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DIMET

Circulating levels of soluble Receptor for Advanced Glycation End-products (sRAGE) decrease with aging and may predict age-related cardiac remodeling

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Table of contents

General Introduction

Aging

Population aging is accelerating rapidly worldwide, from 461 million people older than 65 years in 2004 to an estimated 2 billion people by 2050[1].

The substantial gain of approximately 30 years in life expectancy in western world countries such as Spain and Italy and even larger gains in Japan, stands out as one of the most important achievement of the 20th century. The oldest-old group (aged >85 years) has been, during the last years, the most rapidly expanding part of the population in developed countries. This group is also the most susceptible to disease and disability[2,3].

Increase of mortality, disease, and disability rates in old people will therefore have a primary effect on sustainability of modern society. This outstanding reduction in elderly mortality was unprecedented and unexpected[4].

Since the 1970s, mortality at ages of 80 years and older has continued to decrease and, in some countries, even faster[5,6,7,8].

The most problematic expression of the aged population increase is the clinical state of frailty. Frailty is a condition of increased vulnerability and hard restoration of homoeostasis after a stress or event, which increases the risk of adverse outcomes, including falls, delirium disability and cardiac events[9,10,11]. Frailty is a long-established clinical expression that implies concern about an elderly person's vulnerability and

outlook; an apparently insignificant insult (eg, a new drug, minor infection, or little injury) results in a striking and disproportionate change in health state. Notably, frailty has also been associated with loss of physiological reserve in the respiratory, cardiovascular (CV), renal[12,13,14], haemopoietic and clotting systems[15,16].

Aging is believed to be the result of the time-dependent accumulation of molecular and cellular damage caused by different mechanisms that are regulated by an intricate maintenance and repair network[17,18,19].

Molecular mechanisms of aging

DNA Damage and instability

Time-dependent accumulation of damage in cells and organs is associated with gradual functional failure and aging[17].

Enzymatic reactions increase their error rate, and their aberrant products (including free radicals, such as reactive oxygen and nitrogen species)[17,20] can have potent effects on other molecules. Furthermore, elements in the environment like x-rays, ultraviolet radiation, and many chemicals unceasingly increased DNA damage[21]. The stability of the genome is maintained by an intricate machinery of repair, damage tolerance and checkpoint pathways that counteracts DNA damage[22]. To recognize the different kinds of DNA damage, cells are armed with multiple DNA repair pathways responsible for a specific type of damage (Fig1).

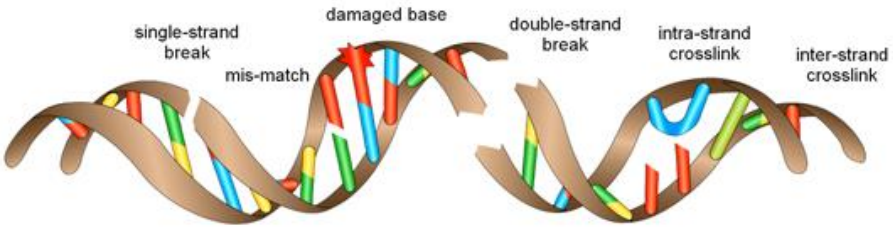

Fig1: Genomic DNA is continually being damaged. This figure shows the types of damage that can occur *(Department of Biochemistry – University of Oxford)*

Unrepaired genomic injury enables the formation of mutations that can be transmitted to new cells during duplication.

This could be involved in the typical changes that occur during aging, as demonstrated in humans and animal models with inefficient DNA maintenance[23].

To avoid the deleterious consequences of genomic instability like cancer, complex organisms have developed protective cellular mechanisms; such as apoptosis and cellular senescence.

DNA damage and apoptosis

Apoptosis is defined by cellular and nuclear shrinkage, chromatin condensation, blebbing, nuclear destruction and the formation of apoptotic bodies[24]. At the biochemical level, apoptosis of mammalian cells is characterized by mitochondrial-membrane permeabilization and/or strong caspase activation[25,26,27].

Numerous cellular mechanisms follow the rule ‘better dead than wrong’, which means that cells with DNA damage or that are afflicted by disorders will be killed by apoptosis preventing the

propagation of pathologic mutations. However, when apoptosis is inhibited, the risk of chromosomal instability at different levels increases, and cells that are conform enough to survival, can take a growth advantage, which can lead to deseases[28].

Telomere shortening

As mentioned before, the stability of the genome is critical for the survival and health of an organism. Telomeres, the ends of linear chromosomes, are specialized nucleoprotein structures consisting of DNA and shelterin protein complexes. Their function is in part to ensure the proper completion of replication of DNA after each cell cycle and in combination with shelterin proteins, protect the ends from DNA double-strand breaks[29]. Mammalian telomere hold a variable number of tandem repeats (10–15 kb long in human) of double-stranded DNA sequence 5'-(TTAGGG)n-3', followed by a terminal 3'G-rich single-stranded protrusion (150–200nucleotides long). Non functionally active telomeres lead to a DNA damage response through the stimulation of DNA-dependent protein kinase, Ataxia-Telangiectasia Mutated (ATM), and Ataxia Telangiectasia and Rad3-related (ATR)[30].

Aging is accompanied by telomere shortening in mammals[31]. Moreover, it has been observed that pathological telomere dysfunction accelerates aging in mice and humans, while experimental stimulation of telomerase can delay aging in mice[32]. Telomere shortening can be accelerated or caused by different environmental factors as air pollution[33], tobacco exposure[34], stress[35], asthma and COPD[36]. Telomerase is a

cellular enzyme able to compensate the progressive telomere attrition by de novo addition of TTAGGG repeats to the chromosome ends[37].

Telomerase activity in adult tissues, however, is not sufficient to avoid telomere shortening associated with aging. Generation of null cells and mice showed an increase in telomere shortening that can leads to loss of telomere protection and end-to-end chromosome fusions[38,31,39]. Many projects are rising to try to inhibit telomerase as a therapeutic target against cancer (Fig2).

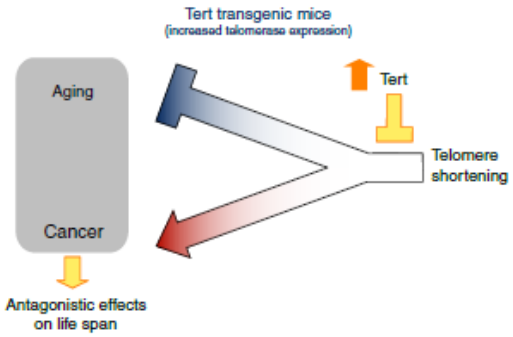

Fig2: Antagonistic effects of telomerase in cancer and aging. Increased telomerase (Tert) expression by means of mouse transgenesis results in decreased aging at the expense of increased tumorigenesis. *(Maria A Blasco – Nature Chemical Biology)*

Epigenetic Alterations

Chromatin is the polymer of nucleosomes composed of DNA wrapping the histone proteins. Chromatin and epigenetic factors regulate the access of transcriptional machinery to DNA influencing gene expression. There are many evidence suggesting that aging is characterized by epigenetic changes[40]. In fact about 20–30% of human lifespan seems to be attributable to genetic effects, but the major part of the variation is caused by non-genetic factors[41,42,43]. However, it is becoming

progressively clear that some aging-associated pathologies have an epigenetic cause[44,45].

