UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di ricerca in:

Medicina Molecolare e Biotecnologie

Dipartimento di Biomedicina, Neuroscienze e Diagnostica avanzata

Settore Scientifico Disciplinare:

MED/04 – Patologia Generale

I centenari, modello di invecchiamento di successo e la malattia di Alzheimer, modello di invecchiamento senza successo

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CICLO XXXII

ANNO CONSEGUIMENTO TITOLO: 2020

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Introduzione - *Premessa*

Il miglioramento delle condizioni igienico-sanitarie, a partire dal XIX secolo, ha favorito un aumento dell'aspettativa di vita nei paesi industrializzati, che nel corso del secolo, fino alla seconda metà del successivo, ha subìto un continuo incremento dovuto ad un cambiamento dei regimi nutrizionali e ad un miglioramento delle condizioni di salute. Oggi, infatti, la fetta di popolazione in costante crescita è costituita da ultraottantenni, e recenti ricerche dimostrano inoltre che vi è una grande prevalenza di centenari, i quali raggiungono i limiti estremi della vita senza gravi patologie correlate all'età. Se prima infatti il termine invecchiamento veniva associato a soggetti nella fascia d'età tra i 60 e i 70 anni, oggi è associato a soggetti di 80-90 anni (Caruso C., Candore G. 2016).

1. L'invecchiamento

Definito come declino funzionale, l'invecchiamento è un processo multifattoriale naturale ed inevitabile, prodotto dell'interazione tra fattori genetici, epigenetici e ambientali, che colpisce tutti gli organismi eucarioti multicellulari e probabilmente anche molti organismi unicellulari, come i lieviti (Sadowska-Bartosz Izabela and Bartosz Grzegorz 2014), ed è caratterizzato da una diminuita capacità di adattamento all'ambiente. Nonostante ciò, tutti gli organismi invecchiano in maniera diversa, e ciò dipende da una combinazione di caratteristiche genetiche ed ambientali, positive o negative. Per questo motivo ci si riferisce ad "**invecchiamento di successo**" o un "**invecchiamento senza successo**"; nel primo caso si parla di soggetti che raggiungono l'età avanzata senza sviluppare particolari patologie età-correlate mentre nel secondo caso di soggetti che, al contrario, sviluppano malattie legate all'età come quelle neurodegenerative, la malattia di Alzheimer (MA) o il Parkinson, malattie metaboliche come la sindrome metabolica (SM), diabete mellito di tipo 2 (T2DM), malattie cardiovascolari (CVD) e il cancro (Sadowska-Bartosz Izabela and Bartosz Grzegorz 2014; Caruso C., Candore G. 2016). L'invecchiamento è anche associato all'accorciamento dei telomeri e alla perdita delle attività delle proteasi, ai cambiamenti e agli effetti associati che si verificano a livello intercellulare e, di conseguenza, a livello organico e di tessuti.

Lo stato infiammatorio generale cronico dell'organismo è alla base del processo dell'invecchiamento, ed è causato da una continua esposizione agli antigeni per un tempo prolungato, che comporta una maggiore suscettibilità alle patologie età-correlate (Franceschi Claudio, MD 2007).

I numerosi studi fino ad ora effettuati hanno permesso di comprendere la stretta correlazione tra longevità e background genetico e stile di vita, e ciò ha portato ad elaborare delle strategie volte ad aumentare le possibilità di raggiungere la longevità in salute modulando i meccanismi chiave dell'invecchiamento. In particolare, hanno suggerito che, anche nell'uomo, le mutazioni a livello di geni correlati al mantenimento e metabolismo di base della cellula sono essenziali per modulare la durata della vita. Alcuni esempi sono i geni coinvolti nella riparazione del DNA, nella conservazione dei telomeri e nella protezione dai radicali liberi. Oltre questi, è stato osservato che hanno un forte impatto sull'invecchiamento e sulla longevità anche geni coinvolti in processi organici importanti, ad esempio quelli implicati nel metabolismo delle lipoproteine, nell'immunità e nell'infiammazione (Brooks - Wilson Angela R. 2013).

È noto da diversi studi che i soggetti che rappresentano un esempio selezionato di popolazione in buona salute sono i centenari, in cui l'insorgenza di gravi malattie legate all'età è ritardata o del tutto assente. Lo studio di questi soggetti consente quindi di distinguere la genetica dai meccanismi ambientali, responsabili del raggiungimento di questo fenotipo positivo, guidando alla comprensione della prevenzione e/o riduzione di alcune malattie, anche quelle neurodegenerative, correlate all'età (Caruso C., Candore G. 2016).

1.1 Teorie dell'invecchiamento

Sono state formulate diverse ipotesi per spiegare i meccanismi cellulari e molecolari alla base dell'invecchiamento e della longevità, e studi recenti hanno confermato che la sinergia tra le variabili genetiche, epigenetiche ed ambientali, può causare un accumulo di danni nelle cellule, nei tessuti e nell'intero organismo. Questo ha portato alla formulazione di diverse teorie dell'invecchiamento (Testa Roberto, Olivieri Fabiola, Ceriello Antonio, La Sala Lucia 2011; Caruso C., Candore G. 2016).

Diverse ricerche hanno messo in relazione i tassi metabolici dell'organismo, inteso come metabolismo basale, con la durata della vita; ad esempio, animali con metabolismo basale alto hanno una durata della vita minore rispetto a quelli con metabolismo basale basso, e ciò ha portato alla formulazione dell'ipotesi del tasso della vita in cui si afferma che il tasso metabolico definisce la durata della vita di una specie e la sua aspettativa di vita. A metà degli anni '50, un teorico,

Denham Harman, formulò la "*teoria dei radicali liberi dell'invecchiamento*" secondo cui i radicali liberi e altre specie reattive dell'ossigeno fossero generati nelle cellule, in particolare a livello dei mitocondri, e ciò risultasse in un danno cumulativo. A questo danno contribuiscono anche le reazioni di metaboliti come zuccheri e aldeidi reattive ed errori spontanei nei processi biochimici, risultando nella comparsa di fragilità e conseguente esordio di diverse malattie (Testa Roberto, Olivieri Fabiola, Ceriello Antonio, La Sala Lucia 2011).

I mitocondri producono e consumano la maggior parte dell'energia nella cellula, in particolare l'ossigeno intracellulare, e quindi la teoria dei radicali liberi dell'invecchiamento e l'ipotesi del tasso di vita sono spesso considerate sinonimi; maggiore è il tasso metabolico di un organismo, maggiore è la produzione di specie reattive dell'ossigeno (ROS) e quindi minore è la durata della vita. Nel corso degli anni, studi più approfonditi hanno però dimostrato che ciò non è vero per tutti gli esseri viventi, ad esempio nei primati, ad un determinato tasso metabolico, i mitocondri tendono a produrre meno ROS, associando la longevità alla loro produzione, invece che al tasso metabolico (Finkel Toren & Holbrook Nikki J. 2000). Oggi tra le teorie che cercano di comprendere e di spiegare quali siano i fattori chiave dell'invecchiamento vi sono, oltre la teoria dei radicali liberi, quella della senescenza cellulare, quella immunologica ed infiammatoria, e quella della regolazione genica. La *teoria della senescenza cellulare* nasce dall'osservazione di Leonard Hayflick che fibroblasti messi in coltura vanno incontro ad un numero finito di replicazioni, fino ad arrestarsi. Il fenomeno poteva essere dato, secondo Hayflick, dalla presenza di geni che regolano il processo della senescenza, o dall'accorciamento dei telomeri; infatti egli osservava una maggiore espressione di proteine come la p53, che causa l'arresto del ciclo cellulare in fase G1 se l'accorciamento dei telomeri è massivo e non controllato, ed i telomeri più corti. L'accorciamento dei telomeri è comunque regolato dalla telomerasi, l'enzima che permette di rigenerarli, e diversi esperimenti hanno mostrato come la sua espressione regoli la durata della vita della cellula e contribuisca quindi all'invecchiamento. Infatti mettendo in coltura cellule di soggetti anziani e giovani, le divisioni cellulari dei primi erano limitate rispetto a quelle dei secondi, suggerendo una correlazione tra potenziale replicativo ed età del donatore. La senescenza può anche essere prematura poichè indotta da stress fisiologici, come le radiazioni, lo stress ossidativo o la mancanza di nutrienti o il danno al DNA (Testa Roberto, Olivieri Fabiola, Ceriello Antonio, La Sala Lucia 2011).

La teoria immunologica è stata proposta da Roy Walford, il quale riconduce l'invecchiamento agli errori del sistema immunitario. Sembra che i soggetti anziani siano più suscettibili a diverse patologie, tra cui quelle autoimmuni, e sembra che questo derivi dalle disfunzioni del sistema immunitario, che subisce dei cambiamenti dopo l'età adulta, come l'atrofia del timo in cui maturano

i linfociti T (Ferrara N., Corbi G., Scarpa D., Rengo G., Longobardi G., Mazzella F., Cacciatore F., Rengo F. 2005). Successivamente Claudio Franceschi, nel 1989, propose la *teoria dell'immunosenescenza* secondo cui, col tempo i linfociti T memoria tendono ad aumentare a discapito di quelli vergini, con conseguente attivazione cronica dell'immunità innata e dell'infiammazione. Infine è stata proposta la *teoria della regolazione genica* la quale si basa sul fatto che l'espressione di alcuni geni varia con l'età. Un esempio è il pathway di attivazione del gene regolatore dell'insulina (IGF-1), che sembra regolare la durata della vita, infatti in C. elegans, in risposta alla diminuzione del segnale mediato dal recettore per IGF-1, si aveva l'aumento dell'aspettativa di vita. Inoltre lo studio dei centenari ha permesso di identificare una forte componente genetica alla base di un invecchiamento di successo, come i geni del pathway dell'insulina/IGF-1 e i geni che codificano per citochine pro- e anti-infiammatorie, i quali possono interagire con diversi processi metabolici cellulari. Inoltre questi soggetti longevi hanno delle particolari caratteristiche, come un basso indice di massa corporea (BMI) una bassa glicemia a digiuno, bassi livelli di insulina nel plasma e di resistenza all'insulina (Testa Roberto, Olivieri Fabiola, Ceriello Antonio, La Sala Lucia 2011).

2. I centenari, modello di invecchiamento di successo

Gli studi sulla longevità e sull'invecchiamento di successo prendono in considerazione i centenari, come esempio di biologia positiva, poiché godono di buona salute ed hanno un'età pari o superiore a 100 anni. Essi infatti rappresentano una popolazione selezionata "naturale" che è in grado di rispondere bene ai fattori di stress e di riparare i danni, grazie ad un insieme di caratteristiche favorevoli genetiche ed ambientali, e su cui studiare l'effetto di determinati polimorfismi e loci genetici associati o meno alla longevità (Avery P, Barzilai N, Benetos A, Bilianou H, Capri M, Caruso C, Franceschi C, Katsiki N, Mikhailidis DP, Panotopoulos G, Sikora E, Tzanetakou IP, Kolovou G 2014).

2.1 Genetica dell'invecchiamento

Tra longevità e invecchiamento in buona salute c'è una differenza, poiché la prima si concentra solamente sulla durata della vita, mentre il secondo è incentrato sulla salute; entrambi i concetti sono comunque intimamente correlati poiché gli individui che vivono molto a lungo tendono anche ad essere sani per gran parte della loro vita. Numerosi studi hanno mostrato l'esistenza di geni che possono prolungare la durata della vita e gli effetti delle mutazioni su di essi. In particolare, associate alla longevità, sono state identificate delle varianti dei geni dell'Apolipoproteina E

(ApoE) una molecola trasportatrice di lipidi, polimorfismi dei geni Forkhead box 3A (FOXO3A) un fattore di trascrizione coinvolto nella regolazione del ciclo cellulare, e le sirtuine, proteine con azione istone deacetilasi che influenzano il metabolismo di organismi eucariotici e procariotici. Uno studio sulla salute dei supercentenari (110-119 anni), semisupercentenari (105-109 anni), centenari (100-104 anni), nonageriani e controlli più giovani ha messo in luce che maggiore è la fascia di età e maggiore è il ritardo nell'insorgenza di patologie. Da analisi geniche e studi di sequenziamento sembra che questi soggetti non manchino dei geni implicati nell'insorgenza delle malattie ma che posseggano delle varianti genetiche che sono in grado di proteggere da specifici alleli a rischio. I geni esaminati per l'associazione con longevità ed invecchiamento in buona salute fanno parte di categorie diverse, e sono stati già studiati in organismi modello poiché in grado di estendere la durata della vita. Questi sono geni coinvolti nel metabolismo lipidico, nella risposta immunitaria e nell'infiammazione, e nella risposta allo stress. Tra tutti i geni analizzati, i più significativi nello studio sono state delle varianti alleliche dei geni ApoE e FOXO3A (Brooks - Wilson Angela R. 2013).

ApoE è sintetizzata principalmente nel fegato, ma è stato trovato anche in altri tessuti come i reni, la milza e il cervello, nel quale è prodotta dagli astrociti e dalle cellule della microglia. È una componente di una lipoproteina capace di rendere i lipidi solubili nel circolo ematico e favorisce le interazioni tra neuroni e cellule gliali e la riparazione neuronale, consentendo il trasporto di lipidi all'interno del tessuto nervoso e lungo i nervi periferici. Si conoscono 3 varianti alleliche: ApoE ε2 (cys112, cys158), ApoE ε3 (cys112, arg158), e ApoE ε4 (arg112, arg158). In uno studio su 1.344 italiani sani, di età compresa tra 22 e 90 anni, la variante allelica ε4 del gene ApoE è stata trovata ad una frequenza più bassa in soggetti centenari rispetto a quella ε2, che al contrario è stata trovata ad una frequenza più alta in soggetti giovani e centenari. In questo contesto ApoE ε2 è un fattore "protettivo", mentre ApoE ε4 un fattore di "fragilità", infatti alcuni studi mostrano che è fortemente associato alle malattie cardiovascolari, al declino cognitivo associato all'età e alla demenza, in particolare la MA (Garatachea N, Marín PJ, Santos-Lozano A, Sanchis-Gomar F, Emanuele E, Lucia A. 2015; Brooks - Wilson Angela R. 2013). Diversi lavori hanno dimostrato che essa rallenta la clearance di Aβ, frammento amilodogenico, che avviene grazie al recettore LRP1 (LDLR related protein) altamente espresso nel cervello. I meccanismi attraverso cui attua questo processo sono diversi, cioè potrebbe sopprimere la clearance di Aβ (apoE4 > apoE3) competendo con essa per il legame con i recettori o trattenendo Aβ dalla sua clearance attraverso la barriera emato-encefalica (BEE), e inoltre favorisce la deposizione come placche amiloidi nello spazio extracellulare (Bu Guojun 2009; Kanekiyo Takahisa, Xu Huaxi, Bu Guojun 2014).

FOXO3A è un fattore di trascrizione (FT) che regola diversi processi biologici coinvolti nella normale omeostasi e sviluppo cellulare come apoptosi, proliferazione, progressione del ciclo cellulare, sopravvivenza e danno al DNA, ed inoltre risponde a diversi segnali di stress cellulare come radiazione UV e stress ossidativo (Liu Ying, Ao Xiang, Ding Wei, Ponnusamy Murugavel, Wu Wei, Hao Xiaodan, Yu Wanpeng, Wang Yifei, Li Peifeng & Wang Jianxun 2018); sembra quindi essere associato alla longevità nell'uomo. Il suo omologo, il gene Daf-16 in *C. elegans*, protegge le cellule dallo stress ossidativo favorendo l'aumento della durata della vita (Hansen M, Hsu AL, Dillin A, Kenyon C. 2005). Il principale regolatore di FOXO3A è il pathway di segnalazione IGF-1-insulina/PI3K/Akt, poiché inibisce FOXO3A che traslocherà, a causa della fosforilazione, nel citoplasma dove verrà ubiquitinato e degradato; quindi la riduzione della segnalazione di questo pathway regola positivamente questo FT. Numerosi studi hanno rilevato polimorfismi a singolo nucleotide specifici di FOXO3A associati alla longevità umana, in particolare l'allele G FOXO3A rs2802292 (G > T) (Aiello A, Accardi G, Candore G, Gambino CM, Mirisola M, Taormina G, Virruso C, Caruso C 2017).

Pochi studi hanno individuato altri geni associati alla longevità e all'invecchiamento in buona salute, e tra questi vi sono le sirtuine, classe di proteine ad attività enzimatica che operano come istone deacetilasi o mono-ribosiltransferasi. Le sirtuine mediano fenomeni quali l'invecchiamento, la regolazione della trascrizione, l'apoptosi, la resistenza allo stress e influiscono inoltre sull'efficienza energetica durante situazioni di restrizione calorica (RC) (Brooks-Wilson Angela R. 2013).

Uno studio ha mostrato che l'estensione della durata della vita nei lieviti tramite RC richiede Sirt2; invece nei mammiferi si è visto che Sirt1 è la principale sirtuina che media gli effetti metabolici della RC, anche se ancora non ci sono conferme di questo ruolo, e Sirt3, localizzata nei mitocondri, regola i livelli di ATP e l'attività del complesso I della catena di trasporto degli elettroni, e quindi può svolgere un ruolo nella riprogrammazione metabolica mediata dalla RC. Studi recenti hanno mostrato come la RC aumenti i livelli di Sirt3 nei mitocondri epatici, infatti topi privi di questa sirtuina mostrano i segni distintivi dei disturbi da ossidazione degli acidi grassi, indicando che è proprio a questo livello che Sirt3 svolge il suo ruolo. Inoltre sembra che questa sirtuina aumenti con la RC nei cardiomiociti di topi, e che la sua sovraespressione protegge le cellule dalla morte indotta da stress ossidativo (Someya Shinichi, Yu Wei, Hallows William C., Xu Jinze,. Vann James M, Leeuwenburgh Christiaan, Tanokura Masaru, Denu John M. and Prolla Tomas A. 2010).

2.2 Immunologia dell'invecchiamento

Insieme al progressivo invecchiamento della popolazione emerso nelle società benestanti, c'è stato un incremento delle malattie correlate all'età, causato non solo da fattori genetici ma anche da fattori ambientali. Gli errori a livello molecolare e cellulare che nel corso della vita si accumulano sono il risultato di influenze esogene ed endogene, che si rispecchiano nel diminuito funzionamento delle difese anti-invecchiamento. Tra queste, il sistema immunitario sembra essere particolarmente implicato nel processo dell'invecchiamento, poiché con l'avanzare dell'età questo subisce un rimodellamento in base al quale si adatta a decenni di esposizione ad agenti dannosi interni ed esterni, cambiando il microambiente dell'organismo. In particolare, i linfociti deputati a rispondere agli antigeni con cui l'organismo era già venuto in contatto, aumentano, restringendo lo spazio immunologico (Franceschi Claudio, MD 2007).

I soggetti anziani infatti sono maggiormente soggetti ai tumori, alle malattie infettive e ai fenomeni autoimmunitari, e non rispondono efficientemente ai vaccini, poiché vi è una riduzione della risposta immunitaria a nuovi antigeni, dato che più l'immunità acquisita che quella innata subiscono delle alterazioni. L'impatto dell'invecchiamento sulla funzione del sistema immunitario è chiamato immunosenescenza, che insieme ad un conseguente stato infiammatorio cronico di basso grado, partecipa all'insorgenza di malattie a carattere infettivo ed infiammatorio correlate all'età. Questa condizione è nota come "inflammaging" (Nevalainen Tapio, Autio Arttu, Kummola Laura, Salomaa Tanja, Junttila Ilkka, Jylhä Marja, and Hurme Mikko 2019). L'immunosenescenza viene caratterizzata da marcatori specifici, individuati grazie allo studio delle modifiche che subisce il sistema immunitario nel tempo, ed analizzare le modifiche che questi marcatori subiscono fornisce informazioni sull'invecchiamento e sull'aspettativa di vita.

In un contesto di immunosenescenza, la ridotta capacità di innescare una risposta immunitaria efficace dopo esposizione ai vaccini rappresenta una sfida per riacquistare la capacità di proteggersi da nuovi patogeni durante l'invecchiamento. La necessità è quindi quella di creare dei vaccini "potenziati" capaci di stimolare in maniera ottimale il sistema immunitario degli anziani, per ottenere un invecchiamento in salute (Pinti M, Appay V, Campisi J, Frasca D, Fülöp T, Sauce D, et al. 2016). Di recente è stato dimostrato che per ottenere questo effetto la stimolazione dei recettori Toll-like (TLR), tipici recettori dell'immunità innata che sono responsabili delle prime risposte immunitarie, con ligandi stimolatori può migliorare l'efficacia del vaccino grazie a meccanismi come l'attivazione delle cellule dell'immunità innata e la conseguente produzione di citochine infiammatorie (Lal H, Cunningham AL, Godeaux O, Chlibek R, Diez-Domingo J, Hwang SJ 2015). Questi recettori sono infatti espressi da polimorfonucleati, macrofagi, cellule dendritiche (DC),

cellule NK e linfociti T e B (Delneste Y, Beauvillain C, Jeannin P. 2007). Quindi una possibile strategia è l'attivazione di più TLR, usando adiuvanti che attivino in sinergia la risposta immunitaria. Gli adiuvanti sono molecole che possono promuovere l'attivazione di cellule che presentano l'antigene (APC), in particolare le DC, reclutandole al sito di vaccinazione in cui riconosceranno l'antigene e lo presenteranno alle cellule che attiveranno la risposta immunitaria (Collin M, Mc Govern N, Haniffa 2013). L'attivazione immunitaria mediata dalle cellule T negli anziani non è purtroppo efficace, e servono quindi degli adiuvanti, solitamente composti da diversi componenti come sali d'alluminio o emulsioni a base di squalene, immunostimolanti come forme di lipopolisaccaridi, più efficenti e che possano attivare le cellule T degli anziani, sia direttamente che attraverso le DC (Sharma S, Dominguez AL, Hoelzinger DB, Lustgarten J. 2008).

Un soggetto anziano ha infatti una bassa produzione di linfociti, poiché gli organi principali dove vengono prodotti, cioè il timo, i linfonodi, la milza ed il midollo osseo subiscono, già dall'età adulta, un'involuzione. È stato evidenziato, grazie a numerosi studi, una riduzione dei **linfociti T**, più precisamente dei linfociti T vergini, i quali non hanno ancora incontrato l'antigene, ed un incrementato numero dei linfociti T memoria oligoclonali (che rispondono solamente agli antigeni per i quali i recettori sono specifici), con conseguente restringimento del repertorio recettoriale. La molecola co-stimolatoria CD28 non viene espressa dai linfociti T, e si ha una ridotta espressione anche della molecola CD154 (CD40L) necessaria per la collaborazione con i linfociti B; ne risulta quindi una riduzione della funzione "helper". Al contrario, analizzando il sistema immunitario di centenari in salute, si nota che il compartimento di linfociti Helper (T $CD4^+$) e linfociti T Citotossici (T $CD8^+$) tende ad aumentare e ciò li rende un buon marker della longevità. Anche il compartimento dei **linfociti B $CD19^+$** subisce una variazione nell'anziano, e diversi lavori hanno dimostrato che questo è correlato ad un cattivo stato di salute in età avanzata. Fisiologicamente, le cellule B maturano nel midollo osseo, ed esprimono le immunoglobuline M e D (IgM e IgD) al primo contatto con l'antigene. Successivamente a questa risposta primaria, che permette di eliminare l'antigene, il livello degli anticorpi diminuisce, e quando si ha una seconda esposizione allo stesso antigene, si assiste alla risposta second0aria, in cui vengono prodotti altri anticorpi (switch isotipico) più affini all'antigene, cioè le IgA e le IgG. Nel caso di soggetti anziani, si osserva un mal funzionamento di questo processo e una perdita della risposta immune specifica umorale verso nuovi patogeni; in particolare si ha riduzione dei livelli sierici delle immunoglobuline prodotte, poiché probabilmente, anche in questo caso si ha una maggiore produzione dei linfociti B memoria, rispetto a quelli vergini, ma questo non è stato ancora definito con certezza. Sicuramente è confermata la minore produzione di Ig della risposta primaria rispetto a

quelli della risposta secondaria, poiché nel siero di soggetti anziani sono state ritrovate concentrazioni più elevate di IgG ed IgA (risposta secondaria), rispetto alle IgM ed IgD (risposta primaria). Il caso dei linfociti B è diverso da quello dei linfociti T, poiché se questi ultimi risultavano maggiormente espressi nei soggetti centenari rispetto agli anziani, la stessa cosa non si può dire per le cellule B. Purtroppo i numerosi studi effettuati non hanno dato risultati certi, ma analizzando il compartimento cellulare dei linfociti B dei figli dei centenari, sembra che questi abbiano livelli di linfociti B più simili ai soggetti giovani che ai coetanei che non hanno genitori centenari, probabilmente perché sono geneticamente avvantaggiati (Caruso, C. Candore, G. Castiglia, L. Rizzo, C. Vasto, S. 2009).

Infine, per quanto riguarda i **linfociti Natural Killer** (NK), diversi lavori hanno evidenziato che queste cellule diminuiscono nei soggetti anziani, i quali hanno un rischio di mortalità superiore rispetto a quelli con un elevato numero di linfociti NK. L'importanza di queste cellule dipende dalla loro attività fisiologica, infatti dato che sono deputati al controllo delle infezioni, la diminuzione del loro numero e della loro attività rende l'organismo meno resistente alle infezioni ed aumenta il rischio di mortalità. Analizzando il compartimento di queste cellule nei centenari si nota un aumento, tanto da rendere i linfociti NK (insieme ai linfociti T) dei marcatori dell'invecchiamento di successo. Gli studi fino ad ora effettuati suggeriscono che il sistema immune dei soggetti centenari, a differenza dei soggetti anziani, è complessivamente ben funzionante, nonostante si notino anche in essi le fisiologiche modificazioni presenti nell'anziano. Molti sono gli interventi proposti per restaurare un'efficiente risposta immunitaria, e quindi compensare la mancanza di linfociti nel sistema immune dell'anziano e diversi lavori hanno suggerito che l'attività fisica (moderata e costante) potrebbe potenziare la risposta immunitaria, come anche la dieta sembra avere un ruolo determinante in molte malattie infiammatorie legate all'età, tra cui ricordiamo l'aterosclerosi o la MA (Caruso C., Colonna Romano G. & Candore G. 2016).

