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Universidad del País Vasco
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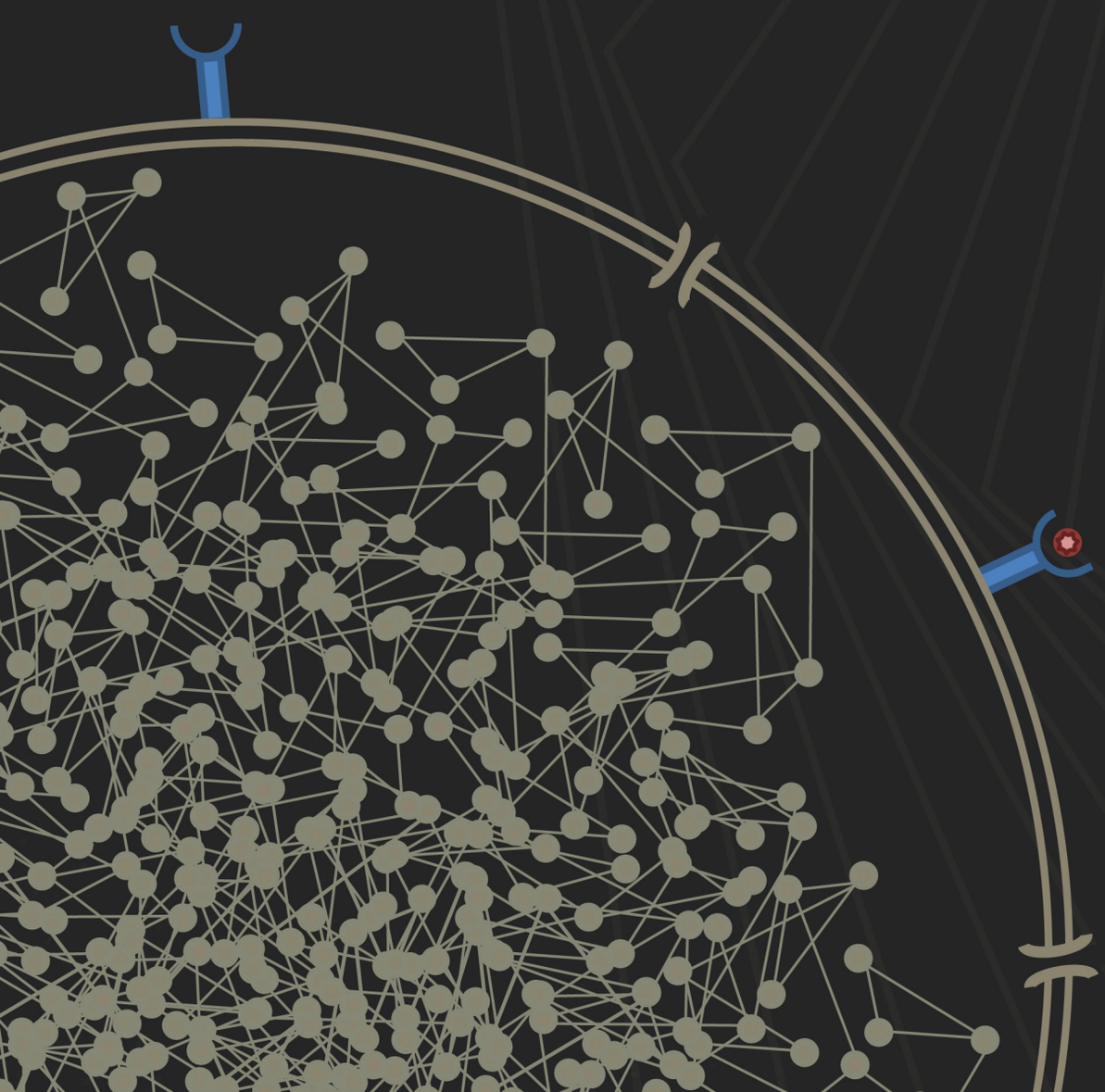
Screening for genetic variants associated with Familial Hypercholesterolemia. Unravelling the epigenetic mechanisms leading to statin-induced Type 2 Diabetes Mellitus

Unai Galicia Garcia

Doctoral Thesis 2021



Screening for epigenetic variants associated with Familial Hypercholesterolemia.

Unravelling the epigenetic mechanisms leading to statin-induced Type 2 Diabetes Mellitus.

Tesis Doctoral para optar al grado de Doctor, presentada por:

Unai Galicia García

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Screening for genetic variants associated with Familial Hypercholesterolemia. Unravelling the epigenetic mechanisms leading to statin-induced Type 2 Diabetes Mellitus.

The purpose of this Doctoral Thesis has been to study the molecular mechanism by which novel genetic variants associated with familial hypercholesterolemia and to decipher the pleiotropic effects associated to statins, which constitute the "gold standard" treatment of hypercholesterolemic patients.

It is well established that mutations in *LDLR, APOB* and *PCSK9* genes are responsible of developing Familial Hypercholesterolemia and that long-term statin exposure occurring in these patients is associated with a 13% higher risk of developing Type 2 Diabetes Mellitus (T2DM). Therefore, in order to present the results in a clearly and affordably the obtained results have been divided into two parts.

Part I covers the screening of novel genetic variants in *LDLR, APOB* and *PCSK9* associated with familial hypercholesterolemia. This part contains a General Introduction covering familial hypercholesterolemia, it diagnosis and treatment, followed by the General Objectives, Material and Methods, Results and Discussion of the results Obtained in this section. In addition, a brief section of the results obtained in the "virtual stay", a work in collaboration with Drs. Giuliana Fortunato and Mª Donata di Taranto has ben included in the format of a brief research article.

In order to explore the pleiotropic effects already described in hypercholesterolemic patients exposed to long term treatment with statins, and due to the complexity and extension of the subject, the results obtained in this experimental section have been included in Part II, which is not independent of Part I but the natural consequence of exploring the effects associated to the adverse secondary effects of patients with Familial Hypercholesterolemia. This part contains a General Introduction covering Pathophysiology of T2DM and the diabetogenic effects of statins. Although being a long general introduction, it has been considered mandatory to deeply explain these processes. Them, General Objectives are exposed, Material and Methods, Results and Discussion of the results Obtained in this section.

In sum, the two parts of these Doctoral Thesis constitutes a whole of molecular mechanisms that lead to Familial hypercholesterolemia and the epigenetic modulation of genes induced by the associated treatment of these patients.

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PART I:

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   5B Functional Characterization of six ApoB-100 and one PCSK9 missense variants found in Italian Population (INTERNATIONAL STAY).

5. **Discussion/**Eztabaida

The results presented in PART I have been published at:

1. **Benito-Vicente A, Uribe KB, Jebari S, Galicia-Garcia U, Ostolaza H, Martin C. Familial Hypercholesterolemia: The Most Frequent Cholesterol Metabolism Disorder Caused Disease.**
   Int J Mol Sci. 2018. 19(11):3426. doi: 10.3390/ijms19113426.

2. **Unai Galicia-Garcia, Jone Amuategi, Shifa Jebari, Asier Larrea-Sebal, Kepa B Uribe, Helena Ostolaza, César Martín, Asier Benito-Vicente. Hiperkolesterolemia Familiarra: Patofisiologia, Diagnostikoa eta Tratamendua. OSAGAIZ: OSASUN-ZIENTZIEN ALDIZKARIA.2020.** 4, zk: 1.

3. **Galicia-Garcia U, Benito-Vicente A, Uribe KB, Jebari S, Larrea-Sebal A, Alonso-Estrada R, Aguilo-Arce J, Ostolaza H, Palacios L, Martin C. Mutation type classification and pathogenicity assignment of sixteen missense variants located in the EGF-precursor homology domain of the LDLR. Sci Rep. 2020. 10(1):1727. doi: 10.1038/s41598-020-58734-9.**

4. **Ana Catarina Alves, Unai Galicia-Garcia, Sílvia Azevedo, Asier Benito-Vicente, Rafael Graça, Patrícia Barros, Peter Jordan, Cesar Martin and Mafalda Bourbon. LDLR variants functional characterization improves variant classification. (Human Molecular Genetics: sent December 2020).**

5. **Galicia-Garcia U, Benito-Vicente A, Giacobbe C, Guardamagna O, Gentile M, Fortunato G, Martín C, Di Taranto MD. Identification and in vitro characterization of six new APOB100 variants found in Italian patients with Familial Hypercholesterolemia (under preparation).**

6. **Galicia-Garcia U, Benito-Vicente A, Giacobbe C, Guardamagna O, Gentile M, Fortunato G, Martín C, Di Taranto MD. Identification and in vitro characterization of one new PCSK9 variant found in Italian patients with Familial Hypercholesterolemia (under preparation).**

1. General Introduction / Sarrera Nagusia

HIPERKOLESTEROLEMIA FAMILIARRA: PATOFISIOLOGIA, DIAGNOSTIKOA ETA TRATAMENDUA

Kolesterola

Kolesterola 1789. urtean deskribatu zen lehenengo aldiz eta ordutik ona ikerketa asko egin dira molekula honen inguruan. Gaur egun, bere egitura eta funtzioaren inguruko informazio asko dago eskuragarri, baita hainbat gaixotasunen garapenean daukan garrantziari buruz ere [1].

Kolesterola zelularen mintzen sintesian eta seinaleztapen prozesuetan ezinbesteko osagaia da, mintzen jariakortasuna kontrolatzen eta lipido eta proteina ezberdinekin elkarrekiten baitu [2,3,4]. Gainera, mintzaren propietate biofisikoak baldintzatzeko gai da, mintzaren lipidoen albo-ordena eta paketamendua handituz eta honen jariakortasuna eta iragazkortasuna gutxituz [5]. Bestalde, proteina ezberdinen funtzioa erregulatu dezake, hauekin zuzenean elkarrekinez [4] edo mintzaren propietateak aldatuz. Kolesterolarekin zuzenean elkarrekiten duten proteinen artean, besteak beste, hurrengoak aurkitu ditzakegu: kolesterola substratu moduan erabiltzen dutenak (Azil-CoA azil-transferasa (ACAT)) [6], kolesterolean aberatsak diren mintz guneekin soilik elkarrekiten dutenak (kolesterol-menpeko zitolisinak) [7], esterol mota ezberdinak lotzen dituzten domeinuak dauzkatenak (mozketa bidezko aktibazioan parte hartzen duten proteinak (SCAP) edo hidroximetilglutaril-KoA erreduktasa (HMG-KoA *reductase*)) [8,9], kolesterol ezagupen sekuentziak (CRAC; ingelesatik, Cholesterol recognition/interaction amino acid consensus sequence) dauzkatenak [10].

Kolesterola beste molekula askoren aitzindaria ere bada, hala nola, behazun gatzena, hormona esteroideoena edo zenbait bitaminena. Behazun gatzak, kolesteroletik abiatuz, gibelean sintetizatu eta lipidoak disolbatzeko detergente moduan erabili daitezke [11,12]. Hormonen kasuan, kolesterola hormona esteroide talde nagusien aitzindaria da: progestagenoak, glukokortikoideak, mineralokortikoideak, androgenoak eta estrogenoak [12]. Bestetik, kaltzio eta fosforo metabolismoa kontrolpean mantentzeko beharrezkoa den D bitamina sintetizatzeko ezinbestekoa da [13].

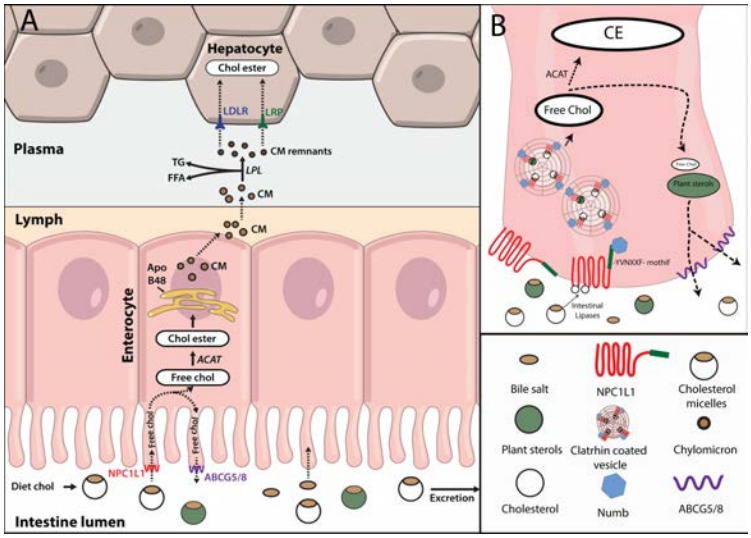
1. **Kolesterolaren metabolismoa**

1.1 Kolesterolaren sintesia

Zeluletan dagoen kolesterol gehiena bi bidezidorretatik lortzen da; dietan jaso [14] edo zelulan bertan sortu daiteke [15]. Azkenengo hau, erretikulu endoplasmatikoan sintetizatzen da mebalonatoaren bidezidorrean. Behin sintetizatuta, zitoplasmara askatzen da eta hortik kolesterol ester moduan biltegiratzen edo zelulatik kanpo lipoproteinetan jariatzen da. Ehun gehienek kolesterola *de novo* sintetizatzeko gaitasuna daukate. Hala ere, gehiengoa gibelean ekoizten da [16,17]. Barne kolesterolaren sintesia zorrozki erregulatuta dago; izan ere, hainbat proteina aktibatu edo inaktibatu egiten dira zelularen beharren arabera. Esaterako, zelula barneko kolesterol mailak behar fisiologikoak gaindituz gero, elementu esterol-erregulatzaileei lotzen zaizkien proteinak (SREBP) erretikulu endoplasmatikoan inaktibatzen dira. Honela, barne kolesterolaren *de novo* sintesia galarazten da, HMG-KoA erreduktasa entzima inhibituz.

1.2 Dietatik datorren kolesterolaren xurgapena

Zelulak bere kabuz sortzen duten kolesterolaz gain, giza gorputzak dietatik datorren kolesterola hartzeko gaitasuna dauka [20]. Kolesterol hau gantz azido libre eta triglizeridoekin batera xurgatzen da hesteetan, enterozitoetan zehazki [21]. Hauek, ordea, ez dira kolesterola bere kabuz era eraginkor batean xurgatzeko gai. Arrazoi horregatik, kolesterola behazun gatzekin nahasten da kolesterolaren barneraketa eraginkorragoa izan dadin. Kolesterol eta behazun gatzak nahastean, kolesterol-behazun gatz mizelak sortzen dira eta hauek hesteetan Niemann-Pick C1 motako (NPC1L1) hartzaileen bitartez barneratzen dira [22]. NPC1L1 hartzaileak kolesterola lotzeko gai den sekuentzia bat dauka heste-lumenera begira dagoen N-muturrean; C-muturra, berriz, mintzari lotuta dago. Kolesterola hartzaileari lotzen zaionean, proteinaren egitura berrantolatu egiten da, C-muturra mintzetik zitosolera mugituz. Berrantolaketa honi esker Numb proteina egokitzailea zitosolera begira geratu den C-muturrera lotuko da, klatrina bidezko barneraketa ahalbidetuz (1. Irudia) [22,23].

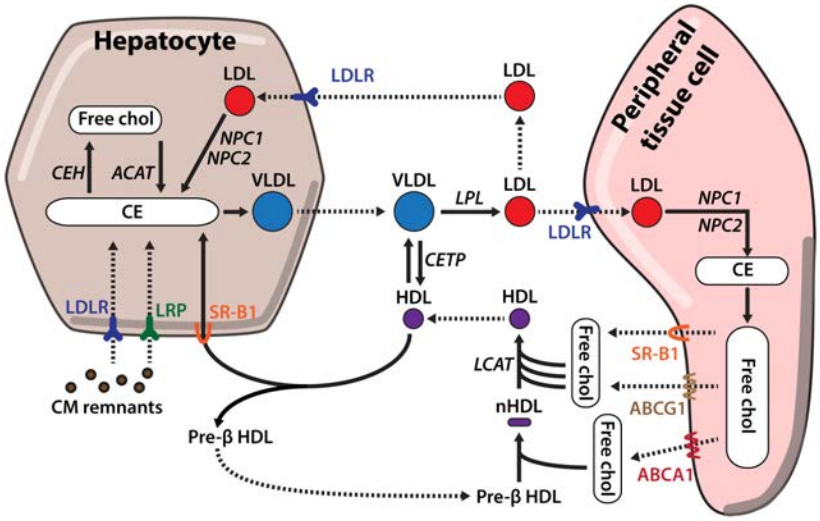


1. Irudia. Dietatik datorren kolesterolaren xurgapena. A) Kanpotik datorren kolesterola behazun azidoekin batu eta mizelak sortzen ditu. Mizela hauek esteetako lumenetik bidaiatzen dute eta enterozitoen mintzetan dauden NPC1L1 hartzaileekin batzen eta barneratzen dira. Barneratutako kolesterola ABCAG5/8 garraiatzailearen bitartez heste lumenera bueltatu edo ACAT entzimaren bitartez zelulan esterifikatu eta pilatu daiteke. Esterifikatutako kolesterola, beste lipido batzuekin batera, CMetan paketatu eta linfara jariatzen da. Linfatik odolera pasatu ostean, LPLri esker, kilomikroi hondar bilakatuko dira. **B)** Kolesterola NPC1L1 hartzailera lotzean, bere aldaketa konformazionala eragiten du. Honen bitartez, mintzera atxikituta dagoen C-muturra zitoplasmara mugitzen da Numb proteina lotuz eta klatrina bidezko barneraketa baimenduz.

Behin barneratua, kolesterola erretikulu endoplasmatikora garraiatzen da. Ondoren, heste-lumenera itzuli daitekeen ABCG5/8 garraiatzaileen bidez edo ACAT proteinak esterifikatu dezake. Esterifikatutako kolesterola lipido tantetan biltegiratu edo triglizerido eta B48 apolipoproteina (ApoB48) bildu daiteke kilomikroiak (CM) eratuz [24].

CMak soilik hesteetan sintetizatzen diren proteinak dira, bereziki baraualdian. Partikula hauek ApoB48 apolipoproteina daukate bereizgarri moduan [26]. Halere, beste hainbat apolipoproteina bereganatzen dituzte biosintesi prozesuan edota beste lipoproteinekin elkarrekinez, esaterako, ApoA-I, ApoA-II, ApoA-IV, ApoA-V, ApoC-I, ApoC-II, ApoC-III eta ApoE [27]. Sintetizatu berri diren kilomikroiak linfara jariatzen dira [28] eta hortik odolera doaz torax aldean dagoen isurbidetik [29]. Behin odolean, kilomikroiek lipoproteina lipasarekin (LPL) elkarrekiten dute [30], triglizeridoak hidrolizatuz eta gantz-azido libreak askatuz [31,32]. Hauek, ehun periferikoetako garraiatzaile espezifikoen bitartez barneratuko dira, muskulu eta gantz ehunetan bereziki. Prozesu honetan lipoproteinak txikiagotu egin eta kolesterol esterretan aberasten dira. Gainerako kilomikroiek soberan dauzkaten

apoliproteinak odolean dauden beste lipoproteinei ematen dizkiete (ApoA eta ApoC HDL partikulei konkretuki) eta bidean ApoE apolipoproteina bereganatzen dute [33]. Azkenik, gibelean LDLR eta LDLR familiako proteinek ApoE apolipoproteinaren bitartez kilomikroiak barneratzen dituzte(2. Irudia) [34].



2. Irudia. Lipoproteinen erregulazio metabolikoa. Kolesterola gibeletik odolera jariatzen da triglizeridoetan aberastuta dauden lipoproteinetan, VLDLk. Odolean VLDL hauek beste plasma proteinekin (LPL) eta lipoproteinekin elkarrekiten dute triglizerido eta apolipoproteina gehienak galduz. Prozesu honetan, kolesterolean aberastuta dauden LDLk sortzen dira, ehun periferikoetan aurkitzen diren LDLRren bidez barneratuko direnak. Bestetik, ehun periferikoetan dagoen kolesterol soberakina HDL partikuletara pasatzen da ABCA1, ABCG1 eta SR-B1 mintz proteinen bitartez eta bueltan doa gibelera.

1.3 Gibeleko kolesterolaren jarioa

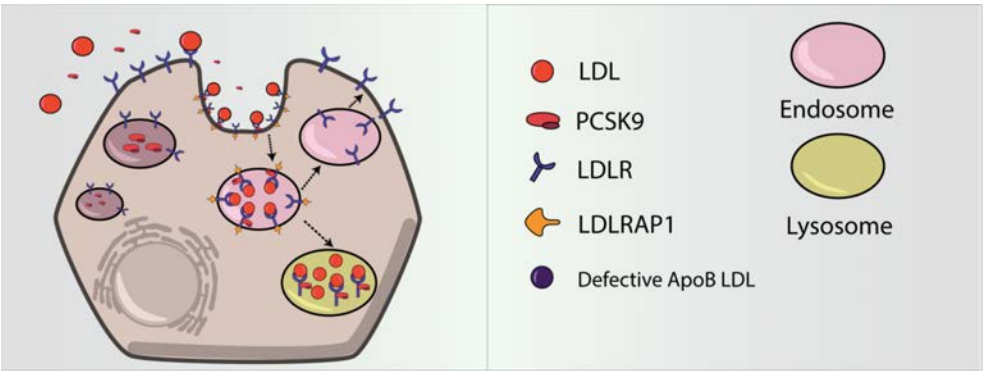
Gibelaren funtzio nagusien artean kolesterol homeostasiaren mantenua dago, bertan gertatzen baitira kolesterol maila egonkor mantentzen duten prozesu gehienak: kolesterol sintesia, lipoproteinen sintesi eta jariaketa edo kolesterolaren iraizketa, besteak beste [16,35,36,37]. Sintetizatu ostean, dentsitate oso baxuko lipoproteinetan (VLDL) jariatzen da. Aipaturiko sintesi eta jariatze prozesuak, kolesterol maila egonkor mantendu ahal izateko, mekanismo zorrotzen menpe daude. VLDL partikulen sintesia 2 urratsetan gertatzen da. Lehenengo urratsean, ApoB100 apolipoproteina erretikulutik zitosolera translokatzen da mikrosometako triglizerido transferasak (MTP) lipidatzen duen bitartean [38]. Pausu hau VLDL partikulen sintesirako bereziki garrantzitsua da, edonolako akatsen bat egonez gero ez direlako egoki sintetizatuko eta degradatuak izango direlako [39]. Sintesiaren bigarren pausuan, partzialki lipidatutako VLDL partikulak Golgi aparatura garraiatuko dira [40]. Bertan, ApoA-I eta ApoE apolipoproteinak eta beste hainbat

lipido ezberdin bereganatuko dituzte [41]. Azkenik, VLDL helduak odolera jariatuko dira eta lipidoak gibeletik ehun periferikoetara garraiatuko dituzte [42].

Ezinbestekoa da kolesterolaren jariatzea zorrozki kontrolatua egotea; izan ere, VLDLen gehiegizko jariatzeak dentsitate baxuko lipoproteina (LDL) maila emenda dezake, eta ondorioz, gaixotasun kardiobaskularrak (CVD) pairatzeko arriskua areagotu [43]. Bestalde, VLDLk behar baino gutxiago jariatuz gero, lipido pilaketak sor daitezke gibelean, gibel koipetsuaren gaixotasuna sortuz [43,44].

1.4 Zelulen kolesterol barneraketa

De novo sintetizatzen duten kolesterolaz gain, zelulek odolean dauden lipoproteinetan garraiatzen den kolesterola hartzen dute, LDL hartzaile (LDLR) bidezidorraz baliatuz [4]. Odolean, VLDLek LPL, gibeleko lipasa (HL) eta beste lipoproteinekin elkarrekiten dute, kolesterol esterrak eta ApoB100 ez diren apolipoporteinak galduz. Prozesu honen ondorioz, kolesterol esterretan aberastuta dauden lipoproteina txikiagoak sortzen dira, LDLk. LDLk kolesterola gibeletik ehun periferikoetara garraiatzeaz arduratzen dira, eta bertan, LDL hartzaileei batu eta klatrinaz inguratutako besikula endozitikoetan barneratzen dira [45]. LDL hauen barneraketa hartzaileak lipoproteina lotzen duen momentuan hasten da. Partikulak batzen direnean, LDLRren proteina egokitzaileak (LDLRAP1) LDLRren C-muturrean dagoen NPXY sekuentzia lotzen du, klatrina bidezko endozitosia baimenduz [46]. Behin zelula barruan, besikula endozitikoetan gertatzen den pH jaitsieraren ondorioz, LDLRk bere egitura aldatzen du, LDLR-LDL konplexua desegonkortuz eta LDL partikulak askatuz (3. Irudia). Prozesu honek, LDLRren birziklapena ahalbidetzen du, LDL partikulak lisosoman degradatzen diren bitartean. LDLen degradazio prozesuan, lisosomako lipasen eraginaren ondorioz, kolesterol esterrak hidrolizatzen dira, esterifikatu gabeko kolesterola askatuz [47-49]. Azkenik, kolesterol librea, Nieman-Pick C1/C2 (NPC1/NPC2) proteinei esker, lisosomatik erretikulu endoplasmatikora joaten da eta zelulen beharren arabera erabiliko da [50].



3. Irudia. LDLR-ren erregulazioa. LDL hartzaileak lipoproteina lotzen du, LDLRAP1-k LDLRren C-muturrean dagoen NPXY sekuentzia lotzen du eta klatrina bidezko endozitosia baimentzen du. Behin zelula barruan, besikula endozitikoetan gertatzen den azidifikazioaren ondorioz LDLR-LDL konplexua desegonkortuz eta prozesu honek, LDLRren birziklapena ahalbidetzen du, LDL partikulak lisosoman degradatzen diren bitartean.

1.5 Kolesterol barneraketaren erregulazioa

Kolesterol maila egonkor mantendu behar da, bai zelula barruan, bai odolean. Hori dela eta, koordinatuta dauden hainbat mekanismok kolesterol barneraketa kontrolatzen dute [18,51,52]. SREBP-2 erretikuluan ageri den eta kolesterol mailarekiko sentikorra den proteina da. Kolesterol maila behar fisiologikora heltzen ez bada, nukleora traslokatzen da, *LDLR* genearen transkripzio eta itzulpena aktibatuz eta kolesterol barneraketa mekanismoa sustatuz [18,53]. Bestalde, gehiegizko barne kolesterol maila ekiditeko, proproteina konbertasa subtilisina kexina 9ren (PCSK9) [54] edota LDLRren degradatzaile induzigarriaren (IDOL) sintesia sustatzen da, LDLRren birziklapena galaraziz eta LDLR degradazioa bultzatuz [55].

PCSK9 2002. urtean lehenbiziko aldiz deskribatutako zen eta bere familian deskribatutako azkena izan da. Modu inaktiboan sintetizatzen da eta automozketa prozesua jasan ostean aktibatzen da, proteina heldu bihurtuz [56]. Zelularen mintzean dauden LDLRek PCSK9 proteina heldua lotzen dute, LDLren ApoB100 apolipoproteina lotzeko erabiltzen ez duen leku batean, eta LDLRrekin batera endozitatzen da, endosomara iritsiz. Bertako medio azidoak LDL eta hartzailearen arteko afinitatea jaisten duen bitartean, PCSK9 eta hartzailearen artekoa areagotzen du. Honek, LDLRren konformazio aldaketa galarazten du. Ondorio gisa, hartzailea ezin da mintzera itzuli eta lisosoman degradatzen da [58,59].

IDOL, PCSK9 bezala, LDLRren bidezidorra erregulatzeko gai da. IDOL E3 ubikitina ligasa bat da eta LDLRri ubikitina marka bat jartzeaz arduratzen da, honen degradazioa bultzatuz [60]. IDOLaren adierazpena zelula barneko kolesterolaren menpe dago horrela, kolesterol maila altua denean honen adierazpena emendatzen da mintzeko LDLR kantitatea murriztuz [51,60].

Mekanismo hauez gain, epigenetikak eta transkripzio ondoko erregulazioak bere garrantzia daukate LDLR menpeko LDL partikulen barneraketan [62-64]. mikroRNAk (miRNA), beste molekula batzuen artean, kolesterol maila kontrolpean mantentzeko gai dira LDLRren itzulpena erregulatuz. RNA sekuentzia txiki hauek 70-100 nukleotido dauzkaten sekuentzietatik sortzen dira (pri-miRNA) eta zenbait mozketen eta aldaketen ostean 20 nukleotido inguruko sekuentzia bihurtzen dira [65], RNAk zuzendutako isilarazpen konplexuetan (RISC) txertatzen direnak [66]. Azken urteotan, miRNA hauek eta LDLR menpeko LDL barneraketa erlazionatzen dituzten hainbat ikerketa argitaratu dira. Esaterako, miR-27b [67], miR-27a [68], miR-148 [69], eta miR-128-1 [64] besteak beste, LDLR itzulpena galarazteko gai dira.

1.6 Kolesterolaren alderantzizko garraioa (RCT)

RCT kolesterolaren homeostasirako funtsezko mekanismoa da. Honen bitartez ehun periferikoetan dagoen kolesterol soberakina gibelera bueltan garraiatzen da, honek metabolizatu eta gorputzetik kanporatzeko [70]. Prozesu honetan, ApoA-I daukaten dentsitate altuko lipoproteinak (HDL) ehun periferikoetan dagoen kolesterol soberakina hartu eta gibelera garraiatzeaz arduratzen dira [71].

RCT prozesua lipido gabeko ApoA-I (pre-β-HDL) eta mintzeko ABCA1 kolesterol garraiatzailearen elkarrekintzarekin hasten da [71]. Elkarrekintza honek zelula barneko kolesterolaren kanporaketa sustatzen du, pre-β-HDL partikulak lipidoz kargatuz eta hauen egitura aldatuz; honela, HDL jaioberriak sortzen dira. Kolesterol librea lipoproteina hauetara gehitzean, lezitina-kolesterol aziltransferasa (LCAT) entzimak esterifikatzen du, HDL esferiko helduak eratuz [72]. HDLk heltzean, ABCA1 garraiatzaileaz gain, ABCG1 eta SR-B1 kolesterol garraiatzaileekin ere elkarrekiteko gai dira, ehun periferikoetan dagoen kolesterol soberakina era eraginkorrean ateraz [36,72,73]. Bestalde, mintzeko kolesterol garraio pasiboak ere HDL helduak kolesterolez kargatzen laguntzen du [74]. Behin kolesterolez beteta daudenean, HDLek ehun periferikoetatik gibelera bidaiatzen dute, non SR-BI hartzailearen bitartez kolesterol esterrak barneratzen diren behazunetik kanporatzeko (2. Irudia) [36].

RCT prozesuan HDL eta beste lipoproteinen arteko elkarrekintza nahiko arrunta da, HDL helduek proteinak eta lipidoak trukatzen baitituzte beste lipoproteinekin. Gehienetan, kolesterol esterrak transferitzeko entzimak (CETP) HDL partikulen kolesterol esterrak VLDL partikuletara trasferitzen ditu eta VLDLen triglizeridoak, berriz, HDLtara transferitzen dira [75]. Mekanismo honek gainerako VLDLk LDL bihurtzen ditu gibeleko LDLR hartzaileen bitartez barneratu eta behazunetik kanporatzeko [76].

1.7 Behazunaren bidezko kolesterolaren iraizketa

Behazunaren bidezko kolesterolaren iraizketa kolesterola gorputzetik kanporatzeko beharrezko azken urratsa da [76]. Prozesu honetan, behazun gatzak, behazunaren osagai nagusia izanda, funtsezko molekulak dira. Hauek, kolesterolaren inguruko prozesuetan modu erabakigarrian hartzen dute parte, hala nola, hesteetako kolesterolaren xurgapenean eta baita honen iraizketan ere [77]. Behazun gatzak, kolesteroletik abiatuz, hertsiki kontrolatua dagoen bidezidor metaboliko batean sintetizatzen dira gibelean [78]. Ondoren, gibelean dagoen behazun hodiko plasmara garraiatzen dira zenbait kolesterol eta fosfolipidoekin mizelak sortu ditzaten. Urrats honetan, ezinbestekoak dira fosfolipidoak, haiei esker kolesterol-mizela egonkorrak sortzen baitira [37]. Hori dela eta, mintz-zeharreko fosfolipido garraioan akatsen bat egonez gero kolesterol kanporaketa ia guztiz oztopatzen da [81].

Aipaturikoa kontuan izanik, kolesterolaren homeostasiaren mantenua era askotako mekanismo konplexuen menpe dagoela esan daiteke, eta beraz, hauen arteko koordinazio zorrotza ezinbestekoa da bere funtzionamendu egokirako. Egiaz, mekanismo hauen funtzionamendu akastunek eta bidezidor hauetan gertatzen diren mutazioek hainbat eta hainbat gaixotasunen garapena eragin dezakete. Hauen artean, LDLren metabolismoa oztopatzen duten mutazioak dira ohikoenak, konkretuki hiperkolesterolemia familiarra (HF) sortzen dutenak.

2. Hiperkolesterolemia Familiarra eta genetika

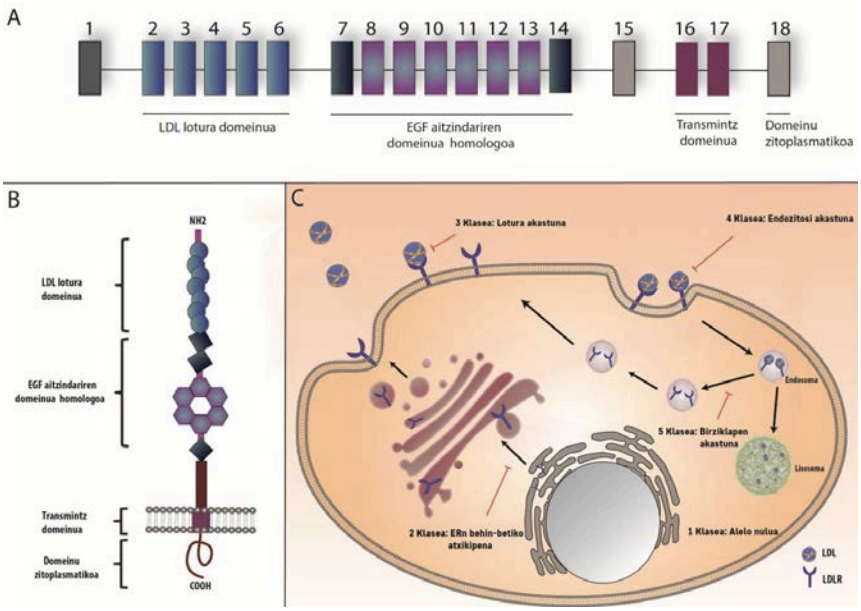
HF zelulek kolesterolaren barneraketa-mekanismoan duten akatsen ondorio da. HF pairatzea eragiten duten mutazioen artean arruntenak LDL hartzaile (*LDLR)* (mutazioen %80-85), ApolipoproteinaB-100 (*APOB100)* (%5-10), Proproteina Konbertasa Subtilisina/Kexina familiako bederatzigarren kidea (*PCSK9)* (%2) edo

LDL hartzailearen proteina egokitzaile (*LDLRAP1)* (<%1) geneetan ematen dira [75]. Hauez gain, nahiz eta prebalentzia oso txikia izan, Apolipoproteina E (APOE), seinaleak itzultzen dituzten egokitzaileen familiako lehengo kidea (STAP1), lisosomako lipasa azidoa (LIPA) eta G5/8 azpi-familiako kasete ATP-lotzailea (ABCG5/8) geneetan aurkeztu daitezkeen zenbait mutaziok ere HFren antzeko fenotipoa sor dezakete [76,77].

2.1 LDLR

*LDLR*n, HF kasu gehienen erantzulea den genean, dagoeneko 3000 aldaera baino gehiago deskribatu dira (Clin_Var database, 2020). LDLR plasmako LDL partikulak lotu eta barneratzeaz arduratzen da, eta funtzionamenduan galerak izanez gero LDL-Cren igoera eragiten du. LDLR proteina erretikulu endoplasmatikoan sintetizatzen eta mintzera garraiatzen da. Bertan, LDLren ApoB-100 proteina estrukturala lotzen du klatrina menpeko endozitosi prozesuari hasiera emanez. LDL barneraketan, endosomak azidifikatzen dira LDLR-APOB arteko afinitatea murriztuz eta hauen arteko lotura ahulduz. Honi esker, LDL partikulak endosoma lumenera askatzen dira lisosomara bideratuz. Bestalde, LDLR mintz plasmatikora bueltatzen da birziklapen prozesu bati esker (4A eta 4B Irudiak). Prozesu honetan edonolako akatsik gertatuz gero, HF garatzen da [78].

Deskribatutako LDLR aldaera patogenikoen artean luzera handiko DNA sekuentzien bikoizketak (CNV), nukleotidoen txertaketak eta delezioak, moztitsasketa guneetako mutazioak edo nukleotido ordezkapenak aurkitu daitezke. Nukleotido txertaketen eta delezioen garrantzia azpimarratu beharra dago, nukleotido ordezkapenekin alderatuz ondorio nabarmenak sortzen dituztelako. LDLR mutazioak 5 klase ezberdinetan banatu daitezke LDLR bidezidorrean eragiten dituzten arazoen arabera (4C Irudia). Horrela, proteinaren sintesia oztopatuz gero, lehenengo motan sailkatzen dira; mutazioek proteina erretikuluan atxikituta geratzea eragiten badute, bigarrenean sailkatzen dira; hirugarren motako mutazioek LDLR eta LDL arteko lotura akastuna eragiten dute; laugarren motakoek, ordea, LDLR-LDL konplexuaren barneraketa oztopatzen dute eta bosgarren motakoek LDLRren birziklapen eraginkorra galarazten dute [79].



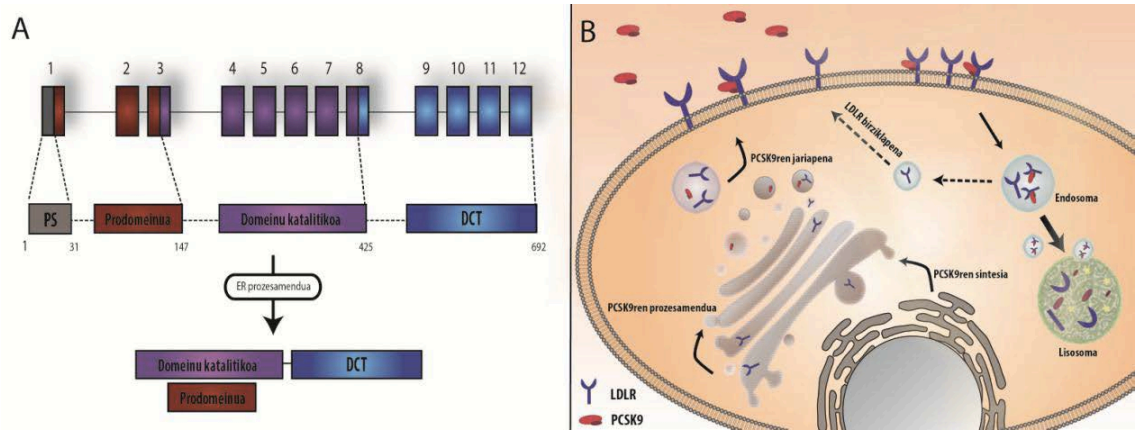
4. Irudia. LDLR eta bere bidezidorra. 4A) LDLRa 18 exoi ezberdinetan kodetutako genea da; **4B)** LDLRaren domeinu ezberdinen irudikapen eskematikoa; **4C)** LDLRa erretikulu endoplasmatikoan sintetizatzen da. Ondoren, Golgi aparatuan eraldatzen da eta, azkenik, zelula-mintzera garraiatzen da. Zelula-mintzean LDLRak LDL partikulen ApoB100 lipoproteina lotzen du eta horrekin batera barneratzen da klatrina bidezko endozitosi-prozesu baten bidez. Endozitosi-prozesuan pHa jaisten da, eta horrek LDLR-LDL arteko loturak ahulduz bi proteinak banatu egiten ditu. Bukatzeko, LDLa lisosomara bideratzen da eta LDLRa, berriz, birziklapen-prozesu baten bitartez mintzera bueltatzen da.

2.2 APOB100

APOB genean gertatzen diren mutazioak HF izateko bigarren arrazoi nagusia dira eta LDL eta LDLR arteko lotura oztopatzen dute. ApoB100, 4536 aminoazidoz osatutako proteina monomerikoa da. Alde batetik, LDL partikulak inguratzen eta egonkortzen ditu eta bestetik LDLR lotzeko gaitasuna dauka [80]. Hortaz, ehun arteko lipido garraiorako ezinbesteko proteina da. APOB100 mutazio patogenikoak, lotura domeinuari dagozkion eta oso kontserbatuta dauden, 26. eta 29. exoietan deskribatu ziren lehenbiziko aldiz. Gaur egun, deskribatutako APOB mutazio gehienak exoi horietan dauden arren, hauetatik kanpo dauden zenbait mutazio patogeniko ere deskribatu dira. Honek LDLR-LDL interakzio gune klasikoetatik kanpo dauden sekuentzien analisiaren garrantzia azpimarratu du. Mutazio hauek sortzen duten fenotipoa APOB akastun familiarra (AAF) izenarekin ere ezagutzen da, eta gehienetan, ez da LDLR mutazioek sortzen duten fenotipoa bezain kaltegarria izaten [81].

2.3 PCSK9

PCSK9 genearen aldaerak mende honen hasieran hasi ziren deskribatzen, *PCSK9* eta HFren arteko erlazioa lehenbiziko aldiz karakterizatu zenean [82]. PCSK9 hiru domeinuz osatutako proproteina konbertasa bat da: prodomeinua (31-152 aminoazidoak), domeinu katalitikoa (153-449 aminoazidoak), eta C-muturreko domeinua (CTD) (449-692 aminoazidoak) dituena (4A Irudia). Proteina erretikulu endoplasmatikora iristen denean, 152 eta 153. aminoazidoen artean auto-mozketa bat gertatzen da. Honela, 14 kDa pisatzen dituen peptido bat eta 60 kDa pisatzen dituen proteina heldua sortzen dira. Aipatutako peptidoa proteinari atxikituta geratzen da 152-226 aminoazidoen artean, 75 kDa-tako proteina bat sortuz. Atxikidura honek proteinaren gune aktiboa estaltzen du proteinaren konbertasa funtzio inaktibatuz [83].



5. Irudia. PCSK9aren prozesamendua eta funtzioa. 5A) PCSK9aren sintesiaren eta prozesamenduaren irudikapen grafikoa. **5B)** PCSK9 aldaeraren funtzio areagotzailearen zelulaz kanpoko funtzioaren irudikapena. PCSK9 aldaera areagotzaile batzuek LDLRarekiko kanpo-afinitate handiagoa daukate eta, ondorioz, endozitosi-prozesuan, pHaren jaitsiera gertatzean, LDLR-PCSK9 konplexua ez da askatzen. Horrek, LDLRaren degradazioa bultzatzen du haren birziklapena ekidinez.

PCSK9 aldaerei dagokienez, bi motatakoak izan daitezke: funtzio areagotzaileak (GOF) edo funtzio galtzaileak (LOF). GOF aldaerak HF kasuekin erlazionatuta daude, zelularen mintzeko LDLR kopurua murrizten dutelako kanpo (LDLRekiko afintitate gehiago) edo barne mekanismoen (LDLRren mintz garraioa oztopatu) bidez. 2019. urtera arte 30 GOF mutazio deskribatu dira, gehienak nukleotido ordezkapenaren ondorio dira, eta PCSK9ren hiru domeinuetan zehar banatuta daude. Hauen artean transkripzioa areagotzen duten, automozketa prozesu ezberdina jasaten duten edo LDLRekiko afinitate handiagoa duten PCSK9 aldaerak deskribatu dira (4B Irudia). LOF mutazioen kasuan, aldiz, gutxi batzuk deskribatu dira orain arte. Hauek LDL-C kontzentrazio baxua eragin ohi dute eta honek CVDren aurkako babesa eskaintzen du [84].

2.4 LDLRAP1

LDLRAP1 proteinak LDLR-LDL konplexuaren barneraketan parte hartzen du, LDLRren domeinu zitoplasmatikoaren eta klatrina molekulen arteko bitartekaria izanik. *LDLRAP1*en mutazio ez-funtzionalek, bi aleloetan aldi berean gertatuz gero, LDL partikulen klatrina bidezko barneraketa oztopatzen dute, plasmako LDL maila emendatuz [85].

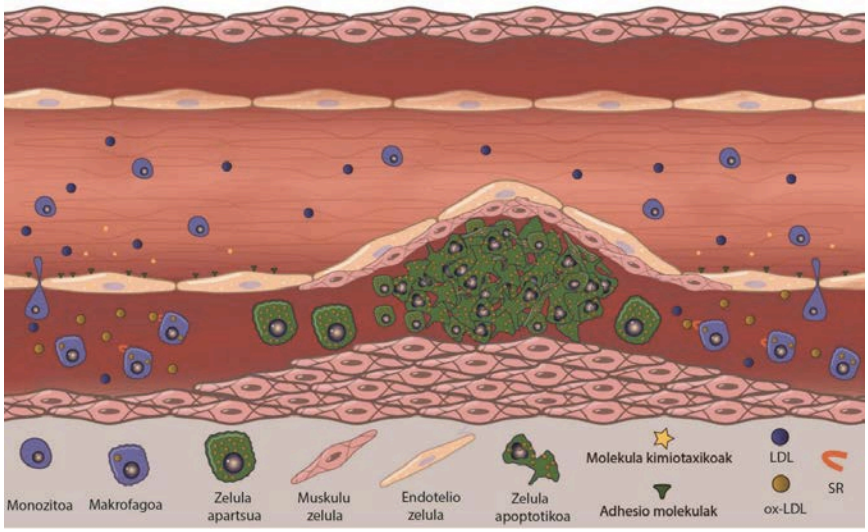
3. Bigarren mailako Hiperkolesterolemia Familiarra

LDL-C maila altuak LDL partikulen katabolismoan parte hartzen duten gene akastunekin lotuta egon ohi dira. Zenbait kasutan, ostera, LDL-C maila beste gaixotasun batzuen edota inguruko faktoreen ondorioz ere igo daiteke. Esaterako, *ABCG5/8* gene akastunek sitosterolemia sortzen dute. Kasu hauetan, HFean gertatzen den moduan, pazienteek LDL-C maila altuak eta arrisku kardiobaskular handia izan ohi dute. Hala ere, gaixotasun hau ez dago LDL metabolismoari lotuta, landare-esterol pilaketei baizik. Horregatik, landare-esterol hauen xurgapena blokeatzeko era askotariko tratamenduak jarraitu behar dituzte[86].

Sitosterolemiaz gain, beste gaixotasun batzuek ere LDL-C maila igo dezakete LDLren katabolismoan inolako eraginik izan gabe: sindrome nefrotikoak, gibel aparatuaren funtzio galerak, kolestasiak edo hipotiroidismoak, esaterako [87–89].

4. Hiperkolesterolemia Familiarra eta gaixotasun kardiobaskularra

HFk eragindako kolesterol maila altuek CVDren agerpena emendatzen dute, batez ere aterosklerosiaren garapen goiztiarra eragiten baitute [90,91]. Aterosklerosia CVDren eragilerik ohikoena da, eta normalean, arterien diametroa txikitzen du, bihotzekoak eta gaixotasun baskular ezberdinak pairatzeko arriskua areagotuz. Odolean pilatzen diren LDLek eta VLDLek pareta endoteliala gurutzatzen dute eta endotelio azpian dagoen intima izeneko geruzan metatzen eta oxidatzen dira. Metaketa hauek endotelioa ahulduta dagoen lekuetan bereziki nabariak dira, hau da, arterien bihurguneetan eta adarkaduretan. Bertan, odolak daukan fluxu zurrunbilotsua dela eta, hodien hormek estres mekaniko handiagoa jasaten dute [92]. Gainera, endotelioko zelulek jasaten duten estres mekanikoaren ondorioz, molekula kimiotaxikoak eta adhesiorako molekulak sintetizatzen eta kanporatzen dira, monozitoen migrazioa eta endotelioan zeharreko garraioa sustatuz [93]. Behin intima barruan, monozitoak makrofago heldu bilakatzen dira eta bertan dauden oxidatutako lipoproteinak barneratzen dituzte, erregulaziorik gabeko *scavenger* hartzaileen (SR) bitartez [94]. Kontrolik gabeko kolesterolaren barneraketak honen metaketa handiak eragiten ditu intimako makrofagoetan, hauek zelula apartsu bihurtuz. Denborarekin, zelula apartsuak ugaritzeak makrofago berriak erakartzen ditu eta intima azpiko geruzan dauden muskulu zelulen (SVMC) migrazioa eragiten du. Honela, zelulak pilatzen dira (aterosklerosi plaka) arterien diametroa murriztuz (5. Irudia). Aterosklerosiaren azken faseetan intima azpian pilatutako zelulek metaloproteinasa ezberdinak sintetizatzen eta kanporatzen dituzte [95]. Hauek aterosklerosi plaka desegonkortu eta apurtu dezakete, bihotzekoak edo gaixotasun baskularrak pairatzeko arriskua areagotuz.



5. irudia. Ateroma-plakaren garapena. Plasman pilaturiko LDL partikulak endotelioa zeharkatu eta bertan oxitadu egiten dira. Hauen oxidazioak, kimioerakarle zein adhesio molekulen sintesia bultzatzen du, monozitoen erakarpena eragiten dutelarik. Monozitoak makrofago bilakatzen dira gune azpiendotelialean eta oxidaturiko LDL modu ez erregulatuan. Gehiegizko kolesterol kantitateak makrofagoak zelula aprtsuetan bilakatu, VSMCen, ingelesetik "smooth vascular muscle cell", migrazioa eragin eta eremu fibrotikoaren sorrera bultzaten du. Azkenik, emendaturiko makrofagoen heriotzaren ondorioz, plakaren tamaina handitu eta arteriaren diametroa murrizten da.

5. Hiperkolesterolemia Familiarraren diagnosia

HFren diagnosi egokia eta ziurra egiteko zenbait gida daude eskuragarri. Haien artean, *Simon Broome Register Gropu* (SBRG) [96], *Male Early Diagnosis to Prevent Death eta Duch Lipid Clinic Network* (DLCN) [75] dira erabilienak. Gida gehienek antzeko irizpideak erabiltzen dituzte HF diagnostikatzerako orduan, haien arteko ezberdintasun nabarmenena irizpide bakoitzari emandako garrantzia izanik. Irizpide ezberdinen artean sintoma fisikoak (tendoi xantomak eta korneako arkua), plasmako kolesterol maila, HF aurrekari familiarrak, pazientearen historia klinikoa edo DNA analisiak agertzen dira.

Irizpideen artean, DNA analisiak dudagabeko diagnosirako parametrorik fidagarrienetarikoak dira, hauekin HF sortu dezaketen mutazioak erraz detektatu baitaitezke. Hala ere, test genetikoen bidez diagnosi zehatz bat lortzeko informazio osagarria ezinbestekoa da [97]. Analisi funtzionalek, kosegregazio analisiek bezala, test genetikoek eskeintzen duten informazioa osotzen dute, honela, HFren diagnosi fidagarri bat baimenduz. Gainera,

kosegregazio analisiak ez bezala, ikerketa laborategi batean erraz burutu daitezke, ez daukate pazienteekiko inolako menpekotasunik eta HF sortzen duten mutazioen mekanismoak ikertzeko aukera ematen dute [98,99].

