression of hepatic FGF21 needs further studies.
- The mechanisms responsible for the establishment of the FGF21-resistant state have to be proven.
- The effect of different dietary interventions on the expression of FGF21 receptors in target tissues needs future evaluation.

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Hipótesis y Objetivos

HIPÓTESIS Y OBJETIVOS

Hipótesis

Los alimentos ricos en compuestos bioactivos muestran efectos saludables sobre determinadas patologías metabólicas y estos efectos se deben, como mínimo en parte, a su impacto en la expresión génica y la capacidad de regular el metabolismo favoreciendo el mantenimiento de la homeostasis metabólica.

Objetivos Generales

Definir los mecanismos moleculares involucrados en el impacto metabólico de alimentos ricos en compuestos bioactivos (sofrito y maqui) en modelos animales de obesidad.

Objetivos Específicos

1.-Analizar el papel de la dieta suplementada con sofrito al 2% sobre la expresión y señalización de FGF21 usando ratas obesas Zucker.(Artículo científico I)

2.-Analizar el efecto de maqui-berry liofilizado en tejido adiposo subcutáneo de ratones C57BL6/J con obesidad inducida por la dieta mediante una dieta rica en grasas (HFD 45%).(Artículo científico II)

3.- Analizar el efecto de maqui-berry liofilizado en el hígado de ratones C57BL6/J con obesidad inducida por la dieta mediante una dieta rica en grasas (HFD 45%).(Resultados no publicados)

Resultados

RESULTADOS

Artículo científico I

Mediterranean Tomato-Based Sofrito Sauce Improves Fibroblast Growth Factor 21 (FGF21) Signaling in White Adipose Tissue of Obese ZUCKER Rats

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La obesidad se describe como un estado de resistencia a FGF21 y algunos compuestos bioactivos se han definido como capaces de regular la producción y la señalización de esta hormona peptídica, la cual ha sido identificada como un candidato potencial para el tratamiento de la obesidad. En este trabajo analizamos el impacto de la suplementación de la dieta con sofrito, una preparación típica mediterránea de salsa a base de tomate, cebolla y aceite de oliva, sobre la expresión y señalización de FGF21 en el tejido adiposo blanco visceral (vWAT), y sobre la sensibilidad a la insulina en Ratas Zucker obesas (OZR).

Las OZR fueron alimentadas con una dieta estándar suplementada al 2% con sofrito w/w o una dieta estándar, durante 8 semanas y los resultados indican que el sofrito mejora la sensibilidad a la insulina y la expresión y señalización de FGF21 en OZR suplementadas.

En concreto, la suplementación de la dieta con sofrito redujo el área bajo la curva del test de tolerancia a la insulina (ITT) de las OZR respecto a las alimentadas con la dieta control y aumentó los niveles de mRNA de los receptores FGFR1 y FGFR4 y de los genes diana corriente debajo de la vía de transducción de la señal de FGF21: Egr-1, c-Fos y Ucp1 indicando una mejor señalización de FGF21 en el vWAT. Además, la inducción de UCP1 y el peso corporal sin cambios a pesar del comportamiento hiperfágico de las ratas alimentadas con sofrito, sugiere que el aumento en la señalización de FGF21 se podría correlacionar con un aumento en el gasto de energía.

Los datos de este estudio permiten concluir que parte de los efectos saludables del sofrito pasarían por la señalización de FGF21.

FOOD & FUNCTION

Mediterranean Diet

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Mediterranean Tomato-Based *Sofrito* Sauce Improves Fibroblast Growth Factor 21 (FGF21) Signaling in White Adipose Tissue of Obese ZUCKER Rats

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Scope: Obesity is a fibroblast growth factor 21 (FGF21)-resistant state. Since FGF21 production and signaling are regulated by some bioactive dietary compounds, we analyze the impact of Mediterranean tomato-based *sofrito* sauce on: (i) the FGF21 expression and signaling in visceral white adipose tissue (vWAT), and (ii) the insulin sensitivity of obese Zucker rats (OZR).

Methods and results: OZR are fed with a *sofrito*-supplemented diet or control diet. Insulin sensitivity and FGF21 signaling are determined. We observed that *sofrito* is able to improve the responsiveness to both hormones in obese rats. *Sofrito*-supplemented diet increases FGF21 signaling in vWAT by inducing the expression of the FGF receptors (FGFR1 and FGFR4) that promotes the expression of canonical target genes, like Egr-1, c-Fos and uncoupling protein 1 (Ucp1).

Conclusions: A *sofrito*-supplemented diet improves insulin and FGF21 sensitivity in OZR, explaining part of *sofrito*'s healthy effects on glucose metabolism. In addition, induction of UCP1 and the unchanged body weight despite the hyperphagic behavior of the *sofrito*-fed rats suggests that the increase in FGF21 signaling correlates with an increase in energy expenditure (EE). Further studies in humans may help to understand whether *sofrito* consumption increases the EE in obese individuals.

1. Introduction

Fibroblast Growth Factor 21 (FGF21) or derivatives are promising therapeutic agents for the treatment of metabolic diseases such as obesity, type 2 diabetes, or metabolic syndrome due to its beneficial effects on glucose/lipid homeostasis and body weight control by increasing energy expenditure (EE) and inducing, at least in part, browning and uncoupling protein 1 (UCP1) overexpression in adipose tissues.[1–4] Pharmacological infusion of FGF21 in different genetic or diet-induced animal models of obesity or diabetes causes an improvement in insulin sensitivity, a reduction in glucose and serum lipids levels, and weight loss.[5–9]

FGF21 shows endocrine, paracrine, and autocrine properties and its target tissues are essentially white and brown adipose tissue (WAT, BAT), skeletal muscle, heart, and brain. FGF21 is predominantly produced by the liver but also by other tissues, such as WAT, BAT, skeletal muscle, and pancreas[10–13] in response to different stimuli such as cold[14] and different nutritional challenges.[15] The effects of FGF21 on its target tissues occur through the fibroblast growth factor receptor (FGFR) together with the co-receptor β-klotho (KLB).[16,17] It has been proposed that obesity is an FGF21-resistant state due to a downregulated expression of FGFRs and KLB that impairs FGF21 signaling.[18,19] Most of the data show

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that pharmacological administration of FGF21 has beneficial metabolic effects; a remaining question is whether it is possible to restore the endogenous FGF21 signaling in obese people to improve their metabolic parameters and reduce the risk of developing obesity-associated pathologies.

The Mediterranean diet as part of a lifestyle is considered a model of healthy eating, with protective properties in terms of morbidity and mortality from different metabolic diseases.[20–22] The health-promoting effects of this diet have been in part attributed to a high intake of bioactive dietary compounds, such as polyphenols and carotenoids present in high amounts in fruits, vegetables, cocoa, and some beverages such as tea, coffee, or wine. Various epidemiological studies show that their regular consumption is associated with many beneficial effects on metabolic diseases.[22,23] Several lines of evidence support the notion that polyphenol-rich diets play a key role in regulating lipid and glucose metabolism and are thus pivotal in the prevention and treatment of pathologies related to energy homeostasis.[24–29] Besides polyphenols, consumption of lycopene, a bioactive compound that belongs to the carotenoid family, or tomato extracts as a lycopene source, has been related to healthy effects in cardiovascular diseases (CVD), diet-induced obesity (DIO), metabolic disorders, inflammation, and adiposity.[30]

Within the Mediterranean diet, tomato and tomato sauces are typical and *sofrito* is one of the most consumed. *Sofrito* is a cooked sauce made with tomato, onion, olive oil, and in some cases garlic, which, due to its composition and cooking method (mechanical and thermal treatments), has a high bioavailable content of diverse bioactive compounds that includes carotenoids such as lycopene and polyphenols belonging to different chemical groups.[31–37]

In terms of the healthy properties of bioactive dietary compounds, one of the most important questions concerns the molecular mechanism by which they exert their beneficial effects. It has been recently described that in various rodent models of DIO there exists a positive correlation between the beneficial effects of polyphenol-rich fruit extracts and FGF21 activity due to an induction of FGF21 levels[38,39] or an improvement of the FGF21 signaling by increasing the expression of FGF21 receptors and KLB.[40,41] Taking into account *sofrito*'s rich bioactive compound content, here we analyzed the impact of a regular consumption of *sofrito* on insulin sensitivity in an obese rat model and identified FGF21 signaling as a mechanism by which *sofrito* could exert, at least in part, its healthy effects. This work is closely related to a previous one where the authors described that *sofrito* protects against vascular alterations that could precede major cardiometabolic complications in obesity.[42]

2. Experimental Section

2.1. Animals and Diets

The animal procedures used in this article were previously described in Rodríguez-Rodríguez et al.[42] Briefly, 8-week-old male obese Zucker rats (OZR) were randomly divided into two groups: (1) OZR fed a chow diet (CD) ($n = 7$); and (2) OZR fed a *sofrito*-supplemented diet (CD with *sofrito* 2% (w/w) ($n = 8$)). We used 8-week-old male lean Zucker rats (LZR) fed a CD as initial control ($n = 7$). Chow diet (Teklad Global 2018) was provided by Harlan Laboratories (Milan, Italy) and *sofrito* by Gallina Blanca-Star (Barcelona, Spain). *Sofrito's* nutritional composition is described in Supporting Information Table S1. The percentage of *sofrito* supplementation (2%) was calculated considering a human consumption of one serving of *sofrito* per day with the meals and the lycopene content in liver was used as a biomarker of *sofrito* intake.

Body weight and food intake were evaluated weekly. After 8 weeks of nutritional intervention, animals were sacrificed and blood samples, liver, and visceral adipose tissue (vWAT: perirenal and retroperitoneal) were collected. The protocol for animal handling and experimentation was approved by the Committee of Ethical Experimentation of the University of Barcelona (557/16).

2.2. Serum Measurements

FGF21 in serum samples was measured with a mouse/rat FGF21 ELISA (ref. EZRMFGF21-26K) obtained from EMD Millipore (Germany). The assay was conducted following the manufacturer's protocol. Briefly, a calibration curve was constructed by plotting the difference in absorbance values at 450 and 590 nm versus the FGF21 concentrations of the calibrators. Concentrations of unknown samples (performed in duplicate) were determined using this calibration curve. The Millipore's protocol indicates that the lower limit of sensitivity is 10.0 pg mL^{-1} and the measurement error in the range of concentration of our samples is 6%.

2.3. Insulin Tolerance Test

Before the end of the treatment, insulin tolerance test (ITT) was performed on rats after fasting for 3 h. Blood samples were collected from the tail vein before and after 30, 60, and 120 min of insulin intraperitoneal administration (0.75 U kg^{-1} insulin solution, Sigma–Aldrich Chemical Co, USA). Plasma glucose concentration was determined using a blood glucose commercial monitoring meter (Contour NEXT, Bayer, Spain). The area under the glucose curve (AUC) was calculated using Prism GraphPad 5.01 software.

2.4. RNA Isolation and Relative Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the frozen tissues using total RNA isolation (TRI) reagent solution (AM9738, ThermoFisher Scientific, USA) followed by DNase I treatment (AM1906, ThermoFisher Scientific, USA). To measure the relative mRNA levels, qRT-PCR was performed using SYBR Green or TaqMan probes. cDNA was synthesized by MMLV reverse transcriptase (28025021, ThermoFisher Scientific, USA) with random hexamers (11034731001, Roche Diagnostics, Germany). The TaqMan Gene Expression Master Mix (4369514) and SYBR Green PCR Master Mix (4364344), supplied by ThermoFisher Scientific (USA), were used for the PCR step. Amplification and detection were performed using the BioRad CFX96 touch (BioRad, USA). 18S and beta-actin were used to normalize the mRNA levels. The

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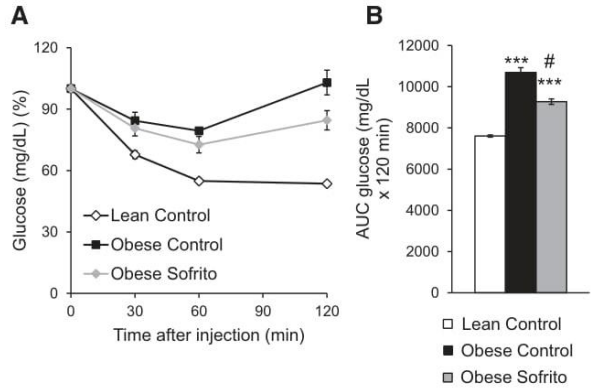

Figure 1. Insulin resistance is attenuated by *sofrito*-supplemented diet in OZR. A) ITT showing plasma glucose after intraperitoneal administration of insulin (0.75 U kg^{-1}) in lean control rats, obese control rats, and obese *sofrito*-supplemented rats after 7 weeks of diet administration. B) AUC of glucose levels. Data are presented as the mean ± SEM. ***$p < 0.001$ versus lean control rats; #$p < 0.05$ versus obese control rats. (n = 6–8/group).

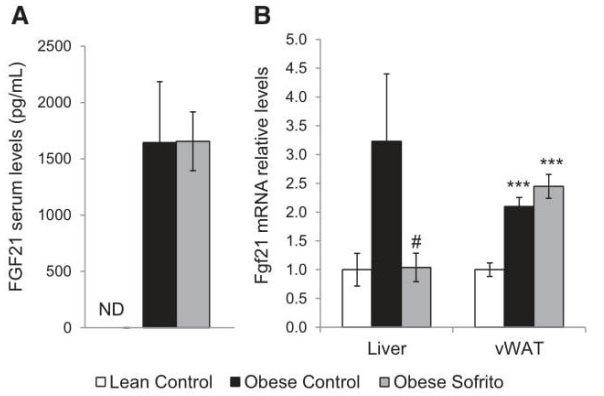

Figure 2. FGF21 serum levels are increased in obese phenotype due to higher expression in liver and vWAT but not influenced by *sofrito*. A) FGF21 protein levels (pg/mL) were measured by ELISA in plasma of lean control rats, obese control rats, and obese *sofrito*-supplemented rats. B) The mRNA relative levels of *Fgf21* were measured by qRT-PCR in liver and vWAT of lean control rats, obese control rats and obese *sofrito*-supplemented rats. Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus lean control rats; #$p < 0.05$ versus obese control rats (n = 6–8/group).

primer sequences or Taqman Probes used are shown in supplemental information (Supporting Information Table S2). Results were obtained by the relative standard curve method and expressed as fold increase compared to the experimental control.

2.5. Data Analysis/Statistics

All data are expressed as mean ± SEM. The gene expression assays are expressed as mRNA relative levels and referred to 1 assigned to LZR control or OZR control, as indicated. Significant differences were assessed by a two-tailed Student's *t*-test.

3. Results and Discussion

3.1. Insulin Resistance is Attenuated by *Sofrito* Supplemented-Diet in OZR

Obesity is usually associated with insulin resistance and glucose intolerance. OZR showed increased levels of blood glucose and those values are not significantly changed by a CD supplemented with *sofrito*.[42] To test the effect of *sofrito* on insulin sensitivity we performed an ITT with the different experimental groups. Both groups of OZR showed insulin resistance as demonstrated by a significant increase in the AUC (**Figure 1**A) compared to LZR, whereas the *sofrito*-supplemented diet was capable of partially counteracting this insulin resistance in OZR (Figure 1B).

