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PROCEEDINGS OF THE SYMPOSIUM UPDATES IN PATHOBIOLOGY: Causality and Chance in Ageing, Age-related diseases and Longevity

Edited by **Giulia Accardi** & **Calogero Caruso**

4. Atti e Convegni

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Calogero Caruso, MD | Full Professor of General Pathology, Department of Pathobiology and Medical Biotechnologies, School of Medicine, University of Palermo.
He was Director of Post-Graduate Schools in Clinical Pathology and in Immunopathology as well as Coordinator of Medical Degree Course. He is Coordinator of the PhD Course in Molecular Medicine and Biotechnologies. His scientific interests concern mechanisms of ageing, longevity and age-related diseases. At this regard, he is Editor in Chief of the online journal Immunity & Ageing and national coordinator of the project "Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and comorbidities", supported by Ministry of University (PRIN: progetti di ricerca di rilevante interesse nazionale – Bando 2015 Prot. 20157ATSLF). He is Author of 346 papers indexed on Scopus with 9810 quotations (H-Index 53).

Giulia Accardi, PhD | Adjunct Professor of General Pathology, Department of Pathobiology and Medical Biotechnologies, School of Medicine, University of Palermo.
She is responsible for recruitment and nutritional assessment in the project "Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and comorbidities", supported by Ministry of University (PRIN: progetti di ricerca di rilevante interesse nazionale – Bando 2015 Prot. 20157ATSLF). Her scientific interests concern nutrition and its link with mechanisms of ageing, longevity and age-related diseases.
She is Author of 20 papers indexed on Scopus with 209 quotations (H-Index 8).

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Atti e Convegni

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Proceedings of the Symposium "Updates in Pathobiology: Causality and Chance in Ageing, Age-related diseases and Longevity"

2017, march 24th, Palermo

(Sala delle Capriate, Palazzo Chiaramonte Steri)

Edited by

Giulia Accardi & Calogero Caruso

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Atti e Convegni - 4
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Introduction

Introduction

The Symposium "Updates in Pathobiology: Causality and Chance in Ageing, Age-related diseases and Longevity" was held on March 24 in Palermo, 2017 (Sala delle Capriate, Palazzo Chiaramonte Steri), the day after exams for obtaining the title of PhD in Molecular Medicine and Biotechnology XXIX, Cycle (Coordinator Prof. Calogero Caruso) by seven PhD candidates; four of them have been awarded the title of Doctor Europaeus. For not completing the PhD thesis, two candidates have achieved the title later.

The following pages contain the Editorial and the papers of the Symposium (chairpersos were the Professors Giuseppina Candore, Giuseppina Colonna Romano and Maurizio Leone).

At the end of the books, there are the abstracts of PhD thesis and the photos of the ceremony with the participation of Professor Fabrizio Micari, Rector of University of Palermo as well as the Symposium.

For the title of Doctor Europaeus in Molecular Medicine and Biotechnologies the candidate were Anna Aiello, Valentina Guarnotta, Dario Spigolon, Chiara Tudisca (Board Johannes Grillari, University of Natural Resources and Life Sciences, Vienna; Ferdinando Nicoletti, University of Catania; Annibale Puca, University of Salerno).

For the title of Doctor in Molecular Medicine and Biotechnologies the candidate were Beatrice Belmonte, Valeria Ingrassia, Lorenzo Volpe (Board Ferdinando Nicoletti, University of Catania; Annibale Puca, University of Salerno; Valeria Vetri, University of Palermo).

Obtained the title later Angela Aronica, Antonio Carlino.

Calogero Caruso
Giulia Accardi

Editorial

Updates in Pathobiology: Causality and Chance in Ageing, Age-Related Diseases and Longevity

Giulia Accardi, Calogero Caruso

Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy.
Correspondence to: Calogero Caruso, Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; e-mail: calogero.caruso@unipa.it.

State of the art

Democritus said that everything that exists in the universe is the result of chance and necessity (http: //www.normalesup.org/~adanchin/causeries/Atomists.html#N1). Epicurus tried to visualize chance by saying that, occasionally, the normally straight paths of atoms in the universe bend a little, and the atoms "swerve." If one considers mispairing during DNA replication, perhaps he was not far off [Luzzatto & Pandolfi, 2015].

The relationship between causality and chance is an open discussion in many disciplines. Often, the boundary among these events is thin to understand if an occurrence is related to one or to both. In particular, ageing, the related diseases, and longevity are difficult to define as consequence of causality, chance or both. Surely, it is known that longevity is based for 25% on genetic background; conditioning factors, that arise in the first part of life (socio-economic state of parents, education and month of birth, which has been found to reflect the environmental conditions during the prenatal and early postnatal period), account for another 25% of such variability; life circumstances at adult and old age (including socio-economic status and medical assistance) may account for about the remaining 50% [Caruso et al., 2012]. However, the possibility to inherit longevity increases with age: for centenaries it reaches up to 33% for women and 48% for men [Brooks-Wilson, 2013]. In any case, the concrete possibility to become centenarian, i.e. to manifest a longevity phenotype, is strictly related to the stochastic interaction, due to accidental events, with genes with a role in ageing and longevity processes [Caruso et al., 2012].

Stochastic processes are accidental phenomena due to causal factors. They play a role in physiological and pathological events, alongside genetics, epigenetics and environment. Intrinsic stochasticity in biological occurrences contributes, in fact, to the individuality of each living organism, including human beings, influencing phenotypic variability, as suggested by the role of the chance in the creation of immunological repertoire and neuronal synapses. So, probability and causality play an important role in living beings. In fact, they are subject to twofold causality: nature laws and genetic programs where Brownian random motion, disordered motion of particles present in fluids or suspensions due to the impact of water

molecules that move in different directions, and crossing over contribute to leave space for the chance. It is precisely the randomness of variability that constitutes the characteristic of evolution, hence of living beings [Kirkwood et al., 2005].

There is increasing evidence of the intrinsic stochastic nature of gene expression and macromolecular biosynthesis, since many genes are transcribed to minimal amounts of mRNA per cell, which can cause large fluctuations in biosynthesis. Genomic instability, which results in somatic mutations and chromosomal abnormalities, is another important source of intrinsic variability, as shown in aged mice, which have a mutation frequency up to 10^{-4} per gene per cell. Epimutations may also occur through loss or disruption of DNA methylation patterns, affecting gene expression [Kirkwood et al., 2005; Kirkwood, 2008].

The role of stochastic processes in the ageing of individuals is clearly demonstrated by experimental studies conducted on inbred mice. They have the same genome as well as the same housing condition but show different lifespan, up to 50% higher, contrary to expectations. This proves that in living organisms there is a stochastic component that results in very little fluctuations in the genetic, epigenetic, environmental and interaction components. This involves continuous microvariations that, accumulating over time, amplify the differences between individuals, manifesting in a striking way in older ages [Kirkwood et al., 2005].

In ageing, age-related diseases and longevity, we can define chance as the occurrence of events in the absence of any obvious intention or cause. So, it must be distinguished from life circumstances that are events or facts that cause or help to cause something to happen (as an example, the death in war of a potential centenarian).

A typical example of age-related disease where chance plays a relevant role in addition to genetics and environment is cancer. The current model of cancer development is based on the notion that each of a succession of somatic mutations confers onto the mutant cell a growth advantage over normal cells in a particular microenvironment. Somatic mutations are stochastic events by their nature because they result from mispairing, which in turn originate from the equilibrium that exists in solution between tautomeric forms of the purine and pyrimidine bases. The neoplastic cell, having accumulat-

ed a number of causal mutations, is now capable of aberrant growth in a given microenvironment. There are also risk modulators such as: genetics, for presence or absence of more or less efficient alleles involved in detoxification, DNA repair, immune-inflammatory responses; diet, for presence or absence of carcinogens or antioxidants, hyper or hypocaloric diet, rich or poor in red meat; and the immune system, for efficient responses against oncogenic viruses. Thus, at the somatic cell level, oncogenesis recapitulates a Darwinian process in which mutations are the innovative force, whereas the environment selects the mutations that are advantageous. Hence, in this process, chance and necessity are strictly linked. Of course, the events might be represented by an epigenetic rather than a somatic mutation [Greaves, 2017; Luzzatto & Pandolfi, 2015].

Epigenetics is used to describe phenotypic variations that may occur in cells following a different expression of individual genes without altering the DNA sequence. These phenotypic changes are stable and inheritable from cell progeny, through DNA replication and cell division cycles. Thus, during the proliferation that occurs in the normal homeostatic replacement, a cell expresses the characteristic genes of the corresponding tissue, and not that characteristic of another one. In a broader sense, epigenetic processes are called to explain the changes in the regulation of the transcription of individual genes. Considering this broader definition of epigenetics, it is not surprising that the profound changes that occur with age in cells and tissues are also due to epigenetic processes as well as accumulation of nuclear and mitochondrial DNA mutations [Peaston & Whitelaw, 2006].

The level of complexity is very high if we consider that not only the effect of each variation but also their combinations must be evaluated. For example, lysine methylation at positions 4 and 9 in H3 has opposite effects: the first increases the expression of genes; the second reduces this action. miRNAs are also involved in gene transcription. They also regulate histone modification processes. A poor folate diet, for example, may increase the expression of specific miRNAs. Moreover, it is known that these effects might be also mediated by exogenous miRNAs, such as that from plants. So, we may hypothesize that in future we will modulate our gene expression choosing specific foods and, consequently, we will modulate ageing towards successful outcome [Bannister & Kouzarides, 2011; Dellago et al., 2017; Vaucheret & Chupeau, 2012].

How might stochastic epigenetic model underlie disease? An obvious example, as previously suggested is cancer, which arises in part from repeated changes to the microenvironment of the tissue. Most cancers arise from cycles of repeated injury and repair. Hepatocellular cancer arises directly from this process whether the injury is induced by viruses or by aflatoxin. Similarly, skin cancer arises from repeated cycles of blistering sunburn and repair. The epigenome sits at the intersection of the environment, genetic mutation and tumour cell growth. Environmental factors, such as carcinogens or diet, as well as injury and inflammation, cause epigenetic reprogramming. The epigenome also accumulates damage stochastically and through ageing [Feinberg, 2014; Sen et al., 2016].

The stochastic epigenetic model has important implications for evolutionary biology considering environmental effects that may be consistent for many generations but can then change stochastically, for example in response to an environmental crisis such as drought or famine. At this regard, during the winter of 1944–1945, a famine affected the western Netherlands. It was the result of an embargo on transport of food supplies imposed by the German occupying forces in early October 1944 in reprisal for a wave of partisan activity. It lasted for approximately 5 months and ended abruptly with the liberation by allied forces in May 1945. Several studies addressed the effects of maternal malnutrition during the different periods of gestation on health in adult life. Individuals conceived in Holland occupied by the Nazis showed at a distance of sixty years a higher incidence of diabetes, obesity and other diseases when compared to subjects age and sex matched whose mothers followed a non-famine dietary regimen in Holland occupied by allied armies. The association was present in subjects, whose exposure to famine occurred around the time of conception, proving the strict relation between causality and chance intrinsic to epigenetic changes. So, research over the past 3 decades has shown that exposure to famine during gestation has life-long effects on health, and that these effects vary depending on the timing of exposure as well as evolution of the recovery period. The effects of famine during gestation thus differ from those of adult exposure, which has only short-term effects [Heijmans et al., 2008; 2009].

The Book

To gain insight into the role of chance and causality in ageing, age-related diseases and longevity, several papers have been assembled in these Proceedings of a Symposium held in Palermo, March 24, 2017.

Ageing affects different body tissues and is known to have a negative impact on the physiology of cells, tissues and organs, resulting in reduced functionality and regeneration capacity. It is clear that circulating factors are important contributors to the ageing phenotype. Consequently, circulating miRNAs have been carefully studied in the context of ageing in recent years. miRNAs are part of the secreted phenotype associated with senescence and are transferred by microvescicles in a paracrine manner. So, they might be an ideal target for modulating healthy ageing [Accardi et al., 2017b].

To discuss the relevance of genetics and lifestyle in the attainment of longevity, three papers mostly focused on Italian centenarians with aim to understand how to prevent and/or reduce elderly frailty and disability. In their review, Ferrario & Puca [2017] pointed out the genetic origin of exceptional longevity: healthy ageing is a complex, hereditable trait that correlates with the presence of protective alleles (FOXO3A rs2802292) and lack of some of the detrimental ones (i.e. APOEε4). Furthermore, they summarized the potential problems of the genetic approaches and possible future evolution based on new technologies. The phenotypic aspects were instead approached in the papers by Bulati et al.,[2017] and Accardi et al., [2017a]. In particular, in this last report the Authors discussed new approaches as bioelectrical impedance to characterize phenotypically elderly and centenarians. Centenarians were also studied in a preliminary report on triggering of Toll-like receptors in the elderly, however TLR agonists significantly enhanced the activation of dendritic cells in the peripheral blood isolated from healthy elderly, but not from centenarians, likely due to the exhaustion of immune system [Gambino et al., 2017].

Three papers by Aiello et al., [2017], Bova et al., [2017], and Vetri [2017] were devoted to the role of genetics, environment and stochasticity in chronic diseases. In particular, it was pointed out the pleiotropic nature of KIR on different diseases in that a given KIR genotype affording protection against one disease may actually predispose to another unrelated disorder [Aiello et al., 2017].

Finally, two papers concerned the pathophysiology of a typical age-related disease, i.e. cancer. In the paper by Cocciadiferro & Carruba [2017], by analysing the role of estrogens in the development of liver cancer, it was pointed out that mutations and epigenetics affect the role of estrogens. Libra & Nicoletti [2017] instead pointed out the role of life style, in particular of diet in the development of cancer.

Conclusion

We can conclude that ageing and longevity as well as cancer as a model of age-related diseases, in themselves, are produced by a complex combination of random events and genetic background. The ageing process is driven by a lifelong accumulation of molecular damage, resulting in gradual increase in the fraction of cells carrying defects. After sufficient time has passed, the increasing levels of these defects interfere with both the performance and functional reserves of tissues and organs, resulting in a breakdown of self-organizing system and a reduced ability to adapt to the environment. It follows age-related frailty, disability and disease [Kirkwood et al., 2005; Kirkwood, 2008].

Stress, adverse environment and poor nutrition can increase the rate at which molecular damage arises. Intrinsic maintenance mechanisms, such as DNA repair and antioxidants, slow the rate of accumulation. Contributing factors are cultural, anthropological, socio-economic, sexual, linked to gender, ethnic differences, healthcare, genetics, and life occupation. In the case of nutrition, for example, a poor diet containing excess sugar and saturated fats contributes directly to the burden of damage with which cells have to deal, whereas a Mediterranean-style diet may contribute protective factors such as dietary antioxidants, a reduced amount of animal proteins and a low glycaemic index [Aiello et al., 2016; Vasto et al., 2014].

Different combinations of these events create the possibility to avoid age-related pathologies and become centenarian.

References

Accardi G., Aiello A., Vasto S., Caruso C. *Bioimpedance: a new approach for studying longevity*. In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G.& Caruso C., eds. Palermo University Press, 2017b; 67-78.

Accardi G., Caruso C., Grillari J. *From cellular senescence to age-associated diseases: miRNAs as tools and targets for healthy ageing*. In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G. & Caruso C., eds. Palermo University Press, 2017b; 27-37.

Aiello A., Accardi G., Candore G., Carruba G., Davinelli S., Passarino G., Scapagnini G., Vasto S., Caruso C. *Nutrigerontology: a key for achieving successful ageing and longevity*. Immun Ageing. 2016; 13: 17.

Aiello A., Accardi G., Candore G., Gambino C.M., Caruso C., Di Bona D. *The importance of the interactions between KIRs and HLA ligands in the development of human autoimmune and viral diseases*. In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G. & Caruso C., eds. Palermo University Press, 2017; 91-110.

Bannister A.J., Kouzarides T. *Regulation of chromatin by histone modifications*. Cell Research. 2011; 21: 381-395.

Bova M., Scola L., Lio D. *Potential new genetic and biochemical markers of chronic kidney failure*. In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G. & Caruso C., eds. Palermo University Press, 2017; 111-123.

Brooks-Wilson A.R. *Genetics of healthy aging and longevity*. Hum Genet. 2013; 132: 1323-38.

Bulati M., Caruso C., Candore G., Colonna-Romano G. *The role of immune response in ageing and longevity. A focus on B cell compartment*. In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G. & Caruso C., eds. Palermo University Press, 2017; 53-66.

Caruso C., Passarino G., Puca A., Scapagnini G. "Positive biology": *the centenarian lesson*. Immun Ageing 2012; 9: 5.

Cocciadiferro L., Carruba G. *Estrogens in Liver Cancer: Friends or Foes?* In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G. & Caruso C., eds. Palermo University Press, 2017; 131-144.

Dellago H., Bobbili M.R., Grillari J. *MicroRNA-17-5p: At the Crossroads of Cancer and Aging - A Mini-Review*. Gerontology. 2017; 63: 20-28.

Feinberg A.P. *Epigenetic stochasticity, nuclear structure and cancer: the implications for medicine*. Journal of internal medicine. 2014; 276: 5-11.

Ferrario A., Puca A.A. *Genetic studies in long living individuals: potentials and limitations*. In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G.& Caruso C., eds. Palermo University Press, 2017; 39-52.

Gambino C.M., Vasto S., Ioannou K., Candore G., Caruso C., Farzaneh F. *Triggering of Toll-like receptors in the elderly. A pilot study relevant for vaccination*. In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G. & Caruso C., eds. Palermo University Press, 2017; 79-90.

Greaves M. Darwinian medicine: a case for cancer. Nat Rev Cancer. 2007; 7: 213-21.

Heijmans B.T., Tobi E.W., Lumey L.H., Slagboom P.E. *The epigenome: archive of the prenatal environment*. Epigenetics. 2009; 4: 526-31.

Heijmans B.T., Tobi E.W., Stein A.D., Putter H., Blauw G.J., Susser E.S., Slagboom P.E., Lumey L.H. *Persistent epigenetic differences associated with prenatal exposure to famine in humans*. Proc Natl Acad Sci U S A. 2008; 105: 17046-9.

Kirkwood T.B., Feder M., Finch C.E., Franceschi C., Globerson A., Klingenberg C.P., LaMarco K., Omholt S., Westendorp R.G. *What accounts for the wide variation in life span of genetically identical organisms reared in a constant environment?* Mech Ageing Dev. 2005; 126: 439-43.

Kirkwood T.B. *Understanding ageing from an evolutionary perspective*. J Intern Med. 2008; 263: 117-27.

Libra M., Nicoletti F. *Diet, Inflammation and Cancer: A Journey from Prevention to Treatment.* In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G. & Caruso C., eds. Palermo University Press, 2017; 145-162.

Luzzatto L., Pandolfi P.P. *Causality and Chance in the Development of Cancer*. N Engl J Med. 2015; 373: 84-8.

Peaston A.E., Whitelaw E. *Epigenetics and phenotypic variation in mammals*. Mammalian Genome. 2006; 17: 365-374.

Sen P., Shah P.P., Nativio R., Berger S.L. *Epigenetic Mechanisms of Longevity and Aging*. Cell. 2016; 166: 822-39.

Vasto S., Buscemi S., Barera A., Di Carlo M., Accardi G., Caruso C. *Mediterranean diet and healthy ageing: a Sicilian perspective*. Gerontology. 2014; 60: 508-18.

Vaucheret H., Chupeau Y. *Ingested plant miRNAs regulate gene expression in animals*. Cell Research. 2012; 22: 3-5.

Vetri V. Protein *Aggregation at the Membrane: insights on Molecular Mechanisms underlying Neurodegenerative diseases.* In "Updates in Pathobiology: causality and chance in ageing, age-related diseases and longevity". Accardi G. & Caruso C., eds. Palermo University Press, 2017; 125-130.

Papers

From cellular senescence to age-associated diseases: miRNAs as tools and targets for healthy ageing

Giulia Accardi[1], Calogero Caruso[1], Johannes Grillari[2]

[1]Department of Pathobiology and Medical Biotechnologies, University of Palermo, Italy; [2]Christian Doppler Laboratory on Biotechnology of Skin Aging, Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria.

Correspondence to: Johannes Grillari, Christian Doppler Laboratory on Biotechnology of Skin Aging, Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria; e-mail: johannes.grillari@evercyte.com.

Abstract

miRNAs are the most abundant RNA species to be found in cell-free blood, encapsulated within microvesicles or bound to proteins. miRNAs play essential roles in the regulation of various biological processes. Moreover, specific changes in miRNA transcription levels or miRNA secretory levels have been linked to the development and progression of certain age-related diseases. So, they might be an ideal target for modulating healthy ageing.

Key Words

Ageing, Age-related diseases, miRNA, Senescence.

State of the art

Ageing of the population

The current growth of the world population has major implications for humanity: growing poverty and famine, depletion and pollution of natural resources essential for human survival and migratory pressure from the poor South to the rich North. In absolute terms, the world population has reached 7 billion in 2011. According to the United Nations, it will exceed 8 billion in 2025 and 9 billion by 2045. To be sustainable, the long-term growth rate, i.e. the difference between birth and death rates, should not differ much from 0% [Van Bavel, 2013].

Even though the world population will continue to grow in absolute figures for a certain period, the rate of growth in percentages is decreasing, a result of most countries experiencing a demographic transition from relatively high to low birth and death rates [Canning, 2011]. The decline in mortality and fertility induces the ageing of the population. The percentage of people over 60 in the world has risen from 9.2% in 1990 to 11.7% in 2013 and will be 21% by 2050. It is expected to exceed the number of children for the first time in 2047. Currently about two-thirds of them live in developing countries. They will be concentrated in less developed regions of the world [Christensen et al., 2009].

The ageing of the population has great social and economic consequences. The number of working-age adults per older person is already low in the more developed regions and in some developing countries and should continue to decline in the coming decades with consequent fiscal pressure on support systems for elderly. The increasing mean lifespan of the population is a big success story of humanity, but also poses a challenge that industrialized countries are currently facing, since ageing is associated with increased susceptibility to many diseases like cancer, type 2 diabetes and neurodegenerative disorders [Avery et al., 2014]. So it is necessary to fully understand the mechanisms of ageing in order to prevent its detrimental aspects.

Discussion

Ageing of the cells

A prominent mechanism strongly linked to cellular ageing is cellular senescence, i.e. the arrest of irreversible cellular growth of normal

human cells after serial passages in vitro [Hayflick & Moorhead, 1961]. Critically short telomeres, DNA damage, oncogenic signalling or cellular stress can cause this blockage. Senescent cells differ from other non-dividing cells, such as quiescent ones, by various markers, and morphological changes (however, senescent cells in vivo maintain the normal morphology dictated by tissue architecture). It is interesting to note that senescent cells are characterized by an irreversible growth arrest, altered function / differentiation status, which is reflected by an altered intracellular protein expression and secretion profile, called senescence associated secretory phenotype (SASP). Biomarkers of senescence include absence of proliferative markers, expression of tumor suppressors, cell cycle inhibitors like p21 and/or p16, and often also of DNA damage markers as well as senescence-associated β-galactosidase activity due to GLB1 upregulation [Lee et al., 2006], concomitant with an increase of the lysosomal content of the senescent cells, which allows the lysosomal β-galactosidase to be detected at a suboptimal pH (pH 6.0). This probably reflects the increased autophagy occurring in the senescent cells together with an enlargement of the lysosomal compartment. As previously stated, senescent cells secrete various extracellular factors, including transforming growth factor-β, insulin-like growth factor 1-binding proteins, plasminogen activator inhibitor 1, and inflammatory cytokines and chemokines that can enhance and propagate senescence in autocrine and paracrine mode, as well as tissue remodelling factors. Therefore, senescent cells contribute to the well known pro-inflammatory status of ageing [Campisi & d'Adda di Fagagna, 2007; Muñoz-Espín & Serrano, 2014].

Several studies over the last ten years have clearly shown that senescence has beneficial and harmful effects. In general, transient induction of senescence followed by tissue remodelling is advantageous because it contributes to the elimination of damaged cells. In contrast, persistent senescence or the inability to eliminate senescent cells are harmful. This is particularly relevant in cancer and ageing, both characterized by the accumulation of severe cell damage. Accordingly, senescence is a crucial barrier to cancer progression and senescent cells accumulate with ageing. In summary, senescence is a response selected to eliminate damaged cells. However, with ageing, the complete sequence of senescence-clearance-regeneration is not fully accomplished and senescence may become part of the problem

rather than its solution. Thus, senescence is considered an example of antagonistic pleiotropy and has been classified as an antagonistic sign of ageing [Muñoz-Espín & Serrano, 2014].

On the other hand, an increasing number of studies have been published showing that senescent cells accumulate with age in vivo, contributing to overall ageing and age-related diseases in an organism. The influence of senescent cells in ageing has been demonstrated by reactivating telomerase in mouse tissues, which subsequently became "rejuvenated". Studies in the models clearly show that the elimination of senescent cells delay the ageing process and the onset of age-related diseases. So many scientists are looking for substances that can eliminate senescent cells, that is, senolytic drugs [Jaskelioff et al., 2011; Weilner et al., 2015a,b].

miRNA and ageing

In recent years, the role of miRNAs in ageing has become increasingly evident. They are small non-coding RNA sequences that regulate gene expression through repression of translation. Only recently, however, miRNAs have been found to be secreted in systemic and local environments in which they are protected from RNAses by either carrying proteins or by being packaged into extracellular vesicles (EVs). EVs are vesicles budding from cell membranes (ectosomes), are shedded from multivesicular bodies (exosomes) or derive from apoptotic cells (apoptotic bodies) and contain, depending on their origin, proteins, different RNA species including mRNAs and miRNAs and/or DNAs. Unlike well-known protein-based signalling systems, EVs have the advantage of providing multiple messages, potentially in a synergistic way. The miRNAs are then taken up by recipient cells, modifying the cellular behaviour by the classical miRNA induced silencing of target mRNAs (Figure 1) [Hromada et al., 2017].

The origin of circulating miRNAs, however, is in many cases unclear, although senescent cells emerge as the possible source of such secreted miRNAs. SASP of different types of cells is probably reflected into circulating miRNAs [Weilner et al., 2013]. In vitro, senescent cells secrete more EVs per cell than their quiescent control cells and the amount of secreted vesicles increases over time after induction of stress induced premature senescence. Since differences in circulating miRNAs have been found in a variety of age-related diseases and the accumulation of senescent cells in the elderly emerges as a possible adverse factor in ageing, it is possible to hypothesize

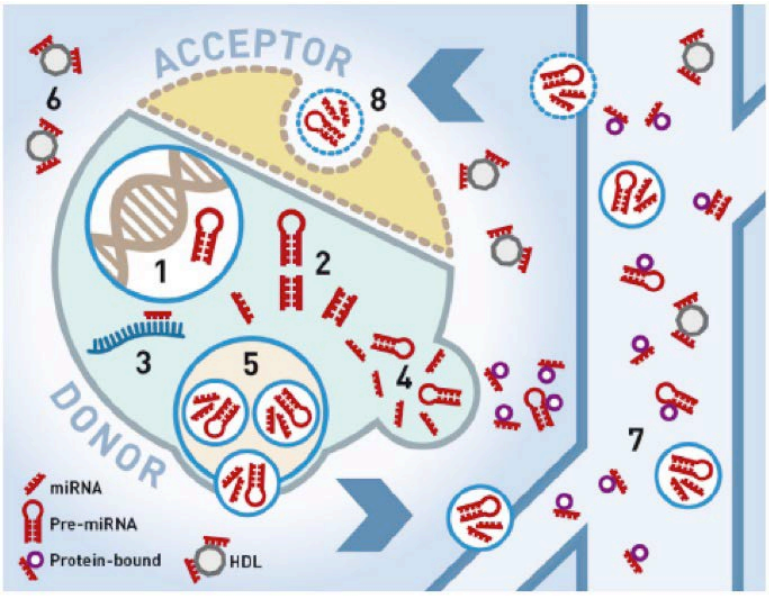

Figure 1. **The concept of circulating microRNAs**. (1) microRNAs are derived from intergenic or intronic genomic regions. In case of intergenic miRNAs, the initial primary transcripts (not shown) are cleaved by Drosha/Dgcr8 to form precursor miRNAs (Pre-miRNA). Alternatively, splicing of intronic miRNAs can give rise to pre-miRNAs. (2) Pre-miRNAs are shuttled via Exportin-5 into the cytoplasm where cleavage by Dicer into double-stranded miRNA duplexes occurs. (3) Single-stranded miRNAs guide the RISC protein complex to target mRNAs resulting in translational repression. (4) mature miRNAs as well as Pre-miRNAs are sorted into ectosomes, which bud from the cell membrane. (5) Intracellularly, multivesicular bodies (MVBs) are formed, which contain exosomes of 50–100 nm size. MVBs fuse with the cell membrane to release miRNA loaded exosomes from the Donor Cell into the supernatant. (6) Lipoprotein or Ago-2 associated miRNAs are present in cell-free liquids. (7) Protein-bound or encapsulated miRNAs are transported with the blood stream. (8) Uptake of extracellular RNA by an Acceptor Cell that is different from the Donor Cell, and potentially located in a different tissue. Reproduced from Hackl M, Heilmeier U, Weilner S, Grillari J. Circulating microRNAs as novel biomarkers for bone diseases - Complex signatures for multifactorial diseases? Mol Cell Endocrinol. 2016; 432: 83-95 under the terms of the Creative Commons Attribution License.

that these miRNAs may contribute to a functional decline observed during ageing [Dellago et al., 2017]. In senescent mesenchymal bone marrow stem cells, the majority of miRNAs are up-regulated, with the exception of miR-199b-5p. This is decreased, thus enhancing the translation of its target, LAMC1, which encodes laminin proteins necessary for cell adhesion and migration as well as signal transduction [Yoo et al., 2014; Hackl et al., 2016].

In an effort to identify miRNAs commonly regulated during ageing, microarray studies were conducted comparing four human replicative cell-ageing models and three organismal ageing models. These diverse model systems shared a set of commonly down-regulated miRNAs, among them members of the miR-17-92 cluster. Several studies using different model systems have confirmed the down-regulation of the miR-17-92 cluster during ageing, and its up-regulation in centenarians considered successful agers [Gombar et al., 2012; Dellago et al., 2017].

On the other hand, in human beings a pilot study analysed 365 circulating miRNA in young, old and centenarians, identifying three general models for circulating miRNAs in young, aged, and long-living individuals. In particular, the miR-21-5p levels were demonstrated to increase in aged, including centenarians. This was replicated in an independent sample group. Based on the expected involvement of miR-21-5p with transforming growth factor-ß signalling and its correlation with other circulating inflammatory molecules, miR-21-5p was proposed as " inflamma-miR", likely linked to the systemic pro-inflammatory status of old people [Olivieri et al., 2012; 2013, Hackl et al., 2016].

In addition to that we recently identified miRNA signatures as biomarkers of an important age-related disease: of osteoporosis. From the notion, that miR-31 is secreted by senescent endothelial cells in vitro, we found that upon EV mediated transfer to mesenchymal stem cells block osteogenic differentiation of these recipient cells [Weilner et al., 2016a]. This prompted us to also test, if other factors from senescent cells might have a synergistic effect and indeed found that Galectin-3, that seems to be involved in modulating Wnt signalling, is pro-osteogenic, but found at low levels in serum as well as in EVs of elderly [Weilner et al., 2016b]. Finally, we set out to identify a signature of miRNAs in serum of osteoporotic fracture patients, which by now is based on more than 700 individually that were analysed using serum based qPCR methods [Heilmer et al., 2016; Kocijan et al., 2016].

Conclusion

Ageing affects different body tissues and is known to have a negative impact on the physiology of cells, tissues and organs, resulting in reduced functionality and regeneration capacity. Since observation in a murine parabiosis models, which, linking the circulation of old animals with young animals, may improve the regenerative potential of old tissue [Conboy et al., 2005], it was clear that circulating factors were important contributors to the ageing phenotype. Consequently, circulating miRNAs have been carefully studied in the context of ageing in recent years [Hackl et al., 2016]. miRNAs are part of the SASP, and are transferred by EVs in a paracrine manner. So, they might be an ideal target for assessing and modulating healthy ageing, especially in the context of osteoporotic fracture risk assessment.

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Conflict of interest

J.G. is a co-founder of Evercyte GmbH and TAmiRNA GmbH, HD is an employee of TAmiRNA GmbH.

References

Avery P., Barzilai N., Benetos A., Bilianou H., Capri M., Caruso C., Franceschi C., Katsiki N., Mikhailidis D.P., Panotopoulos G., Sikora E., Tzanetakou I.P., Kolovou G. *Ageing, longevity, exceptional longevity and related genetic and non genetics markers: panel statement*. Curr Vasc Pharmacol. 2014; 12: 659-61.

Campisi J., d'Adda di Fagagna F. *Cellular senescence: when bad things happen to good cells*. Nat Rev Mol Cell Biol. 2007; 8: 729–40.

Canning D. *The causes and consequences of demographic transition. Popul Stud Camb*. 2011; 65: 353-61.

Chen J., Patschan S., Goligorsky M.S. *Stress-induced premature senescence of endothelial cells. J Nephrol*. 2008; 21: 337–44.

Christensen K., Doblhammer G., Rau R., Vaupel J.W. *Ageing populations: the challenges ahead*. Lancet. 2009; 374: 1196-208

Conboy I.M., Conboy M.J., Wagers A.J., Girma E.R., Weissman I.L., Rando T.A. *Rejuvenation of aged progenitor cells by exposure to a young systemic environment*. Nature. 2005; 433: 760-4.

Dellago H., Bobbili M.R., Grillari J. *MicroRNA-17-5p: At the Crossroads of Cancer and Aging - A Mini-Review*. Gerontology. 2017; 63: 20-28.

Gombar S., Jung H.J., Dong F., Calder B., Atzmon G., Barzilai N., Tian X.L., Pothof J., Hoeijmakers J.H., Campisi J., Vijg J., Suh Y. *Comprehensive microRNA profiling in B-cells of human centenarians by massively parallel sequencing*. BMC Genomics 2012; 13: 353.

Hackl M., Heilmeier U., Weilner S., Grillari J. *Circulating microRNAs as novel biomarkers for bone diseases - Complex signatures for multifactorial diseases?* Mol Cell Endocrinol. 2016; 432: 83-95.

Hayflick L., Moorhead P.S. *The serial cultivation of human diploid cell strains*. Exp Cell Res. 1961; 25: 585–621.

Heilmeier U., Hackl M., Skalicky S., Weilner S., Schroeder F., Vierlinger K., Patsch J.M., Baum T., Oberbauer E., Lobach I., Burghardt A.J., Schwartz A.V., Grillari J., Link T.M. *Serum miRNA Signatures Are Indicative of Skeletal Fractures in Postmenopausal*

Women With and Without Type 2 Diabetes and Influence Osteogenic and Adipogenic Differentiation of Adipose Tissue-Derived Mesenchymal Stem Cells In Vitro. J Bone Miner Res. 2016; 31: 2173-2192.

Hromada C., Mühleder S., Grillari J., Redl H., Holnthoner W. *Endothelial Extracellular Vesicles-Promises and Challenges*. Front Physiol. 2017; 8: 275.

Jaskelioff M., Muller F.L., Paik J.H., Thomas E., Jiang S., Adams A.C., Sahin E., Kost-Alimova M., Protopopov A., Cadiñanos J., Horner J.W., Maratos-Flier E., Depinho R.A. *Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice*. Nature. 2011; 469: 102–6.

Kocijan R., Muschitz C., Geiger E., Skalicky S., Baierl A., Dormann R., Plachel F., Feichtinger X., Heimel P., Fahrleitner-Pammer A., Grillari J., Redl H., Resch H., Hackl M. *Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures*. J Clin Endocrinol Metab. 2016; 101: 4125-4134.

Lee B.Y., Han J.A., Im J.S., Morrone A., Johung K., Goodwin E.C., Kleijer W.J., DiMaio D., Hwang E.S. *Senescence-associated beta-galactosidase is lysosomal beta-galactosidase*. Aging Cell. 2006; 5: 187-95

Muñoz-Espín D., Serrano M. Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol. 2014; 15: 482-96.