6. LDL-C murrizteko terapiak eta hauen bilakaera

Kolesterol maila altuak ez dauka sintoma zuzenik eta, horregatik, askok ez dakite HF pairatzen dutela. CVD pairatzeko aukerak, atalase maila batetik gora (kolesterol maila totala 200 mg/dL eta LDL-C maila 100 mg/dL), asko handitzen dira. Hortaz, HFren prebentzioa eta tratamendua erabakigarriak dira. Gaur egun, estatinak dira farmakorik erabilienak. Hala ere, denboran zehar beste hainbat farmako zein farmako-konbinazio erabili dira (4. Irudia). HoHF eta HeHF kasu larri batzuetan adibidez, estatinetan oinarritutako tratamendua kolesterola murrizteko beste farmako batzuekin batera erabili behar da emaitza egokiak lortzeko [100].

6.1 Estatinak

Estatinak odoleko lipido maila murrizteko mundu mailan gehien preskribatutako medikamentuak dira. HMG-CoA erreduktasaren eta mebalonatoaren bidezidorrean ekoiztutako metabolitoen sorrera inhibitzen dute, biak gibeleko kolesterol sintesian parte hartzen duten konposatuak izanik. Horri esker, zelula barneko kolesterol ekoizpena asko murrizten da, hepatozito zelulen mintzeko LDLR adierazpena bultzatzean odoleko LDL-C mailaren gutxitzen baitu. Beraz, estatinek plasmako LDL eta VLDLen zeharkako desagerpena bultzatzen dute, gibelean eta ehun periferikoetan LDLRen adierazpena handituz [101]. Gainera, beste parametro lipidiko batzuetan ere, eragin onuragarria daukate, besteak beste, HDL maila handitzen edota triglizeridoena txikitzen baitute. Estatinak Akira Endok deskribatu zituen 70eko hamarkadan, baina ez ziren salmentan jarri 1986 arte. Orduan merkaturatu zen lovastatina, HMG-CoA erreduktasaren inhibitzailea. Gaur egun, gehien erabiltzen diren estatinak lovastatina, fluvastatina, atorvastatina, sinvastatina eta rosuvastatina dira. Beraien artean eraginkorrenak, azken hirurak, HoHF gaixoetan ere LDL maila murrizteko gai dira, ziur aski gibeleko LDL-C ekoizpena murrizten dutelako [102].

Estatinak bi motatan taldekatu daitezke: lehenengo motakoak, jatorri naturalekoak edo beraien eratorriak (lovastatina, simvastatina, mevastatina

eta pravastatina); eta bigarren motakoak (atorvastatina, fluvastatina, rosuvastatina, cerivastatina eta pitavastatina), sintetikoak direnak. Azken hauetan, fluorofenil talde batek lehengo motakoen butiril taldea ordezkatzen du gehienetan. Estatinen hidrofilikotasuna beraien hepato-hautakortasunak zehazten du, hortaz, bi taldetan sailkatu daitezke: Hidrofilikoak (rosuvastatina eta pravastatina) eta hidrofoboak (atorvastatina, simvastatina, fluvastatina, lovastatina eta cerivastatina). Bi motak selektiboki barneratzen dira hepatozitoetan; ehun periferikoetan, berriz, bi era ezberdinetan barneratzen dira. Estatina hidrofobikoek eragin handiagoa izaten dute ehun ez-hepatikoetan, era pasiboan barneratu daitezkeelako mintzean zehar. Estatina hidrofilikoak, aldiz, gibelarekiko espezifikoagoak dira, garraio aktibo bidez barneratzen direlako hepatozitoetan [103].

Nahiz eta estatinen eraginkortasuna eta segurtasuna frogatuta egon, gaixo batzuetan denboraldi luzeko eta dosi handiko tratamenduen ondoriozko eragin kaltegarriak deskribatu dira. Ohikoenak estatina lipofilikoei lotutako muskulu sintomak dira, mina edo ahultasuna, adibidez. Horregatik, uretan disolbagarriak diren estatinak (pravastatina, rosuvastatina) hobesten dira. Estatinek bigarren motako mellitus diabetesean (DMII) daukaten eragina ere aztertzen ari dira, dosi handiko tratamenduak IIMDren garapenarekin lotu baitira [104]. Estatina lipofilikoek, hidrofilikoekin alderatuz, eragin metaboliko kaltegarriak sortu ditzakete, intsulinaren jariapen okerra edo intsulinarekiko erresistentziaren garapena besteak beste. Azkenik, kontuan izan behar dira estatinek, zenbait kasutan, funtzio hepatikoan, giltzurrunen funtzioan edo funtzio kognitiboan kalteak eragin ditzaketela [105].

6.2 Niazina

Niazina, B3 bitamina edo azido nikotiniko izenekin ere ezagutua dena, HFren tratamendurako erabili zen lehenengo farmako lipido-eraldatzailea izan zen. Niazinak gantz azido libreen (FFA) ehun adipotsutik kanporako garraioa murrizten du, bertako proteina lipasa sistema inhibituz. Hortaz, gibeleko FFAren erabilgarritasuna mugatzean, kolesterolaren eta triglizeridoen ekoizpena murrizten du. Niazinaren ohiko albo-kalteak basodilatazioa eta entzima hepatikoen mailaren gehiegizko igoera dira [106].

6.3 Azido biliarraren bahitzaileak

Azido biliarraren bahitzaileak 1975. urtean merkaturatu ziren. Molekula hauek, azido biliar-kolesterol mizelekin elkartzean, konplexu disolbaezin bat eratzen dute. Honela, enterozitoetan barneraketa ekiditen eta kolesterol kanporaketa sustatzen dute. Enterozitoek kolesterol gutxiago barneratzen dutenez, hauen eta gibelaren arteko kolesterol garraioa partzialki inhibituta dago. Ondorioz, gibelera iristen den kolesterol maila txikiagoa da. Hori dela eta, LDL eta VLDL jariaketa murriztu egiten da, lipoproteina hauen odoleko maila jaitsiz. CVDk eta haren ondorioek eragindako heriotza tasak murrizteko gai direla frogatu da, hauek ordea, askotan ez dira ondo jasaten. Izan ere, gantzetan disolbagarriak diren bitamina batzuen xurgapena eta azido biliarraren berxurgapena, eragotzi dezakete. Hartzailearen funtzioa guztiz galdu den HoHF kasuetan, azido biliarren bahitzaileak ez dira erabilgarriak izaten [107].

6.4 Ezetimibe

Ezetimibe kolesterolaren heste-xurgapena era selektiboan inhibitzen duen molekula da. NPC1L1 inhibitzen du, bai enterozitoen lumenean bai interfase hepatobiliarrean. Honek, kolesterolaren xurgapena modu eraginkorrean inhibitzen du, baina triglizerido eta gantzetan disolbagarriak diren bitaminen absortzioan, berriz, ez du eraginik. Hesteetan kolesterolaren xurgapena inhibitzeak, kolesterol-behazunen berxurgapena inhibitzeaz gain, kilomikroi gutxiago sortzea eta jariatzea eragiten du. Honek, hepatozitoetako kolesterol gordekinak xahutzea dakar. Gibelean kolesterol gutxiago izateak LDLR adierazpena bultzatu eta VLDL sintesia murrizten du plasma LDL-C murrituz [108].

6.5 Giza jatorriko anti-PCSK9 antigorputz monoklonalak

Giza jatorriko anti-PCSK9 antigorputz monoklonalak, arrisku altuko gaixoetan bereziki, LDL-C mailak jaisteko eta CVD arriskua murrizteko gai direla frogatu da. Beraien erabilpena, estatinekin eta ezetimibekin tratatutako pazienteetan, kolesterol maila behar beste murrizten ez denean gomendatzen da. Gaur egun bi antigorputz ezberdin erabiltzen dira, Alirocumab eta Evolocumab. Biak giza IgG azpitaldekoak dira eta odolean dagoen PCSK9ri

batzen zaizkio, hau LDLRra batu ez dadin. Honek, PCSK9 urritasuna eragingo du, LDLR birziklapena bultzatuz. Ondorioz, mintzean LDL hartzaile gehiago egongo da eta LDL gehiago barneratuko da. Nahiz eta anti-PCSK9 antigorputzek albo-kalte gutxi eduki eta oso eraginkorrak izan, terapiaren kostu handiak bere erabilpena zabaltzea ekidin du [109].

6.6 Beste tratamendu batzuk

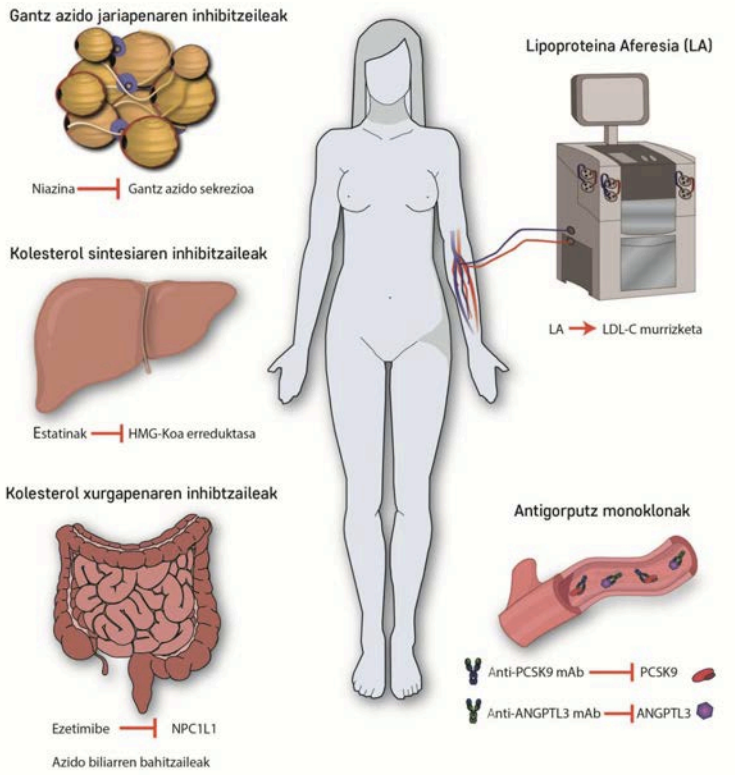
Azken aldian lipoproteinen ekoizpenean eragiten duten hainbat farmako agertu dira. Lomitapide MTP inhibitzaileak APOB apolipoproteinaren lipidazio ezegokia eragiten du hepatozitoetan, VLDL lipoproteinen jariapena ekidinez. Mipomersen, berriz, *APOB* mRNArekin batzen diren oligonukleotidoz osatuta dago eta hauek gibeleko LDL eta VLDL sorrera murrizten dute. Aipatutako bi farmakoek hainbat albo-kalte eragiten dituzte eta HoHF edo arrisku kardiobaskular handiko kasuetan bakarrik gomendatzen dira [110].

HFren tratamendurako fase klinikoan dauden farmakoen artean, Evinacumabek, 3. motako angiopoietina (ANGPTL3) inhibitzaileak, emaitza nabarmenak lortu ditu. ANGPTL3 proteinak LDL-C, triglizerido eta HDL mailak emendatzen ditu LDLR bidezidorrean inolako eraginik izan gabe. Hori dela eta, fase klinikoa gaindituz gero, oso farmako eraginkorra izatea espero da bereziki HoHF pazienteen tratamendurako [111].

Lipoproteina aferesia arrisku kardiobaskular oso handia duten gaixoengan, beste terapia guztiek kale egiten dutenean, erabiltzen den tresna terapeutikoa da. Estatinek edo PCSK9ren aurkako antigorputzek oso eragin txikia dutenean edo LDLR adierazpenik ez duten HoHF gaixoengan, adibidez. CVD arrisku handia eta iragarpena txarra denean, terapia hau erabili daiteke; izan ere, kostu ekonomiko handia dakarrenez eta tratamendua luzea eta irisgarritasun txikikoa denez, lipoproteina aferesia beste teknika guztiak erabilgarriak ez direnean bakarrik gomendatzen da [112].

Orain arte estatinak izan dira kolesterola murrizteko erabilitako tratamendu nagusia, beraien eraginkortasunarengatik eta prezio baxuengatik. Beste farmako guztiak, estatinekiko errefusa agertzen denean edo hauekin hobekuntzarik ez dagoenean bakarrik erabiltzen dira. Estatinek eragin txikia badute, normalean Ezetimibe, PCSK9 inhibitzaileak edo biak gehitzen dira

tratamendura, emaitza esanguratsuak lortuz. MTP inhibitzaileak edo ApoB100en itzulpena inhibitzen duten oligonukleotidoak, tolerantzia txikiko eta kostu ekonomiko handiko teknikak diren arren, LDLR sintetizatzen ez den HoHF kasuetan LDL mailak murrizteko aukerarik erabilienak dira [113].



6. Irudia. Hiperkolesterolemia familiarraren tratamendurako erabiltzen diren edo erabili diren tratamendu ezberdinak.

6. Hiperkolesterolemia Familiarra eta nutrazeutikoak

Nutrazeutikoak onura medikoak eskaintzen dituzten jatorri naturaleko sustantziak dira. HFren tratamenduan erabiltzen diren nutrazeutikoek lipido maila jaisteko gaitasuna daukate eta haien erabilpena, aurretiaz aipatutako tratamenduekin batera, dislipemia ezberdinak kontrolpean mantentzeko oso hedatua dago. Nutrazeutikoek kolesterol metabolismoaren pausu ezberdinetan eragin dezakete, HF tratamendu ezberdinen emaitzak hobetuz. Landare esterolek, adibidez, dietatik datorren kolesterolaren xurgapena murriztu dezakete; berberinak, aldiz, PCSK9ren efektua partzialki inhibitu

dezake eta arrozaren legamian aurkitzen diren monokolinek, estatinen antzera, kolesterol endogenoaren sintesian eragiten dute [114].

7. Hiperkolesterolemia Familiarraren gaur egungo egoera

Denboran zehar mantendutako plasma kolesterol maila altuek aterosklerosi plakaren garapena dakarte. Horren ondorioz, tratatu gabeko HFk CVD pairatzeko arriskua areagotzen du [115]. Gaur egun, kolesterol maila egonkor mantentzeko terapia ezberdinak daude. Hauen eraginkortasuna, ordea, nabarmenki handiagoa da gaixotasuna era goiztiar batean diagnostikatzen bada [116]. Diagnosi goiztiarra garrantzitsua den arren, gaur egungo diagnosi tasa oso baxua da. Herrialde gehienetan HF diagnosia ez da populazioaren % 1era iristen eta Herbeheretan soilik populazioaren % 50 baino gehiagori diagnostikatu zaio. Espainian berriz, populazioaren % 6 inguru diagnostikatu da. Diagnosi maila eta gaixotasunak daukan prebalentzia kontuan hartuta, munduan HF diagnostiko zehatzik ez duten 24-36 milioi pertsona inguru daude.

Historikoki, HeHF hainbat ezaugarri kontuan hartuta diagnostikatu izan da: LDL-C maila, HFren sintoma fisikoak eta pazientearen eta bere familiaren historia klinikoa, esaterako. Diagnosi mota honek, ordea, HF kasu nabarmenenak soilik detektatzeko balio du, tarteko fenotiporik hauteman gabe [117]. Gaur egun, *next generation sequencing* tekniken garapenak, tarteko fenotipo hauen detekzio arina eta erraza ahalbidetu du. Honek gaixotasunaren genetikan sakontzeko aukera eman du eta diagnosi klinikoarekin batera HF kasu berri ugari azaleratu ditu**.**

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2. Objectives / Helburuak

General objectives

FH is the most serious commonly inherited metabolic disease and, with an estimated frequency of 1:200 - 1:250, remains severely underdiagnosed. Autosomal dominant mutations in LDLR, APOB, and PCSK9 genes account for most of the FH cases and although genetic testing can contribute to patients' benefit, only the identification of functional FH-causing mutations provides a definitive diagnosis. To date, there have been described more than 2,500 genetic LDLR variants. Since the great majority of them do not have functional studies, identification of the pathogenic variants causing FH is priority, as it would provide a definitive diagnosis, which is essential to improve patient prognosis.

Therefore, the main objective of the present work has been to assess pathogenicity of LDLR, APOB10 and PCSK9 variants by *in vitro* functional characterization.

Specific objectives:

1. Mutation type classification and pathogenicity assignment of sixteen missense variants located in the EGF-precursor homology domain of the LDLR.

2. Functional characterization and classification of LDLR variants located at the first cysteine rich domain (LR1) of the ligand binding domain (LBD).

3. Functional characterization of 12 LDLR missense variants and p.(Gly207_Ser213Ser) variant found in Portuguese FH patients and in other worldwide populations.

4. Functional characterization of six APOB100 missense variants and one PCSK9 missense variant found in Italian FH patients.

3. MATERIALAK ETA METODOAK / MATERIALS AND METHODS

1. LDLR aldaeren aukeraketa

ClinVar datu basea erabili da (https: //clinvarminer. genetics.utah.edu) EGF aitzindariaren domeinu homologoan dauden eta hartzailearen aktibitatean eragina izan dezaketen LDLR aldaerak aukeratzeko asmoz. Hurrengo aldaerak aukeratu dira: p.(Ser326Cys), p.(Cys338Phe), p.(Cys368Tyr), p.(Gln378Pro), p.(Ala399Tr), p.(Tr413Met), p.(Asp492Asn), p.(Ser584Pro), p.(Ala606Ser), p.(Aps622Gly), p.(Arg633Cys), p.(His656Asn), p.(Tr659Asn), p.(Cys698Tyr), p.(asp700Gly), eta p.(Asp707Tyr). Izan ere, aldaera hauek hiperkolesterolemia familiarra duten pazienteetan deskribatu dira, baina hauen interpretazio klinikoa eta karakterizazio funtzionala egin gabe daude oraindik. Aipaturiko aldaerak CE kalifikazioa duten Progenika Biopharmaren (Derio, Espainia) LIPOchip® edo/eta SEQPRO LIPO S® plataformak erabiliz identifikatu dira.

1. Taula. Ikertutako aldaeren deskribapen, kontserbazio eta *in silico* iragarpenak.

Izen Genetikoa	HGVS Izendapena	Nt Kontserbazioa	AA Kontserbazioa	Grantham Distantzia	GVGD Lerrokapena	SIFT	PolyPhen-2	MutationTaster2
c.977C>G	p.(Ser326Cys)	1.00	0.97	112	C65	Ez Onartua	Kaltegarria seguruenik(1)	Gaixotasun eragilea (prob:1)
c.1013G>T	p.(Cys338Phe)	1.00	1.00	205	C65	Ez Onartua	Kaltegarria seguruenik(1)	Gaixotasun eragilea (prob:1)
c.1103G>A	p.(Cys368Tyr)	1.00	1.00	194	C65	Ez Onartua	Kaltegarria seguruenik (1)	Gaixotasun eragilea (prob:1)
c.1133A>C	p.(Gln378Pro)	1.00	0.24	76	C25	Ez Onartua	Ez kaltegarria (0.155)	Gaixotasun eragilea (prob:0.994)
c.1195G>A	p.(Ala399Thr)	0.86	0.94	58	C0	Ez Onartua	Kaltegarria seguruenik(0.991)	Gaixotasun eragilea (prob:0.996)
c.1238C>T	p.(Thr413Met)	0.94	0.89	81	C15	Ez Onartua	Kaltegarria seguruenik (1)	Gaixotasun eragilea (prob:1)
c.1474G>A	p.(Asp492Asn)	1.00	1.00	23	C0	Ez Onartua	Kaltegarria seguruenik(1)	Gaixotasun eragilea (prob:1)
c.1750T>C	p.(Ser584Pro)	0.85	0.85	74	C0	Ez Onartua	Kaltegarria seguruenik(0.953)	Gaixotasun eragilea (prob:1)
c.1816G>T	p.(Ala606Ser)	0.87	0.87	99	C0	Onartua	Ez kaltegarria (0.067)	Gaixotasun eragilea (prob:0.999)
c.1865A>G	p.(Asp622Gly)	1.00	1.00	94	C65	Ez Onartua	Kaltegarria seguruenik(1)	Gaixotasun eragilea (prob:1)
c.1897C>T	p.(Arg633Cys)	0.92	0.95	180	C55	Ez Onartua	Kaltegarria seguruenik(0.987)	Gaixotasun eragilea (prob:1)
c.1966C>A	p.(His656Asn)	1.00	1.00	68	C65	Ez Onartua	Kaltegarria seguruenik(0.597)	Gaixotasun eragilea (prob:1)
c.1976C>A	p.(Thr659Asn)	0.72	0.69	65	C0	Onartua	Ez kaltegarria (0.003)	Polimorfismoa (prob: 0.997)
c.2093G>A	p.(Cys698Tyr)	1.00	1.00	194	C65	Ez Onartua	Kaltegarria seguruenik (0.998)	Gaixotasun eragilea (prob:1)
c.2099A>G	p.(Asp700Gly)	1.00	1.00	94	C0	Onartua	Kaltegarria seguruenik(0.999)	Gaixotasun eragilea (prob:1)
c.2119G>T	p.(Asp707Tyr)	1.00	0.97	160	C65	Ez Onartua	Kaltegarria seguruenik(1)	Gaixotasun eragilea (prob:1)
c.139G>A	p.(Asp47Asn)	1.00	1.00	23	C0	Ez Onartua	Kaltegarria seguruenik(1)	Gaixotasun eragilea (prob: 1)
c.185C>T	p.(Thr62Met)	0.973	0.947	81	C0	Ez Onartua	Kaltegarri seguruenik(1)	Gaixotasun eragilea (prob:1)
c.136T>G	p.(Cys46Gly)	1.00	1.00	159	C0	Ez Onartua	Kaltegarria seguruenik(1)	Gaixotasun eragilea (prob:1)

p.(Asp47Asn) eta p.(Thr62Met) aldaeren karakterizazio funtzionala burutzea erabaki da, LDL lotura akastuna eragitearekin lotuta egon daitezkeelako. Horrez gain, bi aldaera hauek hiperkolesterolemia familiarra pairatzen duten pazienteetan deskribatu dira, baina horien efektuak ez dira

karakterizatu oraindik. Aldaerak ClinVar datu basea erabiliz identifikatu eta 2 eta 9 indize-kasutan deskribatu dira, hurrenez hurren.

p.(Cys46Gly) aldaera hiru erabiltzailek sartu dute ClinVar datu basean (https://clinvarminer.genetics.utah. edu) (sarrera kodea: RCV000238380). Gainera, Progenika Biopharmak (Derio, Espainia) 3 hiperkolesterolemia familiar indize-kasutan topatu du aldaera. Azterturiko aldaeren deskripzioa, kontserbazio maila eta *in silico* iragarpenak hurrengo taulan adierazi dira (1. Taula).

p.(Gly592Glu), p.(Gly207_Ser213del), p.(Cys184Tyr), p.(Glu288Lys), p.(His211Asp), p.(Ala606Ser), p.(Asp601Val), p.(His656Asn), p.(Asp221Tyr), p.(Ile473Val), p.(Ile764Thr), p.(Gly20Arg) eta p.(Gln92lu) aldaerak, Portugaleko populazioan eta karakterizazio funtzionalik gabe topaturikoak dira (2. Taula).

2. Taula. LDLRren zikloaren karakterizazio funtzionalik gabeko ikertutako aldaera portugaldarren ezaugarriak

Paziente Kopurua (Familiak)	Kokapena	cDNA	Proteina	Domeinua	Kosegregazioa	Talde Normolipedemiko Portugaldarra	ACMG Klasifikazioa *†
38 (17)	Exon 12	c.1775G>A	p.(Gly592Glu)	EGF aitzindari homologia	17/18;0/3	0/190 FH gabeko aleloak	VUS
27 (10)	Exon 4	c.618_638del	p.(Gly207_Ser213del)	Ligando-lotura	14/15;0/7	0/190 FH gabeko aleloak	Kaltegarri seguruenik
15 (7)	Exon 4	c.551G>A	p.(Cys184Tyr)	Ligando-lotura	11/11;0/0	0/190 FH gabeko aleloak	Kaltegarri seguruenik
12 (7)	Exon 6	c.862G>A	p.(Glu288Lys)	Ligando-lotura	2/3;0/2	0/190 FH gabeko aleloak	VUS
10 (5)	Exon 4	c.631C>G	p.(His211Asp)	Ligando-lotura	4/4;0/4	0/190 FH gabeko aleloak	Kaltegarri seguruenik
6 (4)	Exon 12	c.1816G>T	p.(Ala606Ser)	EGF aitzindari homologia	7/7;0/2	0/190 FH gabeko aleloak	VUS
5 (1)	Exon 12	c.1802A>T	p.(Asp601Val)	EGF aitzindari homologia	5/5;0/2	0/190 FH gabeko aleloak	VUS
5 (1)	Exon 13	c.1966C>A	p.(His656Asn)	EGF aitzindari homologia	4/7;1/2	0/190 FH gabeko aleloak	VUS
5 (2)	Exon 4	c.661G>T	p.(Asp221Tyr)	Ligando-lotura	5/5;0/2	0/190 FH gabeko aleloak	Kaltegarri seguruenik
3 (1)	Exon 10	c.1417A>G	p.(Ile473Val)	EGF aitzindari homologia	2/2;1/2	0/190 FH gabeko aleloak	VUS
3 (1)	Exon 15	c.2291T>C	p.(Ile764Thr)	O-loturadun azukrea	2/3;1/3	0/190 FH gabeko aleloak	VUS
2 (2)	Exon 1	c.58G>A	p.(Gly20Arg)	Peptido seinalea	2/2;0/0	0/190 FH gabeko aleloak	VUS
1 (1)	Exon 2	c.274C>G	p.(Gln92lu)	Ligando-lotura	1/1;0/0	0/190 FH gabeko aleloak	VUS

Kosegregazioa, Hiperkolesterolemia Familiarraren Ikerketa Plan Portugaldarreko familien kosegregazio analisia: aldaera eramailea/kaltetu totala, aldaera eramaileak/ez-kaltetu totalak; VUS (ingelesetik *Variant of Unknown Significance*); * Chora et al 2017; † azterketa funtzionala burutu arretiko ACMG klasifikazioa.

2. LDLR aldaeren eragin molekularraren *in silico* analisia

Aukeratutako aldaeren eragina aurresateko, lau software desberdin erabili dira: Align GVGD (http://agvgd.iarc.fr), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), SHIFT (http://sif.jcvi.org) eta MutationTaster2 (http://www.mutationtaster.org). GVGD eta SIFTren bitartez, aminoazido aldaketak aztertu dira. Giza LDLR proteinaren sekuentzia (P01130) beste 9 espezieren LDLR proteinaren aminoazido sekuentziekin alderatu da: *Pan troglodytes* (XP_001167447.1), *Macaca mulatta* (NP_001028078.1), *Mus musculus* (P35951.1), *Rattus norvegicus* (P35952.1), *Oryctolagus cuniculus* (P20063.1), *Sus scrofa* (Q28832.2), *Equus caballus* (XP_001490793.2), *Xenopus laevis* (AAI70345.1) eta *Danio rerio* (NP_001025454.1).

Polyphen-2 aurreikuspen softwarea (2.2.2. bertsioa) testatzeko, HumDiv datu basea erabili da. MutationTaster2k, berriz, Bayes sailkapen sistema erabiltzen du aldaera batek gaixotasuna eragiteko duen gaitasuna aurresateko. Bayes sailkapen sistema sarritan berritzen denez, aldaerei buruzko aurreikuspenak denboran zehar alda daitezke. Orokorrean, aurreikuspen programek eredu zehatz batean oinarrituriko aurreikuspenari probabilitate balio bat esleitzen diote. Bi aurreikuspen software hauen kasuan, krisketen artean agertzen den probabilitate balioa, aurreikuspen balioari dagokio. Esate baterako, 1etik hurbil dagoen balioak, aurreikuspena fidagarria dela adierazten du. SIFT softwarearen kasuan, balioa 0tik 1era doa eta 0,05etik beherako balioak aminoazido aldaketa kaltegarria dela adierazten du. 0,05etik gorako balioek, aldiz, aldaketa jasangarria dela adierazten dute. GVGDren kasuan, balioak 0tik 65era doaz, 0k ez-patogenikoa dela eta 65ek segurtasun handiz patogenikoa dela adierazten dute, hurrenez hurren

3. Kontserbazio analisia

Nukleotidoei eta aminoazidoei dagokienez, espezieen arteko kontserbazio analisia burutu da, Kaliforniako Santa Cruz unibertsitateko datuetan oinarrituz. Genome bilatzailea (http://genome.ucsc.edu) 46 ornodun espezieren sekuentzien parekamendu anitzetan oinarritzen da. Kontserbazio balioa giza sekuentziari batutako sekuentzia kopuruaren eta eskuragarri dauden sekuentzia kopuru totalaren arteko zatidura edo ratio moduan adierazten da.

4. Mutagenesi guneratua

LDLR aldaerak dituzten plasmidoak Innoprot (Derio, Espainia) enpresak eraiki ditu eta giza LDLRaren DNA osagarrian (cDNAn) (NM_000527.4) barneratu dira, SV40 promotorearen menpeko pcDNA3 ugaztun bektorea erabilita. Oligonukleotidoek gidatutako mutagenesia burutu da, QuickChange Lightning (Agilent Technologies, AEB) mutagenesi kita erabiliz eta etxe komertzialaren argibideak jarraituz. LDLR aldaera desberdinak daramatzaten plasmidoen oligonukleotidoak *in vitro* sintetizatu eta SacII eta EcoRI errestrikzio entzimak erabilita azpiklonatu dira. Intereseko nukleotidoen aldaera PCR bidez baieztatu da, eta gainera, zati egokiak moztu dira errestrikzio entzimak erabiliz. Plasmidoen LDLR cDNA sekuentzien osotasuna bermatzeko sekuentzia-analisi zuzena erabili da.

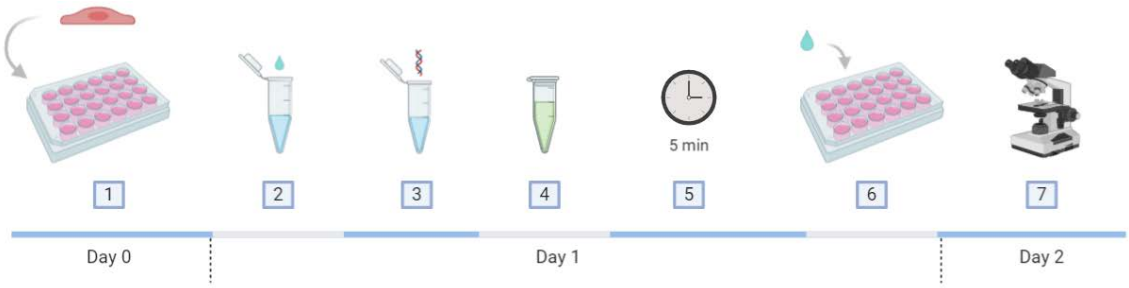
5. Zelula eukariotoen hazkuntza

CHO-*ldl*Δ7 zelulak hamster obulutegietatik isolaturiko fibroblastoak dira. Zelula hauen ezaugarririk bereizgarriena LDLR kodetzeko duten ezintasuna da. Horregatik, LDLR aldaeren karakterizazioa burutzeko zelula aproposak dira. Zelulak M. Kriegerek (MIT, AEB) adeitasunez eskainitakoak dira. Zelula hauek 37 °C-tan eta % 5 CO2–rekin hazten dira. Horretarako, Ham´s F12 (Nutrient mixture F-12 HAM, Sigma-Aldrich, AEB) medioari inaktibatutako % 10 (b/b) FBS (Lonza, Belgika), 100 U/mL penizilina, 100 μg/mL estreptomizina eta 4 mM glutamina (Pen Strep Glutamine, Invitrogen, AEB) gehitzen zaizkio.

HEK293 zelulak giza enbrioi jatorrizko giltzurrun zeluletatik eratorriak dira. Hauek % 10 FBS eta % 1 penizilina/estreptomizinarekin osaturiko DMEM (Dulbeccos´s Modified Eagle´s Medium, Sigma-Aldrich, AEB) medioan hazten dira poli-lisinaz gainestalduriko T175 flasketan, 37 °C-tan eta % 5 CO_2-rekin.

6. CHO-*ldl*Δ7 zelulen lipofektamina bidezko transfekzio iragankorra

LDLRrik gabeko CHO-*ldl*Δ7 zelulak % 5 FBSrekin, 2 mM L-glutaminarekin, 100 unitate/mL penizilinarekin eta 100 µg/mL estreptomizinarekin osatutako Ham's F-12 medioan hazi eta, ondoren, 6-24 putzutako hazkuntza-plakara pasatu dira. Behin % 70eko zelula dentsitatea lortutakoan, Opti-MEMem medioan jarri eta 24 ordu igaro ostean, zelulak lipofektamina (Lipofectamine® LTX eta PlusTM Reagent, Invitrogen, AEB) erabiliz transfektatu dira, etxe komertzialaren argibideak jarraituta. Lipofektaminak negatiboki kargaturiko zelula mintzekin elkar eragiten du. Honela, endozitosi bidez barneratuko da, transfektatu nahi ditugun plasmidoekin batera. Transfekzioa burutu eta 6 ordura, Opti-MEM medioari % 5 FBS gehitu zaio. Esperimentua 24 ordu igaro ostean, adierazpen-maila egokia lortu denean, burutu da (1. Irudia).



1. Irudia. Transfekzio iragankorraren diagrama. CHO-*ldl*Δ7 zelulak 24 putzutako plakan hazi dira. 24 ordu igaro ostean, Lipofectamine® LTX eta DNA Opti-MEM® medioan diluitu dira bakoitza bere aldetik. Diluitutako DNA, diluitutako Lipofectamine® LTX indargetzailera gehitu da 1:1 ratioan. Nahastea giro tenperaturan inkubatu da 5 minutuz, eta ondoren, zelulei esleitu zaie. 24 ordu igaro ostean, transfekzioaren efizientzia aztertu da.

7. Kaltzio fosfato bidezko transfekzio iragankorra

Transfekzioa egin baino 4 ordu lehenago, zelulei hazkuntza medioa berritu zaie. Transfekzioa egiteko, saiodi batean transfektatu nahi den putzuari dagokion 250 mM $CaCl_2$ bolumena eta transfektatu nahi den plasmidoa gehitzen dira. Ondoren, giro tenperaturan dagoen HBS 2X (50 mM HEPES, 1.5

mM Na_2HPO_4 eta 140 mM NaCl, pH 7.05) medioa tantaka gehitu da saiodira. Aldi berean, osagai guztien nahaste homogeneoa bermatzeko, saiodia emeki astindu da. Nahastea giro tenperaturan mantendu da 5 minutuz, eta ondoren, kontu handiz, putzu bakoitzari dagokion bolumena gehitu zaio, zelulak altxatu ez daitezen. Plakak gau osoan zehar 37 °C-tan eta % 5 CO_2-rekin inkubatu dira. Transfekzioa egin eta 24 ordutara, transfekzio medioa kendu eta antibiotikodun medio berria gehitu da.

8. LDLR-ren kanpo domeinuaren ekoizpena eta purifikazioa

C-myc eta His-isatsa dauzkan LDLR-ren kanpo domeinua (1-789 aminoazidoak) ekoiztu eta purifikatu da, LDLR basatia eta p.(Cys46Gly) aldaeraduna, biak ala biak. Horretarako, HEK293 zelulak, Leren irakasleak adeitasunez eskainitako pcDNA3.1-EC-LDLR-His plasmidoarekin, transfektatu dira. Transfekziorako kaltzio fosfatoa erabili da, eta gerora, genetizinarekin (G-418 sulfatoa, Gibco, Invitrogen, AEB) aukeratu dira. Azkenik, LDLRren kanpo domeinua afinitate bidez purifikatu da, pausu bakarreko nikel zutabe bidezko kromatografia erabiliz.

9. Western blot bidezko analisia

Transfekzioa egin eta 24 ordura zelulak PBS hotzarekin garbitu eta lisia burutu da. Zelulak lisatu ahal izateko, 50 mM Tris–HCl (pH 7.5), 125 mM NaCl, % 1 Nonidet P-40, 5.3 mM NaF, 1.5 mM NaP, 1 mM ortobanadato, 1 mg/mL proteasa-inhibitzaile koktela (Roche, Suitza), eta 0.25 mg/mL Pefabloc, 4-(2-aminoetil)-benzenesulfonil fluoride hidrokloruroa (AEBSF) (Roche, Suitza) dauzkan medio hotza erabili dira. Behin lisi-medioa gehituta, zelulak batu eta 4°C–tan biraka mantendu dira ordu batez. Ondoren, 12000 x g-tan 15 minutuz zentrifugatu dira. Liseritutako gainjalkinen proteina kontzentrazioa neurtu da.

Inmunodetekzio semikuantitatiboa burutzeko, lehendabizi proteinak elektroforesi bidez banatu dira, % 8.5-eko SDS-PAGE ez-erreduzitzailea erabilita. Gero, antigorputz-proteina lotura inespezifikoak ekiditeko, mintzak ordubetez inkubatu dira blokeo soluzioan (TBST + % 5 esne gaingabetua),

ondoren, antigorputz primarioarekin inkubatu direlarik. Mintzak gau osoan zehar inkubatu dira, antigorputz primarioarekin (LDL Receptor Polyclonal Antibody, Cayman Chemical, AEB) 4°C–tan. Antigorputz sekundarioaren kasuan (GAPDH Antibody, SantaCruz, AEB) (3. Taula), berriz, mintzak 3 aldiz TBSTrekin garbitu ostean, giro tenperaturan inkubatu dira ordubetez. Seinalea SuperSignal West Dura Extended Substrate (Pierce Biotechnology, AEB) bidez detektatu da, ChemiDoc XRS (Bio-Rad, AEB) erabiliz. Banden intentsitatea kuantifikatzeko, NIH ImageJ softwarea (https://rsbweb.nih.gov/ij/) erabili da eta intereseko proteinen intentsitate mailak GAPDH kontrolaren intentsitatearekiko zuzendu dira.

3. Taula. Western blotean eta Fluxu-zitometrian erabilitzako antigorputzen zerrenda.

Antigorputza	Etxe komertziala	Erreferentzia	Mw (kDa)	Diluzioa	Inkubazioa
LDL Receptor Polyclonal Antibody *	Cayman	10007665	120/160	1:500	16 h/4 °C
GAPDH Antibody	Santa Cruz	SC-47724	35,8	1:1000	16 h/4 °C

*LDLRren Western blotean erabilitako tanpoi indargetzaile guztiek 2 mM $CaCl_2$ daramate.

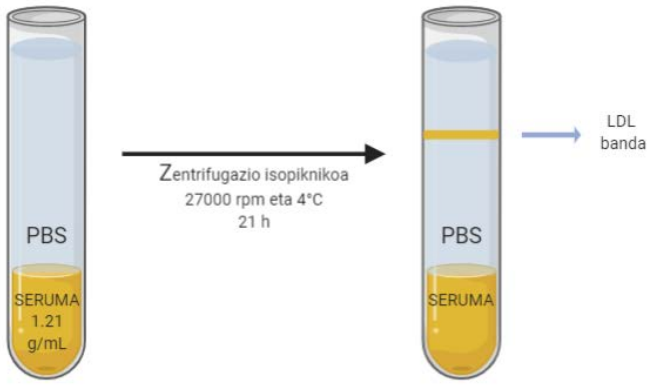
10. Lipoproteinen purifikazioa eta markaketa

Norbanako osasuntsuetatik eskuratutako odol plasma 12000 x g-ko abiaduran eta 4°C-tan zentrifugatu da, 30 minutuz. LDLen purifikazioa (ρ_{LDL} = 1.019-1.050 g/mL) zentrifugazio isopiknikoaren bidez burutu da. Horretarako, lagin bakoitzaren dentsitatea KBrrekin doitu da, 1.21 g/mL-ko dentsitatea lortu arte. Ondoren, lagin bakoitzaren gainean, PBSz (Sigma-Aldrich, AEB) osaturiko indargetzaile hotza gehitu da astiro, honela, bi fasetako gradientea sortuz. Ultrazentrifugazioa burutzeko, SW28.1 errotorea (Beckman Coulter, AEB) erabili da, 27000 rpm-ko abiaduran eta 4°C-tan, 22 orduz (X irudia). Zentrifugazioa amaitzerakoan, LDLeri dagozkion banda jaso eta 4°C-tan gorde dira, handik 2-3 egunetara erabiltzeko asmoz (2. Irudia).

Lipoproteinak fluoreszeina isotiozianatorekin, ingelesez *Fluorescein Isothiocyanate* (FITC) inkubatu dira 0.1 M $NaHCO_3$ indargetzailean (pH 9.0), giro tenperaturan. 2 ordutako inkubazioa burutu da, lipoproteinak biraka mantendu direlarik. Lipoproteina mililitro bakoitzeko 10 µL FITC (1,8 mg/mL, dimetil sulfoxidoan disolbatuta (DMSO)) gehitu dira eta inkubazioa bukatutakoan, lotu ez den FITCa kendu da, PBS EDTA indargetzailean orekaturiko Sephadex G 25 zutabe batez baliatuz. Ondoren, lagin guztien

proteina kontzentrazioa kalkulatu da, BSA estandar moduan erabiliz (DC™ Protein Assay, Bio-Rad, AEB).

Ikerketa hau Euskal Herriko Unibertsitateko Ikerketarako Etika Batzordeak onartu du (*Comite de Ética en la investigación y la práctica docente de la Universidad del Pais Vasco/Euskal Herriko Unibertsitatea*; CEIAB/186/2014/MARTÍN PLÁGARO) eta erabilitako metodologia baimendutakoaren arabera burutu da. Partaide guztiek baimen informatua sinatu dute. Esperimentu guztiak argibide eta erregulazio guztiak jarraituz burutu dira.



2. Irudia. Zentrifugazio isopiknikoaren bidez burututako LDLen purifikazioa. Lagin bakoitzaren dentsitatea KBrarekin doitu da, 1.21 g/mL-ko dentsitatea lortu arte. Ondoren, lagin bakoitzaren gainean, PBSz osaturiko indargetzaile hotza gehitu da astiro, honela, bi fasetako gradientea sortuz. Ultrazentrifugazioa 27000 rpm-ko abiaduran eta 4°C-tan burutu da, 22 orduz.

11. Fluxu-zitometria bidezko analisia

Fluxu-zitometriak zelula populazioei buruzko informazioa eskuratzea ahalbidetzen du, multzo handi bateko zelulak banaka aztertzen dituelarik. Medio isotoniko batean esekita dauden zelulak banan-banan pasarazten dira, eta honela, fluxu zilindriko edo korronte konstante bat eratzen da. Laser izpi batek zelula korrontea zeharkatuko du, izpiaren dispertsioaren eta erreflexuaren iraupena, intentsitatea eta espektroa neurtzea ahalbidetuz. Zelula bakoitzeko hurrengo parametroak aztertu dira: argiaren dispertsio frontala, ingelesez *forward scatter (FSC)*, (zelulen tamainarekiko proportzionala den parametroa) eta angelu zuzeneko dispertsioa, ingelesez *side scatter* (SSC), (zelulen barne konplexutasunarekiko proportzionala den parametroa).

Gainera, zelula bakoitzaren fluoreszentzia intentsitatea neurtu da uhin-luzera desberdinetan. Aipaturikoa zelularen markaketa motak baldintzatuko du.

11.1. LDLRren adierazpenaren kuantifikazioa

CHO-*ldl*Δ7 zelulak % 1 BSArekin osatutako PBS indargetzailea erabilita 3 aldiz garbitu dira, eta ondoren, % 4 paraformaldeidorekin fixatu dira, 10 minutuz. Jarraian, zelulak berriz ere garbitu eta % 5 FBSrekin osatutako garbiketa indargetzailean eta giro tenperaturan inkubatu dira, ordubetez. LDLRren aurkako *mouse anti-human-LDLR* (1:100; 2.5 mg/L; Progen Biotechnik GmbH, Alemania) antigorputz primarioarekin inkubatu dira, 4 °C-tan eta gau osoan zehar. Hurrengo egunean, zelulak berriro ere 3 aldiz garbitu ostean, Alexa Fluor 488 fluoroforoarekin konjokaturiko *goat anti-mouse IgG* (1:100, Molecular Probes, AEB) antigorputz sekundarioarekin giro tenperaturan inkubatu dira ordubetez. LDLRren aurkako antigorputza LDLRra modu espezifikoan lotzen dela bermatzeko, kontrol negatibo moduan plasmido huts batekin transfektaturiko CHO-*ldl*Δ7 zelulak erabili dira. Esperimentu guztiak 3 aldiz errepikatu dira. Lagin bakoitzeko 10000 zelularen fluoreszentzia intentsitatea neurtu da, cytoFLEX (Beckman Coulter, AEB) fluxu-zitometroa erabiliz eta haren argibideak jarraituz. Aztertutako LDLR aldaeren balioak, LDLR basatiaren balio maximoarekiko normalizatu eta ehunekoetan adierazi dira.

11.2. LDLRren eta LDLren arteko lotura-tasaren neurketa

Zelulei medioa kendu eta PBSarekin garbitu ostean, zelulak FITCrekin markaturiko LDLekin inkubatu dira, 4 °C-tan 2 orduz. Inkubazio aldia igaro ostean, zelulak 3 aldiz garbitu dira, % 1 BSArekin osatutako PBS indargetzailea erabiliz. 10 minutuz % 4 paraformaldeidoarekin fixatu ostean, berriz ere garbitu dira. LDLR aldaeren adierazpen-maila kuantifikatzeko egin den moduan, lagin bakoitzeko 10000 zelularen fluoreszentzia intentsitatea neurtu da. Egindako esperimentu guztiak 3 aldiz errepikatu dira eta LDLR-LDL arteko lotura-tasaren balioak LDLR basatiaren balio maximoarekiko normalizatu eta ehunekoetan adierazi dira.

11.3. LDLRren barneraketa-tasaren kuantifikazioa

LDLR aldaerek duten LDL barneraketa-tasa neurtzeko, zelulak FITCrekin markaturiko LDLekin inkubatu dira, 37 °C-tan eta 4 orduz. Behin inkubazio denbora igaro denean, zelulak % 1 BSArekin osatutako PBS indargetzailearekin garbitu dira 3 aldiz. % 4 paraformaldeidoa erabiliz, 10 minutuz fixatu eta berriz ere garbitu dira. Barneratutako LDL-FITC partikulen seinalea bakarrik neurtu ahal izateko, kanpoan geratu diren partikulen seinalea amatatu da Trypan Blue disoluzioa erabiliz, % 0,2 -ko kontzentrazioan. Izan ere, Trypan Blueak ez du mintzak zeharkatzeko gaitasunik, eta beraz, barneratu ez den FITCaren seinale dinamikoan iraungitzeko ahalmena dauka. Horretarako, elkarrekintza fisikoa gertatu behar da. Lagin bakoitzeko 10000 zelulen fluoreszentzia intentsitatea neurtu da berriz ere. Esperimentu guztiak 3 bider errepikatu dira eta LDLR aldaera desberdinek barneratutako LDL-FITC partikulen fluoreszentzia seinalea LDLR basatiaren balio maximoekiko normalizatu eta ehunekoetan adierazi dira.

11.4. pH-aren eragina LDLRren eta LDLren arteko loturan

LDLRren birziklapen akastunak LDLR aldaeren funtzio ezegokia eragin dezakeen ikertzeko, LDLR-LDL lotura aztertu da pH desberdinetan. Honi esker, LDLak barneratu ostean, endosomen azidotze prozesu naturala simulatu da. Horretarako, zenbait LDLR aldaerekin transfektaturiko zelulak 20 μg/mL LDL-FITCrekin inkubatu dira 30 minutuz, pH desberdinetan dagoen eta 0,4 M sakarosadun medioan. Lotuta ez dauden LDL partikulak baztertzeko, inkubazio denbora amaitu ostean, zelulak % 1 BSArekin osatutako PBS indargetzailearekin garbitu dira. Azkenik, zelulak % 4 paraformaldeidoarekin fixatu dira eta lotuta mantendu den LDL-FITC partikulak cytoFLEX fluxu zitometroaren bidez kuantifikatu dira.

11.5. Fluxu-zitometrian erabilitako analisi estatistikoa

Eskuratutako emaitza guztiak gutxienez 3 saiotan egindako esperimentuen batezbestekoei dagozkie. Esperimentu guztien desbideraketa estandarrak, ingelesez *standard deviation*, ere irudikatu dira (±).

12. LDLren eta LDLRren kanpo domeinuaren arteko ELISA bidezko lotura analisia

LDLRren kanpo domeinuaren zatiak ELISA indargetzaile espezifikoan (10 mM Tris-HCl, pH 7.5, 50 nM NaCl eta 2 mM $CaCl_2$) diluitu eta 96 putzutako plaketan fixatu dira, gau osoan zehar 4 °C-tan mantendu direlarik. Hurrengo egunean, plakak blokeatu eta ELISA indargetzailean diluzio seriatu moduan prestaturiko LDLekin inkubatu dira, giro tenperaturan eta 2 orduz. Inkubazioa amaituta, plaka % 0.1 (p/b) Tween 20rekin (Sigma-Aldrich, AEB) osaturiko ELISA indargetzailearekin sakonki garbitu da. Estekatzailearen detekziorako, antigorputzak (g*oat polyclonal anti-apoB*, abcam, Erresuma Batua; *peroxidase-conjugated mouse anti-goat*, ThermoScientific, AEB) % 5 (p/b) BSArekin osatutako ELISA indargetzailerarekin nahastu dira, ondoren, plakan gehitu eta giro tenperaturan ordubetez mantentzeko.

Antigorputz primarioaren eta sekundarioaren inkubazio aldien artean, plaka sakonki garbitu da. Antigorputz lotura zehazteko, antigorputz sekundarioaren inkubazioa amaitu eta plaka garbitu ostean, putzu bakoitzera 50 µL 2,2′-Azino-bis *(3-ethylbenzothiazoline-6-sulfonic acid)* substratu soluzioa (Sigma-Aldrich, AEB) gehitu da eta kolore aldaketa 405 nm-tan neurtu da. Kolore aldaketa denborarekiko lineala da eta neurketak substratua gehitu eta 30-60 minutura burutu dira. Emaitzen analisirako, absorbantziaren balio guztiak lotura inespezifikoarekiko zuzendu eta maximoarekiko erlatibizatu dira. Kurbak 5 parametroko (5-PL) ekuazio logistikora (SigmaPlot 13.0, Systat Software Inc, AEB) doitu ostean, EC50 balioa eskuratu da.

5. RESULTS /EMAITZAK

4A. Mutation type classification and pathogenicity assignment of LDLR variants.