Un altro componente del sistema immunitario implicato nel processo dell'invecchiamento e disturbi ad esso associati è quello delle **cellule della microglia**. Esse sono cellule della glia che si occupano della principale difesa immunitaria del SNC e, a differenza di tutte le altre cellule presenti nel SNC che sono di derivazione embrionale ectodermica, derivano dal foglietto mesodermico come i monociti/macrofagi. Quando gli agenti infettivi riescono ad attraversare la BEE, o sono introdotti direttamente nel cervello, le cellule della microglia reagiscono rapidamente, cambiano morfologia e si spostano verso il sito dell'infezione o lesione, dove incrementano l'infiammazione rilasciando sostanze infiammatorie, come citochine, chemochine e ROS, e distruggono, attraverso fagocitosi, gli agenti infettivi prima che danneggino il tessuto; a volte però anche le cellule cerebrali sane

vengono colpite, e ciò risulta in un risposta infiammatoria generale cronica (Varrassi G., Fusco M., Coaccioli S., Paladini A. 2015).

2.3 Aspetti ambientali dell'invecchiamento di successo

Una dieta, intesa come modello nutrizionale, associato allo stile di vita e alla cultura di un dato popolo, possono significativamente alterare l'effetto di mortalità e morbilità della patologia, meglio se associata a fattori quali pasti consumati in famiglia, socializzazione, adeguato riposo e attività fisica. La stessa longevità subisce un contributo efficace dallo stile di vita (Genovese A., Caporaso N., Villani V., Paduano A., Sacchi R. 2015).

Per studiare il fenotipo dei centenari, sono stati presi in considerazione dei dati su alcune zone del mondo, Okinawa in Giappone, Ogliastra in Sardegna, la penisola di Nicoya in Costa Rica e l'isola di Ikaria in Grecia definite zone blu (ZB), in cui i soggetti centenari erano presenti in grande numero, condividevano uno stile di vita e l'ambiente e godevano di buona salute; in queste aree infatti è diffuso l'uso di diete ipoproteiche tradizionali, come quella Mediterranea. Inoltre queste popolazioni praticano un'intensa attività fisica fino ad un'età avanzata, il cibo è prodotto localmente, hanno ridotti livelli di stress e forte sostegno dalle famiglie (Poulain Michel, Herm Anne and Pes Gianni 2013).

La dieta quindi sembra avere un ruolo importante nel diventare centenari, e uno studio ha suggerito che un regime alimentare sano, costituito principalmente da alimenti ricchi di sostanze con effetti biologici, può migliorare la salute e partecipare ad un invecchiamento efficiente; mentre un regime alimentare inappropriato, in termini di qualità e quantità, diventa un fattore di rischio per le malattie, soprattutto nei soggetti anziani (Puca Annibale Alessandro, Spinelli Chiara, Accardi Giulia, Villa Francesco, Caruso Calogero 2017). Bisogna evidenziare che la dieta Mediterranea è più uno stile di vita, che semplicemente un modello dietetico, ed è particolarmente diffusa in alcune isole del Mediterraneo, come Sardegna, Sicilia e Grecia; è basata su alimenti come frutta e verdura, cereali, carne, pesce, e l'olio extravergine d'oliva (EVO), il quale ha mostrato notevoli capacità nel prevenire patologie come quelle cardiovascolari, e il cancro (Filik L., Ozyilkan O. 2003; Kontogianni M.D., Panagiotakos D.B., C Chrysohoou., Pitsavos C., Zampelas A., Stefanadis C. 2007) grazie alla sua composizione in acidi grassi mono e di-insaturi e la rimanente parte insaponoificabile (2%) contenente squalene (50%), steroli, e polifenoli (Genovese A., Caporaso N., Villani V., Paduano A., Sacchi R. 2015); quindi risulta essere ipocalorica e ricca di fitonutrienti, sottoforma di antiossidanti e flavonoidi. Numerosi studi, infatti dimostrano che il regime dietetico mediterraneo, contribuisce ad una migliore condizione di salute, riducendo il rischio di sviluppare

malattie correlate all'età e, quindi, favorendo la longevità. È noto da diversi studi che i maggiori consumatori di alimenti che costituiscono la dieta mediterranea sono i centenari, la cui dieta tipica è costituita soprattutto da cereali integrali, latte e suoi derivati, uova, frutta e legumi. Comune è il consumo dell'olio EVO e di olive verdi e nere da tavola, ricche di acidi grassi essenziali, apporto principale dei polifenoli in esse contenute (Caruso C., Candore G. 2016).

Nonostante numerosi studi dimostrino la relazione tra genetica e ambiente per una longevità in salute, non è facile condurre gli studi sull'uomo, anche se la presenza di un numero molto elevato di centenari rispetto alla media della zona, in determinate e definite aree geografiche, le ZB, suggerisca che uno stile di vita specifico, che include ambiente e dieta, può favorire la longevità (Puca Annibale Alessandro, Spinelli Chiara, Accardi Giulia, Villa Francesco, Caruso Calogero 2017).

Sembra chiaro quindi che alcuni soggetti, definiti Long-Lived Individual (LLI), o centenari, possono raggiungere la longevità in salute grazie sia alla combinazione fortunata di polimorfismi che consente loro di avere una risposta efficace agli stress ambientali e non, che allo stile di vita, come la dieta, che risulta essere una componente che influenza il risultato (Passarino Giuseppe, De Rango Francesco, and Montesanto Alberto 2016).

3. Un esempio di invecchiamento senza successo: la malattia di Alzheimer

Tra le patologie più diffuse legate all'invecchiamento senza successo vi è la MA, una malattia neurodegenerativa progressiva e multifattoriale su base infiammatoria che colpisce le cellule del sistema nervoso centrale (SNC), in particolare le aree del cervello deputate alla memoria e alle funzioni cognitive, come l'ippocampo, causandone la degenerazione e la conseguente morte. I markers patologici principali sono le placche amiloidi e i grovigli neurofibrillari. Le placche amiloidi sono formazioni proteiche fibrillari di materiale insolubile che si depositano a livello extraneuronale e che comportano infiammazione della zona cerebrale interessata con conseguente morte per apoptosi. La formazione delle placche si deve principalmente all'accumulo ed alla conseguente aggregazione del peptide Aβ, un frammento proteico costituito da circa 42 amminoacidi che deriva da un taglio proteolitico della proteina di membrana APP (proteina precursore dell'amiloide) da parte di due secretasi (β e γ) (Haass Christian and Selkoe Dennis J. 2007). Questo peptide subisce un misfolding trasformando la sua struttura da alfa-elica a foglietti-beta che porta alla formazione di aggregati, fibrille, placche. I grovigli neurofibrillari sono invece depositi intracellulari di materiale proteico fibrillare costituito dalla proteina *tau*. Questa proteina è associata ai microtubuli e svolge funzione di stabilizzazione della struttura citoscheletrica

microtubulare (Dixit Ram, Ross Jennifer L., Goldman Yale E., Holzbaur Erika L. F. 2008.). In condizioni patologiche la proteina *tau* viene iperfosforilata (Terwl D et al. 2002; Flament S., Delacourte A., and Mann D.M.A. 1990) portando alla disgregazione della struttura dei microtubuli i quali formeranno strutture fibrillari e quindi i grovigli. Ad innescare i processi neurodegenerativi non sono solo le placche amiloidi extracellulari, ma anche piccoli aggregati di Aβ che entrano all'interno della cellula e provocano diverse alterazioni come stress ossidativo, disfunzione mitocondriale e del reticolo endoplasmatico, tutti meccanismi che portano anch'essi a morte per apoptosi. Il processo di formazione delle fibrille amiloidi, o fibrillogenesi, viene chiamato polimerizzazione nucleazione-dipendente perché sembra seguire le fasi di una cristallizzazione, infatti nelle cinetiche di aggregazione si presentano tre fasi:

- fase Lag che è il tempo richiesto per la formazione del nucleo (oligomeri), un processo sfavorito termodinamicamente perché consiste nel passaggio dalla struttura nativa alla struttura misfolded delle proteine e l'aggregazione di queste;
- fase esponenziale di crescita in cui più monomeri di proteina contattano il nucleo che si è formato in più punti portando al processo di aggregazione proteica;
- fase di plateau in cui non si ha più formazione di nuovi aggregati.

L'accumulo di proteine sottoforma di aggregati amiloidi è una caratteristica di diverse patologie, non solo di tipo neurodegenerativo (Calamai M, Canale C, Relini A, Stefani M, Chiti F, Dobson CM 2005). Studi in vivo hanno dimostrato che danni significativi a tessuti e sintomi clinici appaiono prima che sia individuato alcun aggregato, il che implica la presenza di un intermedio nel pathway amiloidogenico che potrebbe essere la causa della patogenicità (Zerovnik E, 2002). A conferma di ciò, placche amiloidi sono state trovate anche in individui che non mostrano i sintomi clinici della MA (Katzman R., Terry R., De Teresa R., Brown T., Davies P., Fuld P., Renbing X., Peck A. 1988).

Le cause che portano all'insorgenza della patologia sono varie, a causa del suo carattere multifattoriale. Nella maggioranza dei casi è sporadica, non ereditaria, mentre in una piccola percentuale è ereditaria o familiare. La causa purtroppo non è nota ma si pensa che fattori ambientali ed una predisposizione genetica interagiscano determinando la malattia (Lanoiselée Hélène-Marie, Gaël Nicolas et al, and collaborators of the CNR-MAJ project – 2017); si pensa che siano coinvolti fattori genetici, che hanno un ruolo determinante in quei soggetti che presentano i sintomi della patologia in giovane età e hanno già una mutazione ereditaria secondaria a livello dei geni implicati nella patologia, come l'APP, le Preseniline 1 e 2 (PSEN1 e PSEN2) e l'APOE (Lanoiselée Hélène-Marie, Gaël Nicolas et al, and collaborators of the CNR-MAJ project – 2017),

la cui variante allelica APOE4 influenza il metabolismo del colesterolo e quindi lo stato e la funzione delle membrane plasmatiche (Maurer K., Hoyer S. 2006). Diverse evidenze dimostrano il coinvolgimento dell'acetil-colina (ACh) nella patogenesi della MA. I neuroni comunicano tra di loro grazie ai neurotrasmettitori, molecole racchiuse all'interno di vescicole che veicolano le informazioni attraverso la trasmissione sinaptica. Nel 1983 Coyle e collaboratori notarono infatti che i neuroni che rilasciano ACh degeneravano selettivamente nei cervelli dei soggetti malati, influenzando le aree deputate alle funzioni cognitive, in particolare memoria e apprendimento (Grossberg S 2017). Inoltre alcune evidenze suggeriscono il coinvolgimento dell'enzima che degrada l'ACh, l'acetil-colina esterasi (AChE), nella patogenesi della MA, poiché si è visto che interagisce con il peptide Aβ, favorendo la formazione di fibrille amiloidi, e riduce l'assorbimento della colina a livello dell'ippocampo (Ferreira-Vieira Talita H., Guimaraes Isabella M., Silva Flavia R., and Ribeiro Fabiola M.– 2016).

Studi clinici dimostrano che anche lo stato infiammatorio potrebbe avere un ruolo importante nello sviluppo e nel decorso della MA, poichè risulta anomalo nei soggetti affetti dalla malattia. Sono stati trovati infatti livelli più alti di citochine infiammatorie, come l'interleuchina-1β (IL-1β), l'interleuchina-6 (IL-6) e il fattore alfa di necrosi tumorale (TNF-α), rispetto a soggetti sani, ed in particolare si è osservato che solo nelle zone del cervello colpite dalla malattia c'era un maggiore rilascio di citochine infiammatorie da parte delle cellule della microglia (Varrassi G., Fusco M., Coaccioli S., Paladini A. 2015); probabilmente perché l'accumulo e l'aggregazione del peptide Aβ stimola eccessivamente le cellule della microglia, causandone l'iperattività e producendo grandi quantità di citochine, che oltre a colpire i patogeni, danneggiano anche i neuroni sani. Questo crea un circolo vizioso, per cui più neuroni vengono danneggiati, più le microglia si attivano, le quali producono più citochine infiammatorie, creando un'infiammazione di basso grado e una conseguente neurodegenerazione (Grammas Paula, Ovase Roma 2001).

Fisiologicamente, con l'avanzare dell'età, le cellule della microglia sono meno responsive e la risposta agli stimoli che esse attivano è più intensa e duratura, provocando neuroinfiammazione persistente e danno neuronale irreversibile. Ciò porta inevitabilmente al processo di neurodegenerazione, poiché lo stato infiammatorio cronico di basso grado, può danneggiare le popolazioni neuronali responsabili delle più comuni malattie neurodegenerative, oltre che provocare danni a livello neuroni periferici (Varrassi G, Fusco M, Coaccioli S, Paladini A. 2015).

3.1 Terapie preventive

3.1.1 L'alimentazione (la Dieta Mediterranea)

È stato dimostrato che per intervenire sul decorso clinico della MA e sul rallentamento dell'invecchiamento una possibile soluzione potrebbe essere una corretta alimentazione, nonostante non esistano delle vere e proprie cure. I componenti di una dieta ipocalorica e ricca di fitonutrienti, come quella "Mediterranea", potrebbero infatti intervenire in questo processo (Solfrizzi V, Capurso C, D'Introno A, Colacicco AM, Santamato A, Ranieri M, Fiore P, Capurso A, Panza F. 2008).

Diversi lavori hanno evidenziato che il peptide Aβ è implicato nell'infiammazione, poiché è stato dimostrato che può attivare una risposta infiammatoria umorale e cellulo-mediata. Molte analisi hanno mostrato la presenza, nelle placche amiloidi, di cellule della microglia, e rivelato positività per la citochina infiammatoria IL-1. Quindi si potrebbero mettere a punto dei trattamenti anti-infiammatori (V. Marigliano, G. Viscogliosi 2013); inoltre il tessuto cerebrale è particolarmente vulnerabile allo stress ossidativo, a causa del suo elevato metabolismo del glucosio e del basso livello di antiossidanti (Olanow CW 1993), e l'accumulo del peptide Aβ, sia sottoforma di placche amiloidi presenti extracellularmente nel cervello dei pazienti affetti da MA che a livello intracellulare, è responsabile della formazione di ROS e stress ossidativo e si pensa sia uno dei principali meccanismi che attivano la cascata patogenica che porta alla malattia (Hardy J., Selkoe D.J., 2002). L'utilizzo, quindi, di molecole antiossidanti potrebbe essere un'efficace strategia terapeutica.

Diversi lavori hanno suggerito che una corretta alimentazione può aiutare nella prevenzione e decorso di diverse patologie tra cui quelle neurodegenerative, ed in particolare sembra che i componenti della dieta Mediterranea, possono sinergicamente intervenire in questo processo (Solfrizzi V, Capurso C, D'Introno A, Colacicco AM, Santamato A, Ranieri M, Fiore P, Capurso A, Panza F. 2008). La dieta Mediterranea è un modello nutrizionale ispirato a quelli tradizionali dei popoli del bacino mediterraneo ed è basata su alimenti come frutta e verdura, cereali, carne, pesce, e soprattutto l'olio EVO; quindi risulta essere ipocalorica e ricca di fitonutrienti. Inoltre è stato dimostrato che il regime dietetico mediterraneo, contribuisce ad una migliore condizione di salute, riducendo il rischio di sviluppo di malattie correlate all'età e, quindi, favorendo la longevità (Caruso C., Candore G. 2016).

Riguardo alla MA, l'olio EVO ha un alto contenuto in acidi grassi poli-insaturi, come omega-3, e sembra che questo influenzi il pathway non-amilogenico del processamento dell'APP, che porta alla mancata formazione del peptide Aβ (Van der Beek EM, Kamphuis PJ Eur 2008). Inoltre, come già detto in precedenza, è ricco di polifenoli, i quali hanno attività antiossidante (Genovese A.,

Caporaso N., Villani V., Paduano A., Sacchi R. 2015). Sono stati presi in considerazione anche polifenoli come il resveratrolo, prodotto da mirtilli, pinoli e uva, che li protegge dalle infezioni fungine e dalle radiazioni ultraviolette, la curcumina, polifenolo bioattivo sintetizzato dalla pianta *Curcuma longa*, e il tirosolo, principale fenolo presente nell'olio EVO (Sadowska-Bartosz Izabela and Bartosz Grzegorz 2014).

È proprio grazie a questi composti antiossidanti che la dieta Mediterranea, insieme ad uno stile di vita appropriato, risulta essere un trattamento promettente per consentire l'invecchiamento in buona salute (Sadowska-Bartosz Izabela and Bartosz Grzegorz 2014).

3.1.2 *Gli antiossidanti*

Dal punto di vista chimico, gli antiossidanti sono agenti che prevengono o rallentano il fenomeno dell'ossidazione, mantenendo il bilancio redox cellulare. Se però questo fenomeno viene protratto e non contrastato, nel tempo può trasformarsi in stress ossidativo. Questo processo utilizza l'ossigeno, e nonostante sia necessario per l'utilizzo di alcune molecole, è caratterizzato dalla formazione di un'elevata concentrazione di prodotti secondari, i ROS. La principale reazione endogena che produce ROS avviene a livello della catena di trasporto degli elettroni nel mitocondrio. Durante il trasferimento di elettroni si ha la fuoriuscita di intermedi, i radicali liberi, che, diffondendo nell'ambiente cellulare, reagiranno con qualsiasi molecola si trovi nelle loro prossimità (Testa Roberto, Olivieri Fabiola, Ceriello Antonio, La Sala Lucia 2011). Sono infatti necessari per mantenere l'omeostasi cellulare, ed i processi in cui vengono prodotti sono diversi, tra cui quello messo in atto dalle cellule fagocitiche che producono ROS in risposta ad un meccanismo di difesa dell'ospite per combattere le infezioni; determinati fattori di crescita che producono ROS citoplasmatici dopo stimolazione per regolare la risposta proliferativa; o ancora i mitocondri che, durante situazioni di stress metabolico, producono ROS come molecole di segnalazione (Finkel Toren & Holbrook Nikki J. 2000). Se però risultano in eccesso, danneggiano e compromettono la funzione delle cellule e dei diversi organuli con cui vengono a contatto. I principali componenti cellulari danneggiati da un eccesso di radicali liberi sono DNA, proteine e lipidi, e ciò comporta l'alterazione delle membrane cellulari e del materiale genetico. Inoltre si possono scatenare reazioni a catena poiché i bersagli dei radicali liberi possono essere trasformati a loro volta in altri prodotti altamente reattivi provocando estesi danni nella cellula (Testa Roberto, Olivieri Fabiola, Ceriello Antonio, La Sala Lucia 2011). La formazione dei radicali liberi in eccesso è correlata anche a stimoli ambientali, come una prolungata esposizione ai raggi UV, agenti chemioterapici e persino fattori di crescita, e questo può perturbare il normale bilancio redox delle cellule favorendo lo stress

ossidativo. A contrastare la grande produzione di radicali liberi vi è un intricato sistema di antiossidanti, agenti riducenti che, intervenendo tramite un processo di auto ossidazione, neutralizzano i radicali liberi proteggendo la cellula da ulteriori danni. Sono quindi scavenger che hanno il compito di ri-equilibrare lo stato redox cellulare generando molecole utilizzabili dalla cellula. Quando però la produzione di radicali liberi supera la capacità di riduzione degli scavenger, i ROS vengono veicolati verso altre reazioni producendone di altri e aumentando la concentrazione di radicali liberi circolanti nella cellula, danneggiandola provocando due effetti importanti: uno è quello di danneggiare diversi componenti cellulari, l'altro è quello di innescare l'attivazione di specifiche vie di segnalazione, effetti che possono influenzare numerosi processi cellulari legati all'invecchiamento e allo sviluppo di malattie legate all'età (Finkel Toren & Holbrook Nikki J. 2000).

Gli enzimi antiossidanti più importanti sono la superossido dismutasi (SOD), la catalasi e la glutatione perossidasi. La SOD accelera la conversione del superossido in perossido di idrogeno, mentre la catalasi e la glutatione perossidasi convertono il perossido di idrogeno in acqua.

Risulta quindi necessario assumere una ricca dose di antiossidanti per prevenire questo processo dannoso di ossidazione. Alla base della teoria formulata da Harman vi è l'idea che i radicali liberi e altri ROS, formati durante diversi processi metabolici, e derivanti dall'azione di vari fattori esogeni, danneggino le biomolecole, e l'accumulo di questo danno, insieme alla ridotta capacità del proteasoma di degradare le proteine danneggiate, e ai difetti dei sistemi di riparazione del DNA, sono le cause dell'invecchiamento e delle malattie legate all'età. Quindi, gli antiossidanti dovrebbero rallentare l'invecchiamento e prolungare la durata della vita, ma a questa affermazione è difficile dare ancora oggi una conferma (Testa Roberto, Olivieri Fabiola, Ceriello Antonio, La Sala Lucia 2011).

L'invecchiamento ha la caratteristica di avere perso la capacità di adattarsi all'ambiente, la precisione nella riparazione e una scarsa manutenzione dei sistemi di regolazione dell'organismo, e ciò comporta modifiche che vanno dalla perdita dell'udito o l'aumento dei tempi di reazione, a modifiche più pericolose, come cambiamenti molecolari che rendono vulnerabili nei confronti di malattie come il cancro o di patologie associate all'età come la MA (Caruso C., Candore G. 2016).

Diversi studi hanno preso in considerazione la teoria secondo cui per contrastare gli effetti negativi dell'invecchiamento bisogna integrare con antiossidanti, ad esempio composti sintetici come le vitamine C ed E. La vitamina C, o acido ascorbico, è il principale antiossidante idrofilo e inibisce la perossidazione lipidica. A livello delle membrane riduce i radicali α-tocoferossilici e le LDL per rigenerare α-tocoferolo (vitamina E) e inibire così la propagazione di radicali liberi. La vitamina E,

l'α-tocoferolo, è invece il principale antiossidante idrofobo presente nelle membrane cellulari e nelle lipoproteine circolanti, e la sua funzione è supportata dalla vitamina C che ne promuove la rigenerazione; alcuni studi hanno inoltre dimostrato che la vitamina E è anche in grado di prevenire l'aterosclerosi tramite la modificazione ossidativa (Sadowska-Bartosz Izabela and Bartosz Grzegorz 2014).

3.1.3 *L'acido caffeico*

Tra i vari polifenoli contenuti nella dieta mediterranea, studi in vitro hanno dimostrato che l'acido caffeico (AC) è quello con i maggiori effetti anti-infiammatori ed antiossidanti. Questo polifenolo, derivante dall'acido cinnamico a 9 atomi di carbonio, è contenuto, così come nell'olio EVO, anche nel caffè verde, nelle patate, nel grano e nelle verdure come le carote, cicoria, il carciofo, i piselli e le fragole, e nella frutta come le mele, le prugne, le pere ed i mirtilli. Inoltre è presente nell'erba angelica, nell'arnica, nella bardana, nella fumaria, nella melissa e nel propoli, in elevate percentuali. Per il suo ruolo come antiossidante, l'AC è un candidato promettente nel trattamento delle patologie neurodegenerative come la MA, il morbo di Parkinson, e l'ischemia cerebrale (Sul D, Kim HS, Lee D, Joo SS, Hwang KW, Park SY 2009; Magnani C, Isaac V. L. B., Correa M. A. and Salgado H. R. N. 2014; Jeong CH, Jeong HR, Choi GN, Kim DO, Lee U, Heo HJ 2011).

Inoltre in letteratura è riportata la capacità dell'AC di disaggregare, anche in associazione ad altre molecole, strutture fibrillari, come il lisozima o l'α-synucleina (Fazili Naveed Ahmad, Naeem Aabgeena 2015; Gazova Zuzana, Siposova Katarina, Kurin Elena, Mucˇaji Pavel, Nagy Milan 2013). Tuttavia, è stato dimostrato che le funzionalità antiossidanti ed anti-infiammatorie sono date, non dall'azione del singolo, ma dalla sinergia tra i polifenoli quando presenti contemporaneamente (Schwingshackl Lukas, Morze Jakub, Hoffmann Georg 2019).

4. Scopo della tesi

In un contesto di invecchiamento di successo, il ruolo dell'ambiente è sicuramente importante. Gli stili di vita sono determinanti nel contesto della longevità ed in modo particolare la nutrizione riveste un ruolo preventivo, oramai universalmente riconosciuto, nell'ambito delle patologie croniche età correlate.

Nell'ambito del mio progetto di dottorato ho evidenziato come la nutrizione, in termini di macro e micro-nutrienti, sia un fattore comune nei soggetti che invecchiano con successo. Nel nostro contesto l'analisi dei soggetti Long-Lived Individual (LLI) o centenari, la cui dieta è prevalentemente di tipo mediterraneo ha portato in rilievo un regime nutrizionale costituito da piccole quantità di carboidrati e carne rossa, ricca di alimenti antiossidanti e a basso indice glicemico, come frutta e verdura di stagione, pesce e olio EVO, il quale ricopre un importante ruolo, poiché è ricco di polifenoli, i quali gli conferiscono notevoli capacità antiossidanti; le principali funzioni sono proteggere l'integrità delle membrane cellulari e contrastare l'alterazione delle funzioni cognitive legate all'invecchiamento (Sadowska-Bartosz Izabela and Bartosz Grzegorz 2014). In particolare nella mia tesi valuterò differenti aspetti sotto il profilo nutrizionale, geneticoimmunologico ed ematochimico tra soggetti centenari, i quali invecchiano con successo, e soggetti che invecchiano senza successo, che sono maggiormente suscettibili alle patologie età-correlate. A tal proposito, verranno effettuate analisi sulla popolazione di alcune città dell'entroterra siciliano, in cui la percentuale di centenari è elevato, valutando diverse variabili, come il sesso, l'istruzione, lo stile di vita e la dieta, ed il profilo ematochimico; verranno anche analizzate specifiche metallo-proteasi della matrice, MMP-2, che degrada la fibronectina e la laminina, ed MMP-9, che degrada il collagene di tipo IV e V (Lenz Oliver, Elliot Sharon J. and Stetler-Stevenson William G. 2000), poiché correlate all'infiammazione; in generale queste MMP sono coinvolte nel rimodellamento della matrice durante l'invecchiamento e verrà valutata la loro attività nel siero dei soggetti longevi. Inoltre per studiare quali terapie sviluppare e quali comportamenti assumere per rallentare o ritardare l'invecchiamento, e ridurre o prevenire la fragilità e le disabilità degli anziani verrà valutata l'attività antiossidante ed antiinfiammatoria, in particolari condizioni, di polifenoli naturali (come quelli contenuti nella dieta mediterranea) presenti nell'estratto di un organismo unicellulare come il cianobatterio Aphanizomenon flos-acque (AFA) una particolare microalga con provate proprietà nutrizionali (Kushak R. I., Drapeau C., and Winter H. D. 2001; Nuzzo D., Presti G., Picone P., Galizzi G., Gulotta E., Giuliano S., Mannino C., Gambino V., Scoglio S., and Di Carlo M. 2018).