3.2. FGF21 Serum Levels are not Influenced by *Sofrito*

Taking into account that FGF21 and bioactive dietary compounds are both described as beneficial signals for insulin sensitivity, we decided to evaluate the role of FGF21 expression and signaling on the insulin sensitizing effect of *sofrito* on OZR. We analyzed the FGF21 protein levels in serum by an ELISA assay. The serum levels of FGF21 are higher in both groups of OZR than the corresponding littermates (LZR) (**Figure 2**A). These results are in accordance with previously published studies that have shown increased FGF21 serum levels compared with normal weight littermates in several models of obesity.[18,43,44]

In this case, the FGF21 levels detected in OZR fed the *sofrito*-supplemented diet were almost the same as those in the obese control rats, indicating that *sofrito* is not able to modify the circulating levels of FGF21. It is worth mentioning that the circulating levels of FGF21 in healthy individuals (LZR) were undetectable. These results reinforce the idea that FGF21 production is directly related to metabolic stress conditions such as obesity.

To determine the source of FGF21 in OZR we determined the expression levels of FGF21 in the liver and vWAT as one of the main target tissues of obesity. Figure 2B shows the relative mRNA levels of FGF21 in the liver and vWAT. Contrasting with the unchanged circulating levels of FGF21, OZR fed with the *sofrito*-supplemented diet showed a significant reduction in the mRNA levels of FGF21 in the liver. In vWAT we observed that in both OZR groups the relative mRNA levels of FGF21 were twofold higher than in LZR. These results highlight that an extra hepatic production of FGF21 could be, at least in part, responsible of the higher FGF21 circulating levels detected in OZR. Tissue-specific knockout models would be necessary to elucidate the source of FGF21 under different nutritional conditions.

Since the production of FGF21 is not modified by *sofrito*, we studied the FGF21 signaling as the presumed molecular pathway responsible for the health benefits of *sofrito* on glucose metabolism. As the effects of *sofrito* on insulin sensitivity were observed only in OZR, the next experiments were performed only with those rats.

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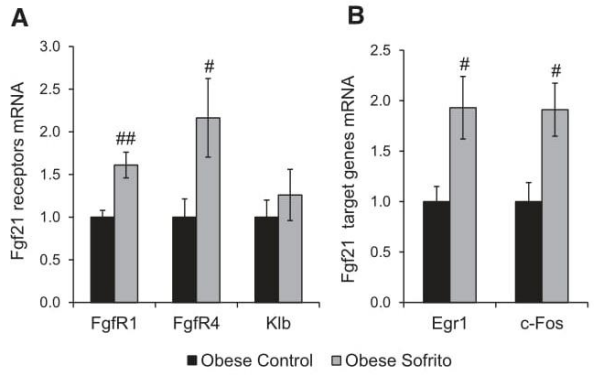

Figure 3. FGF21 signaling is improved in vWAT of OZR fed with a *sofrito*-supplemented diet. The mRNA relative levels of *Fgf21* receptors (*Fgfr1*, *Fgfr4*, *Klb*) A) and the mRNA relative levels of the target genes of the FGF21 signal transduction pathway (*Egr1* and *c-Fos*) B) were measured by qRT-PCR in vWAT of obese control rats and obese *sofrito*-supplemented rats. Data are presented as the mean ± SEM. #$p < 0.05$ ##$p < 0.01$ versus obese control rats. ($n = 6$–8/group).

3.3. FGF21 Signaling is Improved in vWAT of OZR Fed with a *Sofrito*-Supplemented Diet

FGF21 signaling requires the dimerization of the FGFR and KLB and the sensitivity of a tissue or organ to FGF21 is directly dependent on the coexpression of both receptors. In DIO mice, the FGF21-resistant state implies an attenuated responsiveness in the liver and WAT as a result of a reduction in FGFR1, FGFR4, and/or KLB levels.[18,45,46] We tested the FGFR1, FGFR4, and KLB expression in the liver and vWAT. **Figure 3**A shows that the expression of FGFR1 and FGFR4 were significantly increased in rats fed with the *sofrito*-supplemented diet compared to obese control rats, and although KLB expression did not reach statistical significance it shows a tendency to increase. The mRNA levels of the receptors in the liver were unchanged by *sofrito* (data not shown).

Taken as a whole, our data led us to hypothesize that *sofrito* restores the FGF21 signaling in OZR, overcoming the FGF21-resistant state characteristic of obesity. These results were in accordance with previously published data demonstrating that polyphenols or polyphenols-enriched foods are able to increase the expression of FGF21 receptors.[40,41]

To confirm the impact of *sofrito* on the FGF21 signaling pathway we analyzed the mRNA levels of the FGF21 target genes c-Fos and Egr-1 in vWAT. The expression of c-Fos and Egr-1 were significantly induced in rats fed with the *sofrito*-supplemented diet compared to the obese control rats (Figure 3B). This induction represents a direct measurement of FGF21 signaling and tissue responsiveness and led us to confirm the capacity of *sofrito* to improve the sensitivity of vWAT to FGF21 in OZR.

3.4. *Sofrito* Induces UCP1 Expression in the vWAT of OZR

During the nutritional intervention with *sofrito*, the body weight and food intake of the rats were recorded. As previously described, the caloric intake (Kcal d^{-1} per rat) and body weight of OZR were higher than in LZR (Supporting Information Figure S1); curiously, while the caloric intake was also significantly increased by the inclusion of *sofrito* in OZR's diet (Supporting Information Figure S1A), there were no changes in their body weight (Supporting Information Figure S1B). Looking for an explanation for the above-mentioned profile of OZR fed with *sofrito*-supplemented diet compared to control OZR we hypothesized that the differences could be due to changes in EE. It is well-known that FGF21 administration leads to a reduction in body weight through increased EE.[47] Concretely, the injection of FGF21 increases thermogenic capacity by stimulating the expression of UCP1 and type 2 iodothyronine deiodinase protein 2 (DIO2) in BAT, and UCP1 in WAT, where it produces the so-called browning process.[48,49] Because UCP1, an FGF21 target gene[3,4] has been linked to EE, we analyzed the UCP1 expression in the vWAT collected from OZR. The mRNA levels of UCP1 are increased in the vWAT of *sofrito*-supplemented rats compared to control rats (**Figure 4**), suggesting an effect of *sofrito* on the induction of browning. The process of browning causes the change of white adipocytes to beige/brite adipocytes, which are greater energy consumers, and this could explain the unchanged body weight of the hyperphagic OZR fed the *sofrito*-supplemented diet despite they were consuming more calories. We also analyzed pRDM16, PGC1b, and PPARg expression levels but no significant changes were observed.

Our data indicate that *sofrito* modulates FGF21 signaling without modification of FGF21 expression. The effect of *sofrito* on Egr-1, c-fos, and UCP1 expression indicate that FGFR expression renders a more active signaling receptor complex. Recently, it has been described the role of miRNA34a in downregulation of FGFR1 and KLB levels in adipocytes.[50] However, there are no differences in the miRNA34a expression between OZR and OZR rats fed with *sofrito*-supplemented diet (data not shown). As *Sofrito* is a mix of different bioactive compounds, including polyphenols and carotenoids but also oleic acid (MUFA) from

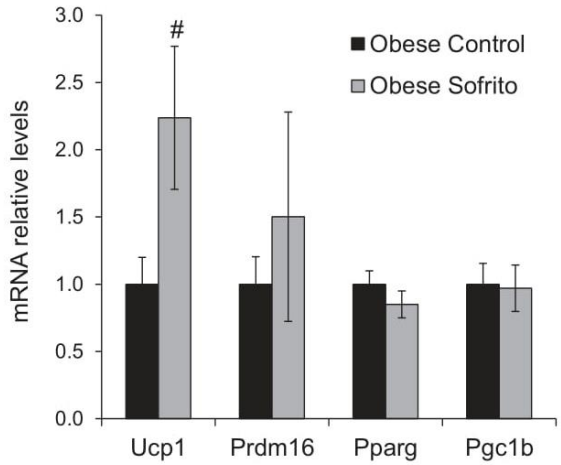

Figure 4. OZR fed with a *sofrito*-supplemented diet show higher UCP1 expression in vWAT. The mRNA relative levels of *Ucp1*, *Prmd16*, *Pparg*, and *Pgc1b* were measured by qRT-PCR in vWAT of obese control rats and obese *sofrito*-supplemented rats. The data are presented in absolute values as the mean ± SEM after 8 weeks of nutritional intervention. #$p < 0.05$ versus obese control rats ($n = 6$–8/group).

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olive oil and a cooking processes that modify some molecules bioavailability,[51] further studies are necessary to determine the molecular mechanisms that produce the metabolic response observed, the role of each component or the necessity of the complete combination in the effects of *sofrito* on FGF21 signaling.

4. Concluding Remarks

The Mediterranean diet is considered a healthy eating pattern but in order to convince society of its benefits, and to make its implementation as a lifestyle easier, it is important to have all the information and this includes the molecular mechanisms that can explain its beneficial effects. *Sofrito* is included in many of the typical Mediterranean dishes and every day brings further evidence of its healthy properties. This manuscript discloses one of the putative mechanisms of *sofrito*'s action specifically the one that could explain the improvement in glucose metabolism described by some bioactive dietary compounds and also reinforce the healthy properties of *sofrito*. **Translation to humans**: we designed our experimental approach using a dose of *sofrito* that may be close to the dose ingested in humans. The percentage of *sofrito* used represents the intake of one serving of *sofrito* per day in humans (https://ndb.nal.usda.gov/ndb/search/list). It is worth to mention that in the Predimed study the intake of two or more servings of *sofrito* per week is considered an indicator of a good adherence to Mediterranean Diet.[52]

Abbreviations

AUC, area under the curve; BAT, brown adipose tissue; CD, chow diet; DIO, diet-induced obesity; Dio2, type 2 iodothyronine deiodinase protein 2; EE, energy expenditure; FGF21, fibroblast growth factor 21; FGFR, fibroblast growth factor receptor; HFD, high-fat diet; ITT, insulin tolerance test; KLB, β-klotho receptor; LZR, lean Zucker rats; OZR, obese Zucker rats; PGC1b, peroxisome proliferator-activated receptor gamma coactivator 1-beta; PPAR, peroxisome proliferator-activated receptor; PRDM16, PR domain containing 16; UCP1, uncoupling protein 1; WAT, white adipose tissue

Supporting Information

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

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V.S. performed the metabolic characterization of the rats, compiled the statistics, and analyzed the results. R.R. designed and carried out the nutritional intervention and the ITT. U.M. performed the assays suggested by the reviewers. C.R. participated in the experimental approaches. R.M. designed the nutritional intervention and discussed the results. P.F.M., D.H., and J.R. designed the experimental approach to characterize the molecular effects of *sofrito*, supervised the study, discussed the results, and wrote the paper. All authors read, approved, and contributed to the final version of the manuscript.

Conflict of Interest

The authors have declared no conflicts of interest.

Keywords

adipose tissue, fibroblast growth factor 21, insulin resistance, Mediterranean diet, uncoupling protein 1

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Artículo científico II

Lyophilized Maqui (Aristotelia chilensis) Berry Induces Browning in the Subcutaneous White Adipose Tissue and Ameliorates the Insulin Resistance in High Fat Diet-Induced Obese Mice

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La baya de Maqui (*Aristotelia Chilensis*) presenta un perfil único de antocianinas que incluye altas cantidades de delfinidina-3-O-sambubiósido-5-O-glucósido y delfinidina-3-O-sambubiósido y ha mostrado efectos positivos en los niveles de glucosa e insulina en ayunas en humanos y modelos murinos de diabetes tipo 2 y obesidad. En este trabajo se investigaron los mecanismos moleculares que subyacen al impacto del maqui en la aparición y el desarrollo del fenotipo obeso y la resistencia a la insulina en ratones con obesidad inducida por una dieta

alta en grasas (HFD) y suplementados con maqui liofilizado. Los animales suplementados con maqui mostraron una mejor respuesta a la glucosa y un menor aumento de peso, pero también una expresión diferencial de genes involucrados en la DNL, la FAO, la formación de gotas de lípidos multiloculares y la termogénesis en el WAT subcutáneo (scWAT). Estos cambios se correlacionaron con una mayor expresión de Chrebp ChREBPβ, Srebp1c y el *cellular repressor of adenovirus early region 1A–stimulated genes 1* (Creg1) y una mejora en la señalización de FGF21. Los datos presentados sugieren que la suplementación dietética con maqui activa el pardeamiento del scWAT y la aparición de un fenotipo de adipocitos marrones en el scWAT que contrarrestarían parte del impacto metabólico insalubre de una HFD. Esta inducción constituye una estrategia putativa para prevenir/tratar la obesidad inducida por la dieta y sus comorbilidades asociadas.

antioxidants

MDPI

Article

Lyophilized Maqui (*Aristotelia chilensis*) Berry Induces Browning in the Subcutaneous White Adipose Tissue and Ameliorates the Insulin Resistance in High Fat Diet-Induced Obese Mice

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Abstract: Maqui (*Aristotelia Chilensis*) berry features a unique profile of anthocyanidins that includes high amounts of delphinidin-3-O-sambubioside-5-O-glucoside and delphinidin-3-O-sambubioside and has shown positive effects on fasting glucose and insulin levels in humans and murine models of type 2 diabetes and obesity. The molecular mechanisms underlying the impact of maqui on the onset and development of the obese phenotype and insulin resistance was investigated in high fat diet-induced obese mice supplemented with a lyophilized maqui berry. Maqui-dietary supplemented animals showed better insulin response and decreased weight gain but also a differential expression of genes involved in de novo lipogenesis, fatty acid oxidation, multilocular lipid droplet formation and thermogenesis in subcutaneous white adipose tissue (scWAT). These changes correlated with an increased expression of the carbohydrate response element binding protein b (*Chrebpb*), the sterol regulatory binding protein 1c (*Srebp1c*) and Cellular repressor of adenovirus early region 1A–stimulated genes 1 (*Creg1*) and an improvement in the fibroblast growth factor 21 (FGF21) signaling. Our evidence suggests that maqui dietary supplementation activates the induction of fuel storage and thermogenesis characteristic of a brown-like phenotype in scWAT and counteracts the unhealthy metabolic impact of an HFD. This induction constitutes a putative strategy to prevent/treat diet-induced obesity and its associated comorbidities.

Keywords: anthocyanins; browning; carbohydrate-responsive element binding protein b; delphinidin; fibroblast growth factor 21; high-fat diet; maqui berry; white adipose tissue

1. Introduction

Stimulation of the brown adipose tissue (BAT) and the induction of browning in white adipose tissue (WAT) as a strategy against obesity and its associated metabolic complications has generated growing interest in recent years. This interest is based on the ability of both BAT and browned-WAT to increase energy expenditure (EE) mainly through fatty acid consumption [1,2], and their pivotal role in the control of energy homeostasis in mammals [3,4]. In addition to classical brown adipocytes located in BAT, thermogenic adipocytes with similar characteristics can be found within white adipose tissue (WAT) [3]. These brite/beige adipocytes are metabolically and phenotypically similar to brown adipocytes and can actively contribute to increasing whole-body EE. Specifically, brite/beige adipocytes show a multilocular phenotype and express genes closely related to BAT metabolism (Ucp1 as a marker of its thermogenic capacity in addition to genes implied in de novo lipogenesis (DNL), fatty acid oxidation (FAO), lipolysis, etc.).

Recent evidence shows that the activation of BAT and the induction of browning in WAT can be induced by cold acclimation but also by nutritional inputs under different signaling cascades [2,5–7]. Cold is a classic activator of BAT and of beige adipocyte development and function [5,8]. Regarding nutritional inputs, we recently demonstrated that low-protein diets and the cooked-tomato sauce called "sofrito" are able to induce Ucp1 expression in WAT, thus indicating a browning phenotype [6,7]. In the same way, other authors published that high-fat diets, bioactive compounds and prebiotics can also induce browning in WAT [9–14].

