Doktorego Tesia - Doctoral Thesis

Erresberatrola eta kertzetinaren metabolitoen efektuak 3T3-L1 adipozitoen adipogenesian eta triglizeridoen metabolismoan, hala nola jatorrizko konposatuen efektuekin konparatzea

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Effects of resveratrol and quercetin metabolites in adipogenesis and triglyceride metabolism of 3T3-L1 adipocytes and comparison to those of the parent compounds

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NUTRICIÓN OBESIDAD

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ESKER ONAK

Doktorego Tesi hau egiteak erakutsi didan guztia ez dago ehundaka orrialde hauetan jasoa. Hein handi batean horrela bada ere, “laborategitik kanpo” gauza asko egin ahal izan ditut, horien artean, dozentzian lehen urratsak eman. Guzti horrek bai profesionalki eta bai pertsonalki “garatzeko” aukera eman dit. Enaiz, inola ere, Tesi hau hasi zuen pertsona hura.

Eskerrak eman nahi dizkizuet denbora guzti honetan zehar alboan eduki zaituztedan guztiei. Laborategian elkarren ondoan batzuk, kafe orduan edo bazkarian besteak… solasaldi luzeetan lankideek irakasten dizutenak ez du preziorik!!

Lana garrantzitsua da, baina are gehiago kanpoko bizitza. Gainera, deskonexioa lanaren parte bezala ulertu daiteke. Hortaz, eskerrak ere nire bizitzaren parte zareten guztiei, “nire zelulak zer moduz dauden” galdetzeaz gain “ezer gutxi” dakizuenak eta deskonektatzen lagundu didazuenak. Horrek ere, Doktorego Tesia aurrera eramaten laguntzen du. Dena den, baten batek asko daki polifenol, adipozito eta laneko abentura guztiez!

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LABURDURAK – ABREVIATIONS

Laburdurak/Abbreviations

3G: erresberatrola-3-*O*-glukuronido / resveratrol-3-*O*-glucuronide

4G: erresberatrola-4´-*O*-glukuronido / resveratrol-4´-*O*-glucuronide

3S: erresberatrola-3-O-sulfato / resveratrol-3-*O*-sulfate

4S: erresberatrola-4´-O-sulfato / resveratrol-4´-*O*-sulfate

ACC: azetil-AKo karboxilasa / acetyl-CoA carboxylase

AGPAT: 1-azilglizerol-3-fosfato aziltransferasa / 1-acylglycerol-3-phosphate acyltransferase

AgRP: agouti-rekin erlazionaturiko proteina / agouti-related protein

AIF: apoptosiaren faktore eragilea / apoptosis-inducing factor

Akt: B proteina kinasa (PKB) / protein kinase B (PKB)

AMPK: AMP-k aktibaturiko proteina kinasa / AMP-activated protein kinase

APAF 1: apoptosiaren proteasa aktibatzen duen 1 faktorea / apoptosis protease-activating factor-1

AS160: 160 KDa dituen AKT-aren substratua / AKT substrate of 160 kDa

ASM: acid soluble metabolites

ATGL: gantz ehuneko triglizeridoen lipasa / adipose triglyceride lipase

ATP: adenosine trifosfatoa / adenosine triphosphate

BAT: brown adipose tissue

Bak: Bcl2 homologoaren erailea / Bcl2 homologous antagonist killer

Bax: Bcl2-ari elkartutako X proteina / Bcl2-associated X protein

Bcl2: B zelulen leuzemia/2 linfoma / B cell leukemia/lymphoma 2

BID: heriotzaren-agonista den BH3-ren interakzio-domeinua / BH3 interacting domain death agonist

BMI: body mass index

cAMP: adenosina 3',5'-monofosfato ziklikoa / cyclic adenosine 3´5´ monophosphate

Cas3: kaspasa 3 / caspase 3

CART: kokainak eta anfetaminak erregulaturiko transkritoa / cocaine and amphetamine-regulated transcript

CD36: txatar-biltzaile 36 hartzailea / scavenger receptor 36

CEBP (α,β,δ): CCAAT-ra lotzen den (α,β,δ) proteina / CCAAT enhancer binding protein (α,β,δ)

ChREBP: karbohidratoen elementu erantzuleari lotzen zaion proteina / carbohydrate response element binding protein

LABURDURAK
ABREVIATIONS

CREB 1: cAMP-ren elementu erantzuleari lotzen zaion 1 proteina / cAMP responding element binding protein 1

CGI-58: genearen 58. Konparaketa izendatzailea / comparative gene Identification-58

CHOP10: CEBP-ren proteina homologoa / CEBP homologous protein

COMT: katekol-O- metiltransferasa / catechol-O-methyltransferase

COUP-TFII: oilaskoaren oboalbuminaren 2 transkripzio faktorearen ur-gorako eragilea / chicken ovalbumin upstream promoter transcription factor II

CPT1: karnitina 1 palmitoiltranferasa / carnitine palmitoyltransferase 1

CREB: cAMP response element binding protein

DAG: diazilglizerola / diacylglycerol

DEXA: dexametasona / dexamethasone

DGAT: diazilglizerol aziltransferasa / diacylglycerol acyltransferase

DISC: heriotza-hasieraren seinalizaziorako konplexua / death initiation signalling complex

DMEM: Dulbecco´s modified Eagle´s medium

DNA: azido desoxirribonukleikoa / deoxyribonucleic acid

ERR: estrogenoekin erlazionaturiko hartzailea / estrogen-related receptor

FABP: gantz azidoetara lotzen den proteina / fatty acid binding protein

$FADH_2$: flabin adenina dinukleotido / flavin adenine dinucleotide

Fas: TNFR familiako 6. kidea / TNF receptor superfamily member 6

FAS: gantz azidoen sintasa / fatty acid synthase

FATP: gantz azidoen proteina garraiatzaileak / fatty acid transport proteins

FBS: behi-fetuaren seruma / fetal bovine serum

FFA: free fatty acid

FGF: fibroblastoen hazkuntza faktoreak / fibroblast growth factors

FOXO1: forkhead box O1

GA: gantz azidoa

GAA: gantz azido askeak

GATA2: GATA-ri lotzen zaion 2 proteina / GATA binding protein 2

G6PDH: glukosa-6-fosfato dehidrogenasa / glucose-6-phosphate dehydrogenase

GEA: gantz ehun arrea

GEZ: gantz ehun zuria

GLUT4: glukosaren 4 garraiatzailea / glucose transporter 4

GMI: gorputz masaren indizea

GPAT: glizerol-3-fosfato aziltransferasa / glycerol-3-phosphate acyltransferase

GR: glukokortikoideen hartzailea / glucocorticoid receptor

GS: glukogeno sintasa / glycogen synthase

GSK3β: glukogeno sintasa 3 kinasa / glycogen synthase kinase 3

HSL: hormonekiko sentikorra den lipasa / hormone sensitive lipase

IBMX: isobutilmetilxantina / isobutylmethylxanthine

IGF-1: intsulinaren antzekoa den 1 hazkuntza hormona / insulin-like growth factor 1

IR: intsulinaren hartzailea / insulin receptor

IRF4: interferona erregulatzen duen 4 faktorea / interferon regulatory factor 4

IRS: intsulinaren substratuaren hartzailea / insulin receptor substrate

ISO: isorhamnetina / isorhamnetin

KDa: kilodalton

KP: konposatu fenolikoak

KLF: krüppel-aren antzekoa den faktorea / krüppel-like factor

LDH: laktato dehidrogenasa / lactate dehydrogenase

LDL: dentsitate baxuko lipoproteinak / low-density lipoprotein

LPH: laktasa floridizina hidrolasa / lactase phloridizin hydrolase

LPL: lipoproteina lipasa / lipoprotein lipase

LXR: gibeleko x hartzailea α / liver x receptor α

MAG: monoazilglizerola / monoacylglycerol

MAPK: mitogenoak aktibaturiko protein kinasa / mitogen-activated protein kinase

MGL: monoazilglizerol lipasa / monoacylglycerol lipase

miRNA: mikroRNA / microRNA

mTOR: rapamizinaren ugaztun-itua / mammalian target of rapamycin

mTORC2: mTOR 2 konplexua / mTOR complex 2

NAD: nikotinamida adenina dinukleotidoa / nicotinamide adenine dinucletide

NADPH: nikotinamida adenine dinukleotido fosfatoa / nicotinamide adenine dinucletide phosphate

LABURDURAK
ABREVIATIONS

NPY: Y neuropeptidoa / neuropeptide Y

NRF: nukleoko arnasketa faktorea / nuclear respiratory factor

OME: Osasunaren Munduko Erakundea

OMI: HtrA 2 serina peptidase / HtrA serine peptidase 2

OXPHOS: fosforilazio oxidatiboaren katearen osagaiak / oxidative phosphorylation chain components

P/S: penizilina/estreptomizina / penicillin/streptomycin

PARP: poli (ADP-erribosa) polimerasa / poly (ADP-ribose) polymerase

PAP: azido fosfatidikoaren fosfatasa / phosphatidic acid phosphatase

P53: tumoreen p53 ezabatzailea / tumor suppressor p53

PC: phenolic compounds

PDE 3: 3 fosfodiesterasa / phosphodiesterase 3

PDK1: fosfoinositidoaren menpeko 1 proteina kinasa / phosphoinositide-dependent protein kinase 1

PEP: fosfenolpirubatoa / phosphoenolpyruvate

PGC1α: peroxisomen ugalketaren 1α hartzailea / peroxisome proliferator-activated receptor gamma co-activator 1α

PHB: proibitina / prohibitin

PI3K: fosfatidilinositol 3-kinasa / phosphatidylinositol kinase-3

PIP2: fosfatidilinositol 4,5-bifosfato / phosphatidylinositol- 4, 5- bisphosphate

PIP3: fosfatidilinositol 3,4,5-bifosfato / phosphatidylinositol- 3, 4, 5- trisphosphate

PKA: cAMP-ren menpeko A proteina kinasa / cAMP-dependent protein kinase A

PKM: muskuluaren pirubato kinasa / pyruvate kinase muscle

POMC: proopiomelanokortina / proopiomelanocortin

PP1: 1 proteina fosfatasa / protein phosphatase 1

PPARγ: peroxisomen ugalketarako γ hartzailea / peroxisome proliferator-activated receptor γ

Q3G: kertzetina-3-O-glukuronido / quercetin-3-O-glucuronide

Q3GA: kertzetina-3-O-β-D-glukuronido quercetin-3-O-β-D-glucuronide

Q3S: kertzetina-3-O-sulfato / quercetin-3-O-sulfate

Q3S+4S: Q3S eta kertzetina-4-O-sulfatoaren arteko nahasketa / Q3S and quercetin-4-O-sulfate mixture

RAR: azido retinoikoaren hartzailea / retinoic acid receptor

RISC: miRNA-k eragindako isilpen-konplexua / miRNA-induced silencing complex

RNA: azido ribonukleikoa / ribonucleic acid

RT-q-PCR: denbora errealean gauzaturiko polimerasaren kate-erreakzio kuantitatiboa / quantitative real-time polymerase chain reaction

RXR: retinoidearen x hartzailea / retinoid-x-receptor

RORa: RAR-ekin erlazionaturiko a zurtz den hartzailea / RAR-related orphan receptor a

ROS: oxigenoaren espezie erreaktiboak / reactive oxygen species

SGLT1: sodioaren menpeko glukosaren 1 garraiatzailea / sodium-dependent glucose transporter 1

SIRT1: 1 sirtuina / sirtuin 1

SMAC: Diablo, IAP-ari loturiko proteina mitokondriala / Diablo, IAP-binding mitochondrial protein

SP1: Sp1 transkripzio-faktorea / Sp1 transcription factor

SREBF1: esterolengatik erregulaturiko elementura lotzen den 1 proteina / sterol regulatory element binding transcription factor 1

SREBP1c: SREBP hausturak aktibaturiko 1 proteina, c isoforma / SREBP cleavage activating protein 1, c isoform

STAT5A: signal transducer and activator of transcription 5A

SULT: sulfotransferasak / sulfotransferases

TAM: tamarixetina / tamarixetin

TFAM: A transkripzio-faktore mitokondriala / mitochondrial transcription factor A

TG: triglizeridoak / triglycerides

TNFα: tumoreen nekrosiaren α faktorea / tumor necrosis factor α

TNFR: tumoreen nekrosiaren faktorearen hartzailea / tumor necrosis factor receptor

TR: hormona tiroideoaren hartzailea / thyroid hormone receptor

TRP53: eraldaketarekin erlazionaturiko 53 proteina / transformation related protein 53

UCP1: 1 proteina desakoplatzailea / uncoupling protein 1

UGT: uridina-5´- difosfato glukuronosiltransferasa / uridine-5´-diphosphate glucuronosyltransferase

VLDL: dentsitate oso baxuko lipoproteinak / very-low-density lipoproteins

WAT: white adipose tissue

WHO: world health organization

SARRERA

1. Obesitatea

Gainpisua eta obesitatea gehiegizko gantz-metaketa bezala definitzen dira [1], haien zorroztasun eta kokapenaren arabera koerikortasun ezberdinen garapenean eragin dezaketenak. Gantz masaren kantitatea, eta bereziki sabel-aldekoa bada, zuzenki proportzionala da obesitatearekin erlazionaturiko koerikortasunen garapenarekin. Horien artean 2 motako diabetesa, gaixotasun kardiobaskularrak, loaldiko apnearen sindromea eta minbizia dira gaixotasun aipagarrienak [2].

Osasunaren Munduko Erakundeak (OME) Gorputz Masaren Indizea (GMI) erabili ohi du gainpisua eta obesitatearen diagnosian. Altuera eta gorputz-pisua erlazionatzen dituen tresna praktikoa da, kalkulatzen erreza eta azkarra, egoera nutrizionalaren lehen balioespena emango diguna (1. taula). Hala eta guztiz ere, kontuan eduki beharra dago ez dela obesitatea diagnostikatzeko tresnarik zehatzena, gorpuzkera, gorputz-konposizioa eta gerri eta aldaka perimetroa bezalakoak kontuan hartzen ez baititu.

1. taula. GMI-an oinarrituta eginiko sailkapena. OME-tik moldatua [3].

Sailkapena	GMI (kg/m^2)
Gutxiegizko pisua	< 18.5
Pisu aproposa	18.5-24.99
Gainpisua (aurreobesitatea)	25-29.99
Obesitatea	≥30

OME-ren zenbatespenean oinarrituta, 2016. urtean munduko helduen %39ak gainpisua zuen, eta %13ak obesitatea. Gainera, 5 urte baino gutxiagoko 41 milioi haurrek gainpisua edo obesitatea zuen eta 5 eta 19 urte bitartekoen kasuan, 340 milioik [1]. Oraindaino, haurtzaroko obesitatea ekonomikoki garatutako herrialdetan ikusi izan da, baina egun, garapen bidean dauden herrialdeetan ere haren prebalentzia modu oso azkarrean igotzen ari da. Haur-obesitateak duen garrantzia ez datza soilik haurtzaroan zehar pairatu daitezkeen gaixotasunetan, baizik eta helduaroan gaixotasun kronikoek eragindako heriotzen probabilitateak handitzean baizik. Alde batetik, obesoa den haur batek helduaroan ere obesitatea pairatzeko arrisku altua du. Bestetik, 2. motako diabetesa edo gaixotasun kardiobaskularrak nozitzeko galzoria oso altua da, beti ere obesitatearen garapenaren hasiera eta iraupenaren arabera baldintzatuta egongo delarik. Aipatutako arrazoi horiengatik, haurtzaro eta nerabezaroko obesitatea epe labur eta luzean eman daitezkeen ondorio larriekin erlazionatzen dela esan daiteke [4,5].

Nahiz eta gainpisu eta obesitatea prozesu konplexu bat izan non faktore askok eragiten dioten (genetika, dieta, estresa, erretzea, etab.), modu orokorrean, hartutako eta xahututako energiaren arteko desorekak eragindako gehiegizko gantz-metaketa dela esan daiteke. Azken urteotan ikusi den prebalentziaren izugarrizko handitzea, ezin zaio kaloria eta lipido askoko elikagai prozesatuen gehiegizko kontsumoari eta

produktu freskoen kontsumo urriari soilik atxikitu, jarduera fisikoaren murrizketak ere garrantzia baitu (lan sedentarioak, bizilekuan mugitzeko erak, aisialdian teknologia berrien gehiegizko erabilera, etab.).

Arrakastatsua izateko, obesitatearen tratamendua diziplina anitzeko profesionalek burutu beharko lukete. Tratamendu dietetikoa, jarduera fisikoa, tratamendu farmakologikoa, kirurgia bariatrikoa edota psikoterapia dira gaixotasun honi aurre egiteko gehien erabiltzen diren bideak. Orohar, tratamenduen bateratzea, gutxienez tratamendu dietetikoa eta jarduera fisikoa barnebiltzen dituena, ohi da terapia erabiliena eta eraginkorrena [6]. Hala ere, epe luzean arrakasta lortzea zaila dela kontuan hartu beharra dago, prebentzioak funtsezko garrantzia duela nabarmentzen duena.

Dieta mediterraneoa 60. hamarkadan definitutako eta Mediterraneo itsasoaren inguruko herrialdeek jarraitutako elikadura-patroia da. Bere ezaugarrien artean sukaldatzeko oliba olioaren erabilera eta landare-jatorriko elikagaien kontsumo altua daude [7]. Minbizi edo gaixotasun kardiobaskularren prebentzioan duen eraginaz gain, elikadura orekatuaren eredu ezinhobea eta obesitatearen prebentzio eta tratamendurako baliabide interesgarri moduan proposatu da [11]. Elikadura-patroi honek hobesten dituen elikagai-motak dira efektu onuragarrien erantzule direnak, karbohidrato konplexu, gantz asegabe, zuntz, bitamina, mineral eta dietako beste osagai onuragarrien iturri ona baitira. Osasunerako mesedegarri izan daitezkeen molekula bioaktiboen artean konposatu fenolikoak (KF) kokatzen dira, besteak beste, fruta, barazki, intxaur, oliba olio eta ardo beltzan aurkitu daitezkeenak (2. taula).

2. taula. Dieta mediterraneoaren elikadura-patroia. Buckland eta kolaboratzaileen artikulutik moldatua [11].

	Dieta mediterraneoaren ezaugarriak
1	Landare-jatorriko elikagaien kontsumo altua (fruta, barazkiak, lekaleak, intxaurrak, haziak eta zereal integralak)
2	Uztaroko eta gertuko elikagai freskoen kontsumoa
3	Oliba olioa (dietako gantzen iturri nagusia)
4	Ardo beltzaren neurrizko kontsumoa
5	Arrain eta itsaski freskoaren kontsumoa
6	Esneki, etxeko hegazti eta arrautzen neurrizko kontsumoa
7	Haragi gorri eta prozesatuaren neurrizko kontsumoa

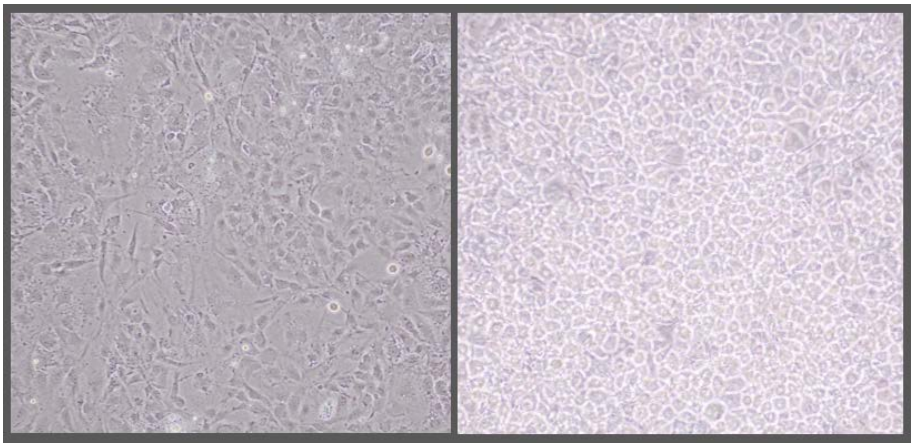
KF landareek defentsa mekanismo moduan estresari aurre egiteko ekoizten dituzten sustantzia antioxidatzaileak dira [12,13]. KF-etan aberatsak diren elikagaiak hartzea gaixotasun kardiobaskularrak, zenbait minbizi mota, gibelaren asaldurak eta diabetesa bezalako gaixotasunen intzidentzia murrizteko baliagarriak dira [13]. Horretaz gain, azken urteotan eginiko ikerketek KF obesitateari aurre egiteko ere erabilgarriak direla agerrarazi dute [14]. Hori horrela, KF dituzten elikagai funtzionalak diseinatzea gaixotasun pandemiko honi aurre egiteko estrategia ona izan daiteke.

2. Adipozitoaren biologia

Giza gorputzak gantza triglizerido moduan metatzen du, gantz ehun arrean (GEA) eta gantz ehun zurian (GEZ) egingo duena. GEZ-ren funtzio nagusia triglizeridoen sintesiaren bidez energia lipido moduan metatzea da, gehiegizko energia dagoenean lipidoak adipozitoetan metatzen baitira. Aldiz, giza gorputzak energiaren beharra duenean, lipolisiaren bidez triglizeridoen mobilizazioa martxan jarriko du. Modu horretan, odolera glizerola eta gantz azido askeak (GAA) jariatuko dira, beste ehun eta organo batzuetara garraitzeko, eta bertan oxidatuak izateko, energia lortzeko helburuarekin. GEA-n ordea, adipozito arreek 1 proteina desakoplatzailea (UCP1) espresatzen duten mitokondrio ugari dituzte, bero moduan energia xahutzeko funtzioa dutenak. Aipaturiko horiez gain, 3. motako adipozitoa badago, “brite” edo “beix” deiturikoa. GEZ-an aurkitu daitekeen zelula mesenkimaletatik eratorritako adipozito arrea da, eragite termogenikoaren ondorioz diferentziatua. Eragile termogeniko ezagunenak hotzarekiko esposizioa, peroxisomen ugalketarako γ hartzailearen (PPARγ) eta hartzaile β-adrenergikoen agonistekin eginiko tratamenduak zein dietako hainbat osagaien ingestioa, dieta hiperlipidikoak edo KF kasu, dira [15]. Aipatzekoa da adipozito arreen zelula-leinu aitzindaria adipozito zurienaren desberdina dela, brite adipozitoek zuriekin, eta ez arreekin, konpartitzen duten bitartean [16]. Gauzak horrela, azken ikerketek adierazi dutenez, brite adipozitoak adipozito zurien transdiferentziaziotik eratorri daitezke [17]. Prozesu hau, GEZ-n adipozito arreen ezaugarriak dituzten adipozitoen agerpenean datza, beti ere hainbat aldagai aktibatzaileren eraginpean.

Gantz ehuna heterogeneoa da, adipozitoez gain beste hainbat zelula baitaude. Besteak beste, aurre-adipozito eta adipozito helduez aparte, zelula mesenkimalak, makrofagoak eta zelula endotelialak proportzio ezberdinetan aurkitu daitezke. Adipozito helduei dagokienez, gantz ehunaren herena suposatzen dutela jotzen da, gainontzeko zelulen konbinazioak gainontzeko bi herenak eratuko duelarik [18].

Gantz ehunaren gorabeherak lipidoen akumulazioan gertatzen diren aldaketengatik eman ohi dira. Gantz masa osoari eragiten dioten prozesuak adipozitoen hipertrofia (tamainaren handitzea) edo adipozitoen hiperplasia (adipozito-kantitatearen areagotzea) dira, gantz ehunaren tamaina hipotrofiak, adipozitoen tamainaren txikitzeak edo hipoplasiak murriztuko dutelarik [19,20]. Hiperplasia ematen denean aurre-adipozitoen ugaritzea eta diferentziazioa emango da, adipogenesia bezala ezagutzen duguna (1.irudia). Are gehiago, hipertrofia, oro har, lipogenesiak ezartzen du eta hipotrofia aldiz, lipolisiak.



1. irudia. 3T3-L1 zelulak. Fibroblasto-itxurako aurre-adipozitoak (ezkerrean) eta diferentziatutako eta lipidoz beteriko adipozito helduak (eskuinean).

2.1 Adipogenesia

Zelula mesenkimalek zelula mota ezberdinen aitzindari izateko desberdintzeko ahalmena dute, hala nola aitzindari miogeniko eta osteoblastoenak, kondrozito edo aurre-adipozitoak, aktibatzaile endokrinoek agintzen dutenaren arabera. Giza eta animali-adipogenesia hazkuntzarekin batera eman ohi da, baina kontuan hartzekoa da, erritmo baxuagoan bada ere, helduaroan ere gertatzen dela [21].

Fibroblasto-itxurako zeluletatik adipozito helduetarako diferentziazio prozesua, bideratutako aurre-adipozitoen ugaritzearekin hasten da, prozesua kontaktu bidezko inhibizioari esker geldituko delarik (3. taula). Horretarako, zelulek bere barneko eta kanpoko matrize zelularrean hainbat eraldaketa abian jartzen dituzte, adipogenesian zehar egingo den lipidoen metaketa ahalbidetuko duen zelulen forma aldatzeko, ardatz-formakoak izatetik zelula handiagoa eta biribilagoa izatera igarotuta. Behin ugaritzearen geldialdia eman dela, aurre-adipozitoak adipogenesiaren lehen fasea hasiko dute klonazio-mitotikoaren hedapenaren bidez, zelulak berriz ere ziklo zelularrean sartu eta bi zelula-zatiketa emango direlarik. Gerora, aurre-adipozitoak adipogenesiaren 2. fasean barna murgilduko dira, non karbohidrato eta lipidoen metabolismoan parte hartzen duten geneen, adipokinen eta beste hainbat geneen espresioa handitzen hasiko den, azkenik lipido asko metatuta dituzten eta intsulinarekiko sentikorrak diren zeluletan bilakatuko direla.

3. taula. Fibroblasto itxurako zelulak adipozito helduetan bilakatzen direneko faseen sailkapena (Lefterova eta kolaboratzaileetatik eraldatua [22]).

Faseak	Ezaugarriak (*in vitro* eginiko ikerketetan oinarrituta)
1. Bideratutako aurre-adipozitoak	Zelulek fibroblasto-itxurako fenotipoa dute eta adipozitoen leinuan barrena ezberdintzen dira.
2. Ugaritzea gelditu duten aurre-adipozitoak	Kontaktugatiko inhibizioak zelulen ugaritzea gelditu du.
3. Klonazio-mitotikoaren hedapena	Zelulak ziklo-zelularrean bersartzen dira eta bi zelula-zatiketa emango dira. Fase honetan, ziklo zelularraren erregulatzaileak eta transkripzio-faktore adipogenikoak (CEBPβ and CEBPδ) osoki espresaturik daude eta bukaerako diferentziazio geneen espresioa erreztu egiten dute.
4. Bukaerako diferentziazioa	Gantz-tantak eratu eta metatu egingo dira. Giltzarri diren PPARγ eta CEBPα erregulatzaileak, hala nola beste adipozito-espezifikoak diren geneak eragin dira.
5. Adipozito helduak	Adipozito-espezifikoak diren geneak osoki espresaturik daude eta adipozitoek gantz-tanta handi bakarra metatuko dute.

CEBP (α,β,δ): CCAAT-ra lotzen den (α,β,δ) proteina; PPAR: peroxisomen ugalketarako hartzailea

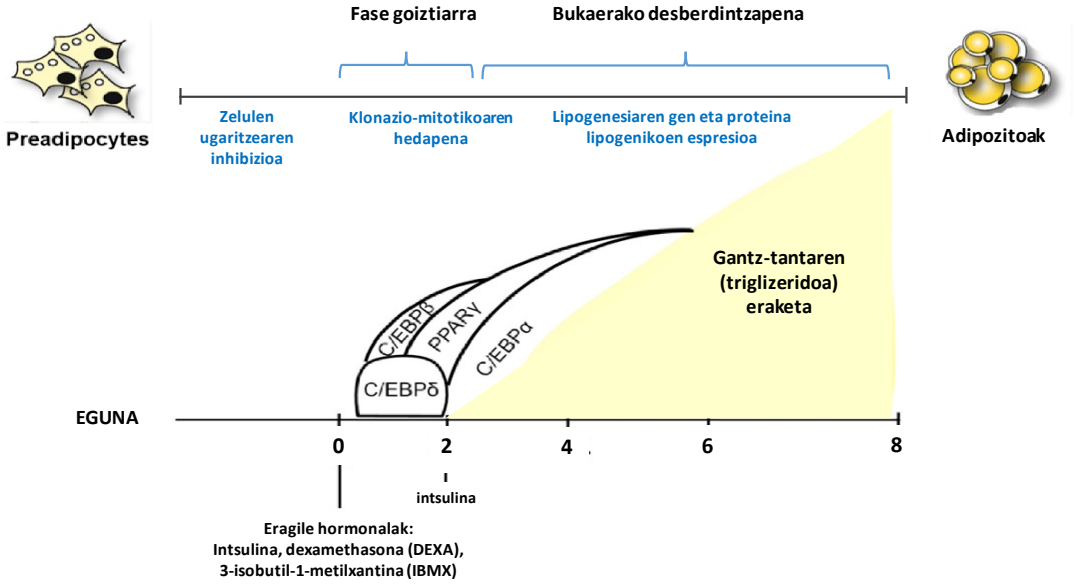
2.1.1 Adipogenesiaren erregulazio transkripzionala

Ikerketa batzuek adenosina 3',5'-monofosfato ziklikoaren (cAMP) elementu erantzuleari lotzen zaion 1 proteina (CREB) adipogenesiaren hasarazle bezala definitu dute, bere gainespresioak triglizeridoen akumulazioa eta markatzaile adipogenikoen espresioa eragiten baitu [23]. Zelula barneko CAMP mailen igoerak fosforilazio bidezko CAMP-ren menpeko A proteina kinasaren (PKA) aktibazioa ahalbidetzen du, honek CREB-en aktibazioa eragingo duelarik. CREB CCAAT-ra lotzen den β proteinaren (CEBPβ) aktibatzailea da, CEBPδ-rekin batera adipogenesiaren hasierako funtsezko transkripzio faktorea dena [24] (2.irudia). 1991.urtean Cao eta kolaboratzaileek frogatu zutenez, CEBPβ eta CEBPδ-ren espresioa estimulu adipogenikoen ondorioz handitzen da, espresio horren maximoa lehen bi egunetan emango delarik [25]. Bestetik, Yeh eta kolaboratzaileek CEBPβ eta CEBPδ-ren espresio ektopikoak, beste inongo hormona adipogenikorik gabe, CEBPα-ren espresioa eragiten duela frogatu zuten, CEBP familiakoek adipogenesian duten ezinbesteko funtzioa aditzera emanez [26]. SREBP hausturak aktibaturiko c proteina (SREBP1c) ere transkripzio faktore garrantzitsua da, bere espresio ektopikoak adipogenesia eragiteko ahalmena baitu. Intsulinak SREBP1c-ren espresioa areagotzeko ahalmena du [27], arinki bada ere PPARγ-ren espresioa eraginez eta haren lotugaien ekoizpena estimulatuz [28,29].

Behin lehen fase hau bukatu dela, adipogenesi-bukaerako transkripzio faktoreen espresioa hasiko da. Horien artean, PPARγ eta CEBPα erregulatzaile garrantzitsuenak direla frogatu izan da. Ikerketa-lan ugarik, PPARγ-ren indukzioa adipogenesia hasteko beharrezkoa eta nahikoa dela erakutsi dute [30-32]. CEBPα-ren kasuan, antzeko ikerketek GEZ-ren eraketarako beharrezkotzat jotzen dute, baina ez ordea GEA-ren sorrerarako[33]. Beraz, PPARγ-k adipogenesia eragin dezake CEBPα-ren beharrik gabe, baina ez ordea alderantziz [34].

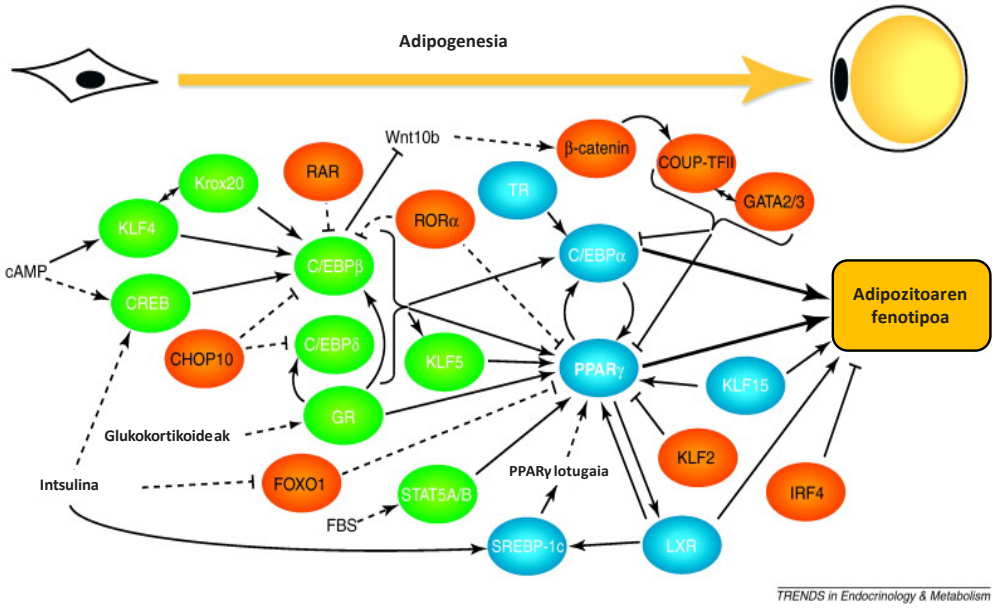
Behin PPARγ eta CEBPα aktibatuta daudela, batak bestearen espresioa mantentzen laguntzen du, hala nola adipozito desberdinduaren fenotipoa ere [29]. Bi faktoreen arteko elkarlanak adipozito helduetan

espezifikoak diren geneen espresioa eragiten du, batik bat triglizeridoen metabolismoan parte hartzen dutenak eta adipozitoen intsulinarekiko sentikortasuna handitzen dutenena, intsulinaren hartzaileak eta glukosaren 4. garraiatzailearena (GLUT4), kasu [35].



2. irudia. Adipogenesiaren bi faseetan ematen den espresio genikoaren profila. Tung eta kolaboratzaileetatik moldatua [36]. C/EBP: CCAAT-ra lotzen den (α,β,δ) proteina; PPARγ: peroxisomen ugalketarako γ hartzailea.

Mikroarrai eta denbora errealean gauzaturiko polimerasaren kate-erreakzio kuantitatiboa (RT-q-PCR) teknikak erabilita eginiko diferentziazio prozesuan zeharreko espresio genikoaren profilaren analisiak, beste hainbat faktore erregulatzaileen garrantzia agerian utzi du [37]. Horien adibide dira Krox20 (edo Egr2) [38], krüppel-aren antzekoa den faktorea (KLF) familiako kideak, 5A transkripzio faktorearen seinalearen transduktore eta aktibatzailea (STAT5A) [41] eta retinoidearen x hartzaileak (RXR) [42]. Testuinguru honetan, Siersbæk eta kolaboratzaileen ikerketa-lan batek [24] adipogenesiaren transkripzio-sarearen konplexutasuna sakonki azaltzen du (3.irudia).



3. irudia. Adipogenesiaren erregulazio transkripzioanala. Siersbæk eta kolaboratzaileetatik hartua. [24]. CHOP10: CEBP-ren proteina homologoa; FOXO1: forkhead box O1; RAR: RAR-ekin erlazionaturiko a zurtz hartzailea; COUP-TFII: oilaskoaren oboalbuminaren 2 transkripzio faktorearen ur-gorako eragilea; GATA2: GATA-ri lotzen zaion 2 proteina; IRF4: interferona erregulatzen duen 4 faktorea; GR: glukokortikoideen hartzailea; TR: hormona tiroideoaren hartzailea; LXR: gibeleko x hartzailea; FBS: behi-fetuaren seruma.

Adipozitoen diferentziazioa eragiten duten hainbat molekula daude, hala nola intsulina, glukokortikoideak, intsulinaren antzekoa den 1 hazkuntza hormona (IGF-1) eta cAMP [43], edo horien guztien konbinazioa. Izan ere, dexametasona (DEXA; glukokortikoideen agonista), isobutilmetilxantina (IBMX; cAMP-ren inaktibazioa ekiditen duena) eta intsulina dituen diferentzioazio medioa, 3T3-L1 zeluletan gehien erabiltzen dena da [44].

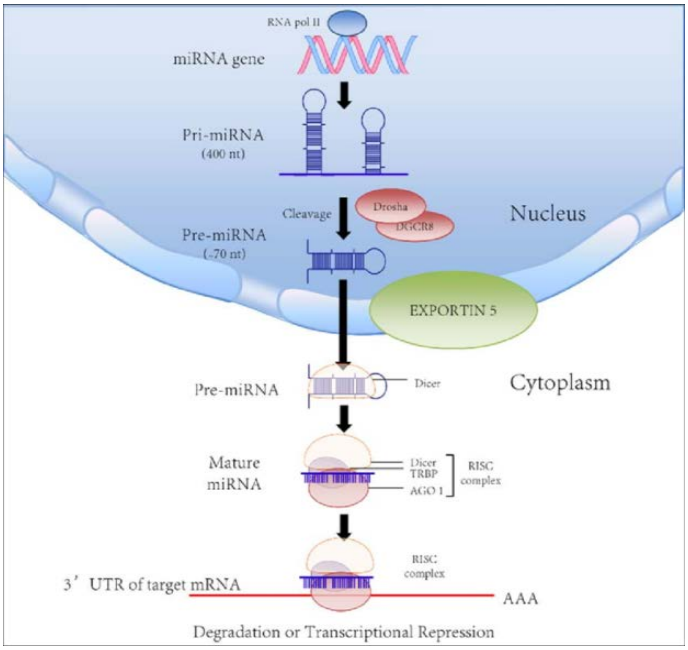
2.1.2 Adipogenesiaren erregulazio post-transkripzionala

Erregulazio post-transkripzionala behin transkripzioa hasi denean ematen den geneen gaineko espresioaren erregulazioa da. Erregulazio transkripzionala bezain ohikoa ez bada ere, gene askoren erregulazioan funtsezkoa da [45]. Erregulazio-mekanismo hauen artean splicing alternatiboa, azido ribonukleikoaren (RNA) edizioa, nukleotik RNA-ren garraioa edo indargabetze transkripzionala kokatzen dira.

Aurrekoaz gain, aldaketa post-translazionalak ere eman daitezke proteinen sintesia (itzulpen prozesua) eman ondoren, hala nola fosforilazioa, glukosilazioa, ubikitinazioa, nitrosilazioa, metilazioa, azetilazioa, lipidazioa eta proteolisi erreakzioak, besteak beste [46]. Ematen diren eraldaketa hauek proteinen funtzio eta ekintzen erregulazioaren gakoa dira, baita seinalearen transdukzio-bideetan ere.

Adipogenesiari dagokionez, PKA-ren fosforilazioak, horren ondorioz diferentziazioaren indukzioa emango delarik, erregulazio post-transkripzionalaren adibide garbia dira. PPARγ ere mekanismo post-transkripzionalaren bidez erregulatzen da, mitogenoak aktibaturiko protein kinasak (MAPK) haren aktibitatea inhibitu baitezake, haren Ser84 hondarraren fosforilazioaren ondorioz [47,48].

Era berean, adipogenesiaren erregulazio post-transkripzionala mikroRNA (miRNA) bidezkoa izan daiteke. Izan ere, haren erregulazioaren mekanismo garrantzitsua dela uste da. MiRNAk 20-22 nukeotido inguruko RNA-labur ez-kodifikatzaileak dira, geneen espresioa modu post-transkripzionalean erregulatzen dutenak. MiRNA-en gehiengoa geneen eremu intergeniko edo intronikoan kokatzen dira [49], baina haien transkripzioa gene horien zonalde eragileen apartekoa dela onartuta dago. RNA II (oro har) edo III polimerasek miRNA aitzindaria sintetizatzen dute, pri-miRNA deiturikoa, 400 nukleotido inguru dituena eta bi katez osatutakoa. Drosha endoerribonukleasak eta DG CR8-k osatutako konplexuak (konplexu mikroprozesatzailea) 70 nukleotidoz eratutako eta “stem-loop” moduan ezagutzen den egiturako pre-miRNA sortzen du, 5 esportina izendaturiko esportatzaile faktore nuklearraren bidez nukleotik zitosolera garraiatua izango dena. Ondoren, bigarren Dicer endoerribonukleasa batek 22 nukleotidotako RNA bikotea eratuko du, harietako bat miRNA-k eragindako isilpen-konplexura (RISC) atxikituko delarik, bestea degradatua den bitartean [50,51] (4.irudia).

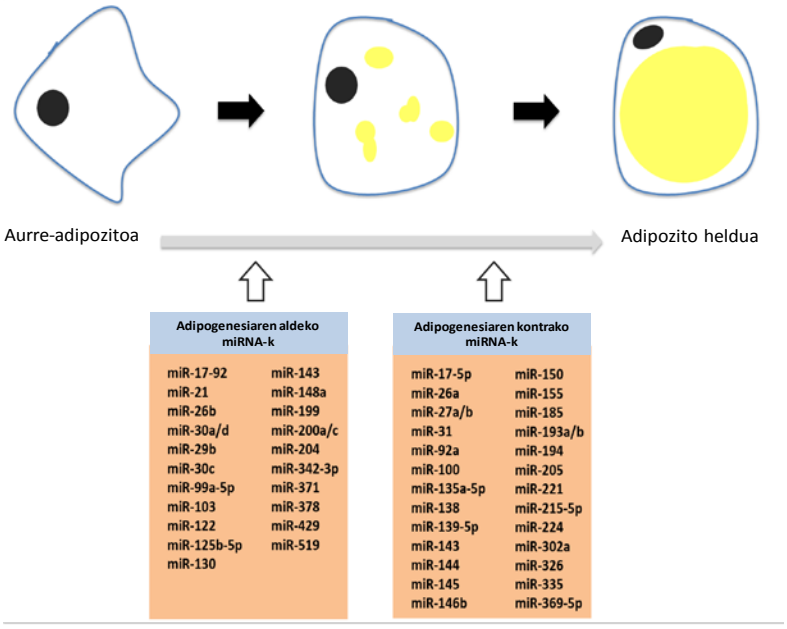


4. irudia. miRNA-en biogenesiaren irudikapena. Pisarello eta kolaboratzaileetatik hartua [50]. Pol II: II polimerasa; DG CR8: DiGeorge sindromearen zonalde kritikoaren 8 genea; RISC: miRNA-k eragindako isilpen-konplexua; TRBP: TAR RNA-ri lotutako proteina; AGO: 1 argonauta.

MiRNA-en erregulazioa, oro har, mRNA-ren translazioaren edo degradazioaren inhibizioan datza, nahiz eta bakoitzak egiten duen ekarpena ez den guztiz ongi deskribatu oraindik [52]. Hala ere, egun ondo ezagutzen da erregulazio mota miRNA eta itu-mRNA-ren arteko osagarritasunaren araberakoa dela. MiRNA-ren nukleotido kopuru minimoa (lehenengo 2-8 nukleotidoak) mRNA-ren 3´UTR eremuarekin lotu beharko da erregulazio post-transkripzionala eman ahal izateko. Horrez gain, lotuko diren nukleotido-osagarrien kopuruak ekintza-mekanismoa definituko du: guztizko lotura ematen bada mRNA-ren degradazioa emango da, aldiz lotura partziala ematen bada, proteinen itzulpen prozesua inhibituko da [53]. Aipatzekoa da gaur egun bi prozesu hauek sekuentzia moduan edo batera ematen diren ez dagoela garbi.

MiRNA-mRNA elkarrekintza eman ahal izateko nahikoa da nukleotido gutxi batzuk osagarriak izatearekin, eta horregatik, miRNA bakoitzerako aurkitu daitezkeen ahalezko itu-mRNA-k asko dira. Dena den, ez dago guztiz frogatua miRNA eta bere itu-mRNA-ren arteko antzematea nola ematen den, hau da, baldintza oso konkretuetan emandakoa edo zorizkoa den [54]. Hori dela eta, miRNA-en itu-mRNA-k identifikatzeko iragarpen konputazionala eta baliospen esperimentala barnebiltzen dituen prozedura ezinbestekoa da. Lehenik eta behin miRNA-en lotura posibleak (geneen 3´UTR eremuan) iragartzen dituzten algoritmoak analizatu behar dira [55]. Gerora, prozedura esperimentalen bidez bioinformatikoki aurreikusitakoa frogatu beharra dago, horien artean luziferasa gene ohartarazlearen entsegua [56] fidagarri era erabilienetariko bat delarik miRNA era mRNA-en arteko elkarrekintza emango den zehazteko. Modu osagarrian edo aldi berean egin daitekeena miRNA-k haren itu-proteinan duen efektua aztertzea da, miRNA-en gainespresio edo inhibizioa eragin eta gero itu-proteinen espresioa neurtzean datzana.

Azken urteotan, adipozitoen diferentziazioan zehar miRNA-en espresio profilean aldaketa ugari ematen direla ikusi izan da [57], gantz ehunaren eraketan ezinbesteko parte-hartzea duela iradokituta. Hipotesi hori frogatzeko nahian, Mudhasani eta kolaboratzaileek miRNA-en biogenesia inhibitu zuten saguen enbrioi-fibroblastoen eta aurre-adipozitoen kultibo primarioen Dicer entzimaren funtzioa ezabatuta, eta aurre-adipozitoen diferentziazio egokirako beharrezkoa dela egiaztatu zuten [58]. Ikerketa-lan honekin, beste batzuekin batera, euren itu-mRNA-tan adipogenesiaren aldeko efektua, adipogenesiaren kontrakoa edo bi funtzioak eragiten dituzten miRNA-k ezagutzera eman ziren (5.irudia). Adipogenesiaren lehen faseetan miR-155-ak efektu bikoitza duela ikusi da, CEBPβ-ren espresioa inhibitzen duenez adipogenesiaren kontrako efektua duelako, baina baita adipogenesiaren aldekoa ere azken batean CEBPβ-ren espresioa eragingo duen CREB-en espresioa handituz [23].



5.irudia. Adipogenesiaren aldeko eta kontrako zenbait miRNA. Zaiou eta kolaboratzaileetatik moldatuta [68].

CREB-k eragindako CEBPβ-ren espresioa modu zuzenean emateaz gain, modu ez-zuzenean ere gertatzen da, miR-448-ren itua den KLF5-aren indukzioaren ondorioz [59]. miR-33b ere giza-adipogenesian parte hartzen du, SREBP1c bere jomuga baita. Hala ere, kontuan hartzekoa da miR-33b ez dela karraskarietan espresatzen, hortaz, *in vitro* edo *in vivo* eginiko esperimentuen bidez miRNA horrek giza-adipogenesian duen eragina ikertzea zaila izanik [60]. Horren harira, aipatzekoa da gaur egun ez dagoela SREBP1c lotzen dituen deskribatutako miRNA-rik arratoi eta saguetan [61].

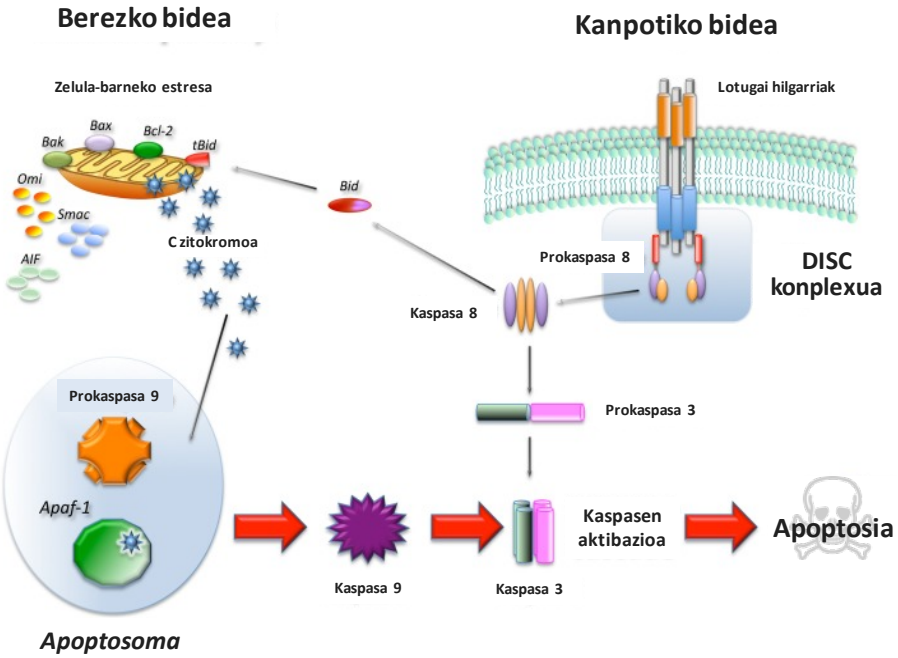
Lehen aipatu bezala, PPARγ adipogenesiaren funtsezko eragile moduan definitu izan da, eta hortaz, haren espresioa erregultzen duten miRNAk sakonki ikertu egin dira. Mir-27a eta miR-27b adipogenesiaren inhibitzaile garrantzitsuak bezala definitu dira, PPARγ-ren gain eragiten baitute [62,63], nahiz eta beste hainbat geneen espresioan ere eragin, CREB eta prohibitina (PHB) kasu [64-66]. Giza aurre-adipozitoekin eginiko ikerketek erakutsi dutenez, miR-130-ak ere adipozitoen diferentziazioa erregulatzen du [67].

2.2 Apoptosia

Apoptosia programatutako heriotza zelularra da, non zelulak auto-suntsitu egiten diren erantzun inflamatorioa edo ehun-kaltea eraginez. Homeostasi zelularraren parte da, tartekari entzimatikoen jauzi bat aktibatzearen bidez aukeratutako zelulak deuseztatzeko metodoa. Prozesu apoptotikoan zelulek hainbat gertakizun morfologiko eta biokimiko jasaten dituzte: zelula-mintzan anpuluak eratzea, plasmolisia eta horrek eragindako zelula-uzkurketa, kromatinaren agregazioa eta apoptosi-partikulak izena ematen zaien zelula-pusketen ekoizpena [69].

Ezberdinak baina bateragarriak diren bi mekanismok parte hartzen dute apoptosi-jauziaren aktibazioan. Heriotza-hartzailearen edo kanpotiko apoptosia, tumoreen nekrosiaren faktorearen hartzaileen (TNFR) familia-kideen, TNFR familiako 6. Kidea (Fas) esaterako, lotugai-bidezko aktibazioaren ondorioz hasten da. Behin aktibazioa emanda, zelula-mintzeko hartzaileek hainbat konformazio-eraldaketa jasaten dituzte, heriotza-hasieraren seinalizaziorako konplexuaren (DISC) lotura ahalbidetuko dutenak, horren ondorioz pro-kaspasa 8-ren bidezko kaspasa-jauziaren aktibazioa emango delarik [70]. Pro-kaspasa 8-k prozesu auto-katalitikoari hasiera emango dio, aktibaturiko kaspasa-8 asko eratu eta gainontzeko kaspasak, kaspasa 3 (cas-3) adibidez, aktibatzeari hasiera emanez [71].

Bestalde, oxidazio-estresa, azido desoxirribonukleikoaren (ADN) kaltea eta beste hainbat estres egoerek bide mitokondrial edo berezkoa estimulatuko dute. Kasu honetan, mitokondrio-mintzaren iragazkortzeak zitosolera zenbait proteinen (C zitokromoa batik bat) jariaketa eragingo du, kaspasa 9-ri atxikitzen zaion apoptosiaren proteasa aktibatzen duen 1 faktorea (Apaf-1) aktibatuko delarik. Apaf-1 – kaspasa 9 konplexua, kaspasa 9 beraren funtziorako beharrezkoa da [72], azken batean erreakzio kateko ondorengoko kaspasak aktibatuz eta zelula heriotzera eramanez (6.irudia). Funtzio mitokondrialaren erregulazioan gainera, B zelulen leuzemiak/2 linfomak (bcl2) ezinbesteko funtzioa du. Bcl2 apoptosi kontrako proteina Bcl2-ari elkartutako X proteinari (bax) lotzen zaio eta mitokondrioan sartzea eragoziko dio. Modu horretan, mintz mitokondrialaren iragazkortasuna bere onean mantenduko da eta C zitokromoa bezalako faktore apoptotikoen jariaketa ekidingo da [73]. Ondorioz, bcl2 eta bax proteina mailak erabakitzaileak dira mitokondrioaren funtzio egokirako eta berezko bide apoptotikoa aktibatu gabe mantentzeko. Bax/bcl2 ratioaren igoerak C zitokromoaren jariaketa eta cas3-ren aktibazioa suposatu lezake, azken batean apoptosia aktibatuz [74].



6. irudia. Apoptosiaren seinalizazioaren jauzia. Flavaloro eta kolaboratzaileetatik moldatuta [70]. BID: heriotzaren-agonista den BH3-ren interakzio-domeinua; OMI: HtrA 2 serina peptidasa; SMAC: Diablo, IAP-ari loturiko proteina mitokondriala; AIF: apoptosiaren faktore eragilea.

Tumoreen p53 ezabatzailea apoptosiaren erregulatzailea dela frogatu izan da, izan ere, 500 itu-gene erregulatu ditzakeela ikusi da [75]. Funtzio homeostatiko ezberdinak baditu ere, apoptosiari hasiera ematea da horietako bat, kanpotiko eta berezko bideetan jardungo duelarik. Are gehiago, p53-ak apoptosian duen eragina heriotzaren agonista den BH3-ren interakzio-domeinuaren (BID) gain eragiten duenean handiagoa dela ikusi da, BID bi bide apoptotikoen arteko lokailua den Bcl2 familiako proteina baita [76].

2.3 Lipolisia

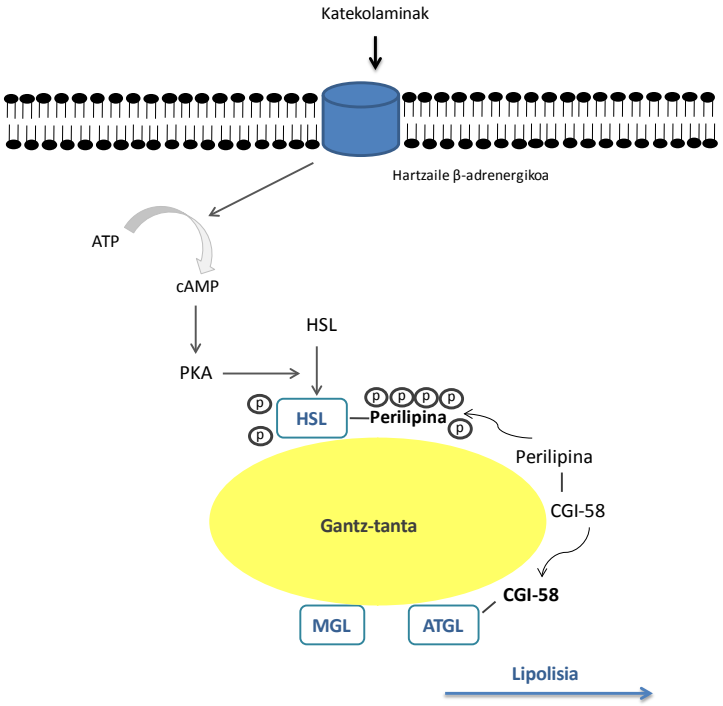
Lipolisia metatutako triglizeridoen zatiketa hidrolitikoa da, hiru lipasen jarduerari esker emango dena: gantz ehuneko triglizeridoen lipasa (ATGL), hormonekiko sentikorra den lipasa (HSL) eta monoazilglizerol lipasa (MGL) [77]. ATGL-ak gehien bat lehen erreakzioa katalizatzen du, triglizeridoen hidrolisia diazilglizerola eta GAA bat askatuz. HSL bigarren erreakzioaren katalizatzaile nagusia da, diazilglizerola (DAG) hidrolizatuz monoazilglizerola (MAG) eta beste GAA bat askatuz. Azkenik, MAG glizerola eta hirugarren GAA moduan hidrolizatuko da MGL entzimari esker. Hiru erreakzioei esker glizerol molekula eta hiru GAA zitosolean askatuko dira, bertan birresterifikatu edo odolera jariatuko dena beste ehunetara garraiatua izateko.

HSL-a, 1960.urtean adrenalinari sentikorra den entzima lipolitiko bezala definitu zen, eta ATGL-aren aurkikuntzararte, lipolisiaren erregulatzaile nagusia zela uste izan zen [78]. HSL-k triglizeridoak hidrolizatzeko ahalmena badu ere, DAG-engatik duen afinitatea 10 aldiz handiagoa da [79]. ATGL hiru ikerketa-taldek identifikatu zuten 2004.urtean [80-82], triglizeridoak hidrolizatzeko kapaza zen lipasa moduan. Bereziki GEZ eta GEA-n aurkitu daitekeen entzima da, nahiz eta beste ehun batzuetan ere topatu daitekeen [78]. MGL 1970.urtean aurkitua izan zen, MAG-en hidrolisirako beharrezkotzat jota eta puntu honetako entzima mugatzailea dela ikusita [84]. MAG-en hidrolisian garrantzi handia badu ere, ez du gainotzeko molekulak hidrolizatzeko gaitasunik.

Lipolisiaren erregulazioa

Lipolisiak egoera nutrizionalaren araberako erregulazioa du, gorputzaren energia falta edo energia gehiegikeriari aurre egiteko mekanismo egokitzailea bezala. Egoera basalean lipido-tantaren gainazalean kokatzen da, eta era ez-aktiboan egonda ere, aktibitate minimoa du [82]. HSL zitoplasman eta fosforilatu gabe aurkitzen da egoera honetan, inolako ekintza lipolitikorik gabe.

Gure gorputzak, energiaren beharra duenean, hainbat hormonen jariaketa bultzatuko du, bide metaboliko honen erregulatzaile nagusiak diren katekolaminenak kasu [85,86]. Katekolaminek, euren hartzaile adrenergikoekin elkarrekintzan, erreakzio-jauzia eragiten dute, cAMP-ren mailak handituta PKA-ren aktibazioa eraginez. Ondoren, PKA-k fosforilazioen bitartez HSL-ren aktibazioa eragingo du zitosoletik lipido-tantaren gainazalera haren garraioa eraginez, non perilipinarekin elkarlana bultzatuko duen. HSL eta perilipinaren arteko interakzioak perilipinaren fosforilazio anitza ekarriko du berarekin, genearen 58. konparaketa izendatzaileak (CGI-58) perilipina askatu eta ATGL-arekin elkartuko dela lipolisiari hasiera emateko [87] (7.irudia).



7. irudia. Katekolaminek eragindako lipolisiaren aktibazioa adipozitoetan. ATP: adenosina trifosfato; cAMP: adenosina 3',5'-monofosfato ziklikoa; PKA: A proteina kinasa; HSL: hormonekiko sentikorra den lipasa; p: fosfato taldea; CGI-58: genearen 58. konparaketa izendatzaileak; ATGL: gantz ehuneko triglizeridoen lipasa; MGL: monoazilglizerol lipasa.

Otordu baten ostean, intsulinak lipolisia inhibituko du bide ezberdinen bidez. Alde batetik, adipozitoaren mintzean intsulina bere hartzailearekin elkartzean, fosfatidilinositol 3-kinasaren (PI3K) bidetik igorriko da energiaren beharrik ez dagoelaren mezua. Ondoren, Akt aktibatu eta 3 fosfodiesterasaren (PDE3) gain eragingo du, cAMP degradatu eta azken batean PKA-ren aktibazioa blokeatuz. Guzti honen ondorioz, ez dira lipasak kitzikatuko eta lipolisia inaktibatuta egongo da [88]. Bide horretaz gain, cAMP-ren menpekoa ez den lipolisi kontrako intsulina bidea deskribatu da, non 1 proteina fosfatasa (PP1) fosforilatzen den eta horren ondorioz HSL inaktibatu defosforilazioz [89].

2.4 Lipogenesia

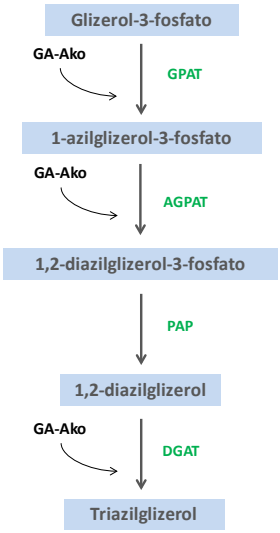
Gantz ehunean ematen den lipidoen metaketa bi prozesuren araberakoa da. Alde batetik, odoleko kilomikroi eta dentsitate oso baxuko lipoproteinetatik (VLDL) triglizeridoak hartzea, lipoproteina lipasari (LPL) esker emango dena. Entzima hau gantz ehunaren endotelio baskularrean, bihotzean edo muskuluan kokatzen da, non GAA eta 2-monoazilglizerolak emango dituen triglizeridoak hidrolizatzean. Odoleko GAA-en jasotzea, bai lipoproteinetan daudenak zein albuminari lotuta garraiatzen direnak, proteinek erreztutako garraioaren bidez ematen da, horien artean txatar-biltzaile 36 hartzailea (CD36) aurkitu daitekeelarik [90]. Coburno eta kolaboratzaileek ikusi zutenez, bihotzean, muskulu eskeletikoan eta gantz ehunean CD36-ren falta zuten saguen GAA-en hartzea kaltetuta zegoen, eta ondorioz, odoleko triglizerido eta GAA-en maila oso handituta [91]. CD36-az gain, gantz azidoetara lotzen den proteinak (FABP) eta gantz azidoen proteina garraiatzaileak (FATP) GAA-en zelularako garraioan parte hartzen dute. Behin adipozitoetan, gantz azidoak triglizeridoak eratzeko biresterifikatzen dira [92].

Karbohidratoak ere, lipogenesiaren iturri izan daitezke. Odoleko glukosa mailak handitzean intsulina jariatzen da odolera, azken batean zeluletan glukosaren jasotzea ahalbidetuko duen erreakzio-jauzi bat emango delarik. Onartuta dago gaur egun adipozitoek glukosa GLUT4-aren bidez hartzen dutela, kontuan hartzekoa delarik garraiatzaile hau muskulu eskeletikoan, kardiomiozitoetan, eta kantitate txikiagoan garun eta giltzurrunetan ere aurkitzen dela [93]. Hartu den glukosak glukolisia jasan dezake, Krebs zikloan sartuko den pirubatoa emango duena, edo energia gehiegikerian *de novo* lipogenesiaren sustratua izan daiteke.

De novo lipogenesia prozesu metaboliko bat da non ehunek azetil-AKo-tik hasita gantz azidoak sintetizatzeko gaitasuna duten, azetil-Ako hori glukosatik, aminoazidoetatik edo gantz azidoen metabolismotik eratorri daitekeelarik. Mekanismo honen bidez gure gorputzak gehiegizko karbohidratoen bidez hartutako energia lipido moduan metatu dezake, hauek modu eraginkorragoan pilatzen baititugu, aipatzekoa izanik animalietan gibel eta gantz ehunean ematen dela batik bat [94]. *De novo* lipogenesia emateko, azetil-Ako molekulen beharra dago, baina baita nikotinamida adenina dinukleotido fosfatoarena (NADPH) ere, ahalmen erreduzitzailea duen kofaktorea. NADPH, glukosa-6-fosfato dehidrogenasa (G6PDH), malato dehidrogenasa, entzima malikoa (ME) eta NADP isozitrato dehidrogenasa entzimek, besteak beste, katalizatutako erreakzioetatik eratorria da.

Krebs ziklotik datorren azetil-Ako molekula mitokondrioan kokatzen da, baina zitosolera garraiatzen da zitrato-anezkaren mekanismoaren bidez [95]. Behin zitosolean, azetil-Ako malonil-Ako molekula bilakatuko da karboxilazioz, azetil-Ako karboxilasa entzimari esker adenosina trifosfatoren (ATP) presentzian. Ondoren, malonil-Ako molekulatik hasita, eta gantz azidoen sintasa (FAS) entzimaren funtzioari esker, kate luzeko gantz azidoak sortuko dira, palmitatoa (16 karbonotako gantz azidoa) bereziki. Horretarako, NADPH-k katalizaturiko errepikatutako sekuentziazko erreakzioa eman beharko da, eta sortutako gantz azidoak asegabetze, luzatze

eta esterifikatze erreakzioak jasango ditu triglizerido moduan metatua izan aurretik [96]. Triglizeridoak eratzeko, hiru gantz azido-Ako molekula glukolisitik eratorritako glizerol-3-fosfato molekularekin esterifikatuko dira DAG aziltransferasa (DGAT) entzimei esker (8.irudia). Bi DGAT entzima daude, DGAT1 eta DGAT2, biek parte hartzen dutelarik aipaturiko prozesuan. Nahiz eta biek triglizeridoen esterifikazioaren azken pausuan parte hartu, haien proteina-sekuentzia eta funtzio biologikoa ezberdina da [97]. DGAT2 entzimaren funtzioa berezko gantz azidoen sintesi eta esterifikazioarekin erlazionatzen den bitartean, DGAT1 hidrolizatutako triglizeridoen birziklapenaz arduratzen da, gantz azidoak biresterifikatuz.



8. irudia. Triglizeridoen sintesiaren bide metabolikoa. Ahmadian eta kolaboratzaileetatik moldatua [98]. GPAT: glizerol-3-fosfato aziltransferasa; AGPAT: 1-azilglizerol-3-fosfato aziltransferasa; PAP: azido fosfatidikoaren fosfatasa; DGAT: DAG aziltransferasa.

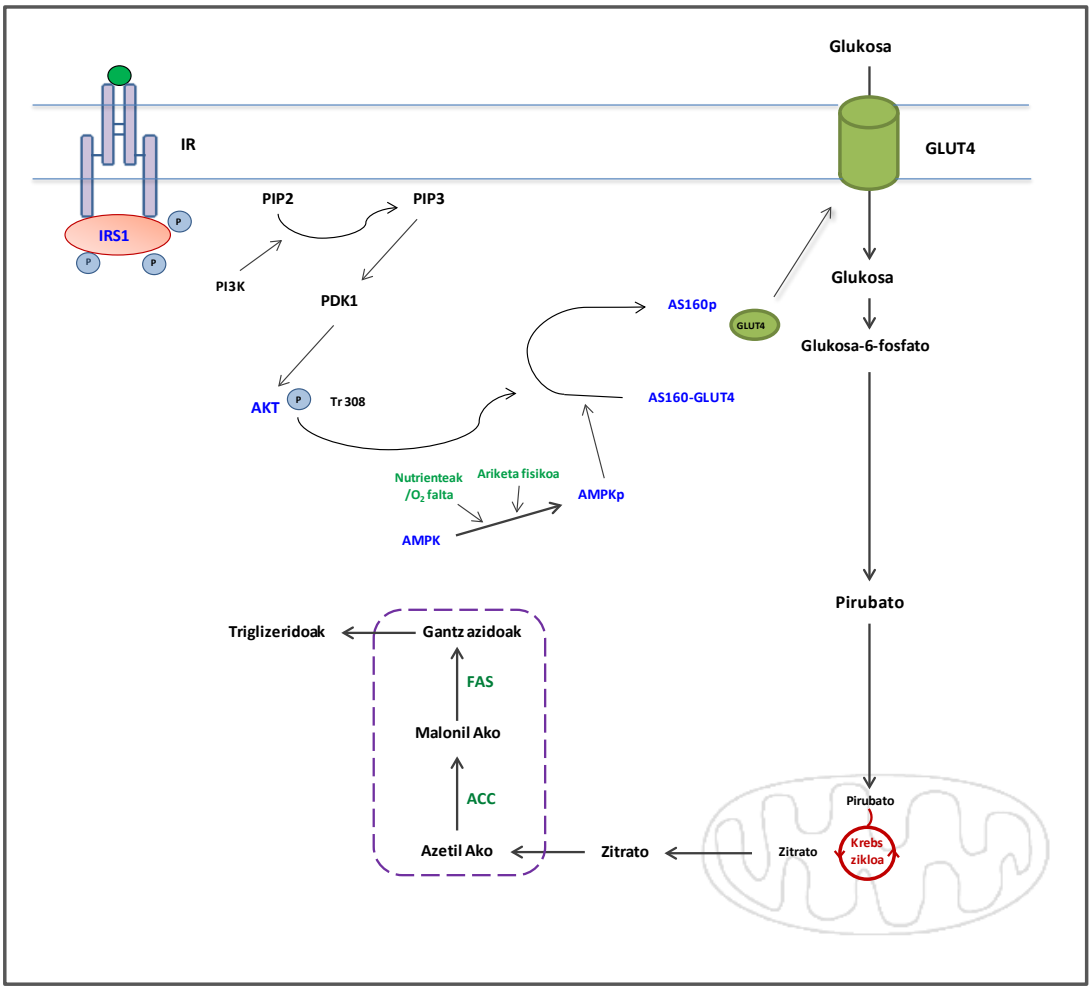
Lipogenesiaren erregulazioa

ACC eta FAS *de novo* lipogenesiaren entzima mugatzaileak dira [95], batik bat mekanismo transkripzional eta post-transkripzionalen bitartez erregulatzen direnak. Entzima hauen lehenengo erregulatzaileak SREBP1c eta karbohidratoen elementu erantzuleari lotzen zaion proteina (ChREBP) dira, gantz ehunean eta gibelean espresatzen direnak [99,100]. SREBP1c, esterolaren elementu erantzulearen bidez gene ugariren espresioa erregulatzen duen transkripzio faktorea da, geneen zonalde eragilean kokaturik [101]. Aldi berean, zenbait kofaktoreren bidez erregulatua izan daiteke, Sp1 transkripzio faktorea (Sp1) kasu, zeinak SREBP1c-ren zein zonalde eragilean kokatzen den arabera efektu aktibatzailea edo inhibitzailea izan dezakeen. Ariketa fisikoa edo KF bezalako zenbait estimuluren aurrean, AMP-k aktibaturiko proteina kinasak (AMPK), zelularen metabolismoa erregulatzen duen energia-sentsorea, bide metaboliko katabolikoak estimulatzen ditu (β-oxidazioa adib.) eta anabolikoak inhibitu (*de novo* lipogenesia adib.). AMPK aktibatzeak SREBP1c-ren inhibizioa eragingo du, azken batean FAS erreprimituta [102], bestetik modu zuzenean ACC fosforilatu eta

inaktibatu egingo duelarik [103]. Hortaz gainera, glukosak berak lipogenesia modulatu dezake ChREBP-en bidezko gene lipogenikoen espresioa erregulatuz [104,105].

Lipogenesia modu zuzen edo ez-zuzenean erregulatzen duten beste hainbat elementu ere badaude. PPARγ-k adibidez, gene lipogenikoen eta LPL-ren espresioa erregulatzen du, azken honek odoleko triglizeridoetatik datozen gantz azidoetarako sarrera moduan jokatzen duena [106,107]. Gibeleko x hartzaileak (LXR) ere *de novo* lipogenesia zuzenean erregulatu dezake FAS-aren zonalde eragileari lotuta, edo modu ez-zuzenean SREBP1c eta ChREBP-en aktibazioa eraginez [104].

Lehen aipatu bezala, *de novo* lipogenesian garrantzi handia dauka glukosaren kaptazioak. Egoera basalean, 160 KDa dituen AKT-aren substratuak (AS160) GLUT4 barnebiltzen duten zelula-barneko besikulak atxikitzen ditu, egoera basalean glukosa bereziki GLUT1-aren bidez hartzen baitu zelulak [108,109] (9.irudia). Aldiz, odoleko glukosa mailek gora egiten dutenean, intsulinak zelula-mintzan kokatzen diren hartzaileetara lotzen da, horrek hartzailearen 6 hondarren fosforilazioa eragingo duelarik. Tyr 960 glukosaren homeostasian eragiten duen intsulinaren hartzailearen hondar garrantzitsuena da. Intsulinaren substratuaren hartzailearen (IRS) isoforma guztietatik, 6-k ezagutuko dute intsulina hartzailea, IRS1 izanik guztietan garrantzitsuena [111]. IRS1 intsulinaren hartzaileari atxikitzen zaionean auto-fosforilatu egiten da, eta ondorioz, beste proteinentzako lotura-uneak eratzen dira. Lotu daitezkeen proteina horien artean fosfatidilinositol 3-kinasa (PI3K) familiako kideak daude, PI3K bidearen bidez seinalea garraiatuko dutenak [112,113]. IRS1 intsulinaren hartzaileari lotuta dagoela, PI3K-k fosfatidilinositol 4,5-bifosfato (PIP2) fosforilatuko du haren 3. kokapenean, modu horretan fosfatidilinositol 3,4,5-bifosfato (PIP3) sortuz, azken batean fosfoinositidoaren menpeko 1 proteina kinasaren (PDK1) aktibazioa eraginez [114]. Erreakzio guzti hauen ondoren Akt-ren aktibazioa emango da, PDK1-k Thr 308 hondarrean fosforilatuko baitu. Akt-k AS160-ren fosforilazioa eraginez GLUT4-a barnebiltzen duten besikulak zelularen gainazalera abiatuko dira, glukosa zelula-barnera sartzeko prest egongo delarik. Azaldutako bide honetaz gain, badago intsulinaren estimulazioaren menpekoa ez den bidea, non AMPK-k AS160-ren fosforilazioa eragiten duen AMP/ATP erratioan aldaketak eman direnean, ariketa fisikoa edo baraua bezalako estimuluek eraginda [115].



9. irudia. Intsulinaren seinalizazioa, glukosaren meatabolismoa eta *de novo* lipogenesiaren eskema. IR: intsulinaren hartzailea; PIP2: fosfatidilinositol 4,5-bifosfato; PIP3: fosfatidilinositol 3,4,5-bifosfato; PI3K: fosfatidilinositol 3-kinasa; PDK1: fosfoinositidoaren menpeko 1 proteina kinasa; Akt: protein kinase B (PKB); AS160: 160 KDa dituen AKT-aren substratua; AMPK: AMP-k aktibaturiko proteina kinasa; ACC: azetil-Ako karboxilasa; FAS: gantz azidoen sintasa.

DGAT1 eta DGAT2 entzimak mekanismo transkripzionalez eta post-transkripzionalez, fosforilazioz alegia, erregulatu daitezkela ikusi da, nahiz eta horren arduradun izan daitezkeen transkripzio faktore espezifikoak ez diren oraindik topatu [97]. Transkripzionalki ematen den DGAT1 eta DGAT2-ren erregulazioa sagu eta arratoien muskulu eskeletiko eta gibelean ikertu da [116,117]. Ikerketek diotenez DGAT-ren aktibitatea egoera nutrizionalaren eta hormonen arabera modulatu daiteke, izan ere, arratoiei glukagoia edo epinefrina eman zaienean ehun ezberdinetan kokatutako DGAT-ren murrizketa ikusi baita [118-120]. Bestalde, arratoien dietan kate luzeko gantz azidoak gehitzean, edo hauen gibeleko kultibo primarioekin eginiko tratamenduetan, DGAT-ren aktibitatearen areagotzea ikusi da [121,122]. Ekintza-mekanismoari dagokionean, Assifi eta kolaboratzaileek AMPK DGAT entzimen erregulazioan parte hartu zezakeela hipotetizatu zuten [122].

Gainera, DGAT1-aren kasuan azetil-Ako-ak alosterikoki erregulatu dezakeela uste da, haren eremu N-terminalari lotuta [97].

2.5 Gantz azidoen oxidazioa

Gantz azidoen oxidazioa energia lortzeko gantz azidoen degradazio-prozesuari deritzo. Peroxisomek oxidazio-ahalmena badute ere [123], batez ere mitokondrietan ematen da. Bide metaboliko honen lehenengo fase mugatzailea gantz azidoak mitokondrietan barneratzea da, 3 pausutan ematen den prozesua.

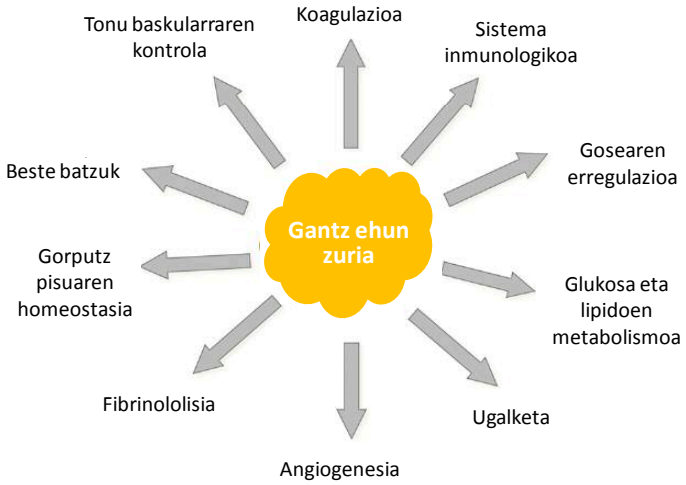
Lehenik eta behin, gantz azidoa Ako taldearekin esterifikatuko da, azil-Ako sintasa entzimak katalizatuko duena ATP molekula baten presentzian. Ondoren, azil-Ako molekulatik hasita azil-karnitina sortuko da, karnitina 1 palmitoiltranferasa (CPT1) entzimaren bidez mitokondrioaren barne-mintzetik mitokondrio-matrizera garraiatuko dena, pausu hau gantz azidoen oxidazioaren fase mugatzailea izanik. Azkenik, azil-karnitina mitokondrio-matrizera eramango da berriz ere gantz azidoa-Ako molekula sortuz, CPT2 entzimak katalizaturik. Erreakzio honetan karnitina molekula askatzen da eta zitosolera bueltatuko da translokasa bati esker. Behin gantz azidoa mitokondrioan dagoela β-oxidazioa hasiko da, azil-Ako dehidrogenasa, enoil-Ako hidratasa, 3-hidroxiazil-Ako dehidrogenasa eta β-ketotiolasa entzimek katalizaturiko 4 erreakzioren bidezko gantz azidoen degradazioa. Erreakzio hauen ondorioz flabin adenina dinukleotido ($FADH_2$), NADH (arnasketa-katera abiatuko dena ATP ekoizteko) eta azetil-Ako molekula eratuko dira, azken hau Krebs zikloaren sustratu nagusia izango dena.

