eman ta zabal zazu

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Characterization of blood markers and their implication in human aging

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Izan zirenei,
direnei,
izango direnei.

Laburpena

Zahartzea definitzean, gure gorputzak denboran zehar jasaten duen aldaketa multzoa dela esaten dugu, gaitasun funtzionalaren galera eta baita gaixotasunekiko eta kanpoko erasoekiko zaurgarritasun handiagoa dakarrena.

Bizi kalitatearen hobekuntzei eta azken hamarkadetan medikuntzan eman diren aurrerapen handiei esker, adinekoen kopurua nabarmen igo da. Batez besteko bizi-itxaropenaren hazkuntza honen eta jaiotza-tasaren beherakadaren ondorioz, biztanleria zentsu gero eta desorekatuagoa daukagu. Gainera, bizi-itxaropena igo den arren, osasuntsu bizi garen urte kopurua ez da hazi eta, beraz, luzeago bizi gara, baina ez hobeto. Horrenbestez, zahartzea orokorrean, eta menpekotasuna bereziki, gure gizartearen erronka bihurtu dira, eta gero eta baliabide gehiago bideratzen dira hauetara. Gauzak horrela, esparru honetan burutzen diren ikerketa medikuen helburu nagusia menpekotasuna murriztea izan behar da, zahartze osasuntsu bat lortzeko.

Zahartze osasuntsuaren bidean, hauskortasun kontzeptuarekin egiten dugu lan. Hauskortasuna adinarekin erlazionatutako sindrome medikoa da, eta bere ezaugarri nagusiak erreserba funtzionalaren galera, sistema fisiologikoen egokitze gaitasun murriztua eta zaurgarritasunaren areagotzea dira. Hauskortasuna hainbat ondorio negatibo garatzeko arriskuarekin erlazionatuta dago, hala nola, erorketak, hausturak, infekzioak, desgaitasuna, ospitalizazioa, menpekotasuna eta heriotza. Beraz, hauskortasuna daukaten pertsonen identifikazioa beharrezkoa da neurriak hartu ahal izateko eta etorkizuneko arazoak saihesten saiatzeko. Baina, orain arte ezin izan da hauskortasuna detektatzeko metodo eraginkorrik garatu, eta hainbat test eta proba funtzional badauden arren, hauek ez dira gai pertsona hauskor guztiak identifikatzeko. Gaur egun eskuragarri dauden tresnak osatu eta emaitza hobeak lortzeko asmoz, hauskortasunaren biomarkatzaileak aurkitzeko ikerketak burutzen ari dira, aurrerago eztabaidatuko dugun moduan. Biomarkatzaileen bilaketa azaldu aurretik, ordea, zahartze prozesuaren oinarri biologikoa laburtuko dugu.

Zahartzea prozesua unibertsala, progresiboa eta heterogeneoa da, eta beraz, modu oso desberdinetan garatzen da. Zahartzearen jatorri molekularrak eta zelularrak ulertzea biologiaren erronka nagusietako bat da, eta helburu horrekin lan ugari egin dira. Ikerketa horiei esker, orain dela urte batzuk zahartzearen 9 ezaugarri nagusiak zehaztu ziren, ondorengoak direlarik: ezegonkortasun genomikoa, aldaketa epigenetikoak, telomeroen laburtzea, disfuntzio mitokondrialak, zelula amen agortzea, zelulen seneszentzia, zelulen arteko komunikazio desberdina, proteostasiaren galera eta mantenugaien hautemate

okerragoa. Hauetariko ezaugarri bakoitza sakonki ikertu da komunitate zientifikoan, baina, jarraian tesi honetan landu direnak azalduko ditugu laburki.

Zahartzaroarekin gertatzen den ezegonkortasun genomikoa, neurri batean, DNAren egituran aldaketak metatzeari zor zaio, hala nola mutazioak, delezioak edo DNAren hausturak harizpi batean edo bietan. Zelulek badauzkate akats horiek kontrolatzeko eta konpontzeko mekanismoak, baina hauek ere adinarekin huts egiten dutela ikusi da. Bestalde, geneen adierazpena aldatu egiten da zahartzean, eta hau beste gene batzuen adierazpena erregulatzen duten transkripzio faktoreetan gertatzen bada, eragina are handiagoa izango da. Gene ez kodifikatzaileen adierazpena ere aldatu egin daiteke, eta transkripzio osteko erregulazioan eragina eduki, mikroRNA molekulen kasuan bezala.

Seneszentziak zelulen proliferazioaren galera dakar, jarduera metabolikoa, bideragarritasuna eta haien berezko funtzio batzuk mantentzen dituzten bitartean. Zelula kaltetu eta potentzialki arriskutsuak izan daitezkeenak seneszentzian sartzea defentsa mekanismoa dela deskribatu da, hauen proliferazioa ekiditen baita. Era berean, badirudi zelula seneszenteek jariatutako seinaleek ehunen birsorkuntzan laguntzen dutela: zelula seneszenteen digestioa eta zelula amen diferentziazioa sustatzen dute, zelula berri helduek ehuna berritzen dutelarik. Adinean aurrera egin ahala, ordea, prozesu honek ere huts egiteko joera dauka, zelula seneszenteak pilatu egiten dira eta kalteak eragin ditzakete. Lan honetan bereziki immunitate-sistemaren seneszentzian (immunoseneszentzian) zentratu gara, adineko pertsonen zaurgarritasunean eragina baitauka.

Aztertu dugun beste ezaugarri bat zelula amen agortzea da. Hau oso lotuta dago seneszentziarekin, izan ere, ehunen birsorkuntza ezin da osatu zelula amarik ez badago edo behar bezala diferentziatzen ez badira. Zelula amak kieszentzia egoeran mantentzen dira eta beharrezkoa denean bakarrik aktibatu, proliferatu eta diferentziatzen dira. Gainera, zelula amek auto-berrikuntza gaitasuna dute, zelula alabetako bat zelula ama moduan mantendu eta bestea bakarrik diferentziatzen bada. Baina zelula amen agortzea zelulak simetrikoki banatzen direnean eman daiteke, edo zahartzearekin lotutako arazoak direla medio kieszentzia egoeratik irten eta aktibatzea lortzen ez dutenean. Izan ere, kieszentziatik irtetzeko prozesua konplexua da eta oso erregulatuta dago, eta zenbait egoerek, hala nola, telomeroen laburtzeak, estres oxidatzaileak edota DNAn eman diren kalteek prozesua eragotzi dezakete.

Azkenik, zahartzaroan ematen den zelulen arteko komunikazio desberdinaren inguruan ere ikertu dugu. Zahartzaroko ezaugarri hau bereziki garrantzitsua da guretzat bi arrazoirengatik. Alde batetik, zahartzerakoan ematen diren aldaketek inflamazio basal kronikoa garatzera eramaten baitute, *inflammaging* izenez ezagutzen dena. Inflamazioa sustatzen duten molekula

hauen jariatzea hainbat zelula motek burutzen dute, eta adinarekin ematen den immunitate-sistemaren funtzionamendu txarra areagotzen dute. Bestalde, zelulaz kanpoko besikulak (EVak, ingeleseko laburdurarekin) dauzkagu, zelula mota gehienek ekoiztu eta jariatzen dituzten partikulak eta zelulen arteko komunikaziorako bide bat direnak. EVak duela 50 urte baino gehiago aurkitu ziren, baina hasieran ez zitzaien garrantzirik eman eta zelulen "zaborra" ateratzeko" modu bat zela pentsatu zen. Azken hamarkadetan hauen funtzioak sakonago aztertzen hasi zen, eta oraindik ere, urtero EVen funtzio berriren bat deskribatzen da. Hala ere, zahartzearen ezaugarri nagusien artean zelulen arteko komunikazio desberdina izendatu zenean, ez ziren EVak kontuan hartu, eta horregatik zahartze prozesuan daukaten inplikazioa erakusten jarraitu beharra daukagu.

Hauskortasun sindromearen biomarkatzaileen gaiari berriro eutsita, lan asko egin izan dira zahartzearen ezaugarri orokorrak hauskortasunarekin erlazionatzen saiatzeko. Ikerketa hauen hipotesia zera da: adinarekin aldaketa edo prozesu kaltegarriak gertatzen dira, eta hauek neurri handiago batetan eman daitezke hauskortasuna edo menpekotasuna pairatzen duten pertsonetan, gaitasun funtzionala mantentzen dutenetan (sendoetan) baino. Ildo horretatik, inflamazioa edo estres oxidatzailearekin lotutako molekulen igoerak, eta hormonen edo metabolismoaren erregulazio galerak hauskortasunarekin izan ditzaketen loturak aztertu dira, adibidez. DNA sekuentzia espezifikoek, geneen erregulazioak edo geneen adierazpen aldaketek hauskortasuna garatzeko joerarekin izan ditzaketen erlazioak ere ikertu izan dira. Biomarkatzaile hauei eta bestelako batzuei buruzko artikulu zientifiko ugari kaleratu dira. Horietako batzuek hauskortasunarekin edo hau garatzeko arriskuarekin harremanak aurkitu dituzte, baina badaude ere proposatutako biomarkatzaileak balioztatu ez dituzten ikerketak, eta horregatik, gaur egun oraindik ez da hauskortasunaren identifikazioan lagundu dezakeen biomarkatzailerik aplikatzen praktika klinikoan.

Gauzak horrela, tesi honen lehenengo kapituluan hauskortasun biomarkatzaileak aurkitzen saiatu gara. Horretarako, adineko pertsonen odol laginak eskuratu ditugu, eta hauek sendo eta hauskor taldeetan banatu ditugu test desberdinetan lortutako emaitzen arabera. Lehenik, inflamazioaren ezaugarri diren molekulak neurtu ditugu. Hauekin emaitza positiboak lortu izan dira aurreko ikerketa batzuetan, baina badaude hauskortasunarekin erlaziorik aurkitu ez duten lanak ere, eta horien antzera, gure laginetan ez dugu inflamazio molekulen kontzentrazio handiagorik topatu pertsona hauskorretan. Gure bigarren estrategia azterketa transkriptomikoa egitea izan da. Horren bidez, pertsona sendoak eta hauskorrak bereizten dituzten 35 gene identifikatu ahal izan ditugu. Hasteko, gene horietako 3ren adierazpena neurtu dugu kohorte zabalago batetan eta EGR1 genearen gainadierazpena balioztatu dugu pertsona hauskorretan. EGR1 prozesu zelular garrantzitsuetan parte hartzen duen

transkripzio faktorea da, hala nola mitogenoekiko erantzunean, proliferazioan, apoptosian eta hainbat zelula moten diferentziazioan, eta estimulu desberdinen ondorioz aktibatua izan daiteke. Gainera, adineko pertsonen hauskortasun egoerari buelta eman edo murrizten saiatzeko esku-hartze bat burutu da, eta 3 hilabetez ariketa fisikoa egin dute partaideek. Esku-hartzearen ondoren, 12 partaideetatik 9k EGR1 genearen adierazpena jaitsi dutela aurkitu dugu. Emaitza nabarmen hauekin, EGR1en adierazpen altua hauskortasun biomarkatzaile bezala proposatzen dugu, eta etorkizuneko ikerketetan kontuan hartu beharrekoa dela deritzogu.

Hauskortasun biomarkatzaileen ikerketan, EVen kontzentrazio plasmatikoa izan da gure azken hurbilketa. Izan ere, zenbait inflamazio prozesuetan, minbizia eta gaixotasun autoimmuneak esaterako, EVen kontzentrazio altua aurkitu da, eta gure helburua hau adinean ematen den inflammaging-arekin ere gertatzen ote den aztertzea izan da. Gure emaitzek erakutsi dutenez, nahiz eta inflamazio basala adineko pertsonetan konfirmatu den, hauek ez dute EV kontzentrazio plasmatiko handiagorik, eta ez dago ezta ere sendo eta hauskorren arteko diferentziarik. Emaitza hauek adierazten dutenez, beraz, ez da EVen kontzentrazio igoera ematen inflamazio prozesu guztietan.

Lan honen bigarren kapituluan EVek zelulen diferentziazioan duten eragina aztertu dugu. Osteogenesi eta miogenesian zentratu gara, zahartzaroan kaltetutako prozesuak baitira eta osteoporosian eta sarkopenian eragina baitute, hurrenez hurren. Adinarekin ohikoak diren bi prozesu hauek ikuspegi desberdinetatik sakonki aztertu izan dira, baina oso gutxik ikertu dute EVen inplikazioa. Gantz ehunetik eratorritako zelula ama mesenkimalekin burututako gure lanak erakusten duenez, plasmako EVek osteogenesi prozesuan laguntzen dute, eta eragin hau nabarmenagoa da helduetatik eskuratutako EVekin, adineko emaileekin alderatuta. Era berean, plasmako EVek miogenesian duten eragina aztertzeko protokolo bat garatzen saiatu gara, eta lehen emaitzek adierazten dutenez, mioblastoen diferentziazio prozesua bultzatzen dute EVek, berriro ere adineko pertsonetatik eskuratuko EVek efektu murriztuagoa dutelarik.

Hirugarren kapituluan immunoseneszentziaren fenomenoari jarri diogu arreta, eta helduak (20-49 urte) eta pertsona adindunak (70-104 urte) aztertu ditugu. Lehenik, seneszentzia zelula mailan neurtu da, eta ikusi dugu CD8 T linfozito seneszenteen proportzioa hazi egiten dela adinarekin. Zahartzaroaren lehen hamarkadetan berdina gertatzen da CD4 T linfozitoetan, baina 90. urtetik gorako pertsonetan zelula seneszente mota honen kantitatea murriztuagoa dela aurkitu dugu. Ondoren, EVek linfozito seneszenteen ezaugarri berdinak erakusten ote dituzten aztertu dugu. Zelula hauen markatzaile berdinak EVen mintzean aurkitu ditugu, baina ez, ordea, “EV seneszenteen” igoerarik adinak aurrera egin ahala. Azkenik, linfozitoen eta EVen *in vitro* esperimentuak burutu ditugu, EVek zelula hauen aktibazioan eragina duten

ebaluatzeko. Emaitzek erakutsi dute EVek ez dela erantzun immunologikorik eragiten, eta are gehiago, zelulen bideragarritasuna hobetzen dutela. Baina, estimulu immunogeniko baten aurrean erantzuteko orduan, EVek T linfozitoen aktibazioa areagotzen dute. Gainera, aktibazioa areagotzeko gaitasun hau ahulagoa da EVak emaile zaharretatik isolatuak badira. Horrela, gure emaitzek adierazten dute, nahiz eta EVen mintzeko molekuletan desberdintasunik ez egon, EVek gaitasun funtzional desberdinak dauzkatela isolatu izan diren emailearen adinaren arabera.

Tesi honen laugarren eta azken kapituluan, immunoseneszentzia aztertu dugu esklerosi anizkoitza duten gaixoetan. Izan ere, proposatu izan da gaixotasun autoimmuneetan ematen den etengabeko immunitate-sistemaren aktibazioa dela eta azken honen agortzea eman daitekeela, eta ondorioz, immunoseneszentzia goiztiarra. Hipotesi hau ebaluatzeko, esklerosi anizkoitza duten pertsonen eta heldu osasuntsuen laginak alderatu ditugu. Esklerosi anizkoitza daukaten gaixoek, tratamendu immunomodulatzaileak hartzen eta erremisioan egon arren, pertsona osasuntsuek baino inflamazio markatzaile kontzentrazio altuagoak dauzkatela aurkitu dugu. Honek inflamazio basala daukatela adierazten du, inflammaging-arekin erlazionatuta egon litekeena. Bestalde, T linfozitoen analisian, esklerosi anizkoitza daukaten gaixoek zelula seneszente gehiagorik ez dutela ikusi dugu, baina gaixo hauen T linfozitoek erantzun desberdina daukate *in vitro* jasotako estimulu baten aurrean, aktibazio murriztuagoa erakusten dutelarik pertsona osasuntsuen zelulekin alderatuta.

Bukatzeko, laburpen modura, doktoretza tesi honek zahartze prozesuaren hainbat alderdiri buruzko ezagutza aurreratzen lagundu duela esan dezakegu. Hauskortasun biomarkatzaileak ikertu ditugu, EGR1 genearen adierazpena etorkizunean kontuan hartu beharreko markatzaile bezala proposatuz. Plasmatik isolatutako EVen ezaugarriak ere aztertu ditugu, osteogenesia eta miogenesia bezalako zelulen diferentziazio prozesuetan lagundu dezaketela erakutsiz, baita T linfozitoen aktibazioa sustatzen dutela estimulu immunogenikoen aurrean, eta EVen funtzio hauek zahartzearekin ahuldu egiten direla. Emaitza hauek guztiek erakusten digutenez, EVek paper garrantzitsuak betetzen dituzte hainbat prozesuetan, eta aintzat hartu beharko lirateke zahartzearen ezaugarri nagusietan. Azkenik, esklerosi anizkoitzaren inguruan burututako esperimentuek adierazten digute gaixotasun hau daukaten pertsonek inflamazio kronikoa badaukatela, eta immunoseneszentzia goiztiarra esklerosi anizkoitzean eta bestelako gaixotasun autoimmuneetan garatzen ote den ikertzen jarraitu behar dugula.

Resumen

El envejecimiento se define como el conjunto de modificaciones que sufre nuestro organismo con el tiempo y que resulta en una pérdida de capacidad funcional, así como una mayor susceptibilidad a enfermedades y vulnerabilidad frente a agresiones externas.

Gracias a la mejora de la calidad de vida y a los grandes avances de la medicina en las últimas décadas, el número de personas de edad avanzada ha incrementado de manera excepcional. Este aumento de la esperanza de vida media y el descenso de la natalidad están produciendo cambios profundos en la pirámide poblacional clásica del último siglo. Además, aunque la esperanza de vida es mayor, no se ha dado un incremento en los años de vida saludable y, por lo tanto, vivimos más pero no mejor. Como resultado, el envejecimiento en general y la dependencia en particular, se han convertido en retos sociales y sanitarios a los que cada vez se dedican más recursos. El principal objetivo de los proyectos de investigación sanitaria que se desarrollan en este ámbito debe ser siempre reducir la incidencia de dependencia para lograr un envejecimiento saludable.

En este contexto, se trabaja con el concepto de fragilidad. La fragilidad se considera un síndrome médico relacionado con la edad que se caracteriza por una pérdida de reserva funcional, una peor capacidad de adaptación de diversos sistemas fisiológicos y un aumento de la vulnerabilidad. La fragilidad resulta en un mayor riesgo de múltiples problemas como caídas, fracturas, infecciones, discapacidad, hospitalización, dependencia y muerte. La identificación de sujetos frágiles es por lo tanto una de las claves para poder tomar medidas a tiempo y tratar de revertir este estadio y futuros problemas. Pero, hasta la fecha, no se ha conseguido dar con un método eficaz de discriminación, y aunque existen diversos formularios y pruebas funcionales, estos no son capaces de reconocer a todos los sujetos frágiles. Para complementar las herramientas disponibles y conseguir mejores resultados, se está investigando la posibilidad de encontrar un biomarcador de fragilidad, como comentaremos más adelante. Pero para poder entender la búsqueda de biomarcadores, debemos primero, repasar la biología del envejecimiento.

El envejecimiento es un proceso universal y progresivo, que tiene una gran heterogeneidad y se desarrolla de maneras muy diferentes. Entender las causas moleculares y celulares del envejecimiento es uno de los retos centrales de la biología, y son muchos los trabajos que se han realizado con este objetivo. Gracias a estos estudios se han podido definir las principales características del envejecimiento: inestabilidad genómica, alteraciones epigenéticas, acortamiento de los telómeros, disfunción mitocondrial, agotamiento de las células madre, senescencia celular, comunicación intercelular alterada, perdida de la proteostasis y alteración

en la percepción de nutrientes. Cada una de ellas ha sido investigada en profundidad por la comunidad científica, pero a continuación vamos a describir brevemente las características sobre las que hemos trabajado en el presente estudio.

La inestabilidad genómica que se observa en el envejecimiento se debe, en parte, a la acumulación de cambios en la estructura del ADN, como mutaciones, deleciones o roturas en una o las dos cadenas de ADN. La célula dispone de mecanismos de control y reparación para estos fenómenos, pero se ha visto que los mecanismos también fallan con la edad. Además, la expresión génica se ve alterada en el envejecimiento, y si esto ocurre en factores de transcripción que regulan la expresión de otros genes, el efecto es todavía mayor. Por otro lado, también se puede ver afectada la expresión de genes no codificantes que influyen en la regulación postranscripcional, como en el caso de los microARNs.

La senescencia se define como la pérdida de capacidad proliferativa de las células, mientras estas mantienen actividad metabólica, viabilidad y algunas de sus funciones. La entrada en senescencia se ha descrito como un mecanismo de defensa que se activa en las células dañadas y potencialmente perjudiciales para evitar su proliferación. Asimismo, se ha visto que las señales producidas por las células senescentes inducen la regeneración de tejidos, un proceso en el que primero se produce la digestión de las células senescentes y después, la diferenciación de células madre a células adultas que renuevan el tejido. Con el envejecimiento este proceso tiende a fallar, las células senescentes se acumulan y terminan por contribuir al daño. En este trabajo, nos hemos centrado en la senescencia del sistema inmune (llamada inmunosenescencia), un fenómeno que incrementa la vulnerabilidad de las personas mayores.

Otra característica con la que hemos trabajado es el agotamiento de las células madre con la edad. Ese proceso está estrechamente relacionado con la senescencia, puesto que la regeneración de tejidos tampoco puede ser completada si no hay células madre o si no se diferencian de manera adecuada. Las células madre se mantienen en un estado de quiescencia y solo se activan, proliferan y diferencian cuando es necesario. Además, tienen capacidad de autorrenovación, si se dividen en una célula que mantendrá en nicho y en otra que se diferenciará. El problema puede surgir cuando las células madre se dividen y diferencian de manera simétrica y no mantienen el nicho, o cuando debido a problemas relacionados con el envejecimiento, como el acortamiento de los telómeros, el estrés oxidativo o el daño en el ADN no consiguen llevar a cabo el complejo proceso de salida de quiescencia y activación.

Por último, estudiamos la alteración de comunicación intercelular en el envejecimiento. Esta característica nos es de interés por dos razones diferentes. Por un lado, por los cambios que se dan al envejecer conducen a un estado de inflamación basal crónico que se conoce como

inflammaging, en el que la secreción de moléculas proinflamatorias es producida por una gran diversidad de células y que contribuye al malfuncionamiento del sistema inmune. Por otro lado, por las vesículas extracelulares (EVs, por sus siglas en inglés). Estas partículas son secretadas por la mayoría de los tipos celulares y participan en la comunicación intercelular. Fueron descubiertas hace más de 50 años y actualmente, se sigue profundizando en su importante papel en el envío y recepción de mensajes entre células y en las funciones biológicas en las que participan. Aun así, cuando se definió la comunicación intercelular alterada en el envejecimiento no se incluyó a las EVs, puesto que su estudio en ámbito del envejecimiento no estaba muy desarrollado, y debemos seguir profundizando en su implicación en los cambios que acontecen durante este proceso.

Volviendo a la búsqueda de biomarcadores de fragilidad, son muchos los trabajos que se han realizado para tratar de relacionar características del envejecimiento con el síndrome de fragilidad. La hipótesis de estos estudios es que si hay modificaciones o procesos perjudiciales que ocurren con la edad, y que éstos pueden tener más peso en los sujetos frágiles o que desarrollan dependencia que en personas que mantienen la capacidad funcional (robustos). En este sentido se ha investigado la posible relación de la fragilidad con un incremento de moléculas relacionadas con la inflamación o el estrés oxidativo y con desregulaciones hormonales o metabólicas. También con cambios a nivel génico como modificaciones en la regulación y expresión de genes, o con la incidencia de polimorfismos de nucleótido único que pudieran predisponer a la fragilidad. Aunque las publicaciones sobre estos y otros posibles biomarcadores son abundantes y han encontrado relaciones con la fragilidad o el riesgo de desarrollarla, también hay estudios que no han validado los biomarcadores propuestos y, a día de hoy, no se ha implementado en la clínica ninguno de ellos.

En el primer capítulo de esta tesis hemos tratado de identificar biomarcadores de fragilidad. Para ello hemos contado con muestras de sangre de personas de edad avanzada clasificadas como robustas o frágiles. Primero comparamos en nuestra cohorte los marcadores clásicos de inflamación que han sido estudiados en otros trabajos. En contra de lo indicado en algunos de ellos, no hemos encontrado un aumento de la concentración de estas moléculas inflamatorias con la fragilidad. Nuestra segunda estrategia ha sido la realización de un estudio transcriptómico, mediante el que hemos podido identificar 35 genes candidatos que están diferencialmente expresados entre robustos y frágiles. En una primera aproximación, hemos comparado la expresión de 3 de ellos en una cohorte más amplia, y hemos validado el aumento de expresión en sujetos frágiles del gen EGR1, un factor de transcripción implicado en importantes procesos celulares (respuesta a mitógenos, proliferación, apoptosis y diferenciación de varios tipos celulares, entre otros) y que puede ser activado en repuesta a

diversos estímulos. Además, se ha llevado a cabo un estudio piloto en el que se trataba de revertir o reducir la fragilidad mediante una intervención física de 3 meses, y hemos visto que en 9 de los 12 participantes se redujo la expresión de EGR1 después de los 3 meses de ejercicio físico. Por ello, proponemos la expresión elevada de EGR1 como un potencial biomarcador de fragilidad que debe seguir siendo estudiado en futuros experimentos.

Como última aproximación a la búsqueda de biomarcadores de fragilidad, investigamos la concentración de las EVs en plasma. Estudios previos han propuesto que la concentración de las EVs aumenta en procesos inflamatorios como el cáncer o las enfermedades autoinmunes, y por lo tanto nuestro objetivo era analizar si este aumento también sucedía en el *inflammaging*. Nuestros resultados han demostrado que la concentración plasmática de las EVs no incrementa en todos los procesos inflamatorios, puesto que los participantes de edad avanzada mostraban signos de inflamación basal crónica o *inflammaging*, pero no tenían mayores concentraciones de EVs que los adultos.

En el segundo capítulo de este trabajo hemos estudiado el efecto de las EVs en la diferenciación celular. Nos hemos centrado en la osteogénesis y la miogénesis, procesos que se ven afectados durante el envejecimiento y que pueden dar lugar a dos de sus principales problemas: la osteoporosis y la sarcopenia. Estos procesos han sido estudiados en profundidad desde diferentes perspectivas, pero son muy pocos los que han investigado la implicación de las EVs. Nuestro trabajo realizado en células madre mesenquimales derivadas de tejido adiposo indica que las EVs de plasma ayudan en el proceso de osteogénesis, y que este efecto es mayor aplicando EVs provenientes de donantes adultos, respecto a las provenientes de donantes de edad avanzada. De manera similar, hemos tratado de poner a punto un protocolo para testar el efecto de las EVs plasmáticas en la miogénesis, y nuestros resultados apuntan a una promoción del proceso de diferenciación en presencia de las EVs, que se ve mermado si las EVs son de personas de mayor edad. El trabajo en este campo parece prometedor, puesto que las EVs podrían ayudar a inducir la diferenciación y regeneración de tejidos, y se podrían aplicar con mayor seguridad y menos efectos secundarios que las terapias celulares.

En el tercer capítulo hemos centrado la pregunta en el fenómeno de la inmunosenescencia, y para ello hemos estudiado individuos adultos (20-49 años) y mayores (70-104 años). Primero se ha medido la senescencia a nivel celular, y hemos visto que las células T CD8 senescentes aumentan con la edad. La misma acumulación se da en las células T CD4, aunque los nonagenarios y centenarios muestran niveles menores de senescencia que los octogenarios en este subtipo de células. También hemos evaluado si las EVs mostraban las mismas características de senescencia que los linfocitos T. La identificación de los mismos marcadores nos indica que las EVs llevan en su membrana algunas de las moléculas características de las

células T, pero que no se observa un aumento de las "EVs senescentes" con la edad. Por último, hemos realizado experimentos de cocultivo de linfocitos y EVs para evaluar el efecto de estas últimas en la activación de las células. Los resultados obtenidos demuestran que la presencia de las EVs no induce respuesta inmune y, en cambio, mejora la viabilidad celular en cultivo. Pero, por otro lado, observamos que las EVs sí promueven la activación de los linfocitos T en presencia de un estímulo inmunogénico. Además, este efecto sobre la activación es mayor en presencia de EVs aisladas de donantes adultos que con las obtenidas de donantes envejecidos. Estos resultados nos indican que, aunque no haya diferencias en las moléculas de membrana estudiadas, las EVs provenientes de participantes de diferentes edades tienen capacidades funcionales distintas.

En el último estudio de esta tesis, que se presenta en el cuarto capítulo, hemos evaluado la inmunosenescencia en la esclerosis múltiple. Este estudio viene motivado por la hipótesis de que la constante activación del sistema inmune en las enfermedades autoinmunes puede llevar a un agotamiento prematuro del mismo y por tanto, a desarrollar una inmunosenescencia prematura. Para evaluar esta hipótesis hemos comparado muestras de pacientes adultos con esclerosis múltiple y donantes sanos de la misma edad. Aunque los pacientes estaban en remisión y bajo tratamientos inmunomoduladores, hemos detectado una mayor concentración de moléculas proinflamatorias en suero respecto a los donantes sanos, lo que indica la presencia de inflamación basal, que podría estar relacionada con el *inflammaging*. Al analizar las células T, hemos visto que los pacientes con esclerosis múltiple no tienen más células T senescentes que los controles sanos, pero, en cambio, sí muestran una activación menor que las células de controles sanos bajo el mismo estímulo en cultivos.

Finalmente, a modo de resumen podemos decir que la presente tesis ha contribuido al avance del conocimiento de diversos aspectos del envejecimiento. Hemos investigado los biomarcadores de fragilidad, proponiendo la expresión de EGR1 como un nuevo candidato que tener en cuenta. También hemos estudiado las características de las EVs aisladas de plasma, observando que pueden ayudar a la diferenciación celular en procesos como la osteogénesis y la miogénesis, así como que promueven la activación de las células T bajo estímulos inmunogénicos, y que estas capacidades de las EVs se ven reducidas con el envejecimiento. Estos resultados nos muestran que las EVs juegan un papel importante en diversos procesos y que deben ser incluidas dentro de las principales características del envejecimiento. Por último, nuestros resultados en esclerosis múltiple indican que los pacientes tienen una inflamación crónica y que tenemos que seguir estudiando la posible incidencia de una inmunosenescencia prematura en esta y otras enfermedades autoinmunes.

Table of contents

Abbreviations

7-AAD	7-aminoactinomycin D
ADL	Activities of daily living
ASC	Adipose tissue-derived stem cells
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BDNF	Brain-derived neurotrophic factor
circRNA	circular RNA
CNS	Central nervous system
CRP	C-reactive protein
CSHA	Canadian Study of Health and Aging
DHEAS	Dehydroepiandrosterone sulfate
EM	Electron microscopy
EV	Extracellular vesicle
FMO	Fluorescence minus one
GFST	Gerontopole Frailty Screening Tool
GS	Gait speed
HC	Healthy control
HSC	Hematopoietic stem cell
HSP	Heat shock protein
IGF-1	Insulin-like growth factor-1
IIS	Insulin and insulin-like growth factor 1
IL	Interleukin
MD	Myogenic differentiation
MHC	Major histocompatibility complex
miRNA	microRNA
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
mtDNA	mitochondrial DNA
MuSC	Muscle stem cell
NSC	Neural stem cell
NTA	Nanoparticle tracking analysis
OD	Osteogenic differentiation
PBMC	Peripheral blood mononuclear cell
PCA	Principal Component Analysis
PHA	Phytohemagglutinin
PRP	Platelet-rich plasma
ROC	Receiver Operating Characteristic
ROS	Reactive oxygen species
RT-qPCR	Quantitative real-time PCR
SASP	Senescence-associated secretory phenotype
sncRNA	small non-coding RNA
SNP	Single nucleotide polymorphism
SPPB	Short Physical Performance Battery
TAC	Transcriptome Analysis Console
TFF	Tangential flow filtration
TFI	Tilburg Frailty Indicator
TNF-α	Tumour necrosis factor alpha
TUG	Timed up-and-go
UPS	Ubiquitin-proteasome system

INTRODUCTION

1. Aging

The process of getting old affects each of us and our society as a whole. Aging is defined as the accumulation of time-related modifications that lead to decreased functional capacity, as well as increased susceptibility and vulnerability to disease or external insults [1]. It is a universal, complex and heterogeneous process. The present work is focused on biological aspects of aging, but we consider we should first outline some general characteristics to better understand the implications and motivations of our research.

Regarding demographics, a dramatic change in the proportions of young and elder people is observed in most of the countries. On one side, the improvements in social, medical and economic conditions have resulted in reduced mortality, better quality of life and consequent increase in life expectancy. On the other side, the reduced birth rate contributes to the overall increase of the population age. In **Figure 1** the population pyramids of the European Union in 2003 and 2018 are depicted, showing a clear drop of the inhabitants younger than 44 years and a rise of the ones over 45 years.

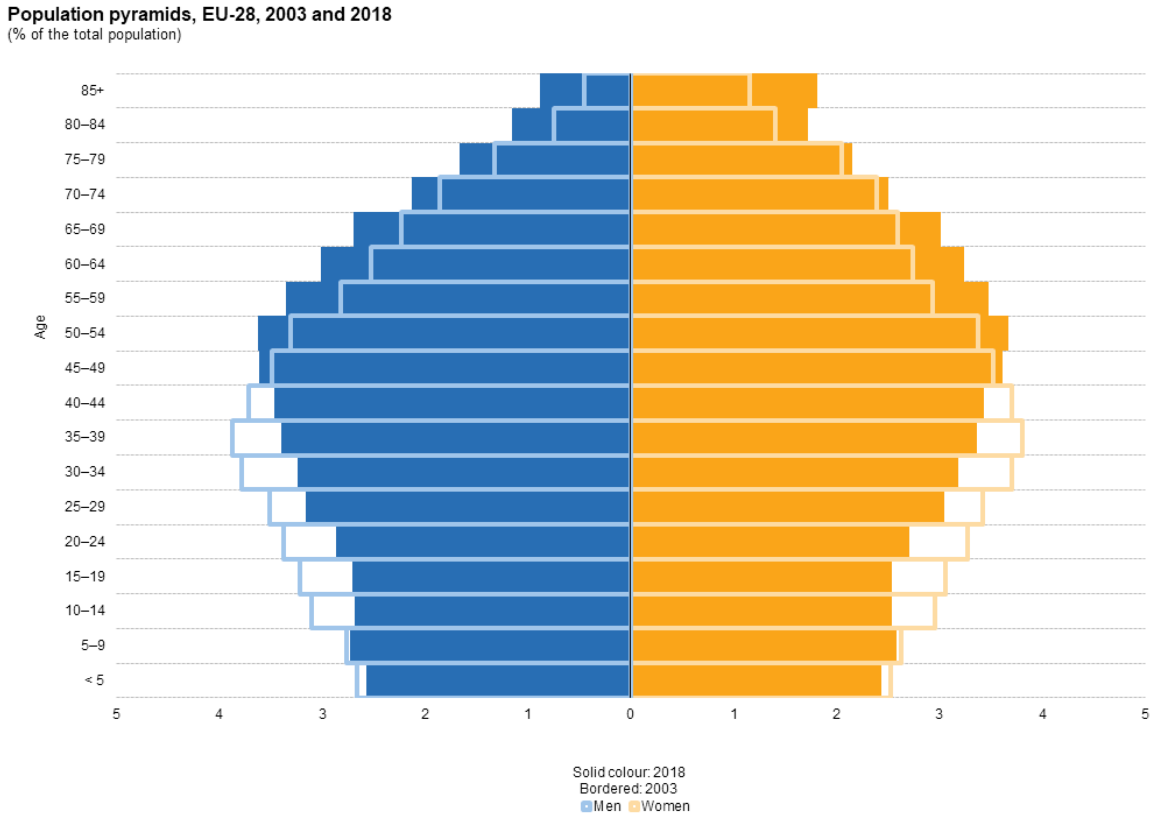


Figure 1. Population pyramids of the 28 member states of the European Union in the years 2003 and 2018. The percentage of people of all age ranges up to 40-44 years has decreased, while all the age ranges over 45-40 years have increased, demonstrating the aging of the population. Source: Eurostat. "Population structure and ageing". Available at: https://ec.europa.eu/eurostat/

Moreover, the current picture will continue evolving and the population is projected to age notably more. In **Figure 2** the present population structure and future projections are shown. Interestingly, we can observe that the proportion of people aged more than 80 years is expected to increase from 5.6% to 14.6% in the year 2100.

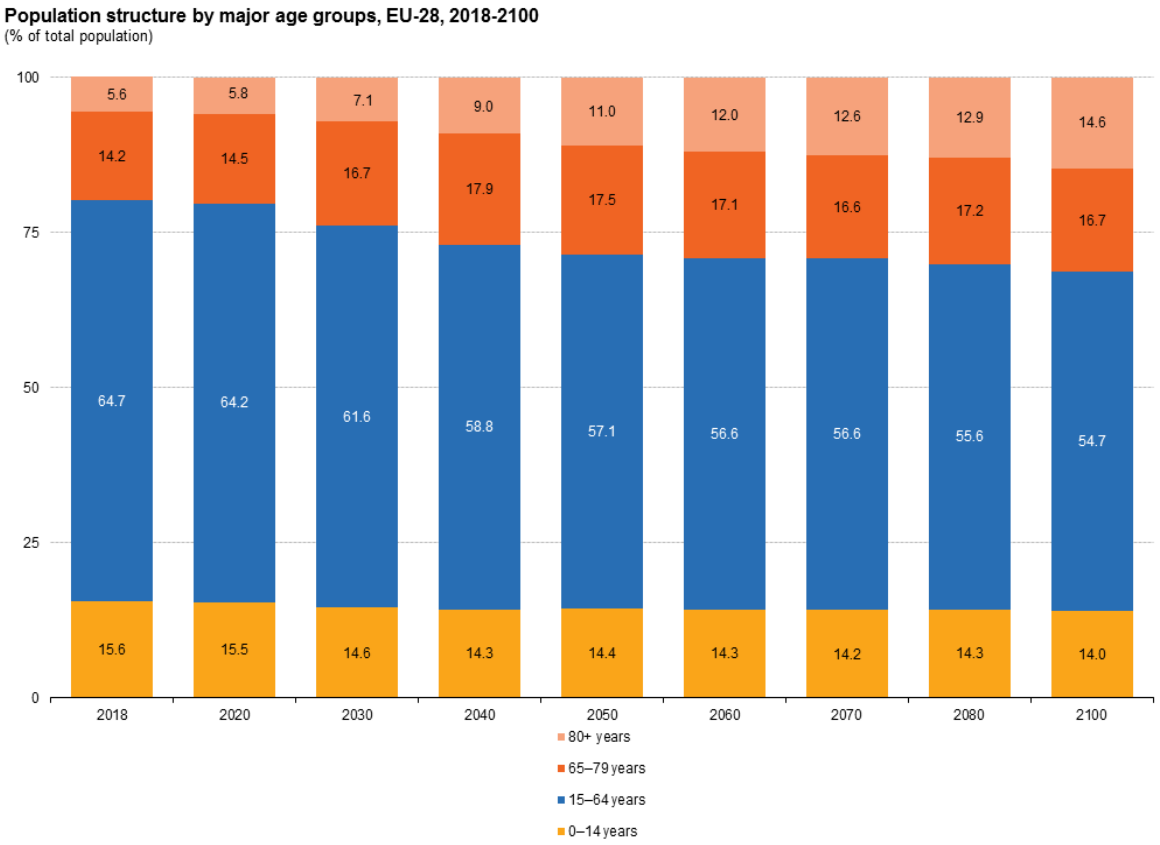


Figure 2. Population structure by major age groups of the 28 member states of the European Union in the year 2018 and the projections for the next decades. The proportion of children (0-14 years) will have only a minor decrease, while a marked decrease of the adults (15-64 years) and increase of elders (65-79 years and 80+ years) is expected. Source: Eurostat. "Population structure and ageing". Available at: https://ec.europa.eu/eurostat/

However, the reports from the European Union also show that life expectancy at birth is increasing, while the healthy life years at birth (also called disability-free life expectancy) is not rising. For instance, in 2016 life expectancy at birth was 83.6 years for women and 78.2 years for men and healthy life years were 64.2 and 63.5 years respectively. This indicates that a woman born in 2016 will live the approximately 77% of her life without disability, while it would be an 81% of his life for a man (https://ec.europa.eu/eurostat/). Furthermore, these percentages would continue to decline as long as life expectancy increases, but no reduction of disability is achieved.

In light of the commented demographic changes and disability data, the importance of taking appropriate and effective actions becomes evident. Moreover, as stated by the World Health Organization, the aging of our societies is one of the major challenges of the 21st Century [2], as it reaches not only sanitary but also many socioeconomic aspects. In consequence, decisions coordinated by experts of different fields should be taken, aiming to achieve the well-being and healthy aging of the population while maintaining financial sustainability.

One of the key actions to face the aging challenge is research. Research on the underlying mechanisms, the age-associated diseases and loss of functions, the interventions and the outcomes are essential to better understand the aging process and to be able to implement innovative treatments and/or interventions. Besides, the primary objective of biomedical research on aging should not be focused on the extension of life, it should aim to improve the quality of life of the elderly, reducing disability and prolonging healthy aging.

1.1. Frailty and other relevant age-related health concepts

Many works have previously studied the loss of functions associated with aging. Notably, there have been different approaches to investigate the age-related dysfunctions, and consequently, multiple terms have also been proposed. In the next lines, the main concepts are presented, and their principal characteristics explained.

1.1.1. Frailty and robustness

Frailty is a common age-related medical syndrome, characterized by a reduced functional reserve, impaired adaptive capacity across multiple physiological systems and increased vulnerability to stressors [3]. The accentuated vulnerability results in high risk of negative outcomes, such as falls, fractures, infections, disability, hospitalization, dependency and death [4]. Frailty syndrome has been widely studied for decades, but still, no consensus has been reached on its definition and identification tools.

Regarding the concept of frailty, a work by Rodríguez-Mañas and colleagues gathered the definitions of experts in the field and presented a list of accepted statements that define frailty [5]. This list included aspects of physical performance, nutritional status, mental health, and cognition. However, they concluded that, even if some concepts of frailty are widely agreed, there is no consensus on an operational definition of frailty.

Despite the lack of a complete definition, as mentioned before, frailty implies a reduced functional capacity of an individual that results in an increased risk of developing dependence. The opposite situation to frailty is most of the times termed robustness. An elder is classified as robust when her/his functional capacity is conserved, and besides, phenotypic stability is

also maintained after the occurrence of a clinical stressor [6]. It is generally accepted that frailty is reversible, and pharmacological, nutritional and physical interventions have been proposed to recover robustness [7]. Taken together, the main objective of frailty identification tools is to detect a person when she/he is at risk of developing dependence, and intervene to improve the functional capacity and to prevent negative outcomes (**Figure 3**).

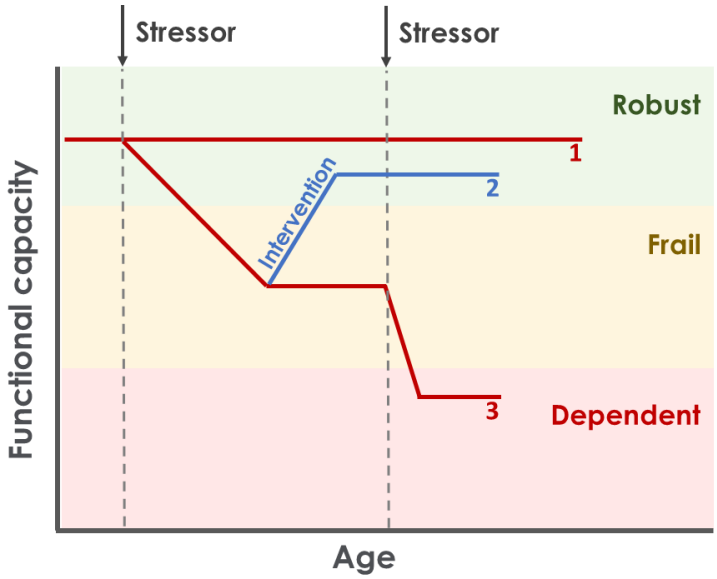


Figure 3. Representation of possible functional capacity progressions with age. Each line represents an individual. The subject 1 has a good function and maintains it after the occurrence of stressors (robust). In contrast, the subjects 2 and 3 lose function after the first stressor. At this point the subjects become frail. If we are able to identify the frail individuals and intervene with a pharmacological, nutritional and/or physical program, functional capacity could be improved, and robustness recovered (subject 2). In contrast, if we fail to identify frailty or we do not perform an intervention, the occurrence of another stressor can result in a more prominent loss of function and subsequent dependence (subject 3).

1.1.2. Frailty scales

As mentioned above, frailty is a complex concept that includes physical, phycological and cognitive aspects. Similar to the open discussions regarding the definition of frailty, the tools and clinical scales applied to identify frail individuals are also debated by geriatricians and researchers. Throughout the last decades, more than one thousand works that studied frailty have been published and tens of frailty screening tools have been proposed [4,8,9].

Each tool has a different approach and focuses on different aspects: clinical score, activities of daily living (ADL), physical performance, self-reported health status, or a combination of several of them. Besides, some of the tools classify individuals as frail or no-frail, while others also distinguish pre-frailty, an intermediate state between robustness and frailty [10,11]. For instance, the systematic literature review performed by Sternberg and colleagues [8] analysed

22 publications describing original frailty screening tools and found that physical function was the most commonly assessed aspect. Moreover, they reported that ADLs were included as identifying components of frailty more frequently in the earlier years of this review, while gait speed and cognition became more common in later years.

The most cited tool for the identification of frailty is the one proposed by Linda Fried and collaborators in 2001 [10] (2635 citation in PubMed, accessed on October 7, 2019) and it is also estimated to be the most applied instrument [12]. It measures 5 aspects: unintentional weight loss, exhaustion or fatigue, physical activity, walking time or walking speed and grip strength. Individuals having none of these components are considered robust, those having 1 or 2 prefrail and those with 3 or more are classified as frail. This tool has been shown to have good predictive value for the incidence of falls, worsening mobility and ADL, hospitalization and death.

However, even if hundreds of studies have applied the classification proposed by Fried *et al.*, many geriatricians and researchers consider that it is a complex tool that cannot be applied in the everyday clinics. In fact, to complete the 5 components of the test, simple measures including body mass index, walking speed and grip strength have to be performed, but also a detailed questionnaire to measure physical activity and to calculate kcal expenditure per week, making it time-consuming and complicating its application in the clinic.

Similarly, the Canadian Study of Health and Aging (CSHA) Frailty Index, which was also published in 2001, has shown good predictive values and it has been cited 259 times (PubMed, accessed on October 7, 2019). It is a continuous scale from 0 to 1, reflecting the proportion of accumulated deficits detected, based on 20 items [13]. Besides, a simpler tool called CSHA Clinical Frailty Scale was published in 2005 by the same research group. They demonstrated that the predictive validity of the CSHA Clinical Frailty Scale was as good as the one of the Frailty Index. This second tool is a 7-point scale based on clinical judgement [14] and it has been cited by 639 works (PubMed, same date). Notably, we can see that when the two identically valid screening tools from the same research group are compared, the one that is simpler and easier to apply has been more widely reproduced.