About epigenetic alterations, it has been demonstrated that, from yeast to humans, in aging cells is present a general loss of histones[46,47] coupled with local and global chromatin remodeling and associated with an imbalance of activating and repressive histone modifications and transcriptional change.

It is still not demonstrated if changes in the activity of epigenetic enzymes impact the expression of important longevity genes or if modifications in these genes lead to strong epigenetic changes.

To overcome the effect of reduction of histone transcripts[48], senescent cells remodel the epigenetic landscape to induce a subset of cellular defense and inflammatory response molecules, many of which secreted from the cells and considered as part of the Senescence-Associated Secretory Phenotype (SASP)[49]. Chronic SASP induction can stimulate tissue aging[50].

Studies performed on monozygotic twins underlined that genomes in young age are epigenetically similar whereas the aged ones are clearly different[51]. In particular, DNA methylation changes associated with aging are strongly different between monozygotic twins who had spent a long period of their lives separately[52,43]. In light of these observations, DNA methylation changes can be mediated by many different factors deriving from lifestyle, diet and environmental exposure.

Mitochondrial dysfunction

Mitochondrial dysfunction is at the basis of aging in mammals[53,54,55].

In fact, impaired mitochondrial function and a late response to environmental stressor factors have been observed to be associated to cells senescence[56,57].

Biogenesis of respiratory chain appears to be directly affected during the senescence, with an impair of oxidative phosphorylation capacity[58,59].

Membrane structures are essential for the activity of mitochondria and during aging. These membranes are mainly composed of phospholipids[60] that together with sterols, influence an important parameter in cellular homeostasis: membrane fluidity or microviscosity[61]. Membrane fluidity is important for the regulation of membrane proteins activity insertion and folding. A stressor factor or aging instead can influence fluidity, affecting the activity of the proteins of a particular membrane[62].

Several diseases are related to highly fragmented mitochondria, a phenomenon observed in particular during aging[63,64]. The opposite has been shown in healty centenarians, who have higher mitochondrial mass associated with hyperfused morphology[65].

It is interesting but not surprising that the replicative and cellular senescence processes largely involve mitochondria and mitochondrial genome (mtDNA). mtDNA mutations could be considered the driving processes at the base of aging.

Stem cells exhaustion

The decline in the regenerative potential of tissues is one of the most obvious characteristics of aging. Stem cell exhaustion, caused by multiple types of aging-associated damages, constitutes one of the ultimate perpetrators of tissue and organismal aging[66]. Mammalian aging results from an age-associated impairment in the replicative function of tissue specific adult stem cells. These uncommon and specialized cells are needed for tissue replacement during the human lifespan, and are characterized by the capacity of self-renewal.

Stem cells in most of the tissues are mainly retained in a quiescent state but can be induced back into the cell cycle in response to extracellular signals, even after prolonged periods of inactivity. Their ability to produce a huge number of differentiated cells from a single stem cell is fundamental in one hand to balance the demand of new cells from the tissues and in the other hand to protect stem cells from mutations and carcinogenesis. In fact, since quiescent stem cells are less metabolically active they may be exposed to low levels of DNA-damage[67].

Some recent papers show that stem cell rejuvenation can reverse the aging phenotype in an organism[68]. It was demonstrated that aging of muscle stem cells is influenced by the microenvironment. In fact, these cells exposed to young mice blood can rejuvenate, showing that the aged environment, influences the replicative function.

Stem cells aging result from cell-intrinsic and extrinsic aging. The discovery that mice young blood can revert cardiac aging phenotype in old mice linked each other by parabiosis was shown by Loffredo et al. in 2013 and is a strong proof of the cooperation of intrinsic and extrinsic factors to induce aging[69] (Fig3).

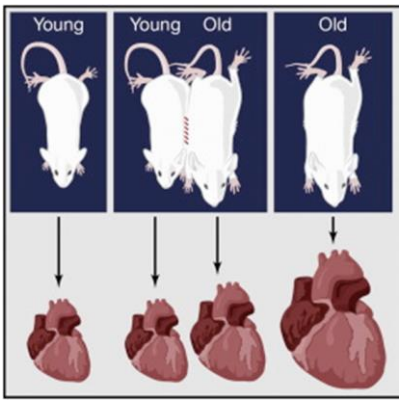

Fig3: Heterochronic parabiosis reverses age-related cardiac hypertrophy in mice. Heart mass is reduced in old mice subjected to heterochronic parabiosis with young mice. *(modified from: Leslie A.- Cell)*

The rejuvenation of cells is the main task in the aging research and many studies are based on the reprogramming of somatic adult cells in Induced Pluripotent Stem Cells (iPSCs) *in vitro*, through the Yamanaka protocol[70].

Despite some successes, it is still hard to enable the reprogramming cells from age-related diseases. The possibility to use iPSCs in regenerative medicine would be fundamental because of all the ethical problems that involve the use of Human Embryonic Stem Cells (hESCs) maintaining the benefits of autologous transplantation and self-renewal.

Biomarkers of aging

The challenge of finding biomarkers able to measure the aging rate in human subjects was articulated long ago. Recently,

progress in the basic biology of aging lets arise the realistic opportunity of interventions that may extend healthy lifespan. This greatly enhances the need for sensitive and specific biomarkers of healthy aging that are validated in both experimental animals and, importantly, in human subjects over the whole age range.

Aging is an intricate and multifactorial process that cannot be predict or identify with specific levels of a single molecule. Ideally, there would be a set of these biomarkers that would identify biological age. These could also be usefull to evaluate whether a healthy life style is able to slow down the aging process.

There has been a longstanding argue on whether biomarkers of aging should (and could) specifically measure only the physiological and unavoidable aging process independent of age-related disease[71]. It is also important to take in account that in the old age the most of people develop different pathologies or unhealthy carachteristcs but still with the realistic possibility to live for many years.

Therefore, a biomarker of healthy aging should not be confounded with a value that is indicator for the healthy part of the aging population only. Rather, it is a parameter that predicts the possibility for maintenance of health with increasing age with high specificity and sensitivity.

Many potential biomarkers have been proposed by recent progresses in the aging research field.

Circulating cell TL was the first marker to be proposed as a

biomarker of human aging[72]. This findings has been clearly validate in many independent studies but this marker lack of specificity/sensitivity and its values depending on methodological variation between laboratories. High levels of p16 (CDKN2A) are related with cell senescence. The levels of p16 was suggested as a biomarker of aging first in mice[73] and after in human in 2006[74].

γ H2AX, the damage-induced phosphorylated form of histone variant H2AX, has recently been indicated as a potential candidate biomarker of aging with clinical potential[75].

Moreover, good correlations have been described between high serum of IL-6, TNF-α and CRP levels and other physical measures influenced by aging and linked to frailty[76,77].

Since inflammatory markers are not commonly used in clinical practice, other markers were mainly adopted.

Large waist circumference, greater body mass index (BMI) and weight-gain in middle age are all associated with higher mortality or lower healthy survival[78,79]. Aging is associated with reduced metabolic capacity exemplified by diminished glucose homeostasis. Raised fasting blood glucose and glycated haemoglobin (HbA1C) are associated with age, CV events and mortality, cognitive impairment, and dementia, in non-diabetics subjects.

