Frailty in the elderly: analysis of the relationship with genomic alterations and cell repair.

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INFORMAN:

Que el trabajo titulado “Frailty in the elderly: analysis of the relationship with genomic alterations and cell repair” presentado por Doña María Sánchez Flores ha sido realizado bajo nuestra dirección y cumple los requisitos necesarios para optar al Grado de Doctor con Mención Internacional. Considerándolo finalizado, autorizamos su presentación y defensa.

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2. Valdiglesias, V., Sánchez-Flores, M., Maseda, A., Marcos-Pérez, D., Millán-Calenti, J.C., Pásaro, E., Lorenzo-López, L., Laffon, B. 2015. *Lymphocyte subsets in a population of non-frail elder individuals.* Journal of Toxicology and Environmental Health, Part A: Current Issues 78:790-804.
3. Sánchez-Flores, M., Marcos-Pérez, D., Costa, S., Teixeira, J.P., Bonassi, S., Pásaro, E., Laffon, B., Valdiglesias, V. 2017. *Oxidative stress, genomic instabily and DNA damage and repair in frail elderly: A systematic review.* Ageing Research Reviews 37:1-15.
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1. 3rd International Congress on Environmental Health (ICEH 2014). September 24-26 2014. Porto, Portugal. Valdiglesias, V., Millán, J.M, Sánchez-Flores, M., Maseda, A.B., Pásaro, E., M., Lorenzo, L., Laffon, B. Frailty in the elderly: current identification and alternative markers.
2. 3rd International Congress on Environmental Health (ICEH 2014). September 24-26 2014. Porto, Portugal. Laffon, B., Maseda, A., Pásaro, E., Millán-Calenti, J.C., Lorenzo-López, L., Sánchez-Flores, M., Valdiglesias, V. Lymphocyte subsets in a population of non-frail elder individuals.
3. 3rd International Congress on Environmental Health (ICEH 2014). September 24-26 1014. Porto, Portugal. Valdiglesias, V., Sánchez-Flores, M., Pásaro, E., Laffon, B. Flow cytometry analysis of γH2AX levels in fresh and cryopreserved human peripheral blood lymphocytes.
4. 1st International Congress on Psychobiology. July 15-17 2015. Oviedo, Spain. Laffon, B.; Millán-Calenti, J.C.; Valdiglesias, V.; Maseda, A.; Sánchez-Flores, M.; Lorenzo-López, L.; Marcos-Pérez, D.; Pásaro, E. Frailty in the elderly: biomarkers for early detection.
5. 3rd International Conference on Occupational & Environmental Toxicology ICOETOX 2016 and 3rd Ibero-American Meeting on Toxicology and Environmental Health IBAMTox. June 21-23 2016. Porto, Portugal. Valdiglesias, V., Sánchez Flores, M., Bonassi, S., Pásaro, E., Laffon, B. H2AX phosphorylation analysis as DNA damage biomarker for human population studies.
6. 59th Congreso de la Sociedad Española de Geriatría y Gerontología (SEGG) and 29th Congreso da Sociedade Galega de Xerontoloxía e Xeriatría (CSGXX). June 7-9 2017. A Coruña, Spain. Cortés, J., Sánchez-Flores, M., Marcos-Pérez, D., Fernández-Bertólez, N., Maseda, A., Bonassi, S., Valdiglesias, V. Revisión sistemática de la asociación del estado de fragilidad en la vejez con biomarcadores de estrés oxidativo, daño genómico y reparación celular.
7. 23rd Reunión Científica de la Sociedad Española de Mutagénesis Ambiental (SEMA 2017). June 14-16 2017. Oviedo, Spain. Sánchez-Flores, M., Marcos-Pérez, D., Maseda, A., Lorenzo-López, L., Millán-Calenti, J.C., Bonassi, S., Pásaro, E., Valdiglesias, V., Laffon, B. Is

micronucleus frequency in peripheral lymphocytes and buccal cells related to frailty syndrome in older adults?

8. 2nd International Congress of Psychobiology. July 19-21 2017. Ávila, Spain. Pásaro, E., Sánchez-Flores, M., Marcos-Pérez, D., Maseda, A., Lorenzo-López, L., Millán-Calenti, J.C., Bonassi, S., Valdiglesias, V., Laffon, B. Association between frailty syndrome and micronucleus frequency in an older adult population. Influence on cognitive impairment.
9. 12th International Comet Assay Workshop (ICAW). August 27-31 2017. Pamplona, Spain. Marcos-Pérez, D., Sánchez-Flores, M., Maseda, A., Lorenzo-López, L., Millán-Calenti, J.C., Pásaro, E., Laffon, B., Valdiglesias, V. Frailty status is not associated with increase in DNA damage or repair alterations in older adults.
10. 2nd International Conference DiMoPEx working groups meeting: Pollution in living and working environments and health. October 30-31 2017. Bentivoglio, Italy. Valdiglesias, V., Sánchez-Flores, M., Marcos-Pérez, D., Maseda, A., Millán-Calenti, J.C., Lorenzo-López, L., Pásaro, E., Laffon, B. Development of biomarkers for identification of frailty in the elderly.

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Nobody looks like what they really are on the inside. You don't. I don't. People are much more complicated than that. It's true of everybody.

The Ocean at the End of the Lane
by Neil Gaiman

Abstract

Frailty is a multidimensional syndrome characterised by an increased vulnerability. Nowadays, frailty identification is based on phenotypical features. Use of biomarkers for frailty identification would provide a more accurate detection of frail subjects in early stages, when frailty can still be potentially reverted.

The main objective of this study was to evaluate the possible association between frailty and several cellular and molecular biomarkers – genomic instability, DNA damage, and DNA repair capacity – so that they can be proposed as frailty biomarkers. To that aim, a cross-sectional study was conducted in a population of older adults (aged 65 or over) classified according to their frailty status.

A systematic review of the literature on genetic outcomes related to frailty was conducted to establish the current knowledge on the topic. Besides, the most critical issues limiting the use of the phosphorylated H2AX assay as DNA damage biomarker in human population studies were addressed.

Results from the population study showed a significant and progressive increase of micronuclei in lymphocytes and phosphorylated H2AX with frailty severity, supporting their use for frailty identification. No association of frailty with micronuclei in buccal cells, frequency of mutation in T-cell receptor, comet assay, or DNA repair capacity was found.

Resumen

La fragilidad es un síndrome multidimensional caracterizado por una vulnerabilidad aumentada. Actualmente, la fragilidad se identifica basándose en características fenotípicas. El uso de biomarcadores para la identificación de fragilidad proporcionaría una detección más precisa de individuos frágiles en sus etapas iniciales, cuando puede ser revertida.

El principal objetivo del presente trabajo fue estudiar la posible relación entre biomarcadores celulares y moleculares - inestabilidad genómica, daño en el ADN y capacidad de reparación del ADN - para su propuesta como biomarcadores de fragilidad. Para este propósito, se realizó un estudio transversal en ancianos (65 ó más años) clasificados según su estado de fragilidad.

Se realizó una revisión sistemática de la literatura sobre biomarcadores genéticos relacionados con fragilidad para establecer el conocimiento actual sobre el tema. Además, se abordaron los puntos críticos que limitan el uso del ensayo de γH2AX en estudios poblacionales humanos.

Los reultados mostraron un aumento significativo y progresivo de micronúcleos en linfocitos y γH2AX con el grado de fragilidad, apoyando su uso como biomarcadores. No se observó relación entre el estado de fragilidad y la frecuencia de micronúcleos en células bucales,

frecuencia de mutación en receptores de células T, ensayo del cometa o capacidad de reparación del ADN.

Resumo

A fraxilidade é unha síndrome multidimensional caracterizada por una vulnerabilidade aumentada. Actualmente, a identificación da fraxilidade baséase en características fenotípicas. O emprego de biomarcadores na identificación da fraxilidade ofrecería unha detetección máis precisa nas sus primeiras etapas, cando pode ser revertida.

O obxectivo principal deste traballo foi estudar a posible relación dunha serie de biomarcadores celulares e moleculares - inestabilidade xenómica, dano no ADN e capacidade de reparación do ADN- co estado de fraxilidade, para a súa proposta coma biomarcadores de fraxilidade. Para este propósito, realizouse un estudo transversal nunha población de anciás (65 ou máis anos) clasificados segundo o seu estado de fraxilidade.

Se realizou unha revisión sistemática da literatura sobre biomarcadores xenéticos relacionados coa fraxilidade, para establecer o coñecemento actual sobre o tema. Ademais, se abordaron os puntos críticosque limitan o uso do ensaio de γH2AX en estudos en poboacións humanas.

Os resultados amosaron unha asociación significativa e progresiva entre micronúcleos en linfocitos e γH2AX co estado de fraxilidade, dando apoio o se uso coma biomarcadores. Non se observou asociación do estado de fraxilidade con micronúcleos en células bucais, frecuencia de mutación dos recepteores das células T, ensaio do cometa ou capacidade de reparación do ADN.

Extended summary in Spanish - Resumen amplio

El envejecimiento poblacional es un fenómeno que en la actualidad se da en las sociedades de todo el mundo. Es debido principalmente a dos causas: un descenso de la fertilidad y un aumento de la esperanza de vida. Esta situación es especialmente pronunciada en Europa, en donde la fertilidad se sitúa por debajo del nivel necesario para regeneración poblacional (alrededor de 2.1 hijos por mujer), mientras que se espera que la esperanza de vida en el momento del nacimiento aumente en 6-7 años en 2045-2050, situándose cerca de los 85 años. En este momento, el porcentaje de personas mayores de 65 años en Europa es el mayor en todo el mundo, alcanzado el 25% de la población. A su vez, se estima que África alcanzará porcentajes similares en 2050, mientras Europa podría alcanzar el 35% de acuerdo a las estimaciones de las Naciones Unidas. Este fenómeno supondría un cambio dramático en la estructura demográfica de las pirámides poblacionales, siendo el grupo de edad de 65-80 años el más numeroso en 2060.

Sin embargo, el aumento de la longevidad no necesariamente lleva consigo un estado de buena salud y bienestar. Muy al contrario, en muchas ocasiones el envejecimiento va acompañado de un aumento del riesgo de mala salud, aislamiento social y dependencia, lo que se traduce en una pérdida de calidad de vida. Es por ello que es necesario un cambio sistemático de las sociedades, no solo en los sistemas de sanidad pública sino en todos los sectores sociales, para afrontar los desafíos y el aumento de los costes que supone el envejecimiento de la población. En este contexto, Europa ha seguido dos principales líneas de acción: (i) el desarrollo de iniciativas que promuevan el envejecimiento saludable y (ii) la mejora de las metodologías de identificación de individuos mayores vulnerables, para prevenir o disminuir el impacto del declive cognitivo y funcional, y para promover la especialización y personalización de la asistencia médica.

A nivel biológico, el proceso de envejecimiento se caracteriza por una acumulación progresiva de un amplio rango de alteraciones moleculares y celulares. Por esta razón y bajo el marco del segundo curso de acción previamente mencionado, el concepto de fragilidad ha surgido en los últimos años como una medida de la edad biológica más precisa que el tradicional concepto de edad cronológica. La fragilidad es un importante síndrome geriátrico cuya prevalencia aumenta con la edad. Es una condición que supone un incremento del riesgo de aparición de efectos adversos para la salud en adultos mayores, incluyendo discapacidad, dependencia y finalmente mortalidad.

La fragilidad en sus primeras etapas es potencialmente reversible, por lo que su identificación temprana resulta de gran importancia. En la actualidad, no existe un consenso en cuanto a la definición de fragilidad o a los criterios específicos para identificar a personas frágiles, existiendo múltiples índices y criterios para ello. Sin embargo, son dos los criterios más comúnmente aceptados y utilizados para la identificación del estado de fragilidad en personas de

65 años o más: El criterio fenotípico, propuesto por Fried y colaboradores en 2001, y el modelo de acumulación de déficits descrito por Mitnitski y colaboradores en el mismo año.

El criterio de Fried se basa en la presencia o ausencia de cinco parámetros fenotípicos: (i) pérdida de peso involuntaria, (ii) actividad física reducida, (iii) reducción de la velocidad al caminar, (iv) pérdida de fuerza muscular medida como fuerza de prensión, y (v) fatiga autorreportada. Aquellos individuos que presentan tres o más de estos parámetros se clasifican como frágiles, los que presentan uno o dos, como pre-frágiles, y aquellos que no presentan ninguno se consideran no frágiles o robustos. Es por lo tanto un método muy sencillo de implementar y aplicar, y por esta razón se utiliza de forma muy amplia tanto en investigación como en clínica.

Por otro lado, Mitnitski, junto con Rockwood y colaboradores, definen la fragilidad como el efecto acumulativo de una serie de déficits que ocurren con la edad, incluyendo síntomas, signos, valores de análisis clínicos anormales, enfermedades y discapacidades. Se trata de un índice cuantitativo en el cual, cuanto mayor es el número de déficits que presente el individuo, maor será la probabilidad de ser frágil.

La fragilidad tiene un fuerte componente biológico, afectando a multitud de sistemas y procesos fisiológicos; sin embargo, su etiología es todavía desconocida. Esto se debe en parte a que no hay una única alteración, sino que todo parece indicar que se trata de una red interconectada de multitud de anomalías a diferentes niveles (celular, sistémico, del organismo) los que llevan a un estado de fragilidad. A nivel celular, la fragilidad se ha relacionado previamente con la acumulación de daño genético como consecuencia de alteraciones en los mecanismos de reparación del ADN. La inestabilidad genómica es una de las posibles consecuencias de esta acumulación de daño. Por lo tanto, un mayor conocimiento de los procesos a nivel celular podría aportar una potencial herramienta para la detención temprana de la fragilidad, ya que el uso de biomarcadores permitiría detectar individuos vulnerables a desarrollar un estado de fragilidad con anterioridad a la aparición de los signos clínicos.

El principal objetivo de este estudio ha consistido en la evaluación de la posible asociación existente entre el estado de fragilidad en personas mayores y varios biomarcadores celulares y moleculares, para que puedan ser propuestos para su utilización como biomarcadores de fragilidad. Para ello, se realizó un estudio epidemiológico transversal en una población de adultos mayores (de 65 años o más) clasificados como frágiles, pre-frágiles o no frágiles, de acuerdo con los criterios fenotípicos propuestos por Fried *et al.* (J. Gerontol. A Biol. Sci. Med. Sci. 2001; 56:M146-156). Además de la determinación de los biomarcadores, se evaluó también la influencia de parámetros clínicos (estado nutricional y estado cognitivo).

Con la finalidad de conocer el estado actual de la cuestión y para entender en mayor profundidad las bases biológicas de la fragilidad, en el Capítulo II de esta tesis se llevó a cabo una revisión bibliográfica sistemática de estudios poblacionales en personas de 60 años o más publicados hasta la fecha en los que se estudia la posible asociación entre fragilidad y biomarcadores de estrés oxidativo, alteraciones genómicas, y reparación del ADN. Como resultado de la búsqueda se encontraron 26 estudios, publicados entre 2006 y 2017, que cumplían los criterios de inclusión y exclusión establecidos. De estos 26 estudios, 8 evaluaron biomarcadores de estrés oxidativo, mientras que 17 incluían biomarcadores genómicos. Además, un estudio evaluó biomarcadores tanto de estrés oxidativo, como genómicos y de reparación del ADN.

De los nueve estudios que relacionan fragilidad y estrés oxidativo, cuatro midieron la capacidad celular antioxidante total o de algún antioxidante específico. Mientras que siete de ellos midieron los efectos directos de las ROS (*reactive oxygen species*) en lípidos (71%), proteínas (57%), y ADN (29%). Además, en dos de ellos se determinaron los niveles d-ROM. (*derivatives of reactive oxygen metabolites*). En cuanto a la relación entre biomarcadores genómicos y fragilidad, de los 17 estudios incluidos en la revisión, seis de ellos (35.3%) evaluaron el contexto genético de individuos frágiles, incluyendo variantes tanto del ADN nuclear (tres estudios) como mitocondrial (otros tres estudios), ocho (47%) investigaron la relación entre inestabilidad genómica y fragilidad, y tres (17%) estudiaron las posibles características epigenéticas del estado de fragilidad. De los ocho estudios que evaluaron biomarcadores de inestabilidad genómica, siete analizaron la longitud telomérica, el octavo estudio analizó la frecuencia de micronúcleos (MN) en linfocitos.

Los resultados de esta revision mostraron que varios biomarcadores de estrés oxidativo, incluyendo sistemas antioxidantes, aumento de los niveles de peroxidación lipídica y daño oxidativo en el ADN, así como metilación del ADN y algunos polimorfismos genéticos específicos, están asociados con el estado de fragilidad en personas mayores. Por el contrario, la inestabilidad genómica, o al menos los dos biomarcadores estudiados hasta el momento – MN y longitud telomérica – no parece estar asociada a la fragilidad. El único estudio que ha evaluado la posible relación entre fragilidad y reparación del ADN tampoco encontró ninguna asociación.

A pesar del número de alteraciones orgánicas inicialmente asociadas con la fragilidad, todavía hay muy pocos estudios y se limitan a unas pocas de las posibles dianas celulares. Por lo tanto es necesaria una mayor investigación encaminada a la exploración de estas alteraciones antes de ser utilizadas como biomarcadores para la identificación de individuos frágiles. Sin embargo, dada la fuerte relación entre la inestabilidad genómica, la capacidad de reparación del ADN y la edad, así como enfermedades relacionadas con el envejecimiento, no se debería descartar esta línea de investigación.

En base a ello, para el estudio epidemiológico se decidió evaluar biomarcadores que atendieran a diferentes dianas a nivel celular. Para el estudio de la inestabilidad genómica se eligió el ensayo de MN, ya que la frecuencia de estas alteraciones es un biomarcador de inestabilidad genómica bien establecido. Este ensayo se realizó en linfocitos, consideradas buenas células indicadoras (reveladoras de lo que sucede en el resto del organismo por sus propiedades circulantes), y en células de exfoliado bucal, obtenidas mediante un procedimiento no invasivo. Además, en el estudio de la mucosa bucal se evalúan otras alteraciones (citoma) que han sido previamente utilizadas como biomarcadores de daño en el ADN (yemas nucleares), defectos en la citoquinesis (células binucleadas), alteraciones en el potencial de proliferación (frecuencia de células basales), y anomalías en la muerte celular (células picnóticas, cariorréxicas, cariolíticas o con alteraciones en la condensación de la cromatina).

El estudio de la relación entre fragilidad y mutagenicidad se llevó a cabo mediante el ensayo de mutación del receptor de las células T (TCR). La frecuencia de mutación en el TCR se ha utilizado previamente como biomarcador de biomonitorizacion y como predictor de riesgo de cáncer. El TCR es un complejo formado por proteínas integrales de la membrana plasmática que participa en la activación de las células T en respuesta a un antígeno. Mutaciones en los genes TCR pueden resultar en la expresión fenotípica de células T defectuosas para TCR, y por lo tanto en una deficiencia en la respuesta de las células T.

El ensayo del cometa y el ensayo de fosforilación de la histona H2AX, se emplearon para estudiar la posible relación entre fragilidad y daño en el ADN. El ensayo del cometa está basado en una electroforesis en microgel de una suspensión celular tras su lisis. El material genético que presente roturas será capaz de migrar a través de los poros del gel por su carga negativa, adquiriendo finalmente la célula la forma de un cometa. Este ensayo es capaz de detectar un amplio espectro de lesiones primarias en el ADN mientras que la γH2AX identifica un tipo de daño específico, las roturas de doble cadena. Como respuesta a las roturas de doble cadena tiene lugar la fosforilación del extremo C-terminal de las histonas H2AX que se encuentran en las proximidades de las roturas, como un mecanismo de respuesta temprana al daño en el ADN. De esta forma, evaluando los niveles de γH2AX es posible cuantificar las roturas de doble cadena en el ADN y/o estudiar la respuesta temprana a un tipo específico de daño genético.

Finalmente, se decidió estudiar la posible relación de la fragilidad con alteraciones en la capacidad de reparación del ADN, utilizando para ello el ensayo de competencia de reparación, basado en el tratamiento de las células (linfocitos periféricos) con un agente genotóxico conocido (bleomicina). Tras permitir la reparación durante un periodo de tiempo prefijado, se evalúa el daño remanente en las células mediante el ensayo del cometa.

Sin embargo, para poder llevar a cabo el ensayo γH2AX fue necesario realizar un estudio previo para determinar las condiciones experimentales óptimas para su aplicación en estudios poblaciones, debido a la gran diversidad de condiciones empleadas en los estudios recogidos en la bibliografía, que dificultan la reproducibilidad del mismo y la comparación de los resultados entre diferentes laboratorios. Este trabajo se recoge en el Capítulo III de esta Tesis. Se testó el uso de linfocitos frescos *vs.* congelados, así como de linfocitos estimulados *vs.* no estimulados, frente a diferentes concentraciones de cuatro agentes genotóxicos con diferentes mecanismos de acción: bleomicina (BLM), agente radiomimético que actúa de forma directa; camptotecina (Campt), genotóxico indirecto que causa roturas de cadena simple que se convierten en roturas de doble cadena durante la replicación; actinomicina D (Act-D), agente intercalante; y metilmetanosulfonato (MMS), agente alquilante que produce roturas de cadena simple.

En los resultados obtenidos se pudo observar que, en el caso de la BLM, para todas las condiciones testadas (linfocitos frescos y congelados, estimulados y no estimulados) se obtuvo un incremento del %γH2AX respecto al control negativo. Los resultados fueron similares para los linfocitos frescos tratados con Campt y Act-D. Sin embargo, en los linfocitos congelados únicamente se observó un incremento significativo en las células estimuladas tratadas con la concentración más alta de Campt y todas las concentraciones de Act-D. En el caso del MMS, sólo los linfocitos congelados estimulados tratados con la mayor concentración mostraron un aumento significativo del %γH2AX, no así los no estimulados ni los frescos.

De acuerdo con los resultados obtenidos, tanto los linfocitos estimulados como los no estimulados se pueden emplear en ensayo de fosforilación de la histona H2AX. Sin embargo, cuando no se estimulan, las células se encuentran en estado quiescente y, por lo tanto, se debe tener en cuenta que las roturas de doble cadena, o más exactamente la respuesta temprana de reparación que se está evaluando, son consecuencia del daño directo sobre el ADN, mientras que en células estimuladas, que se encuentran en proliferación, las roturas de doble cadena que se están analizando puede formarse a partir de otros tipos de daño que se hacen detectables durante la división celular. Por lo tanto, la decisión de estimular o no los linfocitos antes de realizar el ensayo de la γH2AX se debe de tomar en función del tipo de daño que quiera evaluar o que se espere en los individuos del estudio.

Por otra parte, en los estudios poblaciones con humanos no siempre es posible realizar la recogida de muestras y su procesado de forma inmediata. En estos casos, la criopreservación parece ser la mejor solución a este problema. De acuerdo a los resultados obtenidos en este estudio, cuando se utilizan linfocitos congelados para el análisis de la γH2AX, la estimulación de las células es necesaria, ya que el daño basal observado en las células congeladas sin estimular es demasiado alto, probablemente como consecuencia del proceso de congelación y descongelación. En base a todos estos resultados, se decidió utilizar en el estudio poblacional linfocitos de sangre

periférica congelados y estimulados para el análisis de la fosforilación de la histona H2AX como indicador de daño persistente en el ADN.

Como se mencionó con anterioridad, en el Capítulo IV de esta tesis se estudió la posible relación del estado de fragilidad con una serie de indicadores genéticos que han sido previamente empleados como biomarcadores, en una población de 257 personas de 65 años o más, a los cuales se les clasificó como frágiles (34%), pre-frágiles (50.6%) o no frágiles (15.4%), atendiendo al criterio fenotípico de Fried *et al*. Además, se evaluó el estado nutricional y el estado cognitivo de todos los participantes en el estudio, mediante el uso de las escalas Mini Nutritional Assessment-Short Form y la versión española del Mini-Mental State Examination, respectivamente.

La implicación de la inestabilidad genómica, como resultado de un desequilibrio entre el daño producido en el ADN y los mecanismos de reparación, en fenotipos relacionados con la edad ha sido previamente descrita. Además, el ensayo de MN es uno de los más comúnmente utilizados para evaluar daños cromosómicos, siendo la frecuencia de MN un biomarcador de inestabilidad genómica ampliamente reconocido y empleado en diferentes tejidos. El ensayo de MN con bloqueo de la citoquinesis en linfocitos de sangre periférica se utiliza habitualmente para la evaluación de daño en el ADN en estudios de biomonitorización de humanos expuestos a agentes genotóxicos. Además, la frecuencia de MN está fuertemente asociada al proceso de envejecimiento, y se han observado incrementos de este biomarcador en enfermedades relacionadas con la edad. Por otra parte, el ensayo de MN en células bucales es un interesante candidato para el estudio de poblaciones humanas debido a que, además de permitir la identificación de las anomalías indicativas de otros tipos de alteraciones celulares previamente mencionadas, presenta un carácter no invasivo para la toma de muestras.

Los resultados obtenidos mediante análisis estadísticos univariantes mostraron un incremento progresivo de la frecuencia de MN en linfocitos y células bucales binucleadas con el estado de fragilidad. Por el contrario, se observó a su vez un descenso de la frecuencia de células bucales picnóticas en el grupo frágil respecto a los otros dos grupos, y de las células cariolíticas respecto al grupo de no frágiles. Además, se obtuvieron incrementos altamente significativos en la frecuencia de MN en linfocitos en individuos positivos para el criterio “baja actividad física”. Los análisis multivariantes, ajustando por edad, sexo y consumo de tabaco, confirmaron los resultados anteriores.

Por lo tanto, este estudio muestra una posible asociación entre la frecuencia de MN en linfocitos con el estado de fragilidad, al contrario que el único estudio relacionado hasta la fecha. Además, el incremento en la frecuencia de células bucales binucleadas podría indicar a defectos de la citoquinesis en individuos frágiles, mientras que el descenso de las células picnóticas y cariolíticas muestra alteraciones en la muerte celular en individuos frágiles. Todo esto, junto al

hecho de que en nuestro estudio no se encontraron células basales, sugiere que estos individuos presentan una menor capacidad de regeneración de la mucosa bucal.

El estudio de la posible influencia del estado nutricional y el estado cognitivo sobre los parámetros estudiados mostró que los individuos malnutridos o en riesgo de malnutrición presentaban valores significativamente más altos de MN en linfocitos y células bucales binucleadas, y frecuencias de células picnóticas significativamente menores, que aquellos con un estado nutricional normal. Resultados equivalentes se obtuvieron en individuos con deterioro cognitivo respecto a los sujetos con un estado cognitivo normal. Esto sugiere una posible influencia tanto del estado nutricional como del estado cognitivo en los resultados obtenidos en el estudio de la relación con el estado de fragilidad.

Los datos obtenidos del estudio de mutagenicidad, daño primario del ADN y capacidad de reparación celular no mostraron asociación con el estado de fragilidad. Este es el primer estudio, para nuestro conocimiento, que evalúa la relación de estos dos parámetros con la fragilidad, y el segundo en evaluar la capacidad de reparación del ADN. A pesar de que los resultados de la capacidad de reparación muestran una tendencia a descender con la severidad de la fragilidad, no se llegan a observar diferencias significativas, lo que concuerda con los resultados obtenidos previamente.

El análisis de los niveles de γH2AX mostró una asociación con la fragilidad. Además al estudiar su relación con cada uno de los criterios de fragilidad, se observaron resultados paralelos a los obtenidos en el análisis de MN, obteniéndose una importante contribución de la actividad física, velocidad de desplazamiento y fuerza de prensión, mientras que la pérdida de peso involuntaria y el agotamiento apenas contribuyen a la variación del %γH2AX y a la frecuencia de MN. Estos resultados dan mayor apoyo a la relación entre el daño genético fijado y la fragilidad, sugiriendo además que una combinación de criterios fenotípicos y biomarcadores puede mejorar la identificación de la fragilidad.Considerando conjuntamente los resultados de la frecuencia de MN en linfocitos y el ensayo γH2AX, la hipótesis de la existencia de una conexión entre la inestabilidad genómica, entendida como daño genético fijado, y el estado de fragilidad parece plausible y apoyada por los datos de este trabajo. Puesto que ambos biomarcadores, las tasas de γH2AX y MN, aumentaron significativa y progresivamente con la fragilidad, podrían proponerse como herramientas para la identificación o predicción de fragilidad. Sin embargo, la validación posterior de estos resultados es necesaria para su confirmación. Por otra parte, los niveles de γH2AX resultaron alterados tanto en sujetos pre-frágiles como en frágiles, mientras que la frecuencia de MN únicamente se incrementó en el grupo de frágiles. En consecuencia, la combinación de ambos parámetros podría proporcionar información útil sobre la severidad de la fragilidad, permitiendo a los clínicos distinguir entre los estados de pre-fragilidad y fragilidad y

ayudándoles así a proporcionar cuidados personalizados. Por tanto, los resultados del presente trabajo pueden contribuir a mejorar las estrategias de cuidados terapéuticos en pacientes mayores. Sin embargo, se requiere profundizar en la investigación en este sentido para probar si estos resultados son consistentes y reproducibles en diferentes poblaciones y mayores tamaños de muestra, para estandarizar estos biomarcadores antes de que puedan ser utilizados en clínica, y para entender completamente la influencia del deterioro cognitivo sobre estos parámetros.

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List of Abbreviations

%RC percentage of repair capacity
γH2AX phosporilated H2AX
53BP1 p53 binding protein 1
8-OHdG 8-hidroxy-2'-deoxyguanosine
Act-D Actinomycin
ADL Activities of daily living
ATC Anatomical therapeutic chemical
ATM Ataxia telangiectasia mutated
BAP Biological antioxidant potential
BH4 5,6,7,8-tetrahydrobiopterin
BLM Bleomycin
BMI Body mass index
BMNCyt Buccal micronucleus cytome
BN Binucleated cells
Campt Camptothecin
CBMN Cytokinesis-block micronucleus
CES-D Center for epidemiological studies-depression
cf-DNA Cell free DNA
CHS Cardiovascular health study
CI Confidence interval
Cond-chrom-B Condensed chromatin cells in buccal cells
CPT Cell preparation tube
Cyt-B Cytochalasin-B
DDR DNA damage response
DMSO Dimethyl sulfoxide
d-ROM Derivatives of reactive oxygen metabolites
DSB Double strand breaks
E Exhaustion
EDTA Ethylenediaminetetraacetic acid
ELISA Enzyme-linked immunosorbent assay
FACS Fluorescence-activated cell sorting
FBS Foetal bovine serum
FI Frailty Index
FITC Fluorescein isothiocyanate
FRAIL Fatigue, Resistance, Ambulation, Illness and Loss

GDS Geriatric Depression Scale

GI Genomic instability

GSH Glutathione, reduced glutathione

GSSG Glutathione disulfide, oxidized glutathione

HARP Hospital admission risk profile

HAS Healthy ageing sites

HNE 4-hidroxy-2,3-nonenal

HPLC High performance liquid chromatography

HR Homologous recombination

IADL Instrumental activities of daily living

IAGG International Association of Gerontology and Geriatrics

ICAM-1 Intracellular adhesion molecule-1

iPF2 Isoprostane F2

LC Liquid chromatography

LGS Low grip strength

LINE-1 Long interspersed nuclear elements 1

LPA Low physical activity

LpPLA-2 Lipoprotein phospholipase A2

MCP-1 Monocyte chemoattractant protein-1

MDA Malondialdehyde

MDC1 Mediator of DNA damage check point 1

MHC Major histocompatibility complex

MLTA Minnesota Leisure Time Activity

MMS Methyl methanesulfonate

MMSE Mini Mental State Examination

MN Micronucleus

MNA-SF Mini nutritional assessment-short form

MN-B Micronucleus in buccal cells

MN-L Micronucleus in lymphocytes

MR Mean ratio

MRN MRE11-RAD50-NBS1 complex

MS Mass spectrometry

mtDNA Mitochondrial DNA

na Not available

NBUD Nuclear buds

NHEJ Non-homologous end joining

PBL Peripheral blood lymphocytes

PBS Phosphate-buffered solution

PE Phycoerythrin

PHA Phytohaemagglutinin

PI Propidium iodide

PMA phorbol 12-myristate 13-acetate

qPCR Quantitative PCR

RC Repair capacity

ROS Reactive oxygen species

RPMI Roswell Park Memorial Institute Mmeédium

SI Smoking index

SNP Single nucleotide polymorphisms

SOF Study of Osteoporotic Fractures

SSB Single strand breaks

SWT Slow walking time

T/S ratio Mean telomere repeat copy to single gene copy number

TBARS Thiobarbituric binding acid reactive species

TCR T-cell receptor

TCR-Mf TCR mutation frequency

TDNA DNA in the comet tail

TL Telomere length

TTL Total thiol levels

UWL Unintentional weight loss

Vit-E Vitamin E

WHO World Health Organisation

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Chapter I.