So, in the last years, several works have been carried out to find easier and faster tools to evaluate frailty. This is the case, for instance, of the Tilburg Frailty Indicator, a user-friendly questionnaire based on a multidimensional approach, composed by a physical, a psychological and a social domain [15]. Another example is the Gerontopole Frailty Screening Tool, which is based on clinical judgement. This test is composed of 6 yes/no questions that help the physician to evaluate the existence of frailty [16]. Other works have evaluated the use of a

single functional measure, such as gait speed or timed up-and-go, for the identification of frail subjects [17,18]. Besides, the Short Physical Performance Battery (SPPB), which was published already in 1994 by Guralnik and colleagues [19], is gaining importance in the last years for the identification of frailty. It has a total of 1651 citations in PubMed, and 220 of them are from works published in the last year (October 2018 - October 2019). It is a functional capacity test composed of gait speed, a test of balance and a measure of the time needed to stand up from a chair 5 consecutive times. It measures function in three different ways, but it is still a fast and simple test to apply in the clinic.

These examples represent the main trends in frailty assessment: based on the clinical judgement, on self-reported health status and on physical function. All of them seem to be valid for the identification of frail individuals, taking into consideration the wide concept of frailty. However, a single person would be classified as frail by some of the tests, while robust by others. In this context, the use of molecular biomarkers could be helpful, and many researchers are investigating their potential to identify frailty. Indeed, molecular biomarkers could be used in combination with functional tests, questionnaires or the clinical status, to complement the identification process. The current state of the art regarding biomarkers of frailty will be commented in a later section, after the biological aspects of aging are introduced.

1.1.3. Resilience

The concept of resiliency has been widely used in other fields such as ecology, psychology and engineering for a long time, and lastly, it has also been applied to medicine and aging [20]. Resilience is defined as the human ability to adapt in the face of tragedy, trauma, adversity, hardship, and ongoing significant life stressors. More specifically, in the field of aging, resilience is described as the ability to resist or recover from functional decline following the adverse effects of a stressor [6]. The concept of resiliency is closely related to robustness, but there is one relevant difference between them: a robust subject maintains the phenotype quantitatively, while a resilient subject retains the phenotype qualitatively, but there could be some decrease in function. This distinction was nicely represented in a publication by Varadhan and colleagues [6], as shown in **Figure 4**.

Moreover, frailty and resilience are also related, but they are not opposite concepts [6,21]. As explained before, a frail individual has increased vulnerability to stressors. However, in combination with the proposed definition of resilience, a frail person could still be resilient when she/he suffers only a subtle loss of function and maintains the phenotype, whereas a frail person that has a substantial and lasting loss of function would be considered non-resilient. Besides, some authors state that the concept of frailty has a negative connotation, while resilience has been proposed as a more positive term [21,22]. Frailty is seen as the

accumulation of deficits with age, while resilience focuses on the ability to adapt. The negative essence of frailty and the more positive definitions of age-related processes are further detailed in the next section.

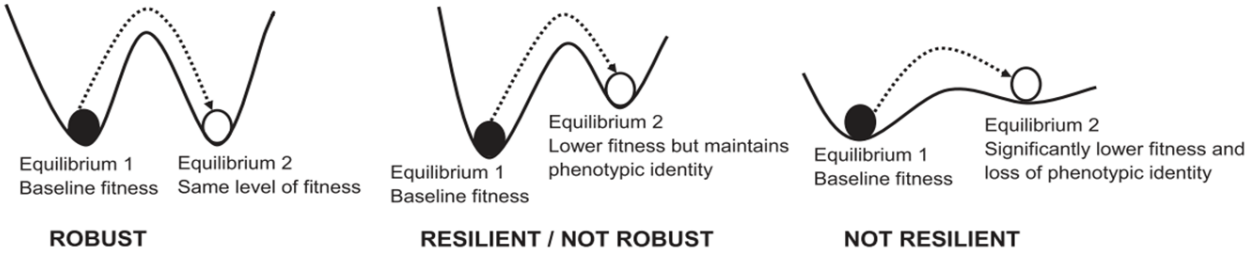


Figure 4. Representation of the possible responses of a system to a stressor. The system is robust when it maintains its functionality intact under the new equilibrium, it is resilient when it maintains its essential functionality under the new equilibrium, and it is nonresilient when it loses its essential functionality under the new equilibrium. Adapted from [6].

1.1.4. Intrinsic capacity

The World Health Organization introduced the concept of intrinsic capacity, defined as the composite of all physical and mental capacities that an individual can draw upon during her/his life, in the World report on ageing and health published in 2015 [2]. Intrinsic capacity is a new model that aims to capture all the individual's functions and capacities. Furthermore, intrinsic capacity is a dynamic construct and should be longitudinally assessed. In this way, the clinician may identify deviations from normality before the onset of clinical manifestations and preventively act to maintain healthy aging. Besides, the longitudinal measures would also serve to evaluate the effectiveness of interventions to improve health status [22].

However, intrinsic capacity is still a theoretical concept. The World Health Organization is coordinating activities to promote its clinical implementation and it is expected that they will soon provide an international validation of the definition and tools to measure intrinsic capacity [23]. For now, the five domains defining intrinsic capacity have been defined: locomotion, vitality, sensory, cognition, and psychological [24]. These domains are interconnected and they are also influenced by environmental factors. In addition, all the mentioned domains are composed of subdomains, increasing the complexity of the system. The possible subdomains of intrinsic capacity were described by Cesari and collaborators [24] and are presented in **Figure 5**.

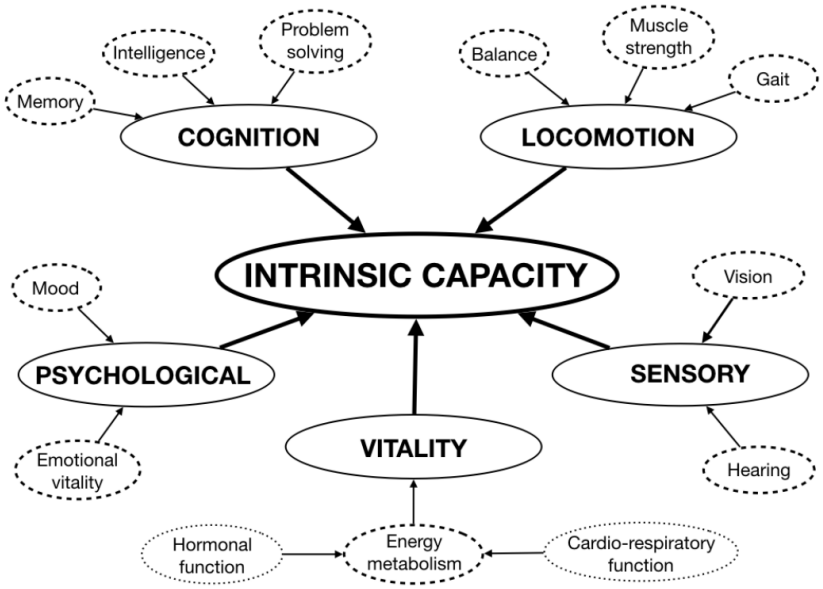


Figure 5. Schematic representation of the five domains of intrinsic capacity and the possible subdomains of each of them [24].

Again, the connections between the aforementioned concepts and intrinsic capacity become evident. Frailty, resilience and intrinsic capacity are multidomain constructs aiming to comprehensively evaluate the individual and implement personalized strategies to prevent, slow or reverse losses. However, frailty is focused on deficits, while resilience and intrinsic capacity concentrate on adaptability and function respectively. In consequence, the two newer concepts have a positive connotation when compared to frailty. In addition, as we mentioned, intrinsic capacity is a longitudinal concept and could be monitored throughout the life course, whereas frailty is an age-related status, reinforcing the idea of the link between aging and negative outcomes. As proposed by Belloni and Cesari, frailty and intrinsic capacity might be complementary, as the monitoring of intrinsic capacity could support the detection of a person's fragilization [22]. Certainly, the accumulation of deficits and reduced function is one of the characteristics of aging, and it should be faced as a natural process, while we will try to minimize it to maintain the quality of life with advancing age.

In any case, frailty is to date the best described term and, we consider that until holistic constructs such as intrinsic capacity could be implemented, we should continue trying to improve the identification of frail individuals.

1.1.5. Healthy aging, successful aging and related concepts

There are also other terms related to aging that are commonly used. For instance, adjectives such as healthy, successful, positive, productive or active are applied to describe the aging process. All of them have a similar meaning, which can be resumed as "aging well". Depending

on the publication, the aim of the work and the authors, the word of choice and the interpretation are slightly different [25].

The World Health Organization, for example, focuses on the definition of the concept of healthy aging, but also employs the successful and active aging terms [2,23]. They define healthy aging as the process of developing and maintaining the functional ability that enables wellbeing in older age. Indeed, the functional ability of a person is determined by intrinsic capacity, the environment and the interactions between the two of them.

With regard to nonagenarians and centenarians, they are typically considered as examples of successful aging. In fact, it has been shown that they have distinct transcriptomic features when compared to septuagenarians and octogenarians [26,27]. However, not all the individuals that reach 90 years are in the same health condition and, for instance, we have previously reported that healthy nonagenarians have longer telomere lengths and lower amyloid β levels in blood when compared to functionally impaired nonagenarians [28]. Moreover, as described before, even if life expectancy is increasing, no changes have been achieved in the healthy life years. Consequently, we can expect rising numbers of nonagenarians and centenarians in our society, but, unfortunately, the successful aging of all of them could not be assumed.

1.2. The biology of aging

To understand the whole process of aging of an individual it is essential to identify and describe the biological alterations that the organism experiences as time goes by. Aging has attracted our curiosity for centuries, but it has been only in the last decades, with the development of modern medical, scientific and technical resources, that the study of the biological aspects of aging has rapidly advanced [29]. Due to the complexity of the aging process, and to the fact that it affects all the tissues and systems of the organism, thousands of investigations have been published, with completely different research approaches and study focuses.

Consequently, to enable a better understanding of the current knowledge, the classification or categorization of the biological aspects of aging becomes important. The characteristics of aging can be categorized, for instance, depending on the organ of study. Indeed, it has been extensively reported that aging affects in a distinct manner and to a different extent to organs, such as the bone marrow, the brain or the bone among others [30–32]. Similarly, the diversity of aging can also be compared between the different cell types that compose an organism. This could be exemplified by contrasting the effects of aging on muscle cells and on lymphocytes: they are radically distinct cells, with disparate maturation, function, regulation and renewal rates, and therefore, the modifications they suffer with advancing age are not the same.

A few years ago, in 2013, a noteworthy classification of the cellular and molecular aspects of aging was proposed by López-Otín and collaborators [33]. They distinguished 9 hallmarks of aging, and further categorized them as primary, antagonistic and integrative hallmarks (**Figure 6**). It is important to mention that the authors proposed this categorization because the hallmarks are tightly interconnected and they cannot be understood individually.

In the next sections, the main characteristics of the proposed hallmarks of aging are explained, with a special focus on the 4 hallmarks that have been investigated in this thesis. Besides, we complemented these sections with information that was not included in the original description of the hallmarks, but that we consider relevant both in the context of this work and for the general understanding of the biology of aging.

Finally, we should bear in mind that thanks to new findings the knowledge about aging is rapidly evolving. The hallmarks proposed in 2013 represented the current state of the art, but probably, modifications such as the inclusion of new categories or further explanations on their connexions and implications will be made in the near future.

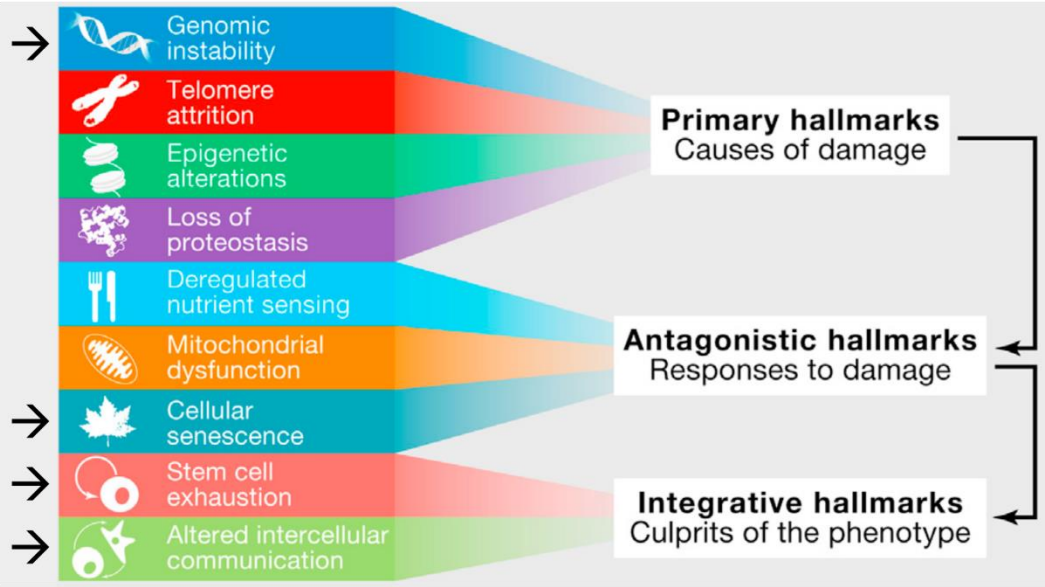


Figure 6. The hallmarks of aging and their interconnections. Genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis are considered the primary hallmarks, the primary causes of cellular damage. Deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence are part of compensatory or antagonistic responses to the damage. These responses are proposed to initially mitigate the damage, but eventually, they become deleterious themselves. Stem cell exhaustion and altered intercellular communication compose the integrative hallmarks, they are the end result of the previous two groups and are ultimately responsible for the functional decline associated with aging. The black arrows indicate the hallmarks that have been investigated in the present work. Adapted from [33].

1.2.1. Genomic instability and gene expression

There is extensive evidence showing that genomic damage accompanies aging. The accumulation of DNA damage is caused by both endogenous and exogenous threats, such as reactive oxygen species (ROS) and ultraviolet radiation respectively [34]. Examples of DNA damage include mutations, single- and double-strand breaks or interstrand crosslinks [33]. The cell has multiple mechanisms for genome maintenance and error repair, which illustrate the importance of genome stability. Whether the accumulation of DNA damage is a cause or a consequence of aging was long debated, but the description of genome instability in diseases of accelerated aging demonstrated the causality, as extensively reviewed by Niedernhofer *et al.* [35]. Indeed, in the same publication, they also reviewed the current data showing that mutations increase and DNA repair capacity decreases with age.

Apart from genomic instability, but directly related to it, gene expression modifications have a relevant role in aging. It has been widely shown that the expression of protein-coding genes changes with age [36–38]. Importantly, transcription factors and related signalling pathways have also been found to affect cell senescence and aging. The insulin and insulin-like growth factor 1 (IIS) pathway is the best studied one, and it would be commented in the section of deregulated nutrient sensing, along with other relevant systems. The interconnections between the proposed hallmarks of aging become evident, as the modification of transcripts leads to deregulated nutrient sensing.

Besides, not only the protein-coding transcripts are affected by aging. Several works have investigated the post-transcriptional regulation of gene expression by microRNAs (miRNAs) and other small non-coding RNAs (sncRNAs). Differentially expressed miRNAs have been found in various organs as well as in the circulatory system of both humans and model animals [39,40]. We also studied the expression of sncRNAs in human leukocytes with age and identified a subset of 69 sncRNAs that gradually increase or decrease. Interestingly, we reported an accelerated change in sncRNA expression between 47-54 years, suggesting that at this age relevant gene expression modifications occur [41]. Notably, works by Borrás and colleagues recently showed that both mRNA and miRNA expression are different when octogenarians and centenarians are compared, and moreover, the data from centenarians are more similar to the ones obtained from adults [26,27]. Furthermore, a longitudinal study performed by Smith-Vikos *et al.* evaluated the expression of serum miRNAs in 16 subjects and identified differentially expressed miRNAs between the short-lived and long-lived subgroups [42]. There is still a long way to get to understand the function of all sncRNAs in aging, but the available data highlight their implication in the process and their potential use as biomarkers of age-related modifications.

In addition, in the last years, a new player has entered the game: circular RNA (circRNA). circRNAs are covalently closed transcripts formed through an RNA back-splicing event and characterized by the presence of a back-splicing junction that makes them distinguishable from their linear counterparts [43]. Although the function of most of the circRNAs remains unknown, it has been found that they can act as miRNA sponges and that they are also involved in gene expression regulation, as circRNAs can regulate the transcription of their parental genes. Moreover, ribosome profiling studies have recently shown that circRNAs can be translated both *in vitro* and *in vivo*, which challenge the stereotypic view of circRNAs as non-coding RNAs [44]. With regard to aging, several studies have investigated these molecules and differential expression of a large number of circRNAs during aging has been found in a wide range of organisms. In humans, for instance, they have been proposed to play a role in Alzheimer's disease and immunosenescence [45]. Even if there are still few reports on the functions of circRNAs, this is an emerging field that will continue developing and circRNAs have to be considered as another piece of the complex puzzle of aging.

Finally, it is worth mentioning that several investigations have been carried out in the last decades to try to find genetic variants related to healthy aging. Studies conducted on exceptionally long-lived individuals and genome-wide association studies revealed many candidate loci and single nucleotide polymorphisms (SNPs) that could be linked to healthy aging and longevity. However, there are controversial results and most of the candidates identified in some works have not been confirmed in other studies. These differences could be due to different study designs, phenotype definitions and inter-ethnic characteristics, as well as by the effects of epigenetics, environmental factors and lifestyle differences [46].

1.2.2. Telomere attrition and epigenetic alterations

As mentioned before, alterations in the sequence of genomic DNA are common in aging, but there are other relevant modifications that affect the structure and transcription of DNA, which include telomere shortening (or attrition) and epigenetic alterations (**Figure 7**).

The shortening of telomeres is caused by the incomplete replication of the terminal ends of genomic DNA. The replication of chromosomes is conducted by replicative DNA polymerases that lack the capacity to replicate completely the ends of DNA molecules. This function is carried out by a specialized DNA polymerase called telomerase. However, most somatic cells do not express telomerase, leading to a progressive and cumulative loss of telomeres in each replication cycle, and consequently, with increasing age [47].

Telomeres are implicated in essential biological functions: they protect chromosomes from recombination, end-to-end fusion, and recognition as damaged DNA, contribute to the

functional organization of chromosomes within the nucleus, participate in the regulation of gene expression, and serve as molecular clocks that control the replicative capacity of human cells and their entry into replicative senescence [48]. Defects in telomerase, telomeres or shelterin (the protein complex that protects telomeres), are linked to diverse problems, including pulmonary fibrosis, premature aging and cancer [49]. Besides, it has been found that telomerase-deficient mice exhibit premature aging, which can be reverted by genetically reactivating telomerase [50].

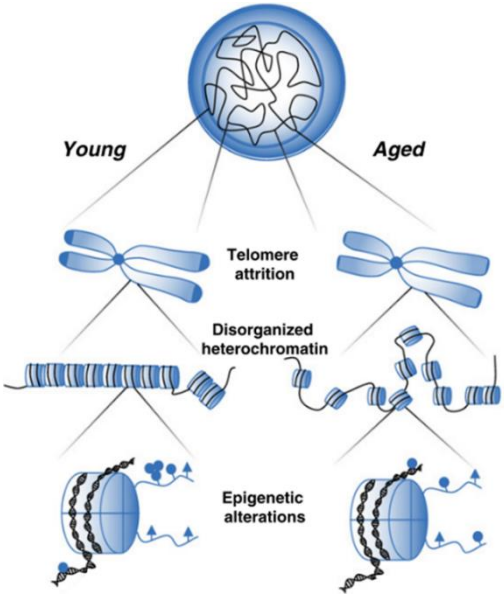


Figure 7. Schematic representation of age-related modifications in DNA structure. Aging affects DNA organization at the chromosome level (telomere attrition) and at the chromatin level (disorganized heterochromatin). In addition, epigenetic alterations, such as methylations or deacetylations can affect both DNA sequences and histones. Adapted from [51].

In humans, the telomere length of leukocytes has been widely studied, and it has been proposed that longer leukocyte telomeres are linked to longevity [52]. However, several authors have investigated the rate of leukocyte telomere attrition and found that telomere length is highly variable at birth, and besides, their shortening is very high during the first years of life, while it slows down considerably during adulthood [53,54]. Moreover, a publication that evaluated the ranking of leukocyte telomere length of four longitudinal studies demonstrated that, despite the interindividual differences in telomere attrition per year, most subjects maintain their classification with respect to their age-matched pairs, meaning that the ones that have shorter telomeres at the age of 30 are the ones that have shorter telomeres one decade later [55]. These results indicate that telomere length is mostly predetermined and environmental or lifestyle changes have only minor effects on telomere attrition. In consequence, the measurement of telomere length early in life is useful for the identification of telomeropathies and as an age-related risk factor, while its utility for intervention monitoring in the elderly is not promising.

On the other hand, the age-associated epigenetic alterations have been found to be partially reversible. The best described epigenetic modification that occurs during aging are methylations, histone modifications and chromatin remodelling [33].

Regarding DNA methylation, only a small fraction of the CpG sites have shown age-related modifications (around 2%), but this fraction represents between 2 and 3 million cytosines in the genome, denoting the complexity of the system. In addition, both hypo- and hypermethylation of the CpG sites happen with age, and the modifications can occur in certain tissues or cell types, or even affect only one part of a specific cell population, adding further complexity [56]. Despite this, robust mathematical methods have been developed and there are reliable algorithms that interpret the methylation pattern of selected CpG sites of an individual and predict chronological age with high accuracy [57]. These tools are called epigenetic clocks, and recent publications indicate that they could also be useful for the detection of accelerated epigenetic aging related to several problems or diseases, including cancer, Alzheimer's disease, frailty and the prediction of mortality risk [56].

Besides, modifications in histones affect the organization of the DNA, as well as gene transcription. Histones can also be methylated, but the best studied characteristic of these structures is the deacetylation performed mainly by sirtuins. Sirtuins are a family of NAD-dependent enzymes able to post-translationally deacetylate histones, which is associated with transcription repression. The increased expression of sirtuins has been related to longevity in humans and model organisms, and moreover, overexpression studies in these animal models resulted in elongated lifespan and healthier aging, while the downregulations of sirtuins increased senescence and accelerated aging [58].

The organization of histones influences in a more general view, the packaging of chromatin into heterochromatin and euchromatin. This organization is coordinated by sirtuins and many other DNA- and histone- modifying enzymes. The proper assembly of histones ensures a packaged heterochromatin, which is, however, partially lost and redistributed with aging [33]. Interestingly, the age-associated chromatin remodelling deeply influences the transcription of multiple genes, as the coding sequence of generally repressed genes can then be accessible for the binding of transcription factors, or the other way around. Indeed, as explained in the previous section, the gene expression modifications that occur with aging are diverse, and chromatin reorganization is just one of the changes that affect it. Interestingly, epigenetic modifications have been found to play a role in inflammaging and immunosenescence [51,59], two of the problems linked to aging that will be addressed later in this work.

Finally, it has to be mentioned that, in contrast to telomere attrition, epigenetic changes are reversible. For instance, several investigations have demonstrated that caloric restriction affects DNA methylation, attenuating some age-related CpG modifications and showing deaccelerated epigenetic aging in mice. Besides, the mTOR inhibitor rapamycin also affects methylation and reduce epigenetic age in treated mice [56]. Similarly, other studies evaluating caloric restriction reported sirtuin mediated slower aging and extended lifespan [60]. Again, compounds that mimic the positive effect of caloric restriction regarding sirtuin modulation are being tested, such as resveratrol and curcumin [58]. In summary, the data from animal models indicate that epigenetic modifications are promising targets, and nutritional or pharmacological interventions could potentially be applied to attenuate age-related changes in humans.

1.2.3. Loss of proteostasis

Proteostasis is defined as the proper control of proteins, including their biogenesis, folding, trafficking, function and degradation. All the mentioned steps are essential for maintaining the correct functioning of each cell and the organism as a whole. Therefore, the processes implicated in proteostasis are tightly regulated, but they can suffer modifications that lead to misfunctioning during aging [61]. For instance, the previously described genomic instability or epigenetic alterations have a direct impact on protein biosynthesis, as coding sequences can be inaccessible, damaged or inappropriately copied.

Besides, even if the biogenesis is completed, many proteins require a specific folding to be functional. Chaperones are a class of heat shock proteins (HSPs) implicated in protein folding and stabilization. The HSPs, and specifically chaperones, have been widely studied in the field of aging. Indeed, chaperones are involved in the response mechanisms against stressors, and have been shown to fail in elders [62]. Furthermore, many studies have been conducted in model organisms, and reports from worms, flies or mice among others have demonstrated the accelerated aging in chaperone deficient animals, while their overexpression elongated lifespan and reduced the accumulation of protein aggregates [62].

The degradation of proteins is another important step to maintain proteostasis. In fact, misfolded, aggregated or non-functional proteins must be removed from the system to prevent the accumulation of toxicity. There are two major pathways for protein degradation: the ubiquitin-proteasome system (UPS) and autophagy. Both pathways have hundreds of different components, including chaperones, and this complexity demonstrates the investment of the cell on proper protein degradation [61]. However, the function of UPS and autophagy declines with age, and despite the defect of one of the pathways can be partially compensated by the other, many cell types accumulate defective proteins [63]. The protein degradation, in

combination with the previously mentioned defects in biosynthesis and folding, are responsible for the age-related loss of proteostasis. Indeed, the deficits of each step contribute to the final failure of the system.

1.2.4. Deregulated nutrient sensing

As presented before, the hallmark describing deregulated nutrient sensing in aging is tightly connected to the gene expression modifications. In this sense, the genetic polymorphisms or mutations that reduce the function of the IIS pathway have been associated with longevity. These include the growth hormone, insulin-like growth factor-1 (IGF-1) receptor, or downstream effectors such as AKT, mTOR and FOXO [64–66]. However, the components of the IIS pathway are multiple and its regulation and interconnections with other signalling pathways are complex. Indeed, apart from being related to longevity, reduced levels of the IIS components are also reported during normal aging and in animal models of premature aging [67]. This could seem contradictory, but it has been proposed that depending on the duration and extent of downregulation the elicited results could be beneficial or deleterious. Thus, the constitutively decreased IIS functioning implies lower cell growth and metabolism, and consequently reduced rates of cellular damage, while acute decreases or extremely low levels lead to premature aging [33]. This process is comparable to other defensive responses that can become deleterious when not properly controlled, as in the case of inflammatory responses.

Other nutrient sensing systems tightly connected to IIS and also associated with aging include mTOR and sirtuins. The mTOR kinases are implicated in anabolic metabolism and the genetic as well as pharmacologic attenuation with rapamycin of this system have been linked to increased longevity in distinct animal models [68,69]. On the other hand, as described in the section of epigenetic alterations, sirtuins are enzymes implicated in the organization of histones and they play a role in the age-associated transcriptional regulation. Notably, sirtuins also respond to nutrient availability, and they get activated under nutrient scarcity. We previously commented the relation between the elevated expression of sirtuins and longevity, and the positive results obtained with caloric restriction or treatments with resveratrol or curcumin and associated with sirtuins [58,60]. Similarly, apart from the pharmacological interventions, the beneficial results of caloric restriction have also been found to be mediated, at least in part, by the reduction of mTOR activity. This link has been demonstrated in animal models under caloric restriction, in which the downregulation or deletion of mTOR genes prevented the otherwise observed extension of lifespan [70].

1.2.5. Mitochondrial dysfunction

The wellbeing of mitochondria is essential for the appropriate functioning of cells. Despite this, there are hundreds to thousands of mitochondria in a single cell (the number depends on the organism, tissue and cell type), and thus, single or small numbers of mutations or deficits can be managed by a cell and maintain proper functioning [71]. However, as we age, the increased damage, reduced respiratory chain functioning, imbalanced fusion and fission, and defective clearance of mitochondria (mitophagy) contribute to cellular and organismal aging [72]. In the last years, the potentially beneficial effects of mild deficiencies in mitochondria have been proposed. Indeed, the elicited mitochondrial defensive response and the low energy state have been found to induce beneficial compensatory responses, and even to extend lifespan in model organisms [33]. This could seem paradoxical, but it is in line with other age-related characteristics, such as cell senescence or inflammation, that could be beneficial when effectively controlled, but detrimental when maintained or abnormally increased.

Mitochondrial dysfunction is closely related to other characteristics of aging. The case of genomic instability becomes evident, as mutations in mitochondrial DNA (mtDNA) are one of the main causes of the severe impairment on energy conversion. Even if only around 1% of the mitochondrial proteome is encoded by mtDNA, these include critical components of the oxidative phosphorylation complexes [71]. And besides, apart from the mutations or deletions in mtDNA, the accumulation of changes in nuclear sequences, also affect the components and dynamics of mitochondria. In addition to the defects in mtDNA that accumulate during the organismal life, it has been demonstrated that single SNPs and mitochondrial haplogroups can influence the aging process. For instance, SNPs in mitochondrial uncoupling protein genes have been related to healthy aging [73], the individuals with mitochondrial H haplogroup showed distinct mitochondrial dynamics [74,75] and the D4, D5 and J haplogroups have been associated with longevity. Moreover, studies performed in mice have shown that the mtDNA haplotype profoundly influences mitochondrial proteostasis and function, as well as ROS generation, insulin signalling and telomere length, resulting in differences in the aging process and median lifespan between conplastic strains [76].

With regard to senescence, the issue of whether mitochondrial dysfunction is causative or just part of the consequences of the entrance of a cell in senescence is still debated. However, the bidirectional link between senescence and mitochondria could be too simple to explain the complex underlying processes, and this interplay could be best outlined as a vicious circle, involving a number of feedback loops between the players. In spite of the triggering mechanisms, it has been widely described that senescent cells have mitochondrial deficits both in genome and proteome maintenance, and consequently present dynamic changes and

dysfunctions [77]. On the other hand, the age-associated problems in mitochondria can also affect stem cells. The accumulation of somatic mtDNA mutations alter stem cell homeostasis and can induce defects such as imbalanced biogenesis, abnormal mitophagy or increased ROS production, which can eventually accelerate stem cell senescence [78,79].

There is also a tight connexion between mitochondrial deficits and the chronic inflammatory state (inflammaging) with aging. The defective or dysfunctional mitochondria can release molecules that promote the activation of the immune system, including mtDNA and ROS among others. The mitochondrial components can boost the immune response through different pathways, as the activation of the inflammasome, the recognition by the cytosolic sensor of dsDNA cyclic GMP-AMP synthase, or the activation of immune cells with secreted metabolites like succinate, as comprehensively reviewed by Jang and collaborators [72]. Remarkably, the contribution of mitochondrial component to inflammaging can result in further injury, as in the case of inflammasome and caspase-1 mediated mitochondrial damage [80].

1.2.6. Cellular senescence

More than half a century ago, the senescence of human cells was described in *in vitro* cultures of fibroblasts [81]. Hayflick and Moorehead found that after a certain number of passages cultured cells lose proliferative capacity. Besides, it was long described that cellular senescence happens also *in vivo* [82]. Furthermore, thanks to all the investigations that have been conducted, nowadays we know that apart from the replicative arrest, senescent cells show many other features distinct from non-senescent cells. Some of the features associated with senescent cells, and commonly used as senescence biomarkers, include the increased activity of lysosomal β-galactosidase, as well as the elevated expression of p53 and $p16^{INK4a}$ [83,84].

Interestingly, senescence can be induced as a controlled mechanism to prevent the proliferation of damaged cells, before they lose the replicative control and develop tumorigenic features. Similarly, the entrance of cells into senescence can be triggered by telomere attrition, accumulation of DNA damage, or ROS, which induce the activation of the DNA damage response mechanism and lead to senescence mainly through the p53 pathway [85,86]. Thus, these processes are linked to the mentioned characteristic expression of p53 and $p16^{INK4a}$ by senescent cells. Indeed, they are tumour suppressor proteins, part of complex signalling pathways that respond to the expression of oncogenes, and consequently induce cellular senescence or apoptosis [87]. This indicates the protective role to prevent the formation of tumours, even if contributing to the accumulation of senescent cells. In consequence, the positive or negative impact of senescent cells is still discussed.

The induction of senescence in damaged and potentially hazardous cells is undoubtedly beneficial. In addition, senescence has been proposed to trigger tissue renewal, but in contrast, this process may not be efficiently completed in aged tissues or pathological contexts, resulting in the accumulation of senescent cells (**Figure 8**) [86]. Therefore, increasing evidence indicates that both pro-senescent and antisenescent therapies can be favourable. For example, in cancer, during active tissue repair and even to prevent age-related damage, controlled pro-senescent therapies could be beneficial, limiting proliferation, accumulation of defective cells and fibrosis [88]. Conversely, antisenescent therapies may help to eliminate the already accumulated senescent cells and to recover tissue function in aged individuals [89].

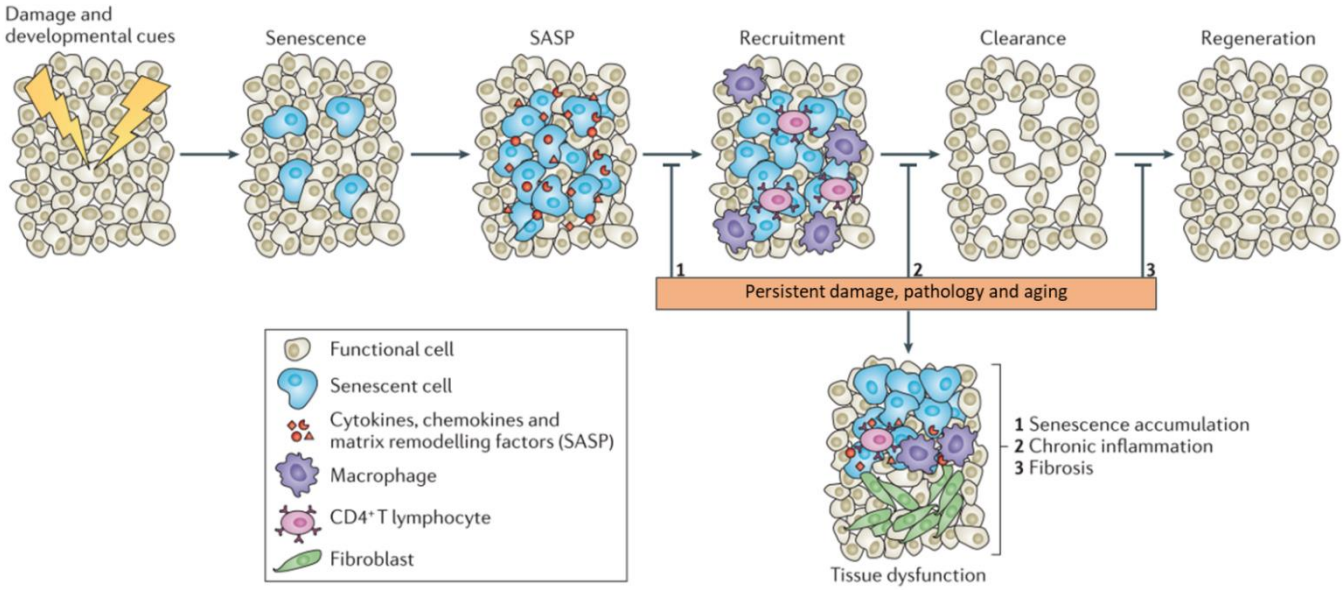


Figure 8. Proposed model of senescence. Senescence initiates a tissue remodelling process by recruiting immune cells through the senescence-associated secretory phenotype (SASP, explained in the section 1.2.8). Macrophages clear the senescent cells, and progenitor cells regenerate the damaged tissue. This sequence is impaired under persistent damage, pathological states or aging. In these cases, senescent cells are not efficiently cleared, the tissue is not fully regenerated, and its functionality diminishes. Resolution of the damage in these cases involves a fibrotic scar with senescent cells, inflammatory cells and fibrotic tissue. Adapted from [86].

Moreover, even if some molecular features –like replication arrest or elevated β-galactosidase activity– are reproduced in most senescent cells, senescence affects in a different manner to distinct animals, individuals, tissues and cell types [89–91]. In this work, we have focused on the senescence of the human immune system, and consequently, in the next lines the alterations of this system are presented.

1.2.6.1. Immunosenescence

The term immunosenescence is used to refer to all the changes that occur to the immune system during aging leading to its dysfunction. However, immunosenescence is not only caused

by the age-associated alterations, as the chronic activation of the immune system associated with virus infections, inflammatory and autoimmune diseases, cancer or organ transplantation also influence the exhaustion of the immune system, in a process called “early” or “premature” immunosenescence [92].

Immunosenescence reaches the components of both the innate and adaptive immune system. In fact, the two subsystems are closely related and the modifications occurring in innate immunity affect the adaptive immunity, and the other way around [93–95]. Regarding the innate immune system, a comprehensive review by Solana and collaborators pointed that aging is associated with changes in the cell numbers and with a decrease in the main functions of these cells, such as antigen presentation or phagocytosis, as a consequence of modifications in the expression of a variety of innate immune cell receptors. These alterations result in a reduced capacity to respond to bacterial and viral pathogens and in an impaired ability of the innate system components to collaborate in the initiation of the adaptive immune response [94].

In the adaptive immune system aging affects the rate at which naïve B and T cells are produced, as well as the composition and quality of the mature lymphocyte pool. Actually, with increasing age, the number of lymphoid-biased hematopoietic stem cells (HSCs) declines, contributing to the reduction of lymphoid progenitors and to oligoclonal expansion. In addition, B cell progenitors in the bone marrow and T cell progenitors in the thymus exhibit reduced proliferation and increased apoptosis and the decline in primary lymphopoiesis results in a reduced number of naïve cells that migrate to secondary lymphoid tissues [96].

In the case of mature B cells, late memory cells (IgD-/CD27-) have been found to be increased in the elderly. Moreover, this subset of B cells is elevated in patients with rheumatoid arthritis, multiple sclerosis, HIV or Alzheimer’s disease among others. They represent the most proinflammatory B cell subset, with activated immune phenotype and transcriptionally active, but with low proliferative capacity, decreased B cell receptor signalling and impaired antibody production [97].

T cells are probably the most dramatically affected immune components, with a decrease in naïve T cells and an accumulation of terminally differentiated T cells with age. Terminally differentiated T cells exhibit features of replicative senescence and lose the expression of the costimulatory molecule CD28 from their membrane [98–101]. Indeed, CD28 plays an essential role in T cell function, taking part in activation, proliferation and survival processes. Hence, CD28 negative T cells present altered molecular features, as well as distinct cytokine

production and effector molecules [102]. The loss of CD28 affects earlier and primarily CD8 T cells, but it has also been described to reach CD4 T cells later in life [103,104].

In consequence, T lymphocytes have a reduced capacity to react against new stimuli, contributing to the aforementioned immune dysfunction. Another feature found in senescent T cells is the enhanced cytotoxicity. Expression of NK cell characteristic receptors such as CD56 and CD57 membrane molecules have been widely reported in these cells, which promote their cytotoxic capacity [105–109]. Additionally, in CD4 T cells, it has been shown that aged cells preferentially differentiate into the Th17 subset, a subset identified by the capacity to generate cytokines of the IL-17 family. This imbalance toward Th17 polarization is thought to account for the general proinflammatory state and autoimmune response in the elderly [110–112] (**Figure 9**). Furthermore, when comparing the numbers of CD4 and CD8 T cells, many authors have found a higher prevalence of an inverted CD4/CD8 ratio among the elders, a feature known as immune risk phenotype, that predicts shorter survival [113–116].

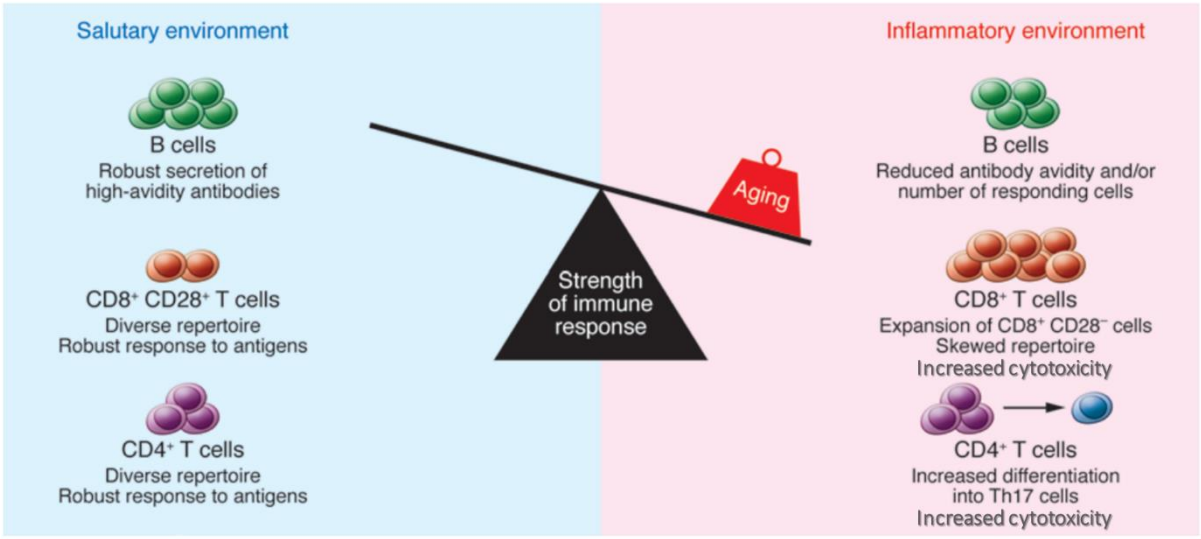


Figure 9. The strength of the immune response declines with age. Multiple age-related changes affect the composition and function of lymphocytes. The number of late memory B cells increases, and consequently, the overall proliferative capacity and antibody production is diminished. CD8 T cells undergo an oligoclonal expansion, lose the expression of CD28 and gain compensatory cytotoxicity, but exhibit impaired function, similar to CD4 cells. Besides, CD4 cells exhibit activation defects and increased differentiation into Th17 cells. In addition, there is an increased concentration of inflammatory cytokines, which may be produced by stromal elements, dendritic cells, or aging B and T cells. Adapted from [96].

1.2.7. Stem cell exhaustion

Similar to the previously addressed cell senescence, stem cell exhaustion is another major cause of tissue and organ dysfunction. Indeed, we presented in **Figure 10** a schematic model

of senescence, in which the accumulation of senescent cells leads to tissue dysfunction. In contrast, senescent cells contribute to the renewal of tissues, when they are properly removed. In this process, the last step for the appropriate regeneration of the tissue is the development of new and functional cells, which is achieved by the differentiation and maturation of stem cells. However, the exhaustion of stem cells is a general problem in aged subjects, contributing to the age-associated loss of function.

Stem cells are tightly regulated and maintained in the protective state of quiescence. This reversible state of temporary cell cycle arrest has been identified in various systems including HSCs, muscle stem cells (MuSCs), neural stem cells (NSCs), intestinal stem cells and mesenchymal stem cells (MSCs) [117,118]. In response to certain intrinsic and extrinsic signals, stem cells can get activated, exit quiescence and perform symmetric or asymmetric divisions. When symmetrically divided, stem cells conduct self-renewal, while asymmetric divisions allow the maintenance of a population of quiescent cells and also yield daughter progenitor cells. Then, a multistep process of controlled proliferation of these progenitor cells leads to the formation of fully differentiated cells [119].

The activation of quiescent stem cells is a highly complex process involving epigenome modulations and the activation of transcription, RNA processing, protein synthesis, DNA replication, mitochondrial biogenesis, and shifts in metabolic pathways among others. All these processes are affected during organismal aging, and therefore, contribute to the age-related stem cell exhaustion [117]. Specifically, it has been described that telomere shortening, oxidative stress, DNA damage, upregulation of $p16^{INK4a}$ and epigenetic changes contribute to the quiescence-to-senescence transition observed in aged stem cells [120,121]. For instance, as mentioned before, the dysfunction of HSCs is one of the causes of immunosenescence. Similarly, NSCs show a reduced proliferation and result in limited numbers of progenitor cells as well as impaired neurogenesis in aged individuals [122]. In the case of skeletal muscles, the reduced self-renewal and exhaustion of MuSCs are also major contributors to age-associated sarcopenia, in which reduced muscle mass with changes in muscle composition and function are observed [119,123] (**Figure 10**). Regarding bone regeneration, the defective osteogenesis has been to shown to be influenced by the preferential commitment of MSCs in the bone marrow to adipogenesis [124]. Besides, MSCs derived from the adipose tissue (ASCs) also have the potential to differentiate into osteoblasts, but the ASCs from elders show a reduced osteogenic potential [125].

All the research studies conducted in the stem cell field and the changes of these cells with aging have improved our knowledge about stem cell exhaustion. This topic is of central interest, as the defects on tissue regeneration are one of the major difficulties of the elderly.

Neurodegenerative diseases, sarcopenia, bone fractures or osteoporosis and immune-related problems have a high prevalence among the aged people, and in most of the cases they result in diminished health status and can even give rise to frailty and dependency. To try to overcome these situations, many studies are being carried out to improve tissue regeneration in elders. They focus both on the potentiation of endogenous factors, as well as on pharmacological interventions or the possible transplantation of functionally active stem cells, for instance for neurogenesis, osteogenesis and myogenesis [122,125,126].

In a different approach, and linked to the accumulation of senescent cells, the use of senolytic drugs for the killing and clearance of senescent cells is proposed. Indeed, the use of senolytics has been demonstrated to selectively target and eliminate senescent cells and rejuvenate HSCs and MuSCs [127], as well as to improve physical function and lifespan in mice [128].

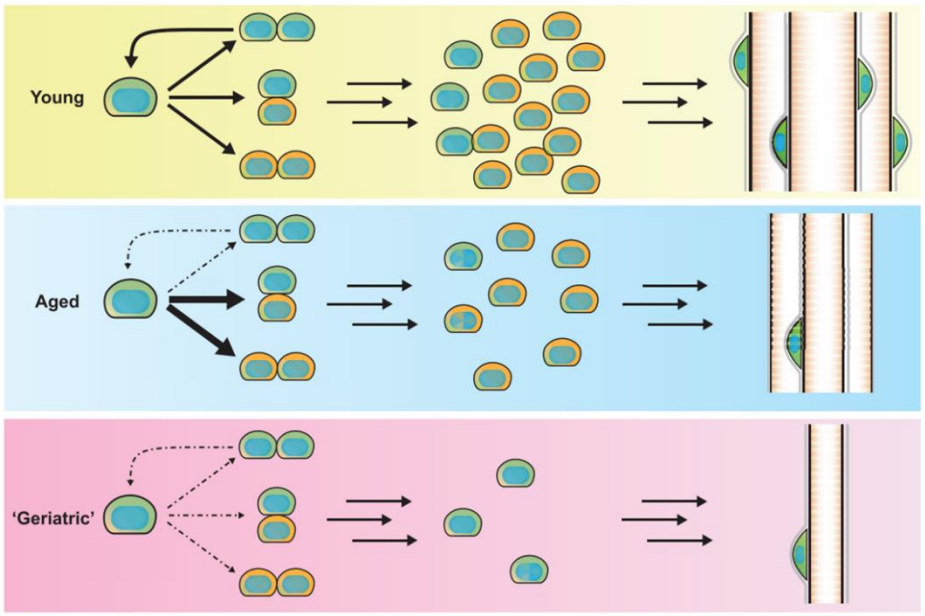


Figure 10. Representation of stem cell exhaustion with aging. Muscle tissue has been taken as an example of an age-related affected tissue. In the upper panel, the optimal situation of a muscle from a young individual is presented. A balance between stem cell self-renewal (green cells) and commitment to myogenic progenitors (orange cells) is maintained. In advanced age (middle panel) muscles show increased lineage commitment (solid arrows) to myogenic progenitors and a lack of self-renewal (dashed arrows), resulting in impaired regeneration and slow exhaustion of the stem cell reserve. Besides, muscle mass starts to decrease. Finally, in the very old or geriatric individuals, most stem cells enter senescence and lose their ability to re-enter the cell cycle. In consequence, the muscle mass continues to decrease and cannot be regenerated. Adapted from [119].