Part of the cold-induced metabolic profile in BAT is regulated by the stimulation of carbohydrate-responsive element binding protein b (ChREBPb) through the Ak strain transforming/protein kinase 2 (AKT2) activity [15]. Besides ChREBP, fibroblast growth factor 21 (FGF21) has shown beneficial effects on glucose/lipid homeostasis and body weight control among other mechanisms by increasing energy expenditure (EE) and inducing browning and UCP1 overexpression in adipose tissues [6,16–19], as well as by promoting the insulin-dependent glucose uptake, mitochondrial biogenesis, and adiponectin secretion in adipocytes [20,21]. In this case, it has been widely demonstrated that FGF21 activity and/or signaling respond to nutritional challenges [22].

Anthocyanidin-rich berries have been proposed for the treatment and prevention of several disorders, including obesity-related metabolic disorders [23–32] but little is known about the molecular mechanisms underlying their beneficial effects. Maqui (*Aristotelia chilensis*) is a native Chilean berry with a unique anthocyanins profile that includes delphinidin-3-O-sambubioside-5-O-glucoside and delphinidin-3-O-sambubioside as the main phenolic compounds [33]. Besides its antioxidant activity, different preparations of maqui have shown positive effects on fasting glucose and insulin levels in humans and murine model of type 2 diabetes and obesity [34–37] and delphinidin-3-sambubioside-5-glucoside has been described as the responsible for hypoglycemic activity in in vivo models [36].

With the global aim of deepening the knowledge of the molecular mechanisms responsible for the metabolic effects of maqui and in some way of the anthocyanidin-rich foods, we investigated the effect of a lyophilized maqui berry preparation on the onset and progression of the diet-induced obesity (DIO) in mice subjected to high-fat diet (HFD) for 16 weeks. We studied the impact of maqui in the metabolic profile of the obese phenotype. We focused on the adipose tissue metabolic phenotype because of its role on the progression of obesity and as a major target to counteract the onset and development of this pathology and its metabolic-associated diseases such as insulin resistance. We analyzed specifically the subcutaneous WAT (scWAT) because growing evidence suggests that this depot is protective to metabolic health while visceral is detrimental [38–43].

Globally, our results highlight the potential role of maqui in the treatment of diet-induced obesity and insulin resistance. Further studies will be needed to identify the effects of maqui on healthy population and the impact of its regular consumption as part of a healthy dietary pattern such as the Mediterranean diet.

2. Materials and Methods

2.1. Anthocyanins Determinations by UPLC-DAD

For sample preparation, 0.1 g of maqui was extracted with 5 mL of a mixture of water and ethanol 80:20 (*v/v*). The extraction was repeated 3 times to increase extractability of the anthocyanidins. The extract was evaporated under vacuum to remove the ethanol and reconstituted to a final volume of 10 mL with MilliQ water. This procedure was done in triplicate. Finally, the sample was filtered through a 0.22 µm PTFE membrane filter into an amber vial for UPLC analysis.

The quantification and identification of anthocyanins was done using a Waters Acquity Ultra Performance Liquid Chromatography H class (Waters Corp, Milford MA, USA) coupled to Photodiode Array (PDA) detector (Waters Corp, Milford MA, USA). The chromatographic separation was performed by an Aquity BEH C18 column 2.1 mm × 100 mm, 1.7 µm. The chromatographic method proposed by Andrés-Lacueva et al. (2005) adapted to the UPLC system was used [44]. Briefly, the injection volume was 10 µL and the gradient elution was performed with water/5% formic acid (*v/v*) (A) and 100% acetonitrile (B) at a constant flow rate of 0.75 mL/min. A decreasing linear gradient of solvent A was used. Separation was carried out in 11 min under the following conditions: 0 min, 98% A; 6 min, 95% A; 9 min, 90% A; 9.1 min, 20% A; 9.3min, 20% A; 9.4 min, 92% A, 11 min, 92% A.

The column was equilibrated for 6 min prior to each analysis. Each maqui anthocyanin was quantified at λ = 520 nm using a calibration curves with pure standard purchased in Extrasynthese S. A. (Delphinidin-3-O-sambubioside-5-O-glucoside, delphinidin-3-O-sambubioside, cyanidin-3-O-sambubioside-5-O-glucoside, cyanidin-3-O-glucoside and cyanidin-3-O-sambubioside), and the identification was made by comparing the retention times of the chromatographic peaks with the retention time of the pure phenolic standards. Results were expressed as millimoles of anthocyanins per kilogram of maqui (mmol/kg).

2.2. Animal Procedures—Dosage Regimen

Animal procedures were approved by the Animal Ethics Committee of the University of Barcelona (CEEA-173/18). C57BL/6J littermates' male mice (n = 23) were housed in a temperature-controlled room (22 ± 1 °C) on a 12-h/12-h light/dark cycle and were provided free access to commercial rodent chow and tap water prior to the experiments. When animals were four-week-old it was confirmed that all animals were normoglycemic before being randomly assigned into two experimental groups (HFD and HFD supplemented with maqui (HFDM)). Both groups were fed a diet of 45% fat-derived calories (HFD) (D12451, Research Diets) for 16 weeks supplemented or not with lyophilized maqui. HFD group (n = 9) had free access to HFD diet and filtered-tap water and HFDM (n = 14) group had free access to HFD diet and filtered-tap water supplemented with maqui (20 mg of lyophilized maqui/mL of filtered-tap water). To prepare the supplemented water, 1 g of the lyophilized maqui was added to 50 mL of tap-filtered water. This mixture was prepared extemporaneously every two days to prevent the oxidation of maqui bioactive compounds.

The dosage regimen of maqui was calculated according to the polyphenol intake recommended as beneficial by the Predimed Study (820 mg in a human diet of 2300 kcal) [45,46]. Mice intake is around 10–15 kcal per day, which means 4–5 mg of polyphenols per day scaling-down the recommended beneficial quantity in humans. Table 1 shown that 1g of lyophilized maqui provides 45 mg of anthocyanins. The dose of maqui was adjusted to achieve 4 mg of polyphenols per day, considering that mice take 2–3 mL of water/day. The nutritional composition of the lyophilized maqui used (Maquiberry, Native for Life, Chile) is indicated in the supplemental information (Table S1).

During the 16-week nutritional intervention, food and beverage intake were recorded every two days and body weight twice a week. At the end of the nutritional intervention, the animals were euthanized. Blood was extracted by intracardiac puncture, and serum was obtained by centrifugation (1500 rpm, 20 min). Epididymal WAT (eWAT), scWAT and BAT were isolated, immediately snap-frozen and stored at −80 °C for future analysis.

2.3. RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from frozen tissues using TRI Reagent™ Solution (AM9738, Thermo Fisher Scientific, Waltham, USA), followed by DNaseI treatment (K2981, Thermo Fisher Scientific, Waltham, USA). cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific, Waltham, USA). Relative mRNA levels were measured by quantitative PCR (qPCR) using SYBR™ Select Master Mix for CFX (4472942, Thermo Fisher Scientific, Waltham, USA). 18S and B2M were used as housekeeping genes. The sequences of the primers used in qPCR are presented in Table S2. Results were obtained by the relative standard curve method, and values were referred to the HFD group.

2.4. Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

Mice were fasted for 6 h in the morning, and then injected intraperitoneally (i.p.) with 1.5 g of glucose (Sigma)/kg mouse (GTT) or 0.5 UI of insulin solution (Sigma)/kg mouse (ITT). Blood samples were collected from the tail vein, and glucose levels were measured using a glucometer (Glucocard SM, Menarini, Florence, Italy) prior to the i.p. injection and at 30, 60 and 120 min postinjection. GTT was performed 14 weeks after the beginning of maqui supplementation and ITT on week 15th.

2.5. Histological Analysis

For the histological analysis, pieces of scWAT of each animal were fixed in 10% formalin (Sigma) and embedded in paraffin. Afterwards, 4 µm-thick sections were cut and stained with hematoxylin and eosin (H&E). Images were acquired using a Digital Upright Microscope BA310 Digital and a Moticam 2500 camera. The selection of the test objects was performed according to color and choosing the same limits for binarization for all images. At least three pictures from different regions of each cut were taken.

2.6. Data Analysis/Statistics

GraphPad Prism version 8.02 (GraphPad, San Diego, CA, USA) was used to perform the statistical analyses. Two tailed Student's Test with Welch's correction when not equal SDs can be assumed was used to determine significant differences among experimental groups. The statistical analysis of body weight progression, body weight/calorie intake and curves of GTT and ITT was performed by 2 factor ANOVA with pot-hoc test (Bonferroni's). In all cases, p-value < 0.05 was considered statistically significant. All data are expressed as the mean ± SEM.

3. Results

3.1. Maqui Anthocyanin Content

The levels of anthocyanins determined in the lyophilized maqui used in our experimental approach (Maquiberry, Native for Life, Chile) (Table 1) were similar to those reported previously [47–50]. In terms of the total content of anthocyanins, the lyophilized maqui used in this work has an extremely high content (45,052 mg/kg = 4.5%) compared to other similar products like a Freeze-Dried Whole Blueberry (2432 mg/kg = 2.4%) [51] or a dried raspberry solids (750 mg/kg = 0.75%) [52]. The predominant (80%) anthocyanins were delphinidin-3-O-sambubioside-5-O-glucose and delphinidin-3-O-sambubioside, as shown in Table 1 and Figure S1 (Supplemental Materials).

Table 1. Anthocyanidin composition of the lyophilized maqui berry. Anthocyanins were determined by UPLC-DAD. The table shows the concentration in mg/g and mmol/kg of the different anthocyanidins detected. The table includes the retention time (min), the limit of detection (LOD) and the limit of quantification (LOQ) for each molecule.

Compound	Conc. (mg/g)	Conc. (mmol/kg)	Retention Time (min)	LOD (mg/L)	LOQ (mg/L)
Delphinidin-3-O-sambubioside-5-O-glucoside	19.645 ± 0.788	24.71 ± 0.99	3.5	1.81	6.02
Delphinidin-3-O-sambubioside	17.770 ± 1.178	28.07 ± 1.86	5.	0.30	1.00
Cyanidin-3-O-sambubioside-5-O-glucoside	2.447 ± 0.063	3.14 ± 0.08	5.5	0.75	2.50
Not identified (quantified as cyd-3-0-glu)	0.402 ± 0.050	0.83 ± 0.10	6.5	-	-
Cyanidin-3-O-glucoside	2.148 ± 0.158	4.43 ± 0.33	7	0.11	0.35
Cyanidin-3-O-sambubioside	2.642 ± 0.201	4.28 ± 0.33	7.3	0.17	0.56
TOTAL	45.052	65.46			

3.2. Maqui Dietary Supplementation Reduces HFD-Induced Body Weight Gain and Improves Insulin Sensitivity in Mice

C57BL6/J mice fed an HFD for 16 weeks put on weight (Figure 1a,b) and displayed glucose intolerance (Figure 1e,f) and insulin resistance (Figure 1g,h). Comparing both experimental groups revealed that mice fed HFD supplemented with maqui (HFDM) showed an attenuated progression in body weight (Figure 1a–c), even the animals were hyperphagic, and their daily caloric intake was higher (Figure 1d). Concretely, the HFDM animals put on less weight after the 16 weeks of nutritional intervention (Figure 1b). Even though no statistical differences were observed in the body weight progression (Figure 1a), there were when the ratio between body weight and caloric intake was calculated (Figure 1c). These data indicated that, in some way, there is an increased energy expenditure in these animals.

Regarding the insulin/glucose responsiveness, HFDM mice shown a significant reduction in fasting glucose at Week 15 of the nutritional intervention (207.6 ± 5.5 vs. 166.5 ± 6.1, $p = 2.7 \times 10^{-5}$). It is worth highlighting that, after the glucose injection, the HFDM animals displayed a better glucose curve (Figure 1e) that corresponded to a significant reduction of the area under the curve (AUC) of the GTT (Figure 1f, $p < 0.05$). In the case of the ITT, the curve of glucose after the insulin injection showed significantly lower levels of glucose at the first time-points (Figure 1g). These differences were minimized but maintained over time, even though they did not reach statistical significance (Figure 1g). Finally, the AUC of the ITT showed a clear tendency to a reduction in HFDM (Figure 1h, $p < 0.08$). Although some of the data are not significant individually, altogether they indicate an improvement on glucose tolerance and insulin sensitivity after 16 weeks of maqui dietary supplementation.

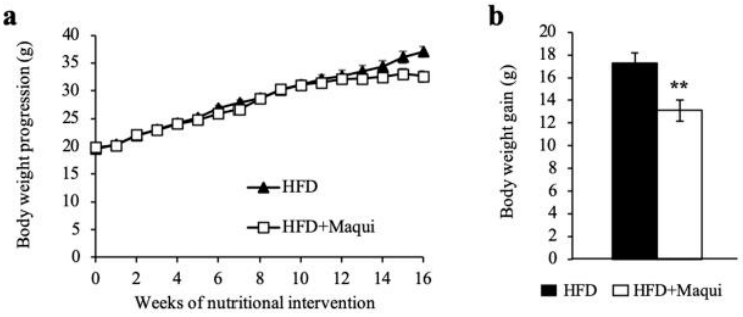

Figure 1. *Cont.*

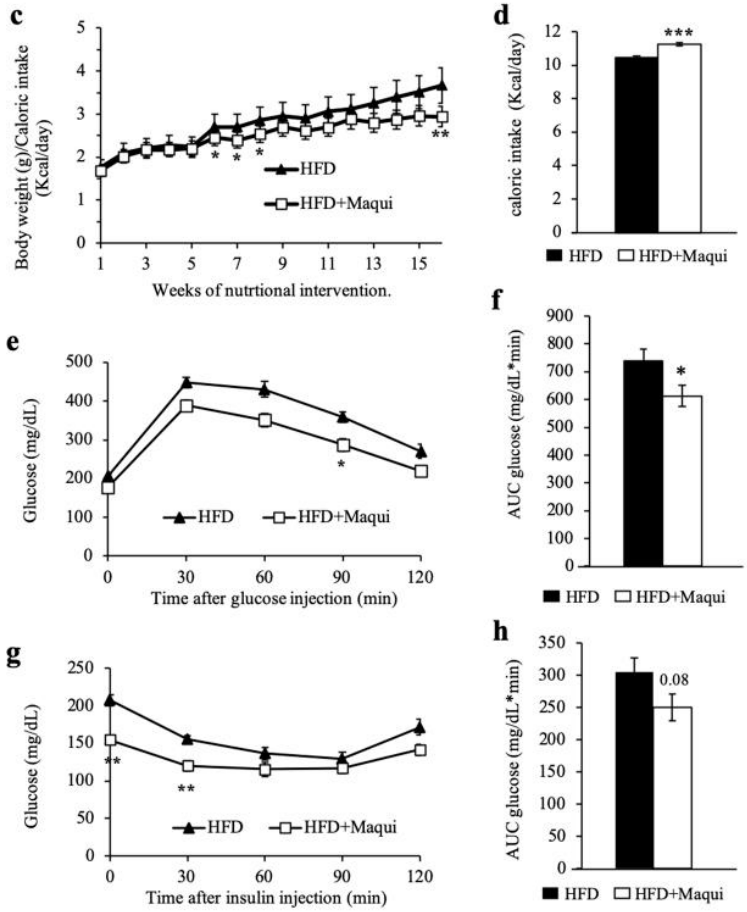

Figure 1. Maqui dietary supplementation reduces HFD-induced body weight gain and improves insulin sensitivity in mice. (**a**) Body weight progression (g) for the 16-week nutritional intervention with maqui. Body weight was recorded twice a week. The graph represents the mean ± SEM of weekly increments in both experimental groups. (**b**) Total body weight increment in grams after 16-week nutritional intervention with maqui. The graph represents the mean ± SEM of the total body weight increment in both experimental groups. (**c**) Body weight (g) related to calorie intake (kcal) per week for the 16-week nutritional intervention with maqui. Calorie intake was calculated based on the energy density of the HFD and the amount of food consumed daily. In HFDM mice, the kcal from maqui were also added. The graph represents the mean ± SEM of the weekly body weight increase related to calorie intake in both experimental groups. (**d**) Calorie intake for both experimental groups during the 16-week nutritional intervention. The graph represents the mean ± SEM. (**e**) GTT curve showing plasma glucose levels after i.p. administration of glucose (1.5 g/kg b.w.) in HFD and HFDM mice after 14 weeks of maqui supplementation. (**f**) AUC of glucose levels in GTT. (**g**) ITT curve showing plasma glucose levels after i.p. administration of insulin (0.5 UI/kg b.w.) in HFD and HFDM mice after 15 weeks of maqui administration. (**h**) AUC of glucose levels in ITT. Data from GTT and ITT are presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the HFD group.

