Association of frailty status in older adults with immunological, endocrine and oxidative stress biomarkers.

Diego Marcos Pérez

Doctoral Thesis

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Thesis supervisors:

Blanca Laffon Lage

Vanessa Valdiglesias García

Cellular and Molecular Biology

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PhD student	Thesis supervisor	Thesis supervisor
Signed.	Signed.	Signed.
Diego Marcos Pérez	Blanca Laffon Lage	Vanessa Valdiglesias García

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2. Valdiglesias, V., Sánchez-Flores, M., Maseda, A., Lorenzo-López, L., **Marcos-Pérez, D**., López-Cortón, A., Strasser, B., Fuchs, D., Laffon, B., Millán-Calenti, J. C., Pásaro, E. 2017. *Immune biomarkers in older adults: Role of physical activity*. Journal of Toxicology and Environmental Health, Part A. 80, 605-620.
3. **Marcos-Pérez, D**., Sánchez-Flores, M., Maseda, A., Lorenzo-López, L., Millán-Calenti, J. C., Strasser, B., Gostner, J. M., Fuchs, D., Pásaro, E., Valdiglesias, V., Laffon, B. 2017. *Frailty Status in Older Adults Is Related to Alterations in Indoleamine 2,3-Dioxygenase 1 and Guanosine Triphosphate Cyclohydrolase I Enzymatic Pathways*. Journal of the American Medical Directors Association (JAMDA). 18, 1049-1057.
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5. Valdiglesias, V., **Marcos-Pérez, D**., Lorenzi, M., Onder, G., Gostner, J. M., Strasser, B., Fuchs,

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7. **Marcos-Pérez, D**., Sánchez-Flores, M., Pásaro, E., Proietti, S., Bonassi, S., Laffon, B., Valdiglesias, V. 2019. *Association of immunological biomarkers with frailty status: results from a systematic review and metanalysis*. (In preparation).
8. **Marcos-Pérez, D**., Sánchez-Flores, M., Pásaro, E., Proietti, S., Bonassi, S., Valdiglesias, V., Laffon, B. 2019. *Systematic review and metanalysis on vitamin D level and frailty status in older adults*. (In preparation).

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2. Valdiglesias, V., Sánchez-Flores, M., **Marcos-Pérez, D**., Maseda, A., Millán-Calenti, J.C., Lorenzo-López, L., Pásaro, E., Laffon, B. 2018. *Development of biomarkers for identification of frailty in the elderly*. Journal of Health and Pollution. 8, S40-S41.
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7. I Congreso Nacional de Jóvenes Investigadores en Biomedicina. III Congreso Biomedicina Predocs Valencia. November 28-29, 2016. Valencia, Spain. **Marcos-Pérez, D**., Sánchez-Flores, M., Maseda, A., Millán-Calenti, J. C., Lorenzo-López, L., Strasser, B., Fuchs, D., Gostner, J. M., Pásaro, E., Laffon, B., Valdiglesias, V. Frailty and immunology in older adults.

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11. II Congreso Internacional de Psicobiología. July 19-21, 2017. Ávila, Spain. Laffon, B., **Marcos-Pérez, D**., Sánchez-Flores, M., Maseda, A., Lorenzo-López, L., Millán-Calenti, J. C., Strasser, B., Gostner, J. M., Fuchs, D., Valdiglesias, V., Pásaro, E. Psychoimmunological depletion in frailty status in older adults is related to alterations in neopterin and tryptophan breakdown.

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15. 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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18. International Association of Gerontology and Geriatrics European Region (IAGG_ER) Congress 2019. May, 23rd – 25th, Gothenburg, Sweden. Valdiglesias, V., Sánchez-Flores, M., **Marcos-Pérez, D**., Fernández-Bertólez, N., Pásaro, E., Laffon, B. Systematic Review of Oxidative Stress, Genetic Damage and DNA Repair Biomarkers in Frailty Syndrome in Older Adults.

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«Success is not always overcome, but never discouraged».

by Napoleón Bonaparte

Abstract

Frailty is a multidimensional geriatric syndrome of loss of reserves and increased vulnerability to stressors. Currently, frailty identification is based on phenotypic characteristics. Due to the well-known reversibility of frailty, early identification is crucial. Biomarkers can be useful tools for the early and accurate detection of frail individuals. Thus, the main objective of this study was to investigate the possible relationship of different biomarkers with frailty in older adults. To that aim, a cross-sectional study was conducted in a population of older adults (aged 65 years and over) classified according to their frailty status, determining a set of biomarkers related to immune activation and inflammation, to endocrine system, and to oxidative stress. Results obtained revealed significant differences in the levels of several immunological biomarkers and cortisol between frail and non-frail individuals, but no association was found between any oxidative stress biomarker and frailty status Data presented in this study provide support to the hypothesis that frailty status in older adults is associated with an additional degree of immune stimulation and inflammation, and with age-related hypothalamic pituitary adrenal axis dysregulation.

Resumen

La fragilidad es un síndrome geriátrico multidimensional caracterizado por una pérdida de homeostasis y un incremento de la vulnerabilidad. Actualmente, la identificación de la fragilidad se basa en características fenotípicas. Dada su reversibilidad, la identificación de la fragilidad mediante biomarcadores sería primordial para posibilitar una detección más temprana y precisa de los individuos frágiles. Debido a esto, el objetivo de este estudio fue investigar la posible asociación de diferentes biomarcadores con la fragilidad en personas mayores. Para ello, se llevó a cabo un estudio transversal en una población de mayores de 65 años, clasificados de acuerdo a su estado de fragilidad, determinando un conjunto de biomarcadores relacionados con la activación inmune y la inflamación, el sistema endocrino y el estrés oxidativo. Los resultados obtenidos mostraron diferencias significativas en los niveles de varios biomarcadores inmunológicos y el cortisol entre individuos frágiles y no frágiles, pero no se encontró asociación alguna entre los biomarcadores de estrés oxidativo y el estado de fragilidad. Los datos presentados en este trabajo apoyan la hipótesis de que el estado de fragilidad en personas mayores está relacionado con la estimulación inmune y la inflamación, así como con la desregulación del eje hipotalámico-pituitario-adrenal dependiente de la edad.

Resumo

A fraxilidade é un síndrome xeriátrico multidimensional caracterizado por unha pérdida de homeostase e un incremento da vulnerabilidade. Actualmente, a identificación da fraxilidade basease en características fenotípicas Dada a súa reversibilidade, a identificación da fraxilidade mediante biomarcadores sería primordial para posibilitar unha detección máis temperá e precisa dos individuos fráxiles. Debido a isto, o obxectivo deste estudo foi investigar a posible asociación de diversos biomarcadores coa fraxilidade en persoas maiores. Para iso, levóuse a cabo un estudo transversal nunha poboación de maiores de 65 anos, clasificados de acordo ao seu estado de fraxilidade, determinando un conxunto de biomarcadores relacionados coa activación inmune e a inflamación, o sistema endócrino e o estrés oxidativo. Os resultados obtidos amosaron diferencias significativas nos niveis de varios biomarcadores inmunolóxicos e o cortisol entre individuos fráxiles e non fráxiles, pero non se atopou asociación algunha entre os biomarcadores de estrés oxidativo e o estado de fraxilidade. Os datos presentados neste traballo apoian a hipótese de que o estado de fraxilidade en persoas maiores está relacionado coa estimulación inmune e a inflamación, así como coa desregulación do eixo hipotalámico-pituitario-adrenal dependente da idade.

Extended summary in Spanish - Resumen amplio

Durante el último siglo, la población mundial ha estado experimentando un rápido, imparable y dramático envejecimiento sin precedentes. Esta situación se deriva del descenso de la fertilidad y del incremento de la esperanza de vida. Respecto a la primera causa, el nivel de fertilidad requerido para la regeneración de la población está situado en 2.1 nacimientos por mujer; sin embargo, el 46% de la población mundial vive en países cuyo nivel de fertilidad está por debajo de este valor, y está previsto que esta situación empeore en 2045-2050, periodo en el que se espera que este porcentaje se incremente hasta el 69%. Aunque en España este rango de fertilidad sigue una tendencia al alza desde 2010 y se prevé que siga aumentando hasta 2100, su rango de fertilidad alcanzará ese año 1.72 nacimientos por mujer, muy por debajo de lo necesario para la regeneración poblacional. La segunda causa de este envejecimiento poblacional es el incremento en la esperanza de vida producida por las mejoras de las condiciones higiénicas, la dieta, los servicios médicos y el descenso de la mortalidad infantil. La esperanza de vida se ha visto sustancialmente incrementada en el último siglo, pasando de los 30-35 años de principios del siglo pasado hasta los 80-85 años que disfrutan la mayoría de los países industrializados actualmente. Hoy en día España es uno de los países con mayor esperanza de vida en todo el mundo (79.9 años en hombres y 85.4 años en mujeres); sin embargo Foreman y colaboradores (2018) apuntan en un reciente estudio que España alcanzará el primer puesto en 2040, superando los 85 años en ambos sexos.

Estos cambios demográficos están provocando alteraciones en las pirámides poblacionales, en las que se están invirtiendo las proporciones de jóvenes y personas mayores por primera vez en la historia. En este sentido, las Naciones Unidas han predicho que en 2050 habrá menos de dos personas de entre 20 y 64 años por cada persona mayor de 65 años en 35 países del mundo, 24 de los cuales serán países europeos, lo cual refleja la importancia del cuidado del sector de población mayor en cuanto a servicios médicos, pensiones, protección social y economía para los gobiernos. Centrándonos en España, nuestro país es el quinto de Europa en cuanto a porcentaje de envejecimiento (European Commission 2015) y Galicia, con un 24.3% de gente mayor de 65 años, es una de las Comunidades Autónomas más envejecidas del estado español según datos proporcionados por el Instituto Nacional de Estadística en su informe de 2017.

Este aumento de la longevidad no necesariamente implica un estado de bienestar y buena salud. Para muchas personas conlleva un aumento del riesgo de aislamiento social y pobreza, con limitaciones en el acceso a los servicios sociales y en la calidad de vida. Por ello es

importante enfocar las investigaciones futuras en la prevención de la discapacidad y de la dependencia, así como en preservar la salud y el bienestar de nuestros mayores.

El envejecimiento consiste en un descenso progresivo y general de las reservas fisiológicas del organismo, que desemboca en un descenso de la capacidad para generar respuestas adaptativas y mantener la homeostasis, haciendo que el organismo incremente su susceptibilidad al estrés y las enfermedades. Debido a la gran heterogeneidad en el deterioro funcional derivado del proceso de envejecimiento, la "edad cronológica" no es un buen indicador de este proceso. Es por ello que el término "fragilidad" se ha propuesto como una aproximación más exacta e individualizada.

La fragilidad se define como un síndrome geriátrico multidimensional caracterizado por la pérdida de homeostasis y el descenso de las reservas biológicas (energéticas, físicas y cognitivas) debidos a la desregulación de diversos sistemas fisiológicos exponiendo a los individuos a un mayor riesgo de sufrir efectos adversos para su salud, incluyendo caídas, fracturas, deterioro funcional, discapacidad, hospitalización y muerte. En la actualidad no existe consenso en cuanto a la definición y criterios específicos para la identificación del estado de fragilidad; sin embargo, dos metodologías han destacado sobre el resto para la clasificación de personas mayores por su aceptación y cotidianeidad de uso tanto en clínica como en investigación. Estos dos criterios son el fenotípico, desarrollado por Fried y colaboradores en 2001, y el criterio de acumulación de déficits, desarrollado por Rockwood, Mitnitski y colaboradores en ese mismo año.

El fenotipo de fragilidad desarrollado por Fried y colaboradores en 2001 está basado en cinco componentes físicos específicos relacionados con el metabolismo y la capacidad física. Estos cinco componentes son: (i) pérdida involuntaria de peso, (ii) reducción de la velocidad al caminar, (iii) reducción de la actividad física, (iv) fatiga autorreportada y (v) debilidad muscular. Los distintos individuos son clasificados como "robustos" o no frágiles si no presentan ninguno de estos criterios, como pre-frágiles si presentan uno o dos de ellos, y como frágiles si presentan más de dos de estos criterios fenotípicos. Por otro lado, el denominado índice de fragilidad fue desarrollado por Rockwood y Mitnitski en 2001, y es un método más multidimensional que el desarrollado por Fried. Está basado en la acumulación de una serie de déficits que incluían inicialmente 92 parámetros, (recomendando utilizar 30 o más en la actualidad) de ámbito físico, neurológico, fisiológico, geriátrico, de enfermedades y de distintos valores de análisis clínicos. Este índice de fragilidad se calcula simplemente mediante la suma de las variables presentes con respecto del total de variables. Estos dos criterios son

claramente distintos, ya que mientras que el fenotipo de fragilidad está enfocado al dominio físico de la fragilidad, el índice de fragilidad se basa en la acumulación de déficits de diversos ámbitos, no sólo físicos.

La prevalencia de la fragilidad es ampliamente variable entre estudios, principalmente debido a los distintos criterios empleados para su identificación. Pero aun empleando el mismo criterio de identificación, esta prevalencia varía en función de variables como el sexo, la edad, la raza o las condiciones socio-económicas de la población. En este sentido, Collard y colaboradores establecieron en 2012 una tendencia de incremento de esta prevalencia conforme aumenta la edad, dividida por rangos de años. La prevalencia de la fragilidad pasa del 4% en personas con 65-69 años al 28% para las personas de más de 85 años, siendo esta tendencia complementada y apoyada por un estudio desarrollado por Ahmed y colaboradores en 2007, en el cual establecen en un 32% la prevalencia de la fragilidad en personas con más de 90 años. En comunidades de personas mayores españolas la prevalencia de la fragilidad empleando el criterio de Fried fue establecida en 8.6% y 16.3% (García-García, 2011 y Abizanda y colaboradores, 2011, respectivamente), llegando a alcanzar el 68.8% en caso de individuos institucionalizados (González-Vaca y colaboradores, 2014).

A nivel celular la fragilidad está relacionada con deficiencias en la capacidad de reparación celular y la consecuente acumulación de daño genético que deriva a su vez en alteraciones de la expresión génica, inestabilidad genómica, mutaciones, pérdida del potencial de división celular, muerte celular, etc. A nivel sistémico, la fragilidad se asocia con la desregulación fisiológica de múltiples sistemas del organismo como el sistema inmunológico, endocrino, musculoesquelético, hematológico y cardiovascular. Todas estas alteraciones, aunque silenciosas, afectan a todo el organismo, que manifiesta signos clínicos de fragilidad como pérdida de masa muscular, deterioro cognitivo y pérdida sensorial, entre otros.

Debido a que el estado de fragilidad presenta características de reversibilidad, sobre todo en sus primeros estadios, la identificación de las personas frágiles es crucial para su prevención. Por ello la investigación en esta dirección es esencial para que las políticas de sanidad pública puedan implementar planes de intervención a distintos niveles, bien sea para la prevención de la fragilidad en personas todavía sanas, o para la paliación o reversión de este síndrome en individuos pre-frágiles o frágiles. Estas intervenciones incluyen la promoción de la actividad física, la estimulación cognitiva, hábitos de vida y dieta saludables, el cese del tabaquismo, etc.

La inflamación es una respuesta necesaria, aguda, transitoria y localizada ante los distintos agentes externos (e.g., radiación ultravioleta y gamma) e internos (e.g., virus, bacterias) que atacan a nuestro organismo. Esta respuesta inflamatoria permite al organismo evitar o atenuar los efectos adversos por los distintos agentes nocivos. Sin embargo, a medida que envejecemos esta inflamación se torna en perjudicial y crónica, debido a que el organismo es incapaz de reparar el tejido sano o de restaurar esa carga inmunológica a niveles normales, dañando el tejido circundante. Este daño continuo, producido por una carga antigénica crónica relacionada con el envejecimiento, da lugar a una serie de cambios a nivel celular y serológico que comprometen la competencia del sistema inmunológico, proceso denominado inmunosenescencia. Esta inmunosenescencia es en parte responsable del fenotipo inflamatorio conocido como "*inflammaging*", el cual está caracterizado por una sobrerregulación sistémica, crónica y de baja intensidad de la respuesta inflamatoria con la edad, que se relaciona con un alto riesgo de consecuencias de salud adversas morbilidad y mortalidad para las personas de mayor edad.

El *inflammaging*, o inflamación crónica asociada a la edad, está considerado como un indicador de la fragilidad, estando actualmente aceptado como factor patogénico en el desarrollo de varias enfermedades dependientes de la edad como la osteoporosis, el cáncer, el Alzheimer y diversas patologías cardiovasculares. El *inflammaging* está asociado con el incremento de los niveles de diversas citoquinas pro-infamatorias como la interleuquina 1 (IL1), la interleuquina 6 (IL6), el factor de necrosis tumoral alfa (TNF-α), así como de proteínas de respuesta aguda como la proteína C-reactiva (CRP).

Durante la activación del sistema inmunológico, diversos factores inflamatorios como el interferón gamma, inducen la expresión de enzimas como la indolamina 2,3 dioxigenasa 1 (IDO) y la trifosfato ciclohidrolasa I (GCH) en monocitos/macrófagos y células dendríticas. La IDO está implicada en el metabolismo del triptófano, aminoácido esencial cuya degradación está relacionada con diversas enfermedades dependientes de la edad, como desórdenes cognitivos, cáncer y enfermedades cardiovasculares. Por otro lado, la GCH es una enzima clave en la biosíntesis de las pteridinas, ya que una vez es activada produce 7,8-dihidroneopterina trifosfato (NH_2TP), que es un precursor de la neopterina y de la tetrahidrobiopteri**na** (BH_4). Este último es un cofactor esencial de las monooxigenasas de aminoácidos, como la fenilalanina 4-hidroxilasa (PHA) y de las óxido nítrico sintetasas (NOS), las cuales están implicadas en la formación de tirosina y óxido nítrico a partir de fenilalanina y arginina, respectivamente.

El sistema immunológico no es el único que sufre senescencia; la endocrinosenescencia representa el descenso substancial de los niveles de hormonas derivadas del sistema endocrino. El eje hipotalámico-pituitario-adrenal (HPA) lleva a cabo una estrecha integración entre los sistemas endocrino, nervioso e inmunológico. La activación del eje HPA constituye: (i) la principal respuesta específica que contrarresta al *inflammaging* (también llamado anti-*inflammaging*), (ii) una explicación para los cambios producidos por inmunosenescencia y (iii) un mecanismo complejo de remodelación licitada por la inflamación, que explica el largo y sinuoso camino que va desde la robustez hasta la fragilidad. El principal producto final del eje HPA es el cortisol, que es uno de los agentes anti-inflamatorios más potentes, mientras que los efectos mediados por las citoquinas y las especies reactivas de oxígeno (ROS) son figuras representativas del *inflammaging*.

Las especies reactivas de oxígeno (ROS) y nitrógeno (RNS) están presentes en todos los organismos aeróbicos, siendo un producto común del metabolismo celular. Están implicadas en numerosas funciones biológicas como la neurotransmisión, la modulación de la presión sanguínea o el control del sistema inmunológico. Por otro lado, los antioxidantes son substancias o moléculas que pueden neutralizar a las especies reactivas aceptando o donando electrones a estos radicales libres, protegiendo a los sistemas biológicos a nivel celular, de membrana o extracelular. Durante el envejecimiento, el equilibrio entre las especies reactivas y la defensa antioxidante puede verse deteriorado, dando lugar a estrés oxidativo. Este estrés puede comprometer las diferentes funciones biológicas de forma directa dañando proteínas, lípidos o al ADN, cambiando la estructura y funciones del organismo o desencadenando factores de transcripción sensibles al estado redox que producen la sobreexpresión de citoquinas inflamatorias.

Por todo lo expuesto, y desde un punto de vista global, la activación inmunológica y la inflamación, las alteraciones del sistema endocrino y el estrés oxidativo actúan como representantes claves del proceso de envejecimiento, estando además implicados en el desarrollo de enfermedades dependientes de la edad y de la fragilidad.

Así, el principal objetivo de este estudio ha consistido en profundizar en el conocimiento sobre la fisiopatología del estado de fragilidad, explorando su relación con los sistemas inmunnológico, endocrino y el estrés oxidativo. Para ello, se han determinado un conjunto de biomarcadores e una población de 259 personas mayores (de 65 años o más), clasificadas según su fragilidad de acuerdo con los criterios fenotípicos de Fried et al. (2001), a

fin de determinar si alguno de estos parámetros puede resultar útil en la identificación temprana de la fragilidad.

Bajo la hipótesis de que la estimulación inmunológica de las rutas enzimáticas de la IDO y la GCH puede estar relacionada con el estado de fragilidad en personas mayores, se analizaron las concentraciones séricas de triptófano (Trp) y kinurenina (Kyn), calculando además la tasa Kyn/Trp como medida de la degradación del triptófano y de la actividad de la enzima IDO. Relacionadas con la ruta de la GCH, se determinaron la concentración sérica de neopterina, considerada un marcador de activación del sistema inmunológico, y las concentraciones de nitrito en plasma, y fenilalanina (Phe) y tirosina (Tyr) en suero, estimando la tasa Phe/Tyr como indicador de la actividad de la enzima PHA. El triptófano y la kinurenina por un lado y la fenilalanina y tirosina por otro fueron analizados mediante cromatografía líquida de alta resolución (HPLC, de *high performance liquid chromatography*) siguiendo los protocolos propuestos por Laich et al. (2002) y Neurauter et al. (2013), respectivamente. Los niveles de nitrito se determinaron mediante el método de Greiss, y los de neopterina mediante inmunoadsorción enzimática (ELISA).

Como biomarcadores representativos de la inmunosenescencia, se analizaron por citometría de flujo diversas subpoblaciones linfocitarias utilizando anticuerpos monoclonales específicos para el reconocimiento de linfocitos T ($CD3^+$), T colaboradores ($CD4^+$), T citotóxicos ($CD8^+$), linfocitos B ($CD19^+$) y células asesinas naturales (NK, de *natural killers*) ($CD16^+56^+$). Como representantes del *inflammaging*, los niveles de citoquinas circulantes en plasma IL6, TNFα y receptor soluble II del TNFα (sTNF-RII), y de CRP se determinaron mediante ELISA.

En cuanto al sistema endocrino, el biomarcador elegido para su estudio, debido a su gran potencial como producto final de la activación del eje HPA y representante del anti-*inflammaging*, fue la concentración de cortisol en suero, evaluado mediante ELISA.