1.2.8. Altered intercellular communication

Finally, to complete the list of the proposed hallmarks of aging, and tightly linked to all the previous characteristics, there is the altered intercellular communication. As its name indicates, this hallmark encompasses all the changes that occur during aging and result in a different communication between cells. The differential communication between cells can be caused both by changes on the secreting cell or by modifications in the receptibility or conditions of the receptor cell. As an example, a cell that enters senescence suffers profound modifications on its inner conditions and its secretome, while the same secreted molecules will produce different effects when received by a non-senescent or a senescent cell.

There are some studies that investigated the differential reception and the elicited cellular changes depending on the characteristics of receptor cells [129,130], but to date, most of the efforts have focused on the description of the changes on secretion during aging. Specifically, the term senescence-associated secretory phenotype (SASP), which was proposed only a decade ago [131], is widely used and includes all the signalling changes that senescent cells suffer. Notably, as seen before, the entrance of a cell into senescence can be influenced by many molecular and functional changes, from genome instability to oxidative stress or loss of proteostasis. In consequence, even if some generally common features have been found, the particular components of the SASP are highly variable depending on the tissue, cell type and surrounding environment [132].

The components of SASP were classified into three major groups: soluble signalling factors (interleukins, chemokines, and growth factors), secreted proteases, and secreted insoluble proteins/extracellular matrix components. In the last years, a new player in cellular senescence and SASP has been proposed: the extracellular vesicles (EVs) [133]. EVs have been found to be secreted by most cells types and to be implicated not only in senescence, but in many other cellular processes. Furthermore, these secreted particles are of central interest in our research, and for that reason, we have included a dedicated section (introduction section 2) to describe their characteristics and functions.

Coming back to the SASP, it is important to mention that it is not necessarily a deleterious process. Indeed, the SASP is a cause of cellular senescence, and as explained before, senescence can have both beneficial effects for example for tissue regeneration and detrimental effects when it is not resolved and senescent cells accumulate [134]. Similarly, SASP can help tumour suppression or promotion. The antitumorigenic role can be driven by the entrance of cells into senescence before losing proliferation control and producing SASP components that will promote cellular clearance. In contrast, some senescent cells can secrete immunosuppressive components that can help tumour progression or relapse [135].

A relevant proportion of the SASP components influence the immune system regulation, and consequently, they are also related to immunosenescence. Particularly, the soluble signalling factors secreted during aging give rise to an inflammatory state termed inflammaging.

1.2.8.1 Inflammaging

The systemic and chronic low-grade inflammation observed in elders is generally referred to as inflammaging. Inflammaging is considered as an "sterile" inflammatory state, as it is present even in the absence of overt infection. Besides, it has been reported that inflammaging is a significant risk factor for morbidity and mortality in aged individuals [136].

There are several potential sources for the increased concentration of inflammatory molecules with aging. For instance, the production of inflammatory mediators can be driven by damaged cells or debris that are not properly eliminated or by the increased number of senescent cells [137]. Furthermore, immunosenescence can be considered both a cause and a consequence of inflammaging: the chronic low-grade inflammation keeps a pressure for immune cell activation, exhaustion and senescence, while the senescent immune cells contribute to inflammation by secreting elevated amounts of inflammatory cytokines. In addition, all the age-associated damages and loss of functions described before, also play a role in the development of inflammaging [33].

Cytokines are one of the major regulators of inflammation. These small proteins are secreted by a wide range of cell types and they can promote or inhibit immune responses. A comprehensive work published by Minciullo and collaborators reviewed the role of proinflammatory and anti-inflammatory cytokines in aging and longevity [138]. Interestingly, the balance between the promoters and inhibitors of immune responses has been related to healthy aging and longevity. On the contrary, the destabilization of the system and the increase of proinflammatory cytokines results in inflammaging (**Figure 11**).

Most cytokines interact with cell surface receptors to initiate intracellular signalling cascades that ultimately activate transcription. One of the targets of inflammatory cytokines is the transcription factor NF-κB. Besides, the NF-κB protein positively regulates many genes that encode proinflammatory cytokines and genes involved the SASP, which can result in a positive feedback loop that enhances inflammation [110,137,139].

Among inflammaging, the most widely studied feature is the circulating concentration of interleukin-6 (IL-6). The concentration of this interleukin is normally low (or non-detectable) in healthy adults, while elevated levels of IL-6 have been reported in the elderly, with increasing concentrations in the very old [138,140]. Moreover, elevated IL-6 has also been associated with disability and mortality in the elderly [141,142]. Other inflammatory

mediators such as tumour necrosis factor alpha (TNF-α) and C-reactive protein (CRP) have also been investigated by many authors, and their concentration have also been found to be elevated in elders [143–145].

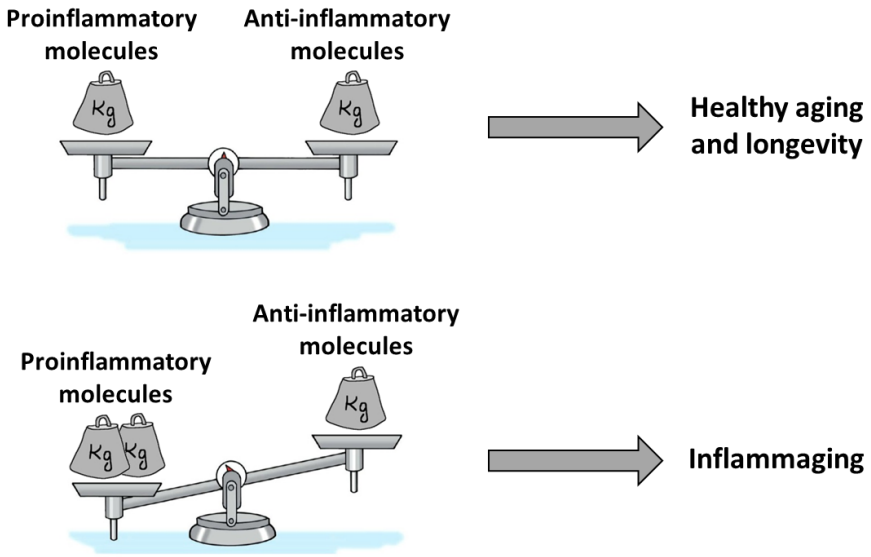


Figure 11. The benefits of maintaining balance. The importance of keeping a functional immune system and a balanced inflammatory state has been shown be related with healthy aging and longevity. On the other hand, when the proinflammatory molecules accumulate and the balance is lost during aging, chronic low-grade inflammation or inflammaging develops.

However, with regard to the roles of cytokines, we should always bear in main the complexity of their functions. Actually, molecules such as the mentioned IL-6 and TNF-α have proinflammatory but also anti-inflammatory functions [146–150]. Their effect depends on the surrounding environment, on the membrane molecules expressed in receptor cells and on the signalling cascaded they elicit. Thus, even if IL-6 and TNF-α are in most of the cases indicators of inflammaging among the old individuals, in some cases there could be other underlying processes and the presence of these molecules could be beneficial [95].

1.3. Molecular biomarkers of frailty

When defining frailty and the main characteristics of the people affected by this syndrome (introduction section 1.1), we commented about the complexity of its identification. Due to the lack of consensus on an operational definition of frailty, many different tests are employed nowadays at primary care services. The physical, cognitive or psychological characteristics that the frailty scales measure, are considered functional biomarkers [151,152]. In this sense, the aim of molecular biomarkers is to complement the already applied tests to help the identification of frail individuals.

Many efforts are being made to try to identify and validate molecular biomarkers of frailty, and even if some have been proposed, to our knowledge none of them are applied in the clinic. In the search of frailty biomarkers, several studies have investigated molecules implicated in the biology of aging and, therefore, included in the hallmarks of aging, aiming to find differences between healthy aging and frailty. In the next, lines we will comment some of the biomarkers that have been suggested to be implicated. A graphical representation of the biological characteristics and biomarkers of frailty is shown in **Figure 12**.

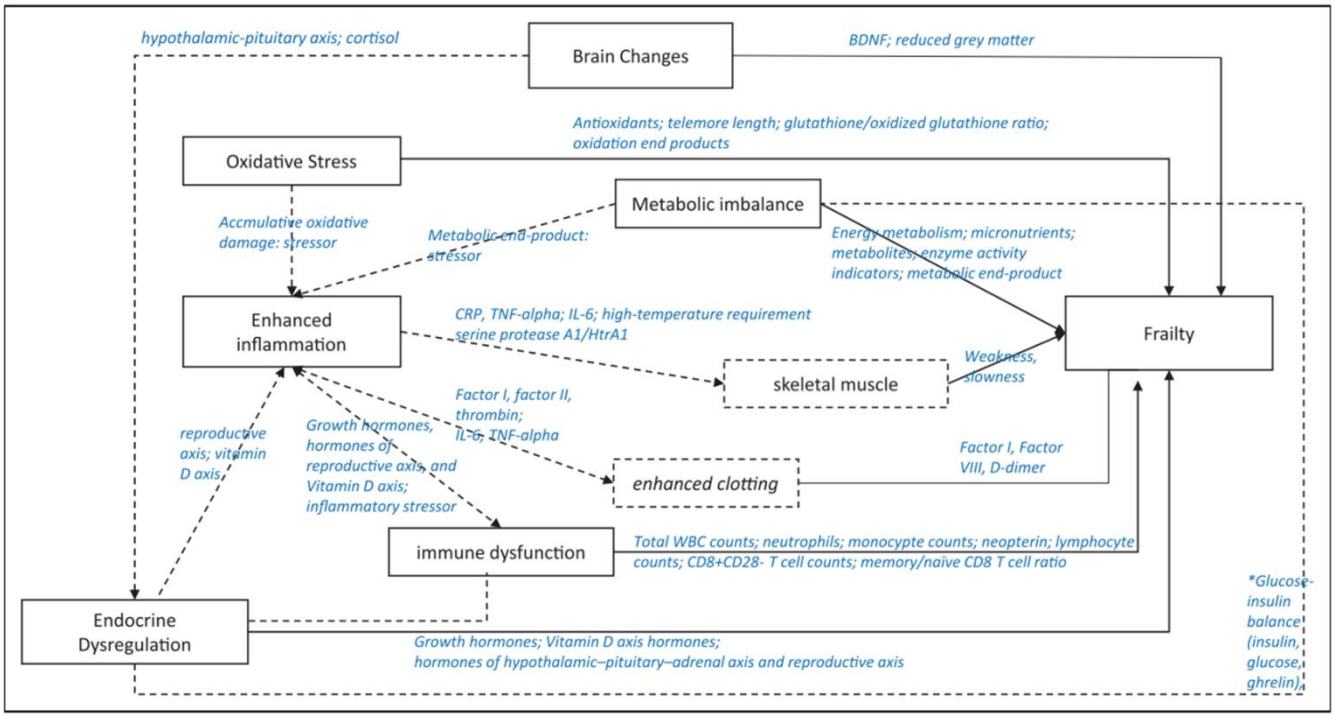


Figure 12. Schematic representation of the main biological processes, their interconnections, the implicated molecules and their relation to frailty syndrome [153].

The current knowledge points, for instance, to a role of oxidative stress in the development of frailty. In this sense, most works have shown increases in oxidative damage indicators, while reduced levels in antioxidant micronutrients, in frail individuals, which gave rise to the recently proposed free radical theory of frailty [154]. This theory suggests a change in the focus of oxidative stress, as diverse studies showed that oxidative damage does not correlate with chronological age, but rather with frailty.

One of the oxidative stress biomarkers of frailty is the elevated levels of circulating protein carbonyls, a well-established indicator of protein oxidative damage. For example, high protein carbonylation correlated to poor grip strength, particularly among older women [155]. Besides, low levels of circulating antioxidants like vitamin E have been found to be associated with frailty [156,157]. However, other authors did not find differences between vitamin E levels and frailty [158], and the fact that vitamin levels can be easily altered with diet or during

disease, complicates even more the interpretation of results. Another vitamin that has been studied in many diseases and processes, including frailty, is vitamin D. Lower levels of this hormone have been linked to frailty, but the normal ranges of vitamin D are highly variable depending on the geographical area and among seasons, and the effects of long-term supplementation with vitamin D are still controversial [3,159,160].

The levels of other hormones have also been investigated as potential biomarkers. Most of the endocrine markers proposed in the context of frailty are those related to the decline in muscle mass and function. During aging, there is a progressive switch from anabolic to catabolic metabolism that affects muscle proteostasis, which has been related to variations in certain hormone levels. Indeed, dehydroepiandrosterone sulfate (DHEAS) is an important regulator of muscle mass and strength that decreases with age and it has been related to sarcopenia [161]. Besides, DHEAS stimulates the production of IGF-1, which is required to muscle regeneration. Some publications have reported lower levels of DHEAS in frail subjects and improved physical function with DHEAS supplementation and exercise [162,163]. In contrast, other authors did not find significant correlations between frailty and the levels of testosterone, DHEAS or IGF-1 individually, while the accumulation of multiple anabolic deficiencies was a good predictor if frailty [164].

Another characteristic generally associated with aging and widely investigated in frailty is metabolic imbalance, and specially glucose and insulin dysregulation. Elevated basal levels of glucose and insulin, insulin resistance and abnormal insulin-glucose dynamics have been related to higher rates of baseline frailty and greater odds of frailty onset [153,165–167]. Notably, it should be mentioned that even if most of the works found some alterations, not all of them obtained the same results. For example, the basal levels of glucose were reported to be elevated in frail subject in some works, while they were not significantly different from non-frails in other publications. In addition, as epidemiological studies indicate that diabetes is a risk factor for developing frailty, and some frail elders without diabetes have elevated levels of glucose, the question of whether this imbalance could be a cause or a consequence of frailty remains open.

The link between chronic inflammation and frailty has been extensively investigated. The concentration of inflammatory mediators in circulation has been measured in many different cohorts aiming to test whether proinflammatory molecules are specially increased in frail individuals when compared to robusts. Certainly, an elevated concentration of IL-6, TNF-α and CRP, among others, have been reported in most of the cohorts in frail elders [144,168–172], but there are also some studies that did not find significant differences between robust and frail individuals [159,173]. Moreover, these three molecules are increased in a vast range of

inflammatory or infectious conditions, so they could not be used as a single measure, and should be combined with other biomarkers that provide information about additional variables related to frailty, such as muscle loss or bone degeneration [174].

Also, the coagulation activity is related to inflammation. Indeed, hypercoagulability both reflects and contributes to enhanced inflammation [153]. Hypercoagulability is generally observed during aging, and elevated levels of coagulation markers, such as fibrinogen, factor VIII, D-dimer and tissue plasminogen activator have also been linked to higher rates of frailty [165,173,175]. However, similar to the previously mentioned biomarkers that have been proposed for frailty, not all the authors obtained the same results. For instance, elevated fibrinogen was related to a higher risk of frailty in women and men by Walston and collaborators [165], while it was only associated with frailty risk in women by Gale and co-workers [175], and the study performed only in women by Reiner *et al.* found no associations between fibrinogen and the risk of incident frailty [173].

Another remarkable source of frailty biomarkers is linked to brain changes. This organ is markedly affected by aging and indeed, the incidence of many brain diseases increases notably in elders. A study by Buchman and collaborators followed nearly 800 aged people and showed that frailty progresses with age, and an accelerated decline was reported in the participants that were found to have brain pathologies in the postmortem evaluation [176]. In an attempt to identify easily measurable brain biomarkers, reduced cerebellar grey matter volume assessed by magnetic resonance imaging have been found in frail elders when compared to robusts [177]. Similarly, the neuroprotective brain-derived neurotrophic factor (BDNF), which protects adult neurons from death during stress and promotes the development of immature neurons, can be measured in plasma, and decreased levels of this protein were linked to higher rates of frailty in women. Moreover, a physical intervention elevated the concentration of BDNF both in robust and pre-frail participants, suggesting its implication in the pathophysiology of frailty [178].

In a different approach, alterations at the genetic level are evaluated. This is the case of telomere length, epigenetic changes and gene expression modifications or even posttranscriptional regulation. With regard to telomere length, many authors have investigated whether there are associations between shorter telomers and frailty syndrome. A systematic review and meta-analysis that was published recently, identified 155 publications on this topic [179]. Interestingly, they selected 9 studies that measured telomere length in leukocytes and concluded that, in accordance with previous reports, telomere length might not be a meaningful biomarker of frailty. In fact, they reported no significant differences in 8 of the selected studies and found only shorter telomeres in the study that was performed in Hispanic

individuals. As discussed in one of the works that investigated the association between telomere length and frailty, even if some aspects that are related to frailty, such as oxidative stress or inflammation contribute to telomere shortening, they may not represent the predominant factors influencing the complex and multicomponent syndrome of frailty [180].

The regulation of gene expression by epigenetics was already commented to be implicated in the biology of aging. Some authors are also working on the question of whether epigenetic modifications, such as DNA methylation influences the incidence of frailty. The investigations performed on the DNA methylation patterns indicate that frailty could be related to accelerated epigenetic aging [181,182], and even specific differences could be observed between twins with a distinct frailty index [183]. However, the cost and complexity of DNA methylation pattern studies in comparison to directed gene expression analyses should be taken into account to evaluate the applicability of these methods in the clinics. Another study approach that focuses on the factors that influence gene expression and function is the identification of SNPs associated with frailty. In this sense, polymorphisms in genes involved in inflammation, muscle biogenesis or apoptosis regulation among others have been related to frailty [7,184].

Notably, the association between gene expression and frailty is probably one of the most widely investigated features, as it englobes all the cellular pathways as well as the studies that measure the expression of thousands of transcripts or only a single one. In consequence, there are hundreds of publications that investigated the expression of certain genes or pathways, and now also 'omics'-based approaches are being developed [185,186]. Recently, a comprehensive review of the biomarkers of frailty was published, in which genes but also proteins and secreted factors related to aging were included [187]. They differentiated the biomarkers in seven categories: inflammation, mitochondria and apoptosis, calcium homeostasis, fibrosis, neuromuscular junction and neurons, cytoskeleton and hormones, and other principles. The authors also classified the biomarkers depending on their priority, with highest scores being attributed to the factors associated with frailty and with more than one hallmark of aging, and with a considerable amount of evidence that the marker is not equally expressed in frail versus non-frail individuals. Finally, they proposed a panel of frailty biomarkers composed of 19 high priority, plus 22 medium priority and 3 low priority markers. Most were proteins or genes, but other emerging biomarker candidates such as miRNAs and microparticles were also included (**Figure 13**). Importantly, the investigation of the emerging biomarkers of frailty continues to develop, as illustrated by the publication of a work focused on miRNAs nearly at the same time that the commented review [188]. Besides, as mentioned before, the case of microparticles (or extracellular vesicles) in age-related processes and also

in frailty is of central interest in our work, and therefore, the following section is dedicated to these particles (introduction section 2).

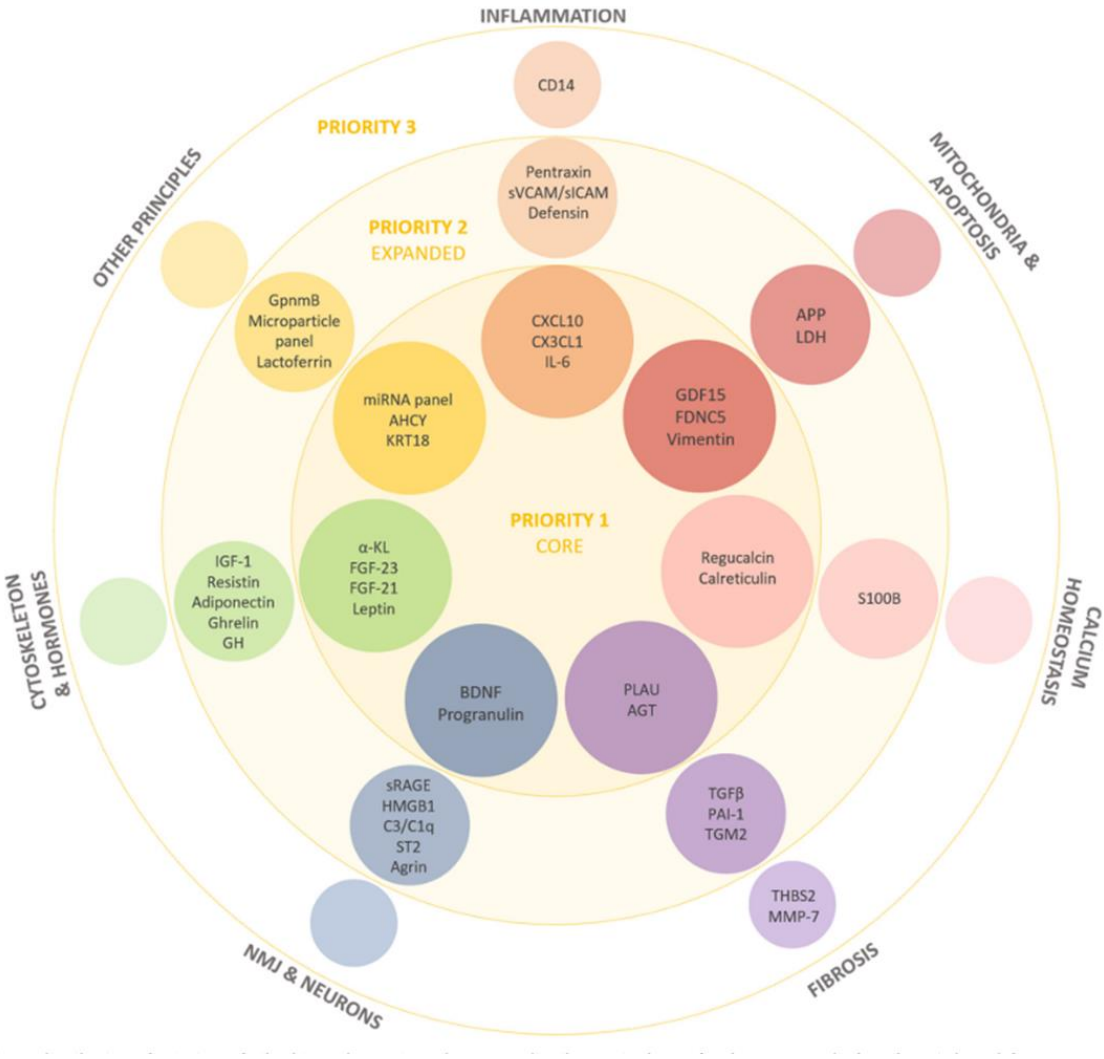


Figure 13. The proposed biomarkers of frailty. The panel is composed of a core of high priority factors and complemented by medium and low priority markers [187].

2. Extracellular vesicles

EVs are membrane-coated particles secreted by almost all cell types. Their first identification was already reported in 1946, as procoagulant platelet-derived particles in normal plasma [189] and more than 20 years later, in 1967, they were referred as "platelet-dust" [190]. Since then, several publications started to report novel particle sources and functions and by the end of the 20th century they were already known to play a role in relevant processes, such as antigen presentation [191]. Importantly, at the beginning of the present century, the research on EVs gained interest among the scientific community, as they were also found to the implicated in other central issues, including the immune system mediated antitumor response [192], and due to the discovery that EVs transfer mRNAs and miRNAs from the donor cell that can induce functional changes in recipient cells [193].

In the last decade, thousands of works have continued describing the characteristics, functions and implications of EVs in intercellular communication. Thanks to all of them, we can now state that EVs are important players in most biological processes. However, as it usually happens in scientific research, the more we know, the more complex the picture is, and the more we need to investigate to understand the molecular processes that govern ourselves and the rest of living organisms.

2.1. Biological characteristics of extracellular vesicles

The term EVs is used to refer to all the particles that cells secrete to the extracellular media. There are two main categories of EVs: exosomes and microvesicles. Besides, apoptotic bodies are also considered EVs. Indeed, apoptotic bodies play an essential role in the proper clearance of the dying cell as well as for the signalling of this programmed cell death to surrounding cells and for the regeneration of the tissue [194]. However, most of the works studying EVs are focused on exosomes and microvesicles, due to their multiple functions and implications.

Exosomes are secreted particles originated by the fusion of a multivesicular body and the plasma membrane, while microvesicles are formed by the direct budding and fission of the plasma membrane. Moreover, apart from their distinct biogenesis, exosomes and microvesicles have also classically been differentiated based on their size. Exosomes were defined to be around 50-100 nm in diameter, and microvesicles from 100 nm up to 1 μm [195]. However, even if this classification was formerly accepted, nowadays we know that there are larger particles originated at multivesicular bodies, as well as smaller vesicles that evaginate from the plasma membrane. Consequently, the International Society of Extracellular Vesicles (ISEV) discourages the use of these terms if the biogenesis pathway of the vesicles is not

known, and recommends the use of EVs or just small, medium or large EVs if we want to refer to their size [196].

With regard to the molecules carried by EVs, we have to consider both their membrane and inner cargo. The membrane of EVs consists mainly of proteins and lipids, but each EV has distinct types of proteins and lipids depending on their origin and function. Furthermore, the composition of the EV membrane influences the fate and internalization by recipient cells [197]. The components of the EV lumen are even more diverse and include proteins and many different nucleic acids. Apart from the above-mentioned mRNA and miRNAs, EVs carry other types of small and long ncRNAs, circRNAs and dsDNA fragments [198,199] (**Figure 14**). Importantly, the investigations about EV secretion and their cargo revealed more than a decade ago, that the sorting of components into a forming particle is a controlled mechanism and not a random packaging of the available molecules in the secreting cell [193,200].

Similarly, the uptake of EVs is thought to be a controlled process. Many authors have studied the binding and internalization of EVs by recipient cells and multiple molecules, such as tetraspanins, integrins, lipids and lectins, have been identified to mediate the uptake. Besides, the integration of EVs can be performed by the fusion of the EV and cellular membranes, or by distinct endocytic pathways (**Figure 14**). An extensive and complete review on the biogenesis, release and targeting mechanisms of EVs was recently published by Niel and co-authors, and it is a recommended read to go into this subject more in depth [197].

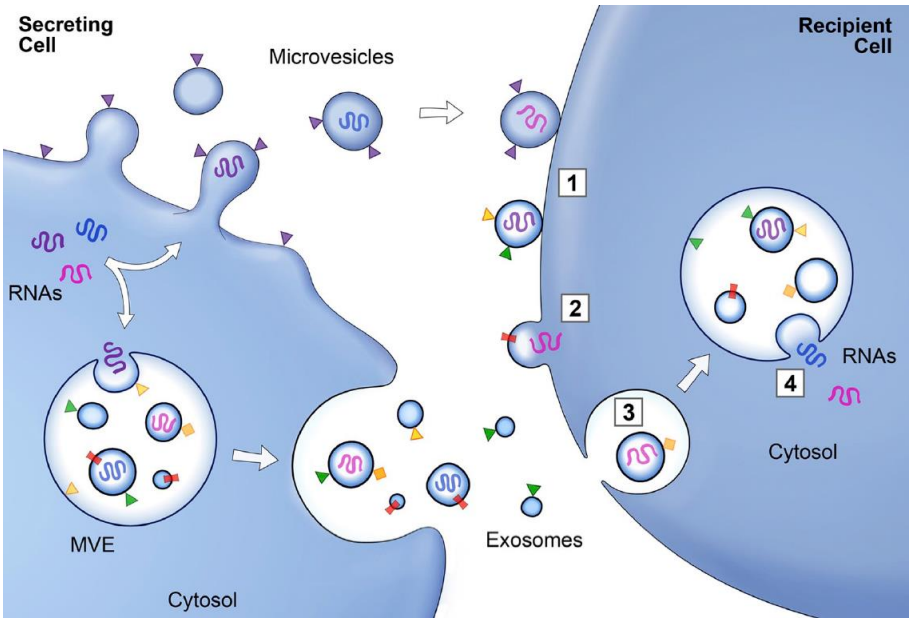


Figure 14. Simplified representation of the secretion (microvesicles and exosomes) and uptake of EVs. For the internalization, EVs can (1) dock and (2) fuse with the plasma membrane, or (3) get endocytosed and (4) eventually fuse with a membrane of the endocytic compartment [195].

Due to the focus of this work, we present here the main characteristics and functions of EVs from human cells, but the intercellular communication mediated by EVs is also present in many other organisms. Furthermore, it has been found that unicellular organisms like bacteria also secrete EVs. Interestingly, the bacteria produce EVs for multiple purposes, including horizontal transfer between intra-species cells, stress response, or biofilm formation. In addition, in mammals, the communication between host and bacterial cells of the microbiota is mediated, at least in part, by EVs [201].

2.2. Isolation and characterization of extracellular vesicles

The first step to take into consideration to obtain EVs is to decide the sample from which we want to isolate the particles, and to perform a proper collection, handling and storage. EVs can be isolated from biological fluids, including blood, urine or cerebrospinal fluid and from cell culture media [202–205]. Depending on the selected source, specific recommendations have been proposed [206]. However, there are many variables that can influence EV secretion and that cannot be completely controlled. For example, when taking blood samples, it has been described that age, sex, diet, infections, treatments or even circadian variations can affect the EVs in circulation [196]. Besides, as we will describe below, there are plenty of EV isolation methods and the choice would depend on the sample characteristics, study objective and available techniques. Thus, it is essential to collect and report all the possible information about the donors, samples and applied steps so that we can take into consideration all our variables, and also to enable the potential replication by other authors.

Regarding EV isolation, it is important to mention that in most of the cases, if not in all of them, it is not possible to achieve a complete separation of the vesicles of interest. Therefore, we have to consider that even if the term isolation is commonly applied, we are probably enriching our samples for EVs. Moreover, this issue is not exclusive for EVs, as other techniques also used for cells, such as sorting, precipitation or immunocapture present good but not perfect yields.

The methods for EV separation are diverse, and besides, each technique can have distinct settings depending on the subtype of EVs aimed to enrich. For instance, differential centrifugation is one of the most widely applied methods, but the centrifugation sequences, forces and times vary among studies. The first steps are usually similar, with centrifugations at low centrifugal forces (< 10,000 g) to pellet cells and debris. Then, some investigators apply middle force centrifugations (15,000-30,000 g) and recover the EVs from the pellet, while the ones that focus on small EVs take the supernatant and perform high speed centrifugations, or ultracentrifugations (usually 100,000-200,000 g). Besides, there are authors that complement differential centrifugations with density gradient centrifugations [206,207].

Ultracentrifugation has been one of the most used methods, but some authors have reported that it can coprecipitate protein aggregates or viruses and can even induce EV clumping and damage [206,208].

There are other classically applied techniques for EV isolation that include size exclusion chromatography, precipitation, filtration and immunocapture. For size exclusion and filtration, the pore size of the matrix and of the membrane, respectively, can be selected. In addition, in the last years, a different filtration method has been introduced: tangential flow filtration (TFF). In contrast to the common filtrations that pressure the sample perpendicularly to the filter, TFF consists on the application of a tangential force, which minimizes pressure and enables the recirculation of the sample into the system. Besides, the pore size of the membranes applied for TFF can also be chosen depending on the desired EVs. The use of TFF is particularly beneficial when large volumes of samples are handled, as cell culture media or urine [209,210]. However, TFF can only separate the EVs based on their diameter.

On the other hand, the immunocapture methods are attractive when a specific subpopulation of EVs want to be separated. This system is based on the use of immobilized antibodies that recognize and bind EV-specific molecules, usually proteins exposed at their membrane. The selected antibodies can be immobilized on a plate, a chip or a magnetic bead, and there are many commercial kits available [207]. Nevertheless, when using immunocapture protocols unwanted soluble ligands can also be recovered, or part of the desired EVs lost if there are more ligands than antibodies available. In addition, immunocapture will always separate a subpopulation of EVs, as no universal marker has been found. For this reason, as an example, we cannot claim to isolate all exosomes from a complex sample when applying an anti-CD63 antibody because not all the exosomes are positive for this tetraspanin. To illustrate the complexity of EVs, a representation of some of the most common molecules identified to be carried by EVs can be seen in **Figure 15.**

Interestingly, new methods are being developed for the isolation of EVs. The microfluidic and acoustic settings are promising techniques, and even the combination of both of them have been shown to be effective to isolate EVs from whole blood [211]. In any case, the election of the EV separation method (or combination of methods) is strongly influenced by the objective of the study, as well as by the required time, costs and applicability, if it is directed for a potential clinical application [202].

With regard to the characterization of EVs, and despite their small size, there are multiple methods available: the ones that could be applied to characterize cells that have been adjusted for EVs, and the ones that have been specially developed for EVs. On one side, we can describe

general features of the obtained sample such as the number, concentration, size or morphology of the particles, among others. For this purpose, two of the most applied techniques are nanoparticle tracking analysis (NTA) and electron microscopy (EM) [212,213]. Furthermore, the advances conducted in the last years have enabled the detection of labelled particles with these techniques.

On the other side, for the more exhaustive characterization of EVs, we try to describe their composition and cargo. For the detection of the membrane markers of EVs, flow cytometry is a reliable technique. The use of flow cytometers is also applied for the quantification of EVs, but besides, it has the potential to detect and differentiate a complex mixture of fluorescently labelled particles. However, the classical flow cytometers were developed for the detection of cells, and cannot accurately identify vesicles smaller than ~500 nm. Aiming to overcome this issue, bead-based detection methods for EVs have been developed [214,215], but also new generation flow cytometers with higher sensitivities able to discriminate individual small vesicles [216] and even imaging flow cytometers [217]. Notably, apart from the new instruments, relevant efforts are being conducted for the analysis and standardization of EV flow cytometry [218].

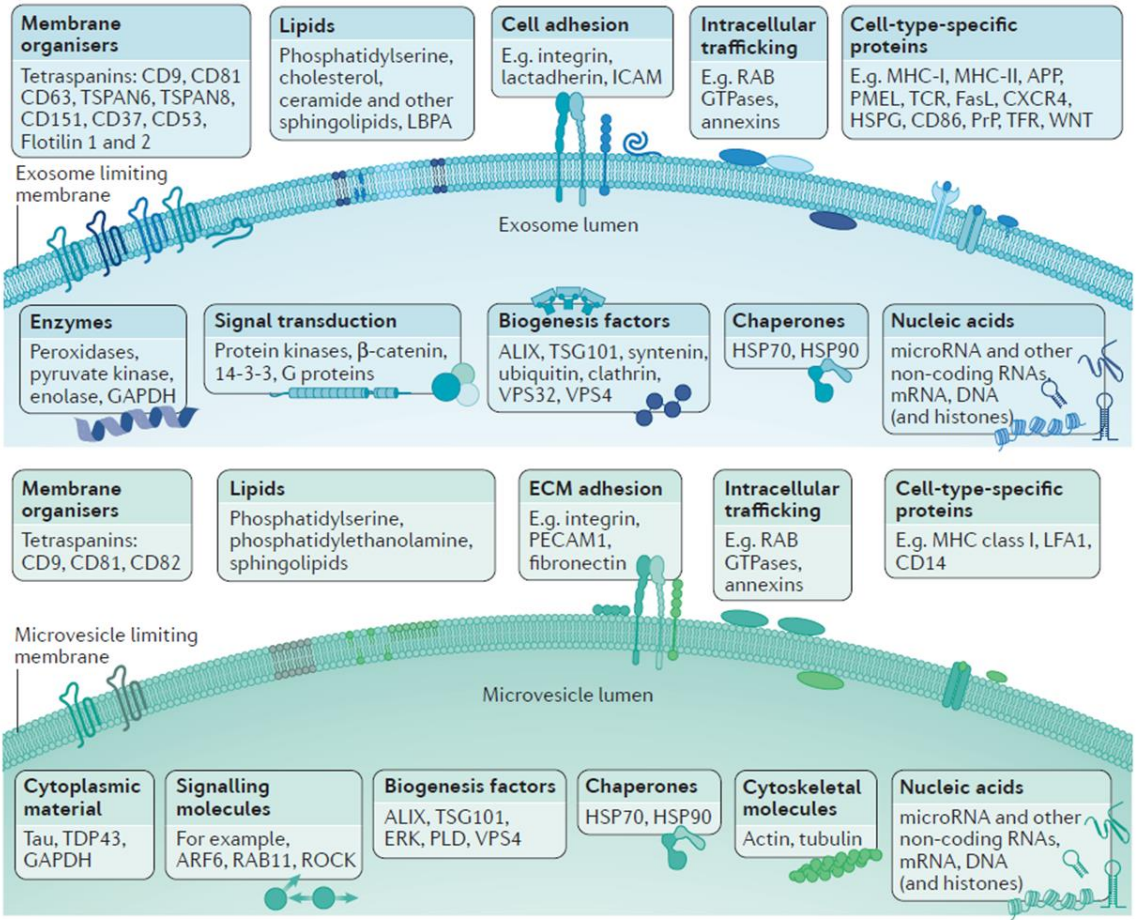


Figure 15. Composition of extracellular vesicles. The two main subtypes of EVs (exosomes and microvesicles) carry hundreds or thousands of molecules. Even if some of the molecules have only been found in one of the subtypes, most of them can be present in both exosomes and microvesicles [197].

There are many other methods for the characterization of EVs. Western blotting is the most widely used method for the identification of selected proteins in EV samples, most of the times just as a targeted approach to demonstrate the enrichment of EV-associated proteins in a given sample [196]. However, the use of 'omics'-based approaches give us the opportunity to study the composition and cargo of EVs, and more and more authors are conducting these experiments to characterize the particles of interest. This approach is used not only for proteins, but also for the identification of nucleic acids and lipids [219–221].

Finally, other aspects of EVs like their biodistribution or functionality can be studied [206,222]. In the next section, the current knowledge about the functions of EVs is presented.

2.3. Extracellular vesicles in physiological and pathological processes

As introduced before, it is long known that EVs are functional particles. However, in the beginning, it was thought that the secretion of particles could be a mechanism of cells to dispose of cellular waste or components no longer needed. Years later, but already more than 60 years ago, EVs were described to play a role in blood coagulation [189,190], and similarly, submicron particles were also observed in the nervous system and particularly in neuron synapses [223,224]. Then, EVs were described to be implicated in immune-related processes [189–192], and the knowledge about the functions of EVs continued to increase. By now, EVs have been found to be secreted by almost all cell types and to play a role in diverse biological processes.

EVs are secreted in physiological and pathological conditions, and depending on their cargo and on the conditions of receptor cells, they can have beneficial or detrimental effects [225]. For example, in the central nervous system (CNS) neurons, microglia, astrocytes, oligodendrocytes and neural stem cells secrete EVs. EVs have been shown to mediate the communication between these cells and to be part of neurogenesis, homeostasis maintenance, neuronal activity control and myelin sheath biogenesis processes [226–228]. In contrast, EVs have been related to Alzheimer's disease, Parkinson's disease or glioma, promoting processes such as neurotoxicity, protein aggregation, inflammation and tumour growth [229].

Similarly, EVs have been studied in many cancer types and hundreds of publications have demonstrated their roles in these pathologies. For instance, EVs secreted by breast cancer cells have been described to help angiogenesis, EVs from hepatocellular carcinoma or ovarian cancer cells promote tumorigenesis and MSC-derived EVs from gastric cancer tissue promote proliferation and migration of recipient cancer cells, as nicely reviewed by Smith and collaborators [230]. Furthermore, one of the most important discoveries about EVs in cancer was probably their implication in the "education" of non-cancer cells and the preparation of

premetastatic niches for the establishment of new metastases [231,232]. This is an essential feature that needs to be taken into account for the treatment and the prevention of new tumour formation.

Another system that perfectly represents the beneficial and detrimental faces of EVs, is the immune system. The implication of EVs in the immune system functioning is one of the most exhaustively investigated fields, and dedicated reviews are perfect to get a deep knowledge on this topic [233,234]. Making a long story short, we will mention some examples that illustrate the relevant roles of EVs. With regard to immune system activation, EVs can carry major histocompatibility complex (MHC) molecules loaded with antigens and perform direct antigen presentation by binding to TCR receptors of T cells. On the other hand, they can mediate indirect antigen presentation if the EV loaded with the MHC-antigen complex is transferred to an antigen-presenting cell, that then presents this complex to a T cell. The EV mediated activation reaches not only T cells, but also B cells, NK cells and macrophages [200,235–237].

In contrast, EVs can also help immune inhibition or regulation. For instance, EVs secreted by activated T cells can induce the apoptosis of other T cells, participating in a regulatory prosses know as activation-induced cell death. Besides, particles secreted by neutrophils and erythrocytes can prevent the secretion of inflammatory cytokines by macrophages and the maturation of dendritic cells, and the EVs secreted by MSCs have also been shown to be immunosuppressive *in vitro* and in animal models (reviewed in [233,234]). Both the activating and suppressing examples mentioned can be beneficial for controlling and conducting appropriate immune system responses.

The harmful effects exerted by EVs through the immune system can be exemplified by their link to cancer and autoimmune diseases, like multiple sclerosis (MS). In the case of cancer, tumour-derived EVs have been shown to be able to suppress T cell response, or even promote their apoptosis by the interaction of T cells with the Fas ligands carried by EVs [238,239]. In MS, EVs are implicated in the blood-brain barrier (BBB) disruption and consequent transendothelial migration of autoimmune cell into the CNS, as well as in the spread of neuroinflammation [240–242].

Finally, the role of EVs in aging and age-associated processes is being investigated. When cell senescence and SASP were described, the implication of EVs was not considered, but the works performed in the last years have shed light on their participation. Indeed, stressed and senescent cells have been shown to secrete more EVs [133,243]. Moreover, we know that the cargo of EVs depends on the condition of the secreting cell, and consequently, these particles bear at least some distinct molecules than the ones secreted from non-senescent cells. As a

representative example, the EVs from senescent endothelial cells are enriched on miR-31 and reduce the osteogenic differentiation capacity on MSCs, which could contribute to the age-associated osteoporosis [244]. Furthermore, the same reduction on osteogenesis was reported when the effect of plasma EVs from aged donors was compared to plasma EVs from young adults, and in this case, the vesicular galectin-3 protein was shown to be implicated [245].

In a different approach, EVs secreted by senescent cells have been found to carry miR-433, promote senescence and prevent apoptosis of surrounding cells, inducing chemoresistance in a model of ovarian cancer [246]. In contrast, experiments performed with non-senescent endothelial cells demonstrated that endothelial EVs suppress senescence and promote angiogenesis in target cells both *in vitro* and *in vivo*, which is mediated by vesicular miR-214 [247]. Again, these works illustrate that the cargo and effects of EVs depend on the status of secreting and receiving cells, and thus, EVs are implicated in physiologic and pathologic processes.

2.4. Potential clinical applications of extracellular vesicles

The use of EVs in the clinic has two main applications. On one side, they can be used as biomarkers. EVs can be obtained from distinct body fluids by minimally invasive techniques and can be used for disease diagnosis or treatment monitoring, among others. On the other side, EVs are promising therapeutic agents. They have been suggested as a good alternative for cellular therapies, due to their potential to carry the molecules of interest while preventing the negative effects that could arise from cell therapy. Moreover, they could outperform nanoparticles and other synthetic particles, as EVs are biologically prepared to be received by target cells.

With regard to EV-based biomarkers, they have been studied in several cancer types. Interestingly, in patients with glioblastoma multiforme, tumour derived-EVs cross the BBB and can be found in the blood. Besides, tumour-specific mRNA mutations and characteristic miRNAs could be detected. These examples show the potential to get molecular information about a CNS cancer with a blood test [248]. In the case of pancreatic cancer, EVs secreted by the tumour are also found in circulation, and importantly, a signature of 5 markers carried by these EVs have shown good accuracy for cancer detection, even better than the commonly used serum marker [249]. Similarly, the plasma EV protein profiling has been suggested to be a promising tool for all stage and histological subtypes of lung cancer [250]. In addition, the potential of EVs for disease and therapy monitoring has been investigated in many other diseases. For example, the investigations about EVs on the two main age-associated neurodegenerative diseases, Alzheimer's and Parkinson's disease, and on the other hand, on

cardiovascular diseases have been recently reviewed [251,252]. In our group, previous works focused on MS patients blood also pointed to the biomarker potential of EVs, as we showed a different EV concentration and cargo depending on MS subtype and treatment [253,254].

The use of EVs as therapeutic agents is the other main proposed application. It is worth mentioning that there are several different approaches, depending on the clinical objective and the EV production procedure. The administration of EVs is directed to improve an specific characteristic of the selected disease or condition: tumour progression inhibition [255], axonal regeneration following peripheral nerve injury [256], remyelination of axons [257], reduction of chronic inflammation [258], muscle regeneration [259] or bone regeneration [260] after traumatic injury. These and other applications are closely related to common age-associated problems, including inflammaging, neurodegeneration, reduced cell differentiation and regeneration capacity, sarcopenia or osteoporosis. For this reason, the study of EVs as therapeutic agents is of central importance.

The use of cell therapies, particularly the ones based on stem cells, was proposed to have great potential. However, in the last years, the possibility of administering EVs have gained interest, because they maintain the beneficial effects of progenitor cells while reducing their risks, such as uncontrolled proliferation or transplant rejection [261]. Furthermore, the use of EVs has other benefits, including the easier storage and distribution, as well as the multitude of possible routes of administration and modes of application [262].

Another advantage of the use of EVs is that they are formed from cells and thus, biologically designed for being taken up by recipient cells. In contrast, other constructs like liposomes or nanoparticles are easily loaded with the molecule or drug of interest, but they could face biodistribution or targeting problems, reducing their efficacy [263]. Moreover, there are several techniques for obtaining EVs enriched in a particular compound. On one side, we can modify the EV producing cell by transfection or transduction, by culturing them under a particular stress or condition, or by incubating them with the molecule of interest. On the other side, EVs can be modified after their production, by electroporation, sonication, extrusion or other methods [263–265] (**Figure 16**).

In summary, EVs have a great potential for future clinical applications. We should continue developing new techniques and standardized protocols for describing their basic characteristics, functions and implications, and consequently, advance our understanding in these promising biological particles.