3.3. Maqui Dietary Supplementation Induces a Multilocular Phenotype in scWAT

HFDM mice were leaner than their littermates (Figures 1a and 2a) and had more BAT and less WAT (Figure 2b,c). Because of the healthier profile observed in HFDM mice regarding body weight and insulin resistance, we hypothesized that maqui could be exerting its effects through the induction of browning in scWAT. The H&E staining of scWAT revealed that maqui supplementation induced the transition of unilocular adipocytes to multilocular ones (Figure 2d). No differences in other WAT depots due to maqui supplementation were observed in any analysis performed.

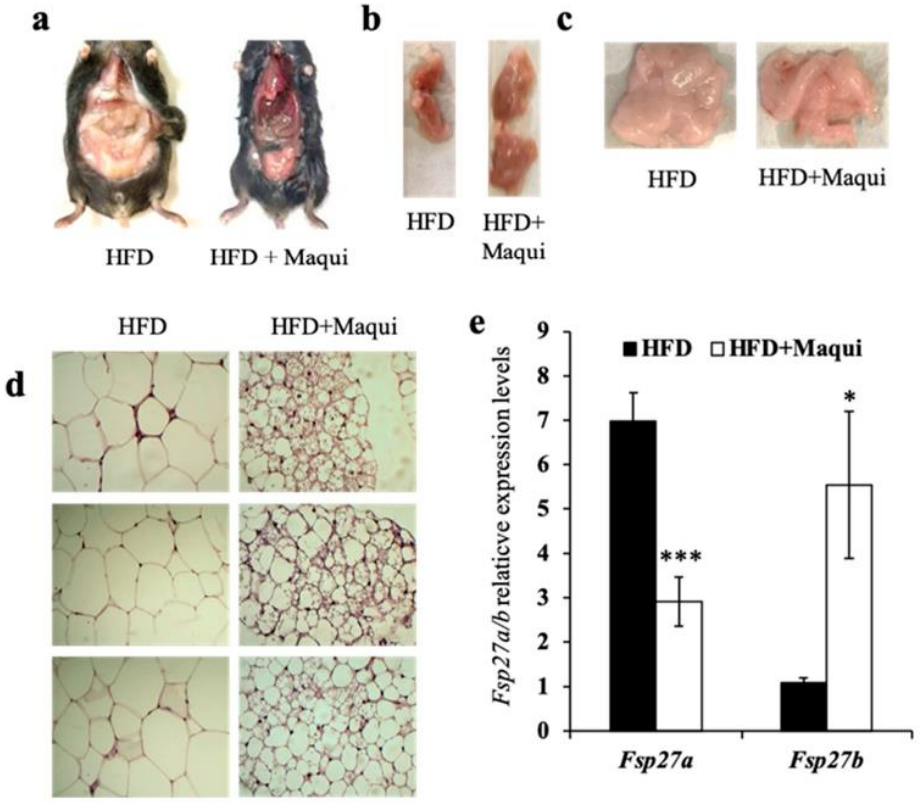

Figure 2. Maqui dietary supplementation induces a multilocular phenotype in scWAT. (**a**) Representative pictures of HFD (n = 9) and HFDM (n = 14) mice after 16 weeks of nutritional intervention. HFDM mice are leaner and show fewer white fat depots. (**b**) Representative pictures of interscapulum BAT of HFD and HFDM mice. For HFDM mice, the BAT depot is larger than the one from HFD mice. (**c**) Representative pictures showing scWAT depots of HFD and HFDM mice. (**d**) Representative hematoxylin and eosin (H&E)-stained scWAT sections from HFD and HFDM (40× magnification). Some multilocular adipocytes are revealed in scWAT of HFDM, but none were seen in the HFD animals. (**e**) *Fsp27a* and *Fsp27b* mRNA levels were measured by qRT-PCR in scWAT of HFD and HFDM mice. Bars represent the relative mRNA levels of both genes in the two experimental conditions in scWAT normalized by the *B2M* gene as housekeeping gene. Data are presented as the mean ± SEM. * $p < 0.05$, *** $p < 0.001$ versus the HFD group.

In the context of browning, recent publications have shown the relevance of fat-specific protein 27 (FSP27) isoforms in the phenotype of unilocular or multilocular lipid droplets. FSP27 is considered to be, at least in part, the protein responsible for the formation and growth of lipid droplets (LDs). Two isoforms have been described with different expression patterns and functions. Fsp27a is expressed in WAT, where it promotes the formation of large LDs. By contrast, BAT expresses the Fsp27b isoform that contributes to the ensemble of smaller LDs [53,54]. To confirm the brown-like phenotype of scWAT in HFDM mice, the mRNA levels of both *Fsp27* isoforms were measured. The results indicated that the dietary maqui supplementation causes a shift in the expression pattern of Fsp27. The scWAT depot of HFDM mice showed a significant reduction in the levels of *Fsp27a* and a significant induction of *Fsp27b* expression (Figure 2e), thus reinforcing the idea that maqui causes a brown-like phenotype in the scWAT.

3.4. Maqui Induces the Expression of Genes from de Novo Lipogenesis, Fatty Acid Oxidation, Thermogenesis and Browning in scWAT

As shown in Figure 3, in the scWAT of HFDM mice, the mRNA levels of genes related to mitochondrial (carnitine palmitoyl transferase 1b (Cpt1b)) and peroxisomal (Acyl-CoA oxidase 3 (Acox3), Enoyl-Coenzyme A, and Hydratase/3-Hydroxyacyl Coenzyme A Dehydrogenase (Ehhadh)) FAO (Figure 3a), DNL (Acetyl-CoA Carboxylase Alpha (Acaca), ATP Citrate Lyase (Acly), Fatty acid synthase (Fasn), Diacylglycerol Acyltransferase I (Dgat1), Sterol regulatory binding protein 1c (Srebp1c) and Glycerol Kinase (GlyK)) (Figure 3b) and thermogenesis/browning (Ucp1, Type 2 Iodothyronine Deiodinase (Dio2), PR-Domain Zinc Finger Protein 16 (Prdm16), Peroxisome proliferator-activated receptor gamma (Pparg), Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Pgc1a) and Cellular repressor of adenovirus early region 1A–stimulated genes 1 (Creg1)) (Figure 3c) increased, suggesting that the scWAT of the HFDM mice were metabolically closer to BAT than WAT. We also analyzed epididymal WAT (eWAT) and BAT. While eWAT did not show any feature of browning (data not shown), BAT showed an increment of size (Figure 2b) but no induction of Ucp1 was observed (data not shown).

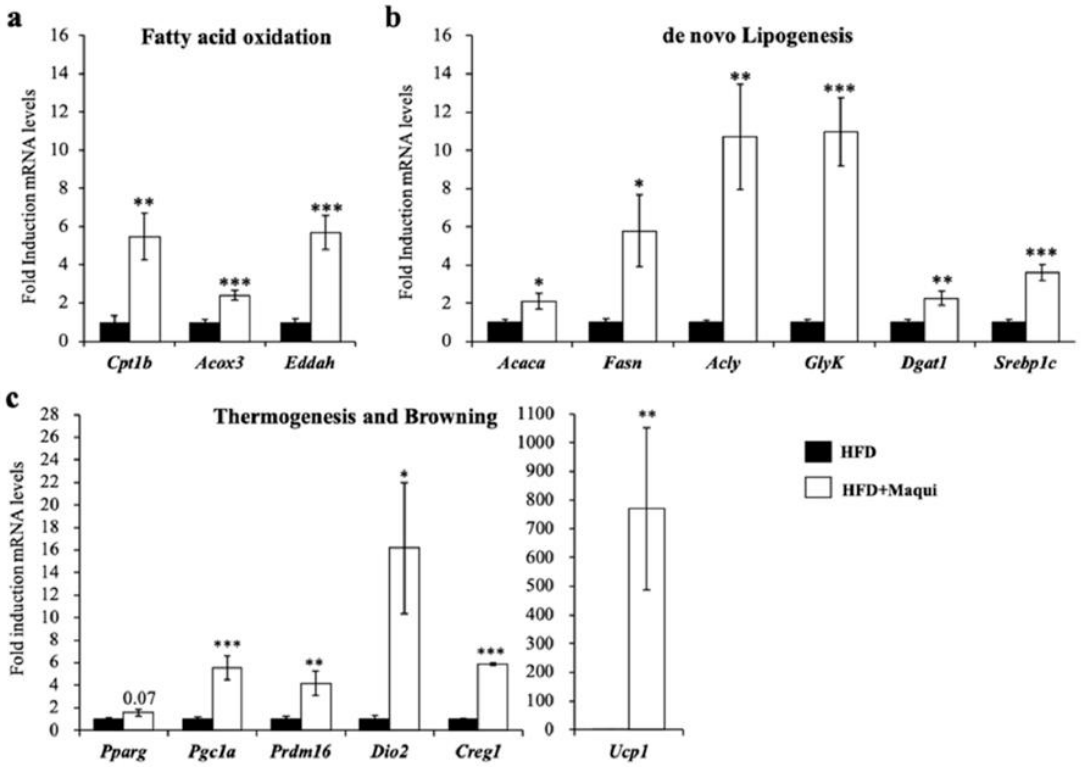

Figure 3. Maqui induces the expression of genes from de novo lipogenesis, fatty acid oxidation, thermogenesis/browning in scWAT. The relative mRNA levels of characteristic genes of (**a**) mitochondrial and peroxisomal FAO (*Cpt1b, Acox3, and Ehhadh*), (**b**) DNL (*Acaca, Acly, Fasn, GlyK, Dgat1 and Srebp1c*) and (**c**) thermogenesis and browning (*Ucp1, Type 2 Iodothyronine Deiodinase (Dio2), PR-Domain Zinc Finger Protein 16 (Prdm16), Peroxisome proliferator-activated receptor gamma (Pparg), Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Pgc1a) and Cellular repressor of adenovirus early region 1A–stimulated genes 1 (CREG1)*) were measured using qRT-PCR in scWAT of HFD and HFDM mice. Bars represent the fold induction in the mRNA levels versus the HFD animals that are considered the control group, which produces an arbitrary value of 1. Data are presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the HFD group.

3.5. Maqui Induces the Expression of Chrebpa, Chrebpb and Glut4 in scWAT

As mentioned above, part of the cold-induced metabolic profile in BAT is regulated by the stimulation of ChREBPb through the AKT2 activity [15]. In WAT, the expression of Chrebp in adipose tissue is regulated by GLUT4-mediated glucose uptake that activates the ChREBPa isoform, which in turn induces the expression of Chrebpb, an alternative-promoter transcribed isoform. Chrebpb expression in human adipose tissue predicts insulin sensitivity, and its induction has been highlighted as an effective strategy for preventing and treating obesity-related metabolic dysfunction and type 2 diabetes [55–57]. Globally, ChREBP is considered the major regulator of DNL in adipose tissue [58]. To analyze the putative role of ChREBPb and GLUT4 in the induction of the metabolic changes observed in scWAT due to maqui supplementation, the mRNA levels of Glut4, Chrebpa and Chrebpb were evaluated. The data show that maqui increases the expression of these three genes (Figure 4), thus providing insight into the molecular mechanism through which maqui induces a brown-like phenotype in scWAT.

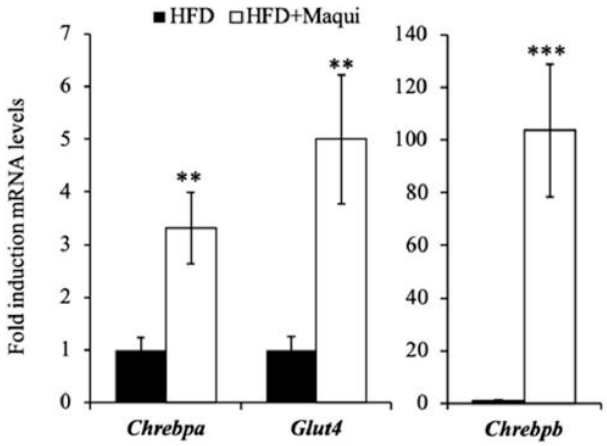

Figure 4. Maqui induces the expression of *Chrebpa*, *Chrebpb* and *Glut4* in scWAT. The relative mRNA levels of *Glut4*, *Chrebpa* and *Chrebpb* were measured by qRT-PCR in scWAT of HFD and HFDM mice. Bars represent the fold induction in the mRNA levels versus the HFD animals that are considered the control group and assigned an arbitrary value of 1. Data are presented as the mean ± SEM. ** $p < 0.01$, *** $p < 0.001$ versus the HFD group.

3.6. Maqui Increases the Expression of Adiponectin and FGF21 and the FGF21 Signaling in the scWAT

Obesity is considered an FGF21-resistant state usually due to a downregulation of the fibroblast growth factor receptor (FGFR) and/or its co-receptor β-klotho (KLB) that impairs FGF21 signaling [59,60]. To analyze the effect of maqui berry supplementation on the FGF21 signaling, the mRNA levels of Fgf21 itself, Fgfr1, Fgfr4 and KLB were determined. Moreover, to analyze the functionality of the FGF21 signal transduction pathway, the expression levels of Egr-1 and adiponectin were measured. Figure 5 shows that, in scWAT, both the FGF21 and its receptors (FGFR1 and FGFR4) were significantly overexpressed in HFDM mice versus the HFD mice (Figure 5). These data, together with the induction of Egr-1 and adiponectin in scWAT (Figure 5), indicate that maqui supplementation improves FGF21 signaling and effectiveness in scWAT.

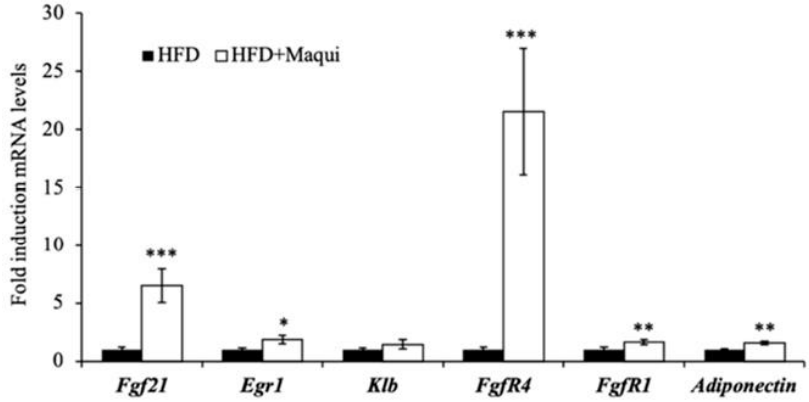

Figure 5. Maqui induces the expression of *Fgf21, Fgf21 receptors* and FGF21 signaling markers in scWAT. The relative mRNA levels of *Fgf21, Fgf21R1, FGFR4, KLB, Adiponectin* and *Egr1* were measured by qRT-PCR in scWAT of HFD and HFDM mice. Bars represent the fold induction in the mRNA levels versus the HFD animals that are considered the control group and assigned an arbitrary value of 1. Data are presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the HFD group.