Adipozito zuriek mitokondrio gutxi dituzte, duten ahalmen oxidatiboa oso baxua baita. Normalean, GEZ-k lipolisiaren ondorioz askatutako GAA-k odolera jariatzen ditu beste ehun batzuek oxidatu ditzaten. Aldiz, adipozito arrek mitokondrio ugari dituzte eta hortaz, gantz azidoak oxidatzeko duten gaitasuna handia da [124]. Hainbat ikerketek frogatu dutenez, bi adipozito motak trukagarriak dira: gantz ehuna bai lipidoak metatzeko zein termogenesirako lipidoak erretzeko moldatu daiteke zenbait estimulupean.

2.6 Gantz ehunaren funtzio endokrinoa

Betidanik gantz ehunari soilik energia pilatzeko funtzioa esleitu zaio, lipidoak triglizerido moduan pilatu eta energia falta dagoenean energia ematen duen ehuna. Azken urteotan eginiko ikerketen emaitzek, aldiz, uste hau aldarazi dute eta gaur egun gorputz-homeostasian funtzio garrantzitsua duen ehun konplexua dela jakina da [125]. Bai adipozitoek zein gantz ehunean dauden beste zelula batzuek ere, funtzio jariatzaile garrantzitsua dute. Adipokinak (adipozitoek jariaturiko molekulak) funtzio biologiko anitzak eragiten dituzten proteina eta peptido bioaktiboak dira, gantz ehunean bertan (funtzio autokrino edo parakrinoa) edo odolaren bidez garraiatuak izatean beste ehun batzuetan (funtzio endokrinoa) eragin ditzaketela euren efektuak.

Adipokinek prozesu fisiologiko asko modulatzen dituzte, hala nola gosearen erregulazioa, glukosa eta lipidoen metabolismoa, intsulinaren seinalizazioa, funtzio kardiobaskularra, funtzio inmunologiko eta inflamatorioa eta ugalketa funtzioa, besteak beste [126] (10. Irudia).



10. irudia Adipokinen funtzio fisiologiko nagusiak. Coelho eta kolaboratzaileetatik hartua [127].

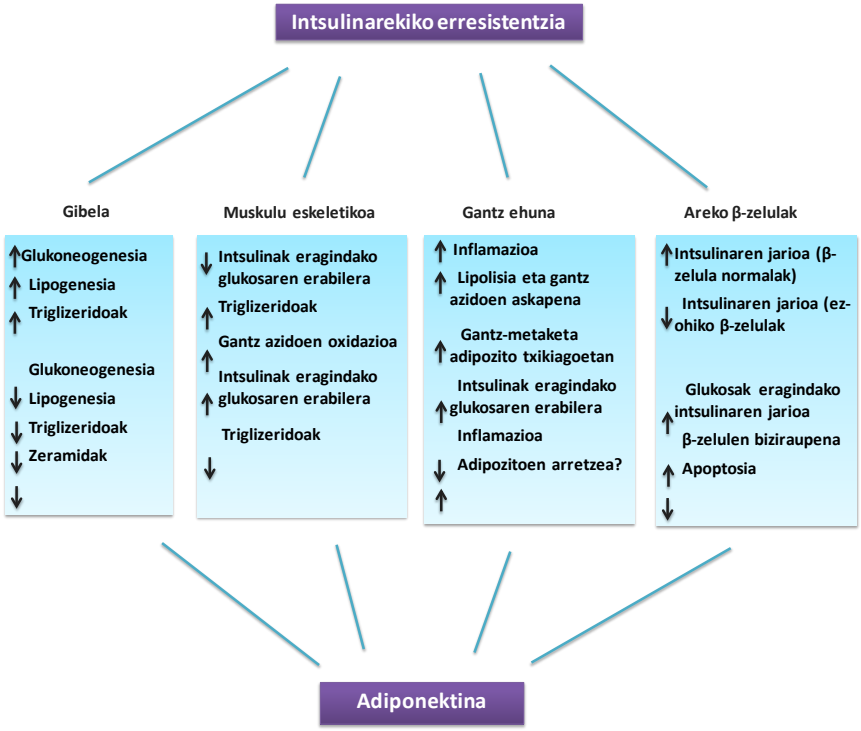
Obesitatea eta haren ko-erikortasunak gantz ehun handituak eragindako maila baxuko hanturarekin estuki erlazionaturik daude, hanturaren aldeko edo kontrako adipokinen jariaketan desorekak ematen baitira [128]. Are gehiago, adipokinak sindrome metabolikoaren egoera inflamatorioa, intsulinarekiko erresistentzia eta endotelioaren disfuntzioaren arteko lotura izan daitezkeela aditzera eman izan da. Adipokinak gantz ehun, muskulu eskeletikoa eta gibelaren intsulinarekiko erresistentziaren erantzule direla uste da, sistema inmunologikoan funtzio erregulatzailea izateaz gain. Gainera, adipokina pro-inflamatorioek aterosklerosiaren garapenean eragina dute, azken batean gaixotasun kardiobaskularrak pairatzeko arriskua handituz [129,130].

2.6.1 Adiponektina

Adiponektina 1995. urtean aurkitu zen lehen aldiz, adipozitoek bakarrik, eta kantitate handian, jariatzen zutela ikusiz [131]. Gizakiek odolean 5-10 µg/mL adiponektina dugu, plasma-proteinen %0.01 dena [132]. Adiponektinaren hartzaileak bi motakoak dira, nonahi aurkitu daitekeen AdipoR1 eta oro har gibelean kokatzen den AdipoR2 [133].

Sindrome metabolikoan zituen onurak aztertuta ezarri zen adiponektina eta osasunaren arteko erlazio onuragarria [132]. Gantz ehunaren masa eta adiponektinaren ekoizpena alderantziz proportzionala dira, obesitatea duten pertsonek odolean duten adiponektina kantitatea baxua dela ikusi den aldetik [134]. Behaketa hau garrantzitsua da odoleko adiponektina maila altuak osasunean dituen efektu mesedegarriekin erlazionatu baitira, bereziki diabetesaren garapenean duen efektu babesgarria nabaria delarik [133, 135].

Adiponektina intsulinarekiko erresistentzian lagungarria da, Ruan eta kolaboratzaileetatik hartutako 11. irudian laburbiltzen diren mekanismoei esker (11.irudia). Sagu osasuntsu eta diabetikoei adiponektina eman zaienean, odoleko glukosa mailak murriztu egiten direla ikusi da [135,137], era berean ikerketa kliniko batean adiponektina maila eta barauko odoleko glukosa maila eta GMI-aren arteko korrelazio negatiboa aurkitu delarik [138]. Ikerketa-emaitzek erakutsi dutenez, adiponektinak diabetesaren kontrako efektua glukosa eta lipidoen metabolismoan duen efektuagatik egiten du, muskulu eskeletikoaren metabolismo lipidikoan eragiten baitu biogenesi mitokondriala eraginez eta gantz azidoen kaptazioa eta oxidazioa handituz [139]. Gainera, adiponektinak zenbait entzima glukoneogenikoen espresioa inhibitzen du, gibeleko glukosaren ekoizpena murriztuz [135]. 2007. urtean, Chow eta kolaboratzaileek odoleko adiponektina mailak hipertentsio arteriala iradokitu zezakeela ikusi zuten, adipokinak presio arterialaren erregulazioan duen garrantzia goraipatuz [140]. Egun, ezaguna da ezarritako harreman hori adiponektinaren hanturaren kontrako efektua eta aterogenesia ekiditeagatik ematen dela [141].



11. irudia. Adiponektinak intsulinarekiko erresistentziaren hobekuntza eragiteko dituen ekintza-mekanismoak. Ruan eta kolaboratzaileetatik moldatua [136].

2.6.2 Leptina

Leptina Zhang eta kolaboratzaileek aurkitu zuten 1994. urtean [142]. 16 kDa-eko proteina da, *ob* (saguetan) eta *LEP* (gizakietan) geneek kodifikatutakoa, LEPR eta Ob-R zitokina hartzaileen familiako I motako hartzaileetara lotzean eragingo dituela haren efektu fisiologikoak. Hartzaile hauek *db* geneak kodifikatzen ditu

eta sei isoformetan aurkitu daitezke, genearen splicing alternatiboak eraginda. Leptina gantz ehunak ekoiztu eta jariatu egiten du nagusiki, baina muskuluak, garunak, urdailak, plazentak, ugatzetako epitelioak eta hainbat fetu-ehunek ere produzitzen dute [143-148].

Leptina sistema neuroendokrino eta inmunologikoaren arteko lotugaitzat hartzen da, T_1 zelulen ugaritzea eta zitokinen ekoizpena handitzen baititu [149]. Aipatutako funtzioetatik harago, leptinaren funtzio nagusia energia homeostasiaren eta gosearen erregulazioa da [150], egoera nutrizionalaren mezulari moduan eginez hipotalamoa informatzen baitu, non bertako neuropeptidoek aipatutako eginkizun nagusia erregulatzen duten. Baraualdian edo gorputz-gantzaren murrizketa dagoenean, odoleko leptina mailak baxu mantentzen dira, gosea eraginez eta energia-gastua murriztuz. Ordea, gantz kantitatea handitzen denean odoleko leptina mailek ere gora egingo dute, langa hematoentzefalikoa gurutzatuz hipotalamora iritsi eta bertako neuropeptidoengan eragingo duelarik. Aldi berean, leptinak peptido orexigenikoak (Y neuropeptidoa (NPY) eta agouti-rekin erlazionaturiko proteina (AgRP) adib.) ihibitzen ditu eta anorexigenikoak (proopiomelanokortina (POMC) eta kokainak eta anfetaminak erregulaturiko transkritoa (CART) adib.) estimulatu, modu horretan elikagaien ingestioa murriztuz eta energiaren gastua handituz [150, 151]. Obesitatea duten pertsona edo karraskarietan leptinak eragindako funtzio nagusiak eragiten ez dituen hiperleptinemia dago, hau da, leptinarekiko erresistentzia deituriko fenomenoa ematen ari da. Egoera honetan, leptinak ezin du langa hematoentzefalikoa gurutzatu edo zelula-barneko leptinaren seinalizazioan akatsen bat dago [152-154].

2.6.3 Visfatina

Adipokina honen izena ingelerazko “visceral fat”-etik dator, eginiko lehen ikerketetan erraien inguruko gantza eta visfatinaren odoleko mailaren arteko harremana zehaztu baitzen [155]. PBEF/Visfatin geneak kodifikatzen duen 52 kDa-eko proteina da, nahiz eta hasiera batean erraietako gantz ehunak sintetizatzen zuela pentsatu, egun leukozito, gantz ehuneko makrofagoak, hepatozitoak eta miozitoek ere ekoizten dutela jakina da [156, 157]. Bi kokalekutan agertu daiteke, zelula-kanpoan eta zelula-barnean. Zelula-barneko formak nikotinamida adenina dinukleotidoaren (NAD) biosintesiaren lehenengo pausuan entzima moduan jarduten du, sirtuinen bidezko histonen deazetilazioan, heriotza zelularra eta oxigenoaren espezie erreaktiboen (ROS) eraketan ezinbesteko funtzioak dituelarik. Zelula-kanpoko formak zitokina eta adipokina funtzioak ditu nagusiki, hala nola zelula-kanpoko aktibitate entzimatikoa [158]. Kontuan hartuta visfatinak zelula-funtzio asko dituela, ikerlariek gaixotasun askoren garapenean eragin zezakeela hipotetizatu zuten, ateroesklerosia, minbizia, gaitz inflamatorioak, 2 motako diabetesa eta obesitatea, besteak beste [159-163]. Visfatinak intsulinaren antzeko funtzioa (intsulina-mimetikoa) duela guztiz frogatuta dago.

Eginiko ikerketa batean saguei zain-barnean visfatina gehitu zitzaien eta odoleko glukosa-mailak txikiagotzen zituela ikusi zuten, murrizketa dosiaren menpekoa izanik. Gainera, intsulina faltan duten sagu diabetikoetan visfatinak eragindako gluzemiaren murrizketa intsulinaren berdina zela ikusi zuten, visfatina intsulinaren hartzaileetara lotzeko (ez intsulinak lotzen duen eremu berean) duen gaitasuna dela eta, intsulinaren seinalizazio-jauzia aktibatuz [156]. Obesitateari dagokionean, Choi eta kolaboratzaileek Koreako emakume obesoek odoleko visfatina maila altuagoak zituztela ikusi zuten, beti ere pisu egokia zutenekin alderatuta, eta pisu galera eraginez visfatina maila horiek tarte egokira bueltatzen zirela [164]. Gainera, *in vitro* eginiko ikerketetan visfatina adipozitoen ugaritzean eta diferentziazioan eragiten duela ikusi izan da [156, 165].

2.6.4 Apelina

Apelina 8.5 kDa-eko mintz-zeharreko proteina da, apln geneak kodifikatua [166]. 2005.urtean Boucher eta kolaboratzaileek GEZ-k sintetizaturiko proteina bezala deskribatu zuten, adipozitoen diferentziazioan haren mailak handitzen direla ere ikusita [167]. Gizakietan, apelina adipozito, kondrozito, zelula endotelialak, bihotza, garuna, barea, timoa eta biriketan kantitate altuan espresatzen da, hala nola muskulu eskeletikoan, kantitate txikiagoetan bada ere. Apelinaren sakabanaketa zabalak funtzio fisiologiko askotan eragin dezakeela iradokitzen du, metabolismo energetikoa, presio arteriala, angiogenesia eta obesitatea, diabetesa, minbizia eta bihotz-gutxiegitasunean, kasu [168]. 2013.urtean Krist eta kolaboratzaileek 2 motako diabetesa zuten pazienteen gantz ehunean apelina gehiago espresatzen zela ikusi zuten, hala nola apelinaren odoleko mailak GMI eta gantz masarekin erlazionatzen zirela. Are gehiago, paziente horiek pisua galtzean apelinaren espresioa ere murriztu egiten zen [169]. Bestalde, apelinak lipolisia inhibitu dezakeela ikusi da [170], baina era berean, esan beharra dago ez dagoela ebidentzia nahikorik eta ikerketa-lan gehiagoren beharra dagoela.

Glukosaren metabolismoarekin erlazionaturik, 2 motako diabetesa duten pertsonetan apelinaren mailak aztoratuta daudela ikusi da, intsulinarekiko sentikortasunean duen garrantzia aditzera emanez. Hau dela eta, ikerketa ugari egin da harreman honen ebidentzia sendotzeko. Zhu eta kolaboratzaileek apelinak 3T3-L1 adipozitoen glukosaren kaptazioa hobetzen zuela ikusi zuten, era berean hobekuntza hau dosiaren menpekoa zela eta mekanismoari erreparatuta, PI3K/Akt bidearen bidez emandako GLUT4-ren translokazioari esker ematen zela [171].

2.6.5 Beste adipokina batzuk

Aipatu diren adipokinez gain, esan beharra dago badaudela adierazgarritasun handia jaso duten beste adipokina batzuk ere, izan ere, egun 50 adipokina baino gehiago identifikatu baitira [128]. Horien artean,

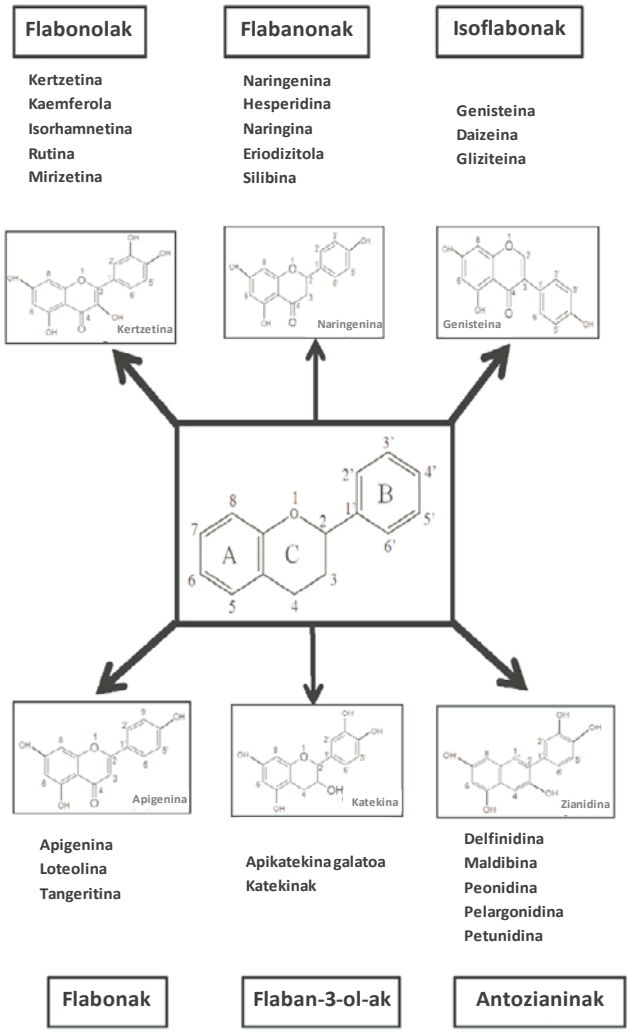
erresistina mailak obesitatea eta diabetesaren eredu diren animali karraskarietan handituta daudela ikusi izan da. Adipogenesian zehar espresatzen hasten den adipokina da eta adipozito, miozito eta beste zenbait zelulen glukosaren kaptazioa murrizten duela ikusi da, intsulinaren aurkako efektua erakutsiz [172]. **Kemerina** proteina kimiko-erakargarria da, gorputzeko funtzio inmunologikoan ezinbestekoa da eta adipozitoen garapenean ere inplikatua. Lotura hori frogatzeko intentzioarekin, Goralski eta kolaboratzaileek diferentziazioan zeuden 3T3-L1 aurre-adipozitoetan kemerina eta haren hartzailea isilarazi zituzten "knock down" tekniken bitartez, eta adipogenesiaren aztoraketa, lipido eta glukosaren homeostasia erregulatzen duten geneen espresioaren inhibizioa eta adipozito helduen bereizgarriak diren funtzioen asaldura ikusi zuten [173]. **Fibroblastoen hazkuntza faktoreak (FGF)** familiak funtzio metaboliko ugari erregulatzen ditu, zehazki FGF-19, FGF-21 eta FGF-23 behazun azidoen, D bitaminaren, kolesterolaren eta glukosaren homestasiaren erregulazioan parte hartzen dute [174-176]. FGF-21-ak adipozitoen glukosaren kaptazioa estimulatzen duela ikusi da, termogenesia, energiaren xahutzea eta lipidoen erabilera estimulatzeaz gain [176]. **Omentina** ere adipokina garrantzitsua da, bereziki erraietako gantz ehunean espresatzen dena eta giza-odolean erraz detektatu daitekeena. Intsulinaren efektuaren erregulatzailea zela uste izan da urteetan zehar, baina egun , beste funtzio metaboliko batzuk ere dituela ikusi izan da. Pertsona Japoniarrekin eginiko ikerketa batean, autoreek omentinaren odol-maila baxuak gerri-perimetro handituarekin, dislipemia, presio arterial altua eta glukosarekiko intolerantziarekin erlazionatzen zela ikusi zuten [177].

Aipatu diren adipokinak erregulatzaile metabolikoak diren adipokina askoren parte bat baino ez dira. Obesitatea eta haren ko-erikortasunen tratamenduan lagungarri izan daitezkeen adipokina berriak identifikatzeko esfortzu handia egin bada ere, ikerketa-lan gehiagoren beharra dago.

3. Konposatu fenolikoak: metabolismoa eta funtzio biologikoak

Landare-jatorriko elikagaiak gaixotasun ugariren prebalentzia murrizten duten KF-en iturri garrantzitsua dira. Elikagai askotan agertzen badira ere, iturri garrantzitsuenetarikoak fruta eta barazkiak, te berde eta beltza, ardo gorria, kafea, txokolatea, oliba eta oliba olioa, sukaldatzeko belarra, ongailuak, intxaurrak eta algak dira [178-179]. Zenbait KF elikagai konkretuetan agertzen dira, soiaren isoflabonak esate baterako, baina gehienak beste batzuekin batera daude elikagaietan kantitate aldakorretan. Are gehiago, kanpo-faktore asko daude elikagai mota berean KF-en kantitatea aldarazi ditzaketenak, hala nola kultibo mota, eguzkiarekiko esposizioa, fruten heltze puntua eta biltegiratzea [180,181].

KF flabonoide eta ez-flabonoide moduan sailkatzen dira, aldi berean azpitalde ugaritan ere sailkatzen direlarik, eta taldekatzea eraztun fenolikoen kantitatearen eta haiei lotutako taldeen arabera egina dago [182]. Flabonoideak flabonol, flabon, proantozianidina, antozianidina, flaban-3-ol (edo katekinak) eta isoflabona moduan sailkatzen dira [183] (12. irudia), ez-flabonoideak azido hidroxizinamiko, tanino hidrosolugarri, azido hidroxibenzoiko eta estilbeno moduan banatzen diren bitartean. Polifenol izenak fenol talde bat baino gehiago duen konposatuari egiten dio erreferentzia, eta kontuan izanda flabonoide guztiek 3 fenol talde dituztela, flabonoide guztiei polifenol esan ohi zaie. Ez-flabonoideak aldiz ezin dira horrela izendatu, talde fenoliko bat edo bi dituzten molekulak baitaude talde horretan.

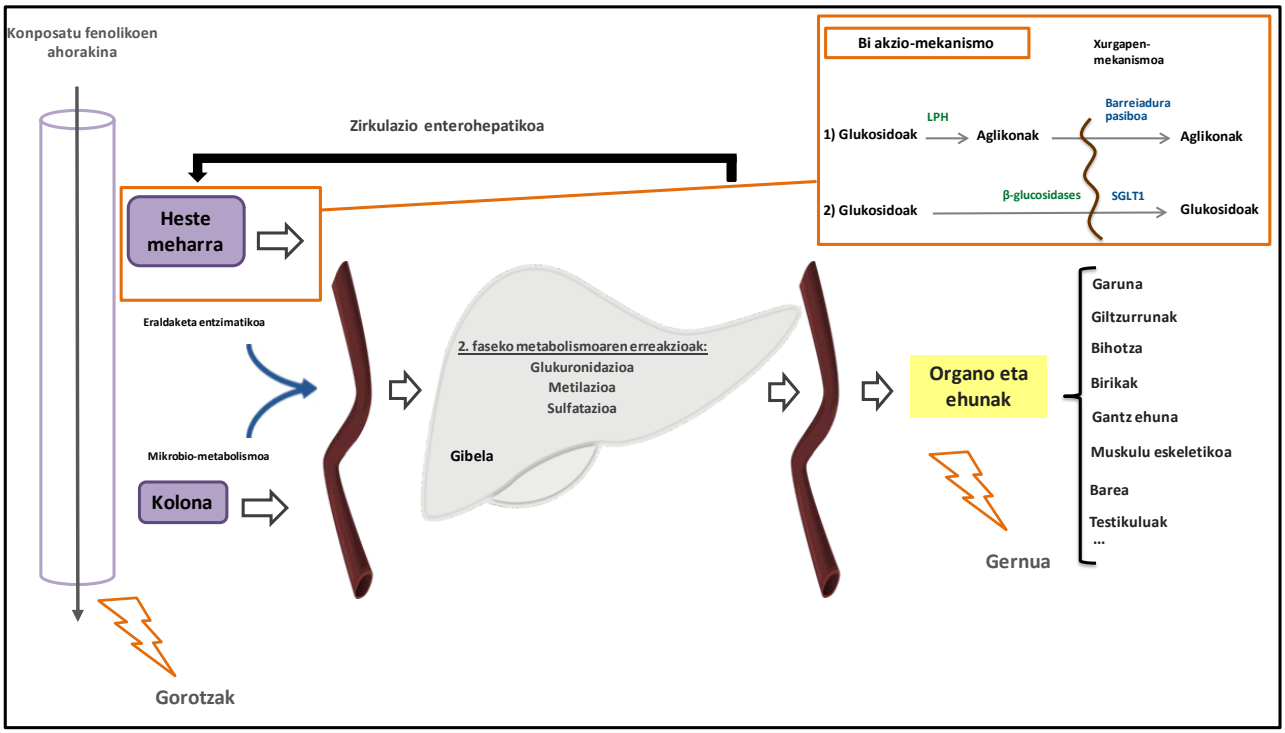


12. irudia. 6 azpitaldeetan eginiko flabonoideen sailkapena, non molekulak eta egitura kimikoen adibideak adierazten diren. Hossain eta kolaboratzaileetatik moldatua [184].

Gizakietan dietatik hartutako KF kuantifikatzeko metodo ezberdinak daude, hala nola elikagaien konposizioa adierazten duten datu-baseak, beti ere KF-en konposizioa erregistratuta dutenean. Informazio honek, elikagaien maiztasun galdeketa edo 24 orduko oroitzapenekin konbinatuta, KF-en ingestioaren kalkulu teorikoa egiten lagunduko digu [185]. Bestalde, odolean, gernuan edo gorotzetan agertzen diren metabolitoen neurketa baliabide egokia da KF-en ingestioa balioztatzeko. Kontuan izanik metodologia bakoitzak dituen indargune eta mugak, bi metodoekin lortutako datuen konbinazioa modurik zehatzena da KF-en ingestioa kalkulatzeko, ikerketa ugaritan egiaztatu den bezala.

Azken hamarkadetan eginiko ikerketetan lortutako datuek erakutsi dutenez, KF-en ingestioa 1.000-1500 mg/pertsona/egun ingurukoa da [186,187], ikerketa batzuek balio baxuagoen berri eman badute ere [188]. Era berean, aipatzekoa da faktore ezberdinek haien bioerabilgarritasunean eragiten dutela, hala nola kanpo-faktoreek (eguzkiarekiko esposizioa, adib.), elikagaien prozesatzearekin erlazionaturikoek (egosketa,

laberatzea, etab.), elikagaiarekin loturikoek (elikagai-matrizea, adib.), beste konposatuekin ager daitezkeen elkarrekintzek (antzeko absortzioa duten polifenolak, adib.), molekularekin erlazionaturiko faktoreek (egitura kimikoa, adib.) eta ostalariarekin lotutako faktoreek (hesteetakoak, adib.) [179]. KF hartu bezain laster metabolizatzen dira (13.irudia), eta prozesu hau antzekoa da gizaki eta karraskarietan ezberdintasun txiki batzuk kenduta. Lehenik eta behin, heste meharrean xurgatu aurretik aglikona formara hidrolizatu behar izaten dira, elikagaietan forma glikosilatuan agertzen baitira. Hidrolisi hori emateko bi mekanismo deskribatu izan dira: lehenengoan, zelula epitelialetan kokatzen den laktasa floridizina hidrolasa (LPH) entzimak KF deglikosilatzen ditu eta modu horretan, aglikona askeak epitelio-zeluletara difusioz barneratzeko prest egongo dira [189]. Bigarren mekanismoan, glukosidoak sodioaren menpeko glukosaren 1 garraiatzailearen (SGLT1) bidez garraiatuko dira, zitosoleko β-glukosidasaren laguntzaz [190]. Heste meharrean xurgatu ez diren konposatuak koloneraino iritsiko dira eta bertan xurgatuko dira egiturazko eraldaketa askoren ondoren. Kasu honetan koloneko mikrobiota ezinbestekoa da, xurgatu aurretik glikosidoak aglikona bihurtzen arduratzen baitira [191]. KF-en hidrolizazioan ematen den norbanakoaren ezberdintasuna erabakigarria da haien bioerabilgarritasunean, izan ere, lehen aipaturiko ostalariarekin lotutako faktore garrantzitsuena baita.



13. irudia. Polifenolen metabolismoaren eskema. LPH: laktasa floridizina hidrolasa; SGLT1: sodioaren menpeko glukosaren 1 garraiatzailea.

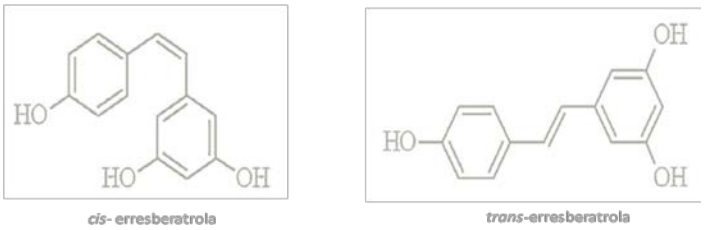
Polifenolek konjugazio erreakzioak jasaten dituzte eta lehenengoa hauek odolera iritsi aurretik gertatzen da, enterozitoetan hain zuzen ere. Behin odol-zirkulazioan, polifenolak porta zainaren bidez gibelera iritsiko dira, bertan konjugazioaren 2. erreakzioa emango delarik [180]. Konjugazio-erreakzioen artean

glukuronidazioa, metilazioa eta sulfatazioa dira garrantzitsuenak, xenobiotikoen metabolismoaren II. faseko erreakzioak. Modu horretan, haien balizko efektu toxikoa murrizten da eta horrela ondorengo gernu eta gorotzen bidezko iraizketa errazten da erraztuz ko. Polifenolak behazun-azidoekin batera jariatzen direnean zirkulazio enterohepatikoan sar daitezke eta heste meharrean birxurgatuak izan [192]. Uridina-5´- difosfato glukuronosiltransferasa (UGT) entzimak UDP-azido glukuronikotik polifenoletara azido glukuroniko taldea eramaten du, lehenengo enterozitoetan eta ondoren gibelean jasotzen den erreakzioa izango delarik. UGT entzimak ehun ugaritan espresatzen dira, zehazki zelulen erretikulu endoplasmatikoan. Katekol-O-metiltransferasa (COMT) entzimek metilazio erreakzioak katalizatzen dituzte, non metilo taldea adenosil-metioninatik polifenoletara transferitzen den. COMT entzimak ehun askotan agertzen diren arren, gibel eta giltzurrunetan dute aktibitate handienetarikoa [193]. Azkenik, nagusiki gibelean gertatzen diren sulfatazio erreakzioak daude, non sulfotransferasak (SULT) polifenolen talde hidroxilora fosfoadenosina fosfosulfato taldea garraiatzeaz arduratuko diren. Konjugazio-erreakzioen ondorioz ehun eta organo ezberdinetara iritsi daitezkeen metabolito asko eratuko dira, egitura kimiko ezberdina dutenez bestelako aktibitate biologikoa izan dezaketela kontuan hartuta. Adibide moduan, kertzetinaren metabolizazioaren ondorioz 20 metabolito ezberdin aurkitu izan dira gizakietan [195, 196].

Gibelean emandako 2. metabolizazioaren ondoren, polifenolak ehun ezberdinetara bideratu daitezke edo iraitziak izan. Zenbait polifenol-konjugatu behazun azidoekin heste meharraren argira jariatuko dira, eta xurgatuak izan ezean, gorotzen bidez kanporatuko dira. Bestetik, odol-zirkulazioan aurkitzen diren hainbat polifenol-konjugatu gernuaren bidez iraitziko dira [192].

3.1. Erresberatrola

Erresberatrola (3,4’,5-trihidroxiestilbenoa) Takaoka eta kolaboratzaileek aurkitu zuten lehenengoz 1939.urtean, *Veratrum grandiflorum* deituriko landarean hain zuzen ere [197]. Erradiazio ultramorea edo onddo, bakterio edota birusek eragindako estresari aurre egiteko landareek era naturalean ekoizten duten estilbenoa da [198], besteak beste mahatsa eta eratorritako edarietan (ardoa eta mahats-zukua), kakahuetean eta baietan aurkitu daitekeelarik. Molekulak etileno-zubi batez loturiko bi eraztun fenoliko ditu, eta horregatik bi isomero formatan aurki daiteke, *cis*-erresberatrola eta *trans*-erresberatrola. Azken hau da biologikoki aktiboena eta hargatik, gehien ikertu dena [199] (14. irudia). Aipatzekoa da ere naturan haren forma glukosidoa (pizeidoa, glukosa molekulari lotua) dela gehien aurkitzen dena.



14. irudia. Erresberatrolaren bi forma isomeroak. Salehi eta kolaboratzaileetatik moldatua [199].

Erresberatrolak gizakietan dituen efektu onuragarriak 1982. urtean deskribatu ziren lehen aldiz behaketa-ikerketa batean, non neurrizko ardo beltzaren kontsumoaren eta gaixotasun kardiobaskularren prebalentziaren arteko alderantzizko harremana ikusi izan zen [200]. Emaitza horiek ikusita mahatsaren konposatuen analisia egin zen, erresberatrola izanik efektu biologiko horien erantzule moduan egiaztatu zutena. Hamarkada bat beranduago, 1991. urtean Serge Renaud frantziar zientzialariak ikusitakoa "Frantses-paradoxa" bezala izendatu zuen [201]. Urteetan zehar antioxidatzaile, hantura aurkako, inmunomodulatzaile, intsulina- sentikortzaile eta minbiziaren kontrako efektuak esleitu zitzaizkion, gaixotasun kardiobaskularrak, minbizia eta diabetesa bezalako gaixotasunetan eragin positiboak izan zitzakeela aldarrikatuz [202, 203]. Horrez gain, erresberatrolak obesitatearen aurkako efektua ere izan dezakeela ikusi da, gorputzeko gantza mekanismo ezberdinei esker murrizten baitu: gosearen erregulazioa, zenbait nutrienteen absortzioaren eraldaketa, adipozitoen bizi-zikloaren doitzea, gantz ehuneko hanturaren murrizketa, funtzio mitokondrialaren erregulazioa, termogenesiaren aktibazioa eta mikrobiotaren modulazioa [204]. Hala eta guztiz ere, duen bioerabilgarritasun baxua zientzialarien kezka handia da, odol eta ehunetara iristen den konjugatu gabeko erresberatrol kantitatea oso txikia baita.

3.1.1. Erresberatrolaren ingestio dietetikoa, absortzioa, sakabanaketa eta metabolismoa

Dietaren bidez hartzen dugun erresberatrol kantitatearen estimazioa egitea erreza ez den arren, Europa mailan estilbenoen kontsumoa 2-3 mg/egun ingurukoa dela balioztatu da, kantitate horren %50 erresberatrol eta pizeidoari dagokiolarik [187]. Erresberatrolaren %70 inguru heste meharrean xurgatzen da, difusio pasiboz edo mintz plasmatikoaren proteinek garraiatuta, integrinak esate baterako [205]. Odolean glukuronido eta sulfatatu forman agertu ohi da (3. eta 4. kokaguneetan konjugatuta batez ere), enterozitoetako II. faseko konjugazio-erreakzioen ondorioz. Aldiz, I. fasekoak diren erreakzioak, hala nola oxidazioa, erredukzioa eta hidrolisia ez dira polifenol honentzat deskribatu. Konjugaturiko molekulez gain, konjugatu gabeko erresberatrolaren kantitate txiki bat ere odolera iristen dela dirudi. Gizaki eta animalietan ikusi izan denez, erresberatrola ahoratu eta 30 minututara agertzen da odolean kantitate handienean, [206-208], albumina, odol-zelula eta dentsitate baxuko lipoproteinei (LDL) lotuta, ondoren gibelera abiatuko delarik porta zainaren bidez [209-211]. Enterozitoetan bezala, erresberatrolak II. motako konjugazio erreakzioak jasango ditu, UGT entzimek katalizatutako glukuronidazioak eta SULT entzimek katalizaturiko sulfatazioak.

Bigarren konjugazio erreakzio horien ondoren, erresberatrolaren metabolitoak ehun ezberdinetara banatuko dira.

Odolean, erresberatrolaren metabolitoak erresberatrola baino 20 aldiz kontzentrazio altuagoan agertzen direla ikusi izan da eta forma glukuronido eta sulfatatuak dira gizakietan kantitate altuagoan agertzen direnak [207,208,212-214]. Are gehiago, erresberatrola-3-O-glukuronido (3G), erresberatrola-4´-O-glukuronido (4G) eta erresberatrola-3-O-sulfato (3S) dira kantitate handienetan aurkitutako metabolitoak [215, 216]. Gutxi gorabeherako kalkuluek diotenez, 25 mg erresberatrol gehigarria hartu duten gizakien odolean agertzen den erresberatrol kantitatea 40 nM baino gutxiago da, haren metabolitoen kantitatearekin batuta 2 µM-eko kontzentrazioa lortzen bada ere [207,217]. Hala ere, beste ikerketa batzuetan kantitate altuagoak ikusi izan dira, Ardid-Ruiz eta kolaboratzaileek eginiko lanean esaterako, non erresberatrolaren gehigarriak jaso zituzten Wistar arratoien odolean 4G metabolitoa 20 µM-eko kontzentrazioan aurkitu zuten [218]. Gizakietan eginiko beste ikerketa batean ere, banaka erresberatrolaren metabolitoen kontzentrazioa neurtuta, bakoitzaren kontzentrazioa 10 eta 20 µM bitartekoa zela ikusi zuten [216]. Ehun eta organoetan metabolito glukuronido eta sulfatatuen proportzioa ehun eta dosiaren menpekoa dela ikusi da eta espezie bakoitzaren araberako metabolismoak ere baldintzatuko du. Horren adibidea Juan eta kolaboratzaileek arratoiekin eginiko ikerketa da, non metabolito glukuronidoak kantitate oso altuetan aurkitu ziren testikulu eta gibelean, biriketan aldiz kantitate txikiak kuantifikatu ziren bitartean [219]. Gainera, erresberatrol-gehigarriekin hartutako dosiak gora egin ahala, odolean topatutako metabolito glukuronidoen kantitatea murriztu egiten da, sulfatatuenak gora egiten duen bitartean [218]. Azkenik, aipatu beharra dago espeziearen araberako desberdintasunak daudela metablismoan: arratoietan metabolito sulfatatuak ugarienak diren bitartean, gizakietan glukuronidoak dira kantitate handienetan topatzen direnak [208,212].

3.1.2. Erresberatrola eta bere metabolitoak obesitatearen kontrako molekula bezala: lipido eta karbohidratoen metabolismoan dituzten efektuak.

Erresberatrola obesitatearen kontrako molekula bezala aspalditik definitu da eta horretarako ekintza-mekanismo desberdinak jarraitzen ditu: adipogenesiaren inhibizioa, lipogenesiaren murrizketa, apoptosia eta lipolisiaren estimulazioa edo mitokondrioen funtzioa eta termogenesia areagotzea [220-224]. Erresberatrolaren funtzio nagusienetariko bat energia murrizketaren efektu mimetikoa da, 1 sirtuinaren (SIRT1) aktibazioaren ondorioz. SIRT1 sirtuinen familiako zazpi kideetako bat da, energia murrizketak eragindako zahartze-prozesua eta bizi-itxaropenaren luzapena erregulatzen dituena [225]. Kontuan izanik energia murrizketa gehiegizko kaloria hartzearen efektu desiragaitzak (gantzaren gehiegizko metaketa eta intsulinarekiko erresistentzia) ekiditeko tratamendu dietetikoan ezinbestekoa dela, efektu honen imitatzaileak diren molekulak topatzea guztiz interesgarria da. SIRT1-aren efektu biologikoen artean, energia

metabolismoaren erregulazioan duen funtzioa nabarmena da, muskuluko glukoneogenesian eta gibeleko glukolisian parte hartzen duten entzimen espresioa handituko duen peroxisomen ugalketaren 1α hartzailearen (PGC1α) aktibazioa, deazetilazioz, eragiten baitu. Are gehiago, energia murrizketa ematen denean PGC1α-ren aktibazioak mitokondrien biogenesia eta gantz azidoen oxidazioaren areagotuko du muskuluan [226]. Gainera, SIRT1-a intsulinarekiko erresistentziaren hobekuntza, adipogenesiaren inhibizioa, lipolisiaren aktibazioa eta gantzaren mobilizazioarekin erlazionatu da PPARγ-rengan duen efektua dela eta [227-229].

Erresberatrolaren metabolitoek SIRT1-en aktibazioan duten efektua ez da orain arte adipozitoetan aztertu. Dena den, 2015. urtean Schueller eta kolaboratzaileek metabolito glukuronido eta sulfatatuek giza U937 makrofagoetan SIRT1-en espresioa areagotzen zutela ikusi zuten [214]. Beste ikerketa batean, Calamini eta kolaboratzaileek aktibitate fluorometrikoaren teknika erabiliz metabolito sulfatatuak (3S eta erresberatrola-4-O-sulfato-4S-) giza SIRT1-aren eraketa aktibatzeko gai zirela ikusi zuten, eta biek erresberatrola bezain besteko aktibazioa eragiten zutela ere [230].

Aurreko atalean azaldu den bezala, hilezkortutako zelula-lerroekin eginiko *in vitro* ikerlanak oso baliagarriak dira molekula batek, erresberatrolak kasu, **adipogenesian** duen efektua ikertzeko. Izan ere, arlo honetan eginiko esperimentu asko sagutik eratorritako 3T3-L1 zelula-lerroan egin izan da. CEBPβ, PPARγ, CEBPα eta SREBP1C transkripzio faktoreek adipogenesian duten rol garrantzitsua kontuan izanda, erresberatrolak haien espresioarengan duten efektua luze ikertu da. Erresberatrolaren dosi eta tratamendu-iraupen ezberdinekin diferentziazioan dauden adipozitoetan gantz-metaketaren murrizketa ahalbidetzen duten gene horien espresioa murrizten dela ikusi da [220,231,232]. Are gehiago, sagu eta giza-adipozitoekin eginiko ikerketek jakinarazi duten bezala, erresberatrolak aurre-adipozitoen ugaritzean eta diferentziazioan duen efektua SIRT1-en menpekoa da [227,233].

Lipogenesiaren murrizketa ere erresberatrolaren obesitatearen aurkako efektuaren parte da. Adipozitoaren biologia azaltzen den atalean aipatu den bezala, lipogenesiaren iturriak odoleko triglizeridotan aberatsak diren lipoproteinetatik (kilomikroiak eta VLDL) harturiko gantz azidoak eta intsulina haren hartzailearekin lotu ostean adipozitoetan barneratutako glukosa izan daitezke. *In vitro* eta *in vivo* eginiko ikerketek erakutsi dutenez, erresberatrolak lipogenesiaren entzima garrantzitsuenak diren FAS eta ACC-ren murrizketaren bidez (maila transkripzional eta post-transkripzionalean) eragiten du bide metaboliko honetan, hala nola lipidoen sintesirako glukosaren erabileran eraginez [220, 233-237]. Adipozitoen sustratu lipogenikoak GAA eta glukosa direnez, **intsulinaren seinalizazioaren jauzia** eta **glukosaren kaptazioa** aintzakotzat hartzea oso garrantzitsua da. Erresberatrola odoleko glukosa mailak jeitsi eta intsulinarekiko erresistentzia hobetzeko gai dela ikusi da animalietan [223,238-240]. Efektu horien erantzule izan daitezkeen mekanismoen artean *in vitro* ikusitako intsulinak eragindako glukosaren kaptazioaren areagotzea eta

intsulinarekiko sentikortasunean eragiten duten adipokinen, adiponektina edo erresistina besteak beste, ekoizpenaren aldaketak daude [241, 242].

Ikerketa askotan lipolisiaren areagotzea ikusi da erresberatrolarekin eginiko tratamenduak eta gero [222,227,234,243,244]. 3T3-L1 eta SGBS zeluletan burututako esperimentu batean, Lasa eta kolaboratzaileek erresberatrolak lipolisia aktibatzeko ATGL entzimaren espresioa mekanismo transkripzional eta post-transkripzionalen bidez erregulatzen zuela adierazi zuten [245]. Zucker eta Sprague-Dawley arratoiekin eginiko bi ikerketetan ordea, erresberatrolak *hsl* genearen espresioa handiagotzen zuen *atgl*-renean eragin gabe [243, 244]. Aipaturiko hiru ikerlanen baldintza esperimentalak ezberdinak izatea kontraesan hauen erantzule izan badaiteke ere, azpimarratzekoa da oro har ez dagoela erresberatrolak mekanismo honengan duen efektuaren inguruko akordiorik, ikerketa gehiagoren beharra dagoela agerian utziz.

Apoptosia erresberatrolak obesitatearen aurka eragiteko duen beste mekanismo bat izan daiteke. 3T3-L1 eta txerri-adipozitoekin gauzatutako esperimentuetan erresberatrolak efektu apoptotikoa 50 µM baino dosi altuagoekin erakutsi zuen [220,246-250]. Esaterako, dosi-menpeko apoptosi efektua duela ikusi da, AMPKα-ren aktibazioaren bitartez AKT-ren inhibizioa eraginda eta horren ondorioz emandako apoptosiaren berezko bidea (mitokondriala) estimulatuta [250]. Gainera, sagu eta txerri adipozitoetan ikusitakoaz gain, erresberatrolaren efektu apoptotikoa giza SGBS zeluletan ere ikusi izan da [251].

Erresberatrolaren hantura-kontrako efektua ere proposatu da zenbait ikerlanetan, hala nola, **adipokinen jariaketarekin** erlazionaturiko efektu biologikoak. Leptinari dagokionez, aurrerago aipaturiko Ardid-Ruiz eta kolaboratzaileen ikerketan, erresberatrolak gorputzeko gantz murrizketaren efektua hein batean gibela, muskulu eskeletikoa eta gantz ehunaren leptinarekiko sentikortasuna hobetuz egin zezakeen ikusi nahi izan zuten. Lortutako emaitzen arabera, erresberatrolak leptinarekiko sentikortasuna berrezarri zuen dietak eragindako arratoi obesoen hiru ehunetan [218]. Adiponektina erresberatrolarekin erlazionatutako beste adipokina bat da. Jimoh eta kolaboratzaileek frogatu zutenez, untxi hiperkolesterolemikoetan erresberatrolarekin eginiko tratamenduak adiponektinaren mailak igo eta leptinarenak murriztu zituen, odoleko intsulina mailak ere murriztu ziren bitartean [252]. Hala ere, esan beharra dago Mohammadi-Sartang eta kolaboratzaileek ikerketa-klinikoekin gauzatutako ebaluazio sistematiko eta meta-analisi batean ez zirela aipatutako efektu onuragarri horiek egiaztatu ahal izan, erresberatrola hartu ondorengo odoleko adiponektina mailan emaitza sendoak lortu badira ere [253].

Visfatinak duen intsulinaren efektu mimetikoa dela eta [156], bere espresioa erresberatrolagatik moldatua izan zitekeela hipotetizatu izan da. Hilabete batez 1 eta 10 mg/kg gorputz-pisu/egun bitarteko dosiekin tratatutako Wistar arratoi diabetikoekin eginiko ikerketa batetan, erresberatrolak eragindako efektu hipogluzemikoa hein handi batean erraietako gantz ehuneko visfatinaren espresioa erregulatzeagatik izan zitekeela ikusi zen [254]. Beste ikerketa batean erresberatrolak arratoi diabetikoen gibelean emandako visftina

mailaren igoera berrezarri zuen, arratoi ez-diabetikoen tratamenduan efektu hori ikusi ez bazen ere [255]. Azkenik, aipatzekoa da Derdemezis eta kolaboratzaileek eginiko ikerketa, non autoreek 25 µM erresberatrolekin tratatutako SGBS zelulen visfatina jarioa murriztu zela ikusi zuten [256]. Zarei eta kolaboratzaileek ere arratoi diabetikoak erabili zituzten erresberatrolak apelinaren espresioan zuen efektua aztertzeko. Arratoiak 5 eta 10 mg/kg gorputz-pisu/egun-eko dosiekin tratatzean apelinaren espresioan aldaketarik ez zegoela aditzera eman zuten, erresberatrolak odoleko glukosa-mailaren jeitsieran zuen efektua intsulinaren jarioa areagotzeagatik eta erresistinaren espresioa modulatzeagatik zela suposatuz [241].

In vitro eta *in vivo* egin diren ikerketak asko izan badira ere, ikerketa-kliniko gutxi egin dira erresberatrolaren obesitatearen kontrako efektua analizatuz. Hilabete batean zehar 150 mg erresberatrol eman zitzaien gizon obesoekin eginiko ikerketa batean, suplementazioak larruazalpeko gantz ehuneko adipozitoen tamaina murrizten zuela egiaztatu zen. Ikerketa sakonagoa egiterakoan, adipogenesiaren sustapena ematen zela ikusi zuten, gantz ehunaren disfuntzioak eragindako intsulinarekiko erresistentziari aurre egiteko mekanismoa izan zitekeela postulatu zutelarik [257,258]. Beste ikerlan batean sindrome metabolikoa zuten pertsonei 4 hilabetetan zehar gramo 1 erresberatrol eman zitzaien gehigarri moduan, ondoren analisi metabolomiko bat egin zitzaielarik. Beste beste, erresberatrolak muskuluaren ordezkapen-tasaren markatzaileen handipena eta lipidoen metabolismoan, zelulen-barneko glizerol eta kate luzeko gantz azidoen metaketan bereziki, eragina zuela ikusi zuten [259]. Egindako ikerketa-kliniko urrietan ezusteko emaitzak lortu badira ere, aipatzekoa da gehiengoetan erresberatrola tratamendu bezala eman zaiela parte-hartzaileei eta ez prebentzio moduan. Hortaz, erresberatrolaren efektu prebentiboa aztertuko balitz, etorkizuneko emaitza hobeak lortzeko aukera egon daitekeela iradoki daiteke.

Erresberatrolaren obesitatearen kontrako efektu positiboak 4. taulan laburbildu dira (4.taula).

4. taula. *In vitro* eta *in vivo* ikerketetan erresberatrolaren obesitatearen kontrako efektuak jasotzen dituen taula.

Bide metabolikoa	***Mekanismoa***	***Zelula edo ehun mota***	***Erreferentziak***
Adipogenesia	↓ PPARγ gene eta proteina espresioa	Saguen 3T3-L1 adipozitoak Giza SGBS zelulak Sagu-obesoen gantz ehun epididimala	[220,231, 232, 233,235]
	↓ CEBPα gene eta proteina espresioa	Saguen 3T3-L1 adipozitoak Sagu-obesoen gantz ehun epididimala	[220, 231, 232, 235]
	↓ SREBP1C gene eta proteina espresioa	Saguen 3T3-L1 adipozitoak Sagu-obesoen gantz ehun epididimala	[220, 231, 232]
	↑ SIRT1 aktibitatea	Giza SGBS zelulak	[233]
	↓ LPL gene espresioa	Saguen 3T3-L1 adipozitoak Sagu-obesoen gantz ehun epididimala	[220, 231]
Gantz azidoen kaptazioa eta lipogenesia	↓ FAS gene eta proteina espresioa eta aktibitatea	Saguen 3T3-L1 adipozitoak Human SGBS cells Sprague-Dawley arratoien gantz ehuna	[220, 233,237, 243]
	↓ ACC gene espresioa eta aktibitatea	Giza SGBS zelulak Sprague-Dawley arratoien gantz ehuna Zucker arratoien gantz ehuna	[233, 243,244]
	↑ GLUT4 gene eta proteina espresioa	Giza SGBS zelulak	[233]
	Egoera basalean eta intsulinak-estimulatutako glukosaren kaptazioa	Giza SGBS zelulak	[233]
	Egoera basalean eta intsulinak-estimulatutako glukosa lipidotan bihurtzea	Arratoien adipozito isolatuak	[234]
	↑ Intsulinaren seinalizazioa	C57BL/6J saguen gantz ehuna	[236]
Lipolisia	↑ ATGL gene eta proteina espresioa	Giza SGBS zelulak	[222]
	↑ HSL gene espresioa	Sprague-Dawley arratoien gantz ehuna Zucker arratoien gantz ehuna	[243,244]
Apoptosia	↑ AMPKα proteina espresioa ↓ Akt aktibitatea	Saguen 3T3-L1 adipozitoak	[250]
	↑ Kaspasa aktibazioa	Gizakien larruazalpeko gantz ehunetik eratorritako aurre.adipozitoen kultibo primarioa	[251]
Mitokondrien biogenesia eta funtzioa	↑ SIRT1 bidezko PGC1α-ren deazetilazioa	C57BL/6J saguak	[223]
Adipokinen modulazioa	↓ Leptina gene espresioa eta odol-maila ↑ Leptinaren sentikortasun indizea	Sagu-obesoen gantz ehun epididimala Untxi hiperkolesterolemikoen odola Wistar arratoien gantz ehuna	[218, 231, 252]
	↓ Resistina gene espresioa	Wistar arratoien gantz ehuna	[241]
	↑ Adiponektina protein espresioa eta odol-mailak	Untxi hiperkolesterolemikoen odola Saguen 3T3-L1 adipozitoak	[252, 242]
	↓ Visfatina jarioa (inkubazio-medioan)	Giza SGBS zelulak	[256]

PPARγ: peroxisomen ugalketarako γ hartzailea; CEBPα: CCAAT-ra lotzen den α proteina; SREBP1c: SREBP hausturak aktibaturiko c proteina; SIRT1: 1 sirtuina; LPL: lipoproteina lipasa; FAS: gantz azidoen sintasa; ACC: azetil-AKo karboxilasa; GLUT4: glukosaren 4 garraiatzailea; ATGL: gantz ehuneko triglizeridoen lipasa; HSL: hormonekiko sentikorra den lipasa; AMPK: AMP-k aktibaturiko proteina kinasa; Akt: B proteina kinasa (PKB); PGC1α: peroxisomen ugalketaren 1α hartzailea.

Azkenik, erresberatrolak jasaten duen metabolismo sakona kontuan hartuta, haren metabolitoen balizko obesitatearen kontrako efektuetan arreta ipintzea oso garrantzitsua da. Minbizia bezalako beste gaixotasun kronikoetan metabolitoen efektuak aztertu izan badira ere, ez dago erresberatrolak obesitatean duen efektuari metabolitoek lagundu diezaioketen ikusteko egin den beste ikerketarik, ezta ere zehazki glukosa eta lipidoen metabolismoa erregulatzeko jarraitu ditzaketen mekanismoak aztertzen duen ikerlanik. Erresberatrolaren metabolitoek minbiziaren prebentzioan edo gaixotasunaren abantzuan izan ditzaketen onurei dagokienez, efektu apoptotikoa, antioxidatzailea eta zitotoxikoa, besteak beste, horien erantzule direla ikusi da [260,261]. Ekintza-mekanismoei erreparatuta, Calleri eta kolaboratzaileek eginiko ikerketa bat goraipatzea garrantzitsua da. Bertan, erresberatrolak eta 3G, 4G eta 3S metabolitoek PPAR transkripzio faktoreak lotzeko zuten gaitasuna aztertu zuten. Eskuratutako emaitzen arabera, bai erresberatrolak zein aztertutako hiru metabolitoek PPARγ lotzen zuten, baina PPARα-k soilik erresberatrolarekin elkartzeko gaitasuna erakutsi zuen [262]. Aintzakotzat hartzekoa da ere zenbait ikerlarik botatako hipotesia. Zientzialari horien ustetan, erresberatrolaren metabolitoak biologikoki aktiboak izan litezkeelako edo ehunetan kokatzen diren glukuronidasa eta sulfatasa entzimei esker berriz ere konjugatu gabeko erresberatrola izatera bueltatu litezkeelako [263]. Hipotesi hau horrela den egiaztatzea eta haren inguruan sakontzea etorkizunean erresberatrola eta haren metabolitoen ikerketa-ildo garrantzitsuenetarikoa izan daiteke.

3.2. Kertzetina

Kertzetina (3,3’,4’,5,7-pentahidroxiflabona) fruta eta barazkiak bezalako lndare-jatorriko elikagaietan era naturalean agertzen den flabonola da, sagar, gerezi, baia, tipula, brokoli eta tean kantitate oso esanguratsuetan agertzen dena [264, 265]. Flabonoidea den aldetik, hidroxilo taldeak atxikita dituzten 3 talde fenoliko ditu bere egituran.

Funtzio biologiko ugari esleitu zaizkio kertzetinari, hala nola hantura, diabetes, hipertentsio, obesitatea, hiperkolesterolemia eta ateroesklerosi kontrako efektuak [266,267]. Hala eta guztiz ere, erresberatrolarekin gertatzen den bezala, duen bioerabilgarritasun baxua bere funtzio biologikoaren muga kontsideratu daiteke.

3.2.1. Kertzetinaren ingestio dietetikoa, absortzioa, banaketa eta metabolismoa

Gizakietan kertzetinaren ingestioa beste polifenol batzuena baino nabarmenki altuagoa dela estimatu da [268], flabonolen ingestioaren %60a delarik. Eguneko kertzetinaren batez besteko ahorakina 5 eta 40 mg artekoa da, baina kantitate hori 200-500 mg izatera irits daiteke fruta eta barazkietan aberatsak diren elikadura-patroiak jarraitzen dituzten pertsonetan, bereziki azalarekin jaten direnean [269]. Olthof eta kolaboratzaileek 9 bolondres osasuntsurekin eginiko ikerketa batean, egunean gutxi gorabeherako 150 mg kertzetina glukosido jan ondoren odoleko kertzetina maila 30 minututara 5 µmol/L ingurukoa zelan ikusi zuten

[270]. Oro har, elikagaiekin hartzen den kertzetina kantitatearekin haren odoleko mailak nanomolar-tartean ageri ohi dira. Aldiz, kertzetina gehigarri moduan hartzen denean kantitate hori µmolar tartekoa izatera irits daiteke. Conquer eta kolaboratzaileek burututako ikerketa-kliniko batean, autoreek 28 eguneko gramo 1-eko suplementazioa eta gero odoleko kertzetina kontzentrazioa 1.5 µM-ekoa izatera heldu zezakeela frogatu zuten [271].

Dietako kertzetina, batez ere, forma glikosilatuan hartzen dugu (kertzetina glukosido eta kertzetina rutinosido) eta aipatzekoa da flabonoideen artean, kertzetinaren glukosidoak direla hoberen absorbatzen direnak. Kertzetina aglikonaren parte bat urdailean xurgatzen dela ikusi da, hau kertzetina glukosidoekin gertatzen ez delarik [272]. Glukosidoak heste meharrean absorbatzen dira lehen aipatutako mekanismoen bidez: LPH entzimaren eta SGLT1 mintzeko garraiatzailearen bidez [273, 274]. Kertzetina rutinosidoaren kasuan aldiz ezberdina da, mikrobiotaren β-glukosidasek burututako deglikosilazio erreakzioen ondorioz kolonean absorbatzen baita [275, 276].

Kertzetina glukuronidazio, sulfatazio eta metilazio erreakzioen bidez konjugatzen da heste meharreko eta gibeleko zeluletan. Ondorioz, odolean kantitate handienean topatzen diren metabolitoak hurrengoak dira: %8,5-11 isorhamnetina (ISO; 3´-O-metil-kertzetina), %10-13 tamarixetina (TAM; 4´-O-metil-kertzetina) eta %78-79 kertzetina-konjugatuak. Konjugatuen artean kertzetina-3-O-glukuronidoa (Q3G) eta kertzetina-3-O-sulfatoa (Q3S) daude, hala nola ISO-3-O-glukuronidoa [277,278]. Kertzetina glukuronidoak kertzetina beraren garraiatzaile bezala jokatu dezaketela uste da, ehunetan aglikona moduan askatzeko ahalmena eduki dezaketelarik [279].

Kertzetinak ehun ezberdinetan duen sakabanaketa ezagutzeko, de Boer eta kolaboratzaileek arratoiak 11 astez 50 eta 500 mg/kg gorputz-pisu/egun eta txerriak 3 egunez 500 mg/kg gorputz-pisu/egun kertzetinarekin elikatu zituzten [280]. Ehunetako kertzetina, ISO eta TAM-ren kantitateak aztertu zituzten. Arratoietan kertzetina eta metabolitoen kontzentrazio altuena biriketan aurkitu zuten, gibel eta giltzurrunetan tarteko kontzentrazioa eta kantitaterik baxuena, aldiz, garunean, GEZ-n eta barean. Txerrien kasuan, kertzetina eta metabolitoen kantitaterik altuenak gibel eta giltzurrunetan topatu zuten, gainontzeko ehunetara kantitate oso txikiak heldu zirelarik. Beste ikerketa batean, Yang eta kolaboratzaileek arratoiei zain-barneko 3G 10 mg/kg gorputz-pisu dosiko tratamendua eman zieten, ondoren giltzurrun, gibel, bihotz eta garuneko distribuzioa aztertzeko helburuarekin [264]. 3G metabolitoaren kantitate gehiena gibelean topatu zuten eta beste organuetako banaketa, kontzentrazio handienetik txikienera, giltzurrunean, bihotzean eta garunean izan zen hurrenez hurren.

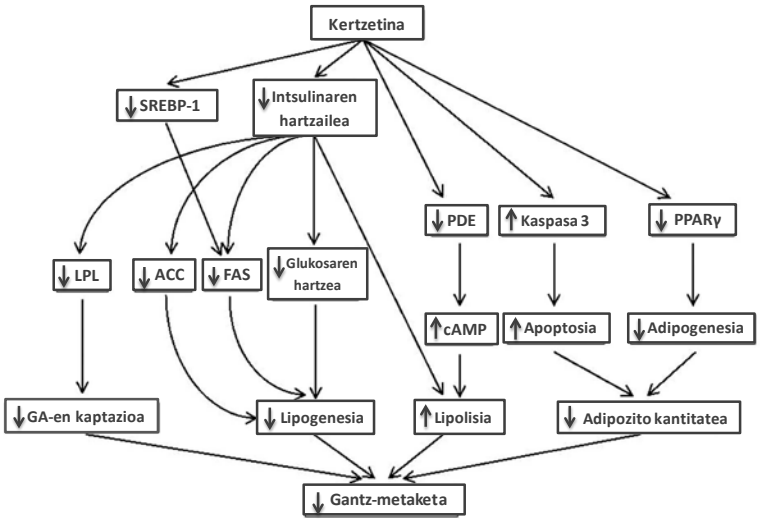
Kertzetinaren kanporaketa gernuaren eta behazun-azidoen (gorotzen) bidez ematen da. Lehen aipatu den bezala, kertzetina zirkulazio entero-hepatikoan sartzen da, eta hargatik, heste meharraren argira bueltatzen da behazun-azidoen bidez. Hestera jariatu den kertzetina berxurgatzen ez bada, koloneraino

jarraituko du, eta mikrobiotak degradatu ostean gorotzen bidez kanporatuko da. Kertzetinaren azken degradazio-prozesuan, gernu eta gorotzen bidez kanporatuko diren azido fenolikoak eratzen dira dekonjugazioz, hala nola arnasketaren bidez botako dugun CO_2-a [281]. Manach eta kolaboratzaileek gizakietan hainbat polifenolek zuten bioerabilgarritasuna aztertu zuten, kertzetinaren batez besteko bizitza 11-28 ordu bitartekoa zela ezarri zutelarik [282].

3.2.2. Kertzetina eta bere metabolitoak obesitatearen kontrako molekula bezala: lipido eta karbohidratoen metabolismoan dituzten efektuak.

Kertzetinari funtzio biologiko ugari egotzi bazaizkio ere, azken urteotan diabetesaren kontrako efektuak interes handia piztu du. β-glukosidasen inhibizioari esker, kertzetinak glukosaren heste-absortzioa murrizten du, intsulinarekiko sentikortasuna handitzen du, intsulinaren jarioa estimulatzen du eta ehunen glukosaren kaptazioa handitzen du, modu horretan odoleko glukosaren jeitsiera esanguratsua eraginez animalia-eredutan ikusi izan den bezala [283-285]. Glukosa-homeostasiaren erregulatzaile garrantzitsuenak **muskulu eskeletikoa** eta **gibela** dira, eta hortaz, kertzetinak miozito eta hepatozitoetan duen efektua sakonki ikertu izan da. Miozitoetan deskribaturiko mekanismo garrantzitsuenetarikoa AMPK-ren aktibazioaren bidez GLUT4-ren mintz plasmatikorako translokazioa da, zelularen glukosa hartzeko gaitasuna nabarmenki handituko duena [286,287]. Animalietan ikusi da kertzetinak mitokondrio kopurua eta hauen funtzioa hobetzen duela, zitokina inflamatorioak eta makrofagoen akumulazioa murrizten duela eta GLUT4-aren espresioa handitzen duela [288-291]. Eid eta kolaboratzaileek arratoien H4IIE hepatozitoekin eginiko ikerketa batean, kertzetinak gibeleko glukosa-ekoizpena murrizteko kapaza zela ikusi zuten [286].

Aurrekoaz gain, kertzetinak gorputzeko gantza murrizteko duen ahalmena ere sakonki ikertu da. Gantz ehun eta gibeleko gantz-metaketan duen efektua eta dietak eragindako obesitatearen ondorioz odoleko zenbait parametroetan eragindako aldaketen berrezarpena, besteak beste, *in vivo* eginiko ikerketa askotan aztertu da [292-295]. Adipozitoen lipidoen metaketan duen efektuaren hainbat mekanismo 15.irudian laburbildu dira (15.irudia).



15.**irudia**. Kertzetinak gantz pilaketa murrizteko jarraitzen dituen ekintza-mekanismoak. Aguirre eta kolaboratzaileetatik 2011 moldatua [296]. SREBP-1: SREBP hausturak aktibaturiko 1 proteina; LPL; lipoproteina lipasa; ACC: azetil-AKo karboxilasa; FAS: gantz azidoen sintasa; GA: gantz azidoa; PDE: fosfodiesterasa; cAMP: adenosina 3',5'-monofosfato ziklikoa; PPARγ: peroxisomen ugalketarako γ hartzailea.

Erresberatrolak bezala, kertzetinak ere **adipogenesiaren kontrako efektuak** dituela ikusi da, adipozitoen diferentziazioan ezinbestekoak diren transkripzio faktoreengan eragiten baitu 3T3-L1 aurre-adipozitoak kertzetinaren dosi ezberdinekin tratatu ostean, SREBP1c, CEBPβ, PPARγ, CEBPα eta FAS transkripzio faktoreen murrizketa ikusi izan da [297,298]. Erresberatrolaren metabolitoekin ez bezala, kertzetinaren metabolitoekin ikerketa gehiago egin dira hauek adipogenesian duten efektua aztertzeko helburuarekin. Zehazki, isoramnetinaren (ISO) efektu adipogenesiaren kontrako efektua aztertu da [299,300], hala nola naturan aurkitzen den kertzetina-3-O-(6´´-feruloil)-b-D-galactopiranosidoarena [301]. Ikerketen autoreek bi metabolitoek adipogenesia, besteak beste, PPARγ eta CEBPα erregulatzaileen espresioaren murrizketa eraginez inhibitzen zutela ikusi zuten.

Kontuan hartuta adipozitoen %10 inguruk berriztatze prozesua jasaten duela, adipogenesia eta apoptosiaren arteko oreka garrantzitsutzat jo dezakegu gehiegizko gantz ehunaren hedapenaz ari garenean [36]. Kertzetinak **apoptosia** eragin dezakeela ikusi da, poli (ADP-erribosa) polimerasa (PARP) eta bcl2 murriztuz pro-apoptotikoak diren kaspasa 3, Bax eta Bak aktibatuz [302].

Adipozito kopuruan izan dezakeen erregulazioaz gain, kertzetinak adipozitoen lipidoen metabolismoan duen efektua ere aztertu izan da. 1992.urtean arratoiaren adipozito primarioekin eginiko ikerketa batean, Kuppusamy eta Das-ek kertzetinaren **efektu lipolitikoa** PDE entzimak lipolisiaren aurka duen efektuaren inhibizioaren bidez eta ondorioz emandako HSL-ren aktibitatea areagotuz ematen dela frogatu zuten [303]. Beste ikerketa batean, entrenatzen ari ziren saguei kertzetina gehigarria ematerakoan,

energiaren iturria glukolisia izatetik lipolisia izatera pasa egiten zela ikusi zuten [304]. **Lipogenesia** ere kertzetinaren jomuga dela dirudi, batez ere FAS eta ACC-ren gene eta proteina espresioan duen efektu murriztatzailea dela eta, saguen adipozitoetan eta hepatozitoetan frogatua izan den bezala [294, 297]. Bestalde, LPL entzimaren aktibitatea inhibitzearen ondorioz odoletik hartutako GAA-en murrizketa ere ematen dela ikusi izan da [305]. Gainera, glukosaren kaptazioak lipogenesian duen garrantzia kontsideratuta, kertzetinak bide hartan eragin lezakeen efektua aztertzea ezinbestekoa da. Hamilton eta kolaboratzaileek L929 gantz ehunetik eratorritako fibroblastoetan frogatu zutenez, GLUT1 garraiatzailea blokeatuta kertzetinak mintz plasmatikoan zehar glukosaren garraioa murrizteko ahalmena zuela egiaztatu zuten [306]. Gainera, Strobel eta kolaboratzaileek arratoien adipozitoen kultibo primarioak 10-100 μM-eko dosian kertzetinarekin tratatu ondoren, glukosaren kaptazioaren inhibizioa ematen zela ikusi zuten [307]. Hala eta guztiz ere, aipatzekoa da arratoiekin eginiko esperimentu bat, non 30 mg/kg gorputz-pisu/egun-eko dosian kertzetinarekin tratatzerakoan, autoreek ez zuten LPL eta entzima lipogeniko garrantzitsuenen aktibitatean aldaketarik ikusi [308].

Kertzetinak **adipokinen** espresioan eta jariaketan duen efektua ere aztertu izan da. 8 astez kertzetinan aberatsa den tipularen erauzkinarekin tratatutako Sprague-Dawley arratoiek gantz ehun mesenterikoaren beherapena jasan zuten, adiponektinaren espresioaren handipenarekin batera [309]. Beste ikerketa-lan batean, kertzetinak Wistar arratoietan gantzetan aberatsa den dietak eragindako adiponektinaren odol-kontzentrazioen murrizketa indargabetzeko kapaza zela erakutsi zuen [310]. Espresuki, kertzetina gehigarriak hartu zituzten arratoiek GEZ-an adiponektinaren mRNA maila altuagoak zituzten.

Sagu diabetikoekin egin berri den ikerketa batean, kertzetinak diabetesaren kontrako efektua duen Sitagliptina farmakoaren ekintza handitzen duela ikusi da. Saguen kontrol gluzemikoan, profil metabolikoan, egoera oxidatibo eta inflamatorioan eta β-zelulen funtzioan hobekuntza behatu da, beti ere farmakoa eta kertzetinaren konbinazioa farmako hutsaren erabilerarekin alderatu denean [311].

Leptinari dagokionean, kertzetinak haren odol-mailak murrizten dituela ikusi izan da animalia ikerketetan [312,313]. Hala ere, esan beharra dago ikerketa-klinikoetan emaitza kontrajarriak lortu direla, kertzetina gehigarriak hartu ondoren ez baita odoleko kertzetina mailaren aldaketarik antzeman [314,315]. Lehen ere aipatu den Derdemezis eta kolaboratzaileen ikerketan, kertzetinak SGBS zeluletan visfatinaren jarioa aldatu zuen. Obesitatea zuten emakumeekin eginiko entsegu kliniko batean ordea, kertzetinan aberatsa den tipularen erauzkinaren gehigarriak (100 mg/egun) hartu zituzten 12 astez eta ez zen odoleko visfatina kantitatea aldatu [314]. Guk dakigula, gaurdaino ez da kertzetinak apelinaren espresioan eta jarioan duen efektua ikertu.

Nahiz eta *in vitro* eta *in vivo* ikerketa asko egin izan diren kertzetinaren obesitatearen kontrako efektua ikertzeko, gutxi batzuk egin izan dira gizakietan. Kertzetinan aberatsak diren erauzkinekin eginiko hainbat

entseguk emaitza ezberdinak eman dituzte. Esate baterako, Japonian emakume gazte osasuntsuekin eginiko bi ikerketetan, emaitza kontrajarriak lortu dira GMI eta gorputzeko gantz portzentajeari dagokionean, tratamenduaren iraupenak emaitzetan izan lezakeen garrantzia agerian utziz [316, 317]. Bestetik, 100 mg/egun-eko dosian 12 astez kertzetinaren gehigarriak hartuta, gainpisua edo obesitatea zuten pertsonek GMI eta gantzaren metaketa modu esanguratsuan murriztu zuten [318]. Aldiz, iraupen bereko eta dosi altuagoko (500 edo 1000 mg/egun) gehigarriak hartutako ikerketa batean, ez zen efektu onuragarririk ikusi [319]. Oro har, kertzetinaren gehigarriak hartzearen esanguratsutasun klinikoa argitzearen helburuarekin ikerketa-kliniko gehiagoren beharra dagoela esan daiteke.

Adipogenesia ikertzeko ISO eta kertzetina-3-O-(6´´-feruloil)-b-D-galactopiranosidoarekin eginiko ikerketak salbu, kertzetinaren metabolitoek jatorrizko konposatuaren obesitatearen kontrako efektuan lagungarri diren ez da aztertu. Hala eta guztiz ere, ematen den metabolizazio sakonaz gain *in vivo* esperimentuetan baieztatu diren gantz murrizketa efektuek, hala nola beste ehun eta gaixotasunen kontra egiaztatu diren akzioek, kertzetinaren metabolitoak biologikoki aktiboak izan litezkeela jakinarazi digute [320-323].

Kertzetinaren obesitatearen kontrako efektu positiboak 5. taulan laburbildu dira (5.taula).

5. taula. *In vitro* eta *in vivo* ikerketetan kertzetinaren obesitatearen kontrako efektuak jasotzen dituen taula.

Bide metabolikoa	***Mekanismoa***	***Zelula edo ehun mota***	***Erreferentziak***
Adipogenesia	↓ PPARγ gene eta proteina espresioa	Saguen 3T3-L1 adipozitoak Sprague-Dawley arratoiak	[297, 298, 309]
	↓ CEBPα proteina espresioa	Saguen 3T3-L1 adipozitoak	[297, 298]
	↓ CEBPβ proteina espresioa	Saguen 3T3-L1 adipozitoak	[297, 298]
	↓ SREBP1C proteina espresioa	Saguen 3T3-L1 adipozitoak	[297, 298]
Gantz azidoen kaptazioa eta lipogenesia	↓ FAS proteina espresioa	Saguen 3T3-L1 adipozitoak	[297, 298]
	↑ ACC fosforilazioa (inaktibazioa)	Saguen 3T3-L1 adipozitoak	[297]
	↓ LPL aktibitatea	Arratoien adipozito isolatuak	[305]
	↓ glukosaren kaptazioa	L929 gantz ehunetik eratorritako fibroblastoak Arratoien adipozito isolatuak	[306, 307]
Lipolisia	↓ PDE aktibitatea ↑ HSL aktibitatea	Arratoien adipozito isolatuak	[303]
Apoptosia	↑ Kaspasa aktibazioa ↑ Cas3 proteina espresioa eta aktibitatea	Saguen 3T3-L1 adipozitoak	[297, 302]
	↓ PARP eta bcl2 proteina espresioa ↓ Bax eta Bak proteina espresioa	Saguen 3T3-L1 adipozitoak	[302]
Adipokinen modulazioa	↑ adiponektina odol-mailak ↑ adiponektina gene espresioa	Zucker arratoie obesoak C57BL/6J saguak Sprague-Dawley arratoiak Wistar arratoiak Wistar arratoi diabetikoak	[292, 294, 309,310,311]
	↓ leptina odol-mailak	C57BL/6J saguak C57BL/6JOlaHsd saguak	[294, 313]
	↓ visfatina jarioa	Giza SGBS zelulak	[256]

PPARγ: peroxisomen ugalketarako γ hartzailea; CEBPα: CCAAT-ra lotzen den α proteina; CEBP β: CCAAT-ra lotzen den β proteina SREBP1c: SREBP hausturak aktibaturiko c proteina; FAS: gantz azidoen sintasa; ACC: azetil-AKo karboxilasa LPL: lipoproteina lipasa; PDE: fosfodiesterasa; HSL: hormonekiko sentikorra den lipasa; Cas3: kaspasa 3; PARP: poli (ADP-erribosa) polimerasa ; bcl2: B zelulen leuzemia/2 linfoma ; Bax: : Bcl2-ari elkartutako X proteina ; Bak: Bcl2 homologoaren erailea.

INTRODUCTION

1. Obesity

Overweight and obesity are defined as an excessive fat accumulation [1] that could influence on the development of co-morbidities, to a greater or lesser extent, depending on its severity and distributin. The amount of fat mass, and especially abdominal fat, is directly related to the number of obesity-associated co-morbidities. Among them, type 2 diabetes, cardiovascular diseases, obstructive sleep apnoea syndrome and cancer are some of the most important ones [2].

World Health Organization (WHO) defines overweight and obesity by using the Body Mass Index (BMI). It is a very practical tool, easy and fast to calculate, which relates height and weight. The interpretation of the result gives an estimation of the nutritional status (Table 1). However, taking into account that it does not consider other parameters such as constitution, body composition or waist and hip perimeters, it is not the most accurate tool for obesity diagnosis.

Table 1. Classification of underweight, normal weight, overweight and obesity based on BMI. Adapted from WHO [3].

Classification	BMI (kg/m^2)
Underweight	< 18.5
Normal range	18.5-24.99
Overweight (pre-obese)	25-29.99
Obese	≥30

According to WHO estimations, in 2016 39% of adults worldwide were overweight and 13% were obese. Furthermore, 41 million children under 5 were overweight or obese and over 340 million in the case of those aged between 5 and 19 [1]. To date, childhood obesity has been observed mainly in developed countries, but its prevalence is increasing rapidly in developing countries as well. The importance of childhood obesity does not only refer to health complications during this period, but it is also associated with a major probability of death caused by related diseases in adulthood. On the one hand, obese children are more prone to continue being obese when they are adults. On the other hand, there is a high risk of suffering diseases such as type 2 diabetes or cardiovascular diseases, whose development is partially conditioned by the onset of obesity and its duration. Therefore, childhood and adolescent obesity is associated to short-term and long-term health consequences [4,5].

Although overweight and obesity development is a complex process in which several factors can influence (genetics, diet, stress, smoking….), in general terms the excessive fat accumulation is due to an unbalance between energy intake and energy expenditure. The increase in prevalence observed in the last decades, could be attributed not only to a high consumption of processed foods -rich in calories and fats- and low consumption of fresh ones, but also to a reduction in physical activity (long sedentary workdays, means of transport, new technologies in leisure time, etc.).