Como biomarcadores de estrés oxidativo se midieron los niveles de especies reactivas de oxígeno y nitrógeno presentes en suero, por medio de la sonda fluorogénica diclorodihidrofluoresceína DiOxyQ (DCFH-DiOxyQ), y el daño oxidativo en el ADN, gracias a una modificación de la versión alcalina del ensayo del cometa que incorpora una incubación con la enzima de reparación OGG1. Además, la capacidad antioxidante total del plasma analizó determinando la capacidad de capturar el catión radical estable ácido 2,2'-azinobis(3-ethilbenzotiazolina-6-sulfónico ($ABTS^+$), cromóforo de color verde cuya intensidad desciende en presencia de antioxidantes.

Los resultados obtenidos en este estudio mostraron incrementos significativos de los niveles de neopterina, así como de las tasas Kyn/Trp y Phe/Tyr en los individuos pertenecientes al grupo frágil con respecto a los no frágiles. Además, se observaron descensos significativos en los niveles de triptófano y tirosina en los individuos frágiles con relación a los no frágiles, mientras que el descenso en los niveles de nitrito en la población de estudio fue progresivo conforme aumenta el grado de fragilidad, y significativo tanto en frágiles como en pre-frágiles. Se encontró también una correlación destacable entre los niveles de neopterina y la degradación del triptófano, representada por la tasa Kyn/Trp, así como entre la neopterina y el metabolismo de la fenilalanina y los niveles de nitrito, lo cual reafirma la relación de la enzima GCH con estas dos últimas rutas enzimáticas de degradación de aminoácidos.

Los resultados obtenidos son consistentes con la idea de que la estimulación crónica del sistema inmunológico en personas mayores frágiles es mayor de lo esperado teniendo en cuenta únicamente su edad. Es decir, el estado de fragilidad en mayores se asocia con un grado adicional de estimulación inmunológica, manifestado por una alteración más intensa de las rutas de la IDO y la GCH que en personas mayores no frágiles o pre-frágiles. En otras palabras, los datos presentados apoyan la implicación de la activación inmunológica en monocitos/macrófagos mediada por Th1 y la alteración de la bioquímica de los aminoácidos en la fisiopatología del síndrome geriátrico de fragilidad.

En el análisis de las subpoblaciones linfocitarias se observó un incremento significativo en la tasa $CD4^+/CD8^+$ y un descenso significativo de los linfocitos B ($CD19^+$) en los individuos clasificados como frágiles con respecto a los no frágiles, sugiriendo una asociación limitada entre la fragilidad y estos biomarcadores de inmunosenescencia.

Las concentraciones de mediadores inflamatorios (IL6, sTNF-RII, TNFα y CRP) se incrementaron significativamente en el grupo de sujetos frágiles respecto al de no frágiles. En el caso del sTNF-RII se obtuvo además una diferencia significativa en la comparación entre pre-frágiles y no frágiles, aumentando la concentración progresivamente con el grado de fragilidad. Estos resultados refuerzan la hipótesis que relaciona la fragilidad en personas mayores con procesos de inflamación crónica. A mayores, se calcularon las curvas ROC (*receiver operating characteristics*) de estos biomarcadores, con el fin de comprobar su capacidad predictiva respecto a la fragilidad. Los resultados obtenidos sugirieron que el sTNF-RII, por encima de los otros biomarcadores, puede tener aplicabilidad clínica como herramienta de identificación de los sujetos frágiles con elevada precisión, pudiendo detectar individuos frágiles por encima de una concentración de 3.461 pg/ml de sTNF-RII con una

elevada sensibilidad (0.94) y especificidad (0.76). Sin embargo, es necesaria la confirmación de estos resultados en otros estudios similares y su posterior estandarización antes de que este biomarcador pueda ser utilizado para tal fin.

Los niveles de cortisol aumentaron significativamente conforme aumentó el grado de fragilidad. Se estudiaron además las correlaciones entre los niveles de cortisol y los diferentes mediadores inflamatorios, dada la estrecha relación existente entre el eje HPA y la inflamación. Los resultados obtenidos confirmaron esta teoría, ya que los niveles de cortisol mostraron correlaciones significativas con todos los mediadores inflamatorios analizados. Los análisis multivariantes confirmaron que los niveles de cortisol aumentan en el grupo frágil, además se observó un leve pero significativo aumento de estos niveles conforme aumentaba la edad. La reactividad del eje HPA frente a estresores externos se manifiesta en último término mediante el aumento en los niveles de cortisol, los cuales también aumentan con la edad. Los resultados obtenidos apoyan esta idea y suman nuevas evidencias a la escasa literatura que relaciona el incremento en los niveles de cortisol tanto con la edad como con la fragilidad. A mayores, estos resultados refuerzan la estrecha relación existente entre el sistema endocrino y el sistema inmunológico dadas las correlaciones, aunque moderadas, encontradas en este estudio.

No se obtuvieron diferencias significativas entre los distintos marcadores relacionados con el estrés oxidativo analizados y el estado de fragilidad en nuestra población de estudio. Aunque diversos estudios han postulado la posible relación de la fragilidad con un aumento del estrés oxidativo y con la posible reducción de las defensas antioxidantes, existe una gran controversia debida a la variabilidad entre los distintos resultados obtenidos en estos estudios, que pude ser debida a muy diferentes factores. Por ello es necesario llevar a cabo más investigaciones que clarifiquen si realmente existe relación entre la fragilidad y el estrés oxidativo en cualquiera de sus manifestaciones.

Este estudio ha establecido por primera vez rangos de referencia para numerosos biomarcadores relacionados con la estimulación inmunológica, las subpoblaciones linfocitarias y el *inflammaging* en una población de personas mayores robustas de acuerdo al criterio de Fried. Además, se han encontrado diferentes asociaciones entre el estado de fragilidad y muchos de los biomarcadores analizados, indicativos de procesos de activación inmunológica, inflamación y alteración endocrina. No obstante, para ratificar la consistencia y reproducibilidad de las asociaciones encontradas en este trabajo y, de este modo, estandarizar la utilización de alguno de los marcadores aquí analizados como biomarcador para la

identificación temprana de la fragilidad, son necesarios más estudios en esta línea, empleando distintas poblaciones de partida y un mayor tamaño muestral.

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LIST OF ABBREVIATIONS

3NT	3-nitrotyrosine
ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACTH	Adrenal-corticotrophic-hormone
ANOVA	Analysis of variance
ATC	Anatomical therapeutic chemical
BH_4	Tetrahydrobiopterin
CD	Cluster of differentiation
CES-D	Center for epidemiological studies-depression
CI	Confidence interval
CMV	Cytomegalovirus
CRH	Corticotropin-releasing-hormone
CRP	C-reactive protein
DAPI	4´,6-diamidine-2´-phenylindole
DCFH	Dichlorodihydrofluorescein
DMSO	Dimethyl sulfoxide
DOPA	3,4-dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FI	Frailty Index
FITC	Fluorescein isothiocynate
FRAIL	Fatigue, Resistance, Ambulation, Illness and Loss
GCH-I	Guanosine triphosphate cyclohydrolase I
HIV	Human immunodeficiency virus
HPA axis	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
IAGG	International Association of Gerontology and Geriatrics
IDO	Indolamine 2,3 dioxygenase I
IL1β	Interleukin 1 beta
IL6	Interleukin 6
IL10	Interleukin 10
KH_2PO_4	Potassium phosphate
Kyn	Kynurenine
Kyn/Trp ratio	Kynurenine/Tryptophan ratio
MLTA	Minnesota Leisure Time Activity
MR	Mean ratio
$NaNO_2$	Sodium nitrite
Neo	Neopterin
NH_2TP	7,8 Dihydroneopterin triphosphate
NK cells	Natural killer cells
NO	Nitrite
NOS	Nitric oxide synthase
OGG1	8-Oxoguanine-glucosilase
PAH	Phenylalanine 4-hydroxylase
PBS	Phosphate buffer solution
PE	Phycoerithrin
PerCP	Peridininchlorophyll-protein complex

Phe	Phenylalanine
Phe/Tyr ratio	Phenylalanine/Tyrosine ratio
ROC	Receiver-operating-characteristic
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
Rpm	Revolutions per minute
SSC	Side Sccater
sTNF- RII	Soluble tumor necrosis factor receptor II
TCA	Trichloroacetic acid
TDO	Tryptophan 2,3 dioxygenase
TNFα	Tumor necrosis factor alpha
Trp	Tryptophan
Tyr	Tyrosine
WHO	World Health Organisation

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I. INTRODUCTION

I. INTRODUCTION

1. Population aging

During the last century, due to improvement in hygienic conditions, proper diet, better health services and decreased infant mortality, life expectancy has increased from 30-35 years at the beginning of the 20th century to figures approaching 85 years in the first half of 21st century in many of the more industrialized countries. Indeed, according to a recent study (Foreman et al. 2018), Spain will be the country with the longest life expectancy in 2040 (85.8 years, range 83.6-87.4 years), exceeding Japan, Singapore or Switzerland, and with ages over 85 years old in both genders.

Fertility level required for population´s replacement is, on average, around 2.1 births per women. During 2010-2015, 46% of the world population lived in countries with a fertility level below this rate of births per women. All countries in Europe are now below this level (United Nations 2017) and this situation will get worse in the next decades. In 2045-2050, it is expected that 69% of the world population will live in countries where women give birth to fewer than 2.1 children on average. Although Spanish fertility rates are expected to follow a raising tendency from 2010-2015 until 2095-2100 (from 1.33 to 1.72 births per women), they are far away from the values of 1975-1980 with 2.55 births per women (United Nations 2017).

The increase in life expectancy, together with this current decline in fertility rates, are the main reasons of an unprecedented, steady, rapid and unstoppable aging world population, which is evident from the most developed countries to the lowest income regions. This situation leads to an increase in future healthcare expenditure, which is increasing faster for older than for younger adults. Such scenario obviously endanger the sustainability of health and social care systems (Cesari et al. 2016). Because of that, researchers and governments are interested in increasing our knowledge about aging and age-related conditions and disorders, in order to reduce healthcare and socioeconomic costs in the future.

These demographic changes are causing alterations of the population pyramids, which will reverse the proportions of young and older people for the first time in history by 2050 (Figure 1) (United Nations 2013). The aging population situation is reflected in numbers; the report "World Population Prospects 2017" by the United Nations predicts that the number of people aged 60 and over in the world will rise from 901 million in 2015 to 1.4 billion by 2030 and to 2.1 billion by 2050. Besides, people aged 80 and above is projected to triple by 2050

(425 million), and to increase its value to nearly seven-fold (909 million) in 2100 regarding to 2017 value (137 million).

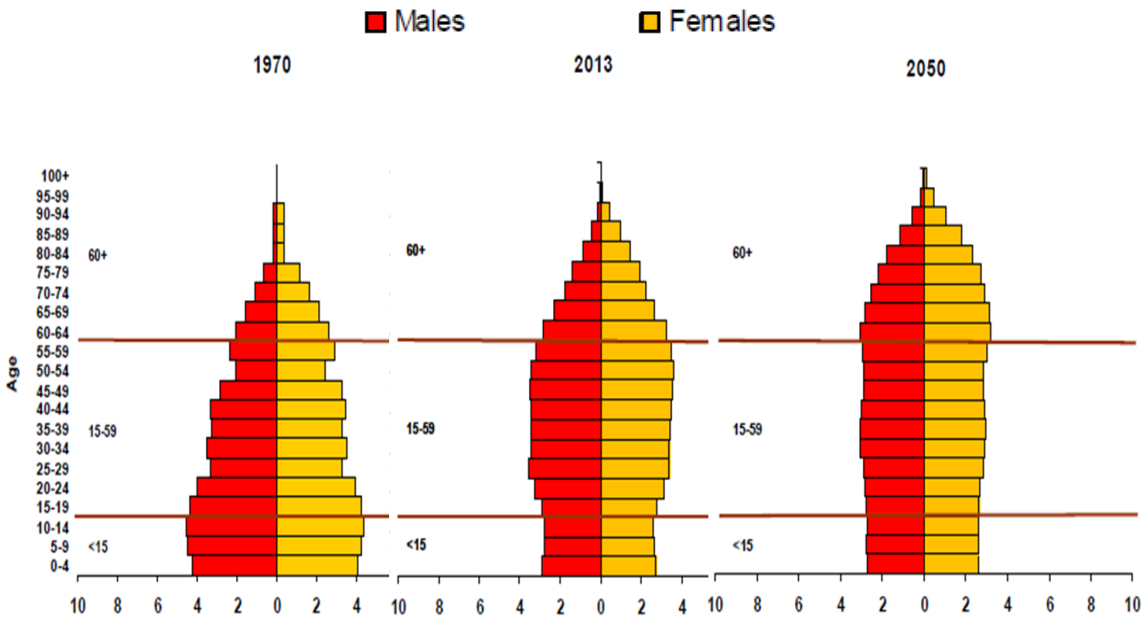

Figure 1. Demographic population pyramids in 1970, 2013 and 2050 (expected) (source: World Population Aging 2013).

In Europe, people aged over 60 years represent 25% of the population and will increase to reach 35% in 2050 and 36% in 2100. In addition, in Europe there are less than 4 people aged 20-64 for every person aged 65 or above, but it is expected to be below 2 by 2050 in 24 European countries (35 countries in the world), reflecting the importance that old-age care will have for these societies concerning healthcare services, pensions, social protection and economy (United Nations 2015).

In Spain, people aged 65 and over will increase 21.5% from 2013 to 2060 (European Commission 2015), being the fifth European country in percentage of older people (8.7%), preceded by United Kingdom, France, Italy and Germany. Particularly, Galicia is, together with Asturias and Castilla y León, the most aged region in Spain, with 24.3% of people aged 65 years and over (Abellán García et al. 2017).

2. Frailty

2.1. Concept and history

Aging is the progressive and overall physiological decline of the organism reserves, which decreases the ability to generate adaptive responses and sustain homeostasis,

becoming the body more susceptible to stress, diseases and injuries (Ruan et al. 2015). The aging process encompasses varied and complex changes at the structural, functional and molecular level of most cells, tissues, and organ systems in the human body. Moreover, it is influenced by different environmental, social, and psychological features.

Life longer does not necessarily mean healthy life-span and welfare. For too many people, old age prompts a high risk of social isolation and poverty, with limited access to affordable, high quality healthcare and social services. Given the difficulty in reversing aging disabling cascades, it is important to act preventively with specifically tailored interventions against signs of disease and disability when these processes are still amenable to effective modification (Kelaiditi 2013). As a consequence, prevention of disability, preservation of health and independence in the elderly is now one of the main targets of healthcare, and should necessarily represent an outstanding focus in future medical research and development. In this pathway from robustness to disability and dependence there is an intermediate stage that has attracted great attention in the last two decades: "frailty" (Angulo et al. 2016), considered as a multidimensional syndrome of loss of reserves (both physical and cognitive) that gives rise to vulnerability. In fact, early identification of frailty would allow professionals to anticipate the adverse health outcomes associated with aging, then acting on frailty through effective interventions that may change the aging trajectories of many individuals from the possible "pathological aging" pattern to the more personally and economically desirable "successful aging" (Morley et al. 2013). Main features related to successful and pathological aging are gathered in Figure 2.

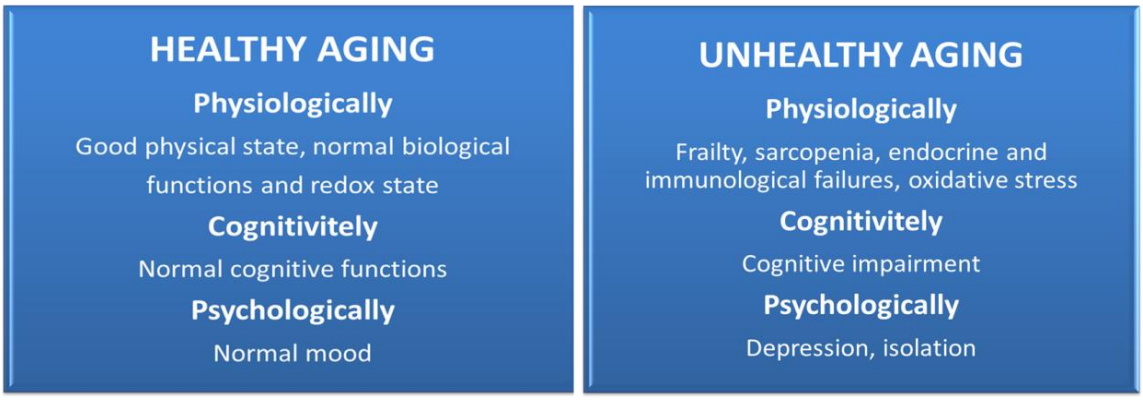

Figure 2. Comparative overview of the common mechanisms of healthy and unhealthy aging.

Chronological age is normally used to classify older people and is only a rough proxy of a person´s vulnerability to adverse health outcomes (Lee et al. 2014). Because of the great heterogeneity of functional decline reported in the aging process, chronological age is not an

accurate indicator of aging signs and symptoms. In this regard, the term "frailty" represents an approach to age-related conditions by replacing the obsolete concept of “chronological age” with the more accurate and person-tailored parameter of “biological age”, that classify older people on the basis of their physiological state (Cesari et al. 2016).

Initially, the concept of frailty appeared first in the research literature in 1968 when O´Brien et al. carried out a study in older adults and outlined the gradual development of frailty as an excessive and disproportionate reaction of these subjects to adverse events. The quantitative and qualitative change in the frailty concept comes at the beginning of this century with two independent studies. Fried and colleagues with the Frailty Phenotype (Fried et al. 2001) on one hand, and Rockwood, Mitniski and colleagues with the cumulative Frailty Index on the other hand (Mitnitski et al. 2001; Rockwood et al. 2005) gave great visibility to frailty in the research community, with the number of scientific papers published on frailty increasing exponentially since then (Figure 3). Throughout all this time to present, the definition of frailty has evolved from a description of dependence to a more dynamic model that encompasses biomedical and psychosocial aspects (Lang et al. 2009). Consequently, a number of definitions and measurements of frailty have arisen in the literature in these last years.

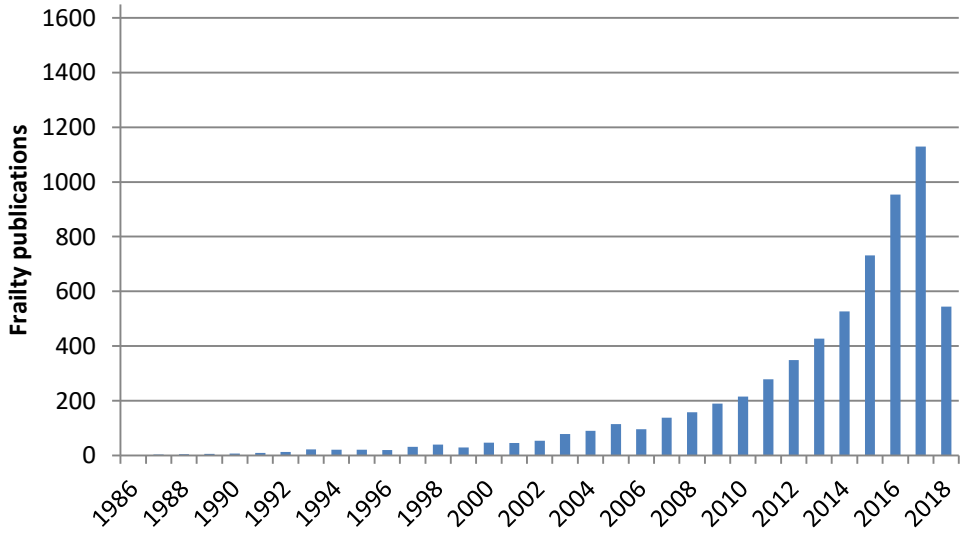

Figure 3. Evolution of publications on “frailty” from 1986 to 2018 in humans aged 65 and over. Data from PubMed Database (reviewed 08/03/19).

2.2. Biological basis of frailty

Frailty, from the French *frêle* and meaning little resistance (Afilalo et al. 2014), does not have an internationally recognized standard definition yet; however, it has been proposed as an age-associated and multidimensional geriatric syndrome characterized by decreased biological reserves (energy, physical ability, cognition, health), due to dysregulation of several physiological systems with diverse consequences (e.g., inflammation, insulin-resistance, neuroendocrine modifications, alterations in coagulation systems, endothelial and vascular dysfunction). Frail subjects have an increased vulnerability, which involves a higher risk of negative health outcomes (falls, fractures, functional impairment or disability, institutionalization, hospitalization and death) when facing with endogenous or exogenous stressors (Alonso-Bouzón et al. 2014; Rodriguez Mañas 2015; Angulo et al. 2016). Another way to conceptualize frailty has recently emerged, in which it is considered as 'primary' or 'secondary'. Primary frailty is not associated with any specific disease or functional decline causing incapacity. In this context, this type of frailty is considered an extension of the physiological aging process in which an older person is even more susceptible to an adverse outcome than what normal aging would predict. On the other hand, frailty may be defined as secondary when it is clearly associated with underlying diseases that are mostly chronic or related to physical disability (Fulop et al. 2015).

Frailty manifestations include outward appearance (consistent or not with age), nutritional status (weight loss), subjective health rating (health perception), performance (cognition, fatigue), sensory/physical impairments (vision, hearing, strength) and current health care (medication, hospital). Although the early stages of the frailty process may be clinically silent, when depleted reserves reach an aggregate threshold leading to serious vulnerability, the syndrome becomes detectable by looking at clinical, functional, behavioural and biological markers (Lang et al. 2009). 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 (Sánchez-Flores et al. 2017) (Figure 4). 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; López-Otín et al. 2013). 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.

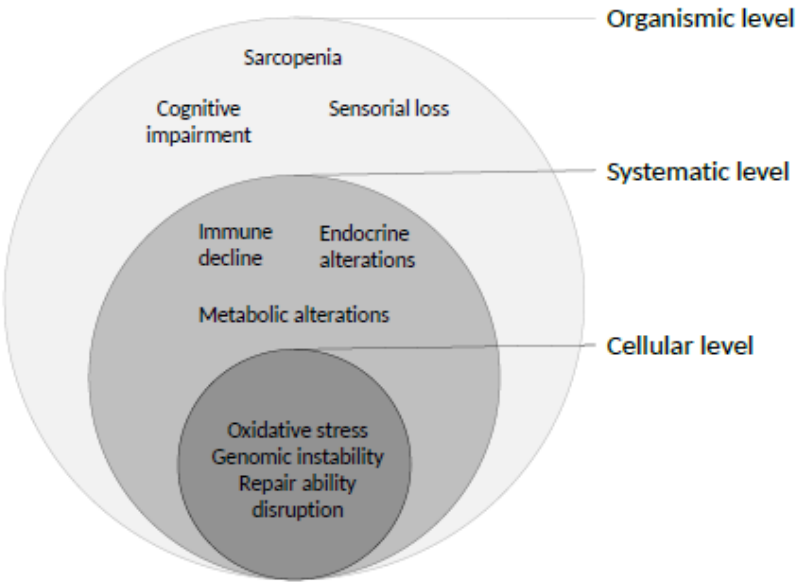

Figure 4. Biological basis of frailty (Sánchez-Flores et al. (2017)).