Introduction.

1. Introduction

The phenomenon known as population ageing is rapidly occurring at a global level. This process is due to two main causes: the decline of fertility and the rise of life expectancy. This situation is especially pronounced in Europe, where fertility is nowadays below the level needed for the replacement of the population (around 2.1 births per woman) whereas life expectancy at birth is projected to rise in 6-7 years by 2045-2050 (from 77.2 years in 2010-2015). At present, the percentage of population aged 65 or over in Europe is the highest in the world (25%); however, the rapid ageing is not a phenomenon exclusive of Europe. It is expected that all regions of the world, with the exception of Africa, will reach similar percentages of population aged 65 or over by 2050, while Europe could reach 35% (United Nations, 2017). In this context, the 2015 Ageing Report from the European Commission, predicts a dramatic change in the structure of demographic population pyramids. In 2013 the most numerous cohorts were reported to be those around 45 years old, for both males and females. This will change in 2060 in favour of the older people groups (65-80 years old) (Figure I.1).

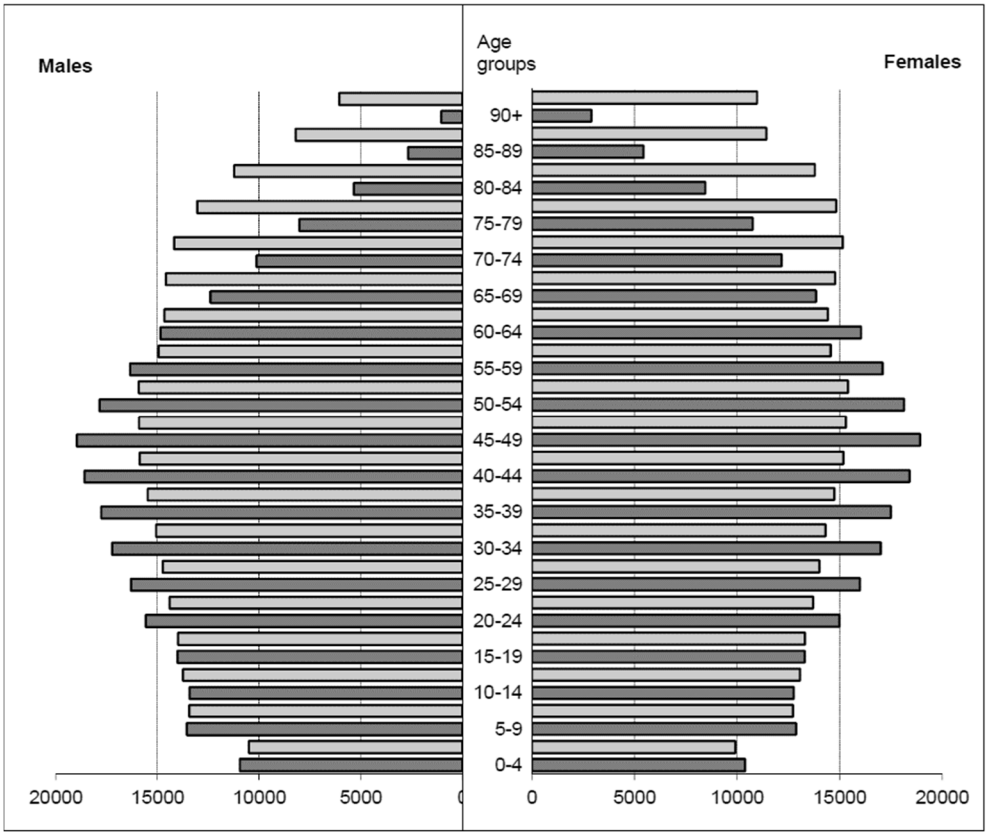

FIGURE I.1. Age structure of the population in 2013 (dark bars) and 2060 (light bars), in the 28 countries of the European Union (modified from The 2015 Ageing Report, Underlying Assumptions and Projection Methodologies, Joint Report prepared by the European Commission (DG ECFIN) and the Economic Policy Committee (AWG), European Economy Series 8/2014).

This increase in longevity does not necessarily mean prolonged health-span and welfare. On the contrary, in many cases old age comes with a high risk of social isolation, poor health and financial privation, accompanied with limited access to affordable, high-quality health care and social services. For this reason, a systematic change in all societies is needed. A comprehensive response, not only in the public-health system but in all social sectors, is required to face the challenges and increased expenditures due to the population ageing, to ensure that the strategies proposed to assist the wellbeing in old age and the healthy ageing process can extend to everyone, regardless where they live or the socioeconomic group they belong to.

In order to achieve this goal, in the last years Europe has followed two main courses of action: (i) developing initiatives (e.g. physical activity, healthy diet, fulfilling social relations, participating in meaningful activities…) to promote healthy ageing, defined by the World Health Organisation (WHO) as *the process of developing and maintaining the functional ability that enables wellbeing in older age*, and (ii) evolving better methodologies for the screening and identification of older subjects in a state of vulnerability, to prevent or diminish the impact of cognitive and functional decline, and to develop specialized healthcare policies and personalized medical assistance.

At a biological level, the ageing process is characterised by a progressive accumulation of a wide range of molecular and cellular alterations that occur in a non-linear or consistent way. For this reason and under the frame of the second course of action previously mentioned, the concept of ***frailty*** has emerged in the last years as a more accurate measure of biological age than the traditional concept of chronological age. Frailty is an important geriatric syndrome with increasing prevalence in advanced age (Topinkova, 2008). Frailty syndrome represents an increased risk of poor health outcomes for those over 65 years old, which offers an interesting and reliable tool to identify people in a state of vulnerability (Cesari *et al.*, 2016). Frailty, especially in its early stages, is potentially reversible, thus early identification of this syndrome may be crucial for the implementation of personalised preventive strategies against age-related conditions (Gill *et al.*, 2006; Espinoza *et al.*, 2012; Roland *et al.*, 2014). Even though the critical time frame for interventions that target frailty has not yet been unmistakably established, frailty prevention should start at early ages in adulthood, and not be exclusively delegated to older age, in order to avoid negative outcomes in the old age (Cesari *et al.*, 2016).

2. Frailty

2.1. Concept and history

As mentioned above, the chronological age criterion traditionally employed for determining whether an individual must be referred to geriatric care or not is no longer reliable (Cesari *et al.*, 2016). Instead, frailty criteria should be used by geriatricians and healthcare professionals in order to provide a proper and personalized care to the individual. However, until this moment there has not been a consensus in an international official definition of frailty, being considered as a condition, syndrome or status depending on the author or publication.

The concept of frailty appeared for the first time in the research literature in 1968 in a study carried out by O'Brien et al. In this study the authors outlined the gradual development of frailty as an excessive and disproportionate reaction of subjects to adverse events. However, it was not until 1988 that a first quantitative measure of frailty was established by Winograd et al. According to their operational definition, frail older adults had one or more of 15 common geriatric clinical conditions including malnutrition, depression, impairment of activities of daily living, incontinence or confusion. Since these pioneer studies, frailty has long been considered synonymous of disability and comorbidity, to be highly prevalent in old age and to confer a high risk for falls, hospitalization and mortality (Lang *et al.*, 2009).

The quantitative and qualitative change in frailty concept comes at the beginning of this century with two independent studies. Firstly, Fried and colleagues (2001) introduced a phenotypical definition of frailty, defining it as the display of three or more out of five physiological deficits (muscle weakness, low gait speed, unintentional weight loss, exhaustion, and low physical activity). Those people presenting one or two of these deficits were classified as pre-frail. Closely, Mitnitski, Rockwood, and colleagues (Mitnitski *et al.*, 2001; Rockwood *et al.*, 2005) defined frailty as the cumulative effect of individual deficits occurring with ageing that include symptoms (e.g., low mood), signs (e.g., tremor), abnormal clinical laboratory values, disease states, and disabilities. The more deficits present in an individual, the more likely to be frail (Rockwood and Mitnitski, 2007).

Throughout all this time to present, the definition of frailty has evolved from a description of a state of dependency to a more dynamic model that encompasses biomedical and psychosocial aspects (Lang *et al.*, 2009). Consequently, numerous definitions and measurements of frailty have arisen in the literature in these last years. However, these two previously described criteria, Fried's and Rockwood's, or their variants, are still the most accepted and used ones.

Trying to reach a consensus, in 2008, the International Academy of Nutrition, Health and Aging postulated a new definition of frailty combining components of both Rockwood's and Fried's definitions in the acronym "FRAIL": **F**atigue, **R**esistance (cannot climb one flight of

stairs), **A**mbulation (cannot walk one block), **I**llnesses (more than five) and **L**oss of weight (>5% over one year or less) (Abellan van Kan *et al.*, 2008). Later, in 2013, a group of experts from both North America and Europe released a consensus on the definition of physical frailty that suggests it is an important medical syndrome, caused by multiple factors, characterized by decreased strength, endurance and physiological function, which increases the vulnerability of a person for greater dependency and/or death (Morley *et al.*, 2013).

Several international groups, including the WHO and the International Association of Gerontology and Geriatrics (IAGG), are currently working on an internationally accepted standard frailty definition (Dent *et al.*, 2016). Nevertheless, there is no consensus yet, although there is an increasing tendency to consider not only physical criteria but also cognitive and sensorial loss, and even biological parameters.

2.2. Prevalence

The prevalence of frailty varies largely between studies mainly due to the different tools employed to identify frailty, but also because of the own features (gender, age, ethnic…) of the populations considered. Collard *et al.* (2012) carried out a systematic review to estimate the prevalence of frailty in the elderly including data from 61,500 older adults from 21 different studies. The reported prevalence varied substantially across studies, ranging from 4.0% to 59.1%.

When analyses were restricted to studies using the phenotype model proposed by Fried and colleagues (2001), the weighted average prevalence was 9.9% and 44.2% for frailty and pre-frailty, respectively. And even using the same criteria, factors as gender, race, or socioeconomic conditions have been reported to influence this prevalence, with higher values in women compared with men (Collard *et al.*, 2012; Theou *et al.*, 2015), in Hispanic and African Americans regarding other ethnic groups (Espinoza and Hazuda, 2008), and in people showing limited education and poverty regarding other more socially favourable populations (Fried *et al.*, 2001). A recent cross-sectional study including 331 Spanish institutionalized older people of both genders, showed that the prevalence of frailty reached 68.8% (González-Vaca *et al.*, 2014).

2.3. Frailty identification

Currently, several tools are used in clinics for frailty screening in older adults. As previously mentioned, the two most well-known instruments to identify frailty are the phenotypic model proposed by Fried and colleagues (2001), and the deficit accumulation model developed by Rockwood, Mitniski and collaborators (Mitniski *et al.*, 2001; Rockwood *et al.*, 2005). The model proposed by Fried *et al.* is based on five criteria that include shrinking or unintentional weight loss, muscular weakness, self-reported exhaustion, slow walk and low physical activity level. Those individuals with three or more of those criteria are considered frail, while those with

one or two are considered pre-frail, and those individuals who do not show any of those are considered non-frail or robust (Fried *et al.*, 2001).

The cumulative model proposed by Rockwood and Mitniski (Mitniski *et al.*, 2001; Rockwood *et al.*, 2005), also called frailty index (FI), is calculated as the ratio between the number of deficits the individual presents divided by the total of deficits considered in the computation. The deficits evaluated are a total of 92 parameters that include physical criteria, neurological examinations, psychological symptoms, and clinical laboratory values, among others. FI is a simple calculation of the presence or absence of each variable as a proportion of the total.

These two instruments are evidently very different in their constructs, but also in their objectives. In particular, frailty phenotype is more focused on screening the physical domain of frailty, while the deficit accumulation model stems from the results of a comprehensive geriatric assessment. Indeed, FI was previously suggested to better identify older adults at high risk for adverse outcomes than the frailty phenotype in the early stages of frailty (Blodgett *et al.*, 2015). Still, a recent study compared these two approaches and confirmed their agreement and their association with falls and overnight hospitalizations (Zhu *et al.*, 2016).

In another study, the capacity to predict future disability and mortality of four different frailty models – including again Fried criteria and FI, plus the FRAIL model proposed by the International Academy of Nutrition Health and Aging, and the SOF (Study of Osteoporotic Fractures) frailty scale – were compared in a longitudinal study on an African American population (Malmstrom *et al.*, 2014). Together with validating the use of FRAIL scale in clinical practice, results from this study concluded that FI and the FRAIL scale exhibited the strongest predictive validity for disability and mortality.

Together with the two more common models, there are many other screening tools to identify frailty, including a number of variants of these pioneer ones. For instance, in a recent review the existence of more than 260 different versions of the frailty phenotype published in the literature was reported. And even though all of them might potentially identify frailty, the modifications introduced in the original phenotype criteria had important impact on its classification and predictive ability (Theou *et al.*, 2015). This observation was later confirmed by Dent *et al.* (2016), who published another complete review collecting all the frailty measurements employed to date to identify this syndrome, and also pointed out that many frailty measurements were modified somewhat from their original validated version, and that this could eventually have a striking impact on frailty classification. Dent *et al.* (2016) reviewed a total of 422 studies classifying older adults (aged ≥65 years) according to frailty status by employing any method, thus reporting 29 different frailty instruments. Fourteen out of these 29 instruments were previously validated to be used in older people and they were deeply compared, concluding that

there is no "one" perfect frailty measurement in existence today; some measurements are better for population-level frailty screening and others are more suitable for clinical use. In that study, authors also claimed the necessity of unifying criteria, or even developing a new gold frailty measurement, in order to establish a standard measurement for frailty, especially to be employed in clinical practice, and to make the different studies comparable. This would also help to know the actual frailty prevalence worldwide. But, as it was also pointed out, several reasons would explain why reaching this objective is so complicated, including the complex frailty aetiology, the differences between populations, or the inherent difficulty in distinguishing frailty from both ageing and disability, among others.

Until a standard criterion to identify frailty is adopted, the choice of the most appropriate frailty instrument has been suggested to rely on the purpose of the evaluation, the outcome for which the definition was originally validated, the validity of the tool, the studied population, and the setting in which the assessment will be conducted (Cesari *et al.*, 2016).

Still, despite the high number of available instruments to measure frailty and their different bases and criteria, it seems that the predictive value of frailty for negative health outcomes, including falls, hospitalizations, disability, institutionalization, and mortality, is consistently confirmed across assessment instruments, target populations, and settings (Fried *et al.*, 2009; Clegg *et al.*, 2013; Theou *et al.*, 2013).

Even though all efforts focused on establishing a common definition and a more accurate measurement of frailty, the truth is that the one proposed by Fried and collaborators is, even nowadays, the most worldwide extended and employed criteria to identify frail individuals in clinical practice and research. Indeed, the 'Frailty Task Force' of the American Geriatrics Society adopted the suggestion of Fried *et al.* as the best current working definition (Lang *et al.*, 2009).

This working definition of frailty, based on Fried's criteria, is very useful; however, it is only based on physical symptoms and signs. It neglects other potentially important components of the syndrome such as mood, cognition, sensory impairments and socioeconomic aspects of older adults' lives (Abellan van Kan *et al.*, 2008; Zaslavsky *et al.*, 2013). Moreover, no biological markers are included in the frailty syndrome defined by Fried *et al.* (2001). Numerous researchers have argued that frailty is a multidimensional and multisystem process that cannot be comprehensively captured by applying physical criteria only (Dent *et al.*, 2016; Zaslavsky *et al.*, 2013).

Consequently, in the last years, more and more authors agree with the fact that it is not satisfactory to define frailty in the physical domain only, highlighting the need for searching for other markers of frailty at different levels, and even several publications started to address

separately physical and cognitive frailty (Dulac and Aubertin-Leheudre, 2016; Kelaiditi *et al.*, 2013; Woods *et al.*, 2013).

2.4. Biological basis of frailty

Although, frailty was initially linked to physical decline and considered as synonymous of disability or comorbidity, currently it is becoming recognized as a distinct clinical syndrome with a biological basis (Lang *et al.*, 2009). Indeed, frailty is commonly accepted to have a strong biological component that result from cumulative cellular damage over the life-course (Dent *et al.*, 2016). As people age, many systems and processes can be modified (reviewed in Fielding, 2015). Similarly, a number of physiological processes/functions have been demonstrated to be altered in frail subjects (reviewed in Walston, 2004).

However, the specific pathophysiological changes involved in frailty aetiology remain undefined. This is mainly due to the fact that no single system impairment characterizes frailty. Instead, it seems that an intertwined network of biological anomalies at different levels is likely to be part of the pathophysiological chain of events leading to frailty (Zaslavsky *et al.*, 2013). In 2009, Lang *et al.* already highlighted the importance of improving our understanding on the complex biological factors leading to age-related muscle loss (sarcopenia, a typical clinical sign in frail subjects) beyond those attributable to a simple decrease in physical activity and to deleterious chronic undernutrition.

All the processes or physiological functions known to be altered in frail patients can be grouped into three different dimensions according to the organizing level affected (Figure I.2). At the cellular level, frailty status has been linked to deficiencies in cellular repair ability and consequent DNA damage accumulation (Dent *et al.*, 2016). The biological consequences of increased levels of this damage can be wide ranging, including altered gene expression, genomic instability, mutations, loss of cell division potential, cell death, impaired intercellular communication, tissue disorganization, organ dysfunctions, and increased vulnerability to stress and other sources of disturbance (Rattan, 2006). At the systematic level, more and more evidence suggests that frailty-associated physiological dysregulation involves multi-organ systems, including the musculoskeletal, immune, endocrine, hematologic, and cardiovascular systems (Fried *et al.*, 2009). Finally, as more systems show abnormal function, frailty severity increases, and all these dysregulations, although initially silent, become physically evident, affecting the whole organism and showing up as the clinical signs of frailty: muscle mass loss, cognitive impairment and sensorial loss, among others.

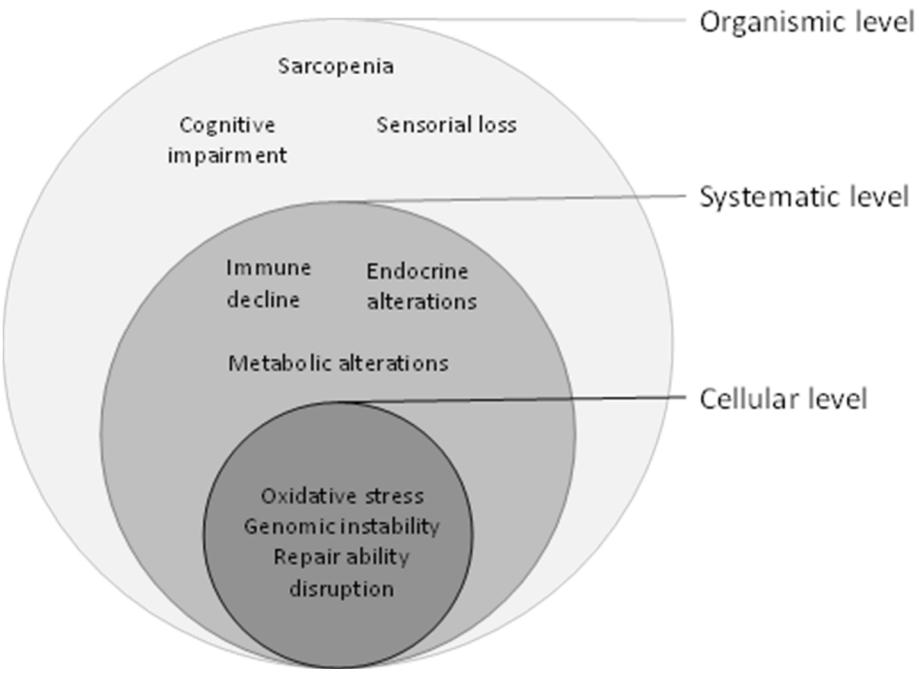

FIGURE I.2. Frailty biological levels.

Recent research efforts have helped to better define the clinical and physiological characteristics of frailty (Lang *et al.*, 2009; Sieber, 2017). However, and despite these last evidences on the biological basis of frailty, up to now no biological feature has been validated to be used as a useful biomarker to identify frailty status.

3. Biomarkers

3.1. Genomic instability

As mentioned above, many physiological processes altered in frail individuals as well in many age-related diseases (cancer, neurodegenerative disorders) and ageing signs are related to a dysregulation between DNA damage and the correct function of cellular DNA repair mechanisms. This loss of balance can lead to a gradual destabilization of the genomic integrity, which is known as genomic instability (GI), one of the ageing process hallmarks (Garm *et al.* 2013; Gorbunova and Seluanov, 2016; Li *et al.* 2016a; Fischer and Riddle, 2017). Cytogenetic assays are often used to detect GI; among them the micronucleus (MN) test is one of the most widely accepted (Maslov and Vijg, 2009). Micronuclei (MN) originate from chromosome acentric fragments or whole chromosomes that lag behind at anaphase during nuclear division (Figure I.3). During telophase, these fragments and whole chromosomes are surrounded by a nuclear envelop, acquiring a morphology similar to a small interphase nucleus or MN. Hence, the presence of MN in the cell cytoplasm is indicative of chromosome damage (Fenech, 2000) and the MN assay provides a reliable measure of both chromosome damage and chromosome loss.

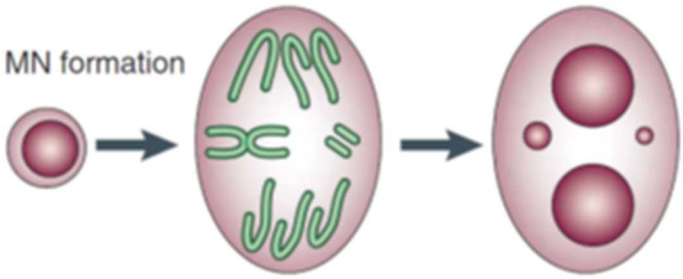

FIGURE I.3. Micronuclei formation from a whole chromosome or chromosome fragments (modified from Fenech *et al.*, 2007).

3.1.1. The cytokinesis-block micronucleus assay

Since one cell division is necessary for the MN to be displayed, it is essential to discriminate those cells that have undergone one mitosis to be considered for the MN scoring. The cytokinesis-block micronucleus (CBMN) assay, firstly described by Fenech and Morley in 1985, allows to identify cells that have experienced a cell division due to their appearance as binucleated cells after blocking cytokinesis with cytochalasin-B (Cyt-B) (Figure I.4). This chemical is an inhibitor of microfilament ring assembly required for the completion of cytokinesis (Fenech, 2007); hence, in its presence cells divide their nuclei normally (mitosis), but cytoplasm division is avoided, thus appearing as binucleated. MN frequency in peripheral blood lymphocytes (PBL) is a reliable measure and widely employed in molecular epidemiology and cytogenetics to evaluate chromosomal damage in human populations (Valdiglesias *et al*, 2015; Hintzche *et al*., 2017). PBL present several biological and practical advantages. These cells are routinely collected and considerable amounts of cells are easily obtained from a small blood sample, cell cycle effects do not interfere since unstimulated lymphocytes are non-cycling (they are quiescent in the G_0 phase of the cell cycle), show reproducible results, and have been demonstrated to be suitable surrogate cells, since their level of DNA damage reflects the level of genetic damage in other types of cells and tissues (Lee *et al*, 2003; Pardini *et al.*, 2017). For these reasons, MN frequency in peripheral blood lymphocytes evaluated by the CBMN assay has traditionally been one of the most used tests for evaluation of chromosomal alterations and is considered a reliable biomarker of GI (Fenech *et al.*, 2007; Bonassi *et al*, 2011a).

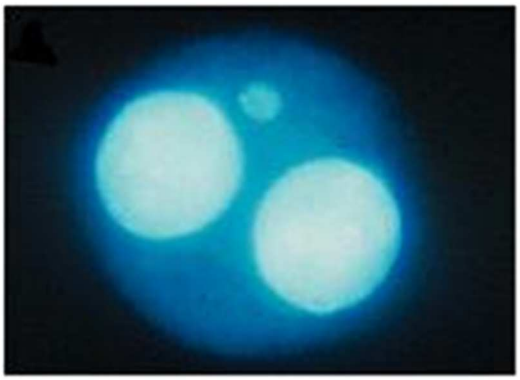

FIGURE I.4. Fluorescence microscopy image of a binucleated lymphocyte with a micronucleus.

It has been previously reported that the ageing process is highly associated with the MN frequency. Also age-related disorders, like cancer, arthrosis, cardiovascular disease and diabetes alongside neurodegenerative diseases (i.e. Alzheimer's or Parkinson's) are characterised by an increase in the MN frequency (Petrozzi *et al.*, 2002; Andreassi *et al.*, 2011; Bonassi *et al.*, 2011; Migliore *et al.*, 2011; Corbi *et al.*, 2014; Franzke *et al.*, 2014). Moreover, high MN frequency in PBL of healthy subjects has been shown to reflect a higher risk of developing cancer later in life, suggesting a predictive role of this biomarker (Bonassi *et al.*, 2007).

3.1.2. The buccal MN cytome assay

In recent years a new alternative assay to evaluate MN frequency as a biomarker of GI in population studies has emerged: the buccal MN cytome (BMNCyt) assay (Figure I.5). This assay was firstly proposed in 1983 by Stich and Rosin, and since then it has been used to assess the impact of nutrition and lifestyle factors (e.g., alcohol, smoking, drugs, and stress) as well as exposure to genotoxic agents. It has also been studied in cancer-associated congenital syndromes, such as ataxia telangiectasia or Bloom's syndrome, and other disorders also characterised by defects in the DNA repair processes (reviewed in Bolognesi *et al.*, 2015). The buccal epithelium is the first barrier for the inhalation or ingestion, main absorption routes of carcinogenic agents; for this reason, buccal cells are likely to be highly exposed to genotoxic agents (Holland et al, 2008). In this context, the BMNCyt assay is nowadays commonly employed in biomonitoring studies assessing environmental or occupational exposures to genotoxic agents (Bonassi *et al.*, 2011b; Benedetti *et al.*, 2013; León-Mejía *et al.*, 2014). Besides, it has been reported an existing strong correlation of MN frequency in buccal exfoliated cells with MN frequency in lymphocytes (Ceppi *et al.*, 2010). This means that buccal cells can also report systemic genotoxic effects present in the bloodstream (Bonassi *et al.*, 2011b).

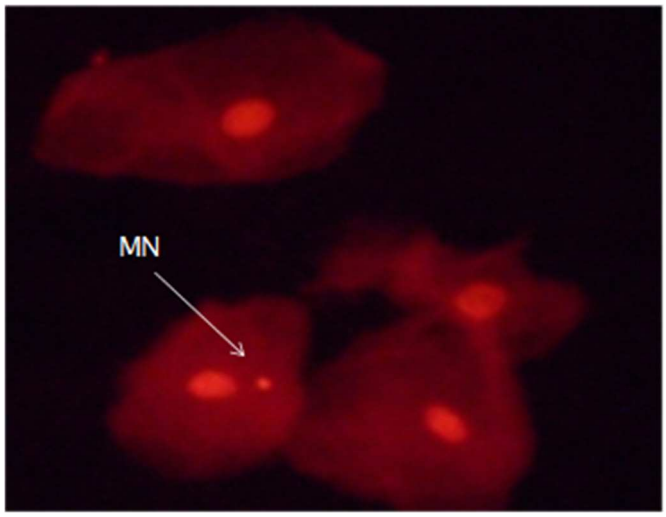

FIGURE I.5. Fluorescence microscopy image of a micronucleus in buccal cells.

In addition, the BMNCyt assay presents a series of advantages. It is a non-invasive and relatively simple technique that do not involve cell culturing, and, therefore, it is well suited for large biomonitoring studies (Bonassi *et al*, 2011b). Moreover, BMNCyt assay allows the evaluation of a variety of complementary toxicological events related to cell proliferation, differentiation and cell death. Hence, the BMNCyt assay provides several biomarkers associated with increased risk of accelerated ageing, cancer and neurodegenerative diseases (Holland *et al*, 2008; Thomas *et al.*, 2009).

The oral epithelium is composed of four strata of structural, progenitor, and maturing cell populations (Figure I.6). These strata, from base to surface, are (i) the lamina propria, connective tissue or *stratum germinativum*, (ii) the basal cell layer or *stratum basale*, (iii) the prickle cell layer or *stratum spinosum*, and (iv) the keratinised layer or *stratum corneum*. The basal cell layer is continuously producing new cells by mitosis that migrate to replace the cells that are constantly shed as a result of the wear and tear of the surface tissue. The stem cells present in the basal cell layer may express DNA damage as chromosome breakage or loss during nuclear division that may or may not originate MN (Thomas *et al.*, 2009).

During this renewal cell process of oral epithelium, several types of cells and nuclear anomalies are originated. For this reason, BMNCyt assay is a useful tool that provides biomarkers for DNA damage (MN and/or nuclear buds), cytokinetic defects (binucleated cells), proliferative potential (basal cell frequency) and/or cell death (condensed chromatin cells, karyorrhectic cells, pyknotic cells and karyolytic cells) (Thomas *et al.*, 2008, 2009).

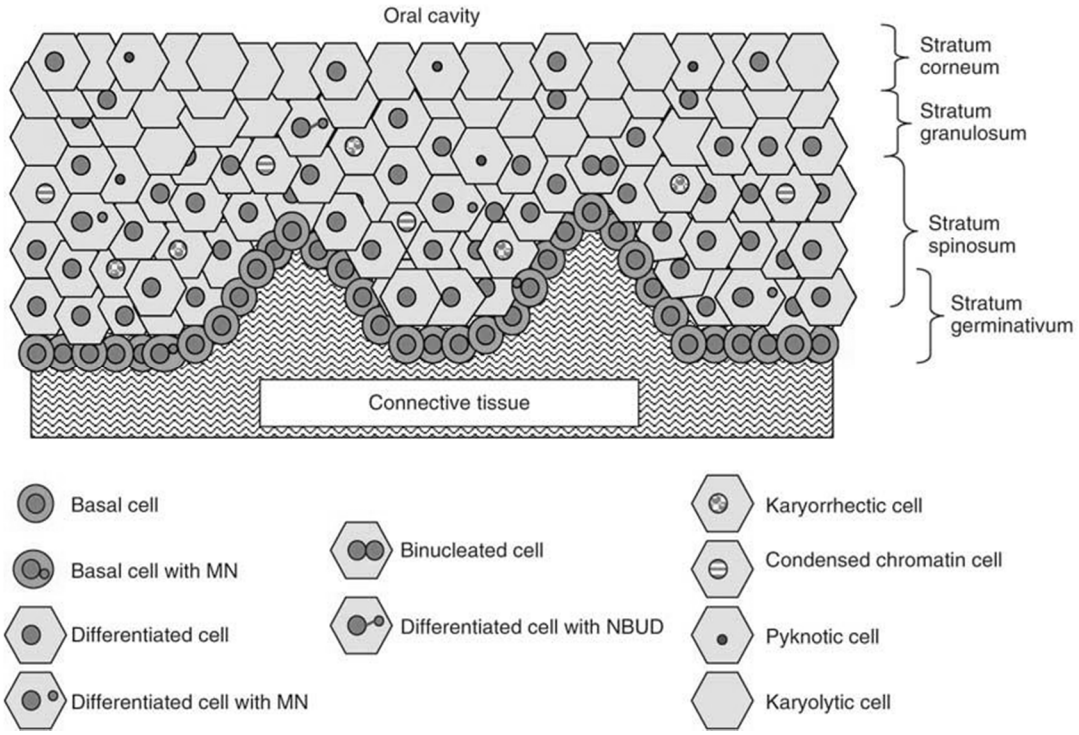

FIGURE I.6. Diagrammatic representation of the different layers and cell types conforming the oral epithelium (source: Thomas *et al.*, 2009).