Olivieri F., Spazzafumo L., Santini G., Lazzarini R., Albertini M.C., Rippo M.R., Galeazzi R., Abbatecola A.M., Marcheselli F., Monti D., Ostan R., Cevenini E., Antonicelli R., Franceschi C., Procopio A.D. *Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging*. Mech Ageing Dev. 2012; 133: 675-85.

Olivieri F., Rippo M.R., Procopio A.D., Fazioli F. *Circulating inflamma-miRs in aging and age-related diseases*. Front Genet. 2013; 4: 121.

Van Bavel J. *The world population explosion: causes, backgrounds and projections for the future, Facts Views Vis Obgyn*. 2013; 5: 281–291.

Weilner S., Schraml E., Redl H., Grillari-Voglauer R., Grillari J. *Secretion of microvesicular miRNAs in cellular and organismal aging*. Exp Gerontol. 2013; 48: 626-33.

Weilner S., Grillari-Voglauer R., Redl H., Grillari J., Nau T. *The role of microRNAs in cellular senescence and age-related conditions of cartilage and bone: A review*. Acta Orthop. 2015; 86: 92–99.

Weilner S., Skalicky S., Salzer B., Keider V., Wagner M., Hildner F., Gabriel C., Dovjak P., Pietschmann P., Grillari-Voglauer R., Grillari J., Hackl M. *Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation*. Bone. 2015; 79: 43-51.

Weilner S., Schraml E., Wieser M., Messner P., Schneider K., Wassermann K., Micutkova L., Fortschegger K., Maier A.B., Westendorp R., Resch H., Wolbank S., Redl H., Jansen-Dürr P., Pietschmann P., Grillari-Voglauer R., Grillari J. *Secreted microvesicular miR-31 inhibits osteogenic differentiation of mesenchymal stem cells*. Aging Cell. 2016a; 15: 744-54.

Weilner S., Keider V., Winter M., Harreither E., Salzer B., Weiss F., Schraml E., Messner P., Pietschmann P., Hildner F., Gabriel C., Redl H., Grillari-Voglauer R., Grillari J. *Vesicular Galectin-3 levels decrease with donor age and contribute to the reduced osteo-inductive potential of human plasma derived extracellular vesicles*. Aging (Albany NY). 2016b; 8: 16-33.

Yoo J.K., Kim C.H., Jung H.Y., Lee D.R., Kim J.K. *Discovery and characterization of miRNA during cellular senescence in bone marrow-derived human mesenchymal stem cells*. Exp Gerontol. 2014; 58: 139-45..

Genetic studies in long living individuals: potentials and limitations

Anna Ferrario[1], Annibale Alessandro Puca[2,3]

[1]Institute for Biomedical Technologies–National Research Council, 20090 Segrate (MI), Italy.; [2]Department of Medicine and Surgery, University of Salerno, Via S. Allende, 84081 Baronissi (SA), Italy; [3]Ageing Unit, IRCCS Multi-Medica, 20138 Milan, Italy.

Correspondence to: Annibale Alessandro Puca, Department of Medicine and Surgery, University of Salerno, Via S. Allende, 84081 Baronissi (SA); email: apuca@unisa.it.

Abstract

Centenarians are a model of successful ageing and the identification of the genetic variants that predispose to long and healthy life is of tremendous interest for the translational medicine. Here we briefly describe the results obtained so far and their meaning. Furthermore, we summarize the potential problems of the genetic approaches and possible future evolution based on new technologies.

Key Words

Centenarian, GWAS, LAV-BPIFB4, Linkage analysis, Longevity.

State of the art

Centenarians are people who can achieve extreme ages of 100 years and beyond [Perls et al., 2002]. At the beginning of 1900 in US the Birth Cohort Study found that life expectancy was 51.5 years for males and 58.3 years for female. In industrialized countries, the increased life expectancy, quantified in 3 month per year in the last 160 years, is due to the improvement of life conditions, diet and a reduced exposure to infection and inflammation [Ferrario et al., 2012].

The quality of food intake was the focus on the Elderly Prospective Cohort Study (EPIC) that identified a correlation between the reduction in mortality in elderly and a diet rich in monounsaturated fatty acids [Trichopoulou et al., 2005].

Although exposed to the same environmental conditions of average population, centenarians show a compression of morbidity and mortality correlated with the enrichment of protective alleles or depletion of disadvantageous ones. This pattern was associated with a strong familial component of reduced mortality in centenarian families as showed by the survival analysis of siblings of 102 centenarians. Indeed, the relative risk to survive to become a centenarian was highly increased for siblings of the centenarians as compared the siblings of a control group [Perls et al., 2002].

The exceptional longevity is an inherited trait that runs across generations. While the hereditability of the age at death in the adulthood in approximately 25%, the chance to inherit longevity increases with age: for centenaries it reaches up to 33% for women and 48% for men [Brooks-Wilson, 2013].

So, healthy ageing is a complex, hereditable trait that correlates with the presence of protective alleles and lack of some of the detrimental ones (i.e. apolipoprotein E-APOE epsilon 4).

Discussion

Candidate gene studies

To this end, more than 20 years of candidate gene studies have identified only two variants that consistently associated with longevity phenotype in APOE and Forkhead box O3A (FOXO3A), even if only the first has been consistently associated in several genome-wide association studies of exceptional longevity.

In particular, Garatachea et al., [2014] showed that for all ethnic groups combined, the likelihood of reaching extreme longevity (EL) was negatively associated with ε4 allele carriage, while the presence of ε2/ε3 genotype was positively associated with EL.

By analysing centenarian offspring, it has been observed that they show a significant delay in the development of cardiovascular disease, diabetes, hypertension, and stroke but not for other age-related diseases such as cancer, osteoporosis, and thyroid disease. Therefore biomarkers for longevity have been identified such as low serum levels of heat shock proteins (HSP), large lipid particle sizes and high membrane palmitoleic acid [Barzilai et al., 2003; Puca et al., 2008; Terry et al., 2004].

Mutations that influence human longevity should modulate cellular signalling that control key aspects of ageing processes such as stress response (HSP), lipid metabolism (CEPT), senescence (P53) and cell survival (PI3K/AKT pathway) [Ferrario et al., 2012].

Also FOXO3A rs2802292 allele was found associated with EL but this polymorphism ha no apparent effect on protein functionality nor is it in LD with functional variants. FOXO3A is part of IGF/1/ PI3K/ PDK1/AKT/FOXO axis, which has been associated with longevity in many species, from worms to humans [Di Bona et al., 2014].

Several studies in lower species from worms to mice have shown that modification that impact on these signals are able to postpone ageing as this pathway regulates many aspects of cell homeostasis, from cell survival to proliferation [Kops et al., 2002].

Summing up, with the exception of APOE and FOXO3A variants, none of the many candidate genetic variants tested to date have been replicated among populations. This is probably due to the different environmental stimuli that generated "private" demographic pressures, which selected unique detrimental/protective variants making results, as a consequence, irreproducible.

Other potential problems that generated false positive and negative results reside in the small sample size of the population adopted for the studies that did not reach sufficient power, and genetic admixture, i.e. different genetic background between cases and controls.

Altshuler et al., [2008] calculated the power of a study based on the number of individuals genotyped, the number of tested hypotheses, and the frequency of the allele tested for a specific Odd Ratio (OR). An important conclusion of their analysis is that, for the OR expected for human exceptional longevity (between 1.2 and 2), the power of a

study is highly dependent upon the number of hypotheses tested. If we consider that many groups test their genetic variants and publish only positive results, the few hundred individuals typically used in a candidate gene approach on exceptional longevity are not sufficient to minimize false-positive findings.

With regard to the availability of an adequate control population in terms of genetic background, it is possible to correct for differences between cases and controls, an effect that can cause false associations, by the application of principal components analysis to the genotyping of thousands of SNPs [Price et al., 2006].

Finally, because of these hypothesis-driven candidate gene studies, the functional validation of the coded protein do not add strength to the finding, while as we will see, this is an opportunity for hypothesis-free approaches, such as the genome-wide association study (GWAS) and sib-pair linkage analysis.

Linkage analysis

For a long time the only tool available for detection of chromosomal regions that potentially harbour genetic variants influencing the phenotype of interest has been sib-pair linkage analysis. The approach is useful for identifying excess allele sharing, and was initially performed with microsatellites. By the identical-by-descendant analysis of very informative markers it is possible to reconstruct the haplotype of parents and how the markers co-segregate in their offspring. We performed such an analysis on a unique collection of sib-pairs and their families, collected by the New England Centenarian Study (NECS), and identified a significant peak on 4q25 [Puca et al., 2001].

Following analysis failed to identify genetic variants that could explain the initial linkage finding. Sibling-pair analysis could eventually capture rare mutations that segregate in centenarians' siblings, but this is not suitable for genetic association studies that loose power as the allele frequency of the tested polymorphisms drops.

Furthermore, is it possible that through linkage studies we can identify chromosomal regions where more causative genetic variants reside, and the sum of their effects determines the linkage results, whereas with follow up genetic approaches only one common polymorphism at time is involved or, eventually, haplotypes.

Attempts to replicate the initial linkage did not succeed, except for an initial replication effort that successfully replicated the linkage at D4S1564 [Beekman et al., 2006; Reed et al., 2004]. The diversity of the population used in terms of genetic background, ages of participants, number of family and genetic markers adopted and an initial false-positive finding could determine the failure of the replication efforts.

Boyden & Kunkel [2010] published a well-performed re-analysis of a part of the sib-pairs used in the initial study. To be noted, some of the largest families that were genotyped and showed a significant linkage on 4q25in the original study were not analysed in this second analysis.

This new analysis, in order to increase the coverage of the genome, used a high-density marker panel of SNPs to genotype the patients but the result of 4q25 finding was not replicated. A significant threshold was reached by a new peak on chromosome 3p24-22 and a second on 9q31-34. This latter peak appears also in the previous analysis with microsatellites, even if less robustly.

In order to identify the genetic variant responsible for 4q25 peak the attention was pointed out on one in the promoter of microsomal triglyceride transfer protein (MTP) gene [Geesaman et al., 2003].

Unfortunately, the finding was not replicated by an independent study and by our analysis that included more controls [Nebel et al., 2005; Novelli et al., 2008]

We hypothesized that the linkage peak was highlighting rare mutations with high penetrance rather than common polymorphisms with low penetrance, and so the follow up analysis needed alternative approaches to the case control studies performed on common polymorphisms.

Thus, new sequencing throughputs, as for Illumina platforms, could give the possibility to compare linkage results and resequencing of the loci of interest for the identification of rare variants. The 4q25 locus hold elongation of very long chain fatty acids protein 6 (ELOVL6), the elongase that transforms C16: 0 into C18: 0 and C16: 1 into C18: 1. Polymorphisms in this gene have been associated with insulin sensitivity; a mouse model deficient for this gene showed high doses of C16: 1 (palmitoleic acid) and did not develop insulin resistance after an high-fat diet [Matsuzaka et al., 2007; Morcillo et al., 2011]. C16: 1 has been identified as an adipose tissue-derived lipid hormone that strongly stimulates muscle insulin action and suppresses hepatic steatosis [Cao et al., 2008].

It has been described in genetically modified long-living worms, a strong correlation between increase in the lifespan and their palmitoleic acid levels. This is very appealing if we consider that we observed an increase of palmitoleic acid level in centenarian offspring and that ELOVL6 is located in the 4q25 longevity locus [Puca et al., 2001; 2008].

Thus re-sequencing in centenarians of this gene could bring to the identification of rare variants able to influence its activity and to explain, at least in part, their predisposition to exceptional longevity. Because of re-sequencing alone generate an enormous amount of information, this would impose the application of a huge statistical correction for multiple testing that could cause the loss of most potential findings. A combination, instead, of old linkage analysis and new technologies such as high massive re-sequencing could produce novel and interpretable results.

Genome-Wide Association Studies (GWAS)

GWASs are hypothesis-free studies and the findings generated must be replicated in independent populations. In case of exceptional longevity studies, differences in participant ages, gender and disease status distribution across the population could influence negatively the success in replication of initial findings. Furthermore, the GWAS approach suffers from multiple-testing statistical penalty that impose the adoption of very low p values of significance, increasing the winning course, i.e. the enrichment of false positive associations among the top findings [Ioannidis et al., 2009]. With exception of APOE locus, recent GWASs were unable to find variants that validate across population. This point out the need of larger studies or alternative study design to find out common polymorphisms with smaller genetic effects and rare variants with high penetrance that influence longevity [Deelen et al., 2011; Nebel et al., 2011]. About the power of capturing true association in GWAS, a cohort of thousands of individuals is needed to identify a sufficient OR in a GWAS on longevity field [Altshuler et al., 2008].

In our GWAS published on Long Living Individuals (LLIs) enrolled in the Southern Italian Centenarian Study (SICS) we not only tried to reduce the number of tested hypotheses, but also evaluating the redundancy of the SNPs represented on the Illumina 317 k SNP screening of the SICS individuals as a hypothesis-generating set, adopting

a Genomic Control (GC)-corrected p-value < 1e-4 threshold (which is a less stringent threshold than p < 5x10-2/317000 = 1.5x10-7) for the replication, evaluating allelic, genotypic, dominant and recessive genetic association models [Malovini et al., 2011].

CAMK4 rs104913334 was one of the first variant individuated in the initial screening of SICS that have been already established among the top 5 SNPs in the Framingham Heart Study on diastolic high blood pressure [Levy et al., 2007]. This evidence points to the conclusion that hypertension and longevity are regulated by common pathways.

In fact, mice knocked out of the angiotensin II type1 receptor, the key regulator of blood pressure, had increased expression of the longevity gene Sirt3 and improved survival [Benigni et al., 2009]. Interestingly, rs10491334 correlated with CAMK4 protein expression, and studies on its function revealed that CAMK4 protein is able to modulate SIRT1 and FOXO3A.

As described above, to identify genes and genetic variants that influence human health through interaction with environmental factors, alternative approaches are needed, such as multistage study designs aimed at reducing the statistical penalty [Ferrario et al., 2012; Levy et al., 2007; Nebel et al., 2011; Sebastiani et al., 2009].

In line with this, Villa et al., [2015] published a study with a combination of a multistage genetic and functional approach to investigate whether a gene was associated with exceptional longevity.

A 2-stage replication effort of 4 variations reported among the top findings of SICS study [Malovini et al., 2011] was designed. To this end, 2 non-synonymous SNPs, that is, rs2070325 and rs571391, and 2 intronic markers, that is, rs7583529 and rs285097, which tagged the functional variants rs7917 and rs16955011 (r2>0.8 in the HapMap CEU panel), respectively, were tested for association in two independent cohorts, the first of which was recruited for the German Longevity Study and the second for a US-based effort. Of the 4 variations tested with TaqMan assays, only rs2070325 was validated, which induces the amino acid change Ile229Val.

Villa et al., [2015] provided impressive evidence that this variant Ile229Val of BPI fold-containing family B, member 4 (BPIFB4/ LPLUNC4) is a gene associated with exceptional longevity. BPIFB4 belongs to the super family of bactericidal BPI/ PLUNC proteins, which are central to the host innate immune response against bacteria

in regions of significant bacterial exposure, like the mouth, nose, and lungs. The expression of the activity-enhanced polymorphic variant LAV-BPIFB4 (Longevity-Associated Variant) might initially produce privileged survival through better resistance to infectious diseases.

Villa et al., [2015] first evaluated BPIFB4 mRNA levels in $CD34^{+}$ circulating cells isolated from LLIs and controls, showing its expression significantly overexpressed in the long-living individuals. Moreover, eNOS phosphorylation levels on serine 1177, a well-characterized phosphorylation site linked to enhanced eNOS function, were higher in mononuclear cells of subjects carrying the a/a BPIFB4 allele as compared to A/A or A/a alleles carriers of rs2070325 polymorphism. This suggest that BPIFB4 may be able to regulate eNOS function and, as a consequence, modulating vascular tone.

Furthermore, aged mice showed reduced BPIFB4 protein level, while overexpressing the longevity associated variant of BPIFB4 in hypertensive rats induced an improvement of endothelial function and a reduction of blood pressure. A beneficial effect was also obtained in the recovery from an induced limb ischemia, with a significant improvement of revascularization as compared to animals treated with either the wild type isoform of BPIFB4 or vehicle. The work was positively commented by an editorial that stressed the importance of BPIFB4 in modulating eNOS [Kraehling & Sessa, 2015].

Finally, by using only the oldest old population, and comparing trough a meta-analysis four different GWAS, Sebastiani et al., [2017] identified a rare mutation in ELOVL6 that reduces gene expression, thus bringing to accumulation of the protective palmitoleic acid (C16:1) in centenarians. This is an example of how genetic predispose to accumulation of a protective molecule that could be also influenced by diet (i.e. trough macadamia administration).

Conclusion

Genetic studies could bring to the identification of mechanisms that protect organism from age related diseases, and, also from diseases unrelated from ageing, as for monogenic disorders that predispose to neuromuscular diseases. Some of these mechanisms could be improved by specific diets, as for palmitoleic acid that could be supplemented by macadamia administration (Figure 1).

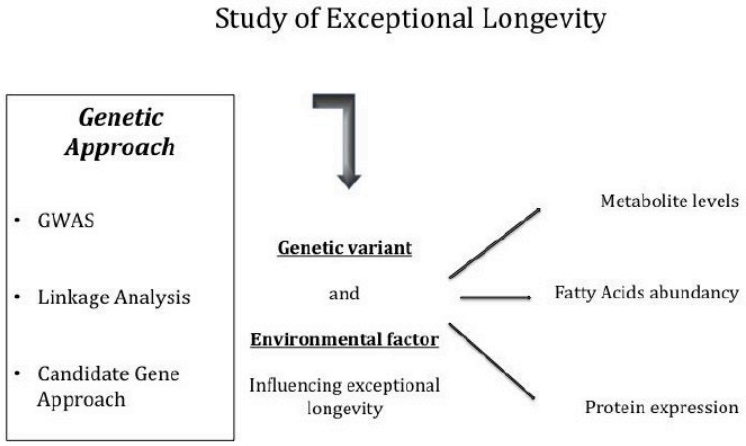

Figure 1. From genetics to environment. Genetic approaches are useful to elucidate which biological targets are modulated in exceptional longevity with the contribution of environmental factors.

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References

Altshuler D., Daly M.J., Lander E.S. *Genetic mapping in human disease*. Science. 2008; 322: 881-8.

Barzilai N., Atzmon G., Schechter C., Schaefer E.J., Cupples A.L., Lipton R., Cheng S., Shuldiner A.R. *Unique lipoprotein phenotype and genotype associated with exceptional longevity*. JAMA. 2003; 290: 2030-40.

Beekman M., Blauw G.J., Houwing-Duistermaat J.J., Brandt B.W, Westendorp R.G., Slagboom P.E. *Chromosome 4q25, microsomal transfer protein gene, and human longevity: novel data and a meta-analysis of association studies*. J Gerontol A Biol Sci Med Sci. 2006; 61: 355-62.

Benigni A., Corna D., Zoja C., Sonzogni A., Latini R., Salio M., Conti S., Rottoli D., Longaretti L., Cassis P., Morigi M., Coffman T.M., Remuzzi G. *Disruption of the Ang II type 1 receptor promotes longevity in mice*. J Clin Invest. 2009; 119: 524-30.

Boyden S.E., Kunkel L.M. *High-density genome-wide linkage analysis of exceptional human longevity identifies multiple novel loci.* PLoS One. 2010; 5: e12432.

Brooks-Wilson A.R. *Genetics of healthy aging and longevity*. Hum Genet. 2013; 132: 1323-38.

Cao H., Gerhold K., Mayers J.R., Wiest M.M., Watkins S.M., Hotamisligil G.S. *Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism*. Cell. 2008; 134: 933-44.

Deelen J., Beekman M., Uh H.W., Helmer Q., Kuningas M., Christiansen L., Kremer D., van der Breggen R., Suchiman H.E., Lakenberg N., van den Akker E.B., Passtoors W.M., Tiemeier H., van Heemst D., de Craen A.J., Rivadeneira F., de Geus E.J., Perola M., van der Ouderaa F.J., Gunn D.A., Boomsma D.I., Uitterlinden A.G., Christensen K., van Duijn C.M., Heijmans B.T., Houwing-Duistermaat J.J., Westendorp R.G., Slagboom P.E. *Genome-wide association study identifies a single major locus contributing to survival into old age; the APOE locus revisited*. Aging Cell. 2011; 10: 686-98.

Di Bona D., Accardi G., Virruso C., Candore G., Caruso C. *Association between genetic variations in the insulin/insulin-like growth factor (Igf-1) signalling pathway and longevity: a systematic review and meta-analysis*. Curr VascPharmacol. 2014; 12: 674-81.

Ferrario A., Villa F., Malovini A., Araniti F., Puca A.A. *The application of genetics approaches to the study of exceptional longevity in humans: potential and limitations*. Immun Ageing. 2012; 9: 7.

Garatachea N., Emanuele E., Calero M., Fuku N., Arai Y., Abe Y., Murakami H., Miyachi M., Yvert T., Verde Z., Zea M.A., Venturini L., Santiago C., Santos-Lozano A., Rodríguez-Romo G., Ricevuti G., Hirose N., Rábano A., Lucia A. *ApoE gene and exceptional longevity: Insights from three independent cohorts*. Exp Gerontol. 2014; 53: 16-23.

Geesaman B.J., Benson E., Brewster S.J., Kunkel L.M., Blanché H., Thomas G., Perls T.T., Daly M.J., Puca A.A. *Haplotype-based identification of a microsomal transfer protein marker associated with the human lifespan.* Proc Natl Acad Sci U S A. 2003; 100: 14115-20.

Ioannidis J.P., Thomas G., Daly M.J. *Validating, augmenting and refining genome-wide association signals*. Nat Rev Genet. 2009; 10: 318-29.

Kops G.J., Dansen T.B., Polderman P.E., Saarloos I., Wirtz K.W., Coffer P.J., Huang T.T., Bos J.L., Medema R.H., Burgering B.M. *Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress*. Nature. 2002; 419: 316-21.

Kraehling J.R., Sessa W.C. *Enhanced eNOS Activation as the Fountain of Youth for Vascular Disease: Is BPIFB4 What Ponce de León Was Looking For*? Circ Res. 2015; 117: 309-10.

Levy D., Larson M.G., Benjamin E.J., Newton-Cheh C., Wang T.J., Hwang S.J., Vasan R.S., Mitchell G.F. *Framingham Heart Study 100K Project: genome-wide associations for blood pressure and arterial stiffness*. BMC Med Genet. 2007; 8 Suppl 1: S3.

Malovini A., Illario M., Iaccarino G., Villa F., Ferrario A., Roncarati R., Anselmi C.V., Novelli V., Cipolletta E., Leggiero E., Orro A., Rusciano M.R., Milanesi L., Maione A.S., Condorelli G., Bellaz-

zi R., Puca A.A. *Association study on long-living individuals from Southern Italy identifies rs10491334 in the CAMKIV gene that regulates survival proteins*. Rejuvenation Res. 2011; 14: 283-91.

Matsuzaka T., Shimano H., Yahagi N., Kato T., Atsumi A., Yamamoto T., Inoue N., Ishikawa M., Okada S., Ishigaki N., Iwasaki H., Iwasaki Y., Karasawa T., Kumadaki S., Matsui T., Sekiya M., Ohashi K., Hasty A.H., Nakagawa Y., Takahashi A., Suzuki H., Yatoh S., Sone H., Toyoshima H., Osuga J., Yamada N. *Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance.* Nat Med. 2007; 13: 1193-202.

Nebel A., Croucher P.J., Stiegeler R., Nikolaus S., Krawczak M., Schreiber S. *No association between microsomal triglyceride transfer protein (MTP) haplotype and longevity in humans*. Proc Natl Acad Sci U S A. 2005; 102: 7906-9.

Nebel A., Kleindorp R., Caliebe A., Nothnagel M., Blanché H., Junge O., Wittig M., Ellinghaus D., Flachsbart F., Wichmann H.E., Meitinger T., Nikolaus S., Franke A., Krawczak M., Lathrop M., Schreiber S. *A genome-wide association study confirms APOE as the major gene influencing survival in long-lived individuals.* Mech Ageing Dev. 2011; 132: 324-30.

Perls T.T., Wilmoth J., Levenson R., Drinkwater M., Cohen M., Bogan H., Joyce E., Brewster S., Kunkel L., Puca A. *Life-long sustained mortality advantage of siblings of centenarians*. Proc Natl Acad Sci U S A. 2002; 99: 8442-7.

Price A.L., Patterson N.J., Plenge R.M., Weinblatt M.E., Shadick N.A., Reich D. *Principal components analysis corrects for stratification in genome-wide association studies*. Nat Genet. 2006; 38: 904-9.

Puca A.A., Andrew P., Novelli V., Anselmi C.V., Somalvico F., Cirillo N.A., Chatgilialoglu C., Ferreri C. *Fatty acid profile of erythrocyte membranes as possible biomarker of longevity*. Rejuvenation Res. 2008; 11: 63-72.

Puca A.A., Daly M.J., Brewster S.J., Matise T.C., Barrett J., Shea-Drinkwater M., Kang S., Joyce E., Nicoli J., Benson E., Kunkel L.M., Perls T. *A genome-wide scan for linkage to human exceptional longevity identifies a locus on chromosome 4*. Proc Natl Acad Sci U S A. 2001; 98: 10505-8.

Reed T., Dick D.M., Uniacke S.K., Foroud T., Nichols W.C. *Genome-wide scan for a healthy aging phenotype provides support for a locus near D4S1564 promoting healthy aging*. J Gerontol A Biol Sci Med Sci. 2004; 59: 227-32.

Sebastiani P., Gurinovich A., Bae H., Andersen S., Malovini A., Atzmon G., Villa F., Kraja A.T., Ben-Avraham D., Barzilai N., Puca A., Perls T.T. *Four Genome-Wide Association Studies Identify New Extreme Longevity Variants*. J Gerontol A Biol Sci Med Sci. 2017. [Epub ahead of print].

Sebastiani P., Timofeev N., Dworkis D.A., Perls T.T., Steinberg M.H. *Genome-wide association studies and the genetic dissection of complex traits*. Am J Hematol. 2009; 84: 504-15.

Terry D.F., Mc Cormick M., Andersen S., Pennington J., Schoenhofen E., Palaima E., Bausero M., Ogawa K., Perls T.T., Asea A. *Cardiovascular disease delay in centenarian offspring: role of heat shock proteins*. Ann N Y Acad Sci. 2004; 1019: 502-5.

Trichopoulou A., Naska A., Orfanos P., Trichopoulos D. *Mediterranean diet in relation to body mass index and waist-to-hip ratio: the Greek European Prospective Investigation into Cancer and Nutrition Study*. Am J Clin Nutr. 2005; 82: 935-40.

Villa F., Carrizzo A., Spinelli C.C., Ferrario A., Malovini A., Maciąg A., Damato A., Auricchio A., Spinetti G., Sangalli E., Dang Z., Madonna M., Ambrosio M., Sitia L., Bigini P., Calì G., Schreiber S., Perls T., Fucile S., Mulas F., Nebel A., Bellazzi R., Madeddu P., Vecchione C., Puca A.A. *Genetic Analysis Reveals a Longevity-Associated Protein Modulating Endothelial Function and Angiogenesis*. Circ Res. 2015; 117: 333-45.

The role of immune response in ageing and longevity. A focus on B cell compartment

Matteo Bulati, Calogero Caruso, Giuseppina Candore, Giuseppina Colonna-Romano

Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy.
Correspondence to: Matteo Bulati, Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; e-mail: matteo.bulati@unipa.it.

Abstract

The improvement of the quality of life of elderly people is going to become a priority because of the continuous increase in the number of centenarians. This render the studies of the processes involved in ageing of critical importance. Centenarians are a widely accepted model of successful ageing, a complex process which is influenced by several biological, environmental and lifestyle factors, because they have reached the extreme limits of life span overcoming the major age-related diseases. In centenarians model, several aspects have been studied, as inflammation, immune system, genetics and metabolism, to understand the secret of their long survival. It has been proposed that centenarians are characterized by more efficient protective molecules and biochemical pathways, and show well preserved immune functions. But a complication in the studies of centenarians is their

extremely variable clinical conditions. Indeed, among centenarians, there are both frail individuals with multiple pathologies, and healthy subjects, though some of them present signs of the advanced ageing process. Concerning the B cell compartment, centenarians seem to show the typical age-related changes observed in the elderly, but, surprisingly, their offspring present a well-preserved humeral immunity. These findings support the hypothesis that centenarian offspring are predisposed to healthy aging and longer survival, making them a suitable target of ageing studies.

Key Words

B cells, Centenarian Offspring, Inflamm-ageing, Immunosenescence.

State of the art

The role of inflammation on immunosenescence

Ageing is characterized by a general decline in physiological functions with an increasing morbidity and mortality, and it has been well established that is the major risk factor for all chronic diseases and geriatric syndromes, which negatively affect health span and longevity [Franceschi et al., 2017]. It is well known that ageing is accompanied by a complex remodelling of tissues and organs, in order to contrast the time-enduring exposure to biological and environmental stressors, whereby longevity depends on efficient mechanisms of adaption capacity [Baluster et al., 2014; Bucky et al., 2014; Candour et al., 2010; Capri et al., 2008]. The most important aspect of ageing is "inflamm-ageing", a chronic low-grade inflammatory status. Given that inflammation is essential for survival, the development of inflamm-ageing can only be explained with the "antagonistic pleiotropic theory", that, in other words, it asserts that inflammation, important for survival in the earlier stage of life to fight infections and for the tissues repair, have adverse and detrimental effects on aged individuals [Goto, 2008]. An opposing mechanism is strictly associated with inflammation, indicated as anti-inflammation, which is its inhibitory counterpart and that have the role to resolve the inflammatory processes. Lifelong depends on the balance between "inflammation" and "anti-inflammation" [Franceschi et al., 2017]. In this way, an important role is played by genetic background, as demonstrated in a large number of studies which have shown that the frequency of several polymorphism involved in inflammation are different between elderly and successfully aged people, as centenarians [Reviewed by Larbi et al., 2008]. As it is known, centenarians show a complex and heterogeneous phenotype, which seems to be the result of the capacity to adapt and remodel their body in response to stressors, that makes them able to delay the ageing process and to escape the major age-related diseases. Moreover, it has been demonstrated that longevity has a strong familial component [Passarino et al., 2016], indeed centenarian offspring, like their centenarian parents, have a significant advantage for longer survival together with a lower risk to develop age-related diseases [Balistreri et al., 2014].

The phenomenon of inflamm-ageing is strictly associated with the deterioration of the immune function, termed "immunosenescence", which is the cause of the increased susceptibility to infectious diseases, cancer, dementia, cardiovascular diseases and autoimmunity, and of the decreased response to vaccination, which characterize elderly people [Bucci et al., 2014; Derhovanessian et al., 2010, 2013; Fulop et al., 2016; Grasse et al., 2016; Pawelec 2014a, b; Salvioli et al., 2013; Strindhall et al., 2007]. The condition of inflamm-ageing provides a continuous mild antigenic challenge that leads to a progressive stimulation, or depletion, of the immune system cells and the filling of the immunological space by activated/exhausted lymphocytes with altered functions [Bulati et al., 2011, 2014; Fulop et al., 2017; Larbi & Fulop, 2014; Naradikian et al., 2016; Pawelec, 2014a,b; Pinti et al., 2016; Rubtsova et al., 2015]. Although T cell compartment has been more extensively studied [Di Benedetto et al., 2015; Wistuba-Hamprecht et al., 2016], age-related changes in B cell number and repertoire have also been described, and data in literature demonstrate that elderly, frequently, do not have protective antibody against recall antigens or newly encountered antigens, so suggesting the impairment of B cell branch [Aberle et al., 2013; Bulati et al., 2011, 2014, 2015, 2017; Cancro et al., 2009; Colonna-Romano et al., 2009; Dunn-Walters & Ademokun, 2010; Frasca & Blomberg, 2016; Naradikian et al., 2016].

B cell and Ageing

It is well known that, with ageing, there is a significant decrease in circulating B lymphocytes [Bulati M et al., 2011; Colonna-Romano G et al., 2009; Pinti M et al., 2016; Strindhall et al., 2007] and a shift from naïve (IgD, IgM) to memory (IgG, IgA) immunoglobulins production [Listì et al., 2006], accompanied by an impaired ability to produce high affinity protective antibodies against infectious agents [Frasca & Blomberg, 2016; Frasca et al., 2017] and the shrinkage of the repertoire diversity [Dunn-Walters, 2016; Gibson et al., 2009]. Moreover, in elderly, it has been demonstrated the increase of a senescent B cell subpopulation, namely DN late memory B cells, at the expense of naïve B cells [Bulati et al., 2011, 2014, 2015, 2017; Colonna-Romano et al., 2009; Frasca et al., 2017]. $IgD^{-}CD27^{-}$ DN late memory B cells have been also reported to be expanded in patients affected by autoimmune diseases, as Systemic Lupus Erythematosus or Rheumatoid

Arthritis, and in HIV-infected people [Fecteau et al., 2006; Mahmood et al., 2015; Palma et al., 2014; Sanz et al., 2008; Wei et al., 2007], suggesting the pivotal role of inflammation as the cause of this increase.

B cell in centenarian offspring, a model of successful ageing

As known, centenarians are able to reach the extreme limits of life slowing down aging processes and are characterized by a lower prevalence of cancer, cardio- and cerebral-vascular diseases and most of the metabolic age-associated diseases, as insulin resistance or diabetes [Caruso et al., 2012]. Moreover, many of them show optimal metabolic (cholesterol, LDL-C, HDL-C, triglycerides), anthropometric (Body Mass Index) and cardiovascular (blood pressure) parameters for their age [Kolovou et al., 2014]. Nonetheless, the study of these exceptional individuals has highlighted several methodological problems. First of all, the difficulty in their recruitment due to their low frequency in the population. Besides, it is not easy to perform population-based cross-sectional studies on centenarians, because of the very high mortality rate at extremely advanced age.

Last but not least, a complication in the studies of centenarians is their extremely variable clinical conditions, as, among centenarians, there are both frail individuals with multiple pathologies, and healthy subjects, though some of them present signs of the advanced ageing process [Arai et al., 2015; Bucci et al., 2014; Gueresi et al., 2013; Salvioli et al., 2013; Strindhall et al., 2007]. It is also known that longevity have a strong familial component and recent studies have suggested that centenarian offspring, like their centenarian parents, have a significant advantage for longer survival together with a lower risk to develop age-related diseases [Balistreri et al., 2014]. At the same time, the study of centenarian offspring resolves the problems occurring with their parents, as being younger, they are easier to recruit, are less frail and less likely to die within a short time. Finally, they can be compared with an appropriate control group, consisting of age-matched elderly people whose parents died at an average life expectancy. For these reasons, offspring of long lived subjects, represent the appropriate model to analyse successful ageing and, consequently, to understand the advantage of centenarian phenotype [Capri et al., 2008; Cevenini et al., 2008; Rea et al., 2005], as centenarian offspring show a favourable lipid, immunological and cardiovascular profile, a

decreased cognitive decline and a protective genetic background [Balistreri et al., 2014]. So, it can be stated that it exists a potential transmission from centenarians to their offspring of the ability to escape or postpone the major age-related disease.

Concerning the B cell compartment, its study in centenarian offspring, has shown that, although there is a reduction in the B cell count, typical of elderly people, they do not present the typical naïve-memory shift observed in the elderly [Balistreri et al., 2014; Buffa et al., 2013; Colonna-Romano et al., 2010]. Indeed, centenarian offspring do not show neither the reduction of $IgD^{+}CD27^{-}$ naïve nor the increase of the $IgD^{-}CD27^{-}$ DN late memory B cells observed in the average elderly population, but it look similar to young people [Colonna-Romano et al., 2010].

This failed age-associated increase of DN B cells, observed in the general elderly population, suggest that in centenarian offspring there is not an exhaustion of the B cell compartment. Furthermore, the evaluation of IgM of centenarian offspring serum shows values that are within the range of the levels observed in young subjects [Colonna-Romano et al., 2010].