5. Manuscripts

Genotypic and Phenotypic Aspects of Longevity: Results from a Sicilian Survey and implication for the Prevention and Treatment of Age-related Diseases

Aging is an inevitable natural process accompanied by a progressive inability to adapt to environmental stress. This involves the reduction of the organism's performance, which uses energy reserves during life to adapt to external and internal changes, increasing the risk of mortality. New studies have shown that this process of functional decline can be modified to delay the onset of age-related diseases [1, 2]. It is important to highlight the aging process is different for every individual, since there are subjects who undergo this process in health, and others that are instead predisposed to various pathologies related to age; we speak of successful and unsuccessful aging respectively, which is linked to neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), metabolic syndrome (MS), type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVD) and cancer. On the other hand, those who age in good health have good physical and mental conditions, are not affected by these diseases and in most cases live long, usually more than 100. These are the centenarians who, despite having the genetic and environmental aspect that distinguishes them from each other, represent the best model for studying longevity in health [1, 3].

Studies on centenarians have suggested that their genetic and epigenetic characteristics, and their positive lifestyles allow them to respond well to stress and in case to repair damage [4], but is not yet possible to understand what is the main feature which allows you to age successfully. The motivation is that the communities with a high number of centenarians present in the world are few, and in 2004 Poulain identified five of them and called them blue zones (BZ) [5]. These groups, which are found in Sardinia (Italy), Ikaria (Greece), Okinawa (Japan), in the Nicoya peninsula (Costa Rica) and in California, in the city of Loma Linda where Seventh-day Adventists live [6], they share lifestyle and environment, but what makes them different is the interaction that the latter has with their different genetic combinations.

The modern era has led, in western countries, to the improvement of hygienic conditions and quality of life, including nutrition and prevention medicine, and this has made it possible to increase the life span to 100 years, and therefore an increase in the elderly population , composed of both healthy nonagerians and centenarians and subjects predisposed to the onset of age-related diseases [1, 7].

We need to clarify how certain subjects can develop diseases in old age and how others can reach that same age, reach it and overcome 100 years, without developing any disease. By studying the biology of these latter subjects we could try to develop prevention therapies by replicating their characteristics in the average person.

Based on previous studies, we analyzed the population of some cities in the Sicilian hinterland, which have a high rate of centenarians, to study genetic and environmental models linked to longevity [8, 9]; and in this work we present the preliminary results obtained from an ongoing study on longevity carried out on sicilian long-lived individuals (LLI) also based on data in the literature, for the prevention and/or treatment of age-related diseases.

In the individuals analyzed in this study the expression of the APO ε4 gene, a negative candidate in longevity, APOE ε2, positive candidate, and the G FOXO3A rs2802292 (G> T) allele associated with longevity were also evaluated. The number of subjects analyzed is not sufficient to make the association of APOE ε2 and allele G FOXO3A rs2802292 (G> T) with longevity, while APO ε4 was found to be little expressed in centenarian subjects. In our study we also evaluated the increase in a subgroup of Natural Killer cells $CD56^{dim}CD16^{+}$, characterized by increased cytotoxicity, which are more expressed in the centenarians than in the elderly (non-centenarians); the same is true for T cells, that showed an increase when compared with ederly. This increase does not occur in the B cell compartment, which are decreased.

Biogerontology has been committed in recent decades to study interventions to slow aging in humans, and has suggested that possible solutions could be different types of dietary restrictions associated with exercise, thus maintaining body weight and hematochemical parameters in normal ranges. Unlike what happens with animal models, it is difficult to verify the efficiency of therapeutic interventions on humans because so many biomarkers have not been identified to characterize aging in health, especially for the centenarians.

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228 *Current Pharmaceutical Design*, 2019, *25*, 228-235

REVIEW ARTICLE

Genotypic and Phenotypic Aspects of Longevity: Results from a Sicilian Survey and Implication for the Prevention and Treatment of Age-related Diseases

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ARTICLE HISTORY

Received: February 13, 2019
Accepted: March 8, 2019

DOI:
10.2174/1381612825666190313115233

Abstract: ***Background:*** It is well known that long living individuals are a model of successful ageing and that the identification of both genetic variants and environmental factors that predispose to a long and healthy life is of tremendous interest for translational medicine.

Methods: We present the preliminary findings obtained from an ongoing study on longevity conducted on a sample of Sicilian long-lived individuals.

Results: We review the characteristics of longevity in Sicily, taking into account lifestyle, environment, genetics, hematochemical values, body composition and immunophenotype. In addition, we discuss the possible implications of our data for the prevention and/or treatment of age-related diseases.

Conclusion: As widely discussed in this review, the explanation of the role of genetics and lifestyle in longevity can provide important information on how to develop drugs and/or behaviours that can slow down or delay ageing. Thus, it will be possible to understand, through a "positive biology" approach, how to prevent and/or reduce elderly frailty and disability.

Keywords: Age-related diseases, body composition, genetics, immunosenescence, longevity, Sicily.

1. INTRODUCTION

Ageing leads to the incapacity to adapt to stress and to a decline in functional capacity. The ageing condition itself changes the performance of physiological systems and increases the susceptibility to death but new pieces of evidence suggest that the process is modifiable, thereby making it possible to delay age-related diseases [1, 2].

There are two main ways to become old, or having good and functioning health (successful ageing), either being with disability and age-related diseases (unsuccessful ageing). The latter concerns old people who develop one or more age-related diseases: neurodegenerative such as Alzheimer's disease (AD) and Parkinson's, metabolic diseases such as metabolic syndrome (MS), type 2 diabetes mellitus (T2DM), cardiovascular (CVDs) and cancer. Centenarians achieve successful ageing since most subjects reach the age of 100 or more without any age-related diseases, in good physical and mental condition. They represent the best model to study successful ageing and longevity although they have different genetic features and lifestyle [1, 3].

Centenarians are able to repair damages and respond well to stressors. That is due to a combination of "positive features", *i.e.* genetic and epigenetic characteristics and a favourable environment with social involvement [4]. Although many studies exist on centenarians, it has not yet been possible to identify the longevity signature. One of the reasons is that communities with a high number of centenarians are relatively rare in the world. These are five worldwide, and in 2004, Poulain *et al*. called them as blue zones (BZs) [5]. They are defined as a rather limited and homogenous geographical area where the population shares the same lifestyle and environment and its longevity has been proved to be exceptionally high. The validated BZs are so far in Sardinia (Italy), Ikaria (Greece), Okinawa (Japan), in Nicoya Peninsula (Costa Rica), and in California, the Loma Linda town where the Seventh-day Adventists live [6].

Such a wide distribution makes it difficult to repeat the data. This is probably due to the different genetic combinations, the genetic mosaic, and the interaction with the environment.

The increased ability to reach the age of 100 in Westernized countries over the last years and the reduction in the overall mortality clearly reflect the improvement of hygienic condition and quality of life, including the attention to diet and the advent of preventive medicine. The increased average life-span led to an increase in the number of nonagenarians and centenarians worldwide. It has also increased the number of elderly affected by age-related diseases [1, 7].

While the reason why human beings can develop a disease in late-life is a puzzle that is worth seriously studying, an even more fascinating enigma lies in understanding how some subjects are able to live for a century without a disease. For the prevention of

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1381-6128/19 $58.00+.00

age-related diseases, it would be interesting to develop medical interventions that would allow replicating the biology of centenarians in the average person.

In the last years, we have surveyed the population of some Sicilian inland towns, characterized by a high rate of centenarians to investigate the genetic and environmental patterns related to longevity [8, 9].

We present the preliminary findings obtained from an ongoing study on longevity conducted on a sample of Sicilian long-living individuals (LLIs), i.e. individuals that belong to the 5 percentile of the survival curve. We discuss data according to the available literature, for their possible implication for the prevention and/or treatment of age-related diseases.

2. LONGEVITY IN SICILY: AN OVERVIEW

A demographic survey performed in 2007 clearly showed that some small municipalities, with a low number of inhabitants, of unpolluted inland areas of Sicily were characterized by a reduced men mortality at the age of 80 and over. This implied an increase in the number of male centenarians in those zones. The rate of longevity was not increased in women, likely because of different conditions and educational level, implying different access to prevention or to health facilities [10].

A few years later, we conducted a pilot study in the Sicani Mountains, a chain of mountains sited in the Sicilian inland, where, at that time, the number of centenarians (people aged 100 to 107) per inhabitants was up to four-fold higher (10.37/10, 000) than the one documented for Italy (2.4/10, 000). In particular, male centenarians were 14 times more than in Italy (12.46/10, 000 inhabitants vs. 0.89/10,000 inhabitants). The nutritional assessment showed high adherence to the Mediterranean diet (MedDiet), with low glycaemic index and low animal protein intake. The meals were frugal and three times a day, comprising a small amount of carbohydrates and meat, while an abundant consumption of seasonal fruits and vegetables dressed with homemade extra virgin olive oil was reported. This is not surprising because, since the beginning of the 1990s, increasing evidence suggest that the MedDiet has a beneficial influence on several diseases (such as CVDs, MS, T2DM, atherosclerosis, cancer), favouring health and longevity at the same time. The evaluation of daily living activities, conducted on these subjects through instrumental activities of daily living, and the mini-mental state examination highlighted, for both genders, a good level of independence and cognitive functioning. The hematochemical profile and the evaluation of the main risk factors for age-related diseases confirmed their good health status, although a reduction in sight and hearing was measured. The importance of social engagement and light physical activities has to be highlighted, due to the mountainous region surrounding the village [8, 9, 11].

We conducted a survey in a different population of Sicily in mountains, including some villages in the Palermo province part of the Madonie Mountains, which partly confirmed the results obtained in Sicani Mountains. We documented the presence of 4.33 centenarians/10,000 inhabitants in the Madonie villages, as compared to 2.6 centenarians/10,000 in Italy. High rate of young people's emigration over time should be considered, thus to ascertain the true longevity rate, it is necessary to study the birth and death records. We checked the death age of about 37,000 newborns between 1881 and 1917 in a sample of five small municipalities (Petralia Soprana, Petralia Sottana, Geraci Siculo, Bompietro, Castellana Sicula) located in Madonie area. About 1,700 individuals died at the age of 90 years and over and about one hundred were centenarians. Therefore, the probability to reach 90 and 100 years old was of 4.6% and 0.22% respectively (Poulain, Busetta and Caruso unpublished observations). We cannot conclude that these small towns exhibit an exceptional level of longevity as that observed in Sardinia. However, the populations of these municipalities are experiencing higher longevity as compared to other places in Sicily and in Italy.

To better investigate the epidemiological context, we compared the Standardized Mortality Ratio (SMR) with respect to whole Sicily, of the two Palermo province Districts, namely Cefalù and Petralia Sottana, including the municipalities of interest, compared to the urban area of the province (Palermo city). In Table **1**, we present the ratios between the observed number of deaths in the population under study and the expected number of deaths, based on the age- and sex-specific rates of the population of Sicily at the last census available [12]. Estimates are provided separately for males and females. To this end, the regional Death Nominal Causes Register has been accessed with regard to the last available period 2004-2011 [13]. The SMRs estimates reported for Cefalù District and Petralia Sottana District were observed to be systematically lower than those reported for Palermo city. In particular, Cefalù District and Petralia Sottana District document statistically significant lower mortality rates for all causes of death and for all other selected death causes, except for diabetes mellitus in Petralia Sottana District and for lung cancer in Cefalù District, but only in females. Furthermore, Palermo City has a statistically higher mortality rate for all causes of death, for respiratory diseases and for lung cancer both in males and females, and for diabetes mellitus, only in males. Conversely, we documented a statistically lower mortality rate for circulatory system diseases in females. These mortality outcomes confirm that the Madonie municipalities belong to a zone with a high rate of longevity.

The ongoing survey based on preliminary analysis on the nutritional habits confirmed the possible association of longevity phenotype with a Mediterranean lifestyle but not during ageing or extreme longevity, rather during young age. In fact, long-lived people used to follow MedDiet but not during the ageing period, suggesting an interesting and effective role of epigenetics in the attainment of longevity.

Thus, longevity concerns people living in small villages, without pollution, likely because of different working conditions, different lifestyles, *i.e.* reduced smoking and alcohol abuse and MedDiet (presently or in the past). The reason because longevity has been observed particularly in small municipalities is not surprising. Individuals with greater access to social support and family network have better health care and lower levels of mortality, particularly when there are adult daughters in the family. All the LLIs studied in these two surveys lived on the mountains in multi-storey homes, thus throughout their life, they were constantly engaged in physical exercise. This is in agreement with the evidence obtained from studies that support the positive association between increased levels of physical activity, exercise participation and improved health in older adults [14]. Most LLIs individuals of the BZs in Sardinia, Costa Rica and Ikaria and in the Italian region of Cilento live on the mountains, with an environment characterized by a low degree of pollution. The average high slope of the terrain, quite common in the mountain zone, should be responsible for a long life with intense physical activity, hence improved cardio-respiratory fitness of the inhabitants. These populations have preserved a traditional lifestyle with an ideal social context as habitat, economic activity, intensive community and family support for their elderly, as well as the consumption of locally produced food. This has likely facilitated the accumulation of ideal conditions capable of limiting the factors that negatively affect health in the Western world [5]. As pointed out by Poulain *et al.* [5], the emergence of LLI phenotypes should be due to a balance between the benefits of traditional lifestyle and those of modernity as the increase in wealth and in better medical care.

In addition, prolonged and short repeated intense exercise can lead to significant reductions in human skeletal muscle mtDNA content, which might function as a signal stimulating mitochondrial

Table 1. Comparison of Standardized Mortality Ratio (SMR) for all causes and specified causes in males and females between Palermo City, Cefalù District and Petralia Sottana District, period 2004-201 (respect to whole Sicily).

MORTALITY		Cefalù District	Petralia Sottana District	Palermo City
		SMR (95%C.I.)	SMR (95%C.I.)	SMR (95%C.I.)
For All Causes	Male	89.4 (85.5;93.4)	85.1 (80.8; 89.6)	107.2 (105.8; 108.5)
	Female	87.2 (83.4; 91.1)	90.5 (86.0; 95.2)	102.4 (101.2; 103.7)
Diabetes Mellitus	Males	57.1 (43.3; 74.0)	79.6 (61.0; 102.1)	115.2 (108.7; 121.9)
	Females	69.8 (56.6; 85.3)	115. 9 (96.0; 138.6)	96.1 (91.3; 101.0)
Circulatory System Diseases	Males	90.3 (84.2; 96.7)	91.7 (84.7; 99.1)	97.2 (95.2; 99.3)
	Females	85.7 (80.3; 91.3)	92.4 (85.9; 99.2)	90.6 (89.0; 92.3)
Ischaemic Heart Diseases	Males	75.8 (65.6; 87.0)	76.3 (64.7; 89.3)	101.0 (97.4; 104.8)
	Females	51.0 (41.9; 61.4)	74.8 (61.9; 89.5)	90.4 (86.7; 94.2)
Respiratory Diseases	Males	78.7 (66.2;93.0)	59.4 (47.2; 73.7)	108.2 (103.3; 113.1)
	Females	71.9 (56.8; 89.7)	70.7 (53.4; 91.8)	112.3 (106.4; 118.4)
Lung Cancer	Males	78.5 (65.5; 93.3)	52.7 (40.7; 67.2)	121.7 (116.9; 126.7)
	Females	68.2 (44.1;100.7)	29.6 (12.7; 58.3)	151.2 (140.5; 162.4)
Breast Cancer	Males	-	-	-
	Females	71.9 (54.2; 93.6)	72.0 (51.5; 98.1)	95.3 (89.4; 101.4)

Font: DASOE (Department for Health Activities and Epidemiological Observatory Health Department - Sicily Region.) Health Atlas of Sicily 2004-2011 Monographic supplement, April 2012.). A district is an aggregation of municipalities used for health purposes.
Cefalù District (45274 people, 21974 males and 23300 females) includes nine small towns: Campofelice di Roccella, Castelbuono, Cefalù, Collesano, Gratteri, Isnello, Lascari, Pollina, San Mauro Castelverde; Petralia Sottana District (27546 people, 13121 males and 14425 females) includes nine small towns: Alimena, Blufi, Bompietro, Castellana Sicula, Gangi, Geraci Siculo, Petralia Soprana, Petralia Sottana, Polizzi Generosa. Palermo city (655875 people, 311121 males and 344754 females).
Statistical significance of SMRs has been explored by using the 95% confidence interval (data in the text).

biogenesis with exercise training and this component can positively influence health [15].

Older people may be encouraged to increase their activities if influenced by clinicians, family or friends, facilitating group-based activities and raising self-efficacy for exercise [16].

3. GENETICS OF LONGEVITY: APOE AND FOXO3A

LLIs are genetically predisposed to reach extreme ages. There are many possible candidate genes for human longevity, however, of the many genes tested, only APOE and forkhead box o3 (FOXO3*)* survived on association in independent populations [7, 17].

APOE expresses Apolipoprotein E, the principal cholesterol carrier that drives lipid transport and repairs injuries in the brain, and plays a central role in plasma lipoprotein metabolism and in lipid transport within tissues. APOE shows a genetic polymorphism determined by three common alleles known as ε2, ε3 and ε4, defined by combinations of genotypes of the single nucleotide polymorphisms (SNPs) *rs7412* and *rs429358*. *APOEε2* frequency fluctuates with no apparent trend while *APOEε3* is the most frequent allele in all the human groups, especially in populations with a long-established agricultural economy. The product of the three alleles differs in several functional properties. The frequency of the ancestral allele, *APOε4,* is higher in countries where foraging still exists, or food supply is (or was until the recent past) scarce and sporadically available. This allele, linked to elevated cholesterol and pro-inflammatory activity, is strongly associated with AD and to a lesser extent, with CVD. This allele could be identified as a 'thrifty' allele. The exposure of people carrying the *APOε4* allele to the new environmental conditions of the Western world should render them more prone to develop AD and CVD. *APO ε4 allele* has emerged as a negative candidate gene in longevity since Schachter *et al.* showed that French centenarians have a very low frequency of this allele. In addition, an increased frequency of the allele *APOEε2* in centenarians was observed [17-19].

Due to our small sample, we did not observe an association of *APOε2* allele with longevity. However, considering the detrimental *APOε4* allele, we did not find this allele in our LLI population.

Several studies have noted specific *FOXO3* SNPs associated with human longevity, particularly with *FOXO3A rs2802292* G-allele (G>T). Some studies support the role of *Daf-16* (ortholog of FOXO) in *C. elegans* longevity by protecting cells from oxidative stress that constitutes a nerve centre in ageing, increasing life-span [20]. Daf-16 is a transcription factor (TF) that modulates the expression of SOD2, acting as a free radical scavenger [21]. The role of FOXO3A in humans might be the same, acting as a TF on multiple homeostatic genes in response to decreased insulin/IGF-1 signalling and consequently increasing life-span [22]. It is conceivable to speculate that hyper or hypoactivation of this signalling pathway, due to genetic mutations that under or overexpress regulative molecules, leads to different expression of homeostatic genes.

A meta-analysis of over 7900 cases and 9500 controls confirmed the association of the G allele of the SNP *rs2802292* with exceptional longevity, especially in males [23]. This datum confirms the results of a previous one, including the sex-specific differences in the association of a genetic variation with survival in old age [24]. This is not surprising because males and females follow different strategies to attain longevity and several association studies show positive results only in males [25, 26]. The reason is multifactorial, with a socio-cultural component distinguished from biological trait linked to longevity.

In our analysis of this SNP in Sicilian LLIs, we did not observe an association with longevity, likely because of the relatively small number of LLIs. Another possibility refers to the diet of Sicilian LLIs, rich in vegetables and fruits and poor in refined sugars, responsible for a hypoactivation of insulin/IGF-1 pathway. However, analyzing survival to extreme longevity in four centenarian studies, Bae *et al*. showed that their results confirmed the previous association of common variants of *FOXO3* with older age, but these common variants did not modify the risk for mortality at ages beyond the oldest 1 percentile age of survival [27].

These two longevity-associated genes have been related to cardiovascular health. As previously stated, *APOE* has been linked to cardiovascular disease, but also *FOXO3A* has been implicated in coronary heart disease [18, 23].

Therefore, it is not surprising that in male Sicilian centenarians, recruited in a precedent survey, we observed a higher frequency of the anti-inflammatory alleles of CC chemokine receptor 5, 5-lipoxygenase, cyclo-oxygenase 2, Toll-like-receptor-4 and cytokine genes that protected them from CVD [28]. Genetic pro-/anti-inflammatory variations in innate immune response influence the susceptibility to all age-related diseases since these diseases have an inflammatory component. Pathogen load determines the type and intensity of inflammatory responses, according to the pro-inflammatory status and tissue injury, implicated in the pathophysiology of these diseases [29]. Adequate control of inflammatory responses might reduce the risk of these diseases, and, reciprocally, might increase the chance of extended survival in an environment with reduced pathogen load [30].

These data concern two essential points closely related to the achievement of successful ageing, *i.e.* the control of inflammation responsible for the development of age-related diseases and the control of nutrient sensing pathways (NSPs) [22, 31]. Control of inflammation can be pursued in different ways: i) anti-inflammatory treatments with statins and non steroidal anti-inflammatory drugs could be useful to counteract and reduce the age-dependent inflammatory status, preventing the development of age-related inflammatory diseases [32]; ii) probiotic treatment might also be useful since optimal gut microbiota plays a role as an anti-inflammatory agent [33]; iii) physical exercise since exercise-deprivation induces a cluster of physiological abnormalities, similar to MS (such as insulin resistance, impaired glucose uptake and hyperlipidemia) [34]; iv) anti-inflammatory diet, i.e. diet rich in fruit and vegetables and poor in meat and in refined sugar [35].

Diet rich in fruit and vegetables and poor in meat and in refined sugar also targets NSP responsible for downregulation of the signals that leads to the inhibition of FOXO, favouring the transcription of homeostatic genes involved in survival and longevity. Nutraceuticals, defined as "naturally derived bioactive compounds found in foods, dietary supplements and herbal products", are constituents of different dietary patterns, such as the Mediterranean and the Asiatic diets, and can modulate NSPs. They explicate their action as hormetins, activating cellular stress response pathways, like the nuclear factor (erythroid-derived 2)-like 2, and leading to the transcription of antioxidant genes [22].

In this context, extra virgin olive oil (EVOO) is a nutraceutical and functional food, an essential element of the MedDiet. Thanks to its bioactive compounds, EVOO modulates different processes linked to ageing and age-related inflammatory diseases [36-38].

4. PHENOTYPIC SIGNATURE

4.1. Hematochemical Values

A study published in 2008 evaluated laboratory parameters in a sample of 120 healthy centenarians and 381 old persons (between 65 and 85 years old) of Sicilian and Italian ancestry. Significant differences were observed in blood glucose, cholesterol and platelet levels, reduced in centenarians as compared to the old subjects, whereas blood urea nitrogen levels were found to be significantly increased in centenarians [39]. In the ongoing survey on Sicilian LLIs (mean age 101.3±4.9), preliminary results suggest no differences concerning lipid profile, glucose and insulin levels when compared to young (mean age 30.7 ±4.8) individuals, whereas creatinine was increased. As expected, several differences were observed between young and elderly (not LLIs), concerning albumin, insulin, and glycaemia (Ciaccio, Caldarella and Caruso, unpublished observations).

Unfortunately, the existing range of laboratory parameters is often not restricted for age with a possible under or overestimation of some values. The reason is due to the ageing process that itself is characterized by a low-grade chronic inflammatory status called inflamm-ageing [31] and reduced response to adaptive mechanisms. We hope that at the end of our study, there will be available reference values for old and oldest old people, allowing a better understanding of health or diseased states of these subjects.

4.2. Body Composition

In young and adults, the accumulation of abdominal fat mass is a risk factor for all-cause and cardiovascular mortality [40-42].

The progressive dysfunction of the white adipose tissue is an important hallmark of the ageing process, which in turn contributes to metabolic alterations, multi-organ damage and a systemic pro-inflammatory state. Obesity shares numerous biological similarities with the normal ageing process such as chronic inflammation and multi-system alterations. There is an interplay between accelerated ageing related to obesity and adipose tissue dysfunction; 'adipageing' illustrates the common links between ageing and obesity [43].

The obesity paradox is an inverse or null relationship between overweight and obesity and, conversely, a protection from fat accumulation, observed in elderly [44-49]. The characteristics of fat distribution in elderly involve the reduction of lean mass and redistribution of adipose tissue, thus maintaining the same body mass index (BMI) [50]. This remodelling of body composition and distribution leads to the hypothesis that the BMI range 18.5-24.9 (for a person with normal weight) could not be suitable for LLIs in which <23 values were associated with a greater mortality risk. This datum was not observed in the younger population in the overweight range [51].

The analysis of body composition (fat mass, fat-free mass, and total body water divided into intra and extra cellular) by bioimpedance (BIA) is a non-invasive technique to monitor health status. Its variation seems to be linked to oedema, response to a specific drug, clinical treatment, and the onset of age-related diseases, sarcopenia [52].

We performed an anthropometric evaluation with BIA 101 in several individuals (mean age of 101.5) recruited in the ongoing survey1 (Fig. **1** and Table **2** show the results of typical studies). The preliminary results demonstrate that this population has a mean BMI of 24.35 Kg/h2, and mean phase angle of 3.2° (mean ref. val. 6.5°). Na^+/K^+ ratio of subjects was above 1. All people followed a strict MedDiet during ageing but not at present. In aged people, abdominal fat accumulation is often a marker of resilience, better

functional reserve and lower subclinical disease prevalence, characterizing the "healthy cohort" effect.

The limit of BIA is the absence of a range of values for restricted population, as LLIs, with different body composition. One of the aims of our study was to identify a longevity anthropometric phenotype. Results of studies on adults and younger elderly led to an over- or underestimation of risks. Understanding the changes in body composition and distribution with ageing and their health implications is important for nutritional support and pharmacological treatment and for the development of appropriate health guidelines for the elderly.