3.2. T-cell receptor mutation assay

The T-cell receptor (TCR) is a heterodimeric cell surface receptor, composed by alpha and beta chains, present in almost every mature T lymphocyte (Kronenberg *et al.*, 1986). These alpha and beta chains contain each, one constant and one variable domain to specifically recognise an enormous number of peptide antigens bound to and presented by major histocompatibility complex (MHC) proteins (Hou *et al.*, 2016). The recognition of the antigenic peptide/MHC complexes by TCR is a crucial step in the activation and regulation of the adaptive immune response (Li *et al.*, 2016b).

Mature T cells are believed to experience a mechanism of allelic exclusion similar to the one which occurs to immunoglobulin genes in B lymphocytes; as a consequence, they only actively express one of the two TCR alleles (they are phenotypically hemizygous) (Kronenberg *et al.*, 1986). Hence, a single mutation at the functional TCR gene will lead to the absence of the phenotypic expression of TCR in the cell surface (Kyoizumi *et al.*, 1992). Moreover, TCR does not possess itself signalling domains, it needs to form a non-covalent bound with the surface protein CD3 (Figure I.7). T cell mutants lacking one or more of the TCRαβ chains are able to form TCR/CD3 complexes in the cell cytoplasm but are unable to transport these complexes to the cell membrane surface (Clevers *et al.*, 1988).

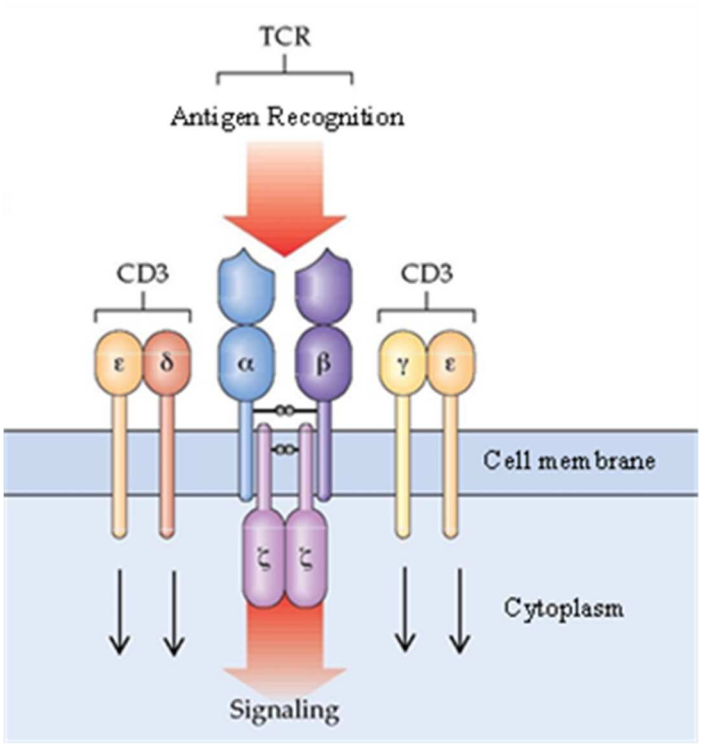

FIGURE I.7. Diagrammatic representation of the structure of TCR/CD3 complex, involved in antigen recognition and T-cell activation.

Mutations in any of the TCRαβ chains can be detected by flow cytometry employing antiCD3 specific antibodies. This technique allows to identify and quantify TCRαβ mutants ($CD3^-$ cells) within the population of T helper cells (Th cells) expressing CD4 ($CD4^+$) (Akiyama *et al.*, 1995). Total mutations in TCR genes are considered, without differentiating between α or β chains. Besides, the analysis is relatively rapid and only requires a small sample of fresh whole peripheral blood.

TCR mutation frequency (TCR-Mf) has been previously employed as a biomarker of mutagenicity in occupational exposure biomonitoring studies as well as a predictor of cancer risk (Lanza *et al.*, 1999; Vershenya *et al.*, 2004; Chen et al., 2006; Taooka et al., 2006; García-Lestón et al, 2011, 2012).

3.3. Alkaline comet assay

The single cell gel electrophoresis assay, commonly known as comet assay, is a simple, fast and sensitive technique for DNA damage identification and quantification in single cells (Singh *et al.*, 1988). The comet assay protocol was originally proposed by Ostling and Johanson in 1984 and later modified by Singh *et al.* (1988).

Depending on the pH employed, the comet assay allows detection of several types of DNA damage, such as single and double strand breaks, incomplete excision repair sites, crosslinks, and alkali-labile sites (Collins *et al.*, 2014). The alkaline version of the comet assay is

the most commonly used. In brief, this assay consists of obtaining nucleoids displayed in an agarose layer over a slide by cellular lysis employing a solution containing a detergent, to remove the membranes, and high salt concentrations, to eliminate the nuclear proteins. Subsequently, the nucleoids are incubated in an alkaline solution to facilitate DNA unwinding. After running the electrophoresis, DNA is stained with a fluorescent dye. During the electrophoresis, DNA fragments migrate to the anode wandering off from the nucleoid due to their negative charge, forming a comet shape (Figure I.8). The more damaged the DNA, the farther migration to the anode. Length and intensity of the comet tail is proportional to the number of breaks in the DNA. Not damaged cells will not show a tail.

FIGURE I.8. Fluorescence microscopy image of leucocyte nucleoids after comet assay: A) not damaged nucleoid, B) mildly damaged nucleoid, C) highly damaged nucleoid.

The comet assay is widely and commonly employed to evaluate primary DNA damage. It is used in (i) genotoxicity testing, to screen novel drugs, cosmetics, or chemicals for potential carcinogenic properties, both *in vivo* and *in vitro*, (ii) in human biomonitoring, to evaluate the effects of toxic agent at DNA level, the effect of lifestyle factors, or its involvement in diseases or individual variations, for instance in DNA repair capacity, (iii) in ecogenotoxicology, as a marker of genetic damage by pollutants, and (iv) in basic research into mechanisms of DNA damage and repair (Azqueta and Collins, 2013).

As mentioned before, both ageing and processes involved in frailty development, such as loss of muscle mass, are associated with high levels of DNA damage (Franzke *et al.*, 2015). So far, several authors have studied the relationship of DNA damage and age, by means of the alkaline comet assay, obtaining different results: however, the use of this methodology in population studies on frailty is still an unexplored field.

3.4. γH2AX assay

DNA double strand breaks (DSB) can be the result of several endogenous processes (i.e. normal cellular procedures, senescence, generation of reactive oxygen species) and exogenous exposures (i.e. ionizing radiation or genotoxic compounds) (Mah *et al.*, 2010). Due to the severity of this kind of DNA damage, the organism has DNA damage response (DDR) mechanisms that are quickly initiated at the side of the DSB with the aim of repairing it. The phosphorylation of

the C-terminal of the histone variant H2AX (Figure I.9) is an early response to DNA DSB and an important step in the DDR process that has been employed as a biomarker of DNA damage (Siddiqui *et al.*, 2015).

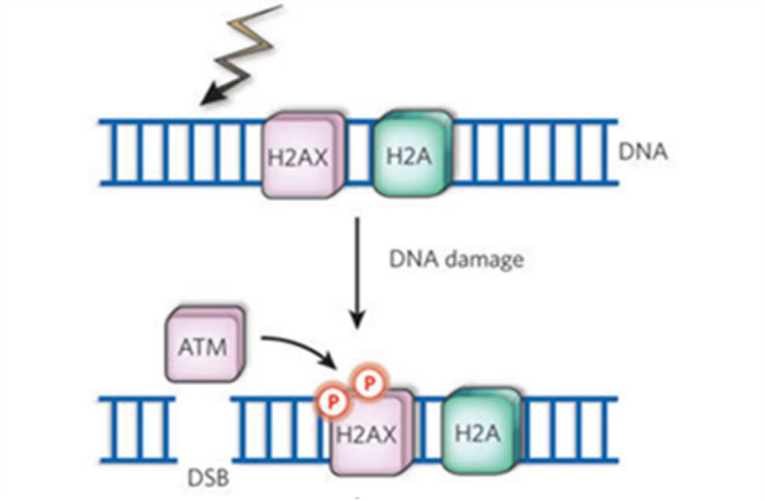

FIGURE I.9. Scheme of H2AX phosphorylation as response to double strand breaks (DSB). ATM, ataxia telangiectasia mutated (modified from Hoeller and Dikic, 2009).

The H2AX histone was firstly reported by West and Bonner in 1980, as a specific variant of the H2A histone family. H2AX is ubiquitously distributed along the genome and differs from the other members of the family by the presence of an evolutionarily conserved C-terminal motif: KKATQASQEY (Rogakou *et al.* 2000; Takahashi and Ohnishi 2005). In response to the formation of DSB, H2AX flanking the DSB sites are rapidly phosphorylated at the serine 139 residue to become γH2AX (Redon *et al.* 2011). For each DSB, a γH2AX focus is formed, making it possible to estimate the number of DNA DSB by measuring the γH2AX foci (Ivashkevich *et al.*, 2011). Hence, the formation of γH2AX foci is a sensitive marker for DSB, for both number and location (Banath and Olive, 2003). Under normal conditions, they appear within few minutes after the lesion, reach maximum levels after about 30 min and then decline and disappear after approximately 24 h (Rogakou, *et al.*, 1999; Bourton *et al.*, 2011). In the main, the half-life of γH2AX foci after DNA damage has been estimated to be 2–7 h (Bouquet *et al.*, 2006). Therefore, H2AX phosphorylation represents an early event in the DNA damage response against DSB and plays a central role in sensing and repairing these lesions (Matsuzaki *et al.* 2010; Scarpato *et al.* 2013). Two different types of γH2AX foci were detected in cells: one is transitory while DSB repair is being carried out, and the other one remains after DSB repair suggesting that it may represent DNA lesions with unrepairable DSB due to cellular senescence (Sedelnikova *et al.*, 2004; Siddiqui *et al.*, 2015).

According to the idea of a gradual accumulation of DNA damage with age leading to a lack of DNA integrity, recent studies have reported the implication of H2AX phosphorylation and DDR in age-related diseases, such as Werner syndrome, Alzheimer's disease, obesity, diabetes,

prostate cancer, hypertension, and Hutchinson-Gilford progeria syndrome (Sedelnikova *et al.*, 2008; Schurman *et al.*, 2012).

However, there is a notable lack of standardization in the methodological procedure used to determine the levels of γH2AX, which leads to a wide heterogeneity in the results obtained and their interpretation. This heterogeneity is mainly due to extensive variability in the experimental procedures, affecting the reliability of the assay. Therefore, it is necessary to standardize γH2AX assay and to determine the main experimental factors influencing its results, in order to establish this technique as a routine biomarker in population studies.

3.5. DNA repair competence assay

DNA repair mechanisms are the cell defence system to protect and maintain the genome integrity. DNA repair involves three main mechanisms: direct reversal of the damage, excision repair – which involves three pathways, according to the type of DNA damage induced: base excision repair, nucleotide excision repair and mismatch repair – and DSB repair, which may be conducted by two routes, depending on the cell cycle phase: homologous recombination and non-homologous end-joining (Walker and Rapley, 1997).

Deficiencies in these systems are often considered the cause of the development of age-related diseases or cancer (Valdiglesias *et al.*, 2011a). It has also been proposed that frailty syndrome might be driven by alterations in the cell repair mechanisms (Dent *et al.*, 2016). DNA repair capacity of human leucocytes has been previously investigated as a biomarker in human biomonitoring and cancer prediction (Bausinger and Speit, 2015).

The DNA repair competence assay, or challenge assay, is a cytogenetic approach to measure the repair competence of cells. In this technique, cells are challenged by exposure to a defined dose of a genotoxic agent that induces a finite amount of DNA lesions (e.g., γ rays or bleomycin). After an additional incubation in fresh medium, during which DNA repair is allowed, the remaining damage is measured by using different cytogenetic techniques (Au, 1993).

The use of comet assay to measure DNA damage in the DNA repair competence assay provides a powerful tool to detect repair ability. Besides, it offers the advantage of quantifying the repair as progress of the DNA damage levels since, in contrast with other cytogenetic techniques such as chromosome aberrations, it allows evaluating the damage in different time points (i.e., after damage induction and after the incubation period in fresh medium) (Rajaee-Behbahani *et al.*, 2001; Schmezer *et al.*, 2001).

On the basis of what was explained in this memory so far, it is necessary to develop biomarkers that may help identify individuals in the early stages of frailty or at risk of developing this syndrome, when it can be prevented or even reverted. Due to their association with ageing and age-related diseases, biomarkers related to genetic outcomes are promising for this objective.

Thus, as the first step of this work, a systematic review of the epidemiological studies published till date evaluating the association of frailty with biomarkers of oxidative stress, genomic alterations and DNA repair was carried out (Chapter II). According to the results obtained in the systematic review, and in order to acquire a better understanding of the biological basis of frailty, a set of biomarkers of genomic instability, and DNA damage and DNA repair was selected to be applied in a cross-sectional study with older adults aimed at determining their possible relationship with frailty status and thus their potential as biomarkers of frailty (Chapter IV). Since one of these biomarkers was phosphorylated H2AX histone, a quite novel assay for DSB evaluation, optimization of the γH2AX assay experimental conditions to be applied to human population studies was necessary (Chapter III).

Objectives.

The rapid ageing of the global populations and the social, economic and health challenges that come with it require a comprehensive response to ensure the wellbeing of the older people through a healthy ageing. Frailty represents an increased risk of poor health outcomes in the old age. In this context, the main objective of this work was to improve the understanding of the biological basis of frailty evaluating alterations at the cellular level in a population of people aged 65 years and over classified according to their frailty status following the Fried *et al*. (2001) criteria.

This overall goal will be achieved through the following specific objectives:

1. To carry out a systematic review of the literature published containing epidemiological studies conducted in older adults, evaluating any alteration at the cellular level, including biomarkers of oxidative stress, genomic alterations and DNA repair, in relation to frailty status.
2. To optimise the experimental conditions of the γH2AX assay for being used as DNA damage biomarker in human population studies.
3. To study the relationship of the frailty status with a set of genetic outcomes (genomic instability, DNA damage and DNA repair) in a population of older adults classified into frail, pre-frail and non-frail according to Fried *et al*. (2001) criteria, in order to test their potential to be established as biomarkers of frailty.

Each one of these objectives will be fully explored in chapters II, III and IV of this Thesis, respectively.

Chapter II.

Systematic review on oxidative stress, genomic features and DNA repair in frail elderly.

1. Introduction

The concept of *frailty* is getting more and more attention as a new and more accurate way to define biological age as well as to identify vulnerability in elderly. Identifying frail people as early as possible is socially and economically crucial since evidence from different studies suggests that frailty status, particularly at its very earliest stages might present characteristics of reversibility (Espinoza *et al.*, 2012; Gill *et al.*, 2006; Roland *et al.*, 2014). Thus, frailty criteria should be used by geriatricians and healthcare professionals in order to provide a proper and personalized care to the older individual. However, currently there is no consensus in an international official definition of frailty.

Due to this lack of an official definition, several screening tools are currently employed to identify frailty. However, the two most commonly used instruments are the frailty phenotype (Fried *et al.* 2001) and the frailty index (FI) (Mitnitski *et al.*, 2001; Rockwood *et al.*, 2005). Nevertheless, multiple variations of the Fried's criteria (260 according to Theou *et al.* 2015) and FI, employing different deficits and/or diverse number of deficits (Searle *et al.*, 2008; Dent *et al.*, 2016), exist which can affect the predictive ability of the specific tool. As it has been mentioned in the Introduction of this memory, the instrument chosen in every case depends on a combination of factors, such as the study population, the purpose of the evaluation, or the available resources.

Some examples of these different screening tools are as follows. Montesanto scale is a population-specific survey consisting of a cluster analysis based on three phenotypic parameters [Mini Mental State Examination (MMSE) (Folstein *et al.*, 1975), hand grip strength, and Geriatric Depression Scale (GDS) (Sheikh and Yesavage, 1986)], adjusted by physical and clinical parameters (height, weight, knee-to-floor height and waist and hip circumferences, functional activity and health status) (Montesanto *et al.*, 2010). Hospital admission risk profile (HARP) is another different instrument for stratifying older patients at the time of hospital admission, according to their risk of developing new disabilities in activities of daily living (ADL) following acute medical illness and hospitalization (Sager *et al.*, 1996). This index is based on demographic information (age, gender, mental status, living arrangement, race), ability to perform six ADL (bathing, dressing, transferring, walking, toileting, and eating) and seven instrumental activities of daily living (IADL) (managing finances, taking medications, telephoning, shopping, using transportation, preparing meals, and doing housework) two weeks before admission. An abbreviated MMSE (range 0–21) is also obtained during the admission interview.

Frailty has been reported to have an important biological basis (Lang *et al.*, 2009); still, the extent of this basis and its aetiology is still quite unknown. The study of the biological causes of frailty and the alterations and interrelations between the different physiological systems

affected in the frailty status will contribute to understand the development of frailty, to reduce variability among different studies, and to reach a consensual definition of frailty.

In this frame, the use of biomarkers to identify frail subjects not only would be a more precise and objective method for frailty identification, but also would allow to compare epidemiological studies and to draw suitable conclusions from them. Due to its link to age and age-related disorders, frailty has been suggested to be associated with loss of genome integrity caused by an unbalance between DNA damage and the correct functioning of the DNA repair mechanisms (Gorbunova and Seluanov, 2016); however, this hypothesis has not been clearly demonstrated so far. For this main reason, a thorough search of the literature published related to clinical/epidemiological studies conducted in older frail adults, evaluating any alteration at the cellular level – including biomarkers of oxidative stress, genomic alterations and DNA repair – was performed, and the findings were reviewed in this chapter, in order to identify parameters that could be associated with frailty condition and consequently be proposed as biomarkers of frailty.

2. Material and Methods

2.1. Bibliographic search

The identification and selection of studies to be included in the review was carried out through an extensive literature search using the PubMed database (National Library of Medicine, National Institutes of Health, Bethesda, MD, USA; http://www.ncbi.nih.gov/PubMed), and was updated to October, 2016.

The search strategy developed comprised two terms that were intersected using the Boolean term "AND". The first one included descriptors related to frailty ('frail', 'frailty' or 'frailty elderly'), and the second one included descriptors related to biomarkers at the cellular level ('cellular damage', 'DNA', 'genomic', 'oxidative', or 'DNA repair'). All searches were focused on title or abstract.

2.2. Selection criteria

Eligible studies to be included in the review were all studies conducted in humans, written in English or Spanish, and focused on populations of older adults (mean age ≥ 60years old). Study individuals must have been classified according their frailty status following any of the currently validated criteria for frailty identification. Moreover, any cellular or molecular biomarkers must have been evaluated using any methods. In particular, the studies selected for this review can be classified within the following subgroups:

- Those evaluating oxidative stress biomarkers in frail and non-frail older adults

- Those evaluating genomic biomarkers in frail and non-frail older adults
- Those evaluating DNA repair ability in frail and non-frail older adults

Studies not considering frailty but ageing, reviews, studies carried out in animals, and articles not written in English or Spanish were excluded. Finally, studies using criteria not validated to identify frail individuals, or employing frailty as a confounder instead of outcome, were also not included in this review. Whenever the same group of authors published papers on the same group of patients, only the most recent or most complete report was considered.

Two hundred and sixty-six citations (after excluding duplicates) were initially obtained and manually reviewed (Figure II.1). Among them, 75 resulted eligible after an initial revision of abstracts. The whole publications of all these studies were fully reviewed, finding 21 studies that fulfilled the selection criteria. Other five additional publications were identified and included in the revision after reviewing the references section of published articles.

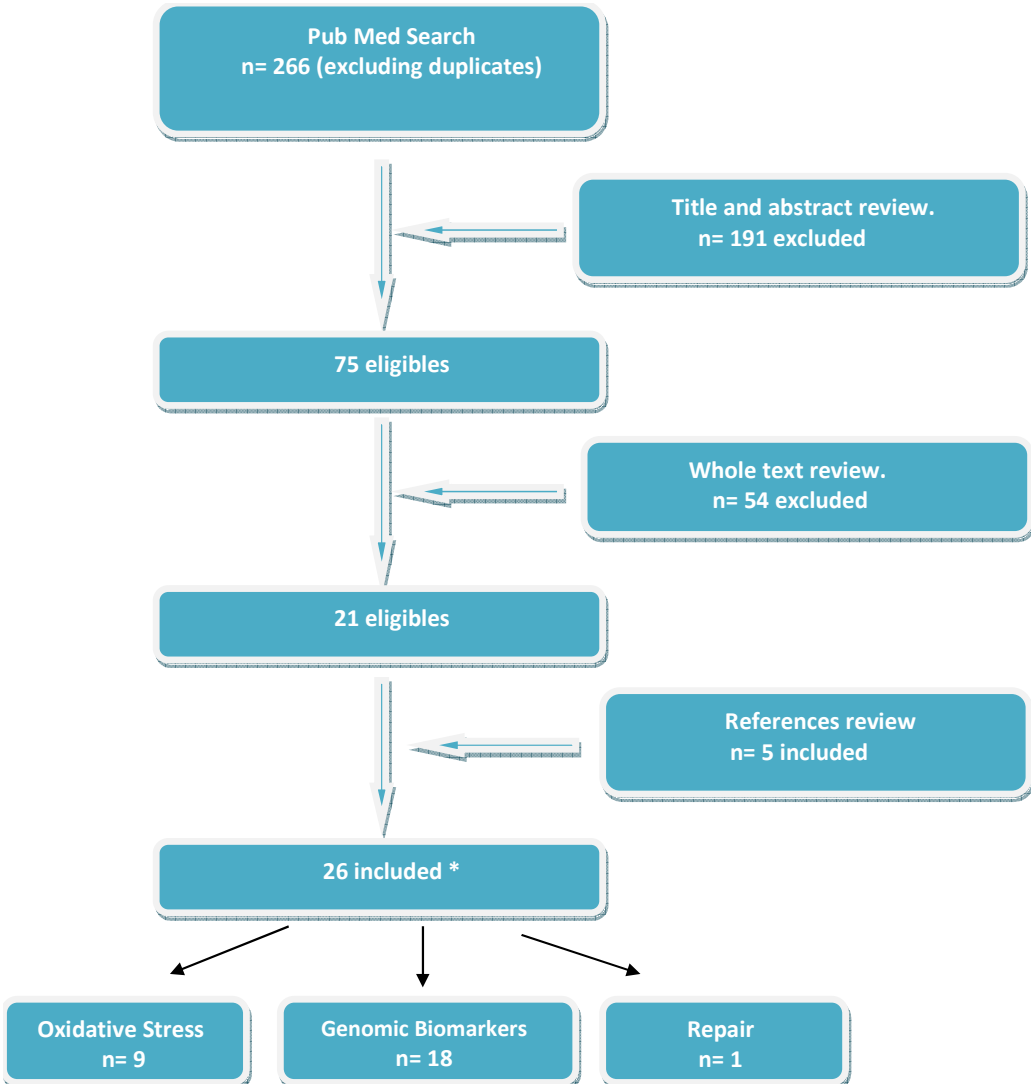

FIGURE II.1. Flow chart of the systematic review conducted. *One paper (Collerton *et al.*, 2012) addressed the three aspects considered in this review.

3. Results and Discussion

As a result of the searching process, a total of 26 studies published from 2006 to 2017 suited inclusion/exclusion criteria and, consequently, were included in this review. Among them, 8 evaluated oxidative stress biomarkers and 17 genomic biomarkers. Besides, one study (Collerton *et al.*, 2012) evaluated oxidative stress and genomic biomarkers, plus DNA repair ability on the same population of older adults. All studies but one (Pereira *et al.*, 2016) were written in English. According to the affiliation of the first author, these studies were mostly conducted in Europe (54%), USA (27%), and Asia (15%) (Figure II.2). The number of individuals analysed per study ranged from 15 to 5,275, with a mean sample size of 499 per group. Four papers only (15.4%) included less than 100 individuals, 73% included more than 300, and in eight out of 26 reviewed studies (30.8%) the sample population size was larger than 1,000 subjects, confirming the robustness of the data evaluated in this review (Figure II.3). The mean age of the studied individuals ranged from 61.9 to 99 years per group, with a total mean age of 66.8 years. Considering all studies in which patients were classified by frailty, the mean age in frail groups was 78.1 years, in pre-frail groups 76.4 years, and in non-frail groups 74.4 years.

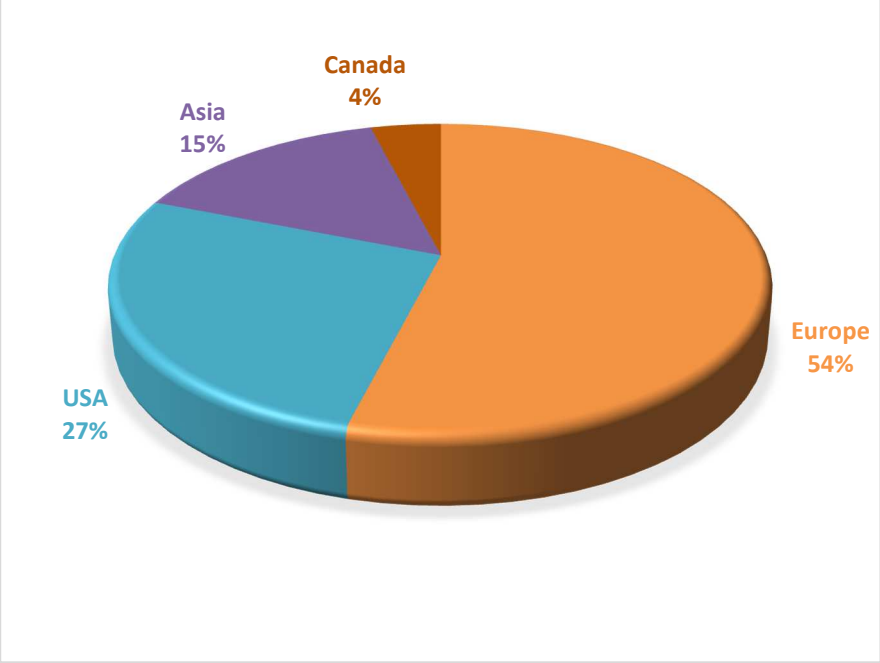

FIGURE II.2. Geographical distribution of the studies included in this systematic review, according to the affiliation of the first author.

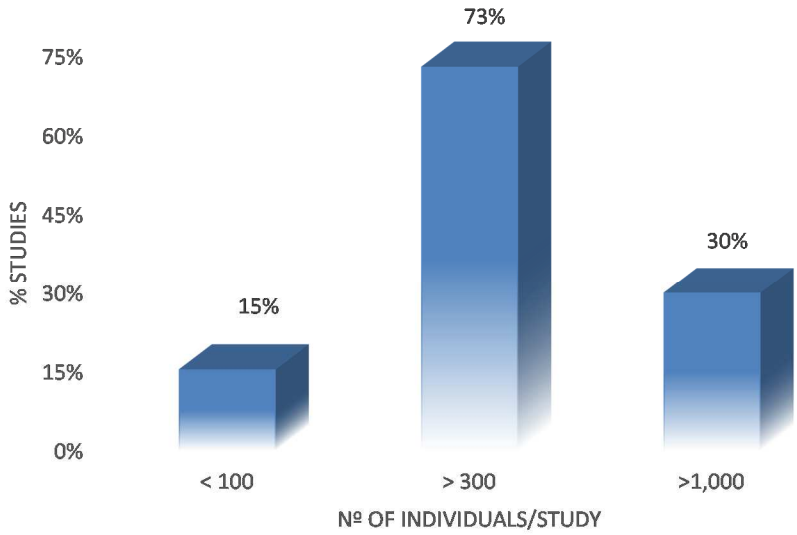

FIGURE II.3. Distribution of studies regarding the size of the population analysed.

Regarding the criteria employed to identify frail people, almost all studies (24 out of 26) employed either Fried's or Rockwood's criteria (FI), or even both of them. Among these studies, the great majority (67%) employed Fried's criteria for frailty identification, and only in two studies a modified version was used instead of the original one. Five out of 24 studies (21%) used FI to identify frail subjects, and three out of 24 studies (13%) employed both of them. However, the number of items analysed in studies employing FI was always different among studies, ranging from 17 to 40. The two remaining studies not using Fried's or Rockwood's criteria were Bellizi *et al.* (2012) and Pereira *et al.* (2016), which employed the Montesanto *et al.* (2010) method, and the Hospital Admission Risk Profile (HARP) together with FI, respectively (Figure II.4).

All studies included in this review are presented in Tables II.1 and II.2, and described in the following sections according to the type of parameter evaluated. Although performing a meta-analysis of these studies was initially considered, it was not possible to carry this out due to the high variability in the outcomes addressed together with the limited number of studies assessing the same outcome.

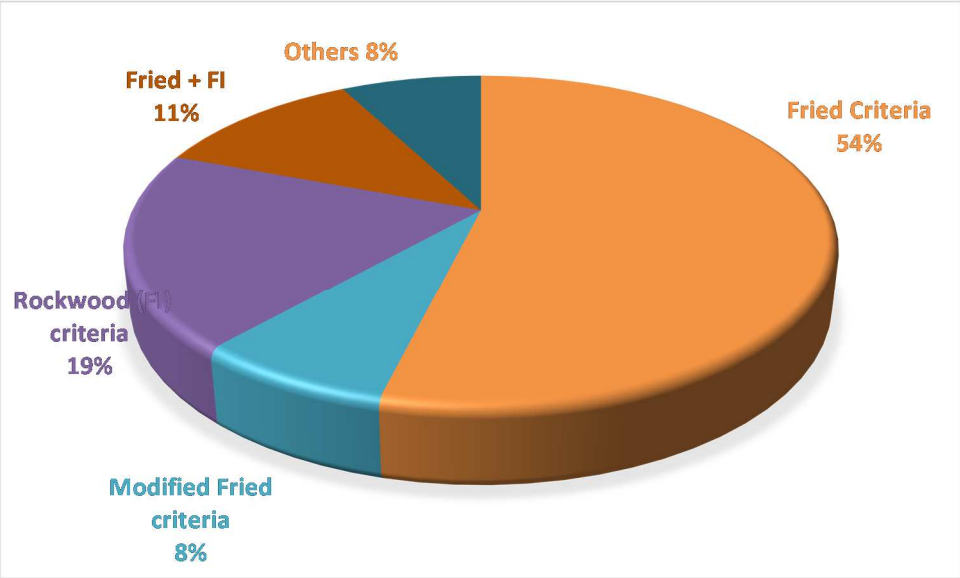

FIGURE II.4. Distributrion of studies regarding the frailty criteria used. FI, Frailty Index.

3.1. Oxidative stress biomarkers

Reactive oxygen species (ROS) are free radicals formed during the cellular metabolism. Generally, these ROS are not harmful for the cell since they are neutralized by cellular antioxidant systems, but their production can also be induced by exogenous agents that include ionizing radiation, air pollution and a wide range of chemicals (Halliwell, 2007). In such cases, the imbalance between increased ROS production and antioxidant defences leads to an oxidative stress state which is highly destructive for the cell and the organism. It has been demonstrated that oxidative stress plays an important role in neurodegenerative diseases, often associated with ageing (Migliore and Coppedè 2002; Perry *et al.*, 2002); indeed, oxidative DNA damage is one of the events that can be detected earlier in the pathogenesis of these diseases. Furthermore, oxidative stress is considered a risk factor for ageing (Coppedè and Migliore, 2009).

Besides, increases in oxidative stress with ageing may also contribute to the development of chronic inflammation and disease (Woods *et al.*, 2012). Indeed, there are a variety of potential mechanisms linking oxidative stress to inflammation, including disturbances in the redox equilibrium, decrease of oxidation-sensitive biomolecules related to immune response, such as vitamins or 5,6,7,8-tetrahydrobiopterin (BH4), or alterations in pattern recognition receptors of the innate immune system, such as toll-like receptors (Gill *et al.*, 2010). Accordingly, a number of previous studies have related immune system alterations to frailty (reviewed in Fulop *et al.*, 2015). Still, this kind of immunological alterations, although may be classified as cellular alterations in some cases, were considered biomarkers at systematic level and, consequently, not included in the present work.