These data, together with the increased number of naïve B cell, suggest a good bone marrow cell reservoir in centenarian offspring. This is an interesting observation, as it has been demonstrated [Cancro et al., 2009] that the bone marrow ability to generate B cells is impaired with age. The "younger" immune profile of centenarian offspring is also supported by data obtained from T cell compartment of these subjects, in which it was not observed the typical shift from naïve to exhausted memory T cells that are a typical feature of immune system ageing [Pellicanò et al., 2014].

Discussion

Aged people are characterized by a chronic inflammatory status, named "inflamm-ageing", which is strictly associated with the deterioration of the immune function, defined as "immunosenescence" [Salvioli et al., 2013]. These events are cause of the increased susceptibility of elderly to infectious diseases, cancer, dementia, cardiovascular diseases, autoimmunity and of the decreased response to vaccination [Bucci et al., 2014; Derhovanessian et al., 2010, 2013; Fulop et al., 2017; Grasse et al., 2016; Pawelec 2014a,b; Salvioli et al., 2013; Strindhall et al., 2007]. It has been widely demonstrated that ageing have a strong impact on the

remodelling of the B cell branch of immune system. Indeed, together with the reduced number of circulating B lymphocytes, the reduction of $IgD^{+}CD27^{-}$ naïve and the simultaneous increase of $IgD^{-}CD27^{-}$ late memory DN B cells have been reported [Bulati et al., 2011, 2014, 2015, 2017; Colonna-Romano et al., 2009; Frasca et al., 2017]. A crucial role in the impairment of B cell branch of the immune system is played by senescent/exhausted $IgD^{-}CD27^{-}$ DN B cells. Indeed, these cells are also increased in other models of chronic inflammation, and they shown a pro-inflammatory trafficking and a senescent associated secretory phenotype [Bulati et al., 2011, 2014, 2015, 2017; Colonna-Romano et al., 2009; Frasca et al., 2017]. Moreover it is also known that there is a considerable variation in the rate of progression of ageing that it has led to make a distinction between successful and unsuccessful ageing. Centenarian offspring, a model of successful ageing, seem to show an optimal B cell profile, which is comparable for several aspects, to that of young subjects [Balistreri et al., 2014; Buffa et al., 2013; Colonna-Romano et al., 2010; Derhovanessian et al., 2010; Pellicanò et al., 2014]. In this way, centenarian offspring behave as the young people, maintaining the ability to respond to new infections and responding to vaccination, differently from their parents [Derhovanessian et al. 2010]. This condition determines a better control of inflammatory response with a reduction of the risk for the major age-associated diseases. All together these factors described, suggest that the positive ageing phenotype of centenarian offspring might be the result of an efficient physical performance. This allows them to have a major chance to extend survival, not only in chronological age, but also in a health and well-being ageing [Balistreri et al., 2014].

Conclusion

The most studies focusing on the "successful pattern of centenarians", reveal that they may easily fail to be considered as successful agers, when objective criteria are applied. Indeed, although they are in relatively good conditions, many centenarians show signs of frailty, due to their extreme age, and present a great heterogeneity in their health and functional status. Nonetheless, these long-lived exceptional individuals have delayed the ageing processes and have escaped the major age-related diseases, probably because they have a favourable genetic background and good metabolic parameters [Paolisso et al., 2001; Motta et al., 2005; Bucci et al.,

2014]. Moreover, it has been widely demonstrated that "IRP" is a real predictor of mortality in elderly individuals and that the survival of centenarians is due to the selection of octo- and nonagenarians without IRP [Stridhall et al., 2007]. All together, these data, led to speculate that centenarians spent the most of their life in good health conditions, but, having overcome the "biological barriers", they are extremely fragile, show rapid evolution of cellular and tissues degeneration, with a compression of their disability into a relatively short period at the end of their exceptional long life. For these reasons, the synonymous "centenarian-successful ageing" somehow collapses, and it becomes of crucial importance to study the differences among individuals which are in the "critical age" in which the physiological decline and the onset of the major age-related diseases occur. This statement is supported by some fundamental concepts. First of all, it has been demonstrated the existence of strong genetic determinants for longevity and also that the favourable modulation of diseases susceptibility is strongly inherited in families with exceptional longevity [Balistreri et al., 2014]. Indeed, offspring of long lived subjects, like their centenarian parents, show a favourable lipid, immunological and cardiovascular profile, associated with decreased cognitive decline and a protective genetic background. So, it exist a potential transmission from centenarians to their offspring of the capacity to escape or postpone the major age-related diseases. Moreover, it has been also demonstrated that the state of health of elderly people, without long-lived parents, is worse compared to that of subjects with at least one centenarian parent [Gueresi et al., 2013], confirming the hypothesis that longevity is a familial genetic trait. So their survival advantage, the higher probability to become long-lived and the lower risk to undergo to major age-related diseases, render centenarian offspring, who are one generation younger than centenarians, the best model for the studies on healthy ageing.

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References

Aberle J.H., Stiasny K., Kundi M., Heinz F.X. *Mechanistic insights into the impairment of memory B cells and antibody production in the elderly*. Age. 2013; 35: 371-381.

Arai Y., Martin-Ruiz C.M., Takayama M., Abe Y., Takebayashi T., Koyasu S., Suematsu M., Hirose N., von Zglinicki T. *Inflammation, But Not Telomere Length, Predicts Successful Ageing at Extreme Old Age: A Longitudinal Study of Semi-supercentenarians*. EBioMedicine. 2015; 2: 1549-1558.

Balistreri C.R., Candore G., Accardi G., Buffa S., Bulati M., Martorana A., Colonna-Romano G., Lio D., Caruso C. *Centenarian offspring: a model for understanding longevity*. Curr Vasc Pharmacol. 2014; 12: 718-725.

Bucci L., Ostan R., Giampieri E., Cevenini E., Pini E., Scurti M., Vescovini R., Sansoni P., Caruso C., Mari D., Ronchetti F., Borghi M.O., Ogliari G., Grossi C., Capri M., Salvioli S., Castellani G., Franceschi C., Monti D. *Immune parameters identify Italian centenarians with a longer five-year survival independent of their health and functional status*. Exp Gerontol. 2014; 54: 14-20.

Buffa S., Pellicanò M., Bulati M., Martorana A., Goldeck D., Caruso C., Pawelec G., Colonna-Romano G. *A novel B cell population revealed by a CD38/CD24 gating strategy: CD38(-)CD24 (-) B cells in centenarian offspring and elderly people*. Age (Dordr). 2013; 35: 2009-2024.

Bulati M., Buffa S., Candore G., Caruso C., Dunn-Walters D.K., Pellicanò M., Wu Y.C., Colonna Romano G. *B cells and immunosenescence: a focus on IgG+IgD-CD27- (DN) B cells in aged humans*. Ageing Res Rev. 2011; 10: 274-84.

Bulati M., Buffa S., Martorana A., Candore G., Lio D., Caruso C., Colonna-Romano G., 2014. *Trafficking phenotype and production of granzyme B by double negative B cells (IgG(+)IgD(-) CD27(-)) in the elderly*. Exp Gerontol. 2014; 54: 123-129.

Bulati M., Buffa S., Martorana A., Gervasi F., Camarda C., Azzarello D.M., Monastero R., Caruso C., Colonna-Romano G. *Double negative (IgG+IgD-CD27-) B cells are increased in a cohort of moderate-severe Alzheimer's disease patients and show a pro-inflammatory trafficking receptor phenotype*. J Alzheimers Dis. 2015; 44: 1241-1251.

Bulati M., Caruso C., Colonna-Romano G. *From lymphopoiesis to plasma cells differentiation, the age-related modifications of B cell compartment are influenced by* "inflamm-ageing". Ageing Res Rev. 2017; 36: 125-136.

Cancro M.P., Hao Y., Scholz J.L., Riley R.L., Frasca D., Dunn-Walters D.K., Blomberg B.B. *B cells and aging: molecules and mechanisms. Trends Immunol*. 2009; 30: 313-318.

Candore G., Caruso C., Jirillo E., Magrone T., Vasto S. *Low grade inflammation as a common pathogenetic denominator in age-related diseases: novel drug targets for anti-ageing strategies and successful ageing achievement*. Curr Pharm Des. 2010; 16: 584-596.

Capri M., Salvioli S., Monti D., Caruso C., Candore G., Vasto S., Olivieri F., Marchegiani F., Sansoni P., Baggio G., Mari D., Passarino G., De Benedictis G., Franceschi C. *Human longevity within an evolutionary perspective: the peculiar paradigm of a post-reproductive genetics*. Exp Gerontol. 2008; 43: 53–60.

Caruso C., Passarino G., Puca A., Scapagnini G. "Positive biology": *the centenarian lesson*. Immun Ageing. 2012; 9: 5.

Cevenini E., Invidia L., Lescai F., Salvioli S., Tieri P., Castellani G., Franceschi C. *Human models of aging and longevity*. Expert Opin Biol Ther. 2008; 8: 1393-1405.

Colonna-Romano G., Bulati M., Aquino A., Pellicanò M., Vitello S., Lio D., Candore G., Caruso C. *A double negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people*. Mech Ageing Develop. 2009; 130: 681-690.

Colonna-Romano G., Buffa S., Bulati M., Candore G., Lio D., Pellicanò M., Vasto S., Caruso C. *B cells compartment in centenarian offspring and old people*. Curr Pharm Des. 2010; 16: 604-608.

Derhovanessian E., Maier A.B., Beck R., Jahn G., Hähnel K., Slagboom P.E., de Craen A.J., Westendorp R.G., Pawelec G. *Hallmark features of immunosenescence are absent in familial longevity*. Immunol. 2010; 185: 4618-4624.

Derhovanessian E., Theeten H., Hahnel K., Van Damme P., Cools N., Pawelec G. *Cytomegalovirus-associated accumulation of late differentiated CD4 T cells correlates with poor humoral response to influenza vaccination*. Vaccine. 2013; 31: 685-690.

Di Benedetto S., Derhovanessian E., Steinhagen-Thiessen E., Goldeck D., Müller L., Pawelec G. *Impact of age, sex and CMV-infection on peripheral T cell phenotypes: results from the Berlin BASE-II Study*. Biogerontol. 2015; 16: 631-643.

Dunn-Walters D.K., Ademokun A.A. *B cell repertoire and ageing. Curr Opin Immunol*. 2010; 22: 514-520.

Dunn-Walters D.K. *The ageing human B cell repertoire: a failure of selection? Clin Exp Immunol*. 2016; 183: 50-56.

Fecteau J.F., Cote G., Neron S. *A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation*. J. Immunol. 2006; 177: 3728-3736.

Franceschi C., Garagnani P., Vitale G., Capri M., Salvioli S. *Inflammaging and 'Garb-aging'*. Trends Endocrinol Metab. 2017; 28: 199-212.

Frasca D., Blomberg B.B. *Inflamm-aging decreases adaptive and innate immune responses in mice and humans*. Biogerontol. 2016; 17: 7-19.

Frasca D., Diaz A., Romero M., Blomberg B.B. *Human peripheral late/exhausted memory B cells express a senescent-associated secretory phenotype and preferentially utilize metabolic signalling pathways*. Exper Gerontol. 2017; 87: 113-120.

Fulop T., Witkowski J.M., Le Page A., Fortin C., Pawelec G., Larbi A. *Intracellular signaling pathways: targets to reverse immunosenescence*. Clin Exp Immunol. 2017; 187: 35-43.

Gibson K.L., Wu Y.C., Barnett Y., Duggan O., Vaughan R., Kondeatis E., Nilsson B.O., Wikby A., Kipling D., Dunn-Walters

D.K. *B-cell diversity decreases in old age and is correlated with poor health status*. Aging Cell. 2009; 8: 18-25.

Goto M. *Inflammaging (inflammation + aging): a driving force for human aging based on an evolutionarily antagonistic pleiotropy theory?* Biosci Trends. 2008; 2: 218-230.

Grasse M., Meryk A., Schirmer M., Grubeck-Loebenstein B., Weinberger B. *Booster vaccination against tetanus and diphtheria: insufficient protection against diphtheria in young and elderly adults*. Immun Ageing. 2016; 13: 26.

Gueresi P., Miglio R., Monti D., Mari D., Sansoni P., Caruso C., Bonafede E., Bucci L., Cevenini E., Ostan R., Palmas M.G., Pini E., Scurti M., Franceschi C. *Does the longevity of one or both parents influence the health status of their offspring?* Exp Gerontol. 2013; 48: 395-400.

Kolovou G., Barzilai N., Caruso C., Sikora E., Capri M., Tzanetakou I.P., Bilianou H., Avery P., Katsiki N., Panotopoulos G., Franceschi C., Benetos A., Mikhailidis D.P. *The challenges in moving from ageing to successful longevity*. Curr Vasc Pharmacol. 2014; 12: 662-73.

Larbi A., Franceschi C., Mazzanti D., Solana R., Wikby A., Pawelec G. *Aging of the Immune System as a Prognostic Factor for Human Longevity*. Physiology. 2008; 23: 64-74.

Larbi A., Fulop T. *From "truly naïve" to "exhausted senescent" T cells: when markers predict functionality*. Cytometry A. 2014; 85: 25-35.

Listì F., Candore G., Modica M.A., Russo M., Di Lorenzo G., Esposito-Pellitteri M., Colonna-Romano G., Aquino A., Bulati M., Lio D., Franceschi C., Caruso C. *A study of serum immunoglobulin levels in elderly persons that provides new insights into B cell immunosenescence*. Ann NY Acad Sci. 2006; 1089: 487-495.

Mahmood Z., Muhammad K., Schmalzing M., Roll P., Dörner T., Tony H.P. *CD27-IgD-memory B cells are modulated by in vivo interleukin-6 receptor (IL-6R) blockade in rheumatoid arthritis*. Arthritis Res Ther. 2015; 17: 61.

Motta M., Bennati E., Ferlito L., Malaguarnera M., Motta L. *Italian Multicenter Study on Centenarians (IMUSCE). Successful aging in centenarians: myths and reality*. Arch Gerontol Geriatr. 2005; 40: 241-251.

Naradikian M.S., Hao Y., Cancro M.P. *Age-associated B cells: key mediators of both protective and autoreactive humoral responses*. Immunol Rev. 2016; 269: 118-129.

Palma P., Rinaldi S., Cotugno N., Santilli V., Pahwa S., Rossi P, Cagigi A. *Premature B-cell senescence as a consequence of chronic immune activation*. Hum Vaccin Immunother. 2014; 10: 2083-2088.

Paolisso G., Barbieri M., Rizzo M.R., Carella C., Rotondi M., Bonafè M., Franceschi C., Rose G., De Benedictis G. *Low insulin resistance and preserved beta-cell function contribute to human longevity but are not associated with TH-INS genes*. Exp Gerontol. 2001; 37: 149-156.

Passarino G., De Rango F., Montesanto A. *Human longevity: Genetics or Lifestyle? It takes two to tango*. Immun Ageing. 2016; 13: 12.

Pawelec G. *Immunosenenescence: role of cytomegalovirus*. Exp Gerontol. 2014a; 54: 1-5.

Pawelec G. *T-cell immunity in the aging human*. Haematologica. 2014b; 99: 795-797.

Pellicanò M., Buffa S., Goldeck D., Bulati M., Martorana A., Caruso C., Colonna-Romano G., Pawelec G. *Evidence for less marked potential signs of T-cell immunosenescence in centenarian offspring than in the general age-matched population*. J Gerontol A Biol Sci Med Sci. 2014; 69: 495-504.

Pinti M., Appay V., Campisi J., Frasca D., Fülöp T., Sauce D., Larbi A., Weinberger B., Cossarizza A. *Aging of the immune system: Focus on inflammation and vaccination*. Eur J Immunol. 2016; 46: 2286-2301.

Rea S.L., Wu D., Cypser J.R., Vaupel J.W., Johnson T.E. *A stress-sensitive reporter predicts longevity in isogenic populations of Caenorhabditis elegans*. Nat Genet. 2005; 37: 894-898.

Rubtsova K., Rubtsov A.V., Cancro M.P., Marrack P. *Age-Associated B Cells: A T-bet-Dependent Effector with Roles in Protective and Pathogenic Immunity*. J Immunol. 2015; 195: 1933-1937.

Salvioli S., Monti D., Lanzarini C., Conte M., Pirazzini C., Bacalini M.G., Garagnani P., Giuliani C., Fontanesi E., Ostan R., Bucci L., Sevini F., Yani S.L., Barbieri A., Lomartire L., Borelli V., Vianello

D., Bellavista E., Martucci M., Cevenini E., Pini E., Scurti M., Biondi F., Santoro A., Capri M., Franceschi C. *Immune system, cell senescence, aging and longevity--inflamm-aging reappraised*. Curr Pharm Des. 2013; 19: 1675-1679.

Sanz I., Wei C., Lee F.E., Anolik J. *Phenotypic and functional heterogeneity of human memory B cells*. Semin Immunol. 2008; 20: 67-82.

Strindhall J., Nilsson B.O., Löfgren S., Ernerudh J., Pawelec G., Johansson B., Wikby A. *No Immune Risk Profile among individuals who reach 100 years of age: findings from the Swedish NONA immune longitudinal study*. Exp Gerontol. 2007; 42: 753-761.

Wei C., Anolik J., Cappione A., Zheng B., Pugh-Bernard A., Brooks J., Lee E.H., Milner E.C., Sanz I. *A new population of cells lacking expression of CD27 represents a notablecomponent of the B cell memory compartment in systemic lupus erythematosus*. J Immunol. 2007; 178: 6624-6633.

Wistuba-Hamprecht K., Haehnel K., Janssen N., Demuth I., Pawelec G. *Peripheral blood T-cell signatures from high-resolution immune phenotyping of γδ and αβ T-cells in younger and older subjects in the Berlin Aging Study II*. Immun Ageing. 2015; 12: 25.

Bioimpedance: a new approach for studying longevity

Giulia Accardi[1], Anna Aiello[1], Sonya Vasto[2], Calogero Caruso[1]

[1]Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; [2]Department of Biological Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Italy.
Correspondence to: Giulia Accardi, Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; e-mail: giulia.accardi@unipa.it.

Abstract

Centenarians are the best model to study successful ageing. Unfortunately, they are rare and do not have an aged-matched control population to compare their exceptional characteristics with non-longevous people.
Considering the complexity of molecular studies, the opportunity to analyse the centenarian phenotype with anthropometry could be an easy and no invasive interesting solution to identify peculiar measurable variables. In addition to the classic measurements, the bioelectrical impedance could be considered. This method permits to analyse the body composition, in terms of fat free mass and fat mass. Ageing is related to reduced fat free mass and increased body fat. The

reduction of the first one is directly responsible for the onset of frailty and sarcopenia. These are typical conditions in elderly that leads to reduced physical strength and loss of immune competence. Thus, it is mandatory to identify specific values and range and perform multiple measurements in aged people and centenarians to address to weight variation modifiable causes, preventing premature death and avoiding diseases.

Key Words

Bioimpedance, Elderly, Life Style, Longevity.

State of the Art

On 2012, a scientific consensus meeting to summarize knowledge and develop a consensus statement on ageing and intervention to slow it was held [Longo et al., 2015]. Although many molecular aspects have been revealed, the actual policy demonstrates that we do not have action plan to guarantee long-lived people needs. So, it is necessary to develop new studies to characterize this population, also from the anthropometric point of view.

In fact, disorders related to body fat accumulation, especially abdominal fat mass, are known risk factors for all-cause and cardiovascular mortality [Carmienke et al., 2013; Cerhan et al., 2014; Hotchkiss & Leyland, 2011]. Although, it has been highlighted a possible inverse or null relationship between overweight and obesity with mortality in old people [Beleigol et al., 2012; Flegal et al., 2013]. It is likely that, in certain population, such as elderly, fat accumulation protects from death. This is the so-called obesity paradox that implies an inverse correlation between body mass index (BMI) and mortality, as demonstrated by several studies. Consequently, it is associated with low specificity of BMI for certain population and for body fat distribution [Gallagher et al., 1996; Kouvari et al., 2017; Romero-Corral et al., 2000; Snijder et al., 2006]. In elderly it is possible to observe reduction of lean mass and redistribution of adipose tissue, maintaining the same weight and the same BMI [De Lorenzo et al., 2013].

To solve this problem, direct and indirect measurements can be made: waist circumference, waist-hip ratio and waist-to-height ratio, percentage of fat free mass and fat mass to assess fat accumulation in abdomen and in other districts.

However, studies have shown opposite results exposing uncertainties regarding the predictive ability of anthropometric measurements in the elderly [de Hollander et al., 2012; Katzmarzyk et al., 2013]. The differing conclusion from the available evidence could be explained by the great heterogeneity between studies, including the wide age range and the clinical characteristics [Chang et al., 2012]. Next to the bias linked to anthropometric measurement due to operator and cut-off point for variables, there is consensus about the inappropriateness of the use of the same weight and abdominal circumference range values for adults and elderly [de Hollander et al., 2012; Donini et al., 2012; Molarius et al., 2000].

Indeed, in aged people the abdominal fat accumulation is often a marker of resilience, better functional reserve and lower subclinical

diseases prevalence, characterizing the so called "healthy cohort" effect [Lopez-Jimenez, 2009]. Changes in body composition, together with specific characteristics in relation to health conditions, make the long-lived a peculiar subgroup.

Therefore, the results found in studies with adults and younger elderly individuals can create an over- or underestimation of risk in this population.

Associations between overweight, abdominal obesity, and mortality have been less studied, in elderly population over 80 years old, especially taking into account important confounding factors that may interfere with the associations between body fat and mortality [David et al., 2017]. In fact, a meta-analysis developed on 2014, showed a greater mortality risk for old people with a BMI under 23 compared with younger people in the overweight range (over 24.9) [Winter et al., 2014].

Understanding the changes in body size, shape and composition with ageing and their health implications is important for nutritional support, pharmacologic treatment and development of appropriate health guidelines targeting the well being of the elderly.

Discussion

Role of life style in attaining healthy ageing

Considering the growing interest in identify possible strategies to improve health in ageing and to live longer in active condition and considering the difficulty to understand molecular aspects of life, possible approaches to prevent age-related disease with daily habits are under study. One of the easiest and more studied approach is the dietary modulation of oxidative stress and inflammation that constitute the basis of ageing process and associated pathologies [Caruso et al., 2012].

Many studies demonstrated the power of different dangerous dietary compounds in the promotion of these events and, on the contrary, the power of the use of dietary restrictions or nutraceutics in the promotion of health-span and in the increase of lifespan in model organisms, form yeast to mice [Aiello et al., 2016].

Unfortunately, the conduction of the same studies in humans are not easy so we need to analyse measurable biomarkers and, when these are not present, we can only analysed retrospectively the best outcome, in this case, the centenarian phenotype.

Worldwide exist five hot spots of longevity, the so called blue zones. These are located in California (Loma Linda); in Japan (Okinawa); in Italy (Sardinia); in Greece (Ikaria); and in Costa Rica (Nicoya). People living in these areas present different cultural traditions but a common characteristic: the healthy lifestyle. Some of them are vegetarians, others follow occasionally fasting and live the life with a positive mood, socially engaged and physically active [Buettner, 2008].

Similar situation was observed in a mountainous zone in Sicily, the Sicani Mountain area, where a group of scientists evidenced, in 2012, the presence of a blue zone-like. It is located in the country side of the island and showed a great number of centenarians: 10.38 on 10000 inhabitants versus 2.4 on 10000 in the rest of Italy [Vasto al., 2012a].

Analysing the life of these people, similar characteristics to blue-zone inhabitants were found. They lived an active life, socially involved, they ate seasonally food, especially fruit and vegetables, and whole grain and they had low calorie intake. Overall, they followed Mediterranean life-style and, consequently, the traditional Mediterranean diet [Vasto et al., 2012b].

Recent Istat data (http: //www.istat.it) showed that in Sicily we have more than 1000 centenarians. Their analysis could be done by the use of specific nutritional and habit questionnaire that could highlight peculiarities of this population. These could constitute the starting point for the identification of a longevity phenotypic signature but measurable variables are needed: anthropometry and bioimpedance could give interesting information.

Anthropometric measurements

Range to classify body composition exists but not specific for longevous people, often characterized by significant changes in mass distribution and nutritional status. Moreover, information about the nutritional status of very old people, such as centenarians, is limited and, probably, the existing range related to BMI, measured as weight in kilograms divided by the square of the height in meters, is not suitable. It is not an exception because it is well known BMI cannot be used for athletes, presenting high percentage of the weight composed by muscle mass, so high BMI, similar to the one of obese people.

Ageing is related to reduced fat free mass and increased body fat, especially in the trunk. The fat free mass or lean mass is composed by all

masses except the fat. Its reduction is directly responsible for the onset of frailty and sarcopenia, typical conditions in elderly, that leads to reduced physical strength and loss of immune competence [Cesari et al., 2016].

Today is possible to analyse body composition using a no invasive method: the bioimpedance. Also called bioelectrical impedance, it provides a measurement of body composition, in terms of free fat mass and fat mass. It is the measure of resistance and reactance of the body. The total impedance is the total sum of impedance of different tissues. When we exposed human cell membrane to an alternating current, we can measure two values: resistance and reactance. Theoretically, the first is an indirect measure of the intracellular volume or body cell mass. The electric resistance is a force opposed by body fat, total body water and extra cellular water to electrical current [Foster & Lukaski, 1996].

The relationship between resistance and reactance give a phase angle that could be used as measurement of health status and diseases. In fact, lower phase angles appear to be consistent with low reactance and either cell death or a breakdown in the selective permeability of the cell membrane. There is a significant difference in phase angle between healthy and disease states. The higher is phase angle valued the better is the healthy condition [Cowen et al., 1998; Guglielmi et al., 1999; Gupta et al., 2004; Schwenk et al., 2000].

So, anthropometric analysis by bioimpedance could be an interesting choice in centenarian and old people but reference values are needed, since the change in body composition seem to be involved in decreased ability to perform daily life activities.

The bioimpedance

To obtain informative and reproducible body composition analysis, the body scan technology or bioimpedance have to be conducted by trained professionals. In particular, for elderly these data might be useful to prevent or evaluate the muscular decline and hydroelectrolytic changes.

Special skin electrodes are placed on the hand and foot of one body side, connected to the device by electrode cables, the red and the black. Detecting electrode edge is placed on an imaginary line bisecting the ulnar head and medial malleolus. The signal electrode is placed on the first joint of the middle finger and on the base of the second toe of the foot. It is important to choose the same side of the body, i.e. right hand and foot, not right foot and left hand.

When a low-voltage is applied within seconds, two values are measures: resistance and reactance of human tissue. Phase angle and body compartments are derived using specific software through medically validated algorithms, adding the resistance and reactance measured.

Then, results are reported in an impressive report that contain information about body composition but also analysis of nutrition and hydration states [Khalil et al., 2014] (Figure 1).

Moreover, using a dynamometer, it is possible to obtain an evaluation of the muscle strength, so an estimation of the onset of sarcopenia.

This method is a direct measurement, differently from the determination of body compartments based on co-predictors such as weight, age, and gender.

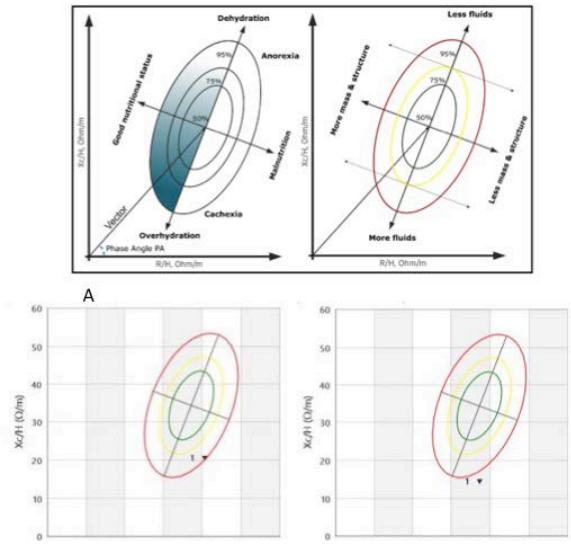

Figure 1. Nomogram of centenarians. The nomogram, or nomograph, is a two-dimensional diagram designed to allow the approximate graphical computation of a mathematical function. It is a graphical, qualitative representation of a bi or multiple variables function. The figure shows reference nomograms (A) and two nomograms of centenarian female, 104 (B) and 101 (C) years old. The first patient was frail and fully assisted in daily activity. Differently, the second one was autonomous. Comparing (B) and (C), referring to (A), it is possible to speculate that both are affected by cachexia. Moreover, in C we can see that the patient have more fluid and more mass and structure.

Conclusion

The lifestyle constitutes an important modifiable risk factor and growing evidence demonstrates the reduction of onset of pathologies improving the quality of the diet. Centenarians and nonagenarians are populations with much peculiarity, including reduced metabolic rate and fat distribution. For this reason, it is fundamental to fix anthropometric range that permits to identify risk factor to classify the population. Notwithstanding, these features might be informative to allow the implementation of adapted programs to optimize centenarians quality of life.

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References

Aiello A., Accardi G., Candore G., Carruba G., Davinelli S., Passarino G., Scapagnini G., Vasto S., Caruso C. *Nutrigerontology: a key for achieving successful ageing and longevity*. Immun Ageing. 2016; 13: 17.

Beleigoli A.M., Boersma E., Diniz Mde F., Lima-Costa M.F., Ribeiro A.L. *Overweight and class I obesity are associated with lower 10-year risk of mortality in Brazilian older adults: the Bambui Cohort Study of Ageing*. PloS one. 2012; 7: e52111.

Buettner D. *The Blue Zone: Lessons for Living Longer From the People Who've Lived the Longest*. Washington, DC: National Geographic Society; 2008.

Caruso C., Passarino G., Puca A., Scapagnini G. "Positive biology": *the centenarian lesson*. Immun Ageing. 2012; 9: 5.

Carmienke S., Freitag M.H., Pischon T., Schlattmann P., Fankhaenel T., Goebel H., Gensichen J. *General and abdominal obesity parameters and their combination in relation to mortality: a systematic review and meta-regression analysis*. Eur J Clin Nutr. 2013; 67: 573-85.

Cerhan J.R., Moore S.C., Jacobs E.J., Kitahara C.M., Rosenberg P.S., Adami H.O., Ebbert J.O., English D.R., Gapstur S.M., Giles G.G., Horn-Ross P.L., Park Y., Patel A.V., Robien K., Weiderpass E., Willett W.C., Wolk A., Zeleniuch-Jacquotte A., Hartge P., Bernstein L., Berrington de Gonzalez A. *A pooled analysis of waist circumference and mortality in 650,000 adults*. Mayo Clin Proc. 2014; 89: 335-45.

Cesari M., Prince M., Thiyagarajan J.A., De Carvalho I.A., Bernabei R., Chan P., Gutierrez-Robledo L.M., Michel J.P., Morley J.E., Ong P., Rodriguez Manas L., Sinclair A., Won C.W., Beard J., Vellas B. *Frailty: An Emerging Public Health Priority*. J Am Med Dir Assoc. 2016; 17: 188-92.

Cowen S., Hannan W.J., Ghosh S. *Nu trition index determined by a portable multifrequency bioelectrical impedance analysis machine*. GUT 1998; 42: 144-152.

David C.N., Mello R.B., Bruscato N.M., Moriguchi E.H. *Overweight and Abdominal Obesity Association with All-Cause and Cardiovascular Mortality in the Elderly Aged 80 and Over: A Cohort Study*. J Nutr Health Aging. 2017; 21: 597-603.

de Hollander E.L., Bemelmans W.J., Boshuizen H.C., Friedrich N., Wallaschofski H., Guallar-Castillón P., Walter S., Zillikens M.C., Rosengren A., Lissner L., Bassett J.K., Giles G.G., Orsini N., Heim N., Visser M., de Groot L.C. *WC elderly collaborators. The association between waist circumference and risk of mortality considering body mass index in 65- to 74-year-olds: a meta-analysis of 29 cohorts involving more than 58 000 elderly persons*. Int J Epidemiol. 2012; 41: 805-17.

De Lorenzo A., Bianchi A., Maroni P., Iannarelli A., Di Daniele N., Iacopino L., Di Renzo L. *Adiposity rather than BMI determines metabolic risk*. Int J Cardiol. 2013; 166: 111-7.

Donini L.M., Savina C., Gennaro E., De Felice M.R., Rosano A., Pandolfo M.M., Del Balzo V., Cannella C., Ritz P., Chumlea W.C. *A systematic review of the literature concerning the relationship between obesity and mortality in the elderly*. J Nutr Health Aging. 2012; 16: 89-98.

Flegal K.M., Kit B.K., Orpana H., Graubard B.I. *Association of all-cause mortality with overweight and obesity using standard body mass index categories: a systematic review and meta-analysis*. JAMA. 2013; 309: 71-82.

Foster K.R., Lukaski H.C. *Whole body impedance- what does it measure?* Am J Clin Nutr 1996; 64: 388-396.

Gallagher D., Visser M., Sepulveda D., Pierson R.N., Harris T., Heymsfield S.B. *How useful is body mass index for comparison of body fatness across age, sex, and ethnic groups?* Am J Epidemiol. 1996; 143: 228-39.

Guglielmi F.W, Mastronuzzi T., Pietrini L., Panarese A., Panella C., Francavilla A. *Electrical bioimpedance methods: applications to medicine and biotechnology*. Ann N Y Acad Sci 1999; 873: 105-111.

Gupta D., Lammersfeld C.A., Burrows J.L., Dahlk S.L., Vashi P.G., Grutsch J.F., Hoffman S., Lis C.G. *Bioelectrical impedance phase angle in clinical practice: implications for prognosis in advanced colorectal cancer*. Am J Clin Nutr 2004; 80: 1634-1638.

Hotchkiss J.W., Leyland A.H. *The relationship between body size and mortality in the linked Scottish Health Surveys: cross-sectional surveys with follow-up*. Int J Obes (Lond). 2011; 35: 838-51.

http: //www.istat.it.

Khalil S.F., Mohktar M.S., Ibrahim F. *The theory and fundamentals of bioimpedance analysis in clinical status monitoring and diagnosis of diseases*. Sensors (Basel). 2014; 14: 10895-928.

Katzmarzyk P.T., Mire E., Bray G.A., Greenway F.L., Heymsfield S.B., Bouchard C. *Anthropometric markers of obesity and mortality in white and African American adults: the Pennington center longitudinal study*. Obesity. 2013; 21: 1070-5.

Kouvari M., Chrysohoou C., Tsiamis E., Kosyfa H., Kalogirou L., Filippou A., Iosifidis S., Aggelopoulos P., Pitsavos C., Tousoulis D. *The "overweight paradox" in the prognosis of acute coronary syndrome for patients with heart failure-A truth for all?* A 10-year follow-up study. Maturitas. 2017; 102: 6-12.

Longo V.D., Antebi A., Bartke A., Barzilai N., Brown-Borg H.M., Caruso C., Curiel T.J., de Cabo R., Franceschi C., Gems D., Ingram D.K., Johnson T.E., Kennedy B.K., Kenyon C., Klein S., Kopchick J.J., Lepperdinger G., Madeo F., Mirisola M.G., Mitchell J.R., Passarino G., Rudolph K.L., Sedivy J.M., Shadel G.S., Sinclair D.A., Spindler S.R., Suh Y., Vijg J., Vinciguerra M., Fontana L. *Interventions to Slow Aging in Humans: Are We Ready?* Aging Cell. 2015; 14: 497-510.

Lopez-Jimenez F. *Speakable and unspeakable facts about BMI and mortality*. Lancet. 2009; 373: 1055-6.

Molarius A., Seidell J.C., Visscher T.L., Hofman A. *Misclassification of high-risk older subjects using waist action levels established for young and middle-aged adults-results from the Rotterdam Study*. J Am Geriatr Soc. 2000; 48: 1638-45.

Romero-Corral A., Montori V.M., Somers V.K., Korinek J., Thomas R.J., Allison T.G., Mookadam F., Lopez-Jimenez F. *Association of bodyweight with total mortality and with cardiovascular events in coronary artery disease: a systematic review of cohort studies*. Lancet. 2006; 368: 666-78.