2.3. Frailty identification

Several international institutions, including the World Health Organization (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, a consensus has not been reached yet, although there is an increasing tendency to consider not only physical criteria but also cognitive and sensorial loss, and even biological parameters. In this sense, a plethora of frailty measurements are currently in existence. Dent and colleagues in the aforementioned work, reviewed 422 studies and identified 29 different frailty identification tools together with a considerable number of measurements modified from original validated scales. Moreover, the existence of more than 260 different versions published in the literature of the Fried´s frailty phenotype (explained below) was also reported (Theou et al. 2015). And even though all of them might potentially identify frailty, modifications introduced in the original phenotype criteria have important impact on its accuracy and predictive ability. Among the different frailty measurements identified by Dent

and colleagues, 14 out of the 29 instruments were previously validated to be used in older people [Fried's frailty phenotype; Rockwood and Mitnitski's Frailty Index (FI); the Study of Osteoporotic Fractures (SOF) Index; Edmonton Frailty Scale (EFS); the Fatigue, Resistance, Ambulation, Illness and Loss of weight (FRAIL) Index; Clinical Frailty Scale (CFS); the Multidimensional Prognostic Index (MPI); Tilburg Frailty Indicator (TFI); PRISMA-7; Groningen Frailty Indicator (GFI), Sherbrooke Postal Questionnaire (SPQ); the Gérontopôle Frailty Screening Tool (GFST) and the Kihon Checklist (KCL), among others]. However, the two frailty measurement tools most commonly accepted and used in clinics and in medical studies are the phenotypic model proposed by Fried and the deficit accumulation model developed by Rockwood and Mitniski.

The Frailty Phenotype model was developed by Fried et al. (2001), and it is based on the presence or absence of five very specific components related to physical fitness and metabolism. According to this definition, the major criteria for characterizing the phenotype of frailty as a clinical syndrome are the following: unintentional weight loss, muscle weakness, self-reported exhaustion, slow walking speed and low physical activity level. An individual with three or more of these characteristics is considered frail, while the presence of one or two of them indicates a pre-frailty state, and the absence of all five indicates a non-frail or robust.

The cumulative model proposed by Rockwood and Mitniski (Mitnitski et al. 2001; Rockwood et al. 2005), also called Frailty Index (FI), is a much more holistic and multidimensional approach which is calculated as a ratio between the number of deficits that the individual actually presents divided by the total of deficits considered in the computation. The deficits evaluated were initially a total of 92 parameters that include physical criteria, neurological examinations, psychological symptoms, diseases, geriatric syndromes and clinical laboratory values, among others, being a simple calculation of the presence or absence of each variable as a proportion of the total.

These two frailty measurements are evidently different in their construct, but also in their objectives. Concretely, frailty phenotype is more focused on screening the physical domain of frailty, and the deficit accumulation model stems from results of a comprehensive geriatric assessment. In spite of being complementary tools, frailty phenotype is more often used in both research and clinical settings due to its simplicity and ease of impementeation.

2.4. Prevalence

The prevalence of frailty is widely variable among studies, mainly due to the different tools employed to identify frailty. But even using the same criteria, factors as gender, age, race, or socio-economic conditions have been reported to influence this prevalence, with higher values in women compared with men (Collard et al. 2012; Theou et al. 2015), and in people with limited education and poverty regarding other more socially favourable populations (Fried et al. 2001). In this sense, Collard et al. (2012) structured frailty prevalence regarding age ranges, reporting a prevalence of 4% in people aged 65-69 years, 8% in those aged 70-74 years, 10% for 75-79 years, 17% for 81-84 years, and 28% in people aged over 85 years, demonstrating that frailty increases with age. In accordance with this trend, Ahmed et al. (2007) reported 32% of frailty prevalence in people aged 90 years and above. Collerton et al. (2012) conducted a systematic review to estimate the prevalence of frailty in older adults including data from 61,500 subjects from 21 different studies. The reported prevalence varied substantially across studies, ranging from 4.0% to 59.1%; later studies showed that this prevalence can be even higher. When analyses were restricted to studies using the phenotype model proposed by Fried and colleagues (2001), the weighted average prevalence was 9.9% for frailty and 44.2% for pre-frailty. In Europe, people aged 65 years or above present prevalence of 17% and 42.3% for frailty and pre-frailty, respectively; data classified by gender were 21.0% and 42.7% in women, and 11.9% and 41.9% in men (Santos-Eggimann et al. 2009). In Spain, in community-dwelling older populations, it was established to be 8.6% (García-García et al. 2011) and 16.3% (Abizanda et al. 2011) in different studies employing Fried's criteria for frailty identification. However, according to a cross-sectional study with 331 Spanish participants of both sexes, this prevalence can reach 68.8% in the case of institutionalized older people (González-Vaca et al. 2014).

2.5. Socio-economic implications and interventions

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, opening new targets in disability prevention and elderly care (Gill et al. 2006; Lang et al. 2009; Roland et al. 2014). Thus, early identification of frailty seems to be essential for implementing multidimensional preventive interventions against age-related and disabling conditions. However, the critical time window for interventions that target frailty has not been clearly established yet (Cesari et al. 2016). Research in this direction will further inform public health policies to implement evidence-

based research findings for the development of prevention plans and clinical trials. Initially, a list of preventive interventions, not only directed to older people, may be considered. These may include promotion of physical activity, cognitive stimulation and training, healthy dietary habits (e.g. the Mediterranean diet), smoking cessation, promotion of emotional resilience, active and socially integrated lifestyles, optimal daily sleep, maintenance of optimal body weight, and metabolic control (including control of dyslipidemia, diabetes and blood pressure) (Kelaiditi 2013). Furthermore, to reach the health, social and economic benefits associated with the early identification of frailty, interventions must be carried out at different levels, from older people, who need to be aware of the signs of pre-frailty or frailty, to healthcare professionals, who need to be trained to confirm these signs and the frailty status. But also public authorities must be conscious of the increasing burden of age-related conditions and their effects on the healthcare and social systems, and become proactive in planning and implementing proper strategies to overcome them. Summarizing, early frailty identification and early intervention can reduce its adverse health consequences and improve the older individuals´ quality of life, thereby helping to achieve the goal of healthy aging (Ruan et al. 2015).

3. Immune system

Generally speaking, the immune response comprises two different, but closely interrelated parts: the innate and the adaptive immunity. The innate response generates an unspecific cellular response to counteract harmful agents which is mediated by neutrophils, monocytes/macrophages, natural killer cells and dendritic cells. The adaptive immune response provides an antigen specific response mediated by T and B lymphocytes.

3.1. Immunosenescence

Through evolution our ancestors were set to live 30-50 years; however, the extraordinary and dramatic increase in lifespan (80-120 years), joined to the reduction in early mortality rate reached in the last century, has leaded modern societies to a huge amount of older people. As a result, the immune system has to be active longer, and older people have to cope with a lifelong antigenic load encompassing several additional decades of antigenic exposure, evolutionarily unpredicted (De Martinis et al. 2005). This continuous attrition caused by the aging chronic antigenic load is accompanied by several changes, both cellular and serological, that compromise the competence of the immune system and that is called “immunosenescence” (Castelo-Branco and Soveral 2014).

Immunosenescence is characterized by multiple alterations in the phenotypes and functions of the innate and adaptive immune cells (Fulop et al. 2016). This antigenic stress can be caused by (i) persistent infections such as cytomegalovirus, human immunodeficiency virus and herpes simplex virus 1, which can be accelerated and aggravated by immunosenescence; (ii) endogenous host-derived cell debris; (iii) senescent cells and their senescence-associated secretory phenotype; (iv) harmful products and metabolites produced by gut and other microbiota (oral) that undergo profound changes with age; and (v) the coagulation system and its increasing activation with aging (Claesson et al. 2012; Fulop et al. 2015, 2016).

Age-related immunosenescence has been extensively documented in the adaptive immune system. This is considered to be responsible, at least in part, for the inflammatory phenotype or "inflammaging", shrinkage of the T cell repertoire, filling up of the immunological space with memory/effector cells, poor immune response to vaccination, and overall immune functional decline observed in older adults (De Martinis et al. 2005; Leng et al. 2011). Inflammaging is a state characterized by a low-grade, chronic and systemic up-regulation of the inflammatory response in aging, which is associated with a high and significant risk factor for morbidity, mortality and detrimental health outcomes in elderly people. Inflammaging is associated with increases in the level of pro-inflammatory cytokines, such as interleukin-1 beta (IL1β), interleukin-6 (IL6) and tumor necrosis factor alpha (TNFα), acute-phase reactants such as C-reactive protein (CRP), and decreases in the anti-inflammatory cytokine interleukin-10 (IL10), just to name a few, impairing the maintenance of immunological homeostasis (Bartlett et al. 2012). Inflammaging is considered a predictor of frailty, and this condition is currently accepted as a pathogenic factor in the development of several age-related diseases, such as cardiovascular disease (Libby et al. 2010), cancer (Grivennikov and Karin 2011), osteoporosis (Lencel and Magne 2011) and Alzheimer's disease (Morales et al. 2010) (Figure 5).

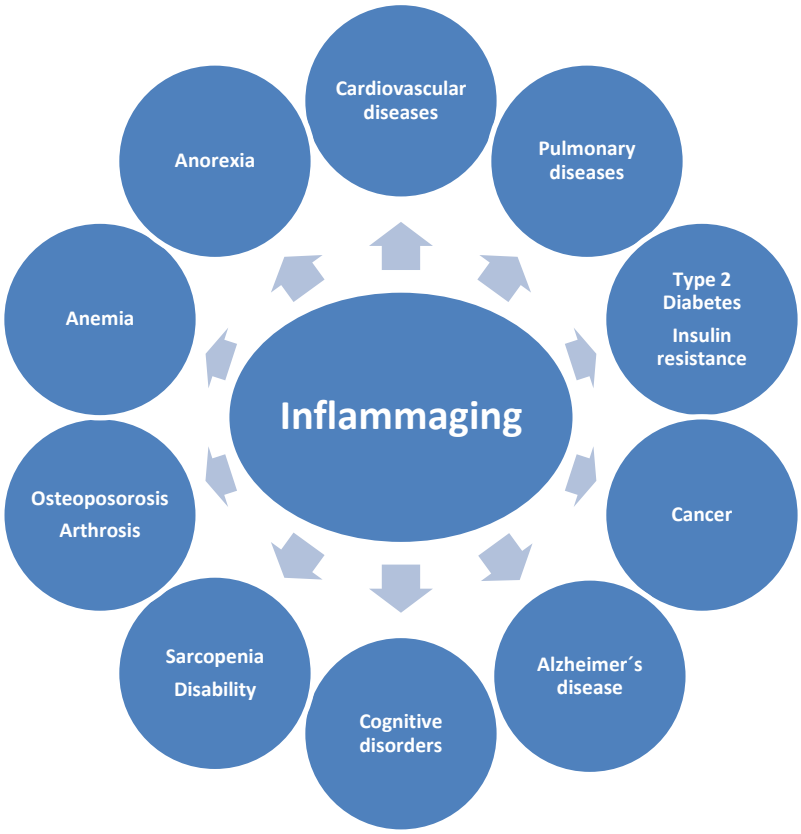

Figure 5. Age-related diseases for what inflammaging is considered to be a pathogenic factor. Modified from De Martinis et al. (2006).

3.2. Immune stimulation

Under chronic inflammation and immune activation conditions, alterations in the metabolism of tryptophan and tyrosine are activated by macrophage-induced pro-inflammatory cytokines. These two amino acid metabolisms are regulated by indolamine-2,3 dioxigenase (IDO) and guanosine-triphosphate-cyclohydrolase-1 (GCH) pathways, respectively.

Tryptophan (Trp) is an essential amino-acid, required for the biosynthesis of proteins and precursor for several biological compounds: the neurotransmitter serotonin (5-hydroxy-tryptamine) and the formation of kynurenine (Kyn) derivatives and nicotinamine adenine dinucleotides (NAD and NADH), the latter by the so called kynurenine pathway, which is responsible for approximately 95% of whole tryptophan degradation (Badawy 2002).

The aromatic amino-acid phenylalanine (Phe) is essential for humans and is the precursor of tyrosine (Tyr), another import amino-acid. Approximately 67-90% of Phe enters the pathway of Tyr biosynthesis (Scholl-Bürgi et al. 2011) which is the precursor for the

biosynthesis of 3,4-dihydroxyphenylalanine (DOPA) and the catecholamines dopamine, epinephrine and norepinephrine, crucially related to neurobehavioral aspects.

Upon immune activation, inflammatory factors (eg, Th1-type cytokine interferon-γ) induce the expression of the enzymes indoleamine 2,3-dioxygenase 1 (IDO) and guanosine triphosphate cyclohydrolase I (GCH) in monocytes/macrophages and dendritic cells (Figure 6). IDO is involved in transforming tryptophan into kynurenine. *In vivo*, kynurenine/tryptophan (Kyn/Trp) ratio reflects tryptophan breakdown, and in inflammatory conditions it is considered to represent IDO enzyme activity (Widner et al. 1997). Several clinical conditions associated with increased immune activation are characterized by intensified tryptophan degradation (e.g., several infections including human immunodeficiency virus infection, autoimmune syndromes, a number of cancers, neurodegenerative disorders, and cardiovascular disease, among others) (Schröcksnadel et al. 2006).

When GCH, the key enzyme of pteridine biosynthesis, is activated, it produces 7,8-dihydroneopterin triphosphate (NH_2TP), which is a precursor of neopterin and tetrahydrobiopterin (BH_4) (Figure 6). BH_4 is an essential cofactor of amino acid monooxygenases, including phenylalanine 4-hydroxylase (PHA), involved in the conversion of phenylalanine to tyrosine, and nitric oxide synthases (NOS), which catalyze the conversion of arginine to nitric oxide (NO) (Neurauter et al. 2008b). In conditions of immune activation, neopterin is released by activated human monocytic cells at the expense of the formation of BH_4 (Widner et al. 2000). Thus, neopterin concentration in body fluids, including serum, urine, and cerebrospinal fluid, is considered a sensitive marker of immune system activation. In fact, neopterin levels are increased in malignant tumors, in autoimmune, cardiovascular, infectious, and neurodegenerative diseases, and during rejection episodes in allograft recipients (Murr et al. 2002). Likewise, the spectrum of diseases in which elevated serum phenylalanine levels, as consequence of low PHA activity, have been reported, including sepsis, HIV infection, cancer, burns, and trauma (Geisler et al. 2013), is very similar to the one with increased degradation of tryptophan and neopterin production.

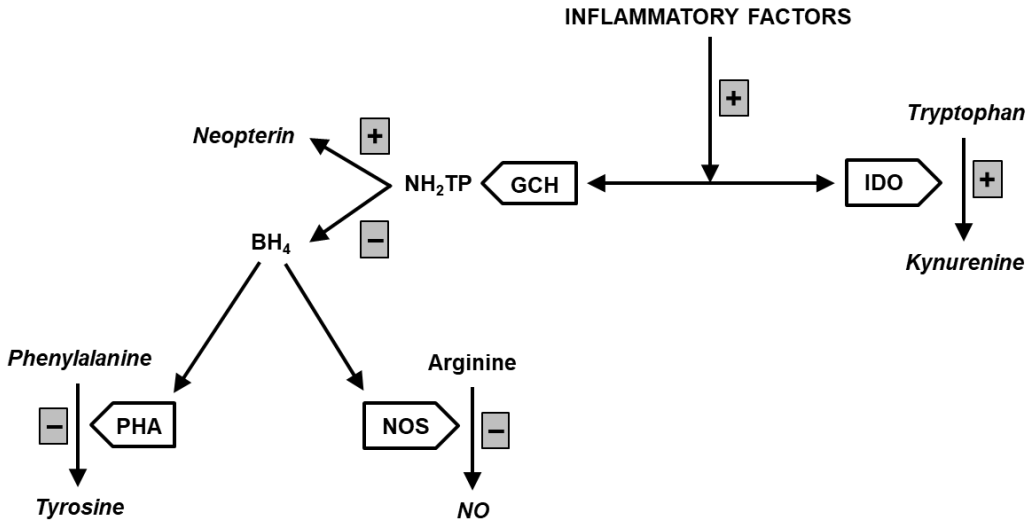

Figure 6. Immune stimulation through inflammation factors involves activation of IDO and GCH pathways, which leads to increase in tryptophan breakdown, increase in neopterin production from 7,8-dihydroneopterin triphosphate (NH_2TP) at the expense of BH_4 and, consequently, decrease in PHA and NOS activities, resulting in decline of tyrosine and NO production. Italic letter indicates immune biomarkers analyzed in this study. GCH: Guanosine triphosphate cyclohydrolase I; IDO: Indolamine 2,3 dioxygenase I; NO: Nitric oxide; NOS: Nitric oxide synthase; PHA: Phenylalanine 4-hydroxylase.

Progressive increase in Trp catabolism is also a part of normal aging process (Frick et al. 2004). In this regard, neopterin, Kyn/Trp ratio, and all kynurenine metabolites were reported to be 20-30% higher in the older group (70-72 years) as compared to younger group (46-47 years) whereas Trp was 7% lower (Theofylaktopoulou et al. 2013).

3.3. Lymphocyte subpopulations

Lymphocytes are the main cells involved in adaptive immunity and integrate 20-40% of blood cells in humans. They are highly specialized cells that interact with other cells to initiate an immune response. The two main types of lymphocytes are B and T, which in turn can be divided into different subpopulations, based on the surface markers they express. Receptor specificity and functional heterogeneity allows lymphocyte subpopulations to respond to almost any antigen (Descotes 2004; Tryphonas et al. 2005).

T lymphocytes constitute approximately 50-70% of peripheral blood lymphocytes in humans and express the T-cell surface receptor (TCR), in addition to the cell differentiation markers CD2 and CD3. In general, this type of lymphocytes is divided into two subpopulations, $CD4^+$ and $CD8^+$, differentiated by their functions and surface markers. The helper T cells (Th)

express the CD4 marker on their surface and their main function is to provide assistance to B lymphocytes, through the release of cytokines, mainly helping them to produce antibodies in response to an antigenic attack. This type of T lymphocytes is subdivided in Th1 and Th2 subtypes depending on the cytokine profiles they secrete. Th1 subtype mainly secretes IL2 and interferon-γ, inducing cellular response which increases microbicide activity. Th2 subtype secretes IL4, IL5 and IL10 in order to produce antibodies (Descotes 2004; Tryphonas et al. 2005). Cytotoxic T lymphocytes (Tc) express the CD8 marker on their surface, and their function is to capture the target cells by adhesion mechanisms and release the content of their granules towards these cells. Maturation of T lymphocytes occurs in the thymus. One of the most remarkable changes in immunosenescence is the involution of the thymus, and consequently a decrease in the proportion of T lymphocytes with age (Goronzy and Weyand 2005; Chinn et al. 2012). Moreover, the aging process in T lymphocytes may be accelerated by several factors, including repeated exposure to antigens, environmental chemicals, and pathogens like cytomegalovirus (CMV), herpes viruses, or polyomaviruses (Weiskopf et al. 2009).

B lymphocytes ($CD19^{+}$) are the precursor cells of the plasma cells secreting antibodies and they constitute approximately 10-20% of peripheral blood lymphocytes in humans. B lymphocytes are activated directly as a consequence of the binding of immunoglobulins, expressed on the cell surface, to an antigen, or indirectly by interaction with T lymphocytes. They play central roles in the establishment and maintenance of protective immunity, including the generation of protective antibodies, antigen presentation, and appreciated regulatory functions (Cancro et al. 2009). B-lymphocytes maturation occurs in the bone marrow. The aging process initiates changes within the bone marrow, leading to a defective production of B-lymphocyte progenitors (Chinn et al. 2012; Pritz et al. 2014). In addition, B-cell functions experience age-related alterations and, consequently, specificity and class of antibodies produced change with age. This entails a rise in susceptibility to infectious diseases and reduces the effectiveness of vaccination in the elderly (Frasca et al. 2011). Further, the proportions of mature human B lymphocytes significantly decrease with age (Frasca et al. 2011).

Natural killer (NK) cells are innate immune cells which play an important role as modulators of tumor, viral, and microbial immunogenicity, as well as in release of pro-inflammatory cytokines and chemokines that facilitate the Th1 driven immune response (Mahbub et al. 2011). NK cells represent 10-15% of peripheral blood lymphocytes, and are closely related to T lymphocytes but lacking the surface receptors characteristic of them.

Instead, they usually express CD16 and CD56 on their surface (Tryphonas et al. 2005). Aging is related to NK-cell alterations, both functional and phenotypical. NK senescent cells seem to present a defective functional capacity with advanced age that is partially compensated by elevating the number of mature NK cells. High levels of NK cells are associated with high NK cytotoxicity, and therefore an age-related increased proportion of NK cells is related to healthy aging and longevity (Weiskopf et al. 2009).

3.4. Inflammatory mediators

Inflammation is a necessary, acute, transient and localized response to damaging agents, both external (e.g., UV and gamma radiation, heat) and internal (e.g., viruses, bacteria, injury or tissue damage) with systemic consequences. Inflammation helps to reduce, sequester or destroy the harmful agents and the wounded tissue. In its acute form, inflammation is characterized by pain, redness, heat, swelling and loss of function. However, normally in later life, immune response becomes detrimental and chronic, continuously damaging the surrounding tissue and being unable to restore healthy tissue or to produce a stable and low-grade response (Vasto et al. 2007).

During a healthy immune response, the elevation of pro-inflammatory cytokines is followed by an increase of anti-inflammatory cytokines, acting to restore the balance in inflammatory mediators (Allison and Ditor 2014). However, under a state of chronic inflammation, the local production of the pro-inflammatory cytokines TNFα and IL1β is the first step that triggers the inflammatory cascade. TNFα and IL1β stimulate the secretion of the pleiotropic cytokine IL6, which firstly inhibits the synthesis of TNFα and IL1β, stimulating the production of soluble TNF receptors. Secondly, IL6 promotes the expansion and activation of T cells and the differentiation of B cells, and thirdly, it induces the hepatic synthesis of acute-phase proteins, such as CRP, amyloid A or fibrinogen. The first one has an anti-inflammatory role, suppressing the production of pro-inflammatory cytokines in macrophages and inducing the synthesis of anti-inflammatory cytokines in circulating monocytes (de Gonzalo-Calvo et al. 2010).