4.3. Immunophenotype

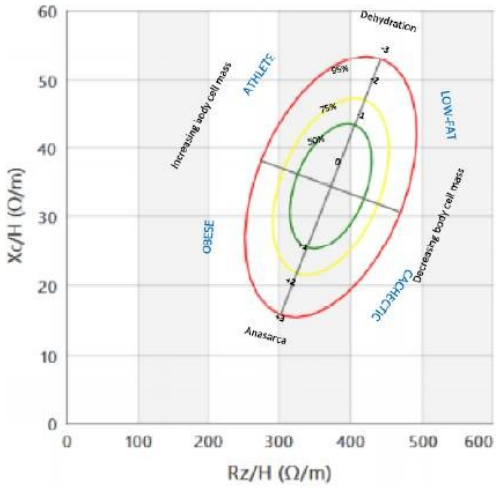
In the elderly, many alterations in innate and acquired immunity have been described and viewed as deleterious, as defined by the term "immunosenescence". Immunosenescence is a complex process involving multiple reorganizational and developmentally regulated changes, rather than the simple unidirectional decline of the whole function. Some immunological parameters are reduced in the elderly and, reciprocally, good function is closely related to health status. Whereas innate immunity is relatively well preserved in the elderly, acquired immunity is more susceptible due to both the functional decline associated with the passage of time and antigen burden to which an individual has been exposed during his/her life. This determines an increase in memory cells and a decrease in naïve cells able to fight new infections [53].

The centenarian's immune system shares characteristics of both young and elderly people. In our ongoing survey, we observe an increase in a subset of NK $CD56^{dim}CD16^{+}$ characterized by increased cytotoxicity, supporting the hypothesis that a well-preserved cytotoxic activity of NK cells represents a biomarker of healthy ageing and longevity. T cells show an increasing trend

Table 2. The table shows some anthropometric and bioelectrical impedance values of the two subjects depicted in Figure 1. Height and weight were measured barefoot and wearing light clothes. Body mass index (BMI) was calculated as weight (in kg) over height squared (in square meters). Fat Mass, free fat mass, Rz (resistance), Xc (reactance) and PhA (phase angle) are obtained by bioelectrical impedance.

Parameters	Centenarian	Old Subject
Age (year)	101.6	68
Sex	Male	Male
Height (cm)	160	180
Weight (Kg)	62.0	92.5
BMI (Kg/m^2)	24.2	28.5
Fat Mass (%)	27.5	41.3
Free Fat Mass (%)	72.5	58.7
Rz (Ω)	541	607
Xc (Ω)	32	35
PhA (°)	3.4	3.3

A)



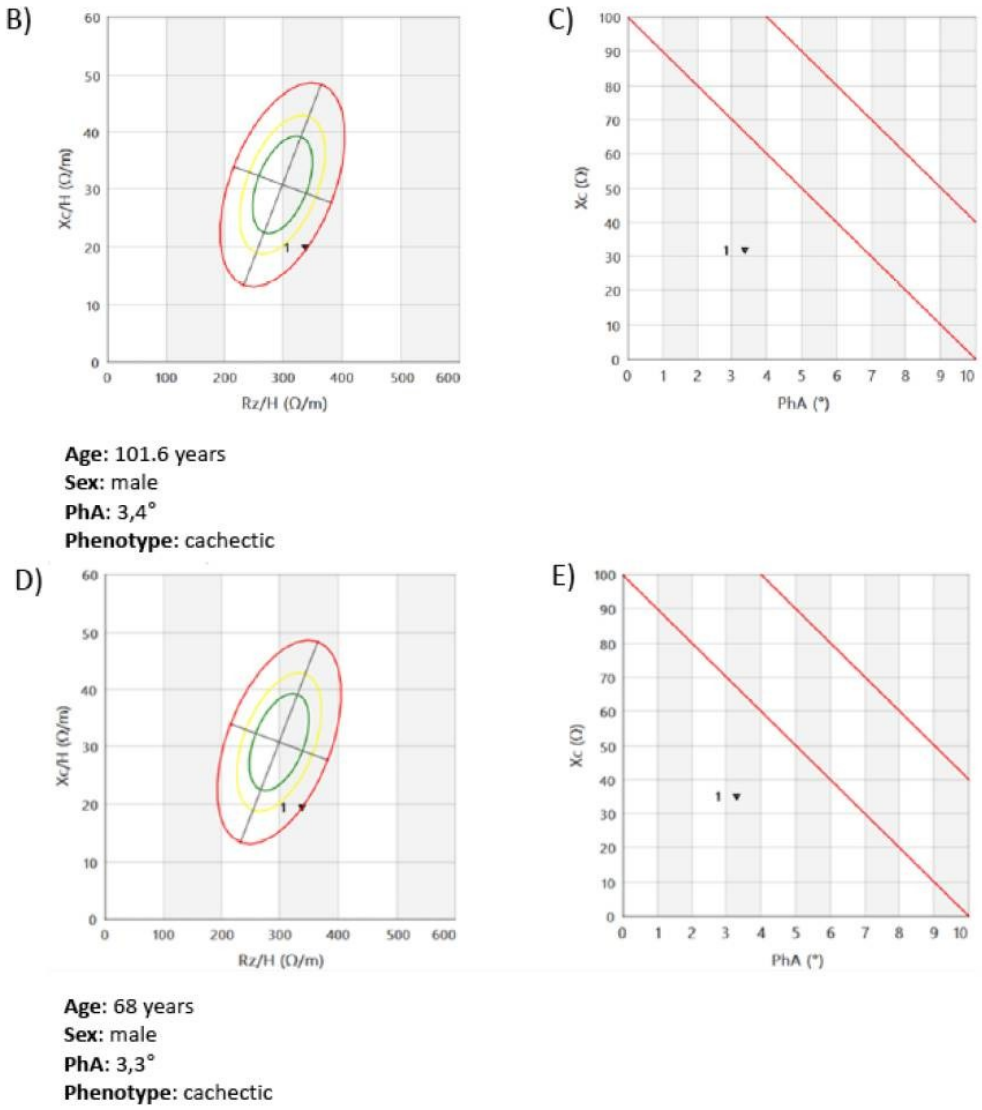


Fig. (1). Figure depicts reference nomogram, one of centenarian male and one of old man. The nomogram, or nomograph, is a two-dimensional diagram. It is a graphical, qualitative representation of a bi or multiple variables function. To get a nomogram, skin electrodes are placed on hand and foot of the same side of body, applying low-voltage and giving two-measures: resistance (Rz), indirectly correlated with amount of body fluids (the higher is Rz, the lower is total body water), and reactance (Xc, directly correlated with cell density in tissues) by human tissues, associated with body composition. A vector quantity, phase angle (Pa), is obtained from Rz and Xc. It permits to evaluate the quality of cell, depending on membrane integrity and body cell mass. Lower Pa is associated with low Xc and cell death or breakdown in selective permeability of membrane (people with low Pa have higher Na+/K+ ratio). The Fig. **A** shows reference nomogram, the Fig. **B** nomogram of centenarian male and Fig. **D** that of old man. Comparing **B** and **D**, referring to **A**, it is possible to speculate that both are cachetic and have similar body composition, although one is centenarians and other is elderly. The Fig. **C** and **E** show the phase angle for centenarian and for old, respectively. Also in this case it is possible to highlight similar results (3.4 and 3.3 respectively).

when compared with elderly, suggesting that this can be considered a predictive marker of longevity. This does not occur for B cells that are decreased, because of the preferential commitment of hematopoietic aged stem cells to the myeloid lineage [54, 55]. A limitation of these studies is the lack of appropriate controls, thus it is better to study centenarian offsprings (CO), which have a significant survival advantage compared to an appropriate control group, *i.e.* age-matched elderly whose parents died at an average life expectancy. In the previous survey, we performed several studies on Sicilian CO with the aim to track immune signatures in CO to test

the hypothesis that these individuals might have an immunological advantage, which may explain their longevity [56, 57].

Our findings documented that CO show significant positive differences in the numbers and proportions of both early- and late-differentiated $CD4^+$ and $CD8^+$ T cells, as well as potentially senescent $CD8^+$ T cells when compared with appropriate controls. This suggests that the acquired T-cell arm of the immune system is more "youthful" in CO than in controls. This might reflect a better ability to mount effective responses against newly encountered antigens, thus contributing to better protection against infection and to greater longevity. Also concerning the B branch, CO does not have the typical trend of memory/naïve B cell subsets observed in elderly people and this is in agreement with the higher levels of IgM in the serum of CO in comparison with data obtained in age-matched controls. This reservoir of naïve B cell might be another cause that makes CO able to keep fighting off new infections, hence prolonging their life [57].

The balance between positive and negative signals dictates the fate of individual T cells and the immune response [58]. Inhibitory molecules play an important role in regulating T cell activation and peripheral tolerance, in particular, the CD28 family, the major regulator of this critical balance. Cytotoxic T-Lymphocyte Antigen(CTLA)-4 is a component of the negative homeostatic control mechanism regulating T cell activation, mediating its inhibitory effects through the coordinated actions with CD28. PD-1 is induced on peripheral $CD4^+$ and $CD8^+$ T cells, B cells, and monocytes upon activation. Programmed cell death protein(PD)-1 signals inhibit T cell proliferation and Interleukin-2 production. CTLA-4 and PD-1 regulate the inhibition and fine-tuning of T cell responses and are used in boosting anti-cancer immunity. Further studies of their functions might be of great therapeutic value in boosting antimicrobial immunity and vaccine responses during ageing [59, 60]

CONCLUSION

In a heterogeneous population, such as the human population, the ability to maintain an adequate response to stressors within a range compatible with a state of good health should have a Gaussian distribution. Centenarians should be the extreme tail of this curve, representing the individuals able to maintain an adequate response to stressors and to repair the damage. They are the individuals better adapted to environmental conditions. In the generation studied here, these factors are represented by inflammatory age-related diseases as cardiovascular ones.

Success in increasing longevity in laboratory organisms has shown that ageing is not an immutable process. The time has come to make serious efforts to slow human ageing or to age successfully. As widely discussed in this review, the explanation of the role of genetics and lifestyle in longevity can provide important information on how to develop drugs and/or behaviours that can slow down or delay ageing. It will be possible to understand, through a "positive biology" approach that seeks to understand the causes of positive phenotypes, trying to explain the biological mechanisms of health and well-being, how to prevent and/or reduce elderly frailty and disability.

Interventions to slow ageing in humans have been the focus of biogerontology in the last decades. We know that different types of dietary restrictions are the possible solutions to increase lifespan in healthy condition. Physical exercise also contributes to control body weight and hematochemical parameters, maintaining them in the normal ranges. The human being is not a model animal, thus it is not easy to verify the efficiency of these approaches because few biomarkers have been identified to characterize healthy ageing, especially for LLI.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

FUNDING

Original studies described in this paper are supported by the grant of Italian Ministry of University (PRIN: progetti di ricerca di rilevante interesse nazionale – Bando 2015 Prot 20157ATSLF "Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and comorbidities") to CC, GC. GA, AA are fellows of this project.

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Triggering of Toll-like receptors in old individuals. Relevance for vaccination

The increase of life and therefore of elderly people in the world, is related both to the reduction of the birth rate and to the mortality rate, and has brought with it an increase in age-related diseases, such as neurodegenerative diseases and cancer. These changes affect different aspects of human life such as social, political and economic. It is therefore necessary to know the aging process and to understand which are the connections between it and the diseases, to allow the successfully aging (2,3). Aging is a functional decline that no longer has the ability to adapt to environmental changes and respond to stress, and is generally associated with a greater risk of morbility and mortality (4). In particular these changes and alterations associated with age at the level of the immune system, both in innate and acquired immunity, have deleterious effects which are defined by the term immunosenescence (5,6). The researcher who coined this term also realized that it was necessary to act with an immunological approach to favor successful aging (5.8). The research confirmed that the immunosenescence includes changes in the cytokine network and the increase in pro-inflammatory mediators, which causes a low-grade chronic inflammatory state called inflammaging (9); however, to generate a state of reduced health there is a general reduction of different immunological functions (6). The aging process causes changes in the number and activity of lymphocytes in the immune system, causing a reduction of naive T and B cells and therefore a lower immune response to new pathogens. This results in an increased incidence of infectious diseases and cancer, and given that immunological protection is reduced, an increase in the incidence of autoimmune diseases following an enhanced inflammatory function (9). Another feature is the reduced efficacy of vaccines in the elderly, given that their immune system does not respond effectively to stimuli (12). Therefore it seems that aging acts more at the level of acquired immunity rather than innate immunity (6, 10). Given that lifespan is increased and the immune system has to defend the body for longer, new vaccination strategies must be created to ensure an increase in immune defense against infections to promote successful aging. Model studies have shown that $CD8^+$ T cells can be generated in immunosenescent aged mice using a multi-factorial adjuvant called CASAC. Our preliminary *in vitro* data on humans have confirmed similar results, therefore an effective production of cytokines by antigen-specific T cells indicating a potential use of a suitable selected combination of TLR agonist in future vaccine approaches for the elderly. Furthermore, the activation of TLRs and other receptors by the appropriate PAMP (pathogen-associated molecular pattern) or other ligands induces changes in innate immunity cells, such as DCs, at long term, as if immunity was trained to recognize the antigen a second time, or recognize

new antigen, and respond more effectively (45). This allows the creation of new vaccines based on different immune modulators that can act synergistically to achieve wider and more lasting protection against pathogens.

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Current Pharmaceutical Design, 2019, *25*, 4163-4167

REVIEW ARTICLE

Triggering of Toll-like Receptors in Old Individuals. Relevance for Vaccination

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ARTICLE HISTORY

Received: October 08, 2019
Accepted: November 07, 2019

DOI:
10.2174/1381612825666191111155800

Abstract: Aging is characterized by a general decline in a range of physiological functions, with a consequent increase in the risk of developing a variety of chronic diseases and geriatric syndromes. Additionally, increasing age is accompanied by a progressive decline in both innate and acquired immune system, referred to as immunosenescence. This impaired ability to mount an efficient immune response after exposure to microorganisms or vaccines represents a major challenge in acquiring protection against pathogens in aging. Therefore, there is still a great need for vaccines that are tailored to optimally stimulate the aged immune system, thus promoting more successful aging. Various strategies can be used to improve vaccine efficacy in old people. Despite this, meta-analyses have clearly shown that the magnitude of protection obtained remains lower in older adults. Recent studies show that stimulation of Toll-like receptors, using stimulatory ligands, can enhance vaccine efficacy by a number of mechanisms, including the activation of innate immune cells and the consequent production of inflammatory cytokines. Therefore, a possible strategy for more effective vaccination in the older population is the triggering of multiple TLRs, using a combined adjuvant for the synergistic activation of cellular immunity. Preliminary *in vitro* data suggest that in humans the presence of multiple TLR agonists can result in the greater stimulation of antigen-specific immune responses in immune cells both in the young healthy and in the immune senescent older donors. These data suggest that appropriately selected combinations of TLR agonists could enhance the efficacy of vaccination mediated immunity in older people.

Keywords: Aging, cytokines, dendritic cells, immunosenescence, TLR, vaccination.

1. INTRODUCTION

People worldwide are living longer. In 2025, there will be about 1.2 billion people over the age of 60, increasing to 2 billion by 2050 [1]. Mechanisms driving this phenomenon are the demographic and epidemiologic transitions. The increased number of aged people is closely related both to reduced birth rate and to the decreased rate of deaths, as well as the epidemiological shift from infectious diseases to non-communicable diseases. The implications of this demographic change are enormous and affect all aspects, social, political, and economic of human life. Furthermore, the health of older people deteriorates with increasing age; hence the increased incidence of diseases such as cancer, cardiovascular disorders and neurodegeneration. It is essential to learn more about the aging process and to understand the intricate connections between aging and disease. This is not to increase longevity *per se*, but rather because such studies could help to understand how to age successfully [2, 3].

Aging is, in fact, a time-dependent functional decline that, due to a diminished homeostatic ability, reduces responsiveness to environmental stimuli; generally is associated with an increased predisposition to illness and death. A complex remodelling of tissues and organs, in response to time-dependent exposure to biological and environmental stressors, plays a key role in the detailed phenotype of old age [4]. In particular, many age-associated alterations in innate and acquired immunity have deleterious effects, which are collectively referred to as immunosenescence, a term coined by Roy Walford [5, 6].

Indeed, in 1969, Roy Walford published his landmark book, "The Immunologic Theory of Aging" [7]. Briefly, he hypothesized that the faulty immune processes play a relevant role in the aging of humans and of all mammals. Therefore, he was the first to note and promote the power of modern immunological approach as a tool for the analysis of aging [5, 8]. Research has repeatedly confirmed the insightful predictions made by Roy Walford regarding the role of the immune system in various pathologies associated with aging [5]. Indeed, in accordance with Roy's original hypothesis on the role of immunosenescence in human aging, there is evidence that the previously identified diseases of aging are closely linked to dysregulation of the immune function and excessive inflammation [5, 9]. In fact, immunosenescence also includes a well characterized and profound modification of the cytokine network. A key feature of this phenomenon is the increase in pro-inflammatory mediators [9]. These circulating inflammatory molecules are associated with a low grade, chronic inflammation, called inflamm-aging [9].

However, immunosenescence is a complex process involving several developmentally regulated changes, rather than a simple unidirectional decline of the whole function. Nevertheless, in older adults there is a decrease in a range of immunological functions, associated with reduced health; reciprocally, immune competence

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1381-6128/19 $58.00+.00

correlates with a range of characteristics associated with a good health status [6].

The impact of aging on the immune system typically includes intrinsic defects within immune cells as well as alterations in the number and activity of the different lymphocyte subsets, and possibly defects in the microenvironment of the lymphoid organs. This phenomenon results in a reduction in naive T and B cells, thus a reduced repertoire of both cellular and immunological responses in the aged population. This reduced complexity in the range and efficacy of response by the immune effector cells to pathogens, underpins the reduced T cell cytotoxicity, proliferation and cytokine production, as well as defective memory responses in the older population [6, 10-12]. Consequently, aged individuals exhibit an increased incidence of infectious disorders and cancer, both associated with reduced immunological surveillance and protection, as well as an increased incidence of autoimmune disease as a consequence of enhanced inflammatory immune functions [9]. In addition, the aged immune system does not efficiently respond to stimuli; therefore, current vaccines are less effective in the older population [12]. Thus, the aging process affects more extensively the acquired, rather than the innate immunity [6, 10]. Age-related changes in antigen uptake, processing and presentation, as well as functional defects of T cells, lead to reduced antibody responses, as well as suboptimal antigen-specific cellular immunity [13].

2. VACCINES IN OLD PEOPLE

Research in immunological aging seeks not only to understand the age-related disorders of immune regulation, but also to identify new efficient strategies for immune rejuvenation and for effective vaccination induced immunity in older people. The severity of many infections is higher in the aged adults. In addition, infectious diseases are frequently associated with long-term consequences such as the onset of frailty and consequent impairment in activities of daily life and loss of independence [14, 15]. Therefore, there is still a great need for vaccines tailored to optimally stimulate the aged immune system in order to promote successful aging [13]. Higher antigen dose, alternative routes of administration, and the use of adjuvants are all strategies to improve vaccination efficacy in old people. However, the already employed strategies induce only moderately higher antibody concentrations in old people vaccinated for influenza [16]. Thus, the identification of more effective adjuvants should be able to enhance the impaired immune responses in order to revert or slow down the age-associated erosion in immunity and to promote healthier aging [12, 13].

Adjuvants are molecules that can stimulate both the non-specific innate immune responses and the direct or indirect activation of antigen presenting cells (APCs), primarily dendritic cells (DCs) [17], by stimulating their recruitment to the site of vaccination, antigen uptake, processing and presentation. Adjuvants are therefore able to promote both the innate and adaptive immunological responses. There has been enormous progress in the last twenty years in the development of new vaccine adjuvants. These adjuvants are made up of different components, such as aluminium salts, emulsions such as MF59 and AS03, both of which are squalene-based, and toll-like receptor (TLR) agonists or a combination of immunostimulants such as detoxified forms of lipopolysaccharides such as MPL. Most adjuvants induce the early activation of innate immunity, in turn potentiating acquired immune responses against the antigens present in the vaccine. Some of these new adjuvants are in clinical use, showing excellent performance in the prevention of infectious diseases, such as influenza and in cervical cancer caused by subtypes of human papilloma virus (HPV) [18].

However, the ineffective induction of T cell mediated immunity in older people remains a persistent challenge for vaccine development. Thus, there is a need for a more efficient and sophisticated adjuvant that will complement novel vaccine strategies for older people. The development and identification of appropriate adjuvants and cytokines might effectively remedy defects in the aged T cell functions, both directly and by better activation of DCs [19, 20].

Various strategies can be used to improve the antibody responses caused by vaccines in older people. Some of these have been used for the anti-influenza vaccination. These include intradermal vaccines [21], high-dose vaccines [22] and vaccines with the squalene based oil-in-water MF59 emulsion as an adjuvant. MF59 contains a synthetic muramyl peptide shown to have significant immunostimulatory activity and low toxicity [23]. These enhanced vaccines show slightly higher immunogenicity in older people when compared with the standard inactivated vaccines [24]. Nevertheless, meta-analyses have clearly shown that the magnitude of protection is lower in older adults than in young people [25, 26].

3. THE DENDRITIC CELLS

DCs are the most potent APCs, specialized in the uptake, processing, transport and presentation of antigens to T cells. After their activation in the periphery, DCs migrate to lymphoid tissues where they interact with T and B cells to initiate and shape the acquired immune responses. According to the expression of various markers, DCs can be divided into three subsets. The CD123 marker characterizes plasmacytoid DCs (pDCs). They possess the ability to produce high levels of type I Interferons (IFN-α/β). In contrast, myeloid DCs (mDCs) express the CD11c marker and are divided into two subsets: CD1c+ mDCs and CD141+ mDCs. Upon stimulation, mDCs secrete mainly interleukin (IL)-6, IL-12, and tumor necrosis factor (TNF)-α [17].

Specific subsets of TLRs are expressed by both innate and adaptive arms of the immune system, including monocytes, macrophages, NK, B, T and dendritic cells. Both pDCs and mDCs, recognize conserved pathogen-associated molecular patterns (PAMPs) on microbes, hence they are key regulators of antimicrobial host defense responses. Recognition of PAMPS by TLRs culminates in the secretion of type I IFNs and pro-inflammatory cytokines that facilitate the linkage of innate to acquired immune responses. Deficiencies in human TLR signalling leads to increased severity of multiple immunological disorders, including sepsis, immunodeficiencies, atherosclerosis and asthma [27].

The cytokine pattern is determined not only by the type of TLR activated, but also by the type of cell. As an example, TLR-7 stimulation induces the expression of IFN-α by pDCs. They are associated with innate antiviral immunity and the development of acquired immunity. By contrast, TLR-7 stimulation induces the expression of IL-12 from mDCs, important for the induction of a T helper (Th)-1 response. Type I IFNs enhance antigen cross-presentation, T cell proliferation, DC maturation and NK cell activation [28].

Data on the influence of aging on human DC activity and cytokine production, in response to *in vitro* stimulation, shows either comparable or reduced DC function in older people. Tan *et al*., [29] report that human DCs isolated from both young and aged individuals exhibit comparable activation in response to most TLR ligands, and are equally capable of direct and cross-presentation of antigens to T cells *in vitro*. On the contrary, You *et al*., [30] demonstrate a reduced production of TNF-α by DCs from old people in response to LPS stimulation. However, in older people there is invariably a marked reduction in cytokine release by pDCs.

As previously mentioned, recognition of microbial components by TLRs induces also the secretion of cytokines. Blood mononuclear cells isolated from young individuals exhibited a quicker and faster response to stimulation with TLR agonists compared with cells obtained from older adults. This resulted in an increased production of cytokines and chemokines [19]. On the other hand, the addition of PAMPs to a subunit vaccine, in order to induce the stimulation of specific TLRs, improves vaccine efficacy in older

people [19, 20, 31, 32]. Thus, DCs and naïve T cells represent the most restrictive elements for the immune response to primary viral infections in older people [33]. Defects in signal transduction appear to be responsible for this impairment since the expression levels of different TLRs remain constant during life [13].

Over the last decade, TLR agonists have emerged as novel vaccine adjuvants [34]. Since TLR stimulation can induce both the production of cytokines by APCs, and the antibody production by germinal centre B cells [13, 35], TLR agonists would be expected to offer a promising strategy to enhance vaccine efficacy. Cytokine production by APCs shows age-related variations, but efficient TLR stimulation may overcome the age-associated TLR signalling dysfunction [36].

Previous studies have shown that the stimulation of human DCs, present in blood mononuclear cells, with two or more TLR agonists, can induce the sustained secretion of IL-12p70. This causes a T1 polarization of the naive T cells, at significantly higher levels than stimulation with single TLR agonists. Activation of DCs with specific combinations of TLRs induces the synergistic production of Th1 polarizing cytokines, up-regulation of co-stimulatory markers of DC and down-regulation of programmed death-ligand 1 (PD-L1) expression [37, 38].

4. DATA FROM MURINE MODELS

A possible strategy for enhanced vaccination efficacy is the activation of multiple TLRs, using a combined adjuvant for synergistic activation of cellular immunity (CASAC). As an example the single-strand oligodeoxynucleotide, characterized by motifs containing cytosines and guanines (CpG), a potent inducer of IFN-α by pDCs, is incorporated in CASAC. This adjuvant also contains a synthetic analogue of viral dsRNA (polyI:C - polyinosinic-polycytidylic acid) that targets TLR3, inducing the production of type I IFNs. In addition, there are IFN-γ and MHC-class I and II peptides. Immunization of young mice with the CASAC adjuvant (containing two or more TLR agonists, anti-CD40, IFN-γ, and surfactant) produced high levels of CD8 responses to peptide or protein antigens and highly polarized Th1 responses [39, 40]. CD40 signalling was required for CD8 expansion but it could be substituted with a concomitant CD4 Th response in place of anti-CD40. Triggering of these pathways activated the migration and activation of mDCs and pDCs and the secretion of IL-12. Therefore, cross-presentation can be exploited to induce potent cytotoxic responses and long-term memory to peptide/protein antigens. When combined with a tumor-associated peptide from tyrosinase-related protein 2, this combined adjuvant approach effectively halted tumor growth in an *in vivo* melanoma model and was more effective than anti-CD40 and a single TLR agonist. Antitumor immunity was associated with long-lived effector memory CD8 T cells specific for the naturally processed and presented tumor antigen; tumor protection was partially but not entirely dependent on CD8 T cells. Thus, CASAC vaccine formulation with two or more TLR agonists, anti-CD40, IFN-γ, and surfactant is more effective than existing adjuvants and provides a technological platform for rapid vaccine development [39, 40].