The treatment of obesity should be carried out by a multidisciplinary group of professionals, in order to obtain a complete approach. Dietary treatment, physical activity prescription, pharmacological treatment, psychotherapy and obesity surgery are the principal factors that need to be studied. Furthermore, their combination, at least that of diet and exercise, is the most used strategy for body weight loss [6]. Nevertheless, long-term success of obesity treatment is difficult to achieve, so the prevention acquires great importance.

The Mediterranean diet is a typical dietary pattern that was described in the 60s by the Mediterranean Sea bordering countries. Its two major characteristics are the use of olive oil and a high consumption of plant-derived foods [7]. Appart from its beneficial effects on the prevention of several diseases, such as cancer or cardiovascular diseases [8-10], it has also been proposed as an interesting tool for obesity prevention or treatment, as a healthy and long-term maintenance dietary pattern [11]. Its beneficial effects may be due to foodstuff recommended by this diet, sources of complex carbohydrates, unsaturated fats, fibre, vitamins, minerals and other dietary components such as phenolic compounds (PC) and other bioactive molecules, present mainly in fruits and vegetables, nuts, olive oil and red wine (Table 2).

Table 2. Dietary pattern of the Mediterranean Diet. Adapted from Buckland *et. al.* 2008 [11].

	Mediterranean Dietary pattern features
1	High consumption of plant-based foods (fruit, vegetables, legumes, nuts, seeds and wholegrain cereals)
2	Seasonally fresh and locally grown foods
3	Olive oil (main source of dietary lipids)
4	Moderate intake of red wine
5	Consumption of fresh fish and seafood
6	Moderate intake of dairy products, poultry and eggs
7	Moderate consumption of red and processed meat

PC are strong antioxidants that naturally occur in plants in response to stress as a defence mechanism [12,13]. It has been shown that the ingestion of PC-rich foods is helpful to decrease the incidence of cardiovascular diseases, several types of cancer, liver disorders and diabetes, among others [13]. Moreover, recent studies have shown the role of dietary PC in the prevention and treatment of obesity [14]. For this reason, their inclusion in functional foods could represent a useful tool to face this pandemic problem.

2. Adipocyte biology

In the human body, lipids can be accumulated as triglycerides in two adipose tissues: white adipose tissue (WAT) and brown adipose tissue (BAT). The main function of WAT is to store energy as fat, through triglyceride synthesis. In a situation of energy excess, fat is accumulated in adipocytes. In energy deprivation situation, triglyceride mobilization starts releasing glycerol and free fatty acids (FFA), which are released to the bloodstream and transported to other tissues to be oxidized to obtain energy. By contrast, in BAT, brown adipocytes exhibit a high number of mitochondria which expresses the uncoupling protein 1 (UCP1), responsible for energy dissipation as heat. In addition , there is a third type of adipocyte called “brite” or “beige”, a brown adipocyte derived from mesenchymal cells present in WAT and differentiated under thermogenic induction. The most known inductors are cold exposure, β-adrenergic and peroxisome proliferator-activated receptor γ (PPARγ) agonist treatments, and the intake of several dietary components such as hyperlipidic diets or PC [15]. It is important to point out that the precursor cell lineage of brown adipocytes differs from that of white adipocytes. By contrast, brites share the same precursors with white adipocytes and not with brown ones [16]. Thus, according to several recent studies, it is thought that brite adipocytes could come from the transdifferentiation of white adipocytes [17]. This process consists ofthe appearance of adipocytes that have brown adipocyte features (“beige” or “brite” adipocytes) in WAT, which occurs under the previous mentioned stimuli.

Adipose tissue is a heterogenic tissue that includes a diverse cell composition as well as adipocytes. Among others, pre-adipocytes, mature adipocytes, mesenchymal stem cells, macrophages and endothelial cells are present in varied proportions. Regarding mature adipocytes, it is considered that they only represent a third part of adipose tissue and the remaining two-thirds, a combination of the rest of cells [18].

Adipose tissue mass fluctuation is given by changes in lipid accumulation. Its total amount is mediated by adipocyte hypertrophy (an increase in adipocyte size) or by hyperplasia (an increase in adipocyte number), whereas its dimension is decreased by hypotrophy, a reduction of adipocyte size or hypoplasia [19,20]. When hyperplasia takes place, pre-adipocyte proliferation and further differentiation is stimulated, which is known as the adipogenic process (Figure 1). Additionally, adipocytes hypertrophy is principally explained by lipogenesis and hypotrophy by lipolysis.

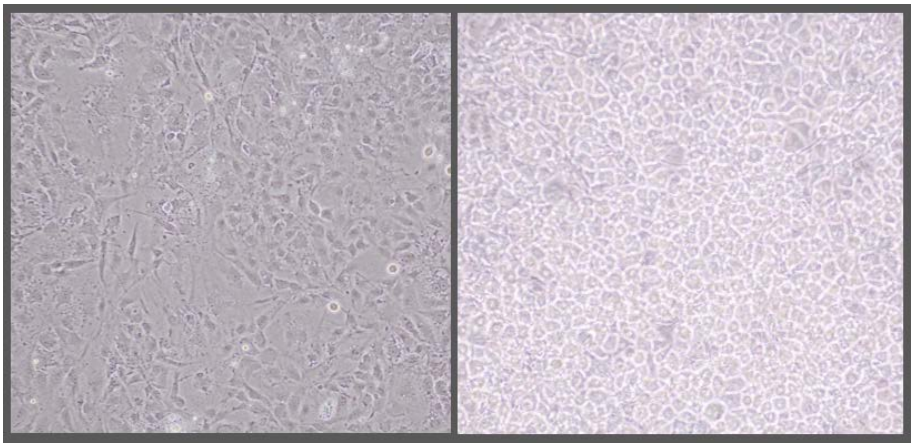


Figure 1. 3T3-L1 cells. Fibroblast-like undifferentiated pre-adipocytes (left) and lipid-full differentiated mature adipocytes (right).

2.1 Adipogenesis

Mesenchymal stem cells have the capacity to differentiate in several cell type-progenitors such as myogenic or osteoblast precursors, chondrocytes or pre-adipocytes, depending on the endocrine stimuli. Human and animal adipogenesis take place mainly during growth. However, although to a lesser extent, it also occurs in adulthood [21].

The differentiation process of fibroblast-like cells to mature adipocytes starts with the proliferation of commited pre-adipocytes until they are growth-arrested by contact inhibition (Table 3). For that purpose, cells undergo several modifications in the intracellular and extracellular matrix and they acquire the capacity to change their fusiform shape to a much bigger and rounded one, as well as to store lipid droplets during the differentiation process. Then, growth-arrested pre-adipocytes enter in the first stage of adipogenesis by mitotic-clonal expansion, where cells re-enter the cell cycle and undergo two cell-divisions. After that, pre-adipocytes continue through the second stage of adipogenesis, a complex process where they differentiate into lipid-full and insulin-responsive mature adipocytes expressing carbohydrate and lipid metabolism genes, adipokines and other specific genes.

Table 3. Classification of the stages in which fibroblast-like cells differentiate in mature adipocytes (modified from Lefterova *et. al.* [22]).

Stage	Characteristics (based on *in vitro* studies)
1. Commited pre-adipocytes	Cells have fibroblast-like phenotype and differentiate along adipocyte lineage.
2. Growth-arrested pre-adipocytes	Cells cease to proliferate by contact inhibition.
3. Mitotic-clonal expansion	Cells reenter the cell cycle and progess to two cell divisions. At this stage, cell-cycle regulators and adipogenic transcription factors (i.e. CEBPβ and CEBPδ) are highly expressed and facilitate the expression of terminal differentiation genes.
4. Terminal differentiation	Lipid droplets are produced and accumulated. Key regulators PPARγ and CEBPα are induced, as well as other adipocyte-specific genes.
5. Mature adipocytes	Adipocyte-specific genes are highly expressed and adipocytes accumulate a big lipid droplet.

CEBP: CCAAT enhancer binding protein; PPARγ: peroxisome proliferator-activated receptor γ.

2.1.1 Transcriptional regulation of adipogenesis

Some studies have implicated cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) as a starter of adipogenesis, due to the fact that its over-expression leads to an expression of adipogenic markers and triglyceride accumulation [23]. An increase inintracellular cAMP levels activates cAMP-dependent protein kinase A (PKA) by phosphorylation, which in turn, activates CREB. CREB is a transcriptional activator of CCAAT enhancer binding protein β (CEBPβ), which has been defined, together with CEBPδ, as the key regulator of the beginning of adipogenesis [24] (Figure 2). In1991, Cao *et. al.* demonstrated that the expression of both transcription factors was induced directly by adipogenic stimuli and that its highest expression was reached during the first two days [25]. In addition, Yeh *et. al*. showed that ectopic expression of CEBPβ and CEBPδ induced CEBPα expression in the absence of extracellular adipogenic hormones, suggesting a key function of CEBP family members on adipogenesis [26]. Another important transcription factor is SREBP cleavage activating protein 1, c isoform (SREBP1c), whose expression is activated in response to insulin [27]. Ectopic expression of SREBP1c is able to stimulate adipogenesis in a modest extent, via induction of PPARγ expression and production of PPARγ ligands [28,29].

Once this initial process is finished, the expression of late-adipogenic transcription factors starts. Among them, PPARγ and CEBPα are considered the master regulators. Several studies showed that PPARγ induction is necessary and sufficient to initiate adipogenesis [30-32]. In the case of CEBPα, similar studies demonstrated that it is required for WAT formation, but interestingly, it is not necessary for that of BAT [33]. So, PPARγ is able to induce adipogenesis in the absence of CEBPα, but CEBPα is unable to do the same without PPARγ [34].

Once both PPARγ and CEBPα are activated, they regulate the expression of each other and maintain a differentiated adipocyte phenotype [29]. The cooperation of both factors activates the expression of mature

adipocyte-specific genes, mainly those implicated in triglyceride metabolism as well as insulin receptors and glucose transporter 4 (GLUT4), which increase insulin sensitivity in adipocytes [35].

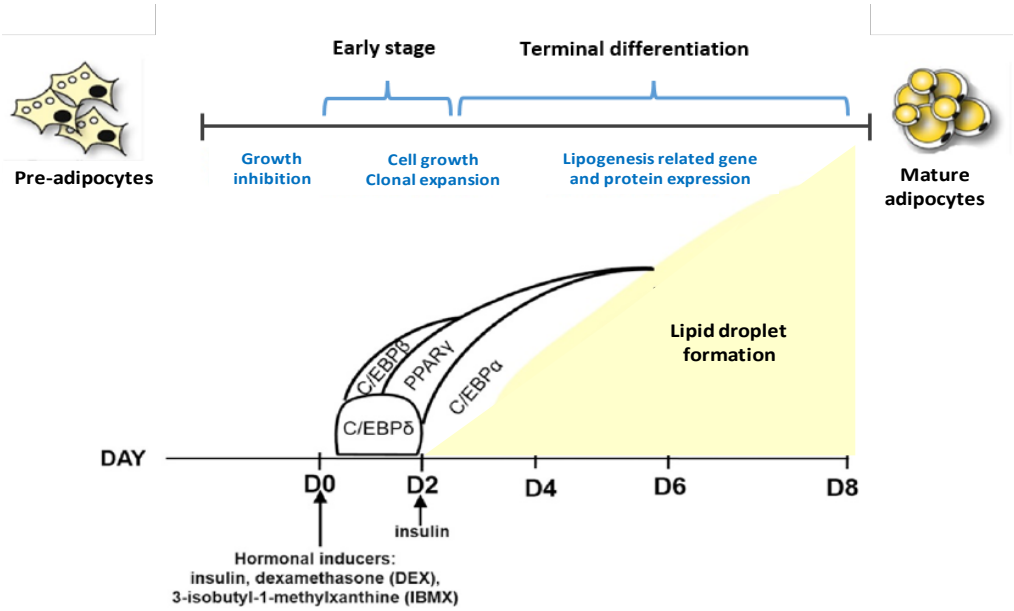


Figure 2. Gene expression profile in both phases of adipogenesis. Modified from Tung *et. al.* [36]. C/EBP: CCAAT enhancer binding protein (α,β,δ); PPARγ: peroxisome proliferator-activated receptor γ.

Microarrays and quantitative real-time polymerase chain reaction (RT-q-PCR) analysis of gene expression profile during the differentiation process, have highlighted the implication of other important transcriptional regulators [37]. An example of that is Krox20 (or Egr2) [38], members of the Krüppel-like factor (KLF) family [39,40], signal transducer and activator of transcription 5A (STAT5A) [41], retinoid-x-receptors (RXR) [42]. In this context, the complexity of the adipogenesis transcription network was extensively represented by Siersbæk *et. al.* [24] (Figure 3).

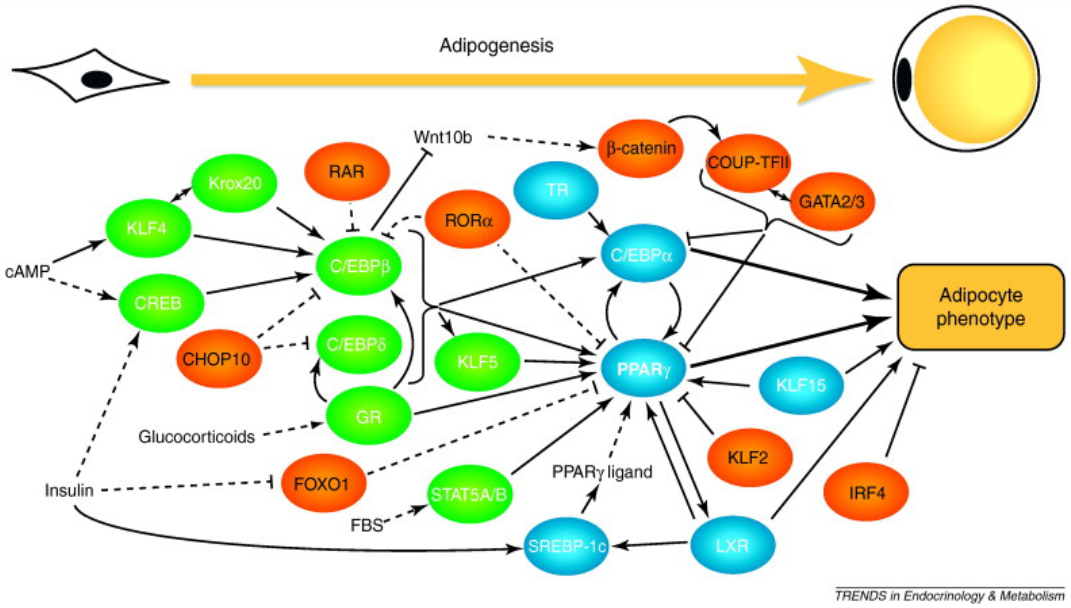


Figure 3. Transcriptional regulation of the adipogenic process, taken from Siersbæk *et. al.* [24]. CHOP10: CEBP homologous protein; FOXO1: forkhead box O1; RAR: retinoic acid receptor; RORa: RAR-related orphan receptor a; COUP-TFII: chicken ovalbumin upstream promoter transcription factor II; GATA 2: GATA binding protein 2; IRF4: interferon regulatory factor 4; GR: glucocorticoid receptor; TR: thyroid hormone receptor; LXR: liver x receptor FBS: fetal bovine serum.

Several agents induced adipocyte differentiation including insulin, glucocorticoids, insulin-like growth factor 1 (IGF-1) and cAMP [42] or the combination of them. In fact, a differentiation medium containing dexamethasone (DEXA; a glucocorticoid agonist), isobutylmethylxanthine (IBMX; prevents cAMP inactivation) and insulin, is the most used in 3T3-L1 cells [43].

2.1.2 Post-transcriptional regulation of adipogenesis

Post-transcriptional regulation refers to the control in gene expression that is exerted once transcription has started. It is less frequent than transcriptional regulation, but crucial for many genes[44]. Within these regulations, there are several mechanisms such as alternative splicing, ribonucleic acid (RNA) editing, the regulation of the RNA transport from the nucleus and transcriptional attenuation.

Moreover, post-translational modifications can occur after protein translation and include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis reactions, among others [45]. These modifications are key in the regulation of protein function and action, as well as in signal transduction pathways.

Regarding adipogenesis, phosphorylations of PKA and its consequent induction of differentiation is a clear example of post-translational regulation. Another example is that of PPARγ. It has been shown that mitogen-activated protein kinase (MAPK) can inhibit PPARγ activity by phosphorylation of its Ser84 residue [46,47].

There is another post-transcriptional regulation of adipogenesis mediated by microRNAs (miRNAs). Actually, it seems to be an important type in its regulation. MiRNAs are non-coding small-RNAs (of around 20-22 nucleotides) that regulate gene expression at post-transcriptional level. The vast majority of miRNAs are located in the intergenic or the intronic region of genes [48], but it is widely believed that in most of them, its transcription is independent on the promotor region of those genes. RNA polymerase II (mostly) or III synthesize a first miRNA precursor that is termed pri-miRNA, a double-stranded molecule of around 400 nucleotides. Endoribonuclease Drosha-DG CR8 complex (also known as microprocessor complex), generates a 70 nucleotide stem-loop pre-miRNA, which is transported from nucleus to cytosol by the nuclear export factor, exportin 5. Then, a second endoribonuclease Dicer produces a 22 nucleotide RNA duplex. One of these strands is assembled to a miRNA-induced silencing complex (RISC), an active post-transcriptional regulator, whereas the other strand is degraded [49,50] (Figure 4).

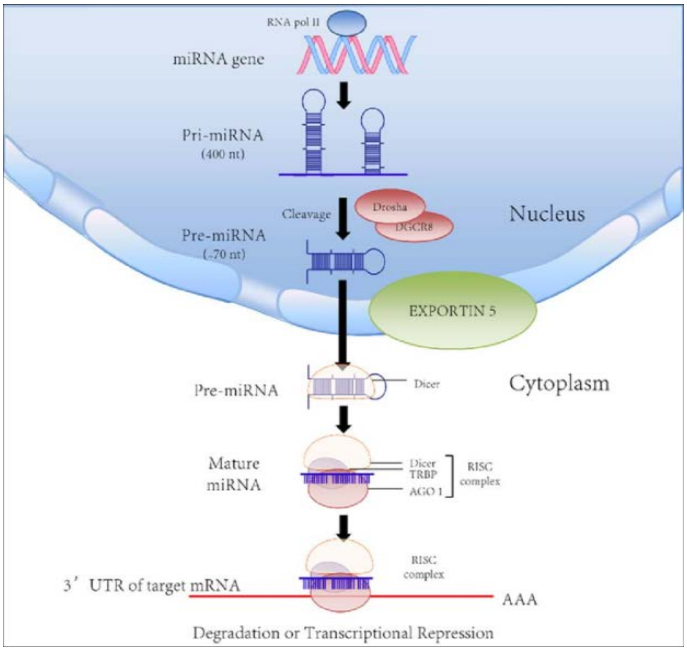


Figure 4. Representation of miRNA biogenesis. Taken from Pisarello *et. al.* [49]. Pol II: RNA polymerase II; DG CR8: DiGeorge syndrome critical region gene 8; RISC: RNA-induced silencing complex; TRBP: TAR RNA-binding protein; AGO: Argonaute 1.

MiRNA regulation mainly consists of the inhibiton of mRNA translation or degradation, but their relative contribution is not yet totally understood [51]. However, it is known that the type of regulation

depends on the complementarity of the miRNA and its target mRNA. A minimum of miRNA seed sequence (first 2 to 8 base pairs) pairing with the 3´UTR region of the mRNA is required to carry out the post-transcriptional regulation. Besides, the matching rate of the complementary nucleotides determine the mechanism of action: a perfect interaction results in mRNA degradation, whereas a partial interaction results in a translational repression [52]. Furthermore, whether both pathways act in sequence or in parallel is under debate.

MiRNA-mRNA interaction requires complementarity of a few base pairs, thus, a very high number of potential targets for each miRNA exist. However, target recognition principles remain unknown and there is an emerging evidence that the impact of some miRNAs upon their mRNA targets depend on specific conditions [53]. For this reason, a double approach of computational prediction and experimental validation of miRNA targets are needed in order to detect certain targets for each miRNA. As a first step, there are various algorithms that predict the probability of functional miRNA binding sites [54]. After that, experimental procedures are needed to verify this bioinformatic prediction. Among them, luciferase reporter gene assay [55] is a very reliable experimental procedure to verify miRNA/mRNA interaction. An additional or parallel step is to verify the miRNA effect on its protein target, which could be carried out by miRNA over- or under-expression experiments and further protein expression analysis.

In recent years, several studies have demonstrated that there is an important change in miRNA expression profile during adipocyte differentiation [56], suggesting an important implication of them in adipose tissue development. In order to prove this idea, Mudhasani *et. al*. blocked miRNA biogenesis by Dicer ablation in mouse embryonic fibroblasts and primary cultures of pre-adipocytes. They confirmed that it is required for an appropriate pre-adipocyte differentiation [57]. That research, as well as others, established a high number of miRNAs exerting pro-adipogenic or anti-adipogenic or both effects depending on their target gene (Figure 5). At the beginning of adipogenesis, miR-155 has shown to exert not only an anti-adipogenic effect by down-regulating CEBPβ [58] but also a pro-adipogenic effect by inducing CREB, which activates CEBPβ expression [23]. CREB induction of CEBPβ expression occurs both directly and indirectly by the induction of KLF5, which is a target of miR-448 [59]. MiR-33b is also involved in human adipogenesis by targeting SREBP1c. However, this miRNA is not expressed in rodents, which hinders the study of mir-33b impact in human adipogenesis through *in vivo* or *in vitro* experiments [60]. Besides, so far there is no validated miRNA targeting SREBP1c in mouse or rats [61].

As explained before, PPARγ is considered the master regulator of adipogenesis and for this reason, potential miRNA implication on the expression of this gene has been widely studied. MiR-27a and miR-27b act as adipogenesis inhibitors by targeting PPARγ [62,63], although recently other adipogenic gene targets have been described for both miRNAS, such as CREB and Prohibitin (PHB) [64-66]. Also miR-130 controls adipocyte differentiation by targeting PPARγ, as it has been demonstrated in human pre-adipocytes [67].

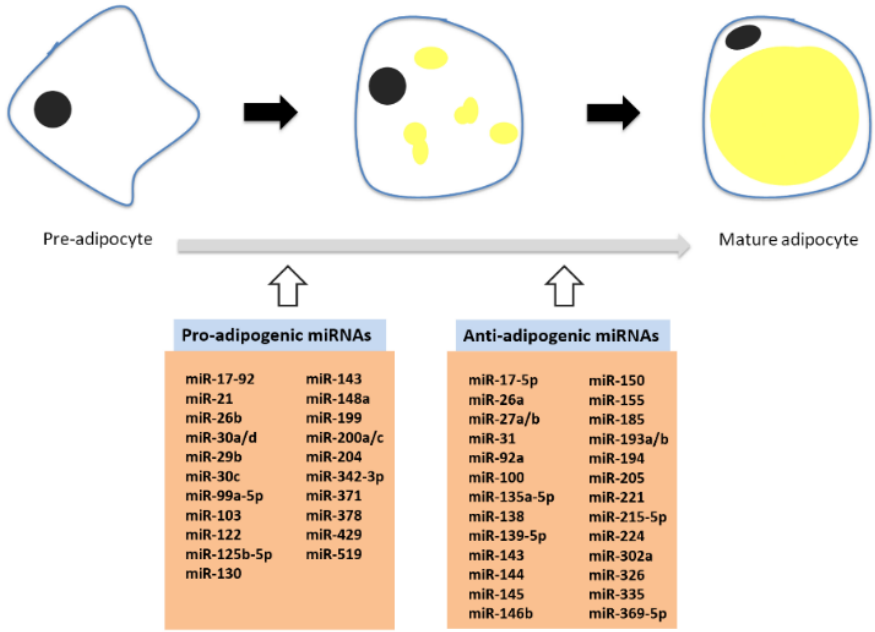


Figure 5. Some of the pro-adipogenic and anti-adipogenic miRNAs. Modified from Zaiou *et. al.* [68].

2.2 Apoptosis

Apoptosis is a programmed cell death process in which cells are self-destructed without leading to an inflammatory response or tissue damage. It is a mechanism within cell homeostasis, where an orchestrated cascade of enzymatic mediators is activated and specific cells are deleted efficiently. In the apoptotic process, cells suffer a sequence of morphological and biochemical events: membrane blebbing, plasmolysis and consequent cell shrinkage, chromatin aggregation and a production of cell fragments called apoptotic bodies [69].

There are two different but convergent mechanisms in the activation of the apoptosis-cascade. The extrinsic or death receptor pathway starts by the ligand-activation of tumor necrosis factor receptor (TNFR) superfamily, such as the TNF receptor superfamily member 6 (Fas) TNFR 1. Then, receptor molecules undergo conformational modifications that allow an assembly of a large multi-protein complex called Death Initiation Signalling Complex (DISC), which leads to the activation of the caspase cascade by the activation of pro-caspase 8 [70]. Pro-caspase 8 initiates an autocatalytic process resulting in a high number of activated caspases 8, that will activate downstream caspases such as caspase 3 (Cas3) [71].

On the other hand, oxidative stress, deoxyribonucleic acid (DNA) damage and other stress conditions stimulate the intrinsic or mitochondrial pathway. In this case, the increase in mitochondrial membrane permeabilization results in the release of proteins (mainly cytochrome C) to the cytosol, activating apoptosis protease-activating factor 1 (Apaf 1) which is linked to caspase 9. The complex apaf 1- caspase 9 is required for caspase 9 function [72], which finally activates downstream caspases and drives the cell to death (Figure

6). Moreover, in mitochondrial function regulation, B cell leukemia/lymphoma 2 (bcl2) plays key roles. Bcl2 anti-apoptotic protein binds the pro-apoptotic Bcl2-associated X protein (bax) and prevents its entrance in the mitochondria. In that way, mitochondrial permeability is mantained and the release of pro-apoptotic factors such as cytochrome C is avoided [73]. Therefore, bcl2 and bax protein levels are crucial to maintain a correct mitochondrial function and to keep clear of the intrinsic apoptotic pathway. An increase in bax/bcl2 ratio can induce cytochrome C release and Cas3 activation, which leads to apoptosis [74].

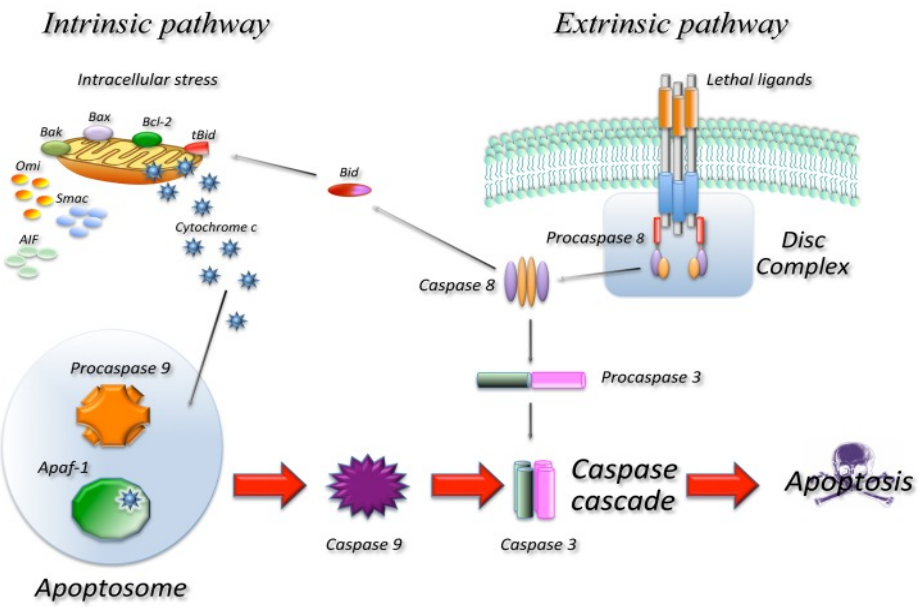


Figure 6. Signaling cascade of apoptosis. Taken by Flavaloro *et. al.* [70]. BID: BH3 interacting domain death agonist; OMI: HtrA serine peptidase 2; SMAC: diablo, IAP-binding mitochondrial protein; AIF: Apoptosis inducing factor.

Tumor suppressor p53 has also been described in the literature as an apoptotic regulator. It is reported to regulate around 500 target genes directly [75]. Among other homeostatic functions, p53 is implicated in the induction of apoptosis by acting through both the extrinsic and the intrinsic pathways. Besides, it has been shown that the impact on apoptosis is even greater when p53 regulates BH3 interacting domain death agonist (BID), a member of the pro-apoptotic Bcl2 protein family which has been described as a linker of the two apoptotic pathways [76].

2.3 Lipolysis

Lipolysis is the hydrolytic cleavage of stored triglyceride that occurs, in general terms, by the actions of three lipases: adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase

(MGL) [77]. ATGL catalyses mainly the first reaction, hydrolysing triglyceride into diacylglycerol (DAG), and releasing a FFA. After that, HSL is the main responsible for the second reaction, forming monoacylglycerol (MAG) and another FFA. At this point, MAG can be hydrolysed by MGL into glycerol and a third FFA. As a result, a glycerol molecule and three FFAs are released to the cytosol which can be re-esterified, used by other organelles or released to the bloodstream and reach other tissues.

HSL was characterized in 1960 as a lipolytic enzyme sensitive to adrenaline and until the ATGL´s discovery, it was considered the main regulator of lipolysis [78]. HSL is capable of hydrolysing triglycerides, but its affinity for DAG is 10-fold greater [79]. ATGL was described for the first time in 2004 by three research groups [80-82], who presented an additional lipase able to hydrolyse triglycerides. It is mainly expressed in WAT and BAT, and to a lower extent in other tissues [78]. MGL was purified in 1970 [83]. MGL is necessary for MAG hydrolysis, it is considered the rate-limiting enzyme in this step [84], but it does not have affinity for DAG or triglycerides.

Lipolysis regulation

Lipolysis is regulated by the nutritional status, in order to adapt the organism to energy deprivation or to an energy excess situation. In basal conditions, ATGL is located in the lipid droplet surface and although it is partially inactive, it maintains basal activity [82]. HSL remains unphosphorylated and it is located in the cytoplasm, without lipolytic action.

In energy demand situations, lipolysis regulation is carried out by several hormonal factors such as catecholamines, the principal regulators of this process [85,86]. The interaction of catecholamines with its adrenergic receptors result in a cascade of reactions, an increase in cAMP levels and consequently, PKA is activated. On its behalf, PKA activates HSL by phosphorylation and subsequent translocation from cytosol to lipid droplet surface, where it interacts with perilipin. This interaction results in perilipin multi-phosphorylation and separation of comparative gene Identification-58 (CGI-58), which interacts with ATGL, starting the activation of lipolysis in the lipid-droplet [87] (Figure 7).

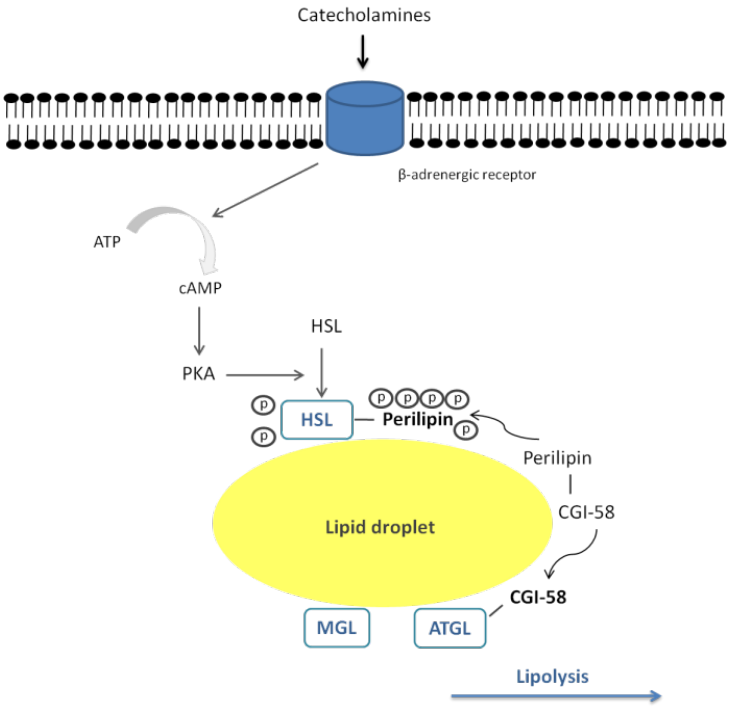


Figure 7. Activation of lipolysis in adipocytes by catecholamines. ATP: adenosine triphosphate; cAMP: cyclic adenosine 3´5´ monophosphate; PKA: protein kinase A; HSL: hormone sensitive lipase; p: phosphate group; CGI-58: comparative gene Identification-58; ATGL: adipose triglyceride lipase; MGL: monoglyceride lipase.

In a post-prandial situation, lipolysis is inhibited mainly by insulin action, which acts though different pathways. On the one hand, it acts via phosphatidylinositol kinase-3 (PI3K) after insulin-insulin receptor union in the adipocyte membrane. In the next step Akt is activated and acts on phosphodiesterase 3 (PDE3), which in turn degrades cAMP blocking PKA activation. In consequence, lipases are not stimulated and the lipolysis pathway is inactivated [88]. In addition, a cAMP-independent anti-lipolytic effect of insulin has been described. In this pathway, protein phosphatase 1 protein (PP1) is phosphorilated and it dephosphorylates and inactivates HSL [89].

2.4 Lipogenesis

Lipid accumulation in adipose tissue is the result of two phenomenon. On the one hand, the uptake of circulating triglycerides from chylomicrons and very-low density lipoproteins (VLDL) by the action of the lipoprotein lipase (LPL). This lipase is found in the vascular endothelium of adipose tissue, heart or muscle and after triglyceride hydrolysis, it provides FFA and 2-monoacylglycerols. The internalization of plasma FFAs (liberated from lipoproteins and those transported by albumin) into cells, is made by protein-facilitated transport. Scavenger receptor CD36 is responsible for this kind of transport [90]. Coburn *et. al.* verified that in

mice lacking CD36 FFA uptake on heart, skeletal muscle and adipose tissue was impaired and consequently, an increase in plasma triglyceride and fatty acid levels [91]. Apart from CD36, fatty acid binding protein (FABP) and fatty acid transport proteins (FATP) take part in FFA transport into the cell. Once in adipocytes, fatty acids are mainly re-esterified to triglycerides [92].

Another source for lipogenesis are carbohydrates. When plasma glucose levels increase, insulin is released to the bloodstream, triggering a complex cascade of reactions that allows glucose uptake by cells. It is generally accepted that glucose transport in adipose tissue is given mainly through GLUT4 protein. In addition to adipose tissue, this transporter is also present in skeletal muscle and cardiomyocytes and toa lesser extent, in brain and kidneys [93]. Thus, uptaken glucose can undergo glycolysis, which results in pyruvate that enters in the Krebs cycle. In situations of energy excess, it can be the substrate for *de novo* lipogenesis.

De novo lipogenesis is the process by which tissues are capable of synthesizing fatty acids from acetyl-CoA, a precursor coming from glucose, amino acid or even fatty acid metabolism. This metabolic pathway serves to transform excessive carbohydrates into lipids, a more efficient storage form. In animals, this process mainly takes place in liver and adipose tissue [94]. For *de novo* lipogenesis, not only acetyl-coA is necessary, but also nicotinamide adenine dinucleotide phosphate (NADPH), a cofactor with reducing power. NADPH origins are reactions catalysed by the enzymes glucose-6-phosphate dehydrogenase (G6PDH), malate dehydrogenase, malic enzyme (ME) and NADP isocitrate dehydrogenase, among others.

Acetyl-CoA molecule, resulting in Krebs cycle, is located in the mitochondria but it is transported to the cytosol by a citrate shuttle transport mechanism [95]. Once in the cytosol, acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC) enzyme with presence in adenosine triphosphate (ATP). After that, malonyl-CoA is transformed, by fatty acid synthase (FAS), in long-chain fatty acids (mainly palmitate, a 16 carbon fatty acid). In order for this step to take place, a repetitive sequence reaction catalysed by NADPH is needed. Finally, generated fatty acid can undergo desaturation, elongation, and esterification reactions before being stored as triglycerides [96]. To form a triglyceride, three fatty acid-CoA molecules are esterified with one glycolysis-derived glycerol-3-phosphate molecule through diacylglycerol acyltransferase (DGAT) enzymes (Figure 8). There are two DGAT enzymes: DGAT1 and DGAT2. Both enzymes are involved in the last and committed step of triglyceride esterification, but their protein sequence and biological functions are different [97]. Whereas DGAT2 function is closely linked to endogenous fatty acid synthesis and esterification, DGAT1 may be involved in the recycling of hydrolyzed triacylglycerols by reesterifying the fatty acids.

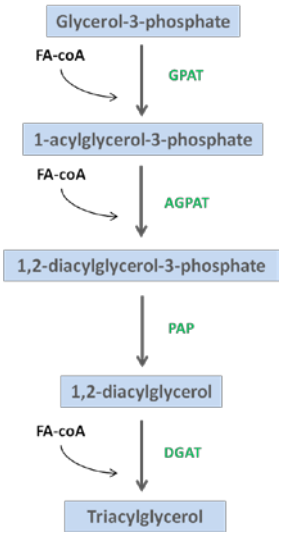


Figure 8. Triglyceride synthesis pathway. Modified from Ahmadian *et. al.* [98]. GPAT: glycerol-3-phosphate acyltransferase; AGPAT: 1-acylglycerol-3-phosphate acyltransferase; PAP: phosphatidic acid phosphatase; DGAT: diacylglycerol acyltransferase.

Lipogenesis regulation

ACC and FAS are two key rate-limiting enzymes in *de novo* lipogenesis [95] and they are regulated mainly by transcriptional and post-transcriptional mechanisms. The primary transcriptional regulators of these enzymes are SREBP1c and carbohydrate response element binding protein (ChREBP), both expressed in adipose tissue and liver [99,100]. SREBP1c is a transcription factor that regulates the expression of several genes by interacting with sterol response elements, located in their promoter regions [101]. At the same time, it can be regulated by some cofactors such as Sp1 transcription factor (SP1), which acts as activator or inhibitor depending on the binding site of the promoter region of SREBP1c. In response to some stimuli such as exercise or some PC, AMP-activated protein kinase (AMPK) (an energy sensor that regulates cellular metabolism) stimulates catabolic pathways (i.e. β-oxidation) and inhibits anabolic processes (i.e. *de novo* lipogenesis). AMPK activation down-regulates SREBP1c and thus, suppresses FAS [102]. In addition, AMPK phosphorylates and inhibits ACC [103]. Furthermore, glucose itself may modulate lipogenesis by the regulation of lipogenic gene expression via ChREBP [104,105].

There are other regulators of lipogenesis that act through direct or indirect mechanisms. PPARγ regulates lipogenic enzyme expression [106] and enhances LPL expression, acting as a gateway for the entry of fatty acids coming from bloodstream triglycerides [107]. Also, liver x receptor (LXR) can modulate *de novo* lipogenesis directly, binding FAS promoter, or via indirect mechanisms such as SREBP1c and ChREBP activation [104].

As it has been mentioned above, the glucose uptake is an important factor to take into account in the regulation of *de novo* lipogenesis. In basal conditions, intracellular vesicles containing GLUT4 are retained by Akt substrate of 160 kilodalton (kDa) (AS160), due to the fact that GLUT1 is the main responsible for glucose cell entrance in that situation [108,109] (Figure 9). However, when blood glucose levels are increased, insulin binding to the insulin receptor leads to its autophosphorylation at 6 residues. Tyr 960 is the most important one in glucose homeostasis [110] and it is recognized by 6 of the identified isoforms of insulin receptor substrate (IRS) proteins. Among these, IRS-1 is the most significant one [111]. When IRS-1 binds insulin receptor, it is phosphorylated and in consequence, new binding sites for other proteins are originated. Some of these proteins are the phosphoinositide 3-kinases **(**PI3K) family members, which transduce the signal through the PI3K pathway [112,113]. After the binding of IRS-1 to the insulin receptor, PI3K phosphorylates phosphatidylinositol- 4, 5- bisphosphate (PIP2) in its third position and thus, there is a synthesis of phosphatidylinositol- 3, 4, 5- trisphosphate (PIP3), which in turn activates phosphoinositide-dependent protein kinase 1 (PDK1) [114]. Afterwards, there is an activation of Akt, which is phosphorylated by PDK1 in its Thr 308 residue, which leads to Akt substrate of 160 kDa (AS160) phosphorylation. Finally, when AS160 is phosphorylated, vesicles containing GLUT4 translocate onto the cell membrane, where they are able to transport glucose into the cell. Furthermore, aside from GLUT4 translocation via Akt -AS160, AMPK-mediated AS160 phosphorylation was demonstrated to modify intracellular AMP/ATP ratio under several stimuli such as exercise or fasting [115].

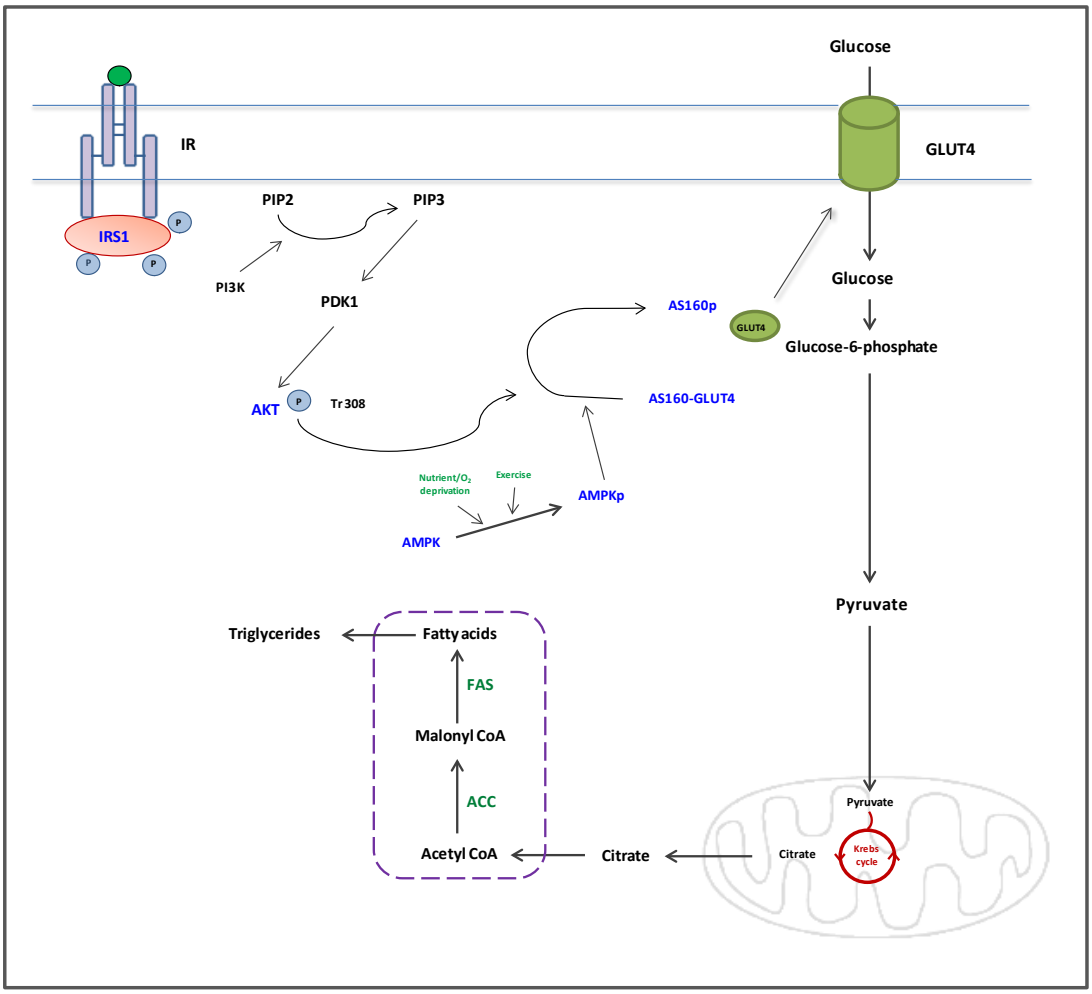


Figure 9. Scheme of insulin signaling cascade, glucose metabolism and *de novo* lipogenesis. IR: insulin receptor; IRS1: insulin receptor substrate 1; PIP2: phosphatidylinositol- 4, 5- bisphosphate; PIP3: phosphatidylinositol- 3, 4, 5- trisphosphate; PI3K: phosphatidylinositol kinase-3; PDK1: phosphoinositide-dependent protein kinase 1; Akt: protein kinase B (PKB); AS160: Akt substrate of 160 kDa; AMPK: AMP-activated protein kinase; ACC: acetyl-CoA carboxylase; FAS: fatty acid synthase.

Although the specific regulatory transcription factors have not been described yet, it has been shown that DGAT enzymes (DGAT1 and DGAT2) may be regulated transcriptionally and post-translationally by phosphorylation reactions [97]. At the transcriptional level, DGAT1 and DGAT2 regulation has been stated in the skeletal muscle and liver of mouse and rat [116,117]. In addition, it seems that DGAT activity could be modulated by nutritional status or hormones. The administration of glucagon or epinephrine in rats, leads to a decrease in DGAT activity of various tissues [118-120]. On the other hand, long-chain fatty acid treatments increased DGAT activity in primary cultured hepatocytes and rats [121,122]. Regarding the mechanisms of action, Assifi and co-workers speculated that AMPK could be involved in the regulation of DGAT enzymes [122]. Furthermore, there is some evidence that suggest that DGAT1 could be allosterically regulated through the binding of acyl-CoA to its N-terminal region [97].

2.5 Fatty acid oxidation

Fatty acid oxidation is the process by which fatty acids are degraded in order to obtain energy. There is an oxidative capacity in peroxisomes [123], but it takes place mainly in the mitochondria. The first limiting step of this process is the transport of fatty acids across the mitochondrial membrane, which occurs in 3 steps. First, fatty acid esterification with CoA takes place, a reaction catalysed by acyl-CoA synthetase in the presence of an ATP molecule. Then, the acyl-CoA molecule is transformed into acyl-carnitine and is transported across the inner mitochondrial membrane into the mitochondrial matrix by carnitine palmitoyltransferase 1 (CPT1), the rate-limiting step of fatty acid oxidation. Finally, acyl-carnitine is transported to the mitochondrial matrix by regeneration of fatty acid-CoA, a reaction catalysed by CPT2. In this reaction, a carnitine molecule is released and it returns to the cytosol by the action of a translocase. Once in the mitochondria β-oxidation starts, consisting in fatty acid degradation by a sequence of four reactions catalyzed by acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and β-ketothiolase. These reactions result in flavin adenine dinucleotide ($FADH_2$), NADH (that travels to the respiratory chain to obtain ATP) and an acetyl-coA molecule, the main substrate of the Krebs cycle.

White adipocytes posses a small number of mitochondria, so its oxidative capacity is very limited. WAT normally releases its lipolytic products to be oxidized by other tissues. However, the high number of mitochondria in brown adipocytes confer them a great ability to oxidize fatty acids [124]. It has been shown that two types of adipocytes are interconvertible, depending on several factors that allow adipose tissue to adapt to situations that require more lipid storage or lipid burning for thermogenesis.

2.6 Adipose tissue as an endocrine organ

Traditionally, adipose tissue has been considered only an energy storage organ, which accumulates fat as triglyceride and supplies energy in an energy-deprivation situation. However, knowledge derived from several studies carried out in the last decades have changed this idea, and it is now considered a complex and active endocrine tissue that plays an important role in body homeostasis [125]. Not only adipocytes but also other cells present in adipose tissue have an important secretory function. Adipokines (secreted exclusively by adipocytes) are defined as bioactive peptides and proteins that exert several biological functions, which can act locally in adipose tissue (autocrine or paracrine function) or they can reach the bloodstream and reach other tissues to exert their biological effects (endocrine function).

Adipokines regulate several physiological processes such as appetite regulation, glucose and lipid metabolism, insulin-signalling, cardiovascular function, inflammatory and immune functions and reproduction, among others [126] (Figure 10).

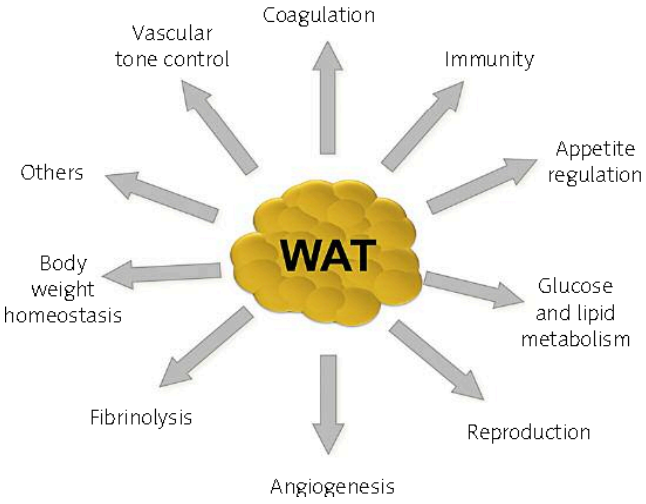


Figure 10. The principal physiological functions of adipokines. Taken by Coelho *et. al.* [127]. WAT: white adipose tissue.

Obesity and its co-morbidities are closely related to the low-grade inflammatory environment created by expanded adipose tissue. In particular, pro-inflammatory and anti-inflammatory adipokine secretion imbalance occurs in the adipose tissue [128]. Moreover, it has been suggested that adipokines could be a connection between the inflammatory state of metabolic syndrome, insulin resistance and endothelial dysfunction. Adipokines have been defined as responsible for insulin resistance in adipose tissue, skeletal muscle and liver, as well as to have regulatory functions in the immune system. Furthermore, pro-inflammatory adipokines are implicated in the development of atherosclerosis and thus, in that of cardiovascular diseases [129,130].

2.6.1 Adiponectin

Adiponectin was firstly discovered in 1995 and is highly and exclusively released by adipocytes [131]. Adiponectin plasma concentration range in humans is between 5-10 µg/mL and it supposes a 0.01% of the total plasma proteins [132]. There are two types of adiponectin receptors. AdipoR1 has a ubiquitous expression, whereas AdipoR2 expression is more restricted to the liver [133].

The clearest association between adiponectin and health has been made by its beneficial function in metabolic syndrome [132]. Adiposity and adiponectin production and secretion are inversely correlated, so it has been shown that obese people have less circulating levels of this adipokine [134]. This is important due to high plasmatic levels of adiponectin related to beneficial effects on health, being especially relevant to its protective effect from diabetes [133,135]. Adiponectin ameliorates insulin resistance by several mechanisms

of action that are summarised in a figure from Ruan *et. al.* [136] (Figure 11). It has been reported that plasma glucose levels are reduced when adiponectin administration is conducted in diabetic and healthy mice [135,137]. Moreover, in a clinical study, negative correlation between plasma adiponectin levels and fasting plasma glucose and BMI was observed [138]. It seems that adiponectin exerts its anti-diabetic effect mainly through glucose and lipid metabolism. It regulates skeletal muscle lipid metabolism by enhancing mitochondrial biogenesis and increasing fatty acid uptake as well as its further oxidation [139]. Besides, adiponectin inhibits the expression of some gluconeogenic enzymes and subsequently, liver glucose production [135]. In 2007, Chow *et. al.* demonstrated that low plasma adiponectin levels are a reliable predictor of hypertension, suggesting its important role in the regulation of blood pressure [140]. Nowadays, it is known that this is due to its anti-inflammatory and anti-atherogenic function [141].

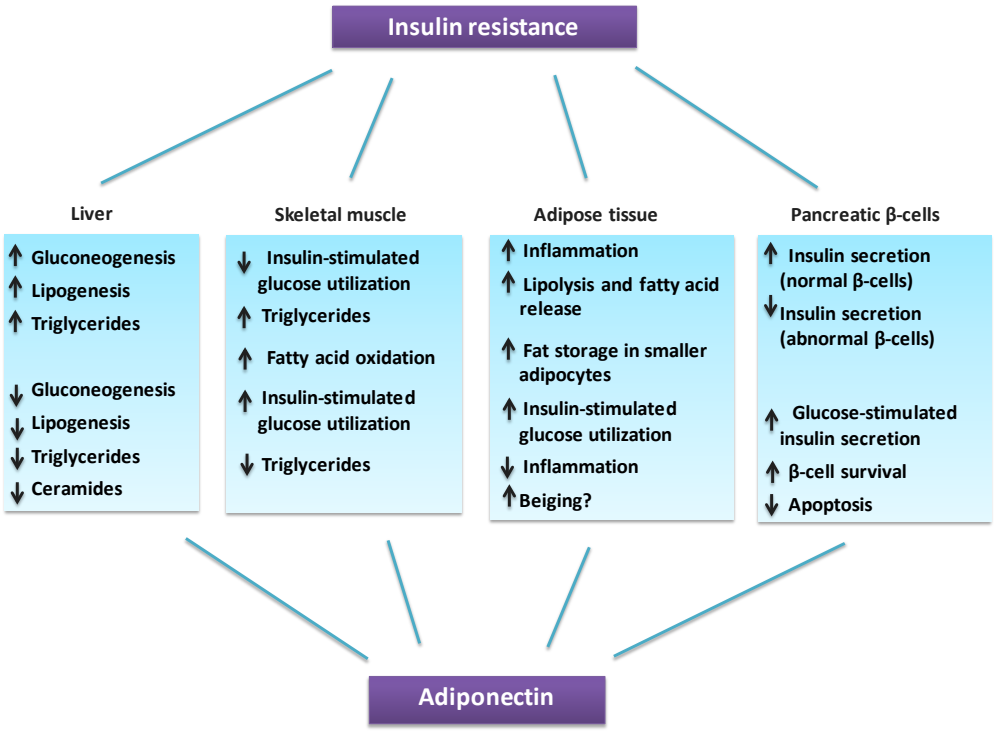


Figure 11. Mechanisms of action of adiponectin for insulin resistance amelioration. Modified from Ruan *et. al.* 2016 [136].

2.6.2 Leptin

Leptin was described by Zhang *et. al.* in 1994 [142] . It is a 16 kDa protein encoded by the *ob* (mouse) and *LEP* (human) genes and it exerts its physiological function by binding to a class I cytokine receptor superfamily receptors LEPR and Ob-R. These receptors are encoded by the *db* gen and they are expressed in six isoforms, products of alternative RNA splicing of the gene. Leptin is mainly produced and released by adipose tissue, but also by other organs and tissues such as muscle, brain, stomach, placenta, breast epithelium and some fetal tissues [143-148].

Leptin is considered a link between neuroendocrine and immune systems, because it enhances T_1 cell proliferation and cytokine production [149]. In any case, it is widely assumed that the main function of leptin is the regulation of energy homeostasis and appetite [150]. It acts as a messenger of the nutritional status in the organism and translates this message to the hypothalamus, where appetite and energy homeostasis is regulated by several neuropeptides. In a situation of fat loss or starvation, plasma leptin levels remain low, food intake is stimulated and energy expenditure is reduced. Contrarily, an adiposity increase leads to a rise in leptin levels in plasma, which is able to cross the hematoencephalic barrier in order to act through hypothalamic neuropeptides. Simultaneously, leptin inhibits orexigenic peptides (i.e. neuropeptide Y (NPY) and agouti-related protein (AgRP)) and stimulates anorexigenic ones (i.e. proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART)). In that way, it reduces food intake and enhances energy expenditure [150,151]. In obese humans or rodents, there is a hyperleptinemia that has not the ability to reduce food intake or to increase energy expenditure, which suggests a failure in leptin action called leptin resistance. In this situation, there is an impairment of leptin transport across the hematoencephalic barrier or a poor intracellular leptin signaling [152-154].

2.6.3 Visfatin

The name of this adipokine comes from “visceral fat”, due to the initial establishment between visceral fat and the adipokine plasmatic concentration [155]. It is a 52 kDa protein that is encoded by the PBEF/Visfatin gene and whereas it was initially reported to be synthesized by visceral adipose tissue, it can be produced in leucocytes, adipose resident macrophages, hepatocytes and myocytes, among others [156,157]. It can be found in both intracellular and extracellular forms. The intracellular form acts as an enzyme that catalyses the first step in nicotinamide adenine dinucleotide (NAD) biosynthesis, which has been shown to have key roles in histone deacetylation through sirtuin regulation, cell death and in the generation of reactive oxygen species. The extracellular form has mainly a cytokine and adipokine function and extracellular enzymatic activity [158]. Taking into account that visfatin has diverse cellular functions, researchers hypothesized that it could be implicated in the development of several diseases, such as atherosclerosis, cancer, inflammatory disorders, type 2 diabetes and obesity [159-163]. The insulin-mimetic activity of visfatin is well established. In mice, intravenous injection of visfatin reduces plasma glucose levels in a dose-dependent manner and it is as effective as insulin in reducing high plasma glucose levels in diabetic insulin-deficient mice. This effect is exerted through visfatin binding to the insulin receptor (but in a different site) and further signaling activation [156]. With regard to obesity, Choi *et. al.* observed that obese Korean women had higher plasma visfatin concentration than lean ones and that this levels could be reverted with a weight loss [164]. Besides, *in vitro* studies showed that visfatin is implicated in adipocyte proliferation and differentiation [156,165].

2.6.4 Apelin

Apelin is an 8.5 kDa transmembrane protein encoded by the *apln* gene [166]. In 2005, Boucher *et. al.* described this protein as a WAT-produced adipokine and they observed that its expression is enhanced by adipocyte differentiation [167]. In humans, apelin is highly expressed in adipocytes, chondrocytes, endothelial cells, the heart, skin, brain, spleen, thymus, and lungs and it is moderately expressed in skeletal muscle. Its wide distribution suggests that it could be implicated in several physiological functions such as energy metabolism, blood pressure or angiogenesis and in the development of obesity, diabetes, cancer and heart failure [168]. In 2013, Krist *et. al.* demonstrated that apelin gene expression was higher in the adipose tissue of type 2 diabetic patients and that plasma levels were positively correlated with BMI and fat mass. Besides, its expression was reduced when these patients underwent a significant weight loss [169]. In addition, it has been shown that apelin could inhibit lipolysis [170]. However, there are no solid results yet and more research in this field is needed.

In relation to glucose metabolism, some studies reported alterations in apelin levels in type 2 diabetic patients, suggesting the importance of this adipokine in insulin resistance. Due to this fact, several studies have been conducted in order to highlight this relationship. Zhu *et. al*. showed that apelin improved glucose uptake in 3T3-L1 adipocytes in a dose-dependent manner and that this improvement was carried out by enhancing GLUT4 translocation via the PI3K/Akt pathway [171].

2.6.5 Other adipokines

Apart from those mentioned above, other adipokines have acquired relevance due to their crucial biological functions. In fact, nowadays more than 50 adipokines are described [128]. Among them, **resistin** has been shown to be raised in rodents models of obesity and diabetes. It is generated in the adipogenic process of adipocytes and it reduces glucose uptake in adipocytes, miocytes and other cells by antagonizing insulin effects [172]. **Chemerin** is a chemoattractant protein which plays an important role in immunity and this could be implicated in adipocyte development. In order to establish this relationship, Goralski and co-workers knocked down the expression of chemerin and its receptor in 3T3-L1 pre-adipocyte differentiation and they showed that the adipogenic process was impaired, lipid and glucose homeostasis genes were downregulated and the metabolic functions that are characteristic of mature adipocytes were altered [173]. The **Fibroblast growth factors (FGF)** family regulate a great number of metabolic functions and the subfamily FGF-19, FGF-21 and FGF-23 have been shown to act like an endocrine molecule regulating bile acids, vitamin D, cholesterol and glucose homeostasis [174-176]. In particular, **FGF-21** has been shown to stimulate the glucose uptake in adipocytes and to increase thermogenesis, energy expenditure and fat utilization [176]. **Omentin** is another

important adipokine, mainly expressed in visceral tissue, that is easily detected in the human bloodstream. It was thought to be an insulin action regulator, but actually, it is known that it exerts other metabolic functions. In a study conducted with Japanese subjects, authors observed that low levels of plasma omentin were correlated with bigger waist circumference, dyslipidemia, high blood pressure and glucose intolerance [177].

The above-mentioned adipokines represent only a small part of very important metabolic regulators. Although sustancial efforts have been done in order to identify novel adipokines that could be therapeutic targets in obesity and its co-morbidities treatment, more studies are needed.

3. Phenolic compounds: metabolism and biological functions

Dietary plant-based foods represent a great source of PC, which are useful to reduce the prevalence of several diseases. They are widely distributed, being fruits and vegetables, green and black tea, red wine, coffee, chocolate, olives and olive oil, herbs, spices, nuts and algae the most important sources [178,179]. Some PC are present in specific foodstuffs, such as soya´s isoflavones, whereas others can be found as a mixture of them. Their quantity in each product can differ significantly. Furthermore, some external factors, such as the type of culture, sun exposure, ripeness of fruits or their storage, can affect the profile and concentration of PC in the same foodstuff [180,181].

PC are classified as flavonoids and non-flavonoids, which are subdivided into even more subclasses. This classification is carried out according to the number of phenol rings and the elements that are bound to these rings [182]. Flavonoids are classified as flavonols, flavons, proanthocyanidins, anthocyanidins, flavan-3-ols (or cathechins), flavanones and isoflavones (Figure 12) [183]. On the other hand, non-flavonoids are classified as hydroxycinnamic acids, hydrolysable tannins, hydroxybenzoic acids and stilbenes. The term polyphenol refers to molecules that contain more than one phenol ring. As all flavonoids contain 3 phenol rings, they can be named polyphenols. This does not occur with non-flavonoid molecules that only have one or two phenol groups in their structure.

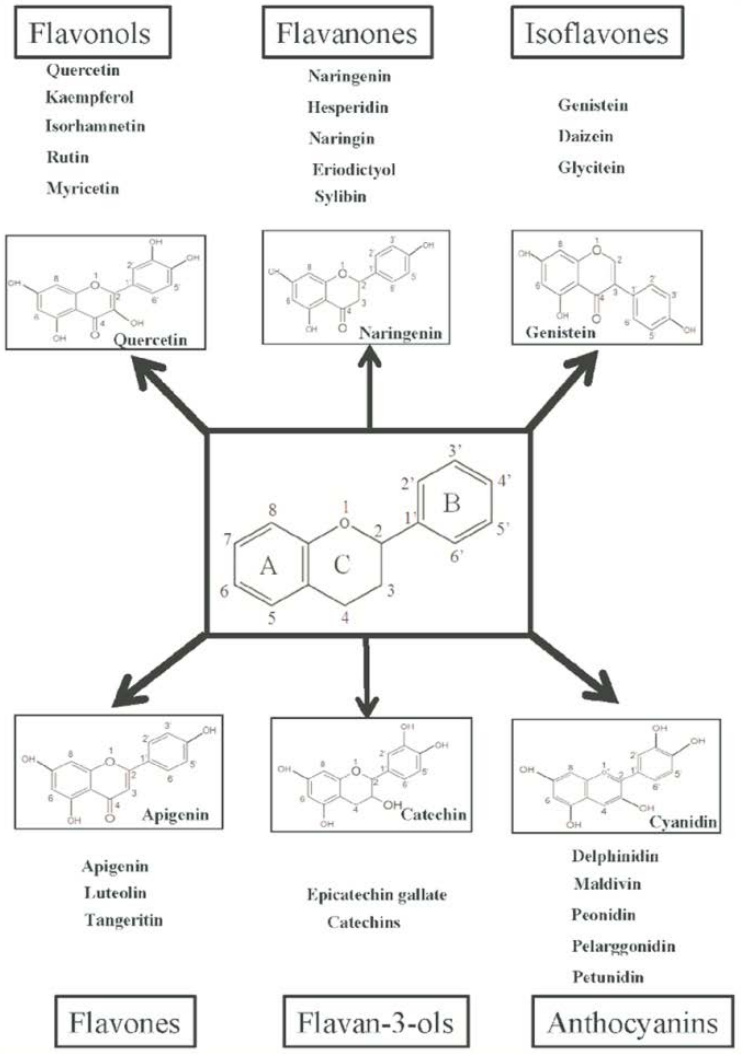


Figure 12. Subdivision of flavonoids in 6 classes, belonging molecules and examples of the chemical structure of each group. Modified from Hossain *et. al.* [184].

To measure the dietary intake of PC in humans different methods can be used. On the one hand, food composition databases, where PC content of foodstuffs is collected. This information, in combination with dietary intake data obtained through food frequency questionnaires or 24-h recalls, make the theoretical calculation of PC intake possible [185]. On the other hand, the analysis of several biomarkers, mainly metabolites present in bloodstream and fecal and urine samples, is a very useful tool to estimate the intake of PC. Keeping in mind the strengths and limitations of each methodology, a combination of data obtained by both methods in different studies represent the best way to estimate the most accurate ingestion.

Data from studies carried out in the last decade show that the total intake of PC oscillates between 1000-1500 mg/person/day [186,187], even though other studies have reported lower values [188]. However, it is important to point out that several factors affect their bioavailability. Among them, there are some external factors (i.e. sun exposure), those related to food processing (i.e. boiling, baking), food-related factors (i.e. the

food matrix), the existence of interactions with other compounds (i.e. other polyphenols with similar absorption mechanism), factors directly related with the molecule (i.e. chemical structure) and the host-related factors (i.e. intestinal factors) [179].

PC undergo rapid metabolism after their oral ingestion (Figure 13). Despite slight differences, this metabolism is similar in humans and rodents. First of all, as most of them befall in glycosylated forms in nature, they have to be hydrolyzed to the aglycone form prior to being absorbed in the small intestine. Two mechanisms have been proposed. In the first one, the epithelial cell-enzyme lactase-phlorizin hydrolase (LPH) deglycosylates PC and after that, the released free aglycones are ready to enter in epithelial cells by passive diffusion [189]. In the second one, glucosides are transported through sodium-dependent glucose transporter 1 (SGLT1) with the involvement of the cytosolic β-glucosidase [190]. Those compounds that are not absorbed in the small intestine reach the colon, where they are absorbed after suffering important structural modifications. The colonic microbiota is crucial because it hydrolyzes glycosides into the aglycone form in order to absorb them [191]. It is important to point out that individual differences in the hydrolyzation of PC suppose a great inter-individual variability in bioavailability, so it is the most important host-related factor.

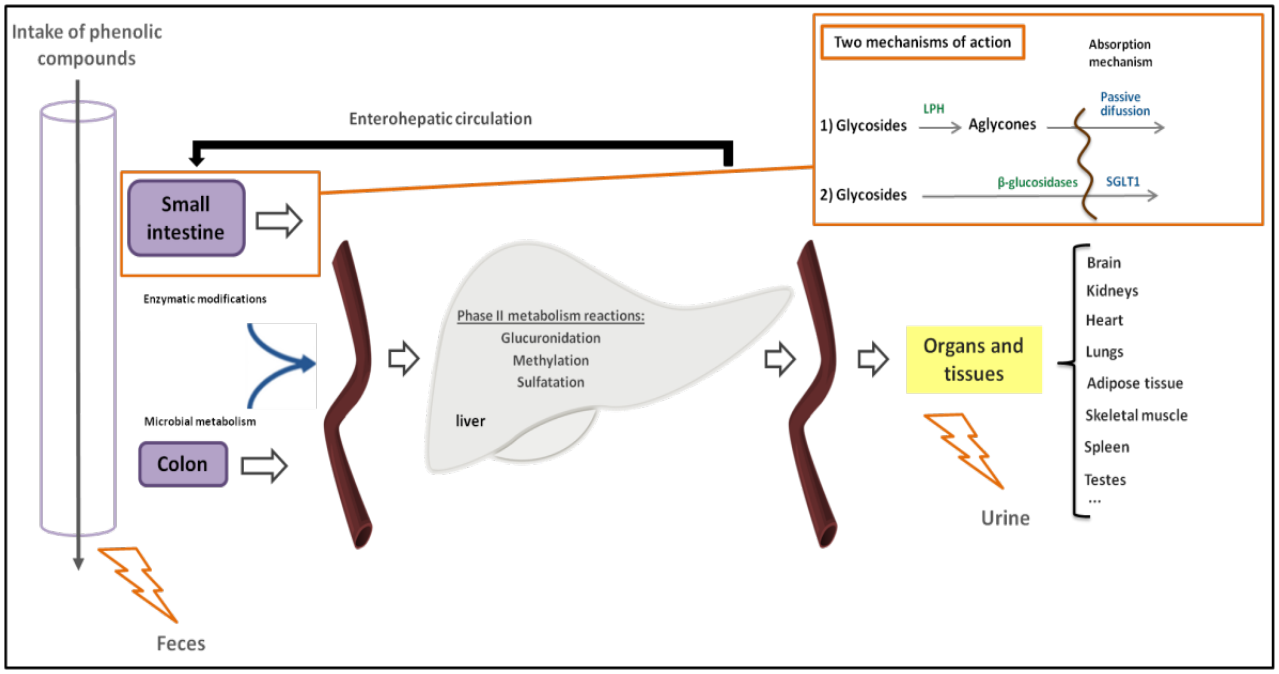


Figure 13. Schematic representation of polyphenol metabolism. LPH: enzyme lactase-phlorizin hydrolase; SGLT1: sodium-dependent glucose transporter 1.

Conjugation reactions occur for polyphenols. The first reaction takes places in enterocytes before reaching the bloodstream. Once in circulation, polyphenols reach the liver by the portal vein, where they undergo a second conjugation reaction [180]. The most important conjugation reactions are glucuronidation, methylation and sulfatation, phase II xenobiotic metabolism reactions, which serve as a detoxication process

in order to reduce their potential toxic effect and facilitate its biliary and urinary excretion. When biliary excretion occurs, some excreted conjugated polyphenols can enter the enterohepatic circulation and be reabsorbed by the small intestine [192]. Uridine-5´-diphosphate glucuronosyltransferase (UGT) enzyme transfers a glucuronic acid from UDP-glucuronic acid to polyphenols, a reaction that takes place first in enterocytes and later in the liver. UGTs are located in a great number of tissues, specifically in the endoplasmatic reticulum of cells. Methylation reactions are catalysed by Catechol-O-methyltransferases (COMT), which transfer a methyl group from adenosyl-methionine to the polyphenol. Even thougth COMT are widely distributed in tissues, their activity in liver and kidneys should be highlighted [193]. Finally, sulfatation reactions occur mainly in liver [194], where sulfotransferases (SULT) transfer a sulfate group from phosphoadenosine phosphosulfate to a hydroxyl group of polyphenols. As a result, polyphenol conjugation reactions lead to a great number of metabolites, that can reach tissues and organs. Considering that their chemical structure is different from the parent compound, their biological effect can differ as well. For example, in the case of quercetin, around 20 different metabolites have been described in humans [195,196].

After the second metabolism reaction in the liver, polyphenols are distributed to several tissues and they can also be excreted. Some polyphenol conjugates are secreted to the intestine lumen as biliary acid components, and unless being reabsorbed, they are excreted via feces. Another part of polyphenol conjugates is eliminated through urine after bloodstream circulation [192].

3.1. Resveratrol

Resveratrol (3,4',5-trihydroxystilbene) was firstly described in 1939 by Takaoka in *Veratrum grandiflorum* flowering plant [197]. It is a stilbene naturally found in several plants produced in response to a stress situation caused by ultraviolet radiation or fungal, bacterial and viral infection [198]. It is found mainly in grapes and their derived beverages (wine and grape juice), peanuts and berries, among others. The molecule contains two phenol rings bound by an ethylene bridge that can be found in two isomeric forms, *cis*-resveratrol and *trans*-resveratrol, being the last one the most studied and biologically active one [199] (Figure 14). In nature, its glucoside form (piceid), bound to a glucose molecule, is widely extended.

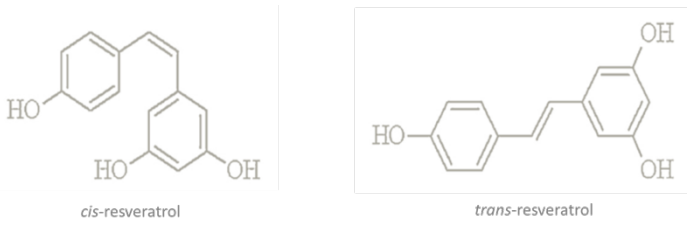


Figure 14. Isomeric forms of resveratrol. Modified from Salehi *et. al*. [199].

The beneficial effects of resveratrol in humans were described for the first time in 1982. In an observational study, an inverse relationship between moderate red wine consumption and the prevalence of cardiovascular diseases was established [200]. After that, grapevine compounds were analysed and resveratrol was described as the main responsible for this biological effect. Ten years later, in 1991, the French scientist Serge Renaud formulated the so-called "The French paradox" [201]. Over the years, antioxidant, anti-inflammatory, immunomodulation, insulin-sensitizing and anti-neoplasic actions have been attributed to resveratrol, claiming the positive effects of the molecule in cardiovascular health, cancer and diabetes, among others [202,203]. Furthermore, it has been shown that resveratrol exerts anti-obesity activity and reduces body fat through several mechanisms, such as modulation of food intake, nutrients absorption modification, adipocyte lifecycle regulation, diminution of adipose tissue inflammation, mitochondrial function regulation, induction of thermogenesis and modulation of gut microbiota [204]. However, its low bioavailability is a matter of concern for scientists, due to the fact that a low amount of the parent compound reaches plasma and tissues.

3.1.1. Dietary intake, absorption, distribution and metabolism of resveratrol

The dietary average intake of resveratrol is difficult to estimate, however it has been reported that in Europe the intake of stilbenes is around 2-3 mg/day, being resveratrol and piceid intake about 50% of this amount [187]. Around 70% of resveratrol is absorbed by the small intestine by passive diffusion or transported by plasmatic membrane proteins, such as integrins [205]. In plasma, it is mainly present in glucuronide and sulfate forms (conjugated primarily in their 3rd and 4th positions), suggesting that enterocytes have the capacity for phase II conjugation-reactions. Contrarily, phase I reactions such as oxidation, reduction, and hydrolysis have not been described for this polyphenol. Apart from the conjugated forms, a small amount of unconjugated free resveratrol seems to reach plasma. As it has been stated in animal models and humans, its peak concentration occurs at 30 minutes [206-208]. Then, the absorbed resveratrol is transported through the portal vein to the liver, and this transport is made outbound to albumin, blood cells and low density lipoproteins (LDL) [209-211]. As it occurs in enterocytes, resveratrol is subjected to phase II conjugation reactions, glucuronidation reactions catalysed by UGT enzymes and sulfatation reactions by SULTs. After these second conjugation reactions, resveratrol metabolites are distributed to different tissues.

It has been observed that comparing to resveratrol, 20-fold higher concentration of its metabolites is found in human plasma. Reseach in humans demonstrate that the most abundant metabolites are glucuronide and sulfate ones [207,208,212-214], and more specifically, resveratrol-3-*O*-glucuronide (3G), resveratrol-4´-*O*-glucuronide (4G) and resveratrol-3-*O*-sulfate (3S) [215,216]. Rough calculations estimate that in humans, the plasmatic concentration of resveratrol is less than 40 nM, even if resveratrol and its metabolites set is around

2 μM, a concentration that was reached after the supplementation of 25 mg of resveratrol [207,217]. Higher concentrations have been detected in some studies, like the one conducted by Ardid-Ruiz and co-workers, where supplemented Wistar rats showed concentrations that reach 20 μM in the case of 4G [218]. Similarly, it has been shown that after resveratrol supplementation in humans, the plasmatic concentrations of each metabolite reach 10 to 20 μM [216]. The proportion of glucuronide and sulfate metabolites in tissue and organs are tissue and dose dependent, and vary depending on species-specific metabolism differences. Juan *et. al.* showed that glucuronide metabolites were highly concentrated in the testes and the liver, but not in the lung [219]. When resveratrol supplementation dose increases, the proportion of glucuronide metabolites in the bloodstream decreases, while that of sulfate metabolites increases [218]. Finally, the metabolism is also influenced by the species, due to the fact that sulfate metabolites are predominantly found in rats, whereas in humans glucuronide metabolites are the main ones [208,212].

3.1.2. Resveratrol and its metabolites as anti-obesity molecules: their effects on lipid and carbohydrate metabolism.

The use of resveratrol as an anti-obesity agent was established several years ago, due to well-defined mechanisms of action, which include inhibition of adipogenesis, diminution of lipogenesis, increase in apoptosis and lipolysis and enhanced mitochondrial function and thermogenesis [220-224]. One of the most important actions of resveratrol is its capacity to mimic calorie restriction by the sirtuin 1 (SIRT1) activation. SIRT1 is a member of 7 sirtuin-family that regulates the aging process and mediates lifespan extension induced by calorie restriction [225]. Taking into account that calorie restriction could be considered as an effective dietary approach preventing the deleterious effects of calorie excess (fat accumulation and insulin resistance), agents that could mimic this effect are especially interesting. Among other biological actions, SIRT1 has an important function in the regulation of energy metabolism. It deacetylates and consequently activates peroxisome proliferator-activated receptor gamma co-activator 1 α (PGC1α) in muscle and hepatic cells, increasing the expression of genes that are implicated in muscle gluconeogenesis and hepatic glycolysis. Furthermore, in calorie restriction situation, PGC1α activation leads to mitochondrial biogenesis and fatty acid oxidation in muscle [226]. Moreover, SIRT1 has been related to insulin resistance improvement, adipogenesis inhibition, lipolysis activation and fat mobilization by targeting PPARγ [227-229].

Regarding the effect of resveratrol metabolites on SIRT1 activation, there is no data about this issue in adipocytes. However, in a study carried out in 2015, Schueller and co-workers observed that glucuronate and sulfate metabolites up-regulated SIRT1 expression in human U937 macrophages [214]. Furthermore, in another research, Calamini *et. al.* performed a fluorometric activity assay in order to observe if resveratrol

sulfate metabolites (3S and resveratrol-4-O-sulfate-4S-) were able to activate human SIRT1 construction, and they demonstrated that both metabolites activated SIRT1 to the same extent that resveratrol did [230].