4. Discussion

Globally, our data show that maqui dietary supplementation ameliorates the unhealthy effects of HFD. By using diet-induced obese mice, the present study demonstrated that supplemented animals displayed better insulin responsiveness, decreased weight gain and increased thermogenic activity. The analysis of gene expression in different tissues showed that scWAT exhibited differential expression of genes involved in browning, DNL, mitochondrial and peroxisomal FAO, multilocular adipocytes and thermogenesis. These changes were probably related with the increased expression of *Chrebpa*, *Chrebpb*, *Glut4*, *Creg1* and *Srebp1c* but also the improvement in FGF21signaling.

Obesity is essentially caused by an imbalance between energy intake and energy expenditure. Some evidence indicates that, at some point, the white adipose tissue (WAT) fails to adequately keep the surplus of nutrients, which together with an insufficient differentiation of new adipocytes leads to an off-WAT accumulation of ectopic lipids in peripheral relevant organs that may cause the metabolic obesity-related metabolic dysfunctions. It seems obvious that defects in WAT functionality together with peripheral lipotoxicity are key in the onset of metabolic syndrome [61–66].

The biomedical relevance of brown and beige adipocytes lies in the ability of these cells to increase EE and counteract metabolic diseases such as obesity or type 2 diabetes [1,2]. Indeed, increasing the activity of brown, beige or both fat depots is considered a promising strategy for the treatment of metabolic diseases [67,68].

Berries are an important source of anthocyanins [27,36,69,70]. Anthocyanins are widely distributed water-soluble polyphenols that have shown important health effects including metabolic effects on glucose metabolism [23–27,36,37,69,71–76]. In this context, maqui berry features a unique profile of anthocyanidins that includes high amounts of delphinidin-3-O-sambubioside-5-O-glucoside and delphinidin-3-O-sambubioside. These anthocyanins with a sambubioside residue are distinctive of maqui berry since they have been not reported in other edible berries such as blueberries [77], acai [78], blackcurrant, elderberry [79], cranberries [80] or other south Patagonian wild berries [81].

Our results clearly confirm the previously described effects of maqui regarding glucose metabolism but also describe the capacity of maqui to induce browning in HFD-induced obese mice. Dietary-supplemented obese mice showed less weight gain despite their hyperphagic behavior, thus indicating that in some way these animals have a higher metabolic rate, probably due to an increased BAT depot and a more energy consuming scWAT. The absence of effects on other WAT depots indicated that scWAT was the target for maqui beneficial effects against diet-induced obesity

and that at least part of the effects of berries on glucose metabolism and insulin sensitivity go through the improvement of adipose tissue functionality.

BAT thermogenesis is stimulated by adrenergic induction of cAMP production, which activates protein kinase A (PKA) to drive transcription of thermogenic genes (including *Ucp1*), lipolysis and FAO. Although less recognized, the activation of BAT thermogenesis paradoxically induces the anabolic DNL pathway [15,82–84]. Several studies support the important role of ChREBP isoforms in the control of insulin signaling and lipogenesis in adipose tissue and describe the induction of both isoforms' activity under the Glut4-dependent glucose uptake [55,85–88]. In WAT, HFD feeding lowers the expression of *Chrebp* and DNL genes in mice [55]. Moreover, the expression of a constitutively active ChREBP in WAT protects mice against obesity and insulin resistance by among others reducing adiposity and increasing the expression of gene related to adipocyte differentiation and browning [89]. In the same context, an adipose-specific *Chrebp* knockout mice show a decrease in DNL and are insulin resistant with an impaired insulin action in the liver, muscle and fat [85]. Beyond mice, in humans, the expression of *Chrebp* and lipogenic genes in WAT shows a strong correlation with insulin sensitivity, and the improvement of insulin sensitivity in insulin-resistant people restores *Chrebp* and glucose transporter type 4 (*Glut4*) expression in adipose tissue [56].

Our key finding is that this characteristic metabolic feature of BAT also appears in the scWAT of obese mice fed a maqui-supplemented HFD where there is an induction of *Chrebpa*, *Chrebpb* and *Glut4* expression and also higher mRNA levels of genes involved in DNL, multilocular LDs formation and thermogenesis/browning. The remaining question is how ChREBP is activated under maqui supplementation. The increased levels of Glut4 in HFDM mice indicated that glucose or glucose metabolites could be the major inducers of Chrebp expression [90,91]. Moreover, although ChREBP was initially identified as a glucose-responsive factor, recent evidence suggests that it is also essential for fructose-induced lipogenesis both in the small intestine and liver [55,92]. The effects of maqui could, therefore, be attributed to its fructose content. However, the absence of Chrebpb induction in liver (data not shown) allows us to rule out this possibility.

SREBP1c is also a key regulator of hepatic DNL [93–98]. It has been demonstrated that, in the liver, SREBP1c and ChREBP are both necessary for the expression of lipogenic and glycolytic genes [88,99] In adipose tissue, the role of SREPB1c is more controversial. In adipose tissue, the mRNA levels of lipogenic genes did not change in animals using a *Srebp1c* loss of function or gain of function approach, thus indicating that in this tissues *Srebp1c* is not essential for DNL activation [58]. By contrast, mice under caloric restriction (CR) showed an SREBP1c/PGC1a-dependant induction of DNL in adipose tissue giving to *Srebp1c* an role on activating this metabolic pathway under this specific nutritional condition [100]. Finally, CREG1 has been described as an inducer of Ucp1 and FGF21 expression in an adipocyte P2–Creg1-transgenic (Tg) mice and globally of BAT adipogenesis and browning [101,102]. Our results indicate that maqui supplementation is able to induce the expression of *Chrebpb*, *Srebp1c*, *Pgc1a* and *Creg1*, thus we cannot discard any of them as possible contributors to the induction of DNL and thermogenic genes observed.

In addition, in the scWAT of dietary-supplemented obese mice, there was an increase in the expression of peroxisomal FAO enzymes that would make possible the contradiction of having FAO and DNL simultaneously active. Peroxisomal FAO is important in this scenario where, despite an increased expression of *Cpt1b*, the rate-limiting enzyme of mitochondrial FAO, this pathway would be inhibited by the malonyl-CoA produced by DNL.

Apart from the abovementioned mechanisms our data also pointed out the improvement of FGF21 signaling as a way through which maqui could exert its beneficial effects. It has been widely described that FGF21 levels are increased in obesity and diabetes in both animal models and humans [60,103,104]. The downregulation of FGF21 receptors in adipose tissue seems to be the key point to explain the FGF21-resistant state described mainly in obese mice as well as, in some studies, in humans [43,59,60,105–108]. In this context, the restoring of FGF21 signaling can be considered as a potential therapeutic strategy to improve the metabolic parameters of obese individuals and to reduce

the risk of obesity-related diseases and some evidence support this hypothesis [7,109]. In various rodent models of diet-induced obesity a positive correlation between the beneficial effects of polyphenol-rich fruit extracts and FGF21 has been described. This correlation with FGF21 can be due to an induction of FGF21 levels [110,111] or an improvement of the FGF21 signaling [7,112,113]. Finally, FGF21-resitance in adipose tissue has been linked to a decreased production of adiponectin [114]. Adiponectin induces fatty acid oxidation leading to a reduction of ectopic lipids and finally the improvement of insulin sensitivity [115]. In our results, the induction of the mRNA levels of *adiponectin*, *FgfR1*, *FgfR4* and *Egr1* in scWAT of HFDM mice indicates that, in obesity, maqui supplementation increases the sensitivity to FGF21 of scWAT.

Although our results do not allow us to discard that other signaling pathways could stimulate the white to beige/brown transition described, the improvement of FGF21 signaling together with the overexpression of ChREBP, CREG1, PGC1a and SREBP1c are at least key players in the induction of browning described under maqui supplementation. Neuroendocrine signaling or molecules such as leptin or Bmp8b will be analyzed in further studies to try to complete the signaling cascade activated by maqui [2,67,116].

To summarize, we demonstrated that a nutritional intervention with maqui partially alleviates the unhealthy effects of HFD in mice. Our results provide evidence that, in mice, a dietary supplementation with maqui added to beverage activates the induction of fuel storage and thermogenesis characteristic of a brown-like phenotype in scWAT. Finally, based on previously published data, our results indicate that maqui could exert its effects, at least in part, through the induction of *Chrebpb* expression and the improvement of FGF21 signaling. Finally, it is worth mentioning that in this work the dose of maqui was scaled-down from the polyphenol intake recommended as beneficial in humans by the Predimed Study [45,46]. This is important because several phenolic compounds such as resveratrol, quercetin, cyanidin-3-glucoside (C_3G), capsaicin, hesperidin have green tea extract have been described as inducers of BAT activity or WAT browning but in most cases the dietary supplementation was performed using high doses and only the active compounds [9–13,117–121].

Limitations of the Present Work and Further Studies

This work clearly demonstrates that maqui is effective in obese mice. The impact of maqui in healthy individual needs to be evaluated. We do not have data about the effects of maqui on normal chow-fed animals where neither obesity nor insulin resistant is present, but this experimental approach will be included in our further studies. Positive results in this follow-up experiment will allow pointing out the efficacy of the consumption of maqui in the prevention of some metabolic diseases and whether to include its regular consumption as part of a heathy dietary pattern.

On the other hand, despite the observed changes in gene expression, together with the scWAT and BAT appearance and the histological analysis to define properly the browning phenotype, in this study, the translation to protein was assumed. To overcome this limitation, Western blot analyses will be performed to reinforce our results and deepen the mechanism of action of maqui.

5. Conclusions

In conclusion, our data provide evidence that, in obese mice, a dietary intervention with a regular dose of an anthocyanidin-enriched berry (maqui) can induce a browning phenotype in scWAT and improve partially the insulin sensitivity, thus ameliorating some of the unhealthy effects of HFD. These effects reinforce the anthocyanidin-enriched foods as a potential strategy to prevent or treat type 2 diabetes and obesity-related diseases and point out maqui as a putative functional fruit to counteract at least in part obesity and its metabolic complications. The data presented in this manuscript reinforce the inclusion of maqui in the diet of obese individuals.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/8/9/360/s1, Table S1: Maqui extract nutritional composition, Table S2: Sequences of the primers used in SYBR Green assays and references of the probes used in Taqman assays, Figure S1: Chromatogram of the anthocyanins identified in maqui samples. 1:Delphinidin-3-O-sambubioside-5-O-glucoside;2:Delphinidin-3-O-sambubioside; 3:Cyanidin-3-O-sambubioside-5-O-glucoside; 4: Unknown; 5: Cyanidin-3-O-glucoside; 6: Cyanidin-3-O-sambubioside.

Author Contributions: V.S., U.M.-G., H.S.-L. and J.R. performed all the animal procedures (nutritional intervention, ITT, GTT, body weight recording, and food and beverage intake measurements). V.S. and A.F. performed the gene expression analysis and compiled the statistics. V.S. and A.F. analyzed the H&E samples. J.M.C., P.Q.-R. and R.M.L.-R. performed the chromatographic analysis and discussed the anthocyanins results. P.F.M., D.H., J.R. and V.S. designed the experimental approach. R.M.L.-R., P.F.M., D.H. and J.R. supervised the study, analyzed and discussed the results and wrote the paper. All authors read, approved and contributed to the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Resultados no publicados

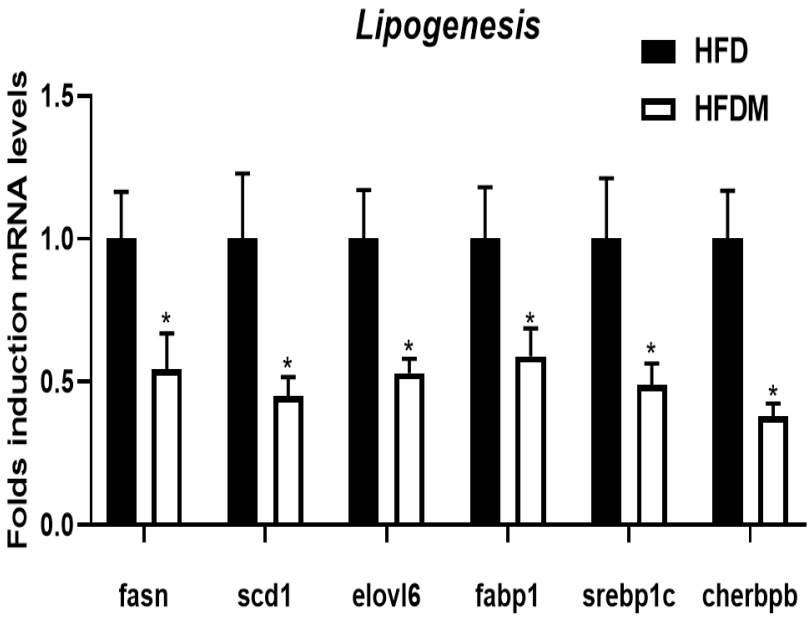
El liofilizado de la baya Maqui (Aristotelia chilensis) mejora la esteatosis hepática en ratones alimentados con una HFD

La suplementación con maqui disminuye la lipogénesis y el tamaño de las gotas lipídicas en hígado

Los ratones alimentados con una HFD y suplementados con maqui liofilizado presentan niveles relativos de mRNA menores de genes que codifican para enzimas de la DNL y de factores de transcripción implicados en la inducción transcripcional de estas enzimas como el ChREBP y el SREBP1c. (Figura A). Así, observamos una menor expresión de la Fasn, Elovl6, Scd1 y del transportador Fatty Acid-Binding Protein 1 (Fabp1). Además, en los ratones con maqui se midieron los niveles de mRNA de Chrebpβ, al ser la forma transcripcionalmente más activa. En global estos resultados muestran un perfil génico que estaría relacionado directamente con una mejora de la esteatosis hepática causada por una HFD.

Para corroborar esta mejora en la esteatosis hepática, se realizó un análisis histológico con tinción de Eosina-Hematoxilina. En los ratones HFD, tal como se espera, hay un gran acúmulo de lípidos en el hígado y se observan LDs grandes características de la NAFLD y que confirman una esteatosis hepática avanzada (Figura B, panel de la izquierda). Por el contrario, el hígado de los ratones obesos suplementados con maqui, presentan una menor cantidad de gotas lipídicas y estas son más pequeñas (Figura B, panel de la derecha)

(A)



(B)

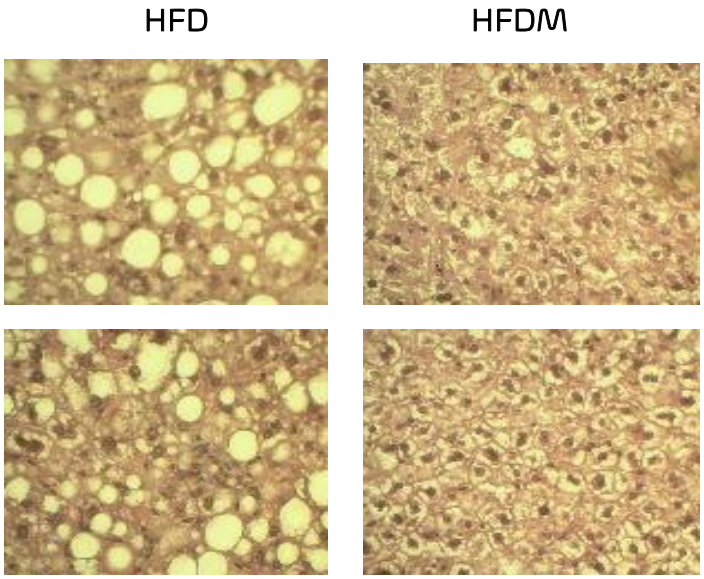


Figura.- A: Niveles relativos de mRNA en hígado de genes lipogénicos: Fatty acid synthase (Fasn), Sterol regulatory binding protein 1c (Srebp1c), Stearoyl-CoA desaturase-1 (scd1), fatty acid elongase 6 (elovl6), Fatty Acid Binding Protein 1 (Fabp1), Sterol regulatory element-binding transcription factor 1 (Srebp1c), Carbohydrate-responsive element-binding protein b (ChERBPb). **B:** Cortes histológicos en secciones de hígado con la tinción hematoxilina-eosina (40x). HFD (n=9) / HFDM (n=14). Se representa promedio de cada grupo durante las 16 semanas +/- SEM. * p<0,05

La suplementación con maqui disminuye la concentración de TAG en el hígado.