Schwenk A., Beisenherz A., Romer K., Kremer G., Salzberger B., Elia M. *Phase angle from bioelectrical impedance analysis remains an independent predictive marker in HIV infected patients in the era of highly active antiretroviral treatment*. Am J Clin Nutr 2000; 72: 496-501.

Snijder M.B., van Dam R.M., Visser M., Seidell J.C. *What aspects of body fat are particularly hazardous and how do we measure them?* Int J Epidemiol. 2006; 35: 83-92.

Vasto S., Rizzo C., Caruso C. *Centenarians and diet: what they eat in the Western part of Sicily*. Immun Ageing. 2012a; 9: 10.

Vasto S., Scapagnini G., Rizzo C., Monastero R., Marchese A., Caruso C. *Mediterranean diet and longevity in Sicily: survey in a Sicani Mountains population*. Rejuvenation Res. 2012b; 15: 184-8.

Winter J.E., MacInnis R.J., Wattanapenpaiboon N., Nowson C.A. *BMI and all-cause mortality in older adults: a meta-analysis*. Am J Clin Nutr. 2014; 99: 875-90.

Triggering of Toll-like receptors in the elderly. A pilot study relevant for vaccination

Caterina Maria Gambino[1,2], Sonya Vasto[3], Kyriaki Ioannou[2], Giuseppina Candore[1], Calogero Caruso[1], Farzin Farzaneh[2]

[1]Department of Pathobiology and Medical Biotechnologies, University of Palermo, Italy; [2]Department of Haematological Medicine, King's College London, Rayne Institute, London, UK; [3]Department of Biological Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Italy.

Correspondence to: Caterina Maria Gambino, Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; e-mail: cmgambino@libero.it.

Abstract

The impaired ability of the elderly to mount an efficient immune response after exposure to microbes or vaccines represents a major challenge in protection against pathogens in ageing. Recently studies have shown that stimulation of Toll-like receptors (TLRs), 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. Since TLR stimulation is a key regulator of the type and magnitude of the immune response, we evaluated cytokine production in dendritic cell populations upon stimula-

tion with two complementary TLR agonists, R848 and MPLA. Our preliminary results demonstrate that TLR activation by this combination of agonists can significantly enhance the activation of dendritic cells in the peripheral blood isolated from healthy elderly donors. This data suggest that the inclusion of appropriate combination of TLR agonists may enhance the efficacy of vaccination in the elderly.

Key Words

Ageing, Cytokines, TLR, Vaccination.

Introduction

Increasing age is accompanied by a progressive decline of both the innate and acquired immune system, noted as "Immunosenescence" [Caruso & Vasto, 2016].

The impact of ageing on the immune system typically includes intrinsic defects within immune cells as well as alterations in number and activity, and possibly defects in the bone marrow and thymic stromal microenvironment. This phenomenon results in a reduction of naive T and B cells, leading to inefficient primary responses of immune effector cells to pathogens, as well as reduced T cell cytotoxicity, proliferation and cytokine production, and defective memory responses in the elderly population [Larbi et al., 2008; Caruso et al., 2009; Nikolich-Zugich & Rudd, 2010]. Consequently, aged individuals exhibit increased incidence of infectious diseases, cancer and autoimmune diseases [McElhaney et al., 2012]. In addition, their aged immune system does not respond to stimuli as efficiently as that of younger adults, therefore current vaccines are less effective in the elderly [Derhovanessian & Pawelec, 2012].

Research in immunological ageing 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 the elderly. One such strategy would be to develop vaccines comprising suitable adjuvants to enhance the impaired cellular immune responses [Wells et al., 2008].

Adjuvants are molecules that stimulate the non-specific, innate immune responses, inducing the activation of antigen presenting cells (APCs) and their recruitment to the site of vaccination. Dendritic cells (DCs) are the most potent APCs, specialized in the uptake, processing, transport and presentation of antigens to T cells [Collin et al., 2013]. 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.

DCs in human blood are defined as Lineage 1 (CD3, CD14, CD16, CD19, CD20, and CD56)-negative and HLA-DR-positive cells. DCs can be divided into three subsets according to the expression of various markers (CD123, CD1c, CD141): one subset of plasmacytoid DCs (pDCs), and two subsets of myeloid DCs (mDCs). pDCs are characterized by the expression of CD123 marker and possess the capacity to produce high levels of type I

Interferons (IFN-α/β). In contrast, mDCs express the CD11c marker and are divided into two subsets: CD1c+ mDCs and CD141+ mDCs [Collin et al., 2013]. Upon stimulation, mDCs secrete mainly IL-6, IL-12, and TNF-α.

Both pDCs and mDCs express toll-like receptors (TLRs) that recognize conserved molecular patterns on microbes and are key regulators of antimicrobial host defence responses. Recognition of microbial components 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 lead to increased severity of multiple immunological disorders, including sepsis, immunodeficiencies, atherosclerosis and asthma [Cook et al., 2004].

Recently, stimulation of TLRs by adjuvants has been shown to be a promising strategy to enhance vaccine efficacy against both foreign and self, tumour-associated, antigens in aged mice by activating innate immune cells and enhancing production of inflammatory cytokines [Tye et al., 2015].

On the basis of these promising results in mice, we have investigated the ability of combined TLR ligands to induce pro-inflammatory responses in the peripheral blood dendritic cells isolated from healthy donors with evidence of immunosenescence.

Material and Methods

Samples

A total of 23 samples, including five centenarians, five centenarian offspring (CO), six old donors and six young donors used as controls, were processed. All participants were in good health according to their clinical history and none of them had infectious, inflammatory, neoplastic or autoimmune diseases at the time of the study. The University Hospital Ethics Committee approved the study, and written informed consent was obtained from all participants according to Italian law. Whole blood was collected by venepuncture in vacutainer tubes containing ethylenediaminetetraacetic acid. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Lympholyte® (Cedarlane, Canada, United States) and viably cryopreserved according to standard protocols.

DCs stimulation assay

Total PBMCs were cultured at $1x10^6$ cells/well on a 96 U-bottom plate. Cells were plated with a combination of two adjuvants, chosen

based on our preliminary experiments, in the presence of 1x Golgi Plug solution (BD Biosciences, Erembodegem, Belgium). Specifically, we tested the following TLR agonists: the TLR7/8 agonist R848, at 3 μg/ml, and the TLR4 agonist MPLA, at 10 μg/ml (InvivoGen, San Diego, California). For each donor, cells were also left unstimulated and served as controls. After 6 hours of incubation cells were transferred to labelled FACS tubes and stained with antibodies. We chose to study total PBMCs, rather than purified DCs, to minimize manipulations that might result in partial activation of DCs, and because purification procedures would substantially decrease the yield of cells for analysis.

Flow cytometry and cell sorting

Cells were stained with antibodies to human CD3, CD14, CD16, CD19, CD20, CD56 (Lineage 1, BD Biosciences, Erembodegem, Belgium), HLA-DR (clone L243, Biolegend, San Diego, California), CD123 (clone 6H6, Biolegend), CD1c (clone L161, Biolegend), CD11c (clone 3.9,Biolegend), and CD141 (clone 1A4, BD Biosciences) for extracellular staining. For intracellular staining, cells were then washed, fixed with permeabilization/fixation buffer (BD Biosciences) and stained with antibodies to human IL-6 (clone MQ2-13A5, Biolegend), TNF-α (clone Mab11, Biolegend), and the p40 subunit of IL-12/23 (clone C11.5, Biolegend). Samples were analysed by flow cytometry on a LSR Fortessa™ (BD Biosciences). FACS data were analysed and plotted using FlowJo software (Tree Star).

Statistics

Statistical analyses were performed using GraphPad Prism software; the significant level of p value was determined using Student t test. (* $p<0.05$, ** $p\leq0.01$, *** $p\leq0.001$)

Results and Discussion

Given that DC activation is a key regulator of the magnitude and nature of the elicited adaptive immune responses, we evaluated whether TLR ligands could effectively activate naturally occurring, circulating DCs. To this end, we stimulated total PBMCs, derived from young, aged, centenarian and CO participants, with a combination of two TLR ligands. The nature and magnitude of cytokine production was assessed using multi-parameter flow cytometry.

Our preliminary *in vitro* screening experiments suggest that from the various TLR agonists tested, the condition that most effectively activated human leukocytes was the combination of TLR7/TLR8 with TLR4. This TLR agonist combination induces significantly greater cytokine production than that induced by each of the individual agonist. This greater stimulation is probably due to the combined activation of both Myd88 and TRIF-dependent signal transduction pathways.

Figure 1 shows an example of our analysis; after excluding doublets, we gated the Lineage (consisting of a cocktail of antibodies against CD3, CD14, CD16, CD19, and CD56)-negative, HLA-DR-positive population, with mDCs and pDCs identified as CD11c and CD123 positive cells, respectively. Subsequently, using intracellular cytokine staining, we evaluated the selected populations for the production of TNF-α, IL-6 and IL-12p40.

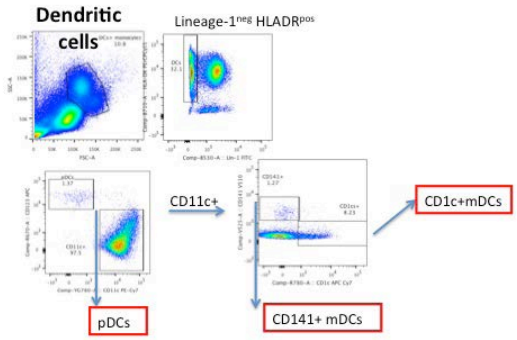

Figure 1. Characterization of human dendritic cells and their subsets. Phenotype of human DCs analysed by flow cytometry. Cells were gated on Lineage-1 negative HLA-DR-positive population by a cocktail of antibodies against CD3, CD14, CD16, CD19, and CD56. Subsequently, mDCs and pDCs were identified using staining against CD11, CD123, CD1c and CD141.

First, we report that the stimulation of cells with the combination of agonists for TLR7/8 and TLR4 is *de facto* an excellent inducer of TNF-α and IL-12/p40 cytokines in the CD141+ mDCs from young, old and CO subjects, with significantly high levels of cytokines compared to unstimulated samples ($p<0.05$, $p\leq0.01$, and $p\leq0.001$; respectively). No significant variation was observed in centenarians (Figure 2A). This, probably, is due to the age-related lower viability and low number of centenarian samples. Nevertheless, we have reported that the difficulties to study centenarians can be overcome by studying CO that are one generation (about 20-30 years) younger than centenarians and are representative of an elderly cohort, characterised by a better functional status and a reduced risk for several age-related pathologies [Balistreri et al., 2014]. Focusing on old people, we observed that both Italian CO and elderly, showed elevated percentage of IL-12/p40 and TNF-α after treatment with the TLR ligands R848 (TLR7/8) and MPLA (TLR4); these differences were highly statistically significant compared to unstimulated cells.

Notably, the combination of R848 and MPLA induce 5–10 fold higher production of IL-12/p40 in CD141+ mDCs isolated from old and CO samples compared with their young counterparts ($p\leq0.01$) (Figure 2A).

In addition, increased amounts of TNF-α, were also observed in CD1c+ mDCs and pDCs from older and CO subjects, in response to R848 and MPLA stimulation. These differences were statistically significant when compared to their unstimulated counterparts (Figure 2B).

Taken together, the data presented suggest that the combination of R848 and MPLA effectively promotes *in vitro* cytokine production in human DCs isolated from elderly, despite their immunosenescent phenotype.

To date, data regarding the influence of ageing on human DCs activity and cytokine production, in response to *in vitro* stimulation, has been inconsistent, showing either comparable or reduced DC function in the elderly [Lung et al., 2000; Pietschmann et al., 2000; Shurin et al., 2007]. Tan et al., [2012] 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., [2013]demonstrated a reduced production of TNF-α by DCs from old people in response to LPS stimulation.

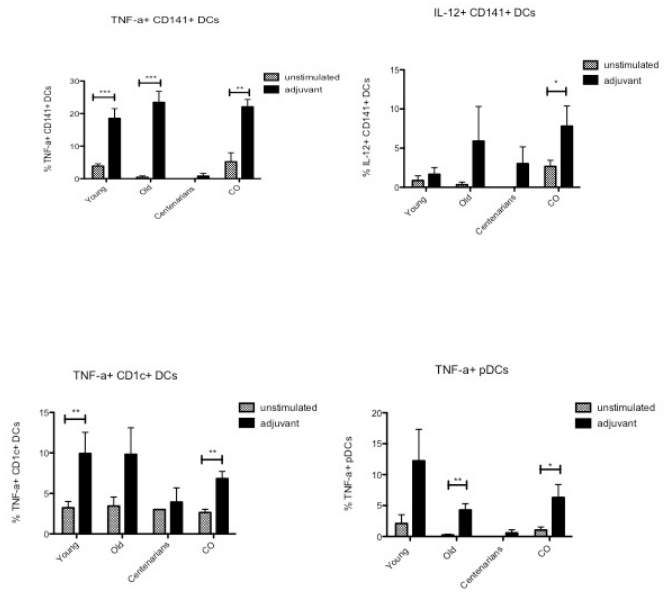

Figure 2. Cytokine secretion by DCs. (A) TNF-α and IL-12 secretion from CD141+ mDCs in response to TLR7/8 and TLR4 stimulation in vitro. PBMCs were cultured in the absence or presence of combined TLR7/8 and TLR4 ligands (unstimulated and adjuvants, respectively). After incubation, CD141+ mDCs were identified by flow cytometry analysis. TNF-α and IL-12 were evaluated using intracellular staining and analysed by FlowJo. The data report the percentage of cytokines produced from young, elderly, CO and centenarian samples after incubation. Statistical significance between the groups has been reported as *p, 0.05, **p, 0.01, ***p, 0.001. (B) TNF-α secretion from pDCs and CD1c+ mDCs in response to TLR7/8 and TLR4 stimulation in vitro. PBMCs were cultured in the absence or presence of combined TLR7/8 and TLR4 ligands (unstimulated and adjuvants, respectively). After incubation, pDCs and CD1c+ mDCs were identified by flow cytometry analysis. The data report the percentage of TNF-α produced from each sample group after incubation. Statistical significance between the groups has been reported as *p, 0.05, **p, 0.01, ***p, 0.001.

In our study, stimulation with the specific combination of TLR agonists, R848 and MPLA, induced significantly higher cytokine secretion by mDCs and pDCs from both elderly and CO subjects. This has potentially important implications, since it has been reported that reduced production of TNF-α by pDCs from old people, caused by defects in TLR signalling pathways, is associated with an ineffective antibody response to influenza vaccination [Panda et al., 2010].

The involvement of TNF-α in DC-induced T cell proliferation is also evident from clinical data of rheumatoid arthritis patients, showing that treatment with anti-TNF-α antibodies cause poor stimulation of T cell activity by DCs [Baldwin et al., 2010; Liu et al., 2012]. Thus, impaired production of TNF-α by older DCs could result in a weak response to vaccination and may contribute to the dysregulation of DC-induced T cell proliferation in the elderly subjects.

Conclusion

Our findings highlight the efficient effect of adjuvant in stimulation of cytokine production, and point towards the potential use of appropriately selected combination of TLR agonists in future vaccination approaches for the elderly.

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References

Balistreri C.R., Candore G., Accardi G., Buffa S., Bulati M., Martorana A., Colonna-Romano G., Lio D., Caruso C. *Centenarian offspring: a model for understanding longevity*. Curr Vasc Pharmacol. 2014; 12: 718-25.

Baldwin H.M., Ito-Ihara T., Isaacs J.D., Hilkens C.M. *Tumour necrosis factor alpha blockade impairs dendritic cell survival and function in rheumatoid arthritis*. Ann Rheum Dis. 2010; 696: 1200-7.

Caruso C., Buffa S., Candore G., Colonna-Romano G., Dunn-Walters D., Kipling D., Pawelec G. *Mechanisms of immunosenescence.* Immun Ageing. 2009; 6: 10.

Caruso C., Vasto S. *Immunity and Aging. Encyclopedia of Immunobiology*. Oxford: Academic Press. 2016; 5: 127–132.

Collin M., Mc Govern N., Haniffa M. *Human dendritic cell subsets.* Immunology. 2013; 140: 22-30.

Cook D.N., Pisetsky D.S., Schwartz D.A. *Toll-like receptors in the pathogenesis of human disease.* Nat Immunol. 2004; 5: 975–979

Derhovanessian E., Pawelec G. *Vaccination in the elderly*. Microb Biotechnol. 2012; 5: 226-32.

Larbi A., Franceschi C., Mazzatti D., Solana R., Wikby A., Pawelec G. *Aging of the immune system as a prognostic factor for human longevity*. Physiology (Bethesda). 2008; 23: 64-74.

Liu W.M., Nahar T.E., Jacobi R.H., Gijzen K., van Beek.J, Hak E., Jonges M., Boog C.J., van der Zeijst B.A., Soethout E.C. *Impaired production of TNF-α by dendritic cells of older adults leads to a lower CD8+ T cell response against influenza*. Vaccine. 2012; 30: 1659-66.

Lung T.L., Saurwein-Teissl M., Parson W., Schönitzer D., Grubeck-Loebenstein B. *Unimpaired dendritic cells can be derived from monocytes in old age and can mobilize residual function in senescent T cells*. Vaccine. 2000; 25; 18: 1606-12.

Mc Elhaney J.E., Zhou X., Talbot H.K., Soethout E., Bleackley R.C., Granville D.J., Pawelec G. *The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines*. Vaccine. 2012; 30: 2060-7.

Nikolich-Zugich J., Rudd B.D. *Immune memory and aging: an infinite or finite resource*? Curr Opin Immunol 2010; 22: 535.

Panda A., Qian F., Mohanty S., van Duin D., Newman F.K., Zhang L., Chen S., Towle V., Belshe R.B., Fikrig E., Allore H.G., Montgomery R.R., Shaw A.C. *Age-associated decrease in TLR function in primary human dendritic cells predicts influenza vaccine response*. J Immunol. 2010; 184: 2518-27.

Pietschmann P., Hahn P., Kudlacek S., Thomas R., Peterlik M. *Surface markers and transendothelial migration of dendritic cells from elderly subjects*. Exp Gerontol. 2000; 352: 213-24.

Shurin M.R., Shurin G.V., Chatta G.S. *Aging and the dendritic cell system: implications for cancer*. Crit Rev Oncol Hematol. 2007; 642: 90-105.

Tan S.Y., Cavanagh L.L., d'Advigor W., Shackel N., Fazekas de St Groth B., Weninger W. *Phenotype and functions of conventional dendritic cells are not compromised in aged mice*. Immunol Cell Biol. 2012; 90: 722-32.

Tye G.J., Ioannou K., Amofah E., Quartey-Papafio R., Westrop S.J., Krishnamurthy P., Noble A., Harrison P.M., Gaensler K.M., Barber L.D., Farzaneh F. *The combined molecular adjuvant CASAC enhances the CD8+ T cell response to a tumor-associated self-antigen in aged, immunosenescent mice*. Immun Ageing. 2015; 12: 6.

You J., Dong H., Mann E.R., Knight S.C., Yaqoob P. *Ageing impairs the T cell response to dendritic cells*. Immunobiology. 2013; 218: 1077-84.

Wells J.W., Cowled C.J., Farzaneh F., Noble A. *Combined triggering of dendritic cell receptors results in synergistic activation and potent cytotoxic immunity.* J Immunol. 2008; 181: 3422–31.

The importance of the interactions between KIRs and HLA ligands in the development of human autoimmune and viral diseases

Anna Aiello[1], Giulia Accardi[1], Giuseppina Candore[1], Caterina Maria Gambino[1], Calogero Caruso[1], Danilo Di Bona[2]

[1]Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; [2]Department of Emergency and Organ Trasplants, University of Bari Aldo Moro, Piazza G. Cesare 11, 70124, Bari, Italy.

Correspondence to: Anna Aiello, Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; e-mail: anna.aiello@unipa.it.

Abstract

Killer immunoglobulin-like receptors (KIRs) regulate the activation of natural killer cells through their interaction with human leucocyte antigens (HLA). KIR and HLA loci are highly polymorphic, and certain KIR/HLA combinations have been found to protect against viral infections or to predispose to autoimmune disorders. In particular, some activating KIR profiles may be detrimental in autoimmune pathogenesis, and specific KIR genes may be particularly aggressive in the clearance of different microorganisms, protecting individuals in the control of a given pathogen. Here we reviewed a growing body of evidence purporting the influence of KIR polymorphism and KIR-HLA interaction in the development of the main human autoimmune and viral diseases.

Key words

Autoimmune diseases, HLA ligand, KIRs, Viral infections.

State of the art

Killer immunoglobulin-like receptors (KIRs) are surface receptors specific for allelic forms of human leukocyte antigen (HLA) class I molecules, which are expressed by natural killer (NK) cells and a subset of CD8 T lymphocytes. The polygenic nature of the KIR locus is particularly consequential in a functional sense because KIR genes encode receptors that can either inhibit or activate both cell types [Campbell & Purdy, 2011].

The KIR gene cluster consists of a segment of about 150 kb situated on chromosome 19q13.4 within the leukocyte receptor complex. To date, 15 KIR genes and 2 pseudogenes have been described. However, variability in gene content at the KIR locus appears largely due to gene duplication and non-allelic homologous recombination [Martin et al., 2003].

KIR genes are organized in two basic haplotypes that have been defined on the basis of gene content, and are termed A and B. Haplotype A is uniform in terms of gene content and is composed of five inhibitory genes (KIR2DL1, 2DL3, 3DL1, 3DL2 and 3DL3), one activating gene (KIR2DS4), and KIR2DL4, which may have both inhibitory and activating capacity. Interestingly, many A haplotypes possess null variants of both KIR2DS4 and KIR2DL4 that are not expressed on the cell surface [Hsu et al., 2002; Witt et al., 2000]. Accordingly with this, these haplotypes do not have functional activating KIR.

The B haplotypes contain variable numbers of activating and inhibitory receptors and are the primary contributors to the extraordinary differences in gene profiles observed in distinct ethnic populations. Although at different frequency, A and B haplotypes have been maintained within the human population, suggesting the occurrence of a balancing selection. A haplotypes seem to be associated with improved responses to pathogens, whereas B haplotypes with improved reproductive fitness [Moffett & Loke, 2006; Parham, 2005; Rajagopalan & Long, 2005].

Much of the variability among KIR haplotypes derives from the presence or the absence of activating KIR, because most of the inhibitory KIRs are present on all or nearly all haplotypes [Uhrberg et al., 1997]. The inhibitory KIR family is characterized by cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs), which recruit the SHP-1/2 tyrosine phosphatases preventing NK cells activation. On the contrary, the activating KIRs have short cytoplasmic tails lacking ITIMs (i.e., KIR2DS and

KIR3DS), which interact with DAP12; this is an adaptor molecule that contains immunoreceptor tyrosine-based activation motifs (ITAMs) linked to protein tyrosine kinase activation pathways [Parham & Moffett, 2013]. The activating KIRs stimulate NK/CD8 T lymphocytes cytokine secretion and target cell cytolysis may be generally beneficial in response to microorganisms and tumor cells. Not all inhibitory or activating receptors are present on the surface of NK or CD8 T cells and it is the balance of these signals that modulates NK cells cytotoxicity and cytokine release.

Most inhibitory KIRs specifically recognize sets of HLA class I (i.e., HLA-A, -B, and -C) allotypes; yet, the ligands for some of them (e.g., KIR2DL5) and for most activating KIRs are unknown.

HLA class I genes map to chromosome 6. These genes are extremely polymorphic, determining functional diversity, and generating variable susceptibility in response to pathogens and other diseases.

The inhibitory KIR2DL1, 2DL2, and 2DL3 recognize HLA-C ligands, that belong to one of two ligand groups based with a dimorphism at position 80: group 1 (HLA-C1), which has asparagine, and group 2 (HLA-C2), which has lysine at position 80 [Winter & Long, 1997]. KIR3DL1 is known to bind HLA-B allotypes with the Bw4 motif, although some low affinity binding with Bw6 has also been reported [Carr et al., 2005; Cella et al., 1994; Gumperz et al., 1995]. Other receptor–ligand relationships among KIRs and HLA include KIR2DL4 specificity for HLA-G, which is primarily expressed on foetal trophoblasts, thymic endothelial cells and cornea, and KIR3DL2 specificity for HLA-A3 and A11 [Dohring et al., 1996; Rajagopalan & Long, 1999].

The activating receptors KIR2DS1, 2DS2 and 3DS1 share sequence similarity in their extracellular domains with their corresponding inhibitory counterparts (KIR2DL1, 2DL2-2DL3 and 3DL1, respectively) and are thought to share HLA ligand binding specificities as well [Kulkarni et al., 2008].

Combinations of HLA and KIR genes have been associated with several diseases such as infectious diseases, autoimmune/inflammatory disorders, cancer and reproduction. Emerging functional data supports a mechanism based on a continuum of inhibition to activation through various compound KIR/HLA genotypes in diseases [Kulkarni et al., 2008]. Moreover, allelic variation also plays a role in determining the strength of the interaction [Yawata et al., 2006]. The diversity of KIR haplotypes, which likely imparts a continuum from relatively strong inhibition to

strong activation, suggests the pleiotropic nature of KIR on different diseases in that a given KIR genotype affording protection against one disease may actually predispose to another unrelated disorder.

In this regard, activating KIR profiles might be detrimental in autoimmune pathogenesis, potentially aggravating the disease process, although, this may be true for only certain autoimmune diseases and quite the opposite for others [Baxter & Smyth, 2002; Flodstromet et al., 2002a]. KIR associations with susceptibility to autoimmune conditions point to the short chain of the activating KIR. Moreover, the NK cell control of viral infections has been the subject of excellent reviews [Brandstadter & Yang, 2011]. Specific KIR genes may be particularly aggressive in the clearance of some microorganisms, and their presence in only a fraction of individuals (as well as their allelic polymorphism) could explain differences observed among individuals in their ability to control a given pathogen.

KIR/HLA association and autoimmune diseases

Currently about 5% of the population of the developed countries is affected by various types of autoimmune diseases [Flodstrom et al., 2002b]. The background of autoimmune diseases is multifactorial and remains unclear. However, the robust associations between the highly polymorphic HLA class I/class II loci and autoimmune diseases are strong [Matzaraki et al., 2017]. It would not be surprising if the strongest effects of KIR variation were also observed in autoimmune diseases. Some previously determined HLA associations with autoimmune diseases might actually be explained by synergistic interactions between KIR and alleles encoding their HLA class I ligands. On the other hand, NK cell activation may be protective against some autoimmune disorders by suppressing or eliminating dendritic cells and monocytes [Geldhof et al., 1998], cells known to stimulate the generation of cytotoxic T lymphocytes (see Table 1 for an overview).

A number of studies have investigated KIR expression in rheumatoid arthritis (RA). RA was the first disease in which an effect of KIR genotype was observed. In RA patients, where $CD4^{+}$ $CD28^{null}$ T cells are expanded and cause endothelial damage, it was showed that these cells expressed KIR2DS2 in the absence of inhibitory KIR2DL2 [Namekawa et al., 2000]. Further, the frequency of KIR2DS2 was increased in RA patients with vasculitis in comparison to normal controls and RA pa-

tients without vasculitis [Yen et al., 2001]. HLA-Cw*03, an HLA-C1 allotype, and therefore a putative ligand for KIR2DS2, was also increased in subjects with vasculitis, although this was not true for other C1 alleles [Yen et al., 2001]. Thus, it is possible that KIR2DS2 recognizes a specific HLA-Cw*03-peptide complex generated during RA vasculitis.

Activating B haplotypes of KIR [Suzuki et al., 2004] and KIR2DS1 alone [Luszczek et al., 2004] or in combination with HLA-Cw6 (a C2 ligand for KIR2DS1) have been reported to associate with psoriasis [Holm et al., 2005]. Based on the data, a model was proposed in which a gradient of more activating to more inhibitory compound genotypes of KIR2DS and HLA-C appear to influence susceptibility to psoriatic arthritis. So, genotypes conferring highest activation (KIR2DS1 and/or KIR2DS2 with either HLA-C1 or C2 homozygosity) are associated with greatest susceptibility, whereas the genotypes conferring maximum inhibition (absence of activating receptors KIR2DS1 and KIR2DS2 and presence of both the inhibitory ligands, such as HLA-C1 and C2) were protective.

Moreover, the association between KIR gene polymorphisms and systemic lupus erythematosus (SLE) risk has been investigated by many case-control studies, but findings are not always consistent. SLE is a multifactorial and highly polymorphic systemic autoimmune disease that predominantly afflicts women in child-bearing age. It is a complex interaction result of genetic, environmental., and hormonal factors, companying a global loss of immune tolerance [Maselli et al., 2016]. In 2007, Pellett et al., first reported that the frequency of KIR2DS1 was significantly increased in SLE patients compared with controls [Pellett et al., 2007]. In addition, a recent meta-analysis shows that KIR2DL1 might be a potential risk factor for SLE in Caucasians and KIR2DL3, KIR2DL5 might be protective factors for SLE in Asians [Liang et al., 2017], indicating that the association between KIR polymorphisms and the risk of SLE may be different in different ethnic populations. In addition, activating KIR profiles, like KIR2DS2/KIR2DS5/KIR2DS1 were significantly higher in SLE patients as compared to healthy persons; however, it was seen that various ethnic and environmental factors might influence susceptibility to disease, which is consistent with previous studies [Pedroza et al., 2016].

An increasing stream of studies has been designed with systemic sclerosis (SSc) and ankylosing spondylitis (AS) patients to investigate the re-

lation between autoimmunity and KIR. SSc is a chronic disease of the connective tissue characterized by the fibrosis of the skin associated with the structural and functional damages of various organs such as the gastrointestinal system, lungs, heart, kidneys. It is a global disease and affects all races, and women are more susceptible than men [Romano et al., 2011]. It was seen that KIR2DS2 was more frequently highlighted in these studies. In fact, it was reported as a risk factor in the absence of KIR2DL2 in SSc patients. Moreover, the KIR2DS3 gene was more frequent in SSc patients than in controls, instead the KIR2DL3 gene was detected more frequently in controls while KIR2DS3 gene was more frequent in the patient group when SLE and SSc were combined [Tozkir et al., 2016].

In AS strong epidemiological evidence of significant genetic associations with HLA has been convincingly identified. AS is a chronic inflammatory disease which primarily affects the sacroiliac joint and is characterized by strong genetic association with HLA-B27 [Mathieu et al., 2008]. HLA-B27 interactions with KIR have been implicated in the pathogenesis of AS, with consistent differences among populations. KIR3DL1, for example, and possibly KIR3DS1, interact with classical B27, whereas KIR3DL2 binds B27 heavy chain dimers [Cauli et al., 2014]. Moreover, it was suggested that reduced HLA-Bw4 genotype with and without its inhibitory receptor KIR3DL1, may influence the inhibitory effect of NK cytotoxicity leading to continued injury in AS.

Further work in this area will help to establish the role of KIR/HLA association in autoimmune disease development.

Table 1. KIR-HLA associations in autoimmune diseases.

Disease	KIR–HLA ligand pair	Effect
Rheumatoid arthritis	KIR2DS2/HLA-Cw*03	Susceptibility
Psoriasis	KIR2DS1/HLA-Cw*06 KIR2DS1; KIR2DL5; KIR haplotype B	Susceptibility Susceptibility
Systemic lupus erythematosus	KIR2DS2; KIR2DS5; KIR2DS1	Susceptibility
Systemic sclerosis	KIR2DS2 in the absence of KIR2DL2 KIR2DS3	Susceptibility Susceptibility
Ankylosing spondylitis	KIR3DL1/ HLA-B27	Susceptibility

KIR/HLA association and viral diseases

Several epidemiological studies have associated KIR/HLA genotypes with susceptibility to some infectious diseases such as human immunodeficiency virus (HIV), human cytomegalovirus (CMV), and hepatitis C virus (HCV) [Cook et al., 2006; Di Bona et al., 2014; Khakoo et al., 2004; Hadaya et al., 2008; Martin et al., 2002; Martin et al., 2007; Stern et al., 2008]. So far, there are limited data on the relationship between KIR genes and their HLA ligands and the outcome of hepatitis B virus (HBV) infection (see Table 2 for an overview).

HIV prevalence is increasing worldwide because people on antiretroviral therapy are living longer, although new infections decreased from 3.3 million in 2002, to 2.3 million in 2012. New insights into the mechanisms of latent infection and the importance of reservoirs of infection might eventually lead to a cure. The role of immune activation in the pathogenesis of non-AIDS clinical events (major causes of morbidity and mortality in people on antiretroviral therapy) is receiving increased recognition [Sharp & Hahn, 2011]. In individuals infected with HIV, the combination of KIR3DS1 with its putative ligand HLA-Bw4-80I was associated with slower progression to AIDS, lower mean viral load, and protection against opportunistic infections [Martin et al., 2007; Qi et al., 2006]. Moreover, a study of 25 HIV exposed uninfected intravenous drug users from Vietnam found transcription of KIR3DS1 to be significantly higher than KIR3DL1 in KIR3DS1/3DL1 heterozygous individuals and there was expansion of NK cells expressing KIR2DL3 in HLA-C1/C1 individuals who were KIR2DS2^{-}/2DL2^{-} [Ravet et al., 2007]. KIR3DS1 homozygosity was also found to be significantly increased in HIV exposed seronegative intravenous drug users and HIV negative partners of sero-discordant couples [Boulet et al., 2008]. These individuals also had an increase in KIR AB haplotypes, which are characterized by increased numbers of activating KIR.

Also the variability in the association of host innate immune response to HCV infection requires the possible role of host KIR and HLA genotypes in HCV-related disorders. The World Health Organization estimates that about 3% of the world population is infected with HCV, and 3 to 4 million individuals are newly infected each year. Although new antiviral treatments are very promising, today

only a minority of patients successfully clear up HCV infections, and the remaining patients (60–85%) develop chronic infection [Afdhal et al., 2014]. NK cells have also been reported to play a role in HCV clearance [Cheent & Khakoo, 2011]. In particular, NK cells were demonstrated to mediate the inhibition of HCV-replication and to exert a targeted cytotoxic action against targeted cells given that NK cells isolated from healthy donors kill HCV-replicating cells and secrete IFN-γ [Larkin et al., 2006; Stegmann et al., 2010].

HLA-C1/KIR2DL3 in homozygosis has been associated with HCV clearance in several studies but the occurrence of this association was not always observed. At the same time, HLA-C1/KIR2DL3 has also been associated with sustained virus response to anti-HCV therapy.

Moreover, a protective role for HLA-Bw4/KIR3DS1 against liver disease progression has been proposed [De Re et al., 2015]. Previous data have also suggested that KIR2DL1/HLA-C2 may confer stronger inhibitory responses than does KIR2DL3/HLA-C1 [Ahlenstiel et al., 2008].

CMV is a member of the herpes virus family (type 5) that is ubiquitous in human populations, reaching a prevalence of 100% in Africa and Asia, and approximately 80% in Europe and the United States, depending on socioeconomic status. Clinically, primary CMV infection is assumed to be asymptomatic in the immunocompetent host, but a minority of subjects (<10%) exhibit symptoms of the infection, such as malaise, fever, sweating, and abnormal liver function [Cannon et al., 2010]. There is increasingly compelling evidence that NK cells play a crucial role in host defence against CMV infection. In order to evade the immune system, CMV encode several proteins that interfere with MHC class I expression, potentially rendering infected cells more susceptible to attack by NK cells [Lin et al., 2007]. In a case study of a child with a novel immunodeficiency syndrome and recurrent CMV infection, the entire population of NK cells from this patient expressed KIR2DL1 and the child also possessed the KIR2DL1 ligand, HLA-C2, raising the possibility that the strongly inhibitory KIR2DL1/HLA-C2 combination crippled NK cell activity and prevented the cells from mounting a protective response against CMV [Gazit et al., 2004]. Recent reports have also documented a role for activating KIRs in the control of CMV infection after hematopoietic stem cell or kidney transplantation, showing that the CMV reactivation rate in patients homozygous for the KIR A haplotype (virtually without activating KIRs) is higher than in patients with the B

haplotype (with a variable number of activating KIRs), suggesting the importance of activating KIRs in the immune surveillance against CMV [Cook et al., 2006; Hadaya et al., 2008; Stern et al., 2008]. Moreover, it was showed that immunocompetent subjects carrying the homozygous A haplotype or the HLA-Bw4^{T} allele are at higher risk of developing symptomatic disease after primary CMV infection. The frequency of the homozygous A haplotype (only KIR2DS4 as activating KIR) was higher in symptomatic patients than controls. By logistic regression, the risk of developing symptomatic disease was associated with the homozygous A haplotype and the HLABw4^{T} allele [Di Bona et al., 2014].