IL6 is a 20-30 kDa glycoprotein produced by immune cells (such as monocytes, macrophages, lymphocytes, etc.), but also produced by non-immune organs and cells (bone marrow stromal cells, chondrocytes, osteoblasts, etc.). It is characterized by its pleiotropy having both pro-inflammatory and anti-inflammatory biological functions, apart from its hematologic, immune, and hepatic effects; it has many endocrine and metabolic actions (Papanicoleau et al. 1998).

CRP is a phylogenetically highly conserved non-glycosylated plasma protein which is a member of the family of "pentraxins". This acute phase protein is produced in the liver under transcriptional control by the pro-inflammatory cytokine IL6 and, to lesser extent, by TNFα and IL1β during inflammation, tissue damage or infection. Its main function is to recognize pathogens and host damaged cells and to mediate their elimination by recruiting the complement system and phagocytic cells, increasing its concentration rapidly over 1000-fold in 24-48 hours as a consequence of tissue damage, infection or inflammation, with a half-life of 19-24 hours when the stimulus is removed (Volanakis 2001).

TNFα is a polypeptide cytokine mainly produced by stimulated monocytes, macrophages, B lymphocytes and T lymphocyte subsets (Zangerle et al. 1994). TNFα is an early potent mediator of inflammatory and immune functions, involved in the production of chemokines, and B cell, T cell, macrophage and neutrophil activities. TNFα exerts its biological functions by binding to specific, high-affinity cell surface receptors. Two distinct TNFα receptors have been identified: TNF receptor type I (TNFR-55 or TNF-RI) is a 55-60 kD protein which is expressed in almost all cell types, while TNF receptor type II (TNFR-75 or TNF-RII) has a 75-80 kD molecular weight and is only located in oligodendrocytes, astrocytes, T cells, myocytes, thymocytes, endothelial cells and in human mesenchymal stem cells (Cabal-Hierro and Lazo 2012). Both receptors are shed by proteolytic cleavage into circulation as soluble TNFα receptors (sTNF-RI and sTNF-RII), which have a stable and longer half-life in the plasma and have been shown to be reliable measurements for the *in vivo* activities of TNFα (Savès et al. 2001). Soluble TNF receptors regulate TNFα function (i) acting as antagonists for TNFα, blocking its action directly or by competition with cell surface receptors, (ii) serving as binding proteins to carry TNFα out of the site of production and (iii) stabilizing and even enhancing the effects of TNFα serving as a reservoir of bioactive TNFα which prolongs the TNFα activity (Diez-Ruiz et al. 1995).

4. Endocrine system

The immune system is not the only one that experiences senescence. Endocrinosenescence represents the substantial decline in the hormonal levels of at least hypothalamic-pituitary-gonadal axis, hypothalamic-pituitary-adrenal (HPA) axis and growth hormone-insulin-like factor 1 (Roshan et al. 1999). Among them, HPA axis performs a tight integration among endocrine, nervous and immune systems. HPA axis is activated by pro-inflammatory mediators after a perceived stress, causing the release of corticotropin-releasing-hormone (CRH) from the hypothalamus, adrenal-corticotrophic hormone (ACTH)

from the pituitary gland and cortisol secretion from the adrenal cortex. The activation of HPA axis constitutes: (i) the main specific response and counterbalance to "inflammaging" (called "anti-inflammaging"), (ii) an explanation for immunesenescence, and (iii) a complex mechanism of remodelling elicited by inflammation, explaining the long and winding pathophysiological road that goes from robustness to frailty (Sergio 2008). An activation of the HPA axis with increased cortisol secretion in response to increased level of pro-inflammatory cytokines of inflammaging acts as a potent immunoregulator and immunosupressor, characterized by the reduced cellular and humoral response of B and T lymphocytes and monocytes (Mazzoccoli et al. 2010) that can prevent the immune system from overacting, causing tissue damage. Temporal dynamics of HPA axis responses to stressors typically consists of three phases: (i) a "basal activity", which reflects non stressed HPA activity, (ii) a "stress reactivity" phase in which cortisol increases from baseline levels following the onset of a stressor, and (iii) a "stress recovery" phase in which cortisol levels return to baseline following the offset of the stressor (McEwen 1998). The hormonal end-product of the HPA axis, cortisol, is one of the most powerful agents of anti-inflammaging, and the cytokines and ROS mediated effects are figures of inflammaging. As a whole, they all represent important key determinants of the aging process, and they are also implicated in the development of age-related diseases and frailty.

5. Oxidative stress

Free radicals refer to atoms or molecules with one or more unpaired valence electron in its atomic orbital, which make them unstable and highly reactive. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) refer to reactive radical and non-radical derivatives of oxygen and nitrogen, respectively. ROS and RNS are present in all aerobic organisms and they are produced as a normal product of cellular metabolism, being involved in several biological functions such as neurotransmission, blood pressure modulation, immune system control, etc. (Pavelescu 2015). The major intracellular site for ROS generation is mitochondria, but they can be also generated by exogenous sources, including cigarette smoke and ultraviolet radiation, among others. Antioxidants are substances or molecules that can neutralize ROS and RNS by accepting or donating electrons to free radicals, protecting biological systems at cellular, membrane and extracellular level against free radical attack. During aging, the balance between oxidative species production and antioxidant defences may deteriorate, resulting in oxidative stress that can compromise biological functions in two general ways: by directly damaging proteins, lipids, DNA and carbohydrates changing the organism's structure and functions, and by providing a trigger to redox-sensitive transcription

factor that up-regulate inflammatory cytokines (Semba et al. 2007; Czerska et al. 2015). Two basic pathways are responsible for the impact of oxidative stress on aging: (i) pathways that affect the amount of ROS/RNS in the whole organism, and (ii) pathways that repair (antioxidant defences) or turnover structures that have been damaged by these ROS/RNS (Bokov et al. 2004). In this sense, given the close relationship between oxidative stress, inflammation and aging the "oxidative-inflammatory theory of aging" (De la Fuente and Miquel 2009) has proposed that aging is a loss of homeostasis due to a chronic oxidative stress that affects especially the regulatory systems, such as nervous, endocrine, and immune systems (Pandey and Rizvi 2010)**.** Indeed, oxidative stress is an often hallmark in a number of age-related diseases, such as diabetes, cancer, cardiovascular diseases, neurodegenerative diseases and chronic kidney disease (Liguori et al. 2018).

II. OBJECTIVES

II. OBJECTIVES

This rapid and unstoppable global situation of population aging leads to an increase in future social, economic and healthcare expenditure. Frailty is a geriatric syndrome of loss of reserves which leads to disability, institutionalization and death. For this reason, identification of frailty as early as possible is crucial to ensure wellbeing in the elderly through a healthy aging. In this sense, the main objective of this work was to increase the knowledge on the physiopathology of frailty status by exploring its relationship with immune and endocrine systems, together with oxidative stress. To this aim, a set of biomarkers were determined in a population of people aged 65 years and above, classified according to their frailty status following the Fried's criteria, in order to determine whether some of the parameters analyzed could be proposed as biomarkers useful for the early identification of frailty.

This main goal was achieved through the following specific objectives:

1. To recruit a population of older adults (aged 65 years and over) and classify them according to their frailty status following the criteria proposed by Fried et al. (2001).

2. To study the relationship of immune activation and inflammaging with frailty status in the selected population.

3. To assess the potential role of endocrine system, in particular, serum cortisol levels, on frailty status in the study population.

4. To determine the possible association of oxidative stress with the development of frailty syndrome in the older adult participants.

III. MATERIALS AND METHODS

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1. Study Population

Participants were recruited from several associations of retired or older people, nursing homes, and day-care centers located in Galicia, North-western Spain. The final cohort included 259 individuals (85 males and 174 females) aged 65-102 years. A clinical evaluation was carried out by interviewers specially trained to unify criteria, and participants completed a questionnaire to collect medical, lifestyle, and demographic information. The small number of current smokers and ex-smokers (*N*=5 and *N*=49, respectively) motivated to join them all in a new category, "ever smokers". Similarly, participants with low comorbidity (Charlson comorbidity index total score = 2, *N* = 47) and high comorbidity (total score ≥ 3, *N* = 33) (Charlson et al. 1987) were grouped together. Participants were excluded if they did not possess the necessary skills to be assessed or denied signing the informed consent. Exclusion criteria also included taking medications included in the Anatomical Therapeutic Chemical category L (antineoplastic or immunomodulating agents) (WHO Collaborating Centre for Drug Statistics Methodology 2012), and having any chronic infection, autoimmune disease or cancer, since these conditions could act as confounders.

Whole blood samples were collected into vacutainer tubes without anticoagulant or containing sodium heparin or ethylenediaminetetraacetic acid (EDTA), and into BD Vacutainer® CPT™ tubes with sodium heparin. All samples were obtained early in the morning to avoid circadian variations in the parameters, and were transported to the laboratory immediately. Fresh whole blood samples were used for the analysis of lymphocyte subsets. Serum and plasma samples were obtained by centrifugation at 950 x g for 10 min, aliquoted, and stored at -80^{o}C until analysis. Blood collected in BD Vacutainer® CPT™ tubes was used for mononuclear leukocyte separation, as described below. All laboratory measurements were performed in a blinded manner because of all samples were coded at the moment of collection.

2. Ethics statement

Ethical approval was obtained from the University of A Coruña Ethics Committee (reference number CE 18/2014). The study was conducted according to the Helsinki Declaration and International Conference of Harmonization guidelines. Written informed consent was obtained from all study participants, or their relatives in case of inability.

3. Frailty Status

Frailty status of each subject was assessed according to the phenotypic criteria proposed by Fried et al. (2001). These criteria are based on the presence or absence of five specific phenotypic components:

(i) Unintentional weight loss (i.e., not due to dieting or exercise): at least 4.5 kg in the past year.
(ii) Self-reported exhaustion: identified by two questions from the Spanish version (Ruiz-Grosso et al. 2012) of the modified 10-item Centre for Epidemiological Studies-Depression (CES-D) scale (Radloff 1977).
(iii) Weakness: grip strength in the lowest 20% at baseline, adjusted for gender and body mass index. It was measured by a handle dynamometer. Three measurements were taken for each hand, and the participant was encouraged to exhibit the best force possible. Each hand's average handgrip strength was calculated, and maximum average value of left or right hand was used as handgrip strength.
(iv) Slow walking speed: the slowest 20% at baseline, based on time to walk 15 ft, (4.6 m) adjusting for gender and standing height.
(v) Low physical activity: the lowest 20% at baseline, based on a weighted score of kilocalories expended per week, calculated according to the Spanish validation (Ruiz-Comellas et al. 2012) of the Minnesota Leisure Time Activity (MLTA) questionnaire (Taylor et al. 1978) according to each participant's report, and adjusting for gender.

Frailty was defined as the presence of three or more of these characteristics, pre-frailty in case of one or two of them present, and the absence of all five determined a non-frail state.

4. Comorbidity and 10-year mortality risk

Charlson comorbidity index (Charlson et al. 1987) was used to assess general comorbidity and number of comorbid diseases. A Charlson comorbidity index age-adjusted score was computed for each participant, coding the absence of comorbid diseases as 0, and the presence as 1 to 6. In addition, this index was used to analyze whether the 10-year mortality rates from comorbid disease differed significantly among frailty categories. A composite comorbidity-age score was computed for each participant, evaluating the 10-year mortality by means of a theoretical low-risk population whose 10-year survival was 98.3% (Hutchinson et al. 1982).

5. Immune stimulation biomarkers

5.1. Neopterin

Serum neopterin levels were determined by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (BRAHMS GmbH). This kit is based in a competitive ELISA quantification (Figure 7). The wells of the microtiter plate are coated with anti-neopterin sheep polyclonal antibodies. After addition of the enzyme conjugate (neopterin/alkaline phosphatase conjugate) to standards, external controls and participant´s plasma samples, the neopterin in the samples competes with the neopterin/enzyme conjugate for the antibody binding sites, thus forming an immune complex bound to the solid phase (anti-neopterin antibody/neopterin or anti-neopterin antibody/neopterin/enzyme conjugate). The subsequent intensive washing steps ensure the complete removal of all unbound components. The addition of the 4-nitrophenyl phosphate substrate solution starts the enzyme reaction in which the alkaline phosphatase contained in the neopterin/enzyme conjugate catalyzes the cleavage of the phosphate of 4-nitrophenyl phosphate, thus forming the yellow 4-nitrophenol.

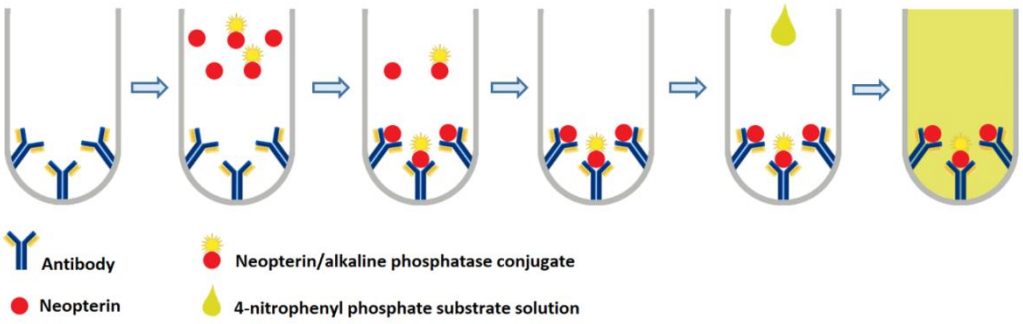

Figure 7. Competitive ELISA diagram.

The enzymatic reaction is stopped by alkalinisation with sodium hydroxide. The color intensity (measured in optical density) depends on the quantity of enzyme bound for a constant reaction time, and consequently, is inversely proportional to the neopterin concentration in the participant's samples. Thus, high neopterin values correspond to a low optical density. The optical density was measured by means of a power wave X microplate reader (Bio-Tek Instruments), equipped with KC4 v.2.5 kinetic analysis software (Bio-Tek Instruments) at an absorption maximum of 405 nm. Sensitivity of the test was 2 nmol/l neopterin.

5.2. Tryptophan and phenylalanine metabolism biomarkers

Serum concentrations of tryptophan metabolism molecules (Trp and Kyn) on one hand, and of phenylalanine metabolism products (Phe and Tyr) on the other hand, were determined simultaneously by high performance liquid chromatography (HPLC), following the general protocols proposed by Laich et al. (2002) and Neurauter et al. (2013), respectively, with minor modifications. An internal calibrator (serum pool) and an internal control (3-nitrotyrosine) were used. Serum pool was composed of a mixture of human serum samples, collected in the same sampling day, which was aliquoted and stored at -20 °C for a maximum three months and never refreeze.

Trp, Tyr and 3-nitrotyrosine were obtained from Sigma; Kyn, Phe and albumin from Serva; trichloroacetic acid from Merck; and KH_2PO_4 and acetonitrile elution buffers were obtained from Carl Roth and Merck, respectively. All chemicals used were of analytical grade. The HPLC pump was a Model 210 (Varian ProStar, Palo Alto, CA). Sample collection was performed by an autosampler Model 400 (Varian ProStar) with a 20 µl sample loop and a cooling unit (4°C). For separation, reversed-phase cartridges LiChroCART RP-18e columns (55 mm length, 3 µm grain size) (Merck) were employed. The software used was the Varian Star Chromatography Workstation, version 6.30.

5.2.1 Chromatographic procedure

The elution buffer used was a degassed potassium phosphate solution (1 M, pH 6.4) containing 27 ml/l acetonitrile with an injection volume of 20 µl for Trp and Kyn measurements, and a degassed potassium phosphate solution (15 mM) with an injection volume of 50 µl for Phe and Tyr measurements. RP18 pre-columns (Merck) were used to protect the column from apolar ingredients of biologic material. They were replaced daily after measurements of approximately 30–40 serum specimens. The HPLC cartridge was rinsed with a gradient from water to methanol and back within 1 h each day. Analyses were carried out at a flow rate of 0.9 ml/min and a temperature of 25°C.

Trp was detected by a fluorescence detector (Varian ProStar, Model 360) at an excitation wavelength of 286 nm and an emission wavelength of 366 nm. Phe and Tyr were detected by the same fluorescence detector at an excitation wavelength of 210 nm and an emission wavelength of 302 nm. A Shimadzu SPD-6A UV-detector (Shimadzu) in flow stream series connection was used for detection of both Kyn and the internal control 3-nitrotyrosine at a wavelength of 360 nm.

5.2.2 External and internal calibrators

For Trp and Kyn measurements one serum pool tube and three standard tubes (one of standard mix, one of 1:1 standard mix dilution in bidistilled water and one of 1:10 standard mix dilution) were prepared and injected per each group of fourteen samples to control the validity of measures as external (standards) and internal (serum pool) calibrators. The external calibrator (standard mix) was prepared from freshly thawed stock solutions of Trp and Kyn (1 mmol/l in bidistilled water, stored at −20°C) and albumin (70 mg/ml), which corresponds to the average physiological protein content in human serum). One hundred microliters of Trp (100 Mm), 20 µl of Kyn (10 µM), and 880 µl of albumin stock solution were mixed together. This mixture was divided in nine aliquots of 100 µl of external calibrator (standard mix); the remaining volume (100 µl approximately) was aliquoted in 1:1 and 1:10 standard mix dilutions in bidistilled water. All serum pools and standard mix preparations were then treated in the same way as the serum specimens.

For Phe and Tyr measurements, one tube of standard 50, one tube of standard 100 and one serum pool tube were prepared and injected per each group of fourteen samples to control the validity of measures. Standard 50 was composed of 15 µl of Tyr and Phe 200 µM, 30 µl of albumin 70 mg/ml, and 300 µl of 3-nitrotyrosine (500 µM). Standard 100 was composed of 30 µl of Tyr and Phe 200 µM and 300 µl of 3-nitrotyrosine.

5.2.3 Sample analysis

Frozen serum specimens were thawed at room temperature, and 100 µl of serum/pool were diluted with 100 µl of internal control 3-nitrotyrosine 25 µM for the analysis of Trp and Kyn. In the case of Phe and Tyr, 30 µl of serum/pool were mixed with 30 µl of K_2PO_4 buffer and 300 µl of internal control 3-nitrotyrosine. Protein was precipitated with 25 or 75 µl of trichloroacetic acid 2 M for Trp/Kyn or Phe/Tyr measurements, respectively. The capped tubes with the precipitate were immediately vortex-mixed and centrifuged for 6 min at 13,000 rpm. Supernatants (370 µl) were transferred into microvials with 400 µl of K_2PO_4 buffer, shook a bit to remove air bubbles, and placed into the autosampling device.

Before measurements were started, two washing samples were pre-injected, pre-conditioning the rinsed column; then a set of external calibrators (standard mix and standard dilutions for Trp/Kyn, and standard 50 and standard 100 for Phe/Tyr) and one serum pool were injected as pre-runs, followed by the analytical runs. After each block of fourteen sample specimens, one external calibrator set and a serum pool were measured.

Peak area counts were used to calculate the concentrations. Trp, Kyn, Phe and Tyr were referred to 3-nitrotyrosine. Thus, the ratios of these biomarkers per 3-nitrotyrosine for the external calibrator were calculated, as well as for the sample specimens, leading to the final results. The reproducibility of the system was controlled by 3-nitrotyrosine and serum pool counts. Considering the total standard deviation of the method, a variation of 3-nitrotyrosine lower than 5% was tolerated.

Trp metabolism retention times were 2.5 min for Kyn, 3.7 min for 3-nitrotyrosine (both by UV detection), and 5.2 min for Trp (fluorescence detection). One single chromatographic run was completed within 7 min. Limits of detection were 0.1 µmol/l Trp, and 0.5 µmol/l Kyn. Phe metabolism retention times were 3.5 min and 2 min for Phe and Tyr, respectively (both by fluorescence detection), and 6 min for 3-nitrotyrosine (UV detection). One single chromatographic run was completed within 10 min. Limits of detection were 0.3 µmol/l for both Phe and Tyr. Flow rates higher than 0.9 ml/min or temperatures higher than 25°C turned out to be less useful, as other compounds tended to co-elute with our target molecules.

5.3. Nitrite

The stable NO metabolite nitrite (NO_2^-) was measured in plasma samples as an estimation of NOS activity and NO production (Kleinbongard et al. 2003), according to the Griess method. A standard curve was prepared with different $NaNO_2$ concentrations; then 50 µl of plasma or standard curve samples and 125 µl of Griess reagent (Merck) were added onto a microplate. After 10 min of incubation at room temperature without shaking, colour development was measured at 562 nm in a power wave X microplate reader (Bio-Tek Instruments), equipped with KC4 v.2.5 kinetic analysis software (Bio-Tek Instruments). Limit of detection was 1.5 µmol/l nitrite.

6. Lymphocyte subpopulations

Peripheral blood lymphocyte phenotypes were determined by three-color direct immunofluorescence surface marker analysis by flow cytometry, as previously described (Valdiglesias et al. 2015). The following lymphocyte subsets were evaluated: T lymphocytes ($CD3^+$), T-helper lymphocytes ($CD3^+$ and $CD4^+$), T-cytotoxic lymphocytes ($CD3^+$ and $CD8^+$), B lymphocytes ($CD19^+$), and natural killer (NK) cells ($CD3^-$ and $CD16^+56^+$). In brief, 100 µl whole peripheral blood was incubated for 15 min in the dark with the following antibodies (Immunostep), according to the manufacturer's instructions: fluorescein isothiocyanate (FITC)-labeled antiCD3, phycoerythrin (PE)-labeled antiCD4, peridininchlorophyl-protein complex

(PerCP)-labeled antiCD8, PerCP-labeled antiCD19, and PE-labeled antiCD16 and antiCD56. One milliliter of FACS lysing solution (Becton Dickinson) was then added and incubated for 8 min in the dark to remove erythrocytes by lysis. After that, cells were centrifuged at 2000 rpm 5 min, supernatants were removed and pellets were resuspended with 1 ml of phosphate buffer solution (PBS) and centrifuged at 2000 rpm 5 min. After repeating this washing-centrifugation step, cells were fixed with 250 µl of CellFix (Becton Dickinson), and analyzed in a FACScalibur flow cytometer using Cell Quest Pro software (Becton Dickinson).