Para corroborar la mejora en la esteatosis hepática y la posible reducción del contenido hepático de lípidos se midió el contenido intracelular de TAG en los hepatocitos (Figura C), los hígados de los ratones alimentados con HFD y suplementados con maqui presentan niveles menores de TG totales respecto a los animales alimentados con la HFD a pesar de que la diferencia entre grupos no alcanza valores significativos (p=0,08).

(C)

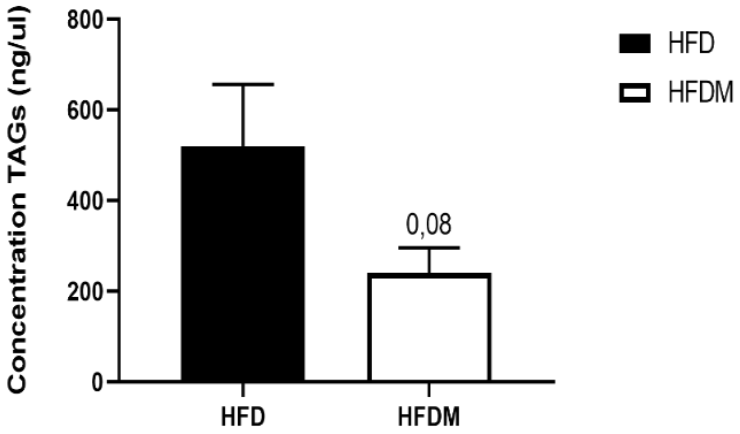


Figura C: Niveles de TAG en el hígado, expresados en ng/ul. HFD (n=9) / HFDM (n=14). Se representa promedio de cada grupo durante las 16 semanas +/- SEM. * p<0,05.

La suplementación con maqui disminuye la expresión del Peroxisome Proliferator-activated receptor alpha (PPARα) y de genes de la oxidación de ácidos grasos.

Con el objetivo de definir el perfil metabólico de los hígados de los ratones alimentados con una HFD y suplementados con maqui se analizaron los niveles de expresión génica de la Cpt1a (FAO), la *Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase (Ehhdah)* (beta-oxidacion peroxisosomal) y *PPARα* que es un receptor nuclear clave en la regulación del metabolismo lipídico a nivel hepático. Los niveles de mRNA relativos de estos tres genes se encuentran disminuidos en los animales con la dieta suplementada con maqui lo que indicaría una reducción de la capacidad oxidativa de ácidos grasos de cadena larga y muy larga por

parte de estos hígados en relación con los animales alimentados con una HFD (Figura D).

(D)

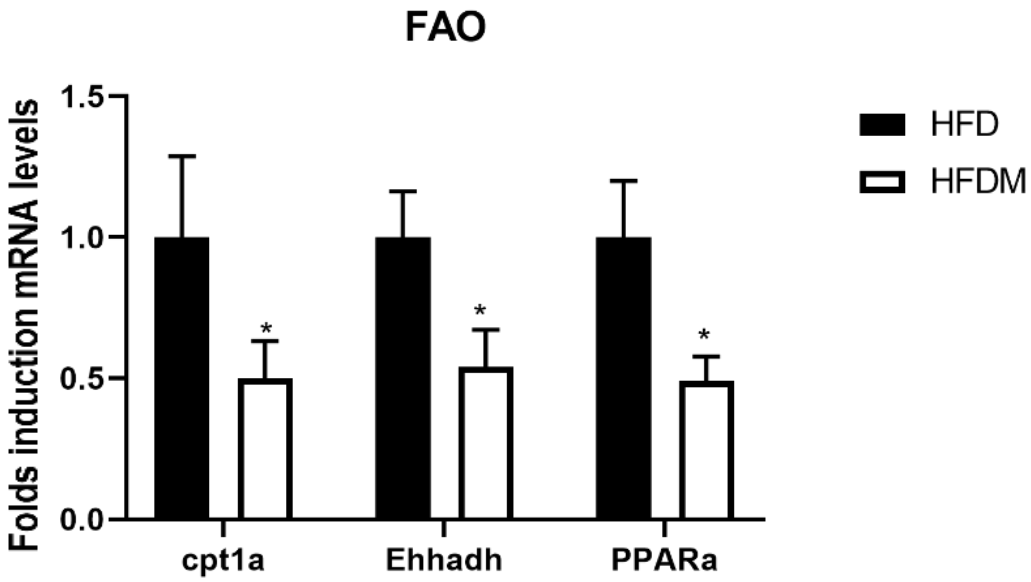


Figura D.-Niveles de mRNA en hígado de Carnitine Palmitoyltransferase 1a (cpt1a), Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase (Ehhdah), Peroxisome Proliferator Activated Receptor Alpha (PPARα). HFD (n=9) / HFDM (n=14). Se representa promedio de cada grupo durante las 16 semanas +/- SEM. * p<0,05.

La suplementación con maqui induce la expresión del co-represor SMILE y reduce la de PGC1α.

A parte de los efectos de maqui sobre la expresión de genes implicados en el metabolismo lipídico la obesidad tiene un fuerte impacto sobre el metabolismo de carbohidratos y una de las vías clave en el hígado es la gluconeogénesis. Es por ello que buscando elucidar el papel de la suplementación nutricional con maqui sobre la síntesis de glucosa a partir de sustratos no carbohidratos se midieron los niveles de mRNA de dos reguladores clave de esta via que son el *Small heterodimer partner–interacting leucine zipper protein (SMILE)*, un potente co-represor que disminuye la expresión del *Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1α) y anula su efecto estimulante sobre

la gluconeogénesis, el propio Pgc1α, la *glucose-6 phosphatase* (G6P) y la p*hosphoenolpyruvate carboxykinase* (PEPCK). Tal y como se muestra en la figura E, en los ratones suplementados con maqui hay un aumento en los niveles de mRNA de *SMILE* y una reducción en los de PGC1α y de G6P, por lo que la gluconeogénesis estaría disminuida respecto a los animales alimentados con HFD. En el caso de la PEPCK no se observan cambios en los niveles de mRNA, pero no podemos descartar efectos en los niveles de proteína.

(E)

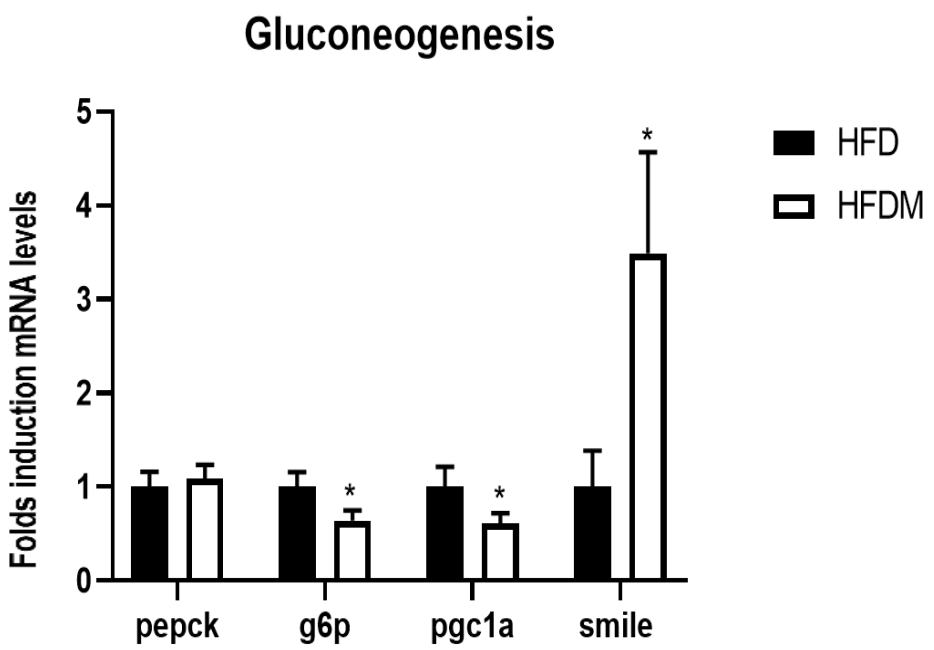


Figura E: Niveles de mRNA en hígado de *phosphoenolpyruvate carboxykinase (pepck), glucose-6-phosphate (g6p), Peroxisome proliferator-activated receptor γ co-activator 1 α (Pgc1a), small heterodimer partner–interacting leucine zipper protein (Smile).* HFD (n=9) / HFDM (n=14). Se representa promedio de cada grupo durante las 16 semanas +/- SEM. * p<0,05.

La suplementación con maqui induce la expresión de FGFR1.

Dado el papel que tiene *FGF21* y su señalización en el control del metabolismo lipídico y en el mantenimiento de la homeostasis energética se analizaron los niveles de mRNA de FGF21, sus receptores y algunos de sus genes corriente abajo en su vía de transducción de la señal. En el caso de la suplementación con maqui no se observan cambios en los niveles de expresión de *Fgf21,* ni en los niveles de genes diana de su señalización como el *early growth response protein 1 (Egr-1)* o el *cellular oncogene fos (c-fos)*. En el caso de los receptores si se observa una inducción en la expresión de *Fgfr1* (Figura F).

(F)

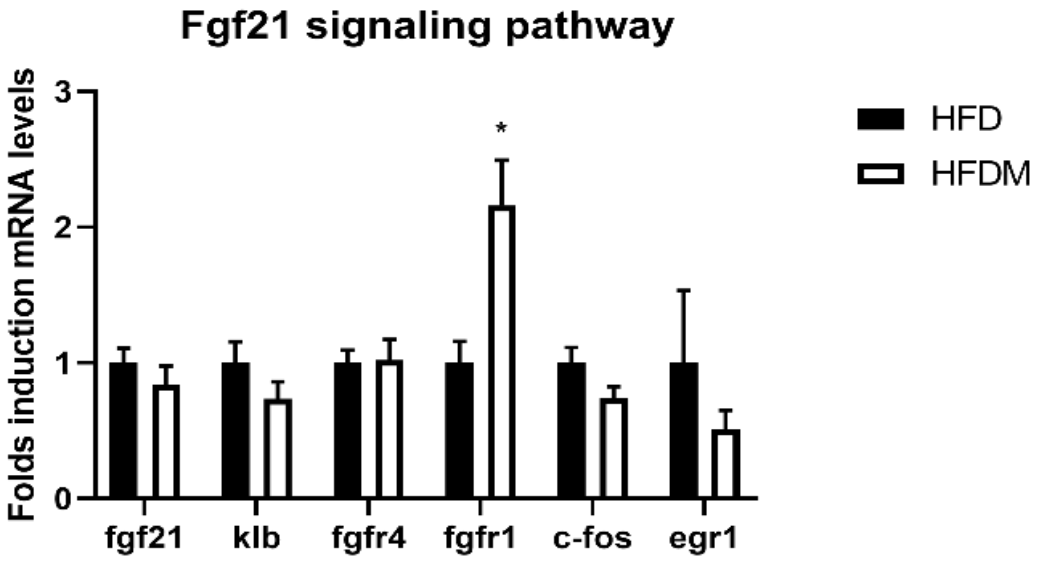


Figura F.- Niveles de mRNA en hígado de fibroblast growth factor 21 (fgf21), co-receptor β-klotho (klb), FGF21 receptor 4 (Fgfr4), FGF21 receptor 1 (Fgfr1), cellular oncogene Fos (c-fos), early growth response protein 1 (Egr1). HFD (n=9) / HFDM (n=14). Se representa promedio de cada grupo durante las 16 semanas +/- SEM. * p<0,05.

Resumen Resultados

RESUMEN RESULTADOS

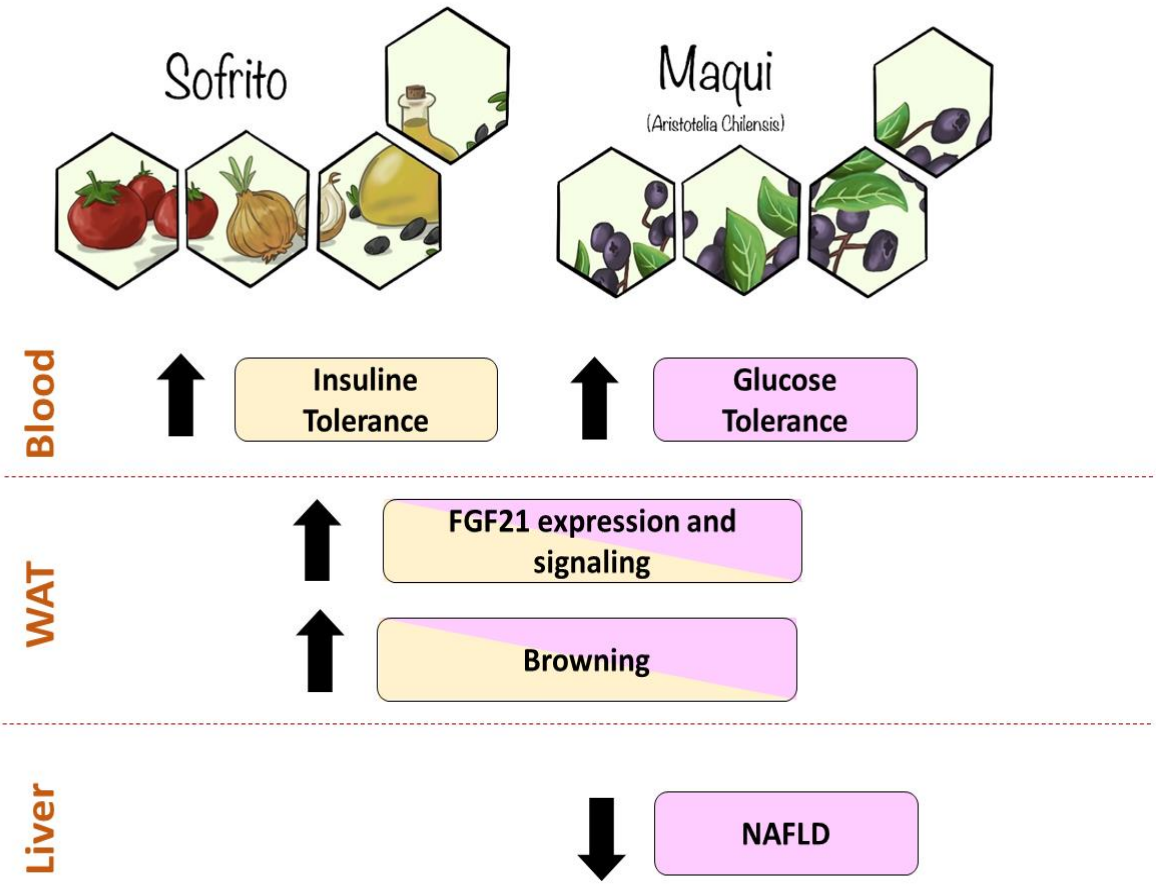


Figura 7: Resumen de los efectos fisiológicos en suero, WAT e hígado, tras la administración a animales obesos de sofrito o maqui, ambos alimentos ricos en compuestos bioactivos.