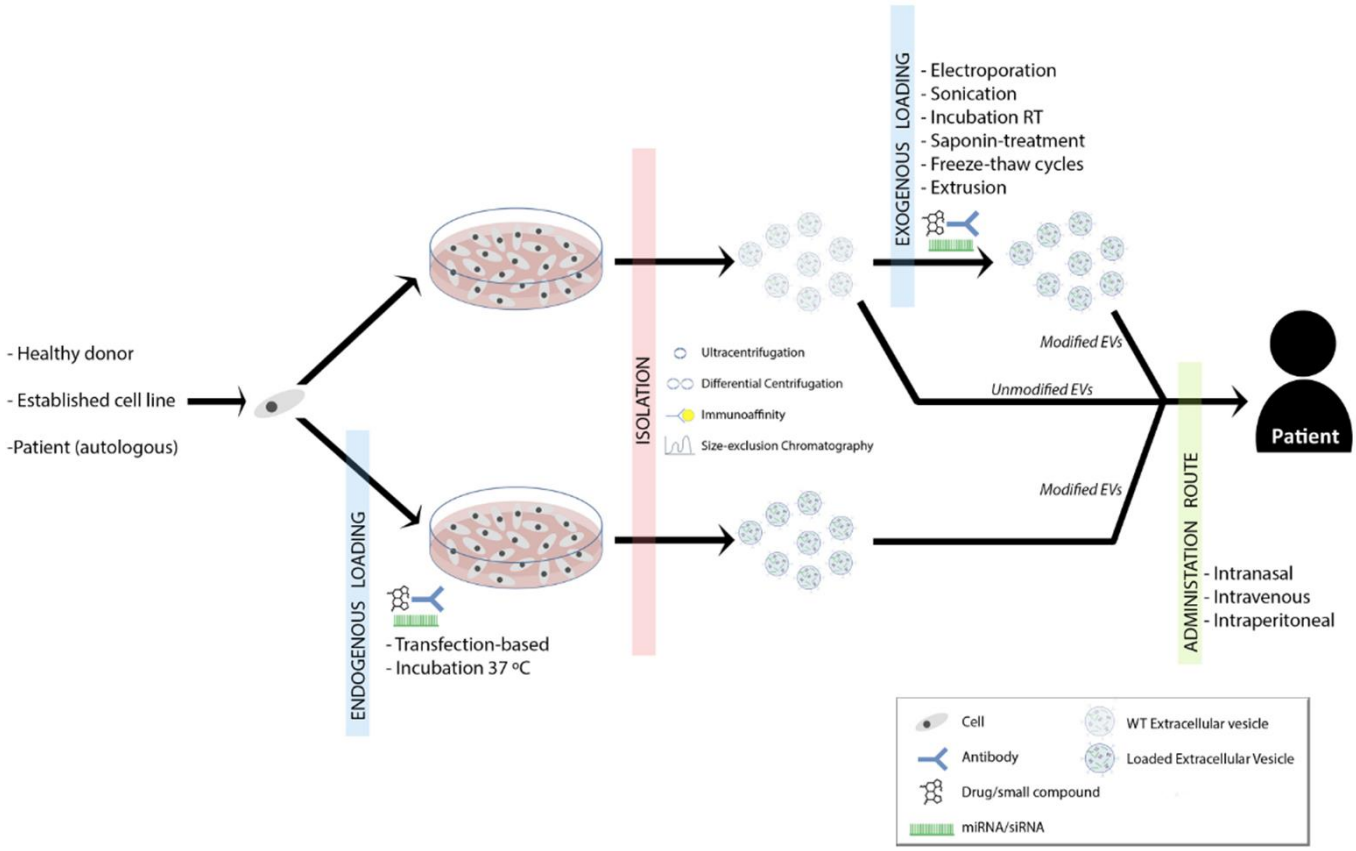


Figure 16. Potential therapeutic approaches with EVs. The producing cells are selected and EVs are produced in controlled cell cultures for the treatment of different disease patients. The cargo of EVs can be modified by controlling the cell culture conditions, by introducing the molecules of interest in producing cells or directly in the EVs. Reviewed in [265].

3. Multiple sclerosis

MS is a chronic autoimmune disease of the CNS, characterized by pathologic demyelination of axons and subsequent neurodegeneration. It is a heterogeneous disease and, clinically, it can follow relapsing-remitting or progressive forms. The relapsing-remitting forms are characterized by outbreaks of neurological disability symptoms lasting at least 24h (relapses) followed by recovery periods (remissions) in which symptoms improve partially or completely. This is the most common disease course at the time of diagnosis, with approximately 85% of patients initially diagnosed with a relapsing-remitting form of MS. In the progressive forms, the disease develops steadily and results in a rapid accumulation of disability. Approximately 50% of patients with relapsing-remitting forms, convert to a secondary progressive phase within 10 years of disease onset [266].

3.1. Etiopathology of multiple sclerosis

MS is a complex disease, and its etiology is not completely understood. It has been found that a combination of genetic, epigenetic and environmental factors, increase the risk of developing MS. Among the genetic factors, the HLA-DRB1*15:01 allele in the MHC class II is the earliest, and most dominant risk factor identified, while cigarette smoking, higher latitudes, low sun exposure, low vitamin D levels and Epstein-Barr virus infection are the principal environmental risk factors. Besides, in the last years, the implication of the microbiome is being investigated, as several studies have shown its influence on the immune system regulation, and some differences in the gut microbiome between MS patients and healthy controls have also been reported [267].

The pathological process of MS is initiated by an inflammatory process mediated by autoreactive T cells. The trigger of the autoimmune attack is thought to be an autoantigen, but it has not been identified yet. The autoreactive T cells get activated in the periphery, start to produce proinflammatory molecules and to express adhesion molecules that favour their attachment to endothelial cells of the BBB. In addition, the BBB is usually damaged in MS patients and, as a result, the autoreactive cells are able to first firmly adhere to endothelial cells, and then migrate through the BBB into the CNS [268]. Once in the CNS, T cells are reactivated by astrocytes or microglia, inflammation spreads and finally, effector T cells damage the myelin sheath of axons, and macrophages and glial cells participate in the digestion process. As a result, there is an impaired isolation of axons and abnormally slow action potential transmission [269]. An illustration of this process is presented in **Figure 17**.

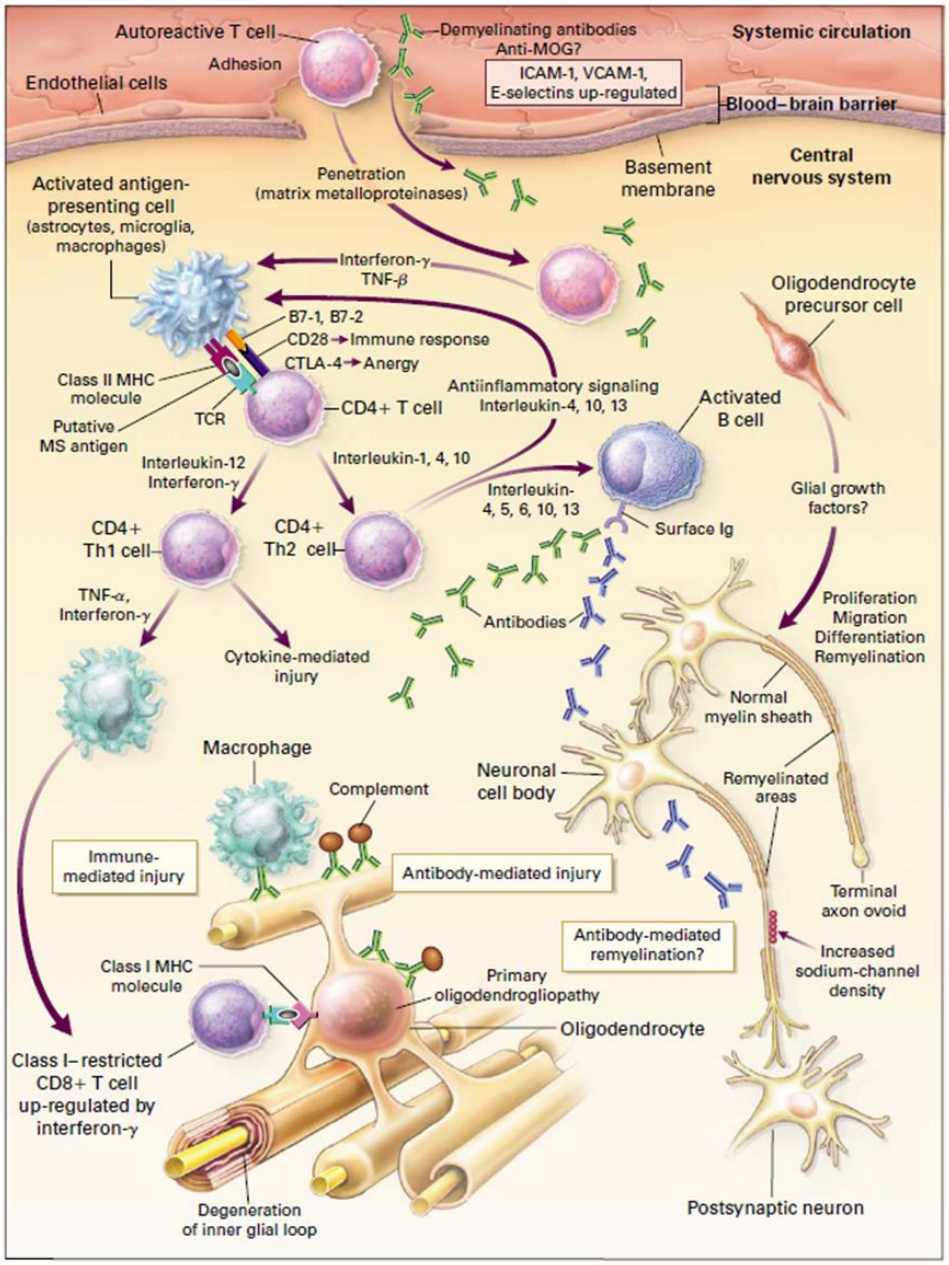


Figure 17. Proposed mechanisms and implicated cells and molecules in demyelination and remyelination processes [269].

In the first stages of MS, the neurologic function is partially or completely restored after a demyelinating event. This process is mediated by oligodendrocyte precursor cells that get activated, migrate to the lesion, proliferate and differentiate to mature myelin-producing oligodendrocytes. However, the newly produced myelin sheath is usually thinner than the original, and besides, the regenerative process becomes less efficient with increasing age. Consequently, the pathologic autoimmune attacks can result in a axonal degeneration and subsequent neurodegeneration, which affects the neurologic function of MS patients and increases disability [270].

3.2. Multiple sclerosis and premature aging

Most of MS patients experience the first symptoms at their 20s or 30s, but it should be mentioned that there are also paediatric or juvenile [271] and late-onset MS cases [272]. Importantly, in the last decades, effective disease-modifying treatments that slow the progression of MS have been developed [267]. Thanks to the beneficial effects of these treatments, MS patients have a slower rate of disability accumulation and thus, a better quality of life than MS patients of previous generations [273]. In consequence, and following the same trend as the general population, the life expectancy of MS patients is increasing, and with it, the mean age of MS patients is also getting higher. The reports of the MSBase registry [274] show that already more than 20% of MS patients are aged ≥60 years (msbase.org). Therefore, an elevated number of patients suffer from the interactions between the MS disease and the aging process.

However, in most of the cases, it is not possible to distinguish between the characteristics of MS and aging in a patient of advanced age. This is due to similarities between the typical features of the two processes, which include cognitive and cardiovascular problems, bowel and bladder dysfunction, or reduced mobility, among others. Of course, not all the MS patients or elders present these problems, but they are common in the two cases. The similarities between MS and aging are also reported at the biological level, as immune system exhaustion and chronic inflammation occur in both processes [275]. Furthermore, it would not the possible to discriminate between the causes of each feature, as the organism has to be understood as a whole entity, in which the dysfunctions accumulate and can influence the other systems (like explained for the biologic hallmarks of aging).

In a different approach, the possible development of premature aging in patients with autoimmune diseases like MS, type 1 diabetes or rheumatoid arthritis has been proposed [276–279]. Particularly for MS patients, the chronic and intense implication of the immune system during MS pathology, as well as the effects of immunomodulatory drugs prescribed, have been suggested to promote the premature exhaustion of the immune system [279]. Indeed, some of these works also alluded to the possibility of an inverse relation, with increased risk of developing autoimmune diseases under premature immunosenescence.

With regard to immunosenescence, some works reported increased levels of CD4+CD28- T cells [280,281], thymic involution [282], altered T cell homeostasis [283] and disturbed regulatory T cell development and function [284] in adult MS patients. In relation to inflammation, elevated levels of TNF-α and IL-6 among other inflammatory markers have been found in the cerebrospinal fluid and serum of MS patients during remission, indicating that

some signs of chronic inflammation, similar to the ones observed the age-associated inflammaging could be present [285,286].

These publications point to the possible premature immune decline in MS. In contrast, there are other works that did not find immunosenescent features in MS patients, or that reported differences in immune characteristics depending on the immunomodulatory drug received by the MS patient [277,287,288]. In any case, the link between premature aging and MS should be further investigated to elucidate whether there is a causative relation, and if such, which steps could be taken to prevent or appropriately treat this situation.

JUSTIFICATION

One of the major concerns of our society is the aging of the population. The rapid increase of life expectancy and the consequent rising incidence of age-associated diseases and dependency have made us aware of the interdisciplinary challenge we face.

Many efforts are conducted in numerous fields to manage the socioeconomic impact of aging and we, as part of the scientific community, are working to describe and understand this complex process from the biological, biochemical and biomedical point of view.

In this context, the present project was outlined in 2015, aiming to advance knowledge and contribute to the ultimate goal of improving the quality of life during the natural process of aging.

HYPOTHESIS AND OBJECTIVES

Hypothesis

- There are several tests and scales that are applied for the identification of frail individuals at primary care services. Moreover, many biomarkers have been proposed. However, frailty is a heterogeneous process and they do not identify all frail individuals. Blood is an accessible biofluid, and it is an interesting source of frailty biomarkers that should be explored.
- EVs circulating in blood are in contact with many tissues and cell types, and they can influence diverse cellular processes in receptor cells. As EVs from adults and elders are different, they could have distinct effects on cell differentiation.
- It has been described that immunosenescence is a progressive process, but it has not been extensively investigated in nonagenarians and centenarians. Besides, EVs have been shown to play an important role in immune system functioning and we hypothesize that they are also implicated in age-related changes.
- In autoimmune diseases, such as MS, the immune system of affected patients is aberrantly stimulated and activated. These episodes are repeated over the years and they could lead to the exhaustion and premature aging of the immune system.

Objectives

- To find a biomarker that could complement the cognitive and functional tests and help the identification of frailty.
- To test the effect of plasma EVs on the differentiation process of different cell types and the influence of age in these processes.
- To describe and compare the immunosenescence status and the implication of plasma EVs in adults and elders.
- To characterise immunosenescence and inflammaging in multiple sclerosis patients and evaluate the presence of premature aging.

CHAPTER ONE

Biomarkers of frailty

General introduction

Life expectancy has increased notably in the last decades, but in most of the cases the last years of life are accompanied by comorbidities, disability and dependency [289]. These problems worsen the quality of life, increase the risk of hospitalization and institutionalization and, consequently, social and healthcare spending. Disability and dependency are usually preceded by frailty, a syndrome characterised by a reduced functional reserve, impaired adaptive capacity across multiple physiological systems and increased vulnerability [3]. Importantly, frailty is the main risk factor for the development of disability among the community-dwelling elders and can precede the deleterious outcomes by several years [290]. Therefore, the identification of frail individuals and the consequent interventions are a key point for preventing dependency.

However, frailty is a heterogeneous state comprising physical, psychological and cognitive impairment, and there is no consensus on the best tool to identify frailty. Several tests based on clinical and functional measures are applied in primary care services, but they fail to identify all frail individuals [8]. Aiming to complement these tests and to understand the biology of frailty, research on biomarkers of frailty is being conducted [186]. Distinct sources of biomarkers, including endocrine, inflammatory, metabolic, genetic and epigenetic markers among others have been proposed, with controversial results [174]. Hence, we should continue investigating new potential biomarkers that could help the identification of frailty. Blood is an interesting biofluid for this search, as it is accessible, can be processed and stored easily and it is routinely obtained at clinical settings.

In this work, we studied three different sources of biomarkers in the blood:

1. Inflammation
2. Gene expression
3. Extracellular vesicles

Each biomarker source is presented in a separate subchapter and the main objective of all of them was to test whether differences between robust and frail elders are present.

Common methods

Participants and Frailty classification

For the present study, samples from 3 different cohorts of elder donors and samples from healthy adults were used. We obtained the samples of the elder cohorts in collaboration with the Primary Care Unit of Biodonostia Health Research Institute and the Neurology department of Donostia University Hospital. Participants are from the province of Gipuzkoa (Basque Country, Spain) and meet the criteria shown in **Table 1**.

Table 1. Inclusion criteria of the cohorts.

Cohort	Description
Cohort 1	Participants from Errenteria and Pasaia Survivors of a previous study were contacted and invited to participate No further inclusion criteria Samples obtained July 2014 – May 2015
Cohort 2	Participants form Errenteria, Irun and Hondarribia Aged 70 or over, community-dwelling and autonomous (Barthel > 90) Samples obtained May 2015 – July 2016
Cohort 3	Participants from Getaria, Urnieta, Zumaia and Zestoa Aged 70 or over, community-dwelling, including autonmomus and non-autonomous Samples obtained August 2016 – May 2017

All participants completed a questionnaire and donors with acute illness were excluded. The study was approved by the hospital's ethics committee and all participants provided written informed consent before blood sampling. Frailty status of elder participants was assessed by primary care services. A battery of tests was conducted. The translated version of frailty tests was applied. A short description of frailty tests is shown in **Table 2** and the main characteristics of each cohort in **Table 3**.

Table 2. Short description of the tests applied to measure frailty.

Frailty assessment test	Description
Tilburg Frailty Indicator (TFI) [15]	A user-friendly questionnaire based on a multidimensional approach. It is composed of a physical, a psychological and a social domain.
Gait speed (GS) [17]	Expressed in meters per second (m/sec). Participants were asked to walk at their usual pace. The test was performed twice and GS was calculated based on the shorter time.
Timed up-and-go (TUG) [18]	The time needed to stand up from a chair, walk 3 meters, turn around, walk back and sit down, with the help of their usual walking aid, if any.
Short Physical Performance Battery (SPPB) [19]	A functional capacity test composed of gait speed, test of balance and time needed to stand up from a chair 5 consecutive times.
Gerontopole Frailty Screening Tool (GFST) [16]	Based on clinical judgement. 6 yes/no questions that help the physician to evaluate the existence of frailty.
Barthel Index (Barthel) [291]	A multiparametric test measuring the performance in activities of daily living and mobility.

Table 3. Information of study participants.

	Elders			**Adults**
	Cohort 1	**Cohort 2**	**Cohort 3**	-
Participants	53	295	91	57
Female/Male	30/23	153/142	56/32	30/27
Age (mean)	79-92 (82.51)	71-91 (79.83)	70-96 (76.98)	24-46 (33.51)
Frailty assesment				-
TFI	X	X		
GS	X	X	X	
TUG	X	X	X	
SPPB		X	X	
GFST		X		
Barthel	X	X	X	

Blood sampling

Peripheral blood was collected by experienced nurses by venipuncture with a 21-gage needle in 8 ml serum separator tubes and 4 ml EDTA tubes (Vacutainer, BD Biosciences) and directly deposited in the Basque Biobank for their processing and storage. Serum separator tubes were allowed to clot for 30 min and centrifuged at 1258 g for 20 min to recover serum from the supernatant. EDTA tubes were kept upright and centrifuged at 1258 g for 20 min to recover plasma. To obtain RNA, samples from EDTA tubes were incubated with Buffer EL (Qiagen) for erythrocyte lysis and then RNA from leukocytes was extracted with QIAamp RNA Blood Mini Kit (Qiagen) following manufacturer's instructions. For DNA, a second EDTA tube was used and the extraction was performed with FlexiGene DNA Kit (Qiagen) following the manufacturer's instructions. RNA and DNA quantity and quality were assessed with a Nanodrop 1000 spectrophotometer (Thermo Fisher). The obtained serum, plasma, RNA and DNA samples were aliquoted and stored at -80 °C. When needed, corresponding request forms were fulfilled to obtain the samples from the Basque Biobank.

Inflammation

Introduction

Chronic low-grade inflammation is one of the best described characteristics of aging. It has been widely shown that elders present elevated levels of inflammatory markers in circulating blood, in the absence of overt infection [137,138,292]. This accumulation of proinflammatory molecules, termed inflammaging, has also been proposed as a potential biomarker of frailty.

Previous studies have measured the concentration of proinflammatory markers such as IL-6, TNF-α and CRP among others in donors with different degrees of dependency. However, the obtained results are controversial, and even if some researchers have found an increased concentration in frail and non-autonomous elders, other studies did not report significant differences [159,169,171,174]. Other molecule related to inflammation and proposed as a frailty biomarker is albumin. The rate of albumin synthesis is affected by both nutrition and inflammation, and inflammation alone is associated with a greater catabolic rate of albumin. Decreased albumin levels have been proposed as a risk factor for frailty, but similar to the above-mentioned inflammatory markers, there is no consensus on its validity [293,294].

The aim of our study is to investigate the validity of inflammatory mediators as biomarkers that could complement the functional and clinical evaluation of elders for the identification of frailty. To that end, we first compared the concentration of the above cited molecules between adults and elder donors, and then, based on the frailty classification of elders, evaluated whether these molecules show different levels with frailty and dependency in our cohorts.

Materials and methods

CRP ELISA and TNF-α Luminex

Plasma samples from elders (cohort 2, n=111) and adults (n=39) were thawed on ice. CRP concentration was measured with Quantikine ELISA (R&D) following the manufacturer's instructions. Plasma samples were diluted 1:150 to fit the standard curve of the kit. On the other side, a panel of 6 interleukins was designed for luminex measurement: IL-6, IL-10, IL-2, IL-1β, IL-1Ra and TNF-α. The Milliplex Map #HCYTOMAG-60K kit (Merck) was used. Manufacturer's instructions were followed and plasma samples were assayed undiluted, but only the measurements of TNF-α were above the lowest point of the standard curve. We performed a second trial with the same kit and obtained similar results. In order to solve this issue, we repeated the assays using the high sensitivity kit #HSTCMAG-28SK (Merck) provided

by the manufacturer, but most of the samples were still non-detectable. Lastly, we also tried a high sensitivity luminex kit from another brand, #FCSTM09-04 (R&D) for IL-6, IL-10, IL-2 and IL-1β. With this kit the measurement of analytes was also non-detectable in many samples (65/160). After the obtained negative results, we decided not to measure more plasma samples with the luminex technique and we analysed only the results from TNF-α, the only analyte that obtained detectable and reliable results (elders n=37 and adults n= 39).

CRP, TNF-α, IL-6 and albumin ELISA

With the objective to test serum samples and to strengthen our results, samples from elders (cohort 3, n=91) and adults (n=18) were used. Samples were thawed on ice, CRP, TNF-α and IL-6 were measured with Quantikine ELISAs (R&D) and albumin with an ELISA kit (Invitrogen, Thermo) following the manufacturer's instructions. Serum samples were diluted to fit the standard curves of each kit: diluted 1:100 for CRP, undiluted for TNF-α, undiluted for IL-6 and diluted 1:500000 for albumin.

Statistical analysis

Statistically significant differences between the study groups and correlations between variables were tested with GraphPad Prism version 6.01 for Windows (GraphPad Software, www.graphpad.com). D'Agostino-Pearson normality test was applied and non-Gaussian distribution was confirmed for all samples. Consequently, Mann-Whitney tests were applied to evaluate differences between two study groups. For correlation analysis, Spearman coefficient was calculated. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Results

Inflammatory markers in plasma

To evaluate the inflammatory status differences between the study groups, CRP and TNF-α were measured in plasma samples from cohort 2. First, results from elders were compared to healthy adults, and we confirmed an increased concentration of both CRP and TNF-α in aged individuals (**Figure 18A** and **Figure 19A**). Then, the correlation between age and inflammatory markers was evaluated, but no significant correlations were found (**Figure 18B** and **Figure 19B**). Similarly, no differences were found based on gender (**Figure 18C** and **Figure 19C**). Considering other possible confounder factors and taking advantage of the available data about the drug number prescribed to the donors in this cohort, we also evaluated the correlation between the number of drugs each participant takes and the concentration of CRP and TNF-α. We found a significant positive correlation between CRP and the drug number – which was corrected by linear regression before further analysis –, while no correlation was found for TNF-α (**Figure 18D** and **Figure 19D**).

Finally, the concentration of both inflammatory markers was compared between robust and frail individuals. The recorded frailty scales for cohort 2 were: TFI, GS, TUG, SPPB and GFST. The classification of each frailty scale was considered, and we did not find any significant differences (**Figure 18E-I** and **Figure 19E-I**). To perform a more robust comparison, the participants that are classified as robust or frail for all the tested scales (n=40) were compared, but no differences were reported (**Figure 18J** and **Figure 19J**). In the last approach, the elders with the same classification for the 3 scales that evaluate the functional status (GS, TUG, SPPB, n=63) were brought into comparison, and as for the previous analyses, no differences were found (**Figure 18K** and **Figure 19K**).

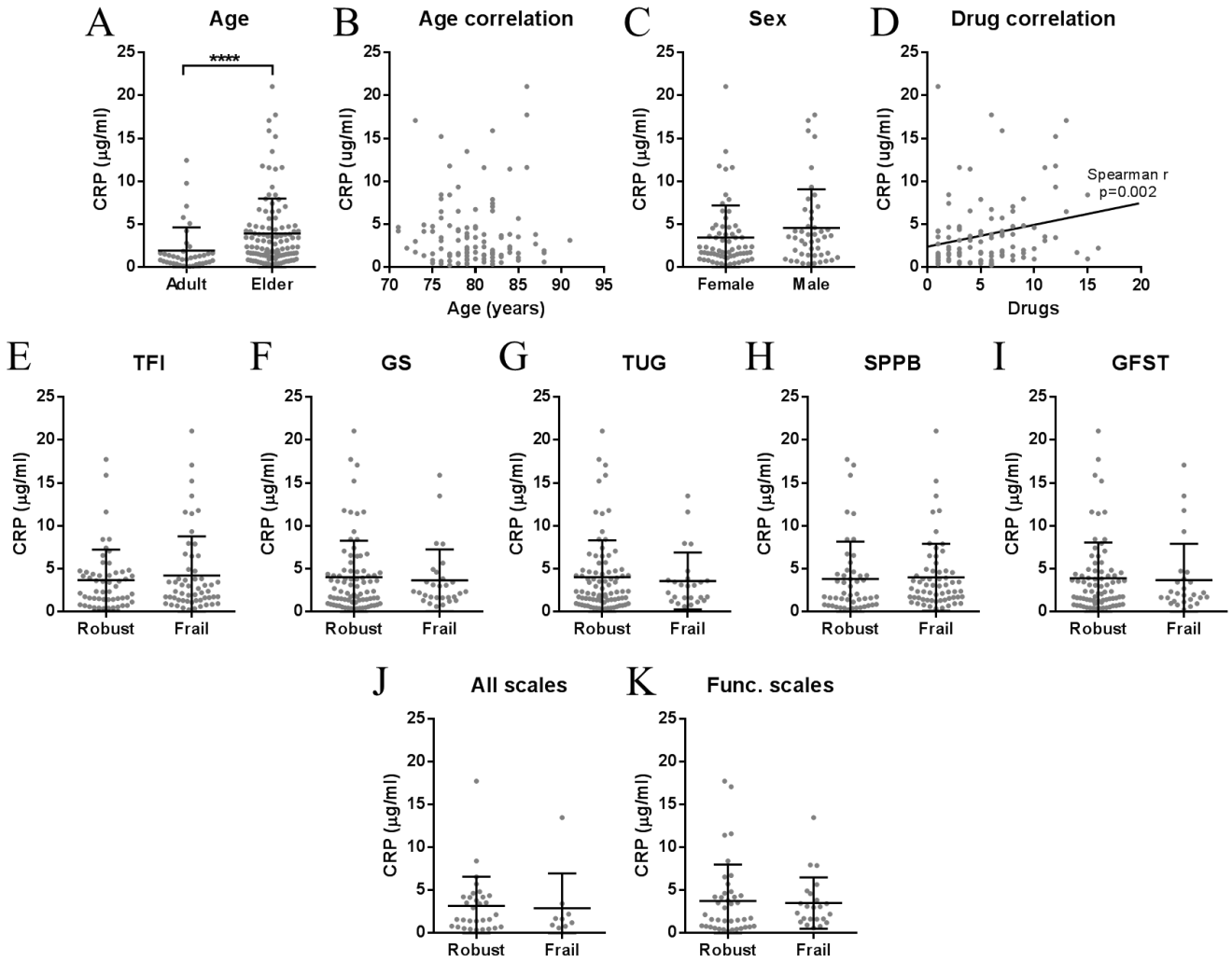


Figure 18. Concentration of CRP in plasma. (**A**) There is elevated CRP in elders compared to adults. (**B**) Among elders, CRP concentration has no correlation to age and (**C**) there is no significant difference between females and males. (**D**) A positive correlation between CRP concentration and drug number was found and corrected. (**E-I**) No differences in CRP levels between robust and frail individuals were found for the 5 analysed frailty scales. (**J**) We also compared the individuals classified as robust or frail with all the available tests or (**K**) with the 3 functional scales (GS, TUG, SPPB), but no differences were found.

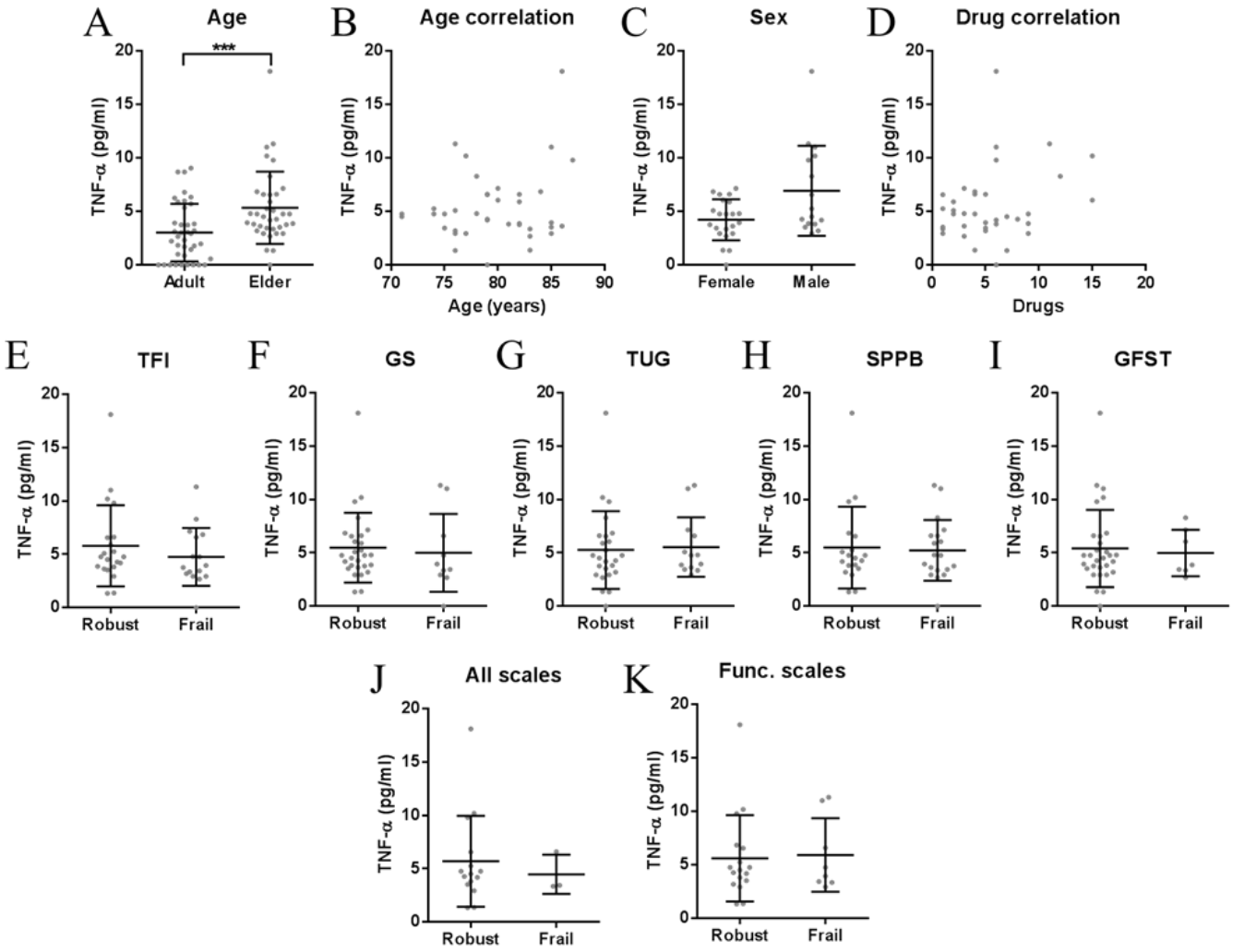


Figure 19. Concentration of TNF-α in plasma. (**A**) There is elevated TNF-α in elders compared to adults. (**B**) Among elders, CRP concentration has no correlation to age, (**C**) there is no significant difference between females and males and (**D**) no correlation between TNF-α concentration and drug number was found. (**E-I**) No differences in TNF-α levels between robust and frail individuals were found for the 5 analysed frailty scales. (**J**) We also compared the individuals classified as robust or frail with all the available tests or (**K**) with the 3 functional scales (GS, TUG, SPPB), but no differences were reported.

Inflammatory markers in serum

For the characterization of inflammatory markers in serum, samples from cohort 3 were used. We studied the previously measured CRP and TNF-α, as well as IL-6 and albumin. We obtained the same results as in plasma, confirming that there is an elevated chronic inflammation in elders when compared to adults: increased CRP, TNF-α and IL-6, while reduced albumin (**Figure 20A**, **Figure 21A**, **Figure 22A** and **Figure 23A**). Moreover, CRP, TNF-α and IL-6 showed a positive correlation to age among elders (**Figure 20B**, **Figure 21B** and **Figure 22B**), which was corrected by linear regression for each analyte. On the other hand, no correlation with age was found for albumin (**Figure 23B**). Regarding gender, no differences were found

for any of the analytes (**Figure 20C**, **Figure 21C**, **Figure 22C** and **Figure 23C**). At last, we made use of the available data of Barthel and TUG scales, performing the comparison between the different dependency statuses of participants in cohort 3: robust, frail and non-autonomous. We found no significant differences for any of the molecules between the analysed groups (**Figure 20D**, **Figure 21D**, **Figure 22D** and **Figure 23D**), following the same trend as the analyses in plasma samples.

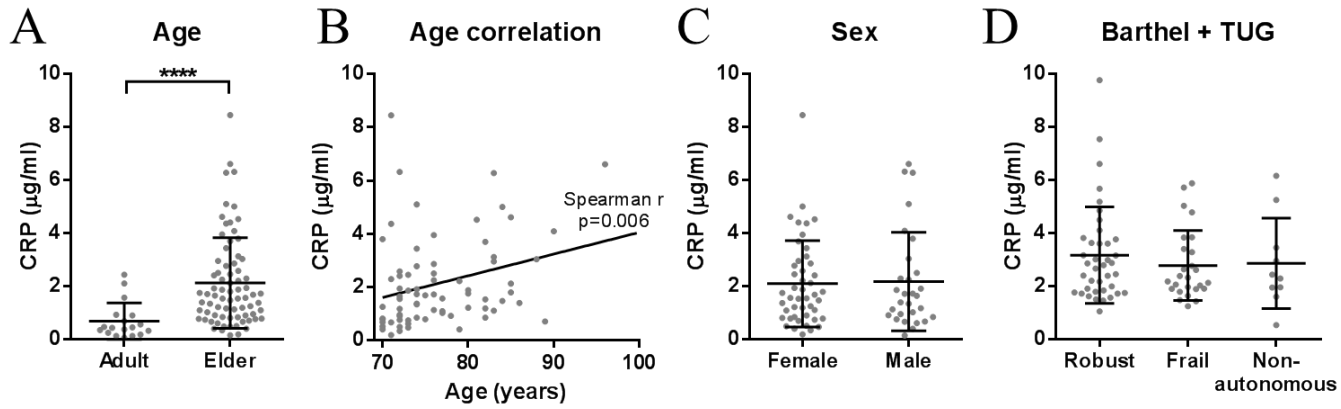


Figure 20. Concentration of CRP in serum. (**A**) There is elevated CRP in elders compared to adults. (**B**) Among elders, serum CRP concentration has a positive correlation to age and (**C**) there is no significant difference between females and males. (**D**) When compared based on Barthel and TUG scales, no differences in CRP levels between robust, frail and non-autonomous individuals were found.

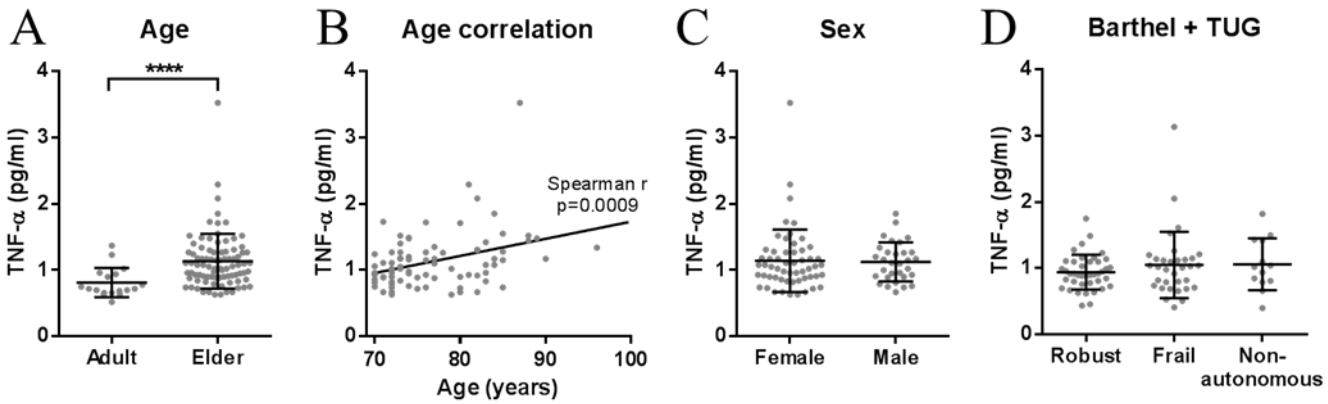


Figure 21. Concentration of TNF-α in serum. (**A**) There is elevated TNF-α in elders compared to adults. (**B**) Among elders, serum TNF-α concentration has a positive correlation to age and (**C**) there is no significant difference between females and males. (**D**) When compared based on Barthel and TUG scales, no differences in TNF-α levels between robust, frail and non-autonomous individuals were found.

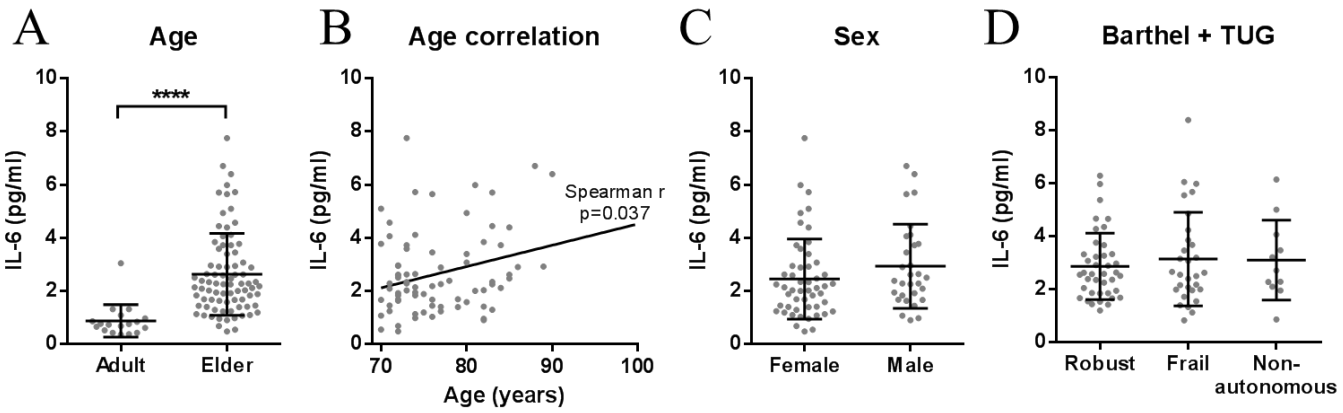


Figure 22. Concentration of IL-6 in serum. (**A**) There is elevated IL-6 in elders compared to adults. (**B**) Among elders, serum IL-6 concentration has a positive correlation to age and (**C**) there is no significant difference between females and males. (**D**) When compared based on Barthel and TUG scales, no differences in IL-6 levels between robust, frail and non-autonomous individuals were found.

Figure 23. Concentration of albumin in serum. (**A**) There are reduced albumin levels in elders compared to adults. (**B**) Among elders, serum albumin concentration has no correlation to age and (**C**) there is no significant difference between females and males. (**D**) When compared based on Barthel and TUG scales, no differences in albumin levels between robust, frail and non-autonomous individuals were found.

Discussion

Inflammaging is one of the main biological characteristics of human aging. This term was proposed in 2000 by Franceschi et al. [136], although a work showing the accumulation of inflammation with age and its relation to mortality was already published in 1991 by Mooradian et al. [295]. Since this term was introduced, many works have investigated the relationship between inflammatory markers and aging, dependency and mortality. However, the obtained results are diverse and many times discordant, so no consensus has been reached [138,144].

The studies investigating the potential role of molecules linked to inflammation as frailty biomarkers encounter the same problem. Many works have been carried out, but no clear association has been found, as some found increased concentrations of proinflammatory markers in frail subjects, whereas others did not report any significant differences [170,173,296]. It should also be considered that the study designs, techniques and the characteristics of included participants are distinct in each investigation. Moreover, the published studies have been carried out in different countries, hence distinctive genetic and environmental aspects should be considered. In addition, the tests employed for frailty assessment evaluate the status of participants based on different aspects, and therefore, the same person can be considered frail based on one scale and robust based on another one. For example, there are tests that focus on clinical aspects [16], while others give more importance to the psychologic domain [15] or the functional performance [19]. This diversity of tests shows the heterogeneity of the "frailty" term, which makes it even more difficult to identify a biological marker of the syndrome.

Regarding the techniques available for the quantification of the molecules of interest, we decided to apply ELISA and Luminex. We obtained good results with the ELISA kits for plasma and serum samples, while we encountered detection problems with Luminex kits. We chose Luminex because it is a technique that enables the detection of a panel of analytes in the same experiment, saving time and reducing the amount of sample needed. Moreover, we have applied this protocol in previous experiments for other projects, and we obtained good results with cell culture supernatants. However, when plasma samples were tested, all the selected analytes, except TNF-α, did not reach detectable levels in most of the samples. We thought that the concentration of our analytes could be lower than the detection limit of the kits, but we dismissed this possibility at least for IL-6, as we have previously measured it with ELISA in samples of the same characteristics and the obtained concentrations are notably higher than the detection limit of the Luminex kits used. Due to this, we decided to continue analysing our plasma and serum samples with ELISA.

We reliably measured CRP, TNF-α, IL-6 and albumin in our cohorts, that include aged subjects from the region of Gipuzkoa (Basque Country, Spain) who have been evaluated with several frailty scales. In these cohorts, our results confirmed the presence of inflammaging by the increased low-grade inflammation in elders when compared to adults. But regarding frailty, which was the main objective of this work, none of the analysed molecules showed significant differences based on the dependency status.

Moreover, to try to overcome the above-mentioned issue of the heterogeneity between frailty tests, and taking advantage of the available data in cohort 2, we also compared the

concentrations of the inflammatory molecules between the participants that were classified as robust or frail for all the recorded scales. Still, no differences were found when the participants that obtained the same classification for the 5 applied scales were considered. Additionally, and following recent recommendations of the WHO [23,297], we focused on the results of the tests that evaluate functional performance (GS, TUG and SPPB), comparing the individuals that obtained the same results for those 3 frailty scales. Again, no differences were reported under these conditions. Therefore, we conclude that in our samples, robust and frail individuals have no differences for the measured inflammatory markers, and they cannot help the identification of frailty.

To our knowledge, this is the first work studying inflammaging and frailty in the region of the Basque Country. Different results were previously reported in other regions of Spain: increased levels of CRP, TNF-a and IL-6 in frails were found in Galicia [298,299], while no differences for IL-6, IL-8 and IL-10 and increased levels only for TNF-α were found in Granada [188], when frailty was evaluated following Fried's criteria.

In short, we think that the utility of inflammatory molecules as biomarkers of frailty needs to be reconsidered since a robust biomarker should be valid in all cohorts. We believe that other approaches such as the development of longitudinal studies would be of great interest, as they enable the follow-up of participants as they age and the loss of functional capacity begins, and the correlation with the levels of inflammatory markers could be evaluated. With this experimental setup, the changes in the concentration of inflammatory molecules could be measured in each participant, and it could be tested individually whether the progression of a specific molecule is related to frailty.

Transcriptomics

Introduction

In a clinical setting, the analysis of gene expression is widely used for the identification of changes induced by diseases or treatments, including the ones that are related to human aging. Additionally, gene expression analysis has been applied to investigate the molecular changes that occur during normal aging, aiming to identify characteristic expression profiles and the mechanisms responsible for the aging process [36].

In the last years, a similar approach is being conducted for the identification of genes related to frailty. In Europe, the FRAILOMICS initiative aims to develop validated biomarkers, which can predict the risk of frailty, improve the accuracy of its diagnosis in clinical practice and provide a prognosis on the evolution from frailty to disability [186]. Studies within this initiative and others, have proposed expression differences, single nucleotide polymorphisms and epigenetic modifications related to frailty, and many different tissues, systems and processes have been shown to be affected, such as the control of inflammation, oxidative stress or hormones [7,183,185,187]. However, the proposed features still need to be further investigated in other cohorts to test their validity, before they could be applied in the clinic.

Taking all these into account and in line with the mentioned applications of gene expression analysis, the aims of our study were to perform a transcriptomic study in a set of robust and frail community-dwelling individuals of our cohort from the Basque Country, identify the most promising transcripts differentially expressed in frail subjects, and try to validate them. Moreover, we also intended to measure the most promising transcripts in the elder participants of a pilot physical intervention study.

Materials and methods

RNA expression arrays

RNA samples of 25 donors from cohort 1 were used for microarray analysis. 300 ng of RNA were labelled with the GeneChip WT reagent kit and hybridized to the HuGene-2_0-st-v1 array, which covers the expression of 48226 transcripts (Affymetrix, Thermo), following manufacturer's instructions. Hybridized arrays were washed and stained in a GeneChip Fluidics Station 450 and scanned in a GeneChip Scanner 7G (Affymetrix, Thermo).

Raw data was analysed with Expression Console software v1.4.1 and Transcriptome Analysis software v4.0 (TAC) (Affymetrix, Thermo). After the quality control check, data was

normalized by robust multi-array average (RMA) and analysed by TAC and BRB-Array Tools software v4.6.0 (https://brb.nci.nih.gov/BRB-ArrayTools). Principal component analysis (PCA) and hierarchical clustering were performed to visualize the data.

To analyse the differential expression between the study groups univariate parametric tests were performed. The inclusion criteria were the p-value (< 0.05) and the fold change (> |1.5|) between the analysed groups.

As a complementary approach, we focused the analysis on the search of classifiers that can be used as biomarkers. We used the class prediction approach that built predictors for classifying experiments into phenotype classes based on expression levels. Six methods of prediction were used: compound covariate predictor (CCP), diagonal linear discriminant analysis (DLDA), Bayesian compound covariate predictor (BCCP), k-nearest neighbors (using k=1 and 3), nearest centroid and support vector machines.

RT-qPCR

Expression of candidate transcripts was measured by quantitative real-time PCR (RT-qPCR). First, technical validation of the selected 12 samples from cohort 1 was performed. Secondly, 120 samples from cohort 1 (n=23) and cohort 2 (n=97) were measured. Finally, the expression was evaluated in the 12 participants of the physical intervention (explained below). A schematic representation of the study workflow is presented in **Figure 24**. Briefly, 200 ng of total RNA was retrotranscribed to cDNA using the miScript II Reverse Transcription kit using the HiSpec buffer (Qiagen) following the manufacturer´s protocol. cDNA samples were stored at -20°C until used. Reactions were prepared with 10 ng of each target cDNA, 5 µl SYBR Green PCR Master Mix (Qiagen) and 1 µl of commercial gene-specific QuantiTect primer assays (Qiagen). RT-qPCR reactions were performed on a CFX384 thermal cycler (BioRad). Beta-2-microglobulin (B2M; QT00088935 assay from Qiagen) was used as the endogenous gene to calculate the relative expression of target transcripts.

Physical Intervention

A subsample of 12 individuals from cohort 2 was invited to participate in a pilot study aimed to assess the impact in terms of physical performance improvement of a community-based physical training program. The program was performed in groups in the sport and leisure facilities of the municipality. Participants received 36 sessions of training including strength, flexibility and aerobic exercises. Blood samples of participants were obtained prior to the start of the training and at the end of the 36 sessions (3 months). This study is coordinated by Itziar Vergara MD PhD and Ander Matheu PhD from the Biodonostia Health Research Institute and the follow up is still ongoing.