As stated before, *in vitro* studies performed in immortalized cells lines resulted useful in order to study the effect of resveratrol in **adipogenesis**. In fact, a great number of experiments in this field have been done in murine cell line such as 3T3-L1 cells. Taking into account the key role of adipogenic transcription factors such as PPARγ, CEBPα, CEBPβ or SREBP1c, the effect of resveratrol on them has been extensively analysed. It has been shown that there is a reduction in their expression after a resveratrol treatment at different doses and durations, leading to a reduction in the fat content of maturing pre-adipocytes [220,231,232]. Furthermore, studies performed in murine and human adipocytes have stated that resveratrol inhibition in pre-adipocyte proliferation and differentiation is carried out in a SIRT1 dependent-manner [227,233].

Another anti-obesity mechanism of action of resveratrol is the **lipogenesis** diminution. As it has been mentioned in the adipocyte biology section, the sources for adipocyte´s lipogenesis could be, on the one hand, **fatty acid uptake** from the circulating triglyceride-rich lipoproteins (chylomicrons and VLDL), and on the other hand, glucose that has been uptaken by adipocytes after insulin union with its receptor. Several *in vitro* and *in vivo* studies have shown that resveratrol reduces lipogenesis mainly via down-regulation of the key lipogenic enzymes FAS and ACC (transcriptional and post-transcriptionally) and measuring the glucose utilization for lipid synthesis [220,233-237]. The lipogenic substrates of adipocytes are FFAs and glucose, so it is important to take into consideration the significance of the **insulin signaling cascade** and **glucose uptake** in adipocytes. It has been stated that resveratrol reduces plasmatic glucose and insulin levels and that improves insulin resistance in insulin-resistant *in vivo* models [223,238-240]. Among the mechanisms by which it exerts this action, insulin-stimulated glucose uptake in cultured cells and the expression variation in those adipokines that modulate insulin-sensitivity, such as adiponectin or resistin have been observed [241,242].

Several studies have observed an increase in **lipolysis** after resveratrol treatment [222,227,234,243,244]. In a study conducted in 3T3-L1 and SGBS cells, Lasa *et. al.* claimed that resveratrol regulates the lipolytic pathway acting mainly on ATGL at transcriptional and post-transcriptional levels [245]. By contrast, in two studies carried out with Zucker and Sprague-Dawley rats, resveratrol increased *hsl* gene expression without changes in *atgl* gene expression [243,244]. Although differences in the experimental conditions could be responsible for this differences, it is important to highlight that there is not a consensus in this mechanism of action and that more studies are needed.

Apoptosis is another potential mechanism of resveratrol. In *in vitro* studies performed with 3T3-L1 and porcine adipocytes, resveratrol played apoptotic effect at higher doses than 50 μM [220,246-250]. Apoptosis induction in a dose-dependent manner via AMPKα activation and the consequent reduction in Akt

activity, leading to intrinsic (mitochondrial) apoptotic pathway activation was also observed [250]. Furthermore, the apoptotic effect of resveratrol in human SGBS was also postulated [251].

An anti-inflammatory effect of resveratrol has been proposed in several studies, as well as other biological effects related to **adipokine secretion**. With regard to leptin, in the previously mentioned study of Ardid-Ruiz *et. al.*, the authors analysed whether resveratrol exerted a part of its body fat-lowering effect by modifying leptin sensitivity in liver, skeletal muscle and adipose tissue. They observed that resveratrol restored leptin sensitivity in rats with diet-induced obesity in the three analysed tissues [218]. Adiponectin, is another adipokine that seems to be related with resveratrol. In a recent study, Jimoh and co-workers observed that resveratrol treatment increased adiponectin levels and decreased those of leptin, at the same time that plasma insulin levels were reduced in hypercholesterolemic rabbits [252]. However, in a systematic review and meta-analysis of clinical trials, Mohammadi-Sartang *et. al.* did not conclude these beneficial effects, even though they reported consistent results on adiponectin concentrations in plasma after resveratrol supplementation [253]. Taking into account the insulin-mimetic activity of visfatin [156], its expression was also linked to the resveratrol effect. Diabetic Wistar rats treated with resveratrol at a range of 1 to 10 mg/kg body weight/day for one month, revealed results suggesting that the hypoglycemic effect of resveratrol was carried out, at least in part, by the modulation of visfatin expression in visceral adipose tissue [254]. In another study, resveratrol also restored the increase of visfatin levels in the liver of diabetic rats, even though resveratrol did not exert beneficial effects when non-diabetic rats were treated [255]. Finally, a study of Derdemezis *et. al.* should be mentioned, where decreased visfatin secretion was observed in SGBS adipocytes after treatment of resveratrol at 25 μM [256]. To study the effect of resveratrol on apelin expression, also diabetic rats were used by Zarei and co-workers. A reduction in serum glucose levels was observed in rats treated with 5 and 10 mg/kg body weight/day, but no differences were observed in apelin expression, suggesting that the effect could be carried out by insulin increase and modulation of resistin expression [241].

In spite of the great number of *in vitro* and *in vivo* studies, no so many **clinical trials** have been conducted in order to elucidate the anti-obesity effects of resveratrol. In a study in which obese men were given 150 mg of resveratrol per day during a month, authors observed that the supplementation reduced the adipocyte size in subcutaneous adipose tissue. Further analysis in adipose tissue suggested an increase in adipogenesis, proposing it as a mechanism for improvement of the adipose tissue impairment-derived insulin resistance [257,258]. In another study, subjects with metabolic syndrome were treated with 1 gr of resveratrol per day for 4 months and they were subjected to metabolomic analysis. Among other results, the authors observed that there was an increase in muscle turnover markers and that lipid metabolism was considerably affected, mainly in terms of an increase of intracellular glycerol and long-chain fatty acid accumulation [259]. In general terms, although unexpected and scarce results have been observed in clinical trials, most of them

have been carried out using resveratrol as a therapeutic treatment, and maybe other promising results could be achieved by using it as a preventive tool.

The positive effects of resveratrol as anti-obesity molecule have been summarized in Table 4.

Table 4. Summary of the observed anti-obesity effects of resveratrol in *in vitro* and *in vivo* studies.

Metabolic pathway	***Mechanism***	***Cell type or tissue***	***References***
Adipogenesis	↓PPARγ gene and protein expression	Murine 3T3-L1 adipocytes Human SGBS cells Epididymal adipose tissue of obese mice	[220,231,232, 233,235]
	↓CEBPα gene and protein expression	Murine 3T3-L1 adipocytes Epididymal adipose tissue of obese mice	[220, 231, 232, 235]
	↓SREBP1C gene and protein expression	Murine 3T3-L1 adipocytes Epididymal adipose tissue of obese mice	[220, 231, 232]
	↑SIRT1 activity	Human SGBS cells	[233]
	↓LPL gene expression	Murine 3T3-L1 adipocytes Epididymal adipose tissue of obese mice	[220, 231]
Fatty acid uptake and lipogenesis	↓FAS gene and protein expression and activity	Murine 3T3-L1 adipocytes Human SGBS cells Adipose tissue of Sprague-Dawley rats	[220, 233,237, 243]
	↓ACC gene expression and activity	Human SGBS cells Adipose tissue of Sprague-Dawley rats Adipose tissue of Zucker rats	[233, 243,244]
	↑GLUT4 gene and protein expression	Human SGBS cells	[233]
	Basal and insulin-stimulated glucose uptake	Human SGBS cells	[233]
	Basal and insulin-induced glucose conversion to lipids	Isolated rat adipocytes	[234]
	↑Insulin signalling	Adipose tissue of C57BL/6J mice	[236]
Lipolysis	↑ATGL gene and protein expression	Human SGBS cells	[222]
	↑HSL gene expression	Adipose tissue of Sprague-Dawley rats Adipose tissue of Zucker rats	[243,244]
Apoptosis	↑AMPKα protein expression ↓Akt activity	Murine 3T3-L1 adipocytes	[250]
	↑Caspase activation	Primary human pre-adipocytes from subcutaneos adipose tissue	[251]
Mitochondrial biogenesis and function	↑PGC1α deacetylation via SIRT1	C57BL/6J mice	[223]
Adipokine modulation	↓Leptin gene expression and blood levels ↑Leptin sensitivity index	Epididymal adipose tissue of obese mice Serum of hypercholesterolemic rabbits Adipose tissue of Wistar rats	[218, 231, 252]
	↓ Resistin gene expression	Adipose tissue of Wistar rats	[241]
	↑ Adiponectin protein expression and serum levels	Serum of hypercholesterolemic rabbits Murine 3T3-L1 adipocytes	[252, 242]
	↓Visfatin secretion (in the media)	Human SGBS cells	[256]

PPARγ: peroxisome proliferator-activated γ receptor; CEBPα: CCAAT enhancer binding protein α; SREBP1c: SREBP cleavage activating protein 1, c isoform; SIRT1: sirtuin 1; LPL: lipoprotein lipase; FAS: fatty acid synthetase; ACC: acetyl-CoA carboxylase; GLUT4: glucose transporter 4; ATGL: adipose triglyceride lipase; HSL: hormone sensitive lipase; AMPK: AMP-activated protein kinase; Akt: AMP-activated protein kinase; PGC1α: peroxisome proliferator-activated receptor gamma co-activator 1α.

Finally, taking into account the large metabolism suffered by resveratrol, it is important to address the potential anti-obesity effects of its **metabolites**. Even though the specific effects of resveratrol metabolites have been extensivey studied in other chronic diseases such as cancer, there is no data of other authors about their contribution to the anti-obesity effect of resveratrol, and specifically, to their effect on the main mechanisms that regulate glucose and lipid metabolism. Regarding the effect of resveratrol metabolites in cancer prevention or the inhibition of its progression, it has been shown that the main mechanisms of action are related to its apoptotic, antioxidant and cytotoxic effects, among others [260,261]. Focusing on their mechanistic features, it is important to highlight a study conducted by Calleri *et. al.*, where they studied if resveratrol, 3G, 4G and 3S binded directly with PPARs. They observed that PPARγ was a target of resveratrol and the three metabolites, whereas only resveratrol bound PPARα [262]. It is very important to bear in mind that some *in vivo* studies have suggested that resveratrol metabolites could be biologically active in themselves or by conversion to the parent compound by glucuronidase or sulfatase enzymes in target tissues [263]. This represents one of the main future research lines to work on the field of resveratrol and its metabolites.

3.2. Quercetin

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonol occurring naturally in plant-derived foodstuffs like fruits and vegetables, and it is especially abundant in apples, cherries, berries, onions, broccoli and tea [264,265]. As it is a flavonoid, it contains three phenol rings with attached hydroxyl groups ((OH) groups).

Several biological functions have been attributed to quercetin, such as anti-inflammatory, anti-diabetic, anti-hypertensive, anti-obesity, anti-hypercholesterolemic and anti-atherosclerotic functions [266,267]. However, similarly to what takes place with resveratrol, its low bioavailability could be considered a limiting step in its biological functionality.

3.2.1. Dietary intake, absorption, distribution and metabolism of quercetin

It has been estimated that quercetin dietary intake is higher than that of other polyphenols in humans [268], and it supposes around 60% of flavonol intake. The average daily intake of quercetin oscillates between 5 and 40 mg, although it can reach values of 200-500mg in fruit and vegetable-rich diets, mainly when they are eaten with skin [269]. In a study carried out by Olthof *et. al.* in 9 healthy volunteers, the ingestion of approximately 150 mg of quercetin glycosides led to a total quercetin plasma concentration of approximately 5 µmol/L in 30 minutes [270]. In general terms, after regular quercetin consumption in the diet, the molecule

appears in nanomolar range in plasma. However, after quercetin supplementation, this amount can increase to µmolar range. In a clinical study carried out by Conquer *et. al.* in 1998, the authors demonstrated that after 28 days of quercetin supplementation (1 g/day), the plasma concentrations of quercetin increased to 1.5 µM [271].

Dietary quercetin intake takes place mainly in glycosylated forms (quercetin glucoside and quercetin rutinoside), and among flavonoids, quercetin glucosides are those that are better absorbed. It has been shown that a part of quercetin aglycone is absorbed in the stomach, but this fact does not occur in the case of quercetin glycosides [272]. The latter are absorbed in the small intestine by the above-mentioned mechanisms: via LPH enzyme and through the membrane transporter SGLT1 [273,274]. In the particular case of glucoside, quercetin grutinoside, the absorption is different. It is absorbed in the colon after the deglycosylation reactions carried out by the microbiota´s β-glucosidases [275,276].

Quercetin is conjugated by glucuronidation, sulfatation and methylation reactions in the intestinal and hepatic cells. Thus, the main metabolites that have been detected in plasma are: 8.5-11% of isorhamnetin (ISO; 3´-O-methyl-quercetin), 10-13% of tamarixetin (TAM; 4´-O-methyl-quercetin) and 78-79% of quercetin conjugates. Among these conjugated metabolites, the most abundant are quercetin-3-O-glucuronide (Q3G) and quercetin-3-O-sulfate (Q3S), as well as ISO-3-O-glucuronide [277,278]. It seems that Quercetin glucuronides, could itself act as quercetin carriers, being further deconjugated in target tissues in order to be released as free aglycones [279].

In a study performed to assess the tissue distribution of quercetin, de Boer and co-workers fed rats with 50 and 500 mg/kg body weight of quercetin for 11 weeks, and pigs with 500 mg/kg body weight for 3 days [280]. Quercetin, ISO and TAM content in tissues were analysed and they found that in rats, the major quercetin summatory concentration was located in the lungs, an intermediate quantity in liver and kidneys and the lowest concentration in the brain, white fat and the spleen. In pigs, the highest quercetin and metabolites concentration was found in the liver and kidneys, whereas in the rest of tissues, only a very low amount of them was found. In another study performed by Yang *et. al*. in 2016, they treated rats with intravenous 3G at 10 mg/ kg body weight and they analysed the kidney, liver, heart and brain distribution [264]. The highest concentration was found in the liver, followed by the kidneys, the heart and the brain.

Quercetin elimination is given by urine and biliary-fecal excretion. As it has been mentioned above, quercetin enters the enterohepatic circulation, and for this reason, the biliary excretion returns it back to the intestinal lumen, where it can be reabsorbed or it continues its way to the colon. Once there, microbiota degrades quercetin and it is excreted via feces. The final degradation process of quercetin occurs by a deconjugation of quercetin and the formation of phenolic acids, eliminated via urine and feces, and CO_2, which

is eliminated via respiration [281]. Manach and co-workers analysed several polyphenol´s bioavailability in humans, establishing that quercetin´s half-life ranges from 11 to 28 hours [282].

3.2.2. Quercetin and its metabolites as anti-obesity molecules: their effects on lipid and carbohydrate metabolism

Among all the biological functions attributed to quercetin, in the last years, its anti-diabetic effect has attracted attention. Quercetin reduces glucose absorption by the inhibition of β-glucosidases, increases insulin sensitivity, stimulates insulin-release, enhances glucose uptake by tissues, resulting in a significant reduction of blood glucose levels in animal models [283-285]. The main regulators of glucose homeostasis are **skeletal muscle** and the **liver**, so the effects of quercetin in myocytes and hepatocytes have been deeply analysed. One of the main mechanism described in myocytes is the GLUT4 translocation to the plasmatic membrane via AMPK activation, which in turn increases the glucose uptake [286,287]. In animal models, it has been shown that quercetin increased the mitochondria number and improved mitochondrial function, reduced inflammatory cytokines and macrophage accumulation, and increased GLUT4 expression as well [288-291]. In the case of the liver, a study performed by Eid *et. al.* showed that quercetin was able to reduce the hepatic glucose production in cultured rat H4IIE hepatocytes [286].

Other extensively studied effect of quercetin has been the one on body-fat accumulation. The effect of quercetin in adipose tissue and liver fat accumulation, the restore of several plasmatic parameters after diet-induced obesity, etc. were stated by numerous *in vivo* studies [292-295]. Some of the mechanisms of action on adipocyte´s fat-accumulation are summarized in figure 15 (figure 15).

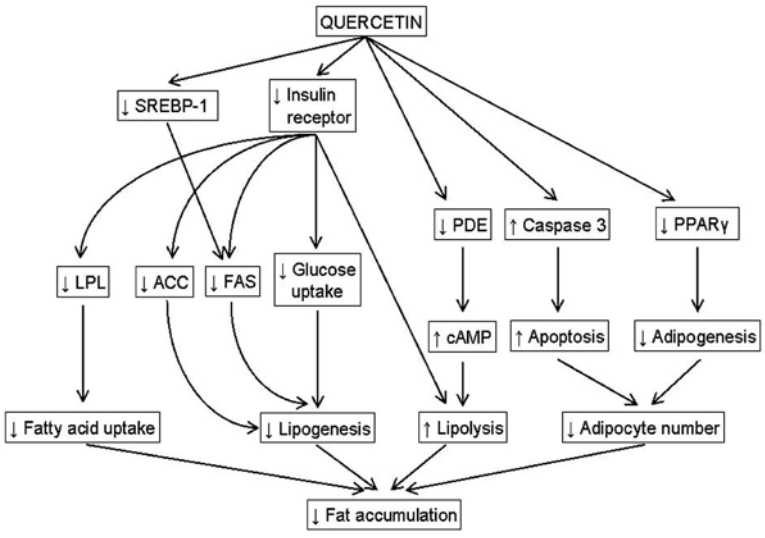


Figure 15. Mechanisms of action of quercetin for reduction of fat accumulation. Taken from Aguirre *et. al.* 2011 [296]. SREBP-1: SREBP cleavage activating protein 1; LPL: lipoprotein lipase; ACC: acetyl-CoA carboxylase; FAS: fatty acid synthetase; PDE: phosphodiesterase; cAMP: cyclic adenosine 3´5´ monophosphate; PPARγ: peroxisome proliferator-activated receptor γ.

As resveratrol does, quercetin also has demonstrated **anti-adipogenic effects** via down-regulation of adipocyte differentiation key transcription factors. Treatment of 3T3-L1 pre-adipocytes with different doses of quercetin during the differentiation process led to reduction of SREBP1c, CEBPβ, PPARγ, CEBPα and FAS expression [297,298]. Unlike resveratrol, the effect of quercetin metabolites in adipogenesis has been more largely studied. Specially, the anti-adipogenic effect of ISO has been observed [299,300], as well as that of the naturally occurring quercetin-3-O-(6´´-feruloyl)-b-D-galactopyranoside [301]. In both cases , authors proposed that these two metabolites inhibited adipogenesis reducing the expression of the key regulators PPARγ and CEBPα, among others.

Bearing in mind that approximately 10% of adipocytes undergo a renewal process, balance between adipogenesis and apoptosis should be considered when looking for the way to control the excessive fat mass expansion [36]. It has been indicated that quercetin is able to induce **apoptosis** via down-regulation of poly (ADP-ribose) polymerase (PARP) and bcl2 and the activation of pro-apoptotic proteins Cas3, Bax and Bak [302].

Apart from the regulation of adipocyte number, the effect of quercetin in adipocytes lipid metabolism has been addressed. In 1992, Kuppusamy and Das demonstrated the **lipolytic effect** of quercetin via inhibition of the anti-lipolytic PDE activity and the subsequent enhancement of HSL activity after the treatment of primary cultured rat adipocytes [303]. In another study conducted using training mice, quercetin supplementation derived the attainment of energy fuel from glycolysis to lipolysis [304]. It seems that **lipogenesis** is another target of quercetin, in fact, it inhibits this procces by acting mainly in the gene and protein expressions of FAS and ACC. These effects on lipogenesis reduction have been observed in both murine adipocytes and liver [294,297]. On the other hand, it has been also reported a reduction in LPL activity and the subsequent diminution of FFA uptake from the bloodstream [305]. Due to its implication in lipogenesis, it is important to consider the potential effect of quercetin in glucose uptake. Hamilton *et. al.* demonstrated that quercetin was able to reduce glucose transport across the plasmatic membrane in L929 adipose-derived fibroblast, due to its capacity to block GLUT1 [306]. Furthermore, Strobel and co-workers observed glucose uptake inhibition after the treatment of isolated rat adipocytes with quercetin ranging 10-100 μM [307].Nevetherelss, it is important to highlight that in a study conducted using rats supplemented with 30 mg/kg body weight/day of quercetin, authors did not observe changes in the activity of the main lipogenic enzymes and LPL [308].

Modulation of adipokine expression and secretion carried out by quercetin has also been reported. Sprague-Dawley rats supplemented with quercetin-rich onion peel extract for 8 weeks showed a reduction in mesenteric adipose tissue, as well as an increase in adiponectin expression [309]. In another study, quercetin was able to counteract the diminished adiponectin plasma concentrations found in high-fat-diet-fed Wistar rats [310]. Specifically, quercetin supplemented rats showed higher adiponectin mRNA levels in WAT. In a recent study conducted with diabetic mice, the authors stated that quercetin enhanced the effect of diabetes drug Sitagliptin, as it was observed by the improvement on glycaemic control, metabolic profile, oxidative and inflammatory status and β-cells function when the combination was compared with the treatment alone. Besides, authors showed that Sitagliptin, quercetin and their combination were able to increase serum adiponectin levels [311]. Regarding leptin, quercetin has reduced serum leptin levels in animal models [312,313]. Contrary results have been obtained in clinical trials, where quercetin did not modify serum leptin levels after quercetin supplementation [314,315]. In the above-mentioned study of Derdemezis *et. al.*, visfatin secretion was modified by quercetin-treatment in SGBS cells [256]. However, in a clinical trial, where overweight and obese women received a quercetin-rich onion peel extract (100 mg/day) for 12 weeks, plasma visfatin concentrations did not change [314]. As far as we know there is no data about the effect of quercetin on apelin expression and secretion.

Despite the great number of *in vitro* and *in vivo* studies performed to analyse the anti-obesity effect of quercetin, only a few studies have been conducted in **human subjects**. Quercetin-rich onion extracts have been used in some clinical trials yielding diverse results. For instance, two studies conducted in healthy young female subjects in Japan showed opposite results in BMI and body fat percentages after extract supplementation. This evidenced the importance of the treatment duration [316,317]. Twelve-week quercetin supplementation (100 mg/day) to overweight and obese subjects decreased BMI and fat depots significantly [318]. By contrast, in another study with the same duration and higher doses of quercetin supplementation (500 or 1000 mg/day), no beneficial effects were observed [319]. Taken as a whole, it can be concluded that more clinical trials are needed in order to clarify the clinical relevance of quercetin supplementation.

With the exception of ISO and Quercetin-3-O-(6´´-feruloyl)-b-D-galactopyranoside in adipogenesis, the potential contribution of quercetin metabolites to the anti-obesity effect of the parent compound has not been addressed yet. However, fat-lowering effects of quercetin in *in vivo* studies, despite its extensive metabolization and the activity of metabolites in other tissues and pathologies, raises the idea that they could be biologically active [320-323].

The positive effects of quercetin as anti-obesity molecule have been summarized in Table 5.

Table 5. Summary of the observed anti-obesity effects of quercetin in *in vitro* and *in vivo* studies.

Metabolic pathway	***Parameter***	***Cell type or tissue***	***References***
Adipogenesis	↓ PPARγ gene and protein expression	Murine 3T3-L1 adipocytes Sprague-Dawley rats	[297, 298, 309]
	↓ CEBPα protein expression	Murine 3T3-L1 adipocytes	[297, 298]
	↓ CEBPβ protein expression	Murine 3T3-L1 adipocytes	[297, 298]
	↓ SREBP1C protein expression	Murine 3T3-L1 adipocytes	[297, 298]
Fatty acid uptake and lipogenesis	↓ FAS protein expression	Murine 3T3-L1 adipocytes	[297, 298]
	↑ ACC phosphorylation (inactivation)	Murine 3T3-L1 adipocytes	[297]
	↓ LPL activity	Isolated rat fat pads	[305]
	↓ glucose uptake	L929 adipose-derived fibroblast Isolated rat adipocytes	[306, 307]
Lipolysis	↓ PDE activity ↑ HSL activity	Isolated rat adipocytes	[303]
Apoptosis	↑ Caspase activation ↑ Cas-3 protein expression and activity	Murine 3T3-L1 adipocytes	[297, 302]
	↓ PARP and bcl-2 protein expression ↓ Bax and Bak protein expression	Murine 3T3-L1 adipocytes	[302]
Adipokine modulation	↑ adiponectin blood levels ↑ adiponectin gene expression	Obese Zucker rats C57BL/6J mice Sprague-Dawley rats Wistar rats Diabetic Wistar rats	[292, 294, 309,310,311]
	↓ leptin blood levels	C57BL/6J mice C57BL/6JOlaHsd mice	[294, 313]
	↓ visfatin secretion	Human SGBS cells	[256]

HIPOTESIA, HELBURU ESPEZIFIKOAK ETA DISEINU ESPERIMENTALA

Hipotesia, helburu espezifikoak eta diseinu esperimentala

1. Hipotesia eta helburu espezifikoak

KF-en inguruan asko ikertu da azken hamarkadetan, giza-osasunerako dituen efektu onuragarriak direla eta. Are gehiago, konposatu mota hauetan aberatsak diren elikagaien kontsumoa eta hainbat gaixotasunen garapena alderantziz proportzionala dela ikusi da, molekula bioaktibo hauekiko interesa izugarri areagotu delarik. Gaixotasun horien artean diabetesa, obesitatea, minbizia eta gaixotasun kardiobaskularrak daude [179].

Hala ere, KF-ek metabolismo zorrotza jasaten dutela kontuan hartu beharra dago eta ondorioz odol eta ehunetara haien kantitate oso txikia iristen da. Ezberdintasunak badaude ere, eskuarki onartuta dago KF iturri dietetikoetatik hartu eta gero, jatorrizko konposatuaren eta metabolitoen odoleko kontzentrazioa nanomolar eta mikromolar tarteen artean kokatzen dela [180,324]. Aitzitik, *in vitro* burututako ikerketa gehienetan erabili izan diren dosiak, giza edo animalien odolean agertzen diren kontzentrazioak baino askoz ere altuagoak dira.

Areago, metabolitoen efektuak jatorrizko molekularenarekin konparatzen dituzten ikerketa gutxi egin izan dira, *in vitro* lorturiko emaitzak *in vivo* gauzatutako esperimentuekin alderatzea biziki zailduz. Dosien harira, KF-ek funtzio bikoitza dutela ikusi izan da, farmakologian oso erabilia den hormesi kontzeptuaren (dosi-erantzun fenomenoa) antzera [325]. Dosi-tarte finko batean behatutako efektu zehatza, beste dosi batzuk erabiltzean bestelakoa izan daitekeela esan nahi du horrek.

Horregatik guztiagatik, lan honen hipotesia 3T3-L1 adipozitoetan erresberatrola eta kertzetinari esleitu zaien triglizerido murrizketa, euren metabolitoei ere egotzi diezaiekegun aztertzea da. Hipotesia onartu edo ukatzearen xedearekin, datozen helburu espezifikoak proposatu ziren:

1. Diferentziazioan dauden eta helduak diren 3T3-L1 adipozitoetan erresberatrola-3-*O*-glukuronido (3G), erresberatrola-4´-*O*-glukuronido (4G) eta erresberatrola-3-O-sulfato-aren (3S) (erresberatrolaren II faseko metabolitoak) lipidoen metaketan duen efektua analizatzea, hala nola ikusitako efektua jatorrizko konposatuarenarekin aldaratzea **(1. eskuizkribua)**.
2. Diferentziazioan dauden 3T3-L1 adipozitoetan, erresberatrolak eta metabolitoek (3G, 4G eta 3S) adipogenesian ezinbesteko erregulazioa ahalbidetzen duten pparγ, cebpβ eta cebpα-ren gain duten efektua miRNA-en bidez ematen den aztertzea **(2.eskuizkribua)**.
3. Diferentziazioan dauden eta helduak diren 3T3-L1 adipozitoetan erresberatrolak eta metabolitoek (3G, 4G eta 3S) adipokinen espresioan eta jariaketan duten efektuaren analisia egitea **(3.eskuizkribua)**.

4. Kertzetinaren dosi baxuek diferentziazioan dauden eta helduak diren 3T3-L1 adipozitoen triglizeridoen metaketan duten efektua aztertzea, hala nola ekintza horren erantzule diren akzio-mekanismoak definitzea **(4.eskuizkribua)**.
5. Diferentziazioan dauden 3T3-L1 adipozitoetan, isorhamnetina (ISO), tamarixetina (TAM), kertzetina-3-O-glukuronido (Q3G), kertzetina-3-O-sulfato (Q3S) eta Q3S eta kertzetina-4-O-sulfatoaren arteko nahasketak (Q3S+4S) -kertzetinaren metabolito nagusiak-, odoleko kontzentraziotik hurbil dauden dosietan, triglizeridoen metaketan duten efektua aztertzea, hala nola kertzetinaren efektuarekin konparatzea **(5.eskuizkribua)**.

2. Diseinu esperimentala

2.1. Kultibo zelularrak

Erresberatrola, kertzetina eta metabolitoen efektua ikertzeko 3T3-L1 zelulak American Type Culture Collection-etik (ATCC; Manassas, VA, Estatu Batuak) eskuratu ziren. 3T3-L1 fibroblastoak *Mus musculus*-etik isolatutakoak dira [326,327], fibroblastotik hasita adipozito heldua izan arte diferentziatzeko duten gaitasunagatik, adipogenesia ikertzeko zelula oso erabiliak direnak. Zelula-lerro hau, adipogenesia edo obesitatearen garapenarekin erlazionaturiko hainbat prozesu ikertzeko egin diren ikerlanen heren batean erabili izan dira [328]. 3T3-L1 zelulak maneiatzen errazak dira, pasatze asko jasaten dituzte, kultibo primarioak baino merkeagoak dira eta tratamenduekiko duten erantzuna nahiko iraunkorra da [328].

3T3-L1 aurre-adipozitoak modu aproposean desberdintzeko oso erabilia izan den protokoloa jarraitu zen [43]. Zelulak, konfluentzia lortu arte, %10-eko behi-fetuaren serumarekin (FBS) eta %1-eko penizilina/estreptomizinarekin (P/S) aberastutako glukosa-maila altuko (4.5 g/L) Dulbecco´s modified Eagle´s medium-ean (DMEM) hazi ziren (hazkuntza-medioa). Konfluentzia lortu eta bi egunetara, zelulak hormona nahasketa (10 µg/mL intsulina, 0.5 mM IBMX and 1 µM DEXA) zeraman hazkuntza-medioarekin hazi ziren bi egunez, hortik aurrera eta zelulak jaso arte, intsulinarekin (10 µg/mL beste 48 orduz eta 0.2 µg/mL gainontzeko egunak) aberasturiko hazkuntza-medioan eduki zirelarik. Aldiro, zelulak 37ºC-tan eduki ziren, hezetutako %5 CO_2 atmosferan. Zelulen heldutasuna mikroskopioz egiaztatu zen, pilatzen dituzten gantz-tantei erreparatuta.

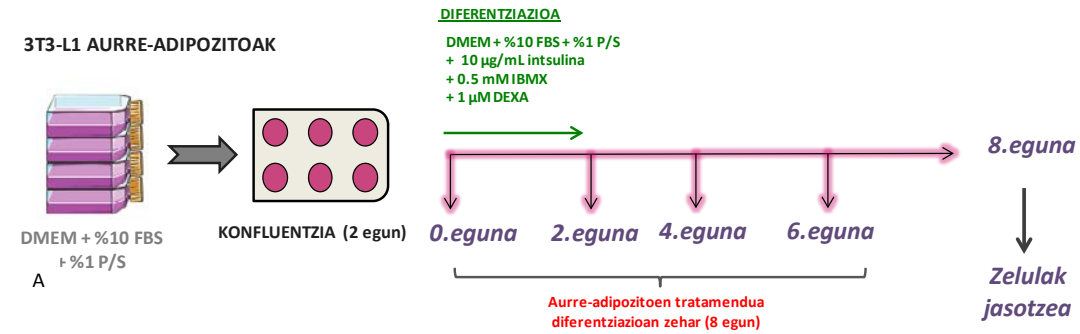
2.2. Zelulen tratamenduentzako polifenolen prestaketa

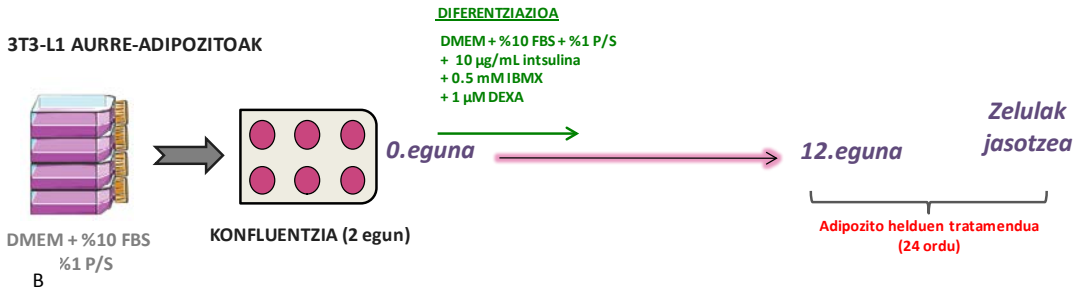
Erresberatrola (%98-ko purutasuna), 3G (%95-eko purutasuna), 4G (%95-eko purutasuna) eta 3S (%98-ko purutasuna) Bertin Pharma-tik (Montigny le Bretonneux, Frantzia) eskuratu ziren. Kertzetina (≥%95-eko purutasuna) Sigma-n (St. Louis, MO, Estatu Batuak) erosi zen eta ISO (≥%99-ko purutasuna), TAM (≥%99-ko purutasuna), eta Q3G (≥%95-eko purutasuna) Extrasynthesen (Genay, Frantzia). Kertzetinaren sulfatoak (Q3S eta Q3S+4S, %96-ko purutasuna) ezin izan ziren komertzialki eskuratu, hortaz, Salamanca-ko Unibertsitateko “Grupo de Investigación en Polifenoles”-eko kideek sintetizatu zituzten, 5. eskuizkribuko metodoen atalean azaltzen den moduan.

Polifenol guztiak %95-eko kontzentrazioa zuen etanolean disolbatu ziren, kontrol taldeari etanol kantitate bera gehitu zitzaiolarik. Gauzatutako tratamendu guztietan, bai polifenolak eta bai kontrol taldearen etanola ere 1000 aldiz diluituta gehitu ziren, zelula-plaken putzu bakoitzeko etanol kontzentrazioa %0.095-ekoa izanik, zelulentzako toxiko bezala finkatutako dosia baino askoz ere baxuagoa dena [329,330].

2.3. Zelulen tratamenduak

Erresberatrolak eta metabolitoek diferentziazio-prozesuan zeuden zelulen triglizerido murrizketan zuten efektua aztertzeko **(1.eskuizkribua)**, 3T3-L1 aurre-adipozitoak erresberatrola, 3G, 4G eta 3S metabolitoekin tratatu ziren 1, 10 eta 25 µM-eko dosian. Molekulak 0, 2, 4 eta 6. egunean gehitu ziren, eta 8. egunean inkubazio medioa gorde eta zelulak jaso ziren, triglizeridoen kantitatea kuantifikatzeko eta RT-q-PCR bidez geneen espresioa aztertzeko (16A irudia). Erresberatrolak eta metabolitoek 3T3-L1 adipozito helduetan triglizeridoen murrizketa eragiten zuten egiaztatzeko, zelulak 12. egunean 24 orduz aurretik aipatutako dosi eta molekula berekin tratatu ziren. Zelulak jaso ostean (13. egunean) inkubazio medioa gorde eta zelulak triglizeridoen kantitatea kuantifikatzeko eta RT-q-PCR bidez geneen espresioa aztertzeko erabili ziren (16B irudia). Triglizeridoen kantitatea neurtzeko, Infinity Triglycerides reagent (Thermo Scientific, Rockford, IL, Estatu Batuak) kit komertziala erabili zen. Triglizeridoen datuak mg triglizerido/mg proteina moduan adierazi zirenez, proteinen kantitatea BCA reagent (Thermo Scientific, Rockford, IL, Estatu Batuak) erabilita kuantifikatu zen. 3T3-L1 aurre-adipozitoetan *cebpβ* eta *cebpα*, *pparγ*, eta *lpl* geneen espresioa neurtu zen eta adipozito helduetan *atgl*, *hsl*, *cpt1*, *lpl*, *fasn*, *acc*, *sirt1* eta *pgc1α* geneena, guztiak RT-q-PCR bidez.

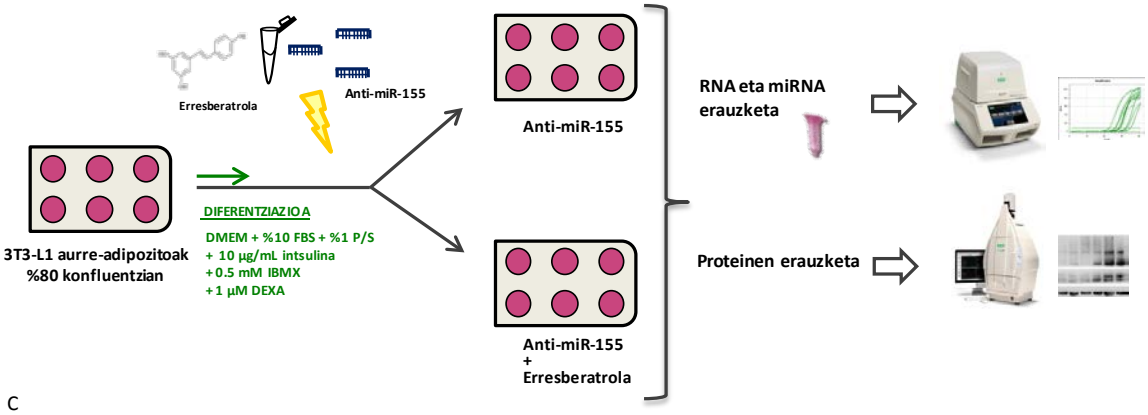




16A eta 16B irudiak. Aurre-adipozitoetan (A) eta adipozito helduetan (B) eginiko tratamenduen diseinu esperimentalaren eskema. FBS: behi-fetuaren seruma; P/S: penizilina/estreptomizina; IBMX: isobutilmetilxantina; DEXA: dexametasona.

Erresberatrol eta metabolitoek adipogenesiaren erregulazioa miRNA-en bidez eman zitekeen frogatzeko **(2.eskuizkribua)**, pparγ, cebpβ eta cebpα lotu zitzaketen miRNA-en iragarpen konputazionala eta bilaketa bibliografikoa egin zen. Horren ondoren, 3T3-L1 aurre-adipozitoak 25 µM-eko dosian erresberatrola, 3G, 4G eta 3S molekulekin tratatu ziren 8 egunez (16A irudia). Aurreko esperimentuetan bezala, kontrol-zelulak etanolarekin (%95) tratatu ziren. 8. egunean, zelulak jaso eta RNA-ren erauzketa egin zen mikroRNA-130b-3p (miR-130b-3p), mikroRNA-155-5p (miR-155-5p), mikroRNA-27b-3p (miR-27b-3p), mikroRNA-31-5p (miR-31-5p), mikroRNA-326-3p (miR-326-3p), mikroRNA-27a-3p (miR-27a-3p), mikroRNA-144-3p (miR-144-3p), mikroRNA-205-5p (miR-205-5p) eta mikroRNA-224-3p (miR-224-3p) mailak eta sterol regulatory esterolengatik erregulaturiko elementura lotzen den 1 proteina (*srebf1*), krüppel-aren antzekoa den 5 faktorea (*klf5*), gibeleko x hartzailea α (*lxrα*) eta cAMP-ren elementu erantzuleari lotzen zaion 1 proteina (*creb1*) mRNA mailak RT-q-PCR bidez neurtzeko.

Erresberatrolak eta metabolito glukuronatuek cebpβ-ren espresioa miR-155-en bidez egiten zuten egiaztatzeko, aurre-adipozitoak anti-miR-155-arekin transfektatu ziren diferentziazioa eragiten zitzaien une berean, eta bestalde, erresberatrola edo etanolarekin (kontrola) ere tratatu ziren. Horren guztiaren ondoren, RNA eta proteinen erauzketa egin zen cebpβ-ren gene eta proteinaren espresioak neurtu ahal izateko, RT-q-PCR eta Western Blot tekniken bidez, hurrenez hurren (16C irudia).



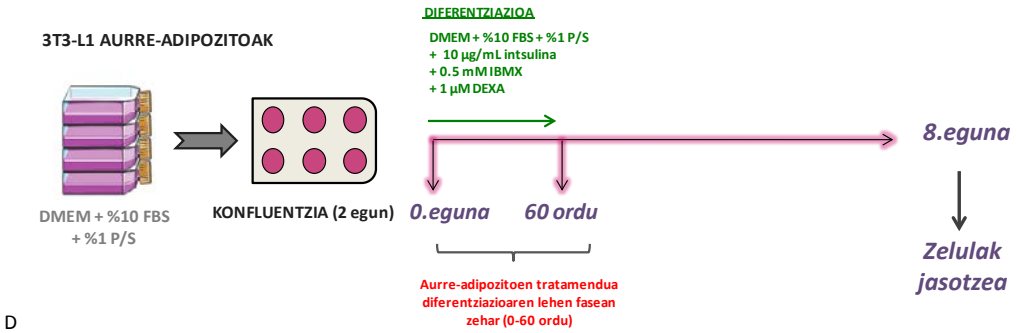
C

16C irudia. miR-155-ak erresberatrola eta metabolito glukuronatuek cebpβ-ren gaineko eraginean duen inplikazioa aztertzeko tratamenduaren diseinu esperimentalaren eskema. 3T3-L1 aurre-adipozitoen diferentziazioa eragin eta aldi berean anti-miR-155 transfektatu zitzaien. Une horretan ere, zelulak erresberatrola edo etanolarekin tratatu ziren, eta 48 orduren buruan, zelulak jaso ziren. FBS: behi-fetuaren seruma; P/S: penizilina/estreptomizina; IBMX: isobutilmetilxantina; DEXA: dexametasona.

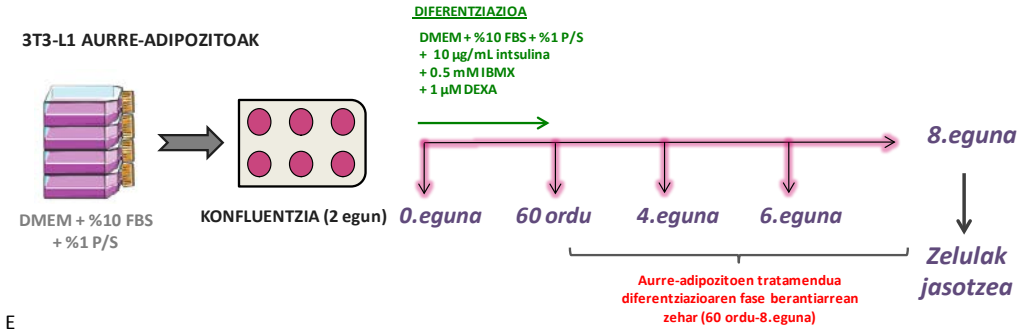
Aurre-adipozito eta adipozito helduetan adipokinen espresioa eta jariaketa aztertzeko xedearekin **(3.eskuizkribua)**, 1. eskuizkribuan erabilitako protokolo bera erabili zen, baina soilik triglizeridoen murrizketan eraginkorrak izan ziren dosiak erabilita: 25 µM aurre-adipozitoetan eta 10 µM adipozito helduetan (16A eta 16B irudiak). Tratamendua eta gero, inkubazio medioa jaso eta adiponektina, leptina, visfatina eta apelina mailak neurtu ziren ELISA teknikan oinarritutako kit komertzialen bidez (RD293023100R, RD191001100 eta RAG004R, Biovendor, Brno, Txekiar Errepublika eta EK-003-80, Phoenix Europe GMBH, Karlsruhe, Alemania). Horrez gain, adiponektina, leptina, visfatina eta apelinaren espresio genikoa RT-q-PCR bidez neurtzeko zelulak jaso egin ziren.

Kertzetinaren dosi baxuek triglizeridoen metaketan zuen efektua analizatzeko **(4.eskuizkribua)**, hiru tratamendu burutu ziren aurre-adipozitoen kasuan. Lehenik eta behin, kertzetinaren efektua 1, 2, 5 eta 10 µM-eko dosiekin aztertu zen aurretik azaldutako protokoloaren modu berean (16A irudia). Ondoren, kertzetinak adipogenesian zuen efektua fase goiztiarrean edo bukaerako fasean eraginda ematen zen ikusi nahian, aurre-adipozitoak bi fase horietan tratatu ziren (16D eta 16E irudiak). Hiru esperimentuak gauzatu ondoren, inkubazio medioa gorde eta zelulen triglizerido kantitatea eta *cebpβ*, *pparγ*, *srebf1* eta *lpl*-ren geneen espresioa RT-q-PCR bidez neurtu zen, hala nola SREBP1C eta PPARγ-ren proteinen espresioa Western Blot teknikaren bidez.

Kertzetinaren dosi baxuek adipozito helduen gantz-metaketan zuten efektua aztertzeko, zelulak 24 orduz 1, 2, 5 eta 10 µM-eko dosiekin tratatu ziren diferentziazioaren 12. egunean, aurretik azaldu den bezala (16B irudia). Inkubazio medioa glizerola eta GAA kit komertzialen (Free Fatty Acids, Half Micro Test,Roche, Basilea, Suedia eta F6428, Sigma, St. Louis,MO, Estatu Batuak) bidez neurtzeko jaso zen eta zelulak triglizeridoak neurtzeko eta *atgl*, *hsl*,*fasn*, *acc*, *sirt-1*, *adiponektina*, *leptina*, *visfatina* eta *apelina* geneen espresioa RT-q-PCR bidez neurtzeko erabili ziren.



D



16D eta 16E irudiak. 4. eskuizkribuan fase goiztiarrean edo bukaerako fasean kertzetinaren efektua analizatzeko egindako tratamenduen eskemak. Aurre-adipozitoak 0 eta 60 ordu bitartean (D) eta 60 ordu eta 8 egun bitartean (E) ere tratatu ziren. FBS: behi-fetuaren seruma; P/S: penizilina/estreptomizina; IBMX: isobutilmetilxantina; DEXA: dexametasona.

Kertzetinaren metabolitoen (ISO, TAM, Q3G, Q3S eta Q3S+4S) triglizeridoak murrizteko ahalmena jatorrizko konposatuarenarekin konparatzeko helburuarekin **(5.eskuizkribua)**, zelulak aurretik aipatu bezala hazi eta 0.1, 1 eta 10 µM-eko dosiekin tratatu ziren (16A eta 16B irudiak). Kontrol taldea etanolarekin tratatu zen (%95). Bi tratamenduak egin eta gero, inkubazio medioa glizerola eta GAA kit komertzialen bidez neurtzeko jaso zen eta zelulak triglizeridoak neurtzeko eta *atgl*, *hsl*, *lpl*, *fasn*, glukosaren 4. garraiatzailea (*glut4)*, diazilglizerol 1 aziltransferasa (*dgat1*), *dgat2*, B zelulen leuzemia/2 linfoma (*bcl2*), eraldaketarekin erlazionaturiko 53 proteina (*trp53)* eta kaspasa 3 (*cas3)* geneen espresioa adipozito helduetan eta *cebpα*, *cebpβ*, *srebf1*, *pparγ*, *glut4*, *dgat1*, *dgat2*, *bcl2*, *trp53 eta cas3* geneen espresioa aurre-adipozitoetan RT-q-PCR bidez neurtzeko erabili ziren.

6. taula. Doktorego tesi honetan eginiko tratamenduen ezaugarri garrantzitsuenak jasotzen dituen taula.

	1. eskuizkribua	**2. eskuizkribua**	**3. eskuizkribua**	**4. eskuizkribua**	**5. eskuizkribua**
Zelula mota	3T3-L1	3T3-L1	3T3-L1	3T3-L1	3T3-L1
Molekulak	Erresberatrola, 3G, 4G eta 3S	Erresberatrola, 3G, 4G eta 3S	Erresberatrola, 3G, 4G eta 3S	Kertzetina	Kertzetina, ISO, TAM, Q3G, Q3S eta Q3S+4S
Dosiak	1, 10 eta 25 µM	25 µM (aurre-adipozitoak) eta 10 µM (adipozito helduak)	25 µM	1, 2, 5 eta 10 µM	0.1, 1 eta 10 µM
Iraupena eta tratamenduaren fasea	Diferentziazioa (8 egun) eta adipozito helduak (24 ordu)	Diferentziazioa (8 egun) eta adipozito helduak (24 ordu)	Diferentziazioa (8 egun)	Adipogenesiaren fase goiztiarra (0-60 ordu), adipogenesiaren fase berantiarra (60 ordu-8 egun), diferentziazioa (8 egun) eta adipozito helduak (24 ordu)	Diferentziazioa (8 egun) eta adipozito helduak (24 ordu)

3G: erresberatrola-3-*O*-glukuronido; 4G: erresberatrola-4´-*O*-glukuronido; 3S: erresberatrola-3-O-sulfato; ISO: isorhamnetina; TAM: tamarixetina; Q3G: kertzetina-3-O-glukuronido; Q3S: kertzetina-3-O-sulfato; Q3S+4S: Q3S eta kertzetina-4-O-sulfatoaren arteko nahasketa.

HYPOTHESIS, SPECIFIC OBJECTIVES AND EXPERIMENTAL DESIGN

Hypothesis, specific objectives and experimental design

1. Hypothesis and specific objectives

PC have been extensively studied in the last decades due to their diverse beneficial effects on human health. In fact, an inverse correlation between the intake of foods rich in these compounds and the development of several diseases has been observed, and thus, the interest for these bioactive molecules has been increased. The protective effects of PC have been established for several pathologies such as diabetes, obesity, cancer and cardiovascular diseases [179].

Nevertheless, it is very important to consider that the extensive metabolism of PC reduces notably their bioavailability, and only a small amount of them reaches plasma and tissues. Although there are differences in their metabolism, it is generally accepted that after oral ingestion, the parent compound and its metabolites appear in nanomolar or micromolar ranges in plasma [180,324]. By contrast, the vast majority of *in vitro* studies use doses far from those concentrations that are found in human or animal plasma.

Apart from this limitation, there are relatively few studies conducted with metabolites that compare the effects to those of the parent compound. This fact makes difficult to extrapolate the results obtained in *in vitro* to *in vivo* studies. Additionally, it has been reported that PC could show a biphasic function, similar to the hormetic concept (a dose-response phenomenon) widely used in pharmacology [325]. This means that a certain action observed in a concrete range of doses could be another one if different doses were used.

So, the hypothesis of the present work was that of evidencing in 3T3-L1 adipocytes whether the triglyceride-lowering effects attributed to resveratrol and quercetin are responsible also for their main metabolites. In order to demonstrate or discard this hypothesis, the following objectives were proposed:

1. To analyse the delipidating effect **(Manuscript 1)** of phase II resveratrol metabolites, resveratrol-3-*O*-glucuronide (3G), resveratrol-4´-*O*-glucuronide (4G) and resveratrol-3-*O*-sulfate (3S), as well as to compare them with that of the parent compound, in maturing and mature 3T3-L1 adipocytes.
2. To determine whether miRNAs are implicated in the regulation of resveratrol and its metabolites (3G, 4G, 3S) in the adipogenic key transcription factors pparγ, cebpβ and cebpα in maturing 3T3-L1 pre-adipocytes **(Manuscript 2)**.
3. To analyse the effect of resveratrol and its metabolites in the adipokine expression and secretion of resveratrol and its metabolites (3G, 4G and 3S) in maturing and mature 3T3-L1 adipocytes **(Manuscript 3)**.
4. To assess the effect of low doses of quercetin on triglyceride accumulation in maturing and mature adipocytes, and to analyse the underlying mechanisms of action **(Manuscript 4)**.

5. To test and compare the triglyceride-lowering effect of predominant quercetin metabolites isorhamnetin (ISO), tamarixetin (TAM), quercetin-3-O-glucuronide (Q3G), quercetin-3-O-sulfate (Q3S) and Q3S and quercetin-4-O-sulfate mixture (Q3S+4S) with that of quercetin in the range of serum concentrations in 3T3-L1 pre-adipocytes as well as in mature adipocytes, and to describe their potential mechanism of action. **(Manuscript 5)**.

2. Experimental design

2.1. Cell cultures

In order to study the effect of resveratrol, quercetin and its metabolites in 3T3-L1 adipocytes, cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). 3T3-L1 mouse fibroblasts are isolated from *Mus musculus* [326,327] and they are widely used as an *in vitro* model to study adipogenesis due to their potential to differentiate from fibroblast to mature adipocytes. This cell line is selected by nearly a third of research studies carried out to study adipogenesis or other processes implicated in obesity development [328].Moreover, 3T3-L1 cells are easily handled, support a high number of passages, they are lower-priced than primary cultures and are very homogeneous with a relatively constant response to treatments [328].

To properly differentiate 3T3-L1 pre-adipocytes, a widely used protocol was employed [43]. Cells were grown in high glucose (4.5 g/L) Dulbecco´s modified Eagle´s medium (DMEM), in the presence of 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin until they reached confluence (growth media). Two days after confluence, cells were stimulated for 2 days with the same growth medium but containing a hormonal cocktail composed by 10 µg/mL insulin, 0.5 mM IBMX and 1 µM DEXA. From day 2 onward, cells were maintained with a growth medium that contained insulin (10 µg/mL for two days more and 0.2 µg/mL for the rest of days) until they were harvested. Incubation media was changed every two days. Cells were maintained at 37ºC in a humidified 5% CO_2 atmosphere. The maturation stage of cells was verified under the microscope since adipocytes show visible lipid-droplets.

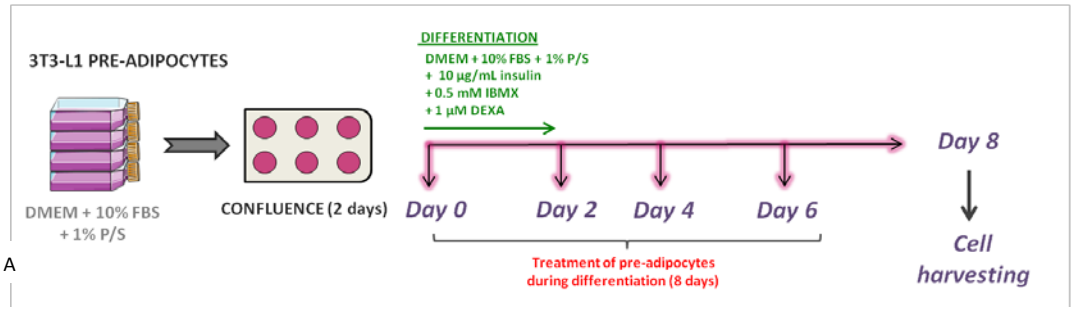
2.2. Preparation of polyphenols for cell treatments

Resveratrol (98% purity), 3G (95% purity), 4G (95% purity) and 3S (98% purity) were provided by Bertin Pharma (Montigny le Bretonneux, France). Quercetin (≥95% purity) was purchased from Sigma (St. Louis, MO, USA) and ISO (≥99% purity), TAM (≥99% purity), and Q3G (≥95% purity) from Extrasynthese (Genay, France). Quercetin sulfates (Q3S and Q3S+4S, 96% purity) were not commercially available and they were synthesized by “Grupo de Investigación en Polifenoles” of the University of Salamanca, as it has been described in the methods section in manuscript 5.

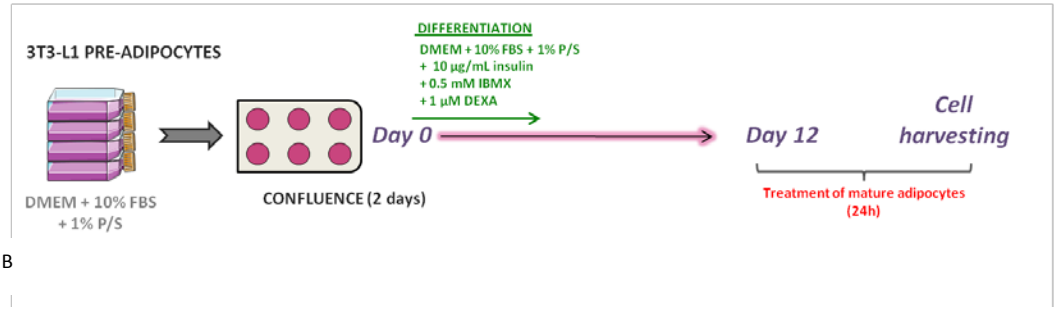
All polyphenols were diluted in 95% ethanol and for the control group, the same volume of the vehicle was used. In the performed cell treatments polyphenols and the vehicle were added a 1000-fold diluted, so the final concentration of ethanol in each well was that of 0.095%, much less than what it has been reported as being toxic for cells [329,330].

2.3. Cell treatments

To study the delipidating effect of resveratrol and its metabolites in the differentiation process **(manuscript 1)** maturing 3T3-L1 pre-adipocytes were incubated with 1, 10 and 25 µM of resveratrol, 3G, 4G and 3S. Molecules were added to the incubation media on day 0, day 2, day 4 and day 6. On day 8 incubation media was removed and cells were harvested to be used for triglyceride determination and gene expression analysis by real-time RT-PCR (Figure 16A). To study the delipidating effect of resveratrol and its metabolites in mature 3T3-L1 adipocytes, cells were incubated with the above-mentioned molecules at the same doses, in a 24-hour treatment on day 12 of differentiation. After cell harvesting (day 13), incubation media was removed and cells were used for triglyceride determination by real-time RT-PCR (Figure 16B). For triglyceride determination, Infinity Triglycerides reagent (Thermo Scientific, Rockford, IL, USA) was used. As triglyceride content was expressed as mg triglyceride/mg protein, the BCA reagent (Thermo Scientific, Rockford, IL, USA) was used for protein quantification. Gene expression of *cebpβ* and *cebpα*, *pparγ*, and *lpl* in maturing pre-adipocytes and *atgl*, *hsl*, *cpt1*, lpl, *fasn*, *acc*, *sirt1* and *pgc1α* in mature adipocytes was measured by Real Time RT-PCR.



A

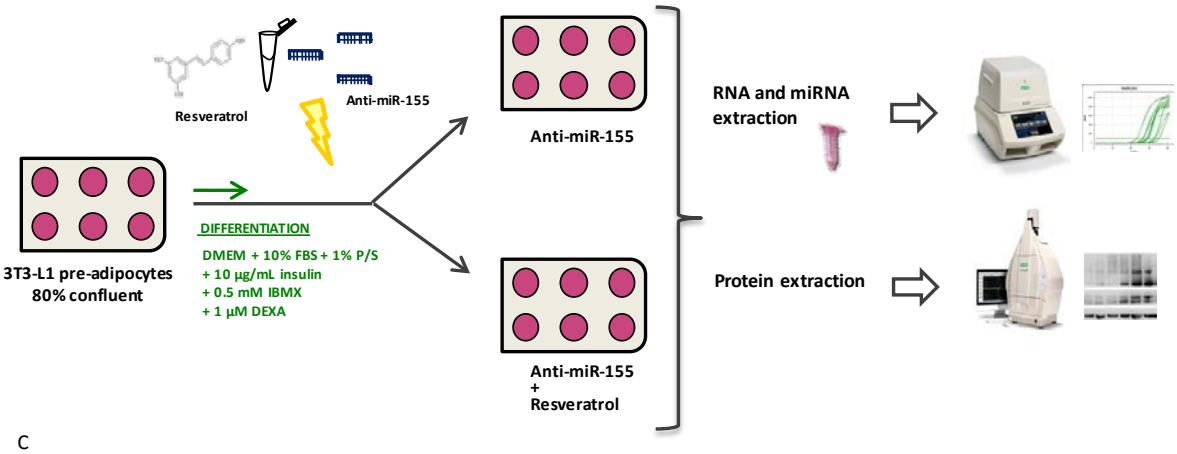


B

Figures 16A and 16B. Schemes of the experimental design of treatments carried out in maturing pre-adipocytes and mature adipocytes. In the first one, cells were treated on days 0, 2, 4 and 6 and harvested on

day 8 (A), and in the second one, cells were treated on day 12 for 24 hours (B). FBS: fetal bovine serum; P/S: penicillin/streptommicin; IBMX: isobutylmethylxanthine; DEXA: dexamethasone.

To determine the implication of miRNAs in the regulation of resveratrol and its metabolites on adipogenesis **(manuscript 2)**, a computational prediction and bibliographic search of miRNAs targeting pparγ, cebpβ and cebpα was performed. After that, maturing 3T3-L1 pre-adipocytes were treated with 25 µM of resveratrol, 3G, 4G and 3S during 8 days (Figure 16A). As in previous experiments, control cells were incubated with ethanol (95%). On day 8, cells were harvested and RNA extraction was carried out to measure the expression of microRNA-130b-3p (miR-130b-3p), microRNA-155-5p (miR-155-5p), microRNA-27b-3p (miR-27b-3p), microRNA-31-5p (miR-31-5p), microRNA-326-3p (miR-326-3p), microRNA-27a-3p (miR-27a-3p), microRNA-144-3p (miR-144-3p), microRNA-205-5p (miR-205-5p) and microRNA-224-3p (miR-224-3p) as well as adipogenic mediators such as sterol regulatory element binding transcription factor 1 (*srebf1*), krüppel-like factor 5 (*klf5*), liver x receptor α (*lxrα*) and cAMP responding element binding protein 1 (*creb1*) mRNA levels by Real-Time RT-PCR. In order to verify whether cebpβ expression was modulated by resveratrol and glucuronide metabolites via miR-155, pre-adipocytes were transfected with an anti-miR-155 (differentiation was simultaneosly induced) and were incubated with resveratrol or the vehicle (ethanol 95%). After that, RNA and protein extractions were carried out in order to measure cebpβ gene and protein expressions by Real-Time RT-PCR and Western Blot techniques respectively (Figure 16C).

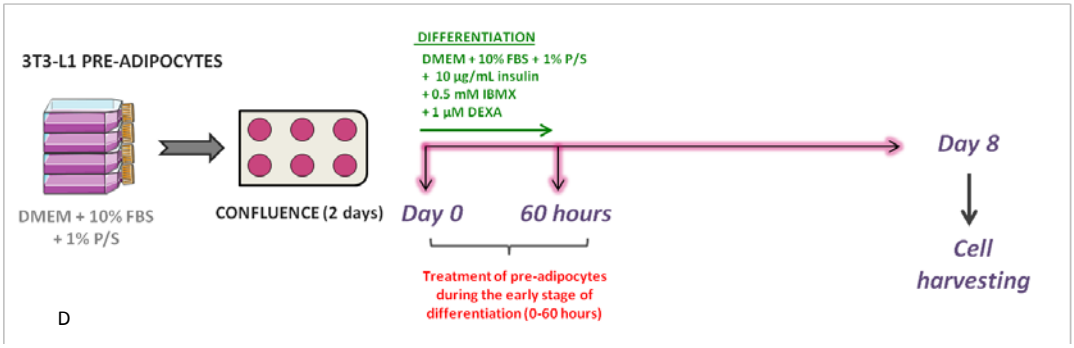


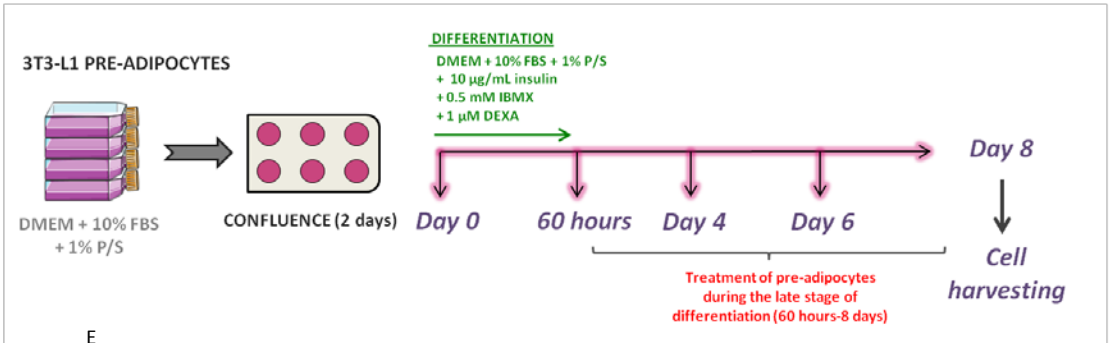
C

Figure 16C. Scheme of the experimental design of the treatment carried out to verify the implication of miR-155 in the modulation of cebpβ by resveratrol and its glucuronide metabolites. 3T3-L1 pre-adipocytes were stimulated to differentiation and transfected with anti-miR-155. In unison, cells were treated with resveratrol or the vehicle. After 48 hours, cells were harvested. FBS: fetal bovine serum; P/S: penicillin/streptommicin; IBMX: isobutylmethylxanthine; DEXA: dexamethasone.

In order to analyse adipokine expression and secretion in maturing and mature adipocytes **(manuscript 3)**, the previously described protocol used in manuscript 1 was used. However, in this case, cells were treated with resveratrol and its metabolites at the doses that were effective in triglyceride reduction: maturing pre-adipocytes were treated at 25 µM and mature adipocytes at 10 µM (Figures 16A and 16B). The incubation media was collected and used for adiponectin, leptin, visfatin and apelin determination using kits based on ELISA techniques (RD293023100R, RD191001100 and RAG004R, Biovendor, Brno, Czech Republic and EK-003-80, Phoenix Europe GMBH, Karlsruhe, Germany). In addition, cells were harvested for adiponectin, leptin, visfatin and apelin gene expression analysis by Real Time RT-PCR.

To assess the effect of low doses of quercetin on triglyceride accumulation (**manuscript 4**) in maturing pre-adipocytes, three treatments were carried out. First of all, the effect of quercetin at 1, 2, 5 and 10 µM was analysed in maturing pre-adipocytes as has been previously explained (Figure 16A). After that, in order to verify if the anti-adipogenic effect of quercetin could take place in the early stage or in the late stage of adipogenesis, cells were treated during those two different phases (Figures 16D and 16E). After the three experiments, culture media was removed and cells were harvested for triglyceride determination by the Infinity Triglycerides reagent (Thermo Scientific, Rockford, IL, USA), gene expression of *cebpβ*, *pparγ*, *srebf1* and *lpl* was analysed by Real-Time RT-PCR and protein expression analysis (SREBP1c and PPARγ) was performed by the means of the Western Blot technique. To study the delipidating effect of low doses of quercetin in mature adipocytes, cells were incubated at mentioned doses during 24 hours on day 12 of differentiation as previously described (Figure 16B). Culture media was collected for the measurement of released glycerol and FFAs by commercial kits (Free Fatty Acids, Half Micro Test,Roche, Basilea, Sweden and F6428, Sigma, St. Louis,MO, USA) and cells were harvested for triglyceride determination by the Infinity Triglycerides reagent (Thermo Scientific, Rockford, IL, USA) and gene expression of *atgl*, *hsl*,*fasn*, *acc*, *sirt-1*, *adiponectin*, *leptin*, *visfatin* and *apelin* was analysed by Real-Time RT-PCR.





Figures 16D and 16E. Schemes of treatments carried out in the manuscript 4 to analyse the effect of quercetin in the early and the late stages of differentiation. For that purpose, cells were treated out between day 0 and 60 hours of differentiation (D) and between 60 hours and day 8 of differentiation (E). FBS: fetal bovine serum; P/S: penicilin/streptommicin; IBMX: isobutylmethylxanthine; DEXA: dexamethasone.

With the goal of comparing the triglyceride-lowering effect of quercetin metabolites (ISO, TAM, Q3G, Q3S and Q3S+4S) with that of the parent compound **(Manuscript 5)**, cells were treated at 0.1, 1 and 10 µM as described above (Figures 16A and 16B). The control group was treated with ethanol (95%). After both treatments, incubation media was collected for glycerol and FFA release measurements (Free Fatty Acids, Half Micro Test,Roche, Basilea, Sweden and F6428, Sigma, St. Louis,MO, USA) and cells were harvested for triglyceride quantification as well as to measure gene expression of *atgl*, *hsl*, *lpl*, *fasn*, glucose transporter 4 (*glut4)*, diacylglycerol acyltransferase 1 (*dgat1*), *dgat2*, B cell leukemia/lymphoma 2 (*bcl2*), transformation related protein 53 (*trp53)* and caspase 3 (*cas3)* in mature adipocytes and *cebpα*, *cebpβ*, *srebf1*, *pparγ*, *glut4*, *dgat1*, *dgat2*, *bcl2*, *trp53 and cas3* in maturing pre-adipocytes.

Table 6. Summary of the main important characteristics of the treatments carried out in the present Doctoral Thesis.

	Manuscript 1	Manuscript 2	Manuscript 3	Manuscript 4	Manuscript 5
Cell type	3T3-L1	3T3-L1	3T3-L1	3T3-L1	3T3-L1
Compounds	Resveratrol, 3G, 4G and 3S	Resveratrol, 3G, 4G and 3S	Resveratrol, 3G, 4G and 3S	Quercetin	Quercetin, ISO, TAM, Q3G, Q3S, Q3S+4S
Doses	1, 10 and 25 µM	25 µM (maturing pre-adipocytes) and 10 µM (mature adipocytes)	25 µM	1, 2, 5 and 10 µM	0.1, 1 and 10 µM
Duration and phase of treatment	Differentiation (8 days) and mature adipocytes (24 h)	Differentiation (8 days) and mature adipocytes (24 h)	Differentiation (8 days)	Early stage of adipogenesis (0-60h), late stage of adipogenesis (60h-day 8), differentiation (8 days) and mature adipocytes (24 h)	Differentiation (8 days) and mature adipocytes (24 h)

3G: resveratrol-3-O-glucuronide; 4G: resveratrol-4´-O-glucuronide; 3S: resveratrol-3-O-sulfate; ISO: isorhamnetin; TAM: tamarixetin; Q3G: quercetin-3-O-glucuronide; Q3S: quercetin-3-O-sulfate; Q3S+4S: Q3S and quercetin-4-O-sulfate mixture.

EMAITZAK – RESULTS

1. eskuizkribua – 1. Manuscript

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Delipidating effect of resveratrol metabolites in 3T3-L1 adipocytes

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Abstract

Scope: Due to the low bioavailability of resveratrol, determining whether its metabolites exert any beneficial effect is an interesting issue.

Methods and results: 3T3-L1 maturing pre-adipocytes were treated from day 0 to day 8 of differentiation and mature adipocytes for 24 hours on day 12 with 1, 10 or 25 μM of resveratrol or its metabolites. Triacylglycerols were assessed by spectrophotometry and gene expression by Real Time RT-PCR. Resveratrol, *trans*-resveratrol-4'-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate reduced triacylglycerol content in maturing pre-adipocytes at 25 μM. In mature adipocytes, both resveratrol and its glucuronide metabolites, though not sulfate metabolite, reduced triacylglycerol content, although resveratrol was more effective than them. Resveratrol and the three metabolites reduced C/EBPβ mRNA levels. *Trans*-resveratrol-3-*O*-sulfate also reduced C/EBPα, PPARγ and LPL expression. In mature adipocytes, resveratrol increased ATGL, CPT-1, SIRT-1 and PGC1α expression. *Trans*-resveratrol-3-*O*-glucuronide reduced mRNA levels of FAS and increased those of SIRT-1. *Trans*-resveratrol-4'-*O*-glucuronide increased HSL and SIRT-1mRNA levels. *Trans*-resveratrol-3-*O*-sulfate did not change gene expression.

Conclusions: The present study shows for the first time the delipidating effect of a) resveratrol metabolites in maturing pre-adipocytes and b) glucuronide metabolites in mature adipocytes. This suggests that both resveratrol and resveratrol metabolites may be involved in the anti-obesity effect of this polyphenol.

Abbreviations: *Trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S).

Key words: Resveratrol, glucuronide metabolites, sulfate metabolites, delipidation, 3T3-L1 maturing pre-adipocytes and mature adipocytes

1 INTRODUCTION

Resveratrol (trans-3,5,4'-trihydroxystilbene) is a phytolaexin polyphenolic compound occurring naturally in various plants, including grapes, berries and peanuts, produced in response to stress, as a defence mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation [1, 2].

A remarkable range of biological functions have been ascribed to this molecule. For example, it acts as a cancer chemoprevention agent, a powerful anti-inflammatory factor and an antioxidant [3, 4]. Its cardiovascular properties have also been described [5]. More recently, resveratrol has been proposed as a potential anti-obesity compound. It seems to mimic the effects of energy restriction, thus leading to reduced body fat and improved insulin sensitivity [6-16].

Most resveratrol undergoes rapid and extensive metabolism into enterocytes, before entering blood. Furthermore, it undergoes rapid first-pass metabolism in the liver [17, 18]. Consequently, resveratrol bioavailability is very low and only a small proportion reaches plasma. The concentrations of glucuronide and sulfate metabolites are relatively higher [17, 19, 20]. For instance, it has been reported that the plasma concentration of this polyphenol after a dietarily relevant 25 mg oral dose of resveratrol is only in the nanomolar range compared with the micromolar range of its metabolites [17, 21, 22]. The proportions of glucuronide and sulfate metabolites depend on the tissue [23] and the species [24]. Juan et al. [23] observed that one minute after intravenous trans-resveratrol administration, trans-resveratrol glucuronide and sulfate were found in rat plasma in percentages of 33% and 8% respectively. In general, glucuronides have been reported to be the main metabolites detected in rodents, whereas sulfates are more abundant in humans [25]. With regard to tissue distribution, Juan et al. [23] reported that glucuronide conjugate concentrations were clearly higher than those of sulfate conjugates in testes and the liver, but not in lungs.

Initially, conjugation is intended for xenobiotic and endogenous inactivation of molecules in order to decrease their cellular permeability and to make them more easily eliminated. Thus, it seems unlikely that glucuronide and sulfate resveratrol metabolites could be as active as resveratrol [7]. However, despite the low concentrations of resveratrol found in tissues, the above mentioned in vivo studies, as well as others devoted to analyze other beneficial effects of this polyphenol, have reported significant effects [26]. This may suggest that some resveratrol metabolites could in fact show biological activities, or that resveratrol metabolites are converted back to resveratrol in target organs via glucuronidases and sulfatases. Thus, considerable controversy exists as to whether resveratrol is the active molecule in vivo.

Recently, a working group on resveratrol research has been created as a result of the 1st International Conference on Resveratrol and Health (www.resveratrol2010.com) held in Denmark in 2010. This group has published a paper in which recommendations for research on resveratrol for the coming years are proposed [27]; one of them is “to elucidate the biological effects of resveratrol metabolites”.

In this line, Halliwell observed that the antioxidant activity of resveratrol metabolites was lower than that of resveratrol [28]. Hoshino et al. (2010) tested the activity of five sulfate metabolites in a set of assays associated with cancer chemoprotective activity and demonstrated that two of them were able to inhibit the activity of COX with nearly the same efficacy as resveratrol, but that in general their citotoxicity towards cancer

cells appeared to be reduced compared to resveratrol itself [29]. Furthermore, Calamini et al. [30] showed that 4'-O-sulfate metabolite was a potent inhibitor of both COX-1 and COX-2, only slightly less than the parent compound. In contrast, resveratrol-3-O-sulfate and -3-O-glucuronide were found to be only weak inhibitors of both enzymes. Moreover, resveratrol-3-O-sulfate and 4'-O-sulfate stimulated SIRT-1 activity to the same extent as resveratrol [30]. Delmas et al., found no effect of resveratrol glucuronide metabolites on colon cancer cell growth [26]. With regard to anti-inflammatory effects, sulfate conjugates have been reported to modulate inflammation pathways in vitro with similar efficacy to the parent compound in some cases, whilst glucuronide conjugates were inactive in vitro at concentrations up to 300 □M [29, 30]. Thus, the literature shows that the activity of resveratrol metabolites depends on the function analyzed. As far as we know, no data have yet been published concerning the effects of resveratrol metabolites on lipid metabolism. This is an important issue to well understand the effects of resveratrol on obesity.

Following the resveratrol working group recommendations, the aim of the present study was to determine whether the following resveratrol phase II metabolites, trans-resveratrol-3-O-glucuronide, trans-resveratrol-4'-O-glucuronide and trans-resveratrol-3-O-sulfate, show delipidating effect in 3T3-L1 maturing and mature adipocytes and to compare this effect with that of the parent compound.

2 MATERIALS AND METHODS

2.1 Reagents

Dulbecco´s modified Eagle´s medium (DMEM) was purchased from GIBCO (BRL Life Technologies, Grand Island, NY). Trans-Resveratrol (98% purity), trans-resveratrol-3-O-glucuronide (95% purity), trans-resveratrol-4'-O-glucuronide (95% purity) and trans-resveratrol-3-O-sulfate (98% purity) were provided by Bertin Pharma (Montigny le Bretonneux, France).

2.2 Experimental design

3T3-L1 pre-adipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10% fetal calf serum (FCS). Two days after confluence (day 0), the cells were stimulated to differentiate with DMEM containing 10% FCS, 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 µM dexamethasone for 2 days. On day 2, the differentiation medium was replaced by FBS/DMEM medium (10%) containing 0.2 µg/mL insulin. This medium was changed every two days until cells were harvested (day 8 in the case of maturing pre-adipocytes and day 12 in the case of mature adipocytes). At day 12 >90% of cells developed mature adipocytes with visible lipid droplets. All media contained 1% Penicillin/Streptomycin (10,000 U/mL), and the media for differentiation and maturation contained 1% (v/v) of Biotin and Panthothenic Acid. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.3 Cell treatment

Maturing pre-adipocytes grown in 6-well plates were incubated with either 0.1% ethanol (95%) (control group) or with trans-resveratrol, trans-resveratrol-3-O-glucuronide, trans-resveratrol-4'-O-glucuronide or trans-resveratrol-3-O-sulfate, all of them at 1, 10 and 25 µM (diluted in 95% ethanol) during the adipogenic phase from day 0 to day 8 of differentiation. The medium was changed every 2 days. On day

8, the culture supernatant was removed and cells were used for triacylglycerol determination and RNA extraction.