In the old immunosenescent mice, serial vaccinations with CASAC or Freund's complete / incomplete adjuvant (CFA/IFA) and a class I epitope, derived from ovalbumin (SIINFEKL, SIL) or melanoma auto-antigen, tyrosinase-related protein-2 (SVYDFFVWL, SVL) were used to get antigen specific CD8+ T cell responses. The analysis conducted by quantification of increase in the antigen specific T cells (by MHC/antigen pentamer staining) demonstrated that the immune senescent animals vaccinated with CASAC/SIL had substantially higher frequencies of CD8 + T cells specific for H-2K(b)/SIL compared with the CFA/IFA vaccinated groups. Similarly, higher frequencies of H-2K(b)/SVL-pentamer^{+} and IFN-γ^{+} CD8^{+} T cells were detected in the aged, CASAC+SVL-vaccinated mice than in their CFA/IFA-vaccinated counterparts. In both antigen settings, CASAC promoted significantly better functional CD8+ T cell activity. These studies demonstrate that functional CD8+ T cells, specific for both foreign and tumour-associated self-antigens, can be effectively induced in aged immunosenescent mice using the novel multi-factorial adjuvant CASAC [40].

5. PRELIMINARY HUMAN DATA

Based on these promising results in mice, the ability of combined TLR ligands to induce the activation of peripheral blood DCs isolated from human aged donors has been investigated. Preliminary *in vitro* screening experiments suggested that the combination of TLR7/TLR8 with TLR4 was the most efficient in activating DCs. This combination induced significantly greater cytokine production than that induced by each of the individual agonists. This greater stimulation is probably due to the combined activation of both MyD88 and TRIF-dependent signal transduction pathways [41]. Stimulation with the specific combination of TLR agonists, imidazoquinoline R848, a TLR-7 ligand, and monophosphoryl lipid A, a TLR-4 ligand, induced significantly higher cytokine secretion by mDCs and pDCs from old people. Notably, the combination of R848 and MPLA induce 5-10 fold higher production of IL-12/p40 in CD141+ mDCs isolated from over 75 years old donors compared with their young counterparts (less than 40 years old). In addition, the increased levels of TNF-α were also observed in CD1c+ mDCs and pDCs from these older donors, in response to R848 and MPLA stimulation. These differences were statistically significant when compared with their unstimulated counterparts. These results have potentially important implications, since the reduced production of TNF-α by pDCs from older people, caused by defects in TLR signalling pathways, is associated with an ineffective antibody response to the influenza vaccination [42]. Thus, impaired production of cytokines by older DCs could result in a weak response to vaccination and might contribute to the dysregulation of DC-induced T cell proliferation in the older people.

The involvement of TNF-α in DC-induced T cell proliferation is also evident from clinical data in rheumatoid arthritis patients, showing that the treatment with anti-TNF-α antibodies causes poor stimulation of T cell activity by DCs [43,44].

CONCLUSION

Due to the increase in the human life span, the immune system must defend the organism for several decades longer than foreseen by evolution; hence it has to work efficiently for a considerable number of years. Old people suffer more frequently from severe infections and experience poorer outcomes from these infections as compared with younger adults. However, vaccine-induced immune responses are frequently less effective in the old people when compared with younger adults [6,10,12,13]. Thus, there is an increasing need to develop new vaccination strategies in order to ensure an enhancement of the immune defense against infections of the older people, as a preventive measure to promote a successful aging.

Data obtained in model studies demonstrate that functional antigen specific CD8+ T cells can be effectively generated in aged immunosenescent mice using the novel multi-factorial adjuvant CASAC. Our preliminary human *in vitro* data highlight a similarly efficient CASAC-mediated stimulation of cytokine production by antigen-specific T cells, indicating the potential use of appropriately selected combination of TLR agonists in future vaccination approaches for the older people.

Interestingly, triggering of TLRs and other pattern recognition receptors by PAMPs and other agonists induces long lasting epigenetic changes in innate immune cells, including DCs. This results in trained immunity, *i.e.*, an enhanced response to a second challenge by the same or unrelated agent [45]. This opens a new avenue in vaccine development based on the use of multiple, synergistically

acting, immune modulators for the stimulation of broader and longer lasting protection against pathogens.

CONSENT FOR PUBLICATION

All authors have provided consent to the publication of the manuscript in Current Pharmaceutical Design.

FUNDING

The original research was funded by Italian Ministry of University (PRIN: progetti di ricerca di rilevante interesse nazionale—Bando 2015 Prot 20157ATSLF "Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and comorbidities") to CC and GC. CASAC vaccination studies were also supported by UK Bloodwise (Programme Grant 13007 - Pre-emptive immune therapy to prevent relapse of myeloid malignancies).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Work in the Molecular Medicine Group at King's is supported by CRUK, the Experimental Cancer Medicine Centre and the NIHR Biomedical Research Centres (BRC) based at the King's Health Partners.

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The Role of Matrix Metalloproteinases (MMP-2 and MMP-9) in Ageing and Longevity. Focus on Sicilian Long Living Individuals (LLIs)

Aging is a functional decline that depends on time and involves a progressive deterioration of the physiological functions of the organisms that increase susceptibility to disease and death. All organisms age differently, in fact we talk about successful aging in the event that the process is free of age-related diseases and with good health, and of aging without success in the event that we have a tendency progressive aging and disability. Among the major age-related diseases are atherosclerosis, Alzheimer's disease, rheumatoid arthritis, diabetes, cancer and the chronic inflammation process of aging itself, which becomes harmful and destructive. Aging is due to intrinsic cell decline, mainly due to genomic instability, telomere shortening, epigenetic alterations and loss of proteases, which are a group of proteins that activate substrates by enzymatic cleavage, and are distinguished into aspartic, metal, cysteine, serine, threonine protease. These proteins control various key physiological and pathological processes, such as DNA replication, cell cycle progression, cell proliferation, cell death, tissue remodeling, wound healing, neurodegeneration and cancer. Therefore, it is not surprising that they play a role on aging. Among the different immune cells, macrophages and neutrophils are responsible for the production of matrix metal-proteases (MMPs) which, during aging, are responsible for the remodeling of the extra-cellular matrix, the maintenance of stem cells, as well as the component of the Senescence Associated Secretory Phenotype (SASP) in human and murine fibroblasts. Among these, MMP-2 and MMP-9 are involved in many diseases such as the onset and growth of breast cancer, muscular dystrophies, neurological disorders including Parkinson's and Alzheimer's and glaucoma. Furthermore, MMP-2 is associated with inflammation of various origins, while MMP-9 has a role in lipid metabolism and in the regulation of cholesterol excretion in the mouse model. Therefore, in our study, in order to understand the bases of longevity and healthy aging, we analyze the hematological, biochemical, immunological, nutritional and anthropometric parameters in a specific population, consisting of long-lived individuals (LLI) who live in Palermo and the surrounding mountain areas. In particular, the presence and quantity of MMP-2 and MMP-9 have been studied in association with inflammatory markers linked to age-related diseases such as CVD, myocardial infarction, type 2 diabetes, stroke or cancer.

A cohort of 154 healthy individuals subdivided into sub-cohorts who had undertaken a series of nutritional and informational tests was recruited. Half of the cohort was female (53%) and the rest was male (47%). The age of the participants ranged from 20 to 112 years, divided into five subgroups: 1 group under 40 (19%), second group ranging from 40 to 64 years (25%), third group

ranging from 65 to 89 years (32%), fourth group ranging from 90 to 94 years (8%) and fifth group over 95 years (16%). On these groups the inflammatory parameters were analyzed such as reactive protein C (CRP), uric acid, lipid profile (LDL, HDL and total cholesterol), triglycerides and metallo-proteases (MMP-2 and 9), and we observed which group falls inside and outside the physiological values of the main inflammatory markers. Regarding the PCR, an acute phase protein and an important biomarker of inflammation, it was found that the levels were significantly lower in the elderly subjects who successfully aged, compared to the elderly with age-related diseases or disabilities. Regarding the lipid profile (LDL, HDL and total cholesterol) and triglycerides, we do not see significant differences between the young population and the LLI, showing that these two cohorts are similar to each other. Finally, regarding the MMP-2 and -9, we see that the amount of the active form of MMP-2 increases significantly with age, and if we observe it by sex we get an association with the male gender. Furthermore, if we correlate the amount of MMP-2 with the studied inflammatory markers, we see a negative association between the levels of MMP-2 and PCR. However, the serum levels of MMP-9 did not show any correlation with age and sex, but appears to be present at any age.

So it seems that the Sicilian centenarians we analyzed, characterized by the absence of serious chronic diseases such as Alzheimer's, cardiovascular and metabolic diseases and cancer, which live in small municipalities and are socially and physically active, have characteristics more similar to those of young people compared to unhealthy peers, and that therefore can contribute to highlighting the combination of "positive characteristics" responsible for lengthening the duration of human life.

Article

The Role of Matrix Metalloproteinases (MMP-2 and MMP-9) in Ageing and Longevity. Focus on Sicilian Long Living Individuals (LLIs)

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Received: date; Accepted: date; Published: date

(This article belongs to the Special Issue Centenarians—A Model to Study the Molecular Basis of Lifespan and Healthspan)

Abstract: Extracellular matrix metalloproteinases (MMPs) are a group of proteins that activate substrates by enzymatic cleavage, and, on the basis of their activities, have been demonstrated to play a role in ageing. Thus, in order to gain insight into pathophysiology of ageing, and to identify new markers of longevity, we analysed the activity levels of MMP-2 and MMP-9 in association with some relevant hematochemical parameters in a population from West Sicily including long living individuals (LLIs). A relationship exists between LLIs and MMP-2 but not between LLIs and MMP-9. However, in LLIs group MMP-2 and MMP-9 values are significantly correlated. Furthermore, in LLIs we find a positive correlation between MMP-2 with anti-oxidant catabolite uric acid and a negative correlation with inflammatory marker C-reactive protein. Finally, in LLIs MMP-9 values correlate with both cholesterol and low density lipoprotein. On the whole, our data suggest that the observed increase of MMP-2 in LLIs might play a positive role in the attainment of longevity. This is the first study that reveals an association of the activity levels of circulating MMP-2 with longevity. It is difficult to make wide-ranging conclusions/assumptions based on these observations in view of the relatively small sample size of LLIs. However, this is an important starting point. Larger scale future studies will be required to clarify these findings including the link with other systemic inflammatory and anti-oxidant markers.

Keywords: Ageing; Longevity; CRP; MMP-2; MMP-9; Positive Biology; Uric Acid.

1. Introduction

Ageing is a time dependent functional decline, which involves a progressive deterioration of organism physiological functions heading to increase susceptibility to disease and death. This process is unavoidable and extremely complex. However, in the last 40 years a lot of efforts has been made to characterize ageing. There are two ways to age, the first is free of age-related diseases and without disability (ageing successfully), while the latter is characterized by a progressive tendency toward inflamm-ageing, disability, and age-related diseases (ageing unsuccessfully) [1]. Major age-related diseases include atherosclerosis, Alzheimer's disease, diabetes, where the inflammatory components prolonged and persisted become damaging [1,2]. On the other hand, long living individuals are considered the best example of successful ageing [3].

Extracellular proteinases are a group of proteins that activate substrates by enzymatic cleavage, and, on the basis of working mechanisms, are classified into aspartic, metallo, cysteine, serine and threonine proteinases [4]. Among different immune cells, macrophage and neutrophils, are the main responsible for matrix metalloproteinases (MMPs) production. This group of proteins control of a large variety of key physiological and pathological processes, including tissue remodelling, neurodegeneration, and cancer [5]. Moreover, MMPs are responsible for the remodelling of extracellular matrix (ECM), a three-dimensional network of extracellular macromolecules, such as collagen, enzymes, and glycoproteins, that provide structural and biochemical support of surrounding cells [6], particularly of stem cell niche [7,8]. Thus, it is not surprising that MMPs can play a role in ageing,

Among others, MMP-2 and MMP-9 are involved in many diseases as cancer pathophysiology [9-11], neurological disorders including Parkinson's and Alzheimer's diseases [12,13]. Furthermore, MMP-2 is associated with inflammatory states such as osteoarthritis and the atherosclerotic plaques [14,15]. MMP-9 is also implicated in lipid metabolism [16].

Thus, in our study, in order to gain insight into pathophysiology of ageing and longevity, we analysed the activity levels of MMP-2 and MMP-9 in association with some relevant hematochemical parameters in a population from West Sicily including LLIs.

4. Materials and Methods

4.1 Subjects recruitment and study design

A cohort of 154 healthy subjects (72 men and 82 women) of different age (age range 20-112) were recruited. Donors were all Sicilians, living in the area of West-Sicily. A group of well-trained nutritionists and physicians administered a questionnaire to collect demographic and anamnestic data of interest. Participants were selected on the basis of their health status since none of them had neoplastic, infective or autoimmune diseases and none was prescribed drugs known to interfere with immune-inflammatory responses. Participants (or their relatives for LLIs) signed an informed consent before the enrolment. To respect the privacy, everyone was identified with an alphanumeric code. A database was created to handle the collected information. The study protocol, conducted in accordance with the Declaration of Helsinki and its amendments, was approved by the Ethic Committee of Palermo University Hospital (Nutrition and Longevity, No. 032017). The suitability of the sample size was checked using free software (http://ps-powerand-sample-sizecalculation.software.informer.com) on the basis of the results of our previous studies. The cohort was divided into five subgroups: the first group with less than 40 years old (19%, group 1-young people, N = 29), the second group ranging from 40 to 64 years old (25%, group 2-adult people, N = 39), third group ranging from 65 to 89 years old (32%, group 3-old people, N = 50), fourth group ranging from 90 to 94 years old (8%, group 4-oldest old people, N = 12) and fifth group with more than 95 years old (16%, group 5-LLIs and centenarians, N = 24). All methods were performed in accordance with the relevant guidelines and regulations.

The recruited participants underwent vein puncture, after a fasting period of 10–12 hours. The fasting blood samples were obtained in the morning (between 8.30 and 10 a.m.) and were collected

in serum tubes with no additives. Hematochemical tests, carried out for all subjects, were performed at the Central laboratory analysis of Palermo University Hospital according to standard procedures.

4.2 Gelatin zymography and polyacrylamide gel electrophoresis

Sera protein concentration was quantified spectrophotometrically, by using the Bradford assay, as previously described [28,29]. For SDS-PAGE, aliquots of 10 µl of sera previously diluted 1:25, containing approximatively 28 µg of total proteins, were mixed with Laemmli buffer (2% w/v SDS, 10% glycerol, 5% b-mercaptoethanol, 62 mM Tris-HCl pH 6.8), boiled for 5 min, and loaded on 10 × 8 cm vertical 8% polyacrylamide gel. The run was performed at 150V for 50 min with a Mini Protean II Xi System (Bio-Rad Laboratories S.r.l., Milano). The running buffer was 25 mM Tris-HCl, 200 mM glycine, 0.1% w/v SDS. Gels were stained with 0.2% Coomassie Brilliant Blue G-250 in 40% methanol and 10% acetic acid and de-stained in 7% methanol and 5% acetic acid. For zymography assay aliquots of 10 µl of sera previously diluted 1:25 were loaded onto 7.5% polyacrylamide SDS-PAGE gels co-polymerized with 0.1% gelatin, under non reducing conditions and run at 150 V in a Tris-glycine buffer, as previously described [30-32]. After electrophoresis, gels were incubated at room temperature for 1 h in a wash buffer (50 mM Tris-HCl pH 7.5 and 2.5% Triton X-100) to remove the SDS and then incubated for 18 h at37 °C with an activation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$), to allow the activation of the gelatinases, as previously described [33,34]. Gels were stained with Blue Coomassie. Band intensity was measured with Image J software. Protein samples extracted from human breast cancer tissues [35-38]were used as reference for MMP-2 and MMP-9 activity levels. All experiments were performed in triplicate.

4.3 Statistical analysis

The data were processed using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, United States). Box plot, displaying the data distribution through their quartiles, was used to describe the distributions of all variables. In the box plot graph, the ends of the box are the upper and lower quartiles, the lines extending parallel from the boxes are used to indicate variability outside the upper and lower quartiles while the median is marked by a vertical line inside the box. Non-parametric tests were applied for statistical analyses; in particular, the Mann–Whitney U-test was used to compare two subgroups of patients, and the Kruskal–Wallis test was used to compare three or more subgroups of subjects. The two-tailed alpha level was set to $p < 0.05$ to indicate a significant difference. The correlations were performed applying the Pearson correlation test.

2. Results

2.1. Activity levels of MMP-2 and MMP-9 in sera samples and their correlation with age

Sera from all subjects enrolled in this study were subjected to gelatin zymography to determine the relative levels of activity attributable to MMP-2 and MMP-9. **Figure 1** shows a panel of 36 zymograms randomly selected among the collection of our samples. Parallel SDS polyacrylamide gels were run in order to ascertain the correct protein loading. The two prominent gelatinolytic bands represent the proenzymatic forms of MMP-2 (Pro-MMP-2) (72 kDa) and of MMP-9 (Pro-MMP-9) (92 kDa). Two additional lytic bands of 200 and 116 kDa, previously identified as MMP-9 dimers (the 220 kDa) and as MMP-9/TIMP1 complex (the 116 kDa), are also evident [17,18]. In all sera samples no activity levels were evident for the active forms of the MMPs.

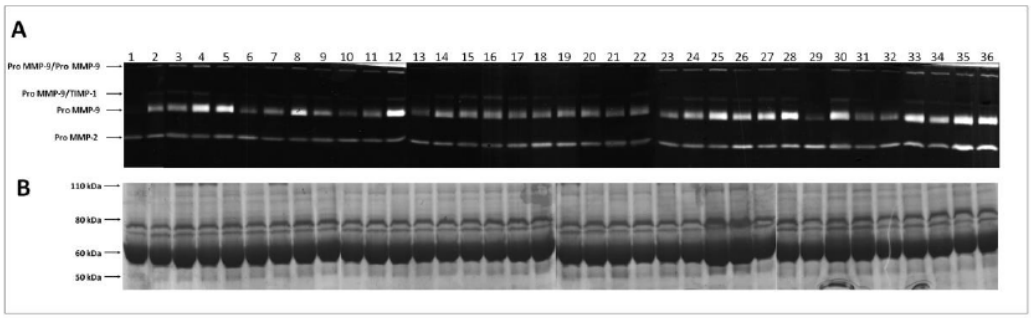

Figure 1: Pro-MMP-2 and Pro-MMP-9 activity levels evaluated by gelatin zymography. A) Prototype of gelatin zymography of 36 randomly selected sera samples used. B) SDS-PAGE electrophoresis of the same sera samples of panel A stained with Blue Coomassie.

The activity levels of Pro-MMP-9 in all samples appear more intense than that of Pro-MMP-2. In order to assess the relative variations of the two Pro-MMPs levels in all subjects (**Figure 2**), gels containing samples run in triplicate were subjected to densitometric analysis by using the Image J software, as described in Materials and Methods.

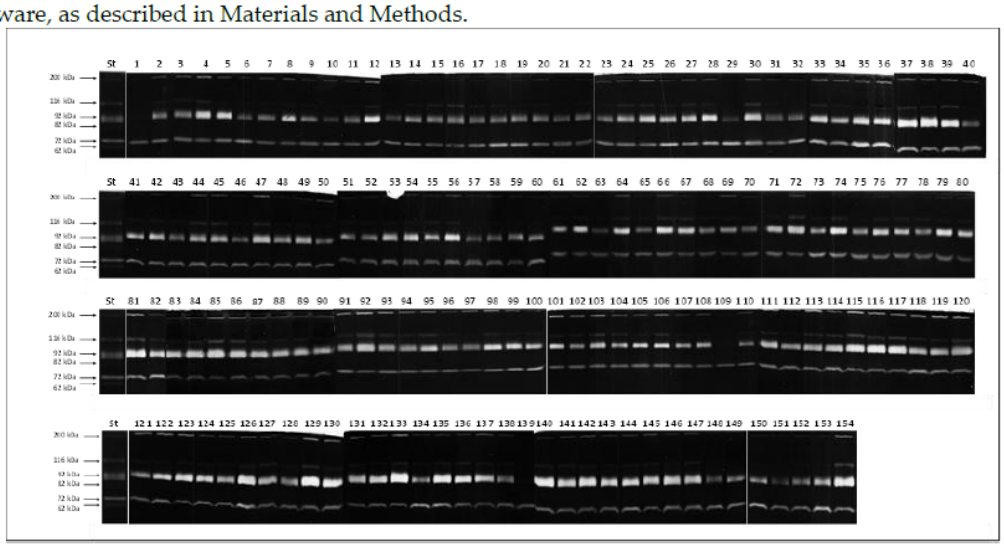

Figure 2: Gelatin zymography of the 154 analysed sera samples. Each lane represents a different subject. Experiments were performed in triplicate and the densitometric bands were quantified by using Image J software. St represent protein samples extracted from human breast cancer tissues used as standard.

In order to assess the possible correlations between levels of gelatinases activity and age, a statistical analysis was performed between the enzymatic activities of the different individual divided in groups as described in Materials and Methods. **Figure 3** reports an overview of the distribution of Pro-MMP-2 and Pro-MMP-9 activities over the 5 groups, corresponding to the average of three measurements per sample. Although the activity levels of each Pro-MMP are variable within the subjects, a significant association was obtained for Pro-MMP-2. In particular, the Pro-MMP-2 activity levels increase with age. Significant differences were observed between the three younger groups and the group of LLIs. No significant differences were obtained between the 4th and the 5th group, probably due to poor representation of the 4th group (8%) and the age proximity with the 5th group.

In contrast, the activity levels of pro-MMP-9 did not show any correlation with age. However, in LLIs group level activity of Pro-MMP-2 was significantly related to Pro-MMP-9 activity ($r=0.53$, $p < 0.01$).

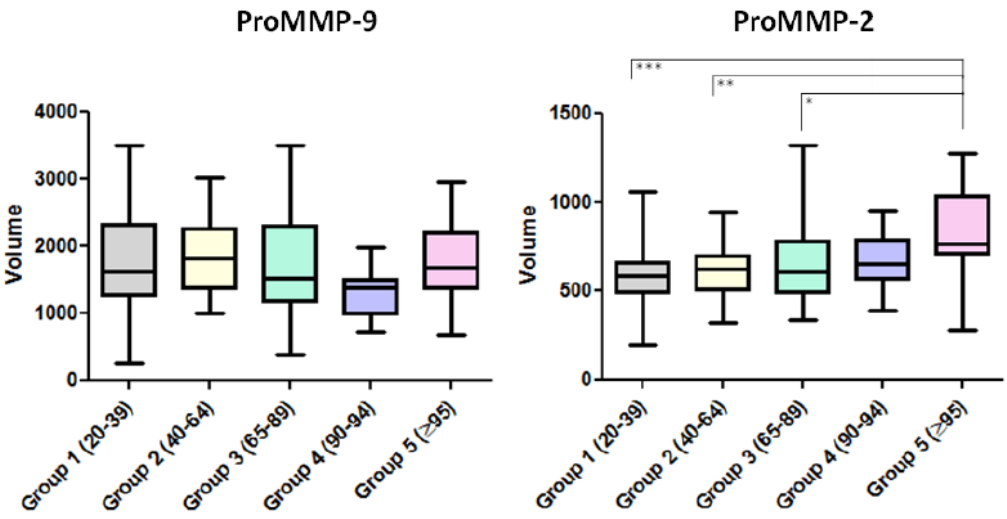

Figure 3: Box plot graphs of Pro-MMP-9 and Pro-MMP-2 activity levels evaluated by gelatin zymography and grouped for age. The densitometric analysis, performed by measuring the intensity levels of each band and the corresponding area, is referred as volume. Statistical analysis was performed applying the Kruskal–Wallis non-parametric test. *p < 0.05, ** p < 0.01, ***p < 0.001.

Interestingly, when the cohorts were analysed according to gender a clear increase of Pro-MMP-2 activity with age was observed in the male gender (**Figure 4**), but not in female gender (data not shown) probably because, especially in women, the expression of MMPs are also dependent on the hormonal status [19,20].

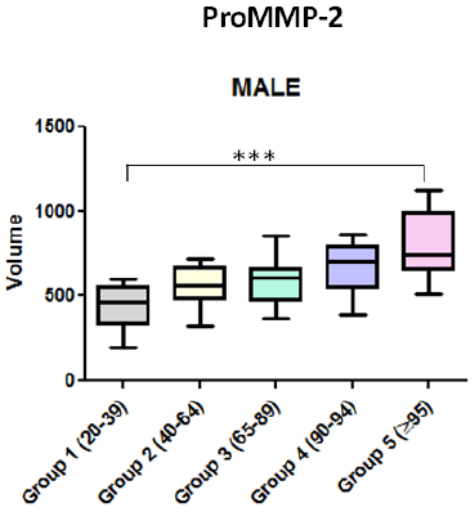

Figure 4: Box plot graphs of ProMMP-2 activity levels in male evaluated by gelatin zymography and grouped for age. The densitometric analysis, performed by measuring the intensity levels of each band and the corresponding area, is referred as volume. Statistical analysis was performed applying the Kruskal–Wallis non-parametric test. *p < 0.05, ** p < 0.01, ***p < 0.001.

2.1. Pro-MMP-2 and Pro-MMP-9 correlation with some relevant hematochemical parameters

Following the association between age and Pro-MMP-2, we analysed the correlation between level activity of Pro-MMP2 and serum levels of C reactive protein (CRP) and uric acid (UA) in LLIs.

In fact, MMP-2 activity plays a relevant role in tissue remodelling and in pathophysiology of inflammation, and CRP is an inflammatory marker [21] whereas serum UA is considered to have an anti-oxidant effect [22]. Notably, 5% of LLIs showed amount of cholesterol above 200 mg/dl (reference values are < 200 mg/dL), whereas 13% of LLIs showed UA levels above 7 mg/dL (reference values are 2.4-7 mg/dL). Interestingly, we found an inverse correlation between CRP levels and Pro-MMP-2 activity in LLIs ($r=-0.39$, $p<0.05$). On the contrary, we found a positive correlation between UA levels and Pro-MMP-2 activity in LLIs ($r=0.41$, $p<0.05$).

Since MMP-9 modulates cholesterol metabolism [16], we analysed the correlation in LLIs between pro-MMP-9 activity and serum cholesterol levels. Notably, 5% of LLIs showed amount of cholesterol above 200 mg/dl (reference values are < 200 mg/dL), whereas 8% of LLIs showed low density lipoproteins (LDL) levels above 129mg/dL (reference values are 70-129 mg/dL). Interestingly, we found a positive correlation between cholesterol levels and Pro-MMP-9 activity in LLIs ($r=0.52$, $p<0.001$). We also found a positive correlation between LDL levels and Pro-MMP-9 activity in LLIs ($r=0.43$, $p<0.05$).