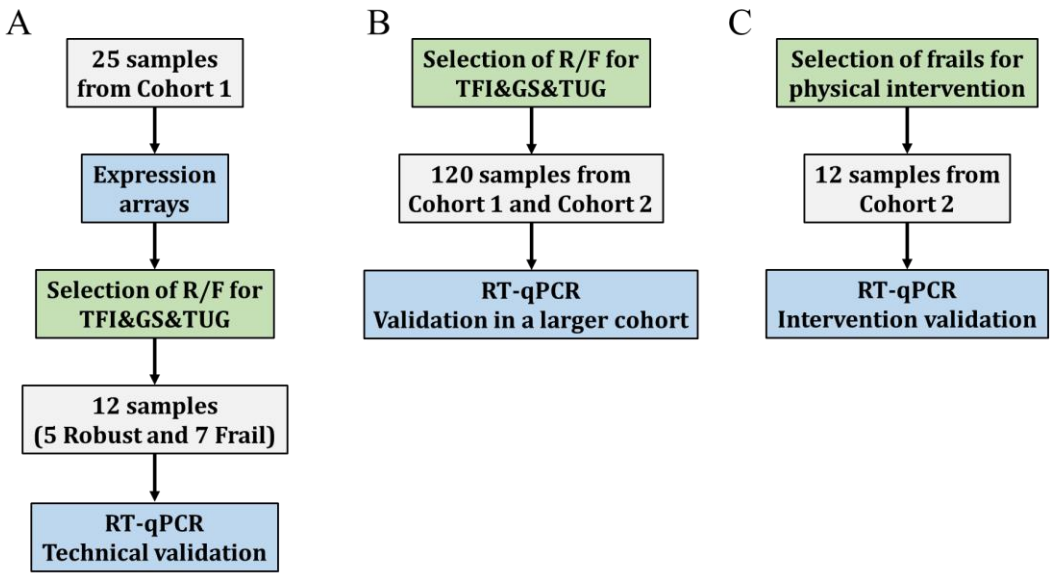


Figure 24. The workflow of the study. (**A**) First, transcriptomic analysis was performed with 25 samples from cohort 1. The samples were separated based on the results of the frailty tests (TFI, GS and TUG). The 12 participants that were classified as robust of frail with the 3 tests were compared, and the technical validation of differentially expressed candidates was performed by RT-qPCR. (**B**) Next, aiming to evaluate the expression of the candidates in a larger sample, we selected 120 participants from Cohort 1 and Cohort 2 that obtained the same result for the TFI, GS and TUG tests and performed an RT-qPCR analysis. (**C**) Finally, a pilot physical intervention study was performed for 3 months. 12 of the donors of Cohort 2 were included in the study, and we measured the expression of the candidate transcripts by RT-qPCR before the intervention and at the end of the 3 months.

Statistical analysis

For the microarray analysis sensitivity and specificity values, as well as Receiver Operating Characteristic (ROC) curves were calculated as part of the class prediction tool by BRB-Array Tools.

For the RT-qPCR analysis, $2^{-\Delta Cq}$ values were calculated for each sample and transcript. The differences between robust and frail groups were evaluated with GraphPad Prism version 6.01 for Windows (GraphPad Software, www.graphpad.com). D'Agostino-Pearson normality test was applied, and non-Gaussian distribution was confirmed. Mann-Whitney test was applied to evaluate differences between the two study groups. For the analysis of the physical intervention results, Wilcoxon matched-pairs signed rank test was applied. *$p<0.05$ and **$p<0.01$.

Results

Identification of a pattern of 35 transcripts differentially expressed in frailty

Expression arrays were performed and the transcriptome of the 25 participants analysed. For the comparative expression analysis, we aimed to separate the individuals classified as frail or robust. However, not all the participants obtained the same classification in the 3 frailty tests employed (TFI, GS and TUG). For this reason, we separated the donors on 3 groups: the ones that were "frail" for all the tests, the ones that were classified as "robust" by all the tests, and the "mixed" ones that got different results depending on the frailty test performed. The PCA of these 3 groups showed that frail and robust groups had a more similar expression pattern, while the mixed group was very heterogeneous (**Figure 25A**). Consequently, only those individuals classified as frail (n=7) or robust (n=5) by the 3 tools considered in this study were compared, in the search of frailty biomarkers.

Next, applying the class prediction approach to obtain the best genes to discriminate between frail and robust, 35 genes were selected (**Figure 25B**). This set of 35 transcripts includes a wide variety of protein-coding genes, pseudogenes and regulatory non-coding RNAs, mainly miRNAs (**Table 4**). Interestingly, a number of these genes are linked to inflammation- and hypoxia-related pathways (EGR1, CXCL8, CISH, LOC644172/MAPK8IP1P2 or CD40LG), immune response (TIA1, IGHV2-26, TRBV3-1 and several members of the T cell receptor alpha locus at 14q11.2 chromosome location) and apoptosis (G0S2). The panel of 35 genes showed an area under the curve of 0.943 with 85.7% sensitivity and 80% specificity (**Figure 25C**), which indicate the potential for detecting frailty of this panel.

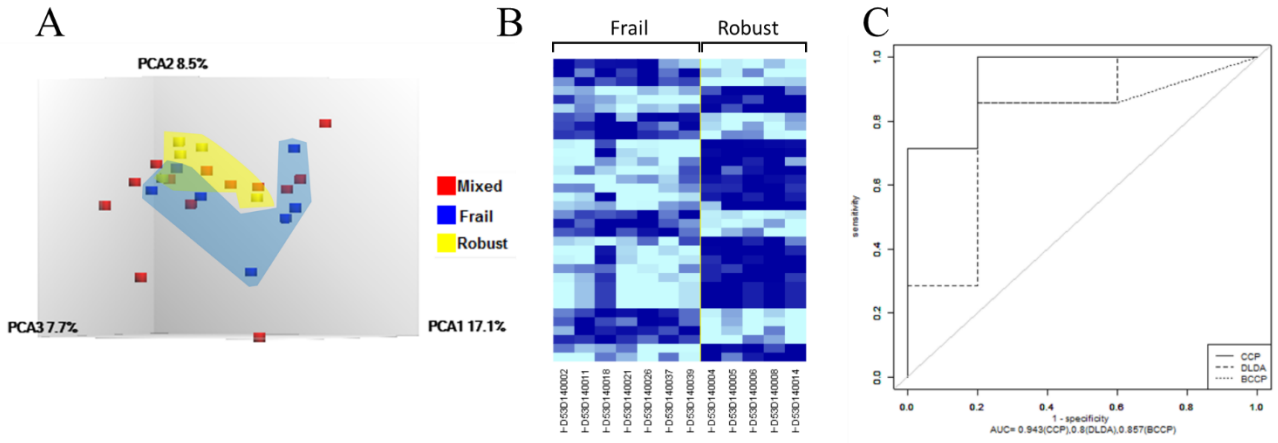


Figure 25. Transcriptomic analysis of robust and frail individuals. (**A**) The PCA of the 25 samples measured were coloured depending on the classification of the frailty tests (TFI, GS and TUG): Frail (classified as frail with the 3 tools), Robust (classified as robust with the 3 tools), Mixed (obtained distinct results depending on the tool). (**B**) Heatmap of the 35 differentially expressed transcripts between frails (n=7) and robusts (n=5). (**C**) ROC curves of the 35 identified transcripts with the best three class prediction approaches (CCP, DLDA and BCCP).

The expression of EGR1 is increased in frail individuals

From the 35 genes identified, we chose 3 genes to start with the validation: early growth response 1 (EGR1), DEAD/H-box helicase 11 like 1 (DDX11L1) and hsa-miRNA-454 (MIR454). First of all, we performed the technical validation of microarray expression data by RT-qPCR in the original cohort (n= 12), which confirmed the downregulation of DDX11L and MIR454, and the upregulation of EGR1 with frailty, while only the expression of EGR1 obtained statistically significant differences (**Figure 26**).

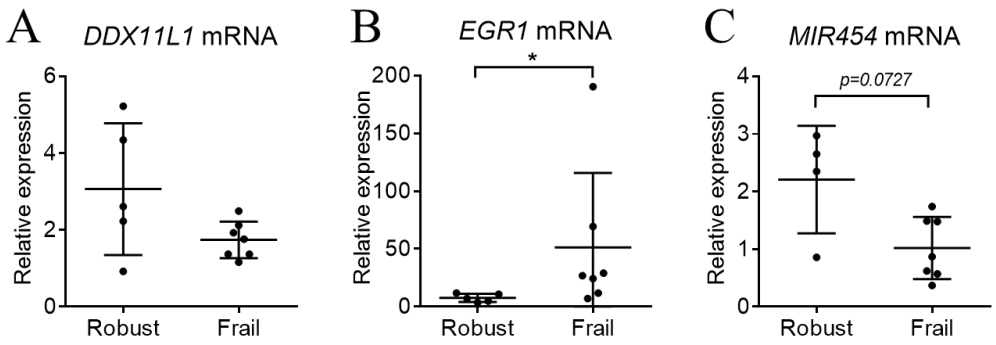


Figure 26. Technical validation of 3 of the candidates. (**A**) The expression of DDX11L1 is reduced in frail elders, but statistical significance was not reached. (**B**) EGR1 is significantly upregulated in frailty. (**C**) MIR454 is downregulated in frailty, but statistical significance was not reached.

Table 4. The 35 genes identified to be differentially expressed between robust and frail elders.

Gene symbol	Entrez Gene ID	Frail Avg (log2)	Robust Avg (log2)	Fold Change	p-value
EGR1	1958	8.08	6.66	2.66	0.0014
DDX11L1	100287102	7.73	9.32	-3.01	0.0039
MIR454	768216	3.13	3.94	-1.63	0.0004
CISH	1154	6.82	7.99	-2.25	0.0033
DDX11L10	100287029	6.25	8.28	-4.11	0.0013
LOC101929775	101929775	5.82	7.22	-2.62	0.0004
LOC644172	644172	5.82	7.22	-2.62	0.0004
NSF	4905	5.58	7.43	-3.59	0.0002
TRAJ17	28738	7.18	8.57	-2.62	0.0017
TRAJ19	28736	6.52	7.6	-2.11	0.0037
TRAV8-3	28683	7.42	8.86	-2.71	0.0004
CD40LG	959	7.14	8.15	-2.01	0.0098
CLDN12	9069	3.13	4.51	-2.6	0.0151
CNTNAP3	79937	6.8	8.18	-2.6	0.0479
CNTNAP3B	728577	6.95	8.21	-2.39	0.0310
CSRNP1	64651	8.01	6.94	2.1	0.0307
CTSLP8	1518	2.14	3.25	-2.16	0.0260
CXCL8	3576	10.17	9.06	2.15	0.0190
G0S2	50486	8.89	7.46	2.69	0.0318
GCNT4	51301	5.8	6.82	-2.02	0.0217
GJB6	10804	5.18	4.09	2.12	0.0057
IGHV2-26	28455	3.63	4.88	-2.38	0.0291
LOC100505530	100505530	5.35	6.64	-2.45	0.0224
LOC105378916	105378916	6.36	7.72	-2.56	0.0333
MIR3941	100500866	3.16	4.43	-2.4	0.0059
MIR487A	619555	2.42	3.62	-2.29	0.0275
MIR626	693211	2.96	4.04	-2.11	0.0244
MTRNR2L2	100462981	5.58	6.84	-2.39	0.0334
RLN1	6013	4.65	5.71	-2.08	0.0092
TIA1	7072	7.46	8.49	-2.05	0.0462
TRAJ14	28741	8.14	9.17	-2.04	0.0128
TRAJ16	28739	7.77	8.78	-2.01	0.0300
TRAJ48	28707	7.83	8.9	-2.09	0.0185
TRAV16	28667	5	6.58	-2.98	0.0172
TRBV3-1	28619	7.21	8.39	-2.26	0.0058

Next, we evaluated whether the expression of the selected candidates is altered in a larger cohort. For this purpose, we selected 120 RNA samples from cohort 1 and cohort 2. To maintain the previously set criteria, only the participants that obtained the same classification in the TFI, GS and TUG tests were selected (robusts n=103, and frails n=17). The RT-qPCR analysis of these 120 samples confirmed the increased expression of EGR1 in frail elders, while no differences were found for DDX11L1 and MIR454 (**Figure 27**).

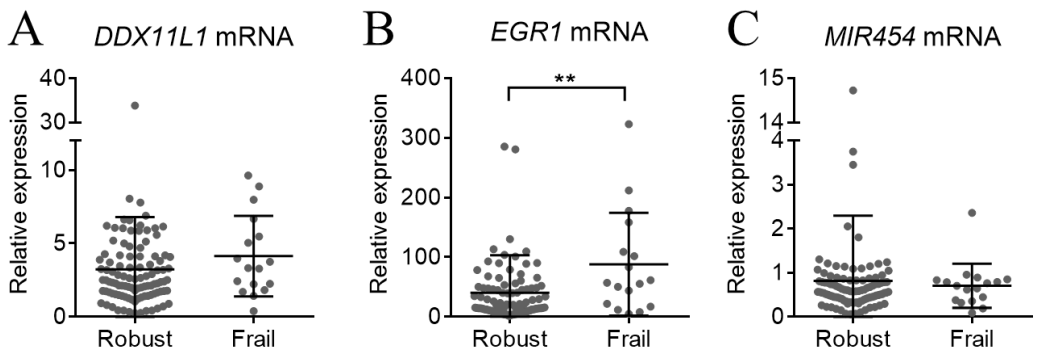


Figure 27. Expression of 3 of the candidates in a validation cohort. (**A**) The expression of DDX11L1 is not different in robust and frail participants. (**B**) EGR1 is significantly upregulated in frailty. (**C**) MIR454 is downregulated in frailty, but statistical significance was not reached.

Physical intervention and EGR1 expression

Some of the donors of cohort 2 were invited to participate in a physical intervention study for 3 months. Blood samples were collected before and after the intervention, and the expression of DDX11L1, EGR1 and MIR454 were determined by RT-qPCR. No clear trends were observed for DDX11L1 and MIR454 (**Figure 28A** and **28C**). In contrast, EGR1 levels were reduced in 9 out of the 12 donors with a p-value of 0.06, reinforcing the potential of this gene as a biomarker of frailty (**Figure 28B**). Furthermore, when the changes in EGR1 expression and TUG performance were compared, we observed that 8 out of the 12 participants obtained concordant results: a reduction of EGR1 accompanied by a better TUG score, or increased EGR1 accompanied by a worse TUG score (**Figure 28D**).

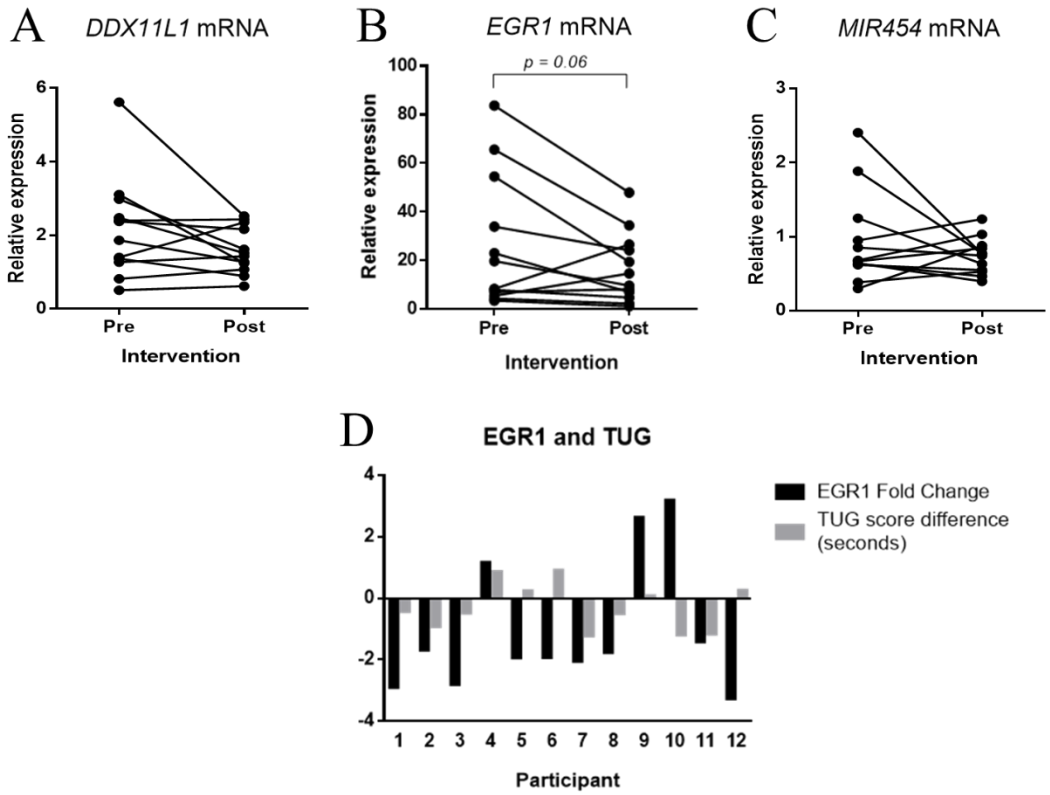


Figure 28. Physical intervention, gene expression and physical performance. (**A**) No differences were reported for DDX11L1 expression. (**B**) EGR1 expression is reduced in 9 out of the 12 participants and a p-value of 0.06 was obtained with the paired-samples statistical analysis. (**C**) No differences were reported for MIR454 expression. (**D**) When the expression of EGR1 and TUG scores were compared, 8 out of the 12 participants obtained concordant results: reduction in EGR1 expression and TUG performance time, or increased EGR1 expression and TUG performance time.

Discussion

In the present study, we have performed a transcriptomic analysis of community-dwelling individuals from the Basque Country. A set of 35 differentially expressed transcripts was found between robust and frail elders. Among them, there were genes linked to inflammation and hypoxia-related pathways, immune response, apoptosis and several members of the T cell receptor alpha locus. These processes have also been related to frailty in several previous studies, but however, our set of genes was different from a recently proposed panel of potential frailty biomarkers [187].

Within the 35 transcripts, we selected 3 for the first validation approach: DDX11L1, EGR1 and MIR454. The technical validation confirmed the microarray results, while when we measured them in a larger cohort, only the increased expression of EGR1 in frail subjects was confirmed. Moreover, we determined the levels of EGR1 in 12 donors before and after a 3-month physical

intervention study, and 9 of the participants reduced the expression of EGR1. Again, no differences were reported for DDX11L1 and MIR454. Our results indicate that EGR1 is a promising biomarker of frailty that should be further investigated.

Indeed, EGR1 is a transcription factor activated in response to a broad range of stimuli that affects directly or indirectly the expression of multiple signalling pathways and tumour suppressors, and it modulates and participates in multiple cellular processes such as mitogen response, growth, proliferation, apoptosis or differentiation of several cell types [300–302]. Besides, some studies also found EGR1 changes to be associated with aging and age-related phenomena, like senescence or immune response regulation [303–306]. Thus, the role of EGR1 in frailty is probably complex and depending on the tissue and context its target genes and the elicited functions may differ.

Moreover, as discussed before, the identification of biomarkers of frailty is challenging due to the lack of consensus its definition and the multiple frailty screening tools available. Thanks to the work performed at primary care services, were had the data of 3 different tests (TFI, GS and TUG) for each of the participants in our study, and after the microarray analysis and the results of the PCA, we decided to focus on the subjects that obtained the same classification in all of the tests, reducing heterogeneity. In addition, it should be mentioned that despite this, the expression levels reported by RT-qPCR were highly variable. In this sense, one of the main advantages of longitudinal studies is that the expression of single participants can be measured over time and the evolution of each of them determined. Indeed, our results from the physical intervention pilot study show that even if the expression of EGR1 was different, most of the subjects reduced the levels of EGR1 after only 3 months. This is an interesting point also from the perspective of the reversibility of frailty. It is generally accepted that frailty is a reversible state [7,307], and the expression of EGR1 could be used as a biomarker of this process.

Finally, we have to point out that the present study was the only first step and we focused on the validation of 3 of the transcripts identified. We are now analyzing more candidates from the presented list of 35 differentially expressed genes between our robust and frail individuals. Our aim is to continue evaluating the expression differences and besides, to try to understand the functions or effects that these changes could be inducing in frail elders.

Extracellular vesicles

Introduction

EVs are membrane-coated particles of endosomal or plasma membrane origin that are secreted to the extracellular environment. They play an essential role in indirect intercellular communication as their membrane and cytosolic proteins, lipids and genetic material can be transferred between cells [195]. Moreover, almost all cell types release EVs and they can be isolated from plasma and other body fluids.

EVs are released both in physiological and pathological conditions and they are implicated in many cellular processes. In particular, EVs play a role in various stages of the immune response and they have been related to inflammatory, autoimmune and infectious disease pathology. EVs can carry and display antigenic material and are able to trigger antigen presentation and modulate immune responses [192]. It has also been reported that there are increased concentrations of EVs in plasma during inflammatory processes, such as in cancer or autoimmune diseases [234,242].

One of the hallmarks of human aging is the chronic low-grade inflammation, the so called inflammaging [136], a phenomenon that modulates intercellular communication. The age-associated immune dysfunction and accumulation of senescent cells promote inflammatory signals, such as elevated secretion of proinflammatory cytokines and activation of NF-κB transcription factor. Among inflammaging, the most widely studied feature is the circulating concentration of IL-6. The concentration of this interleukin is normally low (or non-detectable) in healthy adults, while elevated levels of IL-6 have been reported in the elderly, with increasing concentrations in the very old [138,140]. Moreover, elevated IL-6 has also been associated with mortality in the elderly [142].

Despite all this knowledge, there are many aspects of inflammaging that have not been elucidated, as the implication of EVs in the process. In the present study, and based on the previously mentioned increase of circulating EVs during inflammatory episodes, we proposed that this could also be observed in aged individuals as a result of inflammaging. Furthermore, we also designed an approach to evaluate if the concentration of EVs in plasma could be related to the frailty status of old people—frailty status as defined by the Barthel Index [308] and the Tilburg Frailty Index [15]. These tests are applied to evaluate and measure the frailty and dependence status of the elderly, which could also be related to an increased chronic proinflammatory condition.

Materials and methods

Study participants

Samples from 19 aged individuals (from Cohort 1: 8 males and 11 females, mean age 83.73 years) and 18 adults – classified in three age ranges: 21–30, 31–40, and 41–50 years (3 males and 3 females in each group) were used. Plasma and serum samples were obtained as described above.

EV Isolation

EVs were isolated as described before by our group [202]. Briefly, plasma was centrifuged at 13,000 g for 2 min and supernatant centrifuged again at 20,000 g for 20 min to pellet EVs. The pellet was resuspended with 100 μL of filtered DPBS (GIBCO, Thermo Fisher Scientific), filtered twice through a 0.22 μm-pore filter. Resuspended EVs and serum samples were stored at -80 °C.

Serum IL-6 ELISA Assay

IL-6 concentration was analysed by ELISA (BD Biosciences) following the manufacturer's instructions. Samples were measured in duplicate and results obtained with a microplate reader (Thermo Scientific Appliskan, Thermo Fisher Scientific). IL-6 concentrations were calculated and values above the first standard (>4.7 pg/mL) were considered detectable.

Nanoparticle Tracking Analysis (NTA)

The size distribution and concentration of EVs were measured using a NanoSight LM10 device (Malvern) as described elsewhere [212]. Samples were diluted to appropriated levels to get accurate acquisitions (200–900 recorded tracks) [212] and camera settings were fixed and maintained for all samples. Filtered DPBS was tested and no background signal was detected. For each sample, two videos of 1 min were recorded and analysed with NanoSight NTA software 2.2 (Malvern). Data are shown as the average count of the two duplicates.

Statistical Analysis

Statistical analysis was performed with R version 3.2.2 (R Core Team (2015) [309] in RStudio v0.99.486 (RStudio Team (2015) [309]). A Shapiro-Wilk test was applied to assess normality. As samples did not follow a normal distribution, Wilcoxon signed-rank test and non-parametric Kruskal–Wallis one-way analysis of variance were conducted to evaluate IL-6 and EV concentration differences between groups.

Results

Frailty Status Classification of Aged Individuals

For the present study, adults of different age ranges and elder people were enrolled. Participants were classified based on their age. Additionally, aged individuals (79–92 years) were asked to complete the Barthel and Tilburg Frailty Index questionnaires and were further classified as Robust, Frail or Non-autonomous, as shown in **Table 5**.

Table 5. Classification of enrolled individuals based on their age and frailty status. Samples of a total number of 18 adults and 19 elders were analysed.

Group	Females	Males	Total
Adults			
21–30 years	3	3	6
31–40 years	3	3	6
41–50 years	3	3	6
Elders (79–92 Years)			
Robust	4	3	7
Frail	3	2	5
Non-autonomous	4	3	7

IL-6 Concentration Is Increased in the Elderly

The level of IL-6 in serum was measured and, obtained results demonstrated a very low, nearly non-detectable concentration in adults of different ages, while an elevated concentration in the elderly ($p<0.001$) (**Figure 29A**). This result confirms the low-grade inflammatory condition of aged individuals. Additionally, when IL-6 levels of the elderly were compared depending on their frailty status, an increasing tendency with dependence was found (**Figure 29B**).

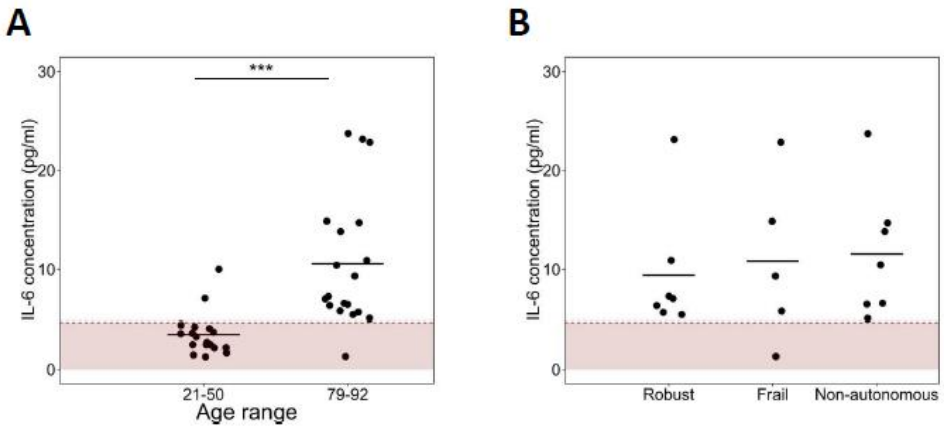


Figure 29. An elevated concentration of IL-6 is observed in aged subjects. IL-6 levels were measured by ELISA and concentration values above 4.7 pg/mL were considered detectable. (**A**) Elderly individuals have a higher concentration of IL-6 than adults (***$p<0.001$); and (**B**) there is a high variability among Robust, Frail and Non-autonomous elderly, but an increasing concentration with dependency can be observed.

The Concentration of EVs Is Not Affected by Age and Frailty Status

To assess the size profile and concentration of circulating EVs, NTA was conducted for all samples. Results showed that, regardless of particle concentration, all samples followed a similar EV size distribution, with most vesicles ranging between 50 and 300 nm in all instances (**Figure 30A**). This result demonstrated that our EV isolation protocol efficiently isolates small EVs, removing larger particles and platelets that can be found in plasma samples. When comparing the EV number, no significant differences were found between groups ($p = 0.505$), indicating that EV concentration is not increased with age (**Figure 30B**). Moreover, the concentration of EVs is also not affected by the frailty status of elder donors ($p = 0.424$), as shown in **Figure 30C**.

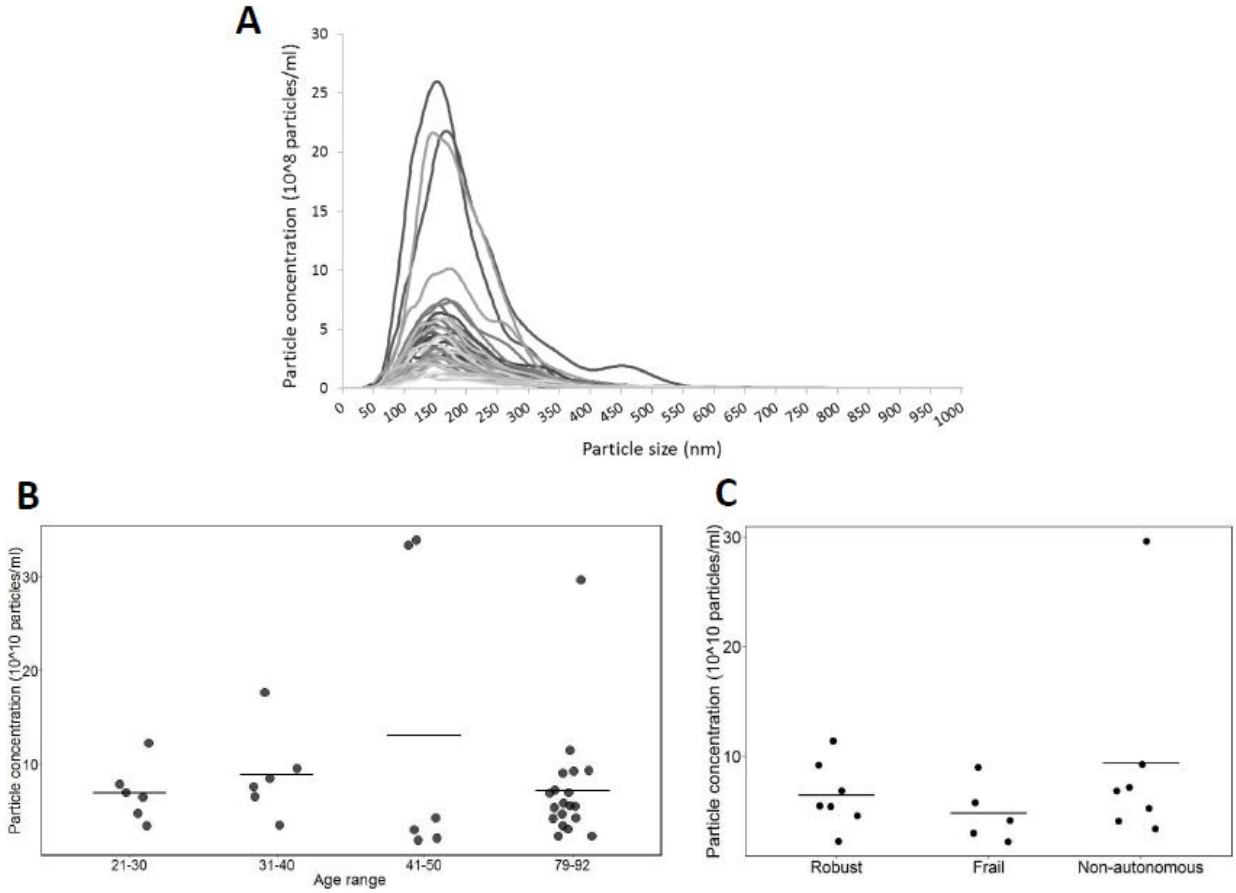


Figure 30. Particle size and EV concentration were measured by NTA. (**A**) Size distribution of EVs. Each line represents one sample. Despite the particle concentration difference, all samples have a similar size distribution—they are enriched in small EVs (50–300 nm); (**B**) EV concentration of different age ranges were compared and samples from elder people (79–92 years) do not show an increased EV number; and (**C**) among elder individuals, the frailty status does also not alter EV concentration.

Discussion

During human aging, a chronic low-grade inflammatory state called inflammaging has been reported [138,140,142], and to our knowledge, this is the first report investigating, specifically, EV concentration in this process. The results presented in this work demonstrate that there are elevated IL-6 levels in the elderly, confirming the basal inflammaging. In contrast to what we hypothesized, and despite inflammaging, EV concentration in circulation is not affected by human aging. Moreover, frailty or dependence did also not alter the EV number. Many authors have previously studied the implication of EVs in diverse inflammatory processes, including cellular senescence, neurodegenerative diseases and cancer, indicating that both the total number of EVs in circulation and also EVs from specific cell origins can be increased [192,230,310]. Our results present a chronic inflammatory process—inflammaging—in which circulating EV levels are not affected. In this work, and when studying EVs, there are several factors that should be taken into consideration.

In our cohort, a high inter-individual EV concentration variability has been found within the same group. Similarly, previous experiments have demonstrated that the protein concentration and content of EVs differ depending on the donor [311]. On the other hand, specific medications may also affect EVs, as there are compounds that can modify EV production and release. For instance, immunomodulatory treatments can affect EV production by immune cells and modulate EV concentration in circulation [241,253,312]. This kind of effects should be considered when measuring EV levels specially in aged people, because nearly all of them have chronic medications. In this study, a representative sample of community-dwelling aged people was analysed and, as expected, all were under chronic treatment. It was ethically not possible to ask the participants in the study to interrupt their medications. Furthermore, the aim of our study was to evaluate whether the low-grade proinflammatory status was sufficient to alter EV concentration in the elderly, despite their medication. Moreover, even if the total number of EVs is not altered, EVs secreted from specific cell types could be affected, both in their concentration and cargo, modulating their function and effect in target cells, as described for other biological processes [230]. Finally, the limited number of samples in the study must be taken into account and results should be validated in a larger cohort. In brief, these results represent a first report and demonstrate that there is no correlation between inflammaging and EV concentration in circulation. More extensive experiments are required to study the specific changes that occur to EVs in regard to human aging, and to further elucidate their role in the process.

CHAPTER TWO

Influence of extracellular vesicles and age on cell differentiation

General introduction

EVs are important mediators of indirect cell communication. In the last years, the study of EVs has increased notably, and consequently, their implication in many biological processes is being described. Cell differentiation has been found to be one of the processes in which EVs have an effect. Recent publications found that EVs enhance or inhibit cell differentiation in many tissues, depending on the particle source and the components they carry [259,313]. However, it should be mentioned, that even if some molecules carried by EVs have been shown to be implicated, the underlying mechanisms in target cells leading to cell differentiation modification are still not completely understood.

On the other hand, as commented before, aging is a multifactorial process that reaches all tissues and affects cell communication, including EVs [133,230]. Moreover, it has been widely shown that cell differentiation is reduced or defective in elders [119,122,125]. Besides, some authors have also found that circulating EVs from aged donors have distinct effects when compared to young donors, such as reduced osteogenic differentiation promotion [245] and CNS myelination [257].

Taking all this into consideration, the aim of the present chapter was to evaluate the potential role of EVs from plasma to induce cell differentiation and to compare the different outcomes of young and aged EVs. We decided to study the influence of plasma EVs on osteogenesis and myogenesis, as the differentiation of both osteoblasts and myoblasts occur in tight communication with the circulatory system. Furthermore, they are active processes throughout the individual's life and defective cell differentiation can result in bone and muscle problems, two of the most common age-related features.

The following two sections describe the study designs, experiments and results obtained in collaboration with:

1. Osteogenesis: The research group led by Prof. Johannes Grillari PhD, Christian Doppler Laboratory for Biotechnology of Skin Aging, Department of Biotechnology, BOKU - University of Natural Resources and Life Sciences, Vienna (Austria).
2. Myogenesis: The research group led by Prof. Adolfo López de Munain MD PhD and Amets Sáenz PhD, Neuromuscular Diseases Group, Neurosciences Area, Biodonostia Health Research Institute, San Sebastian (Spain).

Osteogenesis

Introduction

The bone is a highly dynamic organ. The coordinated bone formation by osteoblasts and bone resorption by osteoclasts ensures its constant remodelling and maintenance. However, this balance is compromised in aged individuals, as reduced numbers and dysfunctions of osteoblasts have been found [120]. One of the main causes of impaired osteogenesis with age is thought to be the increased commitment of MSCs in the bone marrow to adipogenesis. Besides, this process has been shown to be influenced by the bone marrow microenvironment [124].

Taking into account the reduced osteogenesis, as well as the increasing incidence of age-associated bone defects and fractures, in the last years, regenerative treatments to enhance osteogenesis have been proposed [125]. Due to the difficult obtention and poor yield of MSCs from bone marrow, most of the regenerative therapies are based on ASCs. Similar to stem cells in bone marrow, ASCs can differentiate into several cell types, including osteoblasts [314]. Moreover, it is easier to obtain a high number of ASCs from lipoaspirates, they can be taken for autologous transplantations and reduce the donor site morbidity [315]. Many studies have demonstrated the efficiency of ASCs for bone regeneration in animal models, and clinical trials are now ongoing [125].

Regarding the previously mentioned importance of the microenvironment for osteogenesis enhancement, several researchers have reported beneficial effects of the combination of platelet-rich plasma (PRP) with ASCs [125,316], including a recent case report of an early stage avascular necrosis resolution in a 43-aged patient [317]. However, the molecules present in PRP that help the differentiation process are still not understood.

In this line, a study published by Weilner *et al.* found that EVs from plasma enhance osteogenic differentiation of ASCs. Interestingly, they also reported age-related differences, as EVs from elders had a reduced differentiation capacity [245]. We found this work as an important report demonstrating the implication of EVs from plasma in osteogenesis and, consequently, we established a collaboration with the authors. The aim of this collaboration was to try to confirm the effect of plasma EVs on osteogenic differentiation of ASCs with our EV samples, which are obtained with a distinct protocol and come from donors of different ages.

Materials and methods

Obtention and isolation of plasma EVs

Peripheral blood was collected by experienced nurses by venipuncture with a 21-gage needle in 4 ml EDTA tubes (Vacutainer, BD Biosciences). Samples from 5 adults (mean age 37.8 years, 2 females and 3 males) and 5 elders (mean age 85.4 years, 3 females and 2 males) were obtained. Tubes were kept upright and centrifuged at 1258 g for 20 min to recover plasma. To isolate EVs, plasma was centrifuged at 13,000 g for 2 min and obtained supernatant (1 ml platelet free plasma) was centrifuged again at 20,000 g for 20 min to pellet EVs. 900 µl of supernatant were transferred to another tube and the bottom 100 µl with the EV pellet were resuspended with 100 µl of filtered DPBS (GIBCO, Thermo Fisher Scientific, filtered twice through a 0.22 µm-pore filter). The 200 µl of resuspended EVs were stored at -80 °C and thawed on ice when needed.

Obtention and culture of ASCs

Abdominal liposuction was performed in a female donor aged 49 years. With the patient's informed consent, subcutaneous adipose tissue was obtained by outpatient tumescence liposuction under local anesthesia by an experienced physician. ASCs were isolated according to Wolbank *et al.* [318] and cultured in control medium consisting of DMEM-low glucose/HAM´s F-12 (GE-Healthcare) supplemented with 4mM L-glutamine (Sigma-Aldrich) and 10% fetal calf serum (Sigma-Aldrich) at 37°C, 5% CO_2 and 95% air humidity. Culture medium was changed three times a week and cells were passaged once a week at a split ratio of 1:2 to 1:6 according to the growth characteristics.

Coculture of ASCs with plasma EVs and induction of osteogenic differentiation

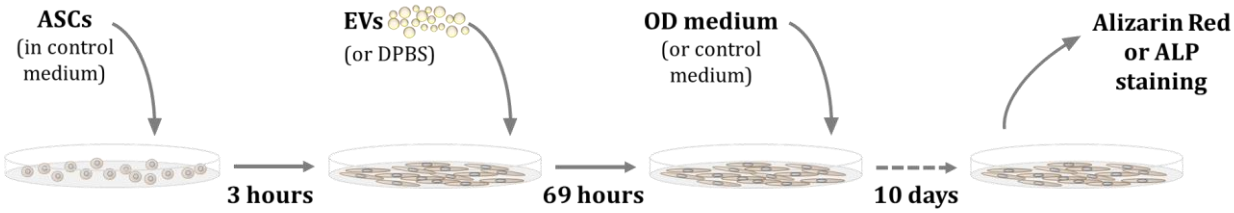
ASCs were seeded in 24-well culture dish wells. 3 days after seeding, osteogenic differentiation was induced by switching the medium to osteogenic differentiation medium consisting of DMEM-low glucose (GE-Healthcare), 10% fetal calf serum (Sigma-Aldrich), 4mM L-glutamine (Sigma-Aldrich), 10nM dexamethasone (Sigma-Aldrich), 150µM ascorbate-2-phosphate (Sigma-Aldrich), 10mM β-glycerolphosphate (Sigma-Aldrich) and 10nM 1.25 Dihydroxyvitamine D3 (Sigma-Aldrich) in a final volume of 1 ml/well.

Before performing coculture experiments to investigate the effect of EVs, culture conditions were optimized. Three different cell concentrations were assayed (4,000/9,000/14,000) and 14,000 ASC cells/well was chosen as the best for osteogenic differentiation measurement. Regarding osteogenic differentiation duration, 10 and 17 days were tested, and 10 days were chosen for subsequent experiments. When investigating the effect of EVs, 4 IU heparin/ml were

added to prevent culture medium jellification. Taken together, the final experimental setup to test the effect of plasma EVs on osteogenic differentiation is presented below and schematically represented in **Figure 31**. Besides, all the experiments performed and obtained results for the optimization of culture conditions are presented in the results section.

- 14,000 ASCs/well were seeded in 24-well culture dishes with 950 µl control medium supplemented with 4 IU heparin.
- 3 hours after seeding the cells, 50 µl of EVs (or 50 µl of DPBS) were added to corresponding wells, reaching a final volume of 1 ml/well, and carefully mixed to ensure a homogeneous EV distribution.
- 3 days after seeding the cells, the culture medium was changed. Osteogenic differentiation (OD) medium or control medium was added to corresponding wells.
- Cells were maintained in culture for 10 days more with media changes every 3 days.
- Alizarin Red or Alkaline phosphatase staining was performed to measure osteogenesis.

A



B

	Cells	Coculture	Media
1	ASCs	DPBS	Control medium
2	ASCs	EVs adult	Control medium
3	ASCs	EVs elder	Control medium
4	ASCs	DPBS	OD medium
5	ASCs	EVs adult	OD medium
6	ASCs	EVs elder	OD medium

Figure 31. Cell culture conditions to test the effect of EVs from plasma on osteogenic differentiation of ASCs. (**A**) Schematic representation of the culture protocol. ASCs were seeded in 24-well culture dishes in 950 µl control medium and 3 hours later, when cells were attached 50 µl of EVs or DPBS were added. 69 hours later (3 days after seeding) culture media were changed, switched to OD medium or to fresh control medium in corresponding wells. Cells were maintained in culture for 10 days more (13 days after seeding) with media changes every three days. Finally, osteogenic differentiation was evaluated by Alizarin Red or Alkaline phosphatase (ALP) staining. (**B**) Schematic representation of the 6 different study conditions. EV samples from 5 adults and 5 elders were tested, and all conditions were assayed in duplicate.

Alizarin Red staining

For quantification of calcified structures, cells were washed 3 times with PBS and then, fixed for 2 h in 70% ethanol (500 µl/well) at -20°C. Then, cells were washed 3 times with dH2O and stained for 10 minutes with 40mM Alizarin Red S solution (pH 4.2, 500 µl/well, Sigma-Aldrich) in an orbital shaker at room temperature. Subsequently, the remaining dye was removed by rinsing the cells with PBS. Finally, the residual dye was extracted by 0.1M HCL/0.5% SDS solution (200 µl/well) for 30 min. The dye signal was quantified in a microplate reader by determining the absorbance at 425 nm.

Alkaline phosphatase (ALP) staining

To determine the activity of ALP, cells were washed 3 times with PBS and then, lysed by incubating with lysis buffer for 1 h (0.25% Triton X-100, 100 µl/well) at room temperature. Next, samples were transferred to 1.5 ml tubes, centrifuged at 13,000 rpm 10 min at 4°C and 90 µl of supernatant transferred to a new tube. Subsequently, a buffer containing 20mM 4-nitrophenyl phosphate disodium salt hexahydrate, 0.5 M 2-amino-2-methyl-1-propanol and 0.2 mM MgCl2 (pH 10.3, 50 µl/tube) was added to each cell lysate and incubated for 20 minutes at room temperature in the dark. The reaction was stopped by adding 50 µl of 0.2M NaOH and ALP activity quantified by determining the absorbance at 405nm (620nm ref).

Statistical analysis

Statistically significant differences between the study groups were tested with GraphPad Prism version 6.01 for Windows (GraphPad Software, www.graphpad.com). Mann-Whitney test was applied to evaluate differences between EVs from adults and elders. **p<0.01.

Results

Osteogenic differentiation settings

The first experiments were directed to establish the best culture conditions to investigate the effect of EVs on osteogenesis. To this end, we tested 3 cell densities (4,000/9,000/14,000 ASCs) and two different end points (10/17 days after osteogenic differentiation induction). Triplicates were performed for all the conditions.

Calcification was measured by Alizarin Red staining and the obtained results are presented in **Figure 32**. We confirmed the induction of osteogenesis in all the tested conditions, as calcium deposition was higher under OD medium than under control medium (basal calcium deposition by ASCs). Moreover, a higher concentration of cultured cells resulted in an increased staining and a higher difference between control and OD medium. Regarding the two tested end points, an elevated differentiation was shown with the prolongation of osteogenesis (17 days).

With these results, we decided to perform the following experiments with 14,000 ASCs/well and to measure osteogenesis 10 days after the induction. We based our decision about the cell density on the prominent differences observed between control and OD medium. On the other hand, for the osteogenesis duration, we took into consideration the possible effects of plasma EVs: previous studies have reported an enhanced differentiation in the presence of EVs, so to be able to see the influence of EVs, we should measure osteogenesis at an intermediate point when an increase of calcification or ALP activity could be reported by the absorbance measurement.

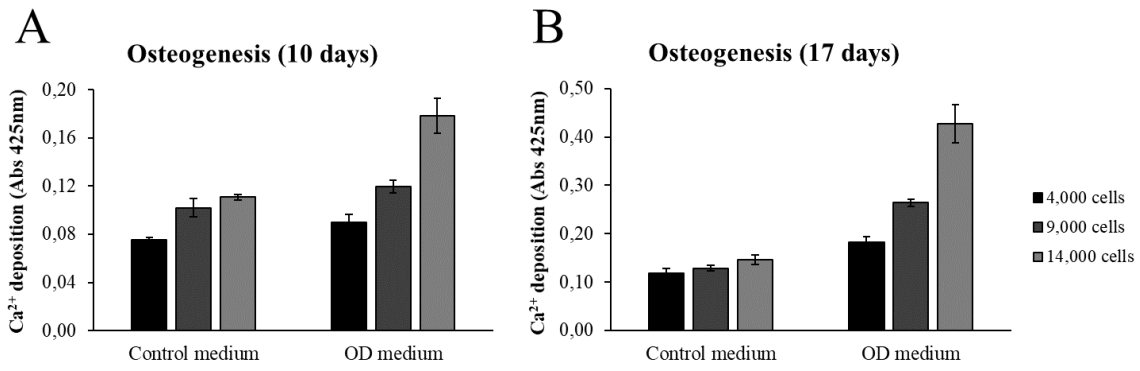


Figure 32. Osteogenic differentiation settings assessed by Alizarin Red staining. 3 different cell densities were seeded and calcium deposition measured 10 days (**A**) or 17 days (**B**) after osteogenic differentiation induction. Wells with control medium in which osteogenesis was not induced were maintained and assayed to measure basal calcium deposition by ASCs. Increasing staining was observed with more cells and with prolonged culture times.

Coculture of ASCs with plasma EVs results in the formation of jelly structures

After establishing the cell culture conditions for ASCs, in the next step we performed the first experiments in which EVs from plasma were added to ASCs. Cells were seeded in 24-well dishes and 3 hours after seeding the cells, 50 µl of thawed EVs were added to each well. 3 days after seeding the cells, culture media were changed and control or OD medium added to corresponding wells. Unexpectedly, at the bottom of the wells where ASCs and EVs were cocultured a jelly layer had formed. Medium change was done by carefully pipetting, but even so, when aspirating the medium, the jelly layer was partially detached in some wells. We still decided to continue with the osteogenesis protocol and try to measure the effect of EVs on calcium deposition.