HBV is a hepatotrophic virus that causes a major global health problem. An estimated 2 billion individuals have been infected with HBV and approximately 350 million have the chronic disease. NK cells are activated in the early response to infection, and there is substantial population variability in the rates of HBV infection [Custer B et al., 2004]. Although detailed genetic and functional analyses exploring KIR influences on HBV in large cohorts are lacking, accumulating evidence supports that NK cell activation contributes to inflammation and liver injury during HBV infection both in HBV transgenic mice and in HBV infected patients [Chen et al., 2007; Dunn et al., 2007; Kakimi et al., 2001]. However, in a Turkish cohort, the rate of inhibitory KIR2DL3 and 3DS1 were higher in the healthy group than in the group composed of chronic HBV patients and patients with spontaneous remission. There were no statistically significant differences between the rate of AA and Bx genotypes of chronic HBV patients and patients with spontaneous remission and the control group. Moreover, a case-control study showed that more copies of HLA-C1 alleles, which resulted in inherently more potent NK cells, were associated with disease progression towards hepatocellular carcinoma (HCC) (one copy associated with cirrhosis; two copies associated with HCC) in HBV-infected patients, suggesting that NK cell activation may play a role in HCC development [Pan et al., 2011]. Finally, in a recent study, the authors compared the frequencies of KIR and HLA gene families in subjects with chronic hepatitis B (CHB) and subjects with resolved infection [Di Bona et al., 2017]. The inhibitory KIR2DL3 gene was less frequent in CHB (81%) than in subjects with resolved infection (98%). The only other KIR gene expressed differently between CHB and subject with resolved infection was the KIR2DS4-Del, which codes

for an inactive receptor. No difference was reported in the frequency of KIR haplotypes between the groups, suggesting that activating receptors likely do not play a role in the control of the infection. These results suggest that a combination of KIR/HLA gene/alleles is able to predict the outcome of HBV infection.

These data suggest that specific KIR and HLA gene segregations were likely the result of a pathogen selective pressure.

Table 2. KIR-HLA associations in viral infections.

Disease	KIR–HLA ligand pair	Effect
HIV	KIR3DS1/HLA-Bw4-80I KIR3DL1*004/HLA-Bw4 KIR3DS1	Slower progression Slower progression Reduced risk of infection
CMV	KIR2DL1 expression on all NK cells >1 activating KIR in donor in bone marrow transplantation KIR A haplotype/HLA-Bw4^{T}	Recurrent CMV infection Protection from CMV reactivation in the recipient Higher risk of developing symptomatic disease
HCV	KIR2DL3/HLA-C1 homozygosity	Resolution of infection
HBV	KIR2DL3; KIR3DS1 KIR2DL3/HLA-A-Bw4 and HLA-C2	Lower in chronic HBV patients Development of chronic hepatitis B

Discussion

Combinations of HLA class I and KIR variants have been associated with pathologies as autoimmunity, viral infections, pregnancy-related disorders and cancer [Parham, 2005; Khakoo & Carrington, 2006]. Thus, interactions between KIR and HLA class I polymorphisms have probably been involved in human during incidences of epidemic infections and have affected reproduction and population expansion. These types of selection pressures might explain the functional coevolution of KIR with diverging HLA class I molecules and why KIR sequences, like the HLA loci, are highly polymorphic and rapidly evolving [Guethlein et al., 2007; Martin et al., 2007].

Multiple factors complicate the interpretation of KIR-HLA disease association, including the extensive polymorphism of the KIR and HLA class I ligands, the incomplete knowledge of KIR ligands, the oversimplification of the structural complexities of their interactions, and limited understanding of KIR gene expression control [Traherne, 2008].

In general., KIR-HLA combinations with a tendency towards stronger NK cell activation or lower levels of inhibition are associated with increased risk of autoimmune diseases but tend to be protective against infectious diseases.

The reviewed data are consistent with the idea that disease susceptibility is modified by specific KIR-HLA ligand interactions. For this reason several studies have examined KIR and HLA class I combinations in disease association studies. Such studies will need to be large, with well-controlled populations, in order to be adequately powered.

Conclusion

In NK cell education, KIR/HLA interactions are required to establish self-tolerance and to shape the KIR repertoire of fully functional NK cells. It is clear that the association of activating KIR genotypes increases risk of autoimmune diseases and decreased risk of some infectious disease outcomes. Further efforts and incremental experiments are necessary to define the role of KIR-HLA interaction in human disease, and in turn, to potentially apply this knowledge clinically, and to define a common threads of KIR involvement across diseases that share some etiological characteristics.

References

Afdhal N., Zeuzem S., Kwo P., Chojkier M., Gitlin N., Puoti M., Romero-Gomez M., Zarski J.P., Agarwal K., Buggisch P., Foster G.R., Bräu N., Buti M., Jacobson I.M., Subramanian G.M., Ding X., Mo H., Yang J.C., Pang P.S., Symonds W.T., Mc Hutchison J.G., Muir A.J., Mangia A., Marcellin P. *ION-1 Investigators. Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection*. N Engl J Med. 2014; 370: 1889-98.

Ahlenstiel G., Martin M.P., Gao X., Carrington M., Rehermann B. *Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses*. J Clin Invest. 2008; 118: 1017–26.

Baxter A.G., Smyth M.J. *The role of NK cells in autoimmune disease*. Autoimmunity. 2002; 35: 1-14.

Biron C.A., Nguyen K.B., Pien G.C., Cousens L.P., Salazar-Mather T.P. *Natural killer cells in antiviral defense: function and regulation by innate cytokines*. Annu Rev Immunol. 1999; 17: 189-220.

Boulet S., Sharafi S., Simic N., Bruneau J., Routy J.P., Tsoukas C.M., Bernard N. *Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals*. Aids 2008; 22: 595–9.

Brandstadter J.D., Yang Y. *Natural killer cell responses to viral infection*. J Innate Immun. 2011; 3: 274-9.

Campbell K.S., Purdy A.K. *Structure/function of human killer cell immunoglobulin-like receptors: lessons from polymorphisms, evolution, crystal structures and mutations*. Immunology. 2011; 132: 315-25.

Cannon M.J., Schmid D.S., Hyde T.B. *Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection*. Rev Med Virol. 2010; 20: 202–13.

Carr W.H., Pando M.J., Parham P. *KIR3DL1 polymorphisms that affect NK cell inhibition by HLA-Bw4 ligand*. J Immunol. 2005; 175: 5222–9.

Cauli A., Piga M., Dessole G., Porru G., Floris A., Vacca A., Desogus E., La Nasa G., Mathieu A. *Killer-cell immunoglobulin-like receptors (KIR) and HLA-class I heavy chains in ankylosing spondylitis*. Drug Dev Res. 2014; 75 Suppl 1: S15-9.

Cella M., Longo A., Ferrara G.B., Strominger J.L., Colonna M. *NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80*. J Exp Med. 1994; 180: 1235–42.

Cheent K., Khakoo S.I. *Natural killer cells and hepatitis C: action and reaction*. Gut. 2011; 60: 268-78.

Chen Y., Sun R., Jiang W., Wei H., Tian Z. *Liver-specific HBsAg transgenic mice are over-sensitive to Poly(I: C)-induced liver injury in NK cell- and IFN-gamma-dependent manner*. J Hepatol. 2007; 47: 183-90.

Cook M., Briggs D., Craddock C., Mahendra P., Milligan D., Fegan C., Darbyshire P., Lawson S., Boxall E., Moss P. *Donor KIR genotype has a major influence on the rate of cytomegalovirus reactivation following T-cell replete stem cell transplantation*. Blood. 2006; 107: 1230-2.

Custer B., Sullivan S.D., Hazlet T.K., Iloeje U., Veenstra D.L., Kowdley K.V. *Global epidemiology of hepatitis B virus*. J Clin Gastroenterol. 2004; 38: S158-68.

De Re V., Caggiari L., De Zorzi M., Repetto O., Zignego A.L., Izzo F., Tornesello M.L., Buonaguro F.M., Mangia A., Sansonno D., Racanelli V., De Vita S., Pioltelli P., Vaccher E., Berretta M., Mazzaro C., Libra M., Gini A., Zucchetto A., Cannizzaro R., De Paoli P. *Genetic diversity of the KIR/HLA system and susceptibility to hepatitis C virus-related diseases*. PLoS One. 2015; 10: e0117420.

Di Bona D., Scafidi V., Plaia A., Colomba C., Nuzzo D., Occhino C., Tuttolomondo A., Giammanco G., De Grazia S., Montalto G., Duro G., Cippitelli M., Caruso C. *HLA and killer cell immunoglobulin-like receptors influence the natural course of CMV infection*. J Infect Dis. 2014; 210: 1083-9.

Di Bona D., Aiello A., Colomba C., Bilancia M., Accardi G., Rubino R., Giannitrapani L., Tuttolomondo A., Cascio A., Caiaffa M.F., Rizzo S., Di Lorenzo G., Candore G., Duro G., Macchia L., Montalto G., Caruso C. *KIRIIND (KIR Infectious and Inflammatory Diseases) Collaborative Group. KIR2DL3 and the KIR ligand groups HLA-A-Bw4 and HLA-C2 predict the outcome of hepatitis B virus infection*. J Viral Hepat. 2017; 24: 768-775.

Dohring C., Scheidegger D., Samaridis J., Cella M., Colonna M. *A human killer inhibitory receptor specific for HLA-A1,2*. J Immunol. 1996; 156: 3098–101.

Dunn C., Brunetto M., Reynolds G., Christophides T., Kennedy P.T., Lampertico P., Das A., Lopes A.R., Borrow P., Williams K., Humphreys E., Afford S., Adams D.H., Bertoletti A., Maini M.K. *Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage*. J Exp Med. 2007; 204: 667-80.

Flodström M., Shi F.D., Sarvetnick N., Ljunggren H.G. *The natural killer cell friend or foe in autoimmune disease*? Scand J Immunol. 2002a; 55: 432-41.

Flodström M., Maday A., Balakrishna D., Cleary M.M., Yoshimura A., Sarvetnick N. *Target cell defense prevents the development of diabetes after viral infection*. Nat Immunol. 2002b; 3: 373-82.

Gazit R., Garty B.Z., Monselise Y., Hoffer V., Finkelstein Y., Markel G., et al. *Expression of KIR2DL1 on the entire NK cell population: a possible novel immunodeficiency syndrome*. Blood. 2004; 103: 1965–6.

Geldhof A.B., Moser M., Lespagnard L., Thielemans K., De Baetselier P. *Interleukin-12-activated natural killer cells recognize B7 costimulatory molecules on tumor cells and autologous dendritic cells*. Blood. 1998; 91: 196-206.

Guethlein L.A., Older Aguilar A.M., Abi-Rached L., Parham P. *Evolution of killer cell Ig-like receptor (KIR) genes: definition of an orangutan KIR haplotype reveals expansion of lineage III KIR associated with the emergence of MHC-C*. J Immunol. 2007; 179: 491-504.

Gumperz J.E., Litwin V., Phillips J.H., Lanier L.L., Parham P. *The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor.* J Exp Med. 1995; 181: 1133–44.

Hadaya K., de Rham C., Bandelier C., Bandelier C., Ferrari-Lacraz S., Jendly S., Berney T., Buhler L., Kaiser L., Seebach J.D., Tiercy J.M., Martin P.Y., Villard J. *Natural killer cell receptor repertoire and their ligands, and the risk of CMV infection after kidney 415 transplantation*. Am J Transplant. 2008; 8: 2674–83.

Holm S.J., Sakuraba K., Mallbris L.,Wolk K., Stahle M., Sanchez F.O. *Distinct HLAC/KIR genotype profile associates with guttate psoriasis*. J Invest Dermatol. 2005; 125: 721–30.

Hsu K.C., Liu X.R., Selvakumar A., Mickelson E., O'Reilly R.J., Dupont B. *Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets*. J Immunol. 2002; 169: 5118–29.

Kakimi K., Lane T.E., Wieland S., Asensio V.C., Campbell I.L., Chisari F.V., Guidotti L.G. *Blocking chemokine responsive to gamma-2/interferon (IFN)-gamma inducible protein and monokine induced by IFN-gamma activity in vivo reduces the pathogenetic but not the antiviral potential of hepatitis B virus-specific cytotoxic T lymphocytes*. J Exp Med. 2001; 194: 1755-66.

Khakoo S.I., Thio C.L., Martin M.P., Brooks C.R., Gao X., Astemborski J., Cheng J., Goedert J.J., Vlahov D., Hilgartner M., Cox S., Little A.M., Alexander G.J., Cramp M.E., O'Brien S.J., Rosenberg W.M., Thomas D.L., Carrington M. *HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection*. Science. 2004; 305: 872-4.

Khakoo S.I., Carrington M. *KIR and disease: a model system or system of models*? Immunol Rev. 2006; 214: 186-201.

Kulkarni S., Martin M.P., Carrington M. *The Yin and Yang of HLA and KIR in human disease*. Semin Immunol. 2008; 20: 343-52.

Larkin J., Bost A., Glass J.I., Tan S.L. *Cytokine-activated natural killer cells exert direct killing of hepatoma cells harboring hepatitis C virus replicons*. J Interferon Cytokine Res. 2006; 26: 854-65.

Liang H.L., Ma S.J., Tan H.Z. *Association between killer cell immunoglobulin-like receptor (KIR) polymorphisms and systemic lupus erythematosus (SLE) in populations: A PRISMA-compliant meta-analysis*. Medicine (Baltimore). 2017; 96: e6166.

Lin A., Xu H., Yan W. *Modulation of HLA expression in human cytomegalovirus immune evasion*. Cell Mol Immunol. 2007; 4: 91–8.

Łuszczek W., Mańczak M., Cisło M., Nockowski P., Wiśniewski A., Jasek M., Kuśnierczyk P. *Gene for the activating natural*

killer cell receptor, KIR2DS1, is associated with susceptibility to psoriasis vulgaris. Hum Immunol. 2004; 65: 758-66.

Martin M.P., Gao X., Lee J.H., Nelson G.W., Detels R., Goedert J.J., Buchbinder S., Hoots K., Vlahov D., Trowsdale J., Wilson M., O'Brien SJ, Carrington M. *Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS*. Nat Genet. 2002; 31: 429-34.

Martin M.P., Bashirova A., Traherne J., Trowsdale J., Carrington M. *Cutting edge: expansion of the KIR locus by unequal crossing over*. J Immunol. 2003; 171: 2192–5.

Martin M.P., Qi Y., Gao X., Yamada E., Martin J.N., Pereyra F., Colombo S., Brown E.E., Shupert W.L., Phair J., Goedert J.J., Buchbinder S., Kirk G.D., Telenti A., Connors M., O'Brien S.J., Walker B.D., Parham P., Deeks S.G., McVicar D.W. *Carrington MInnate partnership of HLA-B and KIR3DL1 subtypes against HIV-1*. Nat Genet. 2007; 39: 733–40.

Maselli A., Conti F., Alessandri C., Colasanti T., Barbati C., Vomero M., Ciarlo L., Patrizio M., Spinelli F.R., Ortona E., Valesini G., Pierdominici M. *Low expression of estrogen receptor β in T lymphocytes and high serum levels of anti-estrogen receptor α antibodies impact disease activity in female patients with systemic lupus erythematosus*. Biol Sex Differ. 2016; 7: 3.

Mathieu A., Cauli A., Fiorillo M.T., Sorrentino R. *HLA-B27 and ankylosing spondylitis geographic distribution versus malaria endemic: casual or causal liaison*? Ann Rheum Dis. 2008; 67: 138-40.

Matzaraki V., Kumar V., Wijmenga C., Zhernakova A. *The MHC locus and genetic susceptibility to autoimmune and infectious diseases*. Genome Biol. 2017; 18: 76.

Moffett A., Loke C. *Immunology of placentation in eutherian mammals*. Nat Rev Immunol. 2006; 6: 584–94.

Namekawa T., Snyder M.R., Yen J.H., Goehring B.E., Leibson P.J., Weyand C.M., Goronzy J.J. *Killer cell activating receptors function as costimulatory molecules on CD4+CD28 null T cells clonally expanded in rheumatoid arthritis*. J Immunol. 2000; 165: 1138–45.

Pan N., Jiang W., Sun H., Miao F., Qiu J., Jin H., Xu J., Shi Q., Xie W., Zhang J. *KIR and HLA loci are associated with hepatocellular*

carcinoma development in patients with hepatitis B virus infection: a case-control study. PLoS One. 2011; 6: e25682.

Parham P. *MHC class I molecules and KIRs in human history, health and survival.* Nat Rev Immunol. 2005; 5: 201–14.

Parham P., Moffett A. *Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution*. Nat Rev Immunol. 2013; 13: 133–44.

Pedroza L.S., Sauma M.F., Vasconcelos J.M., Takeshita L.Y., Ribeiro-Rodrigues E.M., Sastre D., Barbosa C.M., Chies J.A., Veit T.D., Lima C.P., Oliveira L.F., Henderson B.L., Castro A.P., Maia M.H., Barbosa F.B., Santos S.E., Guerreiro J.F., Sena L., Santos E.J. *Systemic lupus erythematosus: association with KIR and SLC11A1 polymorphisms, ethnic predisposition and influence in clinical manifestations at onset revealed by ancestry genetic markers in an urban Brazilian population*. Lupus. 2011; 20: 265-73.

Pellett F., Siannis F., Vukin I., Lee P., Urowitz M.B., Gladman D.D. *KIRs and autoimmune disease: studies in systemic lupus erythematosus and scleroderma.* Tissue Antigens. 2007; 1: 106-8.

Qi Y, Martin MP, Gao X, Jacobson L, Goedert JJ, Buchbinder S, Kirk G.D., O'Brien S.J., Trowsdale J., Carrington M. *KIR/HLA pleiotropism: protection against both HIV and opportunistic infections*. PLoS Pathog. 2006; 2: e79.

Rajagopalan S., Long E.O. *A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells*. J Exp Med. 1999; 189: 1093–100.

Rajagopalan S., Long E.O. *Understanding how combinations of HLA and KIR genes influence disease*. J Exp Med. 2005; 201: 1025–9.

Ravet S., Scott-Algara D., Bonnet E., Tran H.K., Tran T., Nguyen N., Truong L.X., Theodorou I., Barré-Sinoussi F., Pancino G., Paul P. *Distinctive NK-cell receptor repertoires sustain high-level constitutive NK-cell activation in HIV-exposed uninfected individuals*. Blood. 2007; 109: 4296–305.

Romano E., Manetti M., Guiducci S., Ceccarelli C., Allanore Y., Matucci-Cerinic M. *The genetics of systemic sclerosis: an update*. Clin Exp Rheumatol. 2011; 29: S75-86.

Sharp P.M., Hahn B.H. *Origins of HIV and the AIDS pandemic.* Cold Spring Harb Perspect Med. 2011; 1: 006841.

Stegmann K.A., Björkström N.K., Veber H., Ciesek S., Riese P., Wiegand J., Hadem J., Suneetha P.V., Jaroszewicz J., Wang C., Schlaphoff V., Fytili P., Cornberg M., Manns M.P., Geffers R., Pietschmann T., Guzmán C.A., Ljunggren H.G., Wedemeyer H. *Interferon-alpha-induced TRAIL on natural killer cells is associated with control of hepatitis C virus infection*. Gastroenterology. 2010; 138: 1885-97.

Stern M., Elsässer H., Hönger G., Steiger J., Schaub S., Hess C. *The number of activating KIR genes inversely correlates with the rate of CMV infection/reactivation in kidney transplant recipients*. Am J Transplan. 2008; 8: 1312–7.

Suzuki Y., Hamamoto Y., Ogasawara Y., Ishikawa K., Yoshikawa Y., Sasazuki T., Muto M. *Genetic polymorphisms of killer cell immunoglobulin-like receptors are associated with susceptibility to psoriasis vulgaris*. J Invest Dermatol. 2004; 122: 1133-6.

Traherne J.A. *Human MHC architecture and evolution: implications for disease association studies.* Int J Immunogenet. 2008; 35: 179-92.

Tozkır J.D., Tozkır H., Gürkan H., Dönmez S., Eker D., Pamuk G.E., Pamuk Ö.N. *The investigation of killer cell immunoglobulin-like receptor genotyping in patients with systemic lupus erytematosus and systemic sclerosis*. Clin Rheumatol. 2016; 35: 919-25.

Uhrberg M., Valiante N.M., Shum B.P., Shilling H.G., Lienert-Weidenbach K., Corliss B., Tyan D., Lanier L.L., Parham P. *Human diversity in killer cell inhibitory receptor genes*. Immunity. 1997; 7: 753-63.

Winter CC, Long EO. *A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes*. J Immunol. 1997; 158: 4026–8.

Witt C.S., Martin A., Christiansen F.T. *Detection of KIR2DL4 alleles by sequencing and SSCP reveals a common allele with a shortened cytoplasmic tail*. Tissue Antigens. 2000; 56: 248–57.

Yawata M., Yawata N., Draghi M., Little A.M., Partheniou F., Parham P. *Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function*. J Exp Med. 2006; 203: 633–45.

Yen J.H., Moore B.E., Nakajima T., Scholl D., Schaid D.J., Weyand C.M., Goronzy J.J. *Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis*. J Exp Med. 2001; 193: 1159-67.

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Potential new genetic and biochemical markers of chronic kidney failure

Manuela Bova, Letizia Scola, Domenico Lio

Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy.
Correspondence to: Manuela Bova, Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy; e mail: manuela.bova01@unipa.it.

Abstract

Chronic Renal Disease (CKD) most commonly occurs in old age and coincides with a high degree of morbidity and morbidity. In recent years, experimental and clinical data have suggested the possibility of using new biomarkers, in chronic renal failure, such as Klotho and Fibroblast Growth Factor (FGF)23. In fact, the FGF23/Klotho axis is involved not only in cellular ageing process but plays a key role in the mineral metabolism of calcium and phosphate regulation in CKD patients. Patients with CKD exhibit a marked decrease in renal Klotho expression associated with a significant increase in FGF23 levels. Based on this trend, Klotho and FGF23 might be ideal candidates as new biomarkers to be used along with clinical and genetic data, in order to identify patients at risk and to evaluate disease progression. This review focused on pathophysiological role and recent genetic and clinical data acquisitions of FGF23 and Klotho, and their potential role as diagnostic and prognostic biomarkers.

Key words

CKD, CKD-MBD, FGF23, Haemodialysis, Klotho.

State of the art

Chronic kidney disease (CKD) is a condition characterized by a progressive deterioration of renal function with increasing risk of kidney failure and end-stage renal disease (ESRD), the disease stage where dialysis and transplantation are needed [Zoccali et al., 2010].

Chronic kidney disease occurs more frequently in the elderly and coincides with greater mortality and morbidity. CKD can be considered a model of early ageing considering that about half of the CKD cases occur in subjects < 70 years [Kooman et al., 2013; Stenvinkel & Larsson, 2013].

The homeostasis of calcium and phosphate is finely regulated by the kidney, so the loss of functionality determines an imbalance of mineral metabolism.

Chronic kidney disease–mineral and bone disorder (CKD-MBD) is one of the many complications associated with CKD, it is characterized by an abnormal serum levels of minerals (Calcium, Phosphorus) and related hormones, Parathyroid Hormone (PTH) and Vitamin D.

In particular, hyperphosphatemia and secondary hyperparathyroidism are common conditions in dialyzed patients with abnormal bone mineralization and vascular calcification [Danese et al., 2015].

It is well known that calcium homeostasis is regulated by three hormones: PTH, Calcitonin and 1,25-$(OH)_2$ Vitamin D.

Vitamin D stimulates calcium absorption in the gut, and has effects in bone and kidney, as well. Calcium and phosphorus homeostasis is a complex process; several studies in recent years have highlighted new regulation mechanisms of mineral metabolism. In particular great importance has been attributed to the FGF23/Klotho axis.

The FGF23/Klotho axis represents a new endocrine axis alongside the classic homeostasis processes, and it plays an important role not only in calcium and phosphate regulation, but also plays a central role in ageing processes and in the age-related diseases [Shimada et al., 2004a; Shimada et al., 2004 b].

Hyperphosphatemia is the main abnormality in CKD, and is a major contributor to cardiovascular disease, the main cause of mortality in CKD patients. In CKD, vascular calcifications are well represented [Coresh et al., 2004; Kendrick & Chonchol, 2008], so can be considered strong predictor of cardiovascular morbidity and mortality in the CKD patients.

Traditionally, risk factors for cardiovascular mortality in patients with CKD are life-style, dyslipidemia, hypertension and diabetes. In addition to these factors, genetic background, inflammation, oxidative stress and vascular calcifications take on a role of great importance.

Evaluating new molecular and genetic markers is needed to identify the early stage of the disease and to evaluate risk of complications in patients with CKD.

This review will focus primarily on the significance of FGF23 and Klotho as new regulators of phosphate metabolism, and it will review clinical and genetic data of Klotho and FGF23, in patients with CKD, in order to investigate their potential role as diagnostic and prognostic biomarkers.

Discussion

Function of FGF23

FGF23 is a member of the fibroblast growth factor (FGF) family, it was identified 17 years ago [Kenneth et al., 2000]. It acts in the kidney as hormone that regulates Ca and P metabolism [Yamashita et al., 2000]. FGF23 gene encodes a 251 amino-acid protein (molecular weight = 30 kDa) which consist of a hydrophobic signal sequence (24 amino acids), an N-terminal FGF core homology domain (155 amino acids), and a C-terminal domain unique to FGF23 (72 amino acids) essential for interaction with the FGFR-Klotho complex [Goetz et al., 2007]. Between the N- and C-terminal domains, there is a proteolytic cleavage site between Arg^{179} and Ser^{180}.

FGF23 is inactivated by a protease that leads to two inactive fragments, respectively N- and C-terminal. The latter one competes with full FGF23 fragment for binding to the FGFR-Klotho complex and acting as a competitive inhibitor for FGF23 [Goetz et al., 2010].

Therefore, the hormonal activity is played by the native FGF23 and is probably regulated by post-translational modification of FGF23 protein.

FGF23 is produced in the bone, by osteocytes [Pereira et al., 2009]. FGF23 exerts its hypophoshatemic effect through inhibition of luminal sodium-phosphate co-transportes in the proximal tubular epithelial cells [Liu et al., 2007]. The identification of FGF23 has revealed that bone works as an endocrine organ.

Additionally, FGF23 also down-regulates renal proximal tubular expression of 1α-hydroxylase, the rate-limiting enzyme in the synthesis of the vitamin D hormone, 1α,25-dihydroxyvitamin D3 [1,25$(OH)_2$D] [Shimada et al., 2004a,b; Shimada et al., 2005].

FGF23 direct action leads to the increase urinary excretion of phosphate, while indirectly suppresses intestinal phosphate absorption by down-regulating the production of 1,25$(OH)_2$D. In kidney, altogether the effect of direct and indirect hormonal actions decrease the circulating phosphate levels, in order to protect from deleterious effects of hyperphosphatemia. FGF 23 exerts a number of pleiotropic biological actions by binding, dimerizing and activating cell surface FGF receptors (FGFRs) [Mohammadi et al., 2005].

While the affinity of FGF23 for its ubiquitous receptors is quite low, it was shown that this affinity is enhanced by α-Klotho. [Urakawa et al., 2006]. This protein is mainly expressed in the kidneys, so Klotho contributes to organ specific effects of FGF23.

Function of Klotho

The Klotho gene was identified in 1997, originally it was identified as an ageing suppressor. Inactive Klotho gene induces a premature ageing-like syndrome and multiple organ dysfunction in transgenic mice [Kuro-o et al., 1997] whereas overexpression extends mice life span [Kurosu et al., 2005].

There are three different forms of Klotho proteins, which are termed α, β and γ-Klotho, being α-Klotho a transmembrane protein mainly expressed in the kidney, in particular in distal tubular cells [Mian et al., 1998].

Klotho forms complexes with FGF receptors (FGFRs) and works as the high-affinity receptor for FGF23 [Kurosu et al., 2006].

Indeed, experimental study have demonstrated that in the kidney Klotho acts as an obligate coreceptor for FGF23, enhancing and conferring organ specificity to FGF23 hypophosphatemic effect [Kurosu et al., 2006; Urakawa et al., 2006].

Klotho can be enzymatically cleaved, shed, and released as a soluble form in different biological fluids as blood, urine, and cerebrospinal fluid [Imura et al., 2004]. In these cases, soluble Klotho has a paracrine function, having specific effects and FGF23 independent renal and extra-renal effects [Hu et al., 2013].

In particular, the soluble form of Klotho has the ability to inhibit the expression of sodium-phosphate cotransporter NaPi2a in the

proximal tubules, thus inducing a phosphaturic effect enhancing the effect of FGF23 [Hu et al., 2012]. Moreover soluble Klotho leads to an increase reabsorption of calcium inducing the activation of TRPV5 ion channels at the distal tubules levels [Cha et al., 2008]. All together these reports emphasize the role of the Klotho isoforms in the mineral homeostasis regulation [Kuro et al., 2001].

Mice lacking of FGFG 23 and/or Klotho have a similar phenotype, characterized by increases in phosphorus, calcium and vitamin D serum levels, ageing phenotype with vascular calcifications, osteopenia and a shortened life span [Kawaguchi et al., 1999].

Klotho and FGF23 as potential biomarkers for CKD and CKD-MBD

The bone kidney endocrine axis mediated by FGF23 and Klotho has emerged as an essential component in the regulation of phosphate homeostasis.

In patients with stages 4-5 CKD and dialysis hyperphosphatema is often observed, due to an altered renal excretion of P, associated with renal impairment and decrease in number of physiologically active nephrons.

Patients with CKD exhibit a marked decrease in renal Klotho expression associated with a significant increase in FGF23 levels. At this moment, data on Klotho mRNA and protein levels in human CKD are very limited. Experimental models of CKD have shown that there is a significant reduction of renal Klotho transcript and its protein product in CKD with different aetiology [Hu et al., 2012; Barker et al., 2015].

Rotondi et al., [2015] have reported that soluble plasma Klotho levels were significantly decreased in the early stages of CKD, moreover they have found reduced levels of this protein since CKD stage 2. Other studies have also evaluated the urinary Klotho levels, indicating the decrease in urinary levels as one of the earliest abnormalities occurring in patients with CKD. A very significant point is that these levels decrease progressively with the decline of estimated glomerular filtration rate (eGFR) [Hu et al., 2012] and it has been shown that urinary Klotho levels in CKD patients rather than their serum Klotho levels, are related to the number of functioning nephrons [Akimoto T et al., 2012].

Conversely, higher levels of FGF23 have been reported at stage 2 [Rotondi et al., 2015; Shimamura et al., 2012]. On the other hand, the increase of FGF23 was also associated with the decrease of $1,25(OH)_2D$ in patients with CKD [Gutierrez et al., 2005], resulting in increased

secretion of PTH in order to maintain normal serum calcium level, but at the same time leading to a high bone turnover [Nitta et al., 2014; Fliser et al., 2007]. Accordingly, a negative correlation of FGF23 with eGFR levels was observed [Rotondi S et al., 2014].

Several studies in animal models and humans with early native CKD have demonstrated that FGF23 levels are rising in early stages preceding changes in calcium, phosphorus, or PTH levels [Pasquali et al., 2016].

The level of FGF23 was also reported to be associated with various adverse events, particularly in patients with CKD, such as heart failure, stroke, fractures and progression of CKD [Fukumoto & Shimizu, 2011]. In addition, a recent meta-analysis of seven studies, including ESRD patients has reported an association between FGF23 and increased mortality [Yang et al., 2016].

Klotho and FGF23 gene polymorphisms for CKD and CKD-MBD

Single nucleotide polymorphism (SNPs) are DNA sequence variations that commonly occur within a population (e.g., 1%), some genetic variants may correlate with increased or decreased risk of developing complex diseases.

In humans, association of Klotho gene SNPs with several disease such as osteoporosis, stroke, essential hypertension, coronary artery disease have been reported [Friedman et al., 2009; Kalaitzidis et al., 2016].

In the same way studies on FGF23 SNPs suggest their potential role as risk factor for diseases, such as renal failure, cardiovascular failure, and stroke [Itoh, 2015]. Friedman et al., [2009], have found an association between the Klotho gene polymorphism (rs577912) and an increased risk for 1-yr mortality, in patients with end-stage renal disease. In particular, it has been shown that CC genotype is associated with an increased risk of death compared with AA or AC genotypes.

Furthermore this specific genotype was also associated with lower Klotho mRNA expression in lymphoblast cell lines from HapMap subjects genotyped at rs577912 [Friedman et al., 2009]. Although the Klotho gene is closely associated with the calcium, phosphorus, and PTH metabolism, no significant effect of the specific Klotho variant on these measures was observed, in the above study.

Recently, it has been demonstrated, in subjects with disorder of phosphate homeostasis, and calcium stone forming, that a non synony-

mous change in the FGF23 gene, influences the FGF23 biological function and its interaction with FGF receptors and Klotho in phosphate homeostasis [Rendina et al., 2012; Yamazaki et al., 2008]. The sequence variation C716T in exon 3 of the gene changes the normal ACG triplet (encoding threonine) at codon 239 to an ATG triplet (encoding methionine), causing the missense variation designated T239M (rs7955866).

The prevalence of the T allele and of the CT genotype in hypophosphatemic stone former (SF) was significantly higher compared with those observed in normophosphatemic SF. Moreover, SF and controls carrying at least one FGF23716T allele, have shown significantly lower levels of serum phosphate.

Conclusion

CDK is an important public health problem that impinges on health and lifespan of patients. Several studies have focused the attention on Klotho/FGF23 axis, characterized by a typical trend in the levels of Klotho and FGF23 in CKD patients. The decrease in Klotho protein in the blood is an early event in CKD and is progressively reduced along with loss of renal function. Low Klotho level, partially induces FGF23 resistance, causing an initial compensatory increase in blood FGF23 to maintain P homeostasis.

Based on these facts it can be stated that both Klotho and FGF23 could be considered sensitive biomarkers useful in identification of the early stages of the disease. To date, we have very few literature data on the levels of FGF23 and Klotho in CKD. Less is known about possible correlations among genetic polymorphisms of this new regulation axis and CKD and its progression.

In this view, we need other additional studies to better understand the role of FGF23 and Klotho as additional risk factors in the complications of CKD, and to better evaluate the functional correlation between genetic background, biochemical markers and diagnosis and prognosis of patients.

References

Akimoto T., Yoshizawa H., Watanabe Y., Numata A., Yamazaki T., Takeshima E., Iwazu K., Komada T., Otani N., Morishita Y., Ito C., Shiizaki K., Ando Y., Muto S., Kuro-o M., Kusano E.. *Characteristics of urinary and serum soluble Klotho protein in patients with different degrees of chronic kidney disease*. BMC Nephrol. 2012; 23: 13-155.

Barker S.L., Pastor J., Carranza D., Quiñones H., Griffith C., Goetz R., Mohammadi M., Ye J., Zhang J., Hu M.C., Kuro-o M., Moe O.W., Sidhu S.S. *The demonstration of αKlotho deficiency in human chronic kidney disease with a novel synthetic antibody*. Nephrol Dial Transplant. 2015; 30: 223-33.

Cha S.K., Ortega B., Kurosu H., Rosenblatt K.P., Kuro-O M., Huang C.L. *Removal of sialic acid involving Klotho causes cell-surface retention of TRPV5 channel via binding to galectin-1*. Proc Natl Acad Sci U S A. 2008; 105: 9805-10.