Mature adipocytes grown in 6-well plates were also incubated with either 0.1% ethanol (95%) (control group) or with trans-resveratrol, trans-resveratrol-3-O-glucuronide, trans-resveratrol-4'-O-glucuronide or trans-resveratrol-3-O-sulfate, all of them at 1, 10 and 25 µM (diluted in 95% ethanol) on day 12 after differentiation. After 24 hours, supernatant was removed and cells were used for triacylglycerol determination and RNA extraction.

2.4 Measurement of triacylglycerol content in maturing and mature adipocytes

For triacylglycerol extraction, treated cells (maturing and mature adipocytes) were washed extensively with phosphate-buffered saline (PBS) and incubated 3 times with 500 µL of hexane/isopropanol (2:1). The total volume was then evaporated by vacuumed centrifugation and the pellet was resuspended in 200 µL Triton X-100 in 1% distilled water. Afterwards, triacylglycerols were disrupted by a sonicator and the content was measured by Infinity Triglycerides reagent (Thermo Scientific, Rockford, IL, USA). For protein determinations, cells were lysed in 0.3N NaOH, 0.1% SDS. Protein measurements were performed using the BCA reagent (Thermo Scientific, Rockford, IL, USA).

2.5 Extraction and analysis of RNA and quantification by reverse transcription-polymerase chain reaction (Real Time RT-PCR)

RNA samples were extracted from cells by using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using a RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with DNase I kit (Applied Biosystems, Foster City, CA, USA) to remove any contamination with genomic DNA.

One µg of total RNA in a total reaction volume of 20 µL was reverse transcribed using the iScript cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocols. Reactions were incubated initially at 25 °C for 10 min and subsequently at 37 °C for 120 min and 85 °C for 5 min.

Relative CCAAT-enhancer-binding proteins β and α (C/EBPβ and C/EBPα), peroxisome proliferator-activated receptor γ (PPAR□□□and lipoprotein lipase (LPL) mRNA levels in maturing pre-adipocytes and relative adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), carnitine palmitoyltransferase 1(CPT-1), LPL, fatty acid synthase (FASN), acetyl CoA carboxylase (ACC), deacetylase sirtuin 1 (SIRT-1) and peroxisome proliferated-activated receptor γ co-activator 1α (PGC1α) mRNA levels in mature adipocytes were quantified using Real-Time PCR with an iCyclerTM - MyiQTM Real Time PCR Detection System (BioRad, Hercules, CA, USA). β-actin mRNA levels were similarly measured and served as the reference gene. The PCR reagent mixture consisted of 1 µL of each cDNA (10 pmol/µL), SYBR□Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the upstream and downstream primers (300nM each, except in the case of C/EBPβ and C/EBPα whose primer concentration was 600nM). Specific primers were synthesized commercially (Tib Molbiol, Berlin, Germany for 3T3-L1 and Eurofins MWG Operon, Ebersberg, Germany for SGBS cells) (Table 1).

PCR parameters were as follows: initial 2 min at 50ºC, denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 30s, annealing at 60ºC for 30s (except in the case of C/EBPβ and C/EBPα where the annealing was at 68.4ºC and 66.4ºC respectively), and extension at 60ºC for 30s. All sample mRNA levels were normalized to the values of 18S and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [31].

2.6 Statistical analysis

Results are presented as mean + standard error of the mean. Statistical analysis was performed using SPSS 19.0 (SPSS Inc. Chicago, IL, USA). Comparisons between each treatment with the control were analyzed by Student t test. Statistical significance was set-up at the $P < 0.05$ level.

3 RESULTS AND DISCUSSION

3.1 Effects of resveratrol and its metabolites on triacylglycerol content in 3T3-L1 maturing and mature adipocytes

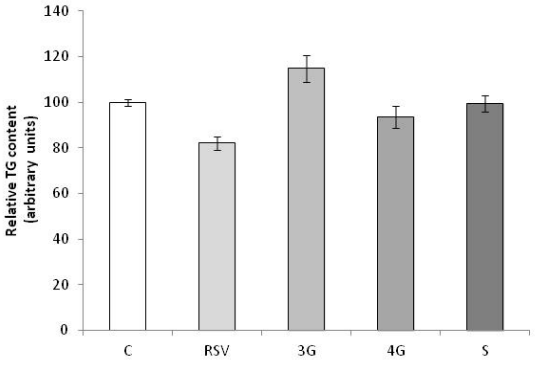
As explained in the Introduction section, following ingestion, most resveratrol undergoes rapid metabolism resulting in up to a 20-fold higher concentration of circulating conjugates, and less than 1% of the parent compound [17, 25]. This is a matter of concern for scientists because this fact could represent a major obstacle to considering resveratrol as an efficient functional ingredient.

Bearing this in mind, enhancement of the bioavailability of resveratrol is the subject of extensive biotechnology research. Different systems have been proposed for this purpose: combinations of resveratrol with other molecules able to inhibit resveratrol metabolism, such as flavonoids [32], or modifications in the chemical structure, such as the introduction of methoxy groups [25].

To properly address the issue of potential solutions to increase resveratrol bioavailability it is important to know whether resveratrol metabolites show biological activities. In this context, the present study focussed on the effects of several phase II resveratrol metabolites, trans-resveratrol-3-O-glucuronide, trans-resveratrol-4'-O-glucuronide and trans-resveratrol-3-O-sulfate, on lipid metabolism in isolated 3T3-L1 maturing pre-adipocytes and mature adipocytes because, as far as we know, these potential effects have not been reported yet. In order to clearly characterize the delipidating effect of resveratrol metabolites, three concentrations were used: 1, 10 and 25 µM. Of these, 25 µM is one of the most commonly used in in vitro studies performed to analyze the effects of resveratrol on adipocytes [33-35]. As far as we know, a concentration as low as 1 µM has never been used in studies devoted to determining the effects of resveratrol on lipid metabolism in adipocytes. Thus, this is an original aspect of the present study. This concentration was used because it is closer to in vivo plasma values reported in humans and rodents.

Biological events leading to obesity include changes in adipocyte number, achieved through a complex interplay between proliferation and differentiation of pre-adipocytes, and changes in lipid metabolism in mature adipocytes. It has been described that resveratrol inhibits both pre-adipocyte differentiation [34-38] and lipid accumulation in mature adipocytes [13, 39, 40]. Thus, in the present study we were interested in the potential effects of resveratrol metabolites on adipogenesis, as well as on triacylglycerol metabolism in mature adipocytes.

With regard to 3T3-L1 maturing pre-adipocytes, exposure to 1 and 10 µM of either trans-resveratrol or resveratrol metabolites did not modify triacylglycerol content (Figures 1A and 1B). By contrast, at a concentration of 25 µM, resveratrol, trans-resveratrol-4'-O-glucuronide and trans-resveratrol-3-O-sulfate led to a significant reduction in this lipid species (-13.0%, -13.8% and -20.0% respectively). Trans-resveratrol-3-O-glucuronide induced a reduction of 11.3% but this effect did not reach statistical significance (Figure 1C). The results concerning resveratrol are in good accordance with those previously reported by other authors who observed no effect with 10 or 12.5 µM of this polyphenol and a significant reduction in triacylglycerol content with 20 or 25 µM [35, 38, 41].



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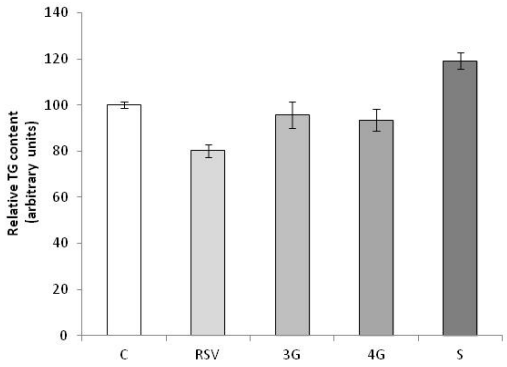

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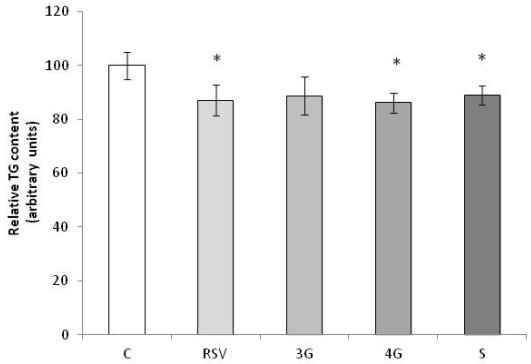

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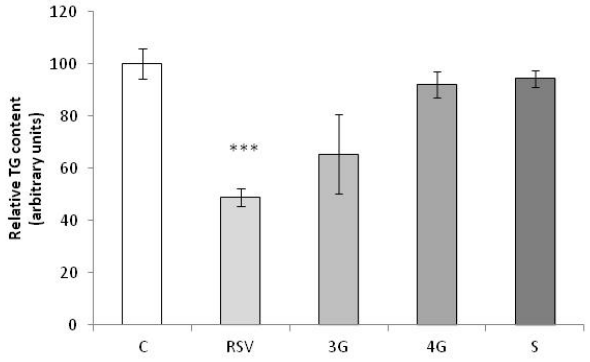
Figure 1. Triacylglycerol amounts in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8 with 1 (A), 10 (B) or 25µM (C) *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S). Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05).

Exposure of mature 3T3-L1 adipocytes to 1 µM resveratrol for 24 hours triggered a significant reduction in intracellular triacylglycerol content (-52.7%). Trans-resveratrol-3-O-glucuronide showed a tendency towards lower values at this dose (P=0.07), and the other two metabolites did not show any delipidating effect (Figure 2A). At a dose of 10 µM resveratrol and the glucoronide metabolites showed delipidating effects (-60.0%, -48.9% and -32.9% respectively). By contrast, trans-resveratrol-3-O-sulfate did not induce significant changes. The effect of resveratrol was significantly higher than that of the metabolites (Figure 2B). At the highest dose (25 µM) additional delipidating effects were not observed (Figure 2C).



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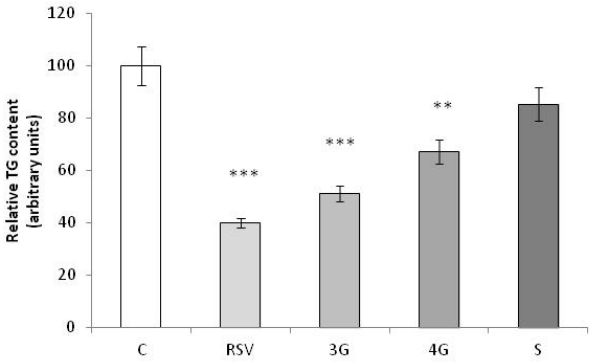

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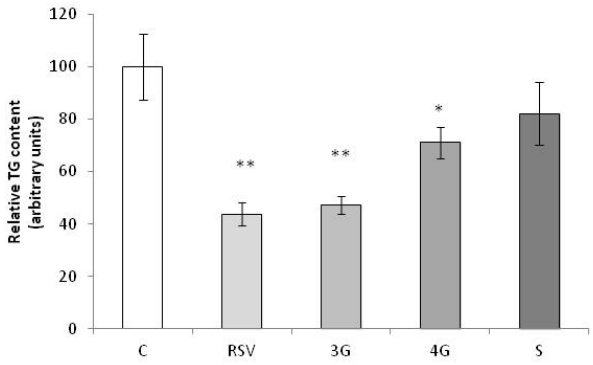

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Figure 2. Triacylglycerol amounts in 3T3-L1 mature adipocytes treated for 24 hours with 1 (A), 10 (B) or 25µM (C) *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S). Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05; ***P*<0.01; ****P*<0.001).

These results demonstrate that both maturing pre-adipocytes and mature adipocytes are targets for glucuronide metabolites. By contrast, the analyzed sufate metabolite seems to be only effective in pre-adipocytes. When comparing the effects of reveratrol with those of its metabolites, it can be observed that both resveratrol and its metabolites only showed the delipidating effect at the highest dose used (25µM) in maturing pre-adipocytes, whereas in mature adipocytes the delipidating effect of resveratrol was found at a lower dose (1 µM) than that of glucuronide metabolites (10 µM). Moreover, in maturing pre-adipocytes the percentage of tricylglycerol reduction was similar in resveratrol and the three analyzed metabolites, whereas in mature adipocytes the delipidating effect of resveratrol was stronger than that of glucuronide metabolites (P<0.05 when comparing resveratrol to trans-resveratrol-3-O-glucuronide and P<0.01 when comparing resveratrol to trans-resveratrol-4'-O-glucuronide).

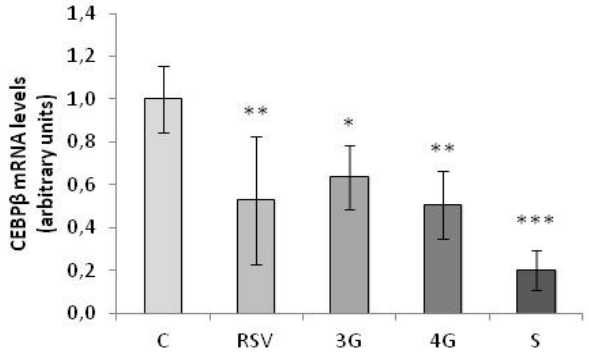
These results as a whole suggest that even if resveratrol, the parent compound, seems to be mostly responsible for the body-fat lowering effects observed when this polyphenol is orally administered in vivo, the analyzed metabolites can contribute to this effect.

3.2 Effects of resveratrol and its metabolites on gene expression in 3T3-L1 maturing and mature adipocytes

It has been demonstrated that C/EBPβ, PPARγ and C/EBPα are transcriptional factors involved in adipogenesis. At the very early stage of differentiation (day 1-2 post-confluence), which corresponds to the period of mitotic clonal expansion, C/EBPβ expression is increased. This change, in turn, triggers high level expression of PPARγ, which is considered the master coordinator of adipocyte differentiation. C/EBP1α is induced during later stages of differentiation and cooperates with PPARγ [42].

The expression of genes related to adipogenesis was analyzed in maturing pre-adipocytes treated with 25 µM of resveratrol or resveratrol metabolites, because of the three concentrations studied this was the only one which led to significant reduction in triacylglycerol content. Resveratrol and the three studied metabolites significantly reduced mRNA levels of C/EBPβ. As far as C/EBPα, PPARγ and LPL expressions are concerned only trans-resveratrol-3-O-sulfate led to a significant reduction (Figure 3). Data in the literature concerning the

effect of resveratrol on adipogenesis are controversial. In several studies perfomed in 3T3-L1 pre-adipocytes the expression of both PPARγ and C/EBPα was reduced, but in others they remained unchanged when using similar doses [34, 35, 38, 41].



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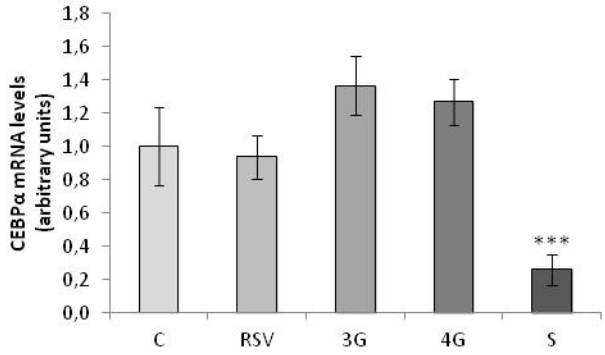

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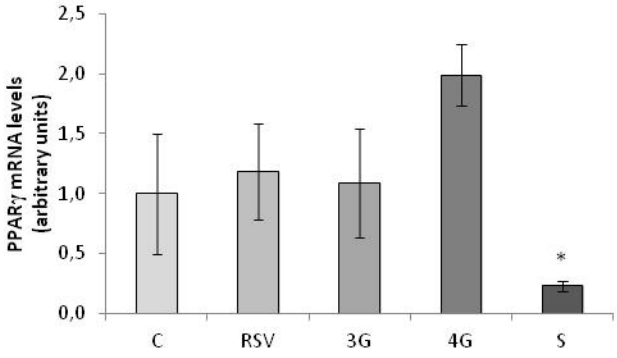

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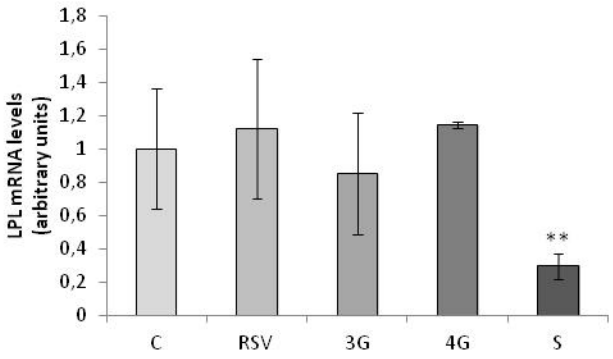

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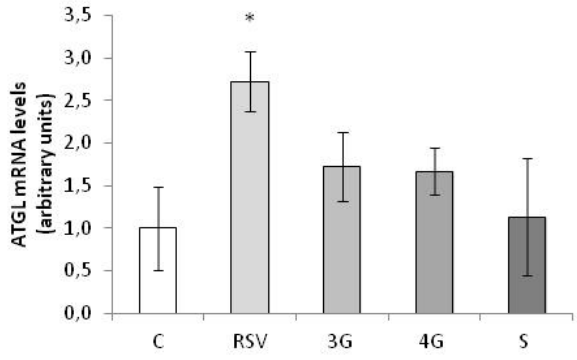
Figure 3. Effects of 25µM (C) *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the expression of C/EBP1β (A), C/EBP1α (B), PPARγ (C) and LPL (D) in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05; ***P*<0.01; ****P*<0.001).

The amount of triacylglycerols stored in mature adipocytes, and hence in adipose tissue, results from the balance among lipid mobilization and oxidation and fatty acid uptake from circulating triacylglycerols and de novo lipogenesis. Thus, the effects of resveratrol metabolites on genes involved in these metabolic pathways were also assessed in mature adipocytes. For this purpose we chose the dose of 10 µM because at 1 µM only resveratrol showed a delipidating effect and at 25 µM the effects were similar to those induced by 10 µM.

The selected enzyme genes were the two main lipases, ATGL and HSL, the oxidative enzyme, CPT-1, the enzyme which allows adipose tissue to uptake fatty acids from circulating triacylglycerols, LPL, and two lipogenic enzymes, ACC and FASN. Moreover, the deacetylase SIRT-1 and PGC-1α genes were included in the analysis.

Resveratrol significantly increased the expression of ATGL and CPT-1b. This suggests that the polyphenol can activate lipolysis and the oxidative pathways. The two glucuronide metabolites showed a trend towards increased values of ATGL (P=0.08 in the case of trans-resveratrol-3-O-glucuronide and P=0.09 in the case of trans-resveratrol-4'-O-glucuronide). Trans-resveratrol-4'-O-glucuronide also increased HSL lipase expression. Trans-resveratrol-3-O-glucuronide, unlike resveratrol or the other glucoronide metabolite, significantly reduced FASN expression. This could suggest that while trans-resveratrol-3-O-glucuronide could be acting at both increasing lipolysis and decreasing lipogenesis, trans-resveratrol-4'-O-glucuronide could be mainly acting on the lipolytic pathway. Trans-resveratrol-3-O-sulfate did not induce significant changes in gene expression (Figures 4 and 5).



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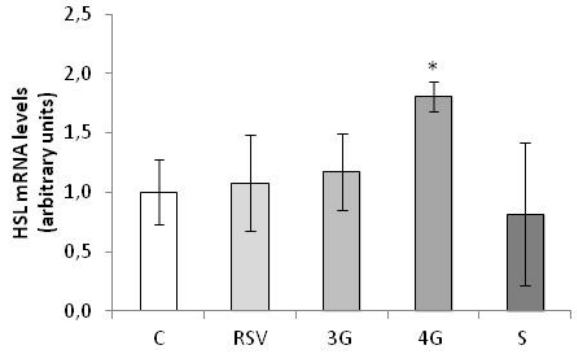

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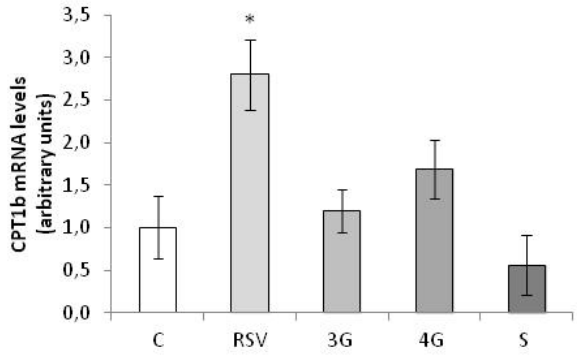
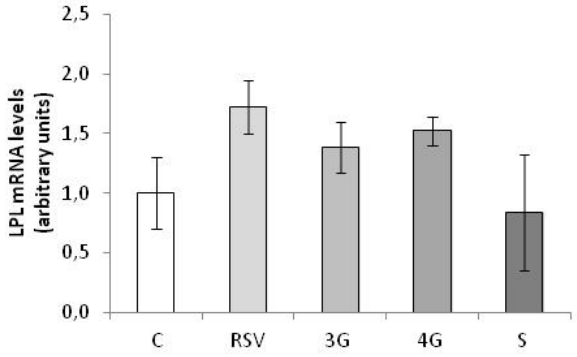
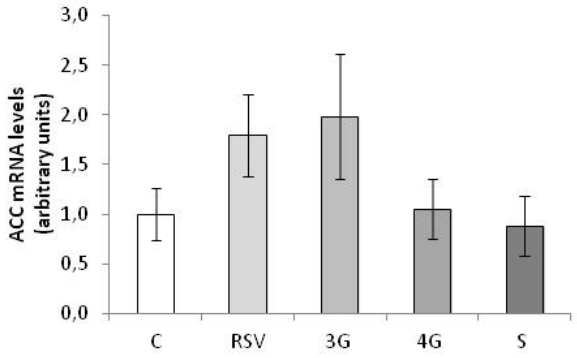


C

Figure 4. Effects of 10 µM (C) *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the expression of ATGL (A), HSL (B) and CPT-1 (C) in 3T3-L1 mature adipocytes treated for 24 hours. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (*P<0.05).



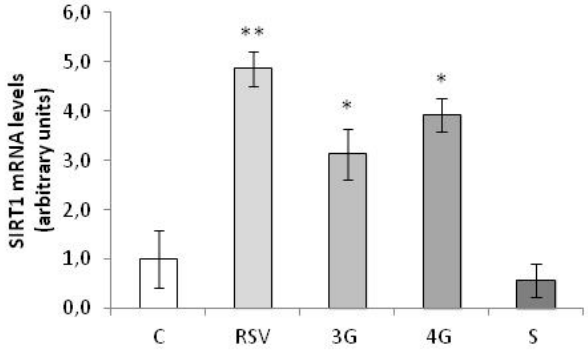
A



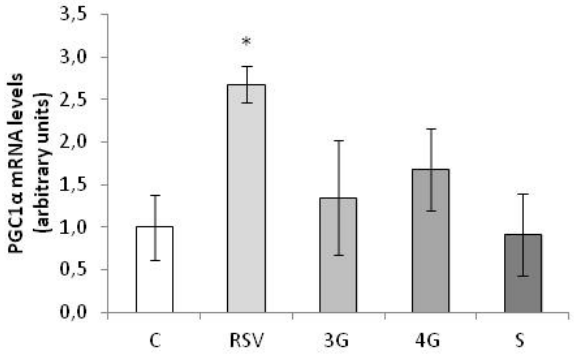
B

Figure 5. Effects of 10 µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the expression of LPL (A), ACC (B) and FASN (C) in 3T3-L1 mature adipocytes treated for 24 hours. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05).

Resveratrol has been proposed to drive its beneficial effects by targeting and activating the NAD+-dependent protein deacetylase SIRT-1, although results have been inconsistent [43-46, 7]. Several authors have also showed that resveratrol can increase the expression of this protein [37]. In the present study resveratrol and its glucuronide metabolites, but not the sulfate metabolite significantly up-regulated this gene. Resveratrol also increased the expression of PGC1α (Figure 6).



A



B

Figure 6. Effects of 10 µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the expression of SIRT-1 (A) and PGC-1α (B) in 3T3-L1 mature adipocytes treated for 24 hours. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05; ***P*<0.01).

The results obtained in the present study can help to better understand the results that we obtained in a previous study carried out in rats by using three doses of resveratrol (6, 30 and 60 mg/kg/d) [14]. We observed that this polyphenol reduced body fat at a dose of 30 mg/kg/d, but not at 6 mg/kg/d. Surprisingly, rats treated with 60 mg/kg/d resveratrol did not show further reduction as compared with those treated with 30 mg/kg/d. This means that a "plateau" was reached when the dose of resveratrol increased. Moreover, it was found in the same study that the amount of sulfate metabolites increased with the doses administered to rats. However, no differences in terms of glucuronide metabolite accumulation were found between rats treated with 30 or 60 mg/kg/d (data submitted). Thus, the lack of increase in glucuronides, active metabolites which may be responsible in part for the body fat-lowering effect of resveratrol, when the dose of this polyphenol increases from 30 to 60 mg/kg/d could help to explain the above mentioned "plateau" effect. Moreover, the results in the present study show that in mature adipocytes there is no significant increase in the delipidating effect when the dose of glucuronide metabolites rose from 10 µM to 25 µM and that of resveratrol from 1 µM to 10 and 25 µM, meaning that in vitro there is also a "plateau" in the effect. Thus, this fact can also contribute to the described in vivo effect.

As stated before, a combination of resveratrol with other molecules able to inhibit resveratrol metabolism has been proposed as an option to increase resveratrol biovailability. It has been proved that some flavonoids are able to inhibit resveratrol metabolism. De Santi and co-workers observed that quercetin inhibited sulfated metabolites production from resveratrol [32]. The results obtained in the present study gives support to the interest in this molecule combination since a decrease in the amount of ineffective metabolites increases the total amount of effective resveratrol+glucuronide metabolites and so the success of resveratrol treatment, at least in adult subjects in which adipogenesis is not an important process underlying obesity development.

4 CONCLUDING REMARKS

In conclusion, the present study shows for the first time that among resveratrol metabolites, trans-resveratrol-4'-O-glucuronide and trans-resveratrol-3-O-sulfate induced similar delipidating effects to resveratrol in maturing pre-adipocytes and that both glucuronide metabolites (trans-resveratrol-3-O-glucuronide and trans-resveratrol-4'-O-glucuronide) show a delipidating effect, although lower than that of resveratrol, in mature adipocytes. Consequently, it may be suggested that both resveratrol and resveratrol metabolites are involved, to greater or lesser extents, in the anti-obesity effect of this polyphenol.

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The authors have declared no conflict of interest.

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Potential miRNA involvement in the anti-adipogenic effect of resveratrol and its metabolites.

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Abstract

Objective: Scientific research is constantly striving to find molecules which are effective against excessive body fat and its associated complications. Taking into account the beneficial effects that resveratrol exerts on other pathologies through miRNA, the aim of the present work was to analyze the possible involvement of miRNAs in the regulation of adipogenic transcription factors peroxisome proliferator-activated receptor *γ (pparγ)*, CCAAT enhancer-binding proteins *α* and *β* (*cebpβ* and *cebpα)* induced by resveratrol and its metabolites.

Methods: 3T3-L1 maturing pre-adipocytes were treated during differentiation with 25 µM of *trans*-resveratrol (RSV)*, trans*-resveratrol-3-O-sulfate (3S), *trans*-resveratrol-3'-O-glucuronide (3G) and *trans*-resveratrol-4'-O-glucuronide (4G). After computational prediction and bibliographic search of miRNAs targeting *pparγ*, *cebpβ* and *cebpα*, the expression of microRNA-130b-3p (miR-130b-3p), microRNA-155-5p (miR-155-5p), microRNA-27b-3p (miR-27b-3p), microRNA-31-5p (miR-31-5p), microRNA-326-3p (miR-326-3p), microRNA-27a-3p (miR-27a-3p), microRNA-144-3p (miR-144-3p), microRNA-205-5p (miR-205-5p) and microRNA-224-3p (miR-224-3p) was analyzed. Moreover, other adipogenic mediators such as sterol regulatory element binding transcription factor 1 (*srebf1*), krüppel-like factor 5 (*klf5*), liver x receptor α (*lxrα*) and cAMP responding element binding protein 1 (*creb1*), were measured by Real Time RT-PCR. As a confirmatory assay, cells treated with RSV were transfected with anti-miR-155 in order to measure *cebpβ* gene and protein expressions.

Results: Of the miRNAs analyzed only miR-155 was modified after resveratrol and glucuronide metabolite treatment. In transfected cells with anti-miR-155, RSV did not reduce *cebpβ* gene and protein expression. 3S decreased gene expression of *creb1, klf5, srebf1* and *lxrα*.

Conclusions: While RSV and glucuronide metabolites exert their inhibitory effect on adipogenesis through miR-155 up-regulation, the anti-adipogenic effect of 3S is not mediated via miRNAs.

Introduction

Obesity is a genuinely serious real health problem. In 2014 about 13% of the world's adult population worldwide (11% of men and 15% of women) suffered from obesity [1]. In addition, this pathology induces a great number of co-morbilities, such as type 2 diabetes, dyslipidemia, hypertension and cancer among others. Consequently, direct and indirect costs associated with these common medical conditions have charted a steady rise in obesity costs over the years, as the epidemic has grown.

Adipose tissue growth in obesity can be mediated by hypertrophy, which is to say an increase in adipocyte size and/or hyperplasia, that is an increase in adipocyte number. When hyperplasia takes place there is a stimulation of pre-adipocyte proliferation and further differentiation. The above process, which promotes pre-adipocyte differentiation into mature adipocytes [2] plays a crucial role in the development of obesity and needs to be highly controlled. It is well established that in the long term continued energy overloading can increase this process, mainly in young subjects [2]. Although several molecular aspects of adipogenesis are still unknown, peroxisome proliferator-activated receptor γ (pparγ) has been identified as the master coordinator of adipocyte differentiation[3]. The control that pparγ exerts over pre-adipocytes for them to reach adipocyte functionally needs the expression of other important genes both at the early and at the latter stages of adipocyte differentiation, such as CCAAT enhancer-binding proteins α and β (cebpβ and cebpα) respectively [4, 5] .

These adipogenic genes are regulated by different mechanisms, microRNAs (MiRNAs) among others. MiRNAs are small non-coding RNAs about 19–23 nucleotides in length that have emerged as important regulators of gene expression [6]. They act by base paring with their target mRNA, which leads to mRNA degradation or translation repression [7, 8]. More than 2500 miRNAs have been described in humans to date [9]. Some of them are involved in numerous physiological and pathological processes, such as energy homeostasis [10], sugar and lipid metabolism [11, 12] and tumorigenesis [13]. As far as adipose tissue is concerned, several studies have concluded that some miRNAs can regulate adipogenesis by targeting genes that regulate this process [14-16].

Scientific research is constantly being undertaken with the aim of finding new molecules, either drugs or food components, which are effective in preventing excess accumulation of body fat and associated complications. This is the case of trans-resveratrol (3,4,5-trihydroxystilbene, RSV), a polyphenol with a stilbene structure that consists of two phenolic rings held together by a double styrene bond. This compound is naturally present in various plants, including grapes, berries and peanuts and is produced in response to stress, as a defence mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation [17]. Most RSV undergoes rapid and extensive metabolism into enterocytes, before entering blood. Furthermore, it undergoes rapid first-pass metabolism in the liver [17]. Consequently, RSV bioavailability is very low and only a small proportion reaches plasma. The concentrations of glucuronide and sulfate metabolites are relatively higher [18-20]. The proportions of glucuronide and sulfate metabolites depend on the tissue [21] and the species [22]. RSV, which shows antioxidant and antiinflammatory properties, is effective in the prevention of several diseases including cardiovascular diseases, diabetes, cancer and recently, obesity. With regard to obesity, a general consensus concerning the body-fat lowering effect of resveratrol in mice and

rats exists [23, 24]. This effect is mainly mediated by a reduction in adipogenesis and lipogenesis and by an increase in energy expenditure, lipolysis and fatty acid oxidation in liver and skeletal muscle [24].

Given the above relating to RSV metabolism, an important question is whether RSV metabolites are active molecules. In a previous study we described how RSV, as well as certain metabolites (trans-resveratrol-3-O-sulfate -3S-, trans-resveratrol-3'-O-glucuronide -3G- and trans-resveratrol-4'-O-glucuronide -4G-) were able to modify the expression of genes related to the adipogenic process [25]. While all of them (RSV, 3G, 4G and 3S) reduced cebpβ mRNA levels, only the sulfate metabolite reduced cebpα and pparγ gene expression.

In this scenario and taking into account that the beneficial effects of RSV on other pathologies, such as cancer and diabetes, are mediated by miRNA [26, 27], the present study focuses on the possible involvement of different miRNAs in the changes induced by RSV and its metabolites in adipogenic transcription factors pparγ, cebpβ and cebpα, a process which has not been analyzed to date. For this purpose, a well-defined pre-adipocyte model, 3T3-L1 murine adipocytes, was used.

Material and methods

Experimental design and cell treatment

The experimental design for 3T3-L1 maturing pre-adipocyte was previously described (25). Briefly, cells grown in 6-well plates were incubated with either 0.1% ethanol (95%) (control group) or with RSV, 3G, 4G or 3S, all of them provided by Bertin Pharma (Montigny le Bretonneux, France), at 25 µM (diluted in 95% ethanol) during the adipogenic phase from day 0 to day 8 of differentiation. The medium was changed every two days. On day 8, supernatant was removed and cells were used for triacylglycerol determination and RNA extraction. Each experiment was performed 3 times.

MiRNAs selection

For miRNAs selection as potential regulators of cebpβ, cebpα, pparγ, two criteria were established: a) to be validated or predicted by five algorithms (miRanda, miRDB, miRWalk, RNA22 and Targetscan algorithms) in miRWalk 2.0. database [28] and b) to be reported in Pubmed search using “miR + adipogenesis” terms (Table 1).

Table 1. miRNAs whose target genes have been predicted or validated by means of the miRWalk 2.0 or reported in the literature.

miRNA	Validated target genes	Predicted target genes (5 algorithms)	Literature Mir+adipogenesis
mmu-miR-31-5p		*cebpα*	[29, 30]
mmu-miR-101a-3p	*cebpα*		-
mmu-miR-101b-3p	*cebpα*		-
mmu-miR-124-3p	*cebpα*	*cebpα*	-
mmu-miR-130b-3p		*pparγ*	[15, 31-33]
mmu-miR-144-3p	*cebpα*		[34]
mmu-miR-155-5p	*cebpβ*		[16, 35-37]
mmu-miR-190a-5p		*cebpα*	-
mmu-miR-190b-5p		*cebpα*	-
mmu-miR-205-5p		*cebpα*	[38]
mmu-miR-224-3p		*cebpα*	[39]
mmu-miR-27a-3p	*pparγ*	*pparγ*	[40, 41]
mmu-miR-27b-3p	*pparγ*	*pparγ*	[42-46]
mmu-miR-326-3p		*cebpα*	[47]
mmu-miR-329-3p	*cebpα*		-
mmu-miR-330-5p		*cebpα*	-
mmu-miR-362-3p	*cebpα*		-
mmu-miR-466f-5p		*cebpβ*	-
mmu-miR-466i-3p	*cebpα*		-
mmu-miR-466m-3p	*cebpα*		-
mmu-miR-466o-3p	*cebpα*		-
mmu-miR-671-5p		*cebpα*	-
mmu-miR-690	*cebpα*		-

CEBP α and β: Relative CCAAT enhancer-binding protein α and β; PPARγ: peroxisome proliferator-activated receptor γ.

miRNA transfection

3T3-L1 pre-adipocytes at a confluence of approximately 80% were transfected with the DeliverX^{TM} Plus siRNA Transfection Kit (Affimetrix, Santa Clara, CA) following the manufacturer´s protocol with mirVana miRNA inhibitor mmu-miR-155-5p or mirVana miRNA inhibitor Negative Control (Applied Biosystems, Foster City, CA, USA). The final concentration of miRNA Inhibitors was established at 30 nM and the transfection period at 48 hours. These optimal conditions were determined in previous experiments carried out at 24, 48 and 72 hours in cells at different confluence statuses, and transfection efficiency was assessed using miRNA probes and fluorescent transfection controls.

At the same time, cells were stimulated to differentiate with DMEM containing 10% FCS, 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 µM dexamethasone and treated with RSV at 25 µM or ethanol 95% (Control group) during 48 hours. Afterwards, the supernatant was removed and cells were used to RNA and protein extraction. Each experiment was performed 3 times.

Extraction and analysis of RNA and quantification by Real Time reverse transcription-polymerase chain reaction (Real Time RT-PCR)

Total RNA sample containing small and large-size RNA from maturing pre-adipocytes was extracted with miRNeasy™ RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Small-size RNA was used for miRNA expression analysis and large-size RNA to quantify the mRNA expression.

1.5 µg of large-size RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Sterol regulatory element binding transcription factor 1 (*srebf1*), krüppel-like factor 5 (*klf5*), liver x receptor α (*lxrα*) and cAMP responding

element binding protein 1 (*creb1*) mRNA levels were quantified using Real-Time PCR with an iCyclerTM – MyiQTM Real-Time PCR Detection System (BioRad, Hercules, CA, USA) in the presence of SYBRGreen master mix (Applied Biosystems, Foster City, CA, USA). All sample mRNA levels were normalized to the values of 18S (Table 2).

Table 2. Primers for PCR amplification of each studied gene.

	Sense primer	Anti-sense primer
srebf1	5´- AAATCTTGCTGCCATTCG -3´	5´- TTGATCCCGGAAGCTCTGTG -3´
klf5	5´- CCGGAGACGATCTGAAACAC -3´	5´- GGAGCTGAGGGGTCAGATACTT -3´
creb1	5´- TTTGTCCTTGCTTTCCGAAT -3´	5´- CACTTTGGCTGGACATCTTG -3´
lxrα	5´- ATCGCCTTGCTGAAGACCTCTG -3´	5´- GATGGGGTTGATGAACTCCACC -3´
18s	5´- GTGGGCCTGCGGCTTAAT -3´	5´- GCCAGAGTCTCGTTCGTTATC -3´

Sterol regulatory element binding transcription factor 1 (*srebf1*); krüppel-like factor 5 (*klf5*); cAMP responding element binding protein 1 (*creb1*), liver x receptor α (*lxrα*); 18S ribosomal RNA (*18s*).

Reverse transcription of 10 ng of small-size RNA and PCR were performed with the

TaqMan® MicroRNA Assay kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). miRNA levels for miR-130b-3p, miR-155-5p, miR-27b-3p, miR-326-3p, miR-31-5p, miR-27a-3p, miR-144-3p, miR-205-5p and miR-224-3p were quantified using TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) for each miRNA and normalized to the values of U6 snRNA. The miRNA assay sequences were as follows:

miR-130b-3p 5'- CAGUGCAAUGAUGAAAGGGCAU -3'

miR-155-5p 5'- UUAAUGCUAAUUGUGAUAGGGGU -3'

miR-27b-3p 5'- UUCACAGUGGCUAAGUUCUGC -3'

miR-326-3p 5'- CCUCUGGGCCCUUCCUCCAGU -3'

miR-31-5p 5'- AGGCAAGAUGCUGGCAUAGCUG -3'

miR-27a-3p 5'- UUCACAGUGGCUAAGUUCCGC - 3'

miR-144-3p 5'- UACAGUAUAGAUGAUGUACU - 3'

miR-205-5p 5'- UCCUUCAUUCCACCGGAGUCUG - 3'

miR-224-3p 5'- AAAUGGUGCCCUAGUGACUACA - 3'

All gene and miRNA expression results were expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [48].

After miRNA transfection assay, total RNA sample containing small and large-size RNA was extracted with miRNeasy™ RNA isolation kit (Qiagen, Hilden, Germany). Small-size RNA was used to mir-155 expression analysis and large-size RNA to quantify the mRNA expression of *cebpβ*. The expression levels of both mir-155 and *cebp*β were analyzed as explained before.

Protein expression analysis

Total protein was isolated from maturing 3T3-L1 adipocytes using 150 µL of lysis buffer (2 nM tris-HCl, 0.1 M sodium chloride (NaCl), 1% Triton, 10% glycerol, 1 mM sodium orthovanadate (OvNa), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium fluoride (FNa) and 1% protease inhibitor) and centrifuged (12.000g, 15 minutes, 4 °C) to remove membranes and other proteic residues. Protein concentration was determined by BCA protein assay kit (Thermo Scientific, Wilmington, DE, USA). Total protein (20 μg) was subjected to 10% SDS-polyacrylamide gel, electroblotted onto PVDF membranes (Millipore, Bradford, MA, USA), and incubated with polyclonal rabbit anti-*cebpβ* (1:1000) and monoclonal mouse anti-tubulin (1:5000) (Santa-Cruz Biotech, CA, USA) overnight and afterwards with polyclonal goat anti-mouse IgG-HRP for *cebpβ* (1:5000) and polyclonal goat anti-rabbit for α-tubulin (1:5000) (Santa-Cruz Biotech, CA, USA) for 2 hours. Bound antibodies were visualized by an ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) and quantified by Chemi-Doc MP imaging system (BioRad, CA, USA).

Statistical analysis

Results are presented as mean $\pm$ standard error of the mean. Statistical analysis was performed using SPSS 24.0 (SPSS Inc. Chicago, IL, USA). Comparisons between each treatment and the controls were analyzed by Student's *t* test. Statistical significance was set-up at the $p < 0.05$ level.

Results and discussion

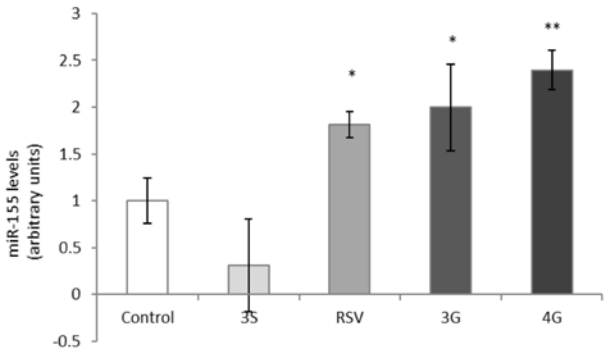
As stated in the introduction section, obesity is a real problem, and functional molecules may be a new effective tool for the management of this disease. Among them, resveratrol has been demonstrated as having beneficial effects in order to face obesity in both *in vitro* and *in vivo* models. Several published *in vitro* studies conclude that this polyphenol is able to inhibit the process of adipogenesis, leading to a lower amount of differentiated adipocytes and thus to a decrease in triglyceride accumulation [49-51]. Along the same lines, we previously demonstrated that resveratrol and its glucuronide and sulfate metabolites are able to block adipogenesis and to reduce triglyceride accumulation to the same extent in 3T3-L1 maturing pre-adipocytes [25].

Adipogenesis is a complex process governed by a tightly controlled network of transcription factors that coordinate a great number of genes [52-55]. At the centre of this network there are two principal adipogenic factors, *pparγ* and *cebpα*, whose expression is regulated by other transcription factors, such as *cebpβ* [27]. In recent years, miRNAs have been described as a potential group of adipogenic controllers. Indeed, a snapshot of miRNA profiling revealed a dramatic change of 21 miRNAs during 3T3-L1 adipocyte differentiation [56]. In this line, miR-155 and miR-27b have been shown to suppress the expression of *cebpβ* and *pparγ* in adipocytes. Therefore, these miRNAs could be considered one of the mechanisms by which the adipogenic process is inhibited [16,35,42].

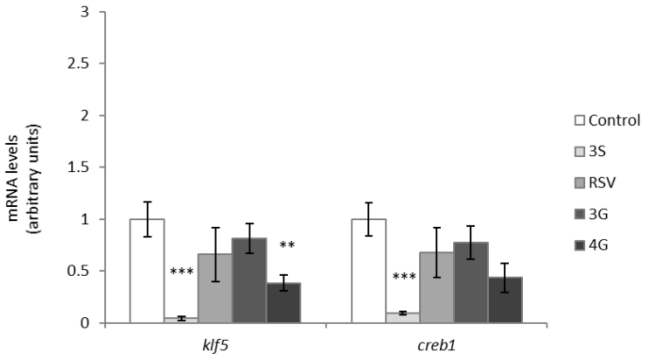
Modulation of miRNA expression by dietary compounds is increasingly being investigated by scientists working in the field of functional ingredients and their potential capacity to prevent pathologies. Indeed, some dietary polyphenols, such as curcumin, epigallocatechin gallate or resveratrol have been demonstrated to suppress different cancer cells growth by up-regulating miRNAs [57]. Resveratrol has also been linked to modifications on miRNAs expression in heart myoblasts, which could explain its cardioprotective effect. Quercetin, coffee polyphenols and grape seed proanthocyanidins can target miR-122 in mice livers and control cholesterol and bile acid synthesis and fatty acid oxidation, and thus, prevent liver steatosis [58, 59]. With regard to regulation of adipogenesis through miRNAs, Zhu et al. demonstrated that epigallocatequines up-regulated the expression of miR-27a and miR-27b and down-regulated that of pparγ and cebpα [60]. The same effects were found by persimmon tannin treatment during adipogenesis [43]. By contrast, it seems that nonivamide, a capsaicin analogue, increases the expression of the miRNA mmu-let-7d-5p, which has been associated with decreased pparγ levels [61]. Other plant or fruit extracts have been also identified as adipogenic regulators via miRNAs [62, 63].

In view of all mentioned above, and considering that miRNAs can play a crucial role in the effect attributed to dietary polyphenols, in the present study we aimed to analyze the mechanisms by which RSV and its metabolites modified the gene expression of adipogenic regulators. For this purpose we focussed on the analysis of those potential miRNA (validated or predicted) targeting pparγ, cebpβ and cebpα, which were selected by using the miRWalk 2.0. database and a literature review.

MiR-155 and other genes that regulate the expression of cebpβ were measured (Table 1). RSV and the glucuronide metabolites increased miR-155 gene expression, but 3S metabolite did not (Fig 1A). These results could suggest that whereas RSV, 3G and 4G exert their effect via miR-155, 3S metabolite does not do so. In order to verify the mechanism of RSV and the glucuronide metabolites, maturing 3T3-L1 adipocytes were transfected with an anti-miR-155 compound while they were cultured in the presence or absence of RSV. After transfection, cebpβ gene and protein expression remained unchanged in treated cells (Fig 2), demonstrating that the polyphenol, and reportedly its glucuronide metabolites, inhibit the process of adipogenesis, at least in part, via miR-155. The modulation of miR-155 by RSV has been extensively studied in monocytes and macrophages. In these cells RSV was shown to increase miR-155 expression, to reduce the inflammatory response and to protect from atherosclerosis and hypertension [64-67]. Nevertheless, studies analyzing this regulatory pathway in adipocytes have not been carried out yet.



A



B

Fig 1. Effects of 25 µM of resveratrol (RSV) on the expression of mir-155 (A) and 25 µM of *trans*-resveratrol-3-O-sulfate (3S), *trans*-resveratrol-3-O-glucuronide (3G) and *trans*-resveratrol-4-O-glucuronide (4G) on *creb1* and *klf5* gene expression (B) in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM (Standard Error of the Mean) of three independent experiments carried out in sextuplicate. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisks represent differences versus the controls (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

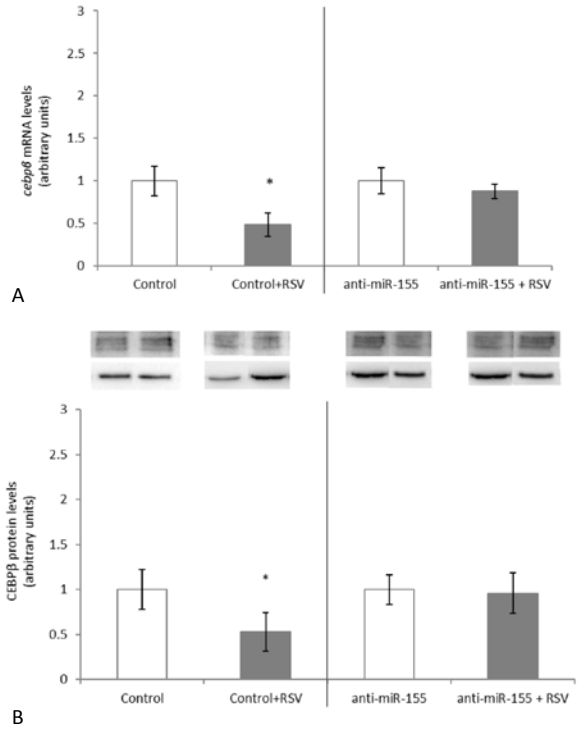


Fig 2. *cebpβ* gene (A) and protein (B) expressions after transfection with miR-155 inhibitor, with or without 25 µM RSV in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 2. Values are means ± SEM (Standard Error of the Mean) of three independent experiments carried out in triplicate. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisks represent differences versus the controls (**P* < 0.05).

Taking into account that the sulfate metabolite did not exert any effect on miR-155 (Fig 1A), other regulatory routes that could lead to the reduction observed in cebpβ gene expression were analyzed. In the network of adipogenic transcription factors creb1 plays a crucial role as cebpβ precursor [68-70]. Moreover,

klf5 is induced by cebpβ/δ and in turn controls pparγ expression, thus mediating both the early and late stages of the differentiation program [71]. In the present study, 3S metabolite reduced gene expression of creb1 and 3S and 4G that of klf5 (Fig 1B). Therefore, it could be suggested that 3S metabolite orchestrated its effects on the initial phase of the adipogenesis in a transcriptional way, apparently without any influence of miRNA. By contrast, the 4G metabolite not only exerted its effect via miR-155, but also through klf5. The modulation of cebpβ was also observed by other polyphenols [72, 73].

MiR-27b, miR-27a and miR-130b were selected by miRWalk database (Table 1) as pparγ regulator. Sulfate metabolite did not change the expression of these miRNAs (Fig 3A). As in the case of cebpβ, other genes that are involved in the regulation of pparγ during adipogenesis (srebf1 and lxrα) [74, 75] were analyzed. 3S metabolite reduced the expression of both genes (Fig 3B), which explains the changed induced by this metabolite in pparγ expression without changes in miR-27b, miR-27a and miR-130b. This fact was also observed with other anti-adipogenic pytochemicals such as apigenin [76], or black adzuki bean [77].

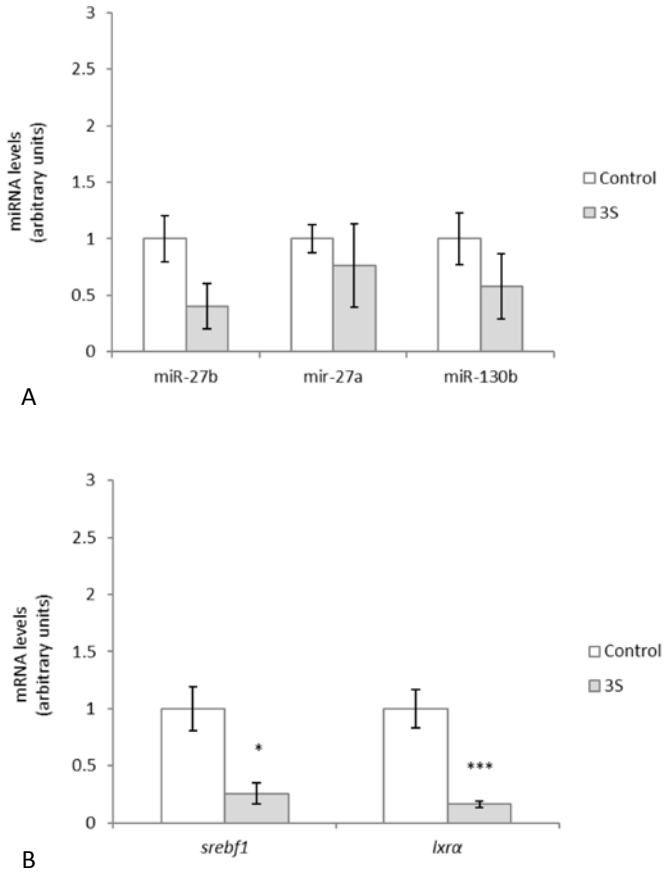


Fig 3. Effects of 25 μM of *trans*-resveratrol-3-*O*-sulfate (3S) on the expression of mir-27b, miR-27a and miR-130b (A) and on gene expression of *srebf1* and *lxrα* (B) in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM (Standard Error of the Mean) of three independent experiments carried out in sextuplicate. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisks represent differences versus the controls (*$P < 0.05$; ***$P < 0.001$).

Finally, we set out to analyze the miRNAs related to *cebpα*. For this purpose, miR-326, miR-31, miR-144, miR-205 and miR-224 were selected as miRNAs targeting *cebpα,* according to the computational analysis and literature (Table 1). None of these miRNAs were modified by 3S treatment (Fig 4), which suggests that its

mechanism of action was not via miRNAs. The down-regulation observed by 3S on *pparγ,* can be considered itself one of the reasons for reduction in *cebpα.* These results, as a whole, suggest that 3S metabolite could exert its anti-adipogenic effect through adipogenic regulatory genes but not through miRNAs, as is the case of resveratrol and glucuronide metabolites (Fig 5).

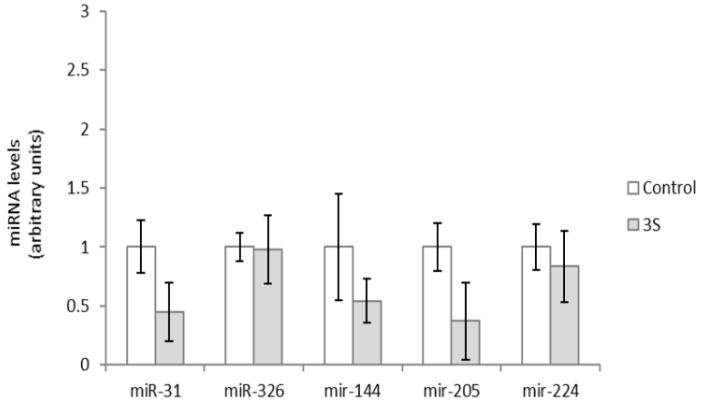
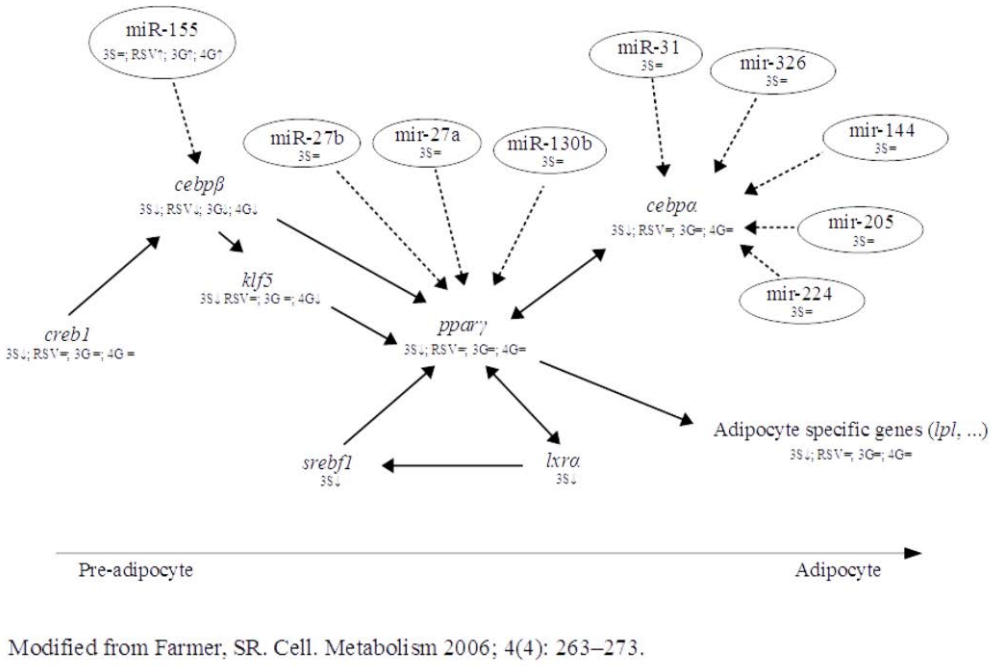


Fig 4. Effects of 25 µM of *trans*-resveratrol-3-*O*-sulfate (3S) on the expression of miR-326, miR-31, miR-144, miR-205 and miR-224 in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM (Standard Error of the Mean) of three independent experiments carried out in sextuplicates. Comparisons between each treatment and the controls were analyzed by Student's *t*-test.



Modified from Farmer, SR. Cell. Metabolism 2006; 4(4): 263–273.

Fig 5. Genes and miRNAs involved in the inhibition of adipogenesis by resveratrol (RSV), *trans*-resveratrol-3-O-sulfate (3S), *trans*-resveratrol-3-O-glucuronide (3G) and *trans*-resveratrol-4-O-glucuronide (4G) in the pathways of the adipogenic process (modified from Farmer *et. al.* 2006).

This study presents the limitation that the experiments were performed in 3T3-L1 adipocytes. Therefore, data extrapolation to human is not completely possible. There are some differences in the metabolism and physiology of mouse and human adipogenesis such as differences in the modulation of *pparγ*

[78]. However, the main species-specific differences in adipogenesis focus on when (and where) the products of the genes are made. However, the role of the master regulators is the same in both species, as far as we know. Taking into account the methodological difficulties that human adipocytes present for transfections and the heterogeneity of results in response to treatments, 3T3-L1 adipocytes were used in the present study.

Conclusions

In summary, our study clearly suggests that the inhibitory effect on adipogenesis attributed to RSV and its glucuronide metabolites (3G and 4G) in 3T3-L1 adipocytes is mediated by the up-regulation of miR-155, which in turn leads to a down-regulation of *cebpβ* gene expression. In the case of 4G, *klf5* also contributed to this regulation. By contrast, the inhibitory effect observed in cells treated with 3S metabolite was not mediated via miRNAs. In this case, changes in *creb1*, *klf5, srebf1* and *lxrα,* explain the effects of this metabolite on adipogenesis.

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Resveratrol metabolites modify adipokine expression and secretion in 3T3-L1 pre-adipocytes and mature adipocytes

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Abstract

Objective: Due to the low bioavailability of resveratrol, determining whether its metabolites exert any beneficial effect is an interesting issue.

Methods: 3T3-L1 maturing pre-adipocytes were treated during differentiation with 25 µM of resveratrol or with its metabolites and 3T3-L1 mature adipocytes were treated for 24 hours with 10 µM resveratrol or its metabolites. The gene expression of adiponectin, leptin, visfatin and apelin was assessed by Real Time RT-PCR and their concentration in the incubation medium was quantified by ELISA.

Results: Resveratrol reduced mRNA levels of leptin and increased those of adiponectin. It induced the same changes in leptin secretion. *Trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-4'-*O*-glucuronide increased apelin and visfatin mRNA levels. *Trans*-resveratrol-3-*O*-sulfate reduced leptin mRNA levels and increased those of apelin and visfatin.

Conclusions: The present study shows for the first time that resveratrol metabolites have a regulatory effect on adipokine expression and secretion. Since resveratrol has been reported to reduce body-fat accumulation and to improve insulin sensitivity, and considering that these effects are mediated in part by changes in the analyzed adipokines, it may be proposed that resveratrol metabolites play a part in these beneficial effects of resveratrol.

INTRODUCTION

In recent years, a remarkable range of biological functions has been ascribed to resveratrol. It shows chemopreventive, anti-inflammatory and antioxidant properties [1], [2]. Beneficial cardiovascular effects have also been described [3]. More recently, resveratrol has been proposed as a potential anti-obesity compound [4-11], which also improves insulin sensitivity [4], [6], [11], [12].

Several studies have attributed the beneficial effects of resveratrol on body fat accumulation and glycemic control [13-16] in part to the changes induced by this polyphenol in adipokines production. Leptin increases energy expenditure and reduces food intake [17], [18]. This adipokine is also related to glycemic control. A physiological increase in plasma leptin levels has been shown to inhibit insulin secretion [19]. Adiponectin increases glucose uptake in muscles and insulin sensitivity, suppresses gluconeogenesis in hepatocytes [20] and increases fatty acid oxidation [21]. With regard to these adipokines, it has been reported that resveratrol reduces leptin expression and secretion and increases adiponectin expression in both *in vitro* and *in vivo* studies [13], [22].

Other adipokines, more recently discovered, such as visfatin and apelin, have been partially involved in obesity and glucose homeostasis. Apelin increases the expression of uncoupling proteins UCP1 and 3, increases energy expenditure and decreases respiratory quotient, resulting in increased fat oxidation [23]. Both of them improve insulin sensitivity and maintain glucose homeostasis in rodents and humans. Visfatin has been shown to play an important role in pancreatic β-cell function by acting as an intra and an extracellular NAD biosynthetic enzyme and regulating glucose-stimulated insulin secretion [24]. Apelin has a close relationship with insulin because it enhances glucose uptake in insulin responsive tissues, such as skeletal muscle [25-28]. Derdemezis *et al.* [29] observed decreased visfatin secretion in SGBS adipocytes treated with resveratrol. No data have been reported to date concerning the effect of resveratrol on apelin production.

One of the main concerns of scientists working in the field of resveratrol is its low bioavailability. It has been described that only a small proportion of this molecule reaches plasma and tissues after its oral intake [30], [31]. The concentrations of glucuronide and sulfate metabolites are relatively higher [32-34]. In order to increase resveratrol bioavailability, and thus its probable effectiveness as a functional ingredient for prevention and treatment of several diseases, different strategies are under evaluation: combination with other molecules which inhibit its metabolization [35], [36], encapsulation with different excipients (microencapsulation or nanoencapsulation), or searching for more resistant structural analogues (pterostilbene). However, to better determine the target for these research strategies, it is very important to know whether these metabolites exert any effect, and to compare the magnitude of these effects with those of resveratrol.

In this line of research it has been reported that several metabolites show biological activities, such as cancer chemoprotection [37], [38] and anti-inflammation [38], [39]. With regard to the effects of resveratrol metabolites on lipid metabolism there is only one study, reported recently by our group [40].

In this context, the aim of the present study was to determine the effect of resveratrol and the following resveratrol phase II metabolites: *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4'-*O*-

glucuronide and *trans*-resveratrol-3-*O*-sulfate, on adipokine production in 3T3-L1 maturing pre-adipocytes and mature adipocytes.

MATERIALS AND METHODS

Reagents

Dulbecco´s modified Eagle´s medium (DMEM) was purchased from GIBCO (BRL Life Technologies, Grand Island, NY). *Trans*-Resveratrol (98% purity), *trans*-resveratrol-3-*O*-glucuronide (95% purity), *trans*-resveratrol-4'-*O*-glucuronide (95% purity) and *trans*-resveratrol-3-*O*-sulfate (98% purity) were provided by Bertin Pharma (Montigny le Bretonneux, France).

Experimental design

3T3-L1 pre-adipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10% foetal calf serum (FCS). Two days after confluence (day 0), the cells were stimulated to bring about differentiation with DMEM containing 10% FCS, 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 µM dexamethasone for 2 days. On day 2, the differentiation medium was replaced by FCS/DMEM medium (10%) containing 0.2 µg/mL insulin. This medium was changed every two days until the cells were harvested. All media contained 1% Penicillin/Streptomycin (10,000 U/mL), and the media for differentiation and maturation contained 1% (v/v) of Biotin and Panthothenic Acid. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Cell treatment

For the treatment of maturing pre-adipocytes, cells grown in 6-well plates were incubated with either 0.1% ethanol (95%) (control group) or with *trans*-resveratrol, *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4'-*O*-glucuronide or *trans*-resveratrol-3-*O*-sulfate, at 25 µM (diluted in 95% ethanol) during differentiation. Media containing resveratrol or its metabolites were changed every two days: on day 0, day 2, day 4 and day 6. On day 8, supernatant was collected for adipokine determination in the media and cells were used for RNA extraction and gene expression measurement. Each experiment was performed 3 times.

For the treatment of mature adipocytes, cells grown in 6-well plates were incubated with the same molecules, at 10 µM (diluted in 95% ethanol) on day 12 after differentiation because at that day >90% of cells were mature adipocytes with visible lipid droplets. After 24 hours, supernatant was collected for adipokine determination in the media and cells were used for RNA extraction and gene expression measurement. Each experiment was performed 3 times.

The reason for using different doses in maturing pre-adipocytes and mature adipocytes was based on the results obtained in the same sets of cells when the delipidating effect of resveratrol metabolites was assessed. In this previous experiment, we analyzed the effects of different doses (1, 10 and 25 µM) and it was observed that in the case of maturing pre-adipocytes the effective dose for reducing triacylglycerol content was 25 µM, and in the case of mature adipocytes this was 10 µM (even though resveratrol also reduced triacylglycerol content at 1 µM) [40].

Adipokine concentration in the media

Commercial kits were used to assess concentrations of apelin, visfatin, leptin and adiponectin in the media by ELISA (EK-003-80, Phoenix Europe GMBH, Karlsruhe, Germany and RAG004R, RD191001100 and RD293023100R, Biovendor, Brno, Czech Republik, respectively).

Extraction and analysis of RNA and quantification by reverse transcription-polymerase chain reaction (Real Time RT-PCR)

RNA samples were extracted from cells by using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using a RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with DNase I kit (Applied Biosystems, Foster City, CA, USA) to remove any contamination with genomic DNA.

One µg of total RNA in a total reaction volume of 20 µL was reverse transcribed using the iScript cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocols. Reactions were incubated initially at 25 °C for 10 min and subsequently at 37 °C for 120 min and 85 °C for 5 min.

Relative leptin, adiponectin, visfatin and apelin mRNA levels were quantified using Real-Time PCR with an iCycler™ - MyiQ™ Real Time PCR Detection System (BioRad, Hercules, CA, USA). β-actin mRNA levels were similarly measured and served as the reference gene. The PCR reagent mixture consisted of 1 µL of each cDNA (10 pmol/µL), SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the upstream and downstream primers (300nM each) were used for leptin and adiponectin. In the case of visfatin and apelin, the reagent mixture consisted of 1 µL of each cDNA, Premix Ex TaqTM (Takara, USA) and the upstream and downstream primers (600 nM for visfatin and apelin; 300nM for β-actin) and probe (1 µM visfatin; 0.5 µM apelin and β-actin). Specific primers and probes were synthesized commercially (Eurogentec, Liège, Belgium) (Table 1).

Table 1. Primers for PCR amplification of each studied gene.

	Sense primer	Antisense primer	Probe
SYBR® Green RT-PCR:			
Leptin	5'- TGG ACC AGA CTC TGG CAG TC -3'	5'- AGG ACA CCA TCC AGG CTC TC -3'	
Adiponectin	5'-TG TAG GAT TGT CAG TGG ATC TG-3'	5'-GCT CTT CAG TTG TAG TAA CGT CAT G-3'	
β-Actin	5'- ACG AGG CCC AGA GCA AGA -3'	5'- GGT GTG GTG CCA GAT CTT CTC -3'	
TaqMan RT-PCR:			
Visfatin	5'- CCG GCC CGA GAT GAA TGC -3'	5'- GGA ATA AAC TTT GCT TGT GTT GGG-3'	5'-FAM- AGC CGA GTT CAA CAT CCT GCT GGC -TAMRA- 3'
Apelin	5'- ATT TAA GGA CAC GCT GAT CAA AGG-3'	5'- AGT CCC GAA AGT ATTCAA AAG CAG-3'	5'- AAA CAG AAG GCA CCC ACC AGG GCT-3'
β-Actin	5'- TCT ATG AGG GCT ACG CTC TCC-3'	5'- CAC GCT CGG TCA GGA TCT TC -3'	5'- FAM- CCT GCG TCT GGA CCT GGC TGG C -TAMRA -3'

PCR parameters were as follows: initial 2 min at 50ºC, denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 30s, annealing at 60ºC for 30s, and extension at 60ºC for 30s. All sample mRNA levels were normalized to the values of β-actin and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the 2-ΔΔCt method [41].

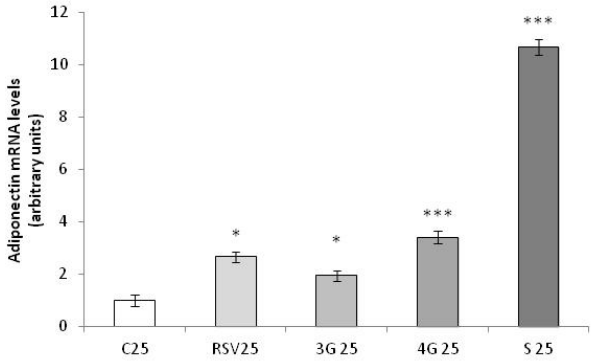
Statistical analysis

Results are presented as mean ± standard error of the mean. Statistical analysis was performed using SPSS 19.0 (SPSS Inc. Chicago, IL, USA). Comparisons between each treatment with the control were analyzed by Student's *t* test. Statistical significance was set-up at the $P < 0.05$ level.

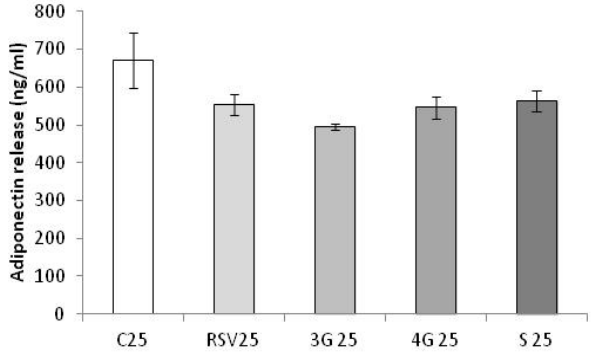
RESULTS

Effects of resveratrol and its metabolites on adiponectin expression

After treating maturing pre-adipocytes from day 0 to day 8, resveratrol and the three analyzed metabolites increased adiponectin expression (Figure 1A). Nevertheless, no changes were observed in the secretion of this adipokine (Figure 1B). In mature adipocytes, only resveratrol increased adiponectin expression but no differences were observed in the concentration in the medium after 24 hours of treatment (Figure 2A and 2B).

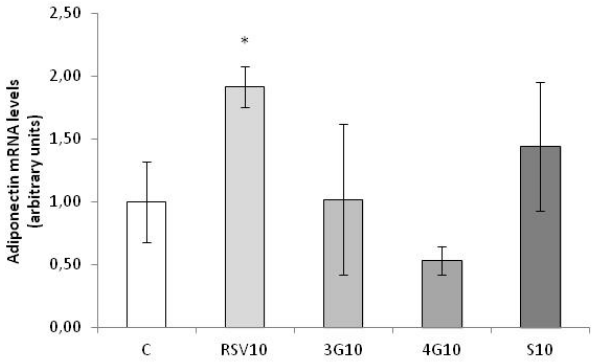


1A

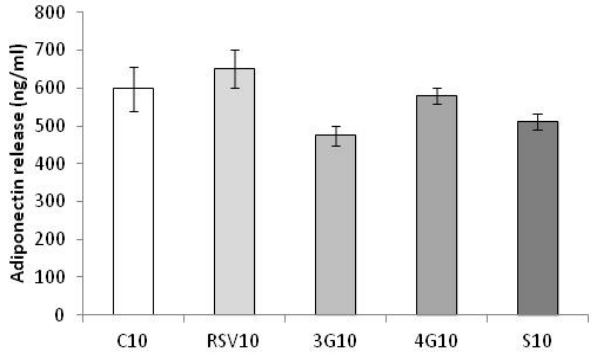


1B

Figure 1. Effects of 25µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the mRNA expression (A) and protein concentration in the culture media (B) of adiponectin in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8 of differentiation. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05, ****P*<0.001).



2A

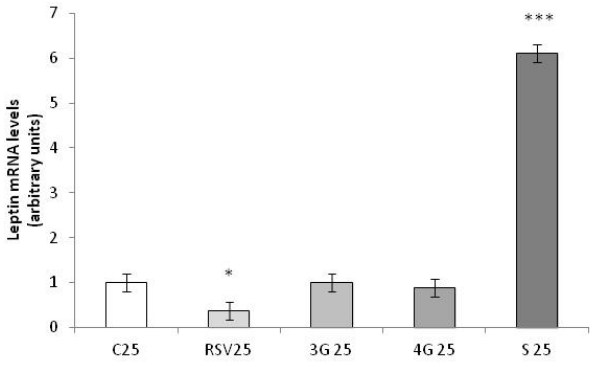


2B

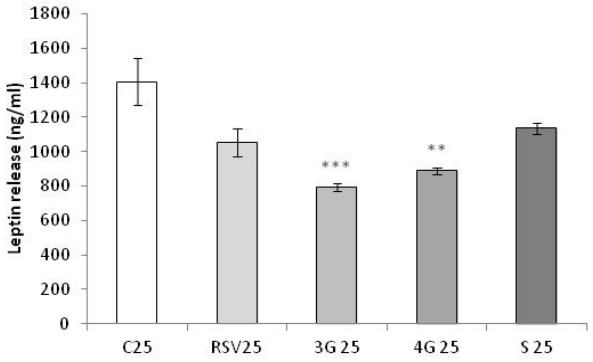
Figure 2. Effects of 10 µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the mRNA expression (A) and protein concentration in the culture media (B) of adiponectin in 3T3-L1 mature adipocytes treated for 24 hours on day 12 of differentiation. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05).

Effects of resveratrol and its metabolites on leptin expression

As far as maturing pre-adipocytes are concerned, resveratrol reduced, while *trans*-resveratrol-3-*O*-sulfate increased, leptin mRNA levels No changes were induced by glucuronide metabolites (Figure 3A). However, glucuronide metabolites reduced leptin secretion to the media, and resveratrol and *trans*-resveratrol-3-*O*-sulfate showed a tendency towards reduced values (Figure 3B). With regard to mature adipocytes, both the expression and secretion of leptin were reduced by resveratrol and by all the metabolites analyzed (Figure 4A and 4B).

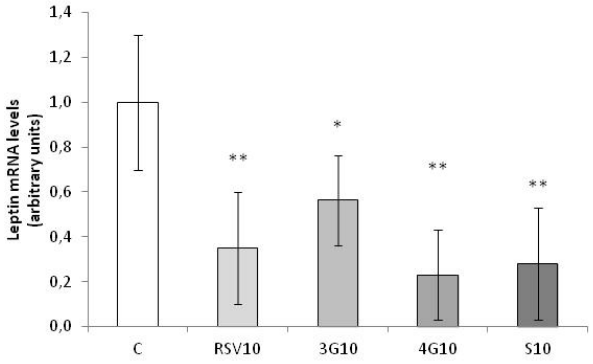


3A

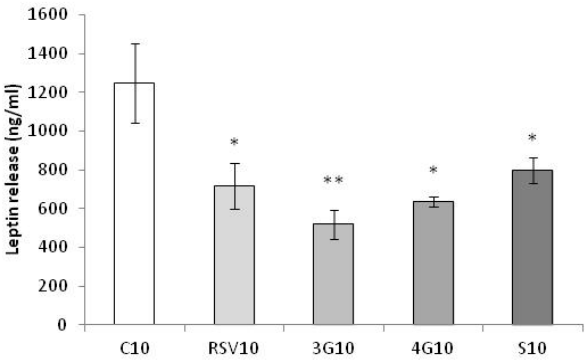


3B

Figure 3. Effects of 25µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the mRNA expression (A) and protein concentration in the culture media (B) of leptin in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8 of differentiation. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05, ***P*<0.01, ****P*<0.001).



4A

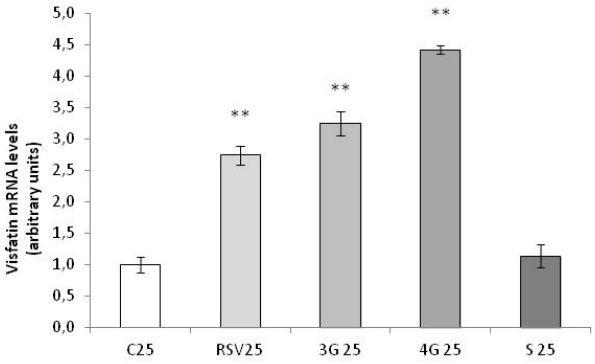


4B

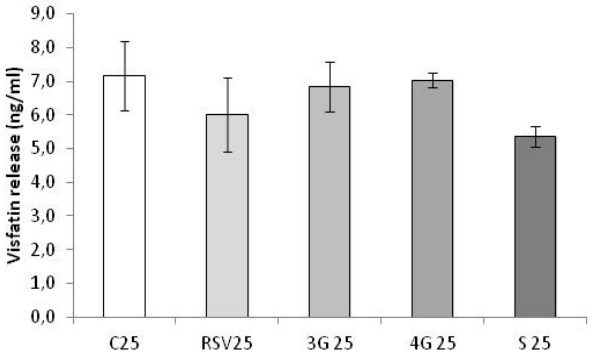
Figure 4. Effects of 10 µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the mRNA expression (A) and protein concentration in the culture media (B) of leptin in 3T3-L1 mature adipocytes treated for 24 hours on day 12 of differentiation. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05, ***P*<0.01).

Effects of resveratrol and its metabolites on visfatin expression

Resveratrol and the glucuronide metabolites increased visfatin expression in maturing pre-adipocytes, without changes in protein secretion. *Trans*-resveratrol-3-*O*-sulfate did not induce any change (Figure 5A). No changes were observed in visfatin secretion (Figure 5B). In mature adipocytes, while resveratrol did not affect the mRNA expression of this adipokine, the three metabolites increased its mRNA levels (Figure 6A). Despite this increase, visfatin concentration in the media did not change (Figure 6B).