The relationship between oxidative stress and frailty has been studied in several studies. Table II.1 shows all human population studies evaluating the possible association between markers of oxidative stress and frailty in old age. A total of 9 studies were found in the literature addressing this issue. The total antioxidant cellular ability or the levels of some specific antioxidants in the frail subjects regarding the pre-frail and non-frail individuals was measured in 4 (44.4%) out of 9 reviewed studies. In 7 studies, direct effects of ROS on lipids (71%) (Collerton *et al.*, 2012; Inglés *et al.*, 2014; Liu *et al.*, 2016; Pereira *et al.*, 2016; Serviddio *et al.*, 2009), proteins (57%) (Serviddio *et al.*, 2009; Inglés *et al.*, 2014; Pereira *et al.*, 2016) and DNA (29%) (Namioka *et al.*, 2016; Wu *et al.*, 2009) were evaluated; evaluation of more than one target in the same study was common. Moreover, just in 2 cases (Namioka *et al.*, 2016; Saum *et al.*, 2015), the levels of derivatives of reactive oxygen metabolites (d-ROM) were determined.

Results from these studies do not clarify if oxidative stress is consequence of frailty (or vice-versa), or if - as suggested by Liu *et al.* (2016) - there is a bidirectional relationship where the presence of one of them increases the risk of the other.

TABLE II.1. Studies assessing oxidative stress biomarkers

Study	Total population (mean age ± SD)	Case population (mean age ± SD)	Control population (mean age ± SD)	Frailty criteria	Outcomes	Assay method	Results
Ble et al. (2006)	n=827 (73.6 ± 6.4) 446 females, 381 males	Frail n=54	Pre-frail n=313; non-frail n=460	Fried et al. (2001)	Plasma Vit-E levels	Reverse-phase HPLC	Levels of vitamin E decreased gradually from the non-frail to the frail group
Serviddio et al. (2009)	n=62 (76.7 ± 5.1) 23 females,39 males	Frail n=43	n=19 non-frail	Fried et al. (2001)	GSSG	Alkaline hydrolysis of *N*-ethylmaleimide	A significant increase in the GSSG was observed in frail patients when compared to non-frail
					Whole blood total GSH level	Spectrophotometry	No association GSH level with frailty Frail patients exhibited an increase in the GSSG/GSH ratio as compared to non-frail
					Plasma MDA and HNE proteins adducts	Spectrofluorimetry	MDA and HNE adducts were significantly higher in frailty as compared to non-frail patients
					Plasma oxidized proteins	Western Blot	An appreciable decrease in oxidized proteins was detected in non-frail subjects when compared to frail patients
Wu et al. (2009)	n=90	Frail n=21 (79.9 ± 5.8)	Pre-frail n=56 (76.8 ± 5.8); non-frail n=13 (73.1 ± 5.3)	Fried et al. (2001)	Serum 8-OHdG	ELISA	Frail subjects had higher serum 8-OHdG level than pre-frail and non-frail individuals
Collerton et al. (2012)	n=552 (85) 332 females, 220 males	Frail n=119 (85) 92 females, 27 males	Pre-frail n=333 (85) 193 females, 140 males; Non-frail n=100 (85) 47 females, 53 males	Fried et al. (2001) FI (40 deficits)	Plasma lipid peroxidation: isoprostanes iPF2 alpha-III and iPF2 alpha-VI	LC/MS/MS	No association lipid peroxidation with frailty
Inglés et al. (2014)	n=742	Frail n=54 (78.8 ± 6.0) 36 females, 18 males	Pre-frail n= 278 (73.8 ± 4.7) 160	Fried et al. (2001)	Plasma lipid peroxidation: MDA	HPLC	Frail people had higher MDA levels than non-frail subjects.

			females, 118 males; n=410 non-frail (72.4 ± 4.2) 237 females, 173 males		Plasma protein carbonylation	Western blot	Frail people had higher levels of protein carbonylation than non-frail subjects
Saum et al. (2015)	n=2518	Frail n=210 (73.7 ± 6.0) 136 females, 74 males	Pre-frail n=1463 (70.3 ± 6.2) 820 females, 643 males; non-frail n=845 (67.8 ± 5.8) 367 females, 478 males	Fried et al. (2001)	Plasma dROM Plasma TTL Plasma BAP	Spectrophotometry	Correlation between dROM levels and frailty was statistically significant, but attenuated after adjustment with multiple covariates. An inverse statistically significant association with frailty was observed for TTL. No association BAP with frailty
Liu et al. (2016)	n=1919	Frail n=142 (77 ± 6) 77 females, 65 males	Pre-frail n=864 (72 ± 7) 495 females, 369 males; non-frail n=913 (69 ± 6) 463 females, 450 males	Fried et al. (2001)	Plasma LpPLA2 activity and mass, serum ICAM-1 and MCP-1, and urine 8-epi-PGFα isoprostanes	ELISA	Frailty was individually associated with isoprostanes, LpPLA2 mass, ICAM-1, and MCP-1. Pre-frailty was individually associated with elevated levels of LpPLA2 activity, ICAM-1, and MCP-1 In those individuals ≥ 70 years old, associations between isoprostanes and frailty, and between pre-frailty and MCP-1 were no longer significant
Namioka et al. (2016)	n=140	Frail n=34 (82.3±6.1) 23 females, 11 males	Pre-frail n=62 (80.5 ± 4.9) 40 females, 22 males; Non-frail n=44 (78.2 ± 6.0) 19, females, 25 males	Fried et al. (2001)	Plasma dROM Plasma BAP Endogenous plasma anti-oxidants: albumin,	Free radical analyzer system (spectrophotometry) Free radical analyzer system(spectrophotometry) Bromocresol purple staining, vanadic acid oxidation, and uricase assay, respectively	dROM levels: frail > pre-frail > non-frail individuals BAP levels were significantly lower in the frail group than in the non-frail group Bilirubin levels were significantly lower in the frail group than in the non-frail group. No association albumin or uric acid with frailty

					bilirubin, uric acid		
					Urinary 8-OHdG and 8-epiPGF2α	HPLC and enzyme immunoassay, respectively	Urinary excretions of 8-OHdG and 8-epiPGF2α were significantly higher in the frail and pre-frail groups than in the non-frail group
Pereira et al. (2016)	n=15 (88 ± 9.78)	High risk of frailty (HARP) n=7	Intermediate or low risk of frailty (HARP) n=7	HARP FI	Lymphocyte membrane lipid peroxidation: conjugated dienes and trienes	UV spectrophotometry	Elderly patients with a higher degree of frailty had significantly higher level of dienes in both frailty scales, and significantly higher level of trienes only in FI
		Intermediate or severe frailty (FI) n=12	Non-frail/mild frailty (FI) n=3		Plasma protein oxidation: MDA protein adducts	TBARS test	Elderly patients with a higher degree of frailty (HARP) presented significantly higher levels of MDA than those with milder levels of frailty

8-OHdG, 8-hidroxy-2'-deoxyguanosine; BAP, biological antioxidant potential; dROM, derivatives of reactive oxygen metabolites; ELISA, enzyme-linked immunosorbent assay; FI, frailty index; GSH, glutathione; GSSG, glutathione disulfide, oxidized glutathione; HARP, hospital admission risk profile; HNE, 4-hidroxy-2,3-nonenal; HPLC,high performance liquid chromatography; ICAM-1,intracellular adhesion molecule-1; LC, liquid chromatography; LpPLA-2,lipoprotein phospholipase A2; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; MS, mass spectrometry; TBARS, thiobarbituric binding acid reactive species; TTL, total thiol levels; Vit-E, vitamin E.

Cells have different mechanisms to limit the levels of ROS and the damage they induce. The cellular defence mechanisms against oxidative stress include both enzymatic (e.g. catalase, superoxide dismutase) and non-enzymatic (e.g. vitamin E [vit-E], glutathione) antioxidants, which play a central role in maintaining the cellular redox balance essential for cell survival (Birben *et al.*, 2012). Several alterations in different antioxidants in association with frailty have been obtained in the studies reviewed. For instance, vit-E, or α-tocopherol, is the major lipophilic antioxidant in humans; low levels of vit-E are considered an indirect biomarker of oxidative stress and, therefore, it has been hypothesized to be associated with an increased risk of frailty. According to this, Ble *et al.* (2006) found a significant decrease of vit-E levels in frail individuals, after adjustment for multiple confounders, in a population of 827 older individuals classified according to Fried's criteria.

Also, increases of both oxidized glutathione (GSSG) and the oxidized/reduced glutathione ratio (GSSG/GSH), but normal reduced glutathione (GSH) levels, were found in 43 frail individuals (Fried's criteria) regarding 19 non-frail subjects (Serviddio *et al.*, 2009).

Investigating the association between oxidative stress and frailty, Saum *et al.* (2015) evaluated three biomarkers of oxidative stress in 2,518 individuals classified according to Fried's criteria: total thiol levels (TTL), d-ROM, and biological antioxidant potential (BAP). Whilst no statistically significant difference was observed in the levels of BAP in frail individuals as compared with the pre-frail and non-frail subjects, the frail group showed higher d-ROM but lower TLL levels. Also, significant positive correlations between d-ROM and BAP, and BAP and TTL were observed, as well as a weak inverse association between d-ROM and TTL. The authors concluded that TTL was the biomarker most consistently associated with frailty in all the regression models.

Namioka *et al.* (2016) studied oxidative stress in a sample of 140 older adults with mild-to-moderate Alzheimer's disease, classified according to their frailty status using the Fried's criteria. To that end, levels of several oxidative stress biomarkers were evaluated including d-ROM, BAP, and endogenous plasma antioxidants (namely albumin, bilirubin, and uric acid). Consistently with the observations by Saum *et al.* (2015), results showed significantly increasing levels of dROM in non-frail < pre-frail < frail groups. On the other hand, BAP and plasma bilirubin levels were significantly lower in the frail group. Plasma albumin and uric acid levels showed no significant differences among the three groups.

ROS attack all biological molecules including DNA, proteins and lipids. Lipids and lipoproteins are particularly susceptible to ROS attack because hydrogen abstraction by a radical can initiate a devastating chain reaction: lipid peroxidation (Anderson and Philips, 1999). Several studies evaluated lipid peroxidation and oxidized protein levels in relation to frailty. Serviddio *et*

al. (2009) analysed plasma levels of malonaldehyde (MDA) and 4-hydroxy-2,3-nonenal (HNE) protein adducts (both of them recognized biomarkers of lipid peroxidation), and plasma oxidized proteins (as expression of oxidative protein damage) in a population of 62 old individuals, classified as frail and non-frail accordingly to the Fried's criteria. Higher levels of MDA and HNE adducts and oxidized proteins were observed in frail individuals as compared with non-frail subjects.

Liu *et al.* (2016) studied the association of frailty (Fried's criteria) with several oxidative stress biomarkers related to cardiovascular disease, namely isoprostanes (accurate markers of lipid peroxidation) and the lipoprotein phospholipase A2 (LpPLA2) (an enzyme involved in hydrolization of oxidized phospholipids) in a population of 1,919 older individuals. They concluded that significantly increased levels of isoprostanes and LpPLA2 mass were related to greater odds of frailty, since they found individual association of frailty with elevated levels of isoprostanes and LpPLA2 mass, and of pre-frailty with elevated levels of LpPLA2 activity.

Inglés *et al.* (2009) employed MDA and protein carbonylation (a circulating indicator of oxidative damage to proteins) to assess the relationship between frailty and oxidative stress, and the capacity of these two parameters as possible biomarkers of frailty. Their results showed significantly higher levels of MDA and carbonylated proteins in older individuals classified as frail, according to Fried's criteria, than in non-frail; no relationship with age or sex was found in any case.

Also, Pereira *et al.* (2016) found in a small population (n=15) that older individuals with a higher degree of frailty, measured by FI or HARP criteria, showed higher levels of lipid peroxidation. In this study, protein oxidation levels (MDA protein adducts) were also evaluated, showing higher levels in individuals with higher risk of frailty determined by HARP criteria, but not by FI.

Collerton *et al.* (2012) carried out a study with 552 older adults classified by means of both Fried's criteria and FI (40 items), evaluating the possible association between frailty status and biomarkers of lipid peroxidation, namely isoprostanes iPF2 alpha-III and iPF2 alpha-VI. No association between frailty and the oxidative stress biomarkers was found in this case.

As previously indicated, ROS may react with different biomolecules in cells, and one of the main targets is DNA. This kind of damage is often referred to as oxidative DNA damage. The consequences include different types of DNA alterations, ranging from simple oxidation of bases to large deletions, through single and double strand breaks (Rao, 2009). Pathogenic roles for DNA oxidation include the induction of mitochondrial dysfunction, promotion of cytotoxicity and modulation of inflammatory responses (Evans and Cooke, 2006). According to our revision, only

two studies have evaluated consequences of oxidative stress on DNA in frail and non-frail older people. Wu *et al.* (2009) evaluated the relationship between oxidative stress and frailty by measuring the level of serum 8-hidroxy-2'-deoxyguanosine (8-OHdG) in a sample of Chinese older individuals (n=90). The frailty status was determined by using the Fried's criteria. Results showed significantly increased serum 8-OHdG levels in frail individuals with respect to the pre-frail and non-frail subjects. More recently, Namioka *et al.* (2016) observed that urinary excretions of 8-OHdG and 8-isoprostane were significantly higher in frail and pre-frail individuals than those determined in the non-frail group.

Together with these 9 selected studies, two additional epidemiological studies, not included in the final reviewed works because they did not strictly fulfil the inclusion criteria, addressed the relationship between frailty and oxidative stress in an indirect way. Firstly, Caballero *et al.* (2014) assessed the role of oxidative stress on deficiencies of functional physical performance of the lower and/or upper body limbs, that could affect a future pre-frailty phenotype (Fried's criteria). To this aim, they studied the concentrations of plasma proteins, carbonylated proteins, lipid peroxidation and plasma total antioxidant activity, as measures of oxidative damage, in a population of 200 individuals aged $\geq$ 70 years. Their results showed significantly lower levels of total antioxidant capacity in women $\leq$ 76 years old with deficiencies in the physical performance of both lower and upper body limbs, but not in women >76 years old or in men of any age, suggesting that deficient oxidative defence in the elderly could significantly affect the functional physical performance and future outcomes of pre-frail individuals.

Secondly, Baptista *et al.* (2012) used the gait speed as a measure of physical performance in older adults, in a population of 280 individuals. The objective of this study was to evaluate the superoxide anion production, and its interaction with physical frailty measured by gait speed in older adults. They found no differences in the baseline levels of superoxide anion production between subjects with slower and faster gait speed. However, after stimulation with PMA (lucigenin and phorbol 12-myristate 13-acetate), the superoxide anion production was significantly higher in slow walkers (gait speed <0.8 m/s). Hence, they suggested that the production of the superoxide anion can be involved in the decline of physical performance in the older age and, therefore, in the frailty process.

Methodologies used in the studies mentioned differ from one another. Four out of these 9 studies used chromatographic assays. Inglés *et al.* (2009) and Namioka et al. (2016) employed high perfomance liquid chromatography (HPLC). This technique allows the elution of a component from a mixture or sample in order to identify and quantify the particular component. The basis of the technique consists of passing a pressurize sample, contained in a liquid solvent, through a stationary solid adsorbent HPLC column. The components of the sample flow through

the column at a different rate due to their different degrees of interaction with the column filling material, allowing their separation. Ble *et al.*, (2006) employed a reverse-phase HPLC. In this case, the mobile phase containing the sample is a polar or aqueous solution that is pumped through a non-polar stationary column. Separation of the components is conducted according to their hydrophobic interactions with the stationary phase (Canene-Adams, 2013).

Collerton *et al.*, (2012) evaluated lipid peroxidation by liquid chromatography (LC) coupled to mass spectrometry (MS). The MS has great sensitivity and is highly specific as compared to other chromatographic detectors. MS operate by converting the analyte molecules to an ionized state. Hence the analysis of these ions, on the basis of their mass to charge ratio (m/z), offers a profile of the components present in a sample (Pitt, 2009).

The enzyme-linked immunosorbent assay (ELISA) was used by Liu *et al.* (2016) to study lipid peroxidation, and by Wu et al. (2009) to assess the effect of oxidative stress on DNA. ELISA tests are immunoenzymatic biochemistry tests that use an enzyme in a liquid sample to detect the presence of an antigen attached to a surface by using specific antibodies. The reaction between the enzyme and the antigen of study produces a reaction (i.e. a change of colour in the enzyme substrate) that can be measured by different means (Lequin, 2005).

In order to analyse oxidative damage in proteins, the Western blot assay was utilized by Serviddio *et al.* (2009) and Inglés *et al.* (2014). This technique is used for immunodetection and quantitation of specific proteins in complex cell homogenates (Taylor and Posch, 2014).

Five studies used spectrophotometry assays to evaluate levels of different plasma biomarkers of oxidative stress (Saum *et al.*, 2015; Namioka *et al.*, 2016; Pereira *et al.*, 2016), and whole blood levels of GSH (Serviddio *et al.*, 2009), as well as peroxidation of lipids of the cell membrane (Pereira *et al.*, 2016). The basic principle of this technique is that each compound absorbs or transmits light over a certain range of wavelength. Thus, a substance present in a sample can be quantified by measuring the intensity of light detected as a beam of light passes through the sample solution. Also, Pereira *et al.* (2016) and Serviddio *et al.* (2009) evaluated the levels of plasma MDA and HNE proteins by a spectrofluorimetric assay, whose basic principle is the same but using fluorescence, conferring a higher accuracy to the method.

3.2. Genomic biomarkers

The contribution of the individual genetic profile to the development of frail is still uncertain. Whether and to what extent the individual genetic features affect the individual susceptibility to frailty is not well established yet. In this regard, studies involving analysis of the contribution of genetic background to frailty and in particular evaluating the association of genomic instability to this condition may help to provide insights into biologically relevant

pathways that contribute to frailty. Among the studies reviewed here addressing genetic alterations, 17 in total, six of them (35.3%) evaluated genetic background of frail individuals, including variations in both nuclear (three studies) and mitochondrial (other three studies) DNA, eight (47%) investigated the relationship between genomic instability and frailty, and three (17,7%) addressed the possible epigenetic characteristics of frailty status (Table II.2).

3.2.1. Individual genetic background

Three out of the 17 studies reviewed here evaluated the individual genetic differences associated to frailty, employing different multiplexing bead chip arrays to genotype whole genomes (Kim *et al.*, 2015) or specific single nucleotide polymorphisms (SNP) (Matteini *et al.*, 2010; Ho *et al.*, 2011).

Kim *et al.* (2015) studied the heritability of healthy ageing as an attempt to find the more beneficial genetic variants or less disadvantageous variants present in healthy, long-lived people. The authors performed a genome-wide linkage analysis and fine-scaled association mapping of linkage regions, using the FI (34 deficits) as a quantitative measure of frailty in a population of 320 older adults. Three healthy ageing sites (HAS) were found at 12q13-14 in intergenic regions (HAS-1 and -2 with enhancer activity, HAS-3 with silencer activity).

Ho *et al.* (2011) studied different biological pathways involved in frailty to try to find variations in genes related to the frailty syndrome. To this aim, they genotyped 1,354 SNP from 134 candidate genes involved in inflammation and muscle maintenance, two of the main phenotypic characteristics of frailty, in a population of 348 subjects classified according to Fried's criteria. Twenty SNP, indirectly related to inflammatory process, were found to be associated with frailty, although statistical significance was not reached.

Matteini *et al.* (2010) also investigated genetic variants of six candidate genes (MTHFR, MTR, MTRR, CBS, TCN1, and TCN2) involved in Vitamin B12 metabolic pathway and their association with frailty (Fried's criteria), in 416 older women. For this purpose, 56 SNP from those six candidate genes were genotyped. The results found SNP in the TCN2 gene showing significant association with frailty. Moreover, two SNP in MTRR gene showed 2-4 times greater odds of being frail compared to robust.

TABLE II.2. Studies assessing genomic biomarkers

Study	Total population (mean age ± SD)	Case population (mean age ± SD)	Control population (mean age ± SD)	Frailty criteria	Outcomes	Assay method	Results
Ashar et al. (2015)	n=4,892; 2,774 females, 2118 males	na	na	Fried et al. (2001)	mtDNA copy number	qPCR	Lower mtDNA copy number was significantly associated with prevalent frailty only in white participants
Bellizi et al. (2012)	Cross-sectional study: n= 318 (S1+S2). S1: n=217 (median age 75 years) 123 females, 94 males, classified in non-frail, pre-frail and frail. S2: n=101(median age 99 years) 51 females,50 males, classified in frail and very frail	na	na	Montesanto et al. (2010)	Global DNA methylation	CpGlobal assay	Global DNA methylation levels were correlated with frailty in S1 but not in S2.
	Longitudinal study 7 years later: n=37, random sample from S1 pre-frail and non frail groups						Lower global DNA methylation levels in pre-frail subjects that became frail after 7 years
Breitling et al. (2016)	Dataset 1: n=969 (62.1±6.5) 484 females, 485 males	na	na	FI (34 deficits)	DNA methylation age acceleration (methylation age minus chronological age)	Infinium HumanMethylation450 BeadChip	Association of DNA methylation age acceleration with FI increased with increasing age acceleration
	Dataset 2: n=851 (63±6.7) 464 females, 387 males				TL	qPCR	Interaction between TL and epigenetic age acceleration did not improve the prediction of FI
Collerton et al. (2014)	n=321 (85) 184 females, 137 males	na	na	Fried et al. (2001) (CHS modified)	DNA methylation in CpG islands	Highly quantitative pyrosequencing	Association CpG island methylation with frailty

	n=231 (85) 148 females, 83 males				LINE-1 methylation levels (surrogate for genome-wide DNA methylation levels)		No association genome-wide methylation (LINE-1) with frailty
Collerton et al. (2012)	n=552 (85) 332 females, 220 males	Frail n=119 (85) 92 females, 27 males	Pre-frail n=333 (85) 193 females, 140 males; non-frail n=100 (85) 47 females, 53 males	Fried et al. (2001) FI (40 deficits)	TL Ionized radiation-induced DNA damage and repair	qPCR Automated fluorimetric alkaline DNA unwinding	No association of TL or ionized radiation-induced DNA damage and repair with frailty
Collerton et al. (2013)	n=1,173 (85.5)	Frail n=696 (FI), n=477 (Fried et al., 2001)	3 ethnically matched population control data sets	Fried et al. (2001) FI (40 deficits)	mtDNA haplogroups	Sequenom Mass ARRAY	No association between common genetic variants of mtDNA and frailty
Jylhava et al. (2013)	n=174	Frail n=144 (nonagerians) 101 females, 43 males	n=30 young controls (range 19-30 years) 21 females, 9 males	Fried et al. (2001)	Total cf-DNA in plasma Unmethylatedcf-DNA Genomic equivalents of the RNase P-coding cf-DNA Alu repeat cf-DNA mtDNA copy number	Fluorimetry (Quant-iT^{TM} DNA high-sensitivity assay kit) ELISA (DNA Methylation Kit) qPCR qPCR qPCR	In nonagenarians: Higher levels of total and unmethylated cf-DNA were associated with increased frailty No association with frailty No association with frailty mtDNA copy number was directly correlated with increased frailty
Marzetti et al. (2014)	n=142 (74.9±6.5) 84 females, 58 males	Frail n=74	Pre-frail/non-frail: n=68	Fried et al. (2001) FI (30 deficits)	TL	qPCR	No association with frailty (Fried or FI)
Moore et al. (2010)	Pilot study: n=315	Frail n=154 (75±4.45) 112 females, 42 males.	Non-frail: n=161 (81.35±3.16) 111 females, 50 males	Fried et al. (2001)	mtDNA variations (SNP)	Oligonucleotide sequencing microarray	Three mtDNA SNPs (mt146, mt204, and mt228) were associated with frailty

	Cross-sectional study: total n=5,275	Frail white: n=262 (77.36±6.36) 174 females, 88 males; frail black: n=102 (75.44±6.76) 76 females, 26 males	Non-frail white: n=4,223 (72.43±5.37) 2377 females, 1,846 males; non-frail black: n=688 (72.05±5.09) 425 females, 263 males			Real-time PCR TaqMan assays for individual SNP selected for follow-up (mt146, mt204, and mt228)	mt204 C allele was associated with greater likelihood of frailty
Saum et al. (2014)	n=3,537 (61.9±6.6) 1963 females, 1574 males. Validation in a subpopulation n=20	na	na	FI (34 deficits)	Relative TL (T/S ratio) Absolute TL in base pairs	qPCR Southern blot	No difference of the FI between the T/S ratio tertiles was observed. TL measurements by the T/S ratio were highly correlated with absolute TL
Woo et al. (2008)	n=2,006. 1,030 females (72.02±5.191), 976 males (72.75±5.026)	na	na	FI (17 deficits)	TL	qPCR	No correlation between TL and frailty
Valdiglesias et al. (2015)	n=180	Frail n=93 (76.9±6.6) 52 females, 41 males	Non-frail n=87 (72.9±6.1) 50 females, 37 males	Fried et al. (2001)	MN frequency	CBMN with automated scoring	No association between MN frequency and frailty
Yu et al. (2015)	n=2,006 (72.4 ± 5.1) 1030 females, 976 males	Frail n=127	Pre-frail n=967; non-frail n=912,	Fried et al. (2001)	TL	qPCR	No association between TL and frailty at baseline, nor in 4 year follow-up
Brault et al. (2014)	n=53 (≥ 75 years old)	na	na	Fried et al. (2001)	TL in leukocytes and aortic tissue		No association between TL and frailty. Association between longer leukocyte and aortic T/S ratio and greater number of clinical frailty criteria.
Kim et al. (2015)	n=320. Parents at least 90 years old and their offspring (50-80 years old)	na	na	FI (34 deficits)	Genome-wide linkage scanning followed by fine-scale association mapping	Genotyping: BeadChip array (Illumina Infinium Linkage 24 set); association mapping:IlluminaGoldenGate assay	They found three sites associated with healthy aging

Ho et al. (2011)	n=348 females (74.2)	Frail n=152	Pre-frail n=165; non-frail n=32	Fried et al. (2001)	SNP variations (134 genes, 1354 SNP)	Bead Array (Illumina custom GoldenGate 1536 SNP panel)	20 SNP (from 11 genes) associated with frailty (not significantly)
Matteini et al. (2010)	n=326 females (74.1)	na	na	Fried et al. (2001)	56 SNP from six candidate genes involved in Vitamin B12metabolic pathway: MTHFR, MTR, MTRR, CBS, TCN1 and TCN2	Illumina BeadArray	SNP in the TCN2 showed significant association with frailty. Two SNP in MTRR showed 2-4 times greater odds of being frail compared to robust
Gao et al. (2017)	Discovery set n=978 (62.1 ±6.5) Validation set n=531 (62.0±6.6)	Frail n=100 Frail n=48	Pre-frail n=443; non-frail n=435 Pre-frail n=220; non-frail n=263	FI (34 deficits)	DNA methylation profiles	Illumina Human Methylation 450 BeadChip	17 smoking-related CpG sites were associated with the FI. 9 of those sites were designated as frailty associated loci. Six of them [cg02657160 (*CPOX*), cg05673882 (*POLK*), cg07826859 (*MYO1G*), cg19859270 (*GPR15*), cg23667432 (*ALPP*), and cg25189904 (*GNG12*)] were mapped showing methylation intensity in frail < pre-frail < non-frail SI, based on those 9 smoking-related CpG sites, manifested a monotonic dose-response relationship with the FI

CBMN, cytokinesis-blocked micronucleus test; cf-DNA, cell free DNA; CHS, cardiovascular health study; LINE-1, long interspersed nuclear elements 1; MN, micronucleus; mtDNA, mitochondrial DNA; na, not available; qPCR, quantitative PCR; SI, Smoking index; SNP, single nucleotide polymorphism; T/S ratio, mean telomere repeat copy to single gene copy number; TL, telomere length.

Mitochondria are involved in several major cell functions, such as cell production of energy, metabolic and apoptotic processes, as well as being a major site of ROS generation. Mitochondrial function experiences alterations with age and these changes are associated with several age-related diseases. Thus, age-related changes and variations in the mtDNA have been proposed as plausible candidates to play a role in degenerative and senescent processes, and to contribute to increases in vulnerability in late life (Moore *et al.*, 2010); hence they are likely to be associated with frailty syndrome. Three studies were found in the literature search that evaluated the possible association between mtDNA alterations (in copy number or in sequence) and frailty. Firstly, Collerton *et al.* (2013) evaluated the association between common genetic variants of mtDNA and frailty as a mean to study heritability of human longevity and healthy ageing, since frailty is considered an "unhealthy ageing" phenotype. They evaluated a population of 1,173 older adults. No association between mtDNA haplogroups and frailty, assessed by both Fried's criteria and FI (40 deficits), was observed.

On the contrary, Ashar *et al.* (2015) evaluated the association between mtDNA copy number, evaluated by quantitative polymerase chain reaction (qPCR) and prevalent frailty (Fried's criteria; n= 4,892). They reported lower mtDNA copy number in frail white, but not in frail black, individuals. They also observed a significant inverse association of mtDNA copy number with age, and higher mtDNA copy number in women relative to men. Finally, Moore *et al.* (2010) studied how mtDNA variations (SNP) with age may increase the susceptibility to frailty. In a pilot study of 315 individuals classified according to Fried's criteria, three mtDNA SNP were associated with frailty (mt146, mt204, and mt228). From these three SNP, mt204 C was confirmed to be the allele associated with greater likelihood of frailty in the cross-sectional further study including 5,275 subjects.

After cell damage or death, DNA is released into the circulation; this is why plasma cell free DNA (cf-DNA) reflects systematic inflammation and cell death, what makes it a potential biomarker of ageing and frailty. For that reason, Jylhava *et al.* (2013) quantified the plasma levels of total cf-DNA, unmethylated cf-DNA, gene-coding cf-DNA, and Alu repeat cf-DNA, and mtDNA copy number to study their potential as biomarkers of frailty (Fried's criteria; n= 174). Their results showed increased levels of total and unmethylated cf-DNA associated with frailty. Also mtDNA copy number was correlated with frailty. However, neither gene-coding cf-DNA nor Alu repeat cf-DNA were associated with frailty. Similarly, the same group (Jylhava *et al.*, 2014) studied the association of these biomarkers with mortality, finding that frailty is detrimental for survival when used to adjust the results obtained.

3.2.2. Genomic instability

As mentioned before, eight out of 17 studies evaluated genomic instability in frailty. Genomic instability refers to a set of genetic events capable of causing temporary or permanent unscheduled alterations within the genome, including diverse types of chromosomal alterations (e.g. inversions, deletions, duplications and translocations of large chromosomal segments) (Migliore *et al.*, 2011; Valdiglesias *et al.*, 2015). Thus, genome instability could lead to altered gene dosage and gene expression as well as contribute to the risk of accelerated cell death in neuronal tissue (Thomas and Fenech, 2007). In accordance, genomic instability is considered a hallmark of a number of ageing-related diseases including cancer, and is related to the ageing phenotype and neurodegenerative disorders (Coppedè and Migliore, 2010; Migliore *et al.*, 2011),

Among the studies evaluating genomic instability, all but one (Valdiglesias *et al.*, 2015) analysed telomere length (TL) in the older subjects by using qPCR. TL analysis is a common approach to evaluate genomic instability in blood samples. Telomeres are regions of repetitive nucleotide sequence at each end of the chromosomes, which contribute to maintain their integrity. They progressively shorten as the cell divides, limiting the number of divisions that normal somatic cells can undergo (Marzetti *et al.*, 2014). The observation that telomeres shorten over the life course has led to the hypothesis that telomere attrition may be a mechanism driving the ageing process (Mikhelson and Gamaley, 2012). Nevertheless, all the studies reviewed here failed in finding an association between frailty and TL shortening: Collerton *et al.* (2012), using both Fried's criteria and FI (40 deficits; n=552), Marzetti *et al.* (2014) (n=142) and Yu *et al.* (2015) (n= 2,006) employing the Fried's criteria, Saum *et al.* (2014) (n=3,537) and Woo *et al.* (2008) (n=2,006) using the FI (34 and 17 deficits, respectively). Lack of association between TL and frailty (Fried's criteria) was also obtained by Brault *et al.* (2014) in older subjects with cardiovascular disease (n=53); however, in this case, they found an unexpected association between longer leucocyte and aortic T/S ratio (mean telomere repeat copy to single gene copy number) and greater number of clinical frailty criteria. Together with these studies, Breitling *et al.* (2016) evaluated the possible association between TL and DNA methylation age acceleration (methylation age minus chronological age) in a population of 851 older adults. They found no correlation between the two parameters, supporting the previously mentioned observations and the idea that TL is not a good biomarker for the identification of frailty.