Familial hypercholesterolemia (FH; MIM# 143890) is a common genetic disorder that leads to severely high low density lipoprotein cholesterol (LDL-C) levels from birth. If untreated, FH significantly increases cardiovascular risk [1, 2] and results in early onset of atherosclerosis which leads to increased risk of premature heart attack, stroke and death [3]. Among all the proteins implicated in the pathology of this disease, the low density lipoprotein receptor (LDLR) (MIM# 606945) is the most common genetic cause, and mutations within it are responsible of approximately 80-85% of FH cases [4] . To date, more than 2600 LDLR variants have been described (ClinVar database).

The LDLR gene is located on the short arm of chromosome 19 (19p13.1-13.3) with a length of approximately 45 kb encoding 18 exons and 17 introns. LDLR is a protein of 839 amino acids that is synthesized in the endoplasmic reticulum (ER), where it folds and is partially glycosylated. Next, LDLR is further glycosylated in the Golgi apparatus, rendering the mature protein [5]. The LDLR is organized in five functionally distinct domains: the N-terminal ligand-binding domain, the epidermal growth factor (EGF)-precursor homology domain, the O-linked sugars containing domain, the trans-membrane and the C-terminal cytosolic domain [6].

Mutations in LDLR can impair LDLR activity at different levels and therefore are classified according to their phenotypic behaviour as: class 1 (no protein synthesis), class 2 (partial or complete retention of LDLR in the ER), class 3 (defective binding to apolipoprotein B (apoB), class 4 (defective endocytosis) and class 5 (diminished LDLR recycling capacity) [7,8].

The physiologic activity of LDLR is to carry lipoproteins into cells, most commonly low density lipoprotein (LDL)[9]. Upon LDL binding to LDLR, the ligand-receptor complex internalizes through clathrin- mediated endocytosis. Cargo is then released by endosome acidification, a process that allows LDLR recycling back to the cell membrane while LDL is degraded in the lysosomes. Failure in cargo release results in lysosomal degradation of the LDL-LDLR complex [10].

The EGF-precursor homology domain (411 amino acids in length) plays a pivotal role in lipoprotein release and receptor recycling processes. It consists of two EGF-like domains (EGF-A and EGF-B), six YWTD repeats that form a six-bladed β-propeller, and a third EGF-like repeat (EGF-C) [11]. Ligand release is an acid-dependent process, where due to endosome acidification, the LDLR conformation switches from an open (ligand-active) to a closed (ligand-inactive) conformation [12]. Although the mechanism underlying this conformational change is still unclear, it has been proposed that three histidine residues located at the interface between the fifth repetition of the ligand-binding domain (LR5) and the β-propeller act as pH sensors that allow the necessary flexing of the LDLR for subsequent conformational change [13]. It has also been proposed that, in endosomes, the β-propeller displaces the bounded lipoprotein ligand thereby acting as an alternative substrate for the ligand-binding domain [12]. Consequently, conformational change could be facilitated by interaction between the β-propeller and the main ligand-binding domain repeats (LR4 and LR5). In addition, it has been shown that the stability of LR5 decreases drastically due to the decreased pH and Ca^{2+} concentrations in the endosome thus triggering LR5 unfolding and consequently LDL release from the receptor [13].

The development of new high throughput screening sequencing technologies allows detection of new variants in different populations continuously [14, 15]; however, in order to provide an accurate genetic FH diagnosis, the LDLR variants must be functionally characterized to avoid misdiagnosis. To date, more than half of the reported missense mutations have not been functionally validated. Although co-segregation studies are very useful in assessing variant pathogenicity [16-18] understanding how alterations disturb the function of the protein is necessary to develop personalized treatment.

To date, 1,108 missense variants localized in exons 7 to 14 (corresponding to the EGF-precursor homology domain) have been annotated in the ClinVar database (as of 10/12/2019), representing the 51.7% of the total missense variants described in the LDLR. The extremely high frequency of missense mutations occurring within the EGF-precursor homology domain is shown in Figure 1, and the specific amino acid substitution at each position are indicated in supplementary Tables S1-S9 together with their clinical significance and review status. As shown in Figure 1, there are variants in

almost all the residues within the EGF-precursor homology domain, in some cases with as many as 5 variants for a given residue. This makes it difficult to assess the activity of all them *in vitro*; however the effort of characterizing as many as possible will provide useful information in order to understand both how these mutations can affect LDLR activity as well as structural information of the domain.

The aim of the present work was to analyse the impact on the LDLR activity of sixteen missense variants located in the EGF-precursor homology domain found in FH patients. Specifically, the activity of the LDLR p.(Ser326Cys), p.(Cys338Phe), p.(Cys368Tyr), p.(Gln378Pro), p.(Ala399Thr), p.(Thr413Met), p.(Asp492Asn), p.(Ser584Pro), p.(Ala606Ser), p.(Aps622Gly), p.(Arg633Cys), p.(His656Asn), p.(Thr659Asn), p.(Cys698Tyr), p.(asp700Gly) and p.(Asp707Tyr) variants.

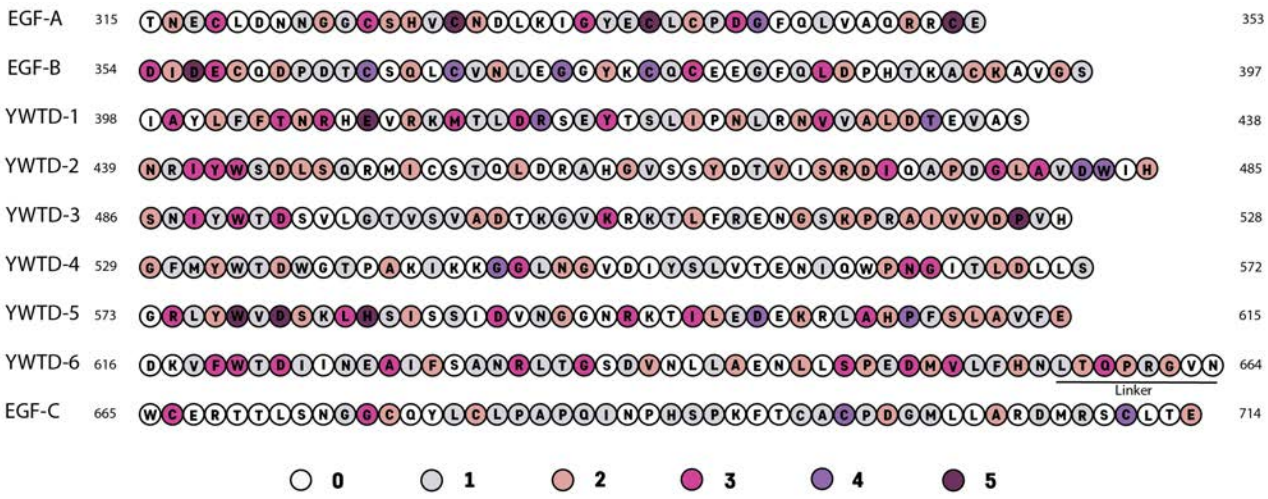


Figure 1. Location and frequency of LDLR variants within the EGF-precursor homology domain according to ClinVar database. Colours represent the number of described variants at a given amino acid.

RESULTS

4A.1 Characteristics of the LDLR studied variants, conservation and *in silico* predictions

The amino acid conservation and *in silico* predictions, determined by different software packages, are shown in Table 1. Most of the variants are classified as damage or possible damage, only the p.(Thr659Asn) change is classified as non-pathogenic by the 4 prediction programs. The *in silico* predictions showed different predictions, even more, for several variants its prediction among programs is the opposite. The inconsistency among algorithms emphasises the importance of performing a functional characterization of the variants.

Table 1: Description of LDLR variants, conservation and *in silico* predictions

HGVS Nomenclature	Conservation Nt	Conservation AA	Grantham Distance	Align GVGD	SIFT	PolyPhen-2	MutationTaster2
p.(Ser326Cys)	1.00	0.97	112	C65	Not tolerated	Pobrably damaging(1)	Disease causing (prob:1)
p.(Cys338Phe)	1.00	1.00	205	C65	Not tolerated	Pobrably damaging (1)	Disease causing (prob:1)
p.(Cys368Tyr)	1.00	1.00	194	C65	Not tolerated	Pobrably damaging (1)	Disease causing (prob:1)
p.(Gln378Pro)	1.00	0.24	76	C25	Not tolerated	Bening (0.155)	Disease causing (prob:0.994)
p.(Ala399Thr)	0.86	0.94	58	C0	Not tolerated	Pobrably damaging (0.991)	Disease causing (prob:0.996)
p.(Thr413Met)	0.94	0.89	81	C15	Not tolerated	Pobrably damaging (1)	Disease causing (prob:1)
p.(Asp492Asn)	1.00	1.00	23	C0	Not tolerated	Pobrably damaging (1)	Disease causing (prob:1)
p.(Ser584Pro)	0.85	0.85	74	C0	Not tolerated	Pobrably damaging (0.953)	Disease causing (prob:1)
p.(Ala606Ser)	0.87	0.87	99	C0	Tolerated	Bening (0.067)	Disease causing (prob:0.999)
p.(Asp622Gly)	1.00	1.00	94	C65	Not tolerated	Pobrably damaging (1)	Disease causing (prob:1)
p.(Arg633Cys)	0.92	0.95	180	C55	Not tolerated	Pobrably damaging (0.987)	Disease causing (prob:1)
p.(His656Asn)	1.00	1.00	68	C65	Not tolerated	Pobrably damaging (0.597)	Disease causing (prob:1)
p.(Thr659Asn)	0.72	0.69	65	C0	Tolerated	Bening (0.003)	Polymorphism (prob: 0.997)
p.(Cys698Tyr)	1.00	1.00	194	C65	Not tolerated	Pobrably damaging (0.998)	Disease causing (prob:1)
p.(Asp700Gly)	1.00	1.00	94	C0	Tolerated	Pobrably damaging (0.999)	Disease causing (prob:1)
p.(Asp707Tyr)	1.00	0.97	160	C65	Not tolerated	Pobrably damaging (1)	Disease causing (prob:1)
p.(Asp47Asn)	1.00	1.00	23	C0	Not tolerated	Pobrably damaging (1)	Disease causing (prob: 1)
p.(Thr62Met)	0.973	0.947	81	C0	Not tolerated	Pobrably damaging (1)	Disease causing (prob:1)
p.(Cys46Gly)	1.00	1.00	159	C0	Not tolerated	Pobrably damaging (1)	Disease causing (prob:1)

4A.2 Expression of LDLR variants in CHO-ldlΔ7 cells

Expression of the sixteen LDLR variants was analysed by Western blot and by flow cytometry in CHO-ldlA7 transfected cells as described in Materials and Methods. Two variants were used as internal-method controls, p.(Trp87)* (a null allele mutant) and, Ex3_4del LDLR variant that is expressed at similar extent than wt LDLR but it is a class 3 variant with 100% impaired binding activity [24]. According to the obtained results, expression of the assessed variants can be classified into three categories, those that are expressed similarly than wt, those with lower expression than wt, and finally those who are not expressed. As shown in Figure 2A and B, expression of p.(Ser326Cys), p.(Cys338Phe), p.(Cys368Tyr), p.(Gln378Pro), p.(Ala399Thr), p.(Thr413Met), p.(Ala606Ser), p.(His656Asn) and p.(Thr659Asn) LDLR variants is similar than wt LDLR expression determined 48 h post-transfection by Western blot, as confirmed by quantification of the relative band intensity of mature LDLR protein expressed as the ratio between the 130 kDa band to that of GADPH by densitometric analysis (Figure 2C). Similar results were obtained when LDLR expression was determined by flow cytometry (Figure 2D).

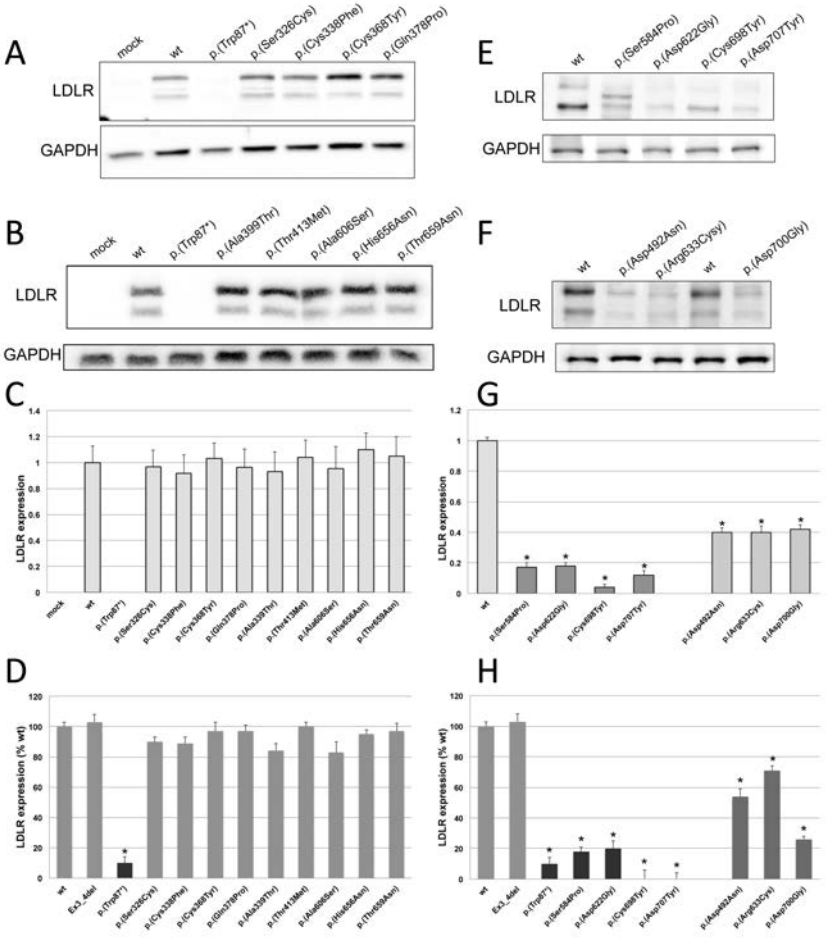


Figure 2. Expression of wt and sixteen LDLR variants in CHO-ldlA7 transfected cells. Expression of LDLR was determined 48 h post-transfection with the plasmids carrying the different LDLR variants by Western blot and flow cytometry. A, B, E and F) Western blot analysis of LDLR expression. C and G) quantification of the intensities of the bands obtained by Western blot by densitometry and, D and H) LDLR expression determined by flow cytometry. A representative blot is shown in panel A, B, E and F. C and G represent the mean of band quantification of three independent Western blots. The values in D and H represent the mean of triplicate determinations (n = 3); error bars represent ±SD. *P < 0.001 compared to wt using a Student's t-test.

On the other hand, p.(Asp492Asn), p.(Arg633Cys) and p.(Asp700Gly) are among the LDLR variants showing diminished LDLR expression, as determined by both Western blot and flow cytometry (Figure 2E, G and H). Finally, expression of mature p.(Ser584Pro), p.(Asp622Gly), p.(Cys698Tyr) and p.(Asp707Tyr) was not detected by Western blot (Figure 2F and G) and consequently, flow cytometry assays showed no expression at cell surface of these three variants (Figure 2H).

4A.3 LDLR activity (LDL binding and uptake) of LDLR variants determined by flow cytometry

Activity of the LDLR variants was assessed in CHO-ldlA7 cells transfected cells as described in Materials and Methods. LDL binding and uptake was determined by FACS as previously described [24]. As shown in Figure 3A and B, both LDL-LDLR binding activity and LDL uptake of p.(Gln378Pro), p.(Ala399Thr), p.(Thr413Met), p.(Ala606Ser), p.(His656Asn) and p.(Thr659Asn) LDLR variants were similar than wt LDLR.

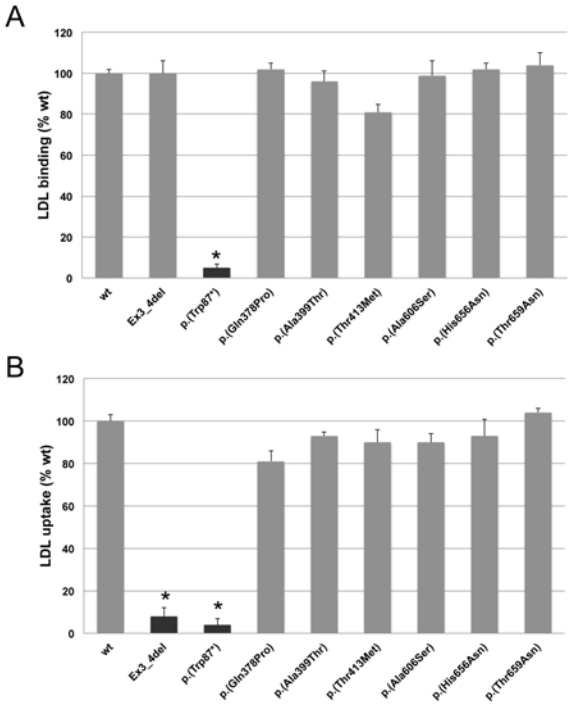


Figure 3. LDLR activity of wt and p.(Gln378Pro), p. (Ala399Thr), p.(Thr413Met), p. (His656Asn), p.(Thr659Asn) and p.(Ala606Ser) LDLR variants. A) LDL-LDLR binding and B) FITC-LDL uptake activity. Assays were performed as described in Materials and Methods. Data show the mean of three independent experiments; error bars represent ±SD. *P < 0.001 compared to wt using a Student's t-test.

On the other hand and as shown in Figure 4A and B, p.(Ser326Cys), p.(Cys338Phe), p.(Cys368Tyr) LDLR variants, which have a similar expression to wt LDLR as determined in the previous section, showed statistically significant reduced LDL-LDLR binding activity (wt: 100±2; p.(Ser326Cys): 50±8, p.(Cys338Phe): 38±10, p.(Cys368Tyr): 39±2) and LDL uptake (wt: 100±3; p.(Ser326Cys): 48±4, p.(Cys338Phe): 38±3, p.(Cys368Tyr): 61±3). On the other hand, and expected due to the LDLR expression assay, p.(Ser584Pro),

p.(Asp622Gly), p.(Cys698Tyr) and p.(Asp707Tyr) showed residual LDLR activity (Figure 4C and D).

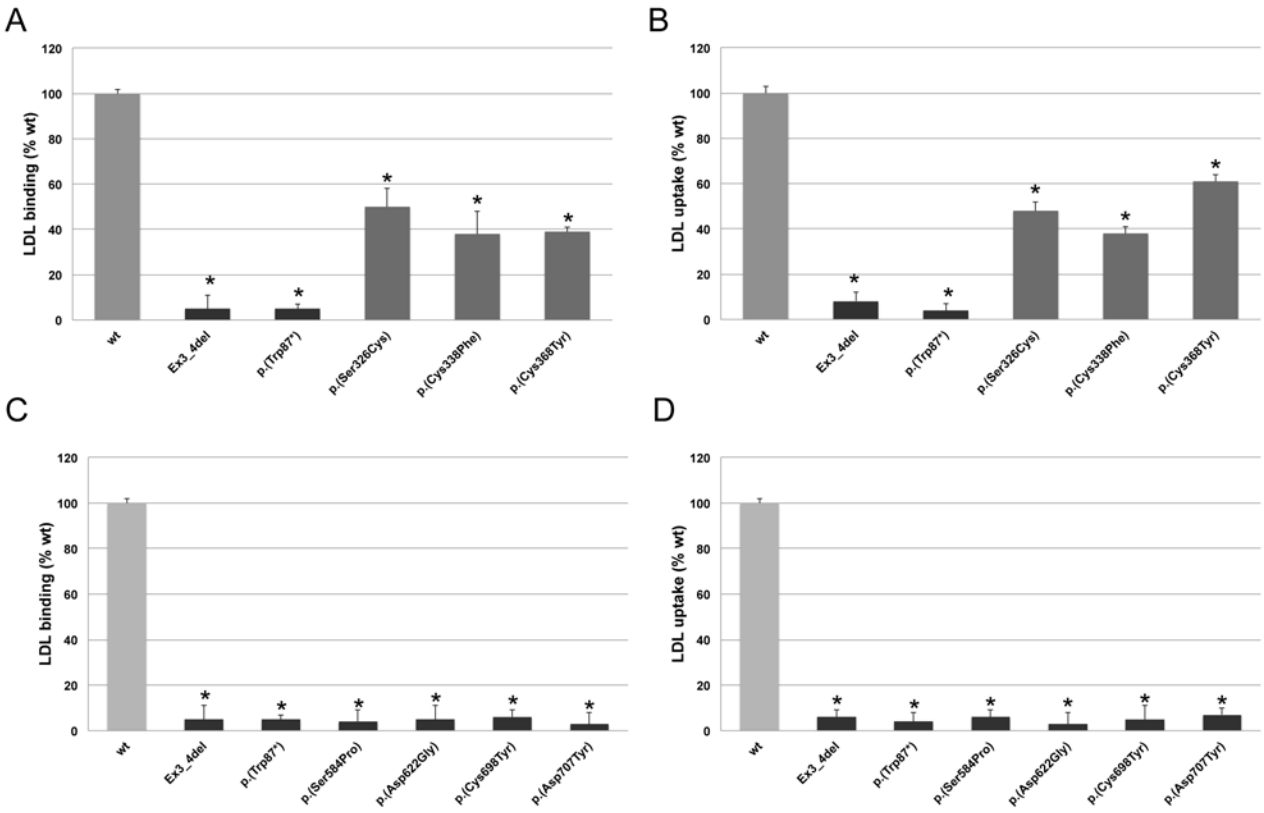


Figure 4. LDLR activity of wt and p.(Ser326Cys), p.(Cys338Phe), p. (Cys368Tyr), p.(Ser584Pro), p.(Asp622Gly), p.(Cys698Tyr) and p.(Asp707Tyr) LDLR variants. A and C) LDL- LDLR binding and B and D) FITC-LDL uptake activity. Assays were performed as described in Materials and Methods. Data show the mean of three independent experiments; error bars represent ±SD. *P < 0.001 compared to wt using a Student's t-test.

Finally, and according to the LDLR expression results, p.(Asp492Asn), p.(Arg633Cys) and p.(Asp700Gly) LDL binding activity was significantly diminished compared to wt LDLR (LDL binding: wt: 100±2; p.(Asp492Asn): 70±4; p.(Arg633Cys): 72±5; and p.(Asp700Gly): 60±7 (Figure 5A). In addition, and corroborating these results, LDL uptake was also significantly diminished in comparison to the uptake values for wt: 100±2; p.(Asp492Asn): 49±6; p.(Arg633Cys) : 75±4; and p.(Asp700Gly): 52±9 (Figure 5B).

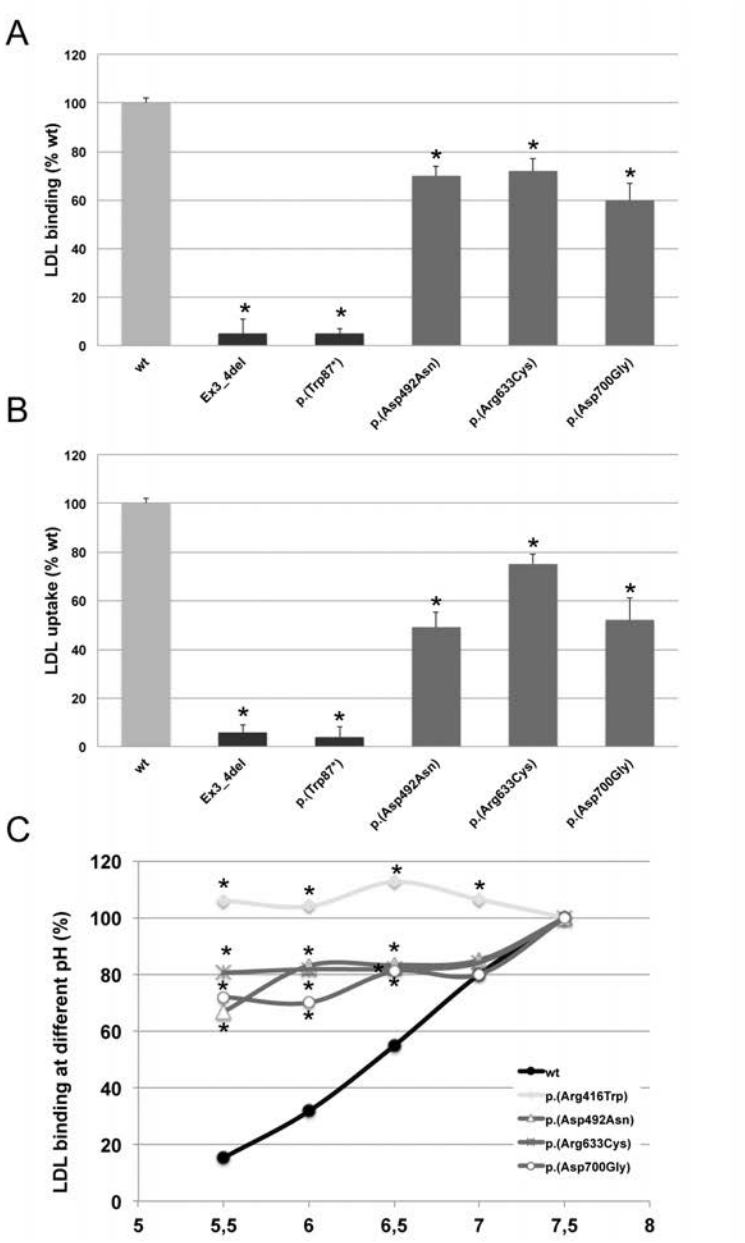


Figure 5. LDLR activity of wt and p.(Asp492Asn), p.(Arg633Cys), and p.(Asp700Gly) LDLR variants. A) LDL- LDLR binding, B) FITC-LDL uptake activity and C) LDL binding at different pH. Assays were performed as described in Materials and Methods. Data show the mean of three independent experiments; error bars represent ±SD. *P < 0.001 compared to wt using a Student's t-test.

4A.4. p.(Asp492Asn), p.(Arg633Cys) and p.(Asp700Gly) LDLR variants affect negatively to receptor recycling

The reduced expression (30-70% compared to wt LDLR) and LDLR activity (≈ 60-70% LDL binding and ≈ 45-75% uptake compared to wt LDLR)

together with the absence of an accumulation of the non-processed LDLR in p.(Asp492Asn), p.(Arg633Cys) and p.(Asp700Gly) LDLR variants (Fig. 2E, F and H, and Fig. 5A and B, respectively) is an indicative of a possible defect in LDLR recycling. Accordingly, we next determined LDL binding capacity of the LDLR variants at different pH (5.5 – 7.4). The rationale of this assay is to mimic the acid-dependent mechanism of lipoprotein release in the endosomal compartment. Diminished LDL binding at pH 5.5 compared to binding at pH 7.4 would resemble the dissociation of the LDL-LDLR complex occurring upon endosomal acidification, which allows the required structural change for the LDLR to be recycled to the membrane. The opposite result, constant LDL-LDLR binding rate, would indicate that acidification does not promote complex dissociation and LDLR would be driven, together with LDL, to lysosomes for degradation.

Accordingly, we studied LDL–LDLR binding at different pH to mimic LDL release induced by pH acidification occurring at endosomes. As shown in Figure 5C, LDL binding was pH dependent for wt, with LDL binding at pH 5.5 being 80% less efficient compared with that at pH 7.5. In this assay we included p.(Arg416Trp) LDLR variant as an internal-control of the method because it has been previously classified as a class 5 LDLR variant [26]. As expected, LDL binding to p.(Arg416Trp) LDLR variant was not affected by acidification (Figure 5C). Similarly to the results obtained with the p.(Arg416Trp) LDLR control, in p.(Asp492Asn), p.(Arg633Cys) and p.(Asp700Gly) LDLR variants, LDL release at acidic pH was not as efficient as in wt. Binding in p.(Asp492Asn) variant at pH 5.5 diminished 37% compared with binding at pH 7.5, binding of LDL to p.(Arg633Cys) at pH 5.5 was 42% less efficient than at pH 7.5 and binding of p.(Asp700Gly) LDLR variant was diminished 36% compared to pH 7.5 (Fig. 5C).

4B. LDLR variants functional characterization improves variant classification

In the Portuguese Familial Hypercholesterolemia Study (PFHS), 142 LDLR alterations were found in 861 index patients and their relatives up to date. Until now 82 of these alterations have already been proved to be mutations causing disease and 15 were shown by *in vitro* studies not to affect LDLR function (9–15). The aim of the present work is to functionally characterize 12 LDLR missense variants and p.(Gly207_Ser213Ser) variant variant found in Portuguese FH patients and in other worldwide populations [15–21].

4B.1 Characteristics of the variants characterized in the Portuguese FH Study

Table 1 shows the 13 variants characterized in this study, ACMG classification and the number of individuals and families with these variants. Altogether, these 13 variants were previously identified in a total of 132 patients, for whom the effect on the LDLR cycle was not known, including 70 (carrying 9 different variants) with an uncertain FH diagnosis due to an inconclusive ACMG classification. In Table 1 we present the *in silico* analysis of the variants under study, based on 3 *in silico* prediction programs, described previously in the methods.

Table 1: Description of LDLR variants, conservation and *in silico* predictions

Genetic name	HGVS Nomenclature	Conservation nt	Conservation AA	Grantham distance	Align GVGD	SIFT	Polyphen-2	Mutation Tester 2
c.977C>G	p.(Ser326Cys)	1.00	0.97	112	C65	Not tolerated	probably damaging (1)	Disease causing (prob: 1)
c.1013G>T	p.(Cys338Phe)	1.00	1.00	205	C65	Not tolerated	probably damaging (1)	Disease causing (prob: 1)
c.1103G>A	p.(Cys368Tyr)	1.00	1.00	194	C65	Not tolerated	probably damaging (1)	Disease causing (prob: 1)
c.1133A>C	p.(Gln378Pro)	1.00	0.24	76	C25	Not tolerated	benign (0.155)	Disease causing (prob: 0.994)
c.1195G>A	p.(Ala399Thr)	0.86	0.94	58	C0	Not tolerated	probably damaging (0.991)	Disease causing (prob: 0.996)
c.1238C>T	p.(Thr413Met)	0.94	0.89	81	C15	Not tolerated	probably damaging (1)	Disease causing (prob: 1)
c.1474G>A	p.(Asp492Asn)	1.00	1.00	23	C0	Not tolerated	probably damaging (1)	Disease causing (prob: 1)
c.1750T>C	p.(Ser584Pro)	0.85	0.85	74	C0	Not tolerated	possibly damaging (0.953)	Disease causing (prob: 1)
c.1816G>T	p.(Ala606Ser)	0.87	0.87	99	C0	Tolerated	benign (0.067)	Disease causing (prob: 0.999)
c.1865A>G	p.(Asp622Gly)	1.00	1.00	94	C65	Not tolerated	probably damaging (1)	Disease causing (prob: 1)
c.1897C>T	p.(Arg633Cys)	0.92	0.95	180	C55	Not tolerated	probably damaging (0.987)	Disease causing (prob: 1)
c.1966C>A	p.(His656Asn)	1.00	1.00	68	C65	Not tolerated	possibly damaging (0.597)	Disease causing (prob: 1)
c.1976C>A	p.(Thr659Asn)	0.72	0.69	65	C0	Tolerated	benign (0.003)	Polymorphism (prob: 0.997)
c.2093G>A	p.(Cys698Tyr)	1.00	1.00	194	C65	Not tolerated	probably damaging (0.998)	Disease causing (prob: 1)
c.2099A>G	p.(Asp700Gly)	1.00	1.00	94	C0	Tolerated	probably damaging (0.999)	Disease causing (prob: 1)

4B.2 Expression of LDLR variants in CHO-ldlA7 cells

The 13 different LDLR variants (Figure 1, suppl. table 1) were transiently transfected in CHO-ldlA7 cells and expression of LDLR was assayed by immunoblotting with a specific antibody against the LDL receptor. For wild-type LDLR two bands were detected, corresponding to the mature form and the precursor form (Figure 2A and 2B, lane 1). As shown in Figure 2B, the

mature form of p.(Asp601Val) variant was not detected by western blot while expression of both mature and precursor form of p.(Gly592Glu) was diminished by ≈50% compared to the wild-type receptor. For the remaining variants the band signal was similar to the wild-type LDLR (Figure 2A). These results were confirmed when relative expression of mature LDLR/GAPDH was quantified by densitometry (Figure 2C).

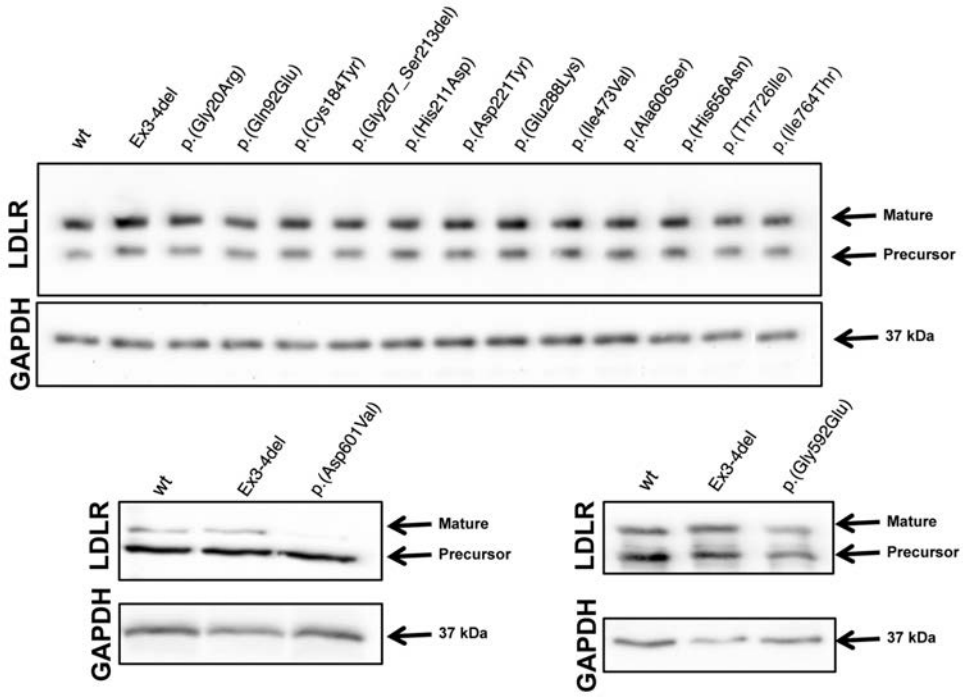


Figure 2. Western blot analysis of wild-type, 13 LDLR variants under study and 2 controls (Ex3-4del and p.(Thr726Ile)) in CHO-ldlA7 cells. Whole cell extracts (30 mg) were fractioned in non-reducing 7% SDS-PAGE, transferred onto nitrocellulose membranes for incubation with a rabbit polyclonal anti-hLDLR antibody or rabbit monoclonal GAPDH antibody and detected by chemiluminescence after incubation with the appropriate secondary antibodies. In non-reducing conditions, mature LDLR runs at an apparent molecular weight of 130 kDa and the precursor form at 90 kDa. Figure 2A and B correspond to a representative western blot of n = 3. Levels of significance in C were determined by a two-tailed Student's t-test. * $p < 0.01$ compared to HDL and # $p < 0.01$ compared to wild-type.

4B.3 Characterization of LDLR activity

We next determined by flow cytometry cell surface LDLR expression, LDL-LDLR binding and LDL uptake. Four controls were used: wt LDLR, p.(Thr726Ile) LDLR variant, which does not affect the normal activity of the LDLR, Ex3_4del LDLR variant, which is expressed at similar levels of wt at cell membrane but does not bind LDL and consequently LDL uptake is

impaired and,finally p.(Trp87*) LDLR variant, which does not express and consequently there is neither LDL binding nor LDL uptake. All values were normalized for wt plasmid transfection, which was considered 100%.

As shown in Figure 3, cells transfected with plasmids carrying p.(Gly20Arg), p.(Gln92Glu), p.(Ile473Val), p.(Ala606Ser), p.(His656Asn), and p.(Ile764Thr) LDLR variants showed cell surface expression, LDL binding and LDL uptake values comparable to cells transfected with the wt LDLR. Thus, they were considered benign variants that do not affect LDLR function. It is important to refer that values between 80% and 100% are considered to be associated to a normal receptor activity, while values lower than 80% are associated to a compromised receptor activity [7]. Furthermore, values lower than 2% were considered to be associated to no receptor activity (null variants).

As shown in Figure 3A, p.(Asp601Val) LDLR variant is not expressed at the cell surface, therefore binding and uptake activities are also compromised (Figure 3B and C). These results confirm those obtained by Western blot and indicate that p.(Asp601Val) LDLR variant shows a complete impairment of activity and can be classified as pathogenic Class 2a LDLR variant.

Although expression of p.(Cys184Tyr), p.(Gly207_Ser213del), p.(Asp221Tyr) and p.(Glu288Lys) LDLR variants is similar to that determined for wild-type LDLR (Figure 3A), the LDLR binding activity results significantly and strongly impaired (Figure 3B). Therefore, p.(Cys184Tyr), p.(Gly207_Ser213del) and p.(Asp221Tyr) LDLR variants lead to a strong impairment of LDLR ability to bind LDL resulting in an impaired LDL uptake. The results indicate that these mutations affect LDLR function and can be classified as pathogenic Class 3 LDLR variants (Figure 3).

Cells transfected with plasmid carrying p.(His211Asp) variant showed similar expression at the cell surface compared to wild-type LDLR (Figure 3A). However, only approximately 50% of binding activity was observed (Figure 3B). Consequently, LDL uptake values also decrease by approximately 50% (Figure 3C). Thus, this mutation leads to a partial impairment of the LDLR binding activity thus this variant can also be classified as a pathogenic Class 3 LDLR variant.

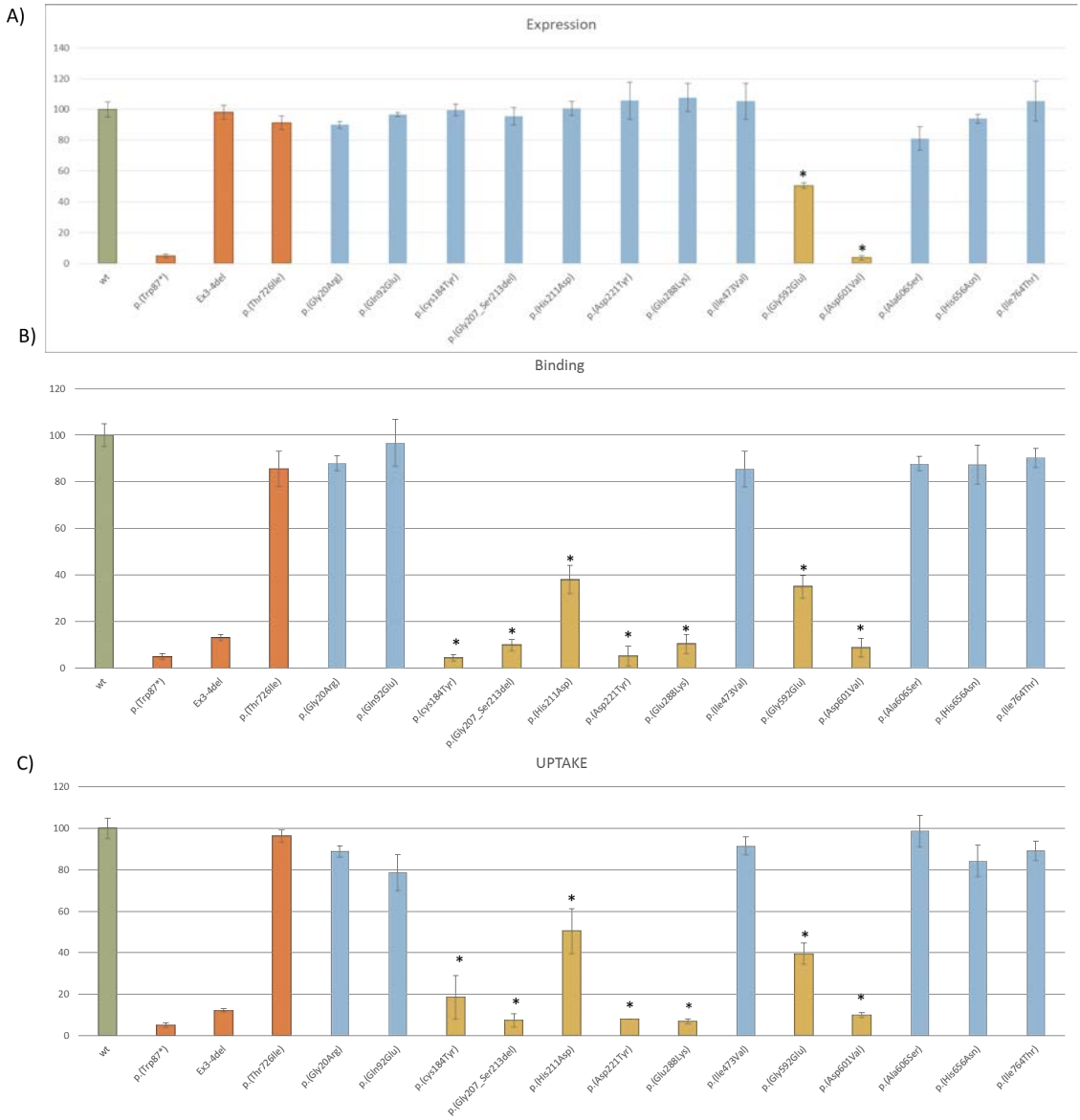


Figure 3. Functional characterization of LDLR variants in transfected CHO-ldlA7 cells. (3A) LDLR expression at the cell membrane. (3B) FITC-LDL binding after 4 h incubation at 4ºC. (3C) FITC-LDL uptake after 4 h incubation at 37 °C. Bars represent values as the mean of three independent measurements. The orange bars represent the LDLR variants used as internal control of the methodology. Error bars represent +/- standard deviation (S.D.). *p-value<0.01 compared to LDLR wt (green) determined by Student's t-test.

Finally we determined that p.(Gly592Glu) LDLR variant presented a milder value, of approximately 50%, for cell surface expression which is in agreement with the previously determined LDLR expression of this variant by Western blot. Consequently, binding and uptake activities also presented approximately half of the activities observed in cells transfected with the wt LDLR. According to these results and the fact that this variant is not being

retained at the ER as precursor form (as demonstrated by Western blot) we sought to determine if this variant affects receptor recycling. Therefore, studies of LDL binding at different pH were performed in order to mimic the acid-dependent mechanism of lipoprotein release that occurs in the endosomal compartment upon acidification. As shown in Figure 4, LDL-LDLR binding for wt receptor was pH dependent, showing an efficient release at pH 5.5 with less than 20% LDL bound to the receptor, which resembles the physiological release of LDL/LDLR upon endosomal acidification. In contrast, for p.(Gly592Glu) LDLR variant up to 50% of LDL remains bound to LDLR at pH 5.5, confirming that LDL release is not completely efficient upon endosomal acidification. According to these results this variant is a pathogenic Class 5 LDLR variant affecting recycling.

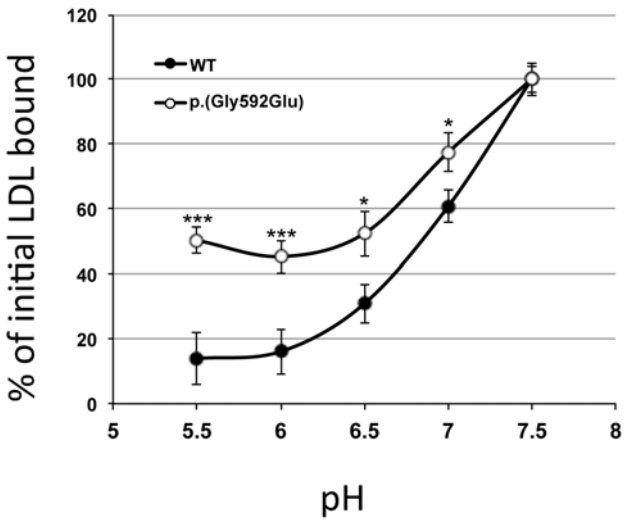


Figure 4. Percentage of initial LDL bound for p.(Glu592Glu) variant vs wt. LDL binding was determined by flow cytometry, 10,000 cells were acquired in a Facscalibur, and values of LDL binding, were calculated as described in Materials and Methods. The values represent the mean of triplicate determinations (n = 3); error bars represent ±S.D. *P < 0.025; ***P < 0.001 compared with the wt using a Student's t-test.

After the functional study of the 13 variants, all variants, except 2, changed ACMG classification: 4 likely pathogenic became pathogenic, 3 VUS were classified as pathogenic and 6 VUS were classified as benign (Table 3).

Table 2. ACMG Classification before and after functional study of the variants in this study.

Localization	cDNA	Protein	ACMG Classification*†	ACMG Classification *¥	Functional study
Exon 1	c.58G>A	p.(Gly20Arg)	VUS	Benign	Not affect LDLR activity
Exon 2	c.274C>G	p.(Gln92lu)	VUS	Benign	Not affect LDLR activity
Exon 4	c.551G>A	p.(Cys184Tyr)	likely pathogenic	Pathogenic	Affect LDLR activity
Exon 4	c.618_638del	p.(Gly207_Ser213del)	Likely Pathogenic	Pathogenic	Affect LDLR activity
Exon 4	c.631C>G	p.(His211Asp)	likely pathogenic	Pathogenic	Affect LDLR activity
Exon 4	c.661G>T	p.(Asp221Tyr)	likely .pathogenic	Pathogenic	Affect LDLR activity
Exon 6	c.862G>A	p.(Glu288Lys)	VUS	Pathogenic	Affect LDLR activity
Exon 10	c.1417A>G	p.(Ile473Val)	VUS	Benign	Not affect LDLR activity
Exon 12	c.1775G>A	p.(Gly592Glu)	VUS	Pathogenic	Affect LDLR activity
Exon 12	c.1802A>T	p.(Asp601Val)	VUS	Pathogenic	Affect LDLR activity
Exon 12	c.1816G>T	p.(Ala606Ser)	VUS	Benign	Not affect LDLR activity
Exon13	c.1966C>A	p.(His656Asn)	VUS	Benign	Not affect LDLR activity
Exon 15	c.2291T>C	p.(Ile764Thr)	VUS	Benign	Not affect LDLR activity

VUS, Variant of Unknown Significance.*Chora et al 2018; † ACMG classification before functional study; ¥ ACMG classification after functional study

SUMMARY

Functionally characterized and classified LDLR variants have been summarized in the following table.

Table 3. Summary of functionally characterized LDLR variants.

LDLR Variant	Classification	LDLR Activity	Reference
p.(Ser326Cys)	Class 3	50%	DOI: 10.1038/s41598-020-58734-9
p.(Cys338Phe)	Class 3	40%	DOI: 10.1038/s41598-020-58734-9
p.(Cys368Tyr)	Class 3	60%	DOI: 10.1038/s41598-020-58734-9
p.(Gln378Pro)	Bening	100%	DOI: 10.1038/s41598-020-58734-9
p.(Ala399Thr)	Bening	100%	DOI: 10.1038/s41598-020-58734-9
p.(Thr413Met)	Bening	100%	DOI: 10.1038/s41598-020-58734-9
p.(Asp492Asn)	Class 5	50%	DOI: 10.1038/s41598-020-58734-9
p.(Ser584Pro)	Class 2a	Residual	DOI: 10.1038/s41598-020-58734-9
p.(Ala606Ser)	Bening	100%	DOI: 10.1038/s41598-020-58734-9
p.(Asp622Gly)	Class 2a	Residual	DOI: 10.1038/s41598-020-58734-9
p.(Arg633Cys)	Class 5	75%	DOI: 10.1038/s41598-020-58734-9
p.(His656Asn)	Bening	100%	DOI: 10.1038/s41598-020-58734-9
p.(Thr659Asn)	Bening	100%	DOI: 10.1038/s41598-020-58734-9
p.(Cys698Tyr)	Class 2a	Residual	DOI: 10.1038/s41598-020-58734-9
p.(Asp700Gly)	Class 5	50%	DOI: 10.1038/s41598-020-58734-9
p.(Asp707Tyr)	Class 2a	Residual	DOI: 10.1038/s41598-020-58734-9
p.(Asp47Asn)	Bening	100%	doi: 10.1038/s41598-018-34715-x.
p.(Thr62Met)	Bening	100%	doi: 10.1038/s41598-018-34715-x.
p.(Cys46Gly)	Class 3	55%	doi: 10.1371/journal.pone.0204771
p.(Gly592Glu)	Class 5	%50	Manuscript in preparation
p.(Gly207_Ser213del)	Class 3	10%	Manuscript in preparation
p.(Cys184Tyr)	Class 3	20%	Manuscript in preparation
p.(Glu288Lys)	Class 3	10%	Manuscript in preparation
p.(His211Asp)	Class 3	50%	Manuscript in preparation
p.(Ala606Ser)	Bening	80%	Manuscript in preparation
p.(Asp601Val)	Class 2a	Residual	Manuscript in preparation
p.(His656Asn)	Bening	100%	Manuscript in preparation
p.(Asp221Tyr)	Class 3	10%	Manuscript in preparation
p.(Ile473Val)	Bening	100%	Manuscript in preparation
p.(Ile764Thr)	Bening	100%	Manuscript in preparation
p.(Gly20Arg)	Bening	100%	Manuscript in preparation
p.(Gln92Glu)	Bening	80%	Manuscript in preparation

6. DISCUSSION / EZTABAIDA

DISCUSSION

A comprehensive assessment of FH patients that combines clinical and molecular data from patients with functional characterization and classification of LDLR variants can elucidate the spectrum of FH phenotypes and provide insight into the development of targeted treatments [27]. Therefore, in this work, in order to align molecular characterization of LDLR variants with clinical severity, we have functionally characterized and classified sixteen LDLR variants located in the EGF-precursor homology domain of the LDLR. These variants have been previously described in FH patients but without prior clinical interpretation or functional characterization.

Due to the role of EGF-precursor homology domain in maintaining the correct folding at cell surface (supported by YWTD repeats) and as a pH sensor that allows receptor recycling, the majority of functional-defective variants are class 2 (partial or complete retention of LDLR in the ER), and class 5 (diminished LDLR recycling capacity) mutations [7, 28]. These findings highlight the importance of characterizing and classifying LDLR variants in order to provide and accurate diagnosis and treatment of FH.

Although laborious and time consuming, a correct assessment of pathogenicity and classification of a large amount of variants cannot just rely on *in silico* characterization due to the lack of a robust computational software able to accurately predict the functional effects of variants. Current software compares amino acid or nucleotide conservation among species and basic biochemical features of the substituted amino acid. However, it does not consider other parameters such as interaction of the substituted amino acid with its surrounding residues and how such interactions can modify protein structure or activity.

Among the sixteen variants selected in this work, p.(Thr659Asn) was the only variant correctly predicted as non-pathogenic by the four softwares tested, which was consistent with our *in vitro* characterization of that variant. For the remaining fifteen LDLR variants, *in silico* analysis showed contradictory interpretations that do not allow drawing any conclusion regarding protein activity. This discrepancy occurs with both non-pathogenic variants and pathogenic variants. In particular, of the non-pathogenic variants identified by *in vitro* experiments, four of the six variants analysed

(p.(Gln378Pro), p.(Ala399Thr), p.(Thr413Met) and p.(His656Asn)) were considered to be pathogenic by at least two predictors.