Recently, aging biomarkers research field met the gender medicine. It is well known that an important feature of human aging is its sexual inequality. In this context it is fundamental that also biomarkers should be evaluated differently between

two sexes, because a strong indicator of healthy aging in males could not represent an important tool for females and viceversa.

Sex-Dependent Differences in aging

Considered an extraordinary human achievement, life expectancy has improved by approximately 3 months per year for the past 160years[80]. This trend of increased lifespan is detected in both men and women, but a strong sexual dimorphism occurs in absolute human life expectancy. Women showed better life expectancy than men[81,82], and this sexual dimorphism is true at all time periods.

The female improvement in life expectancy is multifaceted and it would be unwise to suggest that biological mechanisms alone are responsible for the female advantage in lifespan.

In EU countries, life expectancy at age 50 reached 29.8 years for men and 34.6 years for women in 2010, but the average duration of life free from activity limitation remained practically the same in women (68.6 years) and men (67.9 years)[83], meaning that the almost 5 years of advantage in life expectancy of women are years of diseases and disability.

Although obviously human males and females differ in biological aging, life style and habits (smoking, nutrition, physical activity, type of work and education, among others) could have a fundamental importance. Unfortunately, the real contribution of a particular behaviour is almost impossible to establish. The discovery of molecules with the properties of aging biomarkers can help to study the involvement of certain lifestyles in males and females, ensuring a benchmark of

healthy aging.

The significance of the influence of sexual dimorphism on the outcomes of both ‘basic’ and clinical studies leads to new and, in some cases, unexpected insights.

Several hypotheses have been formulated to clarify the gender gap in life expectancy. For instance, by several analysis women have a more responsive immune system than men[84]. As inflammation is now at the basis of a lot of pathologies, differences in immune response could possibly play a role in the gender gap.

Another option is that sex hormones, like estrogen known to influence the above mentioned pathways, may be involved providing resistance to diseases. On the contrary men’s reproductive hormones increase susceptibility to a host of diseases.

Some evidence supports the hypothesis of the importance of these hormones.

In a study, taking advantage of lifespan data of males and females castrated due to mental illness, it has been observed that life expectancy is strongly influenced by sex hormones[85].

Comparison between castrated men with intact men, revealed that on average the castrated men lived 13.6 years longer than the intact men. Moreover, the earlier in life men were castrated, the longer they lived. For women, the prediction from the sex hormone hypothesis would be that removal of ovaries should reduce longevity.

Switching the argumentation from human to animal model,

female longevity bias is investigated also in models that are currently used to understand human aging mechanisms like mice. This is not easy to address.

In fact, the literature on aging is biased by confusion in the existence of sex differences in survival in mice[86,87]. Some studies found that males are the longer-lived sex[88], other that there is no sex difference[89], and other that females live longer[90]. One obvious explanation for this variation is the mouse genotype and inbreed.

The heart: basic anatomy

The heart is a muscular organ shaped like an inverted cone and located in the centre of the thoracic cavity where it occupies the space between the lungs. It is suspended by its attachments to great vessels within the pericardium, a thin fibrous sac that surrounds the heart. A small amount of fluid in the pericardium lubricates the outer wall of the heart and allows it to move freely during contraction and relaxation[91]. The thicker wall of the heart is called the myocardium and is composed of numerous layers of cardiac muscle fibers. The contraction of the myocardium allows the heart to continuously pump the blood throughout the body in order to transport oxygen and nutrients to the cells and remove carbon dioxide and metabolic waste products from the body. The innermost membrane of the heart, which lines the heart cavity, is the endocardium. It is made of endothelial cells and is continuous with the endothelium of the major blood vessels that attach to the heart.

The heart is composed of four chambers: Right Atrium (RA), Right Ventricle (RV), Left Atrium (LA), and Left Ventricle (LV) (Fig4). The atria are the upper chambers of the heart and are smaller than the two ventricles. The left and the right ventricles are separated by a thick myocardial wall called the intraventricular septum. The walls of the left ventricle are thicker than those of the RV since the left ventricle pumps the blood into systemic circulation, where the pressure is markedly higher compared with the pulmonary circulation to which the blood is pumped by the right ventricle.

In the heart there are four valves: the tricuspid valve between the right atrium and ventricle, the mitral valve between the left atrium and ventricle, the pulmonary valve between the RV and the pulmonary artery, and the aortic valve between the left ventricle and the aorta. Under normal conditions, the valves allow the blood to flow in only one direction in the heart. In particular, the deoxygenated blood coming from the body enters the right atrium from the superior and inferior vena cava. Here it is pumped into the RV through the tricuspid valve from where the blood is pumped into the pulmonary artery through the pulmonary valve. The pulmonary artery transports blood to the lungs where blood cells release CO_2 and take up oxygen. The pulmonary veins carry oxygenated blood to the left atrium where it is pumped through the mitral valve into the left ventricle. The left ventricle contracts to pump oxygenated blood through the aortic valve into the aorta from where it enters into systemic circulation throughout the body until it returns to the heart via the vena cava and the cycle repeats[91].

The four-chambered heart consists of different cell types (Fig4). All these cell types contribute to structural, biochemical, mechanical and electrical properties of the functional heart. More than 50% of the cells of the heart are cardiac fibroblasts. Atrial and ventricular cardiomyocytes form the muscular walls of the heart (that is the myocardium). Endothelial cells form the endocardium, the interior lining of blood vessels and cardiac valves while smooth muscle cells contribute to the coronary arteries and inflow and outflow vasculature. Pacemaker cells

and Purkinje fibres in the conduction system are specialized cardiomyocytes that generate and conduct
electrical impulses. The Sinoatrial Node (SAN), which is composed of a group of pacemaker cells, resides in the right atrium generating impulses to initiate heart contraction. The atrioventricular node (AVN) instead is located between the atria and ventricles, and it conducts an electrical impulse from the atria to the ventricles[92].