Coresh J., Astor B., Sarnak M.J. *Evidence for increased cardiovascular disease risk in patients with chronic kidney disease.* Curr Opin Nephrol Hypertens. 2004; 13: 73-81.

Danese M.D., Halperin M., Lowe K.A., Bradbury B.D., Do T.P., Block G.A. *Refining the definition of clinically important mineral and bone disorder in hemodialysis patients*. Nephrol Dial Transplant. 2015; 30: 1336-44.

Fliser D., Kollerits B., Neyer U., Ankerst D.P., Lhotta K., Lingenhel A., Ritz E., Kronenberg F., MMKD Study Group, Kuen E., König P., Kraatz G., Mann J.F., Müller G.A., Köhler H., Riegler P. *Fibroblast growth factor 23 (FGF23) predicts progression of chronic kidney disease: the Mild to Moderate Kidney Disease (MMKD) Study*. J Am Soc Nephrol. 2007; 18: 2600-8.

Fukumoto S., Shimizu Y. *Fibroblast growth factor 23 as a phosphotropic hormone and beyond*. J Bone Miner Metab. 2011; 29: 507-14.

Goetz R., Beenken A., Ibrahimi O.A., Kalinina J., Olsen S.K., Eliseenkova A.V., Xu C., Neubert T.A., Zhang F., Linhardt R.J., Yu X., White K.E.,

Inagaki T., Kliewer S.A., Yamamoto M., Kurosu H., Ogawa Y., Kuro-o M., Lanske B., Razzaque M.S., Mohammadi M. *Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members*. Mol Cell Biol. 2007; 27: 3417-28.

Goetz R., Nakada Y., Hu M.C., Kurosu H., Wang L., Nakatani T., Shi M., Eliseenkova A.V., Razzaque M.S., Moe O.W., Kuro-o M., Mohammadi M. *Isolated C-terminal tail of FGF23 alleviates hypophosphatemia by inhibiting FGF23-FGFR-Klotho complex formation.* Proc Natl Acad Sci U S A. 2010; 107: 407-12.

Gutierrez O., Isakova T., Rhee E., Shah A., Holmes J., Collerone G., Jüppner H., Wolf M. *Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease*. J Am Soc Nephrol. 2005; 16: 2205-15.

Hu M.C., Kuro-o M., Moe O.W. *Secreted klotho and chronic kidney disease*. Adv Exp Med Biol. 2012; 728: 126-57.

Hu M.C., Kuro-o M., Moe O.W. *Renal and extrarenal actions of Klotho*. Semin Nephrol. 2013; 33: 118-29.

Imura A., Iwano A., Tohyama O., Tsuji Y., Nozaki K., Hashimoto N., Fujimori T., Nabeshima Y. *Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane*. FEBS Lett. 2004; 565: 143-7.

Itoh N. *Endocrine FGFs: Evolution, Physiology, Pathophysiology, and Pharmacotherapy*. Front Endocrinol (Lausanne). 2015; 29: 6-154.

Kalaitzidis R.G., Duni A., Siamopoulos K.C. *Klotho, the Holy Grail of the kidney: from salt sensitivity to chronic kidney disease*. Int Urol Nephrol. 2016; 48: 1657-66.

Kawaguchi H., Manabe N., Miyaura C., Chikuda H., Nakamura K., Kuro-o M. *Independent impairment of osteoblast and osteoclast differentiation in klotho mouse exhibiting low-turnover osteopenia*. J Clin Invest. 1999; 104: 229-37.

Kendrick J., Chonchol M.B. *Nontraditional risk factors for cardiovascular disease in patients with chronic kidney disease*. Nat Clin Pract Nephrol. 2008; 4: 672-81.

Kooman J.P., Broers N.J., Usvyat L., Thijssen S., van der Sande F.M., Cornelis T., Levin N.W., Leunissen K.M., Kotanko P. *Out of control: accelerated aging in uremia*. Nephrol Dial Transplant. 2013; 28: 48-54.

Kuro-o M., Matsumura Y., Aizawa H., Kawaguchi H., Suga T., Utsugi T., Ohyama Y., Kurabayashi M., Kaname T., Kume E., Iwasaki H., Iida A., Shiraki-Iida T., Nishikawa S., Nagai R., Nabeshima Y.I. *Mutation of the mouse klotho gene leads to a syndrome resembling ageing*. Nature. 1997; 390: 45-51.

Kurosu H., Yamamoto M., Clark J.D., Pastor J.V., Nandi A., Gurnani P., Mc Guinness O.P., Chikuda H., Yamaguchi M., Kawaguchi H., Shimomura I., Takayama Y., Herz J., Kahn C.R., Rosenblatt K.P., Kuro-o M. *Suppression of aging in mice by the hormone Klotho*. Science. 2005; 309: 1829-33.

Liu S.J., Yue Q.J., Zhang W. *Structural and functional analysis of an asymmetric bidirectional promoter in Arabidopsis thaliana*. J Integr Plant Biol. 2015; 57: 162-70.

Nitta K., Nagano N., Tsuchiya K. *Fibroblast growth factor 23/klotho axis in chronic kidney disease*. Nephron Clin Pract. 2014; 128: 1-10.

Pasquali M., Tartaglione L., Rotondi S. *New biomarkers of CKD-MBD*. G Ital Nefrol. 2016; 33.

Pereira R.C., Juppner H., Azucena-Serrano C.E., Yadin O., Salusky I.B., Wesseling-Perry K. *Patterns of FGF-23, DMP1, and MEPE expression in patients with chronic kidney disease*. Bone. 2009; 45: 1161-8.

Rendina D., Esposito T., Mossetti G., De Filippo G., Gianfrancesco F., Perfetti A., Magliocca S., Formisano P., Prié D., Strazzullo P. *A functional allelic variant of the FGF23 gene is associated with renal phosphate leak in calcium nephrolithiasis*. J Clin Endocrinol Metab. 2012; 97: 840-4.

Rotondi S., Pasquali M., Tartaglione L., Muci M.L., Mandanici G., Leonangeli C., Sales S., Farcomeni A., Mazzaferro S. *Soluble α -Klotho Serum Levels in Chronic Kidney Disease*. Int J Endocrinol. 2015; 2015: 872193.

Shimada T., Hasegawa H., Yamazaki Y., Muto T., Hino R., Takeuchi Y., Fujita T., Nakahara K., Fukumoto S., Yamashita T. *FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis*. J Bone Miner Res. 2004; 19: 429-35.

Shimada T., Kakitani M., Yamazaki Y., Hasegawa H., Takeuchi Y., Fujita T., Fukumoto S., Tomizuka K., Yamashita T.J. *Targeted ablation of FGF23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism*. Clin Invest. 2004; 113: 561-8.

Shimada T., Mizutani S., Muto T., Yoneya T., Hino R., Takeda S., Takeuchi Y., Fujita T., Fukumoto S., Yamashita T. *Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia*. Proc Natl Acad Sci U S A. 2001; 98: 6500-5.

Shimada T., Yamazaki Y., Takahashi M., Hasegawa H., Urakawa I., Oshima T., Ono K., Kakitani M., Tomizuka K., Fujita T., Fukumoto S., Yamashita T. *Vitamin D receptor-independent FGF23 actions in regulating phosphate and vitamin D metabolism*. Am J Physiol Renal Physiol. 2005: 289; F1088-95.

Shimamura Y., Hamada K., Inoue K., Ogata K., Ishihara M., Kagawa T., Inoue M., Fujimoto S., Ikebe M., Yuasa K., Yamanaka S., Sugiura T., Terada Y. *Serum levels of soluble secreted α-Klotho are decreased in the early stages of chronic kidney disease, making it a probable novel biomarker for early diagnosis*. Clin Exp Nephrol. 2012; 16: 722-9.

Stenvinkel P., Larsson T.E. *Chronic kidney disease: a clinical model of premature aging*. Am J Kidney Dis. 2013; 62: 339-51.

Urakawa I., Yamazaki Y., Shimada T., Iijima K., Hasegawa H., Okawa K., Fujita T., Fukumoto S., Yamashita T. *Klotho converts canonical FGF receptor into a specific receptor for FGF23*. Nature. 2006; 444: 770-4.

Yamashita T., Yoshioka M., Itoh N. *Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain.* Biochem Biophys Res Commun. 2000; 277: 494-8.

Yamazaki Y., Imura A., Urakawa I., Shimada T., Murakami J., Aono Y., Hasegawa H., Yamashita T., Nakatani K., Saito Y., Okamoto N., Kurumatani N., Namba N., Kitaoka T., Ozono K., Sakai T., Hataya H., Ichikawa S., Imel E.A., Econs M.J., Nabeshima Y. *Establishment of sandwich ELISA for soluble alpha-Klotho measurement: Age-dependent change of soluble alpha-Klotho levels in healthy subjects*. Biochem Biophys Res Commun. 2010; 398: 513-8.

Yang H., Luo H., Tang X., Zeng X., Yu Y., Ma L., Fu P. *Prognostic value of FGF23 among patients with end-stage renal disease: a systematic review and meta-analysis*. Biomark Med. 2016; 10: 547-56.

Zoccali C., Kramer A., Jager K.J. *Chronic kidney disease and end-stage renal disease-a review produced to contribute to the report 'the status of health in the European union: towards a healthier Europe*. NDT Plus. 2010; 3; 213-224.

Protein Aggregation at the Membrane: insights on Molecular Mechanisms underlying Neurodegenerative diseases

VALERIA VETRI

Departement of Physics and Chemistry & ATEN Center, University of Palermo, Viale delle Scienze, Edificio 18, Pad. A, 90128, Palermo, Italy.
Correspondence to: Valeria Vetri, Departement of Physics and Chemistry & ATEN Center, University of Palermo, Viale delle Scienze, Edificio 18, Pad. A, 90128, Palermo, Italy; e-mail: valeria.vetri@unipa.it.

Abstract

Protein aggregation and amyloid formation are among the most attractive and challenging themes of current scientific research in neurodegenerative diseases. Studies on aggregation phenomena are directly and with equal importance related to both basic and applied sciences. However, despite growing knowledge of general principles regulating fibril formation, currently little information is yet available on the molecular mechanisms and structural features of aggregate species responsible for toxicity.

Key words

Amyloid fibrils, ß-sheets, Neurodegenerative disease, Spectroscopy.

State of the art

In suitable physicochemical conditions, proteins can be destabilised and may modify their native conformation, this resulting in abnormal aggregation phenomena whose pathway may lead to different aggregate morphologies. In particular, it is now largely accepted that all proteins, in specific conditions, may form peculiar ordered structures called amyloid fibrils [Chiti & Dobson, 2006]. The common structure of these aggregates consists of a regular pattern of intermolecular H bonds that stabilise multiple ß-sheets structure that constitute the fibril backbone.

Importantly, fibrillar state represents the most stable state for the polypeptide chain [Tirumalai & Reddy, 2011; Baldwin et al., 2011]. It is now widely accepted that amyloid fibril formation processes are involved in a growing list of human pathologies like Alzheimer's, Parkinson's, Hungtington diseases [Bucciantini et al., 2002; Walsh & Selkoe, 2004; Di Figlia et al., 1997; Chiti & Dobson, 2006].

Studies on aggregation phenomena are directly and with equal importance related to both basic and applied sciences. In particular, investigating the origin of high impact neurodegenerative disorders cannot be separated from an accurate description of the inter- and intra-molecular interactions involved in the formation of amyloid aggregates. In this context, the formation of soluble and/or insoluble protein oligomers during the aggregation pathways is believed to contribute, being a key factor, in toxicity [Bucciantini et al., 2002; Walsh & Selkoe, 2004]. Within the past decades the complexity of aggregation pathways became evident and it was shown that supramolecular assembly occurs via multiple pathways leading to a variety of different species with different inherent toxicity. The assembly of protein into fibrils and other amyloid-like material can be promoted, regulated or inhibited by the external conditions, i.e., interaction with specific molecules as well as interactions with the environment [Wetzel 2006, Vetri & Foderà, 2015], this being in turn in close relation with their fate in cellular environment and then with their toxic action.

A continuum of aggregates species formed during supramolecular assembly rather than a single uniform species has been indicated as main effectors. Evidence has emerged suggesting that these species share both common structural features and the ability to permeabilise cell membranes potentially initiating multiple processes leading to cell impairment and death, being critical in the onset and progression of disease [Stefani,

2010; Straub & Thirumalai, 2014]. Membranes may act as active surfaces favouring aggregation prone conformations by direct interaction with protein hydrophobic or charged groups or for the accumulation of proteins in a crowded environment at liquid/lipid interface or by favouring nucleation mechanisms [Comellas et al., 2012; Stefani, 2010; Vetri et al., 2011]. Key membrane characteristics may regulate these events promoting or suppressing aggregation and in turn aggregation which may induce disruptive structural alterations in cell membranes. A number of mechanisms have been proposed including specific membrane pores formation, membrane destabilization or thinning or breakage due to induced growth of lipid bilayer rigidity and in some case it was observed amyloidogeneic proteins disintegrate membranes by extracting lipids and incorporate them in the fibrils [Milanesi et al., 2012]. All the above mentioned mechanisms may act cooperatively and be energetically coupled.

We have developed a coupled approach consisting both of bulk spectroscopy and fluorescence microscopy experiments which allow gaining quantitative information on the occurring reaction together with the visualization of intrinsically spatially heterogeneous mechanisms and showing in real time changes in membranes morphology and stability upon protein-membrane interaction. We discuss below results of recent experimental studies in collaboration with Prof. B. Vestegard's group at University of Copenhagen are discussed [Van Maarschalkerweerd et al., 2014; 2015].

Discussion

We analysed α-synuclein interaction with synthetic model membranes aiming at highlighting molecular mechanisms regulating the kinetics of membrane-mediated aggregation and in turn the permeabilisation and disassembly of the lipid bilayer. α-synuclein is a 140 amino acid intrinsically disordered protein, abundant in presynaptic terminals of the human brain [Spillantini et al., 1998]. This protein is able to form amyloid fibrils both in vivo and in vitro, and its deposition with lipids in Levy Bodies is major hallmark of Parkinson's disease [Trojanowski et al., 1998; Spillantini et al., 1998].

These data are drawn from experimental studies by means on the combination of Fluorescence spectroscopy, Circular Dichroism and Small Angle Neutron Scattering and multi-photon fluorescence mi-

croscopy. In particular, we analysed the interaction of Giant lipid vesicles with a-synuclein in native, oligomeric and fibrillar state aiming at highlighting molecular mechanisms underlying lipid membrane-amyloidogenic proteins interactions. The quantification of membrane structural changes was obtained by Generalised Polarisation analysis of Laurdan dye, which allowed us obtaining information on membrane fluidity changes [Parasassi et al., 1990] and to detect spatially separated domains with different lipid organisation. Spectroscopic techniques allowed us to gain information on protein structure and conformation. Results have highlighted the role of lipid charge in triggering interaction with protein. Depending on the lipid composition, model membranes are either unperturbed, disrupt, or undergo dramatic morphological changes and segregate into structurally different components. The extent of the observed modifications in lipid membrane was found to be dependent on protein concentration and aggregation state [Van Maarschalkerweerd et al., 2014].

The presence of new soluble protein-lipid co-aggregates formed in the context of liposome rupture was also assessed. Our results also highlighted the role of cholesterol in favouring the interaction between neutral membranes and oligomeric alphasynuclein leading to changes in lipid packing, bilayer morphology and to segregation of structurally different structures [Van Maarschalkerweerd et al., 2015].

Conclusion

Protein aggregation and amyloid formation is among the most appealing and challenging topics in current scientific research. These phenomena are of fundamental interest in biophysical sciences as they deal many-body transition between an initial stable phase, the functional protein, and a final phase, insoluble aggregates, involving complex intra and intermolecular interactions modulated by initial protein structure and physico-chemical properties of the environment [Uversky et al., 2006; Vetri & Foderà, 2015]. However, in spite of a growing knowledge on general principles that regulate fibril formation a little information is currently available on the molecular mechanisms and on the structural characteristics of aggregates species responsible for toxicity.

References

Baldwin A.J., Knowles T.P., Tartaglia G.G., Fitzpatrick A.W., Devlin G.L., Shammas S.L., Waudby C.A., Mossuto M.F., Meehan S., Gras S.L., Christodoulou J., Anthony-Cahill S.J., Barker P.D., Vendruscolo M., Dobson C.M. *Metastability of native proteins and the phenomenon of amyloid formation*. J Am Chem Soc. 2011; 133: 14160-3.

Bucciantini M., Giannoni E., Chiti F., Baroni F., Formigli L., Zurdo J., Taddei N., Ramponi G., Dobson C.M., Stefani M. *Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases*. Nature. 2002; 416: 507-11.

Chiti F., Dobson C.M. *Protein misfolding, functional amyloid, and human disease*. Annu Rev Biochem. 2006; 75: 333-66.

Comellas G., Lemkau L.R., Zhou D.H., George J.M., Rienstra C.M. *Structural intermediates during α-synuclein fibrillogenesis on phospholipid vesicles*. J Am Chem Soc. 2012; 134: 5090-9.

Di Figlia M., Sapp E., Chase K.O., Davies S.W., Bates G.P., Vonsattel J.P., Aronin N. *Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain*. Science. 1997; 277: 1990-3.

Milanesi L., Sheynis T., Xue W.F., Orlova E.V., Hellewell A.L., Jelinek R., Hewitt E.W., Radford S.E., Saibil H.R. *Direct three-dimensional visualization of membrane disruption by amyloid fibrils*. Proc Natl Acad Sci U S A. 2012; 109: 20455-60.

Parasassi T., De Stasio G., d'Ubaldo A., Gratton E. *Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence*. Biophys J. 1990; 57: 1179-86.

Spillantini M.G., Crowther R.A., Jakes R., Hasegawa M., Goedert M. *Alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies*. Proc Natl Acad Sci U S A. 1998; 95: 6469-73.

Stefani M. *Biochemical and biophysical features of both oligomer/fibril and cell membrane in amyloid cytotoxicity*. FEBS J. 2010; 277: 4602-13.

Straub J.E., Thirumalai D. Membrane-Protein *Interactions Are Key to Understanding Amyloid Formation*. J Phys Chem Lett. 2014; 5: 633-5.

Thirumalai D., Reddy G. *Protein thermodynamics: Are native proteins metastable*? Nat Chem. 2011; 3: 910-1.

Trojanowski J.Q., Goedert M., Iwatsubo T., Lee V.M. *Fatal attractions: abnormal protein aggregation and neuron death in Parkinson's disease and Lewy body dementia*. Cell Death Differ. 1998; 5: 832-7.

Uversky V.N., Kabanov A.V., Lyubchenko Y.L. *Nanotools for megaproblems: probing protein misfolding diseases using nanomedicine modus operandi*. J Proteome Res. 2006; 5: 2505-22.

van Maarschalkerweerd A., Vetri V., Langkilde A.E., Foderà V., Vestergaard B. *Protein/lipid coaggregates are formed during α-synuclein-induced disruption of lipid bilayers*. Biomacromolecules. 2014; 15: 3643-54.

van Maarschalkerweerd A., Vetri V., Vestergaard B. *Cholesterol facilitates interactions between α-synuclein oligomers and charge-neutral membranes*. FEBS Lett. 2015; 589: 2661-7.

Vetri V., Foderà V. *The route to protein aggregate superstructures: Particulates and amyloid-like spherulites*. FEBS Lett. 2015; 589(19 Pt A): 2448-63.

Vetri V., Ossato G., Militello V., Digman M.A., Leone M., Gratton E. *Fluctuation methods to study protein aggregation in live cells: concanavalin A oligomero formation*. Biophys J. 2011; 100: 774-83.

Walsh D.M., Selkoe D.J. *Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration*. Protein Pept Lett. 2004; 11: 213-28.

Wetzel R. *Kinetics and thermodynamics of amyloid fibril assembly.* Acc Chem Res. 2006; 39: 671-9.

Estrogens in Liver Cancer: Friends or Foes?

Letizia Cocciadiferro, Giuseppe Carruba

SIRS, ARNAS-Civico, Di Cristina, Benfratelli - Palermo
Correspondence to: Giuseppe Carruba, SIRS, ARNAS-Civico, Piazza N. Leotta 2, 90127 Palermo, Italy; e-mail: giuseppe.carruba@arnascivico.it

Abstract

There is multiple evidence that supports the implication of estrogen in development and progression of human hepatocellular carcinoma. Notwithstanding, estrogens are recognized as important players in physiological regulation of various target organs and tissues, including liver development and function. In this paper we review data from our own studies and the existing literature that may help understanding this apparent inconsistency.

Key Words

Aromatase, Estrogens, Epigenetics, HCC.

State of the Art

Evidence that sex hormones regulate both morphology and function of human liver dates back to early fifties [Laroche, 1953]. There is multiple, though spare, evidence that estrogens play an important role in human liver development and liver protection from various diseases, including fibrosis, non-alcoholic fatty liver disease (NFLD), hepatitis and hepatocellular carcinoma (HCC). In particular, circulating or locally produced estrogens promote various protective mechanisms, including inhibition of fibrogenesis, preservation of mitochondrial structure and function, inhibition of cellular senescence, increase of innate immunity, and promotion of antioxidant effects [Brady, 2015].

Additionally, estrogen regulates protein synthesis, including lipoprotein and proteins responsible for blood clotting (factors II, VII, IX, X, plasminogen), in the liver [Barros & Gustafsson, 2011]. Estrogen signalling is also essential in the regulation of glucose homeostasis, leading to improve glucose tolerance and insulin sensitivity [Parthasarathy et al., 2009]. Recent studies have suggested the possibility that postmenopausal women with NFLD and longer duration of estrogen deficiency could be burdened by a higher risk of developing severe fibrosis than premenopausal women [Klair et al., 2016]. Estrogen receptor beta (ERβ) is thought to mediate the protective role of estradiol against chronic liver diseases, including cancer, through anti-proliferative and anti-inflammatory activities [Iavarone et al., 2003].

Early studies have indicated that gonadal steroids may play a role in development and progression of HCC as a result of an unbalance of liver hormonal milieu [Nagasue & Kohno, 1992]. HCC represents the sixth leading cancer and the second commonest cause of cancer death worldwide [ACS, 2015]. In Italy, mortality rates have been drastically increasing from 4.8 deaths/100.000 in 1969 up to 11.0/100.000 in 1994, with even higher values in the South of Italy (Italian ISTAT database).

Regardless of race and geography, HCC incidence is two to three times higher in male than in female, suggesting a potential implication of sex steroids in HCC development and progression.

Clinical and epidemiological studies have indicated that chronic viral B and C hepatitis progress more rapidly in males that in females and that cirrhosis is largely a disease of men and postmenopausal women [Poyard et al., 2001]. On the other hand, estrogen receptors

have been previously detected in primary HCC [Nagasue et al., 1986]. Studies in human liver tissues have reported that both ERα and ERβ are expressed at higher levels in patients with chronic liver disease as compared with those with HCC and that an excess of the variant ERβ Δ5 is associated with wild-type ERβ in patients with severe liver disease and/or HCC [Iavarone et al., 2003].

Various clinical trials have assessed the potential impact of antiestrogen drug tamoxifen on the survival of HCC patients, but they have failed to show any significant benefit [Engstrom et al., 1990; CLIP-Group, 1998]. This disappointing finding could however be the result of the expression of mutant ERα forms, as previously described [Villa et al., 1995].

The human liver represents a major site for biotransformation, conjugation and degradation of sex steroids, being featured by the presence of key steroid enzymes, including aromatase. There is epidemiologic evidence for the role of hepatic aromatization of androgen into estrogen in the development of non-viral hepatitis-related HCC [Koh et al., 2011]. We have previously reported that both expression and activity of aromatase are elevated in malignant human liver tissues and cells, as opposed to nontumoral hepatic tissues and nonmalignant liver cells where it remains low or undetectable [Castagnetta et al., 2003; Carruba, 2009]. Locally high estrogen levels may in turn increase both expression and function of a membrane glycoprotein, amphiregulin (AREG), a member of the family of epidermal growth factor receptor (EGFR) ligands, resulting in stimulation of receptor signalling and cell proliferation [Carruba et al., 2011]. AREG activation is mediated by a transmembrane enzyme, TACE that is responsible for cleavage of AREG into EGF-like fragments through a process that is referred to as ectodomain shedding. In our studies we have assessed the expression of AREG in liver cancer tissues and cells, also in relation to expression and activity of aromatase and to the status of wild-type estrogen receptors, either ERα or ERβ, and their splicing variants. Our data suggest that AREG and TACE are expressed correspondingly with aromatase in liver tissues and liver cancer cells. In addition, we have indicated that estradiol (E2) induces both AREG and TACE in human liver cancer cells, implying that estrogen may increase AREG production in the malignant liver either directly (via upregulation of AREG gene) or indirectly (via the induction of TACE enzyme) or both [Carruba et al., 2011]. Moreover, our in vitro studies have demonstrated that proliferative activity of liver cancer cells is significantly

increased by exposure to AREG and that this effect can be abrogated by the simultaneous addition of a neutralizing ant-AREG antibody [Cocciadiferro et al., 2016].

We have also observed that an increasingly higher estrogen formation comparing nontumoral, cirrhotic and malignant liver tissues is strictly associated with a gradual decrease of the wild type ERα (ERα66) and to the progressive appearance of the ERα36 splicing variant, suggesting that this switch of ER forms could represent a distinctive feature of HCC development and progression [Miceli et al., 2011].

More recently, we have investigated the expression of Merlin (neurofibromin 2), the product of the Neurofibromatosis type 2 (NF2) tumor suppressor gene, in nontumoral and malignant human liver tissues and cells [Cocciadiferro et al., 2016]. Merlin belongs to the Band 4.1 family of cytoskeletal linker proteins, containing the ERM (**E**zrin, **R**adixin, **M**oesin) domain and has been implicated in the control of cell growth through signalling from extracellular matrix [McClatchey & Fehon, 2009]. Merlin has been reported to restrict tumor growth and to revert malignant phenotype *in vitro* [Shaw et al., 2001; Kissil et al., 2003; Curto et al., 2007; James et al., 2009]. Recently, in an elegant study, Benhamouche et al., [2010] have proposed that Merlin acts as a regulator of tissue organ size in experimental animal model systems. In particular, the authors, using a conditional NF2 knockout mice, have observed that deletion of the NF2 gene in hepatoblasts eventually leads to liver enlargement as a result of clonal expansion of the putative liver stem cells, the oval cells. Intriguingly, surviving mice invariably develop cholangiocellular and HCC, suggesting that oval cells could be the tumor-initiating cells in liver carcinogenesis. In this context, the authors propose that Merlin plays a pivotal role in the regulation of liver stem cell niche by controlling both EGFR abundance and signalling. In our studies, we have observed that NF2 expression is significantly associated with both aromatase and AREG expression, being elevated in HCC, intermediate in cirrhotic tissues and lowest in nontumoral liver. In addition, NF2 expression is inversely related to wild type hERα66 and proportional to the expression of the membrane-associated hERα36 splice variant.

Discussion

Although sex steroid receptors are expressed in a variable proportion of HCC [Nagasue et al., 1986; Ohnishi et al., 1986], the potential implication of

estrogens in development and/or progression of human liver tumors remains unclear. In the last decades there has been a great deal of interest in the potential use of tamoxifen for HCC patients [Boix et al., 1993]. However, several clinical trials have failed to show any significant increase of overall or disease-free survival in patients treated with tamoxifen [Gallo et al., 2006]. Moreover, it ought to be emphasized that the ER status of liver tissues has only been assessed by immunohistochemistry, using mono- and/or polyclonal antibodies that are often raised against epitopes common to both wild-type and variant ERs. This would imply that, in most cases, immunohistochemical assessment of ER expression cannot distinguish the type(s) of ER expressed in individual tissues or cells.

Based on evidence provided by our own and other studies, we have proposed that locally elevated estrogen formation, resulting from high aromatase expression and activity, may eventually lead to promotion of liver tumor cell growth through the induction of AREG expression and/or activity via an ER-mediated mechanism. AREG has been reported to be undetectable in the normal liver, while it is readily induced during acute liver injury and behaves as a potent pro-regenerative and survival factor [Berasain et al., 2007]. Elevated AREG expression has been observed in a variety of chronic inflammatory diseases and in different human cancers [Berasain & Avila, 2014]. There is consistent evidence that AREG may be implicated in the proliferative regulation of human HCC. Elevated levels of AREG have been described in a variety of human tumors and this growth factor is thought to play a nonredundant unique role in human HCC development and progression [Berasain et al., 2007]. Previous studies have reported that AREG is transcriptionally induced by estrogen via ERα in mammary glands of pubertal mice and in human breast cancer cells [Vendrell et al., 2004; Ciarloni et al., 2007]. Furthermore, AREG has been reported to regulate mammary stem/progenitor cell differentiation and expansion, being implicated in breast cancer initiation and progression [LaMarca & Rosen, 2007]. Evidence from our studies indicates that the aromatase-driven estrogen production we observe in malignant human liver tissues and cells may promote cell proliferation through on ERα-mediated, E2-induced increase of AREG expression and the resulting activation of the EGFR signalling. We have observed in fact that expression patterns of AREG and its converting enzyme TACE (ADAM17) are correspondent to those of aromatase and ERα36 in human liver tissues and liver cancer cells.

Overall, our data suggest that human liver carcinogenesis and tumor progression are associated with a dramatic shift in the expression and balance of ERα66 and ERα36. This implies that ERα36 could be expressed in a subset of ERα66-negative HCC, thus emphasizing the potential shortcomings of routine evaluation of ER status based on immunohistochemistry of ERα66 alone, while it offers new potentially useful information for treatment of "ER-negative" HCC patients. We have reported also that patterns of aromatase expression are inversely related to wild type ERα66, but strictly associated with ERα36 splice variant [Miceli et al., 2011]. The latter lacks ligand-dependent and -independent transactivation regions (AF1 and AF2) of ER, but retains both ligand- and DNA-binding domains and mediates membrane binding and rapid signalling of estrogen. Based on this combined evidence, one could speculate that estrogens, locally produced through aromatase, may favour cell proliferation and therefore be implicated in HCC development also through nongenomic ERα36-mediated signalling. In this framework, the balance of wild-type and splice variant ER may be critical to determine the ultimate efficacy of ER antagonist used as first-line hormone treatment of breast cancer patients and its potential efficacy in other hormone-related tumors, including HCC. This evidence has a two-fold value. In the first place, it suggests that a switch from the wild-type ERα66 to the splice variant ERα36 could be associated with HCC development and/or progression, with a resulting inhibition of estrogen-dependent and independent activation of ERα66-mediated genomic activities and a prevalence of rapid estrogen signalling through the MAPK/ERK pathway, as it has been observed in human breast cancer [Wang et al., 2006]. Secondly, the expression of ERα36 has been associated with tamoxifen-resistance in breast cancer [Viedma-Rodríguez et al., 2014]. In human HCC, failure of antiestrogen (tamoxifen) treatment to produce any survival benefit of patients could be, at least partly, ascribed to the ERα66ERa36 switch. Neither tamoxifen nor the pure antiestrogen ICI-182,780 could in fact induce ERα36 inhibition and/or degradation presumably because this variant ERα lacks both AF1 and AF2 transactivation domains and has a truncated ligand-binding domain lacking the last five helixes (helix 8–12) [Wang et al., 2006]. It is noteworthy that recent studies have highlighted the potential role of ERα36 in the control of breast cancer stem/progenitor cells [Deng et al., 2014].

Based on this combined evidence, we have recently hypothesized a model whereby liver tissue injury and the ensuing establishment of an inflammatory microenvironment results in aromatase-driven estrogen formation that in turn produces, either directly or indirectly, an ERα66-mediated increase of AREG. This latter may be responsible for clonal expansion of liver stem/progenitor cells that may eventually lead to terminal differentiation, repair of tissue damage, of inflammatory process and normalization aromatase expression and function, culminating with the shut-off of the whole mechanism. However, during HCC development, epigenetic alteration may induce a switch in the ERα status and an impairment of the terminal differentiation process, resulting in persistently high aromatase activity and elevated estrogen formation, activation of ERα36-mediated nongenomic estrogen signalling and the consequent abnormal proliferation of liver stem/progenitor cells that are unable to differentiate in a way that tissues injury and repair mechanisms cannot be turned down and, ultimately, lead to either benign or malignant liver tumors (Carruba, Gordon Research Conference 2011 - personal communication).

Evidence is accumulating that the product of the neurofibromatosis type 2 (NF2) gene, referred to as neurofibromin II or Merlin, act as a "wizard" regulator of the liver stem cell niche also through EGFR signalling, being responsible for the maintenance of tissue homeostasis. In particular, using a conditional KO mice model carrying liver-specific deletion of the NF2 gene, it has been revealed that liver of $NF2^{-/-}$ animals is featured by remarkable expansion of the putative liver stem cell, the oval cells, and that mice outliving 30 weeks of age invariably develop either HCC or cholangiocarcinoma [Benhamouche et al., 2010]. In recent studies we have explored the possibility that Merlin could be implicated in our hypothetical model of estrogen-regulated mechanisms of liver injury and repair and HCC development. We found that Merlin expression in liver tissues parallels that of aromatase, ERα36, AREG and ADAM17, suggesting that Merlin could be implicated in estrogen-regulated mechanisms of liver tissue injury and repair, as a gatekeeper protein sensing tissue damage and acting in cooperation with aromatase-driven estrogen formation, eventually leading to regulation of oval stem cells differentiation and tissue repair [Cocciadiferro et al., 2016].

Conclusion

Estrogens are universally recognized as key regulators of many biological functions in target organs. Local estrogen formation is governed by the aromatase enzyme, a member of the P450 superfamily that is responsible for the conversion of C19 androgenic steroids to the corresponding estrogens. Aromatase-driven estrogen production plays an important specific role in classical and non-classical peripheral target tissues, where estrogens regulate an amazing array of physiological processes, including cell growth, protein biosynthesis, intercellular adhesion and communication, cell differentiation, and so forth.

There is multiple, concurrent evidence that aromatase is a key enzyme that has pleiotropic activities in a variety of target tissues. As it occurs at the crossroads of multiple signalling pathways, aromatase is critical for the maintenance of biological homeostasis [Patel, 2017]. There is consistent indication that aromatase deregulation may be implicated in breast and prostate cancer, polycystic ovarian syndrome and ovarian cancer, haemorrhage and thrombosis, endometriosis, chronic liver disease and HCC, neurodegenerative diseases (Alzheimer, schizophrenia, multiple sclerosis), osteoporosis and fracture, obesity and type 2 diabetes [Williams, 2010].

In this framework, according to the model we have proposed, disruption of aromatase control, in combination with epigenetic alteration of cell growth/differentiation, may eventually lead to development and/or progression of various chronic diseases. Although these ailments are of multifactorial origin, pathogenetic mechanism(s) may however, at least partly, recognize a common pattern featured by elevated aromatase/estrogen activity and epigenetic disruption of cell/tissue differentiation, as illustrated in Figure 1a,b.