10 days after osteogenic differentiation induction, Alizarin Red staining was performed. Despite the careful pipetting, we observed that the jelly-like layers were detached and it was not possible to rinse the wells after Alizarin staining (**Figure 33**), so no result was obtained from this assay.

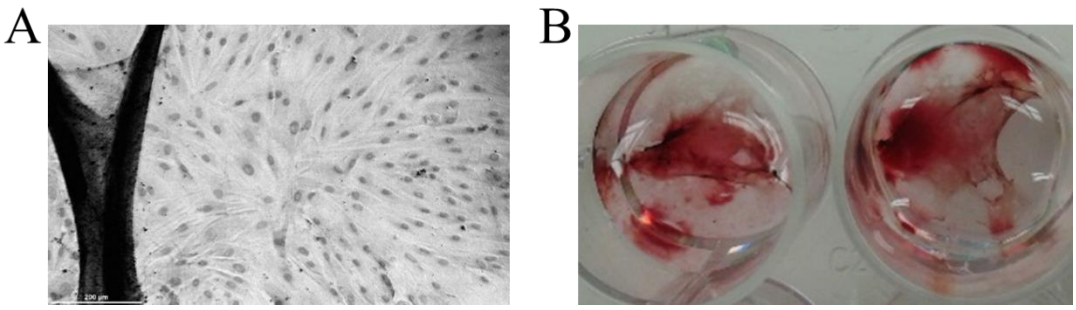


Figure 33. Formation of jelly structures and cell detachment. ASCs were cocultured with EVs isolated from plasma and a jelly layer had formed at the bottom of the wells. (**A**) Representative image of a well where the jelly layer was detached and flipped on top of other cells. (**B**) A picture taken from 2 wells cocultured with EVs. Alizarin Red staining was performed, but no concluding results could be obtained.

After observing this phenomenon, we performed an experiment to investigate the formation of jelly structures and whether heparin could prevent them in our cocultures with plasma EVs. We prepared a 24-well dish as shown in **Figure 34**: EVs alone, ASCs + EVs, EVs in heparin containing medium and ASCs + EVs in heparin containing medium were tested. In all cases, 14,000 ASCs and 4 U of heparin/ml were used, while different EV volumes were added.

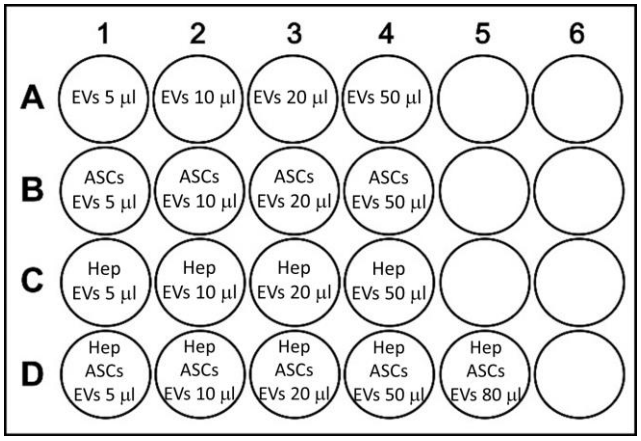


Figure 34. Schematic representation of the 24-well culture dish plan. In *row A* control medium and EVs were mixed, while in *row B* 14,000 ASCs/well were also added. In *row C* control medium with 4 IU heparin and EVs were mixed, while in *row D* 14,000 ASCs/well were also added.

3 days later the formation of gels was evaluated. Culture medium was carefully pipetted and we observed no jelly layers in *row A* and *C*, confirming that the interaction between control medium and EVs do not produce the jelly structures. When medium in *row B* was aspirated, jelly layers were found in all wells, with thicker structures in the wells cocultured with higher volumes of EVs. In contrast, medium pipetting was performed normally in row D, demonstrating that the addition of 4 IU heparin/ml prevents the formation of the gel for all the EV volumes tested. Consequently, we decided to incorporate heparin to the control medium for coculture experiments.

Effect of plasma EVs on osteogenesis

Finally, we tested the effect of EVs isolated from plasma of adult and elder donors on osteogenic differentiation. EV samples from 5 adults and 5 elders were assayed, each of them in duplicate. Our results show that, in all cases, the coculture of ASCs with plasma EVs enhance osteogenesis, and this effect is stronger with EVs from adults (**Figure 35**).

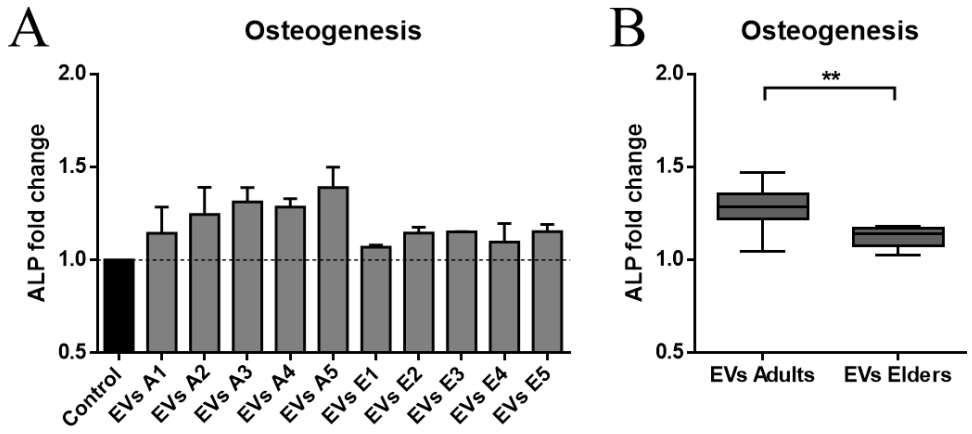


Figure 35. Osteogenesis enhancement by plasma EVs. ASCs were cocultured with EVs for 3 days and then, osteogenic differentiation was induced. 10 days after induction ALP activity was measured. Results are presented in fold change versus the control condition in which no EVs were cocultured. (**A**) Graph showing the results obtained for each EV donor. In all cases, ALP activity was higher than the control. (**B**) Box plot representation of results obtained for EVs from adult and elder donors. EVs from adults enhance ALP activity significantly more than EVs from elders. A = adult and E = elder.

Besides, we also tested whether EVs alone were able to induce osteogenesis. To this end, we cultured ASCs in control medium. The coculture with EVs was performed as before, but instead of inducing osteogenesis with OD medium, control medium was maintained. The obtained results demonstrate that the presence of EVs alone does not induce osteogenesis (**Figure 36**).

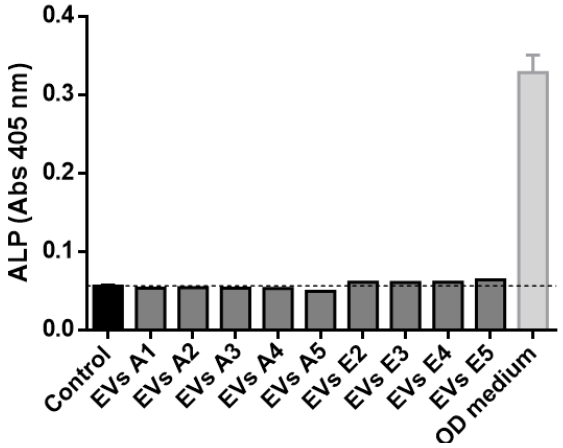


Figure 36. Effect of EVs under control medium. ASCs were cocultured with EVs for 3 days and then, cultured for 10 days more with control medium. Two wells without EVs were cultured with OD medium for 10 days as a positive control of osteogenesis. ALP activity was measured and no differences were reported, indicating that EVs alone do not induce osteogenic differentiation of ASCs. A=adult and E=elder.

Discussion

In this project, we have set the cell culture conditions and investigated the effect of EVs from plasma on the osteogenic differentiation of ASCs. The starting point of this work was based on a publication by the group of Grillari [245], in which they reported an increased osteogenesis enhancement with EVs isolated from plasma of young donors (less than 25 years) when compared to older ones (more than 55 years). Besides, they focused on galectin-3 protein and showed the important role of this molecule during the osteogenic process. Regarding the EV experiments performed by the group, to isolate EVs from plasma they filtered the samples through 0.22 μm pore filters and then applied ultracentrifugation at 100,000 g for 1 hour. With this protocol, they isolated small EVs and discarded bigger EVs. Moreover, when considering potential future applications, their protocol could be difficult to implement in the clinic, as most of the hospitals do not have ultracentrifuges. Taken together, the main objectives of our work were to test the effect of EVs isolated with an easily applicable protocol [202] and to compare the effect of samples coming from adult and elder donors.

First, we obtained ASCs from a healthy donor and conducted experiments to establish the appropriate cell culture settings. In our hands, seeding 14,000 cells/well and maintaining osteogenic induction for 10 days resulted in adequate differentiation. When adding EVs to cultured ASCs, a jelly layer was formed. The addition of only 5 μl of plasma EVs was enough to induce the formation of this structure. On the other hand, a culture medium prepared with 4 IU heparin/ml prevented jellification. The use of human plasma for cell culture has been widely investigated before, and medium clotting was also reported in many cases [319]. Here we applied EVs isolated from plasma, and even if we have demonstrated that our protocol efficiently isolates EVs, when handling a complex fluid as plasma, other small components are probably coprecipitated. In accordance with previous reports, the addition of a low concentration of heparin to our cultures prevented media clotting. It should be mentioned that some authors reported impaired cell proliferation and differentiation of ASCs under high doses of heparin [320], but we applied only 4 IU heparin/ml for 3 days and then, osteogenic differentiation was induced and no issues were reported.

We performed coculture experiments with EVs from 10 different donors: 5 adults and 5 elders. Interestingly, all EV samples boosted osteogenic differentiation, but none of them induced differentiation if OD medium was not used, demonstrating that plasma EVs alone do not induce the differentiation of ASCs to osteoblasts. Moreover, the positive effect of EVs was more prominent when samples from adults were applied. These results indicate that EVs favour osteogenesis, but an age-related exhaustion could be present. Our results are in accordance with the previous study by the group of Grillari, and importantly, we tested more samples,

coming also from older donors (> 80 years, instead > 55 years), and with EVs isolated with a different protocol (final pelleting at 20,000 g, instead 100,000 g).

This work reinforces previous studies and demonstrate the potential application of EVs for osteogenesis enhancement. Furthermore, other authors showed that EVs secreted by ASCs [260,313], or by a specific subset of plasma EVs promote osteogenesis [245], but their application would need *in vitro* cultures of ASCs to produce EVs or complicated protocols to isolate EVs from plasma, respectively. In contrast, our results indicate that EVs easily isolated from plasma, alone or in combination with cell therapies, could help osteogenesis. Furthermore, other authors that applied PRP and ASCs for osteogenesis with positive results were probably also administering EVs, as they would be present in PRP samples [316,317].

Regarding the possible clinical applications to promote osteogenesis, it should be mentioned that, similar to our results with EVs, ASCs from elders have a reduced osteogenic potential [125]. We hypothesize that to overcome the age-associated dysfunction and to avoid allogeneic cell transplantations, regenerative therapies in aged patients could be carried out with autologous ASCs and plasma EVs from a young donor. This combination could boost osteogenesis while preventing potential problems associated with allogeneic cells.

In summary, ASCs are an easily accessible source of MSCs and they can be differentiated into different cell types, including osteoblasts. Due to their therapeutic potential, many efforts are being made to understand the underlying mechanisms of osteogenesis [321]. In parallel, the implication of EVs in osteogenic differentiation is still starting to be investigated. The first reports, including ours, indicate that EVs play an important role and help ASC differentiation, and we consider that they should be taken into consideration for future clinical applications.

Myogenesis

Introduction

The skeletal muscle is the largest organ in the human body. It is a highly adaptable tissue that responds to environmental conditions and physiological challenges by changing fibre size and composition. However, the incidence of skeletal muscle injuries as a consequence of trauma, inherited genetic diseases, pathology or aging is very high and represents relevant socio-economic costs. In the case of aging, muscle wasting, defined by marked muscle mass loss and weakening, is one of the major problems leading to increased risk of falls and development of physical disability [123,322].

It is well known that the skeletal muscle has regenerative potential, which, however, becomes compromised in the case of severe or extended damage as well as with aging [123,126]. In this context, several methods are nowadays applied in the clinic to promote muscle repair and regeneration and, besides, many investigations are being conducted to improve the present techniques or implement new and more effective methods [126]. Among these potential new methods, we are specially interested in the ones investigating the role of EVs in myogenesis.

Indeed, in the last years, many authors have investigated the composition and functions of EVs secreted by myoblasts and myotubes [323]. Regarding their functions, a work by Forterre *et al.* reported that EVs secreted by myotubes reduce proliferation and induce myoblast differentiation [324], while a study by Guescini *et al.* did not obtain the same results [325]. However, the EV concentrations and differentiation endpoint were distinct, which could account for the observed differences. Besides, it should be mentioned that most of the works focusing on the effects of EVs on myogenesis were carried out in the C2C12 immortalized mouse myoblast line, which is an interesting and useful model for studying many processes, but it also presents some differences when compared to humans.

To our knowledge, no works have studied the influence of EVs from human plasma on myogenesis. Nevertheless, many researchers have indirectly applied EVs in their investigations, as EVs are part of the components of PRP and platelet-poor plasma (PPP). These preparations have been widely tested for muscle regeneration, as nicely reviewed by Chellini and colleagues [326]. In any case, they did not consider the presence of EVs and their effects as part of PRP and PPP are still unknown.

Consequently, and taken into consideration the positive effect of plasma EVs reported before for osteogenesis, we wondered whether they could also help myogenesis. To try to solve this question, we collaborated with the group led by López de Munain and Sáenz and tested our EV samples on their human primary myoblasts.

Materials and methods

Obtention and isolation of plasma EVs

Peripheral blood was collected by experienced nurses by venipuncture with a 21-gage needle in 4 ml EDTA and 2.8 ml citrate tubes (Vacutainer, BD Biosciences). Samples from 12 adults (mean age 34.5 years, 5 females and 7 males) and 9 elders (mean age 81.8 years, 5 females and 4 males) were obtained. EDTA tubes were kept upright and centrifuged at 1,258 g for 20 min to recover plasma. To obtain plasma from citrate tubes, we followed they were centrifuged at 2,500 g for 15 min. We isolated EVs coming from both extraction tubes as previously described by our group [202]. Plasma was centrifuged at 13,000 g for 2 min and this supernatant centrifuged again at 20,000 g for 20 min to pellet EVs. The pellet was resuspended with filtered DPBS (GIBCO, Thermo Fisher Scientific). Resuspended EVs were stored at −80 ºC.

Obtention and culture of myoblasts

The skeletal muscle biopsy was obtained at Donostia University Hospital after the donor gave written informed consent, using forms approved by the Ethics Committee. The sample was taken from the triceps brachii of a healthy donor (male, 26 years) that underwent surgery due to bone fracture. The muscle sample was processed, primary myoblasts isolated as previously described by the Neuromuscular Diseases Group [327] and cultured in proliferation medium consisting of DMEM (Lonza) and M-199 (Lonza) supplemented with 10% FBS (Thermo Fisher), 1% insulin 1 mg/ml (Sigma Aldrich), 1% glutamine 200 mM (Life Technologies), 0.5% fibroblast grow factor 5 µg/ml (Prepotech), 0.1% epidermal grow factor 10 µg/ml (Prepotech) and 1% penicillin/streptomycin + amphotericin B (9:1, Thermo Fisher) in 0.5% gelatin-coated plates at 37°C, 5% CO_2 and 95% air humidity. Culture medium was changed three times a week and cells were passaged when 70-75% confluence was reached.

Coculture of myoblasts with plasma EVs and induction of myogenic differentiation

To test the effect of EVs on myogenesis, two coculture experiments were performed. In both of them, myoblasts were seeded in 24-well culture dish wells (previously coated with 0.5% gelatine) with proliferation medium. Then, EVs were added to a final volume of 1 ml/well. When 80-90% confluence was achieved, myogenic differentiation was induced by switching the medium to myogenic differentiation medium consisting of DMEM and M-199

supplemented with 2% FBS, 1% insulin 1 mg/ml, 1% glutamine 200 mM and 1% penicillin/streptomycin + amphotericin B (9:1). Finally, we proceeded to RNA extraction.
The following lines describe the procedure of each of the two experiments, which are also illustrated in **Figure 37**.

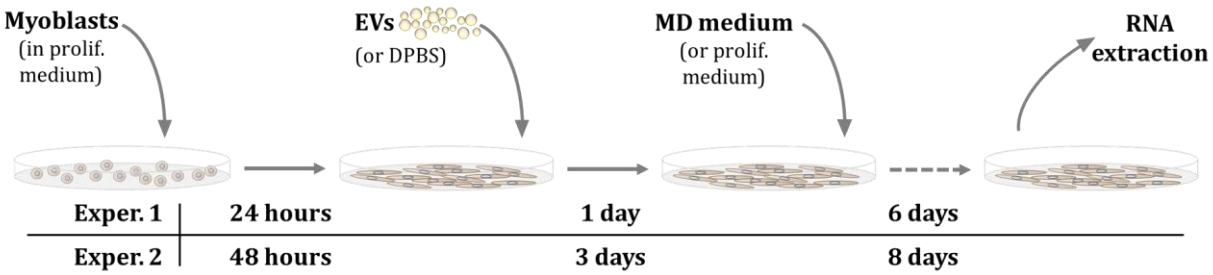
Experiment 1:

- 20,000 myoblasts/well were seeded in 24-well culture dishes with 970 µl proliferation medium.
- 3 hours or 24 hours after seeding the cells, 30 µl of EVs (or 30 µl of DPBS) were added to corresponding wells, reaching a final volume of 1 ml/well, and carefully mixed to ensure a homogeneous EV distribution. EVs isolated from EDTA tubes were used. EVs from 9 adults were pooled and assayed in triplicate. EVs from 6 elders were pooled and assayed in triplicate.
- 2 days after seeding the cells, the culture medium was changed. Differentiation (MD) medium or proliferation medium was added to corresponding wells.
- Cells were maintained in culture for 6 days more with media changes every 3 days.

Experiment 2:

- 150,000 myoblasts/well were seeded in 24-well culture dishes with 970 µl proliferation medium supplemented with 4 IU heparin.
- 2 days after seeding the cells, 30 µl of EVs (or 30 µl of DPBS) were added to corresponding wells, reaching a final volume of 1 ml/well, and carefully mixed to ensure a homogeneous EV distribution. EVs isolated from citrate tubes were used. EVs from 3 adults and 3 elders were individually tested and each of them was assayed in duplicate.
- 5 days after seeding the cells, the culture medium was changed. MD medium or proliferation medium was added to corresponding wells.
- Cells were maintained in culture for 8 days more with media changes every 3 days.

A



B

	Cells	Coculture	Media
1	Myoblasts	DPBS	Proliferation medium
2	Myoblasts	EVs adult	Proliferation medium
3	Myoblasts	EVs elder	Proliferation medium
4	Myoblasts	DPBS	MD medium
5	Myoblasts	EVs adult	MD medium
6	Myoblasts	EVs elder	MD medium

Figure 37. Cell culture conditions to test the effect of EVs from plasma on myogenic differentiation. (**A**) Schematic representation of the culture protocol. Myoblasts were seeded in 24-well culture dishes in 970 µl control medium and then, when cells were attached 30 µl of EVs or DPBS were added. When cells reached 80-90% confluence culture media were changed, switched to myogenic differentiation (MD) medium or to fresh proliferation medium in corresponding wells. Cells were maintained in culture with media changes every three days. Finally, myogenic differentiation was evaluated by extracting RNA from cultures and measuring gene expression by qPCR. (**B**) Schematic representation of the 6 different study conditions.

RNA extraction, cDNA synthesis and qPCR

For the quantification of myogenesis-related genes, we extracted RNA from cultured cells. To this end, wells were washed 2 times with cold PBS and then, 750 µl of TRIzol Reagent (Invitrogen, Thermo Fisher) were added. Cells were detached with cell scrapers, collected on RNase-free tubes and subjected to 1 min vortex. Total RNA extraction was performed following the manufacturer's instructions and resuspended in 20 µl of RNase-free water. The RNA concentration was measured in a NanoDrop ND-1000 spectrophotometer.

RNA was reverse transcribed into cDNA with random primers using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. For the qPCR analysis, 384 well-plates were used. In each well, 5 µl TaqMan Gene Expression Master Mix (Thermo Fisher) and 25 ng cDNA were mixed, together with 0.5 µl of TaqMan probes in a final reaction volume of 10 µl (completed with RNase-free water). GAPDH was chosen as the housekeeping gene for internal control and the expression of desmin (DES, probe ref: Hs00157258_m1), myogenic differentiation 1 (MYOD1, probe ref: Hs00159528_m1) and

myogenin (MYOG, probe ref: Hs01072232_m1) were measured. All the components were maintained on ice and protected from light. Plates were sealed and centrifuged to remove possible bubbles. Then, the cDNA was amplified using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Each sample was run in triplicate and measures with a coefficient of variation higher than 3% were discarded. The presence of a single-peak in the melting curve indicated the specificity of the amplification. For relative expression calculation, the $2^{-\Delta Cq}$ method was used, where ΔCq was calculated by the normalization of the gene of interest with respect to the housekeeping gene.

Statistical analysis

Statistically significant differences between the study groups were tested with GraphPad Prism version 6.01 for Windows (GraphPad Software, www.graphpad.com). Mann-Whitney test was applied to evaluate differences between the study groups. *p<0.05 and **p<0.01.

Results

Effect of plasma EVs on myogenesis – Experiment 1

In the first approach, we cultured 20,000 myoblasts/well. Due to the novelty of our experimental setup, and aiming not to interfere in the attachment of myoblasts to the plate, we decided to try different EV addition times: 3 hours after seeding and 24 hour after seeding. We checked the cells and when they reached the appropriated confluence (48 hours after seeding), we changed the medium to induce cell differentiation with MD media. On the other hand, another set of wells was maintained in proliferation medium, to evaluate the effect of plasma EVs under this medium. At this point, when the media change was performed after the coculture, we noticed the formation of a jelly layer in the wells with EVs (similar to the one described in the osteogenesis experiments). We carefully removed these structures and noticed that, even if some cells could have been removed, most myoblasts were still normally attached. We only lost one of the wells where EV samples from elders were added at 24 hours, as the jelly layer could not be completely removed. We decided to continue with the experiment and 6 days later (8 days after seeding) we extracted RNA from samples and performed the qPCR analysis. Two representative pictures of cells before RNA extraction are shown in **Figure 38**.

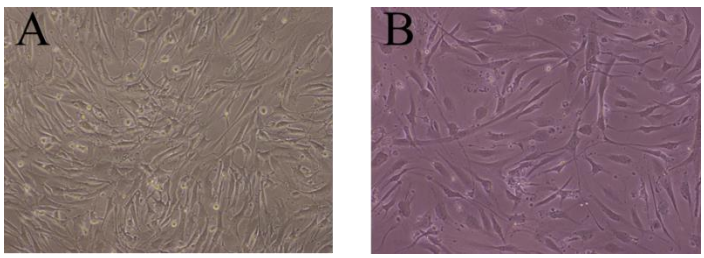


Figure 38. Representative 10x images of the cells at the end of the experiment. (**A**) Proliferation medium and (**B**) differentiation medium.

We could not obtain RNA from the wells where EV samples from adults were added at 24 hours. For the rest of the conditions tested, the obtained results are presented in **Figure 39** and **Figure 40**. As expected, the expression of the myogenic differentiation markers MYOD1, MYOG and DES was higher in the cells where differentiation medium was applied (upper panel vs lower panel). Interestingly, when we evaluated the effect of adding EVs, we observed that the expression of the three markers was increased in the cells cocultured with EVs from plasma and this effect was present under both proliferation and differentiation medium (**Figure 39**). Moreover, a more robust enhancement is obtained with EVs from adults when compared to elders (**Figure 40**), but due to the limited sample size, no statistical analysis was performed.

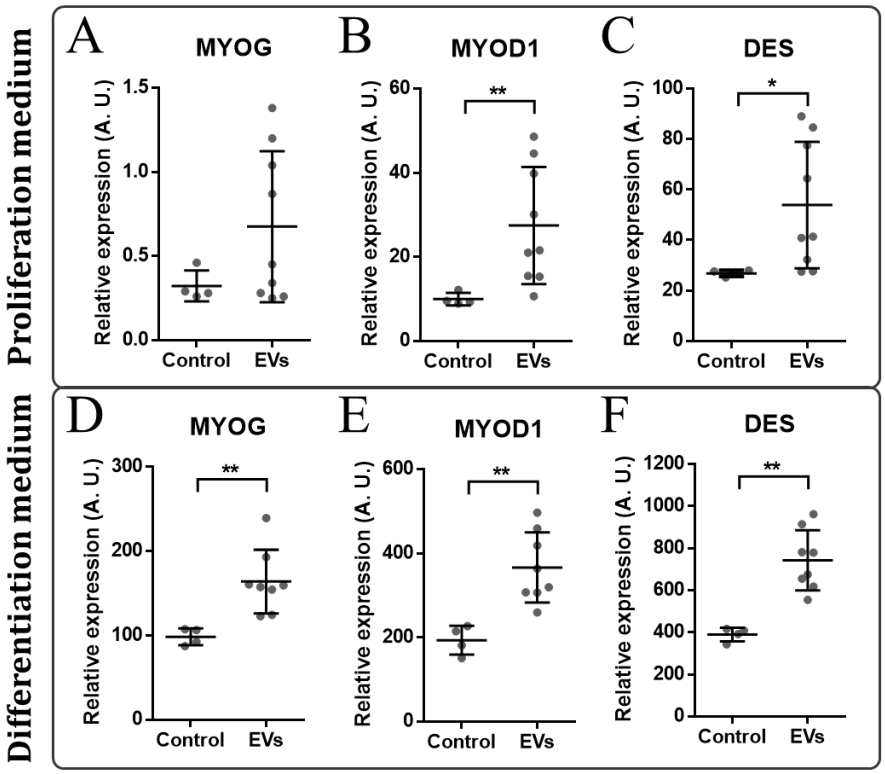


Figure 39. Expression of myogenic differentiation markers analysed by qPCR. The control wells were compared to the ones in which EVs were added. (**A-C**) The coculture of myoblasts and EVs results in higher levels of MYOG, MYOD1 and DES. (**D-F**) The differentiation medium increases the expression of MYOG, MYOD1 and DES, and this effect is enhanced when cells are stimulated with EVs.

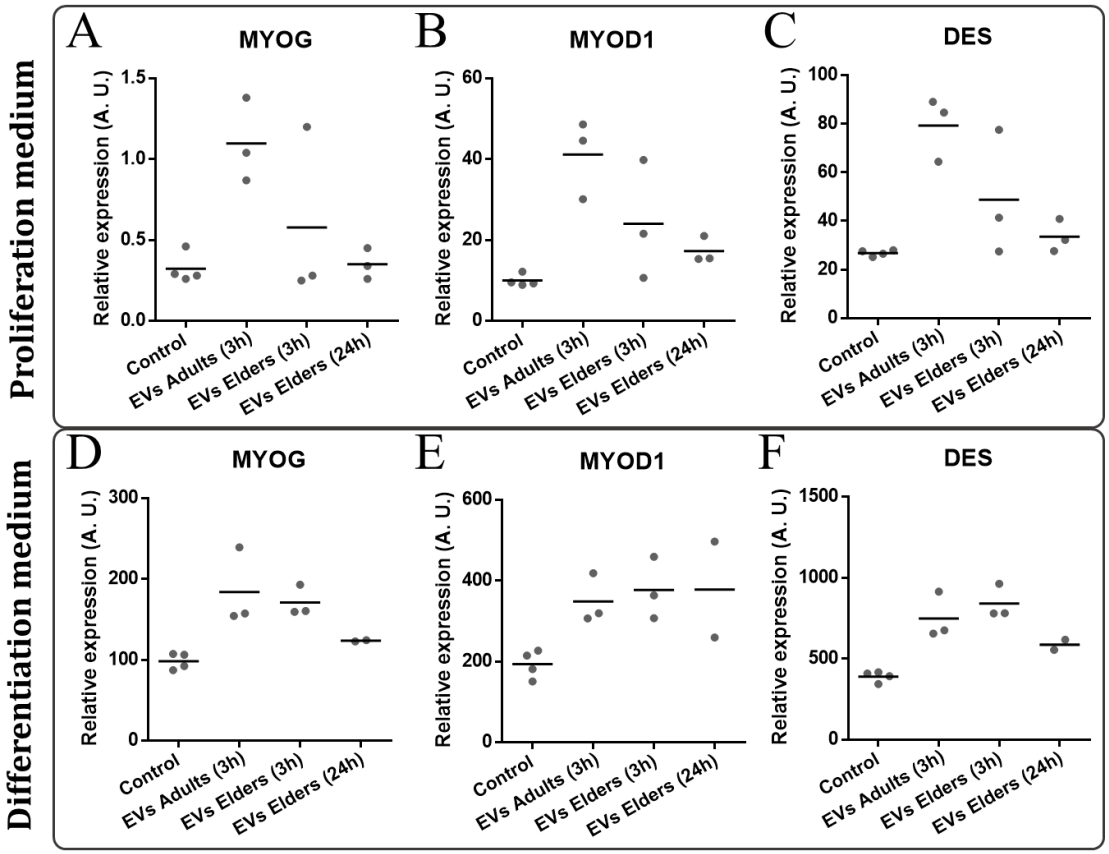


Figure 40. Expression of myogenic markers depending on the EVs added. EVs from adults and elders are compared, and the hours in brackets indicate the time between myoblast seeding and EV addition. EVs from adults stimulate more the expression of myogenic markers, but due to the limited sample size, no statistical analysis was performed.

Effect of plasma EVs on myogenesis – Experiment 2

For the second approach, and aiming to improve the experimental settings, we decided to seed 150,000 myoblasts per well and added 4 IU heparin/ml to prevent clotting. The EVs isolated from plasma were added 2 days after seeding and the coculture was prolonged for 3 days, when myoblasts presented an appropriate confluence to induce differentiation. We changed to MD medium in half of the wells and continued with proliferation medium in the others, to test again the influence of EVs on both conditions. 6 days later, and 13 days after seeding, we extracted RNA from samples and performed the qPCR analysis. Representative pictures of cells before RNA extraction are shown in **Figure 41**.

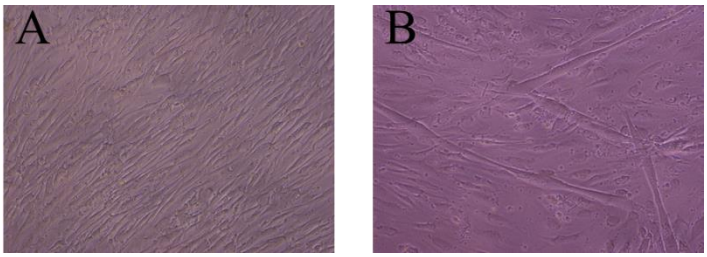


Figure 41. Representative 10x images of the cells at the end of the experiment. (**A**) Proliferation medium and (**B**) differentiation medium.

We satisfactorily isolated RNA from all samples and performed cDNA synthesis and qPCR as usual. However, we did not obtain cDNA amplification for some of the samples, specifically for the ones corresponding to controls and to EVs from adults under differentiation medium. Consequently, no results could be obtained from these samples and, besides, the samples cocultured with EVs from elders with differentiation medium were also not analysed, as there was not any control to compare them with. The results from the rest of the conditions, which included all the samples maintained in proliferation medium, were analysed and the results are shown in **Figure 42**. Similar to the results obtained in the first experiment, we saw that myoblast cocultured with EVs from adults have elevated levels of myogenic differentiation markers when compared to EVs from elders (significant for MYOD1 and tendency for MYOG and DES). In contrast, in this second experiment, we did not observe a significant increase of the myogenic markers between the control wells and the ones cocultured with EVs.

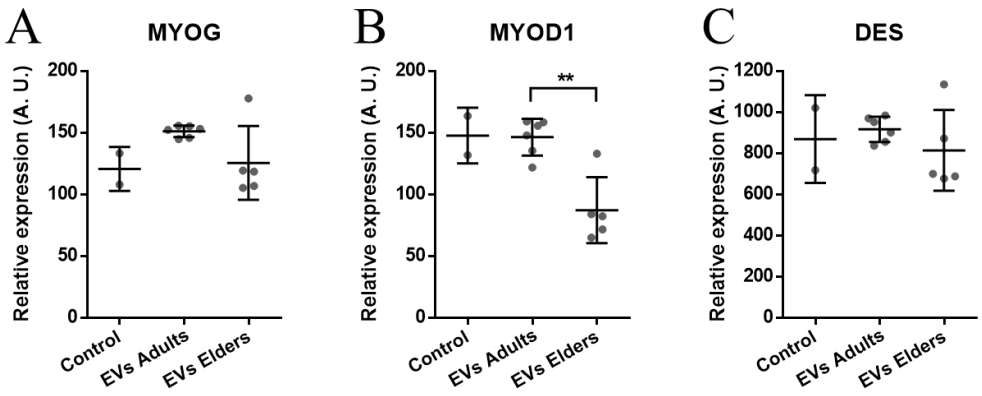


Figure 42. Expression of myogenic differentiation markers. Myoblasts were maintained in proliferation medium and they were cocultured with EVs from adult or elder donors. The samples with EVs from adults showed a higher expression of MYOD1, and the same tendency for MYOG and DES, while these two did not reach statistical significance.

Discussion

The objective of this section was to investigate the effect of EVs isolated from plasma on myogenesis. This is a field that has not been investigated, but it could have potential benefits for muscle regeneration, which is of particular interest for the age-associated sarcopenia. We decided to perform the experiments based on our previous results on osteogenesis, as well as on the literature about myogenic differentiation. Interestingly, a work by Nakamura *et al.* studied the effects of EVs secreted by MSCs [259] and, on the other hand, many investigations have evaluated the effect of PRP and PPP (that contain EVs) on myogenesis [326]. However, none of them specifically evaluated the potential role of EVs from plasma, which we consider that could also be playing a role.

With this in mind, we collaborated with a group from Biodonostia that works with primary myoblasts and performed two experiments with different setups. We tested different cell concentrations, coculture times and endpoints. As in our previous experiments, we evaluated the effect of EVs not only under differentiation medium, but also under proliferation medium, to investigate whether EVs could influence in both conditions. Notably, in the first experiment, we reported an elevated expression of MYOG, MYOD1 and DES myogenic differentiation markers under proliferation and differentiation medium. Besides, when EVs isolated from adult and elder donors were compared in our two experiments, a higher expression of the differentiation markers was observed with EVs from adults, with statistically significant differences for MYOD1 in the second experiment.

Our results are a first report indicating that EVs from plasma could play a role in myogenesis and, moreover, that EVs from adults could have a more robust effect than the ones isolated from elders. Further, we performed the experiments on human primary myoblasts, while most works are performed in murine immortalized myoblasts [323–325]. To our knowledge, one publication investigated before the influence of EVs on myogenesis with human primary myoblasts, but they had a completely different objective, as they studied the effect of EVs present on the foetal bovine serum used for cell culture [328].

In any case, regarding the effects of EVs, we should keep in mind the complexity of biological processes and the limitations of the systems that we and all researchers apply. For instance, we try to model and study myogenic differentiation *in vitro*, by plating myoblasts, coculturing them with EVs from plasma and evaluating the expression of certain genes. We consider that this is a good approach to investigate whether EVs have an effect on the process, which is a novel field that is still in its infancy. However, we do not replicate the microenvironment present when a skeletal muscle of an individual is regenerating, and we would probably never be able to reproduce it exactly. With regard to the potential use EVs, the advantage of EVs with respect to cells is that we can first evaluate their effects in *in vitro* models, and then, test their potential efficacy *in vivo* with less safety concerns.

As discussed in the previous section for osteogenesis, we hypothesize that plasma EVs could be applied in the future alone or in combination with autologous stem cells to enhance myogenesis. However, and even if some treatments with PRP have already been applied to patients with injured skeletal muscles with positive results [326], we are still far from understanding the implications of EVs. Our approach was just the first step and many more should be taken to describe the roles of plasma EVs on myogenesis and whether aging affects them.

CHAPTER THREE

Immunosenescence and the role of extracellular vesicles

Introduction

Human aging is a complex and heterogenic process, in which several cellular mechanisms are affected and modulated, leading to functional decline [33]. One of the most determining consequences of aging is the dysfunction of the immune system, and the subsequent poor response to vaccination, increased susceptibility to infections and age-related diseases observed in the elderly [329].

The molecular and cellular changes that lead to immune dysfunction have been extensively investigated and are generally referred to as immunosenescence [92]. T cells are the most dramatically affected immune components, with a decrease in naïve T cells and an accumulation of terminally differentiated T cells with age. Terminally differentiated T cells exhibit features of replicative senescence and lose the expression of the costimulatory molecule CD28 from their membrane [98–101,330]. CD28 plays an essential role in T cell function, taking part in activation, proliferation and survival processes. Hence, CD28 negative T cells present altered molecular features, as well as distinct cytokine production and effector molecules [102]. The loss of CD28 affects earlier and primarily CD8 T cells, but it has also been described to reach CD4 T cells later in life [103,104]. In consequence, T lymphocytes have a reduced capacity to react against new *stimuli*, contributing to the aforementioned immune dysfunction. Another feature found in immunosenescent T cells is the enhanced cytotoxicity. Expression of NK cell characteristic receptors such as CD56 and CD57 membrane molecules have been widely reported in these cells, which promote their cytotoxic capacity [105–108]. Additionally, many authors have found a higher prevalence of an inverted CD4/CD8 ratio in the elderly, a feature known as immune risk phenotype, that predicts shorter survival [113–115].

The immunosenescent process and the changes that occur in other cell types during aging result in an altered secretion of molecules by cells, termed SASP [131]. The SASP components have been classically divided into three groups: i) soluble signalling factors (ILs, chemokines, and growth factors), ii) secreted proteases, and iii) secreted insoluble proteins/extracellular matrix components [132]. One of the consequences of SASP is the chronic low-grade inflammation seen in the elderly, the so called inflammaging [136]. The age-associated immune dysfunction and accumulation of senescent cells promote inflammatory signals, such as elevated secretion of proinflammatory cytokines like IL-6 [138,140,331]. Another remarkable aspect that is affected by the SASP is intercellular communication. Apart from the three classical SASP components mentioned before, in the last decades EVs have been shown to play a central role in intercellular communication and immune system function [234].

EVs are membrane-coated particles that are secreted by almost all cell types and are present in most body fluids, including plasma. They can be of endosomal or plasma membrane origin and they carry proteins, lipids and genetic material that can be incorporated by the target cell. EVs are released in physiologic and pathologic conditions and are implicated in many cellular processes [195]. As stated before, EVs are also implicated in the immune system function, as they can carry antigenic material and modulate immune responses [234].

Regarding EVs in aging and senescence, the expression of p53 transcription factor have been related to increased EV production [332]. However, some works have studied the concentration of plasma EVs with age, with contradictory results [331,333]. One of these works also examined the EV protein cargo and internalization by immune cells and showed proteins differentially expressed with age and that EVs from older donors are more readily internalized by B cells [333].

In spite of that, there are still many aspects of EVs that have not been elucidated. Similarly, even if immunosenescence at a cellular level has been widely investigated, only a few works have analysed samples from nonagenarians and centenarians [98,334]. It is important to note that only a small percentage of people reach these advanced ages, making it even more difficult to include their samples in study cohorts. Works that studied nonagenarians and centenarians showed that their PBMCs have distinct features at transcriptional and functional levels when compared to septuagenarians and octogenarians [26,27,335].

Taking all this into account, the aims of the present work were to characterize the immunosenescence status of our cohort (donors of 20-49 and 70-104 years), comparing different age ranges at the cellular and EV level and to try to describe the possible immune functions of plasma EVs.

Materials and methods

Participants and blood sampling

For the present study, donors of different age ranges were enrolled. Healthy adults between 20-49 years and elders of 70-104 years were included. Elders were assessed at primary care services and by an experienced neurologist. Both community-dwelling and institutionalized participants and with distinct functional capacities were enrolled, aiming to have a representative sample of age-related heterogeneity. All participants completed a questionnaire and donors with acute illness or immunological disorders were excluded. Samples from 51 donors (29 females and 22 males), 18 healthy adults and 33 aged individuals were collected at Donostia University Hospital. Participants were classified based on their age range: 20-29 (n=6), 30-39 (n=5), 40-49 (n=7), 70-79 (n=6), 80-89 (n=10), 90-99 (n=13) and ≥100 (n=4)

years. The study was approved by the hospital's ethics committee and all participants provided written informed consent before blood sampling.

Peripheral blood was collected by venipuncture with a 21-gage needle. The first millilitre was discarded and then blood collected in a 2.8 ml citrate tube and 4 heparin tubes of 4 ml (Vacutainer, BD Biosciences).

PBMC isolation and storage

Within 1 hour of sampling, peripheral blood collected in heparin tubes (16 ml) was processed. PBMCs were isolated by density gradient centrifugation with Lymphoprep™ (Abbott), following the manufacturer's instructions. Cells were frozen in RPMI medium 1640 with L-Glutamine (Gibco, Thermo Fisher) supplemented with 10% foetal bovine serum, 10,000 U/ml penicillin, 10,000 µg/ml streptomycin and 10% DMSO and stored in liquid nitrogen until used. For flow cytometry and cell culture experiments PBMCs were thawed and immediately washed and resuspended in the fresh RPMI medium to remove DMSO.

EV isolation

Citrate tubes were immediately processed after blood collection. EVs were isolated as previously described by our group [202]. Briefly, tubes were centrifuged at 2,500 g for 15 min, and the obtained plasma was then centrifuged at 13,000 g for 2 min and this supernatant centrifuged again at 20,000 g for 20 min to pellet EVs. The pellet was resuspended with filtered DPBS (GIBCO, Thermo Fisher), filtered twice through a 0.22 µm-pore filter. Resuspended EVs were stored at -80 ºC.

Cryo-electron microscopy (cryoEM)

EVs were vitrified following standard protocols [336]. Glow-discharged Quantifoil holey carbon film grids (Orthogonal Array of 2µm Diameter Holes - 2µm Separation, mounted on a 300M Cu grid, #657-300-CU, Ted Pella) were vitrified in liquid ethane in Vitrobot after deposition of 3 µL of the sample. Cryo-transfer sample holders of the type GATAN Model 626 were used to keep the sample vitrified during electron microscopy analysis. The sample was observed in a JEM-2100F UHR (80-200kV, JEOL) field emission gun transmission electron microscope at different magnifications. Micrographs were recorded on a state of the art TVIPS F216 CMOS camera (2k x 2k).

Nanoparticle tracking analysis

The size distribution and concentration of isolated plasma EVs were measured using a ZetaView (Particle Metrix) instrument following manufacturer instructions. Samples were thawed on ice and diluted with filtered DPBS to get accurate acquisitions. Settings were fixed and maintained for all samples. Filtered DPBS was tested and no background signal was

detected. For each sample, two cycles of analysis at 11 positions were performed and results were analysed with ZetaView 8.04.02 software (Particle Metrix).

PBMC and EV culture

Thawed cells were cultured in 96-well flat-bottom plates in RPMI medium supplemented with 10% exosome-depleted FBS (Gibco, Thermo Fisher), 10,000 U/ml penicillin and 10,000 µg/ml streptomycin. 10^5 cells were plated in each well and immediately after, 100 µg of thawed EVs (measured by protein quantification with Bio-Rad Protein Assay) were added to the corresponding wells. Cells were cultured in 200 µl medium, at a final density of 10^6 cells per ml and incubated for 3 h at 37 °C and 5% CO_2. Then, activation of cells was induced by adding 10 µg/ml phytohemagglutinin (PHA) (Sigma-Aldrich) in corresponding wells. All cultured cells were incubated for 72 h at 37 °C and 5% CO_2. A schematic representation of the coculture protocol is presented in **Figure 43**. PHA was chosen to induce a polyclonal, nonspecific and significant lymphocyte activation, similar to the one produced against infection agents [337]. The 10 µg/ml concentration of PHA was established after titration. In a sample from a healthy adult 8 different concentrations of PHA (1.25-50 µg/ml) were tested and 10 µg/ml was chosen as the best stimulation (**Figure 44**).

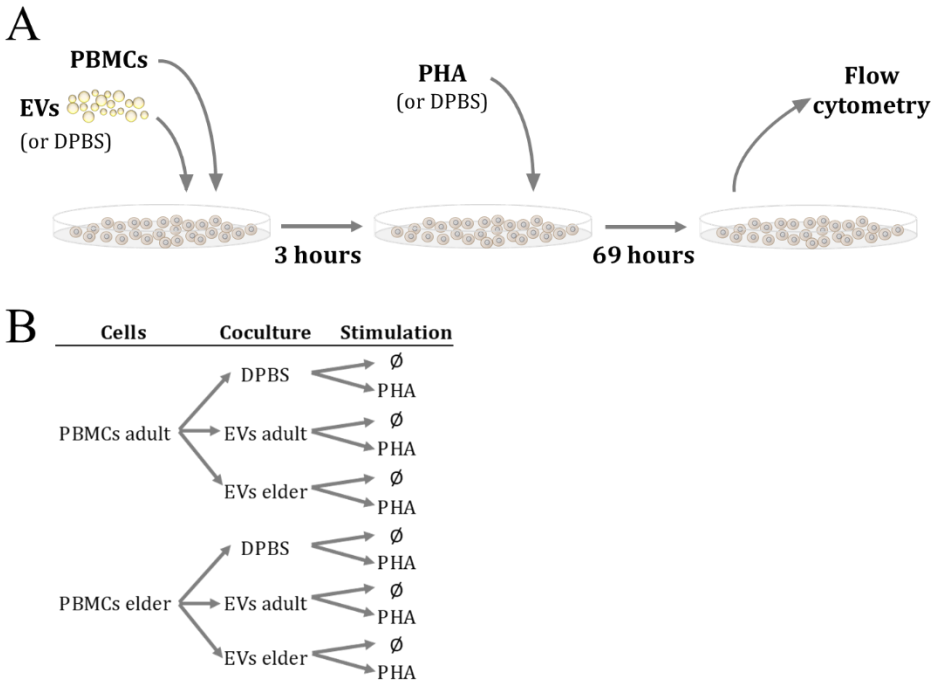


Figure 43. Cell culture protocol to test the influence of EVs on T cell activation. (**A**) 10^5 PBMCs were plated in 96-well dishes and then, 100 µg of EVs were added, while DPBS was added in control wells. 3 hours later, 10 µg/ml PHA were added to induce T cell activation in half of the wells, while the rest was maintained with no stimulation. 3 days after plating, T cell activation was evaluated by flow cytometry. (**B**) Schematic representation of the different study conditions.

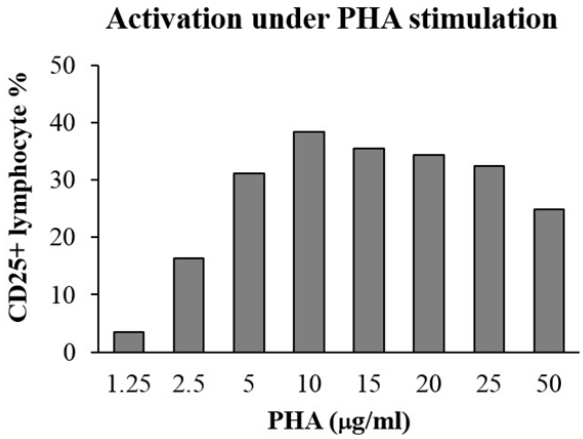


Figure 44. T cell activation under PHA stimulation, measured by flow cytometry. PHA was titrated with a healthy adult lymphocyte sample and the 10 μg/ml concentration was chosen as the best stimulation.