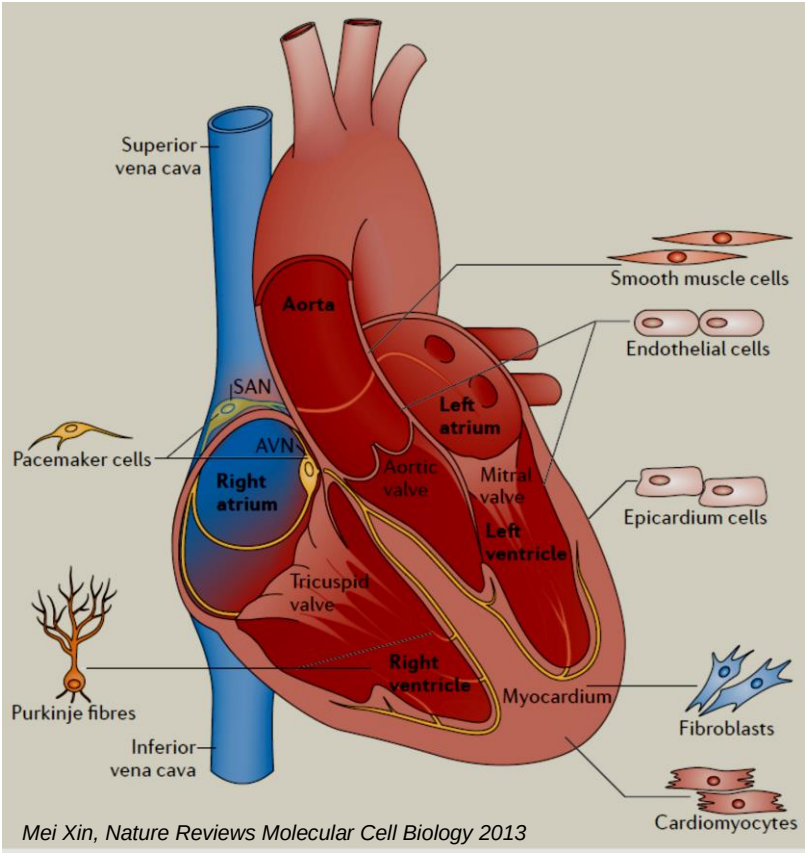

Fig4: Heart anatomy and histology. The heart wall is composed of three layers: the pericardium, a fibrous membrane that surrounds the heart, the myocardium, in the middle, which is the muscular part of the heart, and the epicardium, the inner serous layer that lines the internal chambers of the heart. The heart has four chambers: the left and the right atria on the top and the left and the right ventricle on the bottom. The left and right ventricles are separated by the intraventicular septum. The tricuspid valve connects the right atrium and the ventricle, the mitral valve the left atrium and the ventricle, the pulmonary valve the right ventricle and the pulmonary artery, and the aortic valve the left ventricle and the aorta. The four-chambered heart consists of different cell types: Cardiomyocytes, fibroblasts smooth muscle cells and endothelial cells.

Cardiac Aging

Cardiovascular (CV) disease (CVD) is the main cause of death in aged 65 and above (40%), and 80% of CV events that lead to death occur in this group. It is fundamental, therefore, that clinicians and researchers work to comprehend the changes that occur during aging also in absence of pathologies. The improvement of modern CV imaging techniques has permitted to perform many studies, among which the most significant Baltimore Longitudinal Study of Aging (BLSA) and Framingham, in which healthy populations can be evaluated non-invasively over long time allowing a better separation between the aging and CVD or lifestyle variables effects. Progressive and diffuse myocardial changes that impair the preservation of homeostasis characterized the aging condition. Cardiac alterations associated to physiological aging lead to increased susceptibility to CVD and these changes must be considered specific risk factors for CVD.

The understanding of cardiac structure and functional physiological changes during normal aging is crucial to know the pathophysiology of CVDs in the elderly. As previously mentioned the occurrence of CVDs increases linearly with age, in fact over 80% of Coronary Artery Disease (CAD) and more than 75% of Heart Failures (HF) are observed in old patients[93].

There is a variety of expression of cardiac structural, functional, cellular and molecular alterations that occur during healthy aging in humans and these age-associated changes are important for the onset and progression of Left Ventricle (LV)

Hypertrophy, chronic HF, and Atrial Fibrillation (AF), diseases that are known to increase with aging. Before explaining which are the morphological, functional and molecular changes that arise in aged heart, it is important to focus the attention on the vascular physiology alteration during aging because the heart can not to be considered as an isolated organ since it is in series with the vascular system.

Vascular aging

Many structural changes occur in the arterial system during aging, including thickening and enlargement of large arteries[94]. Taking advantage of echocardiographic techniques it has been observed that the aortic root dilates modestly with age, 6% between the fourth and eighth decades[95]. This, albeit modest, aortic root dilation provides a supplementary stimulus for LV hypertrophy increasing the volume of blood in the proximal aorta that leads to a larger inertial load against which the senescent heart must pump. During aging it was also observed that the carotid artery thickens threefold from the ages of 20 to 90 years in healthy individuals[96]. In fact aging is related with many different structural and functional modifications in the medial layer of arteries (hypertrophy, fibrosis and calcium deposits) and in the vascular endothelium (decrease vasodilators release and increased vasoconstrictors synthesis) that are associated with augmented vascular stiffness[97,98]. Aged vessels are characterized in the medial layer by alteration of the two proteins, collagen and elastin, that physiologically maintain

vasculature strength and elasticity[99]. All these modification lead to reduced arterial elasticity and increased stiffness. The occurrence of permanent non-enzymatic glycation of collagen to produce Advanced Glycation End Products (AGEs) increases with age and is associated with increased arterial stiffness in elderly people[100]. These AGEs can bind their Receptor for Advanced Glycation End-products (RAGE) to arise a substantial cellular activation and increase the synthesis of inflammatory and stress molecules.

The endothelium performs a fundamental role in the regulation of arterial tone by the secretion of Nitric Oxide (NO) and endothelin, whose alteration, defined as endothelial dysfunctions, is present in many CV disorders. Studies conducted on human and animal models revealed that the reduction in endothelial dependent vasodilatation caused by reduced NO production occur during aging[99]. Conversely, a 1000-fold increase in angiotensin II (Ang-II) levels is present in old organism arterial walls[101]. The unbalance between vasodilatation and vasoconstriction stimuli are thought to have a central role in arterial aging. Central arterial stiffening occurs during aging also without clinical manifestation of hypertension. Studies performed in normotensive cohorts demonstrated that Systolic Blood Pressure (SBP), which influences arterial stiffness and cardiac function, increase during aging also in non-pathologic people[102]. Different is the behaviour of Diastolic Blood Pressure (DBP) that typically decrease after the age of sixty.

Cardiac Changes During Aging

Morphologic changes

The improvements in technological approaches, exclusion criteria and statistical analysis have allowed a more deep understanding of LV morphological changes that occur in aging. In early studies based on autopsy without specific screening criteria and subsequently with the arrival of echocardiographic techniques it was found that heart mass increases significantly with aging[103]. The establishment of the best methods to distinguish age-associated and diseases-associated morphological changes in aged hearts is a problem not still completely solved, but with the progress, this limit has become less significant. Results derived from Cardiac Magnetic Resonance Imaging (cMRI) studies, performed in healthy people in the BLSA, and from 3-dimensional echocardiographic studies observation, have demonstrated that there is no difference in LV mass in women but a decrease in LV mass in men during aging among non Chronic Heart Disease (CHD) patients[104]. Instead, cross-sectional studies of subjects without hypertension or in general without afterload increase problems indicate that LV wall thickness, measured via M-mode echocardiography, increases progressively with age in both sexes[95]. Despite the absence of an increase in cardiac mass with aging, an enlargement of myocardial thickness occurs[104] and this could be ascribed to an increase in cardiomyocyte size[105]. The heart of a young adult is formed by approximately 25% of cardiomyocytes and an intricate structure of connective

tissue. It is well known that during aging the total number of cardiomyocytes decrease, likely due to apoptosis, and increase their size becoming hypertrophic[106]. Studies performed on animal and human display that the levels of apoptotic cardiomyocytes increase during aging, and this occurs more in men than in women consistently with the age-related decrease of LV mass in men but not in women[106]. Connective tissue, during aging, starts to produce high levels of collagen, becoming fibrotic. In particular, the loss of myocytes due to cell necrosis or apoptosis is replaced by fibroblasts and by the remaining hypertrophic myocytes (Fig5). In this way fibroblasts start to produce collagen that lead to interstitial fibrosis and to consequent heart stiffness and cardiac functions decline[107,108].