Very interestingly, among the sixteen characterized LDLR variants, in six of them the amino acid alteration did not cause loss of receptor activity thus constituting neutral alterations. Although they were identified in FH patients, p.(Gln378Pro), p.(Ala399Thr), p.(Thr413Met), p.(Ala606Ser), p.(His656Asn) and p.(Thr659Asn) LDLR variants were found to be non-pathogenic thereby indicating that the variants do not account for the clinical presentation. Consequently, the cause of FH in patients carrying these variants should be further investigated in order to identify a cause. Of course, cascade screening to find FH relatives based on these variants is recommended.

We also identified four class 2a LDLR variants in which the amino acid substitution leads to a complete retention of the immature protein at the ER. These identified class 2a variants are p.(Ser584Pro), p.(Asp622Gly), p.(Cys698Tyr) and p.(Asp707Tyr), all showing a residual LDLR activity. A detailed analysis of the impact of the substituted amino acids in the protein structure provides information about the molecular mechanisms by which these variants are pathogenic.

In the case of p.(Ser584Pro), interestingly, in addition to the band corresponding to the non-mature LDLR, we detected by Western blot a band of an intermediate molecular weight between mature and immature LDLR (Fig. 2F). As determined by flow cytometry, there is no detection of LDLR at cellular surface, indicating that the LDLR-form corresponding to that band remains intracellularly retained. Based on the position of the replaced amino acid, there is not any disturbed N-linked site glycosylation, but an O-linked glycosylation site could have been lost. Additionally, since the Western blot was performed under non-reducing conditions, the intermediate band could correspond to a non-correctly folded protein with different secondary or tertiary structure compared to wt LDLR. In agreement with the latter, in the wt LDLR, there is an interaction between the backbones of Ser584 and Asp579, with Asp579 being one of the conserved amino acids within the fifth YWTF motif. As mentioned before, the six YWTD motifs of the β-propeller are packed as six four-stranded β-sheets ("blades") that maintain the domain structure, which is determinant for a correct folding of β-propeller [9]. Moreover, the interaction between Ser584 and Asp579 allows a loop

formation that accommodates a lysine, which in turn interacts with the ligand-binding domain at its closed conformation. Substitution of Ser584 by proline (an amino acid with lower flexibility) may destabilize the highly conserved structure of the YWTD motif causing ER retention.

Regarding the p.(Asp622Gly) LDLR variant, the amino acid substitution is located in the sixth YWTD, in a highly conserved region of the β-propeller. Asp622 interacts with the backbones of Ile623 and Ala627 allowing a loop formation that could be destabilized by a glycine substitution. Moreover, YWTD tetrapeptide repeats are essential for the correct β-propeller structure maintenance and proper receptor folding. Indeed, modifications in these sequences have previously been described as disease causing [25, 29] suggesting that Asp622 modification could not be well tolerated.

The p.(Cys698Tyr) and p.(Asp707Tyr) LDLR variants affect the EGF-C domain and are also retained at ER being class 2 variants. Substitution of Cys698 by a tyrosine leads to a loss of a disulfide bridge between Cys698 and Cys711 probably causing an incorrect folding of the protein resulting in ER retention. Unfortunately, the effect on the LDLR structure caused by the substitution of Asp707 by a tyrosine is not clear because the crystal structure surrounding that position is not well resolved.

On the other hand, we have found three class 3 variants: p.(Ser326Cys), p.(Cys338Phe) and p.(Cys368Tyr) LDLR variants. They showed similar expression to wt LDLR but demonstrated deficient LDL binding. In the wt LDLR, Ser326 backbone interacts with Asn322 and Val328 and its side chain interacts with Arg351. Substitution of Ser326 by a cysteine results in loss of interaction with Arg351 and more importantly, the location of two adjacent cysteines in a position involved in a disulfide bridge formation may disrupt the correct folding of the domain and negatively affect the ligand-binding domain structure. Similarly, the substitution of Cys338 by a phenylalanine causes a disulfide bridge disruption naturally occurring in wt between Cys325 and Cys338. It has been previously described that cysteine substitution at these two positions causes misfolding of EGF-A domain [30] that can affect ligand-binding domain conformation. The Cys368 is located in the EGF-B shortly before the β-propeller and forms a disulfide bridge with the side chain of Cy358 and its backbone interacts with the backbone of Cys364. These interactions favour the generation of a loop that is organized around a

calcium ion. Replacement of Cys368 by a tyrosine disrupts the disulfide bridge thus causing LDLR misfolding.

Finally, we have found three class 5 variants: p.(Asp492Asn), p.(Arg633Cys) and p.(Asp700Gly). Asp492 is located in the fourth repetition of the YWTD motif of the β-propeller. The interactions of Asp492 with the tryptophan located in the YWTD repeat have been described above for the p.(Asp622Gly) and p.(Asp707Tyr) class 2a LDLR variants. In this case, the substitution of Asp492 by asparagine leads to a class 5 LDLR variant probably because the substitute amino acid is relatively similar. In this way, although the protein structure is not affected in LDLR expression or LDL binding and uptake activities, the alteration introduced by the asparagine is enough to cause a defect in recycling of the protein. The p.(Arg633Cys) LDLR variant is located at the end of the β-propeller. In the wt protein, Arg633 interacts with Gln615 through its side chain and with its primary chain with Gln632 side chain. In addition, due to the β-propeller three-dimensional structure, Arg633 interacts with Ser665, an amino acid located between EGF-B and the β-propeller. Substitution of Arg633 by a cysteine completely disrupts the interactions with Gln615 and Ser365.

Finally, Asp700 located in the EGF-C domain, establishes a hydrogen bond with Thr510, which is located in the β-propeller. This interaction seems to be necessary for a correct LDLR recycling because when Asp700 is replaced by a glycine, this interaction is lost and the p.(Asp700Gly) LDLR variant fails to undergo a correct recycling.

Although the FH clinical phenotype is highly variable, it has been suggested that depending on the class mutation, the effects of statin therapy can be affected [26, 31, 32]. Interestingly, although a similar lipid phenotype has been found in heterozygous patients carrying Class 2 and Class 5 variants, statin treatment is more effective at lipid level reduction in subjects with Class 2 LDLR variants than subjects with Class 5 LDLR variants [26]. These results highlight the importance of classification of LDLR variants in order to improve the efficacy or safety of therapy.

Genetic bases of FH are heterogeneous, in the Portuguese population [37, 41, 42, 43] as well as in other studied population, having been found several types of variants spread throughout mostly the LDLR gene.

Although more than 2314 LDLR variants have been identified [34], about 15 % have functional studies proving their pathogenicity and 50% are classified as VUS following ACMG classification [35]. Thus, functional assessment of these variants becomes imperative, as part of the genetic FH diagnosis. Many variants reach a pathogenic or likely pathogenic only when functional information is available.

Furthermore, information obtained with these functional characterizations allows inferences about the relation between the variant and the protein activity, leading to a prediction of the severity of phenotype associated to this variant. This information is important for patient management, especially now with the PCSK9 inhibitor. If an individual is homozygous for variants that produce null receptor activity, the inhibitor will have no effect on reducing cholesterol.

During the past years, several methods have been used to functionally characterize LDLR variants [36, 40, 46, 47], but the most comprehensive method follows Goldstein and Brown original method [33] that allows the characterization of the whole LDLR cycle and thus allows determining with part is affected. A recent guideline from ClinGen has added information on how to perform functional assays in order to be considered valid for variant classification [34].

In the present study, the method used was similar to Goldstein and Brown but using fluorescence labeled LDL, since it is a safer but equivalent method [40]. It was also followed the recommendations of the ClinGen guideline for functional studies: the study was performed in an independent system (variant specific) and several controls were used: wt, a variant that reached a benign ACMG classification without functional studies level 1 p.(Thr726Ile) and a control that reached a pathogenic classification also without functional studies p.(Trp87*). The results obtain are so valid for variant classification and the criteria PS3/BS3 can be used at the strong level.

From the 13 variants under study 7-affected LDLR function and 8 showed the same LDLR activity as the wild type receptor. The most severe variant is p.(Asp601Val) that shows almost no LDLR activity. This variant has only been reported in the Portuguese population [42]. As this variant leads to a complete lack of LDLR function in a heterozygous patient carrying this variant, only approximately 50% of LDLR will be functionally normal, being expected a severe FH phenotype. In the case of an individual being homozygous for this variant, no receptors would be synthesized and this would result in a brutally increased cholesterol levels and so an increased cardiovascular risk at early ages.

There was one particularly interesting variant, p.(Gly592Glu), that presented levels of 50 % for LDLR expression, LDL-LDLR binding and uptake. The data obtained by Western blot showing no retention of the precursor form in the ER suggested that this variant was a class 5 recycling variant. Not many recycling variants have been characterized so far. Therefore, in order to confirm our hypothesis, additional experiments were performed in which LDL-LDLR binding was assessed mimicking the physiologic endosomal acidification that occurs upon internalization of the LDL-LDLR complex. In this case as LDLR recycling is impaired and only newly synthesized p.(Gly592Glu) LDLR variant is present in the cell membrane, leading to values approximately of half of the ones observed in wt. As shown if Figure 5, Gly592 is located in one turn within the fifth blade of the β-propeller. Although, neither Gly592 nor the replacing Glu interact with other amino acids nearby, the bigger size of Glu could affect the local conformation of the blade. In addition, the substitution of a non polar amino acid such Gly by a charged amino acid (Glu) can alter the properties requires for a correct folding of the receptor upon endosomal acidicification in order to be correctly replaced. So, it is expected that patients carrying this variant present a milder phenotype, as the LDLR function is not totally impaired. Also, theoretically, these patients should respond better to statins.

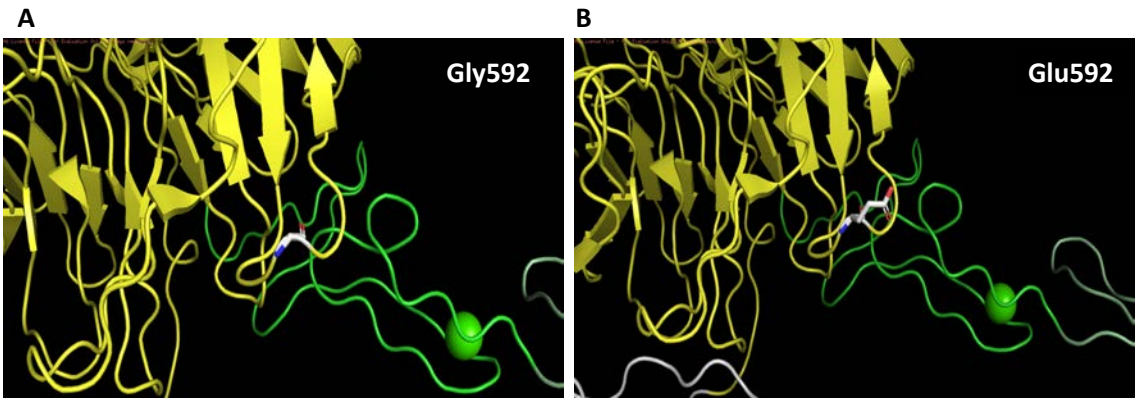


Figure 5. Molecular structures of wt LDLR (A) and p.(Gly592Glu) (B). The figure illustrates location of the substituted amino acid within the fifth blade of the β-propeller and the interactions of the amino acid with the surrounding residues. This figure was prepared with PyMOL (DeLano Scientifics).

LDLR variants c.631C>G p.(His211Asp), c.661G>T p.(Asp221Tyr), c.618_638del p.(Gly207_Ser213del) c.862G>A p.(Glu288Lys) and c.551G>A p.(Cys184Tyr) showed impaired binding activities. All of them are located at the LDL-binding domain, where variants often tend to impair LDLR function [38], due to the incapability of the LDLR variant in recognizing the LDL. As a result of an impairment of binding activity in these variants of LDLR, the uptake was also coherently affected in all cases. However, as uptake activities seem to be similar to binding activities into respective variants, it can be pointed that these variants do not cause any further effect at the uptake level, so lower uptake activities occur as a consequence of lower binding activities firstly performed. Variant c.618_638del p.(Gly207_Ser213del) is a small in frame deletion and have a great impact at the protein function level. This variant is present in 27 individuals in Portuguese FH study and all with a severe phenotype.

As shown in Figure 6, the substituted amino acids or the deletion of some of them within the ligand binding domain cause different molecular interactions with the surrounding residues. p.(His211Asp), in wt LDLR His interacts with a Trp located next to a calcium ion binding site, substitution of

His by Asp a new interaction with a Ser adjacent to the Trp and the charge of the substituting amino acid changes (Figure 6A and B), which could underlie the loss of affinity for LDL observed in this variant. Figure 6C and D show the effects at molecular level of substituting Asp221 by a Tyr. In this case, the interactions of Asp221 with Gly198 and Asp206 which coordinates a calcium ion disappear when Asp is replaced by a Tyr. In addition an interaction of Asp221 with a Lys located in the β-propeller is also missed by Tyr replacement thus causing a defect of receptor conformation.

In the case of p.(Gly207_Ser213del) variant, the 6 amino acids that are deleted are located near the calcium ion binding site of the LR5 thus the loop-like structure responsible for calcium coordination is missing affecting LDL binding (Figure 6E).

According to the data, p.(Glu288Lys) variant also losses LDL binding capacity, as shown in Figure 6F and G, Glu288 is located in the LR7 of the ligand binding domain and interacts with Cys263 this stabilizing the Ca^{2+} ion coordination site of the domain. When replaced by Lys, the interaction disappears thus destabilization of the loop where calcium coordinates is favored, therefore negatively affecting the proper folding of the receptor, which causes a diminished LDL binding.

Finally, loss of affinity for LDL binding in p.(Cys184Tyr) variant could be caused by the loss of interaction of Tyr184 with Ala145 in the LR4 and can thus affect Ca^{2+} coordination (Figure 6H and I).

It is worth mentioning that the majority of the variants are located either in the EGF precursor like domain or in the ligand-binding domain, probably because these are the biggest domains of the LDLR. From the analysis of above referred databases was possible to conclude that the majority of variants occurring in these domains, which have been functionally studied, were proven to be compromise receptor activity. Nevertheless, the justification for this may rely on the fact that, when a variant is proved to have normal receptor activity, these studies are not so often published.

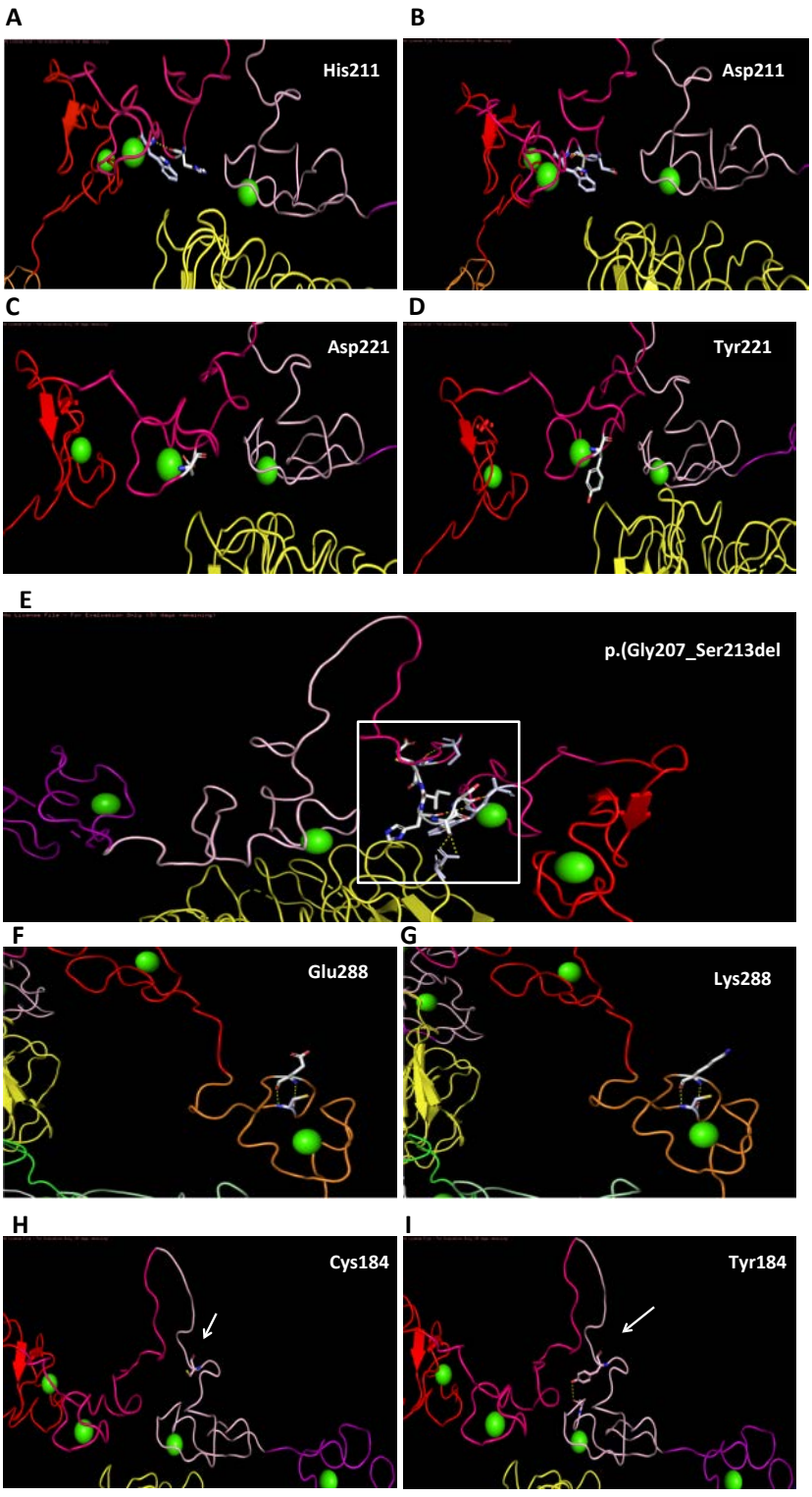


Figure 6. Molecular structures of wt LDLR (A) and p.(His211Asp) (B); molecular structures of wt LDLR (C) and p.(Asp221Tyr) (D); location of the deleted amino acids in p.(Gly207_Ser213del) indicated by an open square (E); molecular structures of wt LDLR (F) and p.(Glu288Lys) (G) ; molecular structures of wt LDLR (H) and disruption of the cysteine bridge in p.(Cys184Tyr) (I) indicated by an arrow. This figure was prepared with PyMOL (DeLano Scientifics).

From the 13 variants under study, functional evidence was not available for 12 variants at the time on this study and for 1 variant (p.Gly20Arg) a functional study existed [47] but only by confocal microscopy, so it was not known the variant effect in the whole LDLR cycle and thus the functional evidence for variant classification could not be used at the level of strong and the variant was classified as VUS. However, recently the functional study of other 2 variants included in this study (p.(Ala606Ser) and p.(His656Asn) were published [48]. For all 3 variants the results obtained before and in this study are concordant, contributing this way for a robust variant characterization.

In conclusion, the data obtained in this work are complementary to clinical data in terms of both determining the extent of protein function impairment and variant classification. Integrating this information with clinical data improves patient clinical management in terms of accuracy of a definite diagnosis and advances in personalized medical care with the ultimate aim of tailoring therapy for maximal patient response.

Functional studies are extremely necessary in order to obtain a final classification of the ACMG and therefore the information given to the clinician is the most accurate and specific. These studies contributed for an update of these variants classification: from the 9 variants classified as variants of unknown significance 7 reached now a final classification (3 pathogenic or likely pathogenic and 4 benign or likely benign) and 4 variants improved classification from likely pathogenic to pathogenic. In Portugal an additional 55 patients received a FH definite diagnosis. Since only likely pathogenic and pathogenic variants are clinically actionable, these studies show the importance of functional studies for variant classification.

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Functional Characterization of six ApoB-100 and one PCSK9 missense variants found in Italian Population

The results presented in this chapter were obtained in collaboration with Dr. Mª Donata Di Taranto and Dr. Giuliana Fortunato. The constituting work was conducted in the context of a "virtual short stay" due to circumstances associated COVID-19, in order to contribute towards an international thesis.

Introduction

Activity determination of the main proteins causing FH

The existence of the LDLR was nicely demonstrated in 1974 by Goldstein and Brown by the radiolabeling of LDL with ^{125}Iodine, which showed the high affinity binding sites for ^{125}I-LDL in normal fibroblast, whereas those derived from FH homozygote patients were not able to bind LDL [1]. Later on they described the physiological pathway of the LDLR by assessing the fate of the surface-bound ^{125}I-LDL and developed the methodology to determine surface-bound from intracellular LDL by using ^{125}I-LDL [2]. Since then, many efforts have been focused on the development of new methodologies to determine LDLR activity that have been lately expanded in terms of determining the pathogenicity of both PCSK9 and apoB-100 variants.

LDLR activity determination

Several strategies allow assessing the pathogenicity of any LDLR variant. Among them, *ex vivo* assays are based in the use of lymphocytes from patients carrying the variant under study, which can be either immortalized by Epstein-Barr virus [3-5] or stimulated by statin [6, 7], mitogens or CD3/CD28 beads [5, 8] to induce upregulation of LDLR expression thus facilitating characterization of receptor activity. Once upregulated, LDLR expression can be easily determined by Western blot and its activity characterized through flow cytometry. The latter constitutes a simple strategy used for LDLR functional assays that yields similar results to those obtained in the past in fibroblast from FH patients using radiolabeled-LDL [9]. Nowadays, LDL labelling with fluorochromes has replaced radiolabeling due to

similar sensitivity and safety of handling fluorescent molecules [5, 9]. Specifically, labelling LDLR with a fluorescent antibody or the LDL with a fluorescent dye/molecule allows determination of LDLR expression levels at the cell surface as well as the efficiency of LDL-LDLR binding [5, 9, 10]. A recent improvement to determine LDL uptake efficiency combines incubation cells with Fluorescein IsoTioCyanate labelled LDL (FITC-LDL) followed by the addition of Trypan-blue dye [9]. This procedure allows determination of LDL uptake in a single step due to the Trypan blue-mediated quenching of the external fluorescence from non-internalized LDL particles [9].

Nowadays, most of the LDLR functional studies are carried out using i*n vitro* cell line model systems. These are particularly helpful to determine the mechanisms underlying pathogenicity of LDLR variants and show many advantages because cell-lines are well-controlled systems, there is no need for clinical samples and as they mimic FH homozygous condition the effect of a pathogenic variant is better determined, even the effect of mild pathogenic LDLR variants that can be masked in heterozygosis. *In vitro* analyses use mostly LDLR-deficient Chinese hamster ovary (CHO) cell line ldlΔ7 (CHO-ldlΔ7) and require transfection with a plasmid carrying the LDLR of interest [9, 11]. Then, LDLR expression is usually evaluated by immunoblotting in order to determine expression of the LDLR and its level of maturation. Quantification of the relative mature-LDLR expression is then determined as the ratio between the sum of band intensities corresponding to the mature and precursor forms of the LDLR to that of a constitutive protein such as GAPDH. LDLR activity (LDLR expression and LDL uptake) is assessed by flow cytometry by using labelled LDL as described above. Generally, activity characterization is complemented by using confocal microscopy that allows determination of the Class type of a given LDLR variant by testing LDLR colocalization with clathrin, lysosomes, or endoplasmic reticulum (ER). Although confocal microscopy is suitable in assessment of Class 1, Class 2a and b, Class 3, Class 4 and Class 5 LDLR variants additional assays can be used to further characterize the pathogenicity extent of a Class 3 variant, and confirm a Class 5 LDLR variant. In order to provide affinity values of a receptor for LDL that allows identify Class 3 mutations from mild to severe pathogenic effect, a modified ELISA binding assay can be performed to assess the affinity of any given LDLR variant to LDL [12]. Finally, to confirm that the defect of a LDLR variant is due to defective LDLR recycling, a LDL binding assay at different pH's mimicking the acidification occurring in the endosome can be performed [13].

LDL activity determination

Several techniques have been applied in order to provide evidence for the existence of functional abnormalities in the ApoB molecule. Studies of restriction fragment length polymorphisms (RFLPs) of ApoB have demonstrated several pathogenic variants [14, 15]. The fast development of NGS has revolutionized research in genetics as well as provided a high number of genetic variants that require pathogenicity validation as occurring with ApoB-100 variants. Initially, LDL binding activity was assessed in lymphocyte stimulated to over express LDLR as described above and then LDL uptake and binding were determined by flow cytometry by using labelled-LDL previously isolated from the patients carrying the ApoB-100 variant. In order to ease the method, the use of HEK293 and hepatome cell lines have been introduced to assess ApoB-100 pathogenicity with the patient's derived labelled-LDL [16]. As a complementary methodology to prove the pathogenicity of ApoB-100 variant, the U937 cells proliferation assay is widely used [13, 17]. U937 is a cell line derived from a histiocytic lymphoma, which has been shown to lack the qualities required for endogenous cholesterol synthesis, and therefore requires extracellular cholesterol supply for proliferation [18]. Accordingly, the proliferation rate of these cells in the presence of LDL carrying wild type ApoB100 or the variants of interest indicates the ability of the LDL to bind the receptor [13, 17]. More recently, direct LDL-binding assays of the LDL carrying the ApoB-100 variants to LDLR have been introduced among the techniques to determine activity defects of LDL. In this case, a modified ELISA binding assay with purified sLDLR and LDL is used and EC_{50} values are determined from binding [19].

PCSK9 activity determination

To date, the methodologies to characterize GOF PCSK9 variants are quite heterogeneous and rely on different approaches such as immunocytochemistry, flow cytometry and biochemistry techniques [20-24].

Prior to PCSK9 functional characterization it is necessary to ascertain that the LDLR is expressed normally in carriers of the PCSK9 variant of interest by assessing LDLR expression at the surface of lymphocytes isolated from these patients. This approach discards potentially undetected LDLR genetic defects and is usually performed in primary lymphocytes isolated from a subset of patients as well as non- affected family members [22]. The LDLR expression in these isolated

lymphocytes is upregulated by statin treatment and then recombinant PCSK9 is exogenously added in the absence or presence of the PCSK9 inhibitor alirocumab in order to confirm the GOF effects of a given variant and be characterized more deeply [22].

Nowadays, PCSK9 synthesis, secretion and impact of the PCSK9 mutations on LDLR cell surface expression and LDL cellular uptake are commonly assessed by transfection of HEK293 cells with plasmids containing the PCSK9 variant under study. Both secreted and intracellular PCSK9 levels are determined by western blot 48 hours after transfection to determine the efficiency of secretion and the ratio of processed/non-processed PCSK9 (65 kDa/75 kDa bands) by desitometric quantification of band intensities. Simultaneously, analysis of furin-mediated PCSK9 cleavage is performed by densitometry in order to detect cleavage-resistant GOF variants. LDLR cell surface expression and uptake of fluorescent labelled-LDL are determined by flow cytometry 48 hours post transfection as described above. These results are further corroborated by assessing the extracellular activity of PCSK9 after addition of purified recombinant-PCSK9 to the extracellular media of HepG2 cells in terms of LDLR expression at cell surface and LDL uptake activity [22-25].

A valuable parameter that gives important information about PCSK9 GOF variants is the affinity for the LDLR. The EC50 of PCSK9 variants for LDLR can be determined by solid-phase immunoassay at pH 7.4 and 5.2 by using a LDLR construct encoding the N-terminal extracellular ectodomain of the LDLR [22, 25] .

Finally, as some GOF variants have been shown to act intracellularly, PCSK9 intracellular activity assessment can be perform using HEK293 cells stably transfected with the PCSK9 variant of interest and transiently co-transfect the cells with a plasmid carrying the LDLR ectodomain. Soluble LDLR secretion onto the medium can be analyzed by Western blot and quantified by densitometry in order to determine the efficiency of transport to the cell membrane mature LDLR in the presence of the GOF variant [24].

Methods

1. Cell culture

HEK293 cells were maintained in DMEM medium (Dulbeccos´s Modified Eagle´s Medium, Sigma-Aldrich, USA) containing 10% FBS (Lonza, Belgium), 4mM L-glutamine and antibiotics (100 u/mL penicillin; 100 μg/mL streptomycin) transfections were carried out when 80% of confluence was reached.

Human lymphoma derived and endogenous cholesterol synthesis lacking U937 cells were grown into 96-well plates, using delipidized RPMI culture medium supplemented with 2mM L-glutamine and antibiotics (100 u/mL penicillin; 100 μg/mL streptomycin).

HUH7 cells were maintained in DMEM medium containing 10% FBS, 4mM L-glutamine, 100 u/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a 5 % CO_2 atmosphere.

2. Lipoprotein purification and labelling with fluorescein isothiocyanate

Blood plasma was collected from healthy individuals after 30 min centrifugation at 2,000 x G at 4 °C. In order to isolate LDL (1.019-1.050 g/mL) by a sequential ultracentrifugation, plasma density was adjusted to 1.21 g/mL adding KBr. Afterwards, a second ice-cold PBS buffer was slowly added to the top of the solution generating a two phase gradient. Ultracentrifugation was carried out in a SW 28.1 rotor (Beckman Coulter, USA) at 27,000 rpm for 22h at 4 °C. Then, the band corresponding to LDL was collected and stored at 4 °C. LDL was used within 2-3 days after purification. LDL was fluorescently labelled with fluorescein isothiocyanate (FITC) as previously described. Briefly, LDL was incubated with 10 μL/mL FITC in 0.1 M $NaHCO_3$ (pH 9.0) at room temperature under slight agitation for 2h. Once incubation was completed, the non-bounded FITC was removed by washing the lipoprotein solution in a previously PBS-EDTA balanced Sephadex G-25 column. Protein concentration was determined in all fractions using BSA as standard (Pierce BCA protein assay, USA). All experiments were carried out according to relevant guidelines and regulations.

3. LDL particle size determination by electron microscopy

Non-labeled LDL particle size was determined using electron microscopy. In order to increase the contrast between the samples and the bracket, negative staining of the particles was carried out using uranyl acetate. Samples were then washed with distilled water and dried. Each sample LDL particle size was quantified by measuring Feret diameter using Image J software. Particle size is represented as the mean value of the measurement from 1500 particles in each sample.

4. Site directed mutagenesis and cloning

Plasmid carrying interest PCSK9 variant was constructed by Innoprot (Derio, Spain). The variant was introduced into the human PCSK9 cDNA (NM_174936.3), in the mammalian expression vector WT-PCSK9 plasmid (pCMV-PCSK9-FLAG) kindly provided by Prof. Horton, by oligonucleotide site-directed mutagenesis, carried out using the QuickChange Lightning mutagenesis kit (Agilent Technologies Inc., USA) and following the manufacturer´s instructions. A 6x His tag was introduced in order to allow purification with no effects on PCSK9 activity. Restriction enzyme digestion of the appropriate fragments and the integrity of the remaining PCSK9 cDNA sequence of the construct were verified by direct analysis.

5. PCSK9 purification from stably transfected HEK293 cells

HEK293 cells grown to sub-confluence were transfected with different PCSK9 plasmids and selected with geneticin (G418 sulphate) (Gibco, Thermo Fisher Scientific, USA) according to the manufacturer´s instructions to obtain stably transfected cells. For PCSK9 purification, stably transfected HEK203 were grown at 80% confluence in complete DMEM medium. Then, the culture medium was replaced with Opti-MEM (Invitrogen, Thermo Fisher Scientific, USA) without geneticin and cells were maintained under these conditions for 48 hours. Finally, the medium was harvested and PCSK9 was purified using one-step nickel affinity chromatography. Purified PCSK9 variants were stored at -80 °C in 50 mM Tris-HCL buffer supplemented with 150 mM NaCl and 10% glycerol, pH 8.0.

6. Solid-phase immunoassay for PCSK9-LDLR ectodomain binding

LDLR ectodomain fragments diluted in working buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$) were coated at a fixed concentration onto 96-well microtiter plates by incubation overnight at 4 °C. Plates were then blocked and incubated with a serial dilution of each of different PCSK9 variants diluted in working buffer. For ligand detection, the antibodies, rat monoclonal anti-DYKDDDDK tag (clon L5) (Cat. No.: MA1-142; Thermo Fisher Scientific, USA) and peroxidise-conjugated goat anti-rat (Cat. No.: 7077S; Cell Signalling Technology® Inc., USA) were diluted in working buffer supplemented with 5% (w/v) BSA, applied directly to the plate and incubated for 1 h at room temperature, with an extensive washing between both incubations. After a final wash, antibody binding was determined using 50 µL per well of 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) substrate solution (Sigma-Aldrich, Germany) and measuring colour change at 405 nm. The time course for colour development was essentially linear and measurements were taken 30-60 min after the addition of substrate. For data processing, all absorbance values were corrected for unspecific binding, relativized to maximum absorbance and EC50 values were extracted from curves after fitting the data to 5-parameter logistic (5-PL) equation (SigmaPlot 13.0, Syat Software Inc, Usa).

7. Solid phase immunoassay for LDL-LDLR ectodomain binding

Such as for PCSK9-LDLR, LDLR ectodomain fragments diluted in working buffer were coated at a fixed concentration onto 96-well microtiter plates by incubating overnight at 4 °C. Plates were then blocked and incubated with a serial dilution of each LDL variant diluted in working buffer. In this case, goat polyclonal anti-apoB (abcam, UK) primary antibody and peroxidase-conjugated mouse anti-goat (ThermoScientific, USA) secondary antibody were used for ligand detection. LDL-LDLR affinity was determined as previously described for PCSK9-LDLR.

8. Flow cytometry

- Cuantification of LDL uptake by flow cytometry

48 h after transfection with the plasmid containing the interest PCSK9 variant, HEK293 cells were incubated for 4 h, at 37 °C with 20μg/mL FITC-LDL and lipoprotein uptake was determined as previously described.

Furthermore, in order to determine the LDL uptake in the presence of extracellular PCSK9 in HEK293 and Huh7 cells, 2 μg/mL of each purified PCSK9 variant was added to the cell culture medium and 2 h post-addition, 20μg/mL FITC-LDL was added to the medium and LDL uptake was determined 4 h post lipoprotein addition. In both experimental approaches, cells were washed twice with PBS-1%BSA after FITC-LDL incubation, fixed with 4 % formaldehyde for 10 minutes and washed again with PBS-1%BSA. LDL uptake was determined as previously described.

HUH7 cells grown into 96-well plates were incubated for 4 hours with the different labelled LDL variants at 37 °C. Later, culture medium was discarded and cells were washed with PBS-1%BSA and fixed with 4 % formaldehyde for 10 minutes. After fixation, cells were washed again and LDL uptake was determined for each LDL variant as previously described.

- Cuantification of LDLR expression

In order to determine LDLR surface expression, cells were incubated with mouse anti-LDLR primary antibody (clone IgG7; 1:100, 2.5 mg/L) (Cat. No.: 61087; Progen Biotechnik GmbH, Germany) and subsequently with Alexa Fluor 488 conjugated goat anti-mouse IgG secondary antibody (1:100) (Cat. No.: A11001; Molecular Probes, Thermo Fisher Scientific, USA), following the previously described methodology.

Fluorescence intensities were quantified in a CytoFLEX (Beckman Coulter, USA) flow cytometer as previously described. LDL particle labelling-level was taken into account and fluorescence intensity values were corrected against each LDL sample´s particle/fluorescence ratio. All measurements were performed at least in triplicates.

9. Inmunodetection of intracellular and extracellular PCSK9

Expression and secretion analysis in HEK293 cells trasnfected with control and variant of interest PCSK9 was performed in cell lysates and culture media by Western blot. For that purpose, proteins from cell lysates or the supernatants were resolved by 8.5 % Tris-Glycine SDS-PAGE. Gels were next blotted onto nitrocellulose membranes (Protan BA 83, Whatman TM, GE Healthcare, Germany), blocked for 1 hour in TBST (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1 % Tween 20) containing 5 % BSA (Bovine Serum Albumin, Sigma-Aldrich, USA) and immunoblotted with a rabbit polyclonal anti-human PCSK9 antibody (1:1000) (Cayman Chemical Company, Cat. No: 10240, USA) overnight at 4 °C. Then, counterstained with horseradish peroxidise-conjugated anti-rabbit antibody (Cell Signalling, Cat No: 7074s, USA). Protein bands where visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology, USA). Densitometric analysis of the gels was carried out using ImageJ software from the NIH (http://rsbweb.nih.gov/ij/).

10. U937 cell proliferation assay

U937 cells were grown up to 1 x 10^4 cell/well density on 96 well plates, using delipidized RPMI culture medium. Simultaneously, cells were incubated with 2 μg/mL of previously purified LDL variants at 37 °C, for 48 h. Cell proliferation-ratio was determined using the CellTiter 96wAQueous Non-Radioactive Cell Proliferation assay (Promega Corporation, USA) kit, following manufacturer´s instructions. Absorbance values corresponding to each lipoprotein variant were measured using a plate reader. All values were standardized to non-LDL sample values and were presented as percentage.

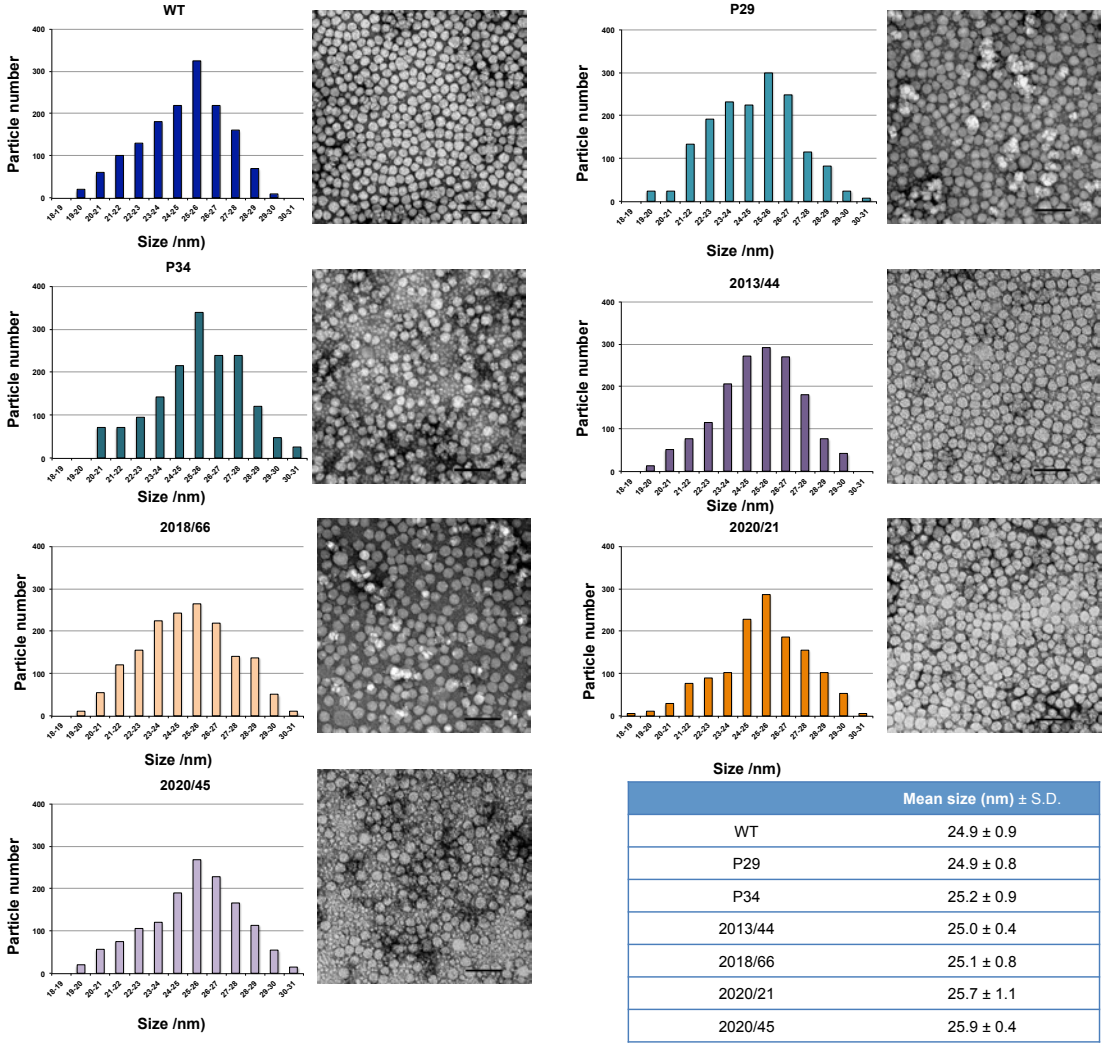
11. Statistical analysis

Data are presented as mean ± SD or means (interquartile range) for continuous variables. Experimental data presented in graphs are reported as percentage relative to the control. Flow cytometry and solid-phase immunoassay were performed in triplicate. Western blots from 3 independent experiments were quantified by densitometry to determine statistical significance. For comparisons between groups the T-test was used. Statistical significance was established at a p value < 0.05.

Results

1. Functional characterization of ApoB-100 variants

Figure 1 shows mean size values of each LDL variant analyzed by negative staining electron microscopy. P29 (24.9 ± 0.8 nm) has the same size as the WT LDL, while P34, 2013/44, 2018/66, 2020/21 and 2020/45 show a small increase in size (25.2 ± 0.9 nm; 25 ± 0.4 nm; 25.1 ± 0.8 nm; 25.7 ± 1.1 and 25.9 ± 0.4 nm, respectively. Despite slight differences in size can be appreciated between some of the LDL variants, there are no statistically significant differences in particle size among any of them when compared to the WT LDL (24.9 ± 0.9 nm).



	Mean size (nm) ± S.D.
WT	24.9 ± 0.9
P29	24.9 ± 0.8
P34	25.2 ± 0.9
2013/44	25.0 ± 0.4
2018/66	25.1 ± 0.8
2020/21	25.7 ± 1.1
2020/45	25.9 ± 0.4

Figure 1. Analysis of LDL particle size using negative staining electron microscopy. Data shown represents mean size values of 1500 particles for each sample.

We tested the binding affinity of the different LDL variants to LDLR using a solid-phase binding immunoassay and compared them to WT LDL. The results shown in Table 1 indicate an EC_{50} value of 0.60 ± 0.05 nM for WT LDL. All the LDL variants showed a higher EC_{50} value, indicating a lower affinity to the LDLR compared to WT. The 2020/21 variant showed the lower EC_{50} among them all (0.77 ± 0.07 nM), whereas the EC50 values of the remaining variants was over 1 nM. Variant P29 showed an EC_{50} value of 1.07 ± 0.18 nM, while 2013/44 and 2020/45 variants showed similar values (1.27 ± 0.13 nM and 1.26 ± 0.35 nM respectively). Affinity of the P34 variant was slightly lower than the last two, showing an EC_{50} value of 1.33 ± 0.08. Lastly, the 2018/66 LDL variant showed 4.6 times lower affinity to LDLR compared to WT LDL, with an EC_{50} value of 2.77 ± 0.28 nM.

Table 1: Affinity of LDL to LDLR determined by solid-phase immunoassay

	EC50		
	Mean	±S.D.	t-student
WT	0,60	0,05	
P29	1,07	0,18	***
P34	1,33	0,08	***
2013/44	1,27	0,13	***
2018/66	2,77	0,28	***
2020/21	0,77	0,07	***
2020/45	1,26	0,35	***

Furthermore, functionality of the different LDL variants was assessed by incubating U937 cells with the different non-labelled LDL variants and measuring their proliferation. For all the LDL variants, cell proliferation was significantly reduced (Figure 2). In the case of P29, 2013/44 and 2020/21 LDL variants, proliferation was diminished up to 40 % (41 %, 40 % and 38 % respectively) when compared to WT. For its part, cell proliferation of the P34 variant was reduced up to 58%, while 2018/66 and 2020/45 LDL variants showed a reduction of 68 % and 71 %, respectively.

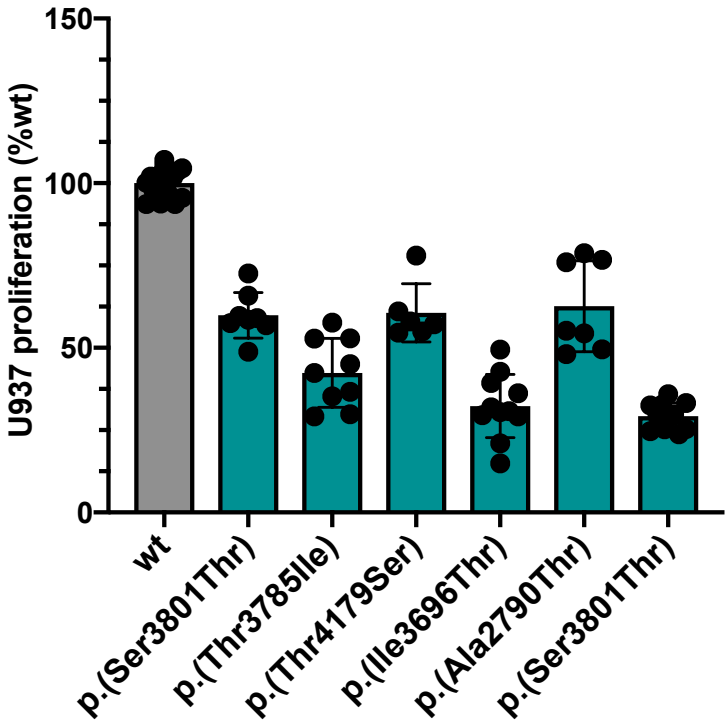


Figure 2. U937 cell proliferation assay performed with the P29, P34, 2013/44, 2018/66, 2020/21 and 2020/45. Data shows mean values of three independent experiments; error bars represent ± SD. * P < 0.001 compared to wt using a Student´s t-test

LDL uptake was determined by FACS as previously described. As shown in figure X, LDL uptake of all the studied variants was statistically reduced when compared to the uptake values for WT LDL. P34 and 2013/44 LDL variants showed uptake values of 52 % ± 6 and 60 % ± 6, while uptake of P29, 2020/21 and 2020/45 variants was reduced to 37 % ± 3, 34 % ± 3 and 40 % ± 2, respectively. The 2018/66 LDL variant exhibits the lowest uptake activity, 17 % ± 5 when compared to WT.

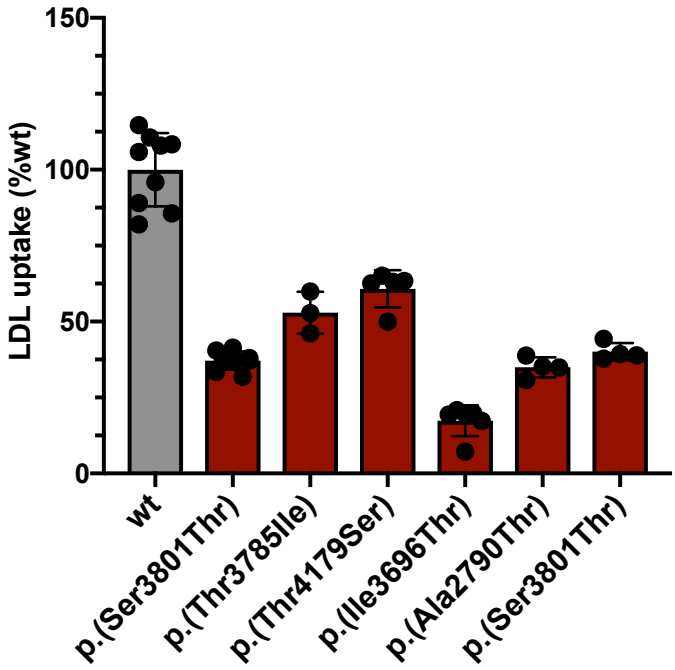


Figure 3. FITC-LDL uptake activity of P29, P34, 2013/44, 2018/66, 2020/21 and 2020/45 LDL variants. Assay was performed as described in materials and methods. Data shows mean values of three independent experiments; error bars represent ± SD. * P < 0.001 compared to wt using a Student´s t-test.

2. Functional characterization of c.1180G>A p.(Gly394Ser) PCSK9 variant

p.(Gly394Ser) PCSK9 variant presents a LOF activity on LDL uptake

In the first experimental approach to determine the activity of the PCSK9 variant, HEK293 cells were transiently transfected with wt, GOF p.(Asp374Tyr) and p.(Gly394Ser) PCSK9 variants. The GOF variant was used as a positive internal control of the method. The efficiency of fluorescent LDL (FITC-LDL) uptake by the cells was measured as described in materials and methods. As shown in Fig. 4A, LDL uptake was significantly higher upon expression of p.(Gly394Ser) PCSK9 variant (≈25%), compared to wt PCSK9. Similar results were obtained in Huh7 (an hepatic cell line) treated exogenously with the PCSK9 variants as described in Materials and Methods (Fig. 4B)

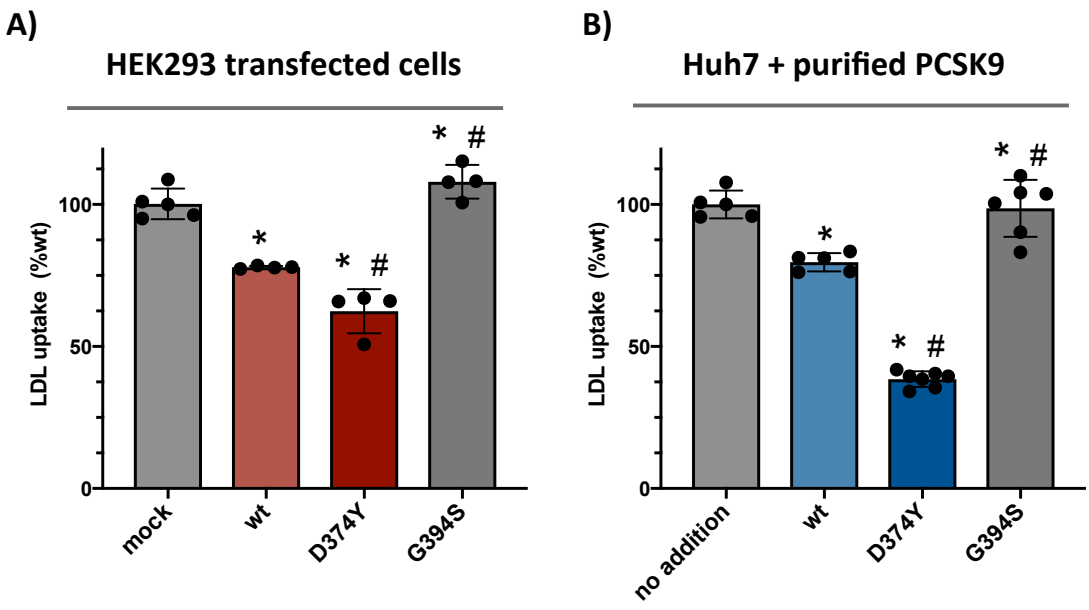


Figure 4. Effect of PCSK9 variants on LDL uptake in (**A**) transiently transfected HEK293 cells, (**B**) in Huh7 cells incubated with purified PCSK9 variants. LDL uptake in transiently transfected HEK293 cells (**A**) was determined as described in Materials and Methods. For LDL uptake assay with purified PCSK9 variants, cells were incubated with the PCSK9 variants at 0.5 μg/mL during 2 h prior FITC-LDL addition (**B**). LDL internalization was determined after 4 h incubation at 37 °C as described in Materials and Methods. Values represent the mean ± standard deviation of 3 independent experiments performed by triplicate. *$p < 0.01$ versus no PCSK9 addition; $^{\#}p < 0.01$ versus wild-type (wt) PCSK9. p.(Asp374Tyr) GOF mutant was used as internal control.