Flow cytometry

For the flow cytometric analysis of PBMCs, the following fluorochrome-conjugated anti-human monoclonal antibodies were used: anti-CD3 APC-Fire750, and anti-CD56 APC from Biolegend; Anti-CD8 FITC, anti-CD28 PE, anti-CD4 PE-Cy7 and anti-CD25 PE from BD Biosciences; for cell viability assessment 7-aminoactinomycin D (7-AAD) dye (Thermo Fisher). Different antibody panels were designed. To assess the T cell population percentages and the senescence state of T cells, the combination of anti-CD3 APC-Fire750, anti-CD56 APC, anti-CD8 FITC, anti-CD28 PE, anti-CD4 PE-Cy7 and 7-AAD was used. T cells were identified by CD3+ staining, NK cells by CD3-/CD56+ staining and B cells as double negative CD3-/CD56-. The same panel without 7-AAD was applied for the flow cytometry of plasma EVs. For cultured PBMC activation measurement anti-CD8 FITC, anti-CD4 PE-Cy7, anti-CD25 PE and 7-AAD were combined.

Directly thawed PBMCs and PBMCs from cell culture were stained following the same protocol. Cells were washed and resuspended in DPBS with 5 % bovine serum albumin (BSA) (Sigma-Aldrich) to block Fc receptor before staining. Corresponding antibodies were added and samples incubated for 20 min at room temperature in the dark. Then, cells were washed to remove unbound antibodies and acquired in a FACS Canto II flow cytometer (BD Biosciences) or in a Guava EasyCyte 8HT flow cytometer (Millipore, Merck). Single staining and fluorescence minus one (FMO) control tubes were used to adjust compensations and set the gating strategy. After gating for singlets, lymphocytes were gated based on FSC and SSC and 20,000 lymphocytes were acquired for each sample. Then, lymphocyte populations were distinguished based on fluorescence and analysis of obtained results was performed with FACS Diva 8.0.1 (BD Biosciences) and InCyte 3.1 (Millipore, Merck) software respectively. The gating strategy for senescent T cells and representative dot plots are presented in **Figure 45**.

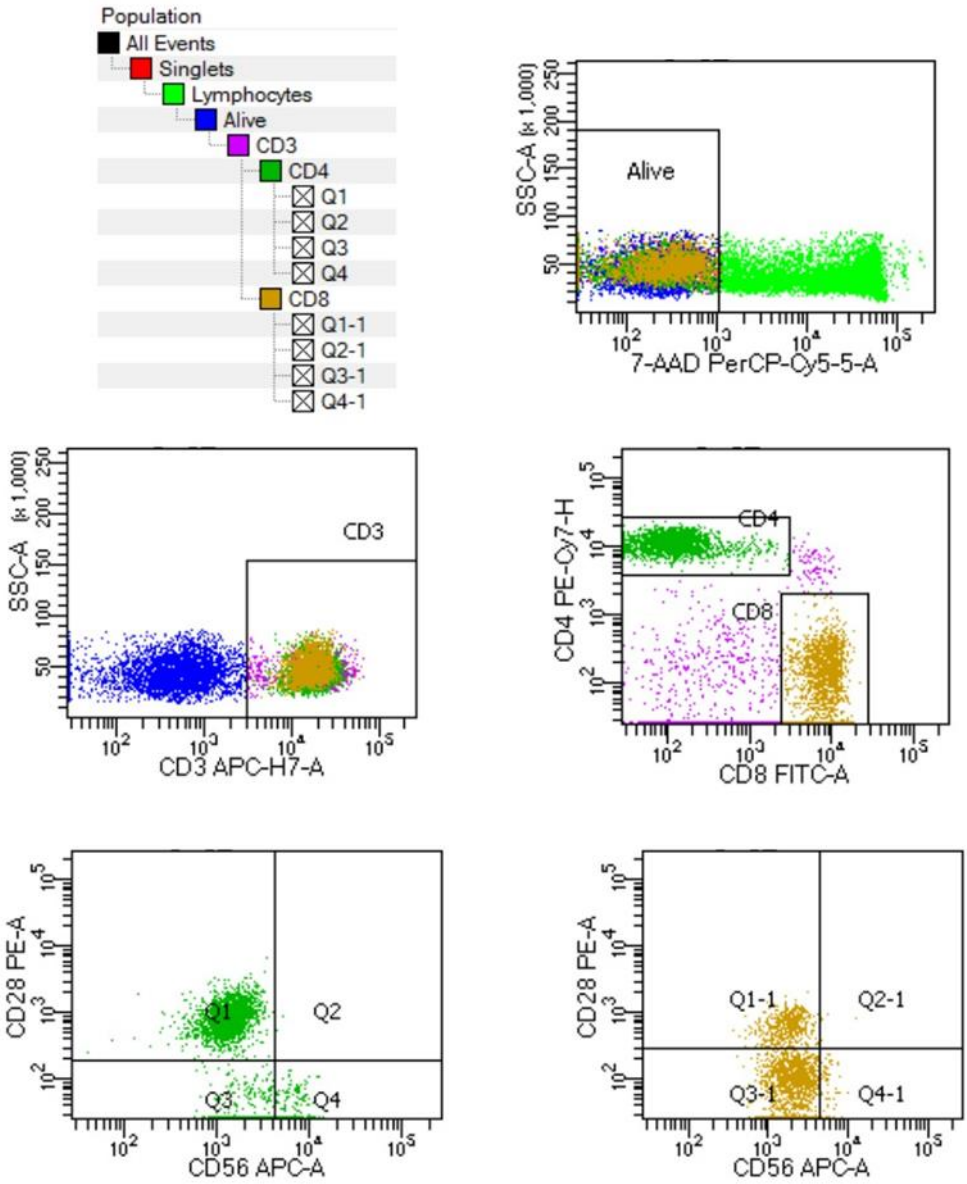


Figure 45. Gating strategy for senescent T cells. After gating the singlets (FSC-H vs FSC-A), and lymphocytes (SSC-A vs FSC-A), events were separated as shown above: negative for 7-AAD, positive for CD3, positive for CD4 or CD8. Finally, senescent CD4+ or CD8+ events were measured by CD28 and CD56 expression.

For the flow cytometry analysis of EVs, samples were thawed on ice, the same staining procedure was applied (starting from 50ul of resuspended EVs) and filtered DPBS was used as staining buffer. An antibody combination of the above-mentioned anti-CD3 APC-Fire750, anti-CD56 APC, anti-CD8 FITC, anti-CD28 PE, anti-CD4 PE-Cy7 was applied to identify senescence markers. For the detection of characteristic EV markers a panel of anti-CD63 FITC, anti-CD81 APC and anti-CD9 PE antibodies (Biolegend) was used. Single staining and FMO control tubes were used to adjust compensations and set the gating strategy. A tube with a combination of DPBS and antibodies (without EVs) was included to discard false positives due to antibody aggregates (**Figure 46**).

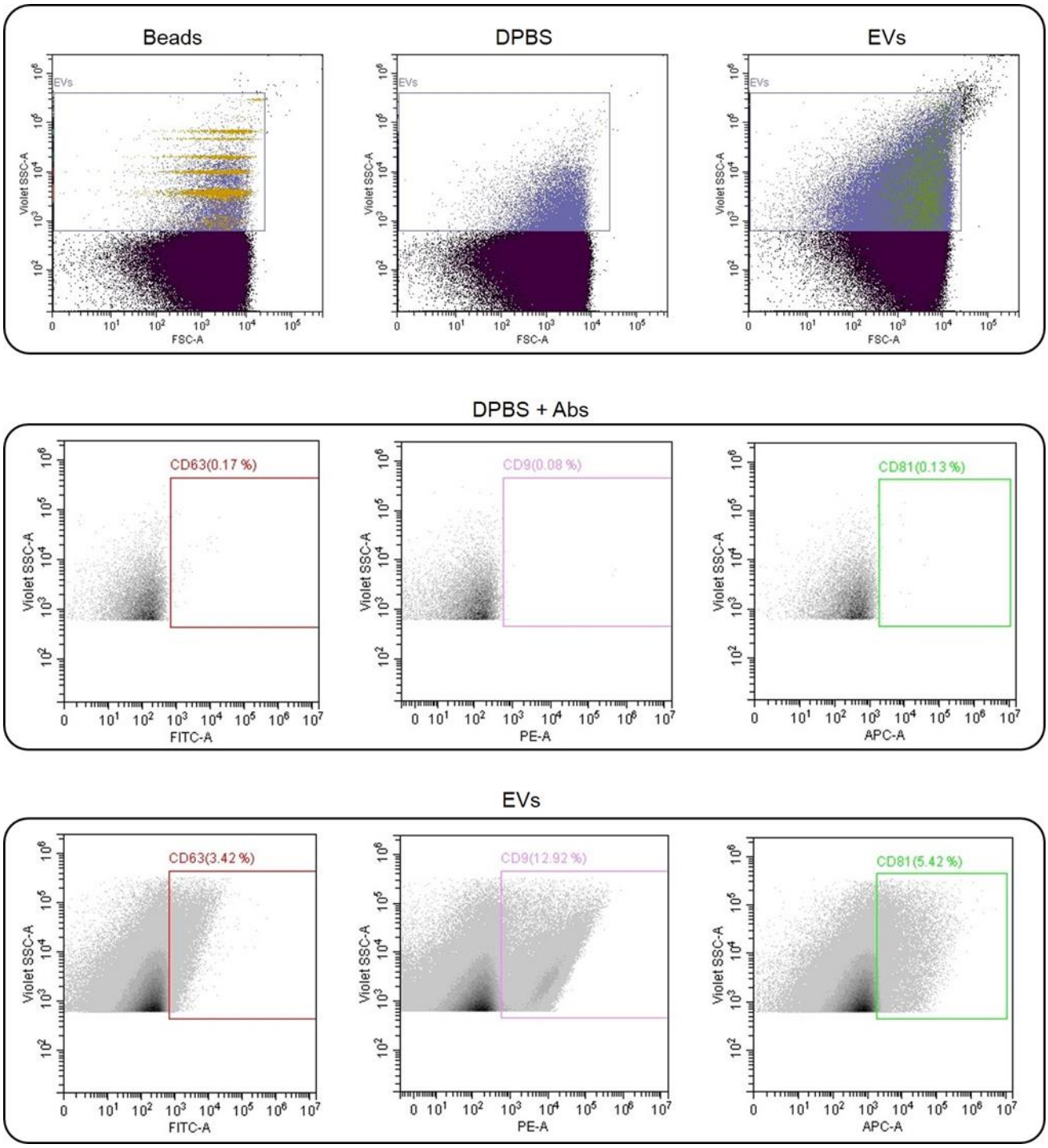


Figure 46. Gating strategy for EVs. The upper panel shows representative dot plots of the Megamix-Plus beads, DPBS and a plasma EV sample. The EV gate was established based on the beads. The middle panel shows the acquisition of a sample with DPBS and antibodies for EV markers, demonstrating that there is no positive signal due to DPBS particles or antibody aggregates. The lower panel shows the positive events for CD63, CD9 and CD81 markers of plasma EVs.

The acquisition was performed in a CytoFLEX flow cytometer (Beckman Coulter) and 500,000 EVs were acquired for each sample. The EV gate was established with a mixture of FITC fluorescent Megamix-Plus SSC and Megamix-Plus FSC beads (100-900 nm) (BioCytex) and the side scatter detector of the violet laser. The analysis was performed with CytExpert 2.1 software (Beckman Coulter). EV samples from nonagenarians and centenarians were obtained several months later, and due to technical reasons, these samples were measured with another CytoFLEX flow cytometer. The staining protocol and parameters were maintained, and as a control for possible technical bias, some samples from adults acquired in the first batch were analysed again. As the fluorescence intensities obtained for these reacquired samples were slightly different with the second CytoFLEX instrument, the samples from the second batch were analysed separately and differences between groups were only assessed among the samples of each batch. Representative dot plots of EV gating and EV markers are presented in **Figure 46**. The gating strategy for EVs with senescent features and representative dot plots are shown in **Figure 47**.

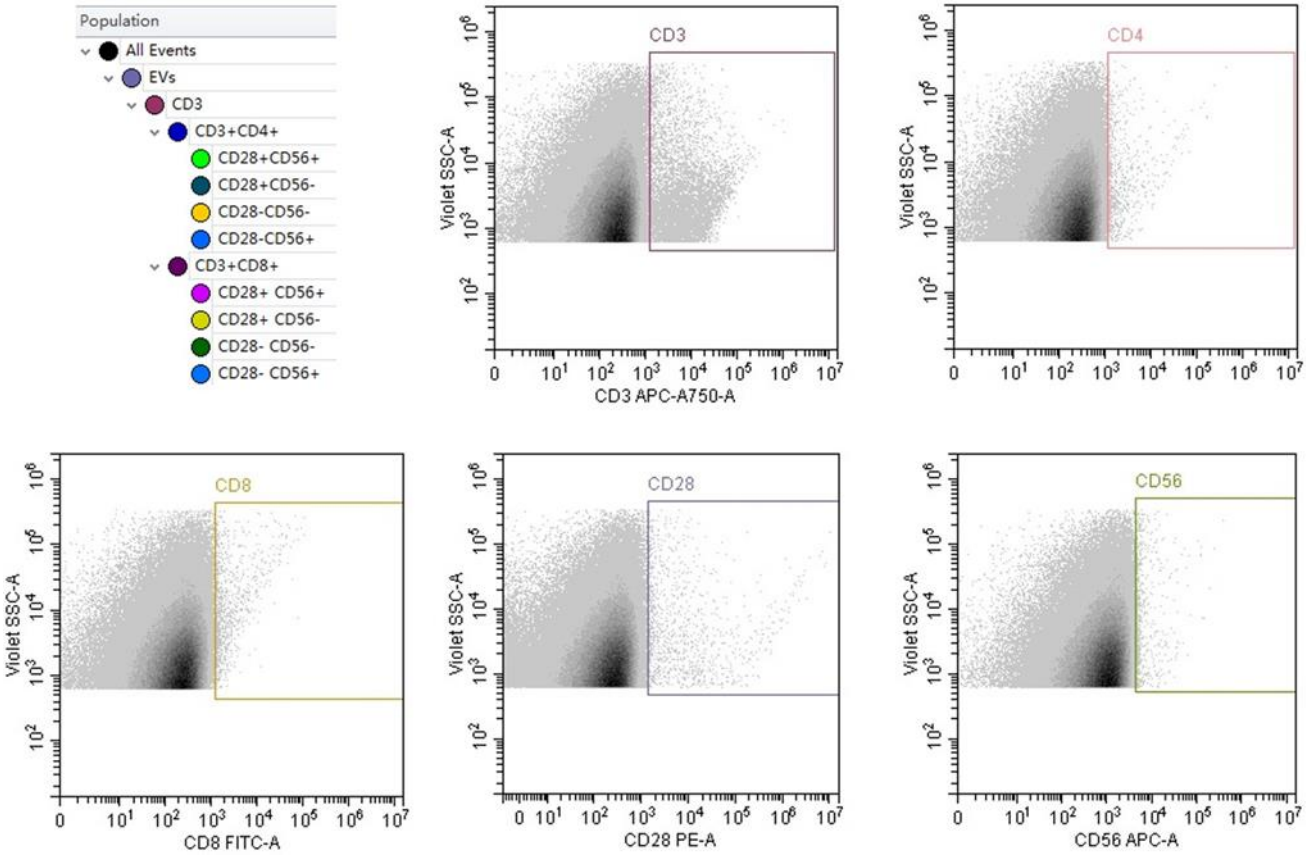


Figure 47. Gating strategy for senescent EVs. After gating the EVs (Violet SSC-A vs FSC-A), events were separated as shown above: positive for CD3, and then positive for CD4 or CD8. Finally, senescent CD4+ or CD8+ events were measured by CD28 and CD56 expression.

Interleukin production measurement

Cell culture supernatants were analysed to measure IL production by PBMCs. The whole content of each well was collected in a microcentrifuge tube, centrifuged at 400 g for 5 min and the supernatant recovered and stored in a new tube.

The concentration of IL-1β, IL-2, IL-6, IL-10 and TNF-α were measured with Milliplex MAP Human Cytokine/Chemokine Multiplex Immunoassay (Millipore, Merck) and Human Magnetic Luminex Assay LXSAHM (R&D systems) following manufacturer's instructions. RPMI culture medium was applied as background. A MAGPIX device with xPONENT software (Luminex Corporation) was used for fluorescence measurement and median fluorescent intensity data were analysed using the 5-parameter logistic method for calculating cytokine concentrations.

Statistical Analysis

Statistical analysis was performed with R version 3.2.2 (R Core Team (2015) [309]) and GraphPad Prism version 6.01 for Windows (GraphPad Software, www.graphpad.com). For assessing lymphocyte population proportions, CD8+CD28-CD56+ cells and T cell activation after coculture with EVs in the whole cohort the Jonckheere-Terpstra test was applied. To probe the increase and subsequent decrease of senescent CD4 cells with age the quadratic effect of the series was tested. Non-parametric Kruskal–Wallis one-way analysis of variance and Wilcoxon rank-sum tests were conducted to evaluate differences between groups. Statistical significance was defined as follows: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Results

Aging results in altered lymphocyte proportions and T cell senescence

First, we tested whether lymphocyte populations were altered by age. To this end, PBMCs from donors were analysed by flow cytometry. Results showed a significant increasing trend in T cell (p<0.001) and a decreasing trend in B cell (p<0.001) and NK cell (p=0.0017) proportions with increasing age (**Figure 48A**).

Next, the senescence of T cells was assessed. The gating strategy was set to identify senescent cells based on their loss of CD28 and gain of CD56 expression. Both CD4 and CD8 T cells were analysed. The results demonstrate a marked increase in the abundance of CD28- cells with age. Moreover, a proportion of CD28- T cells also gained CD56 expression (**Figure 48B-E**). This accumulation of senescent cells affects more severely CD8 than CD4 lymphocytes, reaching nearly 80% of CD28- cells (**Figure 48D**). Interestingly, an increase and subsequent decrease in nonagenarians and centenarians was found for senescent CD4 cells (**Figure 48B**), while the accumulation of senescent cells was gradually occurring with age in CD8 cells (**Figure 48D**).

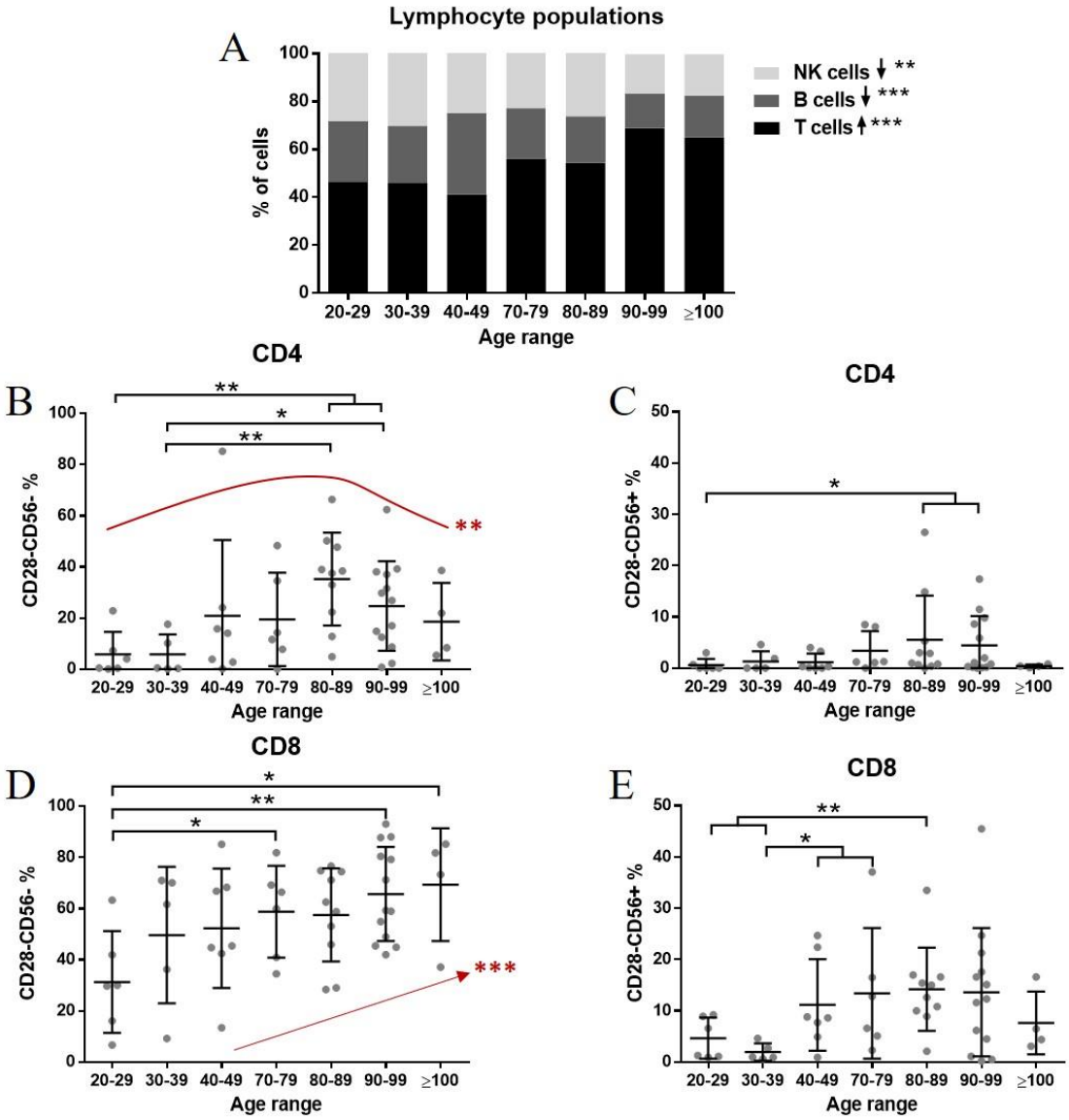


Figure 48. The effect of age on lymphocytes. Lymphocyte subpopulations and T cell senescence were assessed by flow cytometry (n=51 donors). (**A**) The proportion of T cells (CD3+) gradually increases and NK cells (CD3-/CD56+) and B cells (CD3-/CD56-) have a decreasing trend with age (Jonckheere test). (**B**) Among CD4 cells, CD28- cells accumulate in old individuals, while there is a partial reduction in the very old (quadratic effect **). (**C**) A small proportion of CD28- cells express CD56, but some differences are found with age. (**D**) CD28 loss is more pronounced in CD8 cells and a gradual accumulation with age was found (Jonckheere test ***). (**E**) The gain of CD56 is also more pronounced in CD8 cells and increased proportions were found with age. Age range in years.

Circulating extracellular vesicles do not reflect the senescent T cell expression pattern

Isolated plasma EVs were characterized by cryoEM, NTA and flow cytometry. The typical rounded shape was observed (**Figure 49A**) and most EVs were in the size range of 100-200 nm (**Figure 49B**). Moreover, we showed by flow cytometry that a proportion of isolated particles bear EV surface markers CD9, CD63 and CD81 tetraspanins (**Figure 49C**). The

presence of T cell characteristic membrane markers on plasma EVs was assessed by flow cytometry and the same gating strategy applied for cells was followed to identify EVs with senescent features. Among CD3+ EVs, CD4+ and CD8+ were distinguished and then, the presence of CD28 and CD56 was evaluated. Our results show that EVs from the bloodstream carry T cell markers, but contrary to PBMCs, EVs with senescent markers do not accumulate with age (**Figure 49D-G**). Still, a significant decrease of senescent CD4+ EVs was found between nonagenarians and centenarians (**Figure 49D right panel**). Besides, for all age ranges, a higher percentage of senescent markers was observed among CD8+ EVs when compared to CD4+ EVs (**Figure 49D and F**).

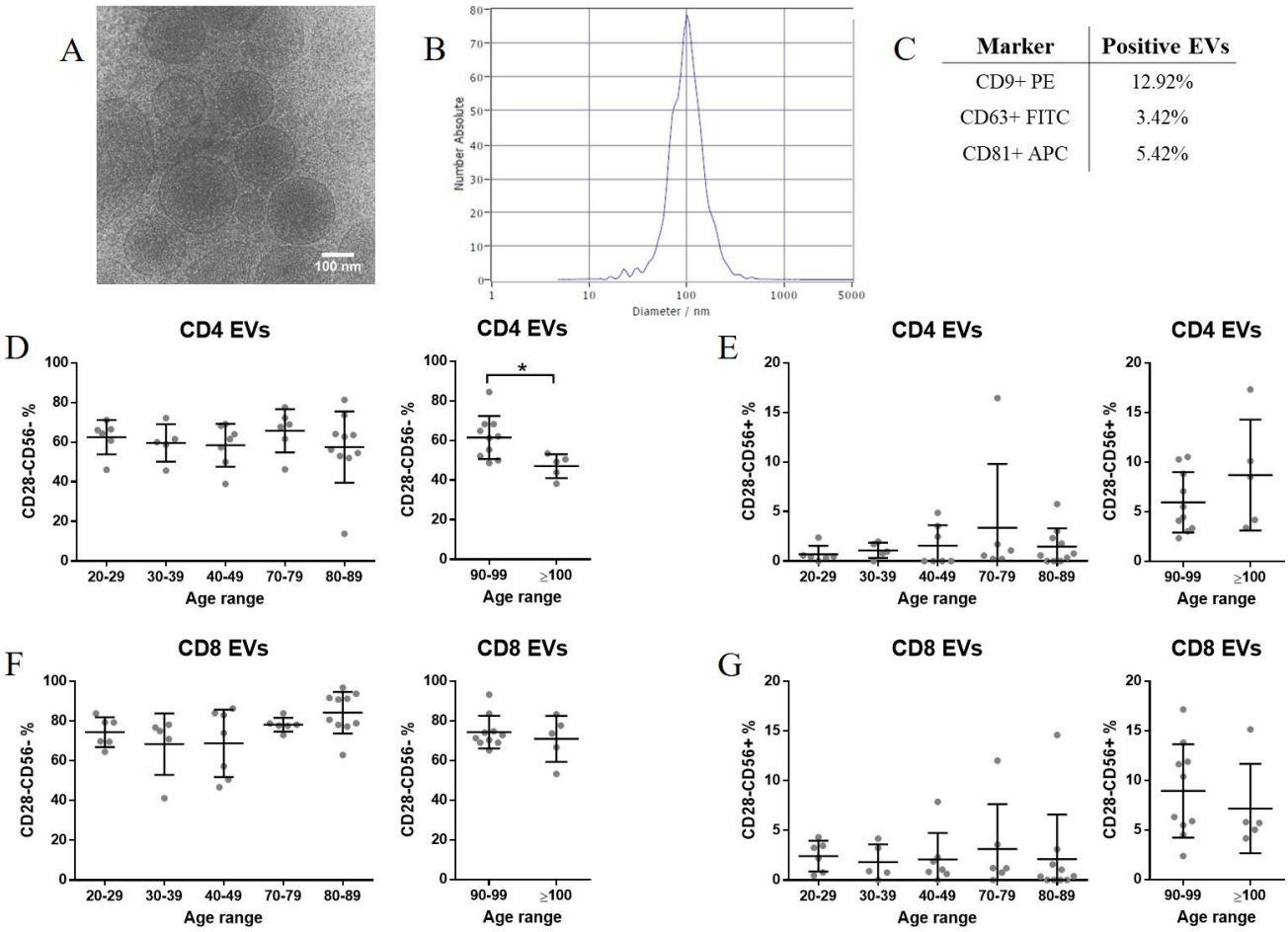


Figure 49. Plasma extracellular vesicle characterization. T cell and senescence markers are present on plasma EVs, but no differences were found between EVs from different age ranges (n=49 donors). (**A**) Representative cryoEM image of isolated EVs. (**B**) Representative figure of particle size distribution of EVs obtained by NTA. (**C**) Percentage of particles positive for EV characteristic markers assessed by flow cytometry. (**D-E**) Senescence markers on CD3+CD4+ and (**F-G**) on CD3+CD8+ EVs assessed by flow cytometry. Age range in years.

The coculture of PBMCs and EVs improves viability and influences cytokine secretion

Next, coculture experiments of PBMCs and plasma EVs were performed. Cell and EV samples of all ages were tested. Four different conditions were assayed: PBMCs alone, PBMCs + EVs, PBMCs + PHA and PBMCs + EVs + PHA. The PHA was applied to stimulate T cell activation, and to test the effect of EVs both under non-stimulated and stimulated conditions. To test whether the addition of EVs affects cell viability, we analysed cells by flow cytometry and compared the 7-AAD negative events between groups after three days in culture. The different conditions of each PBMC donor were normalized to the control wells where only cells were plated. Interestingly, we observed that cell viability improves when EVs are present (**Figure 50A**). Moreover, PHA stimulation significantly reduces viability and this effect is partially rescued when EVs are added (**Figure 50A**). In a further analysis of these results, we compared the effect of plasma EVs on cells of adult (20-49 years) and aged (>80 years) donors. The positive effect of EVs is stronger in PBMCs from adults for all conditions tested (**Figure 50B**).

In order to check whether the coculture with EVs could also affect cytokine production *in vitro*, we performed a luminex assay for TNF-α, IL-6, IL-10, IL-1β and IL-2. Cell conditioned media from all conditions of 2 different individuals (one adult and one aged cell donor, cocultured with EVs from adults and elders) were tested. Importantly, cytokine concentrations were non-detectable in the two conditions where PHA was not added, demonstrating that the only addition of EVs does not induce cytokine production. When compared to PHA stimulation alone, we observed that EV addition influences cytokine production. The secretion of the proinflammatory TNF-α, IL-6 and IL-1β cytokines was reduced, while anti-inflammatory IL-10 was increased and IL-2 not significantly affected (**Figure 50C**).

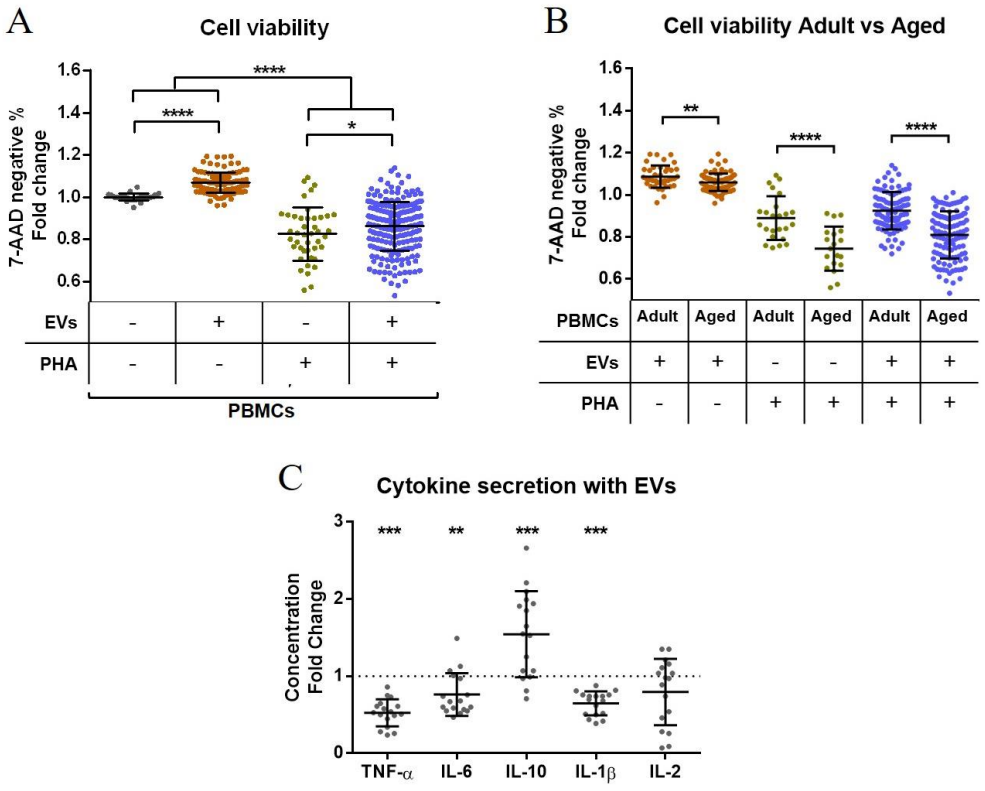


Figure 50. (Previous page) Effect of extracellular vesicles from plasma on PBMC viability and cytokine secretion *in vitro*. PBMCs from donors of all age ranges were cultured for 72h in the presence or not of PHA or/and plasma EVs and then analysed by flow cytometry. (**A**) Cell viability is reduced after stimulation with PHA, while the coculture with EVs improves viability. (**B**) The positive effect of plasma EVs on cell viability is stronger in cells from adults (20-49 years) than aged (80-101 years) individuals. (**C**) The analysis of conditioned media by luminex showed a reduced secretion of proinflammatory cytokines TNF-α, IL-6 and IL-1β and an increased secretion of anti-inflammatory IL-10 by stimulated cells cocultured with EVs compared to stimulated cells without EVs.

T cell activation under PHA stimulation is affected by the coculture of plasma EVs and depends on the age of the EV donor

Finally, the effect of plasma EVs on lymphocyte activation was assessed. Polyclonal activation of T cells was induced with PHA and measured by CD25 expression by flow cytometry. The coculture of lymphocytes with plasma EVs for 72h did not induce T cell activation (**Figure 51C-D**) demonstrating that plasma EVs alone are not immunogenic for non-stimulated cells. PBMC samples of 22 individuals (12 adults and 10 elders) were tested, and each cell donor was assayed with different EV donors (up to 12 different EVs for one PBMC donor, each one in different wells and always in duplicate). The percentage of T cells activated under the same PHA stimulation (and without EVs) was highly heterogeneous for each PBMC donor (33-92 % of CD25+, **Figure 51A-B**). For normalization, control wells with PBMCs + PHA without EVs were used and fold change was calculated.

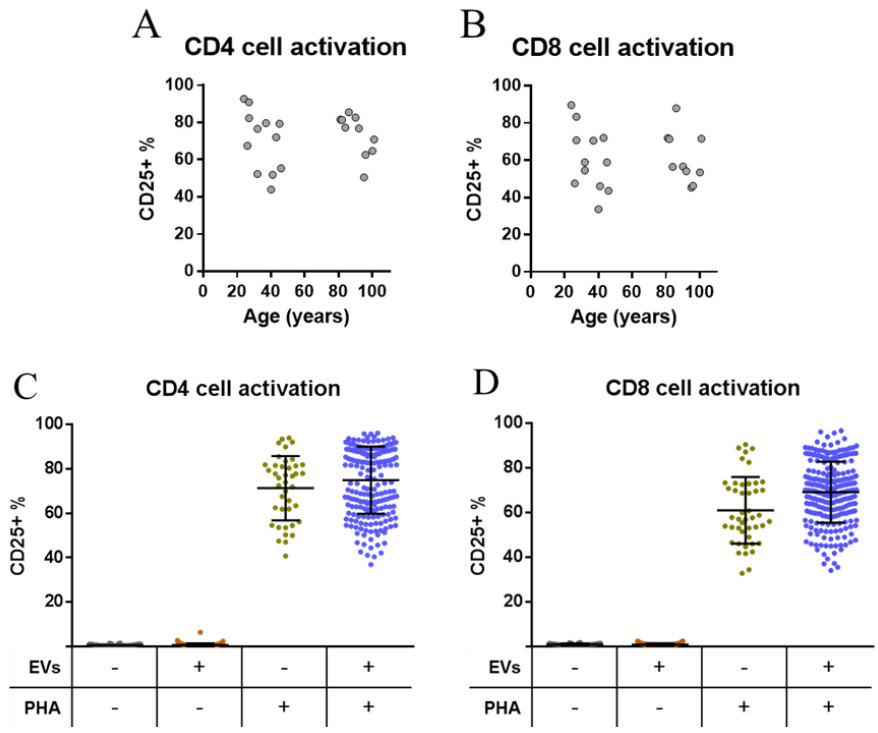


Figure 51. T cell activation measured by flow cytometry. (**A-B**) PBMCs were stimulated with 10 µg/ml PHA and 72h later CD25+ cells measured. The percentage of activated CD4 and CD8 cells is heterogeneous and not correlated to age. (**C-D**) PBMCs were cocultured with plasma EVs. The coculture of EVs alone do not induce T cell activation.

Our results show that EVs modulate T cell activation, but the effect is very heterogeneous and is influenced by the age of the EV donor (**Figure 52A-D**). Taking this into consideration, we performed a separated analysis for plasma EVs from each age range. EVs from adults significantly increase CD4 cell activation, while the ones from nonagenarians and centenarians reduce the activation (**Figure 53A**). In the case of CD8 cells, EVs from adults also enhance cell activation (**Figure 53B**). Importantly, when the tendency of the whole cohort was analysed, we saw that the activation enhancement capacity of EVs significantly decreases with age (**Figure 53**).

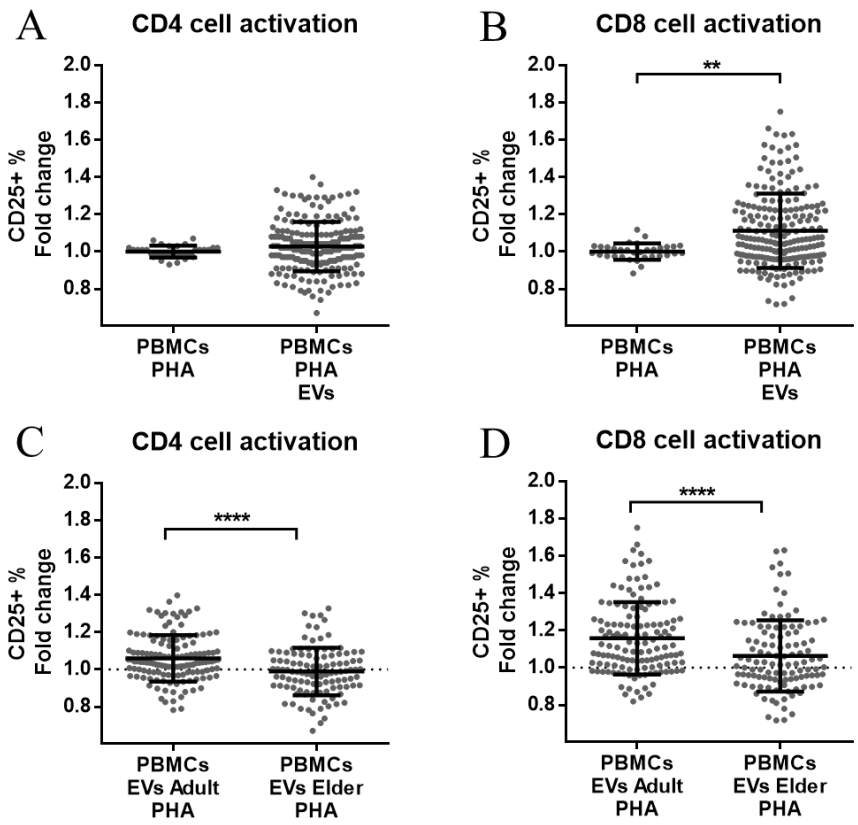


Figure 52. Analysis of activated lymphocytes under PHA stimulation and the influence of the EV donor age. (**A-B**) The coculture of PBMCs with EVs under PHA stimulation affects cell activation in a heterogeneous manner. For each cell donor, wells without EVs were taken as reference for fold change calculation. (**C-D**) Both CD4 and CD8 cells get more activated in the presence of EVs from adult donors when compared to EVs from elder donors. Adults 20-49 and elders 70-104 years.

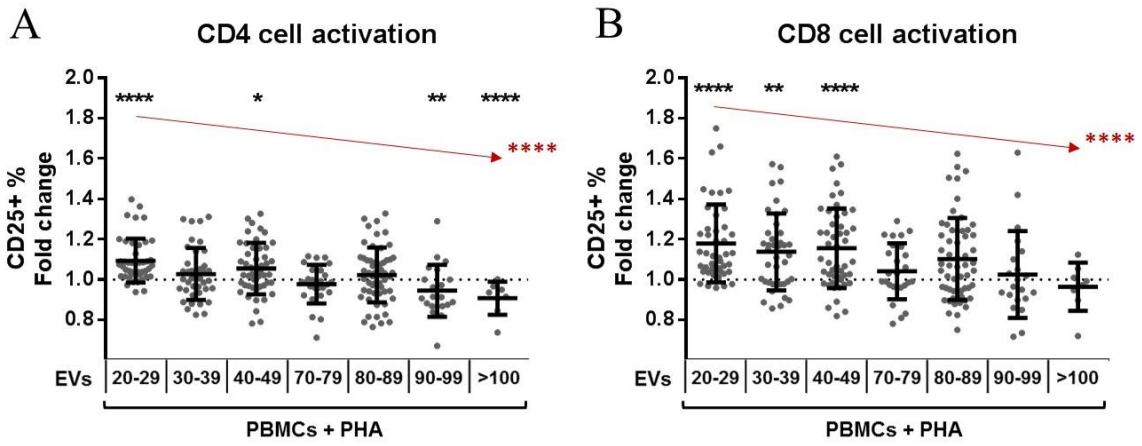


Figure 53. T cell activation under PHA stimulation and the effect of plasma extracellular vesicles. PBMCs from donors of all age ranges were cultured for 72h in the presence of PHA and plasma EVs and then analysed by flow cytometry. Wells without EVs were taken as reference for fold change calculation and Wilcoxon tests. (**A**) The presence of EVs from adult donors resulted in the promotion of CD4 cell activation, an effect that decreases gradually with EV donor age (in red, Jonckheere test ****). (**B**) In a similar way, CD8 cells cocultured with EVs from adults get more activated, but this effect decreases with EV age (in red, Jonckheere test ****). Age range in years.

Discussion

The present study analysed peripheral blood samples from adults and elders of different age ranges, 20-49 and 70-104 years. First, the lymphocyte subsets were compared and an increased T cell and decreased B cell and NK cell proportions were found with age. Several works have studied the lymphocyte subsets with aging, both at the total number and percentage level. Distinct aging patterns have been found between countries and populations, pointing out the complexity and heterogeneity of the immune system and immunosenescence [334,338–342]. To our knowledge, our work is the first investigating lymphocyte populations with age in the Basque Country (in the north of Spain).

On the other hand, some aging-related features have been widely reported, such as the loss of the costimulatory molecule CD28 from T cell membrane and a subsequent gain of NK characteristic markers [100,102,107,343]. This process has been shown to affect both CD8 and CD4 T cells, but earlier and to a greater extent to CD8 cells [104,344,345]. Our results are in accordance with previous reports. Nevertheless, previous studies reported a gradual accumulation of CD4 CD28- cells with age [98,103,346] and here we showed a higher senescent CD4 cell percentage in the 80-89 age range, and interestingly a lower percentage in nonagenarians and centenarians, following a quadratic effect. We hypothesize that rather than CD28 expression recovery, individuals reaching >90 years could be the ones that presented lower senescent cell proportions also earlier in life. However, a longitudinal study with a larger

sample size would be needed to confirm this progression. As mentioned above, other works previously described the loss of CD28 expression in CD4 cells in elders, but to our knowledge, our study is the first one analysing this effect in nonagenarians and centenarians and demonstrates that they follow a distinct aging progression in some aspects.

To further characterize immunosenescence, we focussed on plasma EVs. They are known to carry many molecules in their membrane and among them, EVs can also bear markers of the secreting cell [347]. To test whether plasma EVs resemble the senescence status of T cells, we measured T cell membrane markers on EVs. Our results showed that plasma EVs carry T cell specific molecules, while there is not an increased proportion of "senescent-like EVs" with age. Even if no significant differences were found between age ranges, a higher percentage of CD28- EVs was observed among CD8 EVs when compared to CD4 EVs, which could be linked to the increased CD28- CD8 T cells. Importantly, we also identified the characteristic tetraspanins CD9, CD63 and CD81 of EVs by flow cytometry. A small percentage of circulating EVs in plasma carry these molecules, but it should be noted that observed numbers could be underestimated by other co-isolated particles and that EVs expressing a single or few copies of the surface antigen of interest cannot be detected, as described in previous studies [218,348]. Moreover, it should be mentioned that the detection of EV proteins by flow cytometry is a direct measure that identifies proteins at their physiologic state – at the EV membrane in this case –, in contrast to techniques such as western blotting or proteomics approaches where vesicles are lysed, and the numbers of positive particles for each protein cannot be measured.

Regarding coculture experiments of PBMCs and plasma EVs, we showed that EVs from a different donor are not immunogenic for receptor lymphocytes, and in contrast, they affect cell viability and cytokine secretion. Specifically, plasma EVs enhance cell viability and partially rescue the deleterious effect of PHA, the well-known activation-induced cell death [349]. This positive effect is stronger on cells from adults, when compared to elders. Our results indicate for the first time that the presence of plasma EVs in culture can partially rescue the activation-induced cell death and moreover, that EVs enhance T cell viability when compared to the culture of cells alone. Further, plasma EVs reduce the secretion of TNF-α, IL-6 and IL-1β proinflammatory cytokines and increase anti-inflammatory IL-10 in PHA stimulated cells, but EVs alone do not alter cytokine secretion of PBMCs. It has been widely described that PHA stimulates cytokine production [350], but the effect of EVs is still not understood. A previous study reported a similar effect of mesenchymal cell-derived EVs on IL-10 production [351], and some authors have also studied the effect of EVs on lymphocytes and ILs [333,352]. However, they worked with EVs from other tissues or produced in culture, which can lead to distinct outputs.

And even if plasma EVs are not immunogenic, they influence T cell activation under PHA stimulation, and this effect is different depending on the age of the EV donor. EVs from adults promote T cell activation and this effect decreases with age. These results highlight the influence of circulating EVs on T cells and interestingly, also demonstrate the distinct effects of plasma EV and T cell interactions depending on age. The coculture experiments enable us to more closely resemble the interaction between circulating cells and EVs. Much work is still needed to elucidate the complex pool of particles present in plasma and the triggers of observed effects, but the present work gives a first description of the role that EVs from plasma have on T cells during aging.

In short, our work describes the reduced CD28 loss of CD4 cells in nonagenarians and centenarians, the presence but no accumulation of senescent markers on plasma EVs and the distinct interactions between T cells and plasma EVs with age.

CHAPTER FOUR

Multiple sclerosis and premature aging

Introduction

Aging is a universal process. It affects both healthy individuals as well as the ones that present other syndromes and/or diseases. Importantly, aging and the concomitant health problems interact and influence each other. These interactions are observed in diseases that develop in elder people, such as cancer, but the influence of aging also reaches chronic diseases as patients age, like in the case of MS [275].

MS is a chronic autoimmune disease of the CNS characterised by pathologic demyelination of axons and subsequent neurodegeneration. It is a heterogeneous disease and, clinically, it can follow relapsing-remitting or progressive forms [353]. Most of MS patients experience the first symptoms at their 20s or 30s, but there are also paediatric or juvenile [271] and late-onset MS cases [272]. In the last decades, effective disease-modifying treatments that slow the progression of MS have been developed [267], and thanks to them, patients present increasing age at disability milestones [273]. Consequently, the mean age of MS patients is increasing, with already more than 20% of them aged 60 years or over (msbase.org [274]). Therefore, an elevated number of patients suffer from the aforementioned interactions between MS and aging processes.

It should be noted, that the characteristic features of MS are very similar to the ones observed during aging, as inflammation, and immune alterations. Moreover, the consequences such as mobility and cognitive problems are also found in both processes, which makes it very complicated to separate the effects of MS and aging [275].