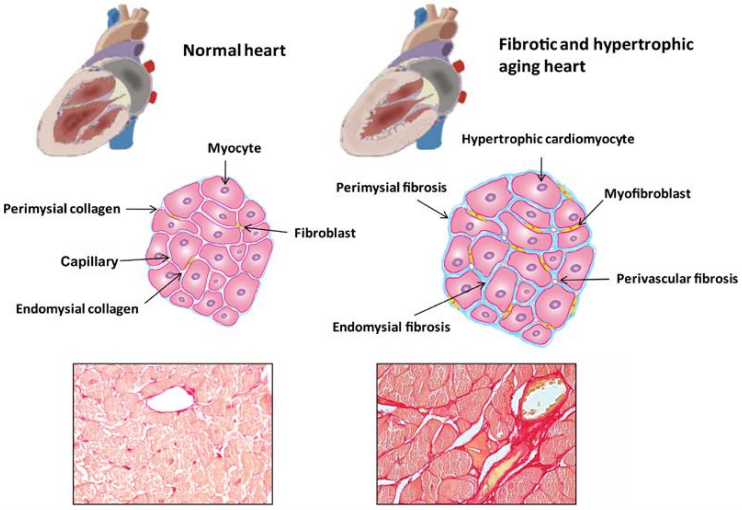

Fig5: Age-dependent remodeling of the heart is associated with cardiomyocyte hypertrophy and interstitial fibrosis.The histopathologic images show fibrotic remodeling of the heart in aging wild type mice. Red staining identifies the collagen network in the myocardium of 2 mo and 24 mo C57BL/6 mice. Senescent hearts display markedly increased collagen content compared to young hearts. *(Anna Biernacka Division of Cardiology, Albert Einstein College of Medicine, Bronx NY)*

In old people, fat accumulates in the SAN, occasionally separating the node from the atrial musculature. Moreover a marked decrease in the number of pacemaker cells in the SAN is generally present after age 60 and furthermore after 75 years occurs a more than a 90% reduction in the cell number compared to young adults. It has also been reported that a reduction to less than 10% of cardiac pacemaker cells, in respect to young adults, results in SAN dysfunction with an augmentation in the nodal conduction time and a decline in the intrinsic heart rate[109]. Heart rate is in turn influenced not only by the defeat of cells in the SAN but also by structural cardiac variations, including fibrosis and hypertrophy, which slow propagation of action potential.

Regarding valves changes during aging, calcific or degenerative aortic valve disease is considered the most common valvular lesion that occurs in old people[110]. The presence of this kind of aortic problem increases during aging, and affects from 2% to 4% of adults over age 65 years[111].

Aortic sclerosis is related with an increase of 50% in the risk of death from CV causes and in the risk of myocardial infarction[111].

Systolic functions

Despite the many changes that happen in LV structure during aging, LV Ejection Fraction (EF), the most frequently used clinical parameter of LV functionality, is conserved. However, very few healthy, sedentary, community-dwelling older individuals that undergo highly screening evaluation to exclude clinical and occult coronary disease have an EF<50%, a value revealing of impaired LV systolic function[112]. The intrinsic contractility declines during aging, partly due to the molecular changes like decreased β-adrenergic responsiveness and impaired Ca+2 handling.

With aging, a lot of changes occur also in the RV and in the pulmonary vascular system. The pressure in pulmonary artery and the vascular resistance increase with healthy aging, because of an augmentation in the arterial stiffness of the pulmonary vasculature[113,114,115]. RV ejection fraction remains relatively well preserved with aging, as LV ejection fraction.

Diastolic functions

While LV systolic function evaluated by EF is typically preserved with aging, sclerosis progressively increases in the cardiac muscle along with a significant decrease in LV diastolic function.

Differently from systolic function, there are many changes in the diastolic phase that happen with aging. The LV early diastolic filling degree gradually decelerates from the age of 20 years to 80 years when the reduction rate is, on average, up to 50%[112,116,117,118]. The diastolic phase, as well as the systolic one is affected by the accumulation of collagen in LV myocardium that lead to fibrosis, stiffer ventricle and less compliant heart.

Persistently elevated afterload from stiff vasculature can lead to LV hypertrophy and protraction in systolic contraction time that in turn, impinges upon early diastole[119,120,121,122].

The two main consequences of arterial stiffening that arise during aging are a diminished aortic elasticity and an increased pulse wave velocity, that is the timespan in which the arterial pulse propagates through the circulatory system[99,123].

Vessel stiffness makes the pulse wave faster. In the younger adult with elastic vessels, the reflected wave comes back to the heart during diastole increasing aortic diastolic pressure and heart perfusion. However, when the pulse wave become faster due to vessel stiffness, the reflected wave comes back during the last part of systole increasing systolic pressure, afterload and pulse pressure width[124]. Thus, arterial changes have a central role in the regulation of diastolic function.

Since most of aged people have normal EF but up to a one-third of them have anomalous diastolic function[125], a deeper understanding of diastolic phase is fundamental to figure out how age-related changes in cardiac structure affect aged heart predisposing it to events.

At the mechanical level diastole starts with aortic valve closing when the pressure within the LV begins to decrease and continues to decrease since it is lower than left atrial pressure.

Quick filling of the LV occurs in this phase. Usually, LV relaxation finishes in the first third of the filling so the most of the filling is supported by LV compliance and pericardial restriction. In the last part of the filling atrial systole contributes to provide the remaining volume.

In the young heart, about 80% of LV filling is provided by the passive filling phase, with only a little part provided by active work of atria. In contrast, during aging, deficiencies in early diastolic relaxation and ventricular elasticity alter the dynamics of ventricle filling making the active atrial transport the central player to diastolic volume[124]. This different diastolic filling pattern can be seen in patients with CVD, as well as in normal healthy older persons who are free of CVD.

Conduction system

As described before, a strong decrement in the number of pacemaker cells occurs in aging and this can influence the right functioning of the conduction system. Despite a general conservation of the resting heart rate during aging, many

changes happen in the cardiac conduction system that impact its electrical properties.

Worsening of the conduction system and nodal pacemaker is thought to start around 70 years of age. Ion channel modifications, along with β-adrenergic receptor reduction that lead to signaling impairment, have been reported to occur in tachyarrhythmias or bradyarrhythmias events in the elderly[126,109,127].

Bradyarrhythmias, characterized by delayed conduction, are common in the very old people, even without apparent heart diseases. These conduction alterations are aggravated by comorbidities or use of drugs leading to a clinical condition that requires pacemaker implantation[128,129,127].

The most of pacemaker receivers for bradyarrhythmias is over 65 years of age[128]. The principal task of pacemaker implantation is to alleviate the symptoms due to bradycardia or chronotropic dysfunctions of sinus node caused by aging-related changes in the atrial pacemaker complex and in conduction system[128,130,126].

Proneness to atrial and ventricular arrhythmogenesis is increased in the senescent cardiac tissue even without evident structural anomalies and is further exacerbated by common comorbidities present during the aging process[131,132]. This is due to structural and functional modification as cell loss, myocyte hypertrophy and interstitial fibrosis, which lead to altered cellular coupling and directional alteration in conduction (anisotropy) and cause heterogeneity in impulse propagation.

This creates regions of functional slowing or block of conduction enhancing vulnerability to arrhythmogenesis[109,133,134].