3. Discussion

Most biomedical researchers are called "negative biology", because the study of the disease is its central heart, focusing on the causes of the diseases. On the contrary, a different approach is possible, called "positive biology". Instead of placing diseases at the centre of research, positive biology searches to explain the biological mechanisms of health and well-being. This means understanding why some individuals, namely the LLIs, have escaped neonatal mortality, infectious diseases in the pre-antibiotic era and the fatal outcomes of age-related diseases, thus living more than 95 years. The knowledge born from this approach could allow modulating the ageing rate by providing valuable information to achieve healthy ageing. In fact, this analysis can give new possible information to delineate a sort of longevity signature. The identification of the factors that predispose to a successfully ageing is, therefore, of enormous interest for translational medicine [3].

In the present study we have focused on the possible role played by MMPs in ageing and longevity. MMPs are a family of structurally and functionally related zinc-dependent proteases with a wide range of substrates, including extracellular matrix components, cytokines, receptors, and cell motility factors. They are recognized as the main proteolytic enzyme group involved in remodelling the extracellular matrix and modifying cell-cell and cell-matrix interactions. In particular, MMP-2 and –9 are gelatinases; thus, their role in the degradation of gelatin leads to the release of signalling molecules from the ECM. MMPs play a role in the pathophysiology of various tissues during growth, development and ageing. Most MMPs are secreted as pro-proteinases and have to be activated. The gelatinase members of the MMP family, MMPs-2 and -9, have traditionally been the easiest to detect using gelatin zymography, therefore there are much more available data on them [23-25].

To best of our knowledge this is the first study on the serum activity levels of MMP-2 and MMP-9 in LLIs. Previous study demonstrated that ageing is associated with increased activities in some kind of cells, either of MMP-2 [26] or of MMP-2 and MMP-9 [27]. Furthermore, it has been shown that the concentration of active MMP-9 decreases with age [24].

Our results show a positive association between LLIs and MMP-2 but not between LLIs and MMP-9. However, in LLIs group MMP-2 and MMP-9 values are significantly correlated. Furthermore, in LLIs we find a positive correlation of MMP-2 with UA and a negative with CRP. Finally, in LLIs MMP-9 values correlate with both cholesterol and LDL.

UA is the end product of both endogenous and exogenous purine metabolism. Epidemiological studies suggest that increased serum levels of UA are a risk factor for age-related diseases where oxidative stress plays an important role. On the contrary, other evidence shows that it might play a role as an antioxidant. UA should function as an anti-oxidant in plasma, but as pro-oxidant in the cell [22].

CRP, primarily secreted by liver, is the most important biomarker of inflammation, commonly evaluated for monitoring treatment response and predicting long-term outcome in inflammatory diseases. The serum levels of CRP increase in an age-dependent manner, and CRP levels are good predictors of physical and cognitive performance and the risk of mortality in both the entire elderly population and in successfully aged individuals [21].

MMP-9 affects cholesterol metabolism, at least in part, through a MMP-9–plasma secreted phospholipase A2 axis that affects the hepatic transcriptional responses to dietary cholesterol. Therefore, it has been proposed that the dysregulation of MMP-9 can contribute to the development of metabolic disorders that could, ultimately, lead to atherosclerosis and coronary heart disease [16].

This is the first study that reveals an association between the activity levels of circulating MMP-2 and longevity. It is difficult to make wide-ranging conclusions/assumptions based on these observations in view of the relatively small sample size of LLIs. However, on the whole, all these data suggest that the observed increase of MMP-2 in LLIs might play a positive role in the attainment of longevity. Accordingly, MMP-2 is considered to act as anti-fibrotic proteases. More interestingly, MMPs have been characterized as bifunctional proteins in Alzheimer's disease, with some of them, such as MMP-2 and MMP-9, displaying protective roles during disease progression, while others promote disease evolution [7]. Larger scale future studies will be required to clarify these findings including the link with other systemic inflammatory and anti-oxidant markers.

5. Conclusion

This is the first study that reveals an association between the activity levels of circulating MMP-2 and longevity. It is difficult to make wide-ranging conclusions/assumptions based on these observations in view of the relatively small sample size of LLIs. However, this is an important starting point. Larger scale future studies will be required to clarify these findings including the link with other systemic inflammatory and anti-oxidant markers.

Author Contributions: Conceptualization: P.C., C.C., S.V.; methodology: P.C.; A.A., R.C.; software: P.C., A.A.; validation: P.C., A.A., G.A., L.C., R.C.; V.S., S.V; formal analysis: P.C., A.A., G.C., M.C, F.D.G, S.V.; investigation: P.C., A.A., S.V.; resources: A.A., G.A., G.C.; C.C., M.C.; data curation: P.C., A.A., S.V.; writing—original draft preparation: P.C., S.V.; writing—review and editing: P.C., C.C., S.V.; visualization: P.C., C.C., S.V; supervision: S.V; project administration: C.C.; funding acquisition: C.C.,G.C. All authors approved the version to be published

Funding: This research was funded by PRIN-20157ATSLF_009 (DESIGN: Discovery of molecular, and genetic/epigenetic signatures underlying resistance to age-related diseases and co-morbidities) to C.C. and G.C.

Conflicts of Interest: Authors declare no conflicts of interest.

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Heat-resistant Aphanizomenon flos-aquae (AFA) extract (Klamin®) as a functional ingredient in food strategy for prevention of oxidative stress

Microalgae include prokaryotic cyanobacteria and eukaryotic photoautotrophic protists that differ in metabolism, cell structure and habitat [1]. They have been the main source of oxygen on the primordial Earth [2], and are of great commercial interest given their ability to produce biomass from which it is possible to obtain bioactive compounds such as pigments, vitamins, proteins, lipids, polyunsaturated fatty acids and carbohydrates [3]. The Aphanizomenon flos-aquae (AFA) is a unicellular cyanobacterial organism with remarkable nutritional properties that, unlike other commercial "microalgae", grows spontaneously in a lake in southern Oregon, called Upper Klamath Lake (USA). This lake is an ideal natural ecosystem for the growth of AFA microalgae since sunny days favor the intense photosynthetic activity of AFA, which is obtained with its various pigments, including phycocyanins and phycoerythrin. Instead, the production of fatty acids, like omega 3, is favored during the winter, when the lake freezes due to low temperatures. Thanks to the volcanic origin of the lake, the AFA contains a complete variety of minerals [4,5] and pigments such as carotene, beta-carotene and chlorophylls [5,6] phycocyanins, phycoerythrin and polyphenols [5,7] with significant antioxidant [8], anti-inflammatory [5,9] and antiproliferative properties [10].

In addition, the AFA extract, which constitutes the commercial food supplement Klamin®, contains concentrated amounts of phenylethylamine and other molecules useful to fight various pathologies of the nervous system, including neurodegenerative diseases such as Alzheimer's disease (MA) [15 -17]. Among the causes of these pathologies there is oxidative stress, inflammation and mitochondrial and neurological dysfunctions [18-20], and in these years it has been shown that neuroprotective agents with antioxidant activity have exerted their benefits [18,21 -24] by improving the activity of antioxidant enzymes or by their scavenger ability which can inactivate reactive oxygen species (ROS). Previous work has demonstrated the scavenging properties of the AFA extract against ROS generation and its ability to inhibit the aggregation process of Aβ, the main peptide involved in MA [5,25,26]. Therefore, since the AFA extract contains high levels of antioxidants, it may be a potential therapeutic agent against neurodegenerative diseases [19, 20]. Neurodegeneration is characterized by a process of oxidative stress at the brain level and the use of antioxidants could help counteract their effects, and the habitual intake of foods rich in substances with antioxidant properties could be an approach in the prevention and treatment of these diseases.

An innovative and natural strategy could be to use food supplements to prevent and treat different diseases, trying to promote the development of new drugs based on supplements [33-35], since

supplements based on plant extracts have proven to be beneficial against some pathologies such as metabolic syndromes, inflammation and neurodegenerative diseases [36-38].

A new more natural approach developed in the pharmaceutical industry is based on functional foods. Numerous studies have shown that food, considered essential for survival, is a tool to reduce the risk of diseases, which are due to the increase in life expectancy, and the growing demand for health care for better quality of life. Of great interest today are therefore functional foods, foods to which are added substances that prevent diseases, controlling metabolic parameters such as cholesterol and blood sugar levels, inflammation and oxidative stress, and promote health [57-59]. So given the demands of a healthy and balanced diet, the AFA extract, composed of plant nutrients and bioactive components, could be a source of substances to make food functional. Furthermore, natural antioxidant compounds can also be used in the field of food packaging [39,40] or as additives, preserving food from deterioration, rancidity or discoloration, allowing the extension of the shelf life without negative effects [41- 43].

The objective of this work is to better analyze the activity of bioactive molecules from the AFA extract and promote further investigations on its application in the food industry. In particular, we analyzed a potential use of the AFA extract as an addition to the bread and/or biscuit mixture, studying whether its antioxidant and prebiotic activities after exposure to high temperatures. In particular, the tests carried out in this work have shown that chlorophyll and carotenoids are slightly influenced by high temperatures, but the antioxidant properties of the AFA extract are maintained, showing that substances that are not damaged act in synergy by a compensatory effect. Furthermore, by exposing the extract to 220°C, there was a slight reduction in the initial weight, as demonstrated by the isothermal analysis, and the ATR-FTIR spectra showed a reduction in the aromatic part and the carotenoids. Therefore AFA could be a valid candidate as an ingredient for functional foods, since satisfies the various requirements even after exposure to high temperatures, such as those reached during cooking. If the endogenous antioxidants are not sufficient to counteract the oxidative processes that could occur in the body, such as during aging or in case of some pathologies, the intake of antioxidants with the diet could be a valid aid [60] . It was shown that, even after exposure to high temperatures (thermal stress), the AFA extract was still able to also have a beneficial effect on neurons in which the toxicity was induced by the Aβ oligomers.

Given that the biological activity of AFA is maintained even after cooking, we can consider using the extract to create other types of functional foods, such as pasta, one of the main ingredients of the Mediterranean diet [63], which is cooked to 100°C. It has already been demonstrated in previous works that pasta, functionalized with plant extracts with antioxidant and anti-inflammatory activity,

was able to have beneficial effects, and to help prevent age-related metabolic disorders [64, 65]. These, like obesity, could lead to imbalance of the microbiota, which plays an important role in maintaining the proper functioning of the gastrointestinal system. Fortunately, there are numerous bioactive compounds in the diet that influence the composition of the microbiota and could therefore be useful against these diseases [66, 68]. In agreement with these studies, it was observed in this work that AFA has a prebiotic effect and an antioxidant activity that are maintained even after thermal stress, indicating that it could regulate the composition of the intestinal microbiota, also modulating its profile. The biscuits that have been produced with the AFA extract have a high content of antioxidant polyphenols, indicating that its properties are maintained and improved after cooking; therefore our preliminary results allow us to hypothesize that the AFA biscuits could be a functional food for the prevention and treatment of pathologies characterized by oxidative stress. Further studies will be needed on healthy volunteers who are given cookies to validate the effect on human health.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2019, Article ID 9481390, 15 pages
https://doi.org/10.1155/2019/9481390

Research Article

Heat-Resistant *Aphanizomenon flos-aquae* (AFA) Extract (Klamin®) as a Functional Ingredient in Food Strategy for Prevention of Oxidative Stress

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Received 19 February 2019; Revised 24 July 2019; Accepted 7 September 2019; Published 11 November 2019

Guest Editor: Maura Palmery

Microalgae are generally considered an excellent source of vitamins, minerals, and bioactive molecules that make them suitable to be introduced in cosmetics, pharmaceuticals, and food industries. *Aphanizomenon flos-aquae* (AFA), an edible microalga, contains numerous biomolecules potentially able to prevent some pathologies including age-related disorders. With the aim to include an AFA extract (Klamin®) as a functional ingredient in baked products, we investigated if its bioactive molecules are destroyed or inactivated after standard cooking temperature. The AFA extract was exposed to heat stress (AFA-HS), and no significant decrease in pigment, polyphenol, and carotenoid content was detected by spectroscopic analysis. Thermal stability of AFA-HS extract was demonstrated by thermogravimetric analysis (TGA), and no change in the morphology of the granules of the powder was noticed by SEM microscopic observation. By Folin-Ciocalteu, ORAC, and ABTS assays, no change in the antioxidant activity and polyphenol contents was found after high-temperature exposition. When added in cell culture, solubilized AFA-HS lost neither its scavenging ability against ROS generation nor its protective role against Abeta, the main peptide involved in Alzheimer's disease. Prebiotic and antioxidant activities of AFA extract that are not lost after thermal stress were verified on *E. coli* bacteria. Finally, AFA-HS cookies, containing the extract as one of their ingredients, showed increased polyphenols. Here, we evaluate the possibility to use the AFA extract to produce functional food and prevent metabolic and age-related diseases.

1. Introduction

Microalgae comprise prokaryotic cyanobacteria and eukaryotic photoautotrophic protists, with significant diversity in their metabolism, cell structure, and habitat [1]. They are the oldest forms of life and have been the main biotic source of oxygen on early Earth [2]. These photosynthetic microorganisms exhibit commercial interest due to their ability to produce biomass from where bioactive compounds can be obtained. The last ones include pigments, vitamins, proteins, lipids, polyunsaturated fatty acids, and carbohydrates [3].

Aphanizomenon flos-aquae (AFA) is a cyanobacterial unicellular organism with remarkable nutritional properties that, unlike other commercial "microalgae," spontaneously grows in Upper Klamath Lake (southern Oregon, USA). This lake is an ideal natural ecosystem for the growth of the AFA microalgae, especially during the period between late summer and early fall, while the cold is gradually starting. Sunny days favor the intense photosynthetic activity of AFA, which is achieved by its various pigments, including phycocyanins and phycoerythrins. Furthermore, the production of fatty acids, such as omega 3, is favored during the winter, while

the lake freezes over due to low temperatures. Thanks to the volcanic origin of the lake, AFA contains a wide and complete variety of minerals [4, 5] and pigments such as carotene, beta-carotene, chlorophylls [5, 6], phycocyanins, phycoerythrins, and polyphenols [5, 7] with significant antioxidant [8], anti-inflammatory [5, 9], and antiproliferative [10] properties.

Numerous physiologically active biomolecules derived from algae have been investigated for their role in disease prevention and health and for their potential use as dietary supplements [11, 12]. For instance, Sabelli et al. [13] demonstrated the therapeutic benefits of phenylethylamine (PEA), an endogenous neuromodulator that is present in AFA algae and which, when deficient, can lead to certain forms of depression and affective disturbances [13, 14].

The nutritional supplement Klamin® is an AFA extract which contains concentrated quantity of phenylethylamine and other supporting molecules, and it has been proven to be effective in countering a wide variety of pathologies of the neurological system, including neurodegenerative diseases [15–17]. Oxidative stress, inflammation, and mitochondrial and neurological dysfunctions are some of the main causes of neurodegenerative pathologies, such as Alzheimer's disease (AD) [18–20]. In these years, the role of natural antioxidants as neuroprotective agents has been investigated in the molecular level, and their benefits have been widely demonstrated [18, 21–24]. These natural molecules can exert their antioxidant activity in two ways: by the improvement of the activity of antioxidant enzymes or by their scavenger capacity that can deactivate the reactive oxygen species (ROS). Therefore, the high levels of antioxidant molecules present in the AFA extract can make it a potential therapeutic agent, especially for those pathologies [19, 20].

In a precedent work, our group demonstrated that Klamin® shows scavenging properties against ROS generation and plays a role in mitochondrial protection [5]. In addition, it inhibits the aggregation process of beta-amyloid (Abeta), the main peptide involved in AD [5, 25, 26]. This peptide is a product of the sequential γ- and β-secretase proteolytic cleavage of the amyloid precursor protein (APP) [27]. Abeta has a high tendency to polymerize and form fibrils, aggregates, and the AD characteristic amyloid plaques, changing its conformation to a β-sheet structure [28]. However, several findings support the hypothesis that soluble oligomers, especially the ADDL aggregates, rather than plaques, are the most neurotoxic agents [29]. On this basis, synthetic or recombinant Abeta oligomers have been administered both in *in vitro* and *in vivo* model systems to mimic the AD pathology and study the possibility to inhibit its progression by using drugs or natural compounds [22, 23, 30–32].

The findings described above support the innovative and natural strategy that recommends the use of dietary supplements as an alternative form of prevention and treatment of several pathologies. So, the development of new medical formulations based on such supplements should be promoted [33–35]. Furthermore, dietary supplements can also be composed of extracts derived from different plants, and their health benefits against metabolic syndromes, arteriosclerosis, burns or chronic wounds, and neurodegenerative diseases have been proven [36–38]. Thus, dietary supplements could be recommended as an alternative form of prevention and treatment of different pathologies and could lead towards a more natural approach in the pharmaceutical industry.

One potential way in this new approach could be the development of functional food. In fact, numerous companies have shown interest in developing new strategies for the functionalization of food for the health and wellness market. Additionally, natural antioxidant compounds can also be used in the field of food packaging [39, 40] or as additives, preserving food from deterioration, rancidity, or discoloration. This allows extension of the shelf life without any adverse effects on the food's sensory or nutritional qualities. Moreover, these natural compounds can replace the currently used synthetic antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) or propyl gallate (PG), that have also shown toxic effects, as food additives [41–43].

Finally, Sun et al. showed that anthocyanins extracted from Chinese purple sweet potato cultivar exert prebiotic-like activity, highlighting that bioactive molecules extracted from plants can be beneficial for the intestinal bacterial flora [44].

The goal of our research is to better analyze the activity of the bioactive molecules from the AFA extract and promote further investigation on its application in the food industry. More specifically, we analyzed a potential use of the AFA extract Klamin® as an addition to bread and/or biscuit dough, by investigating if its antioxidant and prebiotic activities would remain unaffected after its exposure to high temperatures.

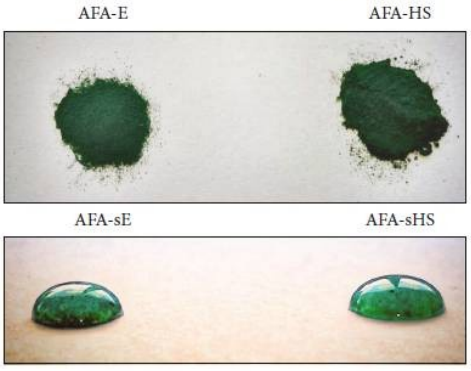
2. Materials and Methods

2.1. AFA Extract Sample Preparation. The AFA extract Klamin® was kindly provided by Nutrigea Research s.r.l. (Republic of San Marino) [45]. The product was pulverized, according to Nuzzo et al. [5]. We called this sample AFA-Extract (AFA-E). For Heat-Stressed sample preparation, AFA-E powder was exposed at 220°C for 10 minutes, and this sample was called AFA-Heat Stressed (AFA-HS). For the experiments with cells and bacteria, a soluble fraction of the AFA extract was prepared. More specifically, 10 mg of AFA-E or AFA-HS powder was dissolved in 10 mL of PBS (pH = 7.4; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_3PO_4). The solutions were sonicated (70% of the maximum power, twice for 30 seconds) and magnetically stirred for an hour. The insoluble fractions were removed by centrifugation at 14,000 rpm for 30 min at 4°C. The supernatants (soluble fractions) were collected, filtered by using a 0.45 μm Sartorius filter, aliquoted (1 mL/vial), and stored at -20°C. These fractions are here named as AFA-soluble Extract (AFA-sE), and AFA-soluble Heat Stressed (AFA-sHS) (Table 1). For the experimental procedure, 10 mg from all the AFA samples was dissolved in 10 mL of PBS to achieve a concentration of 1 mg/mL.

2.2. Fluorescent Analysis of AFA Extracts. 10 μL of AFA-E and AFA-HS solutions was directly spotted onto nitrocellulose membrane strips and incubated at room temperature

Table 1

Abbreviations	
AFA-E	Powder of AFA extract
AFA-HS	Heat-stressed powder of AFA
AFA-sE	Hydrosoluble extract of AFA-E
AFA-sHS	Hydrosoluble extract of AFA-HS



in the dark. After 20 minutes, their fluorescence intensity was measured by using a Typhoon FLA 9500 (GE Healthcare Life Sciences) fluorescence scanner at a resolution of 20 μm. For fluorescence detection, a different wavelength was used, according to the pigment 635 nm (red laser) for phycocyanins, 532 nm (green laser) for phycoerythrins, and 473 nm (blue laser) for carotenoids. The fluorescence intensity was reported in arbitrary units (A.U.).

2.3. Chemical Characterization. The AFA-E and AFA-HS samples were chemically analyzed by Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) and UV-Vis spectroscopy. Infrared spectra were obtained with an ATR accessory (MIRacle ATR, PIKE Technologies) with a diamond crystal coupled to a Fourier-Transform Infrared (FTIR) spectrometer (Equinox 70 FT-IR, Bruker). All spectra were recorded in the range from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}, accumulating 128 scans. UV-Vis spectra of the powder of AFA-E and AFA-HS were taken by using a Cary 300 Scan UV-visible spectrophotometer.

2.4. Folin-Ciocalteu Colorimetric Assay. The phenolic content was calculated by using the Folin-Ciocalteu (F-C) colorimetric assay [5]. Aliquots (0.2 mL) of the AFA-E and AFA-HS extracts were made up to 5 mL with distilled water, and 0.5 mL of Folin-Ciocalteu reagent was added. After 3 min, 1 mL of Na_2CO_3 (20% *w*/*v*) was added to the reaction mixtures that were made up to 10 mL with distilled water. The samples were then stored for 2 hours at room temperature. The absorbance of the solutions was measured at 765 nm by using a spectrophotometer (Shimadzu) and was quantified by using a gallic acid standard curve.

2.5. Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay was performed according to [46, 47], slightly modified. The reaction was carried out by using a 96-well plate: 160 μL of 0.04 μM fluorescein in 0.075 M Na-K phosphate buffer pH 7.0, 20 μL of diluted phenolic extract, or 20 μL of 100 μM Trolox. The mixture was incubated for 10 min at 37°C in the dark. After this incubation, 20 μL of 40 mM 2,2′-Azobis-(2-methylpropionamidine) dihydrochloride (AAPH) solution was added. The microplate was immediately placed in a microplate reader (Thermo Scientific Fluoroskan Ascent F2 Microplate), and the fluorescence was recorded (excitation and emission wavelengths at 485 and 527 nm, respectively) every 5 min for 60 min. The ORAC value refers to the net area under the curve of fluorescein decay in the presence of the Klamin® phenolic extract or Trolox, minus the blank area. The activity of the sample was expressed by μmol of Trolox equivalents (TE)/g of AFA or AFA-Hs by using the following equation:

$$\mathrm{ORAC}(\mu\mathrm{mol\,TE/g}) = k * a * h * \left[\frac{(S_{\mathrm{sample}} - S_{\mathrm{blank}})}{(S_{\mathrm{Trolox}} - S_{\mathrm{blank}})}\right], \quad (1)$$

where k is the final dilution of the water-soluble extract; a is the ratio between the volume (liters) of the water-soluble extract and the grams of AFA-E or AFA-HS; h is the final concentration of Trolox expressed as μmol/L; and S is the area under the curve of fluorescein in the presence of sample, Trolox, or buffer solution. All the reaction mixtures were prepared in triplicates, and at least three independent assays were performed for each sample.

2.6. ABTS Free Radical Cation Scavenging Assay. The ABTS radical cation ($ABTS^{+}$) was generated by the reaction between 7 mM ABTS water solution with 2.45 mM potassium persulfate solution. The reaction took place in the dark at room temperature for 12-16 h. ABTS and potassium persulfate react stoichiometrically at a ratio of 1 : 0.5, resulting in the incomplete oxidation of the ABTS. The oxidation of the ABTS starts immediately, but the absorbance does not become maximal and stable until some hours are elapsed [48]. The $ABTS^{+}$ solution was then diluted with water to obtain a starting absorbance of 1.2 arbitrary units at 734 nm. Right after the dilution, 2 mg of AFA-E and AFA-HS was separately placed in polystyrene cuvettes containing 2 mL of the diluted $ABTS^{+}$ solution. The decrease of the absorbance was measured at 734 nm by using a Cary 300 Scan UV-visible spectrophotometer in the dark at room temperature for 24 h. All measurements were performed in triplicate. A sample with only 2 mL of $ABTS^{+}$ solution was used as a control, to ensure the stability of the solution. The radical scavenging activity (RSA) of AFA-E and AFA-HS was expressed as the inhibition percentage of the free radical of the samples and was calculated by using the following formula:

$$\text{Radical Scavenging Activity (\%)} = \left[\frac{(A)_{\mathrm{control}} - (A)_{\mathrm{sample}}}{(A)_{\mathrm{control}}}\right] \times 100, \quad (2)$$

where $(A)_{\mathrm{control}}$ stands for the absorbance of the control sample at a specific time point and $(A)_{\mathrm{sample}}$ for the absorbance

of the sample at this time point. The results were presented in mean values with ±standard deviation (<1% for 24 h) [33, 49, 50].

2.7. Thermal Characterization. The thermal stability behavior of the AFA-E powder was investigated by a standard thermogravimetric analysis (TGA) method, using a TGA Q500 from TA Instruments. Measurements were performed with 3-5 mg samples in an aluminum pan under air atmosphere with a flow rate of 50 mL/min in a temperature range from 30 to 800°C and with a heating rate of 10°C/min. The weight loss and its first derivative were recorded simultaneously as a function of time/temperature. At the same operative conditions, an isothermal thermogravimetric characterization of the AFA-E sample was carried out, in order to simulate the cooking conditions. The AFA-E sample was exposed to a stable temperature of 220°C for 10 minutes, and the weight loss was evaluated. After 10 minutes, a thermal ramp from 220 to 800° with a heating rate of 10°C/min was performed to complete the thermal analysis.

2.8. Morphological Characterization. Morphology of the AFA-E and AFA-HS samples was analyzed by Scanning Electron Microscopy (SEM), using a variable pressure JOEL JSM-649LA microscope equipped with a tungsten thermionic electron source working in high vacuum mode, with an acceleration voltage of 5 kV. The specimens were coated with a 10 nm thick film of gold using a Cressington Sputter Coater-208 HR. The diameter of the granules of the powder was analyzed and determined by using the ImageJ software. Approximately 170 measurements were taken to obtain the diameter distribution of each algae sample.