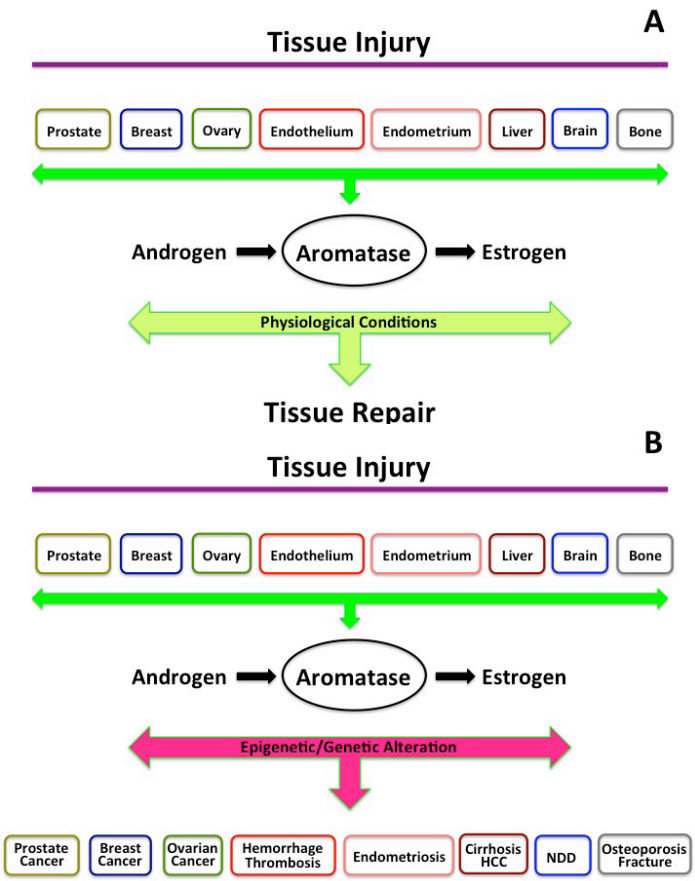

Figure 1. **Hypothetical role of aromatase in tissue injury and repair**. **(A)** Aromatase has pleiotropic homeostatic activities in a variety of target tissues, including prostate, breast, ovary, endothelium, endometrium, liver, brain, bone. In this hypothetical model, tissue injury results in aromatase-driven estrogen formation that is in turn responsible for clonal expansion of liver stem/progenitor cells and the ensuing terminal differentiation, leading to repair of tissue damage. (**B**) However, epigenetic/genetic alteration may induce an impairment of the terminal differentiation process, resulting in persistently high aromatase activity, elevated estrogen formation, ultimately leading to either benign or malignant chronic disease(s), such as breast and prostate cancer, polycystic ovarian syndrome and ovarian cancer, hemorrhage and thrombosis, endometriosis, chronic liver diseases and HCC, neurodegenerative diseases (NDD: Alzheimer, schizophrenia, multiple sclerosis), osteoporosis and fracture.

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References

ACS American Cancer Society 2015. *Global cancer: facts and figures. Barros RP, Gustafsson JÅ. Estrogen receptors and the metabolic network*. Cell Metab. 2011; 14: 289-299.

Benhamouche S., Curto M., Saotome I., Gladden A.B., Liu C.H., Giovannini M., McClatchey A.I. *Nf2/Merlin controls progenitor homeostasis and tumorigenesis in the liver*. Genes Dev. 2010; 24: 1718-1730.

Berasain C., Castillo J., Perugorría M.J., Prieto J., Avila M.A. *Amphiregulin: A new growth factor in hepatocarcinogenesis*. Cancer Lett. 2007; 254: 30–41.

Berasain C., Avila M.A. *Amphiregulin*. Semin Cell Dev Biol. 2014; 28: 31-41.

Boix L., Bruix J., Castells A., Fuster J., Bru C., Visa J., Rivera F., Rodes J. *Sex hormone receptors in hepatocellular carcinoma. Is there a rationale for hormonal treatment*? J Hepatol. 1993; 17: 187-191.

Brady C.W. *Liver disease in menopause*. World J Gastroenterol. 2015; 21: 7613-7620.

Carruba G. *Aromatase in nontumoral and malignant human liver tissues and cells*. Ann NY Acad Sci. 2009; 1155: 187–193.

Carruba G., Miceli V., Cocciadiferro L., Zarcone M., Agostara B., Montalto G., Granata O.M. *Estrogen signalling through amphiregulin may be implicated in human hepatocellular carcinoma*. Horm Mol Biol Clin Investig. 2011; 5: 153–160.

Castagnetta L., Agostara B., Montalto G., Polito L., Campisi I., Saetta A., Itoh T., Yu B., Chen S., Carruba G. *Local estrogen formation by nontumoral, cirrhotic, and malignant human liver tissues and cells*. Cancer Res. 2003; 63: 5041-5045.

Ciarloni L., Mallepell S., Brisken C. *Amphiregulin is an essential mediator of estrogen receptor alpha function in mammary gland development*. Proc Natl Acad Sci USA. 2007; 104: 5455-5460.

CLIP-Group. *Tamoxifen in treatment of hepatocellular carcinoma: a randomized controlled trial*. CLIP-Group (Cancer of the Liver Italian Programme). Lancet. 1998; 352: 17-20.

Cocciadiferro L., Miceli V., Granata O.M., Carruba G. *Merlin, the product of NF2 gene, is associated with aromatase expression and estrogen formation in human liver tissues and liver cancer cells*. J Steroid Biochem Mol Biol. 2016; 172: 222-230.

Curto M., Cole B.K., Lallemand D., Liu C.H., McClatchey A.I. *Contact-dependent inhibition of EGFR signaling by Nf2/Merlin*. J Cell Biol. 2007; 177: 893–903.

Deng H., Zhang X-T., Wang M.L., Zheng H.Y., Liu L.J., Wang Z.Y. *ERa36-mediated rapid estrogen signaling positively regulates ER-positive breast cancer stem/progenitor cells.* PloS One. 2014; 9: e88034.

Engstrom P.F., Levin B., Moertel C.G., Schutt A. *A phase II of tamoxifen in hepatocellular carcinoma*, Cancer. 1990; 65: 2641-2643.

Gallo C., De Maio E., Di Maio M., Signoriello G., Daniele B., Pignata S., Annunziata A., Perrone F. *Tamoxifen is not effective in good prognosis patients with hepatocellular carcinoma.* BMC Cancer. 2006; 6: 196.

Iavarone M., Lampertico P., Seletti C. et al. *The clinical and pathogenetic significance of estrogen receptor beta expression in chronic liver diseases and liver carcinoma*. Cancer. 2003; 98: 529-534.

James M.F., Han S., Polizzano C., Plotkin S.R., Manning B.D., Stemmer-Rachmanimov A.O., Gusella J.F., Ramesh V. *NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth*. Mol Cell Biol. 2009; 29: 4250–4261.

Kissil J.L., Wilker E.W., Johnson K.C., Eckman M.S., Yaffe M.B., Jacks T. *Merlin, the product of the Nf2 tumor suppressor gene, is an inhibitor of the p21-activated kinase, Pak1*. Mol Cell. 2003; 12: 841–849.

Klair J.S., Yang J.D., Abdelmalek M.F. et al. *A longer duration of estrogen deficiency increases fibrosis risk among postmenopausal women with nonalcoholic fatty liver disease*. Hepatology. 2016; 64: 85-91.

Koh W.P., Yuan J.M., Wang R., Govindarajan S., Oppenheimer R., Zhang Z.Q., Yu M.C., Ingles S.A. *Aromatase (CYP19) promoter gene polymorphism and risk of nonviral hepatitis-related hepatocellular carcinoma*. Cancer. 2011; 117: 3383-3392.

Laroche G. *Liver and sex hormones*. Rev. Int. Hépatologie 1953; 3: 579–601.

Mc Clatchey A.I., Fehon R.G. *Merlin and the ERM proteins – regulators of receptor distribution and signaling at the cell cortex*. Trends Cell Biol. 2009; 19: 198–206.

Miceli V., Cocciadiferro L., Fregapane M., Zarcone M., Montalto G., Polito L.M., Agostara B., Granata O.M., Carruba G. *Expression of wild-type and variant estrogen receptor alpha in liver carcinogenesis and tumor progression*. Omics J Integr Biol. 2011; 15: 313–317.

Nagasue N., Ito A., Yukaya H., Ogawa Y. *Estrogen receptors in hepatocellular carcinoma*. Cancer. 1986; 57: 87-91.

Nagasue N., Kohno H. *Hepatocellular carcinoma and sex hormones*. HPB Surg World J Hepatic Pancreat Biliary Surg. 1992; 6: 1–6.

Ohnishi S., Murakami T., Moriyama T., Mitamura K., Imawari M. *Androgen and estrogen receptors in hepatocellular carcinoma and in the surrounding noncancerous liver tissue*. Hepatology. 1986; 6: 440-443.

Parthasarathy C., Renuka V.N., Balasubramanian K. *Sex steroids enhance insulin receptors and glucose oxidation in Chang liver cells*. Clin Chim Acta. 2009; 399: 49-53.

Patel S. *Disruption of aromatase homeostasis as the cause of a multiplicity of ailments: A comprehensive review.* J Steroid Biochem Mol Biol. 2017; 168: 19-25.

Poyard T., Ratziu V., Charlotte F., Goodman Z., McHutchinson J., Albrecht J. *Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis C*. J Hepatol. 2001; 34: 730-739.

Shaw R.J., Paez J.G., Curto M., Yaktine A., Pruitt W.M., Saotome I., O'Bryan J.P., Gupta V., Ratner N., Der C.J., Jacks T., Mc Clatchey A.I. *The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling*. Dev Cell. 2001; 1: 63–72.

Vendrell J.A., Magnino F., Danis E., Duchesne M.J., Pinloche S., Pons M., Birnbaum D., Nguyen C., Theillet C., Cohen P.A. *Estrogen regulation in human breast cancer cells of new downstream gene targets involved in estrogen metabolism, cell proliferation and cell transformation*. J Mol Endocrinol. 2004; 32: 397-414.

Viedma-Rodríguez R., Baiza-Gutman L., Salamanca-Gómez F., Diaz-Zaragoza M., Martínez-Hernández G., Ruiz Esparza-Garrido R., Velázquez-Flores M.A., Arenas-Aranda D. *Mechanisms associated with resistance to tamoxifen in estrogen receptor-positive breast cancer (review)*. Oncol Rep. 2014; 32: 3-15.

Villa E., Camellini L., Dugani A., Zucchi F., Grottola A., Merighi A., Buttafoco P., Losi L., Manenti F. *Variant estrogen receptor messanger RNA species detected in human primary hepatocellular carcinoma*. Cancer Res. 1995; 55: 498-500.

Wang Z., Zhang X., Shen P., Loggie B.W., Chang Y., Deuel T.F. *A variant of estrogen receptor-a, hERa36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling*. Proc Natl Acad Sci U.S.A. 2006; 103: 9063-9068.

Williams G.P. *The role of oestrogen in the pathogenesis of obesity, type 2 diabetes, breast cancer and prostate disease*. Eur J Cancer Prev. 2010; 19: 256-271.

Diet, Inflammation and Cancer: A Journey from Prevention to Treatment

MASSIMO LIBRA, FERDINANDO NICOLETTI

Department of Biomedical and Biotechnological Sciences, Oncologic, Clinic and General Pathology Section, University of Catania; Torre Biologica, Via Santa Sofia, 97,95123 Catania, Italy.
Correspondence to Massimo Libra, Head of the Translational Oncology & Functional Genomics Laboratory, Department of Biomedical and Biotechnological Sciences, Oncologic, Clinic and General Pathology Section, University of Catania; Torre Biologica, Via Santa Sofia, 97, 95123 Catania, Italy; e-mail: m.libra@unict.it.

Abstract

Unhealthy dietary habits are considered as potential risk factors in cancer development and progression. Often, unhealthy diet is associated with overweigh, obesity and endocrine dysregulations that are all included as predictive and prognostic indicators for several cancer types. Accordingly, elevated intake of carbohydrate and fat are associated with initiation of hyperglycaemia, hyperinsulinemia and accumulation of adipose tissue with concomitant release of IGFs, sexual hormones and inflammatory cytokines. In the context of inflammation, accumulating evidences indicate that the pro-inflammatory cytokine MIF (macrophage migration inhibitory factor) may

play a role in specific biological activities related to cancer growth or contributing towards a microenvironment favouring cancer progression. The association between diet and inflammation has been recently confirmed by the identification of the Dietary Inflammatory Index (DII). It has been identified to assign a quantitative value to the pro-inflammatory nutrients. DII may be considered a valuable indicator for cancer prevention and treatment. Further studies are essential to assess whether healthy nutritional attitude, together with physical activity and bioactive compounds consumption, can affect quality of life, response to treatment and individual epigenetics.

Key Words

Cancer, Diet, Genetics, Inflammation.

State of the art

Cancer is a genetic disease, but it is now well known that the genetic abnormalities from which cancer arises are for less than 10% caused by inherited characteristics. This means that more than 90% of the cases are due to several environmental risk factors [Anand et al., 2008]. Up to 30% of the global incidence is addressed to unhealthy dietary habits (Figure 1).

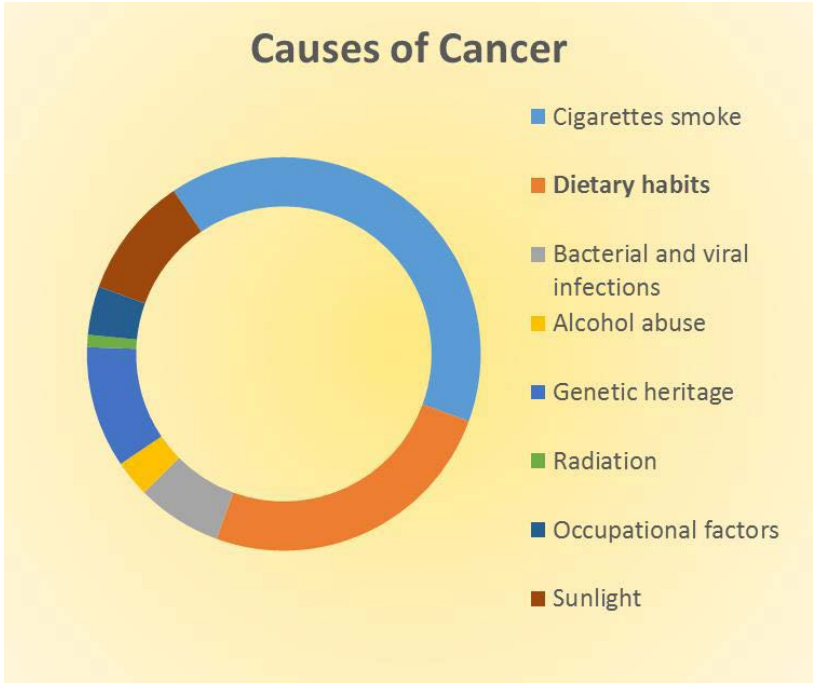

Figure 1. Cancer as multifactorial disease. Cancer is a multifactorial disease associated with several risk factors. Among those, dietary habits account for up to 30% of the cases (orange) while genetic heritage is displayed by less than 10% (blue).

Together with a better clue on how risk factors influence cancer incidence, the last three decades have seen an exponential increased interest towards the relationship between cancer and diet. In vitro and in vivo experiments offer a great insight of specific patterns at the mole-

cular level, but this context suffer of the complex combination between dietary habits, endocrinological conditions, physical activity and overall fitness of each individual. For this reason, case-control epidemiological approach result as the best option to weigh risk factors in cancer.

Major concern orbits around carbohydrates and fat disproportionate consumption compared to the body energy requests, which eventually leads to increased body weight and insulin overproduction due to constant high glucose levels in blood. Purpose of this paper is to underline the molecular mechanisms triggered by these risk factors and what can be done in order to prevent and moderate the latter consequences of unhealthy dietary habits.

Discussion

Once it is assessed that cancer is an environmental disease, we can focus on how diet influence its onset and progression. One direct method to estimate whether this energetic unbalance is present or not, is to characterize patients through the Body Mass Index (BMI) [World Cancer Research Fund,2007]. Even though it does not take in count lean mass and bone weight, a significant link between BMI and cancer risk has been found, particularly to specific cancer types in a gender-related way. A 5 kg/m^2 increase in BMI resulted in a higher relative risk (RR) up to 1.52 ($p<0.0001$) in men and 1.59 ($p<0.0001$) in women [Renehan et al., 2008]. Carbohydrates and fats represent the main energy sources and consequently main reason to weight gain when not used, thus deep investigations have focused on those macronutrients.

Carbohydrates

Whether they are simple or complex, sugar metabolism is a multiorgan concerted process. The easiest way to describe it, is to portray a fasted or fed individual.

A fasting individual experiences a reduction of glucose concentration in the blood, this stimulus induces the release of glucagon by the alpha cells of the Islets of Langerhans of the pancreas. The liver responds to glucagon converting glycogen to glucose and freeing it into the bloodstream. Post-prandial levels of sugar in the blood are high and this leads to the release of insulin by the beta cells of the

pancreas. Insulin reaches target organs, mainly muscles and liver, and through the externalization of specific transporters, cells become competent to glucose uptake. High cytoplasmic glucose concentration trigger increased glycolysis as immediate source of energy and glycogen synthesis and fatty acid synthesis as a store of energy for later use during fasting time.

In obese once, adipose tissue exceeds normal percentage and release supraphysiological concentration of nonesterified fatty acid or free fatty acids (FFAs). In these conditions, their oxidation and usage as energy source is preferred to sugar transport and glycolysis, even when insulin has been released. Thus, tissues become less sensitive to insulin and what is eventually obtained is a constant hyperglycaemia, due to the inability to use glucose as energy and hyperinsulinemia in the attempt to reduce blood glucose levels. Latter consequence is the gain of insulin resistance and development of diabetes mellitus type-2 [Westley & May, 2013].

Low sensitivity to insulin, drives a change in the expression of the insulin receptor (IR) towards its isoforms, A or B depending on different post-transcriptional modification [Ullrich et al., 1985]. The two isoforms have different distribution patterns [Moller et al., 1989; Mosthaf et al., 1990]: the isoform B is prevalent in typical insulin target organs, liver and muscles, while isoform

A is more expressed by non-canonical insulin target tissues, including certain cancer types like breast [Sciacca et al., 1999], ovary [Kalli, 2002], prostate [Cox et al., 2009] and thyroid [Vella et al., 2001]. IR and type I IGF receptors seem to share ancestral origin as they show high grade homology. Their respective ligands also display a certain grade of similarity [Westley & May, 2013] and, even though they bind the receptors with different affinity, free IGF-1 and IGF-2 can bind suitably isoforms of insulin receptors as well as insulin can bind IGF receptors and its heterodimeric forms. In these conditions, through the activation of the type-I IGF receptor, insulin can display not just the endocrine anabolic profile, but also the promotion of cell survival, differentiation and migration [Morcavallo et al., 2011]. On the other hand, the opposite scenario is also seen: where the free IGF-I and IGF-II bind isoforms of IR, mitogen pathways are activated [Alvino, 2011; Morrione,1997] (Figure 2). The proliferation and apoptosis escape mechanisms are achieved through insulin receptor substrates activation which triggers the downstream signalling of both MAPK and AKT pathways.

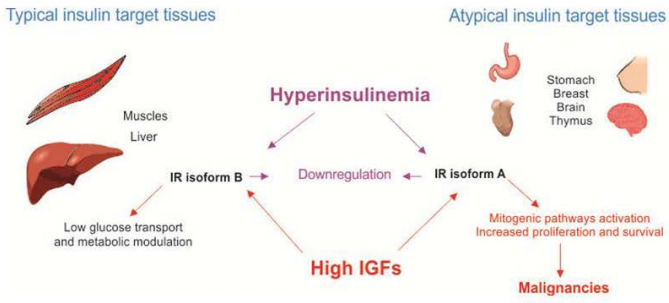

Figure 2. Obesity induced fat oxidation. Obesity induces fat oxidation instead of the glycolytic process leading eventually to hyperglycaemia and hyperinsulinemia. Expression of two different isoforms of the insulin receptor varies depending on the examined tissues. The IGFs bind the isoform A with a significant affinity and lead the activation of mitogenic pathways against apoptosis.

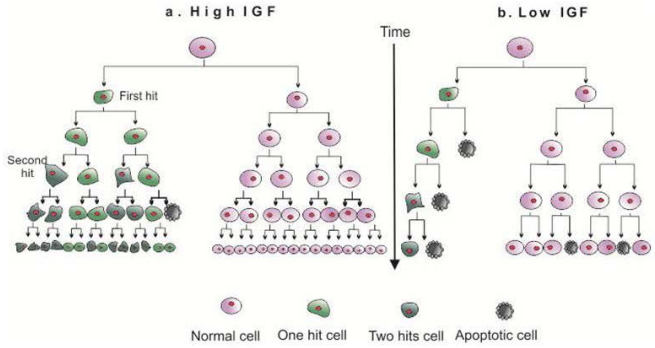

Figure 3. Schematic model of stepwise mutations selection under high or low levels of IGFs. During the same time course, subjects with higher levels of IGFs, especially IGF-1, have higher rate of replication and lower response to apoptosis mechanisms. Consecutively, in terms of probability, there is a higher chance of mutated cells. They might undergo to the first hit, survive cell death and eventually a second hit leading the selection of the neoplastic phenotype.

In normal subject, IGFs are released by the liver under the stimulation of the growth hormone and serum levels of IGF is kept at certain concentrations thanks to insulin-like growth factor binding proteins (IGFBPs). Physi-

ological levels guarantee the balance between proliferation, accumulation of somatic modifications and apoptosis. In overweighed and obese individual, the adipose tissue contributes to the release of free IGFs increasing the circulating concentration. These higher levels shorten the timeframe needed for somatic mutations to accumulate. Mutations give survival advantages to the cells; therefore, these are positively selected and undergo malignant progression [Pollak et al., 2004] (Figure 3). It has been seen that cancer itself can achieve the production of IGFs, enhancing the effects of its high levels in a paracrine and autocrine manner [Pollak et al., 2004].

Lipid

Adipose tissues can be divided in two classes, depending on the role solved in the human body [Saely et al., 2012]: brown adipose tissue, with high catabolic functions breaks down triglycerides basically to maintain thermal homeostasis, and white adipose tissue, believed to be the reservoir of disposable energy in form of triglycerides, is now also known to behave as endocrine regulator. [Trayhurn & Beattie, 2001].

White adipose tissue (WAT) can significantly vary in quantities and distribution across age and gender, but visceral distribution has been correlated to cardiovascular disease and diabetes. Moreover, in obese subjects it has shown to be associated with insulin resistance while subcutaneous adipose tissue has not [McLaughlin et al., 2011].

The endocrine function of WAT is exploited by the so called adipokines which may be typical of the adipose tissue or not [Falcao-Pires et al., 2012]. Macrophages M2 infiltration is present and the enzyme aromatase which is responsible for the transformation of sexual hormones is expressed [Westley & May, 2013]. As obese subjects carry a greater amount of WAT than lean individuals, the significance of the endocrine function is magnified; moreover, they display hypertrophy and hyperplasia of the organ which undergoes a chronic inflammatory state and a shift in the macrophages population, promoted by hyperinsulinemia, from M2 to M1 (pro-inflammatory) is also seen [Olefsky & Glass, 2010] (Figure 4). During these chronic inflammatory conditions, the adipose tissue release specific adipokine as IGF-1, leptin, TNF-α and IL-6 to address signalling for survival and cell proliferation on those targets typically involved in cancer onset and promotion: PI3K, MAPK, IKK/NF-κB and STAT3 [Doyle et al, 2012]. Interesting experiment on rats has been conducted, to elegantly show how population sensitive to diet-induced obesity, are prone to develop a pre-neoplastic en-

vironment in kidneys [Stemmer et al., 2012] and epidemiological study also has shown correlation between increased levels of adipokine and cancer compared to healthy controls [Hillenbrand et al., 2012].

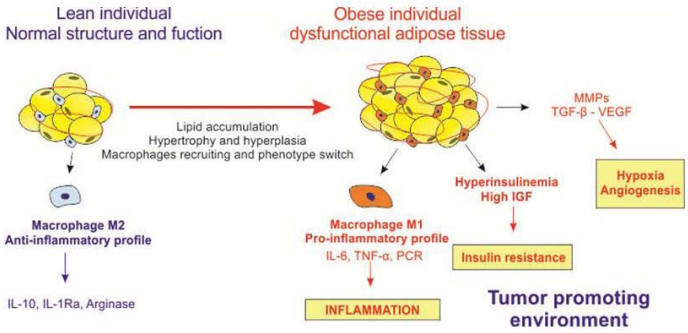

Figure 4. Exceeding adipose tissue displays impaired functionalities. Compared to the anti-inflammatory profile of M2 macrophages, M1 macrophages activate the inflammatory immune response, favouring a pro-tumorigenic ambient.

Macrophage migration inhibitory factor (MIF) and inflammation

MIF is a lymphokine involved in cell-mediated immunity, immunoregulation, and inflammation. In particular, MIF plays a role in the regulation of macrophage function in host defence through the suppression of anti-inflammatory effects of glucocorticoids. Several studies shown that MIF is involved various metabolic pathways that regulate lipid metabolism and insulin secretion and thus influences the processes leading to obesity [Finucane et al, 2012]. Other studies shown that the plasma concentrations of MIF and MIF mRNA are increased in mononuclear cells in the obese [Dandona et al., 2004] suggesting a direct correlation between the consumption of a hyper-lipid diet, the concentration of MIF and the development of obesity due to the acquired insulin resistance. Indeed, it has been shown that obesity induced by hyper-lipid diet causes a state of chronic low-grade inflammation characterised by a progressive infiltration of macrophages, into obese adipose tissue. In first stage of obesity, M2 anti-inflammatory macrophages acquire an M1 pro-inflammatory phenotype which is associated with the production of inflammatory cytokines (IL-6, IL-1β and TNF-α). Finally, this chronic inflammation status may lead to the development of type-2 diabetes mellitus and cancer.

Besides this molecular mechanism, MIF favours tumor microenvironment and tumor growth acting at different molecular levels. MIF is able to inhibit p53 accumulation. Increased levels of MIF down regulated p53 and suppressed its nuclear localization blocking its transcriptional activity. Furthermore, animal models shown that MIF is able to inhibit senescence and apoptosis induced by serum starvation. All this conditions lead to an increased risk of cell neoplastic transformation [Hudson et al., 1999].

Other studies shown that MIF is overexpressed in several cancer types and regulates molecular signalling pathways. In particular, in animal and cell models of intestinal and colorectal cancer MIF enhances tumor cell proliferation via activation of MAPK. Indeed, MIF stimulates of p42/p44 MAPK phosphorylation through PKA resulting in the production of the prostaglandin precursor arachadonic acid, substrate for COX-2. The activation of this molecular pathway results in the further inhibition of p53 sustained by MIFCOX-2 now known to be induced as an important element of p53 inhibition by MIF [Wilson et al., 2005].

Finally, MIF promotes the carcinogenesis supporting hypoxia and angiogenesis, frequently associated with the tumor microenvironment. In particular, the hypoxia promotes the transcription of HIF-1α (hypoxia inducible factor) resulting in an increased production of angiogenetic factor, such as VEGF. Even MIF is a direct transcriptional target of HIF-1α and it is useful to stabilize HIF-1α. The up-regulation of MIF in hypoxic condition enhances angiogenetic processes through MAP kinases MIF-induced activation that leads to the differentiation of endothelial cells to blood vessels [Amin et al., 2003]. This hypothesis is supported by another study showing that MIF over-expression correlates with IL-8 and VEGF expression associated with angiogenesis [Xu et al., 2008; Conroy et al, 2010].

Dietary inflammatory index (DII) and cancer

As we eat, we introduce in our body elements able to affect the metabolic environment in a positive or negative way. Some food can introduce pro-inflammatory agents, while others are known to be antioxidant, photoprotective and so on [Giugliano et al., 2006; Cui et al., 2012]. In the prospective to quantitively state the pro-inflammatory abilities of foods as cancer risk factors, a first attempt of an algorithm elaboration was conducted by Cavicchia et al., [2009]. After a literature research from 1950 to 2009, the algorithm has been formulated based on 929 articles, concerning inflammatory markers as L-1β, IL-4, IL-6, IL-10, TNF-α and CRP, that matched specific criteria

and they eventually scored whole food, common macronutrients, bioactive compounds as well as energy contribution. The validation of the algorithm has been conducted on a total of 519 patients, measuring their concentration of high sensitive CRP (hs-CRP) as indicator of vascular inflammation induced by interleukin, such as IL-6, and cardiovascular dysfunctionality. The results show that a 5 points highly scored regimen, associated with anti-inflammatory nutrients, was significantly correlated to lower hs-CRP, thus diet may have a protective role against those diseases receptive for inflammation. On 2014, the algorithm has been upgraded by Shivappa et al., [2014] in three ways: first including latest articles, second identifying eleven data set from different population to have a comparative parameter of food consumption habit, third using percentile of the food score to generate a food parameter-specific DII scores depending on regional data set (Table 1).

Table 1. DII scoring of common nutrients and bioactive compounds. [Shivappa et al, 2014]

Food parameter	**Raw inflammatory effect Score**	**Overall inflammatory effect score**	**Globaldaily meanintake**	**SD**
Carbohydrate	0.109	0.097	272.2	40.0
Cholesterol	0.347	0.110	279.4	51.2
Garlic	-0.412	-0.412	4.35	2.90
Ginger	-0.588	-0.453	59.0	63.2
Saturated fat	0.429	0.373	28.6	8.0
Vitamin D	-0.446	-0.446	6.26	2.21

SD = standard deviations across the eleven international data sets.

Applications of the DII have already appeared in literature; it performs as a good risk predictor to hepatocellular carcinoma [Shivappa et al., 2016a], nasopharyngeal carcinoma [Shivappa et al., 2016 b] and bladder cancer [Shivappa et al.,2017] but also as a prognostic indicator in prostate cancer [Zucchetto et al., 2016]. Estimation of the overall DII score for each subject may be a reliable tool, for both general practitioners and oncologist, to assess the cancer risk and, on the other hand, to introduce dietary anti-inflammatory compounds as part of the therapy.

Dietary habits as part of the treatment

One of the strongest evidence of the concurrence of obesity-related endocrine impairments and cancer is given by breast cancer. BMI has been inversely associated to overall survival or recurrence free survival, or both [Rock & Demark-Wahnefried, 2002]. The trend followed by subjects with high Waist-to-Hip Ratio, common in diabetic or prediabetic, shows poor prognostic outcomes [Bosetti et al., 2012]. Noteworthy is the weight gain after diagnosis, often reported for those patients treated with adjuvant chemotherapy, which also has been related to decreased survival and risk of recurrence [McTiernan et al., 2010].

The assumptive link between positive energy unbalance and breast cancer seems to be uncovered by three main mechanisms: first, augmented activation of mitogen estrogen receptors due to the higher concentration of circulating sexual hormones, like estradiol and estrone, released by adipocytes expressing aromatase [Westley & May, 2013; De Marco et al., 2015]; second, chronic hyperinsulinemia, often featured by overweighed, obese, type-2 diabetic subject which, as described earlier, triggers anti-apoptotic and hyperproliferative mechanisms through both IR and IGFRs expressed in atypical target tissue and malignancies; third, hyperglycaemia as a carrier of oxidative stress [Fiorentino et al., 2013; Lin et al, 2005], highly involved in free radical-related DNA damage and cancer indeed [Halliwell, 2007; Gilardini et al., 2016].

Stepwise arrangement of the treatments, have eventually led the studies to introduce healthy dietary habits and physical activity as potential key player to ameliorate the underlying mechanisms beneath the increased risk and poorer outcomes for those subjects with endocrine dysregulation, especially for breast cancer patients. Pioneer in this approach has been the Women's Intervention Nutrition Study (WINS) [Blackburn &Wang KA, 2007], which also underline different impact depending on subtypes. More studies merged on the topic, as the Women's Healthy Eating and Living (WHEL) study [Pierce et al., 2002] which investigate whether adding more service per day of fruits and vegetables could affect positively the overall survival and the event-free survival or the Muscle mass, Omega-3, Diet, Exercise and Lifestyle (MODEL) study, that looks for inflammatory markers and improvement of quality of life [McDonald et al., 2014]. Results from all these studies result controversial and often discussed

but, for instance, additional data elaboration on WHEL study deduced that physical activity at baseline and in long term prospective was statistically significant in reduction of mortality and that the adherence to the physical tasks 1-year after intervention were low, probably affecting the results of the study itself. [Cadmus Bertram et al., 2011]. More studies suggest than, that the bearing of healthy eating and exercising might be different on distinct subtypes, having greater effect on those positive to hormone receptors [Pierce et al., 2007; Crispo et al., 2017].

To weigh up the advantages of combining a balanced diet, the improvement of fitness conditions through exercise and the efficacy of bioactive compounds, an ongoing clinical trial on breast cancer patients aims to: determine the magnitude of disease free survival and the epigenetic implications of a life style change, through miRNAs modulation [Augustine et al., 2017].

After the computational analyses on the differentially expressed miRNAs involved in breast cancer development, four of those have been selected as they are involved in both obesity and breast cancer. This study has the advantage to do not rely just on interview and common blood samples, but it collects also details from the epigenome to either check the depth of the intervention.

Conclusion

The dominant influence on cancer development lies on environmental factors. Given that we cannot change the genetic component in this life-threatening disease, we surely can do a lot on preventing it. High BMI and pro-inflammatory dietary habit have been associated significantly to increased cancer risk and worse prognostic outcomes. Introducing diet and lifestyle modifications as relevant parts of the therapy against cancer, health care providers can participate in a new epigenetic approach to treatments and prospectively ameliorate the overall health of the patients.

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References

Alvino C.L., Ong S.C,. McNeil K.A., Delaine C., Booker G.W., Wallace J.C., Forbes B.E. *Understanding the mechanism of insulin and insulin-like growth factor (IGF) receptor activation by IGF-II*. PLoS One. 2011, 6.

Amin M.A., Volpert O.V., Woods J.M., Kumar P., Harlow L.A., Koch A.E. *Migration inhibitory factor mediates angiogenesis via mitogen-activated protein kinase and phosphatidylinositol kinase*. Circ Res. 2003; 93: 321–9.

Anand P., Kunnumakkara A.B., Sundaram C., Harikumar K.B., Tharakan S.T., Lai O.S., Sung B., Aggarwal B.B. *Cancer is a preventable disease that requires major lifestyle changes*. Pharm Res. 2008, 25: 2097.

Augustin L., Libra M., Crispo A., Grimaldi M., De Laurentiis M., Rinaldo M., D'Aiuto M., Catalano F., Banna G., Ferrau F., Rossello R., Serraino D., Bidoli E., Massarut S., Thomas G., Gatti D., Cavalcanti E., Pinto M., Riccardi G., Vidgen E., Kendall C.W., Jenkins D.J., Ciliberto G., Montella M. *Low glycemic index diet, exercise and vitamin D to reduce breast cancer recurrence (DEDiCa): design of a clinical trial*. BMC Cancer. 2017, 17: 69

Blackburn G.L., Wang K.A. *Dietary fat reduction and breast cancer outcome: results from the Women's Intervention Nutrition Study (WINS)*. Am J Clin Nutr. 2007, 86: 878-81.

Bosetti C., Rosato V., Polesel J., Levi F., Talamini R., Montella M., Negri E., Tavani A., Zucchetto A., Franceschi S., Corrao G., La Vecchia C. *Diabetes mellitus and cancer risk in a network of case-control studies*. Nutr Cancer. 2012, 64: 643-51.

Bertram L.A., Stefanick M.L., Saquib N., Natarajan L., Patterson R.E., Bardwell W., Flatt S.W., Newman V.A., Rock C.L., Thomson C.A., Pierce J.P. *Physical activity, additional breast cancer events, and mortality among early-stage breast cancer survivors: findings from the WHEL Study*. Cancer Causes Control. 2011; 22: 427-35.

Cavicchia P.P., Steck S.E., Hurley T.G., Hussey J.R., Ma Y., Ockene I.S., Hébert J.R. *A new dietary inflammatory index predicts interval changes in serum high-sensitivity C-reactive protein*. J Nutr. 2009; 139: 2365-72.

Conroy H., Mawhinney L., Donnelly S.C. *Inflammation and cancer: macrophage migration inhibitory factor (MIF)--the potential missing link*. QJM. 2010, 103: 831-6.

Cox M.E., Gleave M.E., Zakikhani M., Bell R.H., Piura E., Vickers E., Cunningham M., Larsson O., Fazli L., Pollak M. *Insulin receptor expression by human prostate cancers*. Prostate. 2009, 69: 33–40.

Crispo A., Augustin L.S., Grimaldi M., Nocerino F., Giudice A., Cavalcanti E., Di Bonito M., Botti G., De Laurentiis M., Rinaldo M., Esposito E., Riccardi G., Amore A., Libra M., Ciliberto G., Jenkins D.J., Montella M. Risk *Differences Between Prediabetes and Diabetes According To Breast Cancer Molecular Subtypes*. J Cell Physiol. 2017, 232: 1144-1150.