During aging also occurs the impairment of the intrinsic heart rate[135]. This reduction is caused both by the aging-related substitution of pacemaker cells within the SAN and atrioventricular conduction fibers with Extracellular Matrix (ECM), formed by collagen and elastin fibers[136], and by the impairment of β-adrenergic signaling that influence the diminished heart rate response, with a final reduction in aerobic work capacity during aging[128,136,137,109].

Moreover, in the aged heart, the action potential time and repolarization are protracted[138,139], in part because of decrement of potassium currents, including the Ca2+-activated potassium and ATP-sensitive potassium channels and partially due to delay in the inactivation of the calcium current that is needed for contraction[140,141].

Animal models

The most of studies in this field, to understand the mechanisms on which the cardiac aging is based, are performed on animal models and in particular on mice. In fact murine cardiac aging phenotypes closely recapitulate the phenotypes of human cardiac aging[99].

Echocardiography executed on a mouse longevity cohort revealed that left ventricular mass index and left atrial size significantly increased with age. Diastolic function has been described to decrease with age, whereas systolic function showed a modest reduction from young adult to the oldest

group. The myocardial performance index (MPI that includes both systolic and diastolic time intervals defining systolic and diastolic ventricular function) also impairs during aging, which is consistent with the age-related decline in systolic and diastolic function[142]. In addition to the similar cardiac aging phenotypes, the short lifespan and the accessibility of genetically modified mice are the benefits of using mouse models to study the molecular mechanisms of cardiac aging. Despite the presence of the cardiac aging phenotypes similar to humans, laboratory mice do not develop high blood pressure or adverse blood glucose and lipid profiles[143,144].

Molecular changes

Extensive evidences demonstrate that age-related modifications in the expression and function of ion channels, receptors and enzymes involved in different signalling pathways, have a central role in the pathophysiological basis of the aged heart, influencing the mechanical and electrical properties of the myocardium.

Altered Nutrient and Growth Signaling

It is known that the deregulation of neurohormonal pathways, which include in particular Mechanistic Target Of Rapamycin (mTOR) and Insulin-like Growth Factor-1 (IGF-1) signaling, has been associated with cardiac hypertrophy and aging.

Recently, there has been growing interest in the role of the mTOR pathway in nutrient signaling, supported by data implicating mTOR activity in aging. mTOR kinase is an evolutionarily conserved gatekeeper that integrates nutrient and

hormonal cues to modulate growth and longevity[145]. When nutrients are abundant, mTOR activity is high, which favors faster growth and cell division; when nutrients become limiting, mTOR activity is decreased, leading to reduced growth, enhanced resistance to stress, and increased lifespan. There are experimental evidences implicating the major role of mTOR signaling in cardiac aging process[145,146]

It has been showed that a model of increased mTOR signaling developed dilated cardiomyopathy with a lifespan of 6 months[147]. On the other hand no evidence has been provided yet on the positive effects of mTOR signaling decrease in mammalian aged heart. Rapamycin, improved left ventricular end-systolic dimensions, Fractional Shortening (FS), Ejection Fraction (EF), and regressing left ventricular fibrosis in mice with hypertrophy and heart failure[148,149].

Regarding Insulin/IGF-1 signaling, it is one of the most well-known pathways of lifespan regulation in animal models. Impairment of insulin/IGF-1 signaling enhances cardiac functionality with aging in Drosophila and reduces age-related cardiomyocyte alteration in mice[150,151]. However, the role of insulin/IGF1 signaling in the context of cardiac aging has been described as controversial[151].

In fact, human age-dependent decrease in circulating IGF-1 levels[152] correlates with an high risk of HF in old patients without previous presence of CVD[153] or interventions that lead to an increase of IGF-1 signaling, like growth hormone therapy[154,155]. The positive effects of IGF-1 on CVD could result

from mitochondrial protection mechanisms.

Many studies showed that aged rats treated with IGF-1 were protected from mitochondrial oxidative stress[156], and that interventions that aim to increase circulating IGF-1 levels have CV protective effects during aging[157,158,159].

These correlations between lowered IGF-1 and an increased risk of disease complicate our understanding of the role of IGF-1 in aging and lifespan, and as a result, the role of IGF-1 in human cardiac aging remains unclear. Careful studies involving well-controlled participant groups are needed to address the discrepancies and determine the role of IGF-1 signaling in human aging and disease.

Mitochondrial Oxidative Damage and Mitochondrial Dysfunction

Increased inflammation with aging is described by increased gene expressions of interleukin (IL)-1B, IL-6, tumour necrosis factor (TNF)-α, cyclooxygenase (COX)-2, and inducible nitric oxide synthases (iNOS) resulting in augmented oxidative stress through generation of ROS, ultimately contributing to the onset of cardiac aging[160].

The mitochondrial free-radical theory of aging suggests that elevated amount of mitochondrial Reactive Oxygen Species (ROS) damages mitochondrial DNA and proteins involved in redox system, triggering mitochondrial dysfunction and additional increase in ROS production (ROS-induced ROS release). This damage inducted by oxidative stress leads to cellular and organ functional failures that restrict lifespan and

health span[161].

Cardiomyocytes, that are postmitotic cells, are extremely vulnerable to age-related mitochondrial injury. Mitochondria in senescent cardiomyocytes are frequently enlarged with swelling, loss of cristae, and also damaged inner membranes and show a dysfunctional ATP production machinery[162,163].

Indeed, decrease of oxidative stress through overexpression of mitochondrial catalase (mCAT) is protective against cardiac hypertrophy and fibrosis ameliorating the aging phenotype[164].

In addition, increased oxidative stress activates matrix remodeling and profibrotic pathways directly through the activation of Transforming Growth Factor-β (TGF-β1) pathway and the conversion of cardiac fibroblast to myofibroblast[165]

With aging, mitochondrial dysfunction produces oxidative imbalance favouring myocyte hypertrophy, ECM remodeling, and stimulating myofibroblast activity. All these aspects are crucial for the development of the aging phenotype, and interventions on these pathways may prevent the progression of cardiac aging.

Adverse Extracellular Matrix (ECM) Remodeling

ECM is a complex of different proteins located outside the cells that provides structural and biological support to the adjacent cells. Cardiac fibroblasts are the main source of cardiac ECM proteins in the heart[166]. Cardiac ECM has mainly the role to align in the right position cardiomyocytes and to provide structural support to cardiac tissue; however, elevated amount of ECM deposition can increase the stiffness of the myocardium

influencing diastolic function[167].

The balance of the synthesis and degradation of ECM proteins dynamically modifies and controls ECM structure and involves Matrix Metalloproteinases (MMPs) and other proteases. Cardiac aging is related to myocardial fibrosis, and deregulation of ECM balance has been detected in aging hearts.

TGF-β is a family of well-known profibrotic factors that are able to increase the production of ECM proteins, favours transformation of fibroblast into their more active phenotype,the myofibroblasts[168] and prevent protease-driven matrix degradation pathways[169]. Interestingly, in a mouse model of cardiac aging, deletion of MMP-9 has been shown to improve angiogenesis, attenuate inflammation, and ameliorate ECM remodeling[170], underlining the interconnection between endothelial function and ECM composition. It has been demonstrated that the decrement of TGF-β1 expression lead to reduced myocardial fibrosis and enhanced LV compliance in aged 24-months-old TGF-β1 transgenic mice[168]. Connective Tissue Growth Factor (CTGF), another profibrotic factor linked to TGF-β, is known to increase during aging[171]. The function of ECM in cardiac aging consists also in the acceleration of myocardial fibrosis that complements higher TGF-β and CTGF levels in senescence-accelerated mice that exhibit diastolic dysfunction at 6 months of age[172].