The frequency of micronucleus (MN) in peripheral blood lymphocytes is also a biomarker of genomic stability widely employed in molecular epidemiology. Similarly to TL, MN frequency has been previously associated with age-related diseases and the process of ageing (Bonassi *et al.*, 2001; Migliore *et al.*, 2011). Valdiglesias *et al.* (2015) evaluated the frequency of MN to study the association of genomic instability and frailty in a population of 180 older adults classified

according to Fried's criteria. Again, no association between MN frequency and frailty was observed in this case.

Thus, despite the well-reasoned working hypothesis (genomic instability is involved in the pathogenesis of frailty syndrome), all these studies resulted surprisingly fruitless, with no positive association between genomic instability and frailty severity. Two possible explanations were suggested for this lack of association (Valdiglesias *et al.*, 2015). Firstly, the negative findings reported in all these studies may be caused by the high rate of basal genomic damage present in healthy older individuals and reported in previous studies (Mladinic *et al.*, 2010). And secondly, this lack of association may be due to the physiological accumulation of genome damage in the elderly, which could limit the rate of genomic damage production. This condition could, for instance, limit the rate of MN formation.

3.2.3. Epigenetics

It has been previously reported that the genetic material of cells experiences epigenetic variations during the ageing process (reviewed in Sen *et al.*, 2016). A total of 3 studies out of 17 evaluating genomic biomarkers, addressed the study of epigenetics in frail older adults. The three of them showed positive correlation between frailty status and DNA methylation. Bellizi *et al.* (2012) reported the first study investigating the possible correlation between age-related functional decline, including frailty status, and epigenetic modifications. They measured global DNA methylation levels by means of the CpGlobal assay, developed by Anisowicz *et al.* (2008), that utilizes methyl-sensitive restriction enzymes to detect the biotinylated nucleotides incorporated in an end-fill reaction and a luminometer to measure the chemiluminescence. A total of 318 older people, divided in middle/advanced-aged subjects (median age 75 years) and ultranonagenarians (median age 99 years) were evaluated in a cross-sectional study, and in a 7 year follow-up of a subsample of pre-frail and non-frail middle aged subjects. Frailty status was determined using the Montesanto *et al.* (2010) scale, a hierarchical cluster analysis. Higher values of global DNA methylation were observed in frail middle aged subjects respect to pre-frail and non-frail, but no difference between very frail and frail was observed in the ultranonagenarians. In the follow-up study, those individuals that became frail after 7 years showed a significant increase of DNA methylation levels.

More recently, Breitling *et al.* (2016) evaluated the association between DNA methylation age acceleration, assessed by a methylation profiling high throughput platform, and frailty in a population of 969 older adults, classified according their frailty status by using the FI (34 deficits). Their findings showed significantly increasing accumulation of frailty deficits with increasing methylation age acceleration, supporting an association between epigenetic age acceleration and frailty status. Also on this regard, Gao *et al.* (2017) examined the associations of

smoking-related DNA methylation biomarkers and frailty in a population of older adults classified according to FI, and observed that methylation intensity of each locus in the validation panel was significantly lower in the frail, when compared to non-frail, population, whereas intermediate levels of methylation intensity were observed in the pre-frail subjects. On the basis of their results, authors suggested that CpG sites identified could have the potential to be prognostic biomarkers of frailty or frailty-related health outcomes.

Following the same assumption that DNA methylation changes with age, especially in gene promoter regions, Collerton *et al.* (2014) studied the importance of altered DNA methylation in frailty in 552 subjects (Fried's criteria). To this end, methylation at specific cytosine residues within CpG islands associated with gene transcriptional start sites was quantified using highly quantitative pyrosequencing. This technique is based on the sequencing-by-synthesis principle, similar to the one used in the Sanger method, as they both require the direct action of DNA polymerase. Furthermore, to estimate the genome-wide DNA methylation levels, they quantified methylation at LINE-1 repetitive elements as a surrogate, showing no association with frailty. However, a clear association between CpG island methylation and frailty was observed, suggesting a potential role for age-related ranges in CpG island methylation in the development of frailty.

3.3. DNA repair ability

The DNA repair system has been recognized as one of the most important cellular defence mechanisms responsible for the integrity of DNA. Decreased DNA repair ability is exhibited in various clinical conditions and associated with increased frequency of carcinogenesis, since inefficient repair leads to an accumulation of aberrations in the genome that culminate in the genetic instability typical of many malignancies and other pathologies (Valdiglesias *et al.*, 2011a).

Daily exposure to environmental agents (such as oxidizing chemicals, methylating agents, UV light, and ionizing radiation), and even normal physiological processes (such as replication and recombination), all may damage cellular components, including DNA. While modified proteins and lipids can be degraded and resynthesized, DNA must be repaired before replication and cell division take place (Klungland and Bjelland, 2007). Toxic and mutagenic consequences are minimized by distinct pathways of cellular repair that include different enzymes and protein complexes encoded by a number of human DNA repair genes. Genomic instability, previously described, is highly related to failures in cellular repair since aberrant DNA polymerases and other components of the transcriptional and translational machinery are accumulated with age (Rattan, 2012). Thus, during ageing, accumulation of mistakes takes place in the genetic material due to the loss of efficiency of these DNA repair systems, and contributes to the development of genomic instability. Indeed, most age-related diseases and ageing signs are associated with genomic

instability and with unrepaired or erroneously repaired genome damage (Bürkle, 2001). Besides, it is known that the accumulation of DNA damage is involved in premature ageing and neurodegenerative processes, suggesting that alterations in the DNA repair mechanisms may be relevant to these disorders (Coppedè and Migliore, 2010). Indeed, the impact of cellular senescence on ageing of organisms was previously evaluated in different studies revealing an accumulation of DNA damage in both senescent cells and ageing organisms (Sedelnikova *et al.*, 2008). In particular, several works described accumulated γH2AX foci, which reveal persistent DNA double-stranded breaks, in senescing human cell cultures and in ageing mice (Sedelnikova *et al.*, 2004), in early thymocyte subsets of aged as compared to young mice (Hesse *et al.*, 2009), in different organs from ageing C57Bl6 mice (Wang *et al.*, 2009) and in fibroblasts taken from patients with Werner syndrome (Sedelnikova *et al.*, 2008).

To our knowledge, the only study that evaluates DNA damage and repair capacity in association with frailty in human population-based studies is the one reported by Collerton *et al.* (2012), where no association was observed using both Fried's criteria and FI (40 deficits) to identify frailty status.

4. Conclusions

Frailty is gaining attention in the last decades in geriatrics and research areas, with more and more professionals claiming frailty measurement to be incorporated into clinical practice as part of routine care for older patients. However, the currently used criteria identify frailty only after clinical manifestations are obvious. Increasing evidence suggest that the clinical concept of frailty –based mainly on phenotypical signs and symptoms and barely considering its biological basis – is obsolete since no single altered system alone defines frailty, but multiple systems are involved in this syndrome.

In order to achieve a more thorough and objective assessment for early identification of frailty, it is necessary to develop new tools that allow to recognize those individuals more vulnerable and more prone to develop the frailty syndrome. Within this framework, cellular and molecular biomarkers could be used to reach a more accurate identification of frailty, as well as those individuals in early and potentially reversible frailty stages (pre-frail individuals). The development of these new tools and their inclusion in the criteria to identify frailty would facilitate the implementation of personalized care and treatments, as well as improve outcomes by means of prevention and intervention programmes. Additional research is needed to further explore the pathophysiological bases of frailty.

In this review, population studies evaluating alterations associated with frailty status at cellular and molecular level – by means of oxidative stress, genomic and DNA repair biomarkers

– were revised and compared in order to gather all this information and to identify potential biomarkers that could be useful in frailty identification, as well as to point out gaps of knowledge and new research areas needed in this field. Results of this revision showed that several oxidative stress biomarkers –including alterations in antioxidant systems, increased levels of lipid peroxidation and DNA oxidative damage, as well as DNA methylation and some specific genetic polymorphisms –are associated with frailty status in older people. On the contrary genomic instability, or at least the two biomarkers tested so far (telomere length and MN rate) seems not to be linked to frailty. The only study which addressed the possible relationship between DNA repair modulations and frailty status also failed in finding associations.

Despite the number of cellular alterations initially associated with frailty, studies on this regard are still very scarce and limited to some of the possible cellular targets. Additional research is needed to further explore these alterations prior to include any of them in the frailty assessment criteria. However, given the solid link between DNA repair ability, genomic instability, and age and age-related disorders, deeper investigations in this line must be carried out before reaching solid conclusions.

Chapter III.

γH2AX assay as DNA damage biomarker for human population studies

1. Introduction

H2AX phosphorylation is an early event in the DNA damage response (DDR) in the vicinity of double strand break (DSB) sites. H2AX is rapidly phosphorylated, at its highly conserved amino acid Ser 139 present in the C-terminal serine/glutamine motif by PI3 kinases, to become γH2AX (Nakamura *et al*., 2010) (Figure III.1).

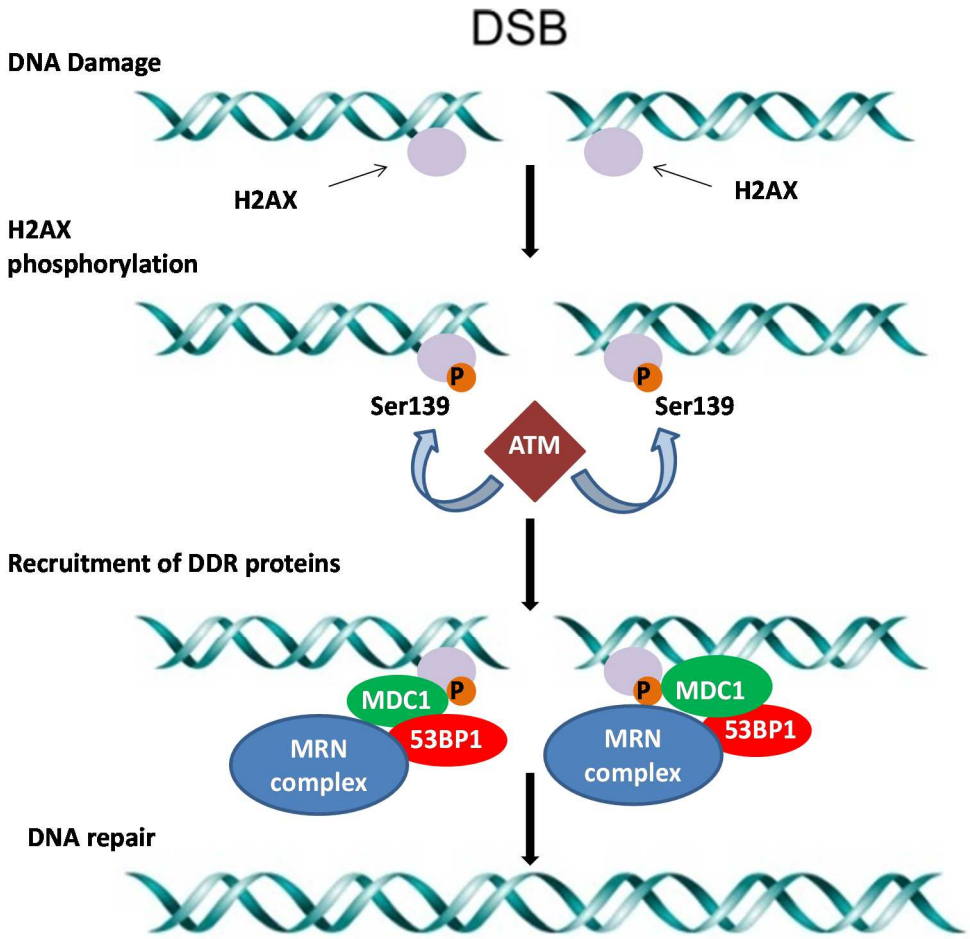

FIGURE III.1. Scheme of H2AX phosphorylation as response to double strand breaks and its involvement in recruitment of the proteins MDC1 (mediator of DNA damage check point), 53BP1 (p53 binding protein 1) and MRN (MRE11-RAD50-NBS1) complex in the early DNA damage response. ATM, Ataxia telangiectasia mutated.

The ataxia telangiectasia mutated (ATM) protein kinase is the main kinase involved in the phosphorylation of H2AX under physiological conditions. This phosphorylation is believed to lead to a change in the chromatin conformation in the damaged area to allow a better access of repair enzymes (Nikolova *et al*., 2014; Siddiqui *et al*., 2015). γH2AX role is to recruit DDR proteins and retain those mediators nearby DSB sites. γH2AX binds to mediator of DNA damage check point 1 (MDC1), which recruits p53 binding protein 1 (53BP1). These two mediators then

interact with the MRE11/RAD50/NBS1 (MRN) complex as part of the signalling pathway that leads to the DDR through either homologous recombination (HR) or non-homologous end joining (NHEJ) (Bouquet *et al*., 2006; Nakamura *et al*., 2010; Siddiqui *et al*., 2015). H2AX phosphorylation occurs within minutes and γH2AX foci disappear after 24 hours. However, residual γH2AX foci can persist permanently in the genome as consequence of defects in the efficiency of repair mechanisms; hence, they can be used as biomarker of fixed DNA damage (Sedelnikova *et al*., 2004).

Since DSB originate in many processes that disturb cellular stability, γH2AX foci detection has several practical uses in both basic research and epidemiological studies. It was used as biodosimeter for drug development (Li *et al.*, 2014), radiation exposure (Beels *et al.*, 2009), and in cancer chemo- and radiotherapy clinical trials (reviewed in Pouliliou and Koukourakis, 2014; Sak *et al.*, 2007). Furthermore, it was used as a detector of toxic environmental agents (reviewed in Geric *et al.*, 2014) and chronic inflammation (Blanco *et al.*, 2007), and as biomarker for ageing and cancer (Garm *et al.*, 2013; reviewed in Redon *et al.*, 2011). Very recently, Nikolova *et al.* (2014) confirmed γH2AX assay as a reliable biomarker for genotoxic exposures after testing 14 well-known genotoxic compounds and comparing them with 10 non-genotoxic chemicals. All chemicals in the first group showed increased levels of γH2AX foci, versus none in the second group, confirming the specificity of this assay for DNA damaging agents.

The analysis of H2AX phosphorylation has a number of advantages that make this assay very suitable to be employed as biomarker of DNA damage in population studies; among others, the specificity in recognizing DSBs and the sensitivity in detecting low frequencies of DSB, the short time frame of the protocol, the small quantity of biological sample required, and the possibility to perform automated scoring. Besides, a recent study compared the reliability of several DNA damage biomarkers by testing a variety of well-known genotoxic agents with different assays, namely Ames test, γH2AX assay, mouse lymphoma assay and chromosome aberration assay (Smart *et al.*, 2011). Among all of them, γH2AX analysis, performed by flow cytometry, showed the highest average sensitivity (91%) and specificity (89%). Nevertheless, despite all applications mentioned and its promising potential as genotoxicity and genomic instability biomarker, there is an important lack of standardization in the methodological procedure that makes it difficult to establish this approach as a routine biomarker in population studies and also hinders the comparison between studies. For instance, the measurement of γH2AX foci formation has been already performed in several previous human population studies using different cell types (isolated leucocytes, exfoliated buccal cells, fibroblasts), different cell culture proliferative state (usually peripheral blood leucocytes [PBL] unstimulated or stimulated with phytohaemagglutinin [PHA]), different cell storage conditions (fresh or cryopreserved samples), different approaches to evaluate the γH2AX foci levels (microscopy, flow cytometry,

Western blot), etc. (reviewed in Valdiglesias *et al.*, 2013). All these dissimilarities between laboratory protocols are to a great extent responsible for the high variability of results regarding this biomarker.

The objective of this study was to address the most critical issues limiting the use of the γH2AX assay as DNA damage biomarker in human population studies. To this aim, differences in γH2AX levels between employing fresh or cryopreserved PBL, as well as the influence of PHA stimulation prior to the γH2AX analysis, were assessed by flow cytometry. Thereby, cells were treated with four known genotoxic agents with well characterized γH2AX foci formation potential (bleomycin [BLM], camptothecin [Campt], actinomycin-D [Act-D] and methyl methanesulfonate [MMS]). All these four agents induce DSB by means of different mechanisms, direct or indirect; thus they were chosen to provide evidence that γH2AX analysis detects DNA damage regardless of the DSB origin or experimental condition tested.

2. Material and Methods

2.1. Chemicals

Bleomycin (BLM) (CAS number 11056-06-7), camptothecin (Campt) (CAS number 7689-03-4), actinomycin-D (Act-D) (CAS number 50-76-0), methyl methanesulfonate (MMS) (CAS number 66-27-3), RNase A, and propidium iodide (PI) were purchased from Sigma-Aldrich Co. BLM and MMS were dissolved in sterile distilled water (dH_2O), and Campt and Act-D were dissolved in dimethyl sulfoxide (DMSO) (CAS number 67-68-5) from Sigma-Aldrich Co.

2.2. Leucocyte isolation and processing

Peripheral blood was collected from three healthy non-smoker female donors (27-40 years old), by venipuncture using BD Vacutainer® CPT™ tubes with sodium heparin (Becton Dickinson) (Fig. III.2). The study followed ethical criteria established by the Helsinki declaration. Written consent was obtained from each donor prior to joining the study.

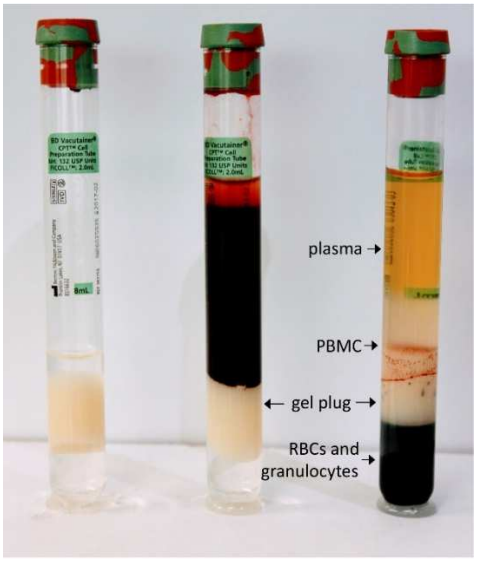

FIGURE III.2. BD Vacutainer® CPT™ tubes: empty (left), after collecting peripheral whole blood (middle), and after centrifugation (right), showing the location of plasma, peripheral blood mononuclear cell (PBMC) buffy coat, gel plug and red blood cells (RBCs) and granulocytes layers.

Mononuclear leucocytes (lymphocytes and monocytes) were isolated following manufacturer's instructions, immediately after blood extraction. In brief, CPT™ tubes were centrifuged at 9,000 rpm for 30 min at 4ºC. Subsequently, the buffy coat containing the leucocytes was transferred to another tube, washed with phosphate-buffered solution (PBS) and centrifuged (10 min 1,500 rpm 4ºC).

After a second washing with PBS, isolated mononuclear cells from each donor were divided in two fractions: cells to be cultured and treated in fresh and those to be frozen. For fresh treatments, cells (5×10^5/ml) were suspended in 900 µl of RPMI 1640 medium containing final concentrations of 15% (v/v) heat-inactivated foetal bovine serum (FBS), 1% (v/v) L-glutamine (200 mM), and 1% (v/v) penicillin (5,000 U/ml)/streptomycin (5,000 µg/ml) (all from Life Technologies), in the presence or absence of 1% (v/v) phytohaemagglutinin (PHA) depending on whether they would be stimulated or not, respectively. The cells to be frozen were suspended in appropriate freezing medium (50% of FBS, 40% RPMI 1640, 10% DMSO) at a concentration of 10^7 cells/ml, and stored at −80ºC in a Nalgene® Cryo 1ºC Freezing Container (Nalgene Nunc International), until use.

2.3. Treatments

To carry out the treatments with the different genotoxic chemicals, both frozen and fresh cells were divided in two subgroups: unstimulated cells and cells stimulated with PHA. Unstimulated cells were treated with the different genotoxic agents right after isolation or after being quickly thawed at 37ºC. Stimulated cells were incubated prior to treatments for 24 h at 37ºC in the presence of PHA.

Cells were then exposed for 4 h at 37ºC with the specific genotoxic agent at four different concentrations (1% of final volume): BLM (1, 5, 10 and 20 µg/ml), Campt (0.17, 0.7, 1.74 and 3.48 µg/ml), MMS (6.5, 13, 32.5 and 65 µg/ml) and Act-D (0.25, 0.5, 1 and 2 µg/ml). The negative control used for BLM and MMS experiments was dH_2O, whereas DMSO was employed for Campt and Act-D experiments. The chemicals used, their respective concentrations, as well as the treatment time, were selected on the basis of previous studies (Mischo *et al.*, 2005; Watters *et al.*, 2009); trypan blue exclusion technique confirmed that cytotoxicity was below 20% in all cases.

2.4. γH2AX analysis

γH2AX analysis was performed following the protocol described by Tanaka *et al.* (2009) and Watters *et al.* (2009), with some modifications (Valdiglesias *et al.*, 2011b). After the treatments, the cell suspensions were centrifuged at 2,000 rpm for 5 min and supernatant was removed. Remaining cell pellets were washed with 1ml of PBS and centrifuged at 2,000 rpm for 5 min. Subsequently, supernatants were removed and cell pellets were fixed in 1% *p*-formaldehyde. After a new centrifugation (2,500 rpm for 5 min), cells were post-fixed with cold 70% ethanol (–20ºC) and stored at 4ºC overnight. Cell suspensions were then centrifuged at 2,500 rpm for 5 min, washed in PBS, and incubated for 15 min in the dark with 100 µl anti-human γH2AX-Alexa Fluor 488-conjugated antibody (Becton Dickinson) (1:20 dilution in 1% bovine serum albumin [BSA] in PBS). Subsequently, cells were centrifuged again (2,500 rpm for 5 min), and suspended in PBS containing 0.1 mg/ml RNase A and 40 µg/ml PI and incubated for 30 min in the dark. The flow cytometry analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson). The lymphocyte population was gated according to size (forward scattering) and complexity (side scattering). A minimum of 10,000 events in the lymphocyte region were acquired, obtaining data from FL1 (γH2AX-Alexa Fluor 488) and FL2 (PI) detectors (Figure III.3). Data were analysed using Cell Quest Pro software (Becton Dickinson); the percentage of gated cells (referred as PBL from now on) positive for both γH2AX and PI were calculated with respect to the total PBL gated and indicated as %γH2AX.

2.5. Statistical Analysis

Three independent experiments were performed for each experimental condition tested, and each experiment was performed in duplicate. Experimental data were expressed as mean ± standard error. Distribution of the response variables departed significantly from normality (Kolmogorov–Smirnov goodness-of-fit test) and therefore nonparametric tests were considered adequate for the statistical analysis. Differences between groups were tested with the Kruskal–Wallis test and Mann–Whitney *U*-test. Associations between two variables were analyzed by Spearman's correlation. A p value of <0.05 was considered significant. Statistical analysis was performed using the IBM SPSS software package V. 20.

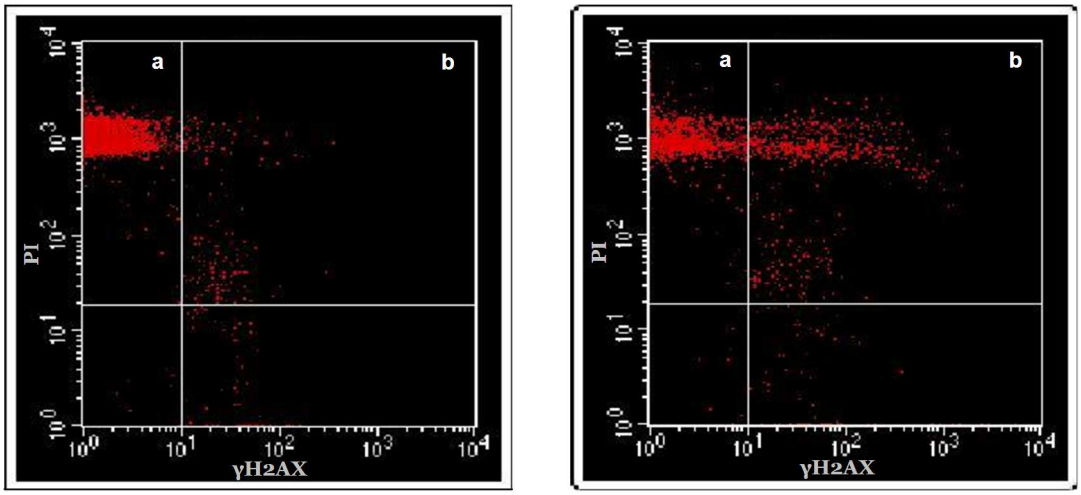

FIGURE III.3. γH2AX-Alexa Fluor 488/PI dot plot showing the regions of negative cells (a) and positive cells (b) for phosphorylated H2AX, in a control cell population (left) and cells treated with BLM (right).

3. Results

Flow cytometry analysis with anti-γH2AX antibodies was performed with the aim of comparing the early DDR against a panel of known genotoxic chemicals, through the detection of γH2AX foci formation in fresh *vs.* cryopreserved lymphocytes, and unstimulated *vs.* stimulated cells. To that ending, human PBL were treated for 4 h with BLM, Campt, Act-D or MMS, at 4 different concentrations, and results obtained are shown in Figures III.4 to III.11, respectively.

As it is shown in Figure III.4 and Figure III.5 respectively, fresh and cryopreserved lymphocytes presented higher %γH2AX in samples treated with BLM when compared with control at all concentrations tested, in both unstimulated and stimulated cells. Also significant dose-dependent increases in the DDR were evidenced in all conditions (fresh unstimulated r=0.847, $P<0.01$; fresh stimulated r=0.708, $P<0.01$; cryopreserved unstimulated r=0.526, $P<0.01$; cryopreserved stimulated r=0.825, $P<0.01$).

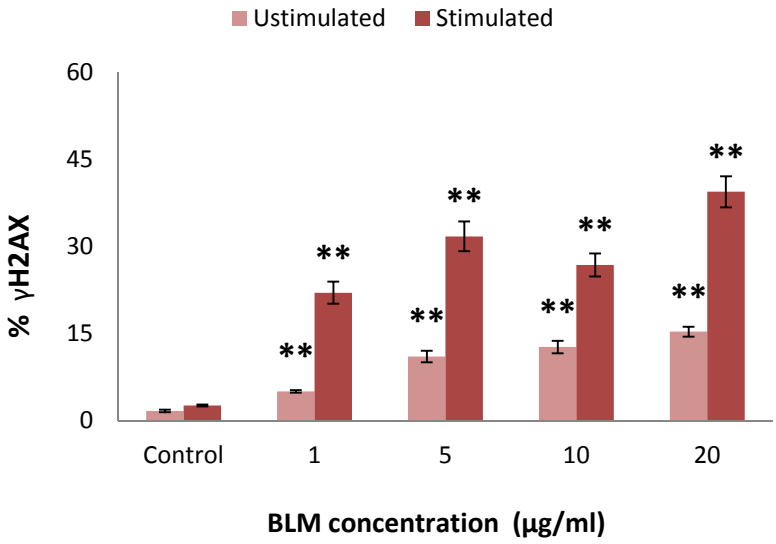

FIGURE III.4. Results of γH2AX assay in unstimulated *vs*. stimulated fresh PBL treated with BLM. Negative control: dH_2O. ***P*<0.01, significant difference with regard to the control (Mann–Whitney *U*-test).

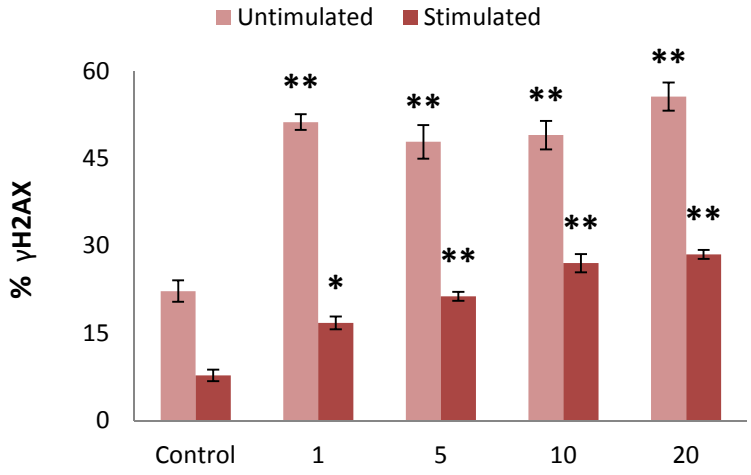

FIGURE III.5. Results of γH2AX assay in unstimulated *vs*. stimulated cryopreserved PBL treated with BLM. Negative control: dH_2O. **P*<0.05; ***P*<0.01, significant difference with regard to the control (Mann–Whitney *U*-test).

A similar response was obtained in fresh lymphocytes treated with Campt (Fig. III.6.) (unstimulated r=0.569, *P*<0.01; stimulated r=0.893, *P*<0.01) and in those exposed to Act-D (Fig. III.8.) (unstimulated r=0.795, *P*<0.01; stimulated r=0.460, *P*<0.05). Nevertheless, only cryopreserved stimulated lymphocytes treated with the highest Campt concentrations, or with all Act-D doses, showed statistically significant increases in the early DDR, and significant dose-dependent relationships (r=0.752, *P*<0.01 for Campt; r=0.515, *P*<0.01 for Act-D) (Fig. III.7 and III.9, respectively).

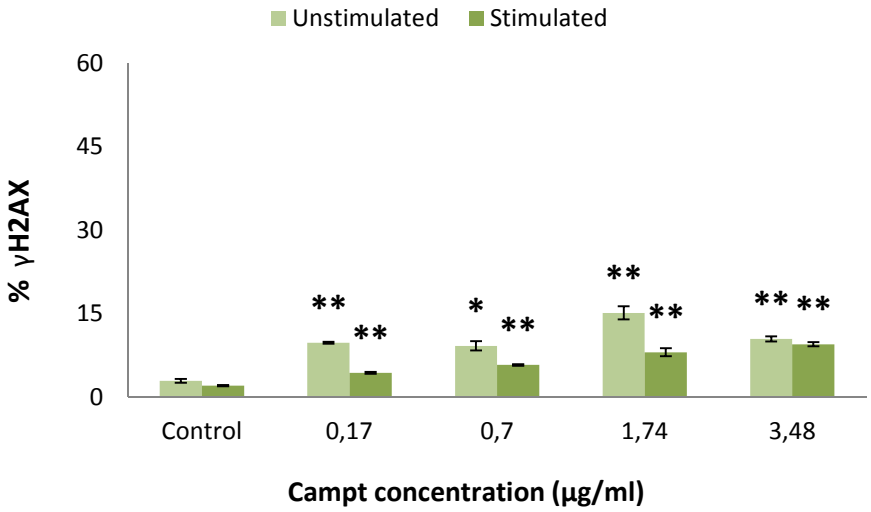

FIGURE III.6. Results of γH2AX assay in unstimulated *vs*. stimulated fresh PBL treated with Campt. Negative control: DMSO. **P*<0.05; ***P*<0.01, significant difference with regard to the control (Mann–Whitney *U*-test).