In order to deeply investigate the action mechanism of the p.(Gly394Ser) PCSK9 variant and to test only its extracellular action, the LDLR assay was performed incubating cells in a culture medium and adding the recombinant purified PCSK9 variants exogenously. For that purpose, HEK293 cells, Fig. 5, were treated with 5 μg/mL PCSK9 variants. Then, cells were incubated with 20 μg/mL FITC-LDL to determine the extent of LDL uptake. As shown in Fig. 5, p.(Gly394Ser) showed a LOF activity in HEK293 cells in which LDL uptake was significantly higher when compared to wt PCSK9.

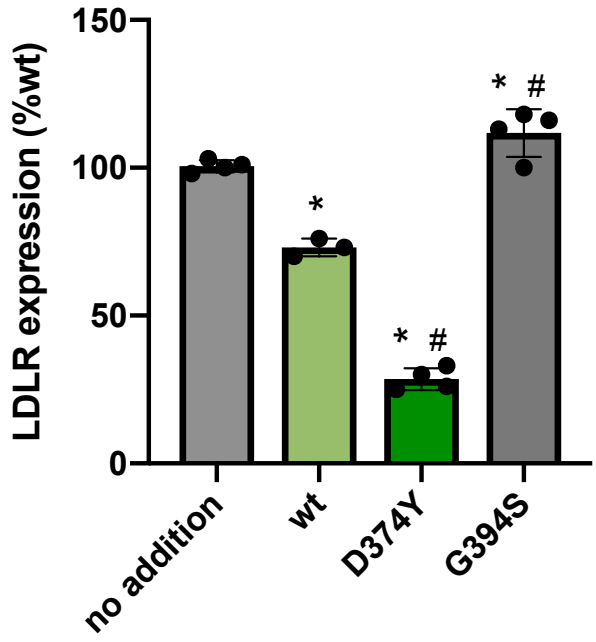


Figure 5. Effect of PCSK9 variants on LDLR expression intransiently transfected HEK293 cells. LDLR expression was determined as described in Materials and Methods. Values represent the mean ± standard deviation of 3 independent experiments performed by triplicate. *$p < 0.01$ versus no PCSK9 addition; $^{\#}p < 0.01$ versus wild-type (wt) PCSK9. p.(Asp374Tyr) GOF mutant was used as internal control

Discussion

Despite mutations in the *LDLR* account for over 80 % of the cases for Familial Hypercholesterolemia, mutations in other genes such as *PCSK9* and *APOB* also contribute to the development of the disease. Apolipoprotein B is the major apolipoprotein in lipoprotein molecules, especially in LDL-C, and is responsible for the LDL-LDLR binding. Thus, mutations in the *APOB* gene that could cause a defective binding between the LDL particle and the receptor, leading to an accumulation of LDL-C in plasma and resulting in the development of the pathogenesis. For its part, PCSK9 regulates plasma cholesterol levels by modulating LDLR expression on cell surface. The higher the affinity between the PCSK9 and the LDLR, the lower the amount of LDLR expression on the cell surface. This reduction in the amount of LDLR will translate into lower LDL-C uptake, increasing its plasma concentrations. Hence, mutations in the *PCSK9* gene that increase the aforementioned affinity, could contribute to the worsening of the pathophysiology.

In this study we have functionally characterized six APOB and one PCSK9 variants found in Italian hyperlipidemic patients. These APOB mutations do not

seem to affect the LDL particle size or morphology. However, they exhibit a lower affinity to the LDLR than the WT, as shown by the solid-phase immunoassay, which lowers the uptake of the LDL-C by the cells, increasing its plasma concentrations and leading to the development of the disease. The proliferation assay clearly shows the lack of functionality of the studied APOB variants, where cells incubated with those LDL samples show a reduced ability to proliferate.

Regarding the PCSK9 variant the preliminary results show that p.(Gly394Ser) PCSK9 variant is a LOF variant that is expressed and secreted similarly than wt PCSK9 but it does not bind to the LDLR receptor and therefore it does not drives the receptor to the lysosomal compartment.

The functional characterization of the variants carried out in this study will help us gain insight into the functional effect of the mutations located in *APOB* and *PCSK9*. This knowledge, combined with the data given by *in silico* analysis, will enable us to move towards earlier diagnosis and tailored treatment, thereby improving the overall management of the disease.

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PART II:

DECIPHERING THE COMPLEX EPIGENETIC NETWORKS THAT ORCHESTRATE STATIN-MEDIATED TYPE 2 DIABETES MELLITUS DEVELOPMENT: ROLES OF MIR-27b AND MIR-33

ESTATINA MEDIATUTAKO 2. MOTAK DIABETES MELLITUS GARAPENA ORKESTRATZEN DITUZTEN SARE EPIGENETIKO KONPLEXUAK: MIR-27b ETA MIR-33 ROLAK

Sections:

1. **General Introduction / Sarrera Nagusia**
2. **Objectives/ Helburuak**
3. **Materialak eta Metodoak / Materials eta Methods**
4. **Results / Emaitzak**
5. **Discussion/Eztabaida**

The results presented in PART II have been published at:

1. **Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, Ostolaza H, Martín C.** Pathophysiology of Type 2 Diabetes Mellitus. **Int J Mol Sci. 2020 Aug 30;21(17):6275. doi: 10.3390/ijms21176275.**
2. **Galicia-Garcia U, Jebari S, Larrea-Sebal A, Uribe KB, Siddiqi H, Ostolaza H, Benito-Vicente A, Martín C.** Statin Treatment-Induced Development of Type 2 Diabetes: From Clinical Evidence to Mechanistic Insights. **Int J Mol Sci. 2020 Jul 2;21(13):4725. doi: 10.3390/ijms21134725.**
3. **Deciphering the complex epigenetic networks that orchestrate statin-mediated type 2 diabetes mellitus development: roles of miR-27b and miR-33. Galicia-Garcia U, Benito-Vicente A, Gonzalez-Moro I, Jebari S, Larrea-Sebal A, Uribe KB, Aspichueta P, Marino A, Ostolaza H, Cenarro A, Civeira F, Suarez Y, Fernández-Hernando C, Santín I, Martín C. (Under preparation 2021)**

1. General Introduction / Sarrera Nagusia

Pathophysiology of Type 2 Diabetes Mellitus

1. Introduction

Type 2 Diabetes Mellitus (T2DM) is one of the most common metabolic disorders worldwide and its development is primarily caused by a combination of two main factors: defective insulin secretion by pancreatic β-cells and the inability of insulin sensitive tissues to respond to insulin [1]. Insulin release and action has to precisely meet the metabolic demand; hence, the molecular mechanisms involved in the synthesis and release of insulin, as well as the insulin response in tissues must be tightly regulated. Therefore, defects in any of the mechanisms involved can lead to a metabolic imbalance that leads to pathogenesis of T2DM.

This review analyses the key aspects of T2DM, as well as the molecular mechanisms and pathways implicated in insulin metabolism and associations between T2DM and cardiovascular pathophysiology. In this Review, we describe the global trends of T2DM, the roles of major risk factors, in particular, obesity, lifestyle factors, genetic predispositions, gut dysbiosis, epigenetics and mitochondrial deregulation. We highlight physiological and molecular mechanisms leading to T2DM and its complications.

2. Type 2 diabetes mellitus: background and epidemiology

According to the World Health Organization (WHO) diabetes mellitus is a chronic, metabolic disease characterized by elevated levels of blood glucose, which leads over time to damage to the heart, vasculature, eyes, kidneys and nerves. Over 90% of diabetes mellitus cases are T2DM, a condition marked by deficient insulin secretion by pancreatic islet β-cells, tissue insulin resistance (IR) and an inadequate compensatory insulin secretory response [2, 3]. Progression of the disease makes insulin secretion unable to maintain glucose homeostasis, producing hyperglycaemia. Patients with T2DM are mostly characterized by being obese or having a higher body fat percentage, distributed predominantly in the abdominal region. In this condition, adipose tissue promotes IR through various inflammatory mechanisms, including increased free fatty acid (FFA) release and adipokine deregulation. The main drivers of the T2DM epidemic are the global rise in obesity, sedentary lifestyles, high caloric diets and population ageing, which have quadrupled the incidence and prevalence of T2DM [4, 5].

The organs involved in T2DM development include the pancreas (β-cells and α-cells), liver, skeletal muscle, kidneys, brain, small intestine, and adipose tissue [6]. Evolving data suggest a role for adipokine dysregulation, inflammation, and abnormalities in gut microbiota, immune dysregulation, and inflammation have emerged as important pathophysiological factors [7].

Epidemiological data show alarming values that predict a worrisome projected future for T2DM. According to the International Diabetes Federation (IDF), in 2019, diabetes caused 4.2 million deaths; and 463 million adults aged between 20 and 79 years old were living with diabetes, a number that will likely rise up to 700 million by 2045. Diabetes was the underlying cause of at least 720 billion USD in health expenditure in 2019. Additionally, the true disease burden of T2DM is likely an underrepresentation as 1 in 3 diabetic people were underdiagnosed, equivalent to 232 million people. The greatest number of people suffering from diabetes is aged between 40 and 59 years old. Incidence and prevalence of T2DM vary according to geographical region, with more than 80% of patients living in low-to-middle-income countries, which pose additional challenges in effective treatment. Patients with T2DM have a 15% increased risk of all-cause mortality compared with people without diabetes with cardiovascular disease (CVD) as the greatest cause of morbidity and mortality associated with T2DM [8]. The association of diabetes with increased risk of coronary heart disease (hazard ratio [HR] 2.00; 95% CI 1.83–2.19), ischemic stroke (HR 2.27; 1.95–2.65), and other vascular disease-related deaths (HR 1.73; 1.51–1.98) has been shown in a meta-analysis [9].

Epidemiology of T2DM is affected both by genetics and environment. Genetic factors exert their effect following exposure to an environment characterised by sedentary behaviour and high calorie intake. Common glycaemic genetic variants for T2DM have been identified by genome-wide association studies, but these only account for 10% of total trait variance, suggesting that rare variants are important [10]. People of different ethnic origin may have different specific phenotypes that increase predisposition to clusters of CVD risk factors, including hypertension, insulin resistance, and dyslipidaemia [11].

3. Risk factors and pathophysiology

T2DM risk factors include a complex combination of genetic, metabolic and environmental factors that interact with one another contributing to its prevalence. Although individual predisposition to T2DM due to non-modifiable risk factors (ethnicity and family history/genetic predisposition) has a strong genetic basis,

evidence from epidemiological studies suggests that many cases of T2DM can be prevented by improving the main modifiable risk factors (obesity, low physical activity and unhealthy diet) [12, 13].

3.1 Ethnicity and family history/genetic predisposition

Globally, the incidence and prevalence of T2DM are found to vary widely depending on ethnicity and geographical region with Japanese, Hispanics and Native Americans having the highest risks [14-16]. It has been shown higher incidence rates in Asians compared with a White American population [17, 18], and white population in the UK [19], where the highest risk is among black population [20]. Whilst no clear reasons have been found, contributing factors such as modern lifestyle factors (which promote obesity), socioeconomic and direct genetic propensity or gene environmental interactions have been postulated.

Genetic predisposition plays an important part in the risk of developing T2DM. Over the past decade, several T2DM genome-wide association studies have shown the complex polygenic nature of T2DM in which most of these loci increase T2DM risk through primary effects on insulin secretion, and a minority act through reducing insulin action [21, 22]. Dimas et al. grouped these variants on the basis of their potential intermediate mechanisms in T2DM pathophysiology, with four variants fitting a clear IR pattern; two reducing insulin secretion with fasting hyperglycaemia; nine lowering insulin secretion with normal fasting glycaemia; and one altering insulin processing [23]. According to these data, the genetic architecture of T2DM is highly polygenic, and additional association studies are needed to identify most T2DM loci [24]. Interactions between susceptibility loci and environmental factors could underlie the missing heritability of T2DM thus the impact of a given genetic variant can be modulated by the environmental factors (and vice versa) as evidenced by both observational studies and clinical trials [25].

3.2. Obesity, low physical activity and unhealthy diet

Obesity (body-mass index [BMI] ≥30 kg/m²) is the strongest risk factor for T2DM [26, 27] and is associated with metabolic abnormalities resulting in IR [28]. There exist an inverse linear relationship between BMI and the age at diagnosis of T2DM [29]. The exact mechanisms by which obesity induces T2DM and IR remain to be elucidated; however, numerous factors have shown a significant role in the development of this pathological process, which involve both cell autonomous mechanisms and inter-organ communications.

Sedentary lifestyle is another risk factor for T2DM as shown by the Women's Health Study and in the Kuipio Ischemic Heart Disease Risk Factor Study, which

showed a reduction of 34% and 56% reduction of developing T2DM in participants walking 2-3 h a week or at least 40 min a week, respectively [30, 31]. There are three primary benefits of physical activity on the delay of T2DM onset. First, contraction of skeletal muscle cells induces an increase in blood flow into the muscle, enhancing glucose uptake from plasma [32]. Second, physical activity reduces the notorious intra-abdominal fat, which is a known risk factor that promotes IR [33]. Finally, moderate intensity exercise has been shown to improve glucose uptake by 40% [34]. Physical activity improves glucose uptake and insulin sensitivity but it can also improve or even reverse inflammation and oxidative stress, which are T2DM predisposing factors [32].

3.3. Pathophysiology

Regarding the pathophysiology of the disease, a malfunctioning of the feedback loops between insulin action and insulin secretion results in abnormally high glucose levels in blood [2]. In the case of β-cell dysfunction, insulin secretion is reduced, limiting the body's capacity to maintain physiological glucose levels. On the other hand, IR contributes to increased glucose production in the liver and decreased glucose uptake both in the muscle, liver and adipose tissue. Even if both processes take place early in the pathogenesis and contribute to the development of the disease, β-cell dysfunction is usually more severe than IR. However, when both β-cell dysfunction and IR are present, hyperglycaemia is amplified leading to the progression of T2DM [35, 36].

4. Mechanisms leading to T2DM and pathophysiology

4.1. Insulin secretion: physiological and dysfunctional mechanisms leading to T2DM

4.1.1. β-cell physiology

To safeguard proper β-cell function, cellular integrity must be ensured and the mechanisms and pathways implicated in the physiology of β-cell must be tightly regulated [35].

β-cells are responsible of insulin production, which is synthesized as pre-proinsulin. In the maturation process, pre-proinsulin undergoes a conformational modification carried out with the help of several proteins in the endoplasmic reticulum (ER) to yield proinsulin [37]. Afterwards, proinsulin is translocated from the ER to the Golgi apparatus (GA), entering into immature secretory vesicles and being cleaved into C-peptide and insulin [38, 39].

Once matured, insulin is stored in granules until insulin release is triggered. Insulin release is primarily triggered by response to high glucose concentrations. It is worth noting that some other factors can also induce insulin release such as amino acids, fatty acids and hormones, [40]. When circulating glucose levels increase, β-cells take in glucose mainly through the glucose transporter 2 (GLUT2), a solute carrier protein that also works as a glucose sensor for β-cells. Once glucose enters, glucose catabolism is activated, increasing the intracellular ATP/ADP ratio, which induces the closing of ATP-dependant potassium channels in the plasma membrane. This leads to membrane depolarization and opening of the voltage dependant Ca^{2+} channels, enabling Ca^{2+} to enter the cell. The rise in the intracellular Ca^{2+} concentration triggers the priming and fusion of the secretory insulin-containing granules to the plasma membrane, resulting in insulin exocytosis [38, 40-42] (Figure 1A).

Additionally, Ca^{2+} signals can be amplified by the RY receptors (RYR) and may play important roles in stimulus- insulin secretion coupling by virtue of their strategic locations within the cell and their ability to mediate Ca^{2+} induced Ca^{2+} release (CICR). RYR amplify Ca^{2+} signals when the channel is sensitized by messenger molecules generated from nutrient metabolism or ligand-binding and are involved in amplification of insulin secretion [43] (Figure 1A).

Nevertheless, other cell signals can also assist or enhance insulin release from β-cells. Among them, cAMP might be the most important messenger potentiating insulin release. Accumulated evidence suggests that cAMP induces insulin containing secretory vesicle mobilization by depleting intracellular Ca^{2+} reservoirs, thereby increasing intracellular Ca^{2+} concentrations [44]. There is also compelling evidence that extracellular ATP is another important regulator of β-cell function. It is well documented that β-cells release ATP through exocytosis of insulin granules upon glucose stimulation. Purinergic signalling via P2Y and P2X purinergic receptors stimulates Ca^{2+} mobilization and regulates insulin exocytosis also independently of glucose. P2Y purinoreceptors have been reported to be coupled to G-proteins [45, 46] whereas P2X-type receptors are ATP-activated ligand-gated ion channels non-selective for cations [47]. In the case of P2Y receptors, it has been proposed that insulin release could be mediated by intracellular Ca^{2+} mobilization in response to inositol-1,4,5-trisphosphate (IP3) formation that triggers release of Ca^{2+} from ER stores, which amplifies the exocytosis-triggering Ca^{2+} signal [48, 49] (Figure 1A).

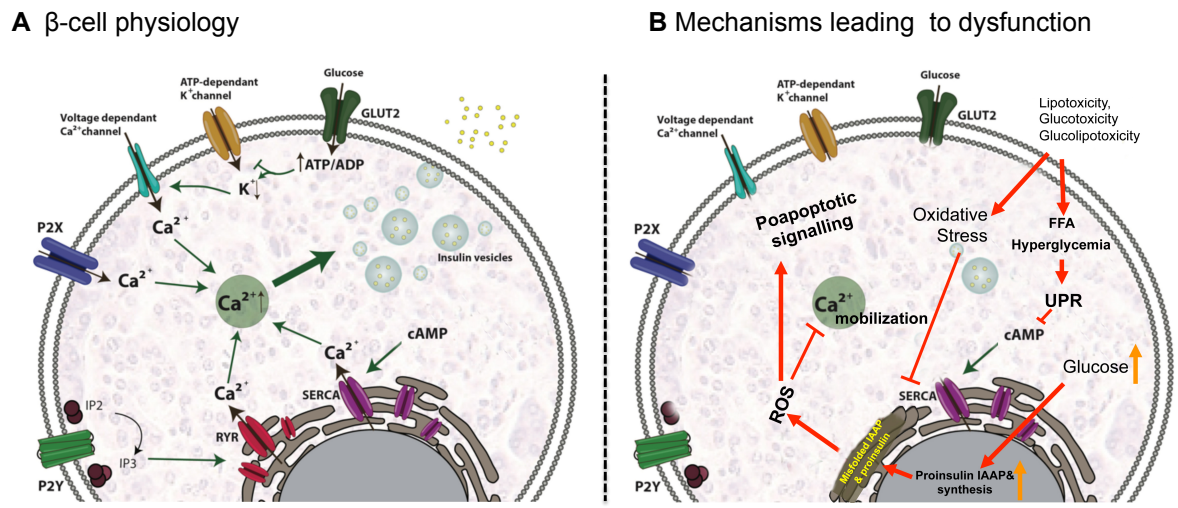

Figure 1. Signalling pathways involved in insulin secretion in β-cells in physiological conditions (A) and mechanisms leading to dysfunction (B). (A) Insulin release is primarily triggered by response to high glucose concentrations and glucose in mainly internalized mainly through GLUT2 transporter. Glucose catabolism increases ATP/ADP ratio, ATP-dependant potassium channels are closed leading to membrane depolarization and opening of the voltage dependant Ca^{2+} channels. The latter enables Ca^{2+} influx triggering insulin exocytosis. Additional Ca^{2+} channels as P2X, P2Y, SERCA and RYR contribute to Ca^{2+} mobilization and insulin secretion. (B) hyperglycaemia and hyperlipidemia promotes oxidative stress leading to ROS generation that inhibits Ca^{2+} mobilization and activates proapoptotic signals. Additionally, an excess of FFAs and hyperglicemia lead to the activation of the apoptotic unfolded protein response (UPR) pathways and generation of ER stress. Sustained high glucose levels increase proinsulin and IAAP biosynthesis, which generate ROS. GLUT2: glucose transporter 2, P2X: purinergic receptor X; P2Y: purinergic receptor Y; IP2: inositol 1,3-bisphosphate; IP3: inositol 1,4,5-trisphosphate; RYR: ryanodine receptor chanel; SERCA: sarco-endoplasmic reticulum Ca^{2+}-ATPase; FFA: free fatty acid, ROS: reactive oxygen species; UPR: unfolded protein response.

4.1.2. Mechanisms leading to β-cell dysfunction

β-cell dysfunction has been traditionally associated to β-cell death [50]. However, recent evidence suggests that the dysfunction of β-cells in T2DM might be due to a more complex network of interactions between the environment and different molecular pathways implicated in cell biology [51]. In an excessive nutritional state, similar to that found in obesity, hyperglycemia and hyperlipidemia are often present, favouring IR and chronic inflammation. Under these circumstances, β-cells, due to differences in their genetic susceptibility, are subject to toxic pressures including inflammation, inflammatory stress, ER stress, metabolic/oxidative stress, amyloid stress, with the potential of ultimately leading to a loss of islet integrity [50].

An excess of FFAs and hyperglicemia lead to β-cell dysfunction by inducing ER stress through the activation of the apoptotic unfolded protein response (UPR) pathways [52]. In fact, lipotoxicity, glucotoxicity and glucolipotoxicity occurring in

obesity, induce metabolic and oxidative stress that lead to β-cell damage [51]. Stress derived from high levels of saturated FFAs can activate UPR pathway by several mechanisms including inhibition of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) responsible for ER Ca^{2+} mobilization; activation of IP3 receptors or direct impairment of ER homeostasis. In addition, sustained high glucose levels increase proinsulin biosynthesis and islet amyloid polypeptides (IAAP) in β-cells, leading to accumulation of misfolded insulin and IAAP and increasing the production of oxidative protein folding-mediated reactive oxygen species (ROS) [52]. These effects alter physiological ER Ca^{2+} mobilization and favour proapoptotic signals, proinsulin mRNA degradation and induce interleukin (IL)-1 β release that recruits macrophages and enhances local islet inflammation [51] (Figure 1B).

As previously mentioned, insulin secretion has to be finely regulated to precisely meet metabolic demand. For that reason, proper islet integrity must be conserved in order to allow β-cells to respond to metabolic needs. Under pathogenic conditions, the mechanism described above can ultimately lead to disruption of islet integrity/organization, impairing optimal cell-to-cell communication within pancreatic islets, contributing to poor regulation of insulin and glucagon release and ultimately exacerbating the hyperglycaemia. Defects in the synthesis of any insulin precursors, or insulin itself, as well as disruption of the secretion mechanism can lead to insulin secretory dysfunction, the primary driver of β-cell failure, and a foundation of T2DM. For instance, reduced expression in the GLUT2 glucose transporter would affect the downstream signalling pathway [53], while failure in the folding of proinsulin is another finding commonly linked to deficient insulin production and diabetes [54].

4.1.3. Pathological conditions perpetuating T2DM

4.1.3.1. Nutritional factors

High-caloric Western diet contains large amounts of fats and carbohydrates that elevate blood glucose and circulating very low-density lipoproteins (VLDLs), chylomicrons (CMs) and their remnants (CMRs) that are rich in triglycerides (TG). This induces a spike in reactive oxygen species (ROS) concentrations, which in turn leads to an abnormal generation of inflammatory molecules. Given that inflammation is a recognized inducer of oxidative stress, a synergistic interaction occurs between the two processes after a heavy meal, with consequent amplification of harmful postprandial effects. The sustained and marked increase in steady-state levels of ROS contributes significantly to the pathogenesis of T2DM

and IR. Therefore, a pro-oxidant environment leads to mitochondrial dysfunction, ER stress, activation of NADPH oxidase (NOX) and superoxide (O_2^-) production. The increase in O_2^- production activates the five major pathways involved in the pathogenesis of diabetes complications: enhancement of the polyol pathway, increased formation of advanced glycation end products (AGEs), increased expression of AGEs receptor and its activating ligands, activation of protein kinase C (PKC) isoforms, and overactivity of the hexosamine pathway [55-57]. Through these pathways, increased intracellular ROS causes defective angiogenesis in response to ischemia, activates a number of proinflammatory pathways, and cause long-lasting epigenetic changes which drive persistent expression of proinflamatory genes even after glycemia is normalized [58]. Additionally, increased blood levels of FFAs also lead to mitochondrial dysfunction through two different mechanisms: (1) FFA metabolism by-products disturb the electron flow throughout the mitochondrial respiratory chain and (2) through incorporation of FFAs into the mitochondrial membranes, thus likely favouring electron leakage [59].

4.1.3.2. Physical Activity

Reduced physical activity and exercise training, and increased sedentary behaviours constitute a link between obesity and T2DM and are associated with increased markers of chronic low-grade systemic inflammation [60, 61]. In this condition, proinflammatory molecules are released into the bloodstream and within specific tissues such as interleukin 6 (IL-6), C-Reactive Protein (CRP), tumor necrosis factor alpha (TNF-α) or IL-1 induce an inflammatory state known as metabolic inflammation [37]. Indeed, IL-1 is involved in the autoimmune response to β-cells in the pancreas, inhibition of β-cell function and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor, thus inhibiting β-cell function and promoting apoptosis [32]. Preclinical data suggest that inflammation resolution could prevent the development of T2DM in obesity and prediabetes, which was substantiated by preclinical animal data showing that deletion of the macromolecular complex NLRP3 inflammasome, responsible for the production of IL-1β and IL-18, resulted in improved insulin sensitivity [62].

Intentional weight loss remains the cornerstone therapy to improve insulin sensitivity and in some circumstances to prevent the incidence of T2DM in individuals with obesity and prediabetes [63]. Regular exercise and increased physical activity enhance the production of anti-inflammatory cytokines such as IL-1 Receptor antagonist (IL-1Ra) and soluble TNF receptor (s-TNF-R) that are antagonists of IL-1 and TNF-α, respectively. Individuals with increased physical activity also show reduced circulating levels of IL-6, IL-18 and CRP, together with

lower levels of leptin, a molecule associated to CRP [64]. Physical exercise can improve T2DM-inducing oxidative stress by inducing the synthesis of antioxidants such as glutathione (GSH), a major non-enzymatic antioxidant [65] and other antioxidant enzymes which lead to a long-term reduction in free radical levels [32].

Finally, irisin is an exercise-regulated myokine, which improves glucose tolerance [66] secreted by skeletal muscle [67] and adipose tissue [57] in response to exercise [68]. T2DM patients have been found to have lower circulating levels of irisin compared to control subjects. Additionally, diabetic patients with CVD had significantly lower serum irisin than non-CVD patients [69]. Low levels of serum irisin have been associated with 1.6 times increased risk of CVD incidence in T2DM patients [70].

4.1.3.3. Gut Dysbiosis

Gut microbiota is composed of many microbial species that impact human physiology and participate in different biological processes [71]. They can modulate the immune system and inflammatory response, regulate gut barrier integrity and human metabolism, take part in the synthesis of metabolites. Gut resident microorganisms produce many metabolites that contribute to physiology in healthy individuals. However, changes due to both inherited and acquired factors such as age, nutrition, lifestyle, genetic predisposition, or underlying diseases can affect the gut microbiota produced metabolite proportion leading to metabolic disturbances that can culminate in disease [72-74]. The better understating of gut microbiota has evidenced its important role in the development of diabetes and recent studies indicate that changes in dysbiosis can promote IR and T2DM [75]. High fat diet can induce up to threefold lipopolysaccharide (from gram negative bacteria) production in mice models, thereby contributing to low grade inflammation and insulin resistance [76, 77]. Furthermore, intestinal dysbiosis can reduce short-chain fatty acid synthesis that promotes gut barrier integrity, pancreatic β-cell proliferation and insulin biosynthesis [78, 79]. Dysbiosis can also compromise the production of other metabolites such as branched aminoacids and trimethylamine thus disrupting glucose homeostasis and triggering T2DM development [80, 81]. Understanding the clinical implications of the gut microbiome is a relatively new field, and requires further research to better elucidate the connection between gut microbiota and T2DM.

4.1.3.4. Metabolic memory

Metabolic memory refers to the persistence of diabetic complications even after maintained glycemic control. This concept arose from the results of multiple large-scale clinical trials, which showed that after diabetes onset, diabetes complications persist and progress even when glycemic control is restored through pharmaceutical intervention [82-84]. Among them, the UKPDS post-trial study and Steno-2 trial showed that specifically early glycemic interventions prevents diabetic complication and has a marked decrease in CVD endpoints in patients that received either standard or intensive treatment following their diagnosis [84]. Later on, animal models of diabetes and *in vitro* cell cultures demonstrated that the initial hyperglycemic period results in permanent abnormalities (including aberrant gene expression) of target organs/cells [85-88]. Metabolic memory involves four mechanisms: epigenetics, oxidative stress, non-enzymatic glycation of proteins and chronic inflammation.

4.1.3.4.1. Epigenetics: microRNAs

Epigenetics involve genetic modulation by factors other than individuals' DNA sequence, and can regulate gene expression and determine which proteins are transcribed [89]. There are different epigenetic regulation mechanisms: direct methylation of cytosine or adenine residues, covalent modifications of histone proteins, higher-order chromatin structure and non-coding RNAs. Disruptions or imbalances in epigenetic mechanisms can lead to the development of diabetic pathophysiology [90].

MicroRNAs (miRNAs) are small non-coding RNA sequences synthesized as non-mature molecules that undergo several processing steps both in the nucleus and in the cytoplasm to become fully matured miRNAs. Once matured, miRNAs bind to their target gene's mRNA, leading to mRNA silencing or degradation [91]. Increasing evidence highlights the importance of miRNA mediated post-transcriptional regulation in different aspects of β-cell biology such as cell differentiation, cytokine and growth factor mediated signalling, glucose metabolism and insulin synthesis and secretion [92]. Deregulation of miRNA expression can directly impair β-cell function leading to the development of T2DM [93]. To date, more than 2600 miRNAs have been described within human genome (miRBase, v.22.1), and multiple miRNAs have been shown to be involved in the pathogenesis of T2DM, including miR-200, miR-7, miR-184, miR-212/miR132 and miR-130a/b/miR-152 [94]. For instance, overexpression of miR-7 results in reduced insulin secretion via inhibition of genes involved in vesicle fusion and SNARE activity such as Snca, Cspa and Cplx1 [95]. In the case of miR-375, over expression

results in impaired exocytosis and thereby reduced insulin secretion. Conversely, it is downregulation of miR-375 expression that causes a reduction in β-cell mass [93, 96, 97].

Several studies have evidenced that deregulation of microRNA (miRNA) profile, (post-translational histone methylation and non-canonical histone variant inclusion in octomers may persist even after normoglycemia restoration [98-101]. MiRNAs participate in metabolic memory by targeting the mRNA of genes encoding enzymes involved in DNA methylation and those tightly regulated at the level of promoter methylation, transcription, and processing [102]. It has been shown that high glucose levels can alter post-translational histone modifications (PTHMs) and the activity of DNA methyltransferases generating irreversible changes that explain the long-term harmful effects of metabolic memory [103-106].

4.1.3.4.2. Oxidative stress, non-enzymatic glycation of proteins and chronic inflammation.

Hyperglycaemia induces an excess of ROS generation by mitochondria, which gives rise to diabetes complications [107] that may persists even when hyperglycemia is controlled. The damage following hyperglycaemia-induced oxidative stress can be prevented when good glycemic control is initiated very early, but is not easily reversed if poor control is maintained for a longer duration [108, 109]. At early stages of T2DM, there is a relationship between hyperglycaemia, increased oxidative stress, and excessive AGE formation. As the disease progresses, there is a persistent protein glycation of the components of the respiratory chain that together with mitochondrial DNA damage can generate a hyperglycemia-independent concatenation of events leading to a synergy between oxidative stress and AGEs [86]. The effects of this metabolic imbalance activate inflammatory processes through receptor binding of AGEs or ROS which can modify the composition and structure of the extracellular matrix [98]. These structural changes may cause endothelial dysfunction and then atherosclerosis [98].

Finally, low-grade inflammation, which is involved in T2DM development and its vascular complications, has shown to mediate metabolic memory. Many environmental factors (age, obesity, sedentarism and diet) that promote T2DM development trigger an inflammatory response leading to IR and endothelial dysfunction [105, 110, 111]. Obesity leads to NF-κB activation, which mediates the expression of inflammatory genes, which enhance monocyte binding to endothelial and vascular smooth muscle cells, subsequently promoting monocyte-to-macrophage differentiation [105]. In addition, NF-κB activation induces expression of inflammatory cytokines that are involved in vascular inflammation,

with subsequent generation of endothelial adhesion molecules, proteases, and other mediators [111]. Another important factor that links inflammation and oxidative stress in obesity condition is the Toll-like receptor, which contributes to hypertension, insulin resistance, and obesity [105].

In summary, T2DM is a heterogeneous and progressive disorder that represents a series of metabolic conditions associated with hyperglycaemia and caused by defects in insulin secretion and/or insulin action due a complex network of pathological conditions. There are many different paths, driven by various genetic and environmental factors that interact and mutually reinforce each other leading to an increased risk of other diseases including heart, peripheral arterial and cerebrovascular disease, obesity and non-alcoholic fatty liver disease, among others. The complex network of pathological conditions leading to T2DM development are summarized in Figure 2.

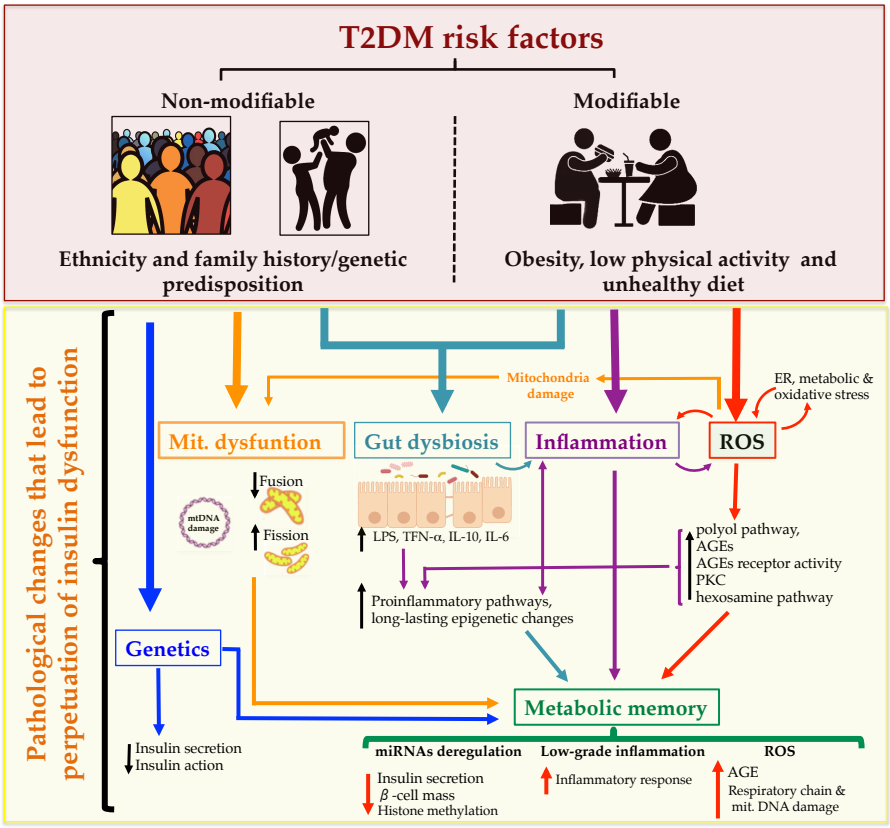

Figure 2. T2DM risk factors and the pathological changes leading to perpetuation of insulin dysfunction. Complex combinations of genetic, metabolic and environmental factors that interact with one another constitute both non-modifiable (ethnicity and family history/genetic predisposition) and modifiable risk factors (obesity, low physical activity and unhealthy diet). These states affect cell function resulting in a complex network of pathological changes that influence mutually and lead to perpetuation of insulin dysfunction. ROS: reactive oxygen species; ER: endoplasmic reticulum; AGEs: advanced glycation end products; PKC: protein kinase C; LPS: lipopolysaccharide; miRNA: microRNA.

4.1.3.5. Mitochondrial dysfunction

There is increasing evidence associating mitochondrial dysfunction with T2DM development, age–related IR and T2DM complications [112]. Indeed, oxidative stress, defective mitochondrial biogenesis, genetic mutations affecting mitochondrial integrity and ageing promote mitochondrial dysfunction and are closely associated with T2DM development (Figure 3) [113, 114].

The main function of mitochondria is ATP synthesis through oxidative phosphorylation in response to metabolic demand [115]. Mitochondria also participate in the production of different metabolites used as precursors of several macromolecules (lipids, proteins, and DNA). In addition, mitochondria play an important role in maintaining ion homeostasis, ROS clearance, the stress response, and serve to integrate multiple signalling pathways [116, 117]. An imbalance between energy intake and expenditure in the mitochondria generates mitochondrial dysfunction, a state characterized by a reduced ratio of energy production to respiration [112]. Under these circumstances, nutrient oxidation efficiency is reduced leading to a decreased ratio of ATP synthesis/oxygen consumption, which increases O_2^- production [118]. In fact, the accumulation of ROS in the mitochondria is one proposed mechanism linking mitochondrial dysfunction to IR [119]. This relationship was corroborated in studies showing decreased mitochondria oxidative capacity in skeletal muscle and impaired lipid metabolism in obese and insulin-resistant individuals compared to healthy controls [120-122]. In addition, patients with T2DM have been found to have downregulation of genes involved in oxidative metabolism that are regulated by the peroxisome proliferator-activated receptor γ co-activator 1α (PGC 1α) [123, 124] and a diminished phosphocreatine re-synthesis rate, both indicative of impaired mitochondrial function [125] (Figure 3). Moreover, some relatives of T2DM patients have been found to have decreased mitochondrial respiration suggesting that mitochondrial dysfunction may precede T2DM development. It has also been proposed that T2DM development may be a direct consequence of defects in the oxidative phosphorylation system and the electron transport chain (ETC) rather than a decrease in mitochondrial content [126].

Generation of ROS is highly implicated in the relationship between mitochondrial dysfunction and insulin resistance. ROS production takes place mainly at complex I and complex III of the ETC and increases when ETC is not able to handle excessive electron input. In these circumstances, as a consequence of nutrient overload, electron supply to the mitochondrial ETC increases and the electron excess is transferred to oxygen generating O_2^- and subsequent hydrogen peroxide [127]. ROS generated in mitochondria oxidize the Cys and Met residues in

proteins, damaging protein structure, impairing their function and eventually causing cell death. ROS species also damage DNA and membrane lipids, thus promoting mitochondrial dysfunction [128]. In addition, ROS overproduction activates the polyol pathway, the formation of AGEs, and the expression of AGEs receptor and its activating ligands. It also activates PKC isoforms and upregulates the hexosamine pathway contributing to T2DM worsening [129, 130]. In sum, excessive ROS generation by mitochondria contributes to accelerated T2DM progression (Figure 3).

Mitochondrial dysfunction includes a reduction in mitochondrial biogenesis, along with a decrease in the expression of mitochondrial oxidative proteins, such as ETC complexes, which leads to decreased substrate oxidation. The damage produced by high oxidative stress in the mitochondria activates mitophagic processes in order to eliminate dysfunctional mitochondria or in case of excessive cellular stress to apoptosis [131]. These two processes reduce substrate utilization and enhance accumulation of lipid intermediates such as diacylglycerols (DAG) and ceramide (CER) that disrupt insulin signalling pathway [132]. DAG induces an increment of the serine/threonine phosphorylation of IRS-1, reducing its insulin-stimulated tyrosine phosphorylation and downstream propagation of the insulin signalling pathway [133] while CER inhibits protein kinase AKT [133]. The accumulation of DAG and CER contributes to the mitochondrial dysfunction seen in IR (Figure 3).

Defects in mitochondrial biogenesis may be mediated by downregulation of PGC 1α that has also been detected in T2DM patients [123, 124]. PGC 1α is a transcription coactivator that regulates the expression of key genes involved in mitochondrial biogenesis, adaptive thermogenesis and metabolic substrate metabolism [134]. Furthermore, some of the genes involved in oxidative metabolism that are downregulated in individuals with T2DM are under the control of PGC 1α [124]. *Mitofusin-2*, a key driver in mitochondria biogenesis is also downregulated in humans with T2DM [135]. Interestingly, *mitofusin-2* levels increase upon weight loss indicating that nutrient and energy oversupply leads to mitochondrial dynamics defects [135].

Mitochondrial homeostasis is maintained via mitochondrial biogenesis and the selective clearance of damaged organelles. Mitochondrial dynamics are crucial to maintain healthy mitochondria and control their quantity. Mitochondria fission promotes the removal of damaged mitochondria in a process known as mitophagy, which has to be efficiently and tightly regulated in order to preserve cell

homeostasis [136]. Thus, mitophagy is considered to be one of the core mechanism controlling mitochondrial quantity and quality [137]. The process of removing damaged mitochondria consists of two steps: the induction of general autophagy and the priming of damaged mitochondria for selective autophagy recognition [138]. Once the degradation process is completed, the products are released back into the cytosol where macromolecular constituents are recycled. This process generates energy to maintain cell viability under unfavorable conditions and protects the cell during stress conditions [136, 139]. When mitophagy is impaired, cellular stress and ROS production increases, contributing to reduced hepatic insulin sensitivity and glucose homeostasis, two of the major pathological branches of T2DM development [112, 140]. Deregulation of mitochondria dynamics with a shift towards fission promotes metabolic dysfunction as demonstrated by the onset of obesity and IR following the ablation of fusion protein in mice [141, 142]. Furthermore, increased mitochondrial fission and mitochondrial fragmentation have been associated with mitochondrial depolarisation, impaired ATP production and decreased insulin-dependent glucose uptake as well as increased mitochondrial ROS and impaired insulin signalling in C2C12 murine cell line and cybrids, respectively [143, 144.] These studies highlight the deleterious effect of unbalanced mitochondrial dynamics on metabolic health. Enhanced mitochondria fission also negatively impacts fatty acid β-oxidation, which is a pivotal metabolic defect in obesity and IR [120, 121] contributing to the accumulation of lipotoxic lipid species. Fusion-shifted mitochondria dynamics has been also associated with an increase in fatty acid utilization putatively preventing lipotoxicity [145].

The role of mitochondrial genetics in the risk of T2DM has been clearly established. Indeed, several mtDNA variants (homoplasmic or heteroplasmic) have been associated with T2DM development. To date, the group of heteroplasmic variants associated with higher risk of T2DM development includes A3243G, T14577C and A5178C [146-149]. The group of homoplasmic variants associated with T2DM risk includes C1310T, G1438A, A12026G, T16189C and A14693G [150-152]. It is important to note that additional studies are necessary to determine whether more metabolically active tissues that generate more mitochondrial ROS have increased rates of mtDNA heteroplasmy in T2DM.

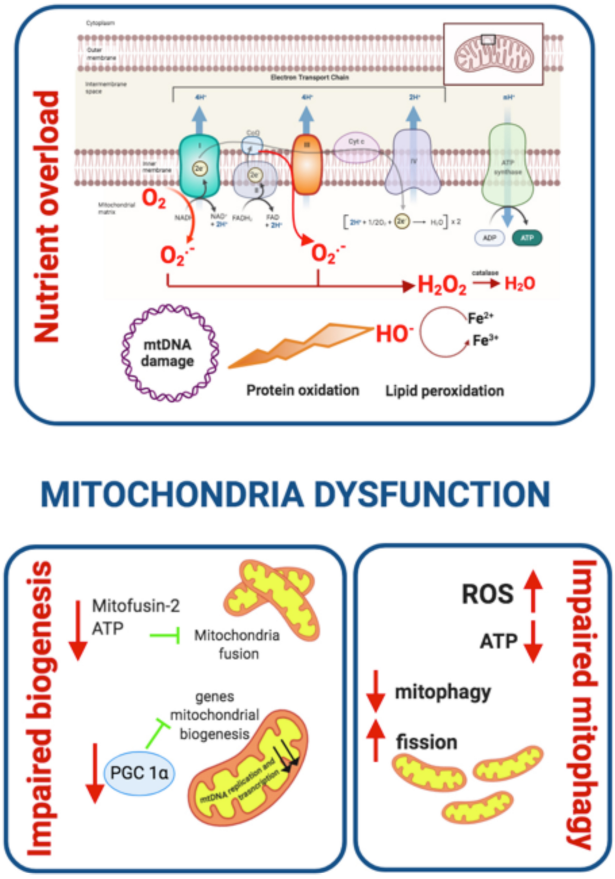

Figure 3. Mitochondrial dysfunction and contribution toT2DM development. Oxidative stress, defective mitochondrial biogenesis and impaired mitophagy promote mitochondrial dysfunction. Generation of ROS links mitochondrial dysfunction and IR. As a consequence of nutrient overload, electron supply to the mitochondrial ETC increases and the electron excess is transferred to oxygen generating $O_2^{\cdot-}$ and H_2O_2. ROS oxidize proteins, damage DNA and membrane lipids. Mitofusin-2 and PGC 1α are downregulated leading to reduced mitochondrial biogenesis. Cellular stress and ROS production contribute to a higher mitochondrial fission and impaired mitophagy. PCG 1α: Peroxisome proliferator-activated receptor-gamma coactivator-1.

To summarize, there is a highly nuanced and bi-directional relationship between mitochondrial dysfunction and T2DM. On one hand, aspects of T2DM such as insulin resistance can lead to mitochondrial dysfunction, such as through nutrient overload leading to ROS accumulation. On the other hand, mitochondrial dysfunction may predispose patients to subsequently developing T2DM, as evidenced by the presence of mtDNA variants associated with T2DM. Additional research is needed to better characterize the relationship between mitochondrial health and diabetes.

5. Insulin resistance

IR refers to a decrease in the metabolic response of insulin-responsive cells to insulin or, at a systemic level, an impaired/lower response to circulating insulin by blood glucose levels [153]. There are three broad categories of IR or insulin-deficient conditions: (1) Diminished insulin secretion by β-cells; (2) Insulin antagonists in the plasma, due either to counter-regulatory hormones or non-hormonal bodies that impair insulin receptors or signalling; and (3) Impaired insulin response in target tissues [154]. The action of insulin is influenced by the interplay of additional molecules including growth hormone and IGF-1 in the fed state. While fasting, the insulin response is mitigated by glucagon, glucocorticoids and catecholamines in order to prevent insulin-induced hypoglycaemia. The ratio of insulin/glucagon plays a major role in this regulation, since it determines the relative degree of phosphorylation of downstream enzymes in the regulatory signalling pathways. While catecholamines promote lipolysis and glycogenolysis, glucocorticoids promote muscle catabolism, gluconeogenesis and lipolysis. Hence, excessive secretion of these hormones may be responsible for inducing IR [155, 156]. Regarding the last category, there are three main extra-pancreatic insulin-sensitive organs that play major roles on the aforementioned processes: skeletal muscle, adipose tissue and liver. A defective action of insulin in these tissues often precedes the development of systemic IR, thus progressively leading T2DM.

5.1. Skeletal muscle

Skeletal muscle IR is considered to be the most important extra-pancreatic factor in the development of T2DM [157]. Under physiological conditions, insulin stimulates muscle glycogen synthesis by enhancing glucose uptake from plasma. There are three primary rate-limiting factors implicated in glucose uptake and glycogen synthesis: glycogen synthase, hexokinase and the glucose transporter GLUT4 [158]. Upon insulin binding to insulin receptor (INSR) in muscle cells, GLUT4 translocates from intracellular compartments (early endosomes (EE), endosomal recycling compartment (ERC) and *trans*-Golgi network (TGN)) to the plasma membrane. This process allows glucose uptake and reduces circulating glucose levels [159].

Mutations that reduce the expression of insulin receptor or GLUT4, as well as any defect in either upstream or downstream signalling pathway would reduce glucose intake into the muscle resulting in a hyperglycaemic state [153, 160]. The activation of INSR tyrosine kinase activity is essential for the action of insulin on glucose metabolism. Insulin binding to the α-subunit of the INSR causes

phosphorylation of the β-subunit on multiple tyrosine residues and allows insulin mediated signalling. Thus, mutations in any of the main phosphorylation sites can impair INSR tyrosine kinase activity, thereby impairing insulin action on skeletal muscle [161]. As mentioned above, mutations in key proteins of the downstream signalling pathway such as IRS-1 and IRS-2 or phosphoinositide 3-kinase (PI3K) also impair insulin action on muscle. Apart from mutations or defective epigenetic regulation, environmental factors can also play an important role in glucose uptake by muscle. Physical activity increases blood flow into skeletal muscle cells and thereby enhances glucose utilization [32]. Obesity, which is associated with chronic inflammation, contributes to IR and T2DM. Increasing evidence suggest that as a consequence of obesity, increased immune cell infiltration and secretion of proinflammatory molecules in intermyocellular and perimuscular adipose tissue leads to skeletal muscle inflammation. This ultimately leads to myocyte inflammation, impaired myocyte metabolism, and contributes to IR via paracrine effects [162].

5.2. Adipose tissue

Adipose tissue is a metabolically dynamic tissue capable of synthesizing a wide range of biologically active compounds that regulate metabolic homeostasis at a systemic level [163]. Indeed, adipose tissue participates in a broad range of biological processes involving, among others, immunity, coagulation, angiogenesis, fibrinolysis, reproduction, vascular tone control, appetite regulation, body weight homeostasis and glucose and lipid metabolism [164].

Insulin acts on adipose tissue in two different ways: (1) stimulating glucose uptake and triglyceride synthesis; and (2) suppressing triglyceride hydrolysis and inducing the uptake of FFA and glycerol from circulation [165]. In the fed state, GLUT4 allows uptake of glucose from the bloodstream into adipocytes, activating glycolysis in which glycerol-3-phospate (glycerol-3-P) is produced and incorporated into lipogenic pathways. Glycerol-3-P, along with the fatty acids coming from VLDLs, is esterified, forming triacylglycerol (TGA) that is stored in lipid droplets. During metabolic stress, TGA droplets the adipocyte are depleted, in order to provide FFA to be used as an energy source in other tissues.