Lymphocytes were gated on the basis of forward/side scatter plots, and fluorescence data from FL1 (FITC), FL2 (PE), and FL3 (PerCP) were obtained to determine percentages of different lymphocyte subsets (Figure 8). At least 10^4 events in the lymphocyte window were acquired. All specimens were analyzed using identical reagents, instruments, and procedures. All measurements were analyzed and interpreted by the same analyst following a standard procedure. Background staining was assessed using appropriate isotype controls (Immunostep). The flow cytometer was calibrated using fluorescent beads (CaliBrite) using FACSComp software (all from Becton Dickinson).

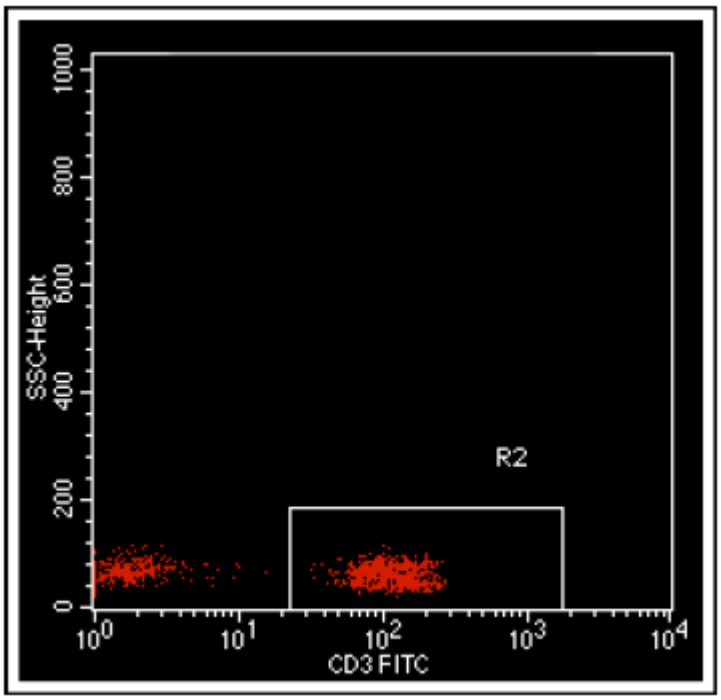

Figure 8. Flow cytometric analysis of CD3-FITC fluorescence intensity *versus* side scatter (SSC) for $CD3^+$ lymphocyte (R2) percentage quantification.

7. Circulating inflammatory molecules

Interleukin 6 (IL6), C-reactive protein (CRP), tumor necrosis factor α receptor II (sTNF-RII), and tumor necrosis factor alpha (TNFα), were measured in plasma samples by using quantitative sandwich ELISA commercial kits (R&D Systems) (Figure 9), following manufacturer´s instructions. The later biomarker (TNFα) could only be measured in 88 plasma samples (20 non-frail, 38 pre-frail, and 30 frail), of whom sufficient residual plasma volume was available. Plasma samples required a 100-fold and 10-fold dilution in their specific diluents for analysis of CRP and sTNF-RII, respectively. Monoclonal antibodies specific for human IL6, CRP, sTNF-RII, and TNFα were pre-coated onto microplates. Standards and plasma samples were pipetted into the wells and any biomarker analyzed present were bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody (IL6, sTNF-RII and TNFα) or monoclonal antibody (CRP), specific for these human molecules, was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of molecules bound in the initial step. After 30 min of incubation for CRP, and 20 min of incubation for the rest of biomarkers, color development was stopped and color intensity was measured within 30 min at 450 nm and with a wavelength correction between 540 nm and 570 nm, using a Power wave X microplate reader (Bio-Tek Instruments) equipped with KC4 v.2.5 kinetic analysis software (Bio-Tek Instruments). Regarding precision of the assay, maximum intra- and inter-assay coefficients of variation were 4.2% and 6.4% for IL6, 8.6% and 7.0% for CRP, 4.8 and 5.1% for sTNF-RII, and 3.0 and 8.4% for TNFα, respectively.

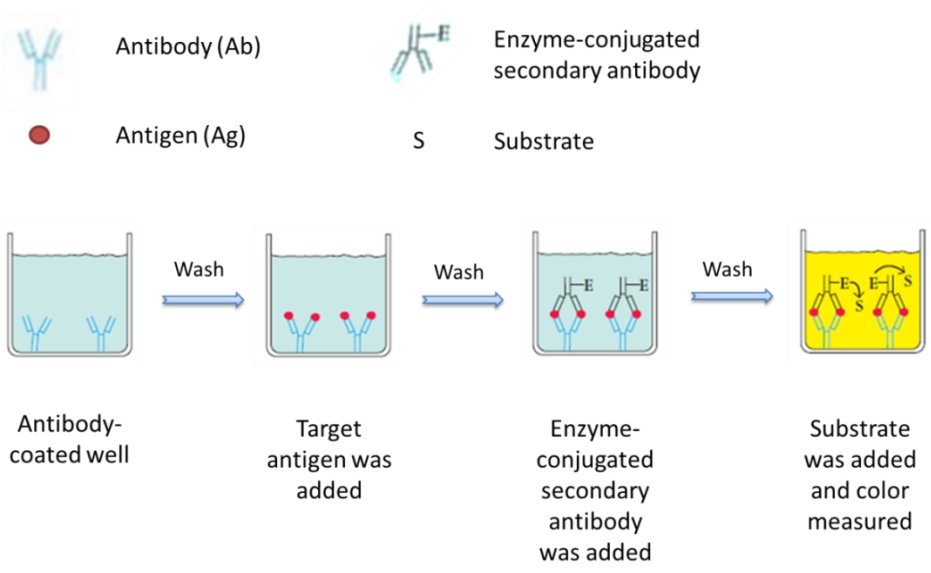

Figure 9. Quantitative sandwich ELISA diagram.

8. Cortisol

Cortisol levels were measured in serum samples by using a quantitative ELISA (Diagnostics Biochem), following the typical competitive scenario (Figure 7). Competition occurred between an unlabeled antigen (present in standards, controls and participant serum samples) and an enzyme-labeled antigen (conjugated cortisol - horseradish peroxidase) for a limited number of antibody binding sites on the microplate. The washing and decanting procedures removed unbound materials. After the washing step, the enzyme substrate was added. The enzymatic reaction was stopped by the addition of stopping solution (1 M sulfuric acid). Absorbance was measured at 450 nm in a Spectrostar Nano microplate reader (BMG Labtech), equipped with Spectrostar Nano Control kinetic analysis software (BMG Labtech). The intensity of the color formed is inversely proportional to the concentration of cortisol in the serum samples. A logistic 4-parameter curve was used for the calculations. The sensitivity of cortisol determination was 0.4 μg/dl.

9. Oxidative stress biomarkers

9.1. Reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species (ROS/RNS) in serum were measured by means of the OxiSelect *In Vitro* ROS/RNS assay kit (Cell Biolabs, Inc.), according to the manufacturer´s instructions (Figure 10). The assay employs a proprietary quenched fluorogenic probe, dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ), which is a specific ROS/RNS probe. The DCFH-DiOxyQ probe is first primed with a quench removal reagent, and subsequently stabilized in the highly reactive DCFH form. In this reactive state, ROS and RNS species can react with DCFH, which is rapidly oxidized to the highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF). Fluorescence intensity is proportional to the total ROS/RNS levels within the sample. The DCFH-DiOxyQ probe can react with hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\cdot}$), nitric oxide (NO), and peroxynitrite anion ($ONOO^{-}$). These free radical molecules are representative of both ROS and RNS, thus allowing for measurement of the total free radical population within a sample. Fluorescence intensity was measured in a TECAN GENios plate reader equipped with XFluor4 analysis software at 480 nm excitation and 530 nm emission wavelengths. Results were expressed as DCF equivalents. The detection sensitivity limit of the assay was 10 pM DCF.

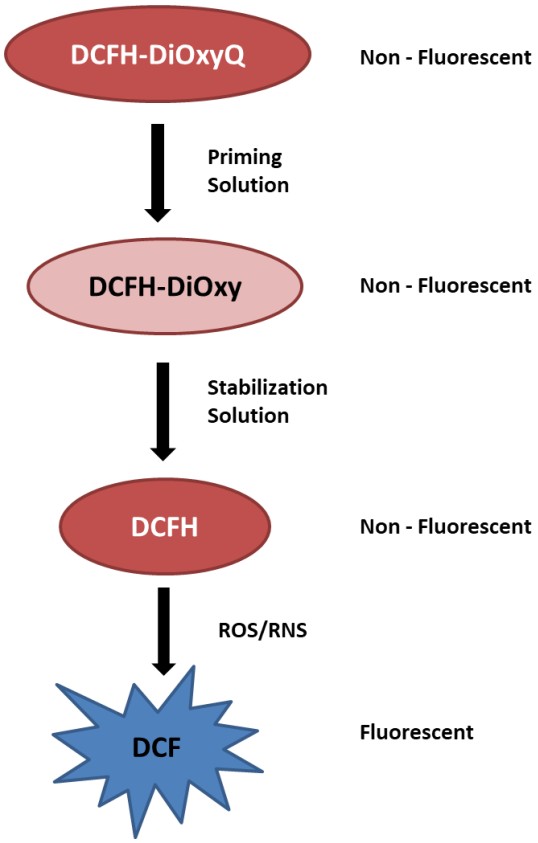

Figure 10. Scheme of ROS/RNS assay principle. DCFH-DiOxyQ: quenched dichlorodihydrofluorescin DiOxyQ; DCFH-DiOxy: dichlorodihydrofluorescin DiOxy; DCFH: dichlorodihydrofluorescein, DCF: dichlorofluorescein.

9.2. Oxidative DNA damage

9.2.1. Leukocyte isolation

Peripheral blood was collected by venipuncture using BD Vacutainer® CPT™ tubes with sodium heparin (Becton Dickinson). These tubes contain a solution which creates a density gradient, along with a gel barrier that allows the separation of the different blood fractions after a centrifugation at 3100 rpm for 30 min at 4°C. Mononuclear leukocytes (lymphocytes and monocytes) were isolated due to this gel barrier since they remained above it after the centrifugation step (Figure 11). Leukocytes were then transferred to another tube and washed twice with PBS pH 7.4 and centrifugation at 1500 rpm for 10 min at 4°C. Cells were suspended at 10^7 cells/ml in freezing medium containing 40% RPMI 1640, 50% fetal bovine serum (FBS) and 10% DMSO, and stored at -80°C until analysis. To minimize possible cellular damage caused by freezing process, a NALGENE® Cryo 1°C Freezing Container (Nalgene Nunc. International) was used to ensure a gradual temperature decrease of 1°C/min.

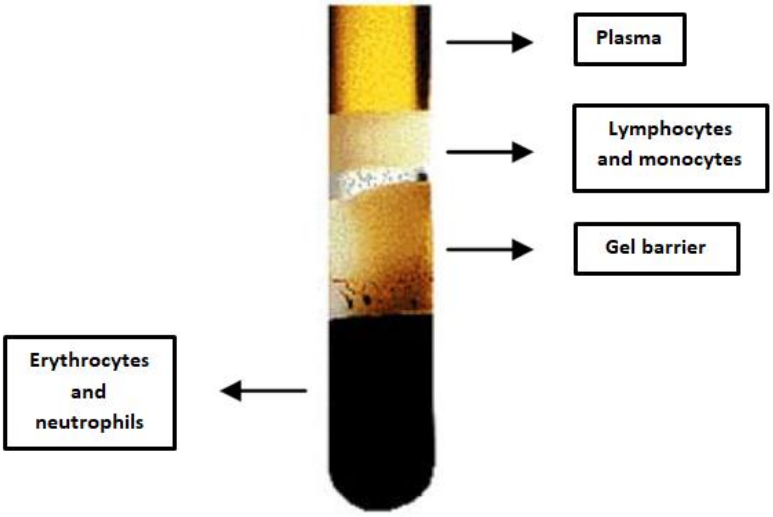

Figure 11. Blood fraction separation in a BD Vacutainer® CPT™ tube.

9.2.2. Internal standard

In order to avoid interassay variability, an internal standard following Cebulska-Wasilewska (2003) was used. Internal standard samples were collected from a young male subject by a single venipuncture using BD Vacutainer® CPT™ tubes, as previously described.

9.2.3. Oxidative comet assay

A modified version of the alkaline comet assay (Figure 12), by incubating with the human 8-oxoguanine-glucosilase (OGG1) repair enzyme, was performed in order to evaluate the oxidative DNA damage following the protocol described by Smith et al. (2006), with minor modifications (García-Lestón et al. 2012).

Slides were previously pre-coated with a 1% layer of normal-melting-point agarose in distilled water. Samples and internal standards were thawed by addition of medium (4ml RPMI 1460, 5ml FBS and 1g dextrose) and immediately centrifuged at 1500 rpm 10 min 4^{o}C. After that, cells were suspended in RPMI 1640 to check cellular viability by Trypan blue exclusion technic, resulting in viability higher than 80% in all cases. Cells were centrifuged at 9,000 rpm 3 min 4^{o}C, suspended in 100 µl of 0.7% low-melting-point agarose in PBS, and dropped onto pre-coated slides. For each individual sample, two slides were prepared. On top of each drop a cover slide was placed and agarose was allowed to solidify on ice for 10 min.

Cover slides were removed and cells were lysed in a Koplin jar filled with lysis solution (NaCl 2.5 M, Na_2EDTA 100 mM, Tris-HCl 10 mM, and NaOH 250 mM, with Triton X-100 1%

added just before use) for 1 h at room temperature in the darkness to avoid additional genetic damage. After the lysis step, slides were washed with buffer (pH 8) composed of 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, 0.1 M KCl and 40 mM Hepes. Then, slides were separated into two groups and treated with either 50 µl of OGG1 (0.0016 U/µl buffer) or 50 µl of buffer, covered with coverslips, and incubated at 37°C for 10 min. After this incubation, coverslips were removed and slides were placed on a horizontal electrophoresis tank and incubated in alkaline electrophoresis solution (1 mM Na_2EDTA, 300 mM NaOH, pH 13) for 40 min. Later, electrophoresis was carried out for 30 min at 25 V and 300 mA (0.83 V/cm). Slides were then washed three times for 5 min with neutralizing solution (0.4 M Tris−HCl, pH 7.5) and stained with 60 µl of 4',6-diamidine-2'-phenylindole (DAPI) 5 µg/ml. The preparations were kept in a humidified sealed box to prevent drying of the gel and analyzed within one week.

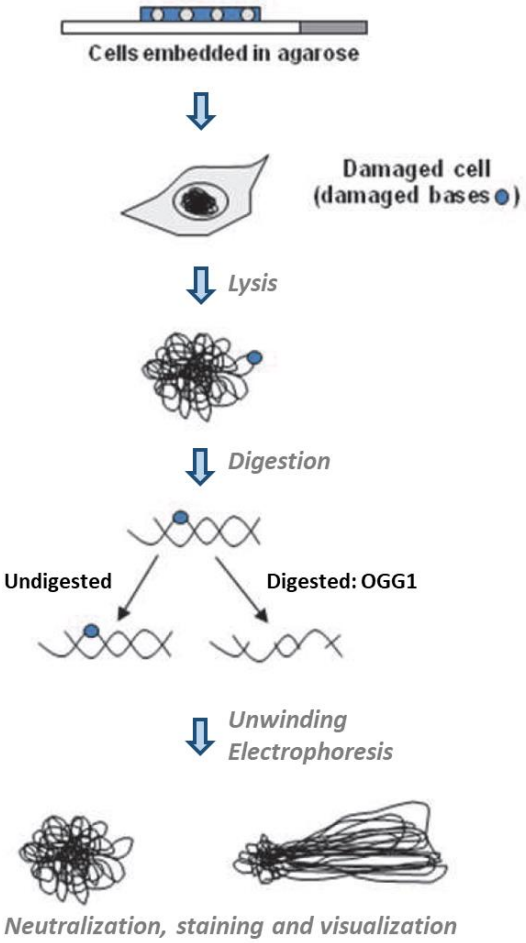

Figure 12. Scheme of the oxidative comet assay (modified from Azqueta et al. 2011).

Slides incubated with enzyme were compared with the corresponding slides incubated with just buffer to estimate the net oxidative DNA damage. Comet IV Software (Perceptive

Instruments) was used for image capture and analysis. In all cases, 100 cells were scored blindly (50 from each replicate slide), and percentage of DNA in the comet tail (%tDNA) was used as DNA damage parameter.

9.3. Total antioxidant capacity

Total antioxidant capacity was measured in plasma samples by using the Antioxidant assay kit (Sigma Aldrich), following manufacturer´s guidelines. The principle of the antioxidant assay is formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation, $ABTS^{\cdot+}$, a soluble chromogen that is green in color and can be determined spectrophotometrically at 405 nm. Antioxidants suppress the production of the radical cation in a concentration dependent manner and the color intensity decreases proportionally. Trolox™, a water-soluble vitamin E analog, served as a standard or control antioxidant. Absorbance at 405 nm was measured with a Spectrostar Nano microplate reader (BMG Labtech) equipped with kinetic analysis software (Spectrostar Nano Control, BMG Labtech). Results were expressed as Trolox™ equivalent antioxidant capacity (TEAC). The sensitivity of this method was 0.015 mM TEAC.

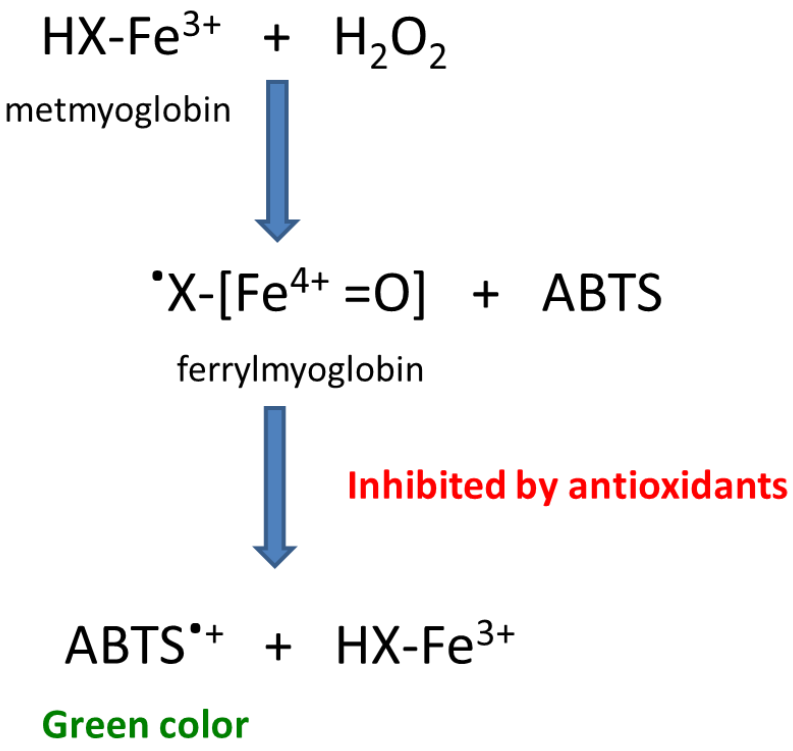

Figure 13. Scheme of total antioxidant capacity determination assay.

10. Statistical analysis

A general description of the study population was conducted by univariate analysis, comparing socio-demographic features (i.e., sex, age, and living conditions), lifestyle factors (i.e., tobacco consumption), and clinical factors (i.e., comorbidity) in the three groups of older adults classified according to their frailty status (non-frail, pre-frail, and frail). Analysis of variance was applied for continuous variables and Chi-square test was used for categorical variables. The effect of frailty status on the different biomarkers determined was preliminarily assessed by ANOVA and Tuckey's *post-hoc* test. Data from tryptophan, kynurenine, tyrosine, %$CD3^+$, %$CD4^+$, %$CD16^+56^+$, TNFα, cortisol and ROS/RNS followed a normal distribution (Kolmogorov-Smirnov goodness-of-fit test). A log-transformation of the data was applied to Kyn/Trp ratio, %$CD8^+$, %$CD19^+$, IL6, and CRP to achieve a better approximation to the normal distribution. As no improvement was achieved with transformation, the Kruskal-Wallis and Mann-Whitney *U*-tests with Bonferroni´s correction were applied for statistical evaluation of neopterin, nitrite, and phenylalanine concentrations, Phe/Tyr ratio, $CD4^+/CD8^+$ ratio, sTNF-RII concentration, antioxidant capacity and net oxidative DNA damage.

Reference ranges were calculated for the inflammatory mediators, lymphocyte subpopulations and immune stimulation biomarkers on the basis of values from non-frail and pre-frail individuals. For those biomarkers following a normal distribution, reference ranges were defined by the mean ± 2 standard deviations. When data were considered to have a non-Gaussian distribution, reference ranges were defined as the central 95% of the area under the distribution curve (from 2.5% to 97.5%).

Linear regression analysis was applied to estimate the effect of frailty status on the immunological, endocrine and oxidative stress parameters. Models were run with log-transformed data, adjusting for age, gender, smoking habit (never/ever smokers) and comorbidity. All results are shown as mean ratios and 95% confidence intervals (95% CI).

Correlation coefficients (Spearman's rho), adjusting for gender, age, and smoking habits, were calculated in order to estimate the possible associations between parameters. Receiver-operating-characteristic (ROC) curves were computed to assess biomarker discriminating ability. Youden's index, which defines the maximum potential effectiveness of a biomarker, was calculated in order to maximize specificity and sensitivity of each parameter. Statistical analyses were conducted by means of the STATA/SE software package V. 12.0 (StataCorp LP, College Station, TX) and the IBM SPSS software package V. 20 (SPSS, Inc, Chicago, IL). Statistical significance was established at a *P* value lower than 0.05.

IV. RESULTS

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1. Study population

The population analyzed (Table 1) was composed of 259 participants, aged from 65 to 102 years. Frail subjects were significantly older, with 62% aged 85 years and over. Female gender was less prevalent in the non-frail group (approximately 1:2), but gender proportions were opposite in the pre-frail and frail groups. The pre-frail group was the most numerous, followed by frail participants. The majority of frail participant lived in nursing homes, whereas all non-frail subjects lived at family home, not attending day-care centers. Proportion of smokers decreased with increasing frailty severity; frail smokers consumed a higher amount of cigarettes per day, but the number of years smoking was not significantly different among the three groups of smokers. Comorbidity increased with frailty severity, from 15% in the non-frail group to 40% in the frail group. Mortality risk at 10 years showed an increasing trend, ranging from 48% in non-frail individuals to 88% in frail individuals.

Table 2 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%). Among all 131 pre-frail participants, 89 (68%) were positive for only one frailty criterion, and 42 (32%) were positive for two frailty criteria. The low grip strength criterion, indicative of muscle weakness, was present in most pre-frail individuals (*N* = 126, 96%).