Cui X., Jin Y., Singh U.P., Chumanevich A.A., Harmon B., Cavicchia P., Hofseth A.B., Kotakadi V., Poudyal D., Stroud B., Volate S.R., Hurley T.G., Hebert J.R., Hofseth L.J. *Suppression of DNA damage in human peripheral blood lymphocytes by a juice concentrate: a randomized, double-blind, placebo-controlled trial*. Mol Nutr Food Res. 2012; 56: 666–670.

Dandona P., Aljada A., Ghanim H., Mohanty P., Tripathy C., Hofmeyer D., Chaudhuri A. *Increased plasma concentration of macrophage migration inhibitory factor (MIF) and MIF mRNA in mononuclear cells in the obese and the suppressive action of metformin*. J Clin Endocrinol Metab. 2004, 89: 5043–5047.

Doyle S.L., Donohoe C.L., Lysaght J., Reynolds J.V. *Visceral obesity, metabolic syndrome, insulin resistance and cancer*. Proc Nutr Soc. 2012; 71: 181-9.

Falcão-Pires I., Castro-Chaves P., Miranda-Silva D., Lourenço A.P., Leite-Moreira A.F. *Physiological, pathological and potential therapeutic roles of adipokines*. Drug Discov Today. 2012; 17: 880-9.

Finucane O.M., Reynolds C.M., Mc Gillicuddy F.C., Roche H.M. *Insights into the role of macrophage migration inhibitory factor in obesity and insulin resistance*. Proc Nutr Soc. 2012, 71: 622-33.

Fiorentino T.V., Prioletta A., Zuo P., Folli F. *Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases*. Curr Pharm Des. 2013, 19: 5695-703.

Gilardini Montani M.S., Granato M., Cuomo L., Valia S., Di Renzo L., D'Orazi G., Faggioni A., Cirone M. *High glucose and hyperglycemic sera from type 2 diabetic patients impair DC differentiation by inducing ROS and activating Wnt/β-catenin and p38 MAPK*. Biochim Biophys Acta. 2016, 1862: 805-13.

Giugliano D., Ceriello A., Esposito K. *The effects of diet on inflammation: emphasis on the metabolic syndrome*. J Am Coll Cardiol. 2006, 48: 677–685.

Halliwell B. *Oxidative stress and cancer: have we moved forward*? Biochem J. 2007, 401: 1-11.

Hillenbrand A., Fassler J., Huber N., Xu P., Henne-Bruns D., Templin M., Schrezenmeier H., Wolf A.M., Knippschild U. *Changed adipocytokine concentrations in colorectal tumor patients and morbidly obese patients compared to healthy controls*. BMC Cancer. 2012, 12: 545.

Hudson J.D., Shoaibi M.A., Maestro R., Carnero A., Hannon G.J., Beach D.H. *A proinflammatory cytokine inhibits p53 tumor suppressor activity*. J Exp Med. 1999; 190: 1375–82.

Kalli K.R., Falowo O.I., Bale L.K., Zschunke M.A., Roche P.C., Conover C.A. *Functional insulin receptors on human epithelial ovarian carcinoma cells: implications for IGF-II mitogenic signaling*. Endocrinology. 2002; 143: 3259-67.

Lin Y., Berg A.H., Iyengar P., Lam T.K., Giacca A., Combs T.P., Rajala M.W., Du X., Rollman B., Li W., Hawkins M., Barzilai N., Rhodes C.J., Fantus I.G., Brownlee M., Scherer P.E. *The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species*. J Biol Chem. 2005, 280: 4617-26.

McDonald C., Bauer J., Capra S., Coll J. *The muscle mass, omega-3, diet, exercise and lifestyle (MODEL) study - a randomised controlled trial for women who have completed breast cancer treatment*. BMC Cancer. 2014, 14: 264.

McLaughlin T., Lamendola C., Liu A., Abbasi F. *Preferential fat deposition in subcutaneous versus visceral depots is associated with insulin sensitivity*. J Clin Endocrinol Metab. 2011; 96: E1756-60.

Mc Tiernan A., Irwin M., Vongruenigen V. Weight. *Physical Activity, Diet, and Prognosis in Breast and Gynecologic Cancers*. J Clin Oncol. 2010, 28: 4074–4080.

Moller D.E., Yokota A., Caro J.F., Flier J.S. *Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man*. Mol Endocrinol 1989, 3: 1263-1269.

Morcavallo A., Gaspari M., Pandini G., Palummo A., Cuda G., Larsen M.R., Vigneri R., Belfiore A. *Research resource: New and diverse substrates for the insulin receptor isoform A revealed by quantitative proteomics after stimulation with IGF-II or insulin*. Mol Endocrinol. 2011; 25: 1456-68.

Morrione A., Valentinis B., Xu S.Q., Yumet G., Louvi A., Efstratiadis A., Baserga R. *Insulin-like growth factor II stimulates cell proliferation through the insulin receptor.* Proc Natl Acad Sci U S A. 1997; 94: 3777-82.

Mosthaf L., Grako K., Dull T.J., Coussens L., Ullrich A., Mc Clain D.A. *Functionally distinct insulin receptors generated by tissue-specific alternative splicing*. EMBO J. 1990; 9: 2409-13.

Olefsky J.M., Glass C.K. *Macrophages, inflammation, and insulin resistance*. Annu Rev Physiol. 2010; 72: 219-46.

Pérez-Hernández A.I., Catalán V., Gómez-Ambrosi J., Rodríguez A., Frühbeck G. *Mechanisms linking excess adiposity and carcinogenesis promotion*. Front Endocrinol (Lausanne). 2014; 5: 65.

Pierce J.P., Stefanick M.L., Flatt S.W., Natarajan L., Sternfeld B., Madlensky L., Al-Delaimy W.K., Thomson C.A., Kealey S., Hajek R., Parker B.A., Newman V.A., Caan B., Rock C.L. *Greater survival after breast cancer in physically active women with high vegetable-fruit intake regardless of obesity*. J Clin Oncol. 2007; 25: 2345-51.

Pierce J.P., Faerber S., Wright F.A., Rock C.L., Newman V., Flatt S.W., Kealey S., Jones V.E., Caan B.J., Gold E.B., Haan M., Hollenbach K.A., Jones L., Marshall J.R., Ritenbaugh C., Stefanick M.L., Thomson C., Wasserman L., Natarajan L., Thomas R.G., Gilpin E.A. *Women's Healthy Eating and Living (WHEL) study group. A randomized trial of the effect of a plant-based dietary pattern on additional breast cancer events and survival: the Women's Healthy Eating and Living (WHEL) Study*. Control Clin Trials. 2002; 23: 728-56.

Pollak M.N., Schernhammer E.S., Hankinson S.E. *Insulin-like growth factors and neoplasia*. Nat Rev Cancer. 2004; 4: 505-18.

Renehan A.G., Tyson M., Egger M., Heller R.F., Zwahlen M. *Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies*. Lancet. 2008; 371: 569-78.

Rock C.L., Demark-Wahnefried W. *Nutrition and Survival after the Diagnosis of Breast Cancer: A Review of the Evidence*. J Clin Oncol. 2002; 20: 3302–3316.

Saely C.H., Geiger K., Drexel H. *Brown versus white adipose tissue: a mini-review*. Gerontology. 2012, 58: 15–23.

Sciacca L., Costantino A., Pandini G., Mineo R., Frasca F., Scalia P., Sbraccia P., Goldfine I.D., Vigneri R., Belfiore A. *Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism*. Oncogene, 1999; 18: 2471–2479.

Shivappa N., Hébert J.R., Rosato V., Rossi M., Libra M., Montella M., Serraino D., La Vecchia C. *Dietary Inflammatory Index and Risk of Bladder Cancer in a Large Italian Case-control Study*. Urology. 2017; 100: 84-89.

Shivappa N., Hébert J.R., Polesel J., Zucchetto A., Crispo A., Montella M., Franceschi S., Rossi M., La Vecchia C., Serraino D. *Inflammatory potential of diet and risk for hepatocellular cancer in a case-control study from Italy.* Br J Nutr. 2016a; 115: 324-31.

Shivappa N., Hébert J.R., Zucchetto A., Montella M., Libra M., Garavello W., Rossi M., La Vecchia C., Serraino D. *Increased Risk of Nasopharyngeal Carcinoma with Increasing Levels of Diet-Associated Inflammation in an Italian Case-Control Study*. Nutr Cancer. 2016b; 68: 1123-30.

Shivappa N., Steck S.E., Hurley T.G., Hussey J.R., Hébert J.R. *Designing and developing a literature-derived, population-based dietary inflammatory index*. Public Health Nutr. 2014; 17: 1689–1696.

Stemmer K., Perez-Tilve D., Ananthakrishnan G., Bort A., Seeley R.J., Tschöp M.H., Dietrich D.R., Pfluger P.T. *High-fat-diet-induced obesity causes an inflammatory and tumor-promoting microenvironment in the rat kidney*. Dis Model Mech. 2012; 5: 627-35.

Trayhurn P., Beattie J.H. *Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ*. Proc Nutr Soc. 2001; 60: 329–339.

Ullrich A., Bell J.R., Chen E.Y., Herrera R., Petruzzelli L.M., Dull T.J., Gray A., Coussens L., Liao Y.C., Tsubokawa M., Mason A., Seeburg P.H., Grunfeld C., Rosen O.M., Ramachandran J. *Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes*. Nature, 1985; 313: 756–761.

Vella V., Sciacca L., Pandini G., Mineo R., Squatrito S., Vigneri R., Belfiore A. *The IGF system in thyroid cancer: new concepts.* Mol Pathol. 2001; 54: 121–124.

Westley R.L., May F.E. *A twenty-first century cancer epidemic caused by obesity: the involvement of insulin, diabetes, and insulin-like growth factors*. Int J Endocrinol. 2013; 2013: 632461.

Wilson J.M., Coletta P.L., Cuthbert R.J., Scott N., MacLennan K., Hawcroft G., Leng L., Lubetsky J.B., Jin K.K., Lolis E., Medina F., Brieva J.A., Poulsom R., Markham A.F., Bucala R., Hull M.A. *Macrophage migration inhibitory factor promotes intestinal tumorigenesis*. Gastroenterology. 2005; 129: 1485-503.

World Cancer Research Fund, American Institute for Cancer Research. *Food, Nutrition, Physical Activity and the Prevention of Cancer: Global Perspective*. 2007.

Xu X., Wang B., Ye C., Yao C., Lin Y., Huang X., Zhang Y., Wang S. *Overexpression of macrophage migration inhibitory factor induces angiogenesis in human breast cancer*. Cancer Lett. 2008; 261: 147-57.

Zucchetto A., Gini A., Shivappa N., Hébert J.R., Stocco C., Dal Maso L., Birri S., Serraino D., Polesel J. *Dietary inflammatory index and prostate cancer survival*. Int J Cancer. 2016; 139: 398-404.

PhD Thesis Abstracts

Healthy eating as a strategy to achieve successful ageing: focus on Mediterranean diet and functional food

Anna Aiello (Tutor Prof. Giuseppina Candore).
Department of Pathobiology and Medical Biotechnologies, University of Palermo, Corso Tukory 211, 90134, Palermo, Italy.

People want to live longer. To date, it is impossible not to grow older, but it is desirable to get older in good health, avoiding age-related disabilities. To do this, it is necessary to have a healthy lifestyle which can limit the damage caused by the environmental hazards that face us each day. In humans, healthy ageing and longevity are modulated by a fortunate interaction between genetic and environmental factors. Regarding the latter, physical activity and healthy dietary habits are the most important modifiable factors that can affect the maintenance of a healthy ageing phenotype. A feasible diet that reduces the risk of ageing-related diseases, promoting health ageing and longevity, should be one that delivers refined carbohydrates and amino acids in a pro-ageing way, in activating or reducing specific molecular pathways. It is not necessary to determine the ideal composition of this diet, because it does already exist. It is the traditional "poor" MedDiet, which rich in nutrients and single foods, can act on ageing. The new findings presented in the experimental studies of this thesis could present a great opportunity for the food and farming industry, especially in Sicily, where local products like extra virgin olive oil, green olives, barley and opuntia ficus Indica, represent a great potential resource. In the era of many expensive and mysterious longevity elixirs, these resources could represent traditional, cheap, and accessible "healthy foods" for everyone. However, it is important to highlight that the interesting effects of nutraceuticals and functional foods could be considered a prevention for many age-related diseases, and not a solution.

Genomic and proteomic evaluation of visceral and subcutaneous adipose derived stem cells and functional roles of pluripotency transcription factors

Valentina Guarnotta (Tutor Prof. ssa Carla Giordano).
Biomedical Department of Internal and Specialistic Medicine, Section of endocrinology, Diabetology and Metabolism.

Introduction

Adipose derived stem cells (ADSCs) are plastic-adherent cells with multilineage capacity, isolated from the stromal vascular fraction (SVF) of adipose tissue. These cells exhibit the potential of unlimited proliferation as well as of differentiation along the mesenchymal lineage to produce adipocytes, osteoblasts and chondrocytes. According to some reports, ADSCs might differentiate into pancreatic beta cells, myocardiocytes and endothelial cells. In addition, ADSCs might be used for several clinical applications such as prevention of metabolic diseases (obesity and type 2 diabetes) through the modulation of adipogenesis and lipogenesis, muscle, bone and tissue regeneration, and transplantation.

Aim

Isolation, phenotypical and functional characterization and differentiation of human ADSCs and evaluation of the functional role of pluripotency transcription factors, Oct4, Sox2 and Nanog.

Materials and methods

Subcutaneous (SAT) and visceral adipose tissue (VAT) were obtained from 42 obese and 28 overweight patients, undergoing elective open-abdominal or laparoscopic surgery. Fat tissue fragments were minced and

digested by collagenase, type I. Material was filtered and adipocytes and free oil were separated from stromovascular components by centrifugation. The floating fraction was placed in culture flasks filled with DMEM/Ham's F12 1: 1 supplemented with 20% fetal bovine serum, and cells were incubated at 37uC in 5% CO2. The primary ADSC grown at the top and bottom of flask, were cultured for 7 days until the confluence (defined as passage 0), and were then split into 60- mm plates. SVF cells were also seeded at 60,000 cell/cm2 in ultra low adherent flasks (Corning, Avon, France). mRNA from VAT and SAT biopsies and spheres was isolated by using an RNeasy kit (Qiagen, Hamburg, Germany). Adipogenic, osteogenic and condrogenic differentiation, was induced by specific StemPro Differentiation Medium. Sphere forming capability was evaluated by plating cells in low adhesion plastic. Stem cell markers CD90 and CD105 were analyzed by flow cytometry and stem cell transcription factors NANOG, SOX2 and OCT3/4 were detected by immunoblotting and Real-Time PCR. Nanog and Sox-2 gene silencing were performed by stealth siRNA.

Results

ASCs isolated from the subcutaneous and visceral fat depots grew as a characteristic cell monolayer in culture dishes after 10 days. In ultra low adherent flasks no cell adhered to the bottom and only spheres were obtained in the supernatant. At RT-PCR spheres had a significant expression all of the stem cells markers such as Oct4, Sox2, Nanog, Thy-1, CD-73, CD-105, ABCG2 compared to SAT and VAT ($p< 0.001$). At Western blot, immunofluorescence and flow-cytometry the PCR data were confirmed. ADSCs differentiated towards adipogenic, chondrogenic, and osteogenic lineages and confirmed by Oil Red-O, Alizarin red and Alcianblu staining, respectively. NANOG silencing induced a significant OCT 3/4 ($70 \pm 0.05\%$) and SOX2 ($75 \pm 0.03\%$) down regulation, whereas SOX2 silencing did not affect NANOG gene expression.

Discussion

Adipose tissue is a good source of mesenchimal stem cells. ADSCs are able to differentiate in chondrocytes, adipocytes and osteocytes and express all of the stem cell markers. Nanog shows a hierarchical role in the complex transcription network that regulates the pluripotency and plasticity.

Biophysical investigation on therapeutic proteins (Chaperonins, Hsp60 and CCT/TRiC) involved in human diseases

Dario Spigolon (Tutor Prof. Maurizio Leone).
Institute of Biophysics, National Research Council, Palermo, Italy; 2Department of Physics and Chemistry, University of Palermo, Palermo, Italy.

Molecular chaperones are indispensable cellular components that assist folding and assembly of newly synthesized proteins, translocation of proteins across membranes, as well as refolding and degrading of misfolded and aggregated proteins. In the last few years, innovative therapeutic strategies targeting stability and functionality of chaperones have received great attention, particularly in the field of neurodegenerative diseases. Moreover, the growing number of diseases found linked to chaperone mutations, testifies to the importance of their role in the cellular protein-quality control mechanism. The investigation of the biophysical interactions between chaperones and specific proteins involved in diseases, including their structural and functional properties, are therefore a crucial step for both validating the chaperones' role in physiological and pathological state, and developing effective chaperones-based treatment approaches. In the present PhD thesis work, we studied two representative examples of human molecular chaperones, Hsp60 and CCT/TRiC, appertaining to the class of the so-called "chaperonins" (Cpns). They are large, hollow, ATP-dependent nanomachines that promote correct folding of a wide range of proteins. Heat shock protein (Hsp60) is a molecular chaperone that assists protein folding in mitochondria. Hsp60 can accumulate in the cytosol, in various pathological conditions (i.e., cancer and chronic inflammatory diseases). Here we studied its functional oligomeric equilibrium as compared to that of its well-known bacterial homolog GroEL. We also show that Hsp60 is capable of

inhibiting the fibrillogenesis of Aβ peptide (the protein involved in Alzheimer's disease). The probable inhibition mechanism operating at molecular level is discussed. CCT/TriC is a chaperon universally found in Archaea and eukaryotes. Several chaperonopathies linked to CCT loci are clinically well characterized and, for those due to non-lethal genetic mutations, there is considerable information on their mode of inheritance. A protein model has been recently developed to investigate the mechanism of a crippling hereditary sensory neuropathy, due to a point mutation (His147Arg) in CCT5, one of the eight subunits of the human CCT. Here we report quantitative information on the loss of structural stability impaired by the pathogenic mutation. Finally, we tested if an efficient inhibition of amyloid formation can be achieved by chaperone-like systems, like as α-Caseins, which is known to exert a stabilizing function through direct interaction (similar to small Hsps). In fact, the evaluation of the mechanism of chaperone-like activity proved helpful for better understanding the structural basis of the substrate binding in sHsps.

Multiparametric-MRIevaluationofcervicalnodesinpatients with head and neck squamous cell carcinoma (HNSCC)"

Chiara Tudisca (Tutor Prof. Massimo Midiri).
Department of Pathobiology and Medical Biotechnologies, Section of Radiological Sciences, University of Palermo, 90127, Palermo, Italy.

Magnetic Resonance Imaging (MRI) allow a multiparametric evaluation of cervical nodes in patients with head and neck (H&N) squamous cell carcinoma (SCC) with the aid of functional MRI sequences, as discriminators of benign from malignant lymph nodes in these patients.
Twenty-four patients with H&N SCC staged for nodal disease were included in the study. 10 healthy volunteer were used as controls. Axial DWI was performed by short tau inversion recovery (STIR) echo planar imaging and trace weighted images obtained b 0, 50, 100, 300, 600 and 1000. Apparent diffusion coefficient (ADC) was calculated by mono-exponential fitting from non-necrotic sections of abnormal and normal nodes using all 6 b-values (ADC_{6b}); b0-50-100 (ADC_{fast}) and b300-600-1000 (ADC_{slow}). For BOLD imaging a T2* weighted imaging was performed, initially whilst breathing room air and repeated after inhalation of 100% oxygen, using a conventional multiple-echo gradient echo sequence. T2* maps (on room air and 100% oxygen) were derived by performing fits of a standard exponential relaxation model ($S = Ke^{-TE/T2^*}$) to the data on a pixel-by-pixel basis. DCE-MRI was performed with a T1 weighted volumetric FLASH sequence. For each node a single slice of the DCE was selected, and a manually ROI was drawn at the level of the external capsule. Values were compared between abnormal and normal nodes using the Mann Whitney test.Median ADC_{6b}, ADC_{fast} and ADC_{slow} for normal nodes was 1.0, 1.76 and 0.98 x $10^{-3}mm^2s^{-1}$ respectively. Correspondingly median ADC_{6b}, ADC_{fast} and ADC_{slow} for abnormal nodes was 1.04, 1.56 and 1.04 x $10^{-3}mm^2s^{-}$

[1]. There was a significant difference of ADC_{fast} (p=0.046) but not for ADC_{6b} (p=0.16) or ADC_{slow} (p=0.098) between normal and abnormal tissue. T2* median values of nodal histograms on 100% oxygen compared with room air were significantly lower for metastatic nodes (p<0.001) but not significantly different for normal nodes (p=0.148). There was a significant difference between normal and metastatic T2* median values of nodal histograms on 100% oxygen (Mann-Whitney p=0.012); but not on room air (Mann-Whitney p=0.549). Among all the PK parameters K_{trans} was the best classifier of head and neck node metastasis.DWI ADC_{fast} differentiates malignant from benign nodes, in keeping with differences in nodal perfusion characteristics as well as K_{trans} value. The observed reduction in T2* in metastatic lymph nodes suggests a paradoxical increase in deoxyhaemoglobin concentration on breathing 100% oxygen.

Dissecting the microenvironment of Splenic Marginal Zone Lymphoma and Diffuse Large B cell Lymphoma to find new stromal and immunological predictive biomarkers and targets

Beatrice Belmonte (Tutor Prof. Vito Franco).
Department of Health Science Tumor Immunology Unit, Human Pathology Section, University of Palermo, Palermo, Italy.

The tumor microenvironment has gained a increasing interest for its recognition as a key factor in multiple stages of tumorigenesis and neoplastic progression, as well as in local resistance, immune-escaping, and distant metastasis both in solid and lymphoid neoplasms. Lymphomas represent an ideal setting to dissect the tumor microenvironment as these neoplasms arise from cells that depend on numerous highly concerted interactions with immune and stromal cells in the course of normal development. My project research aimed at dissecting the tumour-associated immunological and stromal microenvironment of B-cell malignancies, in particular of Splenic Marginal Zone Lymphoma (SMZL) and Diffuse Large B Cell Lymphoma (DLBCL), to identify new tools that could enable a more accurate diagnosis in thr early stages of disease, potential prognostic markers and also therapeutic targets. I have investigated the reciprocal influence of immune cells and stromal elements on the development and progression of SMZL, where the pathogenesis is related with a deregulated immunological stimulation, by focusing on the microenvironment of the bone marrow, which represents an elective disease localization endorsing diagnostic and prognostic relevance. Stromal and immunological compartments of the microenvironment were analyzed to identify new prognostic and therapeutic biomarkers in DLBCL.

For this present study 66 cases of SMZL, and 20 cases of DLBCL were selected, all diagnosed according to WHO criteria. The characterization of stromal and immunological compartments of microenvironment was assessed by morhological analysis and by immunohistochemistry.
On the basis of prognostic clinical score, SMZL patients were divided into three different risk categories based on three parameters, e.g. anemia, elevated LDH levels and hypoalbuminemia. Subsequently statistical analyses were performed to test whether features inherent with the peripheral blood analysis were associated with immunophenotipycal clonal expression. A correlation between low Hemoglobin concentration and CD5 and IgM clonal expression in the bone marrow biopsy of patients belonging to the intermediate/high risk categories, was detected. These data suggest that CD5 and/or IgM expression may be considered as hallmarks of progressive disease. Moreover, in the bone marrow SMZL infiltrates, the neoplastic clone was integrated in the maiority of cases within the pre-exiting vascular niche and in few cases, characterized by progressive disease, the clone formed pseudo-nodular Germinal Center-like structures, displacing the preexisting stroma, with the presence of mesenchymal cells with morphology and phenotype of follicular dendritic cells (CD23+/CD21+). Moreover, within such Germinal Center (GC)-like structures, scattered large B cells with centroblastic morphology and immunophenotypical expression of ki-67, Bcl6, and Bcl2 were detected. These analyses suggested that these stromal modifications might precur disease progression towards towards DLBCLs. Finally, to evaluate the influence of microenvironment in DLBCL, we investigated the stromal and immune tumour- associated microenviroment in the two cell of origin-subtypes of this lymphoma, GC- and non-GC-related. The stromal composition and the quality of the immune infiltrate could be linked with the histotype, although with variability. In fact, trends could be identified for GC and non-GC groups, as far as the infiltrating immune and stromal components were concerned.

A next generation sequencing approach for molecular diagnosis of monogenic dyslipidemias

Valeria Ingrassia (Tutor Prof. Maurizio Averna).
Biomedical Department of Internal and Specialistic Medicine; Molecular Diagnostic Biology Unit "A.O.U.P. of Palermo" Regional Reference Center for the Prevention, Diagnosis and Treatment of Rare Disease of Metabolism.

The main object of my work was the development of the NGS (Next Generation Sequencing) approach for the molecular diagnosis of monogenic dyslipidemias using the Ion Torrent PGM™ platform (Thermo Fisher Scientific, Monza, Italy). In particular, attention has been paid to the study of some primary forms of dyslipidemia: i) high levels of LDL cholesterol (hypercholesterolemia, FH), ii) very low levels of LDL cholesterol (hypobetalipoproteinemia, HBL) and iii) very high levels of triglycerides (severe hypertriglyceridemia, HTG). These are monogenic diseases caused by mutations in several genes, which encode for key proteins involved in different pathways of lipid metabolism. In order to study candidate genes involved in different lipid metabolism pathways, three different NGS panels were drawn using a silicon instrument, the Ion AmpliSeq™ Designer software which allows the design of an oligonucleotide panel for amplifying target genes. The FH panel includes nineteen genes for a total of 81 kb, the HBL panel consists of fifteen genes covering 60 kb and the HTG panel is made up of eighteen genes with a total size of 62 kb. Forty-nine samples of patients with FH clinical diagnosis were analyzed, twenty-one samples were selected among patients with a clinical diagnosis of HBL and thirteen samples were selected among patients with severe HTG clinical diagnosis. For each sample, the different work phases of Ion Torrent technology have been applied: from library building and clonal amplification to sequencing and

data analysis. The NGS panels were validated using positive controls by detecting an analytical sensitivity of 100%, they were very precise and sensitive to the identification of different types of mutations (missense, nonsense, small indels) thus enabling molecular diagnosis in affected patients from different forms of dyslipidemia. Additionally, the NGS approach allows multiple samples and multiple genes to be processed in parallel, substantially reducing costs and working times compared to the classic Sanger sequencing method.

Optimization of a Biotechnological Process for Production and Purification of Two Recombinant Proteins: Col G and Col H

Lorenzo Volpe (Tutor Prof. Giulio Ghersi).
Department of Biological, Chemical and Pharmaceutical Sciences and Technologies; Abiel S.r.l.

Different strategies can be used for increasing production of heterologous recombinant proteins in Escherichia coli. Protein size is often critical for obtaining the best quantity/quality ratio of recombinant protein expression. This study focuses on two recombinant proteins; Class I and class II Collagenases, namely Col G and Col H. Their size is about 150 kDa each. We have developed a method to obtain high levels of cell growth and intracellular expression of each Collagenases in recombinant E. coli BL21(DE3). Batch and Fed-batch fermentation procedures have been performed. Results show that Fed-batch technique was most effective in obtaining the highest cell density for each recombinant bacteria; 28 g/L. We also investigated how to optimize recombinant protein expression; best results were obtained when "multiple shot IPTG induction system" was chosen instead of canonical single shot. By applying a purification protocol based on the use of tangential flow filtration and affinity chromatography we were able to obtain the highest quantity of purified protein: about 13,2 g for Col G and about 12,6 for Col H fermentations. Moreover, by using a stainless steel cooling coil system, we have investigated the effects of low controlled temperature (7°C) during the whole purification process. This system, allowed us to improve the final enzymatic activity of both Collagenases, obtaining 2 fold increase values respect processes performed at room temperature, measured with Pz Grassmann assay. This study shows that, even when the size of a recombinant protein is limiting, is possible to apply a defined Fed-batch protocol

to obtain a very high protein production. Moreover these results can be used as a scale up starting step for industrial production and purification of these kind of recombinant enzymes.

Vascular risk factors in dementia, Alzheimer's disease and mild cognitive impairment: population data from the Zabùt Aging Project

Angela Aronica (Tutor Prof. Calogero Caruso; co-Tutor Prof. Roberto Monastero).
Department of Pathobiology and Medical Biotechnologies, University of Palermo, Palermo, Italy; Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, Palermo, Italy.

Background

Alzheimer's disease (AD) is the most common cause of dementia in older adults, accounting for about 60% of cases. However, autopsy studies suggested that mixed dementia, with vascular and neurodegenerative AD pathology, accounts for nearly 20% of dementia cases.

Aims

Aim of the present study was to evaluate the relationship between isolated or clustered Vascular Risk Factors (VRFs)/diseases and Mild Cognitive Impairment (MCI) or AD. The study was conducted using a Sicilian population-based cohort dataset collected in low-educated, rural subjects, the Zabùt Aging Project (ZAP). The effect-modification by age, sex, education, genetic factor (APOE4 allele carrier), undernutrition, inflammatory status and depressive symptoms was taken into account.

Materials and Methods

VRFs, hypertension, coronary heart disease, atrial fibrillation (FA), previous Transient Ischemic Attack (TIA)/stroke, diabetes, dyslipidemia, obesity and Metabolic Syndrome were identified with a semi-structured questionnaire, physical measurements and laboratory analysis. The

Framingham general cardiovascular disease (CVD) risk profile, the Framingham stroke risk profile, the Cardiovascular Risk Factors, Aging, and Dementia (CAIDE) risk score and Late-Onset Alzheimer Disease (LOAD) vascular risk score were used as markers of vascular burden. Framingham algorithms are multivariable scores that provide a sex-specific absolute risk of cardiovascular events, and CAIDE and LOAD are dementia risk scores. The importance of each VRF in determining the outcome of each diagnosis was modelled by means of a sequential procedure based on the logistic regression, using subjects with normal cognition [NC] as reference group. The contribution of each VRFs in determining the outcome and the mutual relationship among VRFs in separating the diagnoses were studied using canonical discriminant analysis (with Mahalanobis distance).

Results and Discussion

When computed as a crude prevalence, NC accounted for near a half (45.6%) of the total population at follow-up, amnestic MCI (aMCI) and nonamnestic MCI (naMCI) for 27.9% and 11.6%, respectively, AD for 8%, while 6.9% were subjects with other dementias/psychosis. Risk of AD was increased by previous TIA/stroke (OR=2.77, C.I. 95%: 1.21-6.32). Risk of naMCI was increased by atrial fibrillation (OR=2.07, C.I. 95%: 1.00-4.27). AD scored a Framingham general CVD risk profile lower than NC or MCI ($p<0.0001$), whereas Framingham stroke risk profile showed a tendency to increase from NC or aMCI to naMCI or AD ($p=0.0001$). CAIDE risk score was higher in AD than NC ($p=0.003$). The tendency of LOAD vascular risk score to increase among diagnoses were similar to those of Framingham stroke risk profile ($p=0.0002$). In particular, when contemporarily stratifying for APOE4, inflammation, undernutrition and High level of Depressive Symptoms, CAIDE risk score strongly associated with risk of AD (OR =1.38, C.I. 95%: 1.01-1.88), Framingham stroke risk profile with naMCI (OR =1.49, C.I. 95%: 1.02-2.13) and LOAD vascular risk score with aMCI (OR =1.05, C.I. 95%: 1.01-1.09).

Data of this study, collected in low-educated, rural subjects, showed that the presence of multiple VRFs was associated with cognitive decline or AD. This suggests that a cumulative exposure to even mild effects of some VRFs would lead to cognitive decline and dementia more than the presence of an over vascular disease. Furthermore, it is likely that the effect of VRFs on cognition is not attenuated by old age but easier to detect if taking into account

Implementation of advanced methodologies in the commissioning of a Light Ion Beam Therapy (LIBT) facility.

Antonio Carlino (Tutor Prof. Maurizio Marrale; Co-Tutor Prof. Marcus Stock).
Department of Physics and Chemistry, University of Palermo (Italy); EBG MedAustron GmbH, Wiener Neustadt (Austria).

The current study is based on collaboration among the Department of Bio-pathology and Medical Biotechnologies, the Department of Physics and Chemistry of University of Palermo (Italy) and the LIBT facility MedAustron (Austria). It is essentially based on the implementation of innovative methodologies related to medical physics support of radiation therapy with active pencil beam scanning (PBS) technique with protons and ^{12}C ions and accomplished into comprehensive Quality Assurance (QA) program. At MedAustron a very complex and innovative treatment technique in external beam radiotherapy has been commissioned and introduced into clinical practice. The active scanning technique with proton and ^{12}C ion beams allows to build-up the dose as a superposition of many thousands of individually placed and weighted pencil beams. In particular, active scanned ion beams represent a novel irradiation technique taking full advantage from the physical interaction properties of these particles with tissues and advanced deliver modality to generate very sharp dose gradients in three dimensions, with many degrees of freedom available at the treatment planning level.
The project focused on implementation of innovative methodologies applied to the medical commissioning of a LIBT facility consists of three main parts:

1. investigation on Integral Depth Dose (IDD) correction factors for plane-parallel ionization chambers in proton beams by Monte Carlo (MC) simulations;

2. development of the procedures and needed software solution to support the patient specific plan verification in scanned particle beams;
3. development of dosimetric end-to-end test procedures using alanine dosimetry in scanned proton beam therapy.

Regarding the first topic, a commercial dosimetry solution (developed by PTW-Freiburg), based on the use of multiple ionization chambers for a quasi-three dimensional dosimetric verification, has been acquired at MedAustron. However, no specific software to support the selected dosimetric equipment and interface it to the TPS is commercially available. Therefore, the lack of commercial software solution to interface two different medical products (RayStation TPS on one side and the PTW equipment on the other side) has been overcome by the development of in-house software. Furthermore, the system was extensively used for the commissioning of the beam delivery system and the TPS.

Regarding the second topic, MC simulation is a very useful tool in order to support and speed up the medical commissioning of a LIBT facility. At MedAustron the MC particle transport code Gate v7.1 toolkit of Geant4 v10.01p02 is implemented. The primary core of a proton pencil beam is laterally surrounded by a halo mainly due to charged secondaries originating from non-elastic and elastic nuclear interactions. MC particle transport codes are powerful tools in order to investigate the different types of nuclear interactions which contribute to the halo dose deposition. Measurements were carried out at four clinical energies (62, 123.5, 187, 250 MeV) in a water phantom. Transverse dose profiles were acquired at four different depths for each energy and compared with the simulations. Based on the results of the halo measurements the QBBC nuclear model has been selected and used to compute the IDD correction factors for 20 energies selected among the 255 available in the clinical range (from 62 to 252 MeV). The beam model implemented in the RayStation TPS and used for clinical treatments is based on measured depth dose profiles corrected by MC.

Regarding the third topic, the purpose of end-to-end test is to confirm that the entire logistic chain of radiation treatment starting from the patient identification in the Oncology Information System (OIS), immobilization set-up, CT imaging, treatment planning, data transfer from TPS to OIS, dose monitor calibration and beam delivery system (BDS) is efficient and leads to the desired results with sufficient accu-

racy. Especially for new treatment techniques implemented in a centre, the end-to-end test can help to detect and eliminate any possible systematic errors occurring in the treatment chain or dosimetry process. Anthropomorphic phantoms (head and prostate) were customized in order to allocate different detectors such as ionization chambers, Alanine pellets and radiochromic films. One of the challenges of alanine for dosimetry in particle beams is the known response dependency (quenching) on the charge, the fluence and the energy of the particles constituting the mixed radiation field. Corrections for this were implemented in the Monte Carlo dose calculation platform of a non-clinical version of RayStation TPS. The end-to-end test procedures developed at MedAustron showed that the entire chain of radiation treatment works efficiently and with accurate dosimetric results. Our experience shows that alanine pellets are suitable detectors for dosimetry audits and the developed procedures can be used to support implementation of scanning beam delivery technology in clinical practice.

Pictures


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