An impaired response to insulin stimulation by adipose tissue is known as adipose IR (Adipose-IR). Adipose-IR can lead to impaired suppression of lipolysis, impaired glucose uptake, and enhanced FFA release into plasma even in the presence of high insulin levels [166]. Among the signalling elements affected by adipose-IR, we found that defective AKT activation impairs GLUT4 translocation

to the membrane and promotes the activation of lipolytic enzymes that aggravate hyperglycaemia [153]. Adipose-IR, as mentioned before, is associated with glucose intolerance and elevated release of FFA into plasma that accumulates in other tissues such as muscle or liver. In the case of the liver, FFA accumulation results in impaired insulin signalling that promotes hepatic gluconeogenesis and impairs the glucose-stimulated insulin response, inducing T2DM development.

It has been shown that abnormally increased adipose tissue mass and adipocyte size correlate with pathologic vascularisation, hypoxia, fibrosis and macrophage-mediated inflammation [167]. A high fat diet and obesity can activate saturated FFA-stimulated adenine nucleotide translocase 2 (ANT2), an inner mitochondrial protein that results in adipocyte hypoxia and triggers the transcription factor hypoxia-inducible factor-1α (HIF-1α). This culminates in adipose tissue dysfunction and inflammation [1]. Hypertrophied adipocytes as well as adipose tissue-resident immune cells contribute to increased circulating levels of proinflammatory cytokines. This increase in circulating proinflammatory molecules, together with an increase in local cytokine release such as TNF and IL-1β and IL-6 facilitates the emergence of a chronic state of low-grade systemic inflammation, also known as metabolic inflammation [1]. This chronic inflammatory state is considered to be a key part in the pathogenesis of IR and T2DM [168]. The insulin stimulation effects on healthy and hypertrophic adipose tissue are shown in Figure 4.

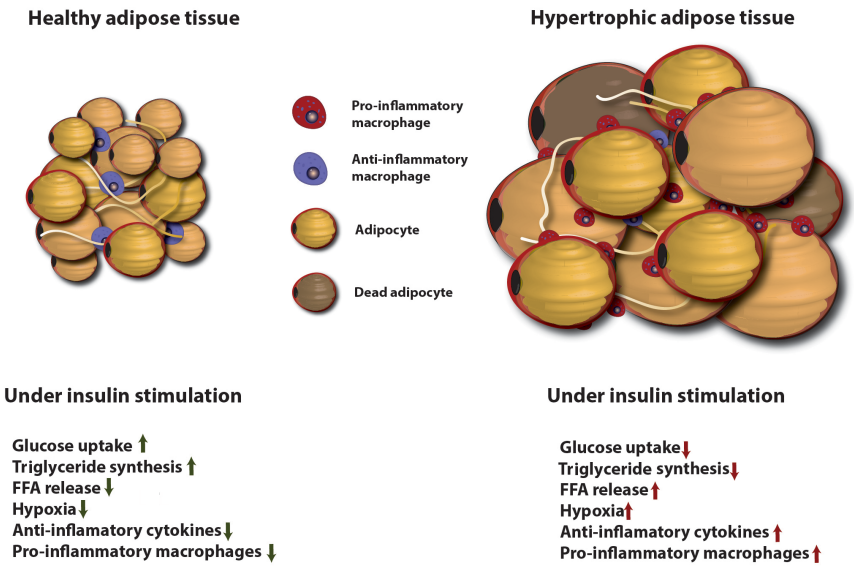

Figure 4. Insulin stimulation effects on healthy and hypertrophic adipose tissue. In healthy adipose tissue insulin stimulates glucose uptake and TG synthesis, induces FFA uptake and diminishes macrophage-mediated inflammation. Hypertrophic adipose tissue leads to a

diminished glucose uptake, TG synthesis and enhances FFA release, hypoxia and macrophage-mediated inflammation. FFA: free fatty acid.

5.3. Liver

In the liver, insulin does not only regulate glucose production/utilization but also affects lipid metabolism more broadly. When circulating glucose levels increase and insulin is secreted by pancreatic β-cells, insulin binding to liver INSR induces autophosphorylation of the receptor. Consequently, insulin receptor substrates (IRSs) are recruited and phosphorylated. In turn, IRSs activate PI3K, which phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2), generating phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3 then activates PDK1, which phosphorylates AKT. In addition, AKT is phosphorylated by mTORC2. Once AKT is fully activated, it participates in several downstream pathways that regulate multiple metabolic processes including glycogen synthesis, gluconeogenesis, glycolysis and lipid synthesis [169].

In physiological states, the combined action of glucagon and insulin allows the precise regulation of hepatic glucose output. While glucagon induces hepatic glucose production, insulin acts as a potent inhibitor of glucose production when its concentration in the blood is elevated [170]. The effect of insulin on hepatic glucose production is due to both direct and indirect mechanisms. However, the relative importance of each of these mechanisms remains unclear [171].

In addition to inducing glycogen synthesis, insulin also inhibits hepatic glucose production by activating FOXO1, resulting in a reduction of hepatic glucose release. FOXO1 is a transcription factor that belongs to a subclass of the forkhead family of transcription factors that possess a forkhead box-type DNA binding domain. FOXO1 recognizes a specific regulatory element termed the insulin response element (IRE) on the promoters of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, both of which play important roles in maintaining glucose level in states of starvation [172-174]. Thus, through inhibition of FOXO1, insulin promotes glucose storage as glycogen and inhibits glucose synthesis and hepatic glucose output [175] (Figure 5).

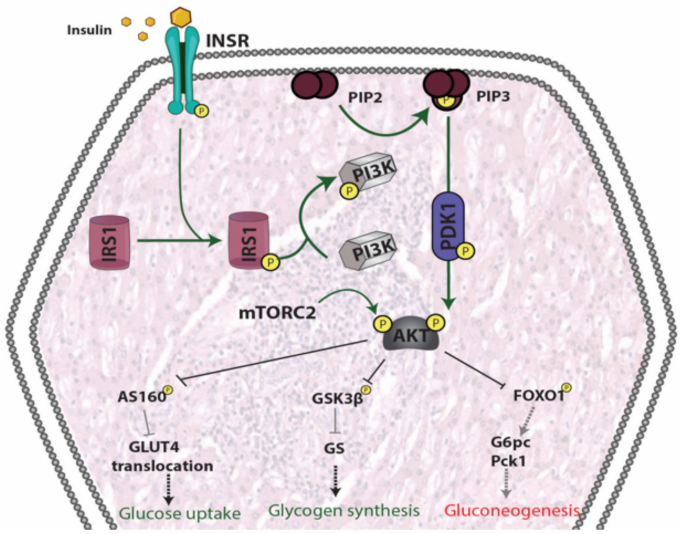

Figure 5. Signalling pathways involved in insulin signalling in hepatocytes. Binding of insulin to INSR induces IRSs recruitment and phosphorylation. Phosphorylated IRSs activate PI3K, generating PIP3 which activates PDK resulting in AKT phosphorylation. AKT fully activated by further mTORC2 phosphorylation and participates in several downstream pathways that regulate multiple metabolic processes including glycogen synthesis, gluconeogenesis, glycolysis and lipid synthesis. INSR: insulin receptor; PIP2: phosphatidylinositol (4,5)-bisphosphate; PIP3: phosphatidylinositol (3,4,5)-triphosphate; IRS1: insulin receptor substrate 1; PI3K: phosphoinositide 3 kinase; mTORC2: mammalian target of rapamycin complex 2; PDK1: Phosphoinositide-dependent kinase-1; AKT: protein kinase B; AS160: Akt substrate of 160 kDa; GLUT4: glucose transporter 4; GSk3β: Glycogen Synthase Kinase 3 Beta; GS: Glycogen synthase; FOXO1: Forkhead box protein O1; G6pc:Glucose 6 phosphate; Pck1: Phosphoenolpyruvate Carboxykinase 1.

Similar to the case in insulin sensitive tissues, in states of IR, physiologic levels of circulating insulin are insufficient to elicit the appropriate insulin response in hepatic cells [176]. In the liver, IR impairs glycogen synthesis, fails to suppress glucose production, enhances lipogenesis, and increases synthesis of proteins such as the proinflammatory CRP. In fact, the abnormal production of proinflammatory proteins such as adipocytokines and cytokines, combined with conditions such as oxidative stress, can lead to an inflammatory state responsible for altered insulin response by the liver [175].

6. T2DM outcomes/complications: cardiovascular risk

As described in the previous sections, T2DM is a multisystem disease with a strong correlation with CVD development [177]. T2DM leads to a two- to four-fold increase in the mortality rate of adults from heart disease and stroke and is associated with both micro- and macro-vascular complications, the latter consisting

of accelerated atherosclerosis leading to severe peripheral vascular disease, premature coronary artery disease (CAD) and increased risk of cerebrovascular diseases [178-180]. These factors lead to T2DM being considered a significant risk factor for CVD [181], likely through the involvement of several molecular mechanisms and pathological pathways. These include the role of IR in atherosclerosis, vascular function, oxidative stress, hypertension, macrophage accumulation and inflammation [182-185]. The following sections describe in detail the main factors implicated in cardiovascular risk outcomes from T2DM and the interactions between them (Figure 6).

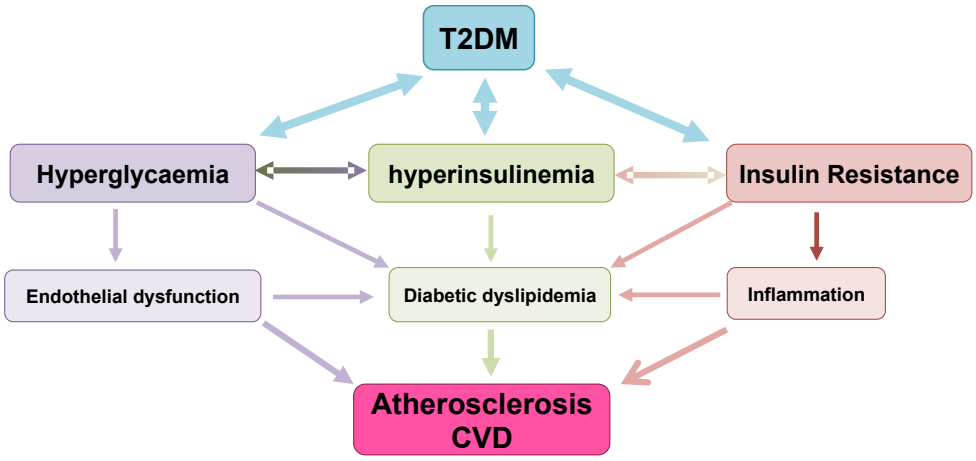

Figure 6. Factors implicated in cardiovascular risk outcomes from T2DM and the interactions between them. T2DM derived hyperglycaemia, hyperinsulinemia and IR causes endothelial dysfuntion, diabetic dyslipidemia and inflammation leading to CVD. The flowchart illustrates the multiple interactions among the implicated factors.

6.1. Diabetic Dyslipidaemia and Atherosclerosis Development

Dyslipidaemia is a common feature of T2DM, and increases the incidence of atherosclerosis and mortality of diabetic patients [186]. The hallmark of diabetic dyslipidaemia is a characteristic dyslipidaemic profile consisting of elevated TG, TG-rich lipoproteins (TRLs), small dense LDLs (sdLDL), and reduced HDL levels [187-189]. Although the pathophysiology of dyslipidaemia in T2DM is not completely characterized, several factors such as hyperglycaemia, insulin resistance, hyperinsulinemia, abnormalities in adipokines and adipocytokines have been implicated [190]. Epidemiological studies indicate that TG-rich lipoproteins and their remnants contribute to atherogenesis and CVD risk [191-195] and experimental studies indicated a connection between cholesterol deposition and inflammation as a result of TRLs entry into the artery wall [196, 197]. TRLs consist of a great variety of nascent and metabolically modified lipoprotein particles including intestine-derived

apoB48 (chylomicrons and chylomicron remnants) and liver-derived apoB100 (VLDL and its remnants). TRLs are highly heterogeneous in size, density, and apolipoprotein composition [198].

Under physiological conditions, chylomicrons deliver dietary lipids and lipid-biliary sources to the liver, which upon entering central circulation acquire apoE, apo-CI, apo-CII and apo-CIII from circulating HDL (Figure 7). Apo-CII, an activator of lipoprotein lipase (LPL), hydrolyzes TG within the chylomicron core, thereby releasing free fatty acids (FFAs). The progressive removal of TGs leads to formation of chylomicron remnants (CR), which upon apoE incorporation, are cleared by hepatocytes (Figure 7). This, together with the uptake of FFA generated by lipolysis in adipose tissue provides the major source of hepatic VLDL assembly and secretion. Once in the circulation, VLDL particles incorporate apo-CII and apoE from HDL allowing VLDL to be progressively lipolyzed leading to generation of smaller VLDL particles (VLDL1, VLDL2 and VLDL3), IDL, and finally LDL (Figure 7). Lipoprotein production, metabolism, and clearance are efficient processes. However, T2DM and IR are among the most important metabolic derangements in these process and they give rise to impaired metabolism and clearance of chylomicrons and VLDLs [199, 200].

6.1.1. Mechanisms leading to T2DM dyslipidaemia and atherosclerosis

Increased hepatic TG content present in T2DM patients leads to elevated hepatic production of VLDL and normal or slightly elevated LDL-C levels, most commonly sdLDLs enriched in TG [189, 201]. One of the primary abnormalities in IR is impaired adipose tissue fat storage, resulting from insulin's inability to inhibit hormone sensitive lipase (HSL). This results in constitutive FFA release from the intracellular TG stores of adipocytes. The released FFAs are taken up by hepatocytes, where they can be directed to the mitochondria and undergo β-oxidation; be re-assimilated into TG to assemble new VLDL particles; shifted to gluconeogenesis resulting in a worsening of hyperglycemia; or stored as TG leading to hepatic steatosis.

The dominant feature of diabetic dyslipidemia is the increased production rate of VLDL-apoB100 by the liver, mainly VLDL1, which is related to insulin sensitivity indices [202]. This highlights the role of insulin on VLDL assembly and secretion by hepatocytes [203]. Insulin plays a role in almost all the steps of VLDL assembly and secretion. It is known that insulin inhibits the transcription of Mttp, the gene coding for the microsomal transfer protein (MTP), the protein responsible of assembling TG with apoB100 [204]. MTP facilitates concerted lipid transfer and apoB100 folding as it enters the ER lumen and lipidation determines the amount of

the active pool of apoB100 [205]. Lipidation of apoB100 is a co-translational event and a rate-limiting step of apoB100 mRNA stability thus low availability of TG leads to apoB100 degradation. Addition of TG to apoB100 generates nascent VLDL particles that are transported to the GA by Sar2/COPII-containing vesicles. Within the GA, VLDL maturation occurs in a process promoted by the phospholipase D1 (PLD1) [203]. Therefore, in insulin resistant condition, MTP expression and activity is increased thus contributing to raise apoB100 lipidation and to its rescue from degradation. Indeed, IR leads to a loss of the acute insulin mediated inhibition of apoB100 secretion [205].

Availability of TGs within hepatocytes is important for VLDL synthesis [206] and the liver uses both *de novo* synthetized FAs and extra-hepatic FFAs as substrate for TG synthesis [207]. *De novo* lipogenesis occurs primarily in the fed state in which the expression of lipogenic genes is regulated by the sterol regulatory element binding protein (SREBP). The SREBP-1c isoform up-regulates almost all the enzymes involved in FA synthesis as well as enzymes that supply acetyl-CoA units and reducing equivalents to the pathway [208]. Insulin regulates SREBP-1c, which explains the lipogenic effect of chronic hyperinsulinemia [208]. FFAs derived from adipose tissue are also a major source of liver TGs and VLDL production. As mentioned above, T2DM is characterized by an increased production of FFAs by adipose tissue [207]. Therefore, in IR, an increase in TG lipolysis in adipose tissue and FFA influx serves as another source of lipid to the liver [207].

As mentioned above, in the IR milieu, insulin has reduced capacity to inhibit VLDL secretion in the fed state, the availability of apo-CII is lower and apo-CIII production is increased [209]. These events result in accumulation of VLDL remnants and IDL due to diminished clearance of TRLs by hepatocytes [210]. Additionally, hepatic IR also impairs LRP1 translocation from intracellular vesicles to the hepatocyte plasma membrane, which contributes to impaired clearance of TRLs [211, 212] (Figure 7).

In an effort to offload TG from remnant lipoproteins (VLDL 2+3 and IDL; RLPs), CETP is activated and promotes an exchange of TG out of RLPs and incorporates CE from HDL and LDL particles [213]. The TG-enriched HDL and LDL particles are better substrates for lipolysis by hepatic lipase leading to reduced levels of circulating HDL-C and an increase in sdLDL particles, which are more atherogenic [214]. The increased movement of CE into circulating TRLs mediated by enhanced activity of CETP [215] plays a key role in generating small dense HDL and LDL particles, the former being less atheroprotective and the latter more atherogenic [216]. TG enrichment of HDL enhances circulating HDL clearance [217].

The lower HDL concentration and modified composition of HDL have an important impact in diminishing the particle capacity of inducing cholesterol efflux from the cells, which is the first step in the reverse cholesterol transport (RCT) [218]. The impaired RCT activity has been associated with increased risk of CAD [219] and with flow mediated vasodilation in T2DM patients [220].

Atherosclerosis is accelerated by the increased permeability of sdLDL into the subendothelial space (Figure 7) [221, 222]. SdLDL particles are characterized by lower affinity for LDLR due to conformational rearrangements occurring in apoB100 as the particle decreases in volume and size [223]. Furthermore, sdLDL particles are more susceptible to oxidation and thus avidly scavenged by activated macrophages in the subendothelial space, giving rise to foam cells [224]. In addition, sdLDL particles show increased proteoglycan binding and facilitated entry into the arterial wall, increased arterial retention, and a longer half-life [225]. sdLDL particles are also more likely to be glycated, more resistant to breakdown, and more susceptible to oxidation by free radicals [226].

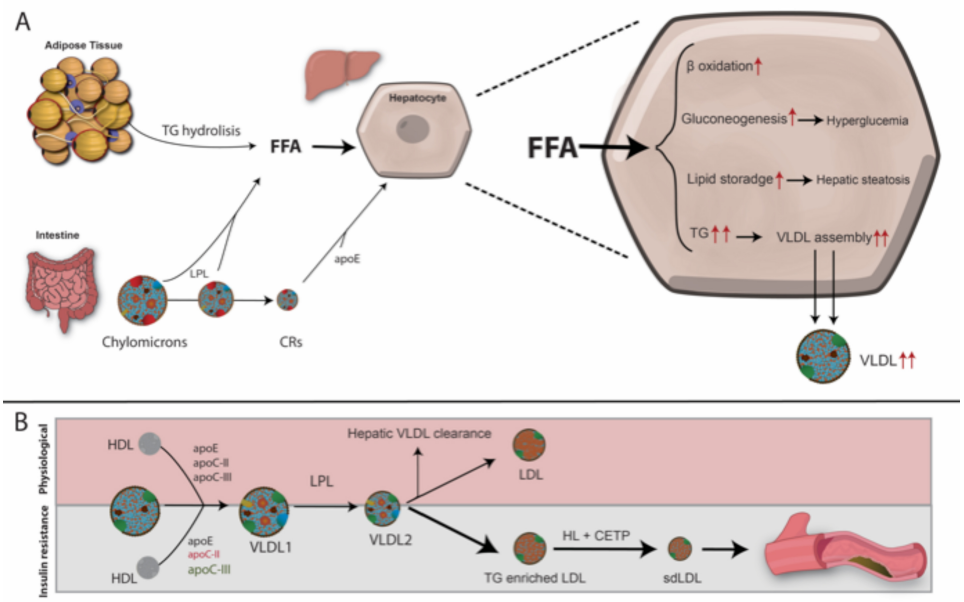

Figure 6. Diabetic dyslipidaemia: mechanisms leading to T2DM dyslipidaemia and lipoprotein clearance in physiological an IR conditions. A) IR leads to an impaired adipose tissue fat storage, resulting in constitutive FFA release from the intracellular TG stores of adipocytes. The released FFAs are taken up by hepatocytes, where they can be directed to the mitochondria and undergo β-oxidation; be re-assimilated into TG to assemble new VLDL particles; shifted to gluconeogenesis resulting in a worsening of hyperglycaemia; or stored as TG leading to hepatic steatosis. **B)** Under physiological conditions, VLDL particles incorporate apo-CII and apoE from HDL allowing VLDL to be progressively lipolyzed leading to generation of smaller VLDL particles (upper panel). T2DM and IR impair metabolism and clearance of chylomicrons and VLDLs. Activation of CETP promotes an exchange of TG out of RLPs and incorporates CE from HDL and LDL particles leading to reduced levels of circulating HDL-C and an increase in the more atherogenic sdLDL

particles (lower panel). TG: triglyceride; FFA: free fatty acid, LPL: lipoprotein Lipase; CR: chylomicron remnants; HL: hepatic lipase; CETP: Cholesteryl Ester Transfer Protein; ApoE: apolipoprotein E; ApoC-II: apolipoprotein CII; apoC-III: apolipoprotein CIII; VLDL: very low density lipoprotein; sdLDL: small dense lipoprotein.

There is an insulin response element in the gene for apoA-I, the primary apolipoprotein constituent of HDL particles [227, 228]. As the liver becomes more insulin resistant, less apoA-I is produced and there is less HDL biogenesis. Adipocytes express the ATP-binding membrane cassette transport protein A1 (ABCA1). IR downregulates expression of ABCA1 on the surface of adipocytes and reduces HDL formation by these cells [229-231]. Chylomicrons are enriched with apoA-I. IR reduces the release of this apoA-I into the serum by inhibiting LPL. In addition, within the milieu of IR of diabetes, HDL particle concentrations are not only quantitatively reduced, but also tend to be dysfunctional and unable to perform their primary functions, including reversal of cholesterol transport and inhibition of oxidative and inflammatory phenomena [232].

This highly atherogenic lipid profile is a pivotal contributor to atherogenic dyslipidaemia, which is causally linked to the development and progression of atherosclerotic CV disease (ASCVD) [233, 234]. The relationship between atherogenic dyslipidaemia and ASCVD is supported by prospective longitudinal cohorts, clinical evidence and genetic linkage studies. As an example, the best predictor of risk of myocardial infarction at the population level in the INTERHEART study was the apolipoprotein (apo) B100/apoA-I ratio, reflecting the correlation between all apoB (atherogenic lipoproteins) and HDL (representing classically anti-atherogenic particles) [235]. The relationship between atherogenic dyslipidaemia and ASCVD has also been demonstrated in prospective randomised clinical trials using statins. Even when treated with statins, patients with the atherogenic dyslipidaemia phenotype have a higher risk of CV events than those without AD [236, 237].

Diabetic dyslipidaemia acts in concert with other metabolic and vascular abnormalities to further compound vascular risk. Chronic hyperglycaemia induces endothelial dysfunction through a variety of mechanisms such as by reducing vasodilation, increasing vasoconstriction, increasing exposure to free radicals and impairing endothelial cell function, with a net effect of facilitating pro-atherogenic conditions [238]. Increased activity of the renin-angiotensin axis has also been found to further increase oxidative stress [239].

6.2. Impaired endothelial function and atherosclerosis development

Endothelium plays an important role in the regulation of vascular tone and structure through a balanced release of endothelial-derived relaxing and contracting factors. This balance is altered in T2DM leading to alteration of the physicochemical properties of the vascular wall via endothelial dysfunction, oxidative stress, platelet hyperreactivity, and inflammation [240, 241]. These abnormalities lead to enhanced vasoconstriction, development of atherosclerosis, and favoured thrombus formation [179, 180].

6.2.1. Mechanisms leading to endothelial dysfunction in T2DM

Vascular endothelial cells are particularly susceptible to developing intracellular hyperglycaemia because glucose diffuses passively through their plasma membrane. In T2DM, the excess of glucose can be metabolized in the sorbitol pathway to sorbitol and fructose by aldose reductase, which activates the aldose reductase secondary metabolic pathway, with concomitant oxidation of NADPH to $NADP^+$ and reduction of NAD^+ to NADH. NADPH depletion and an increased NADH/NAD^+ cytosolic ratio leads to a change in redox potential that accelerates glycolysis and increases *de novo* synthesis of DAG [242]. As a result, protein kinase C (PKC) is activated, nitric oxide (NO) is reduced. These effects cause vascular permeability and increase contractility. Simultaneously, the increased NADH/NAD^+ ratio also results in higher production of O_2^-, LDL oxidation, cytotoxic effects on endothelial cells and reduced NO availability, leading to endothelial dysfunction [241, 242].

The overproduction of aldoses by the sorbitol pathway promotes protein glycosylation that yields the formation of the stable Amadori products (such as glycosylated hemoglobin) and AGEs. AGEs are associated with several molecules that augment oxidant activity and consequently the production of ROS, which increase oxidative stress and prevent the release of NO, resulting in vascular lesions. AGEs may also reduce endothelium-derived NO bioavailability and activity, further compromising vascular activity [243]. In addition, AGEs can trigger an inflammatory and pro-coagulant state and can cause endothelial activation through induction of receptor-mediated gene transcription. AGE binding to the RAGE-receptor, nuclear transcription factor NF-κB [242, 243] is activated leading to transcription of endothelin-1, VCAM-1, ICAM-1, E-selectin, thrombomodulin, TF, vascular endothelial growth factor (VEGF), IL-1, IL-6, and TNF-α [242, 244]. Increased expression of inflammatory and adhesion molecules

amplifies the inflammatory response and aggravates diabetic vascular complications. These pro-inflammatory cytokines stimulate expression and release of pro-coagulant molecules and inhibit the expression of anti-coagulant molecules by endothelial cells [245]. This leads to a pro-coagulant state in the surface of the endothelium and increases growth factor production resulting in a thickening of the basement membrane, thus favouring protein and lipid deposition and impairing vasodilation [242, 246].

6.2.2. Endothelial dysfunction in T2DM and atherosclerosis development

Hyperglycaemia-associated vascular injury, oxidative stress, inflammation and altered hemodynamic balance may initiate atherosclerosis development and formation of arterial thrombus [247]. At early stages of atherosclerosis, circulating LDL binds to matrix proteoglycans where their oxidation is favoured, giving rise to highly pro-inflammatory particles that stimulate the expression of several adhesion molecules by endothelial cells [242, 248]. This promotes selective binding of leukocytes and their transmigration into vascular wall along with recruitment and activation of circulating monocytes that differentiate into macrophages. The excess of oxidized LDL is removed by macrophages by a non-regulated mechanism that leads to formation of foam cells and onset of fatty streaks. Mononuclear cells release inflammatory cytokines, including IL-1 and IL-6, promoting the recruitment of additional inflammatory cells. As a result, smooth muscle cells proliferate and migrate into the intima where they synthesize and secrete extracellular matrix facilitating fibroatheroma formation [242]. As the process progresses, if a fissure or ulceration of the plaque occurs, highly thrombogenic substances are exposed leading to adhesion and aggregation of platelets, which promotes thrombus formation [249]. In addition, platelets can also release pro-inflammatory cytokines and growth factors promoting monocyte recruitment to atherosclerotic plaques, which stimulates fibroblasts and smooth muscle cell proliferation thus accelerating atherosclerotic process.

6.3. Diabetes-Associated Chronic Inflammation and atherosclerosis progression

A critical component of T2DM is a chronic low-grade inflammatory state, referred to as "metaflammation" [250]. This chronic condition involves the same cellular and molecular players of acute inflammatory responses, and has been suggested as an underlying cause of the progression of atherosclerosis in T2DM. Hyperglycaemia can increase circulating cytokines that can lead to chronic inflammation in T2DM [250]. Among them, patients with T2DM have higher levels of IL-1b, IL-6, IL-8, MCP-1, and other major cytokines in both monocytes and macrophages [130]. The underlying mechanisms involved in this process are

ROS-mediated activation of p38 and other proinflammatory kinases, upregulation of NF-kB induction, oxidative stress, and activation of the AGE-RAGE pathway [129, 130]. In addition, exposure to high glucose levels impairs phagocytic activity of macrophages, which partially explains the increased incidence of chronic infection among T2DM patients [251]. Indeed, T2DM is associated with increased activity of the inflammasome, upregulation of the nucleotide-binding oligomerization domain-like receptor 3 (NLRP3), increased levels of IL-1β and IL-18 [252-254]. These events trigger neutrophil extracellular trap activation, or NETosis, a characteristic cell death of macrophages causing chronic inflammation [255]. High levels of these markers have been found in T2DM patients [256], which are enhanced in hyperglycaemic conditions [257].

6.4. Adipokine balance and CVD risk

Adipose tissue dysfunction as a result of T2DM can result in an imbalance between pro-inflammatory and anti-inflammatory adipokines, and is one of the mechanisms of T2DM complications. Several studies indicate that adipokines are related to IR, and can result in endothelial dysfunction, and pro-inflammatory and pro-atherogenic states [258, 259].

Adiponectin is a well-described insulin-sensitizing hormone and its expression and circulating levels are inversely proportional to the extent of adiposity. Adiponectin has insulin-sensitizing properties [260, 261]. Adiponectin acts through ADIPOR1 and ADIPOR2 receptors [262] and the peroxisome proliferator-activated receptor α (PPARα) pathway, leading to decreased hepatic gluconeogenesis, increased liver and skeletal muscle fatty acid oxidation, increased glucose uptake in skeletal muscle and white adipose tissue, and decreased white adipose tissue inflammation [263]. In addition, adiponectin ameliorates β-cell death by neutralizing inflammatory and lipotoxic ceramides and DAGs [264] and shows strong anti-inflammatory effects on other cell types such as macrophages and fibrogenic cells [263, 265, 266]. Low concentrations of adiponectin have been found in T2DM patients and are correlated with increased risk of developing premature arteriosclerosis, and are thus considered an additional CVD risk factor [267]. Notably, adiponectin deficiency is associated with coronary artery disease, hypertension, endothelial dysfunction and greater carotid intima-media thickness [268-271]. Low concentrations of adiponectin leads to an increased expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, promotes differentiation of macrophages into foam cells and enhances the proliferation and migration of smooth muscle cells [272].

Omentin is an adipokine secreted from white adipose tissue and is involved in glucose homeostasis [273, 274]. Omentin circulates in the blood [275, 276], and is associated with reduced levels in T2DM patients [277, 278]. In vitro studies have shown that omentin enhances insulin-stimulated glucose uptake in human adipocytes by AKT signalling pathway activation [273]. In humans, an inverse correlation between omentin levels and IR is seen, both at the protein and mRNA levels [277, 279, 280]. Additional studies show that omentin has anti-inflammatory properties, diminishes cytokine expression [281, 282], and is negatively associated with systemic inflammatory markers such as TNF and IL-6 [283].

Vaspin (visceral adipose tissue-derived serine protease inhibitor) is an adipokine that inhibits proteases responsible for IR and protects against atherosclerosis and plaque development [284, 285]. It has been shown that T2DM patients have higher serum vaspin levels than healthy controls. Higher vaspin levels are associated with a 1.7-fold increased risk of CVD [70]. High vaspin is also associated with increased severity of coronary artery disease [286].

7. Conclusions

The importance of research in the fields of glucose homeostasis, insulin and diabetes has not faded. In fact, due to rapid globalization and the normalization of a sedentary lifestyle, along with increased obesity, diabetes and their consequent co-morbidities, research in this topic must continue to grow. Understanding the mechanisms implicated in every step in the development and complications of T2DM is crucial in order to prevent, control, treat or revert the pathophysiology of T2DM its complications. Although quality outcomes for patients are optimised by early detection of T2DM through screening and intensive patient-centred management, research efforts are needed to define causative factors accounting for correlations among different demographic subsets and the corresponding variable risks for T2DM as well as the drivers of increased risk in individuals of low socioeconomic status. Being the pathophysiology and underlying mechanisms of T2DM increasingly understood, precision medicine should be implemented and treatments individualised and targeted appropriately with the help of molecular genetic tools by identifying specific variants contributing to disease development as well as by searching biomarkers to assess progression and response to therapeutic interventions. Aditional research is needed to determine a direct causal role of the intestinal microbiota in pathogenesis of T2DM and response to therapies needs to be determined.

Taking everything in this review into consideration, it is clear that there is still a long way until we fully understand each of the many stakeholders in glucose homeostasis.

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Statin Treatment-Induced Development of Type 2 Diabetes: From Clinical Evidence to Mechanistic Insights

1. Introduction

Statins are a guideline-directed, first line therapy for prevention of primary and secondary cardiovascular disease (CVD), which is the leading cause of mortality worldwide [1, 2]. Although the principal mechanism of action of statins is inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme-A (HMG-CoA) reductase, statins have been implicated in several other beneficial pleiotropic effects including improving endothelial function, stabilization of atherosclerotic plaques, and anti-inflammatory activities [3]. Despite the safety and relative tolerability of statins, observational studies [4-8], clinical trials [9, 10] and meta-analyses [11-16] have found that statins can increase the risk of new-onset type 2 diabetes mellitus (T2DM). These studies implicated statins in negatively impacting insulin sensitivity, decreasing secretion by pancreatic β-cells and increasing insulin resistance [11, 17, 18]. While the lipid-lowering mechanism of statins is relatively well understood, the mechanisms underlying statin-induced T2DM development seem to be multifactorial and remain unclear. Among experimental studies, multiple works have indicated that statins diminish pancreatic β-cell function via Ca^{2+} signalling pathways impairment [19, 20], compromise insulin signalling, and down-regulate the insulin-responsive glucose transporter 4 (GLUT-4) [21, 22]. In addition, it has also been described that statins impact on epigenetics may also contribute to statin-induced T2DM via differential expression of microRNAs [23].

This review focuses on the evidence and mechanisms by which statin therapy is associated with development of T2DM. Here, we will describe the existing data from clinical studies as well as experimental results that shed some light on the mechanisms of this association.

2. Primary action of statins: cholesterol biosynthetic pathway

Statins are reversible and competitive inhibitors of HMG-CoA reductase, which is the rate-determining enzyme in the cholesterol biosynthetic pathway [24]. The HMG-like portion of statins, which is a modified 3,5-dihydroxyglutaric acid moiety, is structurally similar to HMG-CoA and causes the inhibition of

HMG-CoA reduction reactions [25]. Through this mechanism, the mevalonate pathway is inhibited along with a consequent decrease in downstream products and cholesterol synthesis (Figure 1A). In addition, this statin-mediated decrease in intracellular cholesterol content leads to up-regulation of the LDL receptor (LDLR) in the liver and peripheral tissues, resulting in decreased blood LDL cholesterol (LDL-C) [26]. LDLR is the primary route by which LDL-C is removed from circulation, and its synthesis has been shown to be inversely correlated to the amount of cholesterol synthesized by a cell [27]. Through the action of statins, the cellular cholesterol concentration decreases, stimulating production of more LDLR and promoting LDL-C removal from the bloodstream, ultimately reducing CVD risk [27].

Statins are classified according to their hydrophobicity into hydrophilic statins (pravastatin and rosuvastatin) and lipophilic statins (atorvastatin, cerivastatin, fluvastatin, lovastatin, pitavastatin, and simvastatin) [28, 29]. The solubility and pharmacological properties of statins are determined by the substituents on the ring attached to the active moiety [29]. Hydrophilicity originates from polar substituents added to the active site while the addition of nonpolar substituents leads to lipophilicity [25, 29] (Figure 1B).

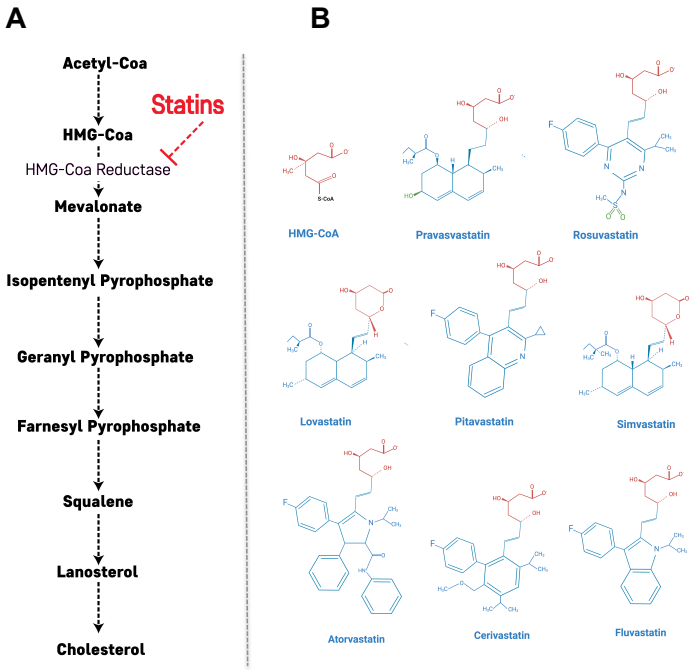

Figure 1: Statin-induced inhibition of the mevalonate pathway and structure of statins. (A) Inhibition of HMG-CoA reductase significantly blocks the production of mevalonate, a necessary precursor for cholesterol synthesis. Mevalonate is the building block for a variety of other compounds. **(B)** Structural formulas of statins and HMG-CoA. The HMG-like

moiety (in red) is conserved in all statins. The polar substituents responsible of pravastatin and rosuvastatin are coloured in green.

Although the target of both types of statins is HMG-CoA reductase, the inhibitory mechanisms are distinct. Hydrophilic statins target the liver more efficiently because their uptake is carrier-mediated, while lipophilic statins passively diffuse through the hepatocellular membrane and similarly are also able to diffuse in extrahepatic tissues, thus showing reduced hepatoselectivity [29, 30]. Their diffuse influence on extrahepatic tissues may explain the higher incidence of adverse effects observed with lipophilic statins. The notable exception to this is rosuvastatin, which is a hydrophilic statin but has a similar activity profile to lipophilic statins [31].

3. Beneficial effects of statins on diabetic complication and/or inflammation in T2DM

There are many factors that contribute to the development of atherosclerotic cardiovascular disease, the main mortality cause in T2DM patients. These include dyslipidemia, increased oxidative stress, enhanced protein glycation or chronic inflammatory state all of them worsen in T2DM [32]. Statins are the gold standard treatment for the prevention and management of cardiovascular disease and their use in T2DM patients is recommended by The American Diabetes Association 2019 guidelines [33]. In addition to the reduction of cholesterol levels and dyslipidemia improvement by reducing lipoprotein levels in plasma, the pleiotropic effects of statins reduce high sensitive C-reactive protein and other pro-inflammatory markers [34], improve endothelial function and reduce oxidative stress [35] which together contribute to a significant CVD reduction in T2DM patients.

Several clinical trials have pointed out the beneficial effects of statins in diabetic patients [36]. The Collaborative Atorvastatin Diabetes Study (CARDS) showed nearly 40% reduction in relative risk of cardiovascular events in diabetic patients aged 45-70 years old with high cholesterol levels and treated with atorvastatin during 4 years [37]. A meta-analysis of 14 randomized trials including more than 18,000 patients confirmed the beneficial effects of statins in diabetic patients showing a 21% reduction in major vascular events per mmol/L LDL-C reduction [38]. Further studies, confirmed the benefits of statin treatment in diabetic patients independently of LDL-C baseline [39].

Unfortunately, in some cases, statin treatment leads to adverse effects such as the decreased insulin sensitivity shown by atorvastatin, simvastatin and

rosuvastatin [40]. For atorvastatin and simvastatin, one proposed explanation is that the higher diffusion rate of lipophilic statins to the intracellular space can interfere with cellular processes, leading to decreased intracellular insulin secretion in response to glucose [41]. For rosuvastatin, despite its hydrophilicity, the higher affinity and efficient transport of rosuvastatin into cells which can underlie it effects on insulin sensitivity [29].

4. Statin therapy and risk of developing T2DM: Observational studies, clinical trials and meta-analysis.

Statins, discovered in the early 70s and commercially available in the mid-80s, have well-characterized benefits in terms of lowering LDL-C and cardiovascular risk reduction. However, 20 years after becoming commonly prescribed, findings from observational studies showed an increased T2DM risk upon statin administration in several populations. Despite the considerable variability among these studies and the statin administered, hazard ratios (HR) were statistically significant ranging from 1.19–1.57, after follow-up durations of 3 to 6 years [4, 6, 7]. Observational studies carried out in Canada, Taiwan and Ireland examining the association between statin administration and T2DM development, showed 10–22%, 15% and 20 % increases in the risk of T2DM associated with statin therapy, respectively [42-44]. Later on, the effects of statin treatment on the risk of T2DM and hyperglycaemia deterioration were assessed in the Metabolic Syndrome in Men (METSIM) study cohort which found that statin therapy was associated with a 46% increased risk of T2DM along with worsening of hyperglycaemia [45]. In addition, the study found statin use to be associated with a 24% reduction in insulin sensitivity and a 12% decrease in β-cell count compared to individuals not taking statin therapy [45]. Notably, treatment with both simvastatin and atorvastatin was associated with reductions in insulin sensitivity and secretion in a dose-dependent manner [5].

Collectively, statin randomized control trials (RCT) were designed and, large, long-term, double blind, placebo-controlled studies were conducted to evaluate the effects of statins in a variety of clinical situations. Although most statin RCTs, including the largest statin RCT trial, were designed primarily to evaluate efficacy in a variety of clinical situations, several RCTs also evaluated the relationship between stain treatment and T2DM development. Among them, the JUPITER, SEARCH and Cholesterol Treatment Trialists trials were un-confounded regarding the intervention and aimed to recruit at least 1000 participants with treatment duration of at least 2 years.

The Justification for the Use of Statin in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial showed a small but significant increase in diabetes incidence rates in patients who received statin treatment when compared to placebo over a median of 1.9 years (absolute increase of 0.6%; relative increase of 24%; P=0.01) [46]. Subsequent meta-analyses of the available randomized controlled trials showed that standard statin dose regimens were associated with a proportional increase of about 10% in reported T2DM. According to the results of the JUPITER trial, treatment with high statin concentrations resulted in a further increase by 10% [16, 47]. In addition, a post-hoc analysis of the JUPITER trial showed that participants with one or more major diabetes risk factor were at higher risk of developing T2DM than were those without a major risk factor. Of note, however, benefits of statin therapy exceeded the diabetes hazard even in participants at high risk of developing diabetes [10]. In patients who had risk factors for diabetes (eg, elevated body-mass index or HbA1c, or impaired fasting glucose), the excess of T2DM diagnoses appeared soon after the start of statin therapy, and did not appear to get larger as treatment continued [10, 48, 49].

Another RCT carried out by the Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) Collaborative Group found that simvastatin treatment was associated with a dose-dependent increased risk of diabetes, with diabetes found in 11.6% participants who received 80 mg simvastatin compared to 10.9% in participants receiving 20 mg simvastatin [50]. Collectively, the findings of multiple RCTs indicate that statin therapy may lead to the development of diabetes [51]. Although results from individual RCTs have shown substantial variability in the association between statin therapy and incident diabetes, they generated a large amount of data that could be more powerfully analysed in meta-analysis. For the most relevant insights, meta-analyses that compile data from several RCTs represent a powerful tool for understanding the impacts of statin therapy.

Consistent with the aforementioned RCTs, The Cholesterol Treatment Trialists' Collaborators meta-analysis (CTT) showed that LDL-C reduction is associated with a 21% reduction in the incidence of any major vascular event in both patients with or without diabetes [38]. In the study, randomised trials were eligible for inclusion if: (i) the main effect of at least one of the trial interventions was to modify lipid levels; (ii) the trial was unconfounded with respect to this intervention (ie, no other differences in modification of risk factors between the relevant treatment groups were intended); and (iii) the trial aimed to recruit 1000 or more participants with

treatment duration of at least 2 years [38]. The study assessed possible variation in the proportional effects of allocation to a statin in different circumstances only for major vascular events. Trial participants were considered to have diabetes if they had a recorded history of diabetes at randomisation, and subdivision of diabetes subtypes was done according to the definitions used in the individual trials [38]. The study showed that statins are directly correlated with an increased risk of developing T2DM. Interestingly, multiple meta-analyses have found that the risk of statin-associated T2DM is higher in participants taking higher doses when compared to patients taking lower doses [15, 16, 47]. Accordingly, the data obtained indicated an excess risk ranging from 9% to 13%, with the highest risk of T2DM seen in patients taking high-intensity statin therapy [13, 15, 16, 43, 47, 52]. Specifically, a recent meta-analysis showed that atorvastatin 80 mg is associated with the highest risk of T2DM, followed by rosuvastatin and simvastatin 80 mg, indicating that statins have varying effects on the risk of T2DM [53]. Overall, meta-analysis studies found a clear association between diabetes and statins across multiple statins, indicating that the diabetogenic property of statins is a class effect. Most importantly, despite the increase of T2DM, it is important to emphasize that the benefits of statin administration in reducing myocardial infarction, stroke, and cardiovascular deaths in high CVD risk patients are enough to warrant statin treatment, although T2DM prevention and screening is important to take into consideration.

As listed above, clinical trials, meta-analyses and observational studies highlight that patients who received statin treatment had a 10-12% increase in T2DM risk [17]. However, the risk is even higher in patients receiving high-intensity statin therapy and among patients with pre-existing risk factors for diabetes. Recent studies indicate a clear correlation between statin type and treatment intensity with T2DM development. Specifically, pravastatin 40 mg/day treatment has been associated the lowest risk of T2DM, while rosuvastatin 20 mg/day and atorvastatin 80 mg/day treatment are associated with increased risks of T2DM. Between rosuvastatin and atorvastatin, rosuvastatin has been associated with the higher risk of T2DM [14].

However, even if statin type and treatment intensity clearly correlate with T2DM development, individual´s risk factors should not be overlooked. Development of T2DM during statin treatment is more frequent among individuals with pre-existing risk factors, including increased adiposity, predisposing dietary patterns, sedentary lifestyle, psychosocial factors and

previous medical history [54], as well as age and gender [55]. In fact, for patients with none to 1 risk factor, the incidence of T2DM is similar between those receiving high dose and moderate dose of statins (3.22% and 3.35%, respectively). Conversely, for patients with 2 to 4 risk factors the incidence is 14.3% in the high dose group and 11.9% in the moderate dose group [17].

5. Proposed mechanisms for T2DM development induced by statins

Overall, the mechanisms by which statin treatment induces T2DM are not fully- understood, but both on-target and off-target effects may be involved. Among these, inhibition of the mevalonate pathway results in a reduction in several cellular biosynthetic pathways including those involved in glucose homeostasis [56]. Over time, chronic statin treatment increases gluconeogenesis by upregulating gene expression of key enzymes that increase glucose production in the liver [57]. In addition, it has been shown that statins can impair the insulin signalling pathway as well as downregulate the GLUT-4 transporter, which is responsible for the uptake of glucose in peripheral cells [22, 58, 59]. Statins can also induce changes in circulating free fatty acids (FFA), changes in hormones such as adiponectin and leptin, impairment of β-cell function, β-cell cell damage, and adipocyte maturation/differentiation [17, 56, 60]. Additional mechanisms involving epigenetic regulation mediated by specific microRNAs have also being involved in the reduction of insulin secretion [56]. These complex pathophysiologic molecular mechanisms of statin-induced T2DM, summarized in Figure 2, are described in more detail in the following sections.

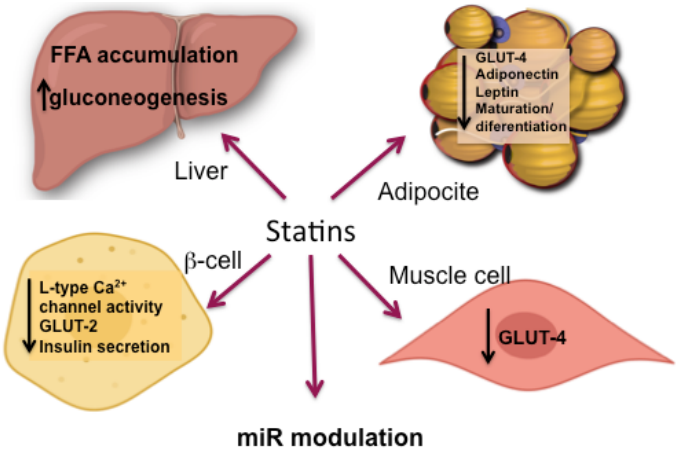

Figure 2: Principal mechanisms for T2DM development induced by statins.

5.1. Dysfunctional effects caused by statins in pancreatic β-cell

Insulin secretion from pancreatic β-cells is initiated by glucose-induced Ca^{2+} entry controlled by voltage-gated Ca^{2+} channels (Figure 3) [61]. Therefore, maintenance of intracellular Ca^{2+} homeostasis is tightly regulated in order to ensure proper insulin secretion and maintain the integrity of the β-cell physiology [62]. Briefly, glucose uptake activates glycolysis in β-cell thus elevating the $[ATP]/[ADP]_i$ ratio [63]. This acts as a signal that closes K_{ATP} channels and depolarizes the plasma membrane, with subsequent activation of voltage-dependent Ca^{2+} channels, entry of extracellular Ca^{2+}, and finally insulin exocytosis (Figure 3) [64]. ATP sensitivity of the K_{ATP} channels is modulated by several effectors including PIP_2 and acyl CoAs [65, 66]. Conversely, a decrease in the metabolic signal causes reopening of K_{ATP} channels and suppresses the electrical trigger for insulin secretion, thereby providing feedback regulation of insulin secretion [67]. In addition, ATP and ADP can act as autocrine activators of β-cell purinergic receptors because they are also within insulin exocytosis granules [68]. Indeed, inhibition of both P2X and P2Y purinergic receptors causes a reduction in glucose-induced insulin secretion [69-72] (Figure 3).

To date, the relationship between statin-mediated inhibition of cholesterol synthesis and impaired L-type Ca^{2+} channel activity remains unclear. However, *in vitro* studies have indicated that simvastatin can directly inhibit L-type Ca^{2+} channels in rat pancreatic islet β-cells [41]. Specifically, because simvastatin was found to immediately inhibit channel activity, it has been suggested that there is a direct interaction between simvastatin and the channel. In contrast, pravastatin lacks L-type Ca^{2+} channels inhibition, possibly because of its lipophilicity [41]. Alternatively, other authors have suggested that the long-term cholesterol reduction caused by statins can lead to incorrect sorting of membrane lipid-raft bound proteins or conformational changes of the Ca^{2+} channel subunits [73]. More recently, it has been suggested that statins can reduce membrane potential by inhibiting mitochondrial complex II activity, which causes oxidative stress [74]. These off-target effects of statins have been very recently corroborated by Curry et al. [75] in experiments showing that simvastatin impairs β-cell function by at least two mechanisms: (1) via direct inhibition of K_{ATP} channels in a mitochondria-independent manner and (2) via interference with mitochondrial respiration, thus decreasing cytosolic ATP levels and inhibiting metabolic upregulation of L-type Ca^{2+} channels [75].

As described before, insulin is secreted by β-cells in response to glucose uptake through GLUT receptors (primarily GLUT-1 to 4), with GLUT-2 being the predominant isoform in β-cells [76, 77]. GLUT-2 represents a high-affinity and low-capacity glucose transporter [78]. It has been shown that treatment of β-cells with atorvastatin and pravastatin inhibited GLUT-2 expression in a concentration-dependent manner [58]. However, rosuvastatin and pitavastatin showed a slight increase in GLUT-2 expression [58]. In addition to this, it has also been observed in mouse pancreatic β-cell line MIN6 cells that simvastatin treatment diminishes GLUT-2 mRNA and protein expression via a dose-dependent reduction of ATP production [79]. Another mechanism through which statins may interfere with glucose metabolism is the statin-mediated LDLR upregulation that increases cholesterol uptake in the β-cell leading to reduced mRNA and protein expression of GLUT-2, consequently limiting glucose uptake [19, 80].