Table 1. General characteristics of the study population.

	Non-frail	Pre-frail	Frail	*P*
Total individuals *N* (%)	40 (15.4)	131 (50.6)	88 (34.0)	
Age (years-old)[a]	73.2±5.5 (65-85)	77.05±7.7 (65-100)	85.8±7.9 (65-102)	**<0.001**[b]
65-69	13 (32.5)	29 (22.1)	2 (2.3)	**<0.001**[c]
70-74	11 (27.5)	26 (19.9)	4 (4.6)	
75-79	10 (25.0)	24 (18.3)	13 (14.9)	
80-84	5 (12.5)	27 (20.6)	14 (16.1)	
≥85	1 (2.5)	25 (19.1)	54 (62.1)	
Gender N (%)				
Males	27 (67.5)	36 (27.5)	22 (25.0)	**<0.001**[c]
Females	13 (32.5)	95 (72.5)	66 (75.0)	
Living conditions N (%)				
Family home	40 (100.0)	113 (86.3)	5 (5.7)	**<0.001**[c]
Family home + daycare center	---	4 (3.1)	23 (26.1)	
Nursing home	---	14 (10.6)	60 (68.2)	
Smoking habit N (%)				
Non-smokers	22 (55.0)	102 (78.5)	76 (90.5)	**<0.001**[c]
Ever smokers	18 (45.0)	28 (21.5)	8 (9.5)	
No. cigarettes/day[a]	16.1±8.8 (3-40)	15.7±13.9 (2-60)	31.4±15.7 (2-60)	**0.020**[b]
Years smoking[a]	19.4±9.1 (10-34)	30.4±18.7 (4-66)	29.3±18.2 (6-52)	0.154[b]
Comorbidity N (%)				
No comorbidity	34 (85.0)	92 (70.2)	52 (59.8)	**0.015**[c]
Comorbidity	6 (15.0)	39 (29.8)	35 (40.2)	
Mortality risk 10 years (%)[a,d]	48.3±27.4 (15.0-100)	59.3±30.7 (15.0-100)	88.3±17.6 (13.8-100)	**<0.001**[b]

[a]mean±standard deviation (range), [b]ANOVA test (bilateral), [c]Chi-square test (bilateral), [d]according to Charlson's comorbidity index.

Table 2. Fried's frailty criteria in the study population [*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 walking speed	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. Immune system

2.1. Immune activation biomarkers

Results of the immune biomarkers analyzed in the non-frail, pre-frail and frail groups are shown in Figure 14. According to univariate analyses, significant and progressive changes were observed in concentrations of several biomarkers. Significant increases were obtained of neopterin concentrations and Kyn/Trp ratio in the frail group with regard to the other two groups. On the contrary, tryptophan, nitrite and tyrosine levels decreased significantly in the presence of frailty; only in the case of nitrite the three population groups were significantly different.

Reference ranges specific for robust older adults were not available for any of the biomarkers of immune activation analyzed in this work. Consequently, and considering that no significant differences between these two groups were observed, with the exception of nitrite values obtained from non-frail and pre-frail subjects were used for calculating the lower and upper limits of the corresponding reference ranges (Table 3). In general, percentages of frail subjects with values out of the corresponding reference ranges for the different parameters oscillated from 1% to 16% for values below and from 1% to 22% for values above the reference ranges. Percentages of frail subjects presenting concentrations out of the calculated reference ranges were notable for Kyn/Trp ratio (above) and tryptophan (below), and moderate for neopterin (above) and nitrite (below). Values exceeding the reference range in both directions were observed for phenylalanine and Phe/Tyr ratio.

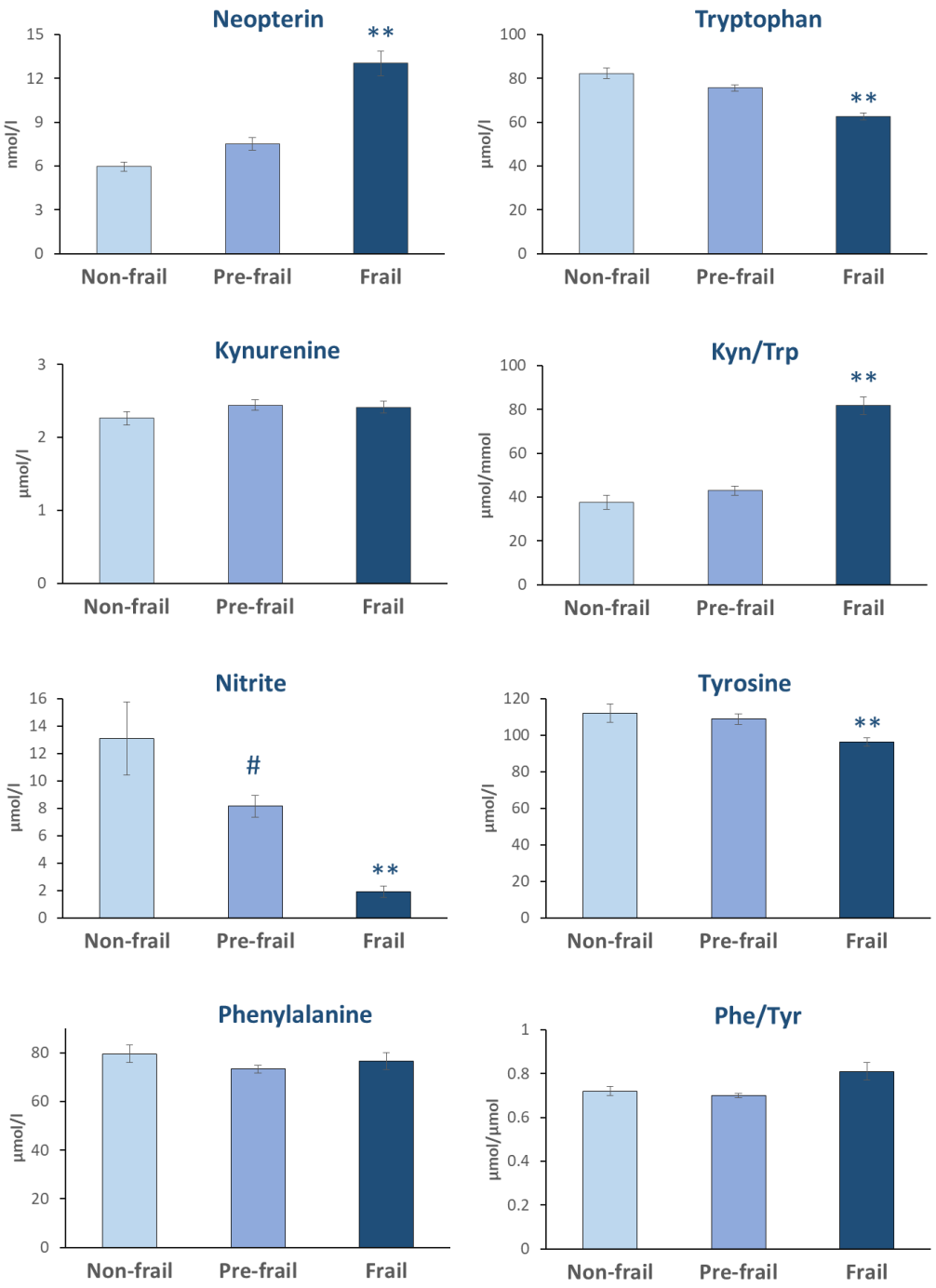

Figure 14. Results of immune activation biomarkers (univariate analyses). ***P*<0.01, significant difference with regard to the non-frail group; #*P*<0.05, significant difference with regard to the pre-frail group. Kyn: kynurenine; Phe: phenylalanine; Trp: tryptophan; Tyr: tyrosine.

Table 3. Reference ranges for the immune activation biomarkers analyzed, calculated on the basis of results obtained in non-frail and pre-frail subjects.

	N	Reference range		%Frail subjects out of the reference range	
		Lower limit	Upper limit	Below	Above
Neopterin (nmol/l)	157	3.50	22.40	----	10.5
Tryptophan (µmol/l)	153	46.14	108.30	16.3	----
Kynurenine (µmol/l)	153	0.88	3.92	----	3.5
Kyn/Trp ratio	153	16.69	99.43	----	22.1
Nitrite (µmol/l)	158	0.59	36.19	9.1	----
Tyrosine (µmol/l)	160	47.95	171.31	----	1.2
Phenylalanine (µmol/l)	160	48.11	118.72	5.8	7.0
Phe/Tyr ratio	160	0.36	1.05	1.2	9.3

Kyn: kynurenine; Phe: phenylalanine; Trp: tryptophan; Tyr: tyrosine.

Remarkable significant correlations were obtained between biomarkers (Table 4). Neopterin showed strong associations with tryptophan breakdown parameters and slight association with nitrite and Phe/Tyr. In turn, nitrite and phenylalanine metabolism products were moderately associated with tryptophan breakdown products. Frailty presented significant direct associations with neopterin and Kyn/Trp, and inverse associations with tryptophan, nitrite and tyrosine.

Table 4. Partial correlation coefficients between immune stimulation biomarkers analyzed, adjusted by age, sex, smoking habits and comorbidity (cells in light blue: moderate associations, cell in dark blue: strong association)

	Neo	Trp	Kyn	Kyn/Trp	Nitrite	Phe	Tyr	Phe/Tyr
Frailty	0.477**	-0.458**	0.041	0.557**	-0.544**	-0.091	-0.211**	0.117
Neo		-0.233	0.365***	0.565***	-0.219**	0.038	-0.122	0.151*
Trp			0.268***		0.119	0.304***	0.410***	-0.094
Kyn					-0.031	0.080	0.167*	-0.078
Kyn/Trp					-0.367***	-0.041	-0.134*	0.086
Nitrite						-0.055	-0.074	0.039
Phe							0.491***	

*P<0.05; **P<0.01, ***P<0.001. Kyn: kynurenine; Neo: neopterin; Phe: phenylalanine; Trp: tryptophan; Tyr: tyrosine.

Table 5 summarizes the results from the multivariate statistical analyses. All models were significant and adjusted by age, sex, smoking habits and comorbidity. Results were essentially in agreement with those obtained from the univariate analyses, i.e., significant increases in neopterin and Kyn/Trp, together with Phe/Tyr levels, and significant decreases in tryptophan, nitrite and tyrosine concentrations in frail individuals as compared with non-frail subjects. No significant differences were observed between non-frail and pre-frail subjects, except in the case of nitrite concentrations, which showed a progressive decline with increasing frailty severity. Significant positive influence of age was obtained in neopterin, kynurenine and Kyn/Trp levels, and inverse influence was observed in tryptophan concentrations. No significant effect of sex, smoking habit, or comorbidity was obtained.

Table 5. Effect of frailty status on immune stimulation biomarkers; models adjusted by age, sex, smoking habit, and comorbidity.

	Neopterin		**Tryptophan**		**Kynurenine**		**Kyn/Trp**	
	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	1.06	(0.88-1.27)	0.93	(0.85-1.01)	1.03	(0.91-1.16)	1.08	(0.91-1.29)
Frail	**1.41****	**(1.15-1.76)**	**0.81****	**(0.73-0.89)**	0.94	(0.81-1.08)	**1.76****	**(1.43-2.17)**
Age	**1.03****	**(1.02-1.03)**	**0.99****	**(0.99-1.00)**	**1.01****	**(1.00-1.02)**	**1.02****	**(1.01-1.03)**
	Nitrite		**Tyrosine**		**Phenylalanine**		**Phe/Tyr**	
	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	**0.65***	**(0.44-10.97)**	0.94	(0.85-1.04)	0.93	(0.84-1.04)	1.00	(0.90-1.10)
Frail	**0.18****	**(0.11-0.30)**	**0.82****	**(0.73-0.92)**	0.92	(0.81-1.04)	**1.12***	**(1.00-1.26)**
Age	1.00	(1.00-1.02)	1.00	(1.00-1.01)	1.00	(1.00-1.01)	1.00	(1.00-1.00)

CI: confidence interval; *P<0.05; **P<0.01. Kyn: kynurenine; Phe: phenylalanine; Trp: tryptophan; Tyr: tyrosine.

Figure 15 shows ROC curves calculated to determine the predictive value of the immune stimulation biomarkers for frailty. They were computed by using non-frail + pre-frail groups as standard when inflammatory parameters increased with frailty status; frail group was used as standard in the case of parameters showing an inverse relationship with frailty. Significant values for areas under the curves (AUC) were obtained for Kyn/Trp ratio (0.85, 95% CI 0.80–0.90, P<0.001), nitrite (0.84, 95% CI 0.78–0.89, P<0.001), neopterin (0.80, 95% CI 0.74–

0.86, *P*<0.001) and tryptophan (0.78, 95% CI 0.71–0.84, *P*<0.001). Optimal predictive values for frailty were 52.80 µmol/nmol for Kyn/Trp with a sensitivity of 0.88 and a specificity of 0.78, 2.05 µmol/l for nitrite with a sensitivity of 0.63 and specificity of 0.92, 7.55 nmol/l for neopterin with a sensitivity of 0.81 and specificity of 0.72, and 63.75 µmol/l for tryptophan with a sensitivity of 0.83 and specificity of 0.62.

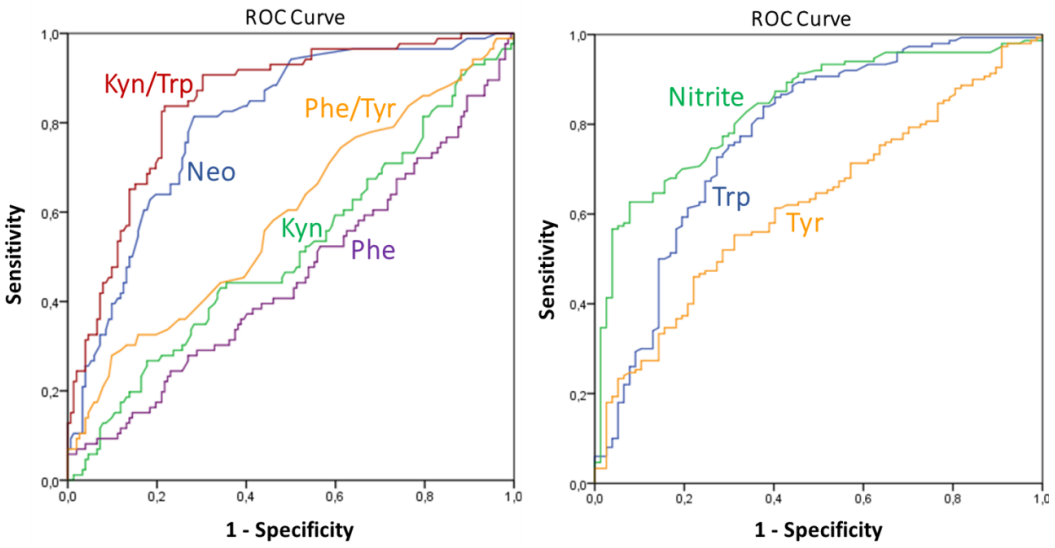

Figure 15. Receiver-operating characteristic (ROC) curves for kynurenine (Kyn), neopterin (Neo), phenylalanine (Phe), and Kyn/Trp and Phe/Tyr ratios (left) and for nitrite, tryptophan (Trp) and tyrosine (Tyr) (right).

2.2. Lymphocyte subpopulations

Figure 16 shows lymphocyte subpopulation values in the three population groups classified according frailty status (univariate analyses). The only lymphocyte subset showing significant changes was %$CD19^+$ cells, which decreased significantly in the group of frail subjects, in agreement with partial correlations results, since these lymphocyte subpopulation was the only one presenting a significant association (inverse) with frailty status (r=-0.159, *P*<0.05).

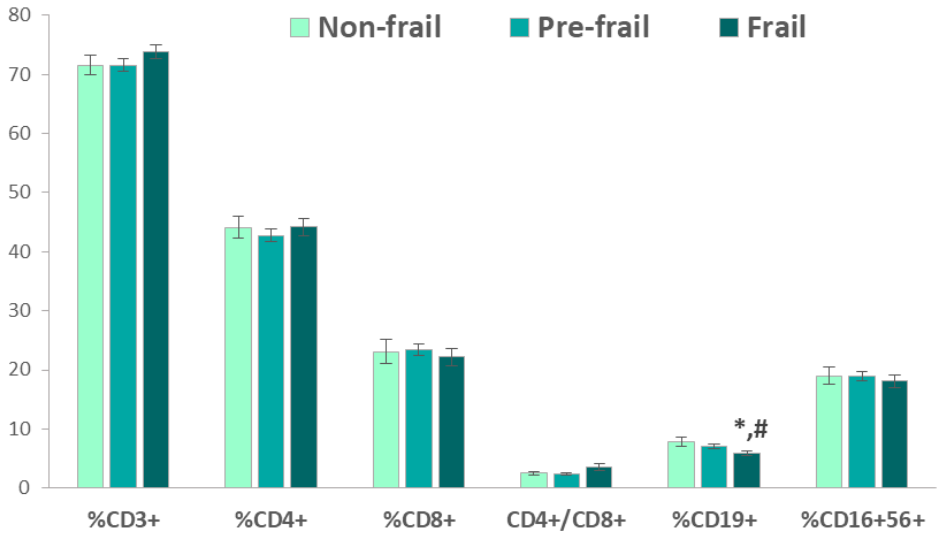

Figure 16. Results of lymphocyte subpopulations (univariate analyses). **P*<0.05, significant difference regarding non-frail group; $^{\#}P$<0.05, significant difference regarding pre-frail group.

Reference ranges for the different lymphocyte subpopulations in older adults in the absence of frailty were not available in the literature so far. Therefore, we calculated those ranges with values obtained in the non-frail and pre-frail subjects, considering that no significant differences were observed between these two groups in any case (Table 6). Percentages of frail participants which presented values out of the different reference ranges were between 81 and 96%, with no significant differences between groups for any particular subset. Values exceeding the reference range only in one direction corresponded to B lymphocytes (%$CD19^+$) and NK cells (%$CD16^+56^+$), with 11% and 6% above the reference range, respectively. Values exceeding the reference range in both directions were observed for the rest of parameters analyzed. Percentages of frail subjects with values exceeding the corresponding reference ranges for the different parameters oscillated from 2% to 9% and from 1% to 12%, in values below and above, respectively.

Table 6. Reference ranges for lymphocyte subpopulations, calculated on the basis of results obtained in non-frail and pre-frail subjects.

	N	Reference range		%Frail subjects out of the reference range	
		Lower limit	Upper limit	Below	Above
%$CD3^+$	237	51.24	92.16	2.4	1.2
%$CD4^+$	216	19.80	64.32	3.2	7.9
%$CD8^+$	229	8.28	54.17	9.1	3.9
$CD4^+/CD8^+$	209	0.46	6.57	6.9	12.0
%$CD19^+$	215	1.94	20.15	----	11.1
%$CD16^+56^+$	239	0.61	36.93	----	6.1

Results obtained from the multivariate statistical analyses regarding frailty status are shown in Table 7, adjusted by age, sex, tobacco consumption and comorbidity. A significant increase in the $CD4^+/CD8^+$ ratio and a significant decrease in %$CD19^+$ lymphocytes were observed in the frail group, with regard to the non-frail group. Significant inverse influence of age was obtained for %$CD3^+$, %$CD4^+$, and %$CD19^+$ cells, and $CD4^+/CD8^+$ ratio. Besides, female gender was associated with higher values of %$CD19^+$ lymphocytes and with lower levels of %$CD16^+56^+$ cells. Neither smoking nor comorbidity influenced significantly any of the lymphocyte subsets.

Table 7. Effect of frailty status on lymphocyte subsets; models adjusted by age, sex, smoking habit and comorbidity.

	%CD3$^+$		**%CD4$^+$**		**%CD8$^+$**	
	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI
Frailty status						
Non-frail	1.00		1.00		1.00	
Pre-frail	1.00	(1.00-1.17)	1.09	(0.86-1.38)	1.00	(0.81-1.24)
Frail	0.98	(0.94-1.03)	1.31	(0.98-1.76)	0.82	(0.64-1.06)
Age	**0.99****	**(0.99-1.00)**	**0.99***	**(0.98-1.00)**	1.01	(1.00-1.02)
Gender						
Male	1.00		1.00		1.00	
Female	1.05	(1.00-1.11)	1.13	(0.93-1.39)	1.00	(0.84-1.19)
	CD4$^+$/CD8$^+$		**%CD19$^+$**		**%CD16$^+$56$^+$**	
	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI
Frailty status						
Non-frail	1.00		1.00		1.00	
Pre-frail	1.09	(0.77-1.55)	0.82	(0.65-1.04)	1.07	(0.85-1.35)
Frail	**1.66***	**(1.09-2.53)**	**0.73***	**(0.55-0.97)**	0.92	(0.70-1.21)
Age	**0.98****	**(0.96-0.99)**	**0.99***	**(0.98-1.00)**	1.01	(1.00-1.01)
Gender						
Male	1.00		1.00		1.00	
Female	1.14	(0.85-1.53)	**1.41****	**(1.15-1.71)**	**0.78***	**(0.65-0.94)**

CI: confidence interval; *P<0.05, **P<0.01.

2.3. Inflammatory mediators

Significant increases with frailty were observed in the univariate analysis for the concentrations of the four inflammatory mediators analyzed, namely IL6, CRP, sTNF-RII and TNFα (Figure 17). No differences were obtained in these markers between non-frail and pre-frail individuals, except for sTNF-RII.