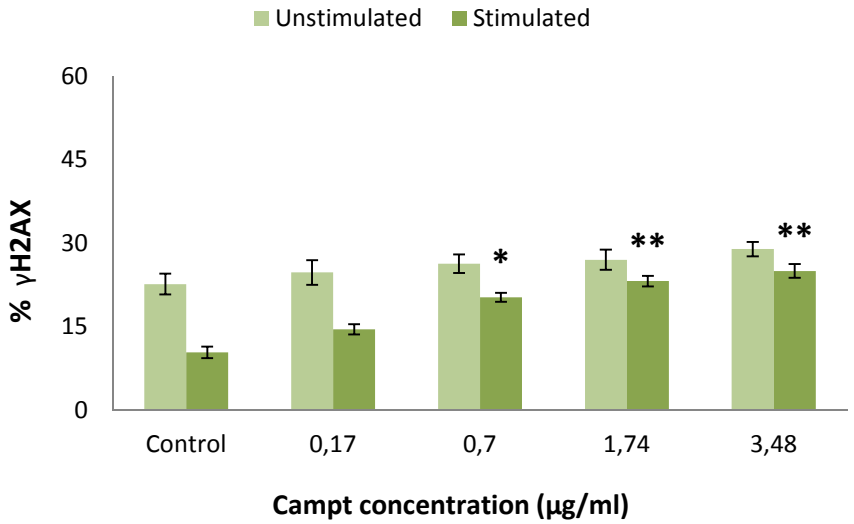

FIGURE III.7. Results of γH2AX assay in unstimulated *vs*. stimulated cryopreserved PBL treated with Campt. Negative control: DMSO. **P*<0.05; ***P*<0.01, significant difference with regard to the control (Mann–Whitney *U*-test).

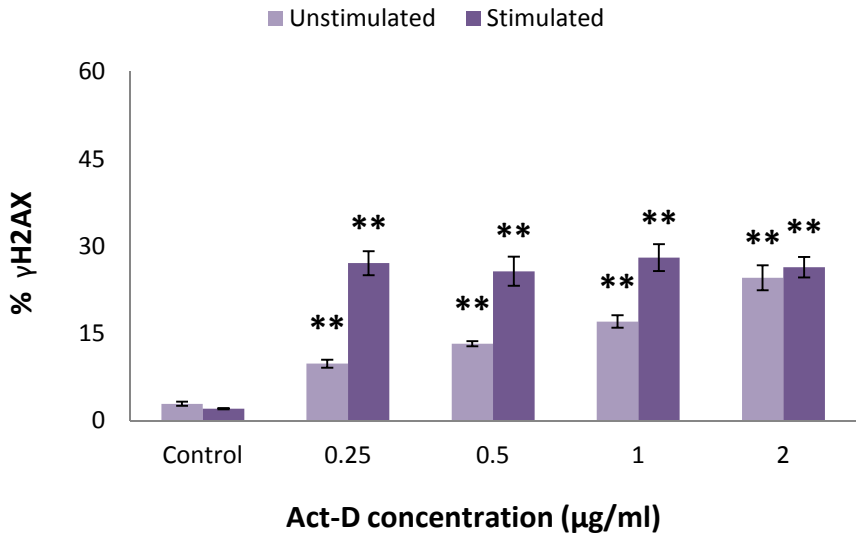

FIGURE III.8. Results of γH2AX assay in unstimulated *vs*. stimulated fresh PBL treated with Act-D. Negative control: DMSO. ***P*<0.01, significant difference with regard to the control (Mann–Whitney *U*-test).

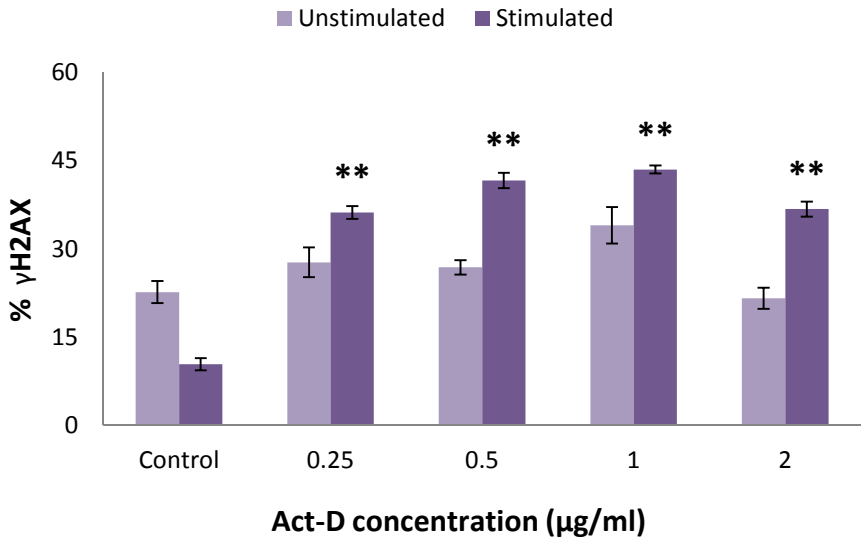

FIGURE III.9. Results of γH2AX assay in unstimulated *vs*. stimulated cryopreserved PBL treated with Act-D. Negative control: DMSO. ***P*<0.01, significant difference with regard to the control (Mann–Whitney *U*-test).

Finally, only stimulated cryopreserved lymphocytes treated with MMS at the highest concentration tested showed a statistically significant increase in H2AX phosphorylation (Fig. III.11), not evidenced in fresh cells (Fig. III.10) or in unstimulated cryopreserved lymphocytes (Fig. III.11).

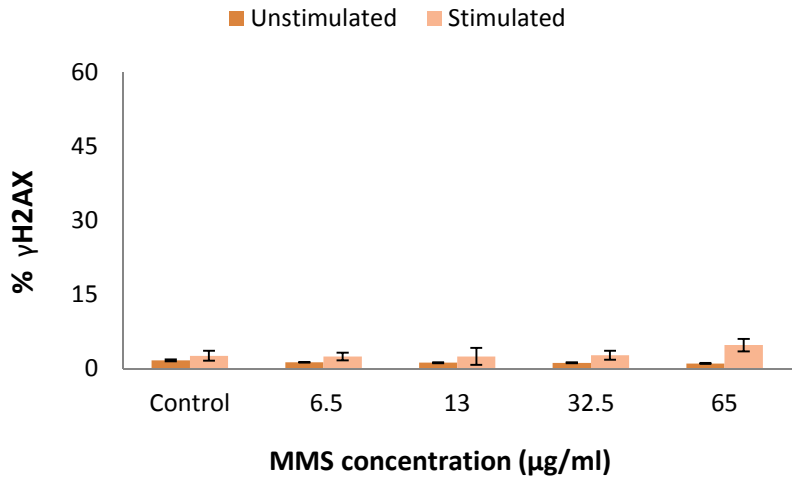

FIGURE III.10. Results of γH2AX assay in unstimulated *vs*. stimulated fresh PBL treated with MMS. Negative control: dH_2O.

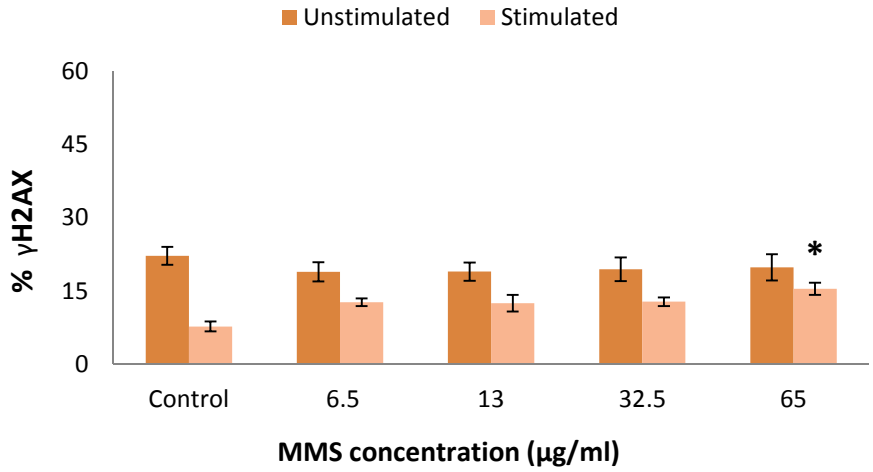

FIGURE III.11. Results of γH2AX assay in unstimulated *vs*. stimulated cryopreserved PBL treated with MMS. Negative control: dH_2O. **P*<0.05, significant difference with regard to the control (Mann–Whitney *U*-test).

Figure III.12. summarizes the comparison of γH2AX levels in negative controls (water and DMSO, see section *2.3. Treatments*) in unstimulated *vs*. stimulated cells and fresh *vs*. cryopreserved cells. No differences were observed between unstimulated and stimulated PBL employing fresh samples; however, γH2AX levels were significantly higher in unstimulated cells when cryopreserved samples were used. Furthermore, %γH2AX were always higher in cryopreserved lymphocytes when compared to those obtained from fresh samples.

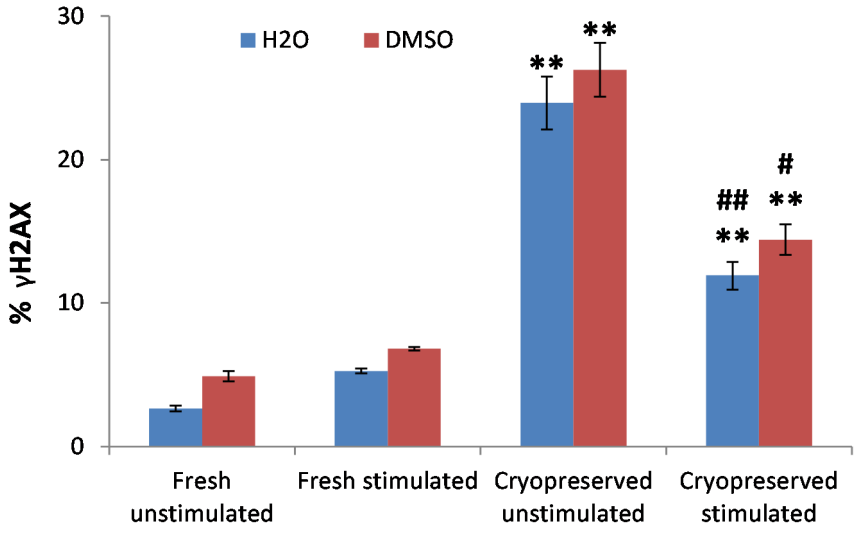

FIGURE III.12. Comparison between the basal γH2AX levels in both negative controls used (dH_2O and DMSO) for all the experimental conditions tested in this study. $^{**}P<0.01$, significant difference with regard to the corresponding fresh sample; $^{\#\#}P<0.01$, $^{\#}P<0.05$, significant difference with regard to the cryopreserved unstimulated sample (Mann–Whitney *U*-test).

4. Discussion

In recent years, an increasing number of epidemiologic studies using γH2AX assay as a biomarker of genotoxicity were reported, especially because this assay provides a valuable and highly sensitive method to monitor DSB presence in the genome. DSB are the most toxic form of DNA damage since a single unrepaired DSB could result in cell death, and inaccurate DSB repair can lead to chromosomal rearrangements (Yamamoto *et al.*, 2011). Besides, since phosphorylation of H2AX is an early event in the DDR that disappears soon, persisting γH2AX even after DNA repair may be considered as indicative of genomic instability (Podhorecka *et al.*, 2010), or cellular senescence (Mah *et al.*, 2010). The residual γH2AX could also be a sign of lethal DNA damage, so that it may be possible to identify drug-resistant tumour cells simply by measuring the fraction of cells that lack residual γH2AX foci (Banáth *et al.*, 2010).

The sensitivity of this assay, its practical accessibility, and its demonstrated utility in detecting early stages of cancer (Sedelnikova and Bonner, 2006) and other chronic and degenerative age-related diseases (Porcedda *et al.*, 2006; Sedelnikova *et al.*, 2008), emphasize the potential of this technique for an extensive use in diagnosis, prevention and management of pathological conditions, in environmental surveillance and, in general, for DNA damage biomonitoring (reviewed in Valdiglesias *et al.*, 2013). Nevertheless, the lack of experimental standardization of γH2AX assay leads to a wide heterogeneity in the results obtained and their interpretation, which affects the reliability of the assay and makes its establishment as routine biomarker in population studies difficult.

On this basis, the aim of the present study was to address the most urgent issues dealing with the validity of this approach to be employed as DNA damage biomarker in human population studies, namely the use of fresh or cryopreserved PBL, and the stimulation of lymphocyte cell cycle progression with PHA. To achieve this, flow cytometry was employed to analyse γH2AX levels in PBL treated with different genotoxic compounds under diverse experimental conditions. PBL are routinely used in human biomonitoring to asses DNA damage and repair due to their availability (Allione *et al.* 2013). They are also the most frequently employed cell type in population studies for the specific case of γH2AX analysis (Valdiglesias *et al.*, 2013).

Antibody-specific immunofluorescence is normally used to visualize the phosphorylated H2AX levels either by microscopy or by flow cytometry. Microscopy is more specific since it allows to locate the breaks within the nucleus, together with providing the measure of the exact number of DSB. However, the assessment of γH2AX using flow cytometry has a number of advantages particularly interesting for population studies. It provides an automated high-throughput platform that is fast, practical, reproducible, and may take into consideration variations due to cell-cycle effects (Watters *et al.*, 2009). Besides, it increases considerably the number of cells evaluated, diminishing the variability and enhancing the statistical power of the results (Brzozowska *et al.*, 2012). In short, the simplicity of flow cytometry, the small quantity of biological sample required, and the short time needed for the analysis offer a great benefit for handling a huge amount of samples. According to these reasons, and also considering that results obtained from these two methods were demonstrated to be correlated (Nikolova *et al.*, 2014; Watters *et al.*, 2009), flow cytometry seems to be the most suitable methodology to be employed in human population studies.

The phosphorylation of H2AX histone occurs after a DSB in the genome, which may be originated from different types of DNA damage –adducts, single strand breaks (SSB), replication or transcription blocking lesions (Sedelnikova *et al.*, 2010) – or even as a consequence of other processes different from DNA damage such as cellular stress, heat or apoptosis (Dickey *et al.*, 2009; Laszlo and Fleischer, 2009). The four genotoxic compounds chosen for this study – BLM, Campt, Act-D and MMS –, were all reported to induce concentration-dependent increases in γH2AX levels in previous works (Takahashi and Ohnishi, 2005; Watters *et al.*, 2009), although through different pathways. BLM behaves as a radiomimetic direct-acting agent, capable of inducing a wide spectrum of mutagenic lesions in mammalian cells, including DNA base damage, abasic sites, and alkali-labile sites (Milic and Kopjar, 2004; Wozniak *et al.*, 2004), which eventually result in DNA SSB and DSB. BLM induces clastogenicity acting in an S-independent manner (Povirk and Austin, 1991). Results obtained in our study for BLM treatments support these observations since it induced dose-dependent increases of γH2AX levels in all circumstances tested, regardless of cell-cycle stimulation or sample storage condition. Still, BLM-

induced H2AX phosphorylation increase was higher in stimulated cells. Although it may seem that this is not true for cryopreserved cells, BLM treatment in stimulated cryopreserved PBL increased γH2AX level by 3-fold in the highest dose tested, while in unstimulated cells this increase was only 2-fold, mainly due to the prominent basal damage in control cells. Indeed, the correlation coefficient for the dose-response relationship in stimulated cells was higher than the one obtained for unstimulated PBL (r=0.825 and r=0.526, respectively, both significant at the 0.01 level). Our results agree with other previous studies that found concentration-dependent increases of H2AX phosphorylation in different cell types after BLM exposure (Banáth and Olive, 2003; Liu *et al.*, 2014).

Campt is a known topoisomerase I S-phase specific inhibitor, frequently used in basic research as apoptosis inducer (Staker *et al.*, 2002). It is an indirect-acting genotoxic agent since it does not cause DSB directly but it binds to topoisomerase I forming a covalent ternary complex that blocks DNA re-ligation (Staker *et al.*, 2005) leading to single strand breaks that are converted to double strand breaks upon replication and, consequently, is associated with extensive H2AX phosphorylation (Banáth *et al.*, 2010). In this study, significant increases of γH2AX levels were found after Campt treatments in all fresh cells and in stimulated cryopreserved cells. These results agree with other previous studies which also reported increases in DSB induction evaluated by the same assay after Campt exposure in HL60, Jurkat and MCF7 cells (Rogakou *et al.*, 2000), and in Chinese hamster V79 and CHO cells (Banáth *et al.*, 2010). Besides, progression of apoptosis was previously found to be paralleled by a decrease in γH2AX immunofluorescence (Huang *et al.*, 2003) which would explain why, in our study, γH2AX levels were higher in unstimulated cells (in the G_0-phase of the cell cycle) compared with stimulated PBL (going through S-phase and so more sensitive to Campt apoptosis induction). Nevertheless, since this compound is also a well-known apoptosis inducer, the increase in γH2AX levels most likely reflects the onset of DNA fragmentation catalysed by nucleases in response to pro-apoptotic stimuli, and therefore may be an artefact of cytotoxicity rather than direct Campt-mediated genotoxicity (Smart *et al.*, 2011).

Act-D is a chemotherapeutic agent commonly used for treatment of childhood cancers such as Wilms' tumour and Ewing's sarcoma (Estlin and Veal, 2003). This is an intercalating agent; it intercalates into DNA strands leading to DNA damage (SSB produced via nucleotide excision repair, and a fraction of them can be converted to DSB) and inhibition of mRNA synthesis by interfering with RNA polymerase (Trask and Muller, 1988; Bensaude *et al.*, 1999). Also, even low concentrations of Act-D are able to prevent the religation step of topoisomerase I inducing both SSB and DSB (Mischo *et al.*, 2005). After treatment with Act-D our fresh PBL, independently on PHA stimulation, and also the stimulated frozen cells showed significant dose-dependent increase of γH2AX levels. Analogous genotoxic effects of this compound were

reported in several previous studies employing the same approach (Mischo *et al.*, 2005; Porcedda *et al.*, 2006), or evaluating micronucleus induction (Kirpnick *et al.*, 2005; Hashimoto *et al.*, 2010). Cell division is required for the conversion of SSB into DSB, since it occurs during the S-phase of the cell cycle: when a replication fork collides with a covalently bound topoisomerase I cleavage complex, the extension of the leading strand is terminated at the 5' -end of the template strand, which generates a DSB (Strumberg *et al.*, 2000). As a result, DNA damage (specifically DSB), and the cellular response to this damage (H2AX phosphorylation), evaluated in stimulated cells treated with Act-D is expected to be higher than that found in unstimulated cells, as actually happened in our study.

MMS is an alkylating compound which methylates DNA bases, mainly guanine producing O^6-methylguanine adducts, in a random manner (Ma *et al.*, 2011). It causes SSB that lead to DSB through either the replication or repair processes (Zhou *et al.*, 2006). Particularly, MMS-induced SSB are considered a source of DSB as a result of collapsed replication forks at the lesions or processed intermediates (Ma *et al.*, 2011). Accordingly, MMS was shown to induce concentration-dependent H2AX phosphorylation in a number of previous studies (Nikolova *et al.*, 2014; Watters *et al.*, 2009). Nevertheless, no increase in γH2AX levels was observed in the current study employing fresh cells, and just stimulated cryopreserved PBL exposed to the highest MMS dose showed a slight effect in this regard. MMS concentrations used in this study were chosen on the basis of Watters *et al.* (2009), who employed these MMS doses and found a positive dose-response relationship for H2AX phosphorylation. However, they used cultured cell lines (namely mouse embryonic fibroblasts and mouse lymphoma L5178Y cells) instead of PBL, which are known to be highly resistant to genotoxic effects (Valdiglesias *et al.*, 2011c). Furthermore, MMS-induced DNA adducts need two cell cycles to be converted into DSB (Quiros *et al.*, 2010). Since cell cycle of cultured cell lines used by Watters *et al.* (2009) is shorter than the leucocytes cell cycle, this fact may also help to explain the differences between studies. As a result, higher concentrations of MMS would likely be necessary to enhance the effects of this compound on PBL.

Phosphorylation of H2AX in response to DNA damage has been observed in both quiescent and cycling cells, and during all phases of the cell cycle, including mitosis (Giunta and Jackson, 2011). However, it usually decreases in quiescent cells (Hamasaki *et al.*, 2007; Tian *et al.*, 2011). Accordingly, taking all our results together, the levels of phosphorylated H2AX induced in stimulated cells in all cases, except for fresh PBL treated with Campt, were higher than those in unstimulated lymphocytes. This observation agrees with other previous studies in which resting cells resulted less sensitive to the induction of DNA damage than proliferating cells (Huyen *et al.*, 2004; Mohrin *et al.*, 2010; Tian *et al.*, 2011). There are two possible explanations for these findings. On one hand, the response to external stimuli of resting unstimulated cells may

be indeed different from proliferating cells response, as it was reported before (Tian *et al.*, 2011). On the other hand, when unstimulated cells are employed, just DSB coming mainly from directly induced DNA damage are revealed, while after PHA stimulation, cell cycle progression favours DSB production from other types of DNA lesions, which are also detected by γH2AX assay.

When designing a population study which includes γH2AX assay, the decision about the advisability of stimulating PBL depends on the type of study to be carried out. In environmental or occupational exposure biomonitoring cohort studies the main aim is to assess the genotoxic effects associated with the exposure. Hence, it is advisable to PHA stimulate lymphocytes in order to detect DSB coming not only from direct DNA damage but also from other types of damage, which become DSB during progression of cell cycle. Consequently, a general view of genotoxicity events will be obtained. Regarding case-control studies, as the main purpose of using H2AX assay in this case is to evaluate the persistent levels of phosphorylated histone as indicative of DNA damage already fixed (Sedelnikova *et al.*, 2004), no previous stimulation is necessary to carry out the assay since the remaining γH2AX will be already present in the DNA without requiring cell division. In such studies, this approach is applied as a biomarker of genomic instability, likely as a result of deficiencies in DNA repair processes, more than as a biomarker of genotoxic effects.

To the best of our knowledge, no studies testing the differences between H2AX phosphorylation in fresh and cryopreserved PBL were published so far. Merely, Porcceda *et al.* (2008) reported that peripheral blood mononuclear cells frozen samples, stored in liquid nitrogen for up to 4 years, showed comparable basal γH2AX levels to fresh samples, although data on the comparison were not provided. Allione *et al.* (2013) compared the influence of different blood storage conditions on DNA damage, and they found a non-significant slight increase of H2AX phosphorylation in isolated PBL after 24 h of blood storage (both at room temperature and at 4ºC) with regard to fresh samples. Similarly, several previous studies reported no significant differences in DNA strand breakage evaluated by means of comet assay between fresh and frozen PBL (Visvardis *et al.*, 1997; Duthie *et al.* 2002). In contrast to these studies, results obtained in the current work for basal γH2AX levels (negative controls) from cryopreserved PBL were always significantly higher than those obtained from fresh cells under the same experimental conditions. Still, basal histone phosphorylation was significantly lower when cryopreserved cells were previously stimulated with PHA than when they were used unstimulated. This behaviour is likely due to the fact that PHA stimulation implies incubation of PBL for 24 with complete culture medium after thawing. Therefore, it is probable that during this time the cells repair their basal DNA damage, caused in part by the storage, freezing and thawing processes.

On the whole, interpretation of the results obtained in γH2AX assay depends on the assay design. When γH2AX measurement is carried out at one single time point, it provides information on early DDR more than on actual repair processes; whereas, when the outcome kinetics is assessed (at two or more different time points). γH2AX loss correlates with DSB repair activity, as indeed was observed in a number of previous studies (Porcedda *et al.*, 2006; Bourton *et al.*, 2011; Brzozowska *et al.*, 2012); thus it allows to evaluate alterations in the DNA repair systems.

5. Conclusions

In conclusion, these findings support that flow cytometry analysis of phosphorylated H2AX histone (γH2AX) levels in human PBL may be used as a rapid screening tool for genotoxicity or genomic instability in human population studies, even though consensus in the methodological procedure should be reached in order to diminish the heterogeneity in the results. According to our results, both unstimulated and stimulated fresh PBL could be employed as cellular material to carry out the γH2AX assay. Yet, when unstimulated quiescent cells are used, it must be considered that DSB evaluated (more precisely the early repair response to DSB) are the consequence of direct damage on DNA, whereas when cells are stimulated to divide with PHA, DSB may also come from other different kinds of damage which become "visible" (i.e. measurable by this technique) during the cell division. Therefore, the decision about stimulating cells prior γH2AX analysis should be taken during the study design according to the kind of damage to be evaluated or that is expected in the individuals.

Furthermore, in human population studies, collecting samples and processing them immediately is not always possible. In such cases, cryopreserving cells seems to be the best option. On the basis of the current results, PHA stimulation is necessary for γH2AX analysis when cells are stored frozen, since basal damage is too high in cryopreserved unstimulated cells, likely as a result of freezing and thawing processes. Further studies are required in order to completely standardize the protocol of γH2AX assay to be employed as biomarker of genotoxicity or genomic instability in human biomonitoring studies.

Chapter IV.

Exploring genetic outcomes as frailty biomarkers.

1. Introduction

Despite being phenotypically well-characterized, the biological basis of frailty still remains fairly unknown. This is due to the fact that this syndrome is not characterised by impairment of a single system, but by several events and anomalies in multiple physiological systems in an intricate process that leads to frailty (Zaslavsky *et al*., 2013).

As it has been mentioned before, to date identification of frail subjects is performed using clinical features, being the most commonly employed diagnostic criteria, due to their simplicity of implementation, those proposed by Fried *et al*. in 2001, based on five phenotypical characteristics (muscle weakness, low gait speed, unintentional weight loss, exhaustion, and low physical activity). The major limitation of this assessment is the late identification of frailty, which is possible only after the onset of clinical manifestations. Therefore, to improve the clinical impact of frailty screening, it is necessary to develop new tools that allow a timely identification of those individuals more prone to develop this syndrome. The availability of these new tools would facilitate the implementation of personalized therapies, as well as the improvement of health outcomes by means of prevention and intervention programs. A deeper knowledge on the biological basis of frailty is required for the development of biomarkers for this syndrome what would allow an earlier and more objective identification of frail individuals.

The involvement of genomic instability in different age-related phenotypes has been previously reported as a consequence of the loss of balance between DNA damage and the correct function of cellular DNA repair mechanisms (Garm *et al*., 2013; Li *et al*., 2016a). However, recent studies reviewed by Gorbunova and Seluanov (2016) suggest the possibility that this imbalance can be the cause of the ageing process and age-related phenotypes, rather than its consequence. These authors suggested that not only mutations accumulate with age but also the rate of mutation accumulation increases with age, which could be due to the DNA repair pathways becoming less efficient (Garm *et al*., 2013).

The evaluation of chromatin alterations could be important for a better understanding of mutations in age-related changes (Gorbunova and Seluanov, 2016). The micronucleus (MN) test is one of the most commonly used methods for assessing chromosome damage. MN assay provides a reliable measure for both chromosome loss and chromosome breakage since MN are formed from chromosome fragments or whole chromosomes that lag behind during anaphase in cell division (Fenech, 2000). MN frequency can be evaluated in different cells and surrogate tissues. For a number of reasons, including ease of sample collection and reproducibility, peripheral blood lymphocytes and exfoliated buccal cells are the most suitable, and consequently, the most frequently employed tissues for MN studies in human populations (Fenech *et al*., 2011).

The cytokinesis-block MN (CBMN) cytome assay performed in peripheral lymphocytes is a comprehensive system for measuring DNA damage (Fenech, 2007); it has been regularly

applied in human biomonitoring of genotoxic exposures and is increasingly used in preventive medicine and nutrition (Lee *et al*., 2003; Fenech *et al*., 2005; El-Zein *et al*., 2006). MN frequency is strongly associated with the ageing process, and increases of this biomarker have been reported in several age-related diseases including cancer, diabetes, neurodegenerative diseases such as Alzheimer's or Parkinson's, and arthrosis (Bonassi *et al*., 2011b; Migliore *et al*., 2011).

The buccal MN cytome (BMNCyt) assay is an attractive candidate for the study of human populations due to its non-invasive nature. This technique, firstly proposed by Stich and Rosin in 1983, has been employed in multiple studies as a sensitive biomarker of genetic damage and cell death caused by lifestyle-related factors such as alcohol and tobacco consumption, or nutritional deficiencies, and environmental exposures to pollutants, medical procedures, as well as inherited genetic defects in DNA repair (Fenech, 2007; Thomas *et al*., 2009). Together with MN formation, this assay allows as well to identify other abnormalities indicative of different kind of cellular alterations. These abnormalities are shown as alterations in the nuclear morphology, such as binucleated cells, nuclear buds, pyknosis, karyorrhexis, abnormally condensed chromatin and karyolysis (Torres-Bugarín *et al*., 2014).

Frailty is commonly accepted to have a strong biological component resulting from cumulative cellular damage over the life-course (Dent *et al*., 2016). Increased levels of damage can lead to different cellular alterations, including genomic instability, mutations, altered gene expression, loss of cell division potential, cell death or impaired intercellular communication, among others (reviewed in Chapter II). These alterations at the cellular and molecular levels could be a good basis to establish frailty biomarkers. Nevertheless, their relationship with frailty has not been established yet. It is not clear whether or in which way genetic outcomes may influence the susceptibility to frailty, and even the few preliminary studies in this regard are not completely clear in finding any association, as it was shown in Chapter II. However, due to the well-founded belief that genome instability and other genetic outcomes are involved in the frailty syndrome, given their strong association with ageing and age-related diseases, further investigations should be carried out in this line.

Hence, in order to improve the understanding of the biological features associated with frailty status, and consequently identify potential biomarkers of frailty, in the present study several genomic instability and genetic parameters, selected according his previous reported association with the ageing process, were evaluated in a population of Spanish older adults (aged 65 and over) classified into frail, pre-frail and non-frail according to Fried *et al*. (2001) criteria. Genomic instability was assessed by MN frequency in both peripheral blood lymphocytes and exfoliated buccal cells, together with other cellular alterations in buccal mucosa. Genetic outcomes analysed included mutation rate (by means of the T-cell receptor [TCR] mutation assay), different types of genetic damage (by employing the comet assay and the γH2AX assay), and cellular repair

capacity (by the DNA repair competence assay). To provide a more comprehensive evaluation of clinical features associated with frailty, the possible association between nutritional status and cognitive impairment with the level of genomic instability and the different genetic outcomes was also evaluated.

2. Material and Methods

2.1. Subjects and sample collection

A total of 257 volunteer donors (84 males and 173 females), aged 65 years or more (79.4±8.8, range 65-102), were recruited from 14 associations of retired older people and nursing homes located in Galicia (NW of Spain) (Table IV.1.). A *post hoc* assessment of the statistical power of the study, based on the MN frequency in PBL, showed that – given the actual size of the three groups – the study had adequate statistical power (80%) to detect with a I type error of 0.05 a minimum increase of 21.6% in frail vs controls and of 20.1% in pre-frail vs controls (Post-hoc Power Calculator by www.ClinCalc.com). All donors, or their relatives in case of inability, signed an informed consent form and completed a questionnaire to collect demographic, lifestyle, and medical information. The study protocol followed the principles embodied in the Declaration of Helsinki and was approved by the University of A Coruña Ethics Committee (reference number CE 18/2014). Qualified staff with extensive experience in the gerontology field (i.e. psychologists, occupational therapists, nurses) was in charge of the clinical evaluation. To unify the criteria in completing the clinical evaluation, all staff members were equally trained prior to the start of the study. Participants were excluded if they were taking medications included in the Anatomical Therapeutic Chemical (ATC) category L (antineoplastic or immunomodulating agents (WHO collaborating centre for drug statistics methodology, 2013)) or they had cancer or any chronic infection (e.g., HIV, HCV, HBV), or if they denied signing the informed consent.

TABLE IV.1. Associations of retired older people and nursing homes

Centre	Subjects	Percentage
ATEGAL (Aulas de tercera edad de Galicia) Santiago	7	2,7
NCG (Novacaixa Galicia) A Coruña	5	1,9
NCG (Novacaixa Galicia) Betanzos	5	1,9
NCG (Novacaixa Galicia) Ferrol	30	11,6
NCG (Novacaixa Galicia) Pontedeume	5	1,9
NCG (Novacaixa Galicia) Santiago de Compostela	6	2,3
UDP (Unión democrática de pensionistas y jubilados de España) A Coruña	27	10,4
UDP (Unión democrática de pensionistas y jubilados de España) Sofán-Carballo	15	5,8
UGT Ferrol	9	3,5
UDP (Unión democrática de pensionistas y jubilados de España) Los Rosales, A Coruña	22	8,5
CSC (Centro Socio Comunitario) Vilalba	28	10,8
Complejo Gerontológico La Milagrosa, A Coruña	86	33,2
Servicio residencial Fundación AdcoR, A Coruña	6	2,3
Sanitas Residencial, A Coruña	8	3,1
Total	259	100,0

Table IV.2 shows the general characteristics of the study population. Due to the small number of current smokers and ex-smokers (*N*=5 and *N*=48, respectively) a new category, "ever smokers", was created combining both conditions. Similarly, a single category was considered including together malnourished individuals (*N*=14) and individuals at risk of malnutrition (*N*=80).