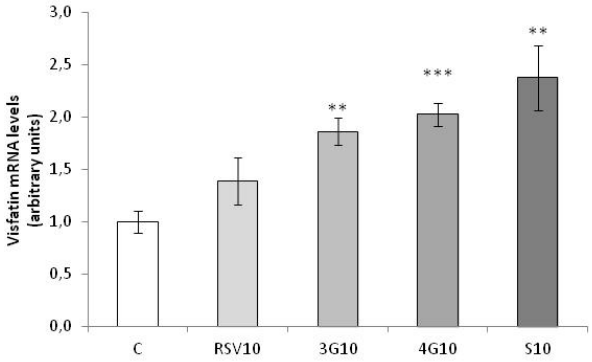


5A

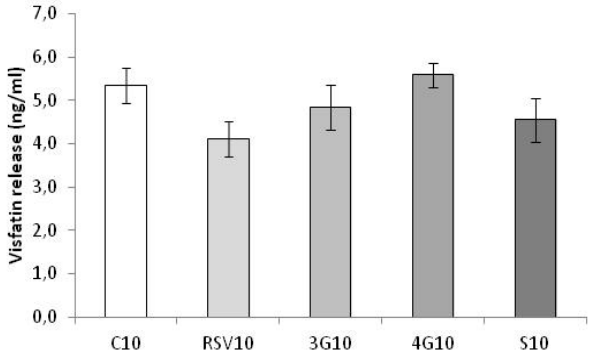


5B

Figure 5. Effects of 25µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the mRNA expression (A) and protein concentration in the culture media (B) of visfatin in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8 of differentiation. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (***P*<0.01).



6A

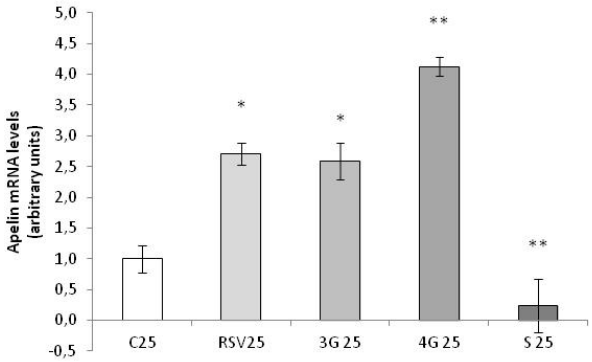


6B

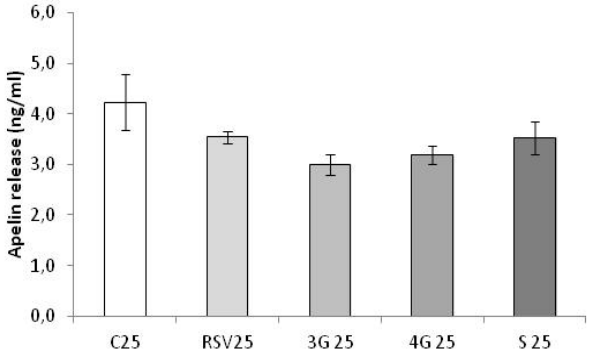
Figure 6. Effects of 10 µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the mRNA expression (A) and protein concentration in the culture media (B) of visfatin in 3T3-L1 mature adipocytes treated for 24 hours on day 12 of differentiation. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (***P*<0.01, ****P*<0.001).

Effects of resveratrol and its metabolites on apelin expression

Resveratrol and glucoronide metabolites increased apelin mRNA levels in maturing pre-adipocytes, whereas *trans*-resveratrol-3-*O*-sulfate reduced them (Figure 7A). Nevertheless, no changes were observed in apelin secretion (Figure 7B). In mature cells, only the metabolites increased apelin expression without changing its secretion (Figure 8A and 8B).

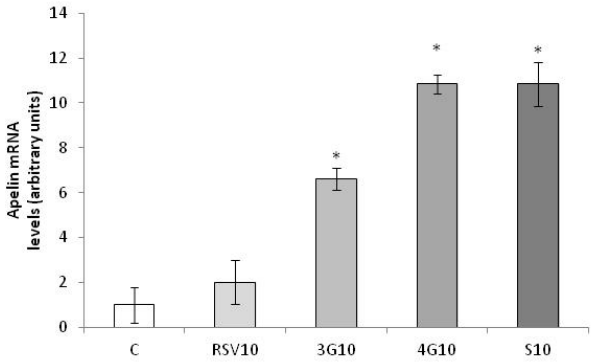


7A

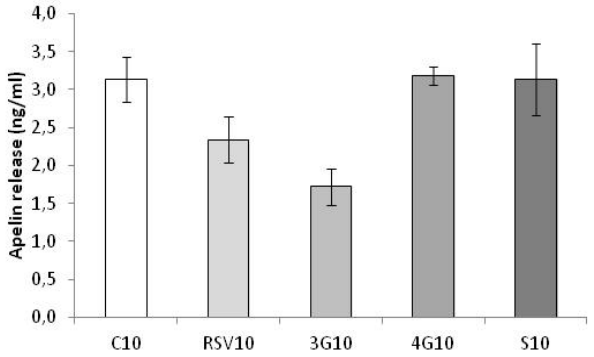


7B

Figure 7. Effects of 25µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the mRNA expression (A) and protein concentration in the culture media (B) of apelin in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8 of differentiation. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (**P*<0.05, ***P*<0.01).



8A



8B

Figure 8. Effects of 10 µM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4'-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (S) on the mRNA expression (A) and protein concentration (B) of apelin in 3T3-L1 mature adipocytes treated for 24 hours on day 12 of differentiation. Values are means±SEM. Comparisons between each treatment with the control were analyzed by Student´s *t* test. The asterisks represent differences *vs.* the control (*P<0.05).

DISCUSSION

Following ingestion, most resveratrol metabolizes rapidly, resulting in up to 20-fold higher concentrations of circulating conjugates, and less than 1% of the parent compound [32], [42]. Thus, it is important to know whether resveratrol metabolites show biological activities and if as a result they contribute to the beneficial effects of resveratrol on health. Some authors have demonstrated the anticarcinogenic effect of resveratrol metabolites [38], [43].

In this context, our group had previously reported the delipidating effect of resveratrol metabolites, by using the same sets of cells as those used in the present study [40]. Specifically, *trans*-resveratrol-4'-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate induced similar delipidating effects to that of resveratrol in maturing pre-adipocytes; *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-4'-*O*-glucuronide showed an important delipidating effect (although lower than that of resveratrol) in mature adipocytes. Consequently, we suggested that both resveratrol and resveratrol metabolites were involved, to greater or lesser extent, in the anti-obesity effect of this polyphenol. We differentiated the effects of resveratrol metabolites on maturing pre-adipocytes and mature adipocytes because these two cell types play different roles in obesity

development. Thus, during childhood and adolescence, obesity is mainly induced by hyperplasia, which implies the differentiation of pre-adipocytes into mature adipocytes. By contrast, in adulthood, obesity is mainly induced by hypertrophy of mature adipocytes,

Several positive effects of resveratrol are mediated by changes in adipokine production. Adipokines produced by adipose tissue can act in an autocrine or in a paracrine manner to modify metabolic pathways involved in lipid metabolism and glucose homeostasis. In view of the interesting effects of resveratrol metabolites on adipocyte triacylglycerol accumulation, and in order to gain more insight into the potential involvement of resveratrol metabolites in the beneficial effects of resveratrol in obesity and insulin resistance prevention, the expression and secretion of several adipokines were analyzed in 3T3-L1 pre-adipocytes and mature adipocytes treated with three resveratrol metabolites. As far as we know, this is the first time that this issue has been addressed. For this purpose, two concentrations were used: 25 µM in maturing pre-adipocytes and 10 µM in mature adipocytes. As explained in the Materials and Methods section, the reason for using these doses was based on our previous study. Moreover, these doses are among those most commonly used in *in vitro* studies performed to analyze the effects of resveratrol on adipocytes [44-46].

The increase in adiponectin expression induced by resveratrol in the present study is in good accordance with the results reported by Wang *et al.* [47]. These authors showed that resveratrol increased adiponectin expression in 3T3-L1 adipocytes in a time- and dose-dependent mode. Nevertheless, other adipocytes, such as SGBS cells, do not respond to resveratrol in the same way. Derdemezis *et al.* [29] did not find any effect on adiponectin secretion when treating these cells with 10 and 25 □M of resveratrol for 24 and 48 hours on day 15 of differentiation. The reduction induced by resveratrol in leptin expression is also in line with other published results. In this context, Skudelszka *et al.* [22] observed that 62.5□□M of resveratrol reduced leptin secretion from isolated rat adipocytes.

To the best of our knowledge, very few data exist in the literature concerning the effect of resveratrol on visfatin and apelin. Only Derdemezis *et al.* [29] described that resveratrol reduced visfatin secretion in mature SGBS cells incubated with 10 and 25 □M of resveratrol for 24 and 48 hours on day 15 of differentiation. With regard to apelin, as far as we are aware, no data have been reported.

A repeated phenomenon in our study is the presence of discrepancies between mRNA and protein concentration in the culture media. Several explanations can be found in the literature to justify differences between gene and protein expression [48-51]: polysome activation on mRNA, alternative mRNA splicing, differences in the half-life of the protein and the mRNA, protein turnover, specific proteolytic processing. Moreover, additional factors regulate protein secretion: translocation across the plasma membrane, sequestration by secretory lysosomes, shedding of microvesicles at the extracellular side of the plasma membrane, etc [52]. In addition, several hormones have also been demonstrated to regulate protein secretion in the cell. In this context, Wang *et al.* [53] described the important effect of insulin on protein secretion but not on protein transcription. They analyzed several secreted proteins regulated by insulin, including adiponectin, and observed that although their gene expression was up-regulated, insulin inhibited their protein secretion.

Taking this into account, it could be hypothesized that in the present study insulin, although present in the culture media at a lower concentration than that used by Wang et al., could have affected protein secretion.

As mentioned in this Discussion section, in our previous study devoted to analyzing the effects of resveratrol metabolites on triacylglycerol content in the same sets of maturing pre-adipocytes and mature adipocytes [40], we observed the delipidating effect of these molecules. It is well known that adipokine expression is related to adipocyte triacylglycerol content. More specifically, lipid accumulation leads to a decrease of adiponectin expression and to a greater expression of leptin. Accordingly, in these cells the reduction observed in triacylglycerol content induced by resveratrol and resveratrol metabolites [40] was associated with the up-regulation of adiponectin and the down-regulation of leptin gene expression.

We also found [40] that the delipidating effect of resveratrol and its metabolites depended on the maturation state of the cells. Following these results, the present study was carried out in both maturing pre-adipocytes and mature adipocytes. In this regard, it is interesting to point out that in the case of adiponectin the effects of the molecules analyzed were greater in maturing pre-adipocytes than in mature adipocytes, whereas in the case of leptin the opposite was observed.

It is important to remember that in our previous study [40], resveratrol and its metabolites reduced the expression of CEBPβ, a gene involved in adipocyte differentiation, in maturing pre-adipocytes. The relationship between adipogenic genes and adipokines has been described in other studies. Mason *et al.* [54] showed that leptin expression was regulated by several transcriptional factors including CEBP, but not by PPARγ or SREBP. In the present study we analyzed the correlations between the expression of leptin and transcription factors (measured in our previous study [40]), and we found that CEBPβ and leptin were positively correlated ($r=0.593$; $P<0.001$). By contrast, no significant correlation was found between PPARγ and leptin expressions. We could carry out this analysis because, although the expressions of transcription factors and adipokines are presented in two different manuscripts, they were measured in the same sets of cells. These results could suggest that the reduced expression of the transcription factor CEBPβ induced by resveratrol, and its metabolites, could have contributed to the reduction of leptin expression. Thus, the changes observed in leptin expression in maturing pre-adipocytes could be due to both direct effects of resveratrol and its metabolites on this parameter as well as to effects derived from changes in CEBPβ expression.

In summary, the present study shows for the first time the effect of resveratrol metabolites on adipokine expression and secretion in 3T3-L1 cells. Consequently, it may be suggested that both resveratrol and resveratrol metabolites are involved, to greater or lesser extents, in the regulation of energy and glycemic homeostasis of this polyphenol.

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4. eskuizkribua – 4. Manuscript

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Doses of quercetin in the range of serum concentrations exert delipidating effects in 3T3-L1 preadipocytes by acting on different stages of adipogenesis, but not in mature adipocytes

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ABSTRACT

Scope: To determine whether doses of quercetin in the range of serum concentrations exert any effect on triacylglycerol accumulation in maturing pre-adipocytes and mature adipocytes. The influence on the expression of adipogenic markers as well as on gene expression and activity of enzymes involved in triacylglycerol metabolism were assessed.

Methods and Results: 3T3-L1 pre-adipocytes were treated during differentiation and mature adipocytes for 24 hours with low doses of (0.1-10µM) of quercetin. Triacylglycerol content in both cell types, and free fatty acid and glycerol in the incubation medium of mature adipocytes were measured spectrophotometrically. Gene and protein expression was assessed by RT-PCR and Western-Blot. LPL and FAS activities were quantified. During differentiation quercetin reduced triacylglycerol content at doses from 0.5 to 10µM. 1µM of quercetin reduced C/EBPβ gene expression, SREBP1 mature protein levels and PPARγ gene expression. 10µM of quercetin reduced LPL gene expression and PPARγ and SREBP1c expression. In mature adipocytes, only 10µM of quercetin reduced triacylglycerol content. Lipogenic FAS expression and activity were reduced at this dose.

Conclusion: Quercetin, in the range of serum concentrations, is able to inhibit adipogenesis, but higher doses, at least 10µM, are needed to reduce fat accumulation in mature adipocytes.

1 INTRODUCTION

Overweight and obesity have become a public health problem in developed societies due to their high prevalence [1-3]. They cause numerous metabolic alterations and co-morbidities such as insulin resistance, diabetes, dyslipidemia and hypertension [4-5]. Scientific research is constantly looking for new molecules which could be used as effective functional biomolecules in the fight against this disease and its co-morbidities.

Among these molecules, flavonoids, a group of natural substances which have a variable phenolic structure and are found in fruits, vegetables, tea and wine have received a great deal of interest in recent years because they have been reported to have beneficial effects on health [6-9]. Quercetin, the most abundant flavonoid [10], is present in onions, broccoli, tomatoes, apples and berries [11]. Its intake in the diet is higher than that of other polyphenols [12]. A wide range of biological effects, such as prevention of oxidation, inflammation and cancer, have been attributed to this compound [13-16]. It has also been reported to improve diabetic status in animal models of either type 1 or type 2 diabetes [17]. With regard to obesity, data concerning its delipidating effect, as well as its mechanisms of action, are scarce.

In this context, the present study aimed to analyze the effect of low doses of quercetin on triacylglycerol accumulation in both maturing pre-adipocytes and mature adipocytes. In order to gain insight into the mechanisms underlying this action, the influence on the expression of adipogenic markers, as well as on gene expression and activity of enzymes involved in triacylglycerol metabolism were assessed.

It is important to underline that our main interest focused on concentrations of this polyphenol which were lower than those used in the reported studies (10 to 500 µM) [18-24], which are far from those reached by this molecule in plasma after oral ingestion [25-27].

2 MATERIALS AND METHODS

2.1 Reagents

Dulbecco´s modified Eagle´s medium (DMEM) was purchased from GIBCO (BRL Life Technologies, Grand Island, NY). Quercetin was purchased from Sigma (St. Louis, MO, USA). Triacylglycerols (TG) were determined by Infinity Triglycerides reagent (Thermo Electron Corporation, Rockford, IL, USA) and protein concentrations of cell extracts were measured with BCA reagent (Thermo Scientific, Rockford, IL, USA). Commercial kits for analyzing free fatty acids and free glycerol were supplied by Roche and Sigma respectively (Free Fatty Acids, Half Micro Test, Roche, Basilea, Sweden and F6428, Sigma, St. Louis, MO, USA).

2.2 Experimental design

3T3-L1 pre-adipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10% foetal calf serum (FCS). Two days after confluence (day 0), the cells were stimulated to differentiate with DMEM containing 10% FCS, 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 µM of dexamethasone for 2 days. From day 4 onward, the differentiation medium was replaced by FBS/DMEM medium (10%) containing 0.2 µg/mL insulin. This medium was changed every two days until cells were harvested. All media contained 1% Penicillin/Streptomycin (10,000 U/mL), and the media for differentiation and maturation contained 1% (v/v) of Biotin and Panthothenic Acid. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.3 Cell treatment

For the treatment of maturing pre-adipocytes, cells grown in 6-well plates were incubated with quercetin, at 0.1, 0.5, 1, 2, 5 and 10 µM (diluted in 95% ethanol) during differentiation. In the case of the control group the same volume of the vehicle (ethanol 95%) was used. Media containing or not containing quercetin was changed every two days: on day 0, day 2, day 4 and day 6. On day 8, supernatant was collected and cells were used for TG determination and RNA extraction. Each experiment was performed 3 times.

In order to distinguish the effects of quercetin in early and late stages of adipogenesis additional cultures were carried out. To assess the effects on early stages, cells grown in 6-well plates were incubated with quercetin at doses of 1, 2, 5 and 10 µM (diluted in 95% ethanol) from day 0 to 60 hours after the induction of differentiation. The effects on late stages were assessed by incubating cells grown in 6-well plates, with the same doses of quercetin, from 60 hours after differentiation to day 8, as reported by Tang et al. [28]. In the case of the control group the same volume of the vehicle (ethanol 95%) was used. Incubation media containing quercetin or not, was changed every two days. Supernatants were collected and cells were used for TG determination. Each experiment was performed 3 times.

For the treatment of mature adipocytes, cells grown in 6-well plates were incubated with quercetin, at 1, 2, 5 and 10 µM (diluted in 95% ethanol) on day 12 after differentiation because at that day >90% of cells developed mature with visible lipid droplets. In the case of the control group the same volume of the vehicle (ethanol 95%) was used. After 24 hours, supernatant was collected and cells were used for TG determination and RNA extraction. Each experiment was performed 3 times.

2.4 Measurement of triacylglycerol content in adipocytes

After treatment, the medium was removed and cell extracts were used for TG determination. Maturing pre-adipocytes and mature adipocytes were washed extensively with phosphate-buffered saline (PBS) and incubated 3 times with 800 µL of hexane/isopropanol (2:1). The total volume was then evaporated by vacuumed centrifugation and the pellet was resuspended in 200 µL Tritón X-100 in 1% distilled water. Afterwards, TGs were disrupted by sonication and the content was measured by means of a commercial kit. For protein determinations, cells were lysed in 0.3N NaOH, 0.1% SDS. Protein measurements were performed using the BCA reagent. TG content values were obtained as mg triacylglycerols/mg protein and converted into arbitrary units.

2.5 RNA preparation and quantitative real-time PCR

RNA samples from cells treated were extracted using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using a RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with DNase I kit (Applied Biosystems, California, USA) to remove any contamination with genomic DNA.

1.5 µg of total RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Relative CCAAT-enhancer-binding protein α and β (C/EBPα and C/EBPβ), peroxisome proliferator-activated receptor γ (PPARγ), lipoprotein lipase (LPL) and sterol regulatory element-binding factor 1c (SREBF1C) mRNA levels in maturing pre-adipocytes, and relative

adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), lipoprotein lipase (LPL), fatty acid synthase (FASN), acetyl CoA carboxylase (ACC), deacetylase sirtuin 1 (SIRT1), leptin, adiponectin, visfatin and apelin mRNA levels in mature adipocytes were quantified using Real-Time PCR with an iCycler™ - MyiQ™ Real Time PCR Detection System (BioRad, Hercules, CA, USA). β-actin mRNA levels were similarly measured and served as the reference gene. The PCR reagent mixture consisted of 4.75 µL aliquot of each diluted cDNA, SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the upstream and downstream primers (300 nM each, except in the case of CEBPα and apelin, whose primer concentration was 600 nM, and ATGL whose primer concentration was 900 nM). In the case of CEBP β, SIRT-1 and visfatin the reagent mixture consisted of 1 µL of each cDNA, Premix Ex TaqTM (Takara, USA) and the upstream and downstream primers (600 nM for CEBP β, visfatin and β-actin and 900 nM for SIRT-1). Specific primers and probes were synthesized commercially (Tib Molbiol, Berlin, Germany and Eurogentec, Liège, Belgium) (Table 1).

RT-PCR parameters were as follows: initial 2 min at 50ºC, denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 30s, annealing at 60ºC for 30s (except in the case of LPL, PPARγ and CEBPα in maturing pre-adipocytes where the annealing was at 62.1ºC, 63.9ºC and 66.4ºC, respectively, and leptin in mature adipocytes where the annealing was at 63.9ºC and 66.4ºC, respectively), and extension at 60ºC for 30s. All sample mRNA levels were normalized to the values of β-actin and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [29].

2.6. Western blot analysis

Total proteins were isolated from maturing 3T3-L1 adipocytes using the modified Trizol (Invitrogen, Carlsbad, CA, USA) method [30], and the protein concentration was determined by BCA assay (Pierce, USA). Total protein (70 µg) was subjected to 7.5% SDS-polyacrylamide gel, electro-blotted onto PVDF membranes (Millipore, Bradford, MA, USA), and immunodetected using mouse anti-SREBP1 (1:1000), mouse anti-PPARγ (1:1000), mouse anti-β-actin (1:5000) (Santa-Cruz Biotech, CA, USA) and goat antimouse immunoglobulin G-horseradish peroxidise conjugate (1:5000) (Santa-Cruz Biotech, CA, USA) with an ChemiDoc MP imaging system (BioRad, CA, USA).

2.7 Enzymatic activity

Lipoprotein lipase enzyme activity was assessed following the method described by del Prado et al. [31] with modifications. For total lipoprotein lipase (LPL) activity determination, 130 µg of protein homogenate (0.3N NaOH, 0.1% SDS) were incubated (15 min, 37°C) with a 2.9 mL of a buffer containing 1.5 mL of dibutyril fluorescein (20 µM), 150 µL of 2-methoxyehanol, and 1.25 mL of phosphate buffer (3 mM NaH_2PO_4 and 50 mM Na_2HPO_4, with or without 2.5 M NaCl). Subsequently the reaction was halted in ice. Finally, fluorescence was measured. Total LPL activity was calculated by subtracting non-LPL lipolytic activity in the presence of NaCl from the total lipolytic activity, determined without NaCl, and expressed as pmol oleate released per minute per µg of protein.

FAS activity was measured by a spectrophotometer at 340 nm of NADPH absorption. The overall reaction system contained 100 µL of 1M KH_2PO_4, 50 µL of 50 mM EDTA, 100 µL of cystein 100 mM, 50 µL of bovine serum albumin 6 mg/mL, 50 µL of 1.2 mM acetyl -CoA, 20 µL of 10 µM malonyl -CoA, 65 µL of 2.4 mM

NADPH, , and 150 µg of protein homogenate (0.3N NaOH, 0.1% SDS pH 7.4) in a total volume of 1.75 mL as previously described [32].

2.8 Measurements of glycerol and free fatty acids in the media

After treatment in mature adipocytes, aliquots of the medium treated with 10 µM of quercetin were removed and analyzed for glycerol and free fatty acid (FFA) quantification by using commercial kits. Results were expressed as µg glycerol/mg protein and nmol FFA/mg protein.

2.9 Statistical analysis

Results are presented as mean ± standard error of the mean. Statistical analysis was performed using SPSS 20.0 (SPSS Inc. Chicago, IL, USA). Comparisons between each treatment and the controls were analyzed by Student's t test. Statistical significance was set-up at the $P < 0.05$ level.

3 RESULTS

3.1 Effect of quercetin on triacylglycerol content in maturing pre-adipocytes

When cells were treated with quercetin from day 0 to day 8 (whole adipogenesis), triacylglycerol content was significantly reduced by all the doses used, with the exception of 0.1 µM which only showed a tendency towards lower values (Table 2). The greatest percentages of reduction in triacylglycerol content were obtained with 1 and 10 µM (-42% and -37% respectively). When cultures were carried out at early and late stages by using these doses, it was observed that 1 µM of quercetin tended to reduce TG accumulation during the early stages of adipogenesis, but not during the late stages (Table 2). By contrast, 10 µM of quercetin was only able to decrease TG content in the late stage of differentiation (Table 2).

3.2 Effect of quercetin on adipogenic gene expression

For this purpose, 1 and 10 µM of quercetin were selected because they induced the greatest delipidating effects. The dose of 1 µM significantly reduced C/EBPβ and PPARγ expression (Figure 1A). The dose of 10 µM significantly reduced the expression of PPARγ, SREBF1c and LPL (Figure 1A).

3.3 Effect of quercetin on adipogenic protein expression

The dose of 1 µM of quercetin did not modify the protein levels of $PPAR\gamma_1$ and $PPAR\gamma_2$, but it did induce a decrease in protein expression of mature SREBP1 (68 kDa) (Figure 1B). The dose of 10 µM significantly reduced.

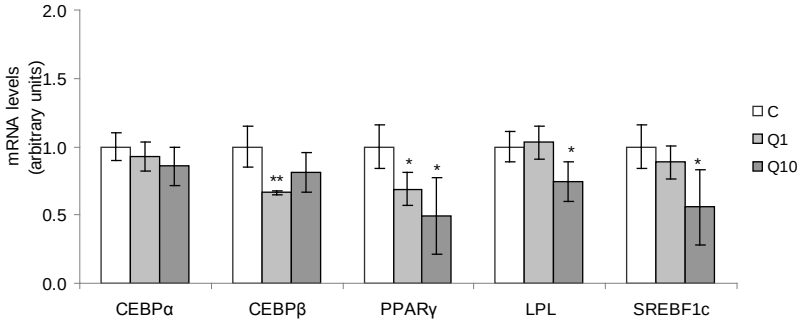
;T CGC AGA AGA CAA TA -3´	5´- GCC GCC GTG CTG TCT CT -3´	
A AGT GCA CAG TG -3´	5´- TGC CAA TGT GTT TTC CCT GA -3´	
'G CAT GGA ATG TTA A -3´	5´- TGA GTG ACT GCC GAA ACA TCT C -3´	
'G AAG CCC TCT TGC -3´	5´- GTT TAG TCT TCC TTT CCT CGT GTC C -3´	
;A CTC TGG CAG TC -3´	5´- AGG ACA CCA TCC AGG CTC TC -3´	
TGT CAG TGG ATC TG -3´	5´- GCT CTT CAG TTG TAG TAA CGT CAT C -3´	
;A CAC GCT GAT CAA AGG -3´	5´- AGT CCC GAA AGT ATT CAA AAG CAG -3´	
C AGA GCA AGA G-3´	5´-GGT GTG GTG CCA GAT CTT CTC-3´	
;A GTA CAA GAT GCG -3´	5´- GCT GCT CCA CCT TCT TCT GC -3´	5´-FAM- TCG TTC TCC GCC GTC AGC TCC AGC- TAMRA- 3´
\G GGC GAG GAG -3´	5´- ACA GGA GGT TGT CTC GGT AGC -3´	5´-FAM- CTG CCG CCG CCG CTG CCG- TAMRA- 3´
A GAT GAA TGC -3´	5´- GGA ATA AAC TTT GCT TGT GTT GGG -3´	5´-FAM- AGC CGA GTT CAA CAT CCT GCT GGC- TAMRA- 3´
G GCT ACG CTC TCC -3´	5´- CAC GCT CGG TCA GGA TCT TC -3´	5´- FAM- CCT GCG TCT GGA CCT GGC TGG C- TAMRA -3´

'eroxisome proliferator-activated receptor, SREBP = Sterol Regulatory Element-Binding Protein, C/EBPα and C/E
)eta, ATGL = adipose triglyceride lipase, HSL = hormone sentitive lipase, FASN = fatty acid synthase, ACC = acetyl-Cc
tivated receptor gamma coactivator, SIRT1 = deacetylase sirtuin 1.

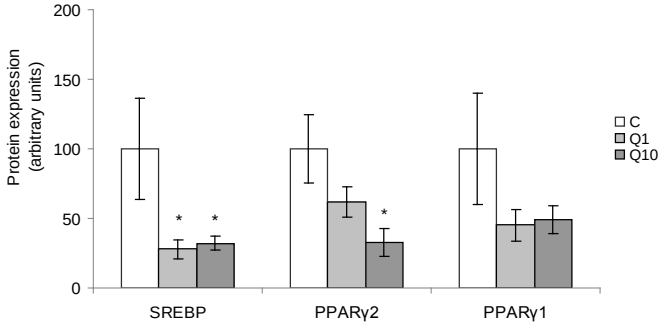
0.1	***P***	**Q 0.5**	***P***	**Q 1**	***P***	**Q 2**	***P***	**Q 5**	***P***	**Q 10**	***P***
±12.8	0.09	75.57±8.6	<0.01	58.4±8.6	<0.001	81.3±7.4	<0.05	79.6±7.2	<0.05	62.9±10.2	<0.01
				86.0±5.7	0.09					95.3±7.3	ND
				92.2±5.6	ND					75.9±10.5	<0.05
				109.3±34.0	ND	86.2±3.4	0.07	79.3±12.8	0.10	76.3±4.8	<0.01

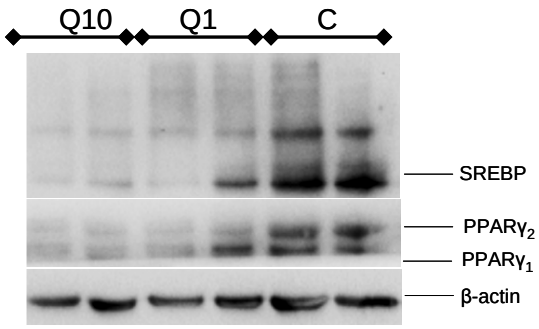
).1 µM of quercetin, Q0.5=0.5 µM of quercetin, Q1=1 µM of quercetin, Q2=2 µM of quercetin, Q5=5 µM of quercetin, Q10=10 µM of
omparisons between each treatment and the controls were analyzed by Student's t-test. The difference was established at $P \leq 0.10$.

protein expression of mature SREBP1 and PPARγ$_2$, but not that of PPARγ$_1$ (Figure 1B).



A





B

Figure 1. Effects of 1 and 10 µM of quercetin (Q1 and Q10) on gene expression of CEBPβ, CEBPα, PPARγ, SREBF1c and LPL (A) and on protein expression of PPARγ$_1$, PPARγ$_2$ and SREBP1 (B) in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisks represent differences versus the controls (**P* < 0.05; ***P* < 0.01).

3.4 Effect of quercetin on triacylglycerol content in mature adipocytes

When mature adipocytes were treated with 1 µM of quercetin no effects on TG content were observed. The doses of 2 and 5 µM induced reductions that did not reach statistical significance, and only the highest dose (10 µM) showed a significant effect (Table 2).

3.5 Effect of quercetin on the expression of genes involved in triacylglycerol metabolism in mature adipocytes

In mature adipocytes, gene expression was analyzed only in cells treated with 10 µM of quercetin because this dose was the only effective one. With regard to lipolytic enzymes, HSL expression was significantly reduced and ATGL remained unchanged (Figure 2). FASN expression was significantly reduced, but no changes were observed in ACC and LPL (Figure 3A).

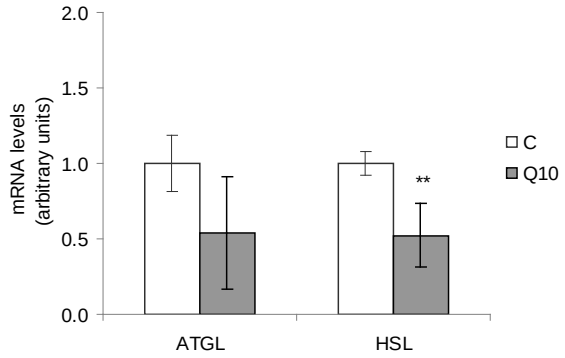


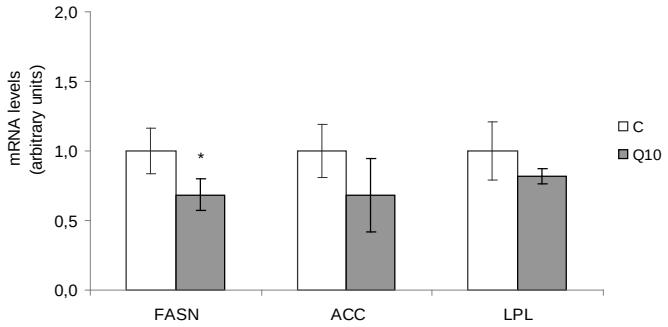
Figure 2. Effects of 10 µM of Quercetin (Q10) on the gene expression of lipases, ATGL and HSL, in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisks represent differences versus the controls (**$P < 0.01$).

When SIRT1 expression was measured, 10 µM of quercetin increased SIRT1 mRNA levels (Figure 4).

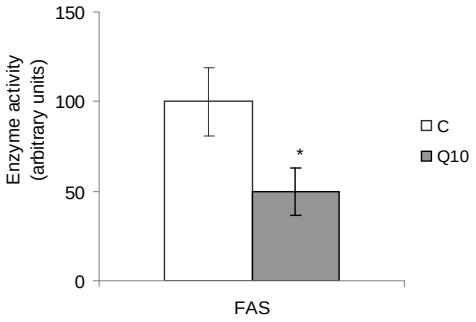
3.6 Effect of quercetin on lipoprotein lipase and fatty acid synthase activities in mature adipocytes

In order to find potential changes in LPL at a post-transcriptional level, the activity of this enzyme was measured in mature cells treated with 10 µM of quercetin and no changes were observed (0.90±0.14 pmol fluorescein/µg protein/min in the case of the control group and 1.15±0.23 pmol oleate/µg protein/min in the case of the group treated with quercetin at 10 µM; P=0.38).

The dose of 10 µM of quercetin had an inhibitory effect on FAS activity. This result is in line with the reduced gene expression of this enzyme (Figure 3B).



A



B

Figure 3. Effects of 10 µM of Quercetin (Q10) on the gene expression of FASN, ACC and LPL (A) and on the activity of FAS enzyme (B), in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisk represent differences versus the controls (*$P < 0.05$).

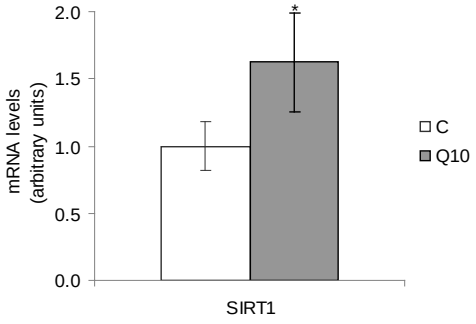


Figure 4. Effects of 10 µM of quercetin (Q10) on the gene expression of SIRT1 in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's *t*-test. The asterisk represent differences versus the controls (*$P < 0.05$).

3.7 Effect of quercetin on glycerol and free fatty acid release in mature adipocytes

In order to know if the decrease in HSL expression was accompanied by changes in the lipolytic pathway, glycerol and free fatty acids were quantified in the incubation media, as markers of lipolysis. Both parameters remained unchanged after treatment of cells with 10 µM of quercetin (65.3±7.5 *vs.* 60.6 ±14.9

nmol FFA/mg protein and 393.05 ± 10.1 *vs.* 429.16 ± 31.6 µg glycerol/mg protein for control and 10 µM quercetin-treated cells respectively).

3.8 Effect of quercetin on adipokine gene expression in mature adipocytes

No changes were observed in gene expression of ADIPONECTIN, LEPTIN, VISFATIN and APELIN in mature adipocytes treated with 10 µM of quercetin (Figure 5).

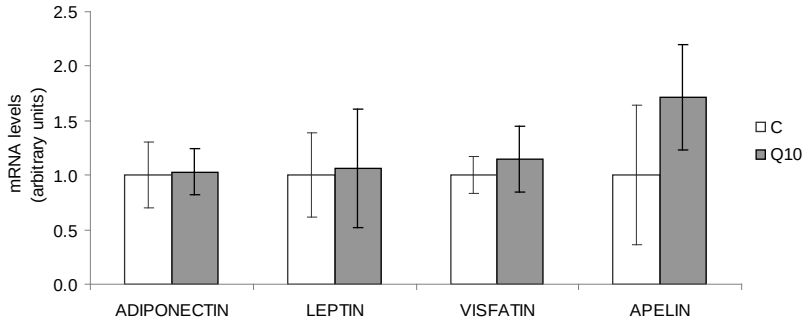


Figure 5. Effects of 10 µM of quercetin (Q10) on the gene expression of ADIPONECTIN, LEPTIN, VISFATIN, and APELIN in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment and the controls were analyzed by Student's t-test.

4 DISCUSSION

In recent years, a great number of studies have been conducted in the field of natural compounds in order to find new tools to combat obesity. In this line, one of the most studied molecules has been resveratrol, a polyphenol (stilbene) present in grapes and wine, which has shown a clear anti-obesity effect in cultured cells [33-41] and in animal models [40, 42-48]. The effects of flavonoids, which show interesting biological effects on cancer and diabetes [7, 9], on obesity prevention or treatment have not been so widely analyzed[49]. The present study focuses on quercetin, the most abundant flavonoid in food stuffs [11].

To date, there are only few studies in the literature demonstrating that quercetin reduces triacylglycerol accumulation in cultured adipocytes and animal models, and very little has been reported concerning the mechanisms of action [18-23, 50]. One advantage of in vitro studies is that they allow one to clearly differentiate between the effects of a molecule on pre-adipocytes (and thus on adipogenesis) and on mature adipocytes. This is the reason for choosing this experimental model for our study.

It is important to point out that previously reported in vitro studies have been performed by using high doses of quercetin (10 to 500 µM). These doses are far from those achieved in plasma in humans and animals. Thus, in the present study we aimed to complete the information provided in the above mentioned studies by analyzing the effects of quercetin on cultured adipocytes at lower doses. In fact doses from 0.1 to 2 µM are in the range of quercetin concentrations found in plasma in several studies performed either in rodents or humans [26, 27, 51, 52].

Quercetin has been demonstrated to exert genotoxicity and mutagenicity in in vitro experiments. Nevertheless, and according to the critical examination of quercetin safety carried out by Harwood et al. [27], 34 µM was the lowest dose to show this effect in mouse cells, specifically in mouse lymphoma L5178Y cells [53]. Thus, we assumed that the doses used in the present study did not exert this toxic effect.

All the quercetin doses tested in the present study (0.1-10 µM), significantly reduced TG content in 3T3-L1 maturing pre-adipocytes, with the exception of 0.1 µM which only showed a tendency towards lower values (8 days of treatment after the confluence). In the experiment reported by Ahn et al. [20] in 3T3-L1 pre-adipocytes, cells were treated with 10, 50 and 100 µM of quercetin during differentiation. The three doses of this flavonoid reduced TG content. Data concerning 10 µM of quercetin are in good accordance with the present study. Yang et al. [21] used 12.5 µM and 25 µM of quercetin for 3T3-L1 pre-adipocyte incubation, and only the highest dose was effective in TG reduction. The difference between the present study and that reported by Yang et al. could be the treatment duration (from day 0 to day 8 in the present study and from day 0 to day 6 in the study reported by Yang et al.). The effect of 5 µM of quercetin is also in the same line as that reported by Bae et al. (2014) [54] by using low doses of quercetin (3.3-6.6 µM). With regard to the lowest doses used in the present study (0.1, 0.5, 1 and 2 µM), as far as we know, this is the first time that they have been used and thus, comparisons with the literature cannot be made.

To compare the delipidating effect of quercetin during adipogenesis with that observed with other polyphenols is a matter of interest. In a recent study performed in our laboratory we analyzed the delipidating effect of resveratrol at 1, 10 and 25 µM, in maturing pre-adipocytes by using the same experimental conditions [37]. No changes were observed in TG content at 1 and 10 µM, but the polyphenol was effective at 25 µM. These results show that quercetin is more efficient in reducing adipogenesis than resveratrol is. There are very few studies where low concentrations of other flavonoids have been tested in 3T3-L1 maturing pre-adipocytes. Yang et al. showed that 3.125, 6.25 and 12.5 µM of xantohumol and isoxantohumol, flavonoids present in hops, reduced TG accumulation during adipocyte differentiation [55]. Nevertheless, other authors have not found this anti-adipogenic effect in the case of other flavonoids (naringenin) or have stated that higher doses are needed to reach this effect (genistein) [22, 56, 57].

Two phases can be distinguished in adipogenesis, the pre-mitotic phase (early stage of differentiation; 60 hours post-confluence) [28], and the post-mitotic phase (late stage). During the early phase C/EBPβ and SREBP1c expressions are increased. This change, in turn, triggers high level expression of PPARγ, which is considered the master coordinator of adipocyte differentiation. C/EBPα and LPL are induced during later stage of differentiation [58]. Interestingly, when the influence of quercetin on the expression of these genes was assessed it was observed that, depending on the dose, the mechanisms of action of this flavonoid to reduce adipogenesis were different (Figure 6). Quercetin, at a dose of 1 µM significantly reduced C/EBPβ and PPARγ gene expressions, but PPARγ1 and PPARγ2 protein levels remained unchanged. Moreover, a reduction in protein expression of mature SREBP1c was observed. As stated before, C/EBPβ and SREBP1c are adipogenic markers expressed in the early stage of the process [28]. Taken together these results suggest that adipogenesis was stopped at this phase. This fact was confirmed by the reduction in TG content (tendency) observed after 60 hours of treatment, but not when pre-adipocytes were treated from 60 hours to 8 days.

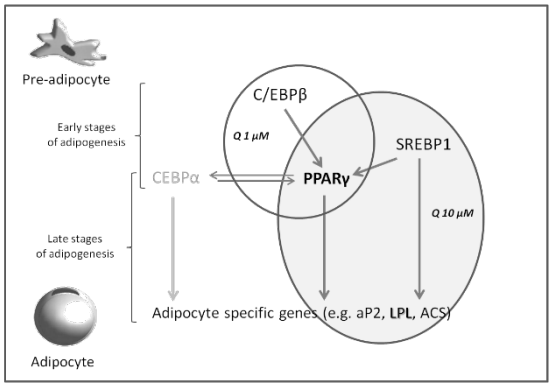


Figure 6. Effects of different doses of quercetin in the pathways of the adipogenic process.

Treatment with 10 µM of quercetin reduced both gene and protein expressions of PPARγ and SREBP1c, as well as LPL gene expression. These results are in good accordance with those reported by other authors [20, 21] who showed that 10 and 25 µM of quercetin reduced PPARγ and SREBP1c expression. Although SREBP1c expression starts at early stages of differentiation, protein expression of mature SREBP reaches its peak at the 5th -6th day after the confluence [59]. For this reason SREBP1c is also considered an important factor in the late stages of adipogenesis, as is the case of PPARγ. The reduction in PPARγ and SREBP1c expression after treatment with 10 µM of quercetin, together with the strong decrease in TG content observed when maturing adipocytes were treated with this dose of quercetin from 60 hours to 8th day after the confluence, suggests that this dose acts primarily at the late stage of adipogenesis.

When the effects on mature adipocytes were assessed, quercetin significantly reduced TGs at the dose of 10 µM. These results are in the same line as those obtained by Park et al. by incubating murine adipocytes, but with a higher dose (25 µM) of this flavonoid [22]. In our previous study, where mature adipocytes were treated with 1, 10 and 25 µM of resveratrol during 24 hours [37], the lowest dose of this polyphenol resulted in a significant reduction in TG content. By comparing the present data with those of this study, it can be suggested that quercetin is less efficient than resveratrol when delipidating mature adipocytes. The effect of other flavonoids at low doses on mature adipocytes has not been widely analyzed. Reported studies show that doses around 100 µM are needed in the case of naringenin and genistein to reach a reduction in TG content [56].

The expression of genes involved in TG metabolism in mature adipocytes was analyzed by Real Time RT-PCR after 24 hours of treatment. Quercetin reduced HSL expression but did not change that of ATGL. Taking into account that HSL is an enzyme regulated mainly at post-transcriptional level, and considering that a reduction in the expression of a lipolytic enzyme is not an expected result for a delipidating molecule, the release of glycerol and free fatty acids to the incubation medium was quantified as an index of lipolytic activity. No changes were observed in treated cells when compared with the controls, and thus a lack of effect of quercetin in HSL activity could be suggested, despite its down-regulation.

Gene expression of fatty acid synthase, as well as the activity of this enzyme was lower after quercetin treatment, suggesting that the delipidating effect of this molecule could be due to its inhibitory effect on de novo lipogenesis. When the expression of LPL, the enzyme which allows adipose tissue to uptake free fatty acids from TGs circulating in lipoproteins, was measured, no changes were observed in quercetin-treated

mature adipocytes. This is a surprising result because, as quercetin reduced LPL mRNA expression in maturing pre-adipocytes, a similar effect could be expected in mature adipocytes. Nevertheless, it is important to highlight that LPL is controlled not only transcriptionally but also post-transcriptionally [60]. Given this, in order to find potential changes in LPL at a post-transcriptional level the activity of this enzyme was measured in mature adipocytes treated with 10 µM of quercetin. No changes were observed and thus it may be suggested that the metabolic process controlled by this enzyme was not affected by the flavonoid.

In previous studies performed in our laboratory, incubation with 10 µM resveratrol increased ATGL expression and reduced FASN gene expression [37, 41]. These data suggest that while 10 µM of quercetin exerts its delipidating effect via inhibition of adipogenesis and lipogenesis, resveratrol acts by increasing lipolysis and inhibiting lipogenesis.

It has been reported that several beneficial effects of polyphenols are mediated by the deacetylase SIRT1 [61]. In the present study the incubation of mature adipocytes with 10 µM of quercetin led to increased SIRT1 expression. SIRT1 can induce SREBP1c deacetylation, which leads to the inactivation of this transcription factor, and thus to decreased expression of lipogenic enzymes [62]. Taking this into account, it can be proposed that the increased expression of SIRT1 observed in mature adipocytes in the present study could be related to the reduced expression of FASN.

In relation to glycaemic control, flavonoids have been reported to improve insulin sensitivity by modifying adipokines secretion. Specifically, quercetin has been demonstrated to inhibit visfatin secretion in SGBS human adipocytes or to increase adiponectin secretion in rats fed a high fat diet [23, 63]. As far as we know, the effect of low doses of quercetin on adipokine expression and secretion has not been analyzed to date. In the present study no changes were observed in adipokine gene expression, suggesting that low doses of this flavonoid do not affect these mediators of insulin signalling and glycaemic control.

It has been reported that quercetin is able to reduce resveratrol metabolism; more specifically it can decrease the formation of sulfate metabolites [64]. Thus, the combination of quercetin and resveratrol has been proposed as a tool to increase the low bioavailability of resveratrol, and consequently its effectiveness. In view of the results obtained in the present study, it can be proposed that the combination of quercetin and resveratrol could be more effective, in terms of body fat reduction, than the administration of these molecules separately. This proposal is justified not only by the effects on resveratrol biavailability, as proposed in the literature, but also because at 1 µM (a dose close to quercetin and resveratrol serum concentrations found in in vivo studies) quercetin is more effective in reducing adipogenesis in pre-adipocytes whereas resveratrol is more effective in inhibiting lipid metabolism in mature adipocytes. Thus, the combination of these two molecules can induce body fat reduction more effectively by targeting both cell types, pre-adipocytes and adipocytes, at the same time.

Taking all the data presented into account, it can be concluded that doses of quercetin in the range of serum concentrations are able to inhibit adipogenesis, but higher doses, at least 10 µM, are needed to reduce fat accumulation in mature adipocytes. In the case of maturing pre-adipocytes, 1 µM of quercetin exerts its anti-adipogenic effect at the early stages of adipogenesis, whereas 10 µM of quercetin acts at the later stages.

5 ACKNOWLEDGEMENTS

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6 CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

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Effects of quercetin metabolites on triglyceride metabolism of 3T3-L1 preadipocytes and mature adipocytes

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Abstract

Quercetin (Q) has rapid metabolism, which may make it worthwhile to focus on the potential activity of its metabolites. Our aim was to evaluate the triglyceride-lowering effects of Q metabolites in mature and pre-adipocytes, and to compare them to those induced by Q. 3T3-L1 mature and pre-adipocytes were treated with 0.1, 1 and 10 μM of Q, tamarixetin (TAM), isorhamnetin (ISO), quercetin-3-*O*-glucuronide (3G), quercetin-3-*O*-sulfate (3S), as well as with 3S and quercetin-4-*O*-sulfate (4S) mixture (3S+4S). Triglyceride (TG) content in both cell types, as well as free fatty acid (FFA) and glycerol in the incubation medium of mature adipocytes were measured spectrophotometrically. Gene expression was assessed by RT-PCR. In mature adipocytes, Q decreased TG at 1 and 10 μM, 3S metabolite at 1 and 10 μM, and 3S+4S mixture at 10 μM. 3S treatment modified the glucose uptake, and TG assembling, but not lipolysis or apoptosis. During differentiation, only 10 μM of ISO reduced TG content, as did Q at physiological doses. In conclusion, 3S metabolite but not ISO, 3G, 4S and TAM metabolites can contribute to the in vivo delipidating effect of Q.

1. Introduction

Quercetin (Q) is a polyphenol classified as a flavonoid, found, mainly in glycoside form, in a variety of foods including berries, onions and shallots, apples, tea and chocolate [1]. Some of its metabolites, such as Isorhamnetin (ISO) and quercetin-3-O-glucuronide (3G), are also present in several food sources [2]. It is estimated that the dietary intake of Q is 5–40 mg/day [3]. However, consumption can reach 200–500 mg/day when fruits and vegetables are abundant in the diet, especially if they are eaten with their skin [4].

Obesity, defined as excess fat accumulation in white adipose tissue, plays a key role as a regulator of lipid storage and release. It can be developed by increasing adipocyte number (hyperplasia) and/or size (hypertrophy) [5]. When hyperplasia takes place, there is a stimulation of pre-adipocyte proliferation and further differentiation. This process, which promotes pre-adipocyte differentiation into mature adipocytes is known as adipogenesis [6]. Nevertheless, this increase in adipocyte number does not necessarily promote obesity directly. Instead, during childhood growth, it determines the lipid-storing capacity of adipose tissue and fat mass in adulthood [7].

For triglyceride synthesis and further storage, mature adipocytes need a source of fatty acids and of glycerol-3-P. Fatty acids can be obtained from triglycerides circulating as lipoproteins, due to the action of lipoprotein lipase (LPL), which can be synthesized de novo from Acetyl-CoA or can be taken-up directly from circulation through specific transporters. Glycerol-3-P comes from glucose metabolism, after glucose uptake from blood through the glucose transporter GLUT-4. These two molecules are assembled into triglycerides in a process catalyzed by several enzymes. TG stored in adipose tissue can be mobilized in the process known as lipolysis, mediated by three lipases, which implies a breakdown of stored triglycerides and subsequent release of fatty acids and glycerol.

Q has recently been shown to be a potential body fat-lowering molecule. Its positive impact on lipolysis, apoptosis, fatty acid uptake, inhibition of adipogenesis and reduction of lipogenesis has been proposed as its mechanism of action [8-12]. In addition, it seems that its effect on white adipose tissue is accompanied by muscle and liver mitochondrial biogenesis and by improved glycaemic control among other effects, resulting in it being a multi-target flavonoid for body fat reduction [13,14]. Not only abundant cell culture experiments [8,10,15,16], but also animal studies have confirmed its usefulness in body fat reduction, mostly in obese animals [17-22]. However, studies in humans remain scarce [19,23-25]. A matter of concern in the use of Q as a bioactive molecule is its rapid metabolism, and thus its low bioavailability. Chen et al. [26] determined that 60% of total quercetin ingested by rats was absorbed, and 55.8% of this absorbed amount was metabolized by the gut and 1.8% by the liver. After ingestion, Q is transformed into an aglycone form in the small intestine, that in turn is further metabolized by glucuronidation, sulfatation and methylation reactions [27]. As a result, only a reduced amount of Q and considerable amounts of metabolites reach the bloodstream. According to the literature, the most predominant metabolites in plasma are ISO, tamarixetin (TAM), 3G and quercetin-3-O-sulfate (3S) [28-31] (Figure 1), with glucuronide metabolites being those that appear in higher concentrations and sulfate and methylated those that appear in lower concentrations [32-34].

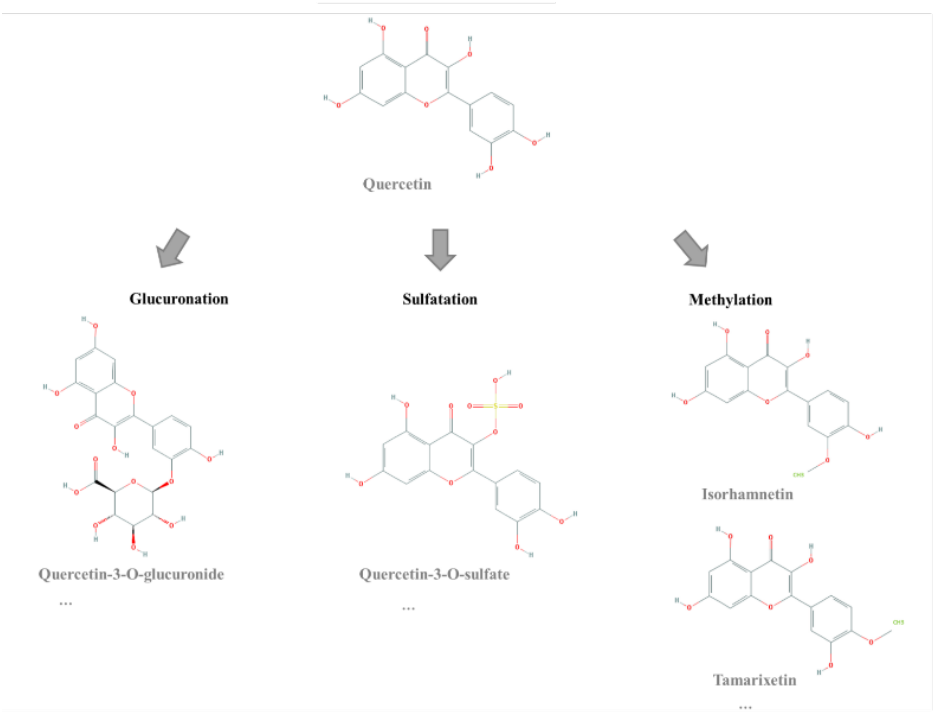


Figure 1. Chemical structures of Q and its metabolites.

Bearing this in mind, it is not possible to be sure that the fat-lowering properties of Q observed in in vivo experiments are exclusively attributable to Q. The potential activity of its metabolites should not be discarded. Data concerning this issue in adipocytes are scarce so far [35-37]. Studies carried out with these molecules in A549 lung cancer cells revealed that Q metabolites could have similar positive effects to those of Q on cell invasion and migration [38].

Considering all these issues, in the present study we wanted to assess whether Q and/or its metabolites are responsible for its beneficial effects in terms of delipidating molecule. For this purpose, the triglyceride-lowering effect of methylated metabolites TAM and ISO, 3G, 3S and a mixture (3S+4S) of 3S and quercetin-4-O-sulfate (4S) in pre-adipocytes and mature adipocytes was evaluated and compared to that induced by Q.

2. Results and Discussion

In order to address the challenge of determining delipidating capacity of each Q metabolite, several approaches with isolated TAM, ISO, 3G, 3S, and 3S+4S mixture dissolved in ethanol were performed in mature and maturing murine adipocytes. With regard to cell treatment, two aspects must be pointed out. First, the final concentration of ethanol per adipocyte was well below 0.1%, a previously reported non-toxic concentration [39-42]. Secondly, the assays were conducted with doses lower than those commonly used in cell culture experiments (0.1, 1 and 10 µM). One of the reasons for choosing these doses is related to the prevention of cell integrity, because 10 µM of ISO and 3G was declared as safe in Raw 2647 cells, but toxic effects were observed for both molecules at 25 and 100 µM, respectively [43]. Moreover, in vivo supplementation studies are commonly carried out by using doses of Q that lead to plasma Q and Q metabolite concentrations in the range of nanomolar and micromolar [44,45]. In addition, in previous studies from our laboratory, doses of Q in the range of serum concentrations (≤10 µM) were tested in adipocytes, revealing a dose-dependent effect on triglyceride (TG) reduction in pre-adipocytes [12].

Apart from commercially available metabolites TAM, ISO and 3G, hemisynthetized quercetin sulfate mixture (3S+4S) and 3S were also used for the present study. Supplementary Figure S1 shows the HPLC chromatograms recorded at 370 nm with the two obtained fractions. In the case of the mixture, 4S and 3S represented 33.4% and 62.5% of the recorded peak areas, respectively.

No significant changes in mature adipocyte TG content were observed when these cells were incubated with the lowest dose (0.1 µM) of the molecules studied. At a dose of 1 µM, only the 3S metabolite, among all the molecules tested, reduced TG content. Finally, at 10 µM, Q, 3S+4S and 3S significantly reduced TG content in adipocytes (21%, 20% and 32%, respectively). By contrast, 3G, ISO and TAM were ineffective (Figure 2). Similar results were reported by Lee et al. with ISO treatment in mature adipocytes [35].

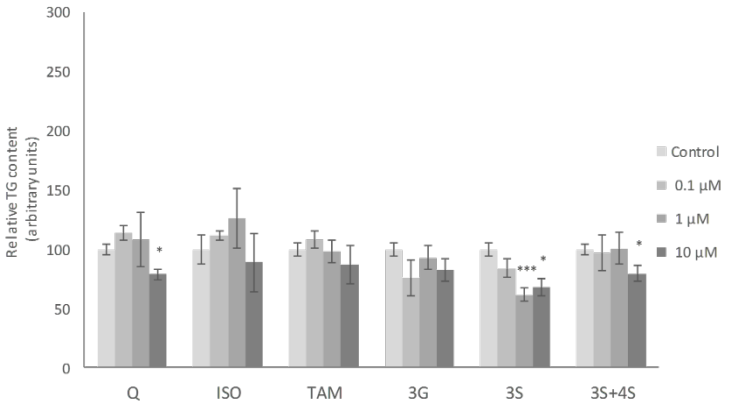
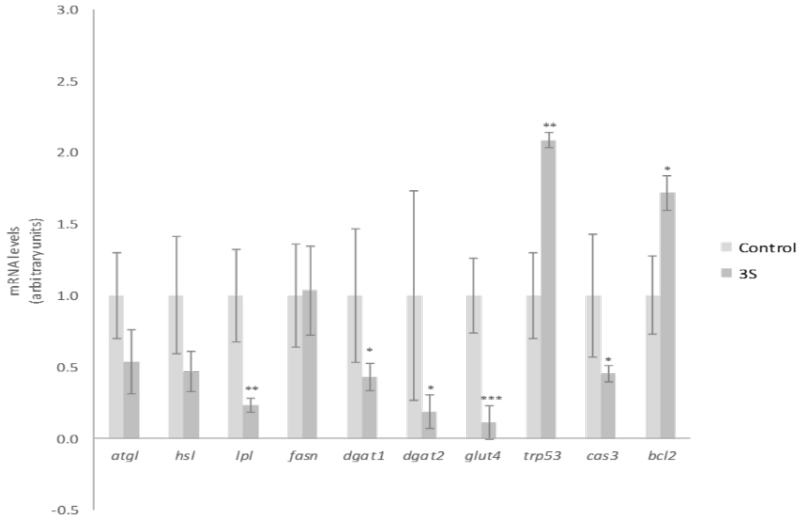


Figure 2. Effects of 0.1, 1 and 10 µM of quercetin (Q), isorhamnetin (ISO), tamarixetin (TAM), quercetin-3-O-glucuronide (3G), quercetin-3-O-sulfate (3S), as well as 3S and quercetin-4-O-sulfate (4S) mixture (3S+4S) on triacylglycerol content of 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparison between each flavonoid dose and the control was analyzed by Student's t-test. The asterisks represent differences versus the controls (* $p < 0.05$; *** $p < 0.001$).

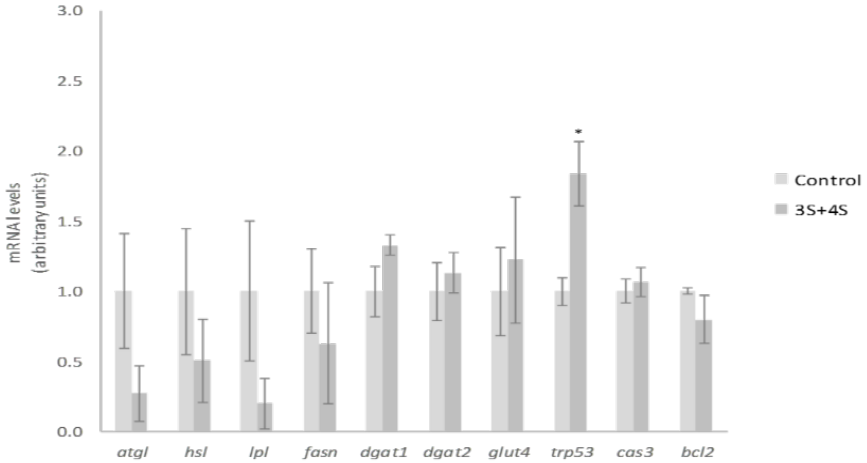
In order to explain the TG reduction observed, gene expression of mature adipocyte-specific genes was analyzed at the dose of 10 µM. We chose the highest dose to carry out this analysis because this was the active one for a great number of the molecules analyzed. Treatment with 10 µM of 3S significantly decreased and 3S+4S tended to reduce lipoprotein lipase (lpl) expression (Figure 3A,B). Evidence confirmed that adipocyte-derived LPL is required for efficient fatty acid uptake and further TG storage in 3T3-L1 adipocytes [46]. It is true that LPL is not determining in in vitro TG accumulation. However, the reduction observed could suggest a positive mechanism of action in in vivo situation. With regard to lipolysis, treatment with 3S+4S tended to reduce adipose triglyceride lipase (atgl) expression ($p = 0.09$) in mature adipocytes and thus, in order to clarify whether this change could result in changes in this metabolic pathway, glycerol and FFA release were measured (Supplementary Figure S2). Given that, as previously reported with Q [12], no changes were observed and consequently, it seems that lipolysis is not involved in TG reduction. On the other hand, even though further analysis is needed in order to confirm this fact, it can be proposed that 3S metabolite, alone or in combination with 4S, could act reducing fatty acid uptake.

According to research conducted with adipose tissue explants from lean, overweight, obese and morbidly obese subjects, body fat mass increase is associated with CASP3 and P53 expression elevation and BCL2

expression reduction [47]. Thus, as the apoptotic pathway is related to adipose tissue homeostasis, the potential involvement of 3S metabolite in apoptosis was studied. It promoted remarkably elevated levels of trp53, a gene that codifies tumor suppressor p53 protein. While p53 is linked with apoptosis, it has many other roles including cell-cycle arrest, DNA repair or senescence [48]. Due to this fact, other apoptosis-related genes such as caspase 3 (cas3) and the anti-apoptotic gene bcl2 were assessed (Figure 3A). The expression of both genes revealed apoptosis reduction, instead of promotion with 3S treatment (bcl2 elevation and cas3 decrease). Thus, apoptosis does not represent a mechanism of action for 3S metabolite in mature adipocytes. In fact, when 4S was included there was no effect on apoptotic genes. Although the mixture 3S+4S raised the expression of trp53, no changes were observed in cas3 or bcl2 genes (Figure 3B).



(A)



(B)

Figure 3. Effects of quercetin-3-O-sulfate (3S) (A) and quercetin-3-O-sulfate and quercetin-4-O-sulfate mixture (3S+4S) (B) at a dose of 10 µM on the expression of atgl, hsl, lpl, fasn, dgat1, dgat2, glut4, trp53, cas3 and bcl2 in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparison of 3S or3S+4S and the control for each gene expression was analyzed by Student's t-test. The asterisks represent differences versus the controls (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Apart from fatty acid uptake, lipolysis and apoptosis, lipogenesis is another crucial metabolic process involved in fat storage. Uptaken fatty acids or new synthesized ones must be assembled with glycerol in order to accumulate triglyceride inside the adipocyte. As a result, facilitated glucose transporter member 4 (glut4), as well as diacylglycerol o-acyltransferase (dgat), genes involved in glucose uptake and TG assembly, can be considered limiting genes for TG synthesis. 3S, but not 3S+4S, reduced glut4, dgat1 and dgat2 gene expression (Figure 3A,B). By contrast, fatty acid synthase (fasn) related to de novo lipogenesis was not modified by the analyzed molecules. These results suggest that the synthesis of fatty acids is not affected by Q metabolite treatment and TG assembly is reduced.

As far as we know this is the first study to reveal the potential effectiveness of Q metabolites in mature adipocytes, postulating that glucose uptake and TG assembling are mechanisms that could justify the TG reduction observed in mature adipocyte after 3S treatment. Consequently, the effects on body fat observed in animals after Q administration would be due not only to the parent compound but also to this metabolite. It is important to highlight that the addition of 4S metabolite to 3S did not confer any additional effect. In fact, the expression of evaluated genes revealed a decrease in their impact (Figure 3B). These results suggest that the TG-lowering effect can be attributed exclusively to 3S metabolite, and that the addition of 4S results in a dilution of the effective molecule.

It has been described that adipocyte turnover rate for lean and obese humans is around 10% [7]. As mature adipocytes do not have the ability to divide, adipocyte precursors with this capacity must exist in adipose tissue. For this reason, in addition to mature adipocyte analysis, the effects of Q metabolites on pre-adipocytes were also assessed in the present study. ISO has been the most studied of all the Q metabolites in maturing adipocytes. It has been demonstrated that this metabolite reduces TG accumulation, with 10 µM the most effective dose in 3T3-L1 and human adipose tissue-derived stems cells [35,49]. Zhang et al. also reported similar results in 3T3-L1 pre-adipocytes, 12.5 µM being the lowest effective dose [37]. In good accordance with these studies, a significant reduction after 10 µM ISO treatment was observed in maturing pre-adipocytes in the present work. Nevertheless, none of the remaining Q metabolites was able to reduce TG at the doses of 1 or 10 µM, as Q did (Figure 4).

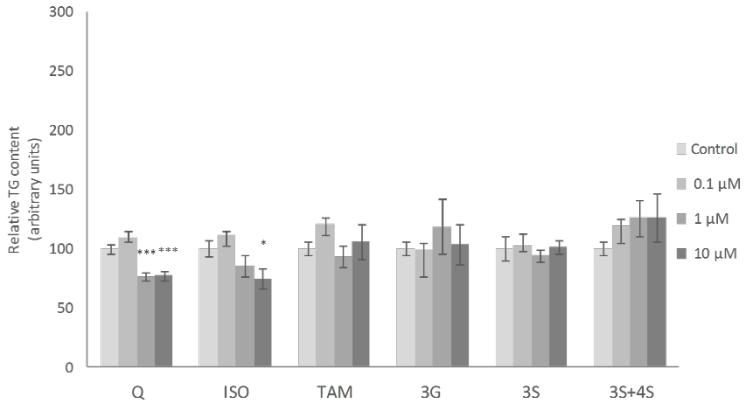


Figure 4. Effects of 0.1, 1 and 10 µM of quercetin (Q), isorhamnetin (ISO), tamarixetin (TAM), quercetin-3-O-glucuronide (3G), quercetin-3-O-sulfate (3S), as well as 3S and quercetin-4-O-sulfate (4S) mixture (3S+4S) on triacylglycerol content of 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM. Comparison between each flavonoid dose and the control was analyzed by Student's t-test. The asterisks represent differences versus the controls (* $p < 0.05$; *** $p < 0.001$).

With regard to the mechanisms of action for ISO, Lee et al. [35] demonstrated that nine days of treatment in maturing 3T3-L1 pre-adipocytes with 50 µM reduced adipogenesis through the inhibition of peroxisome proliferator-activated receptor γ (pparγ) and CCAAT/enhancer-binding protein α (cebpα). In our cell cultures, the dose of 10 µM tended to decrease pparγ gene expression (p = 0.06), but not that of cebpα, cebpβ or sterol regulatory element-binding factor (srebf1) (Figure 5). By comparing both studies, it is clear that higher doses promote greater impact on adipogenesis inhibition. This conclusion was also reached by Zhang et al. [37], who revealed that ISO treatment decreased adipocyte differentiation at a concentration of 12.5 µM and totally blocked this process at 50 µM. Likewise, they proposed the differentiation stage as a limiting step for ISO effect. According to their data, the inhibitory effect on adipogenesis was less prominent when ISO was added at the latter stages of differentiation. Moreover, they indicated that ISO may control the early differentiation stage by inhibiting the transactivation of pparγ. In the present research, we treated 3T3-L1 pre-adipocytes with ISO throughout the adipogenic process (eight days). Therefore, the weak effect on pparγ expression observed could be due not only to the low dose but also to the influence of the differentiating stage.

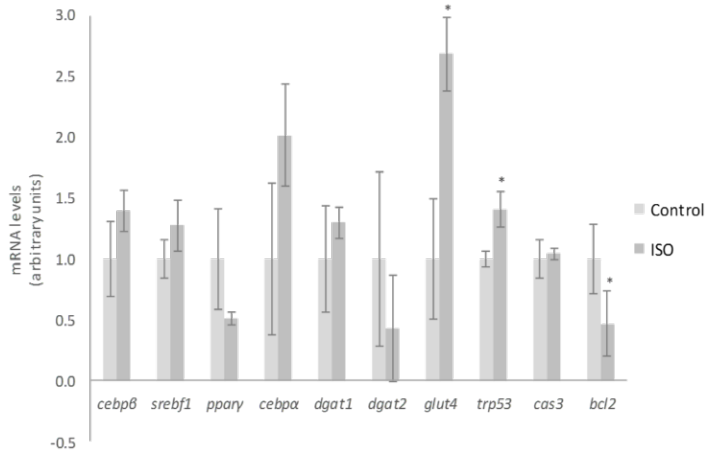


Figure 5. Effects of 10 µM of isorhamnetin (ISO) on the expression of cebpβ, srebf1, pparγ, cebpα, dgat1, dgat2, glut4, trp53, cas3 and bcl2 in 3T3-L1 adipocytes treated for from day 0 to day 8. Values are means ± SEM. Comparison between ISO and the control for each gene expression was analyzed by Student's t-test. The asterisks represent differences versus the control (* $p < 0.05$).

Much as took place with 3S metabolite treatment of mature adipocytes, the expression of trp53 was increased after ISO treatment of differentiating pre-adipocytes, but a diminution of mRNA levels of the death repressor bcl2 was observed (Figure 5). Furthermore, ISO treatment did not promote any change in cas3 expression. These results reveal that the apoptosis pathway was not completely activated by ISO treatment, probably due to the low dose used. Mirroring mature adipocyte gene analysis, the expression of dagt1, dgat2, and glut4 was also evaluated. With the exception of glut4, no changes in the genes mentioned were detected (Figure 5). Therefore, glucose uptake and TG assembly did not justify the observed delipidating effect of ISO.

In spite of the significant effects observed in the expression of several genes the only Q metabolite to induce a significant reduction in pre-adipocyte TG content was ISO, but at 10 µM, a dose higher than that found in serum when animals are treated with Q. Bearing this fact in mind, the involvement of Q metabolites in the anti-obesity effect of this phenolic compound cannot be proposed.

3. Materials and Methods

3.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (BRL Life Technologies, Grand Island, NY, USA). Q was purchased from Sigma (St. Louis, MO, USA) and ISO, TAM, and 3G from Extrasynthese (Genay, France). TG were quantified by Infinity Triglycerides reagent (Thermo Electron Corporation, Rockford, IL, USA) and protein concentrations of cell extracts were measured with bicinchoninic acid (BCA) reagent (Thermo Scientific, Rockford, IL, USA). Commercial kits for analyzing FFA and free glycerol were supplied by Roche and Sigma respectively (Free Fatty Acids, Half Micro Test, Roche, Basilea, Sweden and F6428, Sigma, St. Louis, MO, USA).

3.2. Synthesis of Quercetin-3-O-Sulfate and Quercetin-4-O-Sulfate Metabolites

3.2.1. Synthesis of Quercetin Sulfates

Quercetin sulfates were synthesized as described by Dueñas et al. [50]. Dry pyridine was added to quercetin (500 mg) to remove possible water associated with quercetin. Pyridine was rotary evaporated, and the dry compound was dissolved in dioxane (50 mL) and allowed to react in a water bath (40 °C) for 90 min with a 10-fold molar excess of sulfur trioxide−N−triethylamine complex under nitrogen to avoid contact with air. Products of sulfation precipitated out and stuck to the glass. Dioxane was decanted, and the precipitate was redissolved in 10% methanol in water. The mixtures of quercetin sulfates were fractioned on a Sephadex LH-20 column (350 mm × 30 mm), successively eluted with 10% aqueous ethanol (500 mL) and 20% aqueous ethanol (500 mL). The fractions containing monosulfates were collected, concentrated to dryness under vacuum, redissolved in ultrapure water, and analyzed by high-performance liquid chromatography with diode array and mass spectrometry detection (HPLC−DAD−MS).

3.2.2. HPLC−DAD−MS Analyses

Analyses were carried out with a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a DAD coupled to a HP Chem Station (revision A.05.04) data-processing station. Separation was achieved on an Agilent Poroshell 120 EC-C18 column (2.7 µm, 150 mm × 4.6 mm) thermostatted at 35 °C. Solvents used were (A) 0.1% TFA in water and (B) acetonitrile and the elution gradient was from 10 to 15% B for 5 min, from 15 to 25% B for 5 min, from 25 to 35% B over 10 min, from 35 to 50% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, at a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 250 and 370 nm as preferred wavelengths and in a mass spectrometer connected to the HPLC system via the DAD cell outlet. MS detection was performed in a Finnigan LCQ detector (Thermoquest, San Jose, CA, USA) equipped with an ESI source and an ion-trap mass analyzer, which were controlled by the LCQ Xcalibur software. Both the auxiliary and sheath gases were nitrogen at flow rates of 20 and 80 L/min, respectively. The source voltage was 4.5 kV; the capillary voltage was 11 V; and the capillary temperature was 220 °C. Spectra were recorded in negative-ion mode between m/z 150 and 2000. The MS detector was programmed to perform a series of two consecutive scans: a full scan and a MS−MS scan of the most abundant ion in the first scan, using a normalized collision energy of 45%.

3.2.3. Identification and Quantification of Quercetin Sulfates

Chemical hemisynthesis of the quercetin sulfates was performed as described by Dueñas et al. [50]. The complex mixture of products obtained was fractionated on a Sephadex LH-20 column to separate monosulfates from other products (quercetin and quercetin disulfates). Further fractionation by semipreparative HPLC obtained pure quercetin sulfate and a mixture with two quercetin sulfates that were freeze dried for further use.

Chromatograms peaks showed a pseudomolecular ion [M-H]- at m/z 381 that released a unique fragment at m/z 301 (−80 amu, loss of a sulfate moiety), corresponding to quercetin. The peaks were identified as quercetin 4'-O-sulfate (peak 1) and quercetin 3'-O-sulfate (peak 2) based on their comparison with compounds previously synthesized and fully identified by NMR [50]. The chromatographic purity of the compounds was calculated to be higher than 96% from the area of the peaks obtained in HPLC chromatograms recorded at 370 and 250 nm.

3.3. Experimental Design

3T3-L1 pre-adipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10% fetal calf serum (FCS). Two days after confluence (day 0), cells were stimulated to differentiate with DMEM containing 10% FCS, 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine, and 1 µM of dexamethasone for two days. From day four onward, the differentiation medium was replaced by FBS/DMEM medium (10%) containing 0.2 µg/mL insulin. This medium was changed every two days until cells were harvested. All media contained 1% Penicillin/Streptomycin (10,000 U/mL), and the media for differentiation and maturation contained 1% (v/v) of Biotin and Pantothenic Acid. Cells were maintained at 37 °C in a humidified 5% CO2 atmosphere.

3.4. Cell Treatment

For the treatment of mature adipocytes, cells grown in 6-well plates were incubated with Q, ISO, TAM, 3G, 3S and 3S+4S at 0.1, 1 and 10 µM (diluted in 95% ethanol) on day 12 after differentiation, because at that day >90% of cells developed mature with visible lipid droplets. In the case of the control group, the same volume of the vehicle (ethanol 95%) was used. Vehicle was diluted 1000-fold in each well, reaching a final concentration of 0.095%. After 24 h, the supernatant was collected and cells were used for TG determination, quantification of glycerol and FFA in the media and RNA extraction. Each experiment was performed three times.

For the treatment of maturing pre-adipocytes, cells grown in 6-well plates were incubated with Q, ISO, TAM, 3G, 3S and 3S+4S at 0.1, 1 and 10 µM (diluted in 95% ethanol) during differentiation. In the case of the control group, the same volume of the vehicle (ethanol 95%) was used. Media containing, or not, molecules were changed every two days: on day 0, day 2, day 4, and day 6. On day 8, the supernatant was collected and cells were used for TG determination, RNA extraction and protein extraction. Each experiment was performed three times.

3.5. Measurement of Triacylglycerol Content

After treatment, the medium was removed and cell extracts were used for TG determination. Maturing pre-adipocytes and mature adipocytes were washed extensively with phosphate-buffered saline and incubated

three times with 800 µL of hexane/isopropanol (2:1). The total volume was then evaporated by vacuumed centrifugation and the pellet was resuspended in 200 µL Triton X-100 in 1% distilled water. Afterwards, TGs were disrupted by sonication and the content was measured by means of a commercial kit. For protein determinations, cells were lysed in 0.3N NaOH, 0.1% SDS. Protein measurements were performed using the BCA reagent. TG content values were obtained as mg triacylglycerols/mg protein and converted into arbitrary units.

3.6. RNA Extraction and RT-PCR

RNA samples from cells treated were extracted using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After RNA purity verification, samples were then treated with DNase I kit (Applied Biosystems, Foster city, CA, USA) to remove any contamination with genomic DNA. 1.5 µg of total RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

Relative *atgl*, *hsl*, *lpl*, *fasn*, *glut4*, *dgat1*, *dgat2*, *bcl2*, *trp53* and *cas3* mRNA levels in mature adipocytes and relative *cebpα* and *cebpβ*, *srebf1*, *pparγ*, *glut4*, *dgat1*, *dgat2*, *bcl2*, *trp53* and *cas3* mRNA levels in maturing pre-adipocytes were quantified using Real-Time PCR with an iCyclerTM - MyiQTM Real Time PCR Detection System (BioRad, Hercules, CA, USA). For *atgl*, *hsl*, *lpl*, *fasn*, *cebpβ*, *srebf1* and *bcl2* SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was used. The upstream and downstream primers and probe (TibMolbiol, Berlin, Germany, Eurogentec, Liège, Belgium and Metabion, Munich, Germany) are listed in Table 1. *Cebpα*, *pparγ*, *glut4*, *dgat1*, *dgat2*, *trp53*, and *cas3* were measured by TaqMan® Gene Expression Assays (Mm00514283_s1, Mm00440940_m1, Mm01731290_g1, Mm00436615_m1, Mm00515643_m1, Mm499536_m1, Mm01195085_m1 and Mm02619580_g1) and TaqMan® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA). RT-PCR parameters used were those defined by manufacturer's. β-actin mRNA levels were similarly measured and served as the reference gene.