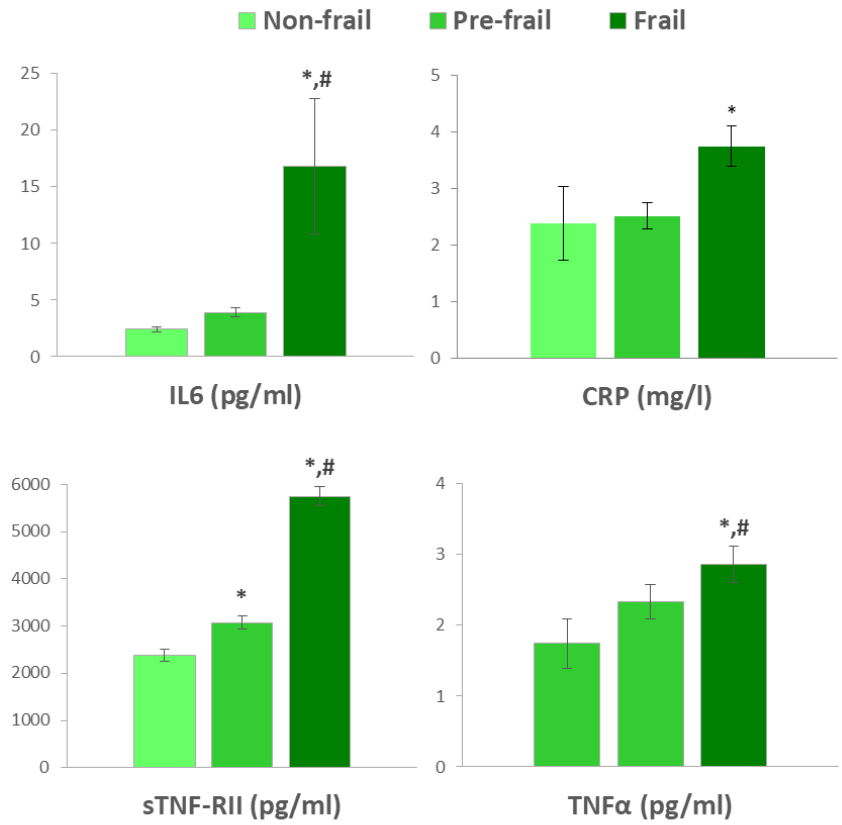

Figure 17. Results of inflammatory mediators (univariate analyses). **P*<0.05, significant differences with regard to the non-frail group; #*P*<0.05, significant differences with regard to the pre-frail group. CRP: C-reactive protein; IL6: interleukin 6; sTNF-RII: soluble tumor necrosis factor alpha receptor II; TNFα: tumor necrosis factor alpha.

Similiarly to immune activation biomarkers, reference ranges for inflammatory mediators were firstly established in this study for robust older adults, since they had not been previously reported in the literature. Thus, concentrations obtained in the non-frail and pre-frail subjects were used for estimating the upper and lower limits of the corresponding reference ranges (Table 8), since no significant differences were observed between these two groups, except in the case of sTNF-RII, where differences regarding frail individuals were much more remarkable. Percentages of frail subjects with values exceeding the corresponding reference ranges for the different parameters oscillated between 9 and 19%, with no values below the reference range in any case.

Table 8. Reference ranges obtained for inflammatory mediators analyzed, calculated on the basis of results obtained in non-frail and pre-frail subjects.

	N	Reference range		%Frail subjects out of the reference range	
		Lower limit	Upper limit	Below	Above
IL6 (pg/ml)	160	0.20	14.65	---	13.4
CRP (mg/l)	160	0.24	9.90	---	9.3
sTNF-RII (pg/ml)	160	1322.80	6563.20	---	18.8
TNFα (pg/ml)	58	0	5.15	---	10.0

CRP: C-reactive protein; IL6: interleukin 6; sTNF-RII: soluble tumor necrosis factor alpha receptor II; TNFα: tumor necrosis factor alpha.

In the analysis of the associations between inflammatory molecules (Table 9), notable and significant correlations were obtained between CRP and IL6, between CRP and sTNF-RII, between sTNF-RII and TNFα, between TNFα and IL6, and between IL6 and sTNF-RII being moderate in the latter case. Frailty was significantly associated with IL6, CRP, sTNF-RII, and TNFα.

Table 9. Partial correlation coefficients between inflammatory mediators analyzed, adjusted by age, sex, smoking habit and comorbidity (cells in light green: moderate associations, cell in dark green: strong associations).

	IL6	CRP	sTNF-RII	TNFα
Frailty	0.243***	0.204***	0.649***	0.325***
IL6		0.405***	0.250***	0.344**
CRP			0.339***	0.113
sTNF-RII				0.433***

***P*<0.01, ****P*<0.001. CRP: C-reactive protein; IL6: interleukin 6; sTNF-RII: soluble tumor necrosis factor alpha receptor II; TNFα: tumor necrosis factor alpha.

Results obtained from the multivariate statistical analyses regarding frailty status are shown in Table 10. Progressive increases with frailty severity were obtained in all inflammatory mediator concentrations, being especially remarkable the 85% increase of IL6 and the 2-fold increase of sTNF-RII in the frail subjects with regard to the non-frail participants. Significant and positive influence of age was observed for IL6 and sTNF-RII concentrations. Female gender

was associated with lower levels of IL-6 and sTNF-RII concentrations. Smoking habit and comorbidity did not significantly affect any parameter.

Table 10. Effect of frailty status on inflammatory mediators; models adjusted by age, sex, smoking habit and comorbidity.

	IL6		**CRP**		**sTNF-RII**		**TNFα**	
	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	1.15	(0.75-1.75)	1.19	(0.80-1.75)	**1.19***	**(1.03-1.38)**	1.60†	(0.96-2.66)
Frail	**1.70***	**(1.03-2.83)**	1.54†	(0.96-2.46)	**2.00****	**(1.68-2.39)**	1.68‡	(0.92-3.09)
Age	**1.03****	**(1.01-1.05)**	1.00	(0.99-1.02)	**1.02****	**(1.01-1.02)**	1.02	(1.00-1.05)
Gender								
Male	1.00		1.00		1.00		1.00	
Female	**0.65***	**(0.46-0.92)**	1.02	(0.74-1.40)	**0.88***	**(0.78-0.99)**	1.23	(0.78-1.93)

*P<0.05; **P<0.01; †P=0.071; ‡P=0.090. CI: confidence interval; CRP: C-reactive protein; IL6: interleukin 6; sTNF-RII: soluble tumor necrosis factor alpha receptor II; TNFα: tumor necrosis factor alpha.

Figure 18 shows the ROC curves computed to test the predictive value of the inflammatory mediators for frailty (except for TNFα, due to the much lower number of data available), using the non-frail group as the standard. AUC obtained were 0.64 (95% CI 0.56-0.71, P<0.01) for IL6, 0.60 (95% CI 0.52-0.68, P<0.05) for CRP, and 0.90 (95% CI 0.85-0.94, P<0.001) for sTNF-RII. A sTNF-RII concentration of 3461.3 pg/ml had the optimal predictive value for frailty, with a sensitivity of 0.94 and a specificity of 0.76.

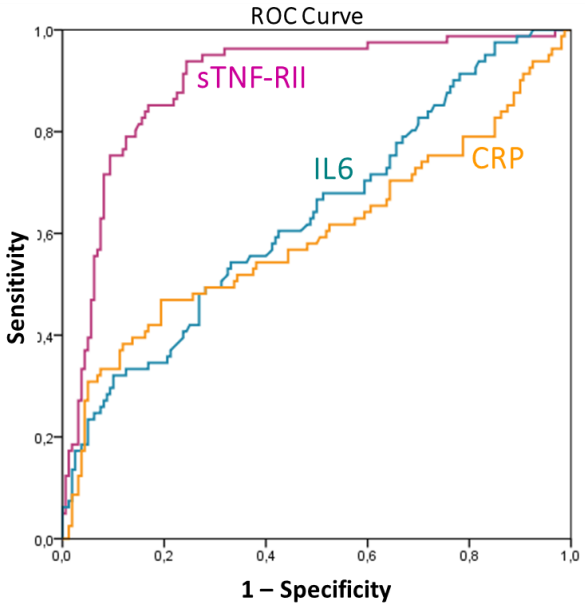

Figure 18. Receiver-operating characteristic (ROC) curves for IL-6, CRP, and sTNF-RII to predict frailty. CRP: C-reactive protein; IL6: interleukin 6; sTNF-RII: soluble tumor necrosis factor alpha receptor II.

3. Endocrine system: cortisol

Figure 19 shows the univariate analysis comparisons of cortisol in the three groups of older adults classified according to their frailty status. Cortisol concentration increased progressively and significantly with frailty burden. When compared with the serum cortisol reference interval for adults in the early morning (171-536 nmol/l, equivalent to 6.20-19.43 µg/dl) (Addison 2012), 16% of non-frail subjects presented cortisol concentrations above the reference interval, while this prevalence was 36.4% in pre-frail individuals and 52% in the frail group.

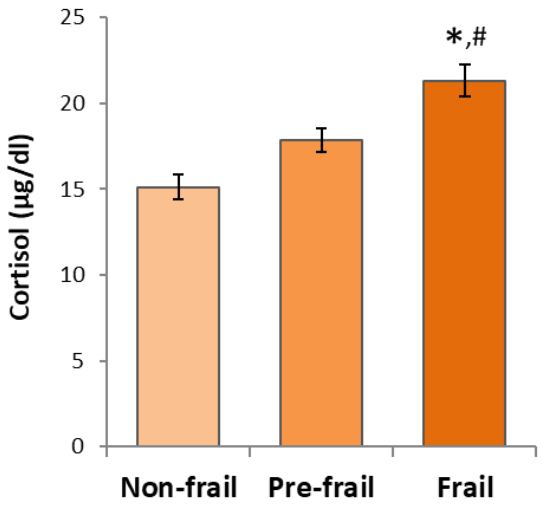

Figure 19. Results of cortisol concentrations according frailty status (univariate analyses). **P*<0.05, significantly differences comparing against non-frail group; #*P*<0.05, significantly differences comparing against pre-frail group.

Due to the close relationship between the HPA axis and the inflammatory mediators, correlation analyses between these mediators and cortisol were carried out (Table 11). Cortisol was significantly associated with all inflammatory molecules analyzed. Nevertheless, these associations were moderate for sTNF-RII, weak-moderate for TNFα and IL6, and weak for CRP. Partial correlations adjusted for age, sex, smoking habit and comorbidity were assessed as well, but statistical significance was not reached for any correlation.

Table 11. Correlation coefficients between cortisol and inflammatory mediators (cells in light orange: weak-moderate associations, cell in dark orange: moderate associations).

	IL6	CRP	sTNF-RII	TNFα
Cortisol	0.250**	0.162*	0.320**	0.265*

**P*<0.05, ***P*<0.01. CRP: C-reactive protein; IL6: interleukin 6; sTNF-RII: soluble tumor necrosis factor alpha receptor II; TNFα: tumor necrosis factor alpha.

Results obtained from the multivariate statistical analysis are shown in Table 12. An increasing tendency with frailty severity was observed in cortisol concentration, although significance was maintained only in the frail group as compared to non-frail individuals. In addition, a very slight but significant influence of age was observed on cortisol level. No

significant effect was observed for sex, tobacco consumption or comorbidity. Moreover, influence of 10-year mortality risk on cortisol concentration was also tested by multivariate analysis, adjusting by age, sex, smoking habit and comorbidity. Mean ratio obtained for this parameter was 1.01 (1.00-1.01 95% CI), significant with a *P*-value lower than 0.05.

Table 12. Effect of frailty status on cortisol concentration; model adjusted by age, sex, smoking habit, and comorbidity.

	Cortisol	
	Mean Ratio	95% CI
Frailty status		
Non-frail	1.00	
Pre-frail	1.12	(0.95-1.31)
Frail	**1.23***	**(1.01-1.49)**
Age	**1.01***	**(1.00-1.02)**

CI: confidence interval; **P*<0.05.

4. Oxidative stress biomarkers

Results obtained for the oxidative stress biomarkers in the three groups of participants are depicted in Figure 20. According to univariate analyses, no significant differences were observed in ROS/RNS levels, net oxidative DNA damage and total antioxidant capacity among frailty groups.

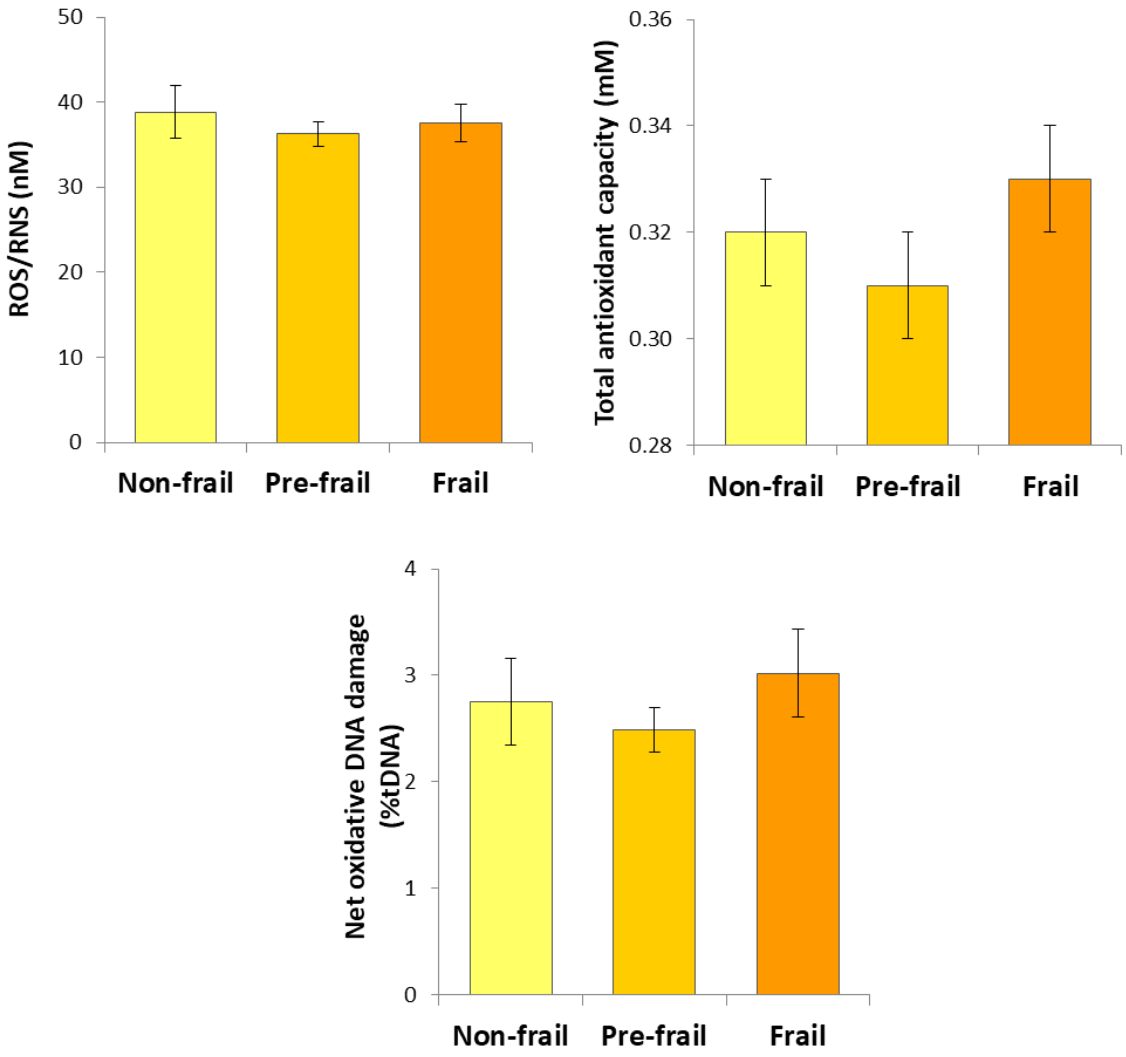

Figure 20. Results of oxidative stress biomarkers (univariate analyses). ROS/RNS: reactive oxygen and nitrogen species.

In accordance with the results from the univariate analyses, no significant differences were found either in any of the oxidative stress biomarkers regarding frailty status in the multivariate analyses (Table 13). Regarding age, a significant and inverse association for ROS/RNS levels and net oxidative %tDNA were found. No significant effect was observed for gender, smoking habit or comorbidity on any oxidative stress parameter.

Table 13. Effect of frailty status on the oxidative stress biomarkers. Models adjusted by age, sex, smoking habit and comorbidity.

	ROS/RNS		Net oxidative DNA damage		Total antioxidant capacity	
	Mean Ratio	95% CI	Mean Ratio	95% CI	Mean Ratio	95% CI
Frailty status						
Non-frail	1.00		1.00		1.00	
Pre-frail	1.02	(0.82-1.29)	0.98	(0.73-1.33)	0.90	(0.74-1.10)
Frail	1.15	(0.88-1.51)	1.12	(0.86-1.77)	0.98	(0.78-1.24)
Age	**0.98***	**(0.97-0.99)**	**0.98***	**(0.97-1.00)**	1.00	(0.99-1.01)

CI: confidence interval; *P*<0.05; ROS/RNS: reactive oxygen and nitrogen species.

V. DISCUSSION

V. DISCUSSION

As above mentioned, world population is experiencing an unstoppable aging situation which inevitably leads to increasing healthcare expenditure for governments. Because of that, researchers and governments are focused on increasing our knowledge about aging and age-related conditions and disorders, in order to improve healthcare and quality of life in elderly and to reduce sanitary and socioeconomic costs in the future. In the last two decades, frailty has been widely investigated; it is a consequence of the biological, physiological, social, and environmental changes that occur with advancing age. These age-related changes increase vulnerability to stressors, which leads to a significant decline of different physiological systems and then to disability, comorbidity, hospitalization and death. Causes of frailty are complex; it is a multidimensional syndrome based on the interplay of genetic, biological (hormonal, metabolic, and immune systems), physical, psychological, social, and environmental factors (Walston et al. 2006; Rockwood and Mitnitski 2007).

Facing the current frailty identification criteria, mainly based on phenotypic and/or clinic parameters, use of biomarkers as feasible endpoints has been proposed for frailty identification (Mitnitski et al. 2015). Biomarkers would provide a more accurate detection of frail subjects in early or previous stages of the syndrome, when frailty can still be potentially reverted. In addition, they will allow to implement preventive interventions or treatments focused on each person (personalized treatments), in order to maintain well-being and improve quality of life in the elderly. For the development of frailty-related biomarkers, different physiological processes disturbed in frailty status must be explored, such as those involving immune system, endocrine system, and oxidation/reduction homeostasis maintenance. A huge body of evidence support the relationship between chronic low-grade inflammation and age ("inflammaging) (Franceschi et al. 2000; Sergio 2008; Franceschi and Campisi 2014). This inflammatory state creates a vicious circle in which chronic oxidative stress and inflammation feed each other and, consequently, increase age-related morbidity and mortality. This relationship was proposed in the oxidative-inflammatory theory of aging (De la Fuente and Miquel 2009) and, in agreement with that, Pandey and Rizvi (2010) reported that chronic oxidative stress affects specially regulatory systems, such as nervous, immune and endocrine system. The later, mainly represented by HPA axis, is also affected by aging, with increasing cortisol levels among different age range populations (Varadhan et al. 2008; Evans et al. 2011). Besides, chronic inflammation, endocrine system alterations and oxidative stress have been considered key underlying mechanisms involved in age-related diseases, such as neurodegenerative diseases (Rothschild 2003; Halliwell 2006; Glass et al. 2010), cardiovascular

diseases (Aviram 2000; Güder et al. 2007; Libby et al. 2010), type 2 diabetes (Buffington et al. 1994; Davì et al. 2005) osteoporosis (Lencel and Magne 2011), or cancer (Thun et al. 2004). Consequently, it has been proposed that immune and endocrine system alterations, as well as oxidative stress, may be closely related to the development of frailty (Lai et al. 2014). Hence, concentrations of parameters indicative of immune activation and proinflammatory molecules, different lymphocyte subpopulation rates, and levels of cortisol and oxidative stress parameters could be suitable biomarkers that provide useful information for an earlier identification of frailty. For these reasons, in this work a set of biomarkers related to immune and endocrine systems, as well as oxidative stress, were evaluated in an older population classified according to frailty, determining the possible association between these biomarkers and frailty status.

1. Immune system

1.1. Immune activation biomarkers

To our knowledge, no studies addressed so far the possible relationship of frailty status with immunologic biomarkers involved in GCH or IDO enzymatic pathways, except for neopterin (Fahey et al. 2000; Leng et al. 2011). Hence, the possible disturbance of the mentioned immune stimulation-related enzymatic pathways was analyzed in the present work.

Although concentrations of the immune biomarkers assessed in this work were previously reported in populations or subpopulations of older adults (Reibnegger et al. 1988; Pitkänen et al. 2003; Frick et al. 2004; Spencer et al. 2010; Capuron et al. 2011; Kouchiwa et al. 2012), frailty status of the participants in these studies was not determined; at most some reports specified they were “healthy.” Thus, it was necessary to establish reference ranges of these biomarkers specifically in the group of robust older adults (i.e., in the absence of frailty). For some of the immune biomarkers, namely neopterin, nitrite, and especially Kyn/Trp ratio and tryptophan, the rate of concentrations in the frail group out of the reference range was remarkable and entirely in the same direction (only above of reference range in the case of neopterin and Kyn/Trp, and only below of reference range in tryptophan and nitrite), indicating a clear tendency of disturbance related to frailty status.

Neopterin concentration in body fluids is considered as a marker of activation of the immune system, in particular of Th1 or cell-mediated response (Murr et al. 2002). Higher concentrations of neopterin in older age were previously reported (Fahey et al. 2000;

Ledochowski et al. 2001; Spencer et al. 2010), and association between increased neopterin concentrations and enhanced tryptophan breakdown (as indicated by Kyn/Trp ratio) has been documented in older adults as well (Solichova et al. 2001; Frick et al. 2004; Pertovaara et al. 2006; Theofylaktopoulou et al. 2013). Similarly, our results showed significant and positive influence of age on neopterin and kynurenine concentrations and Kyn/Trp ratio, and inverse influence on tryptophan levels.

To date, only very few studies evaluated neopterin serum levels in older adults in association with frailty, finding significantly higher neopterin concentrations in frail older adults than in non-frail controls, either equally older (Fahey et al. 2000) or younger (median 38 years) (Leng et al. 2011). Current results support those previous ones and also indicate association of frailty with tryptophan breakdown because significant influence was observed for frailty status on neopterin, tryptophan, and Kyn/Trp ratio. Moreover, our results are also in line with other studies reporting that neopterin urinary concentrations and Kyn/Trp ratio predict mortality in nonagenarians (Solichova et al. 2001; Pertovaara et al. 2006), because frailty is related to increased vulnerability to stressors and increases the risk of death (Morley et al. 2013; Vermeiren et al. 2016).