TABLE IV.2. Description of the study population

	Total	Non-frail	Pre-frail	Frail	*P*-value
Total *N* (%)	257 (100)	39 (15.4)	131 (50.6)	87 (34.0)	
Gender *N* (%)					
Males	84 (32.7)	26 (66.7)	36 (27.5)	22 (25.3)	**<0.001**[b]
Females	173 (67.3)	13 (33.3)	95 (72.5)	65 (74.7)	
Age (years-old)[a]	79.4±8.8 (65-102)	73.3±5.6 (65-85)	77.1±7.7 (65-100)	85.6±7.8 (65-102)	**<0.001**[c]
65-69	43 (16.8)	12 (30.8)	29 (22.1)	2 (2.3)	**<0.001**[b]
70-74	41 (16.0)	11 (28.2)	26 (19.8)	4 (4.7)	
75-79	47 (18.4)	10 (25.6)	24 (18.3)	13 (15.1)	
80-84	46 (18.0)	5 (12.8)	27 (20.7)	14 (16.3)	
≥85	79 (30.9)	1 (2.6)	25 (19.1)	53 (61.6)	
Smoking habits *N* (%)					
Non-smokers	199 (79.0)	22 (56.4)	102 (78.5)	75 (90.4)	**<0.001**[b]
Ever smokers	53 (21.0)	17 (43.6)	28 (21.5)	8 (9.6)	
No. cigarettes/day[a]	18.4±13.8 (2-60)	16.1±8.8 (3-40)	15.7±13.9 (2-60)	31.4±15.7 (20-60)	**0.020**[c]
Years smoking[a]	26.7±16.6 (4-66)	19.4±9.1 (10-34)	30.4±18.7 (4-66)	29.3±18.2 (6-52)	0.154[c]
BMI (kg/m^2) [a]	28.5±5.6 (16.5-53.2)	28.1±3.2 (21.1-35.1)	29.1±5.0 (18.9-47.4)	27.7±7.0 (16.5-53.2)	0.191[c]
Nutritional status*N (%)*					
Normal nutrition status	158 (62.7)	35 (89.7)	106 (80.9)	17 (20.7)	**<0.001**[b]
At risk or malnourished	94 (36.3)	4 (10.3)	25 (19.1)	65 (79.3)	
MNA-SF score[a]	11.8±2.5 (4-14)	13.3±1.4 (8-14)	12.8±1.7 (4-14)	9.7±2.4 (4-14)	**<0.001**[c]
Cognitive status *N* (%)					
No cognitive impairment	174 (69.6)	39 (100)	118 (90.1)	17 (21.2)	**<0.001**[b]
Cognitive impairment	76 (30.4)	---	13 (9.9)	63 (78.8)	
Living conditions *N* (%)					
Family home	157 (61.1)	39 (100)	113 (86.3)	5 (5.7)	**<0.001**[b]
Family home+daycare center	27 (10.5)	---	4 (3.1)	23 (26.4)	
Nursing home	73 (28.4)	---	14 (10.6)	59 (67.9)	
Education years *N* (%)					
≤8	115 (45.3)	19 (48.7)	73 (55.7)	23 (27.4)	**<0.001**[b]
9-17	96 (37.8)	12 (30.8)	32 (24.4)	52 (61.9)	
>17	43 (16.9)	8 (20.5)	26 (19.9)	9 (10.7)	

[a]Mean±standard deviation (range). [b]Chi-square test (two-tails). [c]ANOVA test (two-tails).

2.2. Frailty criteria

All subjects included in the study were classified as frail (N=87), pre-frail (N=131) or non-frail (N=39) according to the Fried's criteria (Fried *et al*., 2001), which included 5 items: (i) shrinking or unintentional weight loss, at least 4.5 kg in the previous year; (ii) muscular weakness: grip strength in the lowest 20% at baseline, adjusted for gender and body mass index (BMI); (iii) self-reported exhaustion, identified by two questions from the modified 10-item Center for Epidemiological Studies-Depression (CES-D) scale (Radloff, 1977), employing the Spanish version (Ruiz-Grosso *et al*., 2012); (iv) slow walk: the slowest 20% at baseline, based on time to walk 4.6 m, adjusting for gender and standing height; and (v) low physical activity level, the lowest 20% at baseline, based on a weighted score of kilocalories expended per week, measured by the Minnesota Leisure Time Activity (MLTA) in its validated Spanish version (Ruiz Comellas *et al*., 2012), according to each participant's report, and adjusting for gender. Individuals positive for three or more of these items were classified as frail; those positive for one or two criteria were classified as pre-frail, meanwhile those with no positive items were classified as non-frail.

Table IV. 3 shows the number of individuals positive for each one of the Fried criteria, being grip strength the most common criteria in the population of study (83%), followed by slow walk (44%) and low physical activity (34%).

TABLE IV.3. Fried frailty criteria in the population of study [N (%)]

Criteria	Negative	Positive
Unintentional weight loss	237 (92.2)	20 (7.8)
Muscular weakness	44 (17.1)	213 (82.9)
Self-reported exhaustion	209 (81.6)	47 (18.4)
Slow walk	144 (56.0)	113 (44.0)
Low physical activity	169 (65.8)	88 (34.2)
Number of positive criteria		
0	39 (15.2)	
1	89 (34.8)	
2	42 (16.4)	
3	45 (17.6)	
4	35 (13.7)	
5	6 (2.3)	

2.3. Clinical assessment

The *nutritional status* of the participants in the study was screened using the Spanish version (Nestlé Nutrition Institute) of the Mini-Nutritional Assessment-Short Form (MNA-SF) (Kaiser *et al*., 2009). This tool includes 6 questions extracted from the full MNA questionnaire (Guigoz *et al*., 1994): declined food intake over the past three months due to appetite loss, digestive problems, chewing or swallowing difficulties; involuntary weight loss during the last three months; mobility; psychological stress or acute disease in the past three months;

neuropsychological problems (severe dementia or depression); and BMI. The sum of the MNA-SF score distinguishes between elderly patients with: i) normal nutritional status (MNA-SF between 12 and 14 points); ii) at risk of malnutrition (MNA-SF 8-11 points); and iii) malnourished (MNA-SF 0-7 points).

The Spanish version (Blesa *et al*., 2001) of the Mini-Mental State Examination (MMSE) scale (Folstein *et al*., 1975) was employed to evaluate the global *cognitive status*. MMSE scores, ranging from 0 to 30, were adjusted for age and level of education, and participants were considered as cognitively impaired if they scored ≤ 24.

2.4. Biological sample collection and leucocyte isolation

Peripheral blood and buccal mucosa samples were collected by nurses and trained technicians. Whole blood was collected by venipuncture into Vacutainer tubes containing heparin as an anticoagulant for MN assay in peripheral blood lymphocytes, and into BD Vacutainer® CPT™ with sodium heparin (Becton Dickinson), for the isolation of peripheral blood mononuclear leukocytes (PBL, lymphocytes + monocytes) following manufacturer's instructions. Fresh PBL were employed in the TCR mutation assay. For the comet, γH2AX and DNA repair competence assays, isolated PBL were frozen at -80ºC in a solution composed of 50% foetal calf serum, 40% RPMI 1640, and 10% DMSO, at 10^7 cells/ml, and stored until analysis.

Exfoliated buccal cells were obtained by gently swabbing oral mucosa on the inner side of both cheeks with a cytobrush, and kept in a buffer solution (see below). Samples were transported to the laboratory immediately, where they were processed within 4 h of collection. All samples were coded at the moment of collection and analyzed under blind conditions**.**

2.5. Lymphocyte micronucleus assay

The CBMN assay was performed in duplicate following the protocol described by Fenech (2007), with minor modifications. In brief, 0.5 ml of whole peripheral blood was suspended in 4.5 ml of RPMI 1640 medium containing final concentrations of 15% (v/v) heat-inactivated foetal bovine serum (FBS), 1% (v/v) L-glutamine (200 mM), 1% (v/v) phytohaemagglutinin (PHA) and 1% (v/v) penicillin (5,000 U/ml)/streptomycin (5,000 µg/ml) (all from Life Technologies). Cell suspensions were incubated at 37 ºC with lids loose in a humidified atmosphere containing 5% CO_2 for 44 h. After this time, 10 µl of cytochalasin-B (final concentration 6 µg/ml) was added to prevent cytokinesis and the cultures were returned to the incubator for another 2 hours, to a total of 68 hours of incubation. Cell suspensions were subsequently centrifuged at 800 rpm for 10 min at 4 ºC. After removal of the culture medium supernatant, cells in mild agitation were hypotonically treated with 4 ml of cold KCl (0.56% at 4ºC) to lyse the red blood cells and centrifuged immediately (800 rpm10 min at 4 ºC). Supernatant was removed and 4 ml of

methanol:acetic acid (3:1) was added with mild agitation for cell fixation. To help the fixation 3 drops of p-formaldehyde were quickly added with a Pasteur pipette. Cells were centrifuged (800 rpm 10 min at 4 ºC) and washed with two further changes of fixative. The remaining pellets were gently resuspended and dropped onto clean glass slides to be air dried. Slides were storaged at -20 ºC until the moment of scoring. Before scoring, slides were stained with 4',6-diamino-2-fenilindol (DAPI) (5 µg/ml).

MN automated scoring was performed using a Metafer4 System fluorescence, connected to an Axio Imager Z2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), equipped with an Automated Slide Feeder x80, controlling the microscope components for automated focusing, light source adjustment (for bright field imaging) and fluorescence filter changes. Slide scanning, focusing, sample capture and image analysis were performed as previously described (Varga *et al*., 2004). A minimum of 2,000 binucleated (BN) cells per individual, 1,000 from each duplicate culture, were automatically scored to determine the number of MN in lymphocytes (MN-L). After the automated scan, the image gallery was visually reviewed by an experienced scorer, following the criteria described by Fenech (2007) for MN and BN cells, in order to reject unsuitable cells and to correct feature values if necessary.

2.6. Buccal micronucleus assay

The BMNCyt assay was performed as described by Thomas *et al*. (2009), with minor modifications. Cytobrushes (Cell sampler peel-pack, Deltalab S.L.U.) were used to collect buccal cells by rotating the brush 20 times in a circular motion against the inner side of each check starting from a central position, gradually increasing the circumference. Separate brushes were employed for each cheek, and suspended in 5ml of a buffer solution (EDTA 0.1M, TrisHCl 0.01M, NaCl, 0.02M) in different tubes. After centrifugation, supernatant was removed and replaced with fresh buffer solution and washed twice more. Cells were placed into slides and air-dried overnight. Slides were fixed with a cold solution absolute ethanol:acetic acid glacial (3:1 v/v;). Air-dried slides were storaged at -20ºC until the moment of staining and scoring.

Fixed slides were treated in 5 M HCl for 30 min and washed in water. Slides were stained with Schiff's reagent (Merck) at room temperature in the dark (1-3 h). After been washed in distillated water, slides were counterstained in 1% Fast Green solution (Merck) for 5 sec, washed in 70% ethanol (3 times, 2 min each) and air-dried.

Slides were scored blindly by a single scorer in a Nikon E-800 fluorescence microscope with ethidium bromide filter. The scoring criteria for the distinct cell types and nuclear anomalies were based on those described by Tolbert *et al*. (1992) and Thomas *et al*. (2009). A minimum of 1,000 cells was scored to determine the frequency of each cell type in the sample, including basal

and differentiated cells, binucleated cells (BN-B), condensed chromatin cells (Cond-chrom-B), karyorrhectic cells (Karyorrhectic-B), pyknotic cells (Pyknotic-B), and karyolytic cells (Karyolytic-B). A minimum of 2,000 differentiated cells was scored to analyse the number MN (MN-B) and the number of cells with nuclear buds (NBUD-B).

2.7. T-cell receptor mutation assay

TCR mutation assay was conducted in duplicate following the protocol proposed by Akiyama *et al*. (1995). In brief, isolated PBL were incubated for 15 min with 7-amino-actinomycin D as a viability marker, and with fluorescein isothiocyanate (FITC)-labelled antiCD3 and phycoerythrin (PE)-labelled antiCD4 antibodies (Becton Dickinson). Cell suspensions were then centrifuged for 5 min at 2,000 rpm, supernatants were removed and cell pellets were resuspended in PBS. This step was repeated twice.

Cell suspensions were analysed using a FACScalibur flow cytometer (Becton Dickinson) with Cell Quest Pro software (Becton Dickinson). The lymphocyte population was gated according to size and complexity. A minimum of 2.5×10^5 lymphocyte gated events were acquired, and TCR mutation frequencies (TCR-Mf) were calculated as the number of events in the mutant cell window ($CD3^-CD4^+$ cells) divided by the total number of events corresponding to $CD4^+$ cells (Figure IV.1).

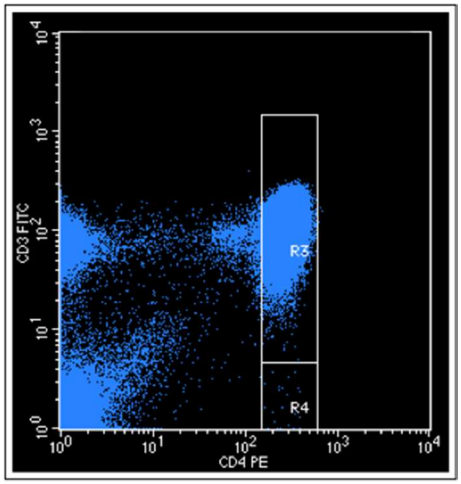

FIGURE IV.1. CD3-FITC / CD4-PE dot plot showing the mutant window (R4) in the determination of TCR mutation frequency.

2.8. Alkaline comet assay

To conduct the alkaline comet assay, following the protocol previously described in Laffon *et al*. (2002), PBL were rapidly thawed at 37ºC and subsequently centrifuged. The supernatant was removed and the remaining pellet was suspended in PBS. Cell viability was assessed by trypan blue exclusion technique being, in all cases, higher than 85%. Cells were then

embedded in 80 µl of 0.7% low melting point agarose in PBS. Cells were then dropped as two drops onto a slide that was previously coated with a layer of 1% normal melting point agarose and covered with coverslips. Slides were placed on ice for 10 min and, after the second layer of agarose was solidified, coverslips were removed. Slides were then immersed in freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 250 mM NaOH, pH 10, and 1% Triton X-100 added just before use) for an hour at 4 ºC in the dark.

After lysis, slides were placed in a horizontal electrophoresis tank (420x300x90 mm) in an ice bath. Slides were completely covered with the unwinding buffer solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13) and left in the dark for 40 min. Subsequently, electrophoresis was carried out for 30 min at 300 mA and 25 V (0.83V/cm). After electrophoresis, slides were washed three times (5 min each) with neutralizing solution (400 mM Tris-HCL, pH 7.5) and air dried for 10 min in the dark. DAPI was employed to stain the slides. The preparations were kept in a humidified sealed box to prevent drying of the agarose gel and were analysed within 48 h.

An internal standard (PBL isolated from whole blood extracted once from a single donor, and stored aliquoted at -80ºC) was introduced in every electrophoresis run as described by Cebuslka-Wasilewska (2003). Comet IV software (Perceptive Instruments) was used for image capture and analysis. For all donors and standards, 50 cells were scored from each replicate slide (i.e. 100 cells in total) by a single scorer. The percentage of DNA in the comet tail (%TDNA) was evaluated as DNA damage parameter.

2.9. γH2AX assay

γH2AX analysis was performed in duplicate following the protocol previously described in Chapter III, section 2.4. Briefly, after being thawed cell suspensions were centrifuged and supernatants were removed. Remaining pellets were suspended in culture medium containing 1% PHA and incubated for 24 h at 37ºC. After fixation, cell suspensions were incubated with anti-human γH2AX-Alexa Fluor 488-conjugated antibody (Becton Dickinson) and stained with propidium iodide (PI). Flow cytometry analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson) with Cell Quest Pro software (Becton Dickinson). A minimum of 10,000 events in the lymphocyte region (gated according to size and complexity) were acquired, obtaining data from FL1 (γH2AX-Alexa Fluor 488) and FL2 (PI) detectors. The percentage of gated events positive for both γH2AX and PI was calculated with respect to the total lymphocytes gated and indicated as %γH2AX.

2.10. DNA repair competence assay

DNA repair competence assay was performed as previously described by Laffon *et al*. (2010). In brief, after being rapidly thawed, PBL were centrifuged and the remaining pellet was incubated for 24 h at 37ºC in culture medium containing 1% PHA. Cells were then treated with

the challenging agent bleomycin (BLM) for 30 min at 37ºC to induce DNA damage. Two duplicate slides were prepared for each donor. The first ones from cells that continued the comet assay protocol as described above directly after BLM treatment (labelled as *before repair*); the second ones from cells that, after BLM treatment, were incubated in fresh culture medium for 15 min at 37ºC to allow DNA repair (labelled as *after repair*), before being processed following the comet assay protocol. An internal standard was introduced as well in each experiment as described by Cebulska-Wasilewska (2003). Final data are shown as percentage of repair capacity (%RC), calculated as follows: $(\%TDNA_{BR} - \%TDNA_{AR}) \times 100 / \%TDNA_{BR}$, where "BR" is before repair and "AR" is after repair.

PBL samples from several individuals (mostly frail and a few pre-frail) were lost due to unexpected problems in storage. Hence, the number of data available in comet assay and DNA repair capacity evaluation are lower than in the rest of assays.

2.11. Statistical analysis

The three groups of older adults (non-frail, pre-frail and frail) were compared by socio-demographic factors (i.e., gender, age, living conditions, and years of education), lifestyle factors (i.e., smoking habit, alcohol consumption, and nutritional status), and clinical characteristics (i.e., BMI, and cognitive status). The Chi-square test was applied for categorical variables and the analysis of variance (ANOVA) for continuous variables.

Statistical analyses were carried out following the recommendations given by Thomas *et al*. (2009) for the buccal MN cytome assay. The effect of frailty status on biological parameters studied was preliminarily tested through ANOVA with the Tuckey's *post-hoc* test. Kolmogorov-Smirnov goodness-of-fit test was applied to assess normal distribution of the data; only MN-L and %γH2AX followed a normal distribution. A log-transformation of the data was applied to BN-B and TCR-Mf, and a square root transformation was applied to Karyorrhectic-B, to achieve a better approximation to the normal distribution. No improvement was achieved with transformation in all other parameters, so the Kruskal-Wallis test with Bonferroni's correction was applied for univariate statistics.

For the analysis of MN tests parameters, best fitting multiple regression models were used to estimate the effect of frailty status, nutritional status and cognitive status. All models included gender, age, BMI, and smoking habit (never/ever smokers). Poisson regression was carried out with NBUD-B, Cond-chrom-B, and Pyknotic-B, and negative binomial regression was fitted for MN-L, MN-B, BN-B, Karyorrhectic-B, and Karyolytic-B. Mean ratio (MR) was used as the point estimate of effect accompanied by its 95% confidence interval (95% CI). For those MN tests parameters significantly influenced by frailty and cognitive status, new models were run including both parameters mutually adjusted, and adjusting also by gender, age, BMI, and smoking habit.

Negative binomial regression models were also used to estimate the effect of frailty status and other clinical parameters on TCR-Mf since this parameter is a count. The same effects on %γH2AX, %TDNA, and %RC were assessed by applying linear regression models on the log-transformed data. All models included gender, age, and smoking habit (never/ever smokers). Similar models were run adjusting also by BMI, but results obtained were very similar in all cases. The results are presented as mean ratios (MR) and 95% confidence intervals (95% CI).

Partial correlation coefficients adjusted by gender, age, BMI, and smoking were used to estimate associations between biological parameters. The threshold of significance was established at 0.05. The statistical software used for the analyses were the IBM SPSS software package V. 20 (SPSS, Inc), and the STATA/SE software package V. 12.0 (StataCorp LP).

3. Results

A total of 257 older adults (age range 65-102, including 31% aged 85 and over) were included in this study. After clinical classification based on Fried's criteria 39 subjects (15.4%) were classified as non-frail, 131 (50.6 %) as pre-frail, and 87 (34.0%) as frail (Table IV.2). Sixty-eight per cent (*N*=89) of pre-frail subjects showed only one frailty criterion, while 32% (*N*=42) showed two frailty criteria. The most commonly reported positive item among them (96%, *N*=126) was muscle weakness (low grip strength). Smoking was more frequent in the non-frail group. Although the number of cigarettes smoked per day was higher in the frail smokers, no significant difference in the duration of smoking was observed among the three groups. The proportion of individuals malnourished or at risk for this condition was much higher among the group of frail (79.2%), than in pre-frail (19.1%) or non-frail (10.3%) and, accordingly, the MNA-SF score was significantly lower in the frail group. Presence of cognitive impairment was observed in 9.9% of pre-frail subjects and in the 78.8% of frail subjects. No case of cognitive impairment was reported in the non-frail individuals. All non-frail subjects and the large majority of pre-frail lived at family home. Most frail subjects lived in nursing homes, although a quarter of them lived at family home but attending daycare centers. The duration of education was similar in the non-frail and pre-frail groups, while frail subjects presented a significantly lower number of years of education.

Since MN evaluation was carried out in different tissues and by means of two different methodologies, analysis of results obtained will be presented, compared and discussed separately from the other genetic outcomes' results.

3.1. Micronucleus evaluation in lymphocytes and buccal cells

Table IV.4 shows the results of both MN assays (in PBL and in buccal cells) in the non-frail, pre-frail and frail groups. Buccal basal cells were not observed in any of the individuals

analyzed, and all micronucleated buccal cells contained only one MN. Univariate analysis of data showed that the frequencies of MN-L and BN-B increased progressively with frailty status, showing significant differences when frail and non-frail subjects are compared. On the contrary, decreases in the frail group were observed in the frequencies of Pyknotic-B, as compared with the other two groups, and Karyolytic-B, as compared to the group of non-frail individuals. No differences were obtained for the frequencies of MN-B, NBUD-B, Cond-chrom-B, or Karyorrhectic-B.

When correlations between MN-L and all parameters obtained in the BMNCyt assay were assessed, a significant association was only found for BN-B (r=0.367, $P<0.001$). In order to determine the single contribution of each frailty criterion to MN-L frequency, this parameter was compared in the groups of subjects negative and positive for each individual criterion (Figure IV. 2). Highly significant increases in MN-L rate were observed in subjects positive for the criteria low physical activity, slow walking time and low grip strength when compared to those individuals negative for the corresponding criterion. No difference was observed in unintentional weight loss, and there was a borderline significant difference in exhaustion.

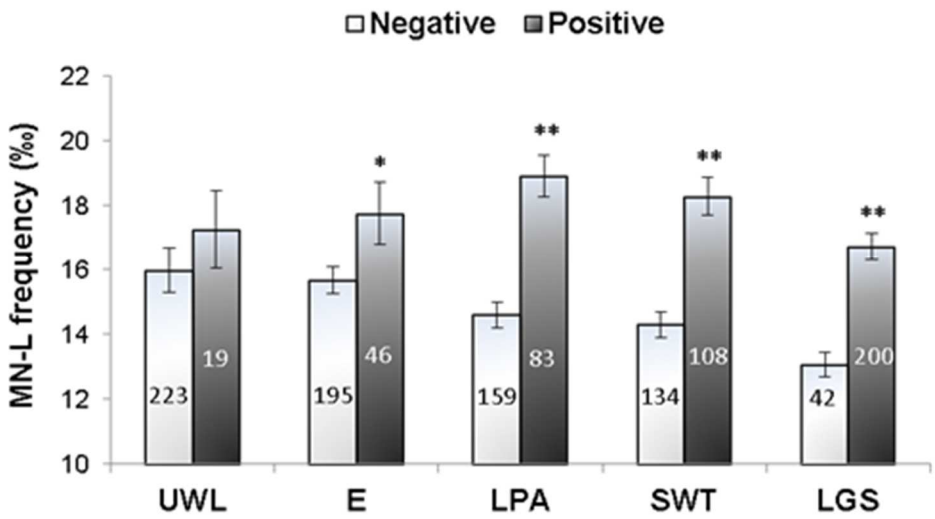

FIGURE IV.2. Micronuclei in lymphocytes (MN-L) frequency in the older adult population, according to each frailty criterion (Fried *et al*., 2001). The number of individuals included in each group is indicated inside each rod. **P*<0.05, ***P*<0.001, significant difference with regard to negative (Student's *t*-test). Bars represent mean standard error. UWL: unintentional weight loss; E: exhaustion; LPA: low physical activity; SWT: slow walking time; LGS: low grip strength.

TABLE IV.4. Results of micronuclei evaluation in lymphocytes and buccal cells in the study group, classified according to frailty status (univariate analysis)

	Non-frail			Pre-frail			Frail			*P*-value[#]
	N	Mean	SE	*N*	Mean	SE	*N*	Mean	SE	
‰MN-L	37	13.07	± 0.78	122	14.87	± 0.45	83	19.16	± 0.66[†,‡]	**<0.001**
‰MN-B	30	0.70	± 0.36	102	0.46	± 0.10	81	0.75	± 0.17	0.582
‰NBUD-B	30	0.10	± 0.06	102	0.03	± 0.02	81	0.02	± 0.02	0.151
‰BN-B	30	36.17	± 2.85	102	43.13	± 2.18	81	82.65	± 3.42[†,‡]	**<0.001**
‰Cond-chrom-B	30	0.53	± 0.40	102	0.04	± 0.02	81	0.02	± 0.02	0.554
‰Karyorrhectic-B	30	22.50	± 3.12	102	18.96	± 1.34	81	22.14	± 2.06	0.537
‰Pyknotic-B	30	0.57	± 0.14	102	0.33	± 0.07	81	0.11	± 0.04[†,‡]	**0.001**
‰Karyolytic-B	30	3.37	± 0.90	102	2.21	± 0.26	81	2.63	± 0.65[†]	**0.018**

MN-L, micronuclei in lymphocytes; MN-B, micronucleus in buccal cells; NBUD-B, nuclear buds in buccal cells; BN-B, binucleated buccal cells; Cond-chrom-B, buccal cells with condensed chromatin; Karyorrhectic-B, karyorrhectic buccal cells; Pyknotic-B, pyknotic buccal cells; Karyolytic-B, karyolitic buccal cells.

[#]Multiple group comparison (ANOVA or Kruskal-Wallis test). [†]Statistically different from non-frail. [‡]Statistically different from pre-frail (Tukey's test or Bonferroni's correction).

Results obtained in the multivariate statistical analyses, adjusting for gender, age, BMI, and smoking habit, confirmed previous univariate analysis results (Table IV.5), i.e., frail individuals showed a 44% significant increase in the frequency of MN in lymphocytes, a significant doubling of binucleated buccal cells, and significant decreases in the frequencies of pyknotic buccal cells, and of condensed chromatin buccal cells. Only in this last outcome, pre-frail subjects presented a significant result, while all the other parameters did not significantly differ from non-frail.

A borderline significant influence of age on the study outcomes was found for the frequency of MN-L and Pyknotic-B (MR=0.99, 95% CI=0.99-1.00, P=0.022, and MR=0.96, 95% CI=0.92-1.00, P=0.029, respectively). Females presented significantly higher rates of MN-L than males (MR=1.18, 95% CI=1.09-1.29, P<0.001), but significantly lower values of Cond-chrom-B (MR=0.16, 95% CI=0.05-0.55, P=0.003). BMI influenced significantly and inversely the frequency of Karyolytic-B (MR= 0.96, 95% CI=0.92-0.99, P=0.014).

The possible influence of nutritional status and cognitive status on the various study parameters was assessed (Table IV.6). Individuals malnourished or at risk of malnutrition presented significantly higher values of MN-L and BN-B, and significantly lower frequency of Pyknotic-B than individuals with normal nutrition. Equivalent results were observed for subjects with cognitive impairment, as compared to subjects with normal cognitive status.

TABLE IV.5. Effect of frailty status on MN tests parameters in lymphocytes and buccal cells; models adjusted by age, sex, BMI, and smoking habit

	‰MN-L		‰MN-B		‰NBUD-B		‰BN-B	
	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI
Frailty status								
Non-frail	1.00		1.00		1.00		1.00	
Pre-frail	1.07	(0.94-1.22)	0.77	(0.24-2.47)	0.39	(0.06-2.42)	1.14	(0.94-1.39)
Frail	**1.44****	**(1.24-1.67)**	1.23	(0.32-4.70)	0.41	(0.04-4.63)	**2.06****	**(1.65-2.57)**

	‰Cond-chrom-B		‰Karyorrhectic-B		‰Pyknotic-B		‰Karyolytic-B	
	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI
Frailty status								
Non-frail	1.00		1.00		1.00		1.00	
Pre-frail	**0.15***	**(0.04-0.50)**	0.84	(0.60-1.16)	0.72	(0.37-1.41)	0.68	(0.35-1.35)
Frail	**0.13****	**(0.02-0.84)**	1.02	(0.70-1.49)	**0.29***	**(0.10-0.81)**	0.85	(0.39-1.86)

CI, confidence interval; MN-L, micronuclei in lymphocytes; MN-B, micronucleus in buccal cells; NBUD-B, nuclear buds in buccal cells; BN-B, binucleated buccal cells; Cond-chrom-B, buccal cells with condensed chromatin; Karyorrhectic-B, karyorrhectic buccal cells; Pyknotic-B, pyknotic buccal cells; Karyolytic-B, karyolitic buccal cells.
$^{*}P<0.05$; $^{**}P<0.01$.

TABLE IV.6. Effect of nutritional status and cognitive status on MN tests parameters in lymphocytes and buccal cells; models adjusted by age, sex, BMI, and smoking habit

	‰MN-L		‰MN-B		‰NBUD-B		‰BN-B	
	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI
Nutritional status								
Normal nutrition	1.00		1.00		1.00		1.00	
At risk or malnourished	**1.23****	**(1.12-1.35)**	1.38	(0.55-3.46)	0.73	(0.11-4.74)	**1.50****	**(1.29-1.75)**
Cognitive status								
No cognitive impairment	1.00		1.00		1.00		1.00	
Cognitive impairment	**1.40****	**(1.27-1.55)**	2.11	(0.91-4.88)	0.81	(0.13-5.18)	**1.87****	**(1.61-2.17)**

	‰Cond-chrom-B		‰Karyorrhectic-B		‰Pyknotic-B		‰Karyolytic-B	
	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI
Nutritional status								
Normal nutrition	1.00		1.00		1.00		1.00	
At risk or malnourished	1.57	(0.17-14.71)	1.27	(1.01-1.61)	**0.39***	**(0.18-0.88)**	1.28	(0.77-2.11)
Cognitive status								
No cognitive impairment	1.00		1.00		1.00		1.00	
Cognitive impairment	0.35	(0.07-1.72)	1.08	(0.84-1.38)	**0.28****	**(0.11-0.73)**	1.15	(0.68-1.94)

CI, confidence interval; MN-L, micronuclei in lymphocytes; MN-B, micronucleus in buccal cells; NBUD-B, nuclear buds in buccal cells; BN-B, binucleated buccal cells; Cond-chrom-B, buccal cells with condensed chromatin; Karyorrhectic-B, karyorrhectic buccal cells; Pyknotic-B, pyknotic buccal cells; Karyolytic-B, karyolitic buccal cells.

*P<0.05; **P<0.01.

When frailty status and cognitive status were mutually adjusted, the presence of frailty and cognitive impairment were independently related to an increase in the frequency of MN-L (MR=1.22, 95% CI=1.02-1.46, $P<0.05$ for frailty, and MR=1.25, 95% CI=1.10-1.43, $P<0.01$ for cognitive impairment) and BN-B (MR=1.59, 95% CI=1.23-2.05, $P<0.001$ for frailty, and MR=1.45, 95% CI=1.21-1.75, $P<0.001$ for cognitive impairment), both of them remaining significant. A decrease of Pyknotic-B frequency remained, not any longer significant (MR=0.54, 95% CI=0.13-2.31, $P=0.404$ for frailty, and MR=0.38, 95% CI=0.10-1.40, $P=0.146$ for cognitive impairment). On the contrary, the inclusion of frailty in models fitting nutritional status reduced the strength of the association between this parameter and all endpoints of the cytome assay, which resulted not any longer significant.