Discusión global

DISCUSIÓN GLOBAL

Debido a la pandemia que es la obesidad[59] se hace urgente encontrar estrategias terapéuticas que permitan prevenirla y/o tratarla. En este sentido, la evidencia creciente indica que el consumo de frutas y verduras está inversamente relacionado con muchos trastornos metabólicos, principalmente la obesidad y sus comorbilidades[69]. Los efectos beneficiosos para la salud de estos alimentos se deben, como mínimo en parte, a su alto contenido de compuestos bioactivos tales como los carotenoides y los polifenoles[46]. Para resolver los objetivos planteados en esta tesis se llevaron a cabo dos aproximaciones experimentales de intervención nutricional con modelos animales de obesidad y se trabajó con el sofrito y el maqui. Parte de la elección de estos alimentos se debe a que ambos son alimentos altos en compuestos bioactivos, carotenoides y polifenoles respectivamente, biodisponibles y que habían demostrado previamente efectos saludables en estudios de intervención nutricional y por tanto bioactividad y donde además la interacción de la matriz alimentaria parece ser fundamental para conseguir estos efectos [72][112-113].

En el caso del sofrito, se sabe que es un preparado con una matriz alimentaria compleja, debido a los ingredientes que lo componen y se ha descrito un efecto sinérgico del tomate, cebolla y aceite de oliva del sofrito en la absorción de los carotenoides[114]. En la investigación base del primer objetivo de esta, el estudio de Rosalía Rodríguez-Rodríguez 2017[91], se observó que los dos subgrupos de ratas alimentadas con sofrito (lean sofrito y obesas sofrito) mostraban mayor contenido de licopeno en las muestras del hígado. El licopeno fue utilizado como un biomarcador del consumo del sofrito, por lo que con este contenido significativamente mayor en ambos grupos intervenidos se puede afirmar que efectivamente el sofrito utilizado en ese estudio contiene

compuestos bioactivos biodisponibles. Los efectos en ese artículo se relacionaban con la protección sobre alteraciones vasculares[91]. Y en el artículo científico I se buscaron efectos del consumo del sofrito relacionados con la obesidad.

Por su parte en el maqui si bien su matriz alimentaria es también compleja ya que contiene fibra, ácidos grasos principalmente poliinsaturados [103],sin duda la particularidad de esta baya pasa por su alta capacidad antioxidante que se relaciona directamente con la gran cantidad de polifenoles que contiene[92][101], la cual es muy superior a otras bayas como arándanos[115], açai[116], grosella negra[117] y otras bayas silvestres del sur de la Patagonia[102]. Otra característica específica del maqui es que en él se combinan varios tipos de antocianinas siendo predominantes las del tipo delfininas (78%) y cianidinas (21%)[107]. El tipo y la mezcla de estas antocianinas del maqui podrían ser las responsables de las propiedades ya estudiadas del maqui tales como antioxidante, antihemorrágico, antipirético, hipoglucemiante e insulino-sensibilizador[96][108–110]. En el artículo científico II se llevó a cabo la caracterización de esta baya, y en él se detalla que el maqui presenta un perfil único de antocianinas que incluye altas cantidades de delfinidina-3-O-sambubiósido-5-O-glucósido y delfinidina-3-O-sambubiósido. La presencia del residuo sambubiósido que ya había sido descrita previamente en Overall 2017[107] es poco habitual en otras bayas, por lo que no podemos descartar que esta molécula sea la responsable de algunos de los efectos del maqui[118]. Si bien no se realizaron estudios de biodisponibilidad en esta investigación, sí se han hecho con anterioridad en otros estudios donde se describe una alta bioactividad de las antocianinas a pesar de la baja biodisponibilidad de estas[82][106].

Con estos antecedentes sobre efectos y biodisponibilidad del sofrito y el maqui se realizaron las correspondientes aproximaciones experimentales para estudiar el potencial de estos alimentos como

estrategias terapéuticas para combatir la obesidad y sobre todo los efectos colaterales de esta patología principalmente en WAT y en hígado.

Los resultados obtenidos con los modelos animales muestran que las dietas enriquecidas en compuestos bioactivos como el sofrito y el maqui podrían ser útiles como estrategia terapéutica contra la obesidad tanto en un modelo obeso genético; ratas Zucker, como en un modelo con obesidad inducida por la dieta, ratones de la cepa C57L6B alimentados con una dieta alta en grasa (45% Kcal). En ambos casos los datos presentados muestran que la suplementación de la dieta con alimentos ricos en compuestos bioactivos contrarresta parte de los efectos nocivos de la obesidad, observándose un perfil metabólico más sano en los animales con la suplementación alimentaria que en los animales control.

La IR es una de las primeras comorbilidades en aparecer con la obesidad. En el **artículo científico I,** vemos como las OZR suplementadas con sofrito muestran una mayor sensibilidad a esta, lo cual es relevante ya que la obesidad y un IR, son grandes factores de riesgo para una DM2, por lo que el sofrito estaría ejerciendo efectos protectores[28]. En el **artículo científico II** se describe un efecto similar con el maqui y su efecto hipoglicemiante, los ratones suplementados con maqui (HFDM) tienen una mejor tolerancia a la glucosa, si bien estos efectos ya se habían descrito previamente en humanos[95][119] y en un modelo de ratas diabéticas inducidas por inyección de estreptozotocina (STZ)[120]. El hecho de corroborar este efecto hipoglucemiante del maqui en un modelo más cercano a la DM2 que el de la inyección con STZ. Con una dieta que induce obesidad y diabetes, hace que los resultados puedan ser más fácilmente aplicables a la población humana, donde son los hábitos alimentarios actuales los que conducen a la alteración del metabolismo de la glucosa. Que los resultados se hayan reproducido en este experimento otorga validez a los efectos del maqui debido a su alta efectividad en distintos modelos. La respuesta a la glucosa es significativamente mejor en

ratones con maqui durante todo el procedimiento, incluso en los puntos basales se observó una mejoría en cuanto a la respuesta a la insulina en ratones suplementados con maqui, aunque el AUC del ITT no alcanza significancia (p =0,08).

En la publicación Rosalía Rodríguez-Rodríguez et al (2018)[91], se menciona que entre las OZR suplementadas con sofrito y sin suplementar no existen diferencias de peso, aunque se señala que las ratas suplementadas con sofrito son hiperfágicas y que sí hay diferencias en el consumo de kcal, siendo significativamente mayor en las ratas con sofrito. Tal y como se menciona en la **revisión de FGF21** la obesidad se define como un estado de resistencia a FGF21, la cual inhabilita las funciones beneficiosas de esta proteína implicada en el metabolismo glucídico, lipídico y en el incremento del gasto energético[121][122]. Dado el papel que se atribuye a FGF21 como diana terapéutica para el tratamiento de la obesidad y la resistencia a la insulina y los efectos beneficiosos de la suplementación con sofrito y maqui sobre los parámetros que miden la resistencia a la insulina y la tolerancia a la glucosa[123] se evaluó la producción de FGF21 y su señalización en los animales suplementados respecto a los controles.

En el **artículo científico I**, vemos como los niveles circulantes en suero de FGF21 no son detectables en ratas Zucker delgadas (LZR), pero si están aumentados en OZR lo que denota el estado de resistencia a FGF21[124]. La administración de sofrito no es capaz de reducir los niveles circulantes de FGF21 a diferencia de otras intervenciones nutricionales que si afectan a la producción de FGF21[125-126]. Alternativamente a la producción de FGF21 diferentes publicaciones han descrito que los compuestos bioactivos pueden afectar principalmente la señalización de FGF21[127] [128]. La suplementación con sofrito sí demostró capacidad para restaurar, como mínimo en parte, la señalización de FGF21 a nivel de tejido adiposo visceral (vWAT). Esta mejora en la señalización de la vía de FGF21 se ve por

medio del incremento en la expresión de los receptores fgfr1 y fgfr4, como así también de los niveles de mRNA de egr1 y c-fos, ambos genes diana de FGF21[124] .Esto indicaría que el sofrito está siendo capaz de contrarrestar la resistencia a FGF21 típica de una obesidad y con ello, en parte, mejorar la resistencia a insulina de las OZR.

En el caso de la suplementación de la dieta con maqui en el **artículo científico II,** los efectos sobre la vía de FGF21 son similares. Efectivamente, los niveles de mRNA de FGF21, egr1 y de los receptores fgfr1, fgfr4, se encontraron aumentados en el tejido adiposo subcutáneo blanco (scWAT), indicando que la expresión y señalización de FGF21 mejoran ante la suplementación nutricional con maqui. y eso puede explicar en parte los beneficios sobre el metabolismo glucídico del maqui y su uso para el control del peso.

Existe un eje entre FGF21-adiponectina que vincula directamente la expresión de ambas. Tanto la transcripción como la secreción de adiponectina son fuertemente inducidas por FGF21, de hecho se ha observado que en un estado de resistencia a FGF21 la adiponectina también se ve disminuida[129]. La adiponectina es una adipoquina con efectos antiinflamatorios[130] y su expresión está directamente relacionada con un fenotipo de un WAT más saludable[131]. Esta adipoquina induce la oxidación de ácidos grasos que conduce a una reducción de los lípidos ectópicos y finalmente a la mejora de la sensibilidad a la insulina[132]. En nuestros resultados, la inducción de los niveles de mRNA de Fgf21, FgfR1, FgfR4 y Egr1 y adiponectina en scWAT de ratones HFDM corroboran un WAT más saludable.

Entre los efectos metabólicos de FGF21 destaca su capacidad para incrementar el gasto energético en parte mediante la activación de la termogénesis en el BAT y del browning en el WAT[39]. Este aumento de la capacidad termogénica pasa por un aumento de los niveles de Ucp1 en BAT/WAT que se considera una proteína marcadora de estos

fenómenos[133]. En las OZR suplementadas con sofrito vemos que UCP1 está sobreexpresada en vWAT **(artículo científico I)**, lo que indicaría que el vWAt de estas ratas podría presentar un perfil metabólico beige según datos previos publicados en que los niveles de Ucp1 se utilizan como marcador de browning[33]. Esos resultados explicarían porque estos animales, a pesar de ser hiperfágicos, no aumentan de peso como las OZR no suplementadas. Se analizaron también otros genes representativos de browning como PRDM16, PGC1b y PPARg, los cuales no presentaron cambios entre los grupos de ratas con sofrito o sin sofrito.

Por otra parte, los ratones suplementados con maqui **(artículo científico II)** mostraron un comportamiento hiperfágico al igual que las ratas con sofrito, pero en este caso sí existieron diferencias en el peso, ya que los ratones con maqui, a pesar de comer más no incrementaban tanto su peso. Además, los ratones suplementados con maqui presentan niveles de mRNA de Ucp1, Prdm16, Pgc1a y Dio2 elevados en el scWAT lo que indica un proceso de pardeamiento o browning en este tejido. Estos genes en las ratas con sofrito no se encontraban aumentados, por lo que al parecer había un incremento en la capacidad termogénica parcial, en comparación a los del maqui.

CREG1, tiene la capacidad de estimular la adipogénesis marrón, incluida la inducción de UCP1, y FGF21[134-135]. Estos resultados concuerdan con la expresión significativamente mayor de CREG1 en el scWAT y el pardeamiento de éste observado en los ratones con maqui.

Estos efectos del maqui en el aumento de su capacidad termogénica y en el control del peso no se habían descrito anteriormente. Estas diferencias pueden deberse a la dosis de maqui utilizada. Si bien en estudios previos el tiempo en semanas fue similar, la dosis que se consideró fue mucho menor. Los datos publicados administraban dosis estimadas en 1,14 mg de antocianinas/día lo que trasladado a humanos corresponde al consumo de 145 mg/día de polifenoles[107], muy por debajo de la dosis

utilizada en nuestra aproximación experimental donde los ratones con maqui recibían una cantidad de 4 mg/día de polifenoles totales. Esta dosis de polifenoles administrados como maqui se calculó en base al consumo recomendado en humanos según PREDIMED de 820 mg/día de polifenoles por una dieta de 2000 Kcal[136].

Tanto la inducción del BAT, como el browning o pardeamiento del WAT, se consideran estrategias terapéuticas contra la obesidad y sus comorbilidades ya que son maneras de conseguir activar un metabolismo más oxidativo mediante el consumo de lípidos. Es conocido que la activación del BAT y el pardeamiento del WAT son procesos inducibles por frío pero también por la dieta[47][137-138]. A pesar de que ambos ayudarían como estrategia terapéutica para el control de la obesidad, es el tejido adiposo beige, es decir el pardeamiento/browning del WAT el que cobra más interés, debido a lo altamente inducible que es por la dieta [33][47] y porque al ser un tejido más extenso en comparación al BAT, tendría mayores implicaciones clínicas al aumentar la capacidad oxidativa y gasto energético del organismo, contrarrestando enfermedades metabólicas como la obesidad y la diabetes tipo 2[139].

Los ratones suplementados con maqui no presentaban activación del BAT a niveles de mRNA en comparación con los de HFD, pero sí se observó un mayor tamaño del BAT. Lo que sí hay es una inducción importante del beige, ya que los adipocitos blancos del scWAT presentan un fenotipo próximo a los adipocitos marrones, con inducción de los genes ya mencionados, como así también de los niveles de mRNA de fsp27b y una histología que muestra gotas multiloculares, lo que significa que son adipocitos más oxidativos que de almacenaje.

En global, los ratones con maqui presentaban un fenotipo más saludable que los no suplementados a pesar de haber consumido durante 16 semanas una dieta alta en grasa.

DNL y FAO son rutas generalmente excluyentes entre ellas, excepto en el BAT donde la activación de la termogénesis lleva a la activación simultanea de la lipogenesis y oxidación de los ácidos grasos[140-141]. Con estos datos se hace evidente que el hecho que estas dos vías estuvieran activadas a la vez en el scWAt de los ratones con maqui corroboraba que los adipocitos del scWAT de estos animales mostraban un fenotipo metabólico parecido al que se ha descrito en el BAT. Además, este perfil metabólico junto a la presencia de gotas lipídicas multiloculares y la expresión de Fsp27b y Ucp1 confirmaban el pardeamiento del scWAT de estos animales.

Estos cambios probablemente estuvieron relacionados con el aumento de la expresión de Chrebpα, Chrebpβ, Glut4, Creg1 y Srebp1c, pero también con la mejora en la señalización de FGF21 (**artículo científico II)**.

Los factores de transcripción ChREBP y Srebp1c tienen efectos opuestos en el scWAT respecto a los que tienen en hígado. Mientras en el scWAT, la expresión y activación de ChREBP y Srebp1c junto con la expresión de enzimas lipogénicas (Acaca, Fasn, Acly, GlyK, Dgat1) se correlacionan positivamente con la sensibilidad a la insulina, en el hígado la expresión de ChREBP y SREBP1c[36] se relacionan con resistencia a la insulina y NAFLD[43][142]. Se ha descrito que pacientes con NAFLD muestran un aumento en los niveles de expresión de Chrebp total en hígado[143].La expresión de Chrebp se induce en respuesta a una dieta alta en carbohidratos y se ha descrito como un regulador central de la glucólisis y la DNL y presenta mecanismos de regulación y acción tejido-dependientes[144].

Esta regulación órgano-dependiente la observamos en los ratones suplementados con maqui. En el caso del scWAT de los ratones HFDM (**artículo científico II)** se observó un aumento en la expresión de ChREBPα y aún mayor de ChREBPβ. Está descrito que ChREBPα induce la expresión de ChREBPβ de manera dependiente de la entrada de glucosa a través de

Glut4[43]. Tal y como muestran los resultados presentados en el **artículo científico II,** en el tejido scWAT de los ratones suplementados con maqui hay un incremento significativo de los niveles de mRNA de Chrebpα, Chrebpβ y Glut4, así como también de enzimas clave de la DNL, como Acaca, Fasn, Acly. La activación de la DNL bajo control de Chrebpβ estaba descrita previamente en el BAT[145] y los resultados aquí presentados permiten también apuntarla en el scWAT lo que refuerza la idea de que la suplementación dietética con maqui provoca un pardeamiento del scWAT. Además, la inducción de la DNL en el scWAT podría explicar, la mejora en la resistencia a insulina observada en estos animales según datos publicados que describen la acción insulino-sensibilizadora de la DNL en el scWAT[146].