2.9. Cell Cultures and Treatment. A549 human epithelial cells or LAN5 neuroblastoma cells were cultured with RPMI 1640 medium (Celbio srl, Milan, Italy) supplemented with 10% fetal bovine serum (Gibco-Invitrogen, Milan, Italy) and 1% antibiotics (50 mg mL^{-1} penicillin and 50 mg mL^{-1} streptomycin). Cells were maintained in a humidified 5% CO_2 atmosphere at 37 ± 0.1°C. For toxicity assays, A549 cells were treated with 0.1, 0.5, and 1 μg of AFA-sE and AFA-sHS for 24 hours or with H_2O_2 (50 μM) pure or combined with AFA-sE and AFA-sHS at different concentrations (0.1, 0.5, and 1 μg) for 24 hours. Untreated A549 cells were used as control. In the experiment that was carried out for the evaluation of the neurodegeneration effect, a recombinant Abeta peptide (60 μM) was produced according to Carrotta et al. [51] and was administered to LAN5 cells under oligomeric form for 24 hours. Small oligomers (ranging between 8 and 67 kDa) were also prepared according to Carrotta et al. [51]. Briefly, after a preliminary treatment with trifluoroacetic acid (TFA), the powder of the recombinant Abeta was dissolved in 0.01 M Tris-HCl buffer, pH 7.2, and the solution was readily characterized by dynamic light scattering (DLS) at T = 15°C [26, 51].

2.10. Determination of Cell Viability. Cell viability was measured by the MTS assay (Promega Italia, S.r.l., Milan, Italy). MTS was used according to the manufacturer's instructions. After cell treatments, the incubation was carried out for 3 hours at 37°C, 5% CO_2. The absorbance was measured at 490 nm on the Microplate Reader Wallac Victor 2 1420 Multilabel Counter (Perkin Elmer, Inc. Monza, Italy). Results were expressed as the percentage of the MTS reduction with the control samples as reference and presented as mean value ± standard deviation (SD).

Table 2

Ingredients	
500 g	Mix of flour
126 g	Margarine
186 g	Sugar
3 g	Yeast
93 g	Eggs
6 g	AFA-E
19.5 mL	Water

2.11. Analysis of Reactive Oxygen Species (ROS) Generation. To assess ROS generation, treated A549 or LAN5 cells were placed in a 96-well microplate. Some of A549 were treated with H_2O_2 (50 μM) alone or with the presence of AFA-sE (1 μg) and AFA-sHS (1 μg) or with AFA-sE (1 μg) and AFA-sHS (1 μg) as control for 24 hours. Then, dichlorofluorescein diacetate (DCFH-DA) (1 mM) was added to each sample, and then the samples were placed in the dark for 10 min at room temperature. After washing them with PBS, the cells were analyzed by a fluorescence microscope (Axio Scope 2 microscope; Zeiss, Oberkochen, Germany) and a fluorimeter Microplate Reader (GloMax, Promega) for fluorescence intensity detection.

2.12. Effect of AFA and AFA-HS on the Probiotic Bacteria Proliferation. The prebiotic activity of AFA-sE and AFA-sHS was tested on *Escherichia coli* (*E. coli*), a gram-negative bacterium, with a harmless serotype. One day before the test, a single colony was inoculated into Luria-Bertani (LB) liquid medium and incubated at 37°C overnight (o.n.). An aliquot (5 μL) of the o.n. bacterial culture, approximately 10^9 CFU/mL, was added to three test tubes containing fresh LB medium (5 mL). AFA-sE (1 μg) or AFA-sHS (1 μg) was added separately to the culture medium at time 0 min or 120 min. Untreated *E. coli* was used as control. For the bacteria oxidative stress experiment, an aliquot (5 μL) of the o.n. bacterial culture was added to different test tubes containing fresh LB medium (5 mL) and H_2O_2 at different concentrations (1, 2, 3, and 4 mM) was added when the bacteria were in the exponential phase of growth (*data not shown*). For the inhibition of the oxidative stress experiment, bacteria were incubated with H_2O_2 (1.5 mM) in three different test tubes, and when the bacteria were in the exponential phase of growth, AFA-sE (1 μg) or AFA-sHS (1 μg) was separately added. *E. coli* treated with H_2O_2 or AFA-sE or AFA-sHS was used as control. In all of the experiments, the exponential growth was determined by reading the absorbance value at 600 nm (OD_{600}) at a spectrophotometer with 30 min intervals.

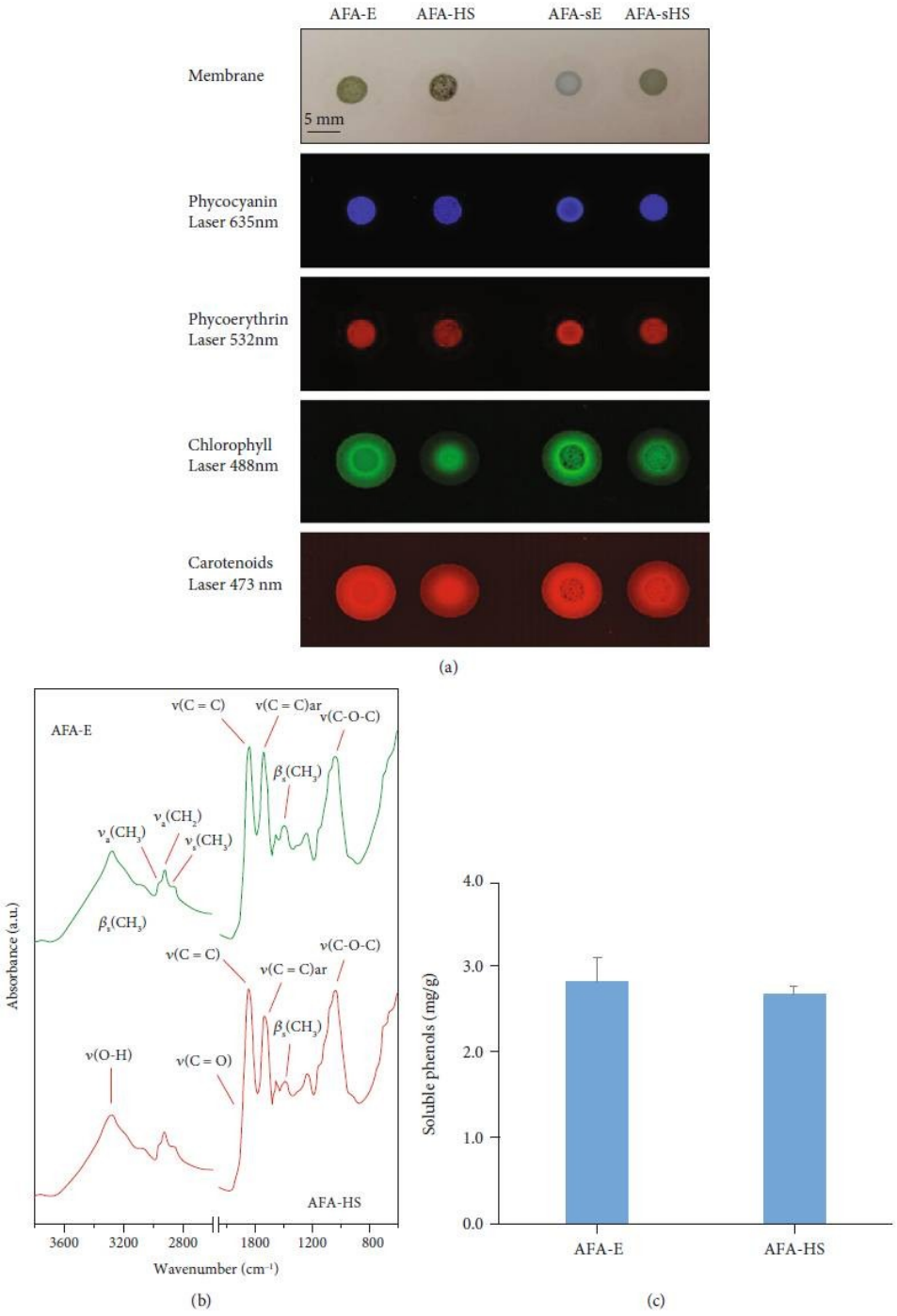


FIGURE 1: Continued.

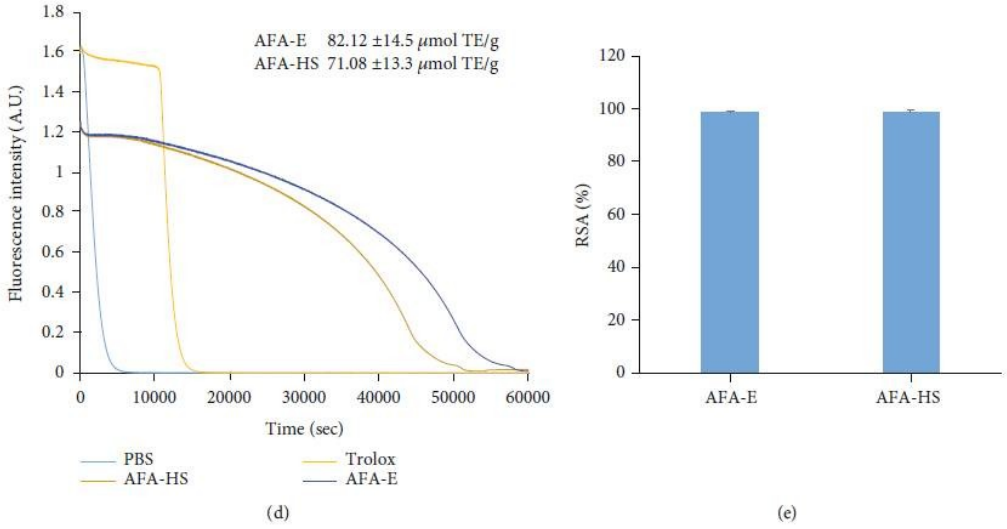


FIGURE 1: Fluorescence measures and absorbance quantification of AFA extracts under no-thermic (AFA-E) and thermic stress (AFA-HS). (a) Fluorescence intensity at 635 nm (phycocyanins), 532 nm (phycoerythrins), 488 nm (chlorophyll), and 473 nm (carotenoids). (b) Chemical analysis, ATR-FTIR spectra of the AFA-E (top) and AFA-HS (bottom) samples. (c) Polyphenol contents of the AFA-E and AFA-HS assayed by Folin-Ciocalteu. (d) Antioxidant capacity of the AFA-E and AFA-HS assayed by ORAC reducing capacity. (e) ABTS assays, comparison between the radical scavenging activity percentages of the AFA-E (left) and the AFA-HS (right) after 24 h.

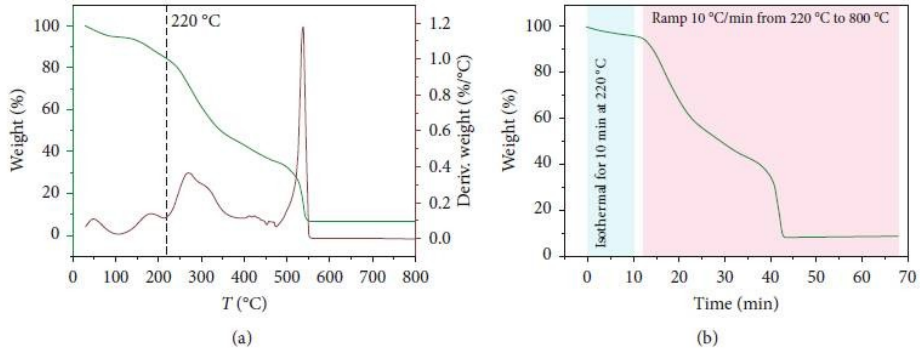


FIGURE 2: Thermal stability analysis. (a) TGA curve of AFA-E expressed as weight percentage (left side) and its first derivate (right side) in the range of temperature between 30 and 800°C. (b) Isothermal thermogravimetric analysis of AFA-E sample at 220°C (blue area) followed by a nonisothermal thermogravimetric analysis (pink area) from 220°C to 800°C at the rate of 10°C/min as a function of the time.

2.13. Effect of AFA and AFA-HS on the Bacteria Reactive Oxygen Species (ROS) Generation. An aliquot of *E. coli* o.n. culture solution, approximately 10^9 CFU/mL, was diluted ($1:10^5$) and 5 μL was placed in a 96-well optical bottom white microplate. H_2O_2 (1.5 mM) with AFA-sE (1 μg) or AFA-sHS (1 μg) was added to the wells. *E. coli* treated with H_2O_2 or AFA-sE or AFA-sHS was used as control. Then, the samples were incubated with 1 mM DCFH-DA for 2 and 4 hours at room temperature. Afterward, the *E. coli* samples were analyzed by using the Microplate Reader (GloMax, Promega) for fluorescence detection.

2.14. Functional Food Design. The cookie dough was prepared according to "*Le Farine dei Nostri Sacchi* s.r.l." (Palermo, Italy) by using classical ingredients with the addition of Klamin® or AFA-E (Table 2) and baking at 220°C for 10 minutes. Each cookie (1.5 g) contains 0.1 g of AFA-E. For the F-C assay, 2 g of biscuit dough raw or cooked was dissolved in 15 mL of water and, after vortexing, centrifuged for 20 min at 1500g. Then, 0.5 mL of the F-C reagent was added to 2 mL of the filtered supernatant and the analysis was performed as described above.

2.15. Statistical Analysis. The significance of the differences in the mean values of multiple groups was evaluated by using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Differences were considered significant when the p value was ≤ 0.05.

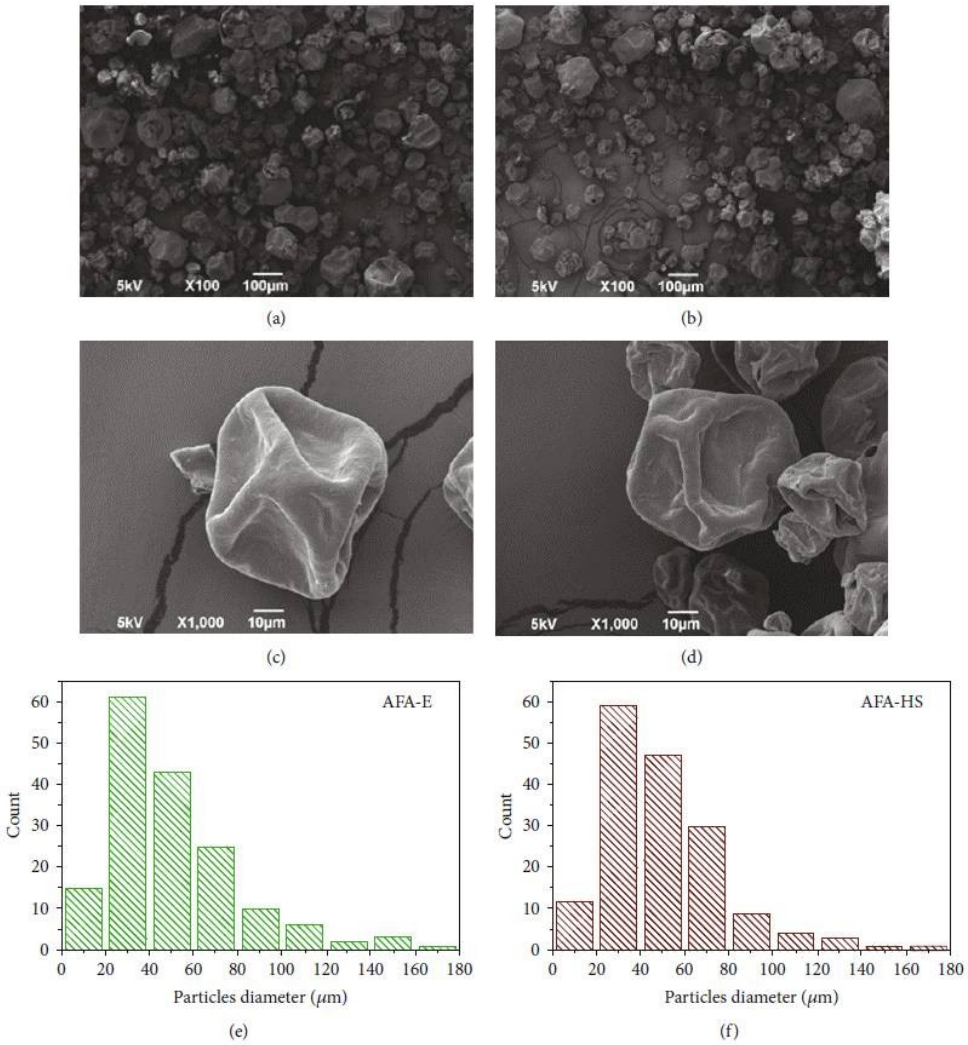


FIGURE 3: Morphological analysis. (a, c) SEM images of the AFA-E powder at different magnifications. (b, d) SEM images of the AFA-HS powder at different magnifications. (e, f) Particle diameter distribution for the AFA-E and AFA-HS samples, respectively.

3. Results

3.1. Heat Stress Does Not Affect AFA Extract Content and Antioxidant Activity. To evaluate if the pigment contents of AFA extracts are maintained under heath stress, we performed an analysis based on their spectroscopic properties and chromophore content [52]. By using appropriate excitation and emission filters, no significant difference in fluorescence intensity and absorption spectra was detected between unheated (AFA-E) and heated (AFA-HS) phycoerythrin and phycocyanin samples. However, light differences were observed for chlorophyll and carotenoids (Figure 1(a)), indicating a minor stability with respect to the other pigments. The ring around the AFA spots (Figure 1(a)) was due to the diffusion of chlorophyll and carotenoids [53].

Infrared spectra of the AFA-E and AFA-HS samples are reported in Figure 1(b). AFA-E is a mix of compounds, so various bands can be associated with different chemical structures, such as polyphenols, pigments, and carotenoids. The two spectra were mainly characterized by the following bands: O-H stretching mode at 3281 cm^{-1}, asymmetric CH_3 stretching mode at 2957 cm^{-1}, asymmetric and symmetric CH_2 stretching mode at 2926 and 2874 cm^{-1}, respectively, C=O stretching mode at 1685 cm^{-1}, C=C stretching mode at 1643 cm^{-1}, aromatic C=C stretching mode at 1533 cm^{-1}, symmetric CH_3 binding mode at 1387 cm^{-1}, and C-O-C stretching mode at 1034 cm^{-1}. Differences between AFA-E and AFA-HS spectra in the intensity of the peak at 1533 cm^{-1} and at 1387 cm^{-1} were observed. The first one is the stretching of aromatic C=C conjugated with aliphatic C=C, and it

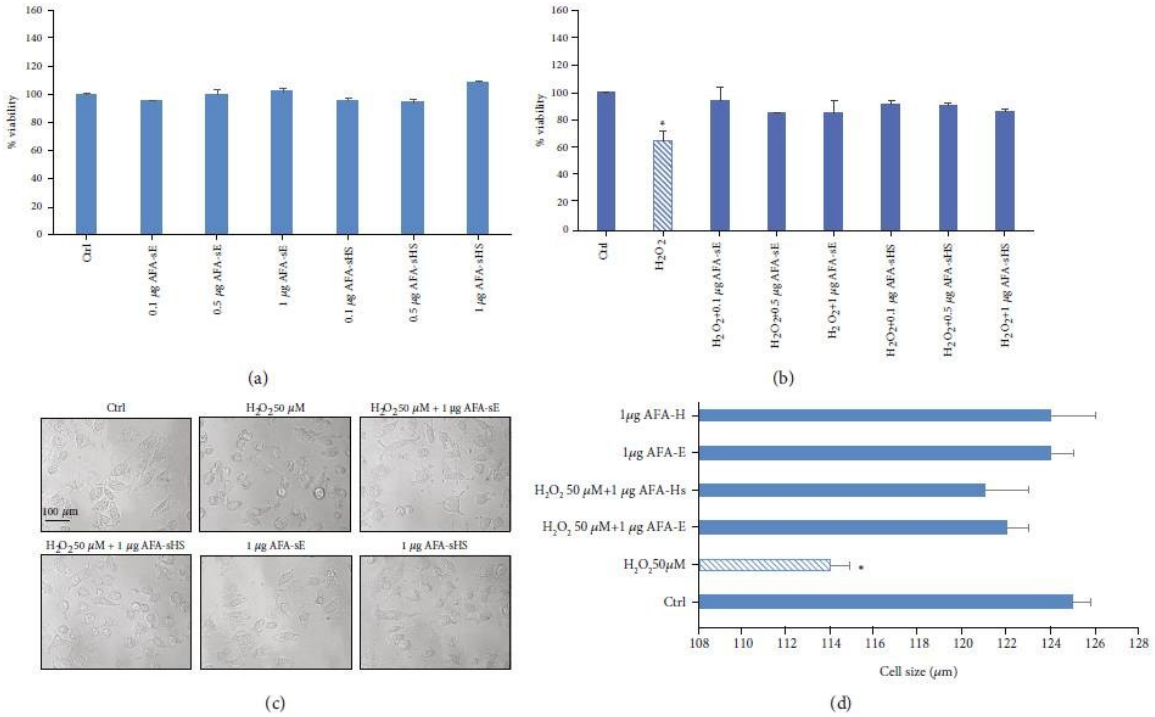


FIGURE 4: Effect of AFA-sE and AFA-sHS on A549 cells. (a) MTS cell viability assay of A4509 cells alone (Ctrl) or incubated with different AFA-sE and AFA-sHS concentrations. (b) MTS cell viability assay without (Ctrl) or after treatment of A549 cells with H_2O_2 alone or in combination with different AFA-sE and AFA-sHS concentrations. (c) Representative morphological images of A549 untreated cells (Ctrl) or treated with AFA-sE and AFA-sHS or with H_2O_2 alone or in combination with AFA extracts. (d) Histogram of A549 untreated cells (Ctrl) or treated with AFA and AFA-HS cell body size. Bar: 100 μm.

is typical of phenolic compounds [54]. Instead, the peak at 1387 cm^{-1} can be assigned to the CH_3 present in the carotenoid structure [55]. Therefore, due to the heating treatment, a decrease in the quantity of these compounds present in the AFA-E powder was observed. After that, the maintenance of polyphenols and bioactive activity after thermic stress was analyzed by Folin, ORAC, and ABTS assays. The values were expressed as mg of gallic acid equivalents per g of extract for the Folin-Ciocalteu assay (Figure 1(c)) and as μmol of Trolox equivalents (TE) per g of extract for ORAC assays (Figure 1(d)), and no significant differences were detected between AFA-E and AFA-HS samples. Finally, by the ABTS radical cation assay, we measured the reduction of the radical cation as the inhibition percentage of absorbance at 734 nm. The comparison between the antioxidant activity of the AFA-E and AFA-HS is presented in Figure 1(e). More specifically, the reaction of our samples with $ABTS^{\cdot+}$ was completed within 24 h, reaching a percentage of 99.1% and 98.6% for AFA-E and AFA-HS, respectively (Figure 1(e)). These results clearly demonstrate that both AFA-E and AFA-HS were able to inhibit successfully the free radicals from the $ABTS^{+}$ solution.

3.2. Thermal Stability and Morphological Analysis. The thermal properties of the AFA-E sample were evaluated by TGA, and the main results are reported in Figure 2. Figure 2(a) shows the thermogravimetric analysis of AFA-E expressed as weight loss percentage (left side) and its first derivate (right side). The powder showed a weight loss of ≈16% when it reached a temperature of 220°C (dash line in Figure 2(a)). Instead, it is decomposed at 550°C, and at the end of the thermogravimetric measurement, a final residual of ≈6.5% was observed.

In Figure 2(b), an isothermal analysis for the AFA-E powder is reported. In order to evaluate the potential weight loss in cooking condition, we maintained the temperature of 220°C for 10 min. After 10 min at the cooking temperature, the powder lost only 4% of its weight, demonstrating its potential compatibility with preparation procedure of bakery products. Afterward, the temperature was increased from 220 to 800°C, and the powder showed the same trend observed in Figure 2(a) with a total decomposition achieved at 550°C. Furthermore, the morphology of the AFA-E and AFA-HS samples was analyzed by SEM. Figures 3(a) and 3(c) show two top-view images at different magnifications of a characteristic AFA-E sample, whereas Figures 3(b) and 3(d) report the top-view images of the AFA-HS sample. No differences in shape were noticed between the sample before and after the thermal treatment, and the "deflated ball" shape was maintained. In Figures 3(e) and 3(f), the diameter size

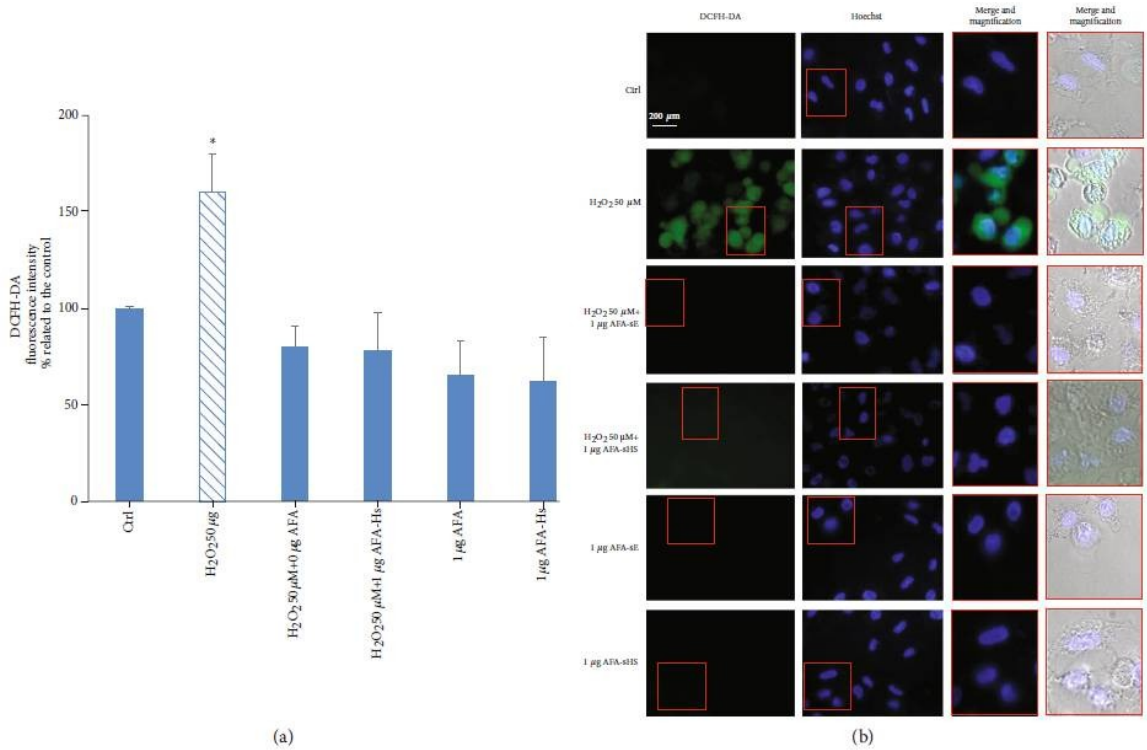


FIGURE 5: AFA extracts protect A549 cells from oxidative insult. (a) Fluorescence intensity of A549 cells alone (Ctrl) or treated with H_2O_2 or AFA-sE or AFA-sHS alone or cotreated with H_2O_2 and AFA-sE or AFA-sHS measured by the DCFH-DA assay. (b) Fluorescence microscopy images of untreated cells (Ctrl) and cells treated with H_2O_2 or cotreated with H_2O_2 and the AFA extracts.

distribution of the particles is presented for the AFA-E and AFA-HS samples, respectively. In both cases, a main distribution between 20 and 80 μm was observed.