Together, these results propose that elevated ECM deposition is an important player in age-related diastolic dysfunction and that low ECM synthesis can improve cardiac aging.

A better understanding of the mechanism underlying the role of myofibroblast in ECM remodeling during cardiac aging is needed in order to help the search of new therapeutic targets to prevent the cardiac manifestation of aging.

Impaired Calcium Homeostasis

Calcium (Ca2+) has complex and wide pleiotropic effects in cardiomyocyte biology. Increased beta adrenergic activity, observed with aging, alters Ca2+ cycling through phosphorylation of different sites, namely phospholamban, Ca2+/calmodulin-dependent protein kinase (CaMK), L-type Ca2+ channels, and Na+/Ca2+ exchanger (NCX), and also through decreased expression of sarcoplasmic/endoplasmic reticulum Ca2+ ATPase (SERCA) and alteration in ryanodine receptors (RyRs)[173,174].

Many studies have explored the effect of age on proteins involved in the excitation-contraction coupling pathways in myocyte, that could provoke contractile failure and diastolic dysfunction in the old heart[138,140,175]. Deceleration of contraction that occur during aging is caused mainly by the modifications in myofilament proteins including a shift from α myosin heavy chain to β myosin heavy chain[175] that leads to a reduction in myosin ATPase activity in the aging heart[175].

Impaired diastolic function can be dependent on alterations in active relaxation of cardiomyocytes[176,177]. During relaxation, calcium ions detach from the actin-myosin complex and are caught up into the Sarcoplasmic Reticulum (SR) or sent outside the cardiomyocyte. Impairment in Ca^{2+} cycling, enhances

myofilament stiffness, decreases Ca^{2+} sensitivity of myofilament proteins, and alters actin and myosin features that could result in impaired cardiomyocyte relaxation[176,177,178]. Hearts of aged mouse show a decrement of Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA2) expression[144] and activity[173].

Moreover calcium–calmodulin (CaM)-dependent pathways activation leads to hypertrophy in human cardiomyocytes[179]. This pathway is activated by high levels of Ca2+ within the cytoplasm, due to increased calcium entry through nonselective cation channels, as transient receptor potential channels (TRPCs), which influence cardiac hypertrophy and fibroblast proliferation both in physiological condition and in aging[180]. Inositol-1,4,5-triphosphate (IP3) receptors, responsible for the release of Ca2+ within the cytoplasm, are also increased in the myocardium of 26-month-old rats compared with 5- and 15-month-old rats[180]. These receptors appear to be upregulated in failing human hearts, probably mediating cell fate in this setting[181].

All this evidences show that age-associated modifications in calcium homeostasis could lead to hypertrophy and in turn alter muscle relaxation mechanics, influencing the reduction in early diastolic filling rate characterizing the aged myocardium.

Circulating biomarkers of cardiac aging

MicroRNAs

Recently microRNAs (miRNAs) started to be considered novel cellular senescence regulators[182,183]. miRNAs are small non-coding RNAs that are approximately 18–25 nucleotides long, and they regulate gene expression at the post-transcriptional level through target mRNA degradation and/or translational suppression via binding to the 3′-Untranslated Region (3′-UTR)[184].

Because of the multiple biological functions of miRNAs, it is not surprising that miRNAs are also involved in cellular and organismal aging and age-related diseases[185,186]. Currently, little is known about the roles of miRNAs in cardiac aging. Although the expression levels of miRNAs altered in aged hearts, have been identified.

In an important paper Boon et al. (2013)[187], demonstrated that miR-34a levels increase in hearts of aged mice and humans, and the silencing of this miRNA for only 1 week is able to rescue the miR-34a age-dependent increase of cardiomyocyte death. Old miR-34a knockout mice had better cardiac function and less hypertrophy compared to wild-type littermates. This suggests that miR-34a upregulation in the aged heart influences cardiac aging and makes miR-34a a sort of biomarker of cardiac aging. Another study indentified miR-22 upregulated during aging in mice heart[188]. A direct target of this miRNA is osteoglycin, a modulator of ECM structure that was described downregulated during aging in heart[189]. The up-

regulation of miR-22 and the subsequent down-regulation of osteoglycin exacerbate cardiac aging via an increase of senescence and cardiac fibroblast activity[188]. Moreover, the levels of other miRNAs have been also associated with an increased lifespan. Interestingly, efficacy of cardiac repair and angiogenesis is affected by loss of miR-126 and miR-130[190].

Taken together, these findings support the potential of miRNAs to act as biomarkers of cardiac aging.

Circulating factors

The evidence that circulating factors are closely related to aging conditions in has generated interest in the scientific world. Many studies, based on parabiosis, a surgical technique to study the effect of a shared circulation in two animals, have shown the potential of humoral factors in influencing the aging process in different tissues[191,192]. Heterochronic parabiosis, where two animals of different ages are joined together, has shown the potential to reverse age-related cardiac hypertrophy in mice[69].

Among all factors, Growth Differentiation Factor 11 (GDF11), a member of the activin/TGF-β superfamily, was identified as a factor that has the potential to reverse cardiac hypertrophy[69]. The results of a following study, however are apparently in conflict with the previous one but the different results can be clarified by the lack of a specific detection reagent that can discriminate GDF11 from its homologous myostatin (GDF8 or MSTN)[193]. It has been showed[194] that GDF8 factor also exerts a dose-dependent effect in reducing cardiac hypertrophy in young mice, extending its therapeutic potential to different forms of

cardiac hypertrophy. These evidences were confirmed by a prospective cohort study showing how GDF11 and GDF8 might have similar cardioprotective properties in humans. Results from this study have shown that older individuals have significantly lower levels of GDF11/8 and that a strong correlation is present between low plasma GDF11/8 concentrations and cardiovascular and mortality outcomes[195], with significantly more cardiac hypertrophy detected by echocardiography in individuals with low levels of the factors. In light of these data plasma levels of GDF11/8 could represent a good biomarker of age-related cadiac remodeling.

Another study reveal the potential ability of MMP-9 (metalloproteasis-9) and MCP-1 (monocyte chemotactic protein-1) as biomarkers of cardiac aging[196]. It has been described that plasma MMP-9 and MCP-1 levels were significantly higher in senescent mice compared with adult mice and positively correlated with end-diastolic dimension of the LV. Moreover MMP-9 plays an important role in cardiac ECM remodeling after MI, and plasma MMP-9 levels have been shown to predict cardiovascular mortality in patients with cardiovascular diseases[197]. The increased plasma MCP-1 levels in the senescent group are consistent with a previous report in humans showing higher circulating MCP-1 levels with age[198]. MCP-1 has also been shown as a plasma biomarker for acute coronary syndromes[199]. TL, in addition being a healthy aging biomarker, appears to be a biomarker of myocardial aging[200].