All gene expression results were expressed as fold changes of threshold cycle (Ct) value relative to controls using the 2−ΔΔCt method [51].

Table 1. Primers for PCR amplification of each studied gene.

Gene	Sense Primer	Anti-Sense Primer	Annealing t[a] (°C)
atgl	5′-GAGCTTCGCGTCACCAAC-3′	5′-CACATCTCTCGGAGGACCA-3′	60.0
hsl	5′-GGTGACACTCGCAGAAGACAATA-3′	5′-GCCGCCGTGCTGTCTCT-3′	60.0
lpl	5′-CAGCTGGGCCTAACTTTGAG-3′	5′-CCTCTCTGCAATCACACGAA-3′	61.5
fasn	5′-AGCCCCTCAAGTGCACAGTG-3′	5′-TGCCAATGTGTTTTCCCTGA-3′	60.0
β-actin	5′-ACGAGGCCCAGAGCAAGAG-3′	5′ -GGTGTGGTGCCAGATCTTCTC-3′	60.0
srebf1	5′- GCTGTTGGCATCCTGCTATC-3′	5′-TAGCTGGAAGTGACGGTGGT-3′	60.0
cebpβ	5′-CAAGCTGAGCGACGAGTACA-3′	5′-CAGCTGCTCCACCTTCTTCT-3′	67.5
bcl2	5′-AGTACCTGAACCGGCATCTG-3′	5′-GGGGCCATATAGTTCCACAAA-3′	60.0

atgl = adipose triglyceride lipase; hsl = hormone sensitive lipase; lpl = lipoprotein lipase; fasn= fatty acid synthase; srebf1 = sterol regulatory element-binding factor 1; cebpβ = CCAAT-enhancer- binding protein β; bcl2 = B cell leukemia/lymphoma 2.

3.7. Measurements of Glycerol and Free Fatty Acids in the Media

After treatment in mature adipocytes, aliquots of the medium treated with 10 µM of 3S+4S were removed and analyzed for glycerol and FFA quantification by means of commercial kits (see Reagents paragraph).

3.8. Statistical Analysis

Results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 24.0 (SPSS Inc. Chicago, IL, USA). After confirming the normal distribution of variables using Shapiro-Wilks normality test, each flavonoid dose effect against the control was checked by Student's t test. Statistical significance was set-up at the $p < 0.05$ level.

4. Conclusions

The results obtained in the present study demonstrate that 3S metabolite may contribute to the delipidating effect of Q by reducing glucose uptake and TG assembling in mature adipocytes. ISO metabolite diminished TG accumulation in pre-adipocytes, but at a concentration of 10 µM, which is higher than that found in plasma from animals treated with Q. Consequently, its contribution to the effect of Q should be discarded, as well as that of 3G, 4S and TAM.

Supplementary Materials: Supplementary materials can be found at: http://www.mdpi.com/1422-0067/20/2/ 264/s1.

Author Contributions: M.P.P., J.M. and A.L. were responsible for the study concept and design. S.G.-M. and C.S.-B. synthetized and analyzed quercetin sulfates. I.E. and A.M.-S. performed the cell experiments and acquired the data. I.E. performed the gene expression and bioanalytical analysis. M.P.P., A.L. and J.M. interpreted data and drafted the manuscript. All authors read and approved the final manuscript.

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

Abbreviations

3G	quercetin-3-*O*-glucuronide
3S	quercetin-3-*O*-sulfate
4S	quercetin-4-*O*-sulfate
atgl	adipose triglyceride lipase
bcl2	B cell leukemia/lymphoma 2
cas3	caspase 3
cebpα	CCAAT-enhancer-binding protein α
cebpβ	CCAAT-enhancer-binding protein β
dgat1	diacylglycerol *O*-acyltransferase 1
dgat2	diacylglycerol *O*-acyltransferase 2
DMEM	Dulbecco's modified Eagle's medium
fasn	fatty acid synthase
glut4	facilitated glucose transporter member 4
FCS	fetal calf serum
FFA	free fatty acid
hsl	hormone sensitive lipase

ISO	isorhamnetin
lpl	lipoprotein lipase
pparγ	peroxisome proliferator-activated receptor γ
Q	quercetin
srebf1	sterol regulatory element-binding factor 1
TAM	tamarixetin
TG	triacylglycerol
trp53	transformation related protein 53

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EZTABAIDA

Eztabaida

Doktorego tesi honen helburua erresberatrola, kertzetina eta bere metabolito nagusien obesitatearen kontrako efektua gantz ehunean aztertzea izan zen. Hori horrela, lortutako emaitzak bi ataletan bana daitezke: 1) erresberatrola eta bere metabolitoen obesitatearen kontrako efektua 3T3-L1 aurre-adipozito eta adipozito helduetan, eta 2) kertzetina eta bere metabolitoen obesitatearen kontrako efektua 3T3-L1 aurre-adipozito eta adipozito helduetan.

Erresberatrola, kertzetina eta bere metabolitoek gantz ehunean duten efektua aztertzeko, 3T3-L1 zelula-lerroa aukeratu zen, saguen 3T3 zelulen klonazio-mitotikoaren hedapenaren bidez sortua [43]. Aurre-adipozitoen zelula-lerroen artean, gehien karakterizatu eta erabilitakoa da. Kontaktugatiko inhibizioa jasan arte ugaritzen ari diren aurre-adipozitoetara bideratutako zelulak dira, hormonen nahasketa (DEXA-IBMX-intsulina) gehitzearen ondorioz diferentziazio prozesua hasiko dutenak. Adipogenesian bi fase ezberdindu daitezke: fase goiztiarra edo klonazio mitotikoaren hedapenaren fasea eta fase berantiarra. Behin diferentziazioa bukatuta, gantz-tanta ikusgarriak dituzten eta adipozito helduen espezifikoak diren geneak espresatzen dituzten adipozito helduak antzeman daitezke mikroskopioz [37,43].

Kontuan hartuta gantz ehunean aurre-adipozito eta adipozito helduen nahasketa dagoela, zelula-lerroen erabilera ezinbestekoa da zelula mota bakoitzean era isolatuan ikerlanak egin ahal izateko. Adipozitoen bolumena lipogenesia eta lipolisiaren arteko orekaren emaitza da, adipozitoen kantitatea aurre-adipozitoen ugaritzea, diferentziazioa eta apoptosiaren artekoa den bitartean [331]. Ugaztun helduen zelula-ordezkapena altu samarra da, urte bakoitzeko %10 gizakien kasuan [20]. Zelula-lerro ezberdinen artean 3T3-L1 zelulak oso erabilgarriak dira, fibroblasto izatetik adipozito heldura desberdintzeko duten ahalmenagatik, maneiatzen errazak direlako eta tratamenduei modu homogeneoan erantzuten dietelako. Gainera, sarreran azaldu den bezala, gantz ehunaren gorabeherak adipozito kantitatearen handipenagatik (hiperplasia) eta adipozitoen tamainagatik (hipertrofia) ematen dira. Guzti hori kontuan hartuta, hala nola Doktorego Tesi hau polifenolek aurre-adipozitoen eta adipozito helduen zelula-ordezkapenean eta lipidoen metabolismoan duten efektuan ardaztu dela, 3T3-L1 zelula-lerroa aukeratu zen esperimentuak burutzeko.

1. Erresberatrola eta bere metabolitoen obesitatearen kontrako efektua 3T3-L1 aurre-adipozito eta adipozito helduetan

Erresberatrola etorkizun handiko polifenola da, azken hamarkadetan oso ikertua izan dena dituen efektu anitzak direla eta. Hargatik, Euskal Herriko Unibertsitateko “Nutrizioa eta Obesitatea” taldeak 2010. urtetik molekula honekin ikerketa-lan asko gauzatu ditu, obesitatearen aurkako tratamendu edo prebentzio moduan lagungarri izan daitekeen konposatu bioaktiboa aurkitzeko xedearekin. Gure laborategian animaliekin eginiko esperimentuetan erresberatrolak bai obesitatean eta bai bere koerikortasunetan, gibel-esteatosian edo inflamazioan kasu, efektu onuragarriak dituela ikusi da. Are gehiago, *in vitro* eta *in vivo* eginiko

esperimentuetan erresberatrolaren ekintza-mekanismo ugari deskribatu dira, hala nola lipogenesiaren inhibizioa edo lipolisia eta β-oxidazioaren areagotzea [222,237,243,244,332-337].

Efektu positibo hauetatik haratago, polifenolak jasaten duen metabolismo zorrotza buruhauste handia da, izan ere, ahoratutako erresberatrolaren kantitate oso txikia iristen da ehunetara. Honengatik guztiagatik, erresberatrolaren metabolitoek efektu biologikoa duten edo ez determinatzea erabakigarria da, eta 3T3-L1 zeluletan egiaztatzea izan zen gure proposamena. Zehaztutako helburu hori lortzeko, iturri bibliografiko gehienek adierazi bezala, 3G, 4G eta 3S metabolito ugarienekin hiru esperimentu burutu ziren [208,338]. Metabolitoen efektuak jatorrizko konposatuarenekin alderatzeko, erresberatrola ere aztertutako molekulen artean sartu zen. Lehenengo esperimentuan, 3T3-L1 zelulak erresberatrola eta bere metabolitoekin tratatu ziren 1, 10 eta 25 µM-eko dosietan. Dosi horiek *in vitro* ikerketa-lanetan erabili diren dosiak baino kantitate baxuagoak dira eta odolean aurkitu daitezkeen kontzentrazioetatik hurbilago daude. 25 µM-eko dosia *in vitro* ikerketetan erabilienetarikoa den bitartean, 1 µM-ekoa ez da adipozitoetan frogatu, honek ikerketa-lanaren berritasuna goraipatzen duelarik. Lehenik eta behin, 3G, 4G eta 3S-ren gantz murrizketa efektua analizatu eta jatorrizko konposatuarenarekin konparatu zen **(1. eskuizkribua)**. Jarraian, aurre-adipozitoen diferentziazioan miRNA-ek eragina dutela ikusita, hainbat miRNA ikusitako efektuen bitartekari izan zitezkeen aztertu genuen **(2. eskuizkribua)**. Bukatzeko, triglizeridoen murrizketan eraginkortasuna erakutsi zuten dosiek adipokinen espresioa eta jariaketa eraldatu zezaketen ikertu nahi izan genuen **(3. eskuizkribua)**.

Gaurdaino, erresberatrolak adipogenesiaren kontrako efektua erakutsi du aurre-adipozitoetan, hala nola lipidoen metabolismoaren aldaketak adipozito helduetan [220,227,228,247,339-341]. Ostera, bere metabolitoek afera honetan izan ditzaketen efektuak ez dira aztertu, eta horregatik, lan honetan 3T3-L1 aurre-adipozito eta adipozito helduak erresberatrola eta 3G, 4G eta 3S metabolitoekin tratatu ziren **(1. eskuizkribua)**. Aurre-adipozitoetan, soilik erresberatrolaren 25 µM-eko dosia izan zen triglizeridoak murrizteko gai, adipozito helduetan 1 µM-eko dositik aurrera eraginkorra izan zen bitartean. Bestetik, aipatzekoa da metabolito glukuronidoak bai aurre-adipozitoetan eta bai adipozito helduetan efektua erakutsi zutela, metabolito sulfatatuaren eraginkortasuna aurre-adipozitoen diferentziazioan soilik eman zelarik.

Oro har, emaitza hauek erakusten dutenez, erresberatrola eraginkorragoa da adipozito helduetan aurre-adipozitoetan baino. Aipagarria da ere metabolitoek gantz murrizketan erakutsitako efektua, 3S metabolitoak aurre-adipozitoetan eta metabolito glukuronatuek bai aurre-adipozitoetan eta bai adipozito helduetan. Gehiago dena, erresberatrolak adipozito helduetan duen efektua metabolito glukuronatuena baino handiagoa dela esan daiteke, 1 µM-eko dositik aurrera eraginkorra delako eta metabolitoek 10 aldiz handiagoa den dosia behar dutelako. Aitzitik, erresberatrolak eta metabolito sulfatatuak duten eraginkortasuna parekoa da **(1. eskuizkribua)**.

Aurre-adipozitoetan eskuratutako emaitzekin bat, beste hainbat autorek ere 10 µM-eko dosiak eraginik ez duela esan dute. 20 eta 25 µM-eko dosietan, ordea, gantz murrizketa ikusi dute [220,231,246]. Beranduago gure ikerketa-taldean eginiko esperimentu batean ere, erresberatrolak 25 µM-eko dosian eraginkortasuna erakusten zuela baieztatu zen [342].

Guk dakigula, lehen aldia da erresberatrolaren lipido murrizketa efektua horren dosi baxuetan frogatzen dela, aurrerago antzeko dosietan ikerketa gehiago burutu badira ere. Hu eta kolaboratzaileek erresberatrolaren efektua 1, 10, 50 eta 100 µM-eko dosietan aztertu zuten, espero ez zitekeen emaitza lortu zutelarik, erresberatrolaren 1 eta 10 µM-eko dosiek adipogenesia aregotzen baitzuten [343]. Kontrako emaitzak ikusi bazituzten ere, esperimentuaren ezaugarri metodologikoak ezberdinak zirela esan beharra dago. Adipogenesia eragiteko erabilitako hormona-nahasketa ez zen arrunki erabiltzen dena izan [43], tratamenduaren iraupena ere egun bat gutxiagokoa izan zelarik. Ezberdintasun sotil hauek bi esperimentuetan eskuratutako emaitzen arteko aldearen erantzule izan daitezke. 2016. urtean Chang eta kolaboratzaileek erresberatrolaren gantz murrizketa efektua oso dosi baxuekin ere ematen zela adierazi zuten, 0.3 µM-tik aurrera (0.3-10 µM) eraginkorra zela ikusi baitzuten [344]. Aranaz eta kolaboratzaileek eginiko beste ikerketa batean, autoreek zenbait KF-ren efektua aztertu zuten adipogenesiaren fase ezberdinetan [345]. Zelulak 10, 50 eta 100 µM-eko dosiekin tratatu zituzten 8 egunez, eta aztertutako dosien artean, eraginkorra soilik 50 µM-ekoa zela ikusi zuten, gure ikerlanarekin bat datorrena. Emaitza guzti hauekin, erresberatrolak adipogenesiaren efektu inhibitzailea badu ere, dosi eraginkorra oraindik zehazteke dagoela esan daiteke. Erresberatrolaren metabolitoei dagokienez, lehen aldia da adipozitoen triglizeridoen murrizketan duten efektua analizatzen dela. Dakigunaren neurrian, ez da beste ikerlanik egin gaurdaino.

Behatutako efektuari erantzuna emango lioketen mekanismoei erreparatuta, *cebpβ* (klonazio-mitotikoaren hedapenaren fasean espresatua) eta *pparγ*, *cebpα* eta *lpl* (fase berantiarrean espresatuak) geneen mRNA mailak neurtu ziren. Erresberatrola, 3G, 4G eta 3S metabolitoek *cebpβ*-ren espresioa murriztu zuten, *pparγ*, *cebpα* eta *lpl* geneen gain soilik 3S metabolitoak eragin zuen bitartean, haien mailak jaitsiz.

Beraz, erresberatrola, 3G eta 4G metabolitoek adipogenesiaren fase goiztiarra blokeatzen duten bitartean, 3S adipogenesi osoa inhibitzeko gai den metabolito bakarra **(1. eskuizkribua)**.

Ikusi izan denez, erresberatrolak eta bere metabolitoek ez dute beti gene berdinengan eragiten, eta beraz duten ekintza-mekanismoa ezberdina izan daitekeela esan daiteke. Esaterako, nahiz eta erresberatrolak tumoreen nekrosiaren α faktorearen (TNFα) espresioa areagotu, ez da ikusi 3G eta 3S metabolitoek ahalmen hori dutenik [346]. Bestetik, Ruotolo eta kolaboratzaileek ikusi zutenez, soilik 3S metabolitoak estrogeno hartzailearen antagonista bezala jokatzen du [347]. Calamini eta kolaboratzaileek eginiko ikerlana ere adibide

ona da, bertan erresberatrola, 3G, 3S eta erresberatrola-4´-O-sulfatoak (4S) estres oxidatiboaren zenbait entzima modu ezberdinean erregulatzen zituztela ikusi baitzuten [230].

Erresberatrolak adipogenesian duen efektuari dagokionean, aipatzekoa da bibliografian emaitza eztabaidakorrak daudela. Kang eta kolaboratzaileek 3T3-L1 aurre-adipozitoak 10, 20 eta 40 µM erresberatrolekin tratatu zituzten 2, 4 eta 6 egunetan zehar, non dosi eta tratamendu-iraupenaren menpeko adipogenesiaren inhibizioa ikusi zuten, hala nola 10 µM-eko dosia adipogenesia inhibitzeko nahikoa zela [348]. Areago, erresberatrolak hainbat transkripzio faktoreen, CEBPβ, PPARγ, CEBPα eta FABP4 kasu, espresioaren gain zuen efektu inhibitzailea dosiaren menpekoa zela behatu zuten. Alabaina, Chen eta kolaboratzaileek antzeko diseinu esperimentalarekin eginiko beste ikerketa batean, 3T3-L1 aurre-adipozitoak erresberatrolaren 10, 20, 40 eta 80 µM-eko dosiekin tratatu zituzten 24 eta 48 orduz [231]. Kasu honetan, autoreek ez zuten erresberatrolaren adipogenesiaren kontrako efektua horren dosi baxuetan finkatu, dosi eraginkor baxuena 20 µM-ekoa baitzen. Aipaturiko emaitzak, gure esperimentuan lorturikoen antzekoak dira. Li eta kolaboratzaileek lorturikoak ere gureekin bat datoz, 8 egunez 25 µM erresberatrolarekin tratatutako 3T3-L1 zelulen gantz kantitatea murrizten zela ikusi baitzuten [221]. *Pparγ* eta *cebpα* geneen espresioari dagokionez ere, emaitza kontrajarriak behatu dira. Ikerketa-lan askotan erresberatrolak haien espresioa murrizteko ahalmena zuela ikusi bazen ere, diseinu esperimental antzekoa zuten beste hainbatetan ez da behaketa hori egin [246,349,350], gure esperimentuan gertatu bezala. Aurreko ikerlanetan lorturiko emaitzek aditzera eman dutenez, badirudi 20-25 µM inguruko dosiak direla *pparγ* eta *cebpα* geneen espresioa murrizteko ahalmena izan dezaketenak. Hori dela eta, dosi hauek antzekotasuna duten esperimentuetan erabili izan direnean, tratamenduaren iraupena edo eginiko neurketa gene edo proteina mailan egin den aintzat hartzea nahikoa izan daiteke emaitza ezberdinak behatzeko.

Molekulek adipogenesiaren erregulazioan zuten efektua sakonki aztertzeko eta kontuan izanik miRNA-k adipogenesiaren kontrolean duten rol garrantzitsua, miRNA-ek erresberatrola eta metabolitoen adipogenesi kontrako efektuan izan lezaketen parte-hartzea aztertu zen **(2.eskuizkribua)**. Xu eta kolaboratzaileak izan ziren miRNA-ek adipogenesiaren erregulazioan zuten paper garrantzitsua deskribatu zuten lehenengoak, Drosophilan miR-14-ren ezabapenak lipidoen handitzea zekarrela ikusi baitzuten, hala nola miR-14 gainespresatzean kontrako emaitzak lortzen ziren [351]. Aurkikuntza hori eta gero, 3T3-L1 zelula-lerroa oso erabilia izan da adipogenesian emandako miRNA-en espresio profila analizatzeko, batik bat microarray analisiez eta ondorengo egiaztapenez [352]. Aipatzekoa da ez dagoela ezarritako microarray-en plataforma orokorra, adipogenesia erregulatu lezaketen balizko miRNA izangaien zehaztean akordiorik ez dagoelarik. 2006. urtean, Kajimoto eta kolaboratzaileek adipogenesian zehar gainespresatu edo inhibitu egiten ziren 21 miRNA identifikatu zituzten, diferentziazioaren 0. egunean edo 9. egunean (zelulek gantz-tanta ikusgarriak dituztenean) aurkitzen ziren adipozitoen miRNA-en profila konparatuta [56]. Besteak beste, miR-21, miR-17-92 klusterra, miR-30, miR-103, miR-125, miR-143 eta miR-378 dira, modu fidagarrienean frogatuta,

adipogenesian gainespresatzen diren miRNA-k. Bestalde, let-7 familia, miR-27a, miR-27b, miR-33, miR-155 eta miR-221/222 familiaren parte hartzea adipogenesiaren inhibizioan ikerlan askotan finkatu da [68].

Erresberatrolak *pparγ*, *cebpα* eta *cebpβ* transkripzio faktoreen gain duen efektuan miRNA-ek izan dezaketen inplikazioari buruz ezer gutxi ezagutzen da, hala nola erresberatrolaren metabolitoen efektua ez den orain arte ikerlan batetan ere ezarri. Hori dela eta, afera honetan sakontzea erabaki zen. Lehenik eta behin, aipaturiko geneen bibliografian deskribaturiko miRNA izangaien iragarpen konputazionala egin zen. Kontuan eduki beharrekoa da miRNA-k euren itu diren mRNA molekulen 3´UTR zonaldera lotu daitezkeen molekula oso txikiak direla. Giltzadura horretarako behar den nukleotido osagarrien minimoa soilik 6koa dela, miRNA bakoitzerako hamaika balizko itu aurkitu daitekeelarik. Hori horrela, miRNA-en aukeraketa egiteko irizpideak ondo definitzea ezinbestekoa da. Gure kasuan, bi irizpide aintzat hartu ziren: 1) 5 algoritmoren bidez mirWalk datu basean iragarri edo egiaztatu bezala azaldu behar ziren, eta 2) “miR+adipogenesis” hitz-gakoak erabilita Pubmed datu basean agertu behar ziren.

Bai CEBPβ eta bai CREB, miR-155-aren egiaztatutako ituak dira [58,353-356]. Costinean eta kolaboratzaileak izan ziren CEBPβ miR-155-aren itua zela balioztatu zuten lehenengoak, miR-155-a falta zuten sagu transgenikoen CEBPβ proteinaren espresioa neurtu zutenean [58]. Gehiago dena, miR-155-aren molekula mimetikoaren presentzian luziferasaren aktibitatea %53-an murriztu zela ikusi zuten. Bestetik, Liu eta kolaboratzaileek 3T3-L1 zelulak TNFα-rekin tratatzearen ondorioz emandako miR-155-aren igoerak CEBPβ eta CREB proteinen espresioaren murrizketa eragiten zuela ikusi zuten, adipogenesiaren inhibizioa eraginez [353]. Chen eta kolaboratzaileek burututako beste ikerketa batean, miR-155-ak eta CEBPβ-k adipozito zurien arretzea eragingo duen feedback-gurpil bat eratzen zutela egiaztatu zen [354]. C*ebpβ* miR-155-aren egiaztaturiko itua dela kontuan hartuta, hala nola adipogenesiaren erregulazioan duen rol garrantzitsua, erresberatrola eta metabolitoen adipogenesiaren kontrako efektua miR-155-*cebpβ*-ren bidezkoa zen aztertu nahi izan genuen. 3S metabolitoak miR-155-aren mailak aldatu ez zituenez, ez genuen mekanismo hau metabolito sulfatatuaren erantzule izan zitekeenik pentsatu. Ostera, erresberatrola, 3G eta 4G molekulekin egindako tratamenduek miR-155-aren mailak handitu zituzten, *cebpβ*-ren gain eragindako murrizketa miRNA bidez izan zitekeela iradokituz. Hipotesia baieztatzeko helburuarekin, lan honetan 3T3-L1 aurre-adipozitoak anti-miR-155-arekin transfektatu ziren erresberatrolaren presentzian edo gabezian, miR-155 isilaraztean tratamenduak CEBPβ-ren gene eta proteina mailak aldatzeko gai ez zela behatuz.

Eskuratutako emaitzak ikusita, erresberatrolaren efektu inhibitzailea, eta ustez metabolito glukuronatuena ere, miR-155-aren erregulazioaren bidezkoa da, hein batean behintzat **(2. eskuizkribua)**.

MiR-155-aren gain erresberatrolak eragindako erregulazioa monozitoak edo makrofagoak bezalako zeluletan ikertu da, inflamazioa, aterosklerosia eta hipertentsioaren kontra dituen funtzioak ezartzeko helburuarekin [357-360]. Aldiz, aipatzekoa da lehen aldia dela erresberatrolak adipozitoetan miR-155-a erregulatzen duela baieztatzen dela.

MiR-155-aren espresioan emandako aldaketak ez ziren 3S metabolitoak *cebpβ*-ren gain eragindako erregulazioa azaltzeko baliagarri suertatu, eta hortaz, beste balizko bide metabolikoak aztertu ziren. CEBPβ eta CEBPδ fase goiztiarraren transkripzio faktore garrantzitsuenak direla zabalki onartua dago [361,362], garrantzitsuak diren beste batzuk badaude ere [24]. Berriz, adipogenesiaren hasiera nola ematen den ez dago guztiz zehaztuta. Aurretik eginiko esperimentu batean oinarrituta, Reusch eta kolaboratzaileek cAMP-k eragindako CREB-en fosforilazioa adipogenesiaren hasiera eragin zezakeela aditzera eman zuten [23]. Ideia horri erantzuna eman nahian, 3T3-L1 aurre-adipozitoak CREB inhibitu edo gainespresatzeko transfektatu zituzten. Triglizeridoen metaketa, zelulen forma eta fase berantiarreko markatzaile adipogenikoak (besteak beste PPARγ eta gantz azidoetara lotzen den proteina (FABP)) neurtuz, adipogenesian zuen efektua aztertu zuten. Ikusi zutenez, kanpoko beste estimuluren beharrik gabe CREB-en gainespresioa adipogenesia eragiteko nahikoa izan zen, haren inhibizioak diferentziazio prozesua guztiz blokeatzen zuen bitartean. Gauzak horrela, CREB-ek adipogenesiaren hasieran duen garrantzia ikusita, bere mRNA mailak neurtu ziren. *Creb*-en espresioa murriztu egin zen 3S tratamenduaren ondorioz, *cebpβ*-aren murrizketan izan zezakeen efektua iradokituz. Areago, KLF5 transkripzio faktorea *CEBPβ*-gatik induzigarria da eta PPARγ-ren aktibazioa ekartzen du [39]. Ikusitako *klf5*-aren mailen beherapena zelulak 3S eta 4G metabolitoekin tratatu ostean, pentsatzekoa da metabolito sulfatatuak adipogenesiaren inhibizioa fase goiztiarrean espresatutako *creb1* eta *klf5* transkripzio faktoreen bitartez eragiten duela. Era berean, 4G metabolitoak bi mekanismo jarraitzen ditu, bata miR-155-aren bidezko *cebpβ*-ren murrizketa eta bestea, *klf5*-aren espresioaren erregulazioa. Ez dago 3S eta 4G metabolitoek KLF5-aren gain eginiko efektuari buruzko daturik, baina erresberatrolak KLF5 modu zuzenean erregulatzen duela esan dute zenbait autorek [363,364]. Nahiz eta gure ikerketan erresberatrolak *klf5*-aren espresioaren gain efekturik ez izan, nabarmentzekoa da zelula mota, erabilitako dosia eta tratamenduaren iraupena nabarmenki ezberdinak direla aipatutako lanetan.

Cebpα eta *pparγ* itu duten miRNA-en artean (miR-326, miR-31, miR-144, miR-205, miR-224 eta miR-27a, miR-27b, miR-130b, hurrenez hurren), ez zen aldaketarik ikusi 3S metabolitoarekin tratatutako zeluletan. Emaitza horiek 3S-k bi geneen espresioaren gain eraginiko efektua miRNA-en bidezkoa ez zela aditzera ematen dute. SREBP1c eta LXRα PPARγ-ren gene erregulatzaile moduan definitu izan direla kontuan hartuta [365, 366], haien espresioa neurtu zen, metabolito sulfatatuak bien espresioak murrizten zituela ikusi genuelarik. Hori horrela, eta miR-27a, miR-27b eta miR-130b-ren mailak aldatu ez zirenez, emandako *pparγ*-ren espresioaren murrizketa SREBP1c eta LXRα bitartez izan daiteke, hein batean bada ere. 3S metabolitoak gene hauengan duen efektuari buruzko bibliografiarik ez dago baina aipatzekoa da biak erresberatrola eta beste polifenolen

(kurkumina, kasu) itu terapeutiko moduan definituak izan direla [35, 367-371]. Bestalde, PPARγ eta CEBPα-ren artean ematen den kooperazio-mekanismoa ondo ulertzen ez dela kontuan hartu beharra dago. Ikerketa ezberdinek adierazi dutenez, bien sinkronizaturiko espresioa beharrezkoa da adipozitoak ondo diferentziatu daitezen, hala nola nahikoa adipogenesiari hasiera emateko [372]. Hortaz, datuek adierazten dutenez, *pparγ*-k *cebpα*-ren murrizketa eragin zuen erresberatrolak transkripzio faktore hauetara lotu daitezkeen miRNA-tan aldaketarik eragin gabe.

Erresberatrola eta metabolito glukuronatuak ez bezala, 3S metabolitoaren adipogenesi kontrako efektua ez da miRNA-n menpekoa **(2. eskuizkribua)**.

Erresberatrola eta metabolitoen 24 orduz tratatutako adipozito helduen triglizeridoen murrizketaren mekanismoei erreparatura **(1. eskuizkribua)**, 10 µM-eko dosian tratatutako zelulen geneen espresioa aztertu zen. Lipolisia aktibatu ote zen aztertzeko helburuarekin, garrantzitsuenak diren bi lipasen mRNA mailak neurtu ziren. Erresberatrolak *atgl*-ren espresioa handitu zuen, gure ikertaldeko beste esperimentuekin bat datorrena, eta 3G eta 4G metabolitoek igotzeko tendentzia erakutsi zuten. Lasa eta kolaboratzaileek saguen 3T3-L1 zelulak, gizakien SGBS zelulak eta ATGL *knockout* (KO) eta HSL KO saguetatik eskuraturiko zelula primarioak erresberatrolarekin tratatu zituzten [222]. Erresberatrolak egoera basalean edo isoproterenolarekin kitzikatutako egoeratan 3T3-L1 zelulen GAA-en askatzea eragin zuen, glizerolaren kantitateak aldatu ez baziren ere. SGBS zelulen kasuan, GAA-en jariaketa isoproterenolarekin suspertutako adipozitoetan ikusi zen soilik. Adipozito primarioetan, erresberatrolak GAA-en jarioa HSL KO saguen zeluletan eragiten zuen, baina ATGL KO saguen adipozitoetan, ordea, ez. Gauzak horrela, ikerlan horretako autoreek erresberatrolak lipolisia ATGL-ren gain eraginda erregulatzen duela aldarrikatu zuten, lipasaren erregulazio maila transkripzionalean eta post-transkripzionalean sorrarazita. Horrez gain, esperimentu honetan 4G–k *hsl*-ren mailak handitu zituen. Lasa eta kolaboratzaileek lortutako emaitzez gain, gure laborategian arratoiekin eginiko ikerketetan erresberatrola, *atgl*-ren espresioan eragin gabe, *hsl* mailak handitzeko gai zela ikusi izan da [243, 244]. Beraz, autore ezberdinek erresberatrolaren efektu lipolitikoa ikusi badute ere, mekanismoa eztabaidagarria da egun [222,228,243,244,373]. Azkenik, aipatzekoa da Chang eta kolaboratzaileek 2016. urtean 3T3-L1 zelulak erabilita eginiko ikerketa [344]. Harrigarriro, erresberatrolak 0.03 eta 10 µM bitarteko dosietan lipolisiaren gaineko efektu inhibitzailea zuela ikusi zuten. Autoreek iradokitu zutenez, erresberatrolaren lipolisia aktibatu edo inhibitzeko efektua dosiaren araberakoa izan daiteke.

Aurrekoaz gain, erresberatrolak *cpt-1b*-en espresioa handitu zuen, hala nola 3G metabolitoak *fasn*-ena murriztu. *Lpl* eta *acc* geneen mailak ez ziren tratamenduen ondorioz aldatu. Lipogenesiaren murrizketan erresberatrolak duen rola beste ikerketa batzuetan behatu izan da [221, 374]. Li eta kolaboratzaileek

gauzatutako esperimentu batean, autoreek 25 eta 50 µM erresberatrolak zelulen triglizeridoen kantitatea murrizten zuela ikusi zuten, beti ere zelulen lipogenesia azido oleikoarekin areagotu eta gero [221]. Bukatzeko, aipatu beharra dago 3S metabolitoak ez zuela geneen espresioan aldaketarik eragin, lipidoen murrizketan izandako eragin ezarekin bat datorrena.

Esperimentu honetan eskuraturiko datuak aztertuta, badirudi erresberatrolak eta 4G metabolitoak lipidoen metaketaren murriztea lipolisiaren bitartez eragiten dutela, erresberatrolak gantz oxidazioa ere handitzen duen bitartean. Aldiz, 3G metabolitoak lipogenesia murriztuz jaisten du adipozitoetako gantz pilaketa **(1. eskuizkribua)**.

Lehenago aipatu izan den moduan, erresberatrolaren gehien deskribaturiko mekanismoa SIRT1-a aktibatzeko duen ahalmena da, PGC1α eta ondorengo geneen aktibazioa eraginez [223, 227, 375]. Lan honetako esperimentuaren baldintzetan, erresberatrolak eta metabolito glukuronatuek *sirt1*-en espresioa handitu zuten. Ostera, molekula guztiek *pgc1α* mailak handitzeko tendentzia erakutsi bazuten ere, erresberatrola izan zen modu esanguratsuan egin zuen bakarra.

Hortaz, erresberatrolaren SIRT1-a aktibatzeko ahalmena 3 metabolitoek ere dute. Ordea, erresberatrola da PGC1α aktibatzen duen bakarra **(1. eskuizkribua)**.

Adipokinen ekoizpenean eragindako aldaketak, erresberatrolak eragindako zenbait efektu osasungarriren erantzuleak izan daitezke, besteak beste gorputzeko gantzaren murrizketa edo intsulinarekiko sentikortasunaren areagotzea [238,376-378]. Erresberatrolaren metabolitoek adipozitoen gantz metaketan efektu onuragarriak zituztela ikusita, molekula horiek adipokinen espresioa eta jariaketa aldatzeko gai ziren aztertu nahi izan zen, era horretan intsulinarekiko sentikortasuna eta glukosaren homeostasiaren hobekuntzan lagungarri izan zitezkeen argitzeko **(3. eskuizkribua)**. Horretarako, lehenengo esperimentuaren emaitzekin **(1. eskuizkribua)** dosi eraginkorrak aukeratuta, 25 µM aurre-adipozitoetan eta 10 µM adipozito helduetan, zelulak tratatu eta adipokinen geneen espresioa eta jariaketa neurtu genituen. Lehenago aipatu den bezala, aukeratutako dosiak *in vivo* ikerketetan eta ikerketa klinikoetan erabilitako dosien ostean animali edo gizakien odolean topatutako kantitateetatik hurbil kokatzen dira [216,218].

Azken hamarkadetan, glukosaren homeostasian eta intsulinarekiko sentikortasunean eragiten duten hainbat adipokina deskribatu dira, horietako batzuk erresberatrolak erregulatuak izan daitezkeelarik. Adiponektina eta leptina erresberatrolaren eraginpeko adipokina ezagunenak kontsideratu dira

[252,253,376]. Bestetik, visfatinak eta apelinak obesitatea eta glukosaren homeostasian duten rola aski ezaguna bada ere [156,167,379,380], gutxi ikertu da erresberatrolaren efektuen erantzuleak diren ezagutzeko helburuarekin [256].

Lan honetako esperimentuan, ustekabeko fenomenoa errepikatu zen: adiponektina, leptina, visfatina eta apelinaren geneen espresioak zelulak erresberatrol eta metabolitoekin tratatzearen ondorioz aldatu baziren ere, horren isladapena izan zitekeen adipokinen jariaketaren aldaketa oso gutxitan gertatu zen. Nabarmentzekoa da adipokinen espresio eta jariaketaren arteko ezberdintasunak beste autore batzuek ere behatu dituztela. Gertakari horren balizko azalpen *bat in vitro* eginiko ikerketetan erabilitako substantziekin erlazionatzen da. Wang eta kolaboratzaileek intsulinak erregulaturiko zenbait proteinen espresioa eta jariaketa aztertu zuten 3T3-L1 adipozitoetan, eta intsulinak adiponektinaren jariaketa inhibitzen zuela ikusi zuten, nahiz eta mRNA mailak handituta egon [381]. Are gehiago, Ryan eta kolaboratzaileek txakurren adipozitoekin eginiko ikerlan batean, DEXA eta rosiglitazonak adipokinen espresioan eta jariaketan eragiten zutela ikusi zuten [382]. DEXA-k IL-6-ren mRNA mailak eta jariaketa berdintsuki aldatzen zituen baina rosiglitazonak IL-6-ren mRNA mailak murrizten zituen inkubazio-medioko kontzentrazioa aldatu gabe. Bestetik, gene eta proteinen espresioaren erregulazio ezberdina azaltzen duten hainbat mekanismo molekular dago, mRNA-ren ordezko splicing-a, mRNA-ren aktibazio polisomikoa, proteinen ordezkapena, edo prozesatze proteolitiko espezifikoa, esate baterako. Horrez gain, besteak beste zelula-mintzerainoko translokazioa, lisosomen menpeko bidea edo mikrobesikulen menpeko bidea dira behatutako ezberdintasunen erantzule izan daitezkeen proteinen jariaketaren erregulatzaileak [383-386]. Mekanismo horiez harago, mRNA eta proteinek duten batez besteko bizitza ezberdinek zelulen tratamenduen benetako efektua estali zezaketela iradokitu zen [387]. Erresberatrola eta metabolitoek adipokinen ekoizpenaren gaineko efektuen mekanismoak hobeto definitzeko, gainontzeko maila molekularretan eginiko neurketak aukera interesgarria izan zitezkeen (proteinen espresioa, kasu). Are gehiago, behatutako kontraesanei erantzuna emateko aukera izan zitekeen. Hala eta guztiz ere, gogora ekarri behar da Doktorego Tesi hau erresberatrolaren metabolitoen efektu bioaktiboa aztertzen ardaztu dela, eta hargatik, lehen hurbilketa bat egin nahi izan dela.

Aurre-adipozitoetan erresberatrolak eta hiru metabolitoek eragindako adiponektinaren espresio genikoaren areagotzea, beste autore batzuek ere ezarri dute. 2011. Urtean, Wang eta kolaboratzaileek erresberatrolak 3T3-L1 adipozitoen adiponektinaren espresioa handitzen zutela ikusi zuten, erabilitako mekanismoa SIRT1-aren menpekoa ez zela egiaztatu zutelarik [242]. Halaber, obesitate edo diabetetaren kasuan, erresberatrolak TNFα-ren gainespresioak eragindako adiponektinaren inhibizioa leheneratu lezake PPARγ –ren aktibazioaren bidez [388-391]. Alabaina, animalia ereduekin eginiko zenbait ikerketek kontrako emaitzak ikusi dituzte, adiponektina benetan erresberatrolaren itu posible bat dela zehaztea zailduz [334, 392-394].

Adipozito helduetan, erresberatrola eta metabolitoek leptinaren espresio genikoaren eta jariaketaren murrizketa eragin zuten modu esanguratsuan, hala nola erresberatrolarekin tratatutako aurre-adipozitoen leptina genearen espresioa. Emaitza hauek Szkudelska eta kolaboratzaileek eginiko esperimentu batekin bat datoz, erresberatrolaren 62.5 μM-ekin tratatutako arratoien adipozito primarioetan leptina jariaketa murrizten zela ikusi baitzuten [234]. Aipatzekoa da hainbat ikerlanek gene adipogenikoen eta adipokinen ekoizpenaren arteko erlazio zuzena topatu dutela. Zehazki, leptina hainbat transkripzio faktoreen itu bezala definitu izan da, CEBPβ-rena esaterako [395]. Hori horrela, erresberatrolarekin tratatutako aurre-adipozitoetan ikusitako leptinaren murrizketa *cebpβ*-ren espresioan emandakoaren bidezkoa izan zitekeela aditzera eman zen lan honetan. Berriki Zhao eta kolaboratzaileek eginiko ikerketa batean, arratoiei 120 mg/kg gorputz-pisu/egun-eko erresberatrolaren dosia emanda, gantz portzentaje altuko dietak eragindako odoleko leptina mailaren handipena berrezartzen zela ikusi zuten [396]. Aldiz, Derdemezis eta kolaboratzaileek ez zuten ikusi erresberatrolak adiponektina edo leptina mailetan eragiten zuenik SGBS zeluletako inkubazio-medioan adipokinen kantitatea neurtu ondoren [256]. Aipatzekoa da ikerlan honetan ez zela adipokinen geneen espresioa neurtu, eta hortaz, ezin da gure emaitzekin konparaketa zuzena egin. Erresberatrolak adiponektina eta leptinaren espresioan dituen efektuak animali ereduetan frogatu izan dira [392,393]. Hala eta guztiz ere, kontuan hartuta Tabrizi eta kolaboratzaileek eginiko meta-analisian autoreek gizakietan erresberatrolak adiponektina eta leptina mailak aldatzen ez dituela aldarrikatzen dutela, ez dago argi datuak estrapolagarriak diren edo ez [397]. Honengatik guztiagatik, erresberatrolaren efektuak adiponektina eta leptinaren bidezkoak diren argitzeko helburua duten ikerketa gehiagoren beharra dago.

Erresberatrolak eta metabolito glukuronatuek visfatinaren mRNA mailak handitu zituzten aurre-adipozitoetan, adipozito helduen kasuan aldiz, aztertutako 3 metabolitoak izan zirelarik visfatinaren espresioa areagotu zutenak. Apelinaren kasuan ere, antzeko emaitzak lortu ziren. Aurretik aipatu den bezala, ebidentzia gutxi dago polifenol honen eta bi adipokina hauen arteko erlazioaren inguruan. Derdemezis eta kolaboratzaileek erresberatrolak visfatinaren jariaketan zuen efektua aztertu zuten SGBS zeluletan, eta 24 eta 48 orduz 10 eta 25 μM-eko dosiekin tratatzean murrizten zela behatu zuten [256]. Apelinari dagokionez, 2016. urtean argitaratutako artikulu batean, autoreek ez zuten ezberdintasunik ikusi arratoi diabetikoei erresberatrola 1, 5 eta 10 mg/kg gorputz-pisu/egun-eko dosian 30 egunez eman zitzaienean [241].

Esperimentu honetako emaitzak kontuan hartuta, erresberatrolak energiaren homeostasian eta kontrol gluzemikoan dituen efektu onuragarriak, hein batean bada ere, adipokinen ekoizpena eragiten duelako direla esan daiteke. Are gehiago, erresberatrolaren metabolitoek efektu honetan laguntzen dute **(3.eskuizkribua)**.

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2. Kertzetina eta bere metabolitoen obesitatearen kontrako efektua 3T3-L1 aurre-adipozito eta adipozito helduetan

Kertzetina ere etorkizunean erabilgarria izango den molekula bezala iragarri izan da, oxidazioaren eta hanturaren kontra dituen ekintzengatik, minbiziaren aurkako funtzioagatik eta aski seguru, ikertuena izan den diabetesaren kontrako egitekoagatik. Hala ere, esan beharra dago obesitatearen aurka dituen efektuei dagokienez, ez dagoela horrenbesteko informaziorik bibliografian. Hori dela eta, "Nutrizioa eta Obesitatea" ikertaldeak bere esperimentuak polifenol honekin ere bideratu ditu. Gantz eta sakarosa askoko dietarekin elikatutako Wistar arratoiei 6 astez 30 mg/kg gorputz-pisu/egun-eko dosiko kertzetina ematean, Arias eta kolaboratzaileek, gorputz-pisuaren murrizketa ikusi ez bazen ere, intsulinarekiko erresistentziaren adierazleetan hobekuntzak antzeman zituzten [284]. Beste autore batzuen esperimentuetan lorturiko datuek diotenez, gorputz-pisua murrizteko gai izateko kertzetina esperimentu honetan baino denbora gehiagoz eman behar zaie animaliei. Hori horrela, autoreek gorputz-pisuaren gain efektuak ikusi ez izana tratamenduaren iraupenagatik izan zitekeela aldarrikatu zuten. Gure ikerketa-taldean eginiko beste esperimentu batzuetan, erresberatrolarekin batera emanda mikrobiotaren konposaketaren hobekuntza [398], GEZ-ko triglizeridoen metabolismoan eta gibelaren gehiegizko gantz metaketaren prebentzioan, efektu positiboak dituela ikusi izan da [399,400].

Kertzetinari gantza murrizteko mekanismo ezberdin egotzi zaizkio *in vitro* eginiko ikerketetan, horien artean lipolisiaren areagotzea, lipogenesiaren murrizketa eta adipogenesiaren inhibizioa [297, 303, 305]. Hala ere, ikerlan hauen mugarik aipagarriena erabili dituzten dosiak 10 eta 500 µM bitartekoak direla da [247,297,303,305,401], gizaki edo animaliei kertzetinaren gehigarriak eman ostean, odolean aurkitutako kertzetina kantitatea baino askoz handiagoak diren dosiak [270, 271, 402]. Gauzak horrela, gure helburua beste ikerlanetan frogatutakoak baino baxuagoak ziren dosietan, odolean aurkitutako kontzentrazioetatik hurbilagokoetan (0.1-10 µM) hain zuzen, kertzetinak 3T3-L1 aurre-adipozito eta adipozito helduen triglizeridoen pilaketan efekturik zuen edo ez aztertzea izan zen, hala nola horren erantzule izan zitezkeen ekintza-mekanismoak definitzea **(4. eskuizkribua)**.

3T3-L1 aurre-adipozitoak diferentziazio prozesu osoan zehar kertzetinaren 0.1 eta 10 µM bitarteko dosiekin tratatu ziren. Aztertutako dosi guztiekin tratatutako zelulen gantz kantitatea murriztu egin zen, 0.1 eta 0.5 µM-eko dosiekin tratatutako zelulen kasuan, murrizketa esanguratsua izan ez bazen ere. 10 µM-eko dosiak eragindako triglizeridoen murrizketa Ahn eta kolaboratzaileek burututako ikerketarekin bat dator, 1, 50 eta 100 µM-eko dosiekin gantz murrizketak ikusi baitzituzten [297]. Bae eta kolaboratzaileek gauzatutako ikerketan, 3.3-6.6 µM kertzetinak 3T3-L1 aurre-adipozitoen gantza murriztu zuen, gure esperimentuko 5 µM-eko dosiarekin ikusitako emaitzekin konparagarria dena [403]. Aldiz, gure esperimentuan ikusitakoaren kontra, Lee eta kolaboratzaileek eta Yang eta kolaboratzaileek 5, 10 eta 20 µM edo 12.5 µM kertzetina, hurrenez hurren, lipidoen kantitatea murrizten eraginkorra ez zela ikusi zuten [247, 298]. Kontrajarriak

diruditen emaitza hauek, esperimentuen diseinu esperimentalean emandako ezberdintasunen ondoriozkoak direla suposatu dezakegu. Esate baterako, Yang eta kolaboratzaileek aurre-adipozitoak diferentziazioaren 6.egunerarte tratatu baitzituzten, gure kasuan baino bi egun gutxiagoko iraupena zuen tratamendua [247]. Kühn eta kolaboratzaileek berriki eginiko ikerketa batean, 10 µM kertzetinak gantz murrizketarako tendentzia bat erakusten zuela ikusi zuten [404]. Dena den, autoreek ez zuten dosi baxuagoren efekturik aztertu, gure emaitzekin konparazio zuzena egitea galaraziz. Guk dakigula, 5 µM baino dosi baxuagoekin adipozitoetan eginiko ikerketarik ez dago.

Adipogenesiko geneen espresioa 1 eta 10 µM-en aztertu genituenean, dosi baxuenarekin tratatutako aurre-adipozitoen *cebpβ* eta *pparγ* mRNA mailak eta SREBP1c proteinaren espresioa murriztu zela ikusi genuen, PPARγ-ren proteina mailak aldaketarik jasan ez bazuen ere. CEBPβ eta SREBP1c adipogenesiaren fase goiztiarrean erabat espresaturiko transkripzio faktoreak izanik, kertzetinak, 1 µM-eko dosian, adipogenesiaren inhibizioa fase honen bidez egiten zuela suposatu genuen. Dosi baxuenarekin ez bezala, 10 µM-eko dosian tratatutako zeluletan ez zen *cebpβ* genearen espresio baxuagoa antzeman, baina bai ordea *srebf1*, *pparγ* eta *lpl* mRNA mailarenarenak, diferentziazio prozesuan beranduago espresatzen diren geneenak alegia. Azpimarratzekoa da SREBP1c fase goiztiarrean espresaturiko genea den arren, proteina helduaren maila gorenena 5. eta 6. egunetan ematen dela, PPARγ erregulatzen duen transkripzio faktorea baita [405]. Gauzak horrela, SREBP1c adipogenesiaren bi faseetan garrantzi handikotzat jo daiteke. Dosiaren arabera, kertzetinak adipogenesiaren fasea ezberdinetan jardun dezakeen edo ez egiaztatzeko, diferentziazioan zeuden aurre-adipozitoak zehazki fase goiztiarrean (0-60 ordu) edo berantiarrean (60 ordu-8 egun) tratatu ziren 1 eta 10 µM-eko dosiekin. Tratamenduen iraupenaren aukeraketa, Tang eta kolaboratzaileek eginiko ikerlanaren araberakoa izan zen [362]. Zelulak dosi baxuenarekin tratatzean, triglizeridoen murrizketa soilik fase goiztiarrean tratatzean ikusi zen, dosi altuena eraginkorra izan zelarik espresuki zelulak fase berantiarrean tratazean. Behaketa honek planteatutako hipotesia baieztatu zuen, kertzetinaren adipogenesiaren kontrako efektuaren mekanismoa dosiaren araberakoa dela, hain zuzen.

Kertzetinaren 10 µM-eko dosiekin tratatutako zelulen transkripzio faktore adipogenikoen espresioari dagokionez, aurretik Ahn eta kolaboratzaileek eginiko ikerketaren emaitzen antzekoak dira, non SREBP1C eta PPARγ proteinen espresioaren murrizketa ikusi zuten. CEBPα-ren gutxiagotzea behatu bazuten ere, aipatzekoa da ez zutela 10 µM-eko dosian behatu, 50 eta 100 µM-en baizik. Areago, ikusitako murrizketa proteina mailan izan zen, eta hortaz, gure datuekin konparaketa zuzena egitea ez litzateke aproposena izango [297]. Moon eta kolaboratzaileek eginiko ikerlan batean, tipula-azalaren erauzkin batekin 3T3-L1 aurre-adipozitoetan eginiko tratamenduak *pparγ* eta *cebpα*-ren espresioa murriztu zuen [406], baina tamalez, erauzkinaren kertzetina kantitatea ezezaguna zen. Bestalde, Mosqueda-Solís eta kolaboratzaileek 15 polifenolen adipogenesiaren kontrako efektua konprobatzeko eginiko miaketa batean, non kertzetinaren efektua ere aztertu zuten, kertzetinak 1 µM-eko dositik hasita 3T3-L1 aurre-adipozitoen triglizerido mailak murriztu zituen [342].

Kertzetinak adipozito helduetan triglizeridoak murrizteko ahalmena, dosi altuenean bakarrik eragin zuen, aurre-adipozitoetan duen efektua bezain eraginkorra ez dela adieraziz. Ez dago kertzetinaren efektua adipozito helduetan aztertzen duen ikerlan gehiegirik, baina aipatzekoa da, dosi altuagoak erabiltzen badituzte ere, kertzetinak 3T3-L1 adipozito helduengan triglizeridoen murrizketa eragiten duela [407]. Ekintza-mekanismoei dagokienez, kertzetinak ez du lipolisiaren aregaotzearen bidezko lipido murrizketa eragiten. *Hsl* lipasaren espresioa murriztu bazuen ere, aldaketa hau ez zen glizerol eta GAA-en inkubazio-mediorako jariaketan islatu. Lipogenesiaren entzimen kasuan, FAS-aren genearen espresioa eta entzimaren aktibitatea murriztu egin ziren, kertzetinaren lipidoen murrizketa efektua lipogenesia urritzearen bidezkoa dela aditzera emanez, hein batean bada ere. Kontuan hartuta entzima lipogenikoak SREBP1c-ek murrizten dituela eta SIRT1-ek SREBP1c-ren desazetilazioa eragin dezakeela [408], SIRT1-aren espresioa aztertzea erabaki genuen. Izan ere, SIRT1 polifenol ugariren jomuga dela ikusi izan da, esate baterako erresberatrola eta kertzetinaren kasuan [409]. Kertzetinak SIRT1-en espresioa handitzen duela ikusi genuen, FAS-aren gene eta entzimaren aktibitatea SIRT1-SREBP1c-ren bidezkoa izan zitekeela iradokitu lezakeena.

Kertzetinaren diabetearen kontrako efektuan oinarrituta eta adipokinek haren erregulazioan duten garrantzia kontuan harturik, polifenolak adipozito helduen lau adipokinen espresioaren gain duen efektua aztertu izan zen. Derdemezis eta kolaboratzaileek SGBS zeluletan eginiko ikerketan, kertzetinaren 10 eta 25 µM-eko dosiek visfatinaren jariaketa murriztu zuten, adiponektina eta leptinaren jarioan aldaketarik ikusi ez zuten bitartean [256]. Esperimentu honetan ordea, ez zen aldaketarik ikusi azterturiko lau adipokinen espresioan, kertzetinaren horren dosi baxuak 3T3-L1 zelulen adipokinen ekoizpena aldatzeko gai ez zirela aditzera emanez.

Honekin guztiarekin, odoleko kontzentrazioaren hurbileko kertzetinaren dosiak adipogenesiaren inhibizioan eraginkorrak direla ondorioztatu daiteke. Are gehiago, efektua dosiaren menpekoa da: aztertutako dosi baxuena adipogenesiaren fase goiztiarraren gain eragiten duen bitartean, dosi altuenak fase berantiarraren gain ere eragiten du. Aldiz, adipozito helduetan, dosi altuagoren beharra dago gantzaren murrizketa eragin ahal izateko, lipogenesiaren gutxitzearen bidez ematen dena **(4. eskuizkribua)**.

Beste KF-ekin gertatu bezala, kertzetinak metabolismo sakona jasateak konposatu bioaktibo moduan erabili ahal izateko muga suposatzen du. Izan ere, dieta bidez hartutako kertzetinaren kantitate oso txikiak dira odolera eta ehunetara iristen direnak, kertzetinaren metabolitoek jatorrizko konposatuaren efektuan eragin dezaketelaren aukera mahaiaren gaineran ipintzen duen gertaera. Ikerketa batzuek diotenaren arabera, kertzetinaren metabolito ugarienak glukuronatuak dira, kantitate urriagoetan bada ere, sulfatatu eta metilatuak kantitate esanguratsuetan agertzen direlarik [277, 410, 411]. Hortaz, 3T3-L1 aurre-adipozito eta

adipozito helduetan kertzetinaren metabolito ugarienen efektua aztertu nahi izan genuen, jatorrizko konposatuarenarekin aztertzeaz gain **(5. eskuizkribua)**.

Esperimentu honetarako, aurrekoan egin genuen bezala, odoleko kontzentrazioaren hurbileko kertzetinaren dosiak aukeratu genituen, 0.1, 1 eta 10 µM hain zuzen. Metabolitoen aukeraketari dagokionez, kertzetina metabolizatu osteko ugarienak aukeratu genituen. Metilatuak (ISO eta TAM) eta glukuronatua (3G) eskuratzeko aukera izan bagenuen ere, ez zen horrela izan metabolito sulfatatuen kasuan, Salamancako Unibertsitateko "Grupo de Investigación en Polifenoles"-eko kideek sintetizatu zituztelarik.

Kertzetinaren metabolitoak adipozito helduetan frogatu zirenean, sulfatatuak izan ziren lipidoen murrizketan eraginkortasuna erakutsi zuten bakarrak, aurreko emaitzekin bat datorren kertzetinaren 10 µM-ek eragindako murrizketarekin batera (4. eskuizkribua). 3S metabolitoa 1 µM-etik gorako dosietan eraginkorra izan zen, 3S+4S metabolitoen nahasketak triglizeridoen beherapena soilik 10 µM-en eragin zuen bitartean. Azterturiko dosietan, gainontzeko metabolitoek ez zuten bioaktibitaterik erakutsi adipozito helduak tratatzerakoan. Kertzetinaren metabolitoek adipozitoen lipidoen metabolismoaren gain duten efektua aztertzen duen *in vitro* eginiko ikerlan gutxi dago bibliografian. Areago, guk dakigunez ez dago kertzetinaren metabolitoen efektua adipozito helduetan aztertzen duen ikerlanik. Hala ere, aipatzekoa da Herranz-López eta kolaboratzaileek eginikoa, non hipertrofiaturiko adipozitoak 100 µM kertzetina edo kertzetina-3-O-β-D-glukuronidoarekin (Q3GA) aztertzen ziren, 0 eta 18 ordu bitarteko tratamenduak izanik [412]. Kertzetina eta Q3GA adipozitoen barnealdera sartzen zirela aldarrikatu zuten autoreek, ondoren molekulak Q3GA eta kertzetina bilakatuko zirelarik, hurrenez hurren. Horrez gain, kertzinaren zelula barneratzea azkarragoa eta eraginkorra zela adierazi zuten, adipozitoen barneko formarik ugariena kertzetina aglikona izanik.

Kertzetinaren metabolitoek jarraitutako ekintza-mekanismoak aztertzeko helburuarekin, 10 µM-eko dosian trataturiko adipozito helduen geneen espresioa aztertu zen, dosi horretan izan baitziren molekula gehien eraginkor. Odoleko gantz azidoen kaptazioaren entzima garrantzitsuenaren mRNA mailak neurtu ziren, alegia *lpl*-renak. Bai 3S zein 3S+4S nahasketak, haren espresioa murriztu zuten, soilik 3S-rena aldaketa esanguratsua izan arren. Kultibaturiko adipozitoetan gantz azidoen hartzea neurtzea pisu gutxikoa dela badirudi ere, garrantzitsua da *in vivo* gerta litekeenaren isla izan daitekeela aintzat hartzen badugu., 3T3-L1 adipozitoek gantz azidoak modu eraginkorrean hartzeko LPL beharrezkoa dela ikusi izan da, azken batean neurketa horren hutsaltasuna zalantzan jartzen duena [413]. Ondorioz, analisi gehiagoren beharra egonik ere, 3S metabolitoak gantz azidoen kaptazioa murriztuz jarduten duela dirudi. Bestetik, zelulak 3S+4S nahasketarekin tratatu eta gero, *atgl*-ren murrizketako joera antzeman zen. Edozein modutan, glizerola eta GAA-en mediorako jarioa ez zenez aldatu, ikusitako triglizeridoen murrizketan lipolisi bideak eraginik ez zuela ondorioztatu zen.

Apoptosia gantz ehunaren homeostasiarekin oso loturik dagoela ikusi izan da. Zehazki, gorputzeko gantz masa Cas3 eta P53-rekin positiboki erlazionatzen da, BCL2 espresioarekin alderantzizko erlazioa dagoela behatu delarik. Beraz, gainpisu eta obesitateak egoera apoptotikoa eragiten du, azken batean intsulinarekiko erresistentziarekin erlazionatzen dena [414]. Hau ikusita, eta triglizeridoen metabolismoan parte hartzen duten geneen espresioa aldatu ez zenez, apoptosia lipidoen murrizketaren erantzule izan zitekeen aztertu genuen. 3S-rekin trataturiko adipozito helduen kasuan, *trp53*-ren mailak izugarri handitu ziren, apoptosiaren aktibazioa iradokitu zezakeena. Hala ere, P53 zelulen gainontzeko zenbait funtzio biologikoetan duen ezinbesteko rola kontuan harturik [415], apoptosiaren beste hainbat erregultzaile neurtu ziren. Apoptosiaren kontrako B*cl2* genearen espresioa handitu egin zen, apoptosiaren azken eragilea den *cas3* generarena aldiz murriztu zen bitartean. Gainera, 3S+4S nahasketarekin eginiko tratamenduaren ostean, soilik *trp53*-ren mRNA mailen handipena ikusi zen. Gauzak horrela, apoptosiak metabolito sulfatatuaren triglizeridoen murrizketa efektuan duen inplikazioa baztertu daiteke.

Lipogenesia ere adipozitoen triglizerido-kantitatearen erregulazioan eragiten duen bide garrantzitsua da. Triglizeridoen metaketarako, glizerola eta triglizeridoen hidrolisitik edo berriki sintetizaturiko gantz azidoen mihiztadura beharrezkoa da, DGAT1 eta DGAT2 entzimek kontrolaturiko prozesua dena. Gantz azidoen sintesian, GLUT4 garraiatzaileak harturiko glukosa gantz azidoen ekoizpenerako erabilia izan daiteke. FAS, *de novo* lipogenesiaren azken entzima mugatzailea da, malonil-Ako-tik hasita eraketa berriko gantz azidoen ekoizpenean ezinbesteko entzima hain zuzen ere. 3S metabolitoak ez zituen *fasn*-en mRNA mailak aldatu, *glut4*-renak aldiz murriztu zituelarik. Gainera, *dgat1* eta *dgat2* geneen espresioaren gutxiagotzea eman zen. Hortaz, 3S metabolitoarekin trataturiko adipozito helduen gantz metaketaren gutxitzearen erantzuna, glukosaren kaptazioa eta triglizeridoen mihiztaduran egon liteke. 3S metabolitoaren efektua 3T3-L1 adipozitoen glukosaren kaptazioan aztertzen zeneko lehen aldia bazen ere, kertzetina eta ISO-rekin trataturiko L6 miozitoetan glukosaren deskribatuta zegoen [416]. Autoreek, kertzetinaren 0.1 eta 1-nMeko dosiek eta ISO-ren 1 nM-eko dosiak glukosaren kaptazioa GLUT4-aren zelula-mintzerako translokazioaren bidez handitzen zela ikusi zuten.

3S+4S nahasketak ez zuen geneen espresioan efektu esanguratsurik eragin, 4S metabolitoak 3S metabolitoari efektu gehigarria ematen ez diola adieraziz. Gauzak horrela, aktibitate biologikoa 3S metabolitoari dagokiola ondorioztatu daiteke.

Emaitza hauekin, adipozito helduetan 3S metabolitoa kertzetina baino eraginkorragoa dela baieztatu daiteke, bere gantzaren murrizpen efektua kertzetinak baino dosi baxuagoan eragin baitzuen. Areago, lipidoen murrizketa glukosaren kaptazioan eta triglizeridoen mihiztaduran eragindako murrizketagatik ematen da, hein batean bada ere **(5.eskuizkribua)**.

KF-ek adipozitoen berritze prozesuan dituzten efektu onuragarriak direla eta, aurre-adipozitoak diferentziazio prozesuan zehar kertzetina eta metabolitoekin tratatu ziren dosi beretan. Metabolito guztietatik, soilik ISO-ren dosirik altuenak murriztu zuen triglizeridoen kantitatea, kertzetinak 1 eta 10 µM-en egiten duen antzeko. Kertzetinak 1 eta 10 µM-en eragindako murrizketa aurretik behaturiko datuekin bat dator **(4. eskuizkribua)**.

Datu hauek ikusita, ISO, kertzetina bezain eraginkorra ez dela ondorioztatu daiteke, aurre-adipozitoen adipogenesia inhibitzeko kertzetinak baino 10 aldiz dosi handiagoa behar izan baitu **(4. eskuizkribua)**.

3T3-L1 aurre-adipozitoekin eginiko ikerlan batean, 12.5 µM ISO-rekin trataturiko zelulen lipidoen kantitatea murrizten zela ikusi zen [300], gure esperimentuan eskuraturiko emaitzen antzerakoak izanik. Aldiz, Lee eta kolaboratzaileek 20 µM ISO-rekin gantz murrizketa ikusi zuten, baina ez 10 µM-ekin [322]. Beste autore batzuek ere ikusi dute nola 3T3-L1 aurre-adipozitoetan gantzaren murrizketa 50 µM ISO-rekin ematen den, baina ez 1 edo 10 µM-ekin [299]. Azpimarratzekoa da diseinu esperimentalaren diferentzia txikiek emaitza ezberdinak bideratu ditzazketela. Hori horrela, aipaturiko bi esperimentuetan tratamenduaren iraupena eta baldintza esperimentalak ezberdinak izatea, kontrajarriak diren emaitzen erantzule izan daitezke. Bestalde, gantz ehunetik eratorritako stem zelulekin eginiko ikerketa batean, Lee eta kolaboratzaileek zelulak 10 µM ISO-rekin tratatzean diferentziazioa inhibitzen zela ikusi zuten, gure esperimentuan ikusitakoarekin bat datorrena [417].

Geneen espresioa aztertzean, *pparγ*-ren murrizpena ikusi genuen, beherapen hori esanguratsua ez bazen ere. Bestetik, *cebpβ*, *srebf1* eta *cebpα* geneetan ez zen aldaketarik antzeman. Lehen aipaturiko Zhang eta kolaboratzaileen ikerketan, autoreek ISO-k adipogenesiaren gain duen efektua, dosiaren menpekoa dela aldarrikatu zuten. 12.5 µM-eko dosiak inhibizioa arina sortarazten zuen bitartean, 50 µM-ekoak berriz ,adipogenesia guztiz blokeatu zuen [300]. Hortaz, ikerketa honetan erabilitako ISO maila baxuek, adipogenesiaren inhibizio zorrotza eragiteko nahikoa izan ez zirela suposatu daiteke. Gainera, ISO-k *trp53*-ren mRNA mailak handitu zituen, 3S-ak adipozito helduetan egin zuen bezala. Ostera, *bcl2* eta *cas3* geneen espresioa analizatzean, ez zen aldaketarik antzeman, apoptosiaren aktibazioa ematen ez zela iradokituz. Adipozito helduen kasuan bezala, triglizeridoen mihiztadura neurtu zen gantza murrizteko mekanismoa izan zitekeen ikusi ahal izateko, baina tratamenduak ez zuen aztertutako gene bat ere ez aldatu.

Kertzetinaren metabolitoen aktibitate biologikoari dagokionean, 3S jatorrizko konposatuaren efektuan lagundu lezakeen metabolito moduan proposatu daiteke. Ostera, gure baldintza esperimentalekin bederen, ISO, TAM, 3G eta 4S eraginkortasunik gabeko metabolitoak dira **(5. eskuizkribua)**.

Doktorego Tesiaren mugak

Ikerketa esperimentalen gehiengoan gertatu bezala, aurkezturiko lanak hainbat muga ditu. Urte hauetan zehar ikasitakoak muga hauetaz jabetu eta horiek aitortzeko zintzoa izateko balio izan dio doktoregaiari. Muga horietako bat, 1,2 eta 3 eskuizkribuetan proteinen espresioa edo entzimen aktibitatea neurtu ez izana da. Analisi hauek, erresberatrola eta metabolitoen ekintza-mekanismoak hobe ulertzeko balio izan zezakeena onartu beharra dago. Polifenolen efektu erregulatzailea maila transkripzional eta post-transkripzionalean ematen dela kontuan hartuta, beste neurketa batzuk egin izan genitzakeen. Hala eta guztiz ere, esan beharra dago lan honen helburua erresberatrola eta bere metabolitoen efektuari buruzko lehen hurbilketa egitea izan zela. Hori horrela, efektuaren azken emaitza (gantz kantitatean aldaketak) eta horren erantzule izan zitekeen ekintza-mekanismo bat (geneen espresioa) aztertzean ardaztu genuen gure lana.

Bestalde, aipatzekoa da ehunetara iristen dena metabolitoen nahasketa dela ,eta ez ordea isolatutako metabolitoak. Nolanahi ere, lan honen helburua metabolito bakoitzak jatorrizko konposatuaren efektuari laguntzen dion aztertzea izan zela. Autore ezberdinek proposatu dutenez, KF jatorrizko konposatura leheneratu litezke zeluletan, metabolitoen bioaktibitatea arrazoi honengatik izan zitekeela iradokituz [412, 418]. Edozein modutan, Doktorego Tesi honetan, ez dugu uste honetan sakondu.

Lan honek duen beste muga bat, lorturiko emaitzak giza gorputzaren fisiologia, metabolismo eta garapenera estrapolatzearen zailtasuna da. Dena den, molekulen efektuak adipogenesian eta adipozito helduen metabolismoan zein bere aldetik aztertu nahi izan genituenez, kultibo zelularren erabilera ezinbestekoa zen. Gainera, 3T3-L1 adipozitoak adipogenesia eta obesitatearekin erlazionaturiko beste ezaugarri batzuk ikertzeko zelula-lerro egokienetarikoa da.

4. eskuizkribuan buruturiko esperimentuei dagokienez, 4S metabolitoaren efektuak zehazki ikertu izana interesgarria izan zitekeen. Are gehiago, kertzetinaren ekintza-mekanismoa adipogenesiaren fasearen araberakoa dela aintzat hartuta, 5. eskuizkribuan antzeko diseinu esperimentala egin izan genezakeen kertzetinaren metabolitoen azterketa egiterakoan.

Aipatutakoa egiteak Doktorego Tesi honen kalitatea hobetu zezakeen, baina era berean Tesi honen helburua erresberatrola eta kertzetinaren metabolitoen efektuari buruzko lehen hurbilketa egitea izan zela kontuan harturik, gure diseinu esperimentalak ez zituen aldagai hauek barnebildu. Gehiago dena, aitortutako muga hauek etorkizunean burutu litezkeen ikerlan gehiagoren abiapuntua izan daitezke, KF-en inguruan gehiago ikertzeari ateak irekiz.

DISCUSSION

Discussion

This Doctoral Thesis aimed to analyse the anti-obesity effect of resveratrol, quercetin and their main metabolites in adipose tissue. Thus, the obtained results have been divided into two parts: 1) anti-obesity effect of resveratrol and its metabolites in maturing and mature 3T3-L1 adipocytes and, 2) anti-obesity effect of quercetin and its metabolites in maturing and mature 3T3-L1 adipocytes.

To study the impact of resveratrol, quercetin and their metabolites in adipose tissue, 3T3-L1 cell line was selected, one of the most extensively characterized and used pre-adipocyte cell line, developed by clonal expansion from murine 3T3 cells [43]. 3T3-L1 cells are committed pre-adipocytes that proliferate until they are growth-arrested by contact inhibition. After the addition of an adipogenic-cocktail (DEXA-IBMX-insulin), the differentiation process starts. Two stages can be distinguished in this process: early stage or mitotic clonal expansion, and terminal differentiation. When differentiation finishes, mature adipocytes can be observed under the microscope, which contain visible lipid droplets and highly expressed adipocyte-specific genes [37,43].

Taking into account that a mixture of both pre-adipocytes and adipocytes can be found in adipose tissue, using cell lines is a very valuable tool because isolated studies, either in differentiating pre-adipocytes or in mature adipocytes, can be carried out. The adipocyte volume is a result of lipogenesis and lipolysis balance, whereas the adipocyte number is determined by the equilibrium of pre-adipocyte proliferation, differentiation and apoptosis [331]. In adult mammals, cell turnover is relatively high and in the case of humans, 10% of adipocyte renovate per year [20]. Among different cell lines, 3T3-L1 mouse fibroblasts are very useful, due to their potential to differentiate from fibroblast to mature adipocytes, they are easily handled and respond homogeneously to treatments. Moreover, and as it has been explained in the Introduction section, it is important to consider that adipose tissue expansion is given by an increase in the adipocyte number (hyperplasia) and size (hypertrophy). In view of this fact, and bearing in mind that this Doctoral Thesis was focused on the effect of polyphenols in cell turnover and lipid metabolism in both maturing and mature adipocytes, the 3T3-L1 cell line was selected to perform the experiments.

1. Anti-obesity effect of resveratrol and its metabolites in maturing and mature 3T3-L1 adipocytes

Resveratrol is a very promising polyphenol, extensively studied in the last decades due to its pleiotropic effects on several biological functions. For this reason, since 2010 “Nutrition and Obesity group” of the University of the Basque Country has focused its research on this molecule, in order to find a bioactive compound for obesity prevention and treatment. Studies carried out in animal models in our laboratory have reported beneficial effects of resveratrol in obesity and its related comorbidities, such as hepatic steatosis or inflammation. In addition, several mechanisms of action of resveratrol have been elucidated in both, in *in vivo*

and *in vitro* studies, such as lipogenesis inhibition or lipolysis and β-oxidation activation [222,237,243,244,332-337].

Beyond the observation of these positive effects, the extensive metabolism of resveratrol is a matter of concern. In fact, only a little amount of intact resveratrol reaches tissues. In this scenario, determining whether resveratrol metabolites exert any biological effect or not, is crucial. Bearing this in mind, we decided to analyse whether the main metabolites of resveratrol could be biologically active in 3T3-L1 cells. In order to achieve this objective, three experiments were carried out using 3G, 4G and 3S metabolites, which were defined as the main metabolites in the literature [208,338]. To compare the effects of these metabolites with that of the parent compound, resveratrol was included among the tested molecules. In the first experiment, resveratrol and metabolites at doses of 1, 10 and 25 μM, closer to physiological levels than those usually used in *in vitro* studies, were tested in maturing and mature 3T3-L1 adipocytes. The dose of 25 μM is one of the most commonly used in *in vitro* studies, and such low doses as 1 μM had not been tested in adipocytes before, highlighting the novelty in the present study. As first approach, the delipidating effect of 3G, 4G and 3S metabolites was analysed and compared to that of resveratrol **(manuscript 1)**. Thereupon, due to the fact that miRNAs had been shown to be implicated in the differentiation process of pre-adipocytes, we wanted to explore if some miRNA could represent a possible mechanism of action for the observed effects **(manuscript 2)**. Finally, doses that resulted effective in reducing triglyceride content in cells, were selected to analyse adipokine expression and secretion **(manuscript 3)**.

To date, resveratrol has shown anti-adipogenic effect in maturing pre-adipocytes, as well as changes in lipid metabolism in mature adipocytes [220,227,228,247,339-341]. However, no data has been reported regarding the effect of its metabolites. In order to clarify this fact, in the present study 3T3-L1 pre-adipocytes and mature adipocytes were treated with resveratrol, 3G, 4G and 3S at 1, 10 and 25 μM (**manuscript 1**). This first challenge showed that in pre-adipocytes only the dose of 25 μM of resveratrol was effective in triglyceride reduction, whereas in mature adipocytes, it was effective from 1 μM. On the other hand, it is important to highlight that glucuronide metabolites showed bioactivity in both maturing and mature adipocytes, whereas the sulfate metabolite did it only during the differentiation process.

In general terms, these results suggest that resveratrol is more effective in mature adipocytes than in maturing pre-adipocytes. Another interesting issue is that resveratrol metabolites also promote delipidating effect in adipocytes; the sulfate one in pre-adipocytes and glucuronide metabolites in both maturing and mature cells. Furthermore, it can be stated that the impact of resveratrol on mature adipocytes was stronger than that of the glucuronide metabolites, because its delipidating effect was reached at 1 μM, whereas

glucuronide metabolites needed 10-fold higher dose. By contrast, resveratrol and the sulfate metabolite showed the same effectiveness in maturing pre-adipocytes **(manuscript 1)**.

In good accordance with our results in maturing pre-adipocytes, some authors reported that doses of resveratrol around 10 µM were ineffective, though those greater than 20 or 25 µM showed delipidating effect [220,231,246]. An experiment carried out later in our group, also confirmed that 25 µM of resveratrol resulted effective in triglyceride reduction [342].

As far as we are concerned, this was the first study where the delipidating effect of resveratrol was established at such a low dose, although later studies have been carried out. In a study performed by Hu and co-workers, authors tested 1, 10, 50 and 100 µM of resveratrol and unexpectedly, they showed that 1 and 10 µM of resveratrol promoted adipocyte differentiation [343]. Even though they observed opposite results, it is important to point out that the experimental conditions were different. They employed an adipogenic cocktail that was not commonly used [43], and their treatment duration was one day shorter than ours. These variances could have been responsible for the different results obtained in both experiments. In a study conducted in 2016, Chang *et. al.* reported that the delipidating effect of resveratrol was given at such low doses from 0.3 µM (0.3-10 µM) [344]. In another study carried out by Aranaz *et. al*., authors analysed the delipidating effect of several PC at different stages of differentiation [345]. They treated cells with 10, 50 and 100 µM of resveratrol during 8 days and they observed a triglyceride reduction from 50 µM, which is in good accordance with our results. With all these results, it could be suggested that even though it seems that resveratrol has anti-adipogenic properties, the effective doses are not well-defined. Regarding resveratrol metabolites, it was the first time that their fat-lowering effect on adipocytes was tested. As far as we know, later studies have not been carried out.

In order to explain the mechanisms for the observed effects, *cebpβ* (expressed in the mitotic clonal expansion) and *pparγ*, *cebpα* and *lpl* (in terminal differentiation) gene expression was analysed. Resveratrol, 3G, 4G and 3S reduced the expression of *cebpβ,* while that of *pparγ*, *cebpα* and *lpl* was decreased only by the 3S metabolite.

Therefore, whereas resveratrol, 3G and 4G metabolites block the early stages of adipogenesis, 3S is the only metabolite that inhibits the whole process of adipogenesis **(manuscript 1)**.

Surprisingly, resveratrol and its metabolites do not always target the same genes, which means that their mechanisms of action should be different. For instance, although strong tumor necrosis factor α (TNFα)

enhancing effect was associated to resveratrol, this effect was not described for 3G and 3S metabolites [346]. Moreover, Ruotolo *et. al.* reported that among resveratrol metabolites, only 3S was able to antagonize an estrogen receptor [347]. Another good example was the study conducted by Calamini *et. al*, where they observed that resveratrol, 3G, 3S and resveratrol-4´-*O*-sulfate (4S) inhibited some enzymes involved in oxidative stress in a different manner [230].