The direct inhibition the mevalonate pathway by statins reduces the intracellular concentration of isoprenoids, the final products of the pathway. Isoprenoids are essential for G protein posttranslational modification, which is important for insulin granule exocytosis [17]. Interestingly, it has been shown that the glucose-induced insulin secretion by lovastatin in normal rat islets is recued by co-incubation with mevalonate [81]. The adverse effects of statins are summarized in Figure 3.

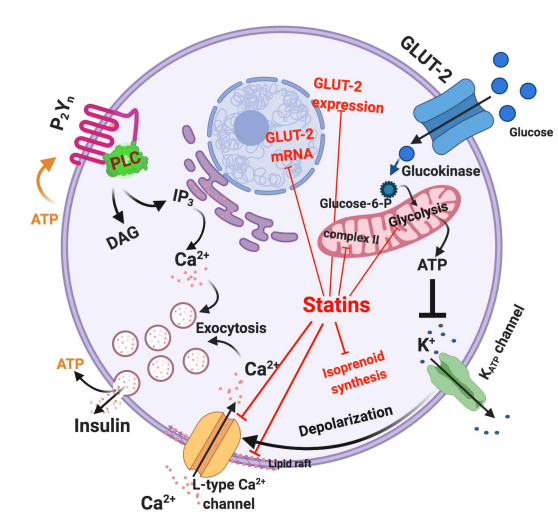

Figure 3: Intracellular actions of statins in β-cells. Red lines indicate the mechanisms affected by statins.

5.2. Statin induced IR

The binding of insulin to the insulin receptor (INSR) triggers insulin signalling with the physiologic objective of normalizing high blood glucose levels [82]. Insulin binding induces structural rearrangements in the INSR leading to auto-phosphorylation of tyrosine residues. The downstream events that follow INSR activation include recruitment of several adaptor proteins, facilitating a suitable binding site for insulin receptor substrates (IRSs) [82] that once phosphorylated, trigger several downstream signals [83]. Among them, IRS-1 is phosphorylated and activates different kinases such as Akt, PKC, SIK2, S6K1, mTOR, ERK1/2, and ROCK1 [83, 84]. IRS-1 activates PI3K which in turn, catalyses the conversion of PIP_2 to PIP_3 which activates Akt, among other targets [85]. Akt activation leads to glucose uptake by facilitating GLUT-4 translocation to the plasma membrane [86]. GLUT-4 is an insulin-dependent glucose transporter primarily expressed in adipose tissue, cardiomyocytes and skeletal muscle cells [87].

Akt also promotes glycogen synthesis by inhibiting glycogen synthase [88]. In addition, insulin also triggers several IRS-independent signalling pathways, among them those mediated by heterotrimeric G protein and SOS-growth factor factor [89].

Several disturbances in insulin signal transduction mediated by statin treatment have been described in different organs and tissues leading to a pathologic insulin resistance. This condition is characterized by a pathophysiologic failure to proper respond to normal circulatory levels of insulin in insulin-sensitive cells, such as adipocytes, skeletal muscle cells and hepatocytes [90]. Below, we review some proposed mechanisms through which statins interfere with the insulin response in each of these tissues.

5.2.1. Adipose tissue

Recently, evidence that statin treatment impairs the insulin signal transduction process in adipocytes, including INSR, GLUT-4, Akt, some small GTP-binding proteins (G-proteins) and caveolae integrity has been demonstrated. Multiple studies have shown that atorvastatin and lovastatin reduce GLUT-4 expression at the plasma membrane in 3T3L1 adipocytes [91, 92] and a similar effect has been described with atorvastatin in mouse-white adipose tissue, thus impairing glucose tolerance [22]. The statin-induced decrease in GLUT-4 translocation to the plasma

membrane has been attributed to inhibition of isoprenoid synthesis [22]. In fact, isoprenylation is essential for the correct functioning of several proteins involved in the GLUT-4 translocation process. As previously described, isoprenylation is impaired due to statin-induced inhibition of the mevalonate pathway. In one illustrative example, it has been described that atorvastatin disrupts plasma membrane colocalization of Rab-4 and RhoA through inhibition of geranylgeranyl pyrophosphate synthesis. Rab-4 and RhoA are isoprenoid-dependent proteins which are involved in the insulin-induced translocation of GLUT-4, thus their atorvastatin-mediated dysfunction may disturb overall insulin signalling [93]. RhoA modulates the activities of IRS-1 in 3T3-L1 adipocytes, in which atorvastatin has been shown to reduce the active membrane fraction of both RhoA and Rab4 [93].

Statins also disrupt the formation of caveolae, plasma membrane microdomains at which GLUT-4 anchors after insulin-stimulated translocation [94]. Furthermore, it has been shown that INSR is highly enriched in adipocyte caveolae [95, 96] through interaction of its beta subunit with Caveolin 1 (Cav-1), one of the essential constituents of caveolae. Thus, it has been suggested that Cav-1 stabilizes the insulin receptor at the protein level, acting as a molecular chaperone necessary for proper insulin signalling in adipocytes *in vivo* [97].

The cholesterol dependence of caveolae in order to acquire their characteristic shape is well known [98]. Caveolae dynamics are tightly regulated by caveolin and cavin proteins. Importantly, cholesterol depletion can disrupt this regulation. The adverse effects of statins within caveolae seem to be partially mediated by the stoichiometric binding of Cav-1 to cholesterol [99] and by cavins, which show an essential cholesterol-dependence for defining caveolar structure [98]. Statin-induced cholesterol depletion leads to proteasomal degradation of cavin-2 and relocation of cavin-1 to the cytosol leading to caveolae disruption [98]. Moreover, statin disruption of caveolar formation seems to reduce secretion of high molecular weight oligomers of adiponectin [100], a mechanism that reduces insulin sensitivity.
Interestingly, statins also affect the preadipocyte to adipocyte differentiation process. The mechanism underlying this effect is likely the lack of secretion of insulin-sensitizing hormones. It has been shown that this is caused by a decrease in the expression of PPARγ (peroxisome proliferator–activated receptor γ) and C/EBP (CCAAT/enhancer-binding protein) transcription factors [56]. The adverse effects of statins are summarized in Figure 4.

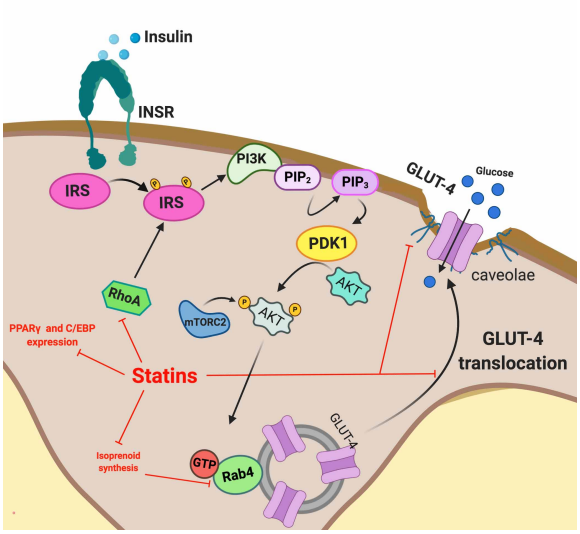

Figure 4: Intracellular actions of statins in adipocytes. Red lines indicate the mechanisms affected by statins.

5.2.2. Skeletal muscle

Skeletal muscle is the major tissue consuming most of the glucose that enters circulation [101], and any impairment in glucose uptake by this tissue may result in T2DM development. GLUT-4 mediates glucose transport into skeletal muscle cells, representing a key factor for blood sugar control [102]. As indicated above, insulin binding to INSR causes Akt activation [103] and translocation of GLUT-4 containing vesicles to the plasma membrane, thus facilitating the transport of glucose [104-106]. Although the mechanism of statin induced T2DM is not completely understood, there are both *in vivo* and *in vitro* studies that shed some light on this phenomenon in skeletal muscle. Some of the mechanisms that have been previously described are statin-mediated inhibition of insulin stimulated glucose uptake, impairment of intracellular signalling of the INSR and thereby of the Akt/mTOR pathway, or an excess of FFA accumulation in skeletal muscle as a consequence of HMG-CoA reductase inhibition.

In support of a role for a statin-induced insulin resistance in skeletal muscle, a decreased GLUT-4 expression has been found in L6 myotubes after simvastatin treatment [107]. Alternatively, it has been more recently shown that atorvastatin diminishes GLUT-4 translocation to the plasma membrane without affecting total GLUT-4 protein expression in C2C12 myotubes [108]. Assessment of the mechanism of simvastatin- or atorvastatin- associated impairment of glucose transport into myotubes suggests that impaired intracellular signalling of the INSR pathway also plays an important role. Indeed, Sanvee et al. [109] have shown that in C2C12

myotubes, simvastatin inhibits both INSR and mTORC2 function leading to impaired Akt activation and decreased translocation of GLUT-4 and consequently, reduced glucose uptake into skeletal muscle [109]. This deficient GLUT-4 translocation is likely caused by impaired Akt-mediated phosphorylation of GSK3β. Additionally, they show that simvastatin treatment induces higher plasma glucose levels in mice despite increased insulin plasma concentrations, consistent with insulin resistance [109]. The sequence of events leading to diminished glucose uptake induced by simvastatin starts with impaired phosphorylation of INSR, specifically the β-chain, which is considered to be essential for action of the receptor [101]. This results in deficient phosphorylation of Akt, which needs to be phosphorylated at both Thr308 (through the insulin signalling pathway) and Ser473 (by mTORC2) to become fully active [101]. Simvastatin treatment significantly impaired only the phosphorylation of Akt Ser473 due to an impaired phosphorylation of mTor, one of the mTORC2 constituents [109, 110]. Since Akt requires both phosphorylations to be fully active, is then unable to activate glycogen synthase kinase 3β (GSK3β), which is involved in the translocation of GLUT-4 to the plasma membrane. Decreased GSK3β phosphorylation in the setting of simvastatin at least partially explains impaired translocation of GLUT-4 to the plasma membrane.

Another adverse effect related to statin-induced T2DM and, similar to in adipocytes, is deficient prenylation of RabGTPases which has been suggested to lead to impaired GLUT-4 translocation [111]. Decreased intracellular cholesterol concentration is also considered a leading mechanism for impaired GLUT-4 translocation [108].

Alternatively, it has been suggested that simvastatin may cause insulin resistance through a novel fatty acid based mechanism independent of its cholesterol lowering effects. In their study, Kain et al. hypothesized that by blocking HMG CoA reductase, simvastatin may lead to accumulation of acetyl CoA, a precursor of fatty acid synthesis that can promote an intracellular build-up of fatty acids. The resulting excess accumulation of FFA in skeletal muscle may inhibit glucose uptake by reducing GLUT translocation [112, 113].

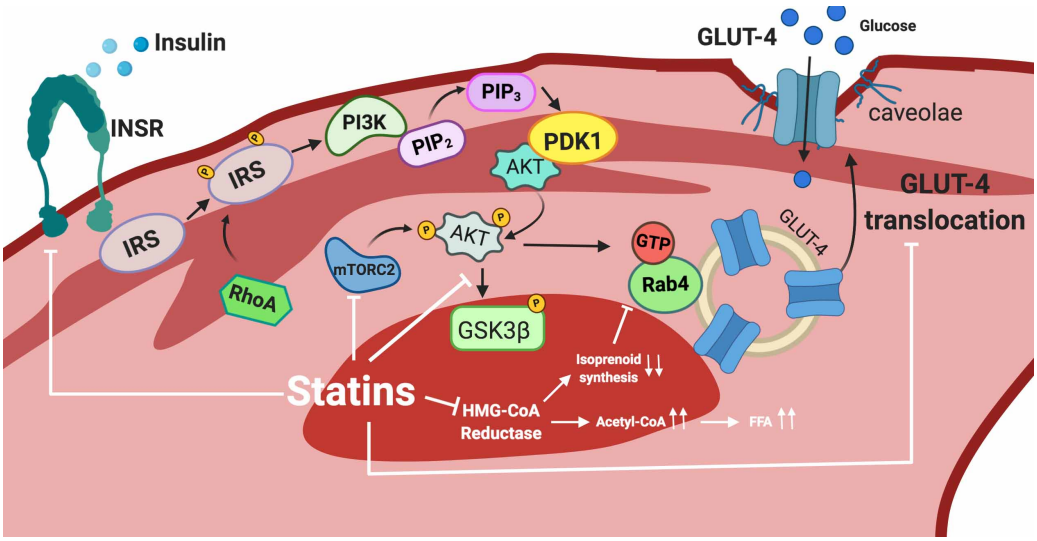

Figure 5: Intracellular actions of statins in muscle cells. White lines indicate the mechanisms affected by statins.

5.2.3. Liver

The liver plays a central role in glucose homeostasis and is exquisitely sensitive to insulin. In fact, insulin regulates many hepatic metabolic pathways ranging from glucose output to lipid synthesis. Therefore, impairment of hepatic insulin sensitivity is rapidly reflected in glucose homeostasis and triglyceride levels. Emerging evidence has demonstrated that statin treatment is associated with worsening glycaemic control in the liver [114]. Several mechanisms possibly involved with the effect of statins on glucose metabolism in the liver are summarized below.

Statin therapy is associated with a small increment in fasting blood glucose levels [115]. It has been shown that statins can stimulate endogenous glucose production by activation of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [116, 117], the major rate-limiting gluconeogenic enzymes in human liver cells. The elevation of hepatic gluconeogenesis contributes to hyperglycaemia, which is characteristic of insulin resistance and T2DM.

Regarding FFAs, it has been shown that an excess of FFA accumulation in liver cells can contribute to the development of T2DM [118, 119]. Interestingly, atorvastatin and rosuvastatin treatment upregulates thyroid hormone-responsive spot 14 protein (THRSP) expression, which is a small protein predominantly expressed in lipid-producing tissues such as those found in the liver. THRSP has been implicated as a regulator of the lipogenic processes by controlling the expression of

lipogenic genes such as fatty-acid synthase (FASN), ATP citrate lyase (ACLY) SREBP and ChREBP [120, 121] or their activity [122].

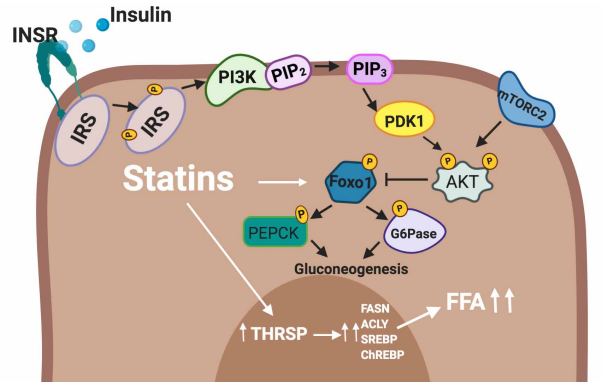

Figure 6: Intracellular actions of statins in hepatocytes. White lines indicate the mechanisms affected by statins.

5.3. MicroRNAs and Impact of Statin Therapy on microRNA expression profile

MicroRNAs (miRs) are small (~22 nucleotide) noncoding regulatory RNAs, which act as post-transcriptional regulators of gene expression [123, 124]. miRs usually silence gene expression through mRNA degradation or sequestration of the target mRNA from translation machinery [125]. It has been shown that miRs are involved in many biological processes including insulin expression, skeletal muscle adaptation to elevated glucose, insulin sensitivity and glucose stimulated insulin secretion (GSIS) [126]. It has been shown that miRs likely mediate the pleiotropic effects of statins via modulation of lipid metabolism, enhancement of endothelial function, inhibition of inflammation, improvement of plaque stability, and immune regulation. More specifically, miRs appear to regulate the fine-tuning of cellular phenotypes rather than serving as molecular on–off switches [127].

Statin therapy has been found to affect the expression of several miRs which play a central role in the regulation of lipid and glucose metabolism [128] and that are associated with development of T2DM.

5.3.1. miR Modulation of Cholesterol and Lipid Homeostasis

miR-33a and miR-33b are encoded within the introns of the Srebp2 and Srebp1 genes, respectively, and modulate intracellular cholesterol and fatty acid homeostasis together with SREBP2 and SREBP1 [129-132]. Specifically, miR-33a targets genes involved in cholesterol export, inhibits ABCA1 and ABCG expression [130-132] and participates in the regulation of HDL levels *in vivo*. On the

other hand, miR-33b modulates metabolic pathways related to of fatty acid metabolism [129, 133]. Importantly, both miR-33a and miR-33b participate in the regulation of fatty acid metabolism and are involved in the regulation of lipid and glucose metabolism [129]. miR-33 also negatively affects IRS2 expression thereby affecting insulin signalling [129]. Collectively, both isoforms of miR-33 participate in the regulation of relevant pathways that impact the primary risk factors of insulin resistance.

It has been demonstrated that simvastatin and atorvastatin induce expression of miR-33a in the liver [134] thus suggesting a link between reduced insulin secretion and, ultimately, the development of statin-incued T2DM. miR-33a is an important regulator of ABCA1 and their expression levels are inversely proportional in β-cells [132, 135]. miR-33a-mediated downregulation of ABCA1 can also alter islet cholesterol homeostasis and impair insulin secretion thus leading to β-cell dysfunction [136, 137]. However, additional studies are needed to further confirm the presence of a causal relationship between statin treatment and miRs in T2DM. Several studies have shown that statin treatment can upregulate miR-33b expression thus suggesting that statins could interfere fatty acid metabolism [138, 139].

Recently, the miR-27 family (miR-27a and miR-27b) has emerged as a new key regulator of cholesterol and lipid homeostasis [140-142]. Interestingly, the miR-27 family has been shown to be upregulated in a dose-dependent manner by simvastatin in HepG2 cells. Alvarez *et al.* demonstrated that miR-27a directly decreases both LDLR RNA and protein levels by binding to the 3'UTR of the *LDLR* mRNA [143]. Moreover, miR-27a also decreases LDLR expression indirectly through upregulation of PCSK9. They suggest that the potential binding site for miR-27a at position -1671 bp relative to the transcription start site of *PCSK9* may be responsible for the upregulation of PCSK9. In addition to the direct and indirect downregulation of LDLR levels, miR-27a also indirectly affects LDLR efficiency through a mechanism in which miR-27a targets the 3'UTR sequence of two genes in the LDLR pathway: LRP6 and LDLRAP1 by downregulating their expression [143]. Both proteins are necessary for correct binding to clathrin and thus are essential for efficient endocytosis of the LDLR-LDL-C complex [144-146]. Therefore, in addition to decreasing LDLR levels at the plasma membrane, miR-27a may also negatively affect LDLR efficiency. Deregulation of miR-27a has been reported in T2DM [147]. Specifically, it has been shown to be upregulated in adipose tissue and in 3T3-L1 adipocytes exposed to increased glucose concentration [147].

5.3.2. Modulation of Hepatic Glucose production

A vast number of miRs have been described to modulate glucose homeostasis through various mechanisms, leading to the question of whether some of them may potentially be involved in statins' diabetogenic effects. Specifically, it has been demonstrated that a direct effect of statins on hepatic glucose production is mediated by upregulation of the miR-183/96/182 cluster by modulating the expression of gluconeogenic enzymes [148]. It has been shown that incubation of hepatocytes with atorvastatin, simvastatin, or pravastatin upregulates the expression the key gluconeogenic enzymes PEPCK and G6Pase [117, 149]. The statin-mediated effects involve miR-183/96/182-mediated downregulation of the transcription factor 7-like 2 (TCF7L2), which modulates hepatic and peripheral glucose metabolism and whole body glycaemic control [148]. In regards to gluconeogenesis, TCF7L2 also reduces hepatic gluconeogenesis likely by decreasing the transcriptional activity of positive regulators of PEPCK and G6PC [150-154]. These results suggest that patients under long-term statin treatment would have persistently elevated expression of the miR cluster and lead to sustained activation of the gluconeogenic pathway, ultimately contributing to T2DM.

5.3.3. Modulation of the Insulin Signalling Pathway

As mentioned above, the activation of INSR by insulin leads to structural rearrangements in the receptor leading to autophosphorylation at tyrosine residues. Within the cell, phosphorylation levels are tightly regulated by protein phosphatases, in this case protein tyrosine phosphatases (PTPAses). These PTPAses negatively modulate insulin signalling by removing phosphate groups from tyrosine residues of the cytoplasmic domain of INSR. Specifically, protein tyrosine phosphatase non-receptor type 1 (PTPN1) has been predicted as an miR-146a target and the expression of PTPN1 is inversely correlated with miR-146a both in skeletal muscle and in liver of a T2DM rat model [155]. Of note, the role of miR-146a has been widely investigated in human T2DM pathogenesis and several studies report that it is downregulated in whole blood, plasma and some peripheral tissues [156]. Notably, it has been shown that simvastatin treatment also downregulates mir-146a expression after 6 months of therapy [157].

As mentioned above, IRSs link INSR activation to insulin metabolic effects through the intermediate modulation of the PI3K/PDK1/Akt pathway. It has been described that expression levels of IRS1 are modulated by miR-145 in hepatocytes [158] whereas in mice, upregulation of miR-145 in the liver leads to insulin

resistance [159]. Atorvastatin treatment has also been shown to differentially upregulate miR-145 and modulates PI3K/Akt signalling pathway [160].

In hepatocytes, miR-33a and miR-33b have been reported to modulate fatty acid and cholesterol metabolism as well as insulin signalling by targeting IRS2 [129, 161]. In one study, miR-33b overexpression in the Huh7 human hepatocytes cell line resulted in reduced Akt and ERK phosphorylation secondary to IRS2 down-regulation [129].

Additional Akt-downstream kinases and phosphatases represent major regulators of insulin signalling. Direct inactivation of AKT is mediated by protein phosphatase 2a (PP2A) [162]. PP2A activity has been shown to be increased in primary rat hepatocytes in insulin resistance condition [163]. Interestingly, insulin resistant Zucker Diabetic Fatty rats, PP2A mRNA is increased in liver, muscle and adipose tissue, thus suggesting a role for the phosphatase in deregulating insulin signaling in T2DM [163]. Importantly, PP2A expression is modulated by several miRs, among them miR-155 [164], whose expression has been found to be altered in T2DM. In addition, high dose rosuvastatin treatment has been shown to reduce the relative levels of serum miR-155 and therefore could lead to increased expression and activity of PP2A [165].

6. Differences in diabetogenic effects between hydrophilic and lipophilic statins

As indicated in previous sections, lipophilic statins (atorvastatin, simvastatin, lovastatin, fluvastatin and pitavastatin) may be more diabetogenic than hydrophilic statins (pravastatin and rosuvastatin) as they can more readily penetrate extrahepatic cell membranes such as β-cells, adipocytes and skeletal muscle cells. Conversely, hydrophilic statins (e.g., pravastatin) are more hepatocyte specific and less likely to enter β-cells or adipocytes [29]. Indeed, a high hepato-selectivity translates into minimal interference with cholesterol metabolism in tissues other than the liver and consequently to a lesser diabetogenicity [29, 30, 56]. Several studies have shown that the detrimental effects of statins are dose and potency dependent and primarily related to their lipophilicity [5, 14, 41, 47, 166].

While lipophilic statins have negative effects on pancreatic β-cell function, for hydrophilic statins such as pravastatin, neutral or improving effects have been observed [40, 41, 167]. As mentioned in **section 4.1**, it has been reported that statins can inhibit glucose-induced cytosolic Ca^{2+} signalling and insulin secretion by blocking L-type Ca^{2+} channels in β-cells. These inhibitory potencies may be particularly

evident for the lipophilic rather than the hydrophilic statins [41, 166, 168]. Indeed, unlike hydrophilic statins, the lipophilic ones have a strong affinity for the cell membrane, and therefore have easier access to the intracellular space [168]. In this context, statins may inhibit the endogenous metabolic pathways described in **section 5.1** that are associated with glucose-stimulated insulin secretion, including endogenous cholesterol synthesis [73, 166] and Ca^{2+}-dependent insulin responses to glucose [168]. It has been shown that atorvastatin (lipophilic) but not pravastatin (hydrophilic) affects insulin release and mitochondrial metabolism due to the suppression of antioxidant defense system and induction of ROS production in pancreatic β-cell models [169].

As described in **sections 5.2.1, 5.2.2 and 5.2.3**, GLUT-4 mediates insulin-stimulated glucose uptake [86] in a process that requires fusion of the transporter with the plasma membranes facilitated by IRS-1 and several kinases [86, 170, 171]. The small GTP-binding proteins are also key players in this process [22, 86] and they require isoprenylation by mevalonate products for their association with the cell membranes. The statin-mediated inhibition of the synthesis of the above products increases insulin resistance in parallel with the mevalonate synthesis inhibitory capacity [21, 172]. Furthermore, several other processes involved in the GLUT-4 signalling pathway may be inhibited by statins. These include IRS-1, insulin receptor β subunit, and Akt phosphorylation [22, 166]. It has been suggested that these effects are relevant only for lipophilic statins (e.g., atorvastatin and simvastatin), but not for hydrophilic statins (e.g., pravastatin) [22, 166]. The capacity of the former to enter adipocytes through passive diffusion can help explain this difference.

7. Conclusions

Taken together, the studies described in this review, ranging from clinical studies to *in vivo* and *in vitro* experimental results, confirm and reinforce the diabetogenic effect of statins. Although a number of questions remain unanswered, the available evidence supports that statins do increase the chances of T2DM with some statins being more strongly related (e.g., simvastatin, rosuvastatin and atorvastatin) than others (e.g., pravastatin). Intense research is currently going on to elucidate the mechanisms of statin induced T2DM at the molecular level. In light of the evidence from multiple observational studies, it is important to emphasize that there is still a favourable risk-benefit ratio for statin therapy, due to the large reduction in cardiovascular risk, despite the adverse effect of T2DM development. Overall, the risk of incident diabetes mellitus with statin therapy is present but

largely outweighed by the actual cardiovascular benefits [16]. Thus, statins should be continued in patients in whom these drugs are prescribed due to high or very high CVD risk, despite the risk of T2DM development until they achieve the target LDL-C levels. Before initiation of statin therapy the risk of diabetes should be assessed [8, 16, 173]. Statin-treated patients at high risk of developing diabetes should be monitored for changes in blood glucose and HbA1c levels, and preventive lifestyle modification should be introduced. If diabetes develops, it should be managed according to the guidelines [16]. Patients should be educated regarding the risk of incident diabetes mellitus with statins as with other risk–benefit of all therapies [174]. Lifestyle modification should be encouraged to lower cardiovascular risk and that for developing T2DN [175] and national guidelines should be used to manage diabetes mellitus [176, 177].

Several mechanisms through which statin treatment causes β-cell dysfunction and insulin resistance in peripheral tissues have been identified. Specifically, these the diabetogenic effects are related both to dose and statin class. In addition, miRs are glucose homeostasis regulators through the specific modulation of insulin signalling components. Growing evidence indicates that statin modulation of miRs expression may also be another mechanism through which statins increase the risk of T2DM. A multifactorial combination of these effects is what most likely contributes to the diabetogenic effects of statins described here. Clinically, these findings should encourage clinicians to consider diabetes monitoring in patients receiving statin therapy in order to ensure early diagnosis and appropriate management. Ultimately, since the risk of statin-induced T2DM is still being characterized, and the efficacy of statins in preventing CVD is very well documented, statins remain a first line treatment for prevention of CVD.

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2. Objectives / Helburuak

General objectives

Clinical studies, *in vivo* and *in vitro* experimental studies confirm that statin treatment has a diabetogenic effec with some statins being more strongly related (e.g., simvastatin, rosuvastatin and atorvastatin) than others (e.g., pravastatin). In light of the evidence, intense research is currently going on to elucidate the mechanisms of statin induced T2DM at the molecular level. To date, several mechanisms through which statin treatment causes β-cell dysfunction and insulin resistance in peripheral tissues have been identified. Interestingly, miRs are glucose homeostasis regulators through the specific modulation of insulin signalling components suggesting that statin modulation of miRs expression may also be another mechanism through which statins increase the risk of T2DM. Therefore the general objective of this Thesis is to study the multifactorial combination of effects is what most likely contributes to the diabetogenic effects of statins and to determine the contribution of microRNAs to the development of T2DM.

Specific objectives:

1. To study the effect of statin treatment in β-cells on the expression of INSR, INS, VDR, MAPK14 and P2Y2 receptor.
2. To analyse *in silico* the microRNA targets of INSR, INS, VDR, MAPK14 and P2Y2 receptor.
3. To determine the mechanisms by which statins modulates microRNA expression and the development of T2DM.

4. MATERIALAK ETA METODOAK / MATERIALS AND METHODS

1. Analisi bioinformatikoa

Ikerketa honetan aztertuiko miRNA miR-27b-3p izan da. miRNA hau hiperkolesterolemia pairatzen duten pazienteetan ematen den diabetesaren agerpenarekin erlazionaturik dagoen frogatzeko asmoz, miRNA horren itu izan daitezkeen geneak aztertu dira. hsa-miR-27b miRNAren izan daitezkeen itu geneak miRWalk datu basearen bidez zehaztu ziren (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). 12 predikzio algoritmoek ematen dituzten itu elkarrekintzak eskaintzen du honek. Kasu honetan, miRWalk, miRanda, RNA22 eta TargetScan erabili ziren espezifikoki.Gene-ituen eta intereseko miRNAren arteko elkarrekintza funtzionalak ikusiarazteko STRING datu basea erabili da (www.string-db.org).

2. Zelula eukariotoen hazkuntza

EndoC- βH zelula pankreatikoak (Univercell Biosolutions, Paris, Frantzia) Matrigel-fibronektinaz, 100mg/mL eta 2 mg/mL, hurrenez hurren, (Sigma-Aldrich, Poole, UK) gaineztatutako plaketan hazi dira. Horretarako, 5.6 mmol/L glukosarekin, % 2 idi-serum albuminarekin (Bovine Serum Albumin fraction V, Sigma-Aldrich, Poole, EB), 50 μmol/L 2-merkaptoetanolarekin (Bio-Rad, CA, AEB), 10 mmol/L nikotinamidarekin (Calbiochem, Darmstadt, Alemania), 5.5 μg/mL transferrinarekin, 6.7 ng/ml selenitarekin (Sigma-Aldrich, Poole, EB), 100 unitate/mL penizilinarekin eta 100 μg/mL estreptomizinarekin osatutako Dubelco´s Modified Eagle´s Medium (DMEM) medioa (Lonza, Leusden, Herbereak) erabili da. Transfekzio protokoloak burutzeko antibiotikorik gabeko eta % 2 behi fetu-serumarekin, ingelesez *Fetal Bovine Serum* (FBS) (Lonza, Belgika), osatutako medio bera erabili da. Zelulak 37 ºC-tan eta % 5 CO_2–rekin hazi dira.

3. Mimiko eta Antago-miRekin egindako EndoC- βH zelulen transfekzioak

EndoC- βH zelulak, lipofektamina (Lipofectamine RNAiMAX Lipid Reagent,Invitrogen, CA, AEB) erabiliz, 30 nM mimiko eta antago-miR-ekin trasfektatu dira, hurrenez hurren. Horretarako, lehenengo, intereseko mimikoa edo antago-miRa eta lipofektamina OptiMEM medioan diluitu eta giro tenperaturan inkubatu dira, 5 minutuz. Lipido-mimiko eta lipido-antago-miR konplexuak giro tenperaturan eratu dira 1 μL lipofektamina mimiko edo antago-miR 30 nM-eko proportzioan. Aipaturuko konplexuak bost aldiz diluitu dira antibiotikorik

gabeko medioan eta gero, zelulei gehitu zaizkie, gau osoan zehar inkubatzeko. Azkenik, zelulak 24 orduz errekuperatzen utzi eta jaso dira.

4. Estatinekin egindako tratamendua

24 putzuko plaketan, 50 x 10^3 zelula erein dira putzu bakoitzean eta lau estatina motarekin (sinvastatina, pravastatina, atorvastatina eta rosuvastatina), 24 orduz, tratatu dira., Dimetil sulfoxidotan (DMSO, Reagent Plus®, Sigma-Aldrich, Poole, UK) disolbatutako 5 μM-estatina gehitu dira, 37 ºC-tan eta % 5 CO_2-rekin. Baina, pravastatina, DMSOtan beharrean, H_2O-tan disolbatu da.

5. Estatinekin trataturiko EndoC-βH zelulen microRNA erauzketa

microRNAren erauzketa NucleoSpin® miRNA (Macherey-Nagel, Düren, Alemania) kita erabiliz burutu da. Zelulei estatinak gehitu eta 24 ordura, putzu bakoitzari medioa kendu eta 800 μL TRIzol® Reagent (Invitrogen, CA, AEB) agente desnaturalizatzailea gehitu zaio. Ondoren, 200 μL kloroformo gehitu eta lagin bakoitza 4 °C-tan eta 12,000 x G-ko abiaduran zentrifugatu da, 15 minutuz. Honela, 3 fase bereizi dira: lipidotan aberatsa den behealdeko fase organikoa, proteinaz osatutako tarteko fasea eta RNAdun goikaldeko fase urtsua. Behin zentrifugazioa amaitutakoan, fase urtsua 500 μL isopropanoletan berreseki da, 10 minutuz giro tenperaturan mantentzeko. Jarraian, 4 ºC-tan eta 12,000 x G-tan, 10 minutuz zentrifugatu da. Zentrifugatu ostean, gainjalkina baztertu eta jalkina % 100 etanol den 1 mL-tan berreseki dira. Azkenik, laginak 4ºC-tan eta 8,000 x G-tan 5 minutuz zentrifugatu ondoren, RNAsarik gabeko 50 μL H_2O-tan tan berreseki dira.

6. Estatinekin trataturiko EndoC- βH zelulen mRNA erauzketa

RNA mezulariaren (mRNAren) erauzketa gauzatzeko, zelulen medioak jaso ostean, 1 ml TRIzol® Reagent (Invitrogen, CA, AEB) gehitu zaio lagin bakoitzari. Ondoren, 200 μL kloroformo gaineratu eta 4 °C-tan eta 12,000 x G-tan zentrifugatu dira 15 minutuz. Zentrifugatzean 3 fase bereiztu dira: organikoa, proteikoa eta urtsua. Azkena, urtsua, izan da mRNA erauzteko jaso dena. Lagin bakoitzari 500 μL isopropanol gehitu eta 10 minutuz giro tenperaturan mantendu dira, jarraian, 4 °C-tan eta 12000 x G-tan 10 minutuz zentrifugatzeko. Zentrifugazioak mRNAren prezipitazioa eragiten du, beraz, behin bukatutakoan, jalkina % 75 etanol den 1 mL-tan berreseki da. Laginak 4 °C-tan eta 8,000 x G-tan 5 minutuz zentrifugatu dira azkeneko aldi batez. Bukatzeko, prezipitatua lehortzen utzi da, RNAsarik gabeko H_2O-tan berresekitzeko gero.

7. Alderantzizko transkriptasadun -polimerasaren kate-erreakzio kuantitatiboa (qRT-PCR)

Proteinen RNA mezulariaren, zein microRNAren, adierazpena neurtzeko alderantzizko transkriptasadun polimerasaren kate-erreakzio kuantitatiboa (ingelesez *quantitative reverse transcription polimerase chain reaction*, qRT-PCR) deritzon teknika erabiltzen da. Aurretik erautzitako mRNAren eta miRNAren cDNA lortzeko Universal cDNA Synthesis kit II (Exiqon Inc, 203301) erabiltzen da. Bertatik RNAsarik gabeko 5 μL H_2O, 2 μL erreakzio buffer, 2 μL lagin eta 1 μL entzima gehitzen zaizkio putzu bakoitzari. Azkenik, plaka film itsaskor batekin estaltzen da eta RT-PCRa burutzen da C1000 Thermal Cycler CFX96 Real-Time System (Bio-Rad Laboratories Inc, CA, AEB.) termoziklatzailea erabilita, kitak ezarritako argibideak jarraituz.

Behin cDNA eskuratutakoan, qRT-PCR bidez lagin bakoitzaren mRNAren eta miRNAren adierazpen maila aztertu da, barne kontrol bat erabiliz. Aipaturikoak zelula guztietan, edozein estatina tratamendu gehituta ere, adierazpen maila bera mantentzen du. Kasu honetan, GAPDH eta U6 barne kontrolak erabili dira, hurrenez hurren. qRT-PCRa 96 putzutako PCR plaka batean egin da (Bio-Rad Laboratories Inc., CA, AEB) C1000 Thermal Cycler CFX96 Real-Time System termoziklatzailean (Bio-Rad Laboratories Inc., CA, AEB). Polimerasaren aktibazioa 95ºC-tan gertatzen denez, erreakzioa tenperatura horretan hasten da eta 10 minutuz mantentzen da, cDNA osatzen duten harizpi biak desnaturalizatu daitezen. Ondoren, 65°C-tara jaisten da minutu batez, primerrak cDNAra lotu eta Taq polimerasa aktibatu dadin. Honi esker, anplifikazioa gertatzen da. Termoziklatzaileak SYBR Green fluoroforoak askatutako fluoreszentzia neurtzen du eta, horretarako, lagina 488 nm-ko uhin luzeran kitzikatzen da eta 522 nm-ko uhin luzeran jasotzen da seinalea. Neurketarekin bukatutakoan, tenperatura berriz ere 95°C-tara bueltatzen da 10 segundoz, prozesua berriz hasteko. Honelako 40 ziklo errepikatu dira, cDNA molekula guztiak anplifikatu direla bermatzeko.

X Taula. miRNAk anplifikatzeko erabili diren hasleak.

Genea	Itu Sekuentzia
hsa-miR27b-3p	5'UUCACAGUGGCUAAGUUCUGC
hsa-miR33a-5p	5'GUGCAUUGUAGUUGCAUUGCA
hsa-miR33b-3p	5'CAGUGCCUCGGCAGUGCAGCCC
hsa-miR23b-3p	5'AUCACAUUGCCAGGGAUUACC

X Taula. Gene ezberdinen mRNA anplifikatzeko erabili diren hasleak

Genea	(5´-3´) *Forward* Haslearen Sekuentzia	(3´-5´) *Reverse* Haslearen Sekuentzia
INS	GCAGCCTTTGTGAACCAACA	CCCCGCACACTAGGTAGAGA
INSR	AGTTTGAGGACATGGAGAATGTG	ATAGGAACGATCTCTGAACTCCAC
MAPK14	TGAAGACTGTGAGCTGAAGATTCTG	CCACGTAGCCTGTCATTTCATC
VITDR	GGCCGGACCAGAAGCCTTT	CAGCCTTCACAGGTCATAGCA
P2RY2	TGCCTGTGAGCTATGCAGTTGTC	GGAACATGTAGGTGGCCGTTG
c-MYC	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

8. Mediora askaturiko intsulinaren detekziorako entseguak

Intsulinaren jariapena neurtzeko, hazkuntza plakako 12 putzu βCOAT®-rekin estali dira eta beste 3 putzu zelula kopurua zenbatzeko erabili dira. Putzu bakoitzerako gomendatutako zelula kopurua 3.75 x 10^5 da, 1 x 10 zelula/zm^2-ko dentsitateari dagokiona. Behin zelulak altxatuta, garbituta eta zenbatuta, OPTIβ1® medioan berreseki dira, 3.75 x 10^5 zelula/mL-ko dentsitatea lortuz. Putzu bakoitzean 1 mL zelula jarri da eta 37°C-tan % 5 CO_2–rekin mantendu dira, 24 orduz.

5 egun igaro ostean, putzuetatik OPTIβ1® medioa kendu, 1 mL OPTIβ2® medioa gehitu eta 24 orduz mantendu dira. 24 orduak igarota, medioa kendu eta bi garbiketa egin dira βKREBS®BSA medioa erabilita. Ondoren, medio berdinaren 1

mL gehitu da eta 37 °C-tan, ordu batez inkubatu dira. Inkubazio aldia igarota, medioa aldatu, βKREBS®BSA medioa gehitu eta 37 °C-tan, 40 minutuz mantendu dira. Beranduago,putzu bakoitzeko 800 μL medio jaso eta 1.5 mL-ko hodietara pasatu dira, hodiak izotzetan mantenduz. Azkenik, hodiak 4 °C-tan eta 700 x G-ko abiaduran zentrifugatu dira, gainjalkina jaso eta intsulinaren jariapena neurtzeko.

Bestalde, zelula barneko edukia neurtzeko plaka espezifikoak erabili dira. Plaka izotzetan mantenduz,putzuetan geratzen den medioa guztiz kendu eta 1 mL lisi-medio gehitu zaio putzu bakoitzari.. Lisatutako zelulak 1.5 mL-ko hodietara transferitu dira eta, ondoren, 4 °C-tan, 700 x G-ko abiaduran, 5 minutuz zentrifugatu dira. Zentrifugatu ostean, intsulina edukia eta jariapena neurtzeko, putzu bakoitzeko gainjalkinaren 400 μL hodi berrietara pasatu dira.

Intsulina kontzentrazioa *Enzyme-Linked ImmunoSorbent Assay* (ELISA) bidez neurtu da, fabrikatzailearen argibideak jarraituz. Gainjalkineko intsulina neurketak mU/L unitatetan adierazten dira eta zelulen intsulina edukia ng/10^6 zelula moduan adierazten da.

9. Ca^{2+} mobilizazio entseguak

Zelulak 12 mm-ko porten gainean hazi dira aldez aurretik aipaturiko baldintzetan. Estatinekin 24 orduz inkubatu ostean, zelula barneko kaltzioa (Ca^{2+}) kontzentrazioa neurtu da. Horretarako, neurketa egin baino 45 minutu lehenago, putzu bakoitzari 1.5 μM Fura-2 AM zunda fluoreszentea gehitu zaio - eta inkubazioa 37 °C-tan burutu da. Zunda kaltzioari lotuta dagoenean, bere kitzikapen balio maximoa 340 nm-ko uhin luzeran dauka. Aske dagoenean, berriz, maximoa 380 nm-tan dauka. Halere, emisioa 510 nm-ko uhin luzeran izango du bi egoeretan.

Zunda gehitu eta inkubazio denbora igaro denean, portak jaso eta mikroskopioaren inkubazio ganbaran kokatu dira, 37 °C-tan, eta bertan 500 μL kaltzio gabeko HBSS medioa gehitu zaie. Interferentziak saihesteko, Nikon TE300 mikroskopioa iluntasunean erabili da, x 40 handipen objektiboaz baliatuz. Mikroskopioan [Ca^{2+}] intrazelularraren neurketak egiten hasi eta minutu batera UTP gehitu da zeluletara, 10 minutuko zinetikak egin direlarik. UTP 5 μM erabili da mintzeko $P2Y_2$ hartzaileak aktibatzeko eta odoren zitosoleko [Ca^{2+}]-aren aldaketa neurtu da estatinen presentzian.

Zelulak, 340 nm zein 380 nm-tan, 350 ms-ro kitzikatu dira, PTI DeltaRAM sistema erabiliz eta 340/380 absorbatzia ratioak neurtu dira denboran zehar.

Absorbantzia ratio hauek hartu dira kontuan, PTI ImageMaster softwerra baliatuz, zelula bizien irudiak lortzeko. Zitosoleko kaltzio kontzentrazioa (R) kalkulatzeko hurrengo formula erabili da:

$$[Ca^{2+}] = K_D \times (R - R_{min})/(R_{max} - R)$$

R_{max} balioa tritoia (Triton X-100 %1 v/v) gehituz kalkulatu da. Tritoiak mintz plasmatikoak apurtzen ditu, eta ondorioz, zelula barneko kaltzio guztia ateratzen da. R_{min} balioa, ordea, EGTA erabilita kakulatu da, EGTAk Ca^{2+} kelatzen baitu. 244 nM-ko K_D balioa erabili da.

UPTk eragindako Ca^{2+} mobilizazioa $P2Y_2$ hartzaileen bitartez ematen dela frogatzeko asmoz, suramina erabili da. Izan ere, suraminak $P2Y_2$ kanalak blokeatzen ditu, eta hortaz, suramina gehitzerakoan UPT ezingo da $P2Y_2$ hartzaileetara lotu, kaltzioaren mobilizazioa eragotziz. Zinetika hauek burutzeko, neurketak egin baino lehen, medioan 20 μM suramina gehitu da. Neurketak hasi eta minutu batzuetara, 0,75 μM UTP gehitu da -eta 10 minutuz neurtu da.

10. Western blot bidezko analisia

Transfekzioa egin eta 24 ordura zelulak PBS hotzarekin garbitu eta lisia burutu da. Zelulak lisatu ahal izateko, 50 mM Tris–HCl (pH 7.5), 125 mM NaCl, % 1 Nonidet P-40, 5.3 mM NaF, 1.5 mM NaP, 1 mM ortobanadato, 1 mg/mL proteasa-inhibitzaile koktela (Roche, Suitza), eta 0.25 mg/mL Pefabloc, 4-(2-aminoetil)-benzenesulfonil fluoride hidrokloruroa (AEBSF) (Roche, Suitza) dauzkan medio hotza erabili dira. Behin lisi-medioa gehituta, zelulak batu eta 4°C–tan biraka mantendu dira ordu batez. Ondoren, 12000 x g-tan 15 minutuz zentrifugatu dira. Liseritutako gainjalkinen proteina kontzentrazioa neurtu da.

Inmunodetekzio semikuantitatiboa burutzeko, lehendabizi proteinak elektroforesi bidez banatu dira, % 8.5-eko SDS-PAGE ez-erreduzitzailea erabilita. Gero, antigorputz-proteina lotura inespezifikoak ekiditeko, mintzak ordubetez inkubatu dira blokeo soluzioan (TBST + % 5 esne gaingabetua), ondoren, antigorputz primarioarekin inkubatu direlarik. Mintzak gau osoan zehar inkubatu dira, antigorputz primarioarekin (LDL Receptor Polyclonal Antibody, Cayman Chemical, AEB) 4°C–tan. Antigorputz sekundarioaren kasuan (GAPDH Antibody, SantaCruz, AEB), berriz, mintzak 3 aldiz TBSTrekin garbitu ostean, giro tenperaturan inkubatu dira ordubetez. Seinalea SuperSignal West Dura Extended Substrate (Pierce Biotechnology, AEB) bidez detektatu da, ChemiDoc XRS (Bio-Rad, AEB) erabiliz. Banden intentsitatea kuantifikatzeko, NIH ImageJ softwarea

(https://rsbweb.nih.gov/ij/) erabili da eta intereseko proteinen intentsitate mailak GAPDH kontrolaren intentsitatearekiko zuzendu dira.

X. Taula. Western blotean eta Fluxu-zitometrian erabilitzako antigorputzen zerrenda.

Ab	Etxe komertziala	Erreferentzia	Mw (kDa)	Diluzioa	Inkubazioa
Anti-INSR	Novus Bio	C18C4	95	1:1000	16 h/4 °C
GAPDH Antibody	Santa Cruz	SC-47724	35,8	1:1000	16 h/4 °C
Anti-MAPK14	Santa Cruz	SC-271120	67	1:1000	16 h/4 °C
Anti-P2Y2	Abcam	ab168984	42	1:1000	16 h/4 °C
Anti-cMYC	Invitrogen	9E10	62	1:1000	16 h/4 °C
Anti-VDR	Santa Cruz	SC-13133	48	1:1000	16 h/4 °C

*LDLRren Western blotean erabilitako tanpoi indargetzaile guztiek 2 mM $CaCl_2$ daramate.

5. RESULTS /EMAITZAK

Deciphering the complex epigenetic networks that orchestrate statin-mediated type 2 diabetes mellitus development: roles of miR-27b and miR-33

1. Introduction

Long-term exposure to high circulating LDL-cholesterol (LDL-C) levels is one of the major risk factors for atherosclerosis and cardiovascular disease (CVD) development. The increase in plasma cholesterol levels induces changes of the arterial endothelial permeability, allowing the migration of LDL-C into the artery wall [1]. Once in the intima, oxidation of the lipoproteins triggers an inflammatory process that leads to foam cell formation, smooth vascular muscle cell (SVMC) migration and fibrous cap synthesis. This all translates to an increase in the size of the atherosclerotic plaque, reducing the diameter of the artery, and therefore, considerably increasing the risk of CVD [2].

In order to reduce plaque related cardiovascular events, individuals with high circulating LDL-C usually receive lipid lowering therapies [3]. Among them, statins are the gold standard therapy due to their effectiveness and low cost [4]. Statins act through inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the cholesterol biosynthetic pathway [5] leading to a decrease in the endogenous cholesterol synthesis [6].

Despite their effectiveness and safety, increasing evidence suggests that statins could be responsible for greater incidence of type 2 diabetes mellitus (T2DM) among treated patients [7, 8]. In fact, meta-analysis of randomized clinical trials show a 10-12 % increased risk of new-onset T2DM associated to statin treatment [8-10]. Although the mechanisms by which statin treatment induces T2DM remain not fully understood, some studies support the hypothesis that statins may disrupt glucose homeostasis through both impaired insulin secretion and diminished insulin sensitivity on target tissues [11-13].

Statin treatment induces β-cell dysfunction by affecting different mechanisms implicated in glucose metabolism and intracellular calcium signaling [8, 14]. Simvastatin has been proven to inhibit calcium input by blocking the L-type Ca^{2+} channels, leading to a decrease in Ca^{2+} input to the cytosol and therefore, disrupting insulin-containing vesicle mobilization [15]. Simvastatin also interferes with mitochondrial respiration and inhibits mitochondrial K_{ATP} channels [16]. Likewise, atorvastatin and pravastatin treatment is associated with a reduction of GLUT-2 expression, the main glucose transporter isoform in the β-cell [17, 18].