In addition, some authors have suggested that several autoimmune diseases, including MS, show premature aging, specially with regards to the immune system. They observed reduced numbers of naïve CD4 T cells in pediatric MS [354] and increased levels of CD4+CD28- T cells in adult MS patients [280,281]. Moreover, the characteristic loss of the costimulatory molecule CD28 from CD4 T cells due to the repeated stimulation and activation is proposed as a sign of senescence and terminal differentiation, but it has been shown that in MS patients these cells remain functional and show increased cytotoxicity [355]. Furthermore, CD4+CD28- T cells have also been found in MS lesions in the CNS, suggesting that they could be implicated in MS pathogenesis [356]. Regarding inflammation, elevated levels of TNF-α and IL-6 among other inflammatory markers have been found in the cerebrospinal fluid and serum of MS patients during remission, indicating that some signs of chronic inflammation are present [285,286].

The aim of this work was to perform a pilot study to evaluate whether differences are found in age-related features due to the presence of MS disease. We tested the above-mentioned inflammaging (by measuring TNF-α, IL-6 and CRP concentrations) and T cell senescence (by

quantifying CD28 and CD56 in T cells) in MS patients and age-matched healthy controls. Besides, and considering the results obtained in the previous chapter, we also performed coculture experiments of PBMCs and plasma EVs from MS patients and controls, to test the influence of EVs on T cell activation.

Materials and methods

Participants and blood sampling

For this study, samples from MS patients and age- and sex-matched healthy controls (HCs) were used. MS patients aged 23-49 years and HCs aged 24-50 years were included. Aiming to have a representative sample of the heterogeneity observed among MS, patients with diverse EDSS scores, treatments and disease evolution times were included. All MS patients were in remission at the time sample obtention.

The study was approved by the Donostia University Hospital's ethics committee and all participants provided written informed consent before blood sampling. Peripheral blood was collected by experienced nurses by venipuncture with a 21-gage needle in 8 ml serum separator tubes, 2.8 ml citrate tubes and 4ml heparin tubes (Vacutainer, BD Biosciences).

Obtention of serum and EV isolation from plasma

Serum separator tubes were allowed to clot for 30 min and centrifuged at 1258 g for 20 min to recover serum from the supernatant. Serum was aliquoted and stored at -80°C.

Citrate tubes were processed immediately after blood collection. EVs were isolated as previously described by our group [202]. Briefly, tubes were centrifuged at 2,500 g for 15 min, and the obtained plasma was then centrifuged at 13,000 g for 2 min and this supernatant centrifuged again at 20,000 g for 20 min to pellet EVs. The pellet was resuspended with filtered DPBS (GIBCO, Thermo Fisher), filtered twice through a 0.22 μm-pore filter. Resuspended EVs were stored at -80 °C.

PBMC isolation and storage

Within 1 hour of sampling, peripheral blood collected in heparin tubes (16 ml) was processed. PBMCs were isolated by density gradient centrifugation with Lymphoprep™ (Abbott), following the manufacturer's instructions. Cells were frozen in RPMI medium 1640 with L-Glutamine (Gibco, Thermo Fisher) supplemented with 10% fetal bovine serum, 10,000 U/ml penicillin, 10,000 μg/ml streptomycin and 10% DMSO and stored in liquid nitrogen until used.

CRP, TNF-α and IL-6 ELISAs

The concentration in serum of CRP, TNF-α and IL-6 was measured with Quantikine and Quantikine High Sensitivity ELISA kits (R&D), following manufacturer's instructions. 38 serum

samples were studied, 19 MS patients (mean age 37.5 years) and 19 HCs (mean age 38.5 years) and the three analytes were measured for all samples. **Table 6** shows the main characteristics of MS patients. Serum samples were assayed undiluted for TNF-α and IL-6 and diluted 1:100 for CRP ELISA.

Table 6. Characteristics of the 19 samples from MS patients used for the analysis of serum.

Sex	Age (years)	Years since MS diagnosis	EDSS	Treatment
Female	23	6	0	Natalizumab
Female	29	5	2.5	Natalizumab
Female	30	10	3.5	Natalizumab
Female	33	15	5.5	Fingolimod
Male	33	7	4	Natalizumab
Female	34	19	1	IFN β-1a
Male	35	11	2.5	Glatiramer acetate
Female	35	9	2.5	Fingolimod
Female	38	8	4.5	Fingolimod
Female	39	15	0	Fingolimod
Female	40	9	1	Natalizumab
Female	41	14	4.5	Fingolimod
Female	42	21	2.5	Glatiramer acetate
Female	42	5	4.5	Natalizumab
Female	42	13	3	Fingolimod
Female	43	7	1	IFN β-1b
Male	43	16	4	Natalizumab
Male	44	16	4.5	IFN β-1a
Female	47	27	3	IFN β-1b

Characterization of senescent T cells by flow cytometry

For the flow cytometric analysis of senescent T cells, the following fluorochrome-conjugated anti-human monoclonal antibodies were used: anti-CD3 APC-Fire750 and anti-CD56 APC from Biolegend; anti-CD8 FITC, anti-CD28 PE, anti-CD4 PE-Cy7 and from BD Biosciences; for cell viability assessment 7-AAD dye (Thermo Fisher).

PBMCs were thawed, washed and resuspended in DPBS with 5 % BSA (Sigma-Aldrich) to block Fc receptor before staining. Corresponding antibodies were added and samples incubated for 20 min at room temperature in the dark. Then, cells were washed to remove unbound antibodies and acquired in a FACS Canto II flow cytometer (BD Biosciences). Single staining and FMO control tubes were used to adjust compensations and set the gating strategy. After gating for singlets, lymphocytes were gated based on FSC and SSC and 20,000 lymphocytes were acquired for each sample. Then, lymphocyte populations were distinguished based on fluorescence and analysis of obtained results was performed with the FACS Diva 8.0.1 software (BD Biosciences). 13 samples from MS patients (mean age 42.4 years and characteristics in

Table 7) were measured. The obtained results were compared to the age-matched HCs measured in the previous chapter (12 samples, mean age 39 years).

Table 7. Characteristics of MS patients' samples used for the analysis of senescent T cells.

Sex	Age (years)	Years since MS diagnosis	EDSS	Treatment
Female	30	4	3.5	IFN β-1a
Female	33	10	0	Glantimer acetate
Male	35	11	4.5	Glantimer acetate
Female	36	7	4.5	IFN β-1a
Female	36	17	3.5	Glantimer acetate
Female	40	9	1	IFN β-1a
Female	40	12	2	IFN β-1b
Female	41	21	2.5	Glatiramer acetate
Female	43	7	1	IFN β-1b
Male	45	18	4	Natalizumab
Female	46	27	3	IFN β-1b
Female	47	18	3	Fingolimod
Female	49	20	4	Glatiramer acetate

Coculture of PBMCs and EVs, activation and flow cytometry analysis

PBMCs were thawed, washed and cultured in 96-well flat-bottom plates in RPMI medium supplemented with 10% exosome-depleted FBS (Gibco, Thermo Fisher), 10,000 U/ml penicillin and 10,000 µg/ml streptomycin. 10^5 cells were plated in each well and immediately after, 100 µg of thawed EVs (measured by protein quantification with Bio-Rad Protein Assay) were added to the corresponding wells. Cells were cultured in 200 µl medium, at a final density of 10^6 cells per ml and incubated for 3 h at 37 °C and 5% CO_2. Then, activation of cells was induced by adding 10 µg/ml PHA (Sigma-Aldrich) in corresponding wells. All cultured cells were incubated for 72 h at 37 °C and 5% CO_2. Then, cells were taken from culture, transferred to 1.5 ml tubes and stained for flow cytometry.

To investigate the effect of EVs on T cell activation and whether the samples from MS patients show distinct features, cells and EVs obtained from HCs and MS patients were used. Cells isolated from both HCs and MS patients were tested, and each of them was cocultured with EVs from the two donor types. A schematic representation of the culture protocol and the assayed combinations are shown in **Figure 54**. PBMC samples from 14 donors (7 HCs and 7 MS patients) and EV samples from 8 donors (4 HC and 4MS patients) were used for the cell culture experiments.

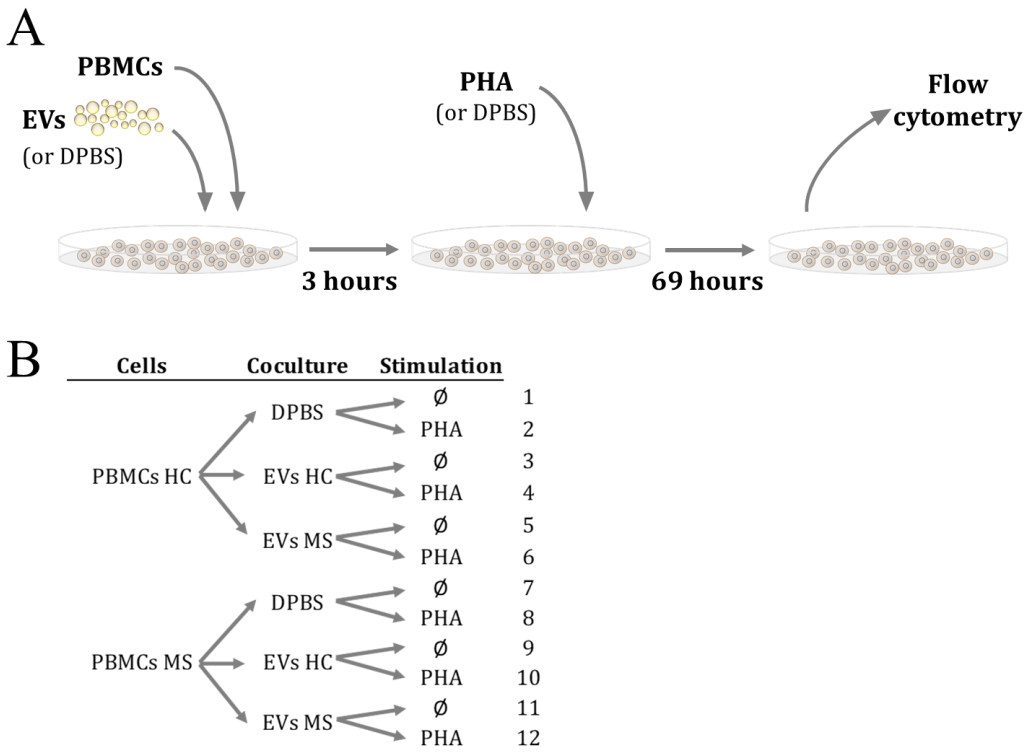


Figure 54. Cell culture protocol to test the influence of EVs on T cell activation. (**A**) 10^5 PBMCs were plated in 96-well dishes and then, 100 µg of EVs were added, while DPBS was added in control wells. 3 hours later, 10 µg/ml PHA were added to induce T cell activation in half of the wells, while the rest was maintained with no stimulation. 3 days after plating, T cell activation was evaluated by flow cytometry. (**B**) Schematic representation of the 12 different study conditions. EV samples from 5 adults and 5 elders were tested, and all conditions were assayed in duplicate.

For the flow cytometric analysis, the following fluorochrome-conjugated anti-human monoclonal antibodies were used: anti-CD8 FITC, anti-CD4 PE-Cy7 and anti-CD25 PE from BD Biosciences; for cell viability assessment 7-AAD dye (Thermo Fisher). Cells were stained following the same protocol as the one explained above for senescent T cells. Samples were acquired in a Guava EasyCyte 8HT flow cytometer (Millipore, Merck). Single staining and FMO control tubes were used to adjust compensations and set the gating strategy. After gating for singlets, lymphocytes were gated based on FSC and SSC and 10,000 lymphocytes were acquired for each sample. Then, lymphocyte populations were distinguished based on fluorescence and analysis of obtained results was performed with the InCyte 3.1 software (Millipore, Merck).

Statistical analysis

Statistically significant differences between groups were tested with GraphPad Prism version 6.01 for Windows (GraphPad Software, www.graphpad.com). The nonparametric Mann-Whitney test was applied. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Results

Inflammatory markers in serum

The first approach to compare MS patients and HCs was to measure 3 of the most relevant inflammatory markers: CRP, TNF-α and IL-6. We performed ELISA assays with serum samples of 19 MS and 19 HCs. Before performing the statistics to compare the two groups of interest, we proved that there was no correlation between the analytes and donor age. Finally, the results obtained demonstrate that there are no significant differences for CRP concentration, while MS patients have an increased concentration of TNF-α and IL-6 in circulation when compared to controls (**Figure 55**).

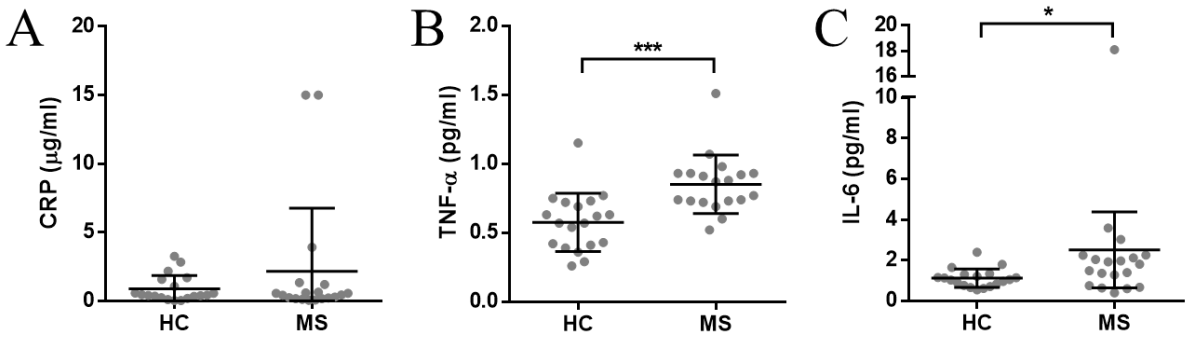


Figure 55. Analysis of inflammatory markers in serum. (**A**) The concentration of CRP shows no differences between HCs and MS patients. (**B-C**) An elevated concentration of TNF-α and IL-6 was reported for MS patients.

Characterization of senescent T cells

We wondered whether there could also be different proportions of senescent T cells in MS patients. To test this, we analysed the PBMCs isolated from MS patients and healthy controls, comparing them by flow cytometry. The absence of the costimulatory molecule CD28 and the gain of CD56 expression in T cells was evaluated.

The obtained results are shown in **Figure 56**. We did not find any significant difference between HCs and MS patients regarding CD4 senescent cells (**Figure 56A-B**). On the other hand, when CD8 senescent cells were compared, a reduced proportion of CD28- CD56+ cells were found in MS patients of 40-49 years with respect to the age-matched controls (**Figure 56D**). A similar trend was reported for the CD28- CD56- CD8 cells of both age ranges, but they did not reach statistical significance (**Figure 56C**). Notably, the proportion of senescent cells was higher for CD8 cytotoxic cells than for CD4 helper cells.

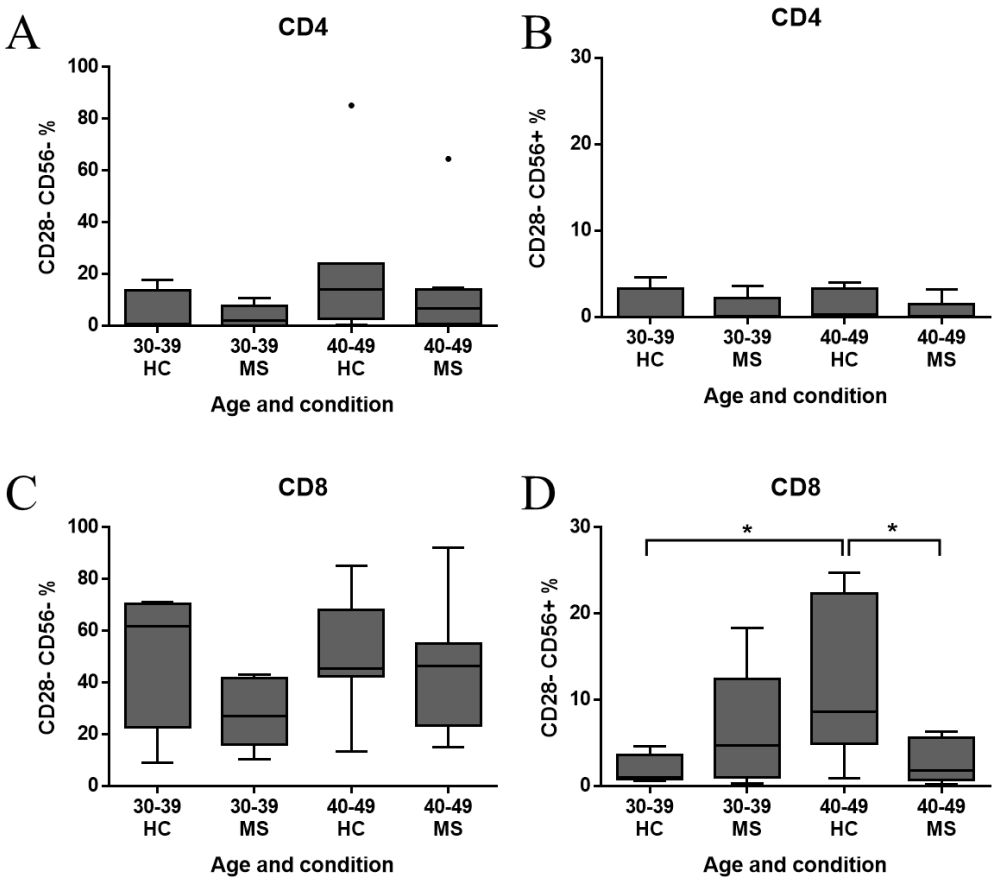


Figure 56. Characterization of senescent T cells. PBMC samples from HCs and MS patients were stained and analysed by flow cytometry. Among the CD3 cells, CD4 and CD8 were distinguished and the expression of CD28 and CD56 molecules evaluated. (**A-B**) For CD4 T cells, no differences were reported among the analysed groups. (**C**) Even if no statistical significance was reached, a reduced proportion of CD28- cells is shown for MS patients. (**D**) There is an accumulation of CD28- CD56+ cells in HCs aged 40-49 when compared to the ones aged 30-39 years. When HCs and MS patients were compared, a reduced percentage of CD28- CD56+ cells was found for 40-49 MS patients compared to the age-matched controls. Age range in years.

Effect of the coculture of PBMCs and EVs on cell viability

Aiming to compare the effect of plasma EVs on T cell activation in controls and MS patients, we performed coculture experiments following the same protocol as in the previous chapter. This experimental setup and the design of the flow cytometry staining panel, with the cell viability marker 7-AAD, also gave us the opportunity to test the effect of EVs on cell viability.

The percentage of viable cells in all the tested culture conditions (PBMCs alone, PBMCs + EVs, PBMCs + PHA and PBMCs + EVs + PHA), were measured for cells from 4 MS and 4 HCs. Each cell donor was assayed in different replicates and cocultured with several EV donors, so the average of viable cells on each condition was calculated for every cell donor. Consequently, the mean values of the 4 culture conditions for each cell donor were plotted and compared. We

showed that the percentage of viable cells is heterogeneous depending on the donor, but all of them improve cell viability in the presence of plasma EVs (**Figure 57**). Moreover, as reported in the previous chapter, PHA reduces viability, and this effect is partially rescued in the presence of EVs.

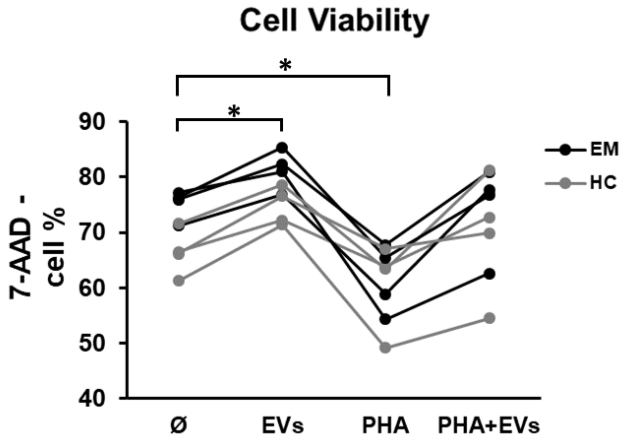


Figure 57. Cell viability differences based on cell culture conditions. The percentage of viable cells after 3 days in culture was assessed by 7-AAD staining in a flow cytometer. PBMCs cultured alone were taken as reference. The coculture of cells with plasma EVs resulted in an elevated proportion of viable cells, while the stimulation with PHA reduced cell viability. In addition, a partial rescue of viability was found when cells were stimulated with PHA in the presence of EVs.

Effect of the coculture of PBMCs and EVs on T cell activation

As mentioned above, we cultured PBMCs and EVs from MS patients and age-matched controls, in order to evaluate whether they present a different T cell activation pattern. We performed several experiments with all the possible combinations: we cultured HC or MS cells, and we stimulated them EVs from HC or MS patients. We tested multiple cell and EV donors for each condition. As explained in the previous section, all culture conditions were tested (PBMCs alone, PBMCs + EVs, PBMCs + PHA and PBMCs + EVs + PHA) and none of the samples assayed showed T cell activation in the presence of EVs alone (without PHA stimulation), demonstrating no immunogenic effect of EVs. In consequence, cells with PHA alone or with PHA + EVs were compared. In all the cases, the wells stimulated with PHA (and without EVs) were taken as reference and fold change differences were calculated with respect to them.

The analysis was performed separately for CD4 and CD8 T cells. For CD4 cells, no differences on cell activation were found between the tested coculture conditions (**Figure 58A**). In contrast, when CD8 T cells were evaluated, we found activation differences between cells from HCs and MS patients (**Figure 58B**). Specifically, CD8 cells from HCs get more activated than cells from MS patients. This difference is statistically significant when cells are cocultured with EVs from HCs, while the observed difference does not reach statistical significance when cells

are cocultured with EVs from MS patients (p = 0.08). Notably, the coculture of cells with PHA + EVs resulted in enhanced activation of both CD4 and CD8 T cells in all conditions, when compared to cells stimulated with PHA alone (fold change > 1).

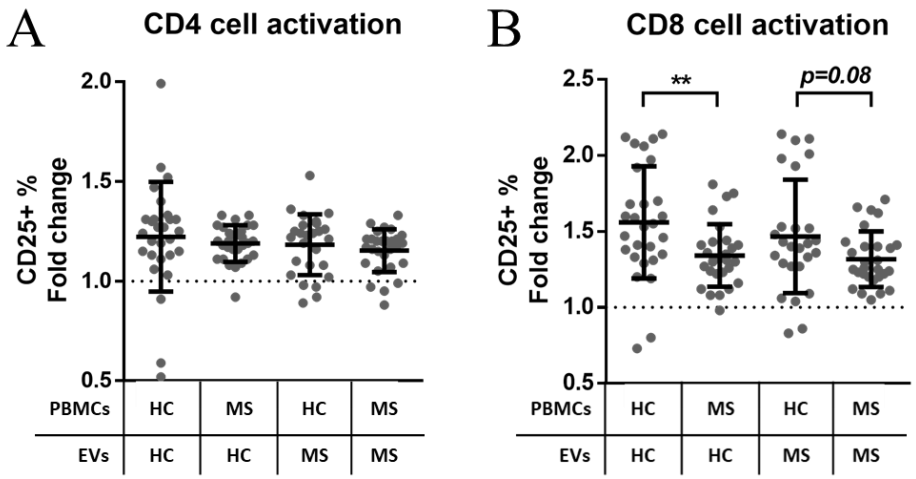


Figure 58. T cell activation under PHA stimulation and EV coculture. (**A**) The activation of CD4 cells was compared depending on the cell donor and the EVs that were added to cultured cells, and no differences were found between any of the conditions. (**B**) When CD8 cells were measured, a reduced activation of cells from MS patients was reported when compared to cells from HCs. This reduction was statistically significant for cells cocultured with EVs from HCs. The results for cells cocultured with EVs from MS patients follow the same trend, but the analysis did not reach statistical significance.

Discussion

MS is a complex disease, in which many systems, tissues and cell types are affected. The main consequence of MS is the demyelination of axons, and even if the triggering factor initiating the damaging cascade is still unknown, the implication of the immune system has been widely reported [357]. Taking into consideration the chronic and intense implication of the immune system during MS pathology, as well as the effects of immunomodulatory drugs prescribed to patients, the possible premature exhaustion of the immune system was proposed [279]. However, as mentioned before, due to the pathology of MS it is not easy to separate the features of the disease from the ones caused by aging.

This is the case, for example, of inflammation. Elevated concentrations in circulation of proinflammatory markers are found in MS patients [286] as well as in aged subjects [138]. In accordance with previous reports, here we showed that in our samples, MS patients have increased IL-6 and TNF-α concentrations in serum when compared to age-matched controls. In contrast, we did not report elevated CRP levels in MS patients. Interestingly, the differences for IL-6 and TNF-α concentrations are found even if MS patients were in remission and despite

the immunomodulatory drugs they take. Other authors also found elevated inflammatory markers in MS patients during relapse when compared to HCs [358,359]. On the other hand, recent studies reported that treatments with Natalizumab, Fingolimod or hormones such as vitamin D or melatonin reduce the concentration of inflammatory mediators (including IL-6 and TNF-α) in MS patients [360–363]. Taken together, and considering that the participants of our study were treated with immunomodulatory drugs, it seems that treatments could partially reduce inflammation, but differences can still be observed when compared to healthy controls.

With the obtained results, we demonstrated that chronic inflammation is present both in elders and MS patients, but we hypothesize that the leading causes and molecular mechanisms are probably different. Moreover, we also consider it should be kept in mind that the observed differences are based on the mean differences between groups, but not all MS patients present elevated inflammation, reflecting disease heterogeneity.

Our second approach was to measure the proportion of T cells with features associated with senescence (loss of CD28 and gain of CD56) in MS patients and compare the results to the ones obtained for HCs. First, we evaluated CD4 T cells and we did not find differences between the studied groups. In contrast, when CD8 T cells were compared, a reduced percentage of CD28-CD56+ cells was found in MS patients aged 40-49 years with respect to age-matched controls. Notably, even if no statistical significance was reached, CD28-CD56- CD8 cells showed the same trend for both 30-39 and 40-49 age ranges. Our results for CD8 T cells are in line with previous reports, in which less CD8+CD28- cells were found in the blood of MS patients with different disease phase and treatments [277,287,288]. Regarding CD4, other authors reported elevated proportions of CD28- cells in the blood of untreated MS patients, while they did not find differences in patients treated with interferon-β1b [355,364]. These results point out the influence of treatments and therefore, could explain the results we obtained, as all samples we tested were from patients treated with different drugs.

Besides, we should consider that even if little differences are found for the analysed membrane molecules, there could be other features that result in distinct functions of T cells. For instance, one of the research groups that found elevated CD28- CD4 T cells in untreated MS patients also demonstrated that both CD28+ and CD28- cells from MS patients express less CD95 Fas death receptor, which could probably contribute to MS pathogenesis by preventing apoptosis and prolonging T cell survival [355]. Moreover, CD4 CD28- cells were found to accumulate in MS lesions and have cytotoxic effects, evidencing that the loss of CD28 expression does not mean lack of function or anergy [356,365]. Therefore, we consider that future experiments in MS

patients should investigate both the characteristic membrane molecules as well as the functions of T cells.

The last objective of this work was to study the influence of EVs on T cell activation. We previously evaluated this effect on samples from healthy controls, and now we wanted to test whether samples from MS patients present different interactions between T cells and plasma EVs. To this end, we followed the same coculture protocol as in the previous chapter and we assayed cells and EVs from MS and HCs. First, taking advantage of the staining panel, we measured the proportion of living cells after 3 days in culture. Interestingly, we replicated our previous results, demonstrating that the coculture of PBMCs with plasma EVs enhances cell viability. Moreover, this effect was shown for cells of MS patients and controls, as well as the partial recovery of cell viability in the presence of PHA stimulation. As commented before, no previous works have investigated the influence of EVs on cell viability so, importantly, we reported an effect of EVs that was not known. Consequently, our results open new questions that should be further investigated in future studies to try to understand the responsible mechanisms.

Then, we evaluated T cell activation by CD25 expression. Importantly, in line with our previous results, the coculture of cells and plasma EVs did not result in T cell activation in any of the cocultures tested, so we can conclude that EVs from controls or MS patients do not induce an immune response. However, EVs influence T cell activation under PHA stimulation, as they enhance CD25 expression in both CD4 and CD8 cells. We investigated the effect of EVs on T cell activation depending on the source of the cell and EV donor. No differences were found between EVs from controls and MS patients. In contrast, when PBMCs from controls and MS patients were compared, we showed that CD8 T cells from MS donors have a reduced activation enhancement than age-matched HCs. This difference was observed when cells were cocultured with EVs from HCs and from MS patients, but it reached statistical significance only for cells cocultured with EVs from HCs. We hypothesize that with advancing age, CD8 T cells of MS patients could have a reduced capacity to respond to new stimulus or insults when compared to healthy individuals.

These last results describe novel features of both T cells and EVs from plasma. We showed that CD4 and CD8 T cell activation is increased in the presence of EVs, but CD8 cells from MS patients have a milder response to the same EV coculture. Linking this to the previous discussion about senescent T cells, in this study we evaluated both T cell membrane characteristics as well as their functioning in MS patients. Moreover, we highlight that EVs must be taken into consideration when trying to understand the immune system, as it has been

shown by us and many other researchers that EVs secreted by different types of cells are present in plasma and influence the immune response [234].

In summary, in this work we have investigated different aspects of the immune system in adult MS patients, aiming to evaluate the presence of features related to premature aging. Despite the effect of immunomodulatory treatments, individuals with MS have elevated levels of inflammatory markers, similar to the ones reported in elders. In contrast, when T cells were analysed, no accumulation of senescence markers was reported. Lastly, the coculture of PBMCs and plasma EVs under PHA stimulation promotes T cell activation, but this effect is reduced in MS patients.

Therefore, there are still many aspects that are not completely understood and the implications between MS and aging, including the possible occurrence of premature aging during MS need to be further investigated. We should first describe and comprehend the molecular changes and consequences that occur, to then be able to apply this knowledge and make the best treatment decisions during the aging of MS patients.

GENERAL DISCUSSION, PERSONAL OPINION AND FUTURE PERSPECTIVE

The aim of these lines is to provide a general overview of the presented data from a personal point of view. I would go through the chapters and comment about what we got and where are we going. Besides, when introducing my opinion, I will try to summarize some of the thoughts that crossed my mind during the writing of this thesis, and also the ones that were there for the last four years.

First, and importantly, a comment about growing old. Aging affects all of us. During the first couple of decades of our life, we notice this process in the people around us: close relatives and people that is a reference for us start to get old. Then, we realize that aging is a universal process, and we have to learn how to face it in our own body. Of course, growing older has both positive and negative sides. On one hand, the older we are, the more we know, the more conscious we are, and the more we appreciate our time. On the other hand, the older we are, the more ailments we have.

The universality of aging makes the research on this field different from other biomedical issues. It is of course very rewarding to investigate any disease and to try to describe the underlying biological processes, contributing to the better understanding and to the possible development of new treatments. Indeed, the principal aim of biomedical research is to advance the knowledge and consequently, to help the affected people. In addition, in the particular case of aging, as commented before, we research on a process that we feel socially closer and that will undoubtedly also reach us (if it has not yet).

Secondly, I would also like to comment on the two main approaches in the research of aging. There are studies that focus on living longer, or lifespan [69,128]. In this field, there are investigations, for instance, trying to describe why some humans live more years than others or attempting to find longevity-related genes in model organisms. In contrast, the other principal strategy focuses on living better, or health span [2]. This approach aims to describe age-associated defects and problems that lead to reduced functional capacity and thus, worsen our quality of life. In fact, we have mentioned previously in this work that we live longer than previous generations, but the health span is not simultaneously increasing. And in the end, why would we want to live longer, if it just means to live more years but with disability? For this reason, I believe that we should first focus on living our last years better, and then we could investigate how to live longer.

With regard to the loss of functional capacity and dependency, a previous state of frailty has been defined. Due to the diverse pathways that can lead to dependency, frailty is comprised of a wide range of symptoms and indicators [5]. The tests that have been developed and are now applied in the clinic identify a part of the frail elders, but fail to accurately identify all of them.

The complementation of the available tests with an easily measurable biomarker is a promising possibility that it is worth exploring. We and many others have tried to find blood biomarkers of frailty, and some interesting candidates have been proposed. However, the validity of the proposed biomarkers has still not been proved in large cohorts of different regions [187]. For instance, some inflammatory mediators like IL-6 or TNF-α have been measured in many works, but not all of them, including ours, found increased levels in frail individuals.

In my view, the differences between studies may be influenced by the inclusion criteria and the frailty screening tool selected. When data was available, we have compared the classification of our participants by several frailty scales and shown that there is a high proportion of people that are identified as frail by only some of the tests. In consequence, it is not easy to search for molecular biomarkers, and the inclusion of participants classified as frail by all the tests could improve the outcomes, as shown in our transcriptome profiling study. On the other hand, I consider that longitudinal studies would be more useful to monitor the proposed biomarkers for several years and evaluate whether they change in accordance with the loss of function and the development of frailty.

However, I am aware of the complexity of performing longitudinal studies, and specially in people of advanced age. Another possibility that I consider that could be useful, is to study the changes of potential biomarkers after an intervention aimed to reverse or reduce frailty, as we did with the pilot physical intervention study and the expression of candidate genes. Indeed, this is a longitudinal approach that can be followed by only several months and provide valuable data. Moreover, longitudinal studies, including an intervention or not, are an interesting tool to follow the progression of biomarkers in a single individual, which could be more useful than the establishment of a single cut-off value, as we have seen that there is high interindividual variability in the concentration or expression levels of biomarkers.

In this work, a special focus has also been set on EVs. In the last decades EVs have cleared their name, and now we know that they are much more than cellular trash. These secreted particles have been found to be produced by almost all cell types and to be implicated in many physiological and pathological processes. In addition, the communication between cells by EVs is not a particular feature of humans or mammals, and a wide range of organisms use these communication media. Furthermore, it has been described that EVs can also mediate inter-organismal communications, as seen between different bacteria, but also between the microbiota and human cells in our bodies. So, we should admit that EVs are relevant mediators of intercellular communication that have not been taken into consideration until recently [201].

This fact, as well as the other new discoveries about molecules, structures or functions that are made every year in the biological field, demonstrate the complexity of the system and the long way we still have to get to understand the complete picture. For instance, there could probably be other molecules or particles that participate in intercellular communication, and even if no other components await to be discovered, we will need time to get to describe all the features and functions of EVs.

One of the main challenges for the investigation of EVs is their small size. In contrast to cells, EVs cannot be easily visualized or measured. Besides, the term EV is applied for vesicles of diverse size, with a diameter that ranges from few nanometres to more than a micrometre. In consequence, some techniques classically designed for cells, like flow cytometry, can only detect large EVs, while other dedicated machines, including NTA, better identify small EVs but do not accurately recognize some of the larger particles. In the last years, thanks to the increasing interest of EVs, novel instruments have been developed. For example, there are new flow cytometers and imaging flow cytometers that detect particles of around 100 nm, and there are protocols to detect and track several fluorescently labelled or engineered EVs *in vivo* [217,366]. These are relevant advances that have enabled the description of previously unknown characteristics of EVs.

On the other hand, the EV cargo has also been studied by many authors and in EVs from different sources. As recorded by dedicated databases like Vesiclepedia, thousands of proteins, lipids and nucleic acids have been reported to be transported by EVs [367]. Some studies have been carried out by evaluating the presence or concentration of just the molecules of interest, while others performed omic approaches and identified hundreds of EV components. Interestingly, the presence of both specific and common molecules has been reported in several works. The molecules found in all the analysed samples have been suggested to be the components that are constitutively secreted in the studied EV subtypes.

However, the issue of whether some of the molecules identified in EV samples could actually be outside and co-isolated is still discussed. Representative examples of this phenomena are the free circulating miRNAs that can get attached to EVs, or the proteins, which are thought to form a protein corona around the EVs in body fluids. Indeed, when isolating EVs from controlled conditions like cell culture, we can get very enriched samples. Specifically, we can ensure the obtention of EVs produced by a single cell type if we perform a monoculture and apply EV-depleted culture media. On the contrary, when handling complex biological fluids such as blood or urine, the isolation of a single type EV or the complete separation from other components is very complicated.

From my point of view, however, complexity is one of the features we have to assume when analysing biological samples. Cell culture experiments are very useful for the characterization of certain aspects of EVs, but we have to keep in mind that EVs are found and operate in a much more complex environment. For this reason, I consider that when working with samples isolated from humans we should focus on the applications EVs can have, even if we do not exhaustively know the components of our samples. An illustrative example of the proposed approach, as commented in the second chapter of this work, is the use of PRP and PPP for skeletal muscle regeneration. Many authors have applied PRP and PPP in animal models and in human patients with positive results, and they were not aware of the presence of EVs in these plasma preparations [326]. Also, the use of some drugs shares some similarities, as there are approved and commercially available drugs for the treatment of distinct diseases, even if their mechanism of action resulting in the desired effect is not fully understood – for instance, interferon beta treatment for MS [368]. Of course, it would be desirable to describe and get to understand all the molecules and their effects, but we should not downplay the significance of an EV discovery if the isolation is not perfect or the description of the sample is not complete.

Thus, I think that the experiments performed in the second chapter show promising effects of EVs from plasma for improving cell differentiation in two of the tissues more affected by aging: skeletal muscles and bones. Additionally, the variance found between EVs from adults and elders point to an age-dependent decline in differentiation promotion capacity that should be further studied. In a different approach, we have also shown that plasma EVs affect T cell activation under PHA stimulation, and our results are in line with previous works that have demonstrated the implication of EVs in the immune system functioning. Finally, and even if it was not the main objective of our experiments, we found that the coculture of plasma EVs improved T cell viability. I believe that this is an exciting result because it indicates that T cells are “happier” and survive more in culture when EVs are present. Furthermore, and knowing that a wide range of EVs circulates in the blood, we hypothesize that probably only some of them are taken up by each cell type and for this reason, EVs isolated from plasma can influence different systems.

Importantly, we characterized our EV samples by cryoEM, NTA and flow cytometry. Probably, the confirmation of EV enrichment does not imply the absence of other molecules from plasma that could be co-isolated in our samples, but as discussed, we consider that our results are relevant despite other components could be present. Indeed, we established our EV isolation protocol more than five years ago [202], and apply it always strictly in the same way, ensuring that the reported differences have a biological basis and are not due to technical or procedural discrepancies. Moreover, we are now trying to characterize the cargo of our plasma EV samples

by proteomics and microarrays. These analyses will provide extensive information and could give us a hint about the responsible molecules for the observed effects.

Another multifaceted and intricate system of central importance in this thesis is the immune system. During the last years, and thanks to the expertise of my supervisors on immunology and multiple sclerosis disease, I have learned a lot about the immune system functioning and misfunctioning. I must admit that the more I learn, the more fascinated I feel about the complexity of this system. I think that I have acquired just basic knowledge and I continue learning new things every day. Particularly in this work, we focused on the relevant role of the immune system functioning during aging. Chronic low-grade inflammation or inflammaging is present in most elders and besides, T cell senescence also increases with age. Interestingly, we had the opportunity to analyse T cell senescence in participants of different ages, and we found lower levels in nonagenarians and centenarians, contributing to the description of the distinctive features of in the very old. However, our results should be confirmed in a larger cohort and, moreover, we should continue investigating to try to find the possible links between these features and longevity.

Regarding the possible incidence of premature aging characteristics in patients with autoimmune diseases, we performed a small study in MS patients. The hypothesis that the chronic activation of the immune system could lead to its premature exhaustion was evaluated in our patients. Despite the MS patients were in remission and under immunosuppressive treatment, they had elevated levels of inflammatory markers, which indicates a state of chronic inflammation and could be linked to inflammaging. In contrast, these same MS patients did not have increased proportions of senescent T cells (CD28-), or even have reduced numbers, when compared to age-matched healthy controls. As discussed before, this feature was observed in previous studies, and some of them also demonstrated the cytotoxic capacity of CD28- T cells in MS patients [356,365]. These findings show again the complexity of the immune system and suggest that CD28- T cell could be implicated in MS pathology. In addition, the strong effect of the immunomodulatory drugs applied for the treatment of MS influence not only the picture we get when performing a cross-sectional study, but they could also contribute to contradictory activating and suppressing signals and elevated immune stress in the patients.

Finally, I would like to finish this general discussion by giving my personal view about completing a PhD thesis in biomedical research. Two of the conclusions that I draw are the importance of study participants and the eager to answer unsolved questions. With regard to the participants, I have to point out that this work would not have been possible if donors were not enrolled. I really appreciate the people that decide to take part in research by donating a biological sample, in our case blood. It might just seem a small puncture, but it can be a great

effort for a person with more than 90 years or with a chronic disease. About the eager to answer questions, I have to acknowledge the colleagues and researchers that have directly or indirectly contributed to this work. This thesis was developed as a consequence of the previous works, and thanks to the collaboration of many researchers around us. We all dedicate our time to try answer questions and to generate new ones.

Getting a PhD might seem long and difficult when we start, but years go fast, and we never have enough time to complete that never ending "to-do list" that is stuck at our lab desk. So, we will have to continue working….

CONCLUSIONS

1. Frailty is a heterogeneous syndrome and molecular biomarkers could help its identification.

2. The concentration of CRP, TNF-α and IL-6 is elevated in elders, while albumin is decreased, and the concentration of EVs is not altered.

3. Despite inflammaging, there are no differences in inflammatory markers between robust and frail elders.

4. The expression of EGR1 in circulating leukocytes is a potential biomarker of frailty.

5. EVs isolated from plasma promote osteogenesis and myogenesis *in vitro*. This effect is reduced when EV samples from elder donors are applied.

6. Senescent CD8 T cells increase with age. Similarly, senescent CD4 T cells increase with age, but in contrast, there is a decrease in nonagenarians and centenarians.

7. EVs isolated from plasma improve lymphocyte viability *in vitro*, both unstimulated or under PHA stimulation. The positive effect of EVs is stronger in cells from adults.

8. Plasma EVs boost T cell activation *in vitro*. This effect is produced only under PHA stimulation, and it gradually decreases with the age of the EV donor.

9. Adult MS patients have increased inflammatory markers in circulation despite being in remission and under immunomodulatory treatments, which indicates a chronic inflammatory state that could be related to inflammaging.

10. Plasma EVs also boost the activation of T cells from MS patients. In the case of CD8 T cells, this effect is stronger in cells from healthy controls than from MS patients.

PUBLICATIONS

Research articles

- **Alberro A**, Sáenz-Cuesta M, Muñoz-Culla M, Mateo-Abad M, Gonzalez E, Carrasco-García E, Araúzo-Bravo MJ, Matheu A, Vergara I, Otaegui D. Inflammaging and Frailty Status Do Not Result in an Increased Extracellular Vesicle Concentration in Circulation. *Int J Mol* Sci **2016**, 17(7):1168. doi: 10.3390/ijms17071168

- **Alberro A**, Osorio-Querejeta I, Sepúlveda L, Fernández-Eulate G, Mateo-Abad M, Muñoz-Culla M, Carregal-Romero S, Matheu A, Vergara I, López de Munain A, Sáenz-Cuesta M, Otaegui D. T cells and immune functions of plasma extracellular vesicles are differentially modulated from adults to centenarians. *Aging (Albany NY)* **2019**, 11(22):10723-10741. doi: 10.18632/aging.102517

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- Álvarez-Satta M, Berna-Erro A, Carrasco-Garcia E, **Alberro A**, Saenz-Antoñanzas A, Vergara I, Otaegui D, Matheu A. Relevance of oxidative stress and inflammation in frailty based on human studies and mouse models. **Under review**

- Osorio-Querejeta I, Sáenz-Cuesta M, Eguimendia E, Oregi A, Sepúlveda L, Irizar H, **Alberro A**, Llarena I, Aiastui A, Muñoz-Culla M, Otaegui D. MiR-219a-5p is a key factor in the cargo of pro-remyelinating exosomes. **Under review**

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Patent

- Request for grant of a European Patent (**EP18382566.0**).
  Applicant: Administración general de la comunidad autónoma de Euskadi.
  Title: Biomarkers for Diagnosis and/or Prognosis of Frailty.
  Inventors: Ander Matheu, David Otaegui, Estafanía Carrasco, **Ainhoa Alberro**, Itziar Vergara, Kalliopi Vrotsou.

Scientific dissemination article

- Sáenz-Cuesta M, **Alberro A**, Otaegui D. Ba al dugu immunitate-sistema hackeatzerik? *Elhuyar* **2017**, 328: 64-68

Scientific communications as first author

- **Alberro A**. Implication of EVs in multiple sclerosis disease and human ageing process. *ME-HaD Training Course - Extracellular Vesicles & Exosomes: Analysis and Properties* (1-3 March 2016) Ioannina, Greece **ORAL COMMUNICATION**

- **Alberro A**, Sáenz-Cuesta M, Muñoz-Culla M, Osorio-Querejeta I, Mateo-Abad M, Gonzalez E, Vergara I, Otaegui D. Aging and Frailty Status Do Not Result in an Increased Extracellular Vesicle Concentration in Circulation. *3rd GEIVEX Symposium: Therapeutic applications of Extracellular Vesicles* (29-30 September 2016) San Sebastian, Spain **POSTER**

- **Alberro A**, Sáenz-Cuesta M, Muñoz-Culla M, Osorio-Querejeta I, Mateo-Abad M, Gonzalez E, Vergara I, Otaegui D. Aging and Frailty Status Do Not Result in an Increased Extracellular Vesicle Concentration in Circulation. *I Congreso nacional de jóvenes investigadores en Biomedicina* (28-29 November 2016) Valencia, Spain **POSTER**

- **Alberro A**. Implication of extracellular vesicles in multiple sclerosis disease and human aging. *1st Euskadi Workshop on Exosomes* (23 March 2017) Derio, Spain **ORAL COMMUNICATION**

- **Alberro A**, Sáenz-Cuesta M, Sepúlveda L, Osorio-Querejeta I, Iparraguirre L, Llarena I, Vergara I, López de Munain A, Otaegui D. Characterization of human plasma extracellular vesicles and their role in aging-related immunosenescence and immune response. *International Society for Extracellular Vesicles Annual Meeting* (2-6 May 2018) Barcelona, Spain **POSTER**

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- **Alberro A**. What do we get when we get old? About the immune system and extracellular vesicles, long friends with new relationships. *UPV/EHUko II. Doktorego Jardunaldiak* (3 July 2019) Bilbao, Spain **POSTER (Best poster award)**

- **Alberro A**, Berna-Erro A, Osorio-Querejeta I, Iparraguirre L, Muñoz-Culla M, Sáenz-Cuesta M, Vergara I, Matheu A, Otaegui D. EGR1 expression is a potential frailty biomarker. *International Congress of the European Geriatric Medicine Society* (25-27 September 2019) Kraków, Poland **POSTER**

- **Alberro A**, Osorio-Querejeta I, Iparraguirre L, Carregal S, Elguezabal N, López de Munain A, Vergara I, Matheu A, Sáenz-Cuesta M, Otaegui D. T cells and plasma extracellular vesicles are differentially modulated from adults to centenarians. *International Congress of the European Geriatric Medicine Society* (25-27 September 2019) Kraków, Poland **POSTER**

- **Alberro A**, Osorio-Querejeta I, Iparraguirre L, Muñoz-Culla M, López de Munain A, Matheu A, Vergara I, Sáenz-Cuesta M, Otaegui D. Heteroplasmic mutations in mtDNA and H haplogroup are related to Frailty and Dependency. *International Congress of the European Geriatric Medicine Society* (25-27 September 2019) Kraków, Poland **POSTER**

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