With regard to the effect of resveratrol on adipogenesis, reported results in the literature are controversial. Kang *et. al.* incubated 3T3-L1 pre-adipocytes with 10, 20 and 40 µM of resveratrol during 2, 4 and 6 days, and they showed anti-adipogenic effect of the compound in a dose and treatment-period dependent manner, resulting the dose of 10 µM enough to inhibit adipogenesis. Besides, they observed that resveratrol reduced in a dose-dependent manner the expression of several adipogenic transcription factors such as CEBPβ, PPARγ, CEBPα and FABP4 [348]. However, in a study performed by Chen *et. al.* with a similar design where 3T3-L1 pre-adipocytes were treated at 10, 20, 40 and 80 µM for 24 and 48 hours, authors did not observe the anti-adipogenic effect of resveratrol at such low doses, being the doses effective from 20 µM [231]. The mentioned results are similar to those obtained in our experiment. In another study conducted by Li *et. al.,* authors observed that 25 µM of resveratrol reduced the lipid content of 3T3-L1 cells treated from day 0 to day 8 of differentiation, which is in good accordance to our results [221]. In relation to the expression of *pparγ* and *cebpα* controversial results have been reported. Although several studies have observed that resveratrol was able to reduce their expressions [220,247], this fact has not been stated in other studies with similar experimental designs, unlike the results yielded in our study [246,349,350]. In view of the obtained results in the aforementioned studies, it seems that at doses around 20-25 µM *cebpα* and *pparγ* expressions are reduced. Thus, when these doses are used in similar experiments, slight differences such as the treatment duration or whether gene or protein expressions have been quantified, could be enough to observe different results.

With the aim of analysing the adipogenic regulation in depth, and taking into account that miRNAs have been defined as important adipogenesis controllers, the potential implication of miRNAs in the anti-adipogenic action of resveratrol and its metabolites was analysed (**manuscript 2**). The first evidence of miRNA implication in adipogenesis was described in Drosophila by Xu *et. al*., where they observed that deletion of miR-14 supposed an increase in lipid content. Opposite results were obtained when miR-14 was overexpressed [351]. After that finding, 3T3-L1 cell line has been extensively used in order to analyse miRNA expression profile during adipogenesis, mainly by microarray analysis and subsequent validations [352]. Nevertheless, there is not a universal microarray platform, leading to a disagreement in the real miRNA candidates in adipogenesis regulation. In a study conducted in 2006, Kajimoto and co-workers identified 21 miRNAs that were up or down-regulated during adipocyte differentiation. Specifically, they compared the miRNA profile in pre-adipocytes at day 0 of differentiation and in mature adipocytes at day 9, when cells had visible lipid droplets [56]. Among

others, miR-21, miR-17-92 cluster, miR-30, miR-103, miR-125, miR-143 and miR-378 were the most characterized up-regulated miRNAs in adipocyte differentiation. On the other hand, the implication of let-7 family, miR-27a, miR-27b, miR-33, miR-155 and miR-221/222 family in adipogenesis inhibition has been stated in numerous studies as well [68].

Little is known about the miRNAs implication in the effect of resveratrol on the main adipogenesis regulators *pparγ*, *cebpα* and *cebpβ,* and the effect of resveratrol metabolites has not been studied yet. Due to this fact, we performed a detailed analysis in this field. As first step*,* computational predictions of miRNA candidates for mentioned genes described in the literature were analysed. It is important to bear in mind that miRNAs are small molecules that can be paired with the 3´UTR region of their target mRNA. The minimum required for this pairing is only a region of 6 nucleotides, resulting in a great number of potential targets for each miRNA. Therefore, it is crucial to define the correct criteria for miRNAs selection. In our case, two inclusion criteria for miRNA were considered: 1) they had to be validated or predicted by five algorithms in mirWalk database and, 2) that they had to be reported in Pubmed search after using "miR+adipogenesis" in the browser.

CEBPβ, as well as CREB, have been defined as validated targets for miR-155 [58,353-356]. Validation of CEBPβ as a target of miR-155 was carried out for the first time by Costinean and co-workers, who measured CEBPβ protein expression in transgenic mice lacking miR-155 [58]. Furthermore, they observed 53% less luciferase activity in the presence of miR-155 mimic. Besides, Liu *et. al.* reported that the up-regulation of miR-155 after TNFα treatment of 3T3-L1 cells resulted in a down-regulation of CEBPβ and CREB protein expressions*,* which in turn led to adipogenesis diminution [353]. In addition, the authors verified that the adipocyte differentiation reduction induced by TNFα was reverted by miR-155 inhibition. In another study carried out by Chen *et. al.,* they established the configuration of a feedback loop between miR-155 and CEBPβ that regulates the browning of white adipocytes [354]. Taking into account that *cebpβ* was a validated target of miR-155 and the implication of its regulation on adipogenesis, we wanted to know if the anti-adipogenic effect of resveratrol and its metabolites were carried out via miR-155-*cebpβ*. As 3S treatment did not modify miR-155 levels, we discarded this mechanism of action for the sulfate metabolite. However, miR-155 levels were increased after resveratrol, 3G and 4G treatment, so it seemed that their effect on *cebpβ* expression was carried out via miRNA. To confirm this issue, we transfected 3T3-L1 pre-adipocytes with anti-miR-155 in the presence or absence of resveratrol, and we observed that when miR-155 was silenced, neither CEBPβ gene nor protein expression were reduced by the treatment.

In view of the obtained results, we demonstrated that the inhibitory effect of resveratrol, and presumably that of glucuronide metabolites, is, at least in part, via up-regulation of miR-155 (**manuscript 2**).

Although up-regulation of miR-155 by resveratrol has been extensively studied in other cell types such as monocytes and macrophages, establishing its implication in the anti-inflammatory, anti-atherosclerotic and anti-hypertensive actions [357-360], is important to point out that this was the first time it was demonstrated in adipocytes.

As changes in miR-155 did not explain *cebpβ* expression modification by 3S, other potential regulatory pathways were analysed. It is thoroughly known that CEBPβ and CEBPδ are the most important transcription factors in the early stage of adipogenesis [361,362], as well as other transcription factors that have been identified [24]. However, the onset of adipogenesis is not clearly stated. Reusch and co-workers, based in a previous study performed in their laboratory, hypothesized that cAMP-induced CREB phosphorylation could be a key at the beginning of adipogenesis [23]. With this idea, they transfected 3T3-L1 pre-adipocytes in order to up-regulate or down-regulate CREB expression and they assessed its effect on adipogenesis by measuring triglyceride accumulation, cell morphology and the expression of later adipogenic markers, such as PPARγ and fatty acid binding protein (FABP). They showed that without other external stimuli, CREB over-expression was enough to initiate adipogenesis, whereas its inhibition totally blocked the differentiation process. Therefore, in view of its importance, CREB expression was measured in the present study. After 3S treatment, mRNA levels of *creb* were decreased, suggesting its potential implication in *cebpβ* reduction. Moreover, KLF5 transcription factor is induced by *CEBPβ* and contributes to PPARγ activation [39]. The observed *klf5* down-regulation after the treatment with 3S and 4G, led us to think that 3S metabolite reduced adipogenesis by acting in the early stage of adipogenesis via *creb1* and *klf5*. Similarly, the 4G metabolite effect could be due to miR-155 up-regulation and consequent *cebpβ* reduction, and through a direct effect on *klf5*. There is no data about the effect of 3S or 4G metabolites on KLF5 expression, but a direct effect of resveratrol on it has been indicated by others [363,364] . Although in this study no effect on *klf5* expression was observed after resveratrol treatment, it is important to consider that the cell type, the used dose and treatment duration were greatly different in the aforementioned research works.

Among the candidate miRNAs targeting *cebpα* (miR-326, miR-31, miR-144, miR-205 and miR-224) and *pparγ* (miR-27a, miR-27b and miR-130b), none of them were modified after the treatment of 3S. This fact suggested that the down-regulation of both genes carried out by 3S was not mediated by miRNAs. SREBP1c and LXRα have been described as regulatory genes of PPARγ [365,366], and thus, their expression was measured. Both genes were reduced after 3S treatment. Therefore, it can be postulated that the reduction in *pparγ* expression without changes in miR-27a, miR-27b and miR-130b expression could be explained, at least in part, by this mechanism. Even though there is no data referring to 3S metabolite, it is important to point out that SREBP1 and LXRα have been described as therapeutic targets for resveratrol and other polyphenols such as curcumin [55,367-371]. Furthermore, it should be taken into account that the cooperation mechanism between PPARγ and CEBPα is not fully understood. It has been addressed that their synchronous expression

is necessary for the correct differentiation of adipocytes and that they are sufficient to initiate it by themselves [372]. So, these data suggests that *pparγ* leads to a reduction in *cebpα* without changes in the expression of those miRNA which resveratrol could act through.

Unlike resveratrol and glucuronide metabolites, the anti-adipogenic effect of 3S metabolite was not mediated via miRNA **(manuscript 2)**.

Regarding the mechanisms by which resveratrol and metabolites reduced triglycerides in mature adipocytes treated for 24 hours (**manuscript 1**), gene expression of cells treated with the compounds at 10 µM was analysed. This dose was selected because it was the lowest one at which resveratrol and glucuronide metabolites reduced triglycerides. In order to verify whether the lipolytic pathway was activated, the expression of the two main lipases was analysed. Resveratrol increased mRNA levels of *atgl*, and 3G and 4G showed a tendency which is in good accordance with the data previously reported by our group. Lasa *et. al*. treated murine 3T3-L1 and human SGBS adipocytes, as well as primary adipocytes from ATGL KO and HSL KO mice with resveratrol [222]. They observed that resveratrol stimulated FFA release, but not that of glycerol, in 3T3-L1 cells in both basal and isoproterenol-stimulated conditions, and exclusively in isoproterenol-stimulated conditions in SGBS adipocytes. In primary adipocytes, they observed that FFA release was maintained in HSL KO mice adipocytes, but not in ATGL KO mice adipocytes. With these results, authors claimed that resveratrol regulates the lipolytic pathway acting mainly on ATGL at transcriptional and post-transcriptional levels. Appart from that, in the present study, 4G metabolite increased the expression of *hsl*. Despite the results obtained by Lasa *et. al.* in other studies carried out in our laboratory using rats, resveratrol increased *hsl* gene expression without changes in *atgl* gene expression [243,244]. Thus, although the lipolysis-enhancing effect of resveratrol has been reported by several authors [222,228,243,244,373], the mechanism of action still remains confusing. Finally, it is worth mentioning the study conducted by Chang *et. al*. in 3T3-L1 adipocytes [344]. Surprisingly, they observed that resveratrol, at doses ranging from 0.03 to 10 µM, had an inhibitory effect on lipolysis. In this study, authors hypothesized that the enhancing or inhibitory effect of resveratrol on lipolysis could be dose-dependent.

What's more, in the present work resveratrol increased *cpt-1b* expression and 3G reduced that of *fasn*, while *lpl* and *acc* levels were not modified by any of the molecules. The diminution of lipogenesis has also been suggested for resveratrol in other studies [221,374]. In an experiment conducted by Li *et. al*., authors observed that 25 and 50 µM of resveratrol reduced the triglyceride accumulation of cells after their lipogenic-stimulation with oleic acid [221]. Finally, 3S metabolite did not modify gene expression on mature adipocytes, which is in good accordance with the lack of effect observed in triglyceride reduction.

In view of the data from the present study, it seems that resveratrol and 4G metabolite reduced lipid content by stimulating lipolysis. Resveratrol could also enhance the oxidative activity of adipocytes, and the effect of 3G was carried out by a diminution of lipogenesis **(manuscript 1)**.

As it has been previously revealed, one of the most described mechanisms of resveratrol is its capacity to activate SIRT1, which in turn deacetylates and activates PGC1α and other downstream genes [223,227,375]. Under these experimental conditions, resveratrol and glucuronide metabolites were able to up-regulate *sirt1*. However, although all molecules tended to increase *pgc1α* levels, only resveratrol did so in a significant manner.

Thus, SIRT1 activation ability described for resveratrol was also carried out by the three analysed metabolites. Nonetheless, the activation of PGC1α resulted only for resveratrol **(manuscript 1)**.

Several studies have reported that some beneficial effects of resveratrol, such as body fat reduction or insulin sensitivity enhancement, could be mediated by changes in adipokine production [238,376-378]. Since promising results of resveratrol metabolites in adipocyte fat accumulation were obtained, we wanted to know whether these molecules modified the adipokine expression and secretion, and thus, if they could be considered as useful tools to improve insulin sensitivity and glucose homeostasis **(manuscript 3)**. For this purpose, the effective doses from the first study (manuscript 1) were chosen (25 µM in differentiating adipocytes and 10 µM in mature ones) and adipokine gene expression and secretion were analysed. As explained before, these doses are close to those achieved in plasma in *in vivo* studies and in clinical trials after resveratrol supplementation [216,218].

In the last decades, several adipokines involved in glucose homeostasis and insulin sensitivity have been identified, and some of them could be regulated by resveratrol. Adiponectin and leptin have been considered as the most characterized adipokines targeted by resveratrol [252,253,376]. Even though the involvement in obesity and glucose homeostasis of visfatin and apelin has have been confirmed [156,167,379,380], scarce data exists in the literature concerning their role in the effect of resveratrol [256].

With regard to adipokines, an unexpected pattern was repeated in the present work: adiponectin, leptin, visfatin and apelin gene expression were modified after cell treatments with resveratrol and the metabolites, but few modifications were observed in adipokine secretion. These discrepancies between adipokine expression and secretion were also observed by other authors. One possible explanation to this fact

could be related to the different agents used in *in vitro* studies. Wang *et. al*. analysed the expression and release of several proteins regulated by insulin in 3T3-L1 adipocytes [381]. They showed that insulin inhibited adiponectin secretion, even though its mRNA levels were up-regulated. Moreover, in a study carried out using canine adipocytes, Ryan and co-workers showed that DEXA and rosiglitazone interfered in adipokine expression and secretion [382]. DEXA reduced IL-6 mRNA and released levels, but rosiglitazone reduced IL-6 mRNA levels without changes in IL-6 concentration in the media. On the other hand, there are several molecular mechanisms that could explain the different modulation of gene and protein expression, such as alternative mRNA splicing, polysomic activation of mRNA, protein turnover or the specific proteolytic processing. In addition, plasmatic membrane translocation, the lysosome-dependent pathway and the microvesicle-dependent pathway, among others, are regulatory factors of protein secretion that could justify the observed discrepancies [383-386]. Along with these mechanisms, we speculated that the different half-life of mRNA and proteins could disguise the real effect of cell-treatments [387]. In order to understand more deeply the mechanisms by which resveratrol and its metabolites modified adipokine production, more measurements at other molecular levels could have been done (i.e. protein expression). Besides, the potential molecular mechanisms that led to the observed discrepancies could have been demarcated. However, this Doctoral Thesis focussed on elucidating whether resveratrol metabolites exerted any effect on adipokine expression and secretion, and therefore, only a first approximation was carried out.

The increase in adiponectin gene expression observed in pre-adipocytes treated with resveratrol and the three analysed metabolites are close to results reported by others. In 2011, Wang. *et. al.* observed that resveratrol increased adiponectin expression in 3T3-L1 adipocytes and this mechanism was SIRT1-independent [242]. Moreover, resveratrol could reverse, through PPARγ activation, the inhibition of adiponectin expression and secretion in adipocytes resulted from TNFα up-regulation in obesity or diabetes states [388-391]. Nevertheless, several studies carried out using animal models reported opposite results, and thus, it is difficult to establish whether adiponectin is a real target for resveratrol [334,392-394].

In the case of leptin, resveratrol and metabolites exerted a significant reduction in both the gene expression and the release to the media in mature adipocytes, as well as in gene expression in maturing pre-adipocytes treated with resveratrol. These results are in accordance with a study performed by Szkudelska and co-workers, where they reported a diminution on leptin secretion in isolated rat adipocytes treated with 62.5 µM of resveratrol [234].Is worth considering the fact that some studies have described a relationship between adipogenic genes and adipokine production. Specifically, leptin expression has been defined as a target for several transcription factors, such as CEBPβ [395]. Thus, we postulated that the reduced leptin expression observed in maturing pre-adipocytes treated with resveratrol could be mediated by the down-regulation of *cebpβ* expression. With regard to other researches, in a recent study conducted by Zhao *et. al*., authors observed that 120 mg/kg/day of resveratrol normalized the increase in leptin plasma levels reported

in rats fed with a high-fat diet [396]. By contrast, Derdemezis *et. al.* did not show an influence of resveratrol in adiponectin and leptin secretion in SGBS cells [256]. These authors did not measure gene expression and thus, it is not possible to make a real comparison with our results. The modulatory effect of resveratrol on adiponectin and leptin expression has been reported in animal models [392,393]. However, and taking into account that in a recent meta-analysis Tabrizi and co-workers claimed that resveratrol intake does not affect adiponectin and leptin levels in humans, it is not clear if the observed effects in cell and animal models could be extrapolated to humans [397]. Therefore, more studies are needed in order to verify if the beneficial effects of resveratrol are mediated by adiponectin and leptin.

Resveratrol and its metabolites, with the exception of 3S, increased visfatin mRNA levels in maturing pre-adipocytes. In the case of mature cells, the analysed metabolites, unlike resveratrol, promoted the increase. Similar results were observed in the case of apelin. As mentioned before, little evidence exists concerning the relationship between the polyphenol and these two adipokines. Derdemezis *et. al.* tested the resveratrol effect on visfatin secretion in SGBS cells, and they observed that it was reduced after 24h and 48h treatment with resveratrol at 10 and 25 μM [256]. With regard to apelin, in a study published in 2016, the authors did not find differences in apelin gene expression in the adipose tissue of diabetic rats supplemented with 1, 5 and 10 mg resveratrol/body weight/day for 30 days [241].

Taking the results of this experiment into account, it could be suggested that the beneficial effects of resveratrol on energy metabolism and glycaemic control could be, at least in part, via regulation of adipokine production. Furthermore, resveratrol metabolites could contribute to this effect **(manuscript 3)**.

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2. Anti-obesity effect of quercetin and its metabolites in maturing and mature 3T3-L1 adipocytes

Quercetin has also been proposed as a good prospect molecule due to its biological properties, such as anti-oxidant and anti-inflammatory functions; its activity against cancer; and probably due to its best-described anti-diabetic function. However, the literature is limited regarding the anti-obesity effect of the molecule. For this reason, the "Nutrition and Obesity" group also focussed their research on this molecule. In a study conducted using Wistar rats fed with a high-fat and high-sucrose diet supplemented with 30 mg/kg body weight/day of quercetin for 6 weeks, Arias *et. al.* observed improvement on insulin resistance parameters even though body fat was not significantly reduced. Data in the literature indicated that quercetin exerted body fat-lowering effects when it was administered during longer periods than the ones used in this study, so authors hypothesized that the treatment length could be the reason for the lack of effects [284]. It is important to highlight that other studies carried out in our group attributed other beneficial effects to quercetin when it was administered in combination with resveratrol, such as gut microbiota composition

amelioration [398], changes in adipose tissue triglyceride metabolism and liver fat accumulation prevention [399,400].

Diverse mechanisms have been attributed to quercetin for its fat-lowering effect *in vitro,* such as lipolysis induction, lipogenesis reduction and adipogenesis inhibition [297,303,305]. Nevertheless, the most important limitation of these studies was that they were carried out with high doses ranging from 10 to 500 μM [247,297,303,305,401]. These doses are significantly farther from those that can be achieved in plasma and tissues after quercetin supplementation in animals or humans [270,271,402]. In this scenario, we aimed to analyse the effect of quercetin in triglyceride accumulation in maturing and mature 3T3-L1 adipocytes, as well as to describe the mechanisms underlying these actions at lower doses than those used in other studies and thus, at doses that are closer to plasma concentrations (0.1-10 μM) **(manuscript 4)**.

3T3-L1 maturing adipocytes were treated during the whole adipogenesis from 0.1 to 10 μM of quercetin. All the doses tested reduced triglyceride content, although 0.1 μM and 0.5 μM did not reach statistical significance. The observed triglyceride reduction after 10 μM quercetin treatment is in good accordance with the study of Ahn. *et. al*., where they observed the same effect at 10, 50 and 100 μM [297]. In another study, Bae *et. al.* reported delipidating effects in 3T3-L1 pre-adipocytes by using 3.3-6.6 μM of quercetin, which is comparable to the reduction observed with 5 μM in the present study [403]. Contrary to our results, Lee *et. al*. and Yang *et. al.* observed that 5, 10 and 20 μM or 12.5 μM of quercetin, respectively, were not effective in lipid content reduction [247, 298]. The divergence between these and our results could be due to the differences in the experimental design of the study, due to the fact that in the case of Yang *et. al.* they treated pre-adipocytes only until the 6th day of differentiation [247]. In a recent study conducted by Kühn *et. al.*, authors observed that 10 μM of quercetin showed a tendency in fat reduction [404]. Notwithstanding the lack of analysis at lower doses, a direct comparison of the effect at low doses cannot be made. As far as we are aware, there is no data in the literature regarding adipocyte-treatments at lower doses than 5 μM.

When adipogenic gene expression was analysed at 1 and 10 μM, we observed that after pre-adipocytes treatment with the lowest dose, mRNA levels of *cebpβ* and *pparγ* and SREBP1c protein levels were reduced, but PPARγ protein expression remained unchanged. Taking into account that both CEBPβ and SREBP1c are transcription factors that are highly expressed in the early stage of adipogenesis, we supposed that quercetin at 1 μM could block the differentiation process at this point. In contraposition to this proposal, when cells were treated with 10 μM of quercetin, *cebpβ* gene expression was not down-regulated and lower mRNA levels of *srebf1*, *pparγ* and *lpl*- genes that are late-expressed- were found. It is important to lighten that although SREBP1c is expressed in the early stage of differentiation, the mature protein maintains its high expression in the 5th and 6th day, and it acts over PPARγ [405]. Thus, SREBP1c is considered crucial in both the stages of adipogenesis. In order to confirm whether quercetin could act differently on adipogenesis process

depending on the dose, maturing pre-adipocytes were treated specifically in the early (0-60 hours) or the late stage (60 hours-8 days) of differentiation at 1 and 10 µM. Stage-duration was selected according to Tang *et. al.*, [362]. The lowest dose only reduced triglycerides after the treatment in the early stage, whereas the highest dose was effective at the late stage, which verified our hypothesis.

Regarding the effect of 10 µM of quercetin in the expression of adipogenic transcription factors, this study presents similar results to the previously mentioned study performed by Ahn and co-workers, where they observed down-regulation of SREBP1c and PPARγ protein expressions. Although reduction of CEBPα was shown, it occurred at 50 and 100 µM, but not at 10 µM. Besides, the reduction they reported was at protein level, and thus, a direct comparison with our data could not be made [297]. In a study performed by Moon and co-workers, an onion-peel extract reduced *pparγ* and *cebpα* expressions in 3T3-L1 pre-adipocytes [406]. Unfortunatety, the quercetin dose in the extract was unknown. Additionally, Mosqueda-Solís *et. al.* conducted a screening of fifteen polyphenols, including quercetin, in order to check their anti-adipogenic activity. Similarly to the present study, quercetin reduced the triglyceride content starting from 1 µM [342].

Quercetin was less effective in reducing triglyceride content in mature adipocytes because its delipidating effect was reported only at the highest dose. There are not many studies performed in quercetin in mature adipocytes, but the available ones, despite using higher doses, demonstrate that quercetin exerts a fat-lowering effect in 3T3-L1 mature adipocytes [407].

With regard to the mechanisms of action, quercetin did not act by increasing lipolysis. Surprisingly, *hsl* gene expression was reduced; nevertheless this change was not reflected in glycerol and FFA release to the media. In relation to lipogenic enzymes, *fasn* gene expression and enzyme activity were down-regulated suggesting that the effect of the molecule was, at least in part, through lipogenesis reduction. Considering that lipogenic enzymes were down-regulated by SREBP1c and that SIRT1 could induce SREBP1c deacetylation, we decided to analyse SIRT1 expression [408]. In fact, SIRT1 has been reported as a potential target for several polyphenols, such as resveratrol and quercetin [409]. We observed that quercetin increased the expression of SIRT1, postulating that FAS gene and the enzyme activity down-regulation could be via SIRT1-SREBP1c.

By virtue of the attributed anti-diabetic effect to quercetin, and considering that adipokines have been reported to be involved in that regulation, the expression of four adipokines was analysed in mature adipocytes. In a study carried out by Derdemezis *et. al.* in SGBS cells, visfatin secretion was reduced in cells treated with 10 and 25 µM of quercetin, although no changes were reported in adiponectin and leptin secretion [256]. However, in the present study, no changes were observed in none of the analysed adipokines, suggesting that these low doses of quercetin could be insuficient to modify adipokine production in 3T3-L1 cells.

With these data, it could be concluded that quercetin, in the range of serum concentrations, is a very effective molecule in adipogenesis inhibition. Moreover, this effect is dose-dependent: whereas the lowest dose acts in the early stage of adipogenesis, the highest one also modifies the later ones. By contrast, in mature adipocytes, higher doses are needed to reduce lipid content, which seems to be carried out by lipogenesis reduction **(manuscript 4)**.

As it occurs with other PC, the rapid metabolism of quercetin is a matter of concern for its utilization as a bioactive compound. In fact, after oral ingestion, a very little amount of quercetin reaches plasma and tissues, and thus, the potential contribution of quercetin metabolites to the biological activity of the parent compound needs to be elucidated. Data in the literature assert that glucuronide metabolites are the most abundant ones, whereas sulfated and methylated ones appear in lower concentrations [277,410,411]. Hence, we aimed at analysing the effect of the most abundant metabolites in maturing and mature 3T3-L1 adipocytes and to compare these effects with that of the parent compound **(manuscript 5)**.

For this experiment, as we did in the previous study, doses in the range of serum concentrations were selected, 0.1, 1 and 10 μM. Regarding the selection of quercetin metabolites, the most abundant ones were chosen. Among them, methylated (ISO and TAM) and glucuronated metabolites (3G) were commercially available, but not sulfate metabolites. Thus, the latter ones (3S and 4S), were chemically synthesized by the "Grupo de Investigación en Polifenoles" of the University of Salamanca.

When quercetin metabolites were tested in mature adipocytes, only sulfate ones had a triglyceride-lowering effect, apart from that of quercetin at 10 μM, which is in good accordance with our previous results (manuscript 4). 3S metabolite showed a delipidating effect from 1 μM, whereas the 3S+4S mixture reduced triglyceride content only at 10 μM. At tested doses, none of the other metabolites reduced triglyceride content in mature adipocytes. Very scarce data exists in the literature regarding the effects of quercetin metabolites in lipid metabolism of adipocytes cultures. As far as we know, no studies are available concerning the effect of quercetin metabolites in lipid metabolism in mature adipocytes. However, it is important to mention an interesting study conducted by Herranz-Lopez and co-workers, where hypertrophied adipocytes were treated with 100 μM of quercetin or quercetin-3-O-β-D-glucuronide (Q3GA) at different incubation periods ranging from 0 to 18 hours [412]. The authors stated that both quercetin and Q3GA were absorbed by 3T3-L1 adipocytes and metabolized to Q3GA or quercetin respectively. In addition, they reported that the absorption was faster and more efficient for quercertin, being the quercetin aglycone the most abundant form inside adipocytes.

In order to analyse the underlying mechanisms of action for quercetin metabolites, the gene expression of mature adipocytes treated at 10 μM, the dose at which more molecules showed delipidating

effects, was measured. mRNA levels of *lpl*, a key enzyme for circulating fatty acid uptake, were down-regulated by both 3S and 3S+4S, but only the reduction exerted by 3S reached statistical significance. In spite of the fact that the fatty acid uptake seems to be inappropriate in cultured adipocytes, this fact is an important issue because it could be a reflection of what would take place *in vivo*. Furthermore, it has been observed that LPL is required for an effective fatty acid uptake in 3T3-L1 adipocytes, questioning the irrelevance of its measurement in cell cultures [413]. Consequently, even though further analysis are needed, it seems that 3S metabolite could act via reduction of fatty acid uptake. On the other hand, after the treatment with 3S+4S, we observed a tendency in the reduction of *atgl*. However,glycerol and FFA release to the media remained unchanged, and thus we believed that this pathway was not implicated in the observed triglyceride reduction.

It has been reported that apoptosis is closely related to adipose tissue homeostasis. More concretely, body fat mass has been positively correlated with Cas3 and P53 expression, whereas an inverse association with BCL2 expression has been observed. Therefore, overweight and obesity lead to a pro-apoptotic situation, which in turn contributes to an insulin resistance situation [414]. In view of this association, and as no changes in genes from the triglyceride metabolism were observed, we decided to evaluate whether apoptosis would be a possible explanation for the reported triglyceride reduction. In mature adipocytes treated with 3S, the expression of *trp53* was greatly increased, which could suggest the activation of the apoptotic pathway. Nevertheless, due to the fact that P53 is involved in other several biological functions in cells [415], other apoptotic regulators were measured. The expression of the anti-apoptotic *bcl2* gene increased and that of *cas3*, the final inductor of apoptosis, was reduced. In addition, after 3S+4S treatment, only an enhancement of *trp53* mRNA levels was observed. Therefore, the implication of apoptosis in the delipidating effect of the sulfate metabolite could be discarded.

Lipogenesis is another important route that contributes to the balance of triglyceride content in adipocytes. For triglyceride accumulation, the assembly between glycerol and fatty acids coming from triglyceride hydrolisis or newly-synthesized ones is required, which is controlled by DGAT1 and DGAT2 enzymes. In the synthesis of fatty acids, the uptaken glucose by the GLUT4 transporter can be used as a substrate for fatty acid production. FAS is the last step-enzyme in *de novo* lipogenesis that converts fatty acids from malonyl-CoA, so it is crucial for the synthesis of new fatty acids. 3S metabolite did not modify neither *fasn* mRNA levels, but it decreased *glut4* gene expression. Moreover, a diminution on *dgat1* and *dgat2* gene expression was observed. As a result, the reduction in fat accumulation observed in mature adipocytes after the treatment with 3S could be due to a reduction in glucose uptake and triglyceride assembly. This was the first time where 3S effect on glucose uptake in 3T3-L1 adipocytes was reported. However, related to lipogenesis, and more specifically to glucose uptake, a recent study with quercetin and ISO in L6 rat myotubes was conducted [416]. The authors demonstrated that 0.1 and 1 nM of quercetin and 1nM of ISO increased glucose uptake by the translocation of GLUT4 to the cell surface.

No significant effect of the 3S+4S mixture in gene expression regulation was observed, indicating that 4S did not confer an additional effect, hence the biological activity could be exclusively attributed to 3S metabolite.

With these results, it can be affirmed that 3S metabolite is more effective than quercentin in mature adipocytes, because its delipidating effect is exerted at a lower dose than that of quercetin. Furthermore, the lipid-lowering effect is, at least in part, caused by a diminution in glucose uptake and triglyceride assembly **(manuscript 5)**.

Due to the potential beneficial effects of PC in adipocyte turnover, maturing pre-adipocytes where treated during the differentiation process with quercetin and its metabolites at the same doses. Among all metabolites, only the highest dose of ISO reduced triglyceride content in pre-adipocytes, similar to the triglyceride reduction observed in cells treated with 1 and 10 μM of quercetin, which is in good accordance with what has been previously reported in manuscript 4.

It could be stated that ISO was less effective than quercetin inhibiting adipogenesis in maturing pre-adipocytes, due to the fact that it needed a 10-fold higher dose **(manuscript 5)**.

In a study conducted in 3T3-L1 pre-adipocytes, ISO was able to reduce lipid content at 12.5 μM, a similar dose to that employed in our study [300], whereas Lee *et. al.* observed delipidating effects at 20 μM, but not at 10 μM [322]. The lipid content reduction effect of ISO in differentiating 3T3-L1 adipocytes at 50 μM, but not at 1 or 10 μM, was also described by other authors [299]. It is important to note that slight differences in the experimental design may yield different results, so the different period of treatment and the experimental conditions in which cells were mantained and differentiated in both studies, could be responsible for the observed discrepancies. On the other hand, in a study performed with adipose tissue-derived stem cells, Lee *et. al.* observed that 10 μM of ISO inhibited their differentiation, which is in good accordance with our study [417].

After gene expression analysis, we observed a great down-regulation of *pparγ*, although no statistical significance was reached. In addition, mRNA levels of *cebpβ*, *srebf1* and *cebpα* remained unchanged. In the above-mentioned study of Zhang and co-workers, the authors postulated that the impact of ISO in adipogenesis is dose-dependent, considering that the dose of 12.5 μM reduced adipogenesis but that of 50 μM totally blocked it [300]. Thus, the low doses of ISO used in this study might not be enough for a dramatical

reduction of adipogenesis. Moreover, ISO increased the mRNA levels of *trp53*, as 3S did in mature adipocytes. However, when the expression of *bcl2* and *cas3* was analysed, no changes were observed, suggesting that apoptosis was not activated. As in mature adipocytes, the triglyceride assembly was measured in order to explain the delipidating effect of the metabolite. Unfortunately, the treatment did not modify any of the analysed genes.

Concerning the biological activity of quercetin metabolites, 3S can be proposed as a potential contributor to the body-fat lowering effect of quercetin. By contrast, under our experimental conditions, ISO, TAM, 3G and 4S proved to be ineffective metabolites **(manuscript 5)**.

Limitations of the present Doctoral Thesis

As it occurs with most of the experimental studies, the present work has some limitations. At this point, the adquired trainning as a researcher leads to the awareness and acceptance of some of them. One of the constrains is that protein expression or enzyme activities were not analysed in manuscripts 1, 2 and 3, data that could have helped to understand in more depth the mechanisms of action of resveratrol and its metabolites. Due to the fact that the regulatory action of polyphenols is exerted at both transcriptional and post-transcriptional levels, other measurements could have been carried out. However, this work was aimed at giving a first approximation to resveratrol and its metabolites effects, and thus, we focused on their final effects (changes in lipid content) and on just one of their mechanisms of action (gene expression).

It is important to consider that a mixture of metabolites does reach target tissues but not isolated ones. It is worthtaking in mind that the aim of the present work was that of analysing the potential contribution of each metabolite to the described effect of the parent compound. In this line, several authors have proposed that PC metabolites could be regenerated to the parent compound in target cells, suggesting that the potential bioactivity of metabolites could be due to this reason [412,418]. In the present Doctoral Thesis, this field has not been adressed.

Another limitation of the present work is the difficulty to extrapolate the obtained results to whole-body phisiology, metabolism and development. However, and as our target was to analyse the effects on adipogenesis and mature adipocytes´s biology separately, cell cultures had to be used. It turns out that the 3T3-L1 is a very useful cell line to the study of adipogenesis and other obesity-related features.

In relation to the experiments performed in manuscript 4, it would have been interesting if we had tested the effect of 4S metabolite specifically. Taking into account that quercetin acts differently depending on the adipogenic stage in which cells are treated, a similar experiment could have been performed to test the effect of quercetin metabolites in manuscript 5.

The consideration of these issues could have improved the quality of the present Doctoral Thesis, but as stated before, this Thesis aimed at giving a first approximation of resveratrol and quercetin metabolites´s activity. For this, the experimental design was not able to contemplate these variables. Nevertheless, these limitations can also be considered as the starting point for further reseach while opening the door to further analysis in the field of PC metabolism.

ONDORIOAK

Doktorego tesi honen ondorioak hurrengoak dira:

Gure diseinu esperimentalarekin lortutako emaitzekin…

1. Doktorego tesi honetan aztertu diren erresberatrolaren metabolitoek, neurri batean polifenolak berak eragindako triglizeridoen murrizketan parte hartzen dute
2. Erresberatrola-3-*O*-glukuronido eta erresberatrola-4´-*O*-glukuronidoak, erresberatrolak bezain besteko triglizeridoen murrizketa eragiten dute aurre-adipozitoetan, efektua dosi berean eragiten baitute
3. Metabolito glukuronatuek adipozitoen gantz murrizketa eragiten dute, erresberatrolak duen eragina baino txikiagoa bada ere
4. Bai aurre-adipozitoetan zein adipozito helduetan, metabolitoek triglizeridoak murrizteko duten akzio-mekanismoa erresberatrolak duenaren ezberdina da
5. Erresberatrolak, eta ustez metabolito glukuronatuek, adipogenesiaren transkripzio faktoreen gain duten inhibizio-efektua miR-155-aren bidezkoa da, baina ez ordea metabolito sulfatatuarena
6. Erresberatrolari egotzi zaion glukosaren homeostasiaren erregulazio-funtzioa, hein batean, erresberatrolak berak eta bere metabolitoek aurre-adipozito eta adipozito helduen adipokinen ekoizpenean duten efektuagatik izan daiteke
7. Odoleko-kontzentrazioen antzerako dosietan kertzetinak adipogenesia inhibitu dezake, baina dosi altuagoak behar dira adipozitoen gantz murrizketa eragiteko
8. Dosiaren arabera, kertzetinak fase goiztiarrean edo fase berantiarrean eraginez inhibitzen du adipogenesia
9. Kertzetinak adipozito helduetan eragiten duen triglizeridoen murrizketa, besteak beste, *de novo* lipogenesian duen inhibizio-efektuagatik da
10. 3S metabolitoak kertzetinak adipozitoen gantza murrizteko duen efektuan eragin dezake, glukosaren kaptazioa eta triglizeridoen mihiztadura murriztuz
11. Kertzetinak aurre-adipozitoetan eragiten duen efektuari, ez diote haren metabolitoek laguntzen

Hortaz, erresberatrolak eta kertzetinak duten triglizeridoen murrizketa efektua, bere metabolitoei esker ere ematen deneko hipotesia partzialki egiaztatu da. Erresberatrolak adipozito helduetan duen efektuari eta kertzetinak aurre-adipozitoetan duen efektuari dagokionean, polifenolen metabolismoa muga ez dela baieztatu daiteke.

CONCLUSIONS

The conclusions of this Doctoral Thesis were:

Under our experimental conditions…

1. Resveratrol metabolites studied in this Doctoral Thesis are involved, to greater or lesser extents, in the triglyceride-lowering effect of this polyphenol
2. Resveratrol-4´-O-glucuronide and resveratrol-3-O-sulfate induced similar delipidating effects to resveratrol in maturing pre-adipocytes, since they have delipidating effect at the same dose.
3. Glucuronide metabolites show a fat-lowering effect in mature adipocytes, although lower than that of resveratrol
4. Both in maturing and mature adipocytes, the mechanism of action by which metabolites reduce triglycerides is different from that of resveratrol.
5. The inhibitory effect of resveratrol on the analysed adipogenic transcriptional factors, and presumably that of its glucuronide metabolites, is mediated via miR-155, unlike the sulfate metabolite
6. The effect on glycemic homeostasis attributed to resveratrol could be, at least in part, due to adipokine modulation caused by resveratrol and its metabolites in maturing and mature adipocytes
7. Doses of quercetin in the range of serum concentrations are able to inhibit adipogenesis, but higher doses are required to reduce fat accumulation in mature adipocytes
8. Depending on the dose, quercetin inhibits both the early and late phases of adipogenesis
9. The delipidating effect of quercetin in mature adipocytes is, among other mechanisms, due to its inhibitory effect on *de novo* lipogenesis.
10. 3S metabolite may contribute to the delipidating effect of quercetin in mature adipocytes by reducing glucose uptake and triglyceride assembling
11. None of the analyzed metabolites contribute to the triglyceride-lowering effect of quercetin in maturing pre-adipocytes

Therefore, the hypothesis that determines whether the beneficial triglyceride-lowering effects of resveratrol and quercetin is only attributed to them or also to their main metabolites has been partially confirmed. Metabolism should not be considered a limitation neither in the case of resveratrol in mature adipocytes nor for quercetin in maturing pre-adipocytes.

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ERANSKINA - ANNEX

Atzerrian eginiko ikerketa-egonaldian burututako lan esperimentala

Doktoregaiak, Toulouseko (Frantzia) Institut des Maladies Métaboliques et Cardiovasculaires (I2MC - UMR1048) ikerketa zentroko “Laboratoire de Recherche sur les Obésités” ikerketa-taldean 3 hilabete eta erdiko egonaldia egin du bere formakuntza osatzeko helburuarekin. Egonaldia 2015. urteko otsailetik maiatzera bitartean izan zen, Cédric Moro Doktorearen gidaritzapean.

Gantz ehunak energiaren homeostasian ezinbesteko funtzioa du, gehiegizko energia triglizerido moduan metatzeko duen gaitasuna dela eta, aurrerago energiaren falta dagoenean gorputzak erabili ahal izateko. Hala eta guztiz ere, glukosaren homeostasiaren erregulazioan eta gantz azidoen oxidazioan muskulu eskeletikoak duen garrantzia aintzat hartzen badugu, aipatzekoa da energiaren kontrolean ehun honek ere garrantzi handia duela. Hortaz, “Laboratoire de Recherche sur les Obésités” ikerketa-taldeak muskulu eskeletikoaren zelulekin lanean duen esperientzia kontuan hartuta, egonaldian zehar erresberatrolak eta kertzetinak giza-miozitoetan zuten efektua aztertu zen.

Experimental work carried out in the international research internship

During the present Doctoral Thesis, an international research internship completed the professional training of the Ph. D. candidate. This stay took place in the "Laboratoire de Recherche sur les Obésités" of the Institut des Maladies Métaboliques et Cardiovasculaires (I2MC - UMR1048) in Toulouse (France), under the supervision of Cédric Moro Ph. D, from February to May 2015.

Adipose tissue plays a key role in energy homeostasis due to its capacity to store energy excess as triglycerides and uses them in energy-privation situations. However, skeletal muscle can contribute significantly to energy control because of its importance in the glucose homeostasis regulation and the fatty acid oxidation. The effect of resveratrol and quercetin in human myotubes was analysed during the stay in the "Laboratoire de Recherche sur les Obésités" which has ample experience working with such cells.

Egonaldiaren eskuizkribua – Internship manuscript

The effect of physiological doses of resveratrol and quercetin on glucose metabolism in human primary myocytes

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Abstract

Background

Phenolic compounds have emerged in recent years as an alternative or synergistic option to promote glucose uptake and therefore, as a tool to face insulin resistance and diabetes. The aim of the present research had two lines. On the one hand, to prove whether resveratrol (RSV) and Q (quercetin), at physiological doses, have any impact on glucose metabolism in human myotubes. On the other hand, to check whether AMPK and Akt pathways are involved in this effect. In addition, the effect of these polyphenols in mitochondrial biogenesis, fatty acid oxidation and fatigue was analysed.

Methods

Myotubes from healthy donors were cultured for 24 h with either 0.1 µM of RSV or with 10 µM of Q. Apart from measuring the main processes that take part in glucose metabolism (glycogen synthesis, glucose oxidation or lactate production) and palmitate oxidation, the expression of key genes and proteins were assessed using RT-PCR and Western blot techniques.

Results

Only *pgc1α* expression increased after cell treatment. Polyphenol treatment neither modified the flow of glucose to oxidation nor that of β-oxidation. Q increased AMPK, IRS-1, Akt and AS160 phosphorylation in basal conditions as well as GSK3β in insulin-stimulated condition. By contrast, the effect of RSV did not reach statistical significance. With regard to the processes involved in fatigue, both polyphenols increased insulin-stimulated glycogen synthesis and diminished lactate production in human myotubes.

Conclusions

Under our experimental conditions that used physiological doses, only Q was useful for the increase of glucose uptake in basal state mainly by AMPK pathway. Nevertheless, both RSV and Q are valuable tools for preventing fatigue.

Keywords: Quercetin, resveratrol, glucose, human primary myocytes

1. Introduction

Insulin resistance and diabetes currently represent pandemic diseases at a global level. In 2016, diabetes caused an estimated 1.6 million deaths, and high blood glucose levels were responsible for 2.2 million deaths [1]. For this reason, big efforts are being made in order to find new strategies to fight this disease.

Skeletal muscle is the main tissue for glycaemic control in the post-prandial state. It contributes to 85% of the whole body glucose uptake, which is essential to avoid insulin resistance development [2]. The most important step in the glucose uptake is the incorporation of glucose transporter 4 (GLUT4)-containing vesicles into the plasma membrane [3]. This process can be promoted in an insulin dependent or independent manner. In the first case, when insulin binds its receptor, the later phosphorylates insulin receptor substrate (IRS) and in consequence, new binding sites for other proteins are originated. Some of these proteins are the phosphoinositide 3-kinases (PI3K) family members, which through the PI3K pathway, stimulates Akt. This fact not only leads to GLUT4 glucose transporter translocation from an intracellular pool to the plasma membrane, but also to glycogen synthase kinase 3 (GSK3β) inhibition and thus, to glycogen synthase (GS) activation [2,4,5]. It should be noted that 80% of glycogen storage is located in the skeletal muscle, so it is the main glycogen contributor to the body.

AMP-activated protein kinase (AMPK) is another GLUT4 translocation promoter, although in an insulin-independent manner [6]. Furthermore, AMPK is also considered as a critical regulator of lipid oxidation [7] which regulates exercise-related metabolic adaptations. Consequently it is considered a therapeutic target for several metabolic disorders, including obesity and diabetes [6].

As it occurs with exercise in AMPK activation, other stimuli such as drugs, energy restriction or dietary compounds can trigger glucose uptake through insulin dependent and independent pathways [8-10]. Among these stimuli, phenolic compounds have emerged in recent years as an alternative or synergistic option. *In vitro* and *in vivo* studies have shown that resveratrol (RSV) and quercetin (Q) have anti-diabetic properties. Although both phenolic compounds are multi-target molecules, it seems that skeletal muscle is relevant to achieve the effect. A clear example of this fact is the great number of pieces of research conducted in the last years with RSV and Q in muscle tissue or muscle cells [11,12]. Nevertheless, the extrapolation of those results to humans have some limitations, such as the low bioavailability of polyphenols [13] and cell or tissue origin. Thus, we had a double aim in the present study: to prove whether RSV and Q, at physiological doses, have any impact on glucose metabolism in human myotubes; and to check whether AMPK and Akt pathways are involved in this effect. In addition, the effect of these polyphenols in mitochondrial biogenesis and fatty acid oxidation was analysed.

2. Material and methods

2.1 Experimental design

Human satellite skeletal muscle cells were isolated as previously described [14] from *vastus lateralis* biopsies of lean, healthy and insulin-sensitive subjects. A pool of cells from several donors was grown in Dulbecco's modified Eagle's medium (DMEM) low glucose-Glutamax™ (GIBCO, BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS) and growth factors. At 90% of confluence, myoblasts were differentiated to myotubes by switching to α-minimum essential medium (α-MEM) low glucose-Glutamax™ (GIBCO, BRL Life Technologies, Grand Island, NY, USA), 2% FBS and fetuin (0.5 mg/ml) until myoblasts were harvested 5 days after the induction of differentiation. Cells were maintained at 37ºC in a humidified 5% CO_2 atmosphere and both incubation media were changed every two days.

2.2 Cell treatment

Myotubes were incubated with either 0.1 µM of RSV or with 10 µM of Q (Sigma, St. Louis, MO, USA) diluted in 95% ethanol for 24 hours and after that, cells were harvested. In the case of the control group the same volume of the vehicle (ethanol 95%) was used.

2.3 Cytotoxicity assay

Cytotoxicity assay was carried out by following instructions in ToxiLight™ bioassay kit (Lonza, Walkersville, MD, USA). The ToxiLight™ bioassay kit measures the release of adenylate kinase (AK) from damaged cells. After cell treatment and harvesting, 30 µl of the incubation media was mixed with ToxiLight™ agent. After a short incubation time, the light intensity was measured using a luminometer.

2.4. Glycogen synthesis assay

As in the case of glucose oxidation assay, myotubes were pre-incubated in a glucose- and serum-free medium for 90 minutes, followed by 3 hours incubation using αMEM supplemented with D[1-^{14}C]glucose (1µCi/ml) with or without 100 mM of insulin. $^{14}CO_2$ was determined by liquid scintillation counting, and data was represented as nmol per hour, and normalized per mg of cell protein.

2.5. Glucose oxidation assay

Myotubes were pre-incubated in a glucose and serum-free medium for 90 minutes. To study basal and insulin-mediated glucose oxidation, cells were incubated with α-MEM supplemented with D[1-^{14}C]glucose (1µCi/ml) and 5.5 mM of non-labelled glucose, in the presence or absence of 100 mM of insulin. The glucose oxidation was determined by the measurement of $^{14}CO_2$. The radioactivity of CO_2 and acid soluble metabolites (ASM) were determined by liquid scintillation counting and data was represented as nmol per hour normalized per mg of cell protein.

2.6. Palmitate oxidation assay

Cells were pre-incubated for 3 hours with α-MEM supplemented with [1-^{14}C]palmitate (1µCi/mL; PerkinElmer, Boston, MA). After the incubation period, the radioactivity of $^{14}CO_2$ (indicating a complete

oxidation) and ^{14}C-ASM (indicating an incomplete oxidation) were determined by liquid scintillation counting, as previously described [15]. Data was represented as nmol per hour and normalized per mg of cell protein.

2.7. Measurement of lactate content in the media

After cell treatment, aliquots of the incubation media were removed and analysed for lactate quantification using a commercial kit and following the manufacturer´s instructions. Results were expressed as mmol/l.

2.8. RNA preparation and quantitative Real Time PCR

RNA samples from treated cells were extracted using RNeasy mini kit (QIAGEN, Valencia, California, USA) according to the manufacturer´s instructions. The integrity of the RNA extracted from all samples was verified and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). 1 µg of total RNA of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using MultiScribe reverse transcriptase method (Applied Biosystems, Foster City, CA, USA) on a GeneAmpPCRSystem 9700. Relative *peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (*pgc-1α*), *mitochondrial transcription factor A* (*tfam*), *nuclear respiratory factor 1* (*nrf1*), c*ytochrome C* (*cycs*), *succinate dehydrogenase complex, subunit alpha* (*sdha*), *ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1* (*atp5a1*), *cytochrome c oxidase subunit 7C* (*cox7c*) and *NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8* (*ndufb8*) were quantified using Real-time PCR with a StepOne-Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). H*exokinase 2* (*hk2*), *pyruvate kinase M1/2* (*pkm*) and *lactate dehydrogenase A* (*ldha*) were measured by TaqMan® Gene Expression Assays (Hs00606086_m1, Hs00761782_s1 and Hs01378790_g1respectively) in MyiQ™ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA, USA). *Rplp0* mRNA levels were similarly measured and served as the reference gene. The PCR reaction was carried out in duplicate on 10 ng of cDNA in 20 µl of reaction and the amplification parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. All sample mRNA levels were normalized to Rplp0 values and the data was expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [16].

2.9. Western Blot analysis

Myotubes were incubated for 20 minutes in α-MEM-low glucose-Glutamax with the presence or absence of 100 nM insulin. Then, cells were harvested in a RIPA buffer (Sigma, St. Louis, MO, USA) complemented with 10 µl/ml protease inhibitor, 10 µl/ml phosphatase I inhibitor and 10 µl/ml phosphatase II inhibitor. Afterwards, protein concentration was determined by bicinchroninic acid (BCA) reagent (Thermo Scientific, Rockfold, IL, USA). 20 µg of total protein were run on 4-15% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, Hercules, CA, USA), electroblotted onto PVDF membranes (Millipore, Bradford, MA, USA) and inmunodetected with ChemiDoc MP imaging system (BioRad, Hercules, CA, USA) using the following primary antibodies: Ser9 pGSK-3β, Thr172 pAMPK, Thr308 pAkt (Cell Signaling Technology, Danvers, MA, USA), Tyr-989 pIRS-1 (Abcam, Cambridge, UK) and Thr642 pAS160 (Gene Tex, CA, USA). Histone H3 (Cell Signaling Technology, Danvers, MA, USA) served as an internal control. Bound antibodies were visualized by an ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) and quantified using Chemi-Doc MP imaging system (BioRad, CA, USA).

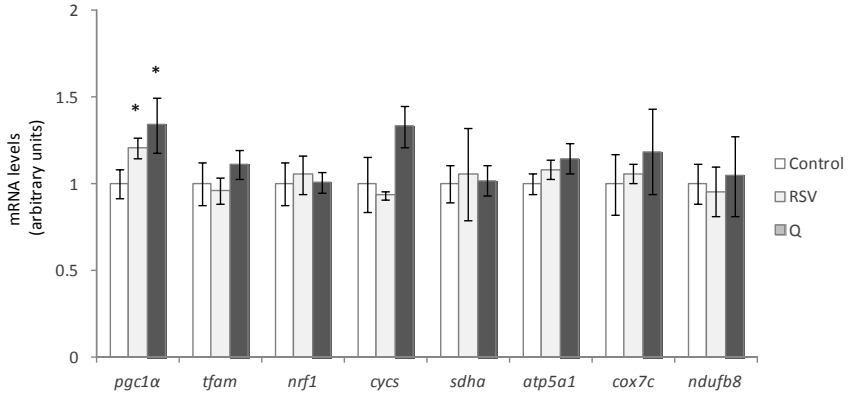
2.10. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 24.0 (SPSS Inc. Chicago, IL, USA). Comparisons between each treatment and the controls were analysed by Student's *t* test. Statistical significance was set-up at $P<0.05$ level.

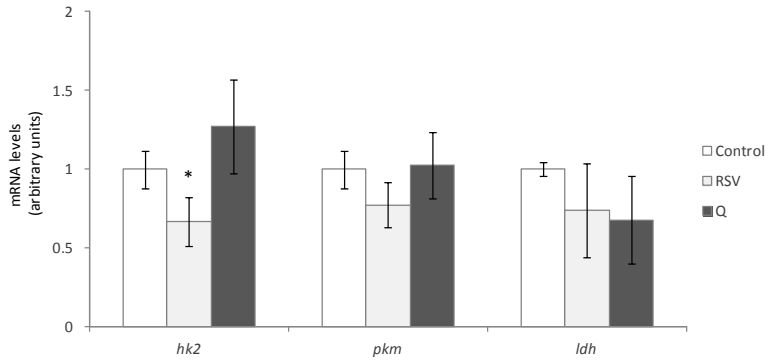
3. Results

3.1. Effects of RSV and Q in the expression of mitochondrial and cytosolic genes

The expression of *pgc1α*, *tfam* and *nrf1*, genes that regulate mitochondrial biogenesis, and that of *cycs*, *sdha*, *atp5a1*, *cox7c* and *ndufb8*, which codify the respiratory chain components, were measured. Among all of them, only *pgc1α* expression increased after cell treatment (Figure 1A). mRNA levels of *hk2*, *pkm* and *ldh* were also measured and a reduction of *hk2* expression after RSV treatment was observed (Figure 1B).



A



B

Figure 1. Mitochondrial (A) and citosolic (B) gene expression in human myotubes treated with 0.1 μM of RSV and 10 μM of Q for 24 hours. Values are means ± SEM. Comparisons between each treatment and the controls were analysed by Student's *t*-test. The asterisks represent differences versus the controls (∗ $p < 0.05$).

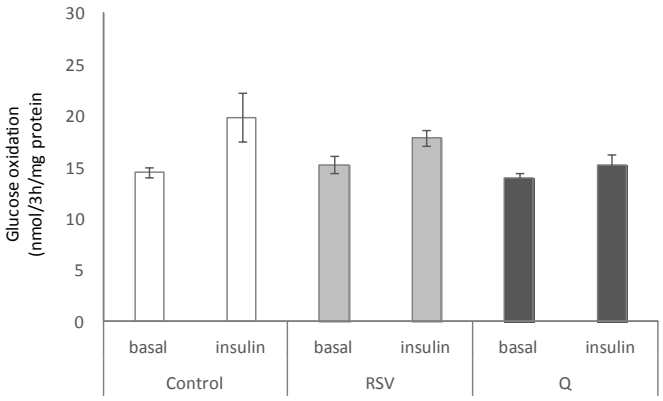
3.2. Lack of citotoxic effect of RSV and Q in human myotubes

Potential citotoxic effect of both polyphenols was analysed, and no changes were observed in the release of AK to the incubation media between the treated and control cells (data not shown).

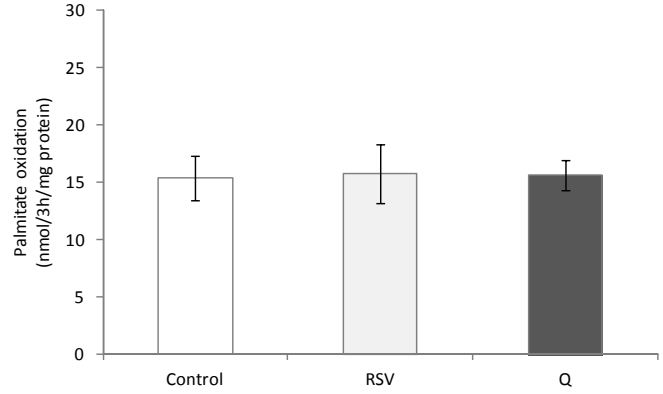
3.3. Effects of RSV and Q on glucose oxidation and palmitate oxidation in human myotubes

With the purpose of testing whether RSV and Q modified glucose oxidation, cells were treated with both molecules in basal and insulin-stimulated conditions, while labelled glucose was added to the media. After CO_2 quantification, polyphenol treatment did not modify the flow of glucose to oxidation (Figure 2A).

In addition, we wanted to analyse if cell treatment could enhance fatty acid β-oxidation, so labelled palmitate was added to incubation media and the resulting CO_2 was quantified. No changes were observed between control and treated cells (Figure 2B).



A



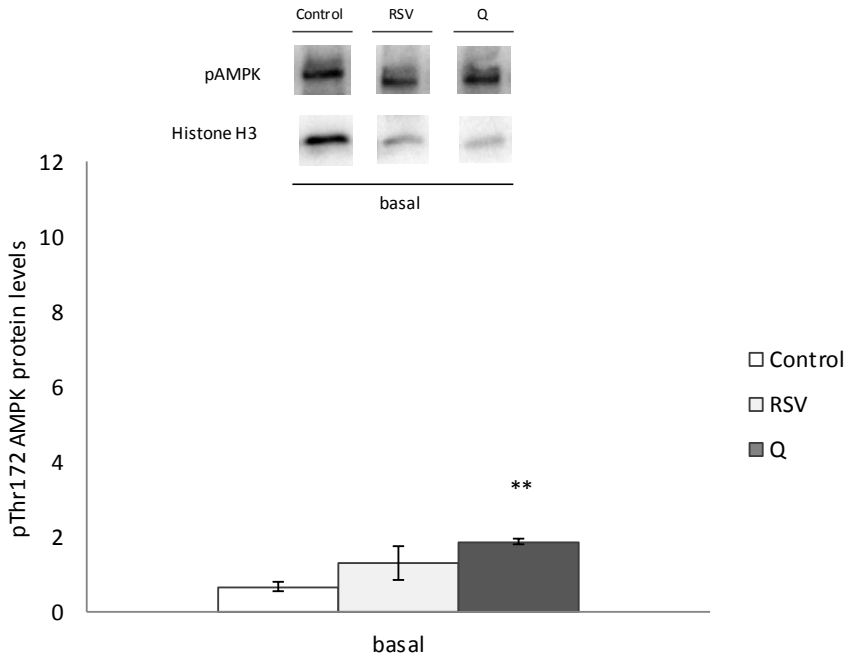
B

Figure 2. Glucose oxidation (A) and palmitate oxidation (B) in human myotubes treated with 0.1 μM of RSV and 10 μM of Q for 24 hours. Values are means ± SEM. Comparisons between each treatment and the controls were analysed by Student's *t*-test.

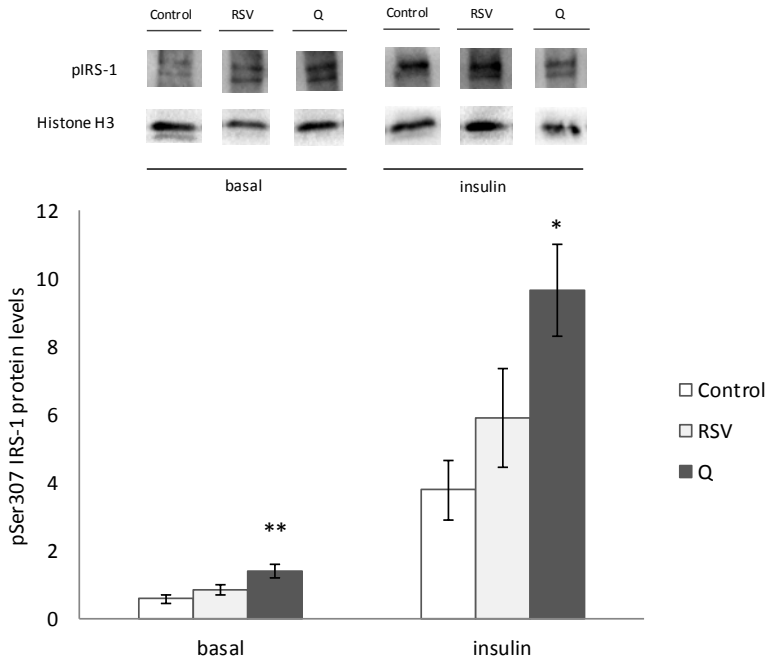
3.4. Effects of RSV and Q in the insulin signalling cascade

To analyse the effect of RSV and Q in the insulin signalling cascade, the protein expression of AMPK, IRS-1, Akt, GSK3β and AS160 was measured. In fact, phosphorylated proteins were measured in order to

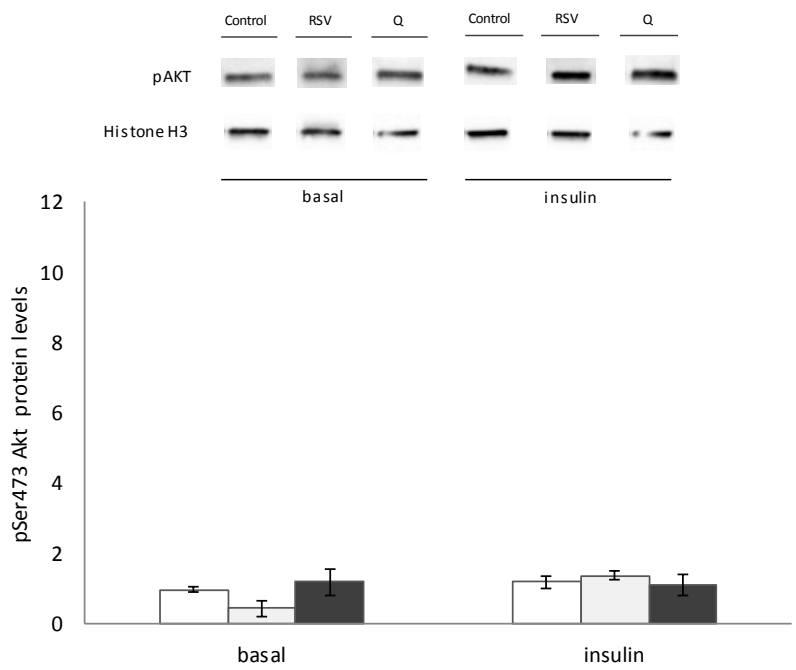
analyse the effect of polyphenols in protein activation. Furthermore, the protein expression analysis was carried out under basal and insulin-stimulated conditions, with the exception of AMPK. Q increased AMPK, IRS-1, Akt and AS160 phosphorylation in basal conditions (Figure 3A, 3B, 3C and 3E), and that of GSK3β in insulin-stimulated condition (Figure 3D). Although RSV increased the phosphorylation of some proteins like AMPK, these enhancements did not reach statistical significance (Figure 3).



A



B


Control
RSV
Q
Control
RSV
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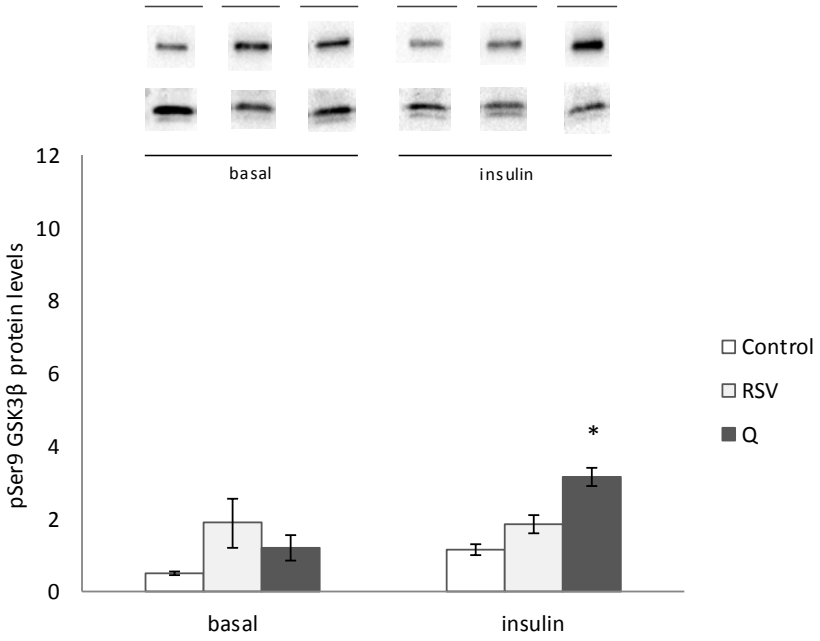
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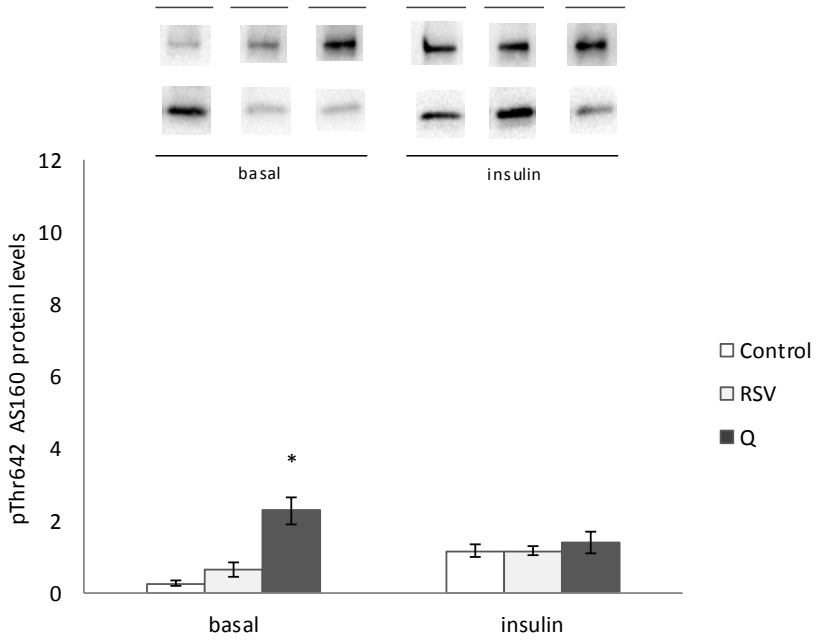


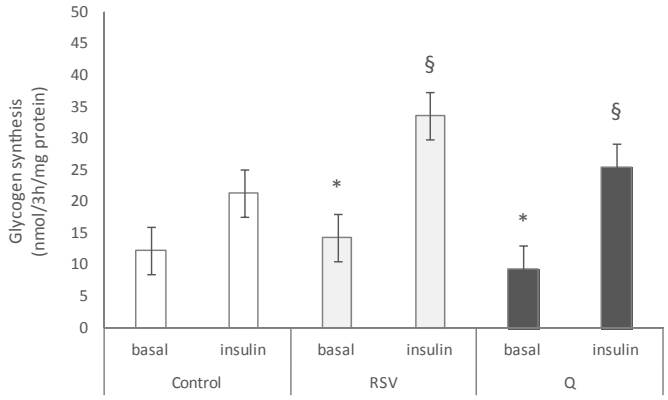
E

Figure 3. Protein expression of AMPK (A), IRS-1 (B), AKT (C), GSK3β (D) and AS160 (E) in human myotubes treated with 0.1 μM of RSV and 10 μM of Q for 24 hours in the presence or absence of insulin, with the exception of AMPK that was measured in basal condition. Values are means ± SEM. Comparisons between each treatment and the controls were analysed by Student's *t*-test. The asterisks represent differences versus the controls (∗ $p < 0.05$; ** $p < 0.01$).

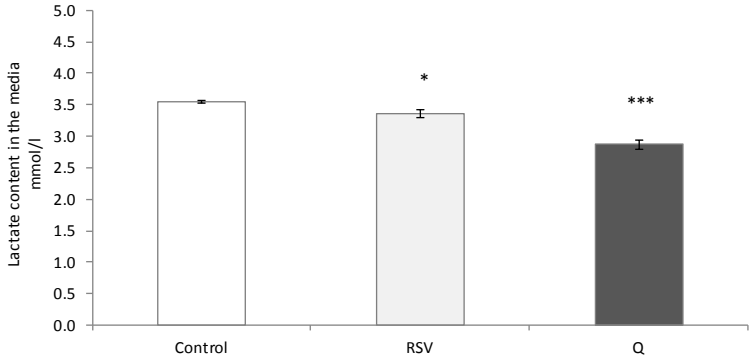
3.5. RSV and Q increased insulin-stimulated glycogen synthesis and diminished lactate production in human myotubes

The effect of RSV and Q on glycogen synthesis was analysed under basal and insulin-stimulated conditions. We observed that both polyphenols increased the insulin-stimulated glycogen synthesis significantly (Figure 4A).

Regarding lactate production in anaerobic circumstances, after cell incubation with 0.1 μM of RSV and 10 μM of Q, the lactate content present in the incubation media was significantly lower than that present in control cells (Figure 4B).



A



B

Figure 4. Glycogen synthesis (A) and lactate content in the media (B) in human myotubes treated with 0.1 µM of RSV and 10 µM of Q for 24 hours. Values are means ± SEM. Comparisons between each treatment and the controls were analysed by Student's *t*-test. * symbol represents differences ($p < 0.05$) between treatment and control in basal condition. § symbol represents differences ($p < 0.05$) of insulin/basal ratio between treatment versus control.

4. Discussion

Phenolic compounds exert healthy cardiometabolic benefits in humans, alone or in synergy with other plant components [17]. In the case of RSV and Q, the anti-diabetic effect emerges as one of the most studied positive effects, even though a total extrapolation to human cannot be totally made yet [18,19]. Here, we wanted to provide more information about the potential activity of both molecules in muscle, through a research conducted in healthy human myotubes. With this intention, our first challenge was to select the dose for cell treatment. In order to test potentially active doses of both polyphenols, in a first approach, PGC1α gene expression was measured after cell treatment with doses ranging from 0.1 to 10 µM of each phenolic compound. PGC1α is the master regulator of oxidative and mitochondrial biogenesis, and a well-defined target for both polyphenols [20,21]. Although there is a discrepancy about their mechanism of action in muscle, it is generally accepted that RSV and Q play a role as mitochondriogenic agents in rodents [22,23]. In the case of RSV, the dose of 0.1 µM for 24h was enough to induce a great increase in PGC1α expression, whereas for Q, 10 µM was the selected dose (data not shown). It is important to highlight that both treatment conditions had

a non-toxic effect for myotubes (data not shown), in agreement with other studies conducted using RSV at 25 μM for 72 h or Q 50 μM for 18 h in C2C12 cells [24,25] and RSV at 100 μM for 4 h in human primary muscle cells [26].

Even though *pgc1α* expression was elevated by RSV and Q treatments, the expression of genes that regulate mitochondrial biogenesis and that of those from the respiratory chain was not modified. This is a clear contraposition of the effect observed in the aforementioned murine cells. Nevertheless, the lack of effect on glucose and palmitate oxidation indirectly confirmed that, in general terms, the number of mitochondria was not altered after RSV and Q treatments in our experiment using human muscle cells. Contrary to our results, Skrobuk *et. al.* reported that 100 μM of RSV for 4 h was able to decrease palmitate oxidation in primary human muscle cells without modification of PGC1α acetylation [26]. Their research was a clear example of RSV hormesis phenomenon [27], due to the fact that RSV at 1 μM for 4h increased PGC1α acetylation. Unfortunately, palmitate oxidation at lower doses than 1 μM was not assessed, making it incomparable with our results. With regard to clinical studies conducted using RSV, while some studies confirmed the mitochondriogenic effect observed in animals [28-30], others did not reveal any effect in muscle mitochondria after RSV intervention [31-33]. In the case of Q, Nieman *et. al.* [34] did not show an effect on mitochondrial biogenesis in thirty-nine trained cyclist after a two-week treatment with 1 g/day of Q and 3 days of heavy exertion. One year later, the same authors described Q influence on moderate exercise performance and muscle mitochondrial biogenesis in physically sedentary young patients who took 1 g/day of Q for two weeks [35]. Like in Nieman´s trials, factors such as exercise, age, previous metabolic state, dose and treatment period, confounded the real effect on mitochondrial biogenesis in muscle. At this point, our results indicated that with low doses of RSV and Q, muscle cells of healthy subjects did not promote mitochondriogenesis or oxidative metabolism.

Apart from mitochondrial biogenesis, glucose uptake in muscle has been the object of study following treatments with phenolic compounds [12,19]. As stated in the Introduction section, this process involves GLUT4 translocation to the membrane by the activation of insulin-dependent pathway Akt being the master regulator, or by the stimulation of insulin-independent pathways with AMPK as the key promoter. Our results demonstrated that in human muscle cells, glucose uptake, measured indirectly as AS160 activation, was enhanced with Q at basal state, but not with RSV. By contrast, none of the phenolic compounds modified the process after insulin stimulation. A more in depth analysis indicated that Q effect on glucose uptake at basal conditions was coherently promoted by AMPK activation. On the other hand, Q raised IRS-1 phosphorylation, but no effect was shown in the activation of its downstream protein Akt, and thus, in the stimulation of total insulin-dependent pathway. Contrary to this data, a study performed in L6 rat muscle cells with low doses of Q (0.1 and 10 nM) showed clear Akt activation [8]. Differences in cell type and Q dose could explain the disagreement between results.

AMPK regulates many physiological processes, including fuel metabolism, insulin secretion and mitochondrial function and biogenesis [19]. Since exercise stimulates AMPK activity in muscle, and RSV has demonstrated to activate molecular mechanisms analogous to exercise training, it might stimulate this insulin independent pathway as well [22]. Nevertheless, studies carried out in murine cells postulate that time and

doses are limiting factors. Breen *et. al.* showed in L6 myotubes that a minimum dose of 25 μM RSV and 30 minutes of treatment were needed to induce a significant glucose uptake enhancement [36]. In accordance to these results, Park *et. al.* [37] found that RSV induced glucose transport is mediated by AMPK in murine muscle cells and not by Akt. Lower doses of RSV (1 μM) also seem to be able to increase glucose uptake by both insulin and AMPK signalling [38]. In contraposition to these results, Kaminski *et. al.* showed decreased *glu4* mRNA levels after 30 μM RSV treatment for 48h in C2C12 myotubes [39]. An explanation for this disagreement among studies could be again, the hormesis phenomenon of RSV in glucose uptake reported by Skrobuk [26]. In fact, authors found that RSV at 1 or 10 μM for 4h in primary muscle cells increased basal glucose uptake whereas exposure to higher RSV concentrations (100 and 200 μM) led to a decrease. A similar impact of the polyphenol was observed in insulin pathway inhibition by Fröjdö *et. al.*, when human primary cells were treated with doses ranging from 5 to 100 μM [40].

In the present study a lack of effect of RSV on Akt and AMPK was observed, which is not in the line with the aforementioned studies [26,40]. However, several factors from the experimental design such as the dose, treatment period or cell origin have been described as important influencing factors for the effect of RSV on glucose uptake. Indeed, Fröjdö *et. al.* did not indicate any effect on insulin pathway inhibition at 5 μM [40] and Skrobuk observed no significant tend toward an increase on AMPK phosphorylation with 24h treatment period [26]. In addition, Barger *et. al.* observed that RSV enhanced insulin-stimulated glucose transport in soleous muscle, but not in extensor digitalis muscle of mice [41]. Therefore, the low dose of RSV used in the present study, the cell line and the treatment length could explain the differences between our data and that from the literature.

With regard to Q, different pieces of research proposed that in L6 and C2C12 murine cells, 18 or 6h treatment length with 25 and 50 μM of this phenolic compound promote glucose uptake via AMPK but not through Akt activation [25,42,43]. Furthermore, Dai *et. al*. demonstrated that in C2C12 myotubes under 24h of treatment, the dose of 5 and 10 μM of Q did not modify glucose uptake, whereas the dose of 20 μM increased it, enhancing Akt and AMPK activation [44]. Therefore, as stated by others, the dose and stimulatory status of muscle cells are crucial factors for Q effectiveness [43]. In the present study, we demonstrated for the first time that treatment in human primary muscles cells of healthy donors with 10 μM of Q for 24h increases glucose uptake mainly by AMPK pathway. In agreement with this proposal, there is a recently published research which has demonstrated that in murine myocytes the dose of 0.1nM is enough for AMPK activation, while 10nM are needed for Akt activation [8].

The literature showed that muscle fatigue is another potential target of Q and RSV [45,46]. Several theories have been postulated as the causes of fatigue [47], such as the exhaustion of blood glucose or muscle glycogen and excessive accumulation of lactic acid among others. In view of the above, promising results were obtained in the present research for Q and RSV. Both compounds were able to enhance glycogen synthesis and to decrease lactic acid content in human muscle cells. In contraposition to our data, Skrobuk *et. al.* observed glycogen content reduction in human primary myocytes after 100 μM RSV treatment for 4h [26]. Nevertheless, in support of our results in glycogen synthesis, we found that GS inhibitor tended to become inactive (increase of the phosphorylated form). In the case of Q, the patent titled “methods for reducing lactate concentration” presented similar results to ours regarding lactic acid in stimulated C2C12 cells [48].

Furthermore, we found a positive regulation of glycogen synthesis through the significant inhibition of GSK3β by phosphorylation.

In view of the above, we conclude that under these experimental conditions, and using physiological doses in human primary myocytes from healthy donors, only Q was useful for the increase of glucose uptake in basal state, mainly by AMPK pathway. Nevertheless, both RSV and Q are valuable tools for preventing fatigue.

5. References

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