Aging process leads to changes in LV structure and diastolic function and is linked with a phenotype of concentric LV remodeling. Telomere attrition is associated with age-related LV diastolic dysfunction.

Sex and age-related cardiac remodeling

During life, the heart undergo to a remodeling and these changes differ between the males and females. Typical age-related remodeling includes increasing of left ventricular (LV) wall thickness, modification of LV dimensions and increasing concentricity. All these modifications are linked to CV risk factors and incidence of adverse events[201,202].

At all ages, men compared with women have higher absolute values of LV mass, wall thickness and chambers dimensions[202]. However, with aging and adjusting for body size, women undergo to an accelerated increase in LV wall thickness than men[203]. Furthermore, in women occur a more age-related concentric remodeling than men[204], which may be accompanied by more pronounced diastolic dysfunction[205]. Regarding cardiac systolic function, LV ejection fraction (EF) increases with age in both sexes, and this augmentation is more marked in women[206].

The epidemiology of age-related CV pathologies is markedly different between genders and changes radically in women after menopause[207].

Surely, both aspects linked to sex (gene expression from the sex chromosomes, sex hormones, metabolism of drugs by sex-specific cytochrome expression) and gender (sociocultural processes, behaviours, exposure to specific environment, nutrition, lifestyle and attitudes towards treatments and prevention) play a fundamental role in determining CVD risk[208].

HF (heart failure) is one of the main disease present in occidental world and affects up to 10% of the aged people, more women than men[209]. However, women overcame better than men HF that in females usually occurs at older age and with less ischaemic aetiology than in men[210]. The difference in the epidemiology of hypertension between men and women depend on which age of population is considered. It has been observed higher prevalence of hypertension in males in young and adult age, while is more common in women than in men in the elderly population[208]. Indeed, falling estrogen production during and after menopause has been associated with hypertension in women[211].

Women are particularly prone to the adverse impact of diabetes and hypertension on cardiovascular health. These conditions were related with higher risk of HF in women with respect to men[212]. In addition, diabetes condition worsens the coronary artery disease outcome more in women than in men[213]. Finally, pregnancy-associated conditions, such as preeclampsia and other hypertensive disorders, further influence the increased risk for future chronic hypertension, CVD, cerebrovascular diseases and death in women[214].

The Receptor for Advanced Glycation End Products (RAGE)

RAGE, that is a cell surface receptor and member of the immunoglobulin superfamily, was identified in bovine endothelial cells and initially described for its ability to bind an heterogeneous class of molecules derived from non-enzymatic glycation of proteins (Advanced Glycation End Products, or AGEs)[215].

The human RAGE gene (also termed AGER) is located on chromosome 6 in the Major Histocompatibility Complex (MHC) class III region and is constituted by a 5' flanking region that controls its transcription, 11 exons and a short 3'-UTR[216]. The resulting mRNA of 1.4 kb is translated into a protein of 404 amino acids with a molecular weight of 55 kDa[215].

In adult tissues RAGE is present at very low levels, except in lung, in particular in type-I pneumocytes, where it is very abundant[215,217]; however, RAGE starts to be rapidly up-regulated at sites where its ligands accumulate, and produces a sustained cellular activation through multiple intracellular signaling pathways. Expression of RAGE could change in a pathological situation like lung tumours and idiopathic pulmonary fibrosis, in which its levels decrease from the high levels present in the healthy tissue[218,219].

In the last fifteen years, it has been demonstrated that RAGE interacts with a variety of structurally and functionally different ligands among which AGEs, HMGB1, amyloid-β-peptides,

members of the S100 protein superfamily[220] and β_2 integrin Macrophage-1 Antigen (Mac-1)[221]. Its particular multi-ligand nature places it in the midst of different chronic inflammatory disease, such as diabetes, atherosclerosis[222], heart ischemia-reperfusion[223], tumor growth/metastasis and Alzheimer's disease[224].

Full Length RAGE (fl-RAGE), the complete translated isoform, is formed by different protein domains: an extracellular region composed by a signal peptide (aa 1–22), followed by three immunoglobulin-like domains, including an Ig-like V-type domain and two Ig-like C-type 1 or 2 domains; a single transmembrane domain, and a short cytoplasmic tail. The V domain is characterized by two N-glycosylation sites and is responsible for the binding of the most (but not all) of ligands[225]. The cytoplasmic tail is fundamental for intracellular signaling and activation of downstream pathway, for example through the binding to diaphanous-1 to mediate cellular migration[226] (Fig6).

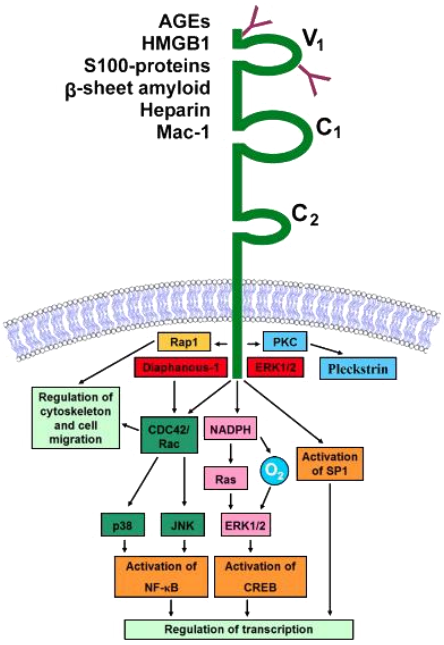

Fig6: Cartoon of RAGE protein and its domains. RAGE is a multi-ligand receptor consisting of three Ig-domains (V, C1 and C2), a transmembrane domain and a cytosolic tail required for RAGE-mediated intracellular signaling. Ligand binding activates multiple signaling pathways and regulates gene expression through the transcription factors NF-kB, CREB and SP1 *(Rauvala H - Biochim Biophys Acta)*.

The signaling events mediated by RAGE are complex due to the diversity of its ligands and their effects in different cell types. In order to activate the downstream RAGE pathways, the receptor needs to establish constitutive multimers within the cytoplasmic membrane[227]. Biochemical analysis of RAGE shows that such multimers include at least four RAGE molecules and might also produce greater assemblies[227] in the plasma membrane. Depending on the intensity and duration of RAGE ligation, specific signaling molecules such as ERK1/2, p38 MAPK, CDC42/RAC, SAPK/JNK and NF-κB have been shown to be regulated in different cell types[228]. The signals downstream to RAGE activate migration, adhesion and inflammation pathways.

Similarly to other members of the immunoglobulin superfamily of receptors, RAGE gene during transcription can be subjected to alternative splicing that lead to different splice variants[229].

Recently, it has been shown that this protein was ancestrally involved in cells adhesion, exhibiting the same genetic and structural qualities of other adhesion molecules, such as Activated Leukocyte Cell Adhesion Molecule (ALCAM), Basal Cell Adhesion Molecule (BCAM) and Melanoma Cell Adhesion Molecule (MCAM)[230].

RAGE is expressed both as fl-RAGE, and as various soluble forms (sRAGE) that lack the transmembrane and cytoplasmic domain produced by either proteolytic cleavage of fl-RAGE or by alternative mRNA splicing[231,232,229].(Fig7) The different soluble isoforms of RAGE are functionally equivalent[231] and act as decoy receptor that neutralize circulating ligands involved in RAGE-mediated disorders[233].