3.3. AFA-sE and AFA-sHS Recover H_2O_2-Induced Cytotoxic Effect. In order to understand if AFA-E is able to release toxic molecules after heat treatment, different concentrations of untreated (control) and high temperature-treated AFA extracts were added to A549 cells, and after incubation for 24 hours, an MTS assay was performed. Figure 4(a) shows that no toxicity was detected at all the concentrations, compared with the control. Furthermore, to assess if unheated or heated AFA extracts can inhibit H_2O_2-induced toxicity, A549 cells were incubated with hydrogen peroxide in combination with different concentrations of AFA-sE and AFA-sHS. As shown by the MTS assay, all of the used AFA extracts are able to inhibit the H_2O_2-induced cell toxicity, and no differences were observed for the heated samples (Figure 4(b)). All these results were confirmed by the morphological observation (Figure 4(c)) and analysis of the cell body size in which a recovery of the altered cell shape due to H_2O_2 treatment was detected (Figure 4(d)).

3.4. AFA-sE and AFA-sHS Inhibit ROS Generation. A clear-cut result about the maintenance of the antioxidant activity of the AFA extract after thermal treatment was evaluated by treating A549 cells with H_2O_2 alone or in combination with unheated and heated AFA extracts and by using the DCFH-DA assay. By fluorometric analysis, we detected that the presence of both of AFA-sE and AFA-sHS decreases H_2O_2-induced ROS generation (Figure 5(a)). Furthermore, these data were confirmed by fluorescence microscope inspection. Indeed, cells treated with H_2O_2 showed green fluorescence due to ROS generation, while cells treated with AFA extracts or H_2O_2 AFA-treated extracts did not show any fluorescence (Figure 5(b)). The result suggests that the components of AFA extract, such as carotenoids, phycoerythrins, phycocyanins, and polyphenols, preserve a significant role as antioxidant agents even if they are heat stressed.

3.5. Neuroprotective Effect of AFA-sE and AFA-sHS. Since a neuroprotective effect was demonstrated for Klamin® supplement [5], we tested if this property is maintained after thermic stress. LAN 5 cells were treated with Abeta oligomers alone or with AFA-sE and AFA-sHS extracts and submitted to the MTS assay. The Abeta-induced toxicity was inhibited by the coadministration of the AFA extracts (Figure 6(a)). Observation of cell morphology confirmed the viability assay results (Figure 6(b)). The antioxidant capacity of both AFA extracts against the Abeta oligomer-induced oxidative stress was evaluated by the DCFH-DA assay. Fluorescence analysis indicated that cells treated with Abeta alone exhibit high

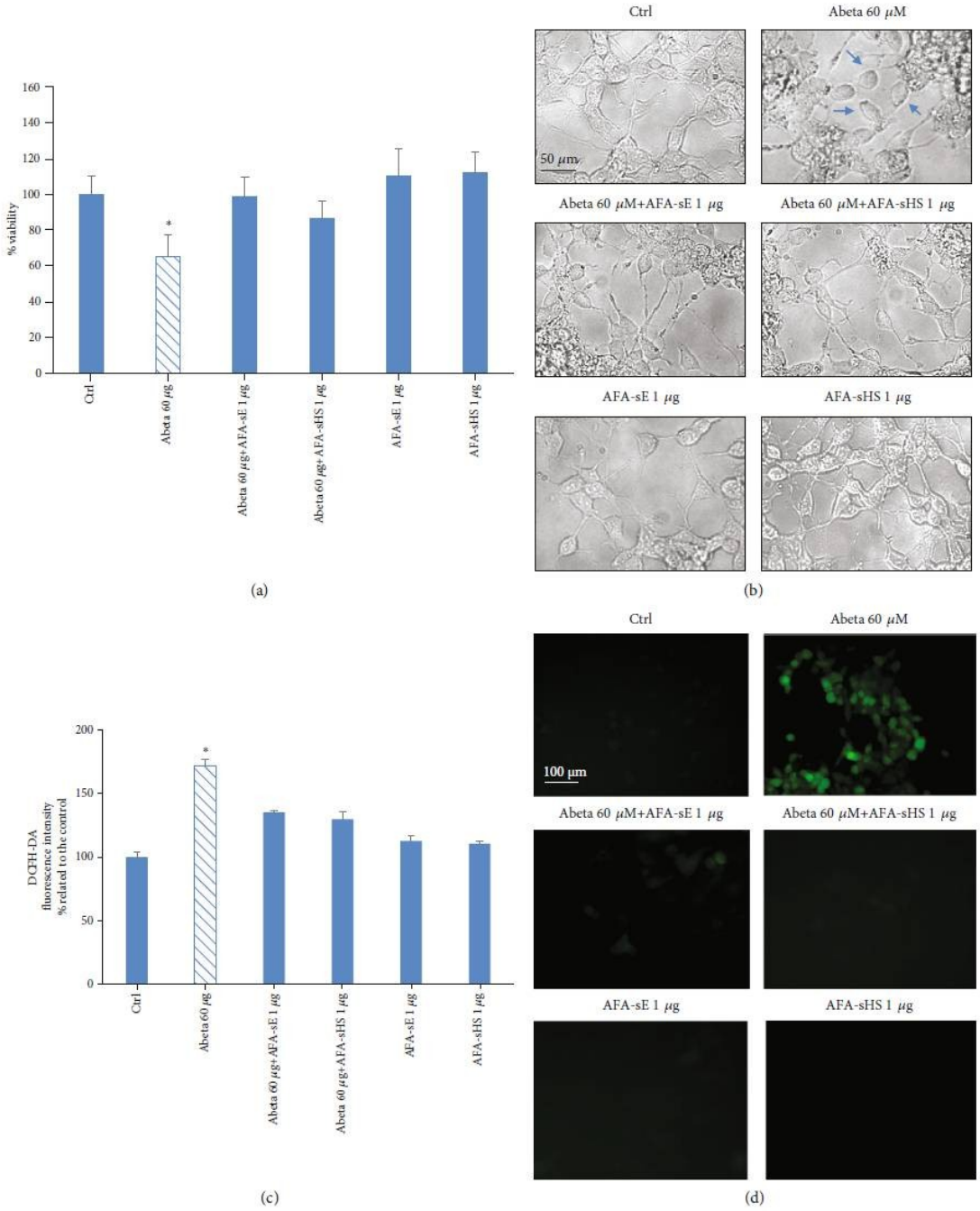


FIGURE 6: AFA-sE and AFA-sHS protect against Abeta-induced toxicity. (a) MTS of untreated LAN5 cells (Ctrl) or cells treated with the AFA extract, with Abeta alone, or with AFA-sE or AFA-sHS. (b) Morphological representative images of samples indicated in (a). (c) DCFH-DA assay of untreated LAN5 cells (Ctrl) or cells treated with Abeta oligomers alone or with AFA-sE or AFA-sHS. (d) Fluorescence representative images of samples indicated in (c). Bar: 50 μm.

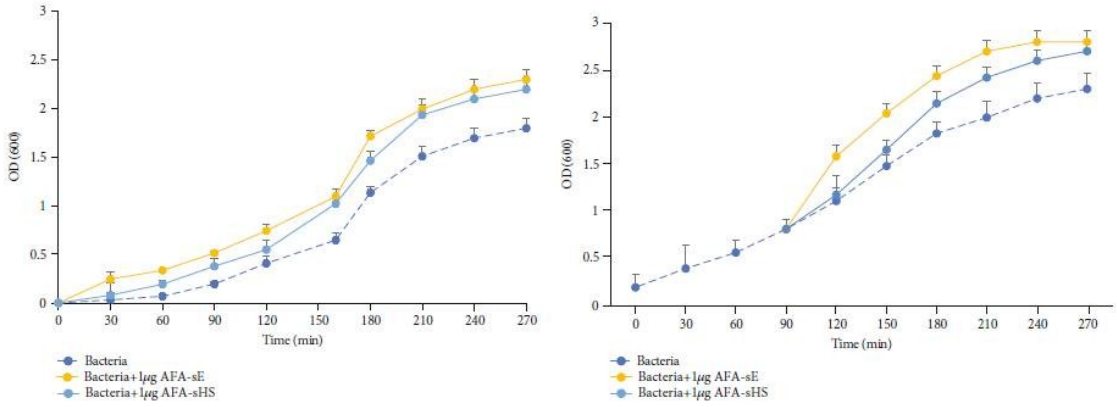


FIGURE 7: AFA and AFA-HS have prebiotic effect. *E. coli* growth curve with the presence of AFA-sE and AFA-sHS added at the beginning (a) or at the exponential phase of growth (b).

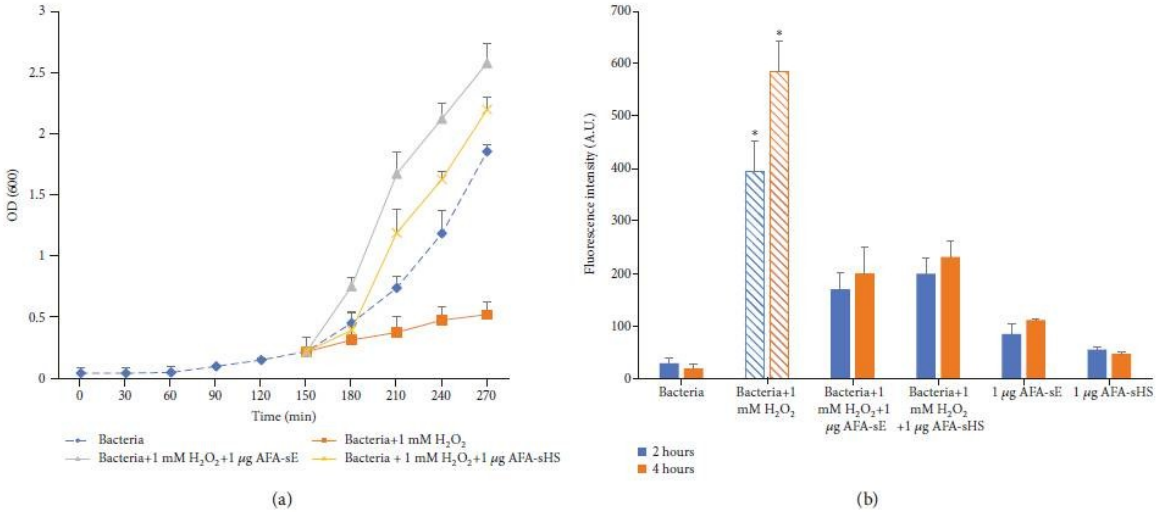


FIGURE 8: AFA extracts protect *E. coli* by oxidative stress. (a) Growth curve of *E. coli* alone (bacteria) or treated with H_2O_2 alone or with H_2O_2 in combination with AFA-sE and AFA-sHS. (b) Oxidation kinetics of *E. coli* alone (bacteria) or in the presence of H_2O_2 or H_2O_2 in combination with AFA-sE and AFA-sHS measured by the DCFH-DA assay after 2 and 4 hours of incubation.

levels of ROS generation, whereas cotreatment of Abeta oligomers and AFA extracts did not produce any fluorescent signal (Figure 6(c)). The same samples were observed at fluorescent microscopy (Figure 6(d)).

3.6. Prebiotic and Antioxidant Effect of AFA-sE and AFA-sHS on Bacteria. Prebiotics beneficially affect the intestinal microbiota, stimulating the growth or activity of helpful bacteria and altering their composition [55, 56]. The prebiotic effect of AFA-sE and AFA-sHS on *Escherichia coli* was evaluated by following the growth curve. AFA-sE and AFA-sHS were added to *E. coli* culture at time 0 (Figure 7(a)) or when the bacteria were in the exponential phase of growth (Figure 7(b)). In both cases and samples, an increase in the growth of bacteria was observed with respect to the control, indicating that AFA extracts induce a prebiotic proliferation activity of bacteria that is not affected by the heat stress.

The bacterial cell envelope is mainly exposed to the oxidizing molecules generated by the extracellular environment or host cells, and although bacteria possess defense mechanisms against oxidative stress, they sometimes could be inadequate. To evaluate whether AFA extracts can inhibit H_2O_2-induced toxicity or not, we exposed bacteria in the exponential phase of growth to peroxide alone or in combination with AFA-sE or AFA-sHS and a recovery of toxicity was observed (Figure 8(a)). Furthermore, AFA-sE and AFA-sHS antioxidant ability was analyzed after 2 and 4 hours by using the DCFH-DA assay. The presence of AFA-

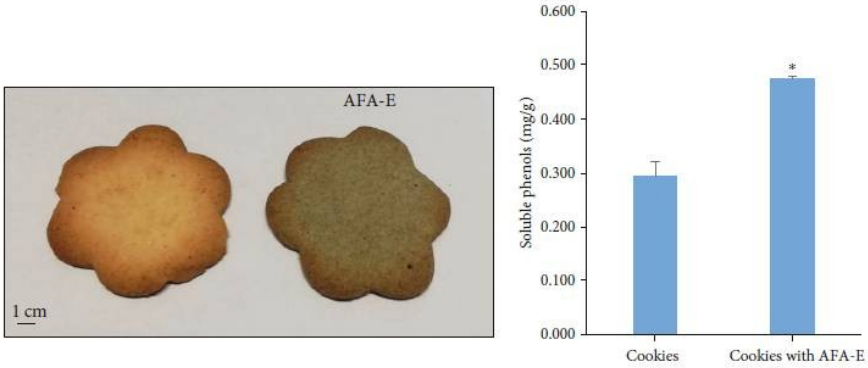


Figure 9: AFA extract is a versatile ingredient for functional food. (a) Biscuit dough without or with AFA-E after cooking. (b) Polyphenol content in cookies without or with AFA.

sE and AFA-sHS decreases H_2O_2-induced ROS generation in a time-dependent manner (Figure 8(b)). No significant differences in the antioxidant activity were observed between the unheated and heated samples.

3.7. Functional Cookies' Preparation. The basic cookie dough was prepared according to a classic recipe with the addition of AFA-E and baked at 220°C for 10 minutes (Figure 9(a)). Furthermore, the presence of polyphenols before and after the baking of the biscuits was analyzed by the F-C assay (Figure 9(b)). AFA addition improved the polyphenol contents, and no significant differences were detected after cooking, indicating that AFA is a useful functional compound.

4. Discussion

On the basis of current knowledge, food may not only be considered as essential for the survival of humans but also as a delight for the palate, promoter of well-being, and help in reducing the risk of diseases. This might be particularly important considering the growing cost of health care, the increase in life expectancy, and the continuous request for a better quality of life. Functional foods are conventional foods with additional functions often related to health promotion or disease prevention. These products are designed to offer a reduction in the risk of developing some diseases and a control of metabolic parameters, such as cholesterol levels, blood sugar concentration, oxidative stress, and inflammation [57–59]. Thus, in a view of a varied and balanced human diet, AFA extract could be an excellent source of vegetable nutrients and bioactive components.

High temperature slightly affects only chlorophyll and carotenoids, but as demonstrated by classical assays, the AFA-E antioxidant properties were preserved, indicating that the undamaged molecules exert a synergic compensatory effect.

As suggested by the isothermal thermogravimetric analysis, the AFA extract can resist the thermal treatment, showing a reduction of just 4% of its initial weight when exposed to a stable temperature of 220°C. Moreover, a slight reduction of the aromatic and carotenoid part of the AFA was observed in the ATR-FTIR spectra. Finally, no morphological changes were noticed in the powder structure. Thus, AFA extract can be a suitable ingredient, which meets the functional food requirements. Taking dietary antioxidants could indeed be useful in cases in which endogenous antioxidants are not sufficient to counteract the oxidative processes that can take place in the human body in case of some pathologies or during the natural aging process [60]. In agreement with our results, studies in which *Vicia narbonensis L.* (Narbon bean) extract was used to produce gluten-free functional crackers have demonstrated that the antioxidant capacity of its compounds is not only unaffected and but also possibly increased during baking [61]. The presence and thermic-stress resistance of AFA bioactive compounds make them suitable as food ingredients and as preservatives against deterioration due to oxidation, improving the stability and extending the shelf life of food products [62].

Neurodegeneration is characterized by oxidative stress process in specific areas of the brain, and antioxidants may have a positive effect in this kind of pathologies. Daily consumption of food containing bioactive molecules with antioxidant properties could be an approach for the prevention and treatment against neurodegenerative diseases. The beneficial effect on neurons in which toxicity was induced by Abeta oligomers was also maintained after thermal stress. This data suggests that the AFA extract could play a significant neuroprotective role if consumed as an ingredient of functional food. However, its protective role could also be exerted by direct interaction on Abeta aggregation and amyloid plaque formation [5].

On the basis of the reported results in which the biological activity of the AFA extract is maintained during baking, we cannot exclude that the same could also be used to produce other kind of functional foods such as pasta, one of the main ingredients of the Mediterranean Diet (MedDiet) [63] that are cooked at 100°C. In a recent study, pasta enriched with 3% of *Opuntia ficus-indica* (OFI) extract, a plant known for its antioxidant and anti-inflammatory properties, was given to volunteers and beneficial effects were observed

[64]. Similarly, pasta with beta-glucans as functional food has been investigated in a pilot study, demonstrating that its consumption could help in the prevention of age-related metabolic disorders [65].

Many studies have proven that microbiota plays a relevant role in the maintenance of the proper function of the gastrointestinal system, and several systemic disorders lead to its imbalance. Obesity and other metabolic-related disorders can cause gut microbiota dysbiosis. Fortunately, numerous bioactive compounds from the diet have a significant influence on its composition and could be useful tools against these pathologies [66]. It has been reported that milk fat globule membrane (MFGM) supplementation is able to modulate gut microbiota composition, demonstrated by the fact that a beneficial effect was observed in mice, after following a diet rich in fats. In addition, the possibility to use MFGM as a potential ingredient in functional food for dietary strategies against metabolic disorders has been suggested [67].

Further, antioxidant molecules such as resveratrol improved the mouse gut microbiota dysbiosis induced by high-fat diet by increasing the *Bacteroides*/*Firmicutes* ratio [68]. In agreement with these findings, we observed that the AFA extract has a prebiotic effect and an antioxidant activity that are not lost after thermal stress, indicating that it could regulate the gut microbiota composition. AFA extract could also exert its protective role by modulating the gut microbiota profile. Furthermore, since metabolic disorders are a risk factor for neurodegeneration [69], we cannot exclude the possibility that AFA extract could also prevent age-related disorders via gut microbiota modulation.

AFA biscuits showed increased polyphenol content with respect to the traditional recipe without AFA, indicating that their antioxidant properties were not only maintained but also enhanced after baking the cookies at a high temperature. Our preliminary results let us speculate that the AFA cookies could be considered as a functional food for the prevention and management of pathologies in which oxidative stress is a risk factor, including metabolic and age-related disorders. Additional studies on healthy volunteers to whom cookies are administered will help us to validate the effect of AFA cookies on human health.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Neither the funding agency nor any outside organization has participated in the study design or have any competing of interest.

Conflicts of Interest

Dr. Stefano Scoglio is the owner and manager of the *Nutrigea* company that provided us the AFA extract. Mrs. Gloria Bosco is the owner and manager of the *Le Farine dei Nostri Sacchi* S.M.E. that provided us the cookies.

Acknowledgments

The authors wish to thank Dr. Alessia Provenzano and Mister Luca Caruana for their useful technical support. We thank Dr. Daniela Giacomazza for critical reading of the manuscript. The research was partially funded by Nutrigea and *Le Farine dei Nostri Sacchi* that had the final approval of the manuscript.

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6. Conclusioni

L'aumento dell'aspettativa della vita e la riduzione della mortalità in età giovanile ha favorito un incremento della popolazione anziana nelle società moderne, e di soggetti centenari, grazie al miglioramento delle condizioni igienico-sanitarie e un cambiamento dei regimi alimentari; questo è però accompagnato da un aumento dell'incidenza di patologie croniche età-correlate, come le malattie neurodegenerative e il cancro.

Nel mio progetto di dottorato ho analizzato la popolazione di alcune città dell'entroterra siciliano, le quali hanno un alto tasso di centenari, per studiare i modelli genetici e ambientali legati alla longevità. I risultati sono preliminari ed ottenuti da uno studio in corso sulla longevità condotto su individui siciliani di lunga durata, definiti LLI (età media 101,3 ± 4,9); in particolare, basandoci su dati di letteratura e valutando il gene APO ε4, è stata confermata la sua correlazione con il fenotipo negativo di longevità, poiché poco espresso nei centenari analizzati, mentre a causa del basso numero di soggetti analizzati non si è trovato una correlazione del gene APO ε2 e dell'allele G FOXO3A rs2802292 (G > T) con i centenari. Per quanto riguarda l'immunofenotipo dei LLI, è stata valutata l'espressione del sottogruppo di cellule dell'immunità innata Natural Killer CD56dimCD16+ che risultano maggiormente espresse nei centenari rispetto agli anziani (non centenari); lo stesso vale per le cellule T ma non per le cellule B, che risultano diminuite. Abbiamo analizzato inoltre che dato il progressivo indebolimento del sistema immunitario, i soggetti anziani sono poco capaci ad innescare una risposta immunitaria efficace dopo esposizione ai vaccini, e vi è quindi la necessità di crearne di migliori, in grado di stimolare in maniera ottimale la risposta immunitaria negli anziani, per avere un invecchiamento di successo. Per questo motivo, in questo lavoro è stato valutato che stimolando specifici recettori dei linfociti, come i TLR, con ligandi stimolatori, e con l'aiuto di adiuvanti, si possono ottenere vaccini più efficaci, in grado di attivare i linfociti con conseguente produzione di citochine infiammatorie capaci di eliminare i nuovi patogeni nei soggetti anziani. Per quanto riguarda i valori ematochimici, dai risultati preliminari non si notano differenze del profilo lipidico, del glucosio e dei livelli di insulina dei centenari rispetto ai giovani (età media 30,7 ± 4,8), mentre la creatinina è aumentata. Un fattore da tenere sotto controllo durante l'invecchiamento è la composizione corporea, in particolare l'accumulo di massa grassa addominale la quale è un fattore di rischio per diverse malattie poiché contribuisce ad uno stato pro-infiammatorio sistemico. Nei soggetti centenari facendo analisi di bioimpedenza (BIA) che valutano massa grassa, massa magra e acqua corporea totale divisa in intra ed extra cellulare, si nota che il loro indice di massa corporea è all'interno di un intervallo normale (tra 18,5 e 24,9 Kg/m2) poichè questi soggetti avevano seguito una dieta mediterranea nella loro giovinezza, ma non attualmente.

Quindi c'è una possibile associazione del fenotipo della longevità con uno stile di vita mediterraneo durante la giovane età, e non durante l'invecchiamento, suggerendo un ruolo interessante ed efficace dell'epigenetica nel raggiungimento della longevità. Pertanto, essa riguarda le persone che vivono in piccoli villaggi, senza inquinamento, probabilmente per le diverse condizioni di lavoro, stili di vita diversi, ovvero riduzione del fumo e dell'uso di alcol e dieta mediterranea (attualmente o in passato). Infine su questi soggetti è stata valutata l'attività di metallo-proteasi nel siero, data la stretta correlazione che l'invecchiamento ha con l'accorciamento dei telomeri e la perdita delle proteasi stesse, che sfocia in cambiamenti a livello intercellulare, organico e di tessuti. In questo studio sono state analizzate le MMP-2 ed MMP-9, e si è valutato che la quantità della forma attiva della MMP2 aumenta significativamente con l'età, e sembra associata al sesso maschile. Inoltre, nella nostra popolazione di LLI, la MMP2 sembra inversamente correlata con il marker infiammatorio come la proteina C reattiva (PCR), , e l'acido urico, con cui vediamo un'associazione positiva, dato che nell'ultimo decennio si è visto possedere un effetto antiossidante diretto a dosi fisiologiche. Al contrario, nei LLI i livelli sierici di MMP9 non hanno mostrato alcuna correlazione con età e sesso, ma sembra essere correlato con il colesterolo totale e il colesterolo LDL. Si nota infine che un alto livello sierico di MMP2 è associato con un alto livello di MMP9 solo nei LLI, probabilmente dovuto al ruolo protettivo di MMP2 e MMP9 nelle malattie neurocognitive come la malattia di Alzheimer, correlato all'invecchiamento senza successo. Infine riguardo a questo e ai possibili trattamenti preventivi, e considerando le basi infiammatorie e ossidanti di molte patologie legate all'invecchiamento, come l'AM, è stato analizzato l'estratto di un microorganismo chiamato Aphanizomenon flos-aquae (AFA), di cui erano già state valutate le proprietà nutrizionali, il potenziale anti-ossidante ed anti-infiammatorio naturale e la capacità di interferire con la cinetica di aggregazione del peptide Aβ, uno dei markers principali della MA. L'estratto di AFA è ricco di polifenoli, tra cui l'idrossitirosolo, l'acido vanillico e l'acido caffeico, il quale sembra essere un'efficace molecola antiossidante e antinfiammatoria contro patologie legate all'età e neurodegenerative, e si è studiato se fosse in grado di conservare le sue proprietà dopo esposizione a temperature alte, fino a 220°C, e di fungere da integratore di alimenti cotti, come pane e biscotti. Dalle analisi effettuate l'estratto AFA dopo la cottura era ancora in grado di avere un effetto benefico sui neuroni, in cui la tossicità era stata indotta dagli oligomeri di Aβ, come è anche mantenuta la sua attività biologica, e quindi si può pensare di utilizzare l'estratto per creare alimenti funzionali, come la pasta, uno degli ingredienti principali della dieta mediterranea.

Sulla base di questi risultati riteniamo che nella nostra popolazioe Sicilia, fin qui studiata, l'influenza della enetica non sia così importante quanto la forza dell'ambiente, sebbene , debbano essere presenti entrambe per ottenere un invecchiamento in salute e di successo.

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