3.2. Other genetic outcomes

Table IV.7 shows the results of the different genetic parameters tested in the study population. According to the univariate analyses, no significant influence of frailty status on TCR-Mf, %TDNA or %RC was obtained, although a certain decrease in DNA damage in frail subjects and a clear tendency to decline in repair capacity with increasing frailty status was observed. Moreover, a significant ($P<0.01$) and progressive increase of %γH2AX with frailty severity was also detected.

Associations between parameters tested were not obtained according to Spearman's correlation. Nevertheless, when associations with MN rate in peripheral lymphocytes were tested, a significant correlation was obtained for the association with %γH2AX (r=0.252, $P<0.001$).

Results obtained in the multivariate statistical analyses, adjusting by gender, age, and tobacco consumption (and alternatively adjusting by BMI), confirmed previous results from univariate analyses on the influence of frailty (Table IV.8), i.e., increasing frailty severity was accompanied by a progressive decrease in repair capacity and increase in H2AX phosphorylation; significance was observed for this last parameter in frail individuals with regard to non-frail ($P<0.05$). TCR-Mf and comet assay results did not show any significant effect. No significant influences were obtained for gender, age or smoking on any parameter tested, and including BMI in the models scarcely changed the results.

Given the positive influence of frailty on γH2AX assay results, and in order to determine the single contribution of each frailty criterion to γH2AX levels, this parameter was compared in the groups of subjects negative and positive for each individual criterion (Figure IV.3). No differences were observed between individuals negative and positive for unintentional weight loss or exhaustion. However, significantly higher values of %γH2AX were observed in individuals positive for the criteria low physical activity ($P<0.001$), slow waking time ($P<0.01$) and low grip strength ($P<0.01$) when compared to those individuals negative for each corresponding criterion.

TABLE IV.7. Results of biomarkers analyzed in the study group, classified according to frailty status (univariate analysis)

	Non-frail			Pre-frail			Frail			*P*-value[#]
	N	Mean	SE	*N*	Mean	SE	*N*	Mean	SE	
TCR-Mf	37	4.99 ±	0.86	119	4.34 ±	0.23	87	4.43 ±	0.39	0.566
%TDNA	37	11.35 ±	2.10	114	11.00 ±	1.23	33	6.78 ±	1.85	0.127
%γH2AX	32	10.20 ±	0.59a	100	12.01 ±	0.48b	86	13.55 ±	0.51c	**0.001**
%RC	35	41.08 ±	3.81	102	35.30 ±	2.62	33	30.60 ±	4.95	0.295

TCR-MF, T-cell receptor mutation frequency; TDNA: DNA in the comet tail; γH2AX, phosphorylated H2AX histone; RC, repair capacity.
[#]Multiple group comparison (ANOVA or Kruskal-Wallis test). Different letter indicates statistically different groups (Tukey's test).

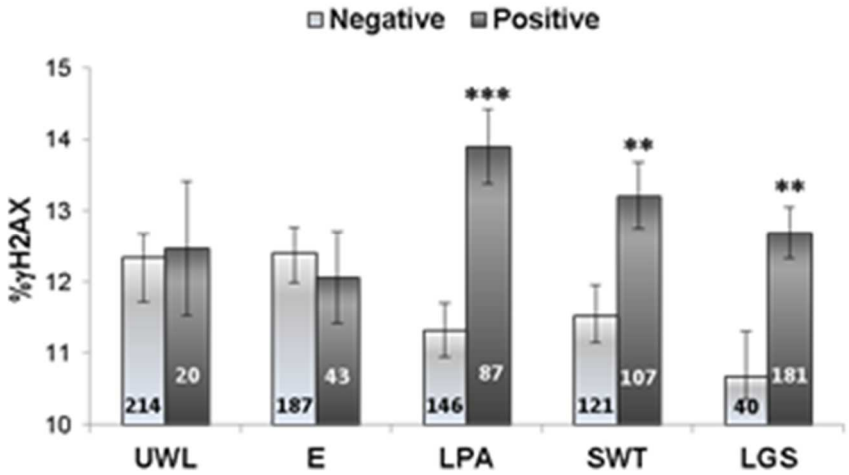

FIGURE IV.3. Percentage of H2AX phosphorylation in the older adult population, according to each frailty criterion (Fried *et al*., 2001). The number of individuals included in each group is indicated inside each rod. Bars represent mean standard error. ***P*<0.01, ****P*<0.001, significant difference with regard to negative (Student's *t*-test). UWL: unintentional weight loss; E: exhaustion; LPA: low physical activity; SWT: slow walking time; LGS: low grip strength.

TABLE IV.8. Effect of frailty status on the biomarkers analyzed; models adjusted by age, sex, and smoking habits.

	TCR-Mf		%TDNA		%γH2AX		%RC	
	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI
Frailty status								
Non-frail	1.00		1.00		1.00		1.00	
Pre-frail	0.99	(0.93-1.06)	0.90	(0.64-1.27)	1.13	(0.95-1.34)	0.58	(0.30-1.15)
Frail	1.08	(0.06-1.00)	0.66	(0.41-1.08)	**1.25***	**(1.03-1.53)**	0.46	(0.18-1.23)
Gender								
Male	1.00		1.00		1.00		1.00	
Female	0.99	(0.81-1.22)	1.27	(0.93-1.72)	1.00	(0.87-1.15)	1.17	(0.63-2.18)
Age	1.00	(0.99-1.01)	1.00	(0.99-1.01)	1.01	(0.99-1.01)	0.98	(0.95-1.02)
Smoking habits								
Non-smokers	1.00		1.00		1.00		1.00	
Smokers	1.16	(0.92-1.47)	0.98	(0.70-1.38)	1.09	(0.93-1.28)	1.03	(0.55-1.95)

CI: confidence interval; TCR-MF, T-cell receptor mutation frequency; TDNA: DNA in the comet tail; γH2AX, phosphorylated H2AX histone; RC, repair capacity.

*P<0.05.

Multivariate analyses were also applied to estimate the influence of other clinical parameters on the biomarkers tested. Nutritional status did not show significant effects on any biomarker. Notably, both TCR-Mf and %γH2AX increased significantly with the 10-years mortality risk estimation ($P<0.001$ and $P=0.026$, respectively), and significant associations were found between these parameters (r=0.140, $P=0.029$ for TCR-Mf, and r=0.231, $P=0.001$ for %γH2AX). Moreover, subjects presenting cognitive impairment showed a significant 16% increase (95%CI 1.02-1.31, $P=0.026$) in the level of H2AX phosphorylation than subjects with normal cognitive status.

4. Discussion

Frailty is a condition of vulnerability involving an increased risk of poor health outcomes in older adults. The use of biomarkers to identify frail subjects not only would be a more precise and objective method for frailty identification, but also would make epidemiological studies more comparable, allowing to draw suitable conclusions from them. Besides, they might have the potential of anticipating the recognition of frail individuals thus helping to prevent or attenuate the negative outcomes of frailty. However, and despite the last evidence supporting the relationship between a number of cellular alterations and frailty (reviewed in Chapter II), up to now no biological feature has been validated to be employed as a biomarker to identify frailty status.

4.1. Micronucleus evaluation in lymphocytes and buccal cells

The association of genomic instability and the ageing process has been widely described (Thomas *et al*., 2008; Garm *et al*., 2013; Gorbunova and Seluanov, 2016). Frailty is considered a consequence of the deregulation of several physiological systems (immune, endocrine, muscular) occurred during the ageing process. For this reason, a direct association between frailty and genomic instability seems to be also plausible, and, accordingly, it has been previously assessed by evaluating different genomic biomarkers, including telomere length (Saum *et al*., 2014), DNA methylation (Collerton *et al*., 2014), or DNA damage and repair impairment (Collerton *et al*., 2012). Nevertheless, all these studies failed in finding a relationship between frailty and any of these genomic parameters.

Since MN frequency is a well-established biomarker of genomic instability, and in order to address its suitability as a potential biomarker of frailty, in the present study MN formation in peripheral blood lymphocytes and exfoliated buccal cells was determined in a population of elder individuals classified as non-frail, pre-frail or frail, according to the five phenotypic criteria proposed by Fried *et al*. (2001). Results obtained showed a significant progressive increase in the frequency of MN-L with frailty severity, while no difference among groups was found in the frequency of MN-B. Although similar results would be initially expected from both approaches,

this discrepancy between results obtained from lymphocytes and buccal cells was previously reported, [e.g., in subjects with Down's syndrome regarding healthy controls (Ferreira *et al*., 2009), and in Alzheimer's disease patients and controls (Migliore *et al*., 2011)]. As suggested by Ferreira *et al*. (2009), this may be due to differences in metabolism and/or apoptosis levels between exfoliated buccal cells and lymphocytes. In addition, buccal epithelial cells are considered short-lived cells due to their continuous renewal; therefore, the presence of MN in buccal mucosa has been mainly linked to recent exposure to genotoxic agents more than to fixed genetic damage (Ceretti *et al*., 2014), a condition which would contribute to explain the differences found in our study between the two tissues.

As previously indicated, MN production was associated with ageing and age-related diseases in both peripheral lymphocytes and buccal cells in a number of previous studies (Thomas *et al*., 2008; Bonassi *et al*., 2011b; Fenech *et al*., 2011). Moreover, this biomarker has also been associated with features of the ageing phenotype, including loss of function, mental retardation, disability, and death (Fenech and Bonassi, 2011). However, to the best of our knowledge, this is the first study evaluating MN formation in buccal mucosa cells from frail older subjects and the second one in applying this approach to lymphocytes of older adults classified according to frailty status. Opposing to our results, this single previous study (Valdiglesias *et al*., 2015) failed in finding a relationship between frailty and MN-L frequency in an Italian elder population. However, in that work, no distinction was considered between pre-frail and non-frail groups, so they were analysed together. This decision could have possibly masked the difference between frail subjects and non-frail controls, due to the lower MN-L frequency in the pre-frail group. Moreover, their sample population size was smaller (N=180), and the regression models applied did not include BMI and smoking habit, which could have consistently contributed to explain the lack of association reported.

Even though in our study the significant increase in MN-L frequency regarding the non-frail subjects was only found in the frail group, pre-frail individuals showed also a slight increase in this parameter, supporting a possible linear association between genomic instability and frailty.

Another original result from this study refers to the different contribution of the five different frailty criteria (Fried *et al*., 2001) to the increase of the MN-L (Figure IV. 1); low physical activity, slow walking time, and low grip strength contribute the most, while unintentional weight loss does not contribute at all. The lack of contribution of this last criterion was in some way unexpected, especially considering the observed effect of nutritional status on the final results (Table IV. 6) and the previous studies linking diet deficiencies and chromosomal damage (Fenech, 2002). However, it must be taken into account that unintentional weight loss in elderly, or ageing-related sarcopenia, has multi-factorial causes including disuse, changing

endocrine function, chronic diseases, etc., being nutritional deficiencies just one of them (Fielding *et al*., 2011).

Although less employed than the CBMN test, the BMNCyt assay in exfoliated cells is a useful and minimally invasive method for monitoring genetic damage in humans. It has been previously employed as a tool to evaluate age-associated genomic instability both in healthy individuals (Thomas *et al*., 2008) and Down's syndrome individuals, that experience premature ageing (Thomas *et al*., 2008; Ferreira *et al*., 2009). In these cases, a positive association between MN production and ageing was found. Besides, an increase in MN-B frequency was previously reported in several age-related diseases such as diabetes (Grindel *et al*., 2017), cancer (Yildirim *et al*., 2006), or rheumatoid arthritis (Ramos-Remus *et al*., 2002).

Tissue regenerative capacity depends on the number and division rate of the proliferating cells, along with genomic stability and propensity to cell death. This process is basic for healthy ageing. Buccal mucosa offers the possibility to study the regenerative capacity of the epithelial tissue, in an easily accessible and non-invasive sampling procedure (Thomas *et al*., 2009). Thus, together with MN evaluation, BMNCyt assay allows studying several endpoints for other nuclear abnormalities that occur during the normal cell division. These abnormalities have been previously employed as a biomarker of DNA damage (NBUD-B), defects in cytokinesis (BN-B) and proliferative potential (basal cell frequency), and/or cell death (Cond-chrom-B, Karyorrhectic-B, Pyknotic-B and Karyolytic-B) (Torres-Bugarín *et al*., 2014).

Even though the rate of BN-B decreased significantly with age, it was found significantly higher in the frail group and showed a progressive increase with frailty severity. Thus, these results indicate alterations in the cytokinesis process, which could lead to alterations in cell proliferation, in frail subjects. Besides, decreases were obtained in buccal cell death parameters (Pyknotic-B, Karyolytic-B, and Cond-chrom-B, the two former significant) in the frail group with regard to the other two. Since significant increases in apoptosis indicative parameters (Cond-chrom-B, Karyorrhectic-B) were previously observed in healthy older subjects (aged 65-70) as compared with younger individuals (aged 18-25) (Thomas *et al*., 2007), our results may reflect important changes in the profile of the buccal mucosa related to frailty and not associated with age.

The proportion of basal cells and cells undergoing cell death in buccal mucosa is an indication of the regenerative capacity of this tissue (Thomas *et al*., 2009). In our study, no basal cells were found in the scored samples, possibly due to the subjects' advanced age and the expected wear of their mucous tissue. Besides, the rate of cells undergoing cell death (Cond-chrom-B, Pyknotic-B, and Karyolytic-B) resulted significantly decreased in frail and pre-frail groups, suggesting a minor regenerative capacity of the buccal mucosa in these individuals.

Together with data on frailty, the possible influence of the nutritional status and the cognitive impairment of the study subjects on the obtained results were analyzed. Malnourishment is a common status in the elderly. In our study population, 14.6% frail individuals were malnourished, while 79.2 % frail and 19.1% pre-frail were at risk of being malnourished. When the influence of nutritional status on MN tests parameters was assessed, higher levels of MN-L and BN-B, and lower levels of Pyknotic-B were observed in individuals malnourished or at risk of malnutrition regarding those with normal nutrition. These results coincide with those for frailty status, which is not unexpected since it has been previously described that those individuals with an impaired nutritional status are more likely to be frail (Dorner *et al*., 2014). Furthermore, the frailty criterion unintentional weight loss is related to nutritional status, indeed 'involuntary weight loss during the last three months' is one of the items included in the MNA-SF questionnaire.

Micronutrient status plays an important role in the protection against genome damage by providing co-factors required for an efficient DNA repair, detoxification or maintenance of genome methylation (Thomas *et al*., 2011). Consequently, and in agreement with our results, vitamin and mineral deficiencies in diet could be associated with increased genomic damage and cancer risk (Ames and Wakimoto, 2002). On this regard, Fenech *et al*. (1997) also reported an increase in MN-L frequency in older men (aged 50-70) with non-optimal values of serum folate and homocysteine regarding subjects with higher levels of these micronutrients. A complete review on the effects of dietary intervention on MN levels concluded that micronutrient supplementation (e.g., with vitamins, antioxidants or wine) could lead to a significant reduction of MN frequency, in both peripheral lymphocytes and buccal mucosa cells, in supplemented subjects (Thomas *et al*., 2011). This observation, together with the influence of nutritional status found in the present study, would support the idea that MN-L frequency associated with frailty or pre-frailty status could be reduced, at least in part, with a proper diet intervention in the elderly.

A similar relationship was found when the influence of cognitive status was assessed. In particular, increases in MN-L and BN-B cells, and a decrease in Pyknotic-B were observed in subjects with cognitive impairment. Supporting this last result, decreases in the buccal cell death parameters (Karyorrhectic-B, Cond-chrom-B) were previously observed in Alzheimer's patients with regard to healthy controls (Thomas *et al*., 2007). However, since the significant effect on MN-L and BN-B cells remained when both statuses were mutually adjusted, it seems that cognitive status has a strong influence on the obtained results. Accordingly, a relationship between frailty status and cognitive impairment has been previously described (Han *et al*., 2014), as well as the association between MN frequency, as a biomarker of genomic instability, and cognitive impairment (Thomas *et al*., 2007). This demonstrated association between both statuses, frailty and cognitive, strongly complicates the distinction between genomic or cellular alterations

related to frailty condition and those due to cognitive impairment. The Fried's criteria, frequently used in clinical settings and employed in the present study to identify frail people, do not consider cognitive features of the individuals and consequently, the initial distinction between subjects with differential cognitive status cannot be conducted. However, more and more authors are increasingly claiming for differentiating physical frailty from cognitive frailty (Kelaiditi *et al*., 2013). Our results would support the need for this distinction in order to enhance reliability when testing the suitability of a potential biomarker for frailty identification.

4.2. Other genetic outcomes

In order to fully understand the association between DNA cumulative damage and frailty status, the present study also addressed the possible relationship between frailty status in older adults and different genomic outcomes, chosen on the basis of their demonstrated link to ageing or age-related diseases (Shao *et al*., 2014; Siddiqui *et al*., 2015).

TCR is a complex of integral membrane proteins that participate in the activation of T-cells in response to an antigen. Induced or spontaneous mutations in TCR genes could result in the phenotypic expression of TCR-defective T-cells and thus contribute to impairment of T-cell response. It has been suggested that TCR variant frequency might be a particularly relevant endpoint in population monitoring for genetic damage (Cole and Skopek, 1994). Accordingly, this endpoint has been previously employed as a mutagenicity biomarker in biomonitoring studies of occupationally or medically exposed subjects (Vershenya *et al*., 2004; García-Lestón *et al*., 2012), as well as a predictor of cancer risk (Taooka *et al*, 2006). In the present study, the first addressing the possible relationship between frailty and mutagenicity, TCR-Mf was not found to be influenced by frailty status or age. Contradictory results have been previously obtained regarding age effect on TCR-Mf in occupationally-exposed populations entirely below 65 years old, with both absence (Lanza *et al*., 1999) and presence (García-Lestón *et al*., 2012) of such influence. Besides, significantly positive and linear association between TCR-Mf and age was observed by Akiyama *et al*. (1995), in a wide age range group of subjects (0-96 years). It is likely that the age range covered by our study population (65-102) was not wide enough to detect variations in TCR-Mf with age, since our results indicate that over the age of 65 mutation rate remains stable as well as independent of frailty status.

DNA repair is one of the most important mechanisms to maintain genome integrity. Consequently, deficiencies in this process are often considered one of the key processes in the development of diseases such as cancer and other age-related pathologies (Valdiglesias *et al*., 2011a). Indeed, it was previously suggested that one of the possible causes or events involved in frailty syndrome is the alteration of the cellular repair mechanisms that would result in the accumulation of genetic damage (Dent *et al*., 2016). In the present study, this possible association

has been evaluated by means of the DNA repair competence assay. However, and despite repair capacity showed a tendency to decrease with frailty severity, no significant differences were reached. To the best of our knowledge, only the study of Collerton *et al*. (2012) has previously evaluated the possible association between repair capacity and frailty status in the elderly (subjects aged over 85), and negative results were also obtained. In both cases, repair capacity was assessed using DNA damaging agents with similar action mechanism, namely ionizing radiation in Collerton's study and the radiomimetic agent BLM in the current study. These agents induce a wide spectrum of mutagenic lesions, including DNA base damage, abasic sites, and alkali-labile sites, which eventually result in DNA single strand breaks and DSB. Considering the demonstrated link between repair capacity and ageing or age-related diseases (Maynard *et al*., 2015;), further investigations in this line, maybe using other assays to assess different repair pathways, are required prior fully rejecting DNA repair influence on frailty status.

Primary DNA damage was determined by means of alkaline comet assay, but no association with frailty status was found in the present study. Similarly, Collerton *et al*. (2012) reported a lack of association between genetic damage (γ ray-induced DNA strand breakage, evaluated by fluorimetric detection of alkaline DNA unwinding) and frailty condition in an older adult population over 85 years old. No other studies evaluating genetic damage in frail subjects are available in the literature; however, there are a number of works addressing the association between this kind of genetic damage and age showing inconsistent results. For instance, Humphreys *et al*. (2007) found a decrease in DNA damage in the oldest group (aged 75-82 years) with respect to the young controls (aged 20-35 years) and to the younger older people (aged 63-70 years); Hyland *et al*. (2002) reported similar levels of DNA damage in older individuals (86-96 years old) that in middle-aged individuals (40-60), and Mladinic *et al*. (2010) (age groups ranges:35-47 and 65-76 years old), Piperakis *et al*. (2009) (age groups ranges: children: 5-10; adults: 40-50; old people: 70-80 years old) and Mutlu-Türkoglu *et al*. (2003) (age groups ranges: 21-40; 61-85 years old) observed an increase of DNA damage with age. Several authors have previously pointed out that results obtained in the comet assay are highly variable and often difficult to interpret since several types of damage are detected – including single and double strand breaks, alkali-labile sites, and breaks generated during repair processes – and can be influenced by a number of variables (season, diet, sample collection time…) (Azqueta and Collins, 2013).

Opposite to comet assay, γH2AX assay determines not a wide spectrum of DNA lesions but a specific kind of damage, namely double strand breaks (DSB). The phosphorylation of the C-terminal of the variant core histone protein H2AX (γH2AX) at the highly conserved amino acid Ser139 is a quickly occurring event in the early DNA damage response to DSB (Siddiqui *et al*., 2015). The half-life of γH2AX after DNA damage induction has been estimated to be 2-7h; after

this time H2AX is again dephosphorylated (Bouquet *et al*., 2006). Still, it has been reported that γH2AX persistent in time represent DNA lesions with unrepairable DSB (Sedelnikova *et al*., 2008). In the present study, a progressive increase in the γH2AX rate with frailty severity not influenced by age was observed, statistically significant in both pre-frail and frail groups when compared with the non-frail. Besides, participants with cognitive impairment showed an increase in %γH2AX with respect to those with normal cognitive status. Silva *et al*. (2014) also reported an increase in γH2AX nuclear expression levels in individuals with Alzheimer's disease with regard to healthy individuals, and the present study also found that the presence of cognitive impairment and frailty were independently related to an increase in the frequency of MN in lymphocytes. These results would suggest a connection between cognitive impairment and frailty status, and give support to the quite recently coined term 'cognitive frailty', introduced in an attempt to encapsulate the cognitive decline that is often observed in non-demented elderly individuals who are physically frail, with an underlying pathophysiology different from that driving the cognitive trajectory in neurodegenerative disorders (Kelaiditi *et al*., 2013).

Although the present study is the first one in evaluating the relationship of H2AX phosphorylation with frailty status, the relationship between cellular senescence and persistent γH2AX, as indicative of unrepaired DSB, has been already suggested by several authors (Sedelnikova *et al*. 2008; Siddiqui *et al*., 2015). Still, Schurman *et al*. (2012) reported that γH2AX endogenous levels increase with age, peaking at ~57 years, which is in agreement with the absence of influence of age in the current study, where all subjects were 65 and older.

Both comet assay and H2AX assay detect DSB, but their results do not always coincide. While γH2AX was found to be associated with frailty in the current study, several reasons could explain the lack of association for comet assay. On one hand, whereas comet assay usually reveals recently induced and easily repairable DNA damage (Collins *et al*., 2014), γH2AX levels reflect fixed genetic damage or DNA damage that could not be properly repaired (Valdiglesias *et al*., 2013). On the other hand, it is not absolutely clear whether γH2AX foci do in fact always reflect the presence of DNA breakage (Rothkamm *et al*., 2015). For example, ageing haematopoietic stem cells have been reported to harbour replication stress-induced nucleolar γH2AX foci which persist due to ineffective H2AX dephosphorylation rather than ongoing genetic damage (Flach *et al*., 2014). Nevertheless, the significant association found in the current older adult population between γH2AX levels and MN frequency in peripheral lymphocytes, indicative of persistent DNA damage, points to unrepaired DSB as the outcome influenced by frailty status and by cognitive impairment, according to the results obtained in the multivariate analyses.

Besides, parallel results between γH2AX assay and MN test were also obtained when analysing each five frailty phenotypic criteria independently. Thus, major contribution of physical

activity, walking time, and grip strength to variation of %γH2AX and MN frequency was observed, whereas unintentional weight loss and exhaustion did not contribute, or contributed only minimally, to both parameter modifications. These similar results in γH2AX and MN assays provide further support to the relationship between fixed genetic damage and frailty, and also suggest that combinations of some phenotypic criteria and biomarkers might improve frailty identification.

5. Conclusions

Different studies support the reversibility of frailty status or its improvement by changes in diet, physical exercise and medications (Espinoza *et al*., 2012; Roland *et al*., 2014). Identifying frail people as early as possible seems, therefore, crucial for geriatricians and healthcare professionals since it would allow to implement interdisciplinary and personalized cares, as well as to improve outcomes by means of prevention and intervention programs. All this would lead to decrease the need for admission to nursing homes and hospitals, lowering the risk of dependence and death, and eventually improve the welfare and personal satisfaction, reducing the health, social and economic costs associated with frailty.

The use of biomarkers could result highly helpful in identifying frailty of pre-frailty status. Given its sensitivity, specificity, objectivity and predictive capacity, several authors have pointed out that cellular and molecular biomarkers may potentially be used for frailty identification (reviewed in Chapter II). However, to date, no specific biological parameter has been identified as a definitive biomarker for frailty.

In the present study, we addressed the possible relationship between different genetic outcomes – namely genomic instability, mutagenicity, genetic damage and cellular repair capacity – and frailty status by evaluating a population of older adults classified as frail, pre-frail and non-frail according to the commonly used phenotypic criteria.

According to our findings, MN frequency evaluated in lymphocytes (as a marker of fixed or accumulated genetic damage), but not in buccal cells (reflection of recent damage) could be considered as a biomarker of frailty. Thus, these results demonstrate for the first time a direct relationship between frailty in older adults and genome instability. Even though this association resulted statistically significant only in the frail group, also individuals with a pre-frail status showed an increase in the MN-L frequency, supporting this relationship and opening the door to further investigations in this line. Moreover, associations between frailty and cell death parameters were obtained from the BMNCyt assay, which supports the use of this minimally invasive method as a complement in frailty identification, at least in its advanced state, where these differences resulted statistically significant.

Besides, no association of TCR-Mf and primary DNA damage with frailty was observed in this study. DNA repair capacity showed a non-significant tendency to decrease with frailty, and the persistent levels of γH2AX increased progressively and significantly with frailty severity.

Taking together the results from MN frequency in peripheral lymphocytes and γH2AX assay, the hypothesis that there is indeed a link between genomic instability, understood as fixed genetic damage, and frailty status seems to be plausible and supported by our data. Since both biomarkers, γH2AX and MN rates, resulted significantly and progressively increased with frailty, they could be proposed as tools for frailty identification or prediction (Figure IV.4); still, further validation is required to confirm our results. Furthermore, as γH2AX level resulted altered in both pre-frail and frail groups, whereas MN frequency was significantly increased only in frail individuals, a combination of both parameters could provide useful information regarding frailty severity, allowing clinicians to distinguish between pre-frail and frail status and helping them to provide personalized care. Consequently, results reported in the present study may contribute to improve healthcare/therapeutic strategies in older patients. Nevertheless, further investigation is necessary to prove whether the current findings are consistent and reproducible in different populations and larger sample sizes, to eventually standardize these biomarkers before they can be used in clinics, and to fully understand the influence of cognitive impairment on the results obtained.

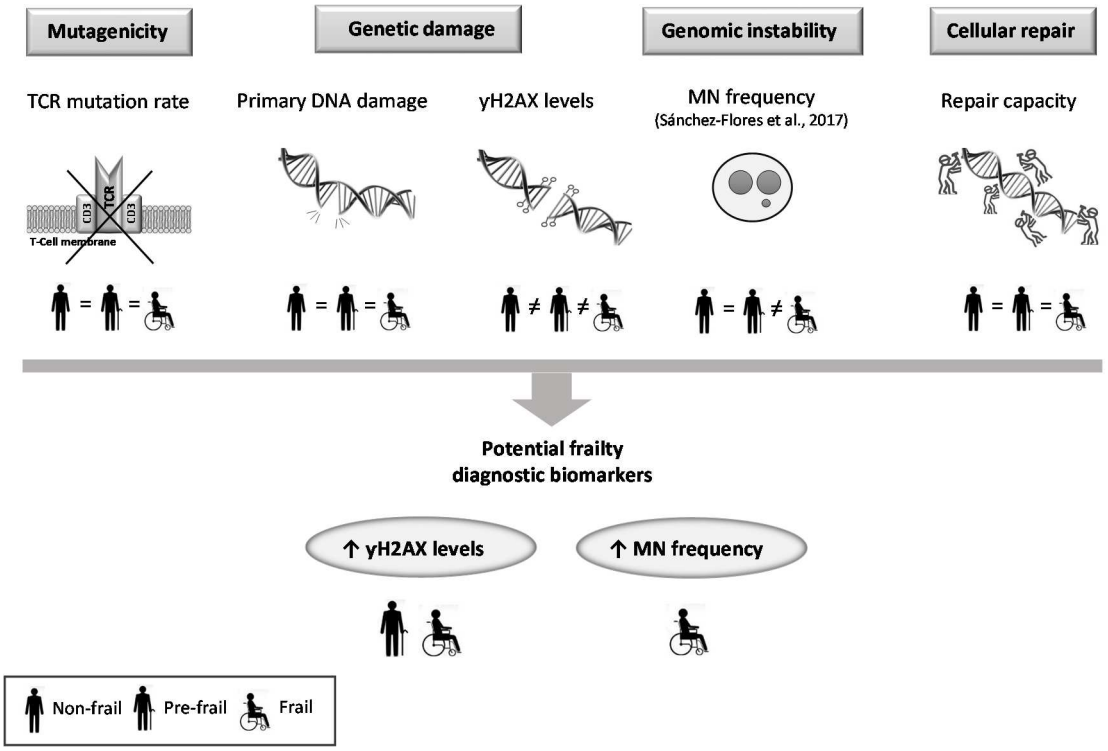

FIGURE IV.4. Relationship between frailty and genetic outcomes analysed in the study population. Biomarkers of mutagenicity, primary DNA damage and cellular repair capacity do not show differences according to frailty status. MN frequency discriminates between non-frail and frail subjects, meanwhile H2AX levels are different in non-frail, pre-frail and frail groups. A combination of both MN and γH2AX rates shows potential to be employed in frailty identification.

Conclusions.

From the results obtained in this study, we may draw the following conclusions:

Systematic review

1. Systematic review of the literature has shown that several oxidative stress biomarkers – including alterations in antioxidant systems, increased levels of lipid peroxidation and DNA oxidative damage, as well as DNA methylation and some specific genetic polymorphisms – are associated with frailty status in older people.
2. Genomic instability, or at least the two biomarkers tested so far (telomere length and MN rate), seems not to be linked to frailty. The only study which addressed the possible relationship between DNA repair modulations and frailty status also failed in finding associations.

γH2AX assay experimental optimization

3. Both unstimulated and stimulated fresh peripheral blood lymphocytes could be employed as cellular material to carry out the γH2AX assay.
4. The decision about stimulating cells with phytohaemagglutinin prior γH2AX analysis should be taken during the study design, according to the kind of damage to be evaluated or that is expected in the individuals.
5. Phytohaemagglutinin stimulation is necessary for γH2AX analysis when cells are stored frozen, since basal damage is too high in cryopreserved unstimulated cells, likely as a result of freezing and thawing processes.

Epidemiological study

6. MN frequency evaluated in lymphocytes (as a marker of fixed or accumulated genetic damage), but not in buccal cells (reflection of recent damage) showed significantly higher values in frail individuals as compared to non-frail subjects.
7. Associations between frailty and cell death parameters were obtained in exfoliated buccal cells, supporting the use of this minimally invasive method as a complement in frailty identification, at least in its advanced state, where these differences resulted statistically significant.
8. Persistent levels of γH2AX increased progressively and significantly with frailty severity.
9. TCR-Mf and primary DNA damage were not associated with frailty status. DNA repair capacity showed a non-significant tendency to decrease with frailty.
10. Both MN in lymphocytes and γH2AX could be proposed as tools for frailty identification or prediction. Besides, since γH2AX level resulted altered in both pre-frail and frail groups, whereas MN frequency was significantly increased only in frail individuals, a combination of both parameters could provide useful information regarding frailty severity

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