Recently, it has been described that some of the pleiotropic effects of statins, such as plaque stability and endothelial function improvements, can be mediated by direct modulation of the cellular miRNome [19-21]. In like manner, deregulation of microRNAs (miRNAs) involved in key processes of lipid metabolism and glucose homeostasis could be responsible for the development of new-onset T2DM [22-24]. Recently, together with biochemical modifications of DNA and histones, miRNAs have raised as important epigenetic regulators of β-cell biology. In fact, several miRNAs have been identified to play a role in pancreas development (cell lineage specification and differentiation) as well as mature β-cell function (insulin expression, secretion and signaling) [25, 26].

miRNAs are small non-coding double stranded RNA sequences that act as post-transcriptional regulators of more than half of protein-coding genes in humans [27]. MicroRNAs (miRNAs) are an abundant class of endogenous noncoding RNAs that have emerged as key posttranscriptional regulators of gene expression. In animals, miRNAs cause repression by base pairing to the 3′ untranslated region (3′ UTR) of their target mRNAs, which contain perfect or near-perfect sequence complementarity to nucleotides (nt) 2 to 8 (the "seed region") of the mature miRNA [28, 29]. The canonical miRNA biogenesis pathway involves the stepwise processing of primary miRNA transcripts (pri-miRNAs) by the RNase III proteins Drosha and Dicer [28, 29]. After processing by Drosha in the nucleus, the pri-miRNA is exported to the cytoplasm and subsequently cleaved by Dicer to produce an 22-nt miRNA/miRNA* duplex [28, 29]. Although both strands of the duplex are necessarily produced in equal amounts by transcription, their accumulation is asymmetric at the steady state [30-32]. By convention, the most abundant duplex strand is defined as the mature miRNA strand, whereas the less abundant strand is known as the "passenger strand," or miRNA star strand (herein referred to as miRNA*) [30, 31, 33]. The mechanisms of miRNA strand selection and RNA-induced silencing complex (RISC) loading are still unclear; however, it is thought that strand selection is

dictated by the relative thermodynamic stability of each duplex end [30, 31]. The strand with unstable base pairs at the 5′ end typically evades degradation and is incorporated into Argonaute (Ago) complexes, where it mediates posttranscriptional gene silencing (Figure 1) [30, 31].

miRNAs perform gene expression silencing by targeting the 3′UTR region of their target mRNA. Once they bind, the target gene is silenced through mRNA degradation or sequestration [34]. On the one hand, several miRNAs can modulate the expression of a single mRNA, while on the other, a single miRNA can target more than one mRNA [35]. Furthermore, even if some miRNAs might be tissue specific, the ability to communicate among tissues has already been described [36-39]. In fact, miRNAs can be secreted to plasma and delivered throughout the body into lipoproteins [40], reaching distant tissues due to their high stability [41, 42]. Thus, an imbalance in single or multiple miRNA expression could lead into a deregulation of several target genes, affecting different metabolic pathways and therefore, triggering disease causing events.

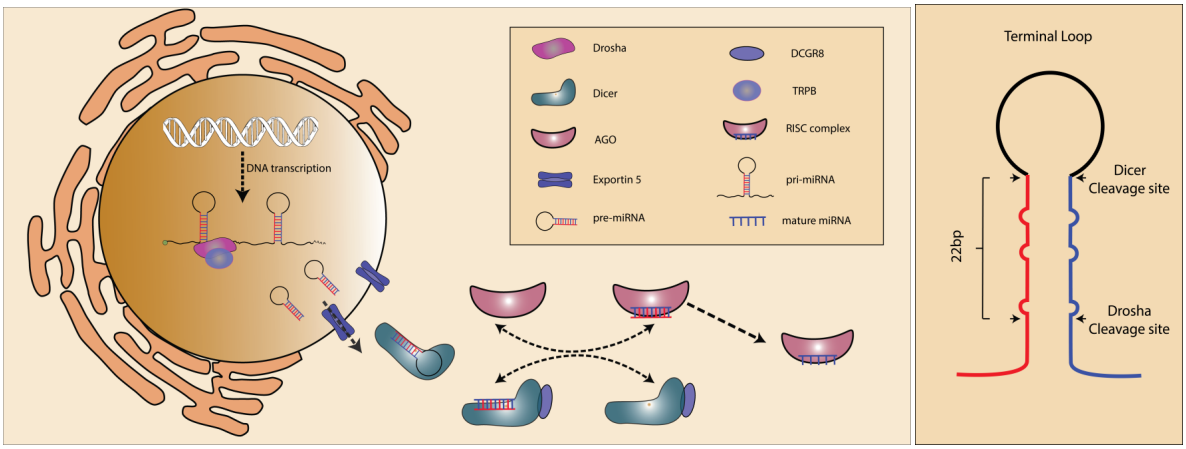

Figure 1. miRNA processing. The miRNA are transcribed by RNA polymerase II (RNA Pol II/III). The synthesized pri-miRNA is processed by Drosha/DGCR8 complex generating the pre-miRNA that is transported out from the nucleus by exportin 5. Once in the cytoplasm the terminal loop is cleaved by Dicer/TRPB and the RNA product is loaded into AGO proteins where the guide RNA along with AGO protein forms the RISC complex once passenger strand has been degraded [43].

In the recent years, the relevance of miRNAs on lipid and glucose metabolism regulation, together with their role on insulin signaling pathway has been highlighted [44, 45]. In the case of the intronic miRNAs miR-33a and –b, which are located within the sterol regulatory binding elements, several studies have demonstrated their implications on fatty acid metabolism and insulin signaling [46].

It has been shown that miR-33 expression can be modulated by statins [47], and that miR-33 induced ABCA1 deregulation can alter pancreatic islet cholesterol homeostasis and impair insulin secretion, causing β-cell dysfunction [48]. At the same time, miR-124 and miR-34 are involved in pancreatic development by targeting genes such as Foxo2, Rhab27a, VAMP2 and Bcl2, whereas miR-29, miR-9 and miR-375 are involved in insulin secretion acting through Mct1, Onecut2, Sirt1, PDK1, and Mtpn [49]. Furthermore, the effect of miRNAs on insulin signaling and glucose homeostasis is not restricted to pancreas; liver, muscle and adipose tissue also being affected [45, 50].

Considering all the evidence gained in the last years about miRNAs and their role in metabolomics and epigenetic modulation, understanding how the miRNome interacts with the genes of any specific metabolic pathway will help us increase our knowledge about diseases affecting those pathways. In addition, modulation of the miRNAs responsible for any given alteration could become a potential therapeutic target. In this study we sought to unravel the molecular mechanism by which statin treatment induces T2DM through modulating the expression of some miRNAs implicated in β-cell insulin signaling and glucose homeostasis.

2. Results

2.1. Statin treatment downregulates INSR, INS, VDR, MAPK14 and P2Y2 expression

To investigate the mechanisms underlying the association of statin therapy with diabetes we performed a gene expression analysis on genes previously associated with statin treatment, β-cell dysfunction and T2DM development. As mentioned above, meta-analyses of randomized controlled trials as well as observational studies have reported that statin therapy is associated with a 12% increased risk of new-onset diabetes [4, 9, 51-53]. It has been shown that treatment with statins has decreased insulin sensitivity [54], decreased insulin secretion [15] and Ca^{2+} channel dysfunction [55], although no clear mechanisms have been suggested for the link between inhibition of insulin sensitivity and statin effects (Figure 1A). Herein, to evaluate the β-cell response to statins, EndoC-βH were incubated during 24 h with 5 μM simvastatin, pravastatin, rosuvastatin or atorvastatin and next, expresión of INSR, INS, VDR, MAPK14 and P2Y2 was assessed both at mRNA and protein levels. As shown in Figure 1B, statin treatment induced a downregulation of *INSR*, *INS*, *VDR*, *MAPK14* and *P2Y2* mRNA levels. Similar effects were observed

when protein expression was assessed by western blot (Figure 1C-F, left panels), INSR, VDR, MAPK14 and P2Y2 levels determined by densitometry were significantly reduced compared to control (non-treated) cells (Figure 1C-F, right panels). Additionally, activity of P2Y2 receptor was assessed by monitorizing Ca^{2+} release from ER stores by using (0.75 μM) as specific agonist of P2Y2 receptor. As shown in Figure 1G, statins reduced Ca^{2+} release to the cytoplasm by different extent, being atorvastatin the most effective statin reducing Ca^{2+} mobilization (% Ca^{2+} mobilization inhibition: simvastatin 35.4 ± 7; pravastatin 36.0 ± 8; rosuvastatin 47.6 ± 3 and, atorvastatin 52.2 ± 5).

Finally, the observed downregulating effects of statin treatment on INS mRNA were further coroborated by determining insulin release into the culture media of EndoC-βH cells treated 24 h with statins. As shown in Figure 1H, insulin secretion in the presence of stains was significantly reduced compared to control cells (% insulin release inhibition: simvastatin 39.1 ± 5; pravastatin 43.0 ± 7; rosuvastatin 49.3 ± 7 and, atorvastatin 54.9 ± 5).

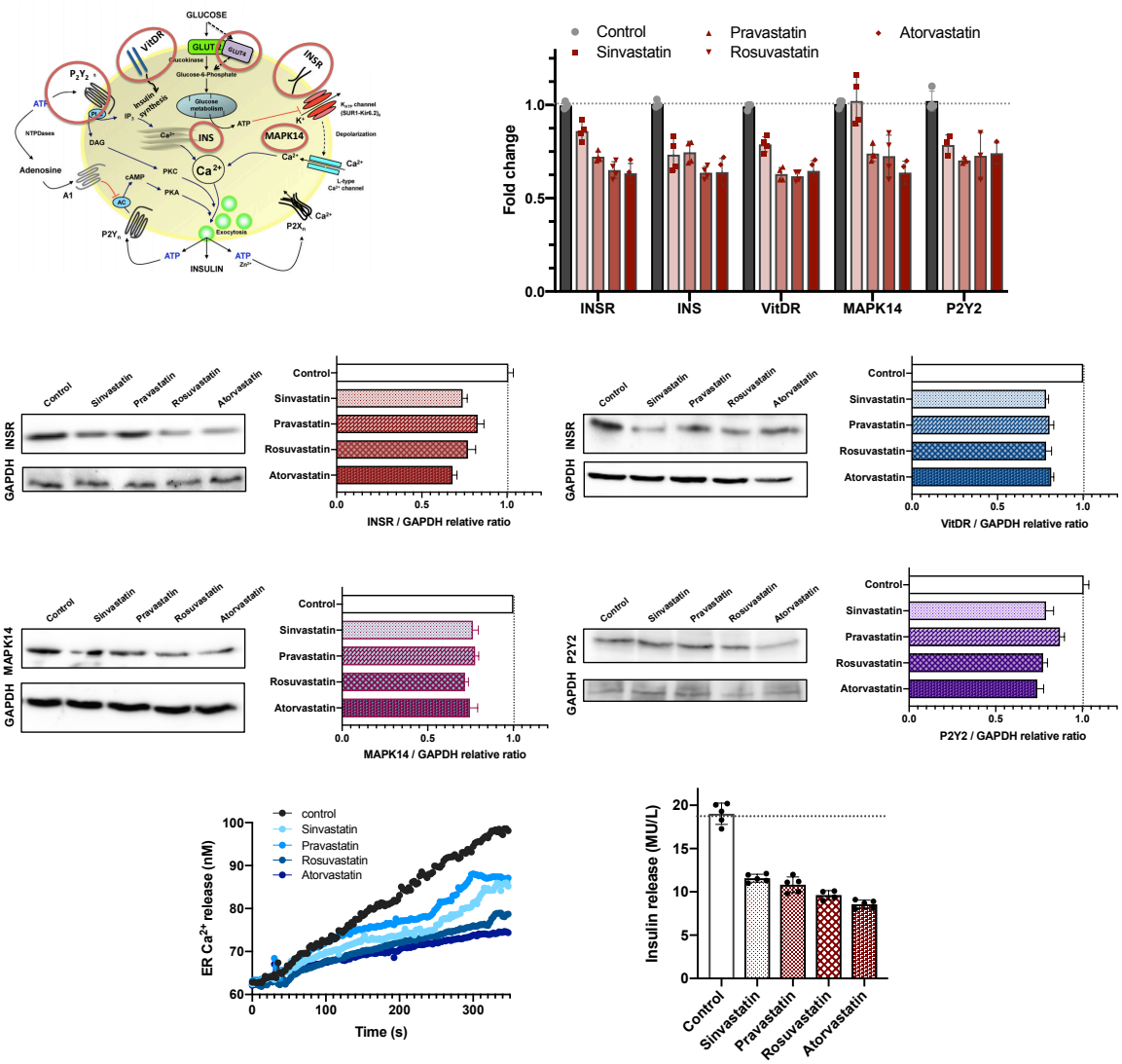

Figure 1: Statins downregulate INSR, INS, VDR, MAPK14 and P2Y2 expression. (A) Mechanisms affected by statins proposed to be the link between insulin sensitivity inhibition and statin effects; (B) effect of 5 μM simvastatin, pravastatin, rosuvastatin or atorvastatin on *INSR, INS, VDR, MAPK14* and *P2Y2* mRNA expression; (C) effect of 5 μM statins on INSR expression determined by Western blot (left panel) and by densitometry (right panel); (D) effect of 5 μM statins on VDR expression determined by Western blot (left panel) and by densitometry (right panel); (E) effect of 5 μM statins on MAPK14 expression determined by Western blot (left panel) and by densitometry (right panel); (F) effect of 5 μM statins on P2Y2 receptor expression determined by Western blot (left panel) and by densitometry (right panel); (G) Ca^{2+} release from ER stores of EndoC-βH by UTP (0.75 μM) in the presence of statins; (H) insulin release into the culture media of EndoC-βH cells treated 24 h with statins. To evaluate the β-cell response to statins, EndoC-βH were incubated during 24 h with the different statins and experiments were done as described in Materials and Methods. * $p <$ 0.05; * $p <$ 0.025; * $p <$ 0.01 (significantly different from control (non-treated) cells. Data are presented as the mean ± SEM of at least $n = 3$ independent experiments (B-H right-handed panels). Data correspond to results from a representative experiment among three that gave similar results in left-handed panels of (C-F).

2.2. Identification of miR-27b as a Potential Regulator of the Insulin Signaling Pathway

In an attempt to identify miRNAs that might affect insulin signaling, we performed *in silico* analysis. Using miRwalk database we check the predicted miRNAs affecting the expression of the previously metioned human genes encoding nuclear receptors and transcription factors known to affect glucose homeostasis. The analysis gived back many miRNA sequences but only miR-27b had predicted binding sites for all of the genes under study (Figure 2A). Previous studies have shown that *INSR* is metabolic target of miR-27b in both human hepatocarcinoma cell line and mice [56], thus showing its capacity to initiate IR development at an early stage by modulating INSR expression.

To gain insight into the function of miR-27b, we performed a bioinformatic analysis to identify all the potential miR-27b target genes using the miRWalk database. These genes were classified according to the pathways in which they were involved. As expected, insulin pathway was one of the pathways overrepresented [57, 58] (Figure 1B)]. We next performed sequence interaction analysis that showed that miR-27b have at least one possible binding site in each of the analyzed 3′UTR sequences (Figure 1C). Moreove, interaction type and site conservation where also included in the analysis as its shown in Figure 1D.

We next performed protein–protein interaction analysis that showed that miR-27b might play an important role in controlling key processes involved in insulin signaling, insulin release and β-cell survival (Figure 1B). Very interestingly, the highest miR-27 score were for the genes participating in insulin pathway (*INSR, INS, VDR, P2Y2)* and β-cell survival (*MAPK14*) (highlighted in red) (Figure 1B) proven to be downregulated by statin treatment in the previous section. The predicted miR-27b binding sites for these genes are shown in Figure 1C and the number, type and conservation of predicted sites of the selected genes are shown in Figure 1D.

Next, statin-induced overexpresión of miR-27b was assessed after 24 h incubation of β-cell with 5 μM of statins (simvastatin, pravastatin, rosuvastatin or atorvastatin). As shown in Figure 1E, statin treatment induced the upregulation of miR-27b expressión levels. The extent of miR-27b upregulation was 21% with simvastatin, 25% with pravastatin, 34% with rosuvastatin and 52% with atorvastatin compared to control (non-treated) cells (Figure 1D).

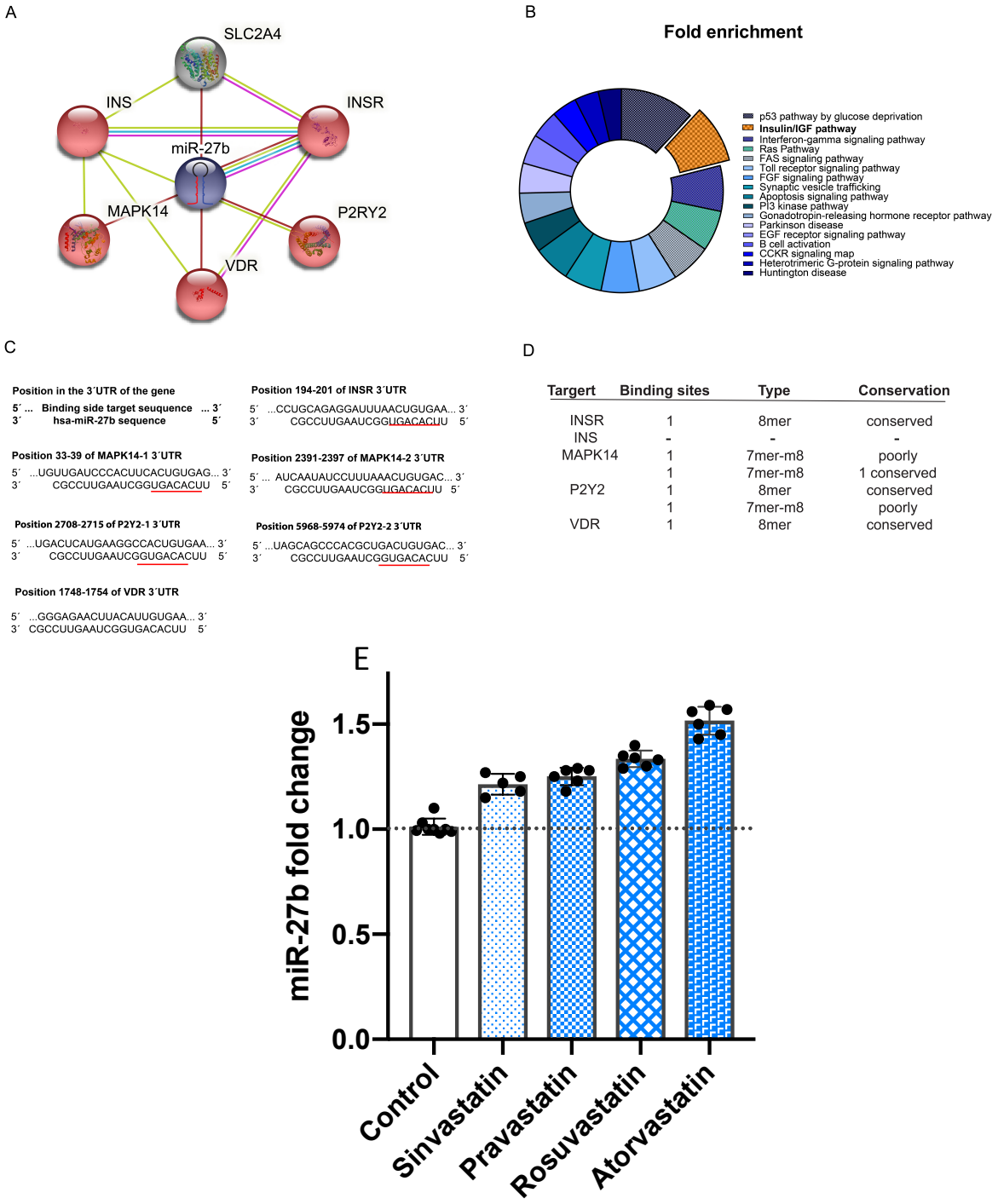

Figure 2: miR-27b is predicted to target many genes involved in insulin signaling pathway and is upregulated by statins. (A) Pathway enrichment of validated targets of miR-27b; (B) interaction gene sequences of the five genes selected in this study with miR-27b; (C) genetic representation of miR-27b interactions with the 3′UTR of the selected genes; (D) number, type and conservation of predicted sites of the selected genes; (E) statin-induced overexpresión of miR-27b. To evaluate the upregulation of miR-27b in response to statins, EndoC-βH were incubated during 24 h with the different statins and experiments were done as described in Materials and Methods. * $p < 0.05$; * $p < 0.01$ (significantly different from control (non-treated) cells. Data are presented as the mean ± SEM of at least $n = 3$ independent experiments (E).

2.3. miR-27b regulates the Expression of INSR, INS, VDR, MAPK14 and P2Y2 Receptor

To assess the role of miR-27b in the regulation of *INSR*, *INS*, *VDR*, *MAPK14* and *P2Y2* receptor expression in β-cells, we overexpressed or inhibited miR-27b levels in EndoC-βH cells using miRNA mimics and inhibitors, respectively. Transfection of EndoC-βH cells with miR-27b mimic markedly increased miR-27b levels (Figure 2A). Overexpression of miR-27b reduced both mRNA and protein levels of INSR, INS, VDR, MAPK14 and P2Y2 receptor (Figure 3B, C, respectively). Conversely, miR-27b inhibition reversed the effects of statins and rescued the mRNA expression levels of *INSR, INS, VDR, MAPK14* and *P2Y2* receptor to those shown by control (transfected with control antagomiR) cells (Figure D-H). These results indicate a physiological role of miR-27b in regulating components of insulin signalling pathway, insulin secretion and β-cell survival. Activity of P2Y2 receptor was also impaired by mimic-27b as determined by Ca^{2+} release from ER to the cytoplasm upon stimulation with 0.75 μM UTP (Figure 3 I). On the other hand, treatment with antagomiR-27b reverted the impaired Ca^{2+} signalling mediated by the upregulation of miR-27b by statin treatment (Figure 3 J).

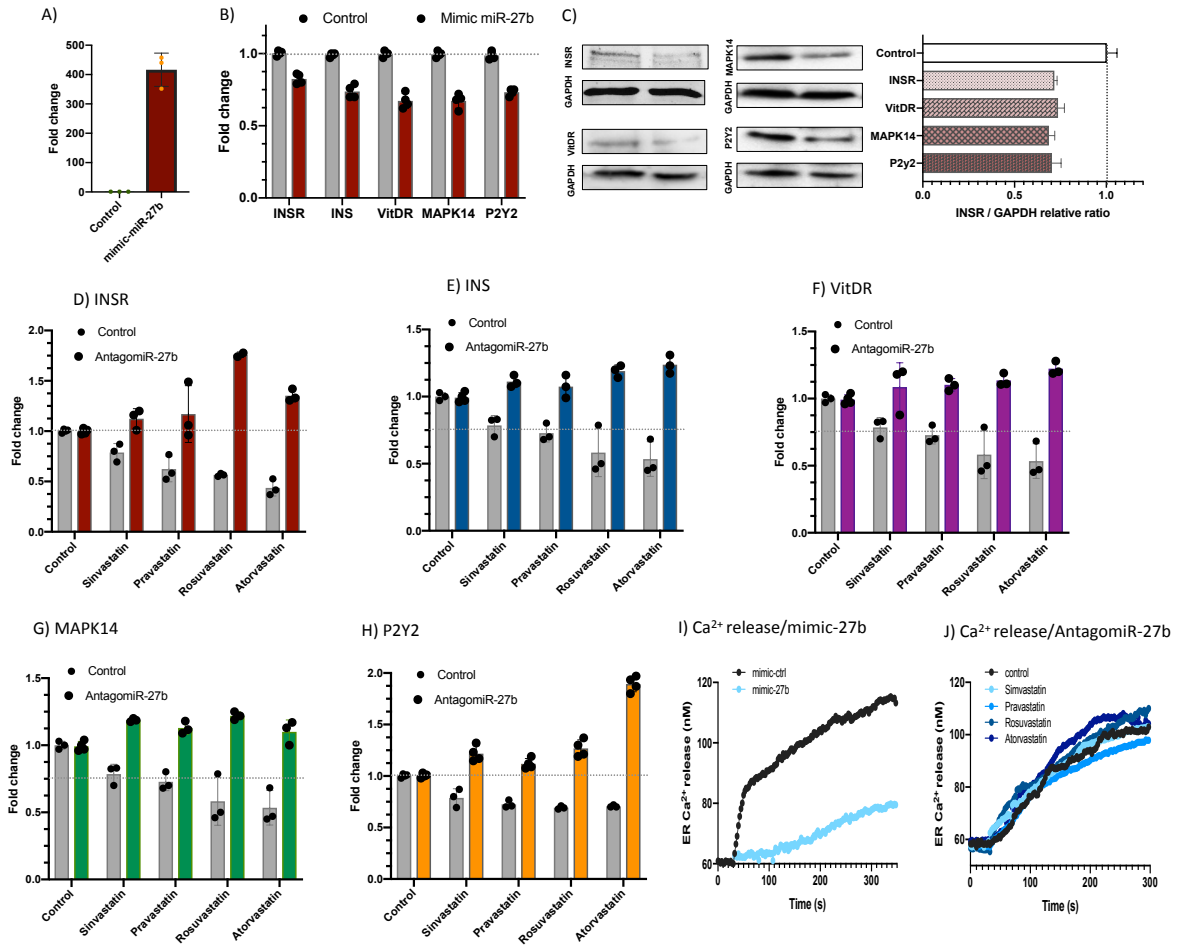

Figure 3: miR-27b modulation affects INSR, INS, VDR, MAPK14 and P2Y2 expression. (A) miR-27b levels in EndoC-βH cells transfected or not with mimic-miR-27b; (B) *INSR, INS, VDR, MAPK14* and *P2Y2* mRNA expression levels in EndoC-βH cells transfected with mimic-miR-27b; (C) INSR, INS, VDR, MAPK14 and P2Y2 protein expression levels in EndoC-βH cells transfected with mimic-miR-27b determined by Western blot (left panel) and densitometry (right panel); (D-H) mRNA expression of *INSR, INS, VDR, MAPK14* and *P2Y2* recpetor in EndoC-βH cells treated with statins and antagomiR-27b; (I) activity of P2Y2 receptor in EndoC-βH cells transfected with mimic-miR-27b; (J) activity of P2Y2 receptor in EndoC-βH cells treated with statins and transfected with antagomiR-27b. * $p < 0.05$; * p< 0,25; * $p < 0.01$ (significantly different from control (non-treated, transfected with the control mimic or control inhibitor) cells. Data are presented as the mean ± SEM of at least $n = 3$ independent experiments (A-B, C right panel, D-J). Western blot images correspond to results from a representative experiment among three that gave similar results (C left panel).

2.4. miR-27b expression is regulated by c-Myc

miR-27b is a conserved miRNA encoded by the miR-23b-27b-24–1 cluster within the 14th intron of *C9orf3* gene, whose expression can be directly downregulated by c-Myc upon binding to the promoter region of miR-23b-27b cluster. It has been shown that c-Myc binds at two of its identified three putative

binding sites matching sequence at 4 kb, 13 kb and 25 kb upstream from the of miR-23b-27b cluster transcriptional starting site (Figure 4A).

To test whether c-Myc could be an upstream regulator of the observed miR-27b upregulation induced by statin treatment, we assessed c-Myc mRNA and protein levels in EndoC-βH cells treated with statins (24 h). As shown in Figure 4B, C, c-Myc expresion both at mRNA and protein was significantly diminished in the presence of statins. These results indicate a role of c-Myc in the miR-27b upregulation induced by statin treatment.

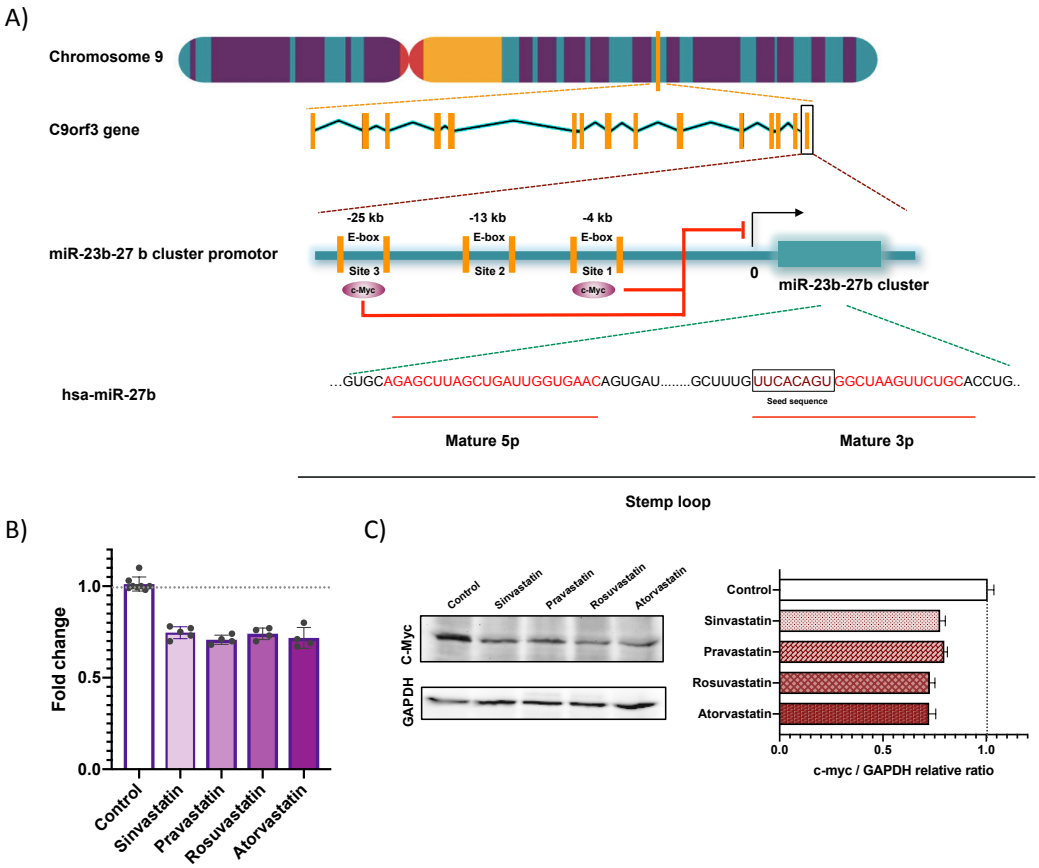

Figure 4: miR-27b expression is regulated by c-Myc. (A) Location of miR-23b-27b-24–1 cluster within the 14th intron of *C9orf3* gene in chromosome 9, c-Myc binding sites in the promoter of miR-23b-27b cluster and, mature sequences of miR-27b; (B) *c-Myc* mRNA levels in EndoC-βH cells treated for 24 h with statins; (C) c-Myc protein levels in EndoC-βH cells treated for 24 h with statins determined by western blot (left panel) and densitometry (right panel). * $p < 0.01$; (significantly different from control (non-treated) cells. Data are presented as the mean ± SEM of at least $n = 3$ independent experiments (B and C right panel). Western blot image correspond to results from a representative experiment among three that gave similar results (C left panel).

2.5. miR-27b expression is regulated by c-Myc

To ascertain if statin-induced miR-27b upregulation occurs *via* c-Myc dysregulation and, taking into account the previously demonstration that miR-33 binding site is present in c-Myc (Figure 5A), we next tested whether miR-33 could be an upstream regulator of c-Myc. It is well established that stains induce miR-33a (Figure 5B) [59].

As shown in Figure 5C, statin treatment of EndoC-βH cells induced miR-33a upregulation in a different extent (% miR-33a upregulation: simvastatin 30.5 ± 4; pravastatin 31.0 ± 3; rosuvastatin 49.6 ± 5 and, atorvastatin 65.9 ± 3). In addition to this, transfection of EndoC-βH cells with mimic-33a reduced the mRNA levels of *INSR, INS, VDR, MAPK14* and *P2Y2* receptor (Figure 5D) (% mRNA downregulation: simvastatin 45.1 ± 1; pravastatin 33.0 ± 9; rosuvastatin 57.5 ± 5 and, atorvastatin 50.2 ± 3).

In addition, transfection of EndoC-βH cells with mimic-33a caused a significant reduction in the mRNA expression levels of c-Myc ((% mRNA downregulation: 40.1 ± 3 compared to control) and a consequent upregulation of miR-27b expression levels (% upregulation: 54.6 ± 10 compared to control) (Figure5 E and F, respectively).

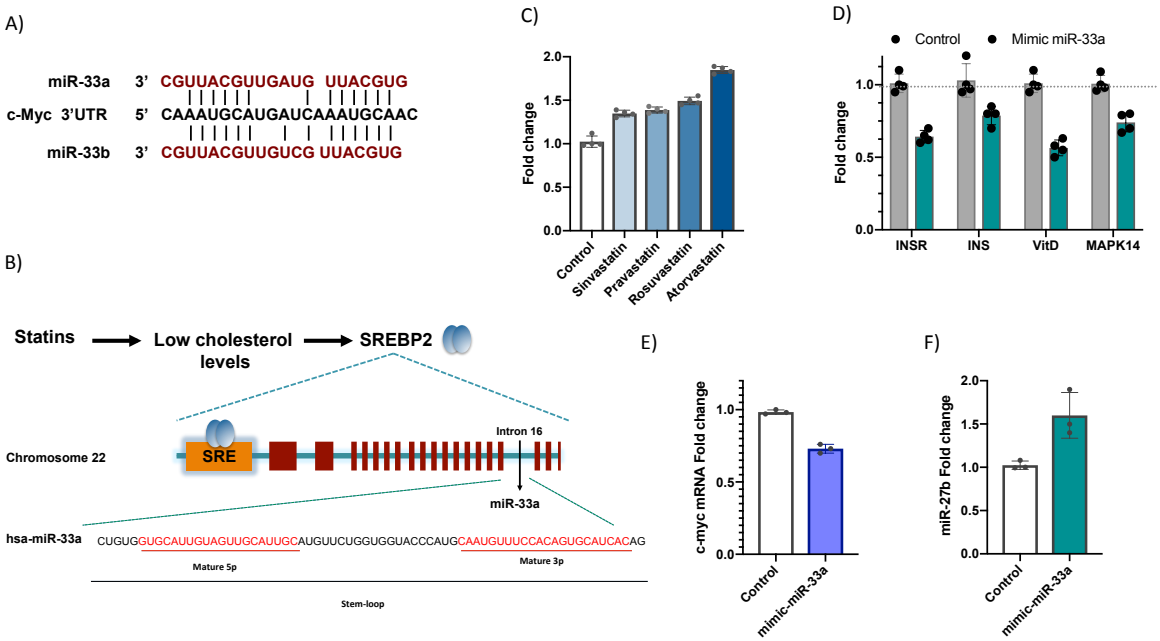

Figure 5: miR-33a modulates miR-27b levels through represssing c-Myc expression. (A) miR-33a and miR-33b binding site is present in c-Myc; (B) Regulation of miR-33a expression by statin treatment; (C) miR-33a levels in EndoC-βH cells treated for 24 h with statins determined by qRT-PCR; (D) *INSR, INS, VDR, MAPK14 and P2Y2 receptor* mRNA expression levels in EndoC-βH

cells transfected with mimic-miR-33a; (E) *c-Myc* mRNA levels in EndoC-βH cells transfected with miR-33a mimic determined by qRT-PCR; (F) *miR-27b* levels in EndoC-βH cells transfected with miR-33a mimic determined by qRT-PCR. * $p < 0.01$; (significantly different from control (non-treated) cells. Data are presented as the mean ± SEM of at least $n = 3$ independent experiments.

3. Discussion

In this study, we demonstrate that statins promote a coordinated microRNA upregulatión in β-cells, which attenuates expression of key genes involved in insulin signalling, insulin secretion and β-cell survival. These findings provide novel mechanistic insight into how statin treatment might regulate the progression of T2DM and suggest that increased levels of miR-33a and miR-27b might be involved in mediating some of the pleiotropic effects of statins accounting for the 12% increased risk of new-onset diabetes [8-10]. Here we have thus identified a molecular mechanism mediated by statins at the levels of upstream *INSR*, *INS*, *VDR*, *MAPK14* and *P2Y2 receptor* in β-cells. We have discovered that reduction of cholesterol concentration mediated by statins increases miR-33a expression, which causes *c-Myc* repression. The low levels of c-Myc in turn, promote miR-27b upregulation, which results in diminised expresion of INSR, INS, VDR, MAPK14 and P2Y2 receptor. Therefore, the results presented here suggest a mechanistical crosstalk that links statin treatment, miR-33, c-Myc and miR-27b to ultimately modulate the expression of key genes that are involved in T2DM and insulin resistance development. The sequential events that regulate this process could be mediated by the mechanisms that are discused below.

It has been well described that statin inhibition of HMG-CoA reductase activity leads to a decrease in intracellular cholesterol content that stimulates maturation of SREBP (sterol regulatory element binding protein) transcription factors [60, 61]. SREBPs reside in the endoplasmic reticulum (ER) as membrane intrinsic, inactive precursors [61] that are transported to the Golgi upon lowering the intra-membrane cholesterol levels [60] where proteases release the transcriptionally active portion [62]. The control of SREBPs activity is complex and responds to a variety of metabolic signals. SREBP-2 is tightly linked to cholesterol synthesis, whereas the SREBP-1a/c isoforms have broader roles [63]. SREBP-2 activates the transcription of genes involved in cholesterol homeostasis, such as HMG-CoA reductase, which catalyses a rate-limiting step in cholesterol biosynthesis, and the LDLr, which imports cholesterol from the blood [62-64]. Profiling approaches from several groups identified miR-33a as a miRNA that regulates cholesterol homeostasis, which is encoded at the 16th intron of the *SREBP2* [65, 66]. Therefore, under low sterol conditions *miR-33a* is

co-regulated with its host gene *SREPB2* [65-68] and works to increase cellular cholesterol levels by reducing cholesterol efflux through the inhibition of the ATP binding cassette (ABC) transporters ABCA1 and ABCG1, as well as the endolysosomal protein Niemann-Pick C1 (NPC1) [65-68]. As shown by the data presented here, treatment of EndoC-βH with statins induces the expected miR-33a upregulation triggered by the lowering of cholesterol levels mediated by HMG-CoA reductase activity.

Very interestingly, it has been described that overexpression of miR-33a and miR-33b modulates negatively c-Myc expression, a transcription factor that has been implicated in the regulation of up to one-third of human genes [69, 70]. Downregulation of c-Myc occurs through direct binding of miR-33 to the 3'UTR of the c-Myc mRNA (positions 80–86 and 92-97; in the inset of Fig. 5) [59, 71]. Here we show that upon statin treatment c-Myc levels are downregulated and that this effect is rescued by antagomiR-33a and stimulated by miR-33a mimic in the absence of statins in EndoC-βH cells (Figure 5). These results indicate that the c-Myc modulation by miR-33 already described in other cell types also occurs also in β-cells.

The bioinformatics analysis performed in this study pointed out miR-27b as potential microRNA with high probability to target genes involved in insulin signaling, secretion and cell survival. miR-27b is located in the miR-23b-27b-24-1 cluster, whose expression is well reported to be negatively regulated by c-Myc, at the transcriptional level via binding to the enhancer sequences (E-box) in the promoter region [72]. This previously described mechanism by others is very well corroborated with the results presented here. We show that statin treatment induces a decreased c-Myc expression and an increased miR-27b levels in EndoC-βH cells. Therefore, the strong possibility of inverse correlation between the expression of miR-27b and c-Myc in statin-treated cells was further confirmed by modulating the expression of c-Myc through its downregulation by miR-33 mimics (Fig. 5). These experimental evidences related to miR-27b modulation by the binding of c-Myc to the promoter region of miR-23b-27b-24-1 indicate its role in the development of T2DM associated to statin treatment.

Being the focus of interest of this work the elucidation of the mechanism that links statin therapy with diabetes, we performed a gene expression analysis on the genes that were predicted to be target of miR-27b: *INSR, INS, VDR, MAPK14* and *P2Y2 receptor* (Figure 2). Consistent with the *in silico* predictions, we find a significant reduction in the expression of all different predicted target genes.

Previous studies have shown that *INSR* and *INS1* are metabolic target of miR-27b in several cell models and mice [56, 73], thus indicating that miR-27b affects modulation of insulin signaling. Here we show that statin-induced upregulation of miR-27b diminishes INSR expression affecting therefore the insulin signalling in EndoC-βH cells (Figure 3). This effect was further corroborated by using a miR-27b mimic, which reduced INSR expression to a similar extent than the observed with statin treatment and, the INSR expression was rescued by transfecting EndoC-βH cells with antagomiR-27 in the presence of statins (Figure 2). In addition, statin treatment reduced significantly INS expresion, in agreement with previously reported works using *in vivo* and *in vitro* models [74]. Although the described mecanisms that induce INS downregulation are different, here we show that INS expression is negatively modulated by miR-27b (Figure 3).

VDR is another gene whose modulation has been shown to be downregulated in β-cell in the pathophysiology of T2DM. VDR is a receptor that belongs to the steroid hormone receptor superfamily, which is expressed in β-cell [75] and also in several insulin-responsive metabolic tissues, as the liver, skeletal muscle, or adipose tissue, where improves insulin sensitivity of these tissues [76]. It has been shown that deficit in VDR is associated with impaired glucose tolerance, defective insulin secretion, and a reduction in insulin mRNA content, suggesting that VDR is a key factor in β-cell function [77]. Here, we also determined that the statin-induced miR-27b upregulation diminished significantly the expression of VDR in β-cell (Figure 1). We clearly demonstrate that VDR expression in β-cells is downregulated by miR-27b as similar effects are obtained upon cell transfection with miR-27b mimic and reversed with antagomir transfection in statin treated cells (Figure 3).

VDR deficiency may have an impact on β-cell function alterations during T2DM as indicated by studies in mice lacking functional Vdr, which show a reduction in INS mRNA levels and a deficit in insulin secretion [77]. In accordance with this, it has been determined a decrease in Ins gene expression levels parallel to Vdr reductions in islets from diabetic mice [75]. These results suggest that VDR may be involved in *Ins* transcription in mouse pancreas [78]. However, so far, no vitamin D response elements have been identified in the human or mouse insulin gene promoters. In addition to INS expression, it has been shown that Overexpressing VDR presented a preservation of β-cell mass in streptozotocin-induced diabetic mice [75]. In agreement with these results, it has been reported that VDR activation induces β-cell replication [79]. VDR overexpression

partially protected transgenic mice with induced experimental diabetes from islet inflammation by reducing the expression of inflammatory markers in islets [75]. In contrast, knockdown of VDR led to an increased cytokine-induced cell death in human β-cell-like cells and to a reduction in the expression of key β-cell genes in cytokine-treated rat β-cell INS1 [79]. These data suggest that VDR may reduce the inflammatory milieu in islets and therefore maintain β-cell mass and function resulting in a protection against diabetes development.

Besides the possible negative effects on β-cell survival that can be mediated by statin-induced downregulation of VDR, the adverse side effects induced by statin-treatment could be potentiated by the diminished MAPK18 expression described in this work. Among MAPK14 activities, which include to promote phosphorylation of hsp25, MAPKAP-K2, MSK1, ATF2 and CREB [80, 81] or to control caspase-3 activity [82], it has been shown that inhibition of MAPK14 results in a significant decrease in *in vitro* cell proliferation [83]. Similarly than the observed effects with VDR expression in the presence of statins, MAPK14 expression was also downregulated by miR-27b as similar effects to those obtained after statin treatments were observed miR-27b mimic transfected cells and reversed with antagomir transfection in statin treated cells (Figure 3).

Finally, as P2Y2 receptor was also predicted to be one of the miR-27b targets by *in silico* analysis, we studied the receptor expression levels and its activity in β-cell treated or not with statins. $P2Y_2$ is a G protein-coupled receptor that selectively responds to exogenous ATP or UTP, UDP [84, 85], as well as their metabolites, but not to ADP [86]. It is well established that P2Y receptors potentiate insulin secretion by a mechanism that involves metabolism of glucose, rise in intracellular Ca^{2+} and possibly cAMP. Activation of $P2Y_2$ receptor results in phosphoinositide hydrolysis (IP_3), which triggers the release of Ca^{2+} from the ER, and diacylglycerol (DAG) ensuing activation of protein kinase C (PKC) among others molecular events [87, 88]. The increase in the cellular Ca^{2+} mediated by P2Y2 receptor is a triggering event that leads to exocytosis of secretory vesicles containing insulin [89]. The results presented here show that P2Y2 receptor is under the expression of miR-27b and the upregulation of this microRNA by statin treatment diminishes P2Y2 receptor expression and consequently its activity (Figure 1). The direct regulation of the recptor by miR-27b was further corroborated by the effect of transfecting β-cells with miR-27b mimic, which significantly inhibited P2Y2 receptor expression and activity (Figure 1), or by transfecting statin-treated β-cell with miR-27b antagomir, which reversed the statins effects.

Statin pleiotropy remains a hot topic for debate due the pathophysiological implications of blockade of mevalonate with multiple mechanisms of cellular physiology. The term "Pleiotropy", originated from the Greek words "pleion," which means more, and "tropy" meaning response or stimuli, is perfectly illustrated by the great scope in the modulation of different gene targets induced by statin-treatment in β-cell *via* overexpression of mir-33a. Thus, statin pleiotropy as a consequence of miR-33 upregulation represents a perfect example of how pleiotropic effects exert modulation of multiple activities through a single gene activity. As mentioned above and in order to summarize the results presented in this work, the statin-mediated lowering of cholesterol concentration in β-cell leads to an upregulation of miR-33a expression (Figure 6). Consequently, c-Myc expression is downregulated and, in turn, the repression that exerts in the promotor of miR-23b-27b-24–1 cluster is no longer so effective thus miR-27b upregulation is promoted. The sequential activation of miR-27b through miR-33 modulation results in diminished expression of INSR, INS, VDR, MAPK14 and P2Y2 receptor hence interfering with their physiological signalling (Figure 6).

In conclusion, the data presented herein tackle statins potential in interfering β-cell metabolism by cooperative mechanisms with the ultimate effect of promoting a T2DM-like cellular milieu (Fig. 6) and, expand our current understanding of molecular mechanism that might underlie diabetogenic effects of statins.

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CONCLUSIONS

According to the obtained results we propose the following conclusions:

PART I: FAMILIAL HYPERCHOLESTEROLEMIA: PHATOPHYSIOLOGY, DIAGNOSIS AND TREATMENT

1. The amino acid alteration in twelve LDLR variants, p.(Gly20Arg), p.(Gln92Glu), p.(Gln378Pro), p.(Ala399Thr), p.(Thr413Met), p.(Ile473Val), p.(Ala606Ser), p.(His656Asn), p.(Thr659Asn), p.(Ala606Ser), p.(His656Asn), and p.(Ile764Thr) does not cause loss of receptor activity thus the mutations do not account for the clinical presentation of FH. Consequently, the cause of FH in patients carrying these variants should be further investigated in order to identify a cause lipidemia.
2. Five LDLR variants, p.(Ser584Pro), p.(Asp601Val), p.(Asp622Gly), p.(Cys698Tyr) and p.(Asp707Tyr) Belong to Class 2 LDLR variants, which are retained completely in the endoplasmic reticulum thus causing a severe FH phenotype.
3. Eight LDLR variants, p.(Cys184Tyr), p.(Gly207_Ser213del), p.(His211Asp), p.(Asp221Tyr), p.(Glu288Lys), p.(Ser326Cys), p.(Cys338Phe) and p.(Cys368Tyr) are Class 3 LDLR variants with different affinities for LDLR thus leading to an intermediate FH phenotype.
4. Four variants, p.(Asp492Asn), p.(Gly592Glu), p.(Arg633Cys) and p.(Asp700Gly), are Class 5 LDLR variants affecting negatively to receptor recycling thus causing FH.
5. We characterize the six novel ApoB100 pathogenic variants, p.(Ser3801Thr), p.(Thr3785Ile), p.(Thr4179Ser) , p.(Ile3696Thr),p.(Ala2790Thr) with defective binding to the LDLR thus causing FH.
6. We characterize one loss of function PCSK9 variant, p.(Gly394Ser), which leads to hypocholesterolemia due its inability to bind LDLR thus maintain high levels of LDLR at plasma membrane.

PART II: UNRAVELLING THE EPIGENETIC NETWORKS LEADING TO STATIN-INDUCED TYPE 2 DIABETES MELLITUS

1. The upregulation of miR-27b induced by statins constitutes an epigenetic mechanisms that might induce T2DM progression.
2. Statin-treatment induces the upregulation of mir-27b, which leads to a direct downregulation of INSR, INS, VDR, MAKP14 and P2Y2 receptor though interaction with their 3'UTR sequences.
3. The mechanism by which statins may contribute to the development of T2DM is initiated by the lowering of cholesterol levels, which induces SREBP-2 expression and consequently miR-33a overexpression. The targeting of miR-33a to the 3'UTR of c-Myc leads to a diminished repression of the cluster miR23b/miR27-B and consequently miR-27b expression is upregulated.

PUBLICATIONS

The results presented in PART I have been published at:

1. **Benito-Vicente A, Uribe KB, Jebari S, Galicia-Garcia U, Ostolaza H, Martin C. Familial Hypercholesterolemia: The Most Frequent Cholesterol Metabolism Disorder Caused Disease. Int J Mol Sci. 2018. 19(11):3426. doi: 10.3390/ijms19113426.**

2. **Unai Galicia-Garcia, Jone Amuategi, Shifa Jebari, Asier Larrea-Sebal, Kepa B Uribe, Helena Ostolaza, César Martín, Asier Benito-Vicente. Hiperkolesterolemia Familiarra: Patofisiologia, Diagnostikoa eta Tratamendua. OSAGAIZ: OSASUN-ZIENTZIEN ALDIZKARIA.2020.** 4, zk: 1.

3. **Galicia-Garcia U, Benito-Vicente A, Uribe KB, Jebari S, Larrea-Sebal A, Alonso-Estrada R, Aguilo-Arce J, Ostolaza H, Palacios L, Martin C. Mutation type classification and pathogenicity assignment of sixteen missense variants located in the EGF-precursor homology domain of the LDLR. Sci Rep. 2020. 10(1):1727. doi: 10.1038/s41598-020-58734-9.**

4. **Ana Catarina Alves, Unai Galicia-Garcia, Sílvia Azevedo, Asier Benito-Vicente, Rafael Graça, Patrícia Barros, Peter Jordan, Cesar Martin, and Mafalda Bourbon. LDLR variants functional characterization improves variant classification. (Human Molecular Genetics: sent December 2020).**

5. **Galicia-Garcia U, Benito-Vicente A, Giacobbe C, Guardamagna O, Gentile M, Fortunato G, Martín C, Di Taranto MD. Identification and in vitro characterization of six new APOB100 variants found in Italian patients with Familial Hypercholesterolemia (under preparation).**

6. **Galicia-Garcia U, Benito-Vicente A, Giacobbe C, Guardamagna O, Gentile M, Fortunato G, Martín C, Di Taranto MD. Identification and in vitro characterization of one new PCSK9 variant found in Italian patients with Familial Hypercholesterolemia (under preparation).**

The results presented in PART II have been published at:

1. **Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, Ostolaza H, Martín C.** Pathophysiology of Type 2 Diabetes Mellitus. **Int J Mol Sci. 2020 Aug 30;21(17):6275. doi: 10.3390/ijms21176275.**
2. **Galicia-Garcia U, Jebari S, Larrea-Sebal A, Uribe KB, Siddiqi H, Ostolaza H, Benito-Vicente A, Martín C.** Statin Treatment-Induced Development of Type 2 Diabetes: From Clinical Evidence to Mechanistic Insights. **Int J Mol Sci. 2020 Jul 2;21(13):4725. doi: 10.3390/ijms21134725.**
3. **Deciphering the complex epigenetic networks that orchestrate statin-mediated type 2 diabetes mellitus development: roles of miR-27b and miR-33. Galicia-Garcia U, Benito-Vicente A, Gonzalez-Moro I, Jebari S, Larrea-Sebal A, Uribe KB, Aspichueta P, Marino A, Ostolaza H, Cenarro A, Civeira F, Suarez Y, Fernández-Hernando C, Santín I, Martín C. (Under preparation 2021)**