Alterations of Kyn/Trp ratio may be due to an enhanced activity of two enzymes, namely IDO and tryptophan 2,3-dioyxgenase (TDO), an IDO isoenzyme not induced by proinflammatory cytokines but rather upregulated by tryptophan itself and corticosteroids (Taylor and Feng 1991; Chen and Guillemin 2009). However, in the presence of immune stimulation, Kyn/Trp together with concentrations of neopterin reflect the degree of Th1-type immune activation (Capuron et al. 2014). The strong correlation found in this study between neopterin concentration and Kyn/Trp points to enhanced IDO activity and immune stimulation as the cause behind tryptophan parameters disturbance. Besides, in many cases, the tryptophan breakdown rate not only correlates with neopterin concentrations, but also with the extent and the activity of the disease (e.g., in viral infections or malignant tumors) (Schröcksnadel et al. 2006; Weinlich et al. 2006; Sucher et al. 2010). The moderate significant correlations obtained in the current study between frailty status and tryptophan breakdown parameters, and also with nitrite and tyrosine, suggest that the level of these markers may be indicative (directly or inversely) of frailty severity. This also indicates that, although neopterin and tryptophan breakdown products are not specific biomarkers for frailty, development of frailty status takes most likely place when these immune biomarkers increase, being the immune system activation a strong driving force for frailty development. In this sense, Kyn/Trp ratio and neopterin may have clinical applicability as identification tools for frail individuals,

regarding ROC curves results obtained in this work. Nevertheless, further studies in other populations are necessary to replicate and standardize these novel results.

Activated human monocytes/macrophages produce neopterin at the expense of BH_4 (Fuchs et al. 1994). BH_4 deficiency affects PHA and NOS enzymatic activities, consequently diminishing tyrosine and NO production and increasing the ratio Phe/Tyr, considered a useful measure to estimate PAH activity (Scriver 2007). Indeed, increases in phenylalanine concentration and in Phe/Tyr ratio have been reported in patients with different chronic inflammatory conditions, and correlations with neopterin concentrations were also found (Neurauter et al. 2008a; Ploder et al. 2008; Mangge et al. 2013). Our results showed a significant influence of frailty status on Phe/Tyr ratio (direct) and on tyrosine and nitrite concentrations (inverse), supporting the view that both PAH and NOS activities are impaired in frail older adults. The significant correlation found between Phe/Tyr and neopterin, and the inverse associations observed of nitrite with neopterin concentrations and Kyn/Trp, also point toward parallel disturbance of GCH and IDO enzymatic pathways caused by Th1-type immune activation in frail older adults.

No significant association was obtained in this work between nitrite and any of the phenylalanine breakdown parameters. Several reasons may help to explain this lack of association. On one hand, even though the majority of plasma nitrite is derived from constitutive NOS activity (Kleinbongard et al. 2003), serum nitrite concentrations only serve as a rough estimate of NO production rates (Mangge et al. 2013); indeed food is an important exogenous factor influencing serum nitrite concentrations (Geisler et al. 2015). And, on the other hand, tyrosine is not an end-product and its concentrations are also influenced by the activity of another BH_4-dependent enzyme (tyrosine hydroxylase), which forms DOPA (3,4-dihydroxyphenylalanine) from tyrosine (Widner et al. 2000).

In summary, this work on immune stimulation biomarkers establishes, for the first time, reference ranges for a number of these biomarkers related to IDO and GCH enzymatic pathways in the population of robust older adults (excluding the presence of frailty). Besides, results obtained are consistent with the idea that chronic immune system stimulation in frail older adults is higher than expected according only to their age (i.e., frailty status in the elderly is associated with an additional degree of immune stimulation, manifested in more intense disturbance of IDO and GCH pathways than in non-frail or pre-frail older adults). In other words, our data support the involvement of monocyte/macrophage mediated Th1 immune

activation and disturbed amino acid biochemistry in the pathophysiology of the frailty geriatric syndrome.

1.2. Lymphocyte subpopulations

The immune response can be divided into an adaptive part, represented by B and T lymphocytes and an innate part, which comprises monocytes, natural killer (NK) cells and dendritic cells. Immunosenescence results in a loss of adaptive immune function with relative preservation of innate immunity, with changes in different lymphocyte subpopulations. Previous studies found age-related decreases in B and T lymphocytes (Jentsch-Ullrich et al. 2005; McElhaney and Effros 2009; García-Dabrio et al. 2012; Lutz and Quinn 2012) and age-related increases in NK cells (Jentsch-Ullrich et al. 2005; Lutz and Quinn 2012; Vasson et al. 2013). Besides, contradictory results were found for $CD4^+/CD8^+$ ratio with increased (García-Dabrio et al. 2012; Muller et al. 2015) and decreased (Semba et al. 2005) age-related results. Thus, analysis of age-related immunological changes, such as alterations in lymphocyte subsets, may provide useful biomarkers for frailty and associated pathologies.

The analyses of the different lymphocyte subpopulations were carried out in this study by means of flow cytometry. This methodology has several advantages: (1) It is a precise and reliable technique for the assessment of immunological status (Al-Mawali et al. 2013); (2) only a small quantity of biological sample is required; and (3) the time needed for the analysis is very short, which is a great benefit in population studies due the vast amount of samples usually handled.

Specific reference ranges for lymphocyte subset rates were calculated focusing on the elderly sector population for the first time in this study. The distribution of lymphocyte subsets found was quite different from those described by Vasson et al. (2013) for a Spanish population (range of differences between means from 6.3% for $\%CD3^+$ lymphocytes to 74.5% for $\%CD19^+$ lymphocytes). These differences are probably related to the broader age range of the participants in that study (20-75 years), and also to the fact that it was restricted to men. Most values obtained in the three population groups fell within the corresponding reference ranges, and no differences were observed regarding frailty severity.

In which regards association of frailty with lymphocyte subsets, our results showed a slight decrease of $\%CD19^+$ cells in the frail group both in the univariate analysis and in the linear regression analysis adjusting for age, gender, smoking habit and comorbidity, and an increase of the $CD4^+/CD8^+$ ratio ($P<0.05$) in frail subjects in the multivariate analysis, not

significant in the univariate analysis. These quite weak results point to a limited strength association of these biomarkers with frailty. Up to now, very few studies have assessed the link between lymphocyte subpopulations and frailty status in older adults. De Fanis et al. (2008) found a significant association between increased $CD8^+$ and decreased $CD4^+$ cell percentages in frail subjects regarding to the non-frail group, although sample size evaluated was very modest (13 frail vs. 13 non-frail participants). Besides, Semba et al. (2005) obtained similar results in addition to a subsequent significant decrease in the $CD4^+/CD8^+$ ratio, with a quite larger population size (*N*= 24, 75 and 28 for non-frail, pre-frail and frail individuals, respectively). None of these studies adjusted for possible confounders in the statistical analysis, what may account in part for the differences with the current study, together with the more restricted sample sizes.

1.3. Inflammatory mediators

Reference ranges for IL6, CRP, sTNF-RII and TNFα in robust adults (excluding the presence of frailty) aged 65 years and over were firstly established in this study. Percentages of frail subjects presenting concentrations of these biomarkers out of the corresponding reference range oscillated between 9% in the case of CRP and 19% in the case of sTNF-RII. These values were always located above the reference range, showing a clear trend to increase with frailty status.

TNFα is an early mediator of inflammatory responses, which is produced by stimulated monocytes, macrophages and T lymphocyte subsets (Diez-Ruiz et al. 1995). During inflammation, TNFα, IL1, and IL6 are secreted, in that order. IL6 then inhibits the secretion of TNFα and IL1, and activates the production of acute phase reactants from liver (CRP) (reviewed in Papanicolaou et al. 1998). TNFα membrane receptors are shed by proteolytic cleavage into circulation as soluble TNFα receptors (sTNF-RI and sTNF-RII) (Figure 21), which have been shown to be reliable measurements for the *in vivo* activities of TNFα (Savès et al. 2001). Results from the present study support the idea of an interrelated activation of the entire inflammatory cascade, since TNFα, sTNF-RII, IL6, and CRP concentrations were significantly correlated with one another.

Data obtained in this work showed positive influence of frailty on IL6, CRP, TNFα and sTNF-RII concentrations. The only study analyzing sTNF-RII concentrations in relation to frailty so far found progressive increase of this biomarker with frailty status and significance was reached in the group of pre-frail subjects (Liu et al. 2016). Still, considerable amount of literature has accumulated concerning the association of high levels of IL6, TNFα and CRP with

frailty in older adults in cross-sectional studies. Among works assessing all these three biomarkers, some of them reported increases in their concentrations with frailty (Collerton et al. 2012; Hubbard et al. 2008; Hubbard et al. 2009; Langmann et al. 2017; Ronning et al. 2010) and with increased risk of death (Giovannini et al. 2011). On the contrary, other studies did not find such significant associations with frailty (Tsai et al. 2013), or obtained mixed results (significance for some markers and no effect for others) (Lai et al. 2014; Namioka et al. 2016). A recent meta-analysis (Soysal et al. 2016) conducted with most abovementioned studies and some others, both cross-sectional and longitudinal, concluded that, on the basis of cross-sectional studies, frailty and pre-frailty are associated with higher inflammatory parameter levels, in particular CRP and IL6. However, these findings were not confirmed in longitudinal trials, supporting the need of further studies to better understand the role of inflammatory markers in frailty status. Our study confirmed the involvement of chronic inflammation on frailty in later life; particularly strong associations were obtained in the regression analysis for IL6 (70% increase in frail subjects with regard to non-frail participants), and for sTNF-RII (19% increase in pre-frail and 2-fold increase in frail individuals; all three categories were significantly different). Moreover, area under the ROC curve obtained for sTNF-RII (0.90) indicates a high accuracy in the predictive value of this biomarker for frailty. At concentrations higher than 3461.3 pg/ml, frail subjects can be identified with quite high sensitivity (0.94) and specificity (0.76).

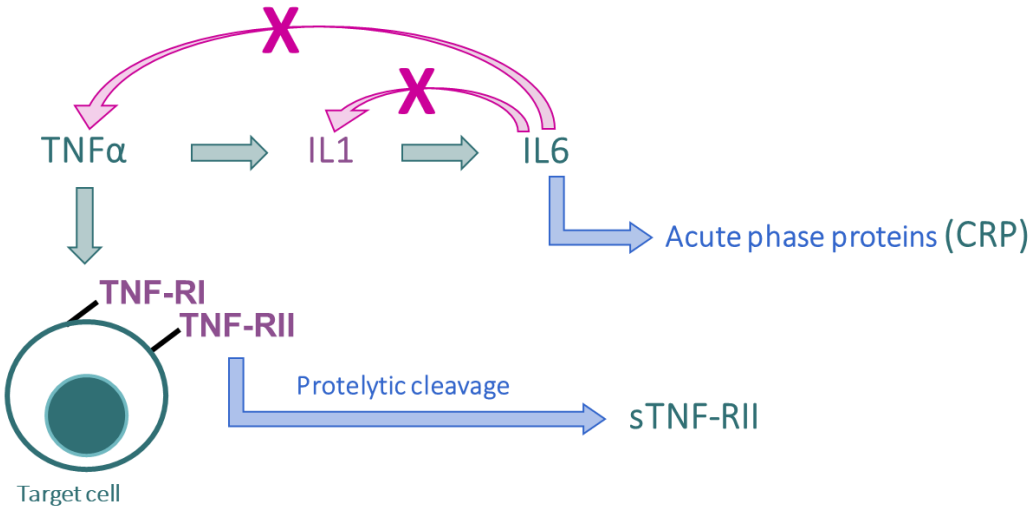

Figure 21. Relationship among the different inflammatory mediators analyzed in the context of the inflammatory cascade.

Numerous studies of older adults showed that levels of several inflammatory mediators increase with age even in apparently healthy individuals and in the absence of acute infection (reviewed in Singh and Newman 2011). Present results show that frailty status in older adults involves an additional increase in these mediators, beyond that related to aging. Chronic inflammation has been proposed as a key underlying mechanism involved in frailty (Fulop et al. 2010; Li et al. 2011). Inflammatory molecules may directly contribute to frailty or its central components (such as decreased muscle mass, strength, and power, and slowed motor performance). But, as frailty is a multidimensional syndrome, the contribution can also be indirect through other intermediate pathophysiologic processes, i.e. its detrimental effects on other organ systems, such as musculoskeletal and endocrine systems, cardiovascular diseases, and nutritional dysregulation (reviewed in Chen et al. 2014).

Increasing evidence suggests that frailty is a useful risk assessment tool for pre-surgery evaluation, for overall immune functional decline, in older patients with cardiovascular conditions, or for risk stratification of older patients with cancer (reviewed in Chen et al. 2014). Hence, the importance of identifying frailty is undeniable. The current study suggests that sTNF-RII may have clinical applicability as a screening tool for identifying frail subjects, although standardization and replication of these results in other populations is necessary before it can be used to that aim.

In summary regarding the analysis of inflammatory mediators, reference ranges for several of these molecules were established for the first time in older adults in the absence of frailty according to Fried's criteria. Associations found between inflammatory molecules confirm their interrelationship in the inflammatory cascade. Data obtained for the different inflammatory mediators provide additional reinforcement to the widely established hypothesis that inflammaging is involved in frailty status in older adults. Hence, frail subjects present an additional degree of chronic inflammation manifestations than what could be expected only according to the normal aging process. This association was more intensively manifested in IL6 and sTNF-RII, and this last biomarker showed a high accuracy for predicting frailty.

2. Endocrine system: cortisol

Once demonstrated the association of frailty in older adults with an additional degree of immune stimulation and inflammation, and considering the relationship of the immune system with the endocrine system, and that cortisol may be influenced by multiple endogenous and environmental factors throughout the lifespan, in this section the possible

relationship of frailty syndrome with cortisol serum concentrations was evaluated in the same cohort of Spanish older adults.

There is a significant consensus that HPA axis reactivity to external stressors, eventually manifested by raising cortisol levels, increases with age. Accordingly, higher cortisol concentrations were reported in patients of several age-related diseases such as Alzheimer´s disease (Lupien et al. 1999), diabetes (Buffington et al. 1994), metabolic syndrome (Reynolds et al. 2003), depression (Rothschild 2003), hypertension (Al'Absi and Wittmers 2003), osteopenia (Dennison et al. 1999), sepsis (Sam et al. 2004), heart failure (Güder et al. 2007), and sarcopenia (Waters et al. 2008). Furthermore, progressive and significant increases of cortisol concentrations with age have been described in the literature in wide age range populations (20-80 years) (Swaab et al. 2005; Evans et al. 2011), and in older adults (women aged 80-90 years) (Varadhan et al., 2008). In line with these previous studies, our results also show a significant slight positive age effect on serum cortisol.

Besides, significant and progressive increases in cortisol concentrations with frailty severity were obtained in the current study. In addition, the rate of subjects out of the reference range established for serum cortisol for the whole adult population increased progressively from the non-frail to the frail group. To our knowledge, only two previous studies addressed the possible relationship between frailty and cortisol levels so far, both of them in saliva samples. Varadhan et al. (2008) measured salivary cortisol over a 24-hour period, and found significant positive associations of frailty burden with evening cortisol and 24-hour mean cortisol, but not with awakening cortisol. And higher salivary cortisol values in the morning and before bedtime among frail aged individuals were described by Holanda et al. (2012).

Since cytokines such as IL6 and TNFα, that resulted increased in frail subjects in this and other previous studies, are well-known activators of the HPA axis (Turnbull and Rivier 1999), increase in cortisol concentrations related to frailty is likely to be mediated by chronic inflammation response. In agreement with that, our results showed significant correlations between serum cortisol and inflammatory mediators. Besides, catabolic effects of cortisol are related to loss of muscle strength and mass, weight loss, and decreased appetite and energy (Attaix et al. 2005). All these effects, which are classic frailty phenotypic traits, provide additional reinforcement to the involvement of cortisol (and HPA axis) upregulation in frailty status.

Frailty is a predictor of a number of adverse health outcomes in the older people, including mortality, with an incidence of up to 45% per year in the frail group (Abizanda et al.

2013). In addition, several studies related increased mortality risk with high cortisol concentrations in patients with different age-related diseases such as stroke (Christensen et al. 2004), heart failure (Güder et al. 2007), sepsis (Sam et al. 2004), and sarcopenia (Waters et al. 2008). Our results support these previous studies, obtaining a statistically significant association between serum cortisol and 10-year mortality risk in the older adult population.

In summary regarding serum cortisol, higher concentrations of this endocrine biomarker were observed related to increasing frailty burden, thus supporting the hypothesis that age-related HPA axis dysregulation is associated with frailty status in the older people.

3. Oxidative stress biomarkers

A growing body of evidence suggests the association between oxidative stress and aging. Firstly, the "free radical/oxidative stress theory of aging" and later the "oxidative-inflammatory theory of aging" (De la Fuente and Miquel 2009) supported this idea and they have proposed that aging is a loss of homeostasis due to a chronic oxidative stress that affects especially the regulatory systems, such as nervous, endocrine, and immune systems (Pandey and Rizvi 2010). These theories, complemented with the revisited "nitric oxide theory of aging" (McCann et al. 2005), assert that levels of oxidative stress and inflammatory cytokines gradually increase with age, whereas antioxidant defenses decrease. Indeed, several studies have reported that ROS/RNS could play a pivotal role in a number of age-related diseases, such as Alzheimer´s disease (Halliwell 2006), diabetes (Davì et al. 2005), Parkinson´s disease (Wood-Kaczmar et al. 2006), atherosclerosis (Parthasarathy et al. 2008), cardiovascular disease (Aviram 2000), and rheumatoid arthritis (Hitchon and El-Gabalawy 2004).

In addition, the possible relationship between frailty in older adults and oxidative stress biomarkers has been also investigated. Significant increases in the concentration of derivate of reactive oxygen metabolites (Saum et al. 2015; Namioka et al. 2016), isoprostanes and lipoprotein phospholipase A2 (Liu et al. 2016), oxidized glutathione (Serviddio et al. 2009), malondialdehyde (MDA) and 4-hydroxy-2,3-nonenal-protein plasma adducts (Serviddio et al. 2009; Pereira et al. 2016), conjugated dienes and trienes (Pereira et al. 2016), serum 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Wu et al. 2009), MDA formed from lipoperoxides and protein carbonylation (Inglés et al. 2014), and urinary 8-OHdG and 8-isoprostane (Namioka et al. 2016) were observed in frail subjects as compared with non-frail individuals. No such effects were detected for MDA and paraoxonase-1 by Goulet et al. (2009) in a rather small population (*N*=54) and for isoprostanes iPF2 alpha-III and iPF2 alpha-VI in a large cohort (*N*=845) (Collerton et al. 2012).

Regarding antioxidant-related biomarkers, frailty has been reported to be associated with significantly lower levels of total thiol levels (Saum et al. 2015), vitamin E (Ble et al. 2006), and total antioxidant potential (Namioka et al. 2016); although other studies did not find significant differences regarding to frailty status for vitamin C and vitamin E (Goulet et al. 2009), or biological antioxidant potential (Namioka et al. 2016). Our results showed neither significant effect of frailty on the levels of oxidative stress biomarkers (ROS/RNS concentration and oxidative DNA damage) nor on total serum antioxidant capacity. Besides, and contrarily to the expected increase in oxidative stress parameters with age, significant decreases were obtained in the current study for both ROS/RNS levels and oxidative DNA damage, although mean ratio values were quite slight.

Controversies among results from the different studies (including this one) reflect the difficulty of assessing this kind of biomarkers in humans, maybe due to the highly reactive nature of some of them, which determines extremely short lifespans, or to the environmental factors that may influence the values of these parameters. Furthermore, limited sample sizes evaluated in several studies, confounders considered (or not) in the statistical analyses, and different criteria used for the definition of frailty status across studies, may also account for the differences in the results reported.

In summary regarding to oxidative stress biomarkers evaluation, although it was previously reported that frailty may be associated with higher oxidative stress and possibly lower antioxidant parameters, no such relationships could be obtained in the biomarkers analyzed in this study.

4. Strenghts and limitations

This work establishes, for the first time, reference ranges for a number of immune biomarkers related to IDO and GCH enzymatic pathways, as well as for several inflammatory mediators in the population of robust older adults (i.e., excluding the presence of frailty) according Fried´s frailty criteria. Furthermore, results from this study provide evidence for the existence of significant influence of frailty status on circulating concentrations of immune biomarkers involved in IDO and GCH enzymatic pathways, proinflammatory molecules involved in the immune activation cascade and endocrine system, represented by cortisol levels. These findings contribute to increase the knowledge on the pathophysiology of frailty status, necessary for the orientation and feasibility of future implementation of therapeutic or, more importantly, preventive interventions in the older adult population, considering that frailty may be reverted in its early stages.

Nevertheless, because this study was carried out in an older adult population, a major limitation is that participants are not completely healthy, but most of them present different pathologic conditions (i.e., comorbidity), from 15% of robust individuals to 40% of frail subjects, and medications were taken to treat them. Although linear regression analyses were adjusted for comorbidity and exclusion criteria included (i) taking antineoplastic or immunomodulating medications, and (ii) having infections, autoimmune disease or cancer, the fact that some of the chronic diseases common in older adults, or the associated medications, may have influenced the parameters evaluated in this study cannot be ruled out.

Further investigation is necessary to prove whether the current findings are consistent and reproducible in larger sample sizes and different populations that may differ in the presence of other factors not considered in the performed analyses. Such investigation would eventually allow to standardize these biomarkers before they can be used in clinics, and to fully understand their relationship with frailty development.

VI. CONCLUSIONS

VI. CONCLUSIONS

Immunological Biomarkers

1. This work establishes, for the first time, reference ranges for a number of immune biomarkers – related to immune activation, lymphocyte subpopulations and inflammaging – in the population of robust older adults (i.e., excluding the presence of frailty).

2. Immune stimulation biomarkers involved in IDO and GCH enzymatic pathways were strongly associated with frailty status, providing evidence for the involvement of monocyte/macrophage mediated Th1 immune activation and disturbed amino acid biochemistry in the pathophysiology of the frailty geriatric syndrome. Besides, the significant correlations found between several parameters point toward parallel disturbance of IDO and GCH pathways caused by Th1-type immune activation.

3. Results obtained for the different lymphocyte subpopulations assessed suggest a limited strength association between frailty and these immunosenescence biomarkers.

4. Frailty was observed to be associated with the inflammatory mediators analyzed, providing additional reinforcement to the widely established hypothesis that inflammaging is involved in frailty status in older adults. Among all these biomarkers analyzed, sTNF-RII showed the most promising predictive ability and may have clinical applicability as a screening tool for identifying frail subjects, although standardization and replication of these results in other populations is necessary before it can be used to that aim.

Endocrine System

5. Serum cortisol concentrations were significantly associated with frailty status, and significantly correlated with inflammatory mediators, providing support to the hypothesis that age-related HPA axis dysregulation is associated with frailty syndrome in older adults.

Oxidative Stress Biomarkers

6. No significant association was found between frailty and oxidative stress biomarkers (reactive oxygen/nitrogen species, oxidative DNA damage, and total antioxidant capacity) in the population of older adults analyzed.

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