Por el contrario, en el hígado **(resultados no publicados)** se observa una reducción significativa de la expresión de Chrebpβ que se puede relacionar con la disminución observada en la expresión de genes de enzimas clave de la DNL. A nivel hepático, y tal como se ha mencionado anteriormente, un exceso de DNL correlaciona con una mayor esteatosis. El hecho de reducir la expresión de los enzimas de la DNL como la Fasn y las de elongación (Elovl6) y desaturación (Scd1) de los ácidos grasos, sugiere que la formación de ácidos grasos complejos se encuentra disminuida[147]. Estos resultados explicarían, como mínimo en parte, la mejora de la esteatosis hepática observada en los ratones suplementados con maqui.

Otro de los factores de transcripción clave en la regulación del metabolismo lipídico es SREBP1c, el cual es un factor de transcripción regulado por insulina y cuya actividad transcripcional induce la expresión de los genes clave de la DNL sobretodo a nivel hepático. En los animales suplementados con maqui se observa el mismo efecto órgano-dependiente descrito para ChREBP respecto a sus niveles de expresión: un incremento en WAT y una reducción en hígado lo que indicaría

diferentes efectos sobre la DNL en el scWAT respecto al hígado y en el mismo sentido que los observados para ChREBP, se encuentra disminuido a nivel hepático SREBP1c el cual es clave para regular la DNL[148] , no sorprende observar la reducción de sus niveles de mRNA en los animales suplementados con maqui, ya que concuerda con la reducción de los niveles de mRNA de algunos de sus genes diana como la Fasn.

Otro gen importante en el metabolismo de los lípidos intracelulares en el hígado es la *Fatty Acid-Binding Protein 1* (FABP1). La FABP1 tiene un papel clave en la absorción de ácidos grasos, la DNL y la acumulación de TAG[149]. En los animales suplementados con maqui los niveles de mRNA de Fabp1 también se encuentran disminuidos lo que participaría también en la mejora de la esteatosis hepática observada.

A parte de la DNL, para analizar el metabolismo lipídico debe evaluarse la FAO. Tal y como se ha descrito en el BAT ambas vías metabólicas coexisten en el tiempo[145] y eso mismo ocurre en el scWAT de los ratones HFDM donde se observa el incremento a nivel de mRNA de *Carnitine palmitoyl transferase 1b* (Cpt1b), *Acyl-CoA oxidase 3* (Acox3) y *Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase* (Ehhdah) a nivel mitocondrial y peroxisomal respectivamente. A nivel hepático por el contrario la expresión de genes relacionados con la FAO está disminuida. En los ratones con dieta suplementada con maqui tanto Pparα como dos de sus genes diana, a Cpt1a y Ehhdah muestran niveles de mRNA más bajos en los animales suplementados con maqui respecto a los alimentados con una HFD. PPARα pertenece a la familia de receptores nucleares y es importante en la regulación del catabolismo de los ácidos grasos ya que regula la expresión de enzimas implicados en la oxidación mitocondrial y peroxisomal de ácidos grasos. En este modelo de intervención nutricional con maqui se observa que a nivel hepático tanto la DNL como la FAO están disminuidas. Estos datos se contradicen con

publicaciones previas donde se describe que en intervenciones con polifenoles hay un aumento de PPARα y como consecuencia en la mejora de la esteatosis hepática[150-151]. Por otro lado, y de acuerdo con nuestros resultados, ratones con deficiencia hepática de *carnitine palmitoyltransferase 2* (Cpt2) son resistentes a la obesidad y a la intolerancia a la glucosa y no presentan daño hepático ante una ingesta crónica de HFD, aunque estos ratones sí presentaban dislipidemia sérica, estrés oxidativo hepático y deficiencia de carnitina sistémica[152], además, la deficiencia de FAO en el hígado de estos ratones suprimió la producción de glucosa hepática (gluconeogénesis), lo que resultó en una mejora de la tolerancia a la glucosa. En este caso, la disminución de adiposidad y daño tras una HFD pareciera estar relacionada con la secreción de hepatoquinas en suero y en niveles de mRNA, como FGF21. Si bien en los ratones suplementados con maqui la vía de FGF21 pareciera estar parcialmente activada es importante corroborarlo a nivel de proteína para aseverar que el efecto beneficioso del maqui a pesar de una FAO disminuida pudiera estar pasando por una estimulación de la producción de hepatoquinas.

Tal y como se ha mencionado en el modelo de deficiencia hepática de Cpt2 se observó una reducción de la gluconeogénesis. La gluconeogénesis es una vía metabólica clave en el mantenimiento de la glucemia y de la homeostasis metabólica. En el caso de los ratones suplementados con maqui se observó una reducción en los niveles de mRNA de la G6pc, enzima clave de la gluconeogénesis **(resultados no publicados)**. Paralelamente, se ha descrito que la activación/represión de la gluconeogénesis depende en parte de dos co-reguladores transcripcionales que compiten: SMILE y PGC1α. SMILE es un co-represor de genes lipogénicos que tiene efectos protectores frente a una dieta alta en grasa[153-154].Por el contrario, PGC1α es un marcador de biogénesis mitocondrial y activador de la gluconeogénesis hepática. En los ratones

HFDM se observa un aumento de los niveles de mRNA de SMILE y una reducción de los de Pgc1α y G6p, que se relacionaría no sólo con que estos animales estén alimentados, sino que pudiera reflejar el correcto metabolismo de la glucosa a causa del consumo de maqui[155-156].

De manera global, los resultados mostrados indican que el maqui tiene efectos beneficiosos sobre el perfil metabólico de la obesidad y confirman datos previos ya publicados sobre su impacto beneficioso en el metabolismo de la glucosa [96][110]. Lo que de momento se desconoce es cual o cuales son los componentes específicos del maqui responsables de estos efectos.

Limitaciones de los estudios

El consumo del sofrito mejora la vía de FGF21, lo que explica en parte el beneficio del consumo de este, pero se necesitan más estudios para determinar otros mecanismos moleculares que pudieran explicar con más precisión la respuesta metabólica observada. Como así también es importante realizar análisis a nivel de proteínas mediante aproximaciones de Western blot (WB) y/o actividades enzimáticas para profundizar en los mecanismos estudiados.

Dado que la inflamación es un punto crítico en la obesidad, evaluar las moléculas inflamatorias de estos animales otorgaría más información en relación con la mejora observada en el perfil de obesidad.

Como perspectivas futuras, si bien se ha realizado el estudio del sofrito en población sana[90], sería interesante estudiar si en población obesa estos resultados son extrapolables, sobre todo considerando que el consumo de dos o más porciones de sofrito por semana, es un marcador de adherencia a Dieta Mediterránea. Según los datos previos y los presentados en este trabajo, incluir el sofrito en una intervención nutricional podría ser una estrategia para acercar a la población a una Dieta Mediterránea con los beneficios que eso conllevaría.

En cuanto al estudio del maqui, se pueden correlacionar los efectos beneficiosos en ratones obesos con los cambios observados a nivel de expresión génica, principalmente los relacionados con el pardeamiento del scWAT. En el mismo sentido que en el estudio del sofrito se deberían ahora corroborar los datos a nivel proteico por medio WB, ya que, aunque los resultados de histología y fenotípicos son claros los niveles de proteínas reforzarían nuestros resultados.

Analizar los parámetros bioquímicos en sangre también es importante, sobre todo la evaluación de una posible dislipidemia, considerando que la FAO en estos ratones se encuentra disminuida; pero dado que estos animales no estaban ayunados realizar la analítica pudiera arrojar resultados confusos y no fiables. El no ayuno de estos animales se justifica ya que un eje fundamental de esta investigación era FGF21, el cual es sensible ayuno[157] e interesaba estudiar su comportamiento en situación de alimentación.

Con el maqui, vemos un WAT mucho más saludable respecto a funcionalidad, pero no se analizó nada en cuanto a inflamación; y ya que la obesidad es una inflamación crónica y de bajo grado[19] este sería un punto de continuación importante. El análisis de genes inflamatorios como TNF-α e IL-6 deben ser analizados para evaluar cambios en el estado de inflamación en los ratones tratados con maqui respecto a los controles.

Perspectivas futuras

Como perspectivas futuras, realizar el estudio en un modelo animal sano suministrando el maqui con una dieta estándar sería importante para conocer si los efectos del maqui se observan tan potentes en un modelo sin alteraciones metabólicas, como así también realizar estudios de bioaccesibilidad/biodisponibilidad del maqui.

En cuanto a humanos, la suplementación con maqui sí se ha estudiado en pacientes prediabéticos, pero principalmente con compuestos aislados como son las delfininas del maqui en dosis agudas[95] y durante tres meses [96], arrojando datos que indican los beneficios de la suplementación en el metabolismo de la glucosa y el perfil lipídico. Por lo que sería interesante ver si el consumo del maqui como alimento completo es extrapolable a humanos por medio de un ensayo aleatorizado controlado; como así también obtener datos de expresión génica por medio de células mononucleares de sangre periféricas para conocer los mecanismos moleculares que están suscritos al consumo de maqui en humanos. Resulta interesante también seguir con la caracterización y el análisis de esta baya, para confirmar si es que los efectos corresponden a una molécula específica o a un efecto sinérgico de los diferentes componentes del maqui.

En global, esta tesis ha conducido a señalar el consumo de sofrito y maqui como una estrategia putativa para contrarrestar los efectos nocivos de la obesidad con una fácil aplicabilidad ya que ambos alimentos son fáciles de incorporar en nuestros patrones alimentarios y por ende con un alto impacto en la promoción, prevención y tratamiento de la obesidad. Nuestra investigación está respaldada por los datos publicados en estudios en humanos sobre los efectos del sofrito y el maqui en la salud metabólica.

Conclusions

CONCLUSIONS

Foods such as sofrito and maqui with a high content of bioactive compounds are effective for the treatment of obesity and its comorbidities. The molecular mechanisms underlying these beneficial effects are partially elucidated in this thesis. The main conclusions are:

- Sofrito dietary supplementation improves the obese phenotype of OZR at least in part by inducing the FGF21 signaling in the visceral WAT.
- Sofrito supplementation improves insulin sensitivity in the OZR
- Maqui intake ameliorates the body weight increment caused by a HFD in mice
- Maqui supplementation improves the glucose tolerance in mice fed a HFD.
- Maqui supplementation improves the obese phenotype of mice fed a HFD at least in part by inducing the FGF21 signaling in the scWAT.
- ChREBPβ is induced in mice fed with maqui-supplemented HFD and this induction may explain, at least in part, the induction of DNL described in scWAT
- Maqui supplementation induces a brown-like phenotype in scWAT.
- Maqui supplementation ameliorates the HFD-induced hepatic steatosis in mice

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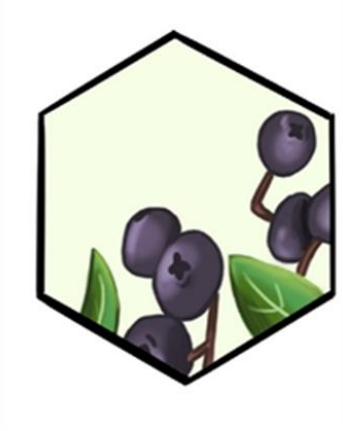

Anexos

ANEXOS

-Información nutricional de intervenciones

Información nutricional de ratas Zuker suplementadas con sofrito 2% (w/w)

Teklad 18% roedent diet

Ingredients:(in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, iodized salt, Llysine, DL-methionine, choline chloride, kaolin, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate,vitamin A acetate, calcium iodate, vitamin B12 supplement, folic acid, biotin,vitamin D3 supplement, cobalt carbonate.

Macronutrients		
Crude Protein	%	18,6
Fat (ether extract)	%	6,2
Carbohydrate (available)	%	44,2
Crude Fiber	%	3,5
Neutral Detergent Fiber	%	14,7
Ash	%	5,3
Energy Density	kcal/g	3,1
Calories from Protein	%	24
Calories from Fat	%	18
Calories from Carbohydrate	%	58
Minerals		
Calcium	%	1
Phosphorus	%	0,7
Non-Phytate Phosphorus	%	0,4
Sodium	%	0,2

Potassium	%	0,6
Chloride	%	0,4
Magnesium	%	0,2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0,23
Amino Acids		
Aspartic Acid	%	1,4
Glutamic Acid	%	3,4
Alanine	%	1,1
Glycine	%	0,8
Threonine	%	0,7
Proline	%	1,6
Serine	%	1,1
Leucine	%	1,8
Isoleucine	%	0,8
Valine	%	0,9
Phenylalanine	%	1
Tyrosine	%	0,6
Methionine	%	0,4
Cystine	%	0,3
Lysine	%	0,9
Histidine	%	0,4
Arginine	%	1
Tryptophan	%	0,2
Vitamin		
Vitamin A	IU/g	15
Vitamin D3	IU/g	1,5
Vitamin E	IU/kg	110
Vitamin K3 (menadione)	mg/kg	50
Vitamin B1 (thiamin)	mg/kg	17
Vitamin B2 (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	70
Vitamin B6 (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B12 (cyanocobalamin)	mg/kg	0,08
Biotin	mg/kg	0,4
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0,7
C18:0 Stearic	%	0,2
C18:1ω9 Oleic	%	1,2

C18:2ω6 Linoleic	%	3,1
C18:3ω3 Linolenic	%	0,3
Total Saturated	%	0,9
Total Monounsaturated	%	1,3
Total Polyunsaturated	%	3,4
Others		
Cholesterol	mg/kg	n/d

Nutritional composition Sofrito

Ingredients: Tomato (pulp and concentrated) 50%, onion 37%, extra virgin olive oil 12%, salt.

	100g	Per serving*	%**
Calories	**536kJ/136Kcal**	**281kJ/68Kcal**	**3%**
Total fat	**12g**	**6,0g**	**9%**
Saturated	1,7g	0,9g	5%
Total Carbohydrate	**5,5g**	**2,8g**	**2%**
Dietary Fiber	1,0g	0,5g	
Sugars	4,0g	2,0g	1%
Protein	**1,0g**	**0,5g**	**4%**
Sodium	0,5g	0,25g	1%

* Per serving: 50g

** Percent daily values are based on 2000 kcal

Información nutricional de ratones C57BL6/J suplementados con maqui 4mg/d

D12451 HFD 45%

Macronutrients		
Protein	24	20
Carbohydrate	41	35
Fat	24	45
TOTAL		100
kcal/gm	4,73	

Ingredients		
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	72,8	291
Maltodextrin 10	100	400
Sucrose	172,8	691
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	177,5	1598
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5,5	0
Potassium Citrate, 1 H2O	16,5	40
Vitamin Mix V10001	10	0
Choline Bitartrate 2 FD&C Red Dye	2	0
FD&C Red Dye #40 0	0,05	0

Nutritional composition of lyophilized maqui

	100g	2g*
Calories	914kJ/232Kcal	19,7kJ/4,64Kcal
Total fat	10,8g	0,2g
Total Carbohydrate	78g	1,6g
Sugar	24,2g	0,5g
Dietary Fiber	50,4g	1g
Protein	6,2g	0,1g
Sodium	20,1mg	0,4mg
Polyphenols (GAE)	6550 mg	131 mg

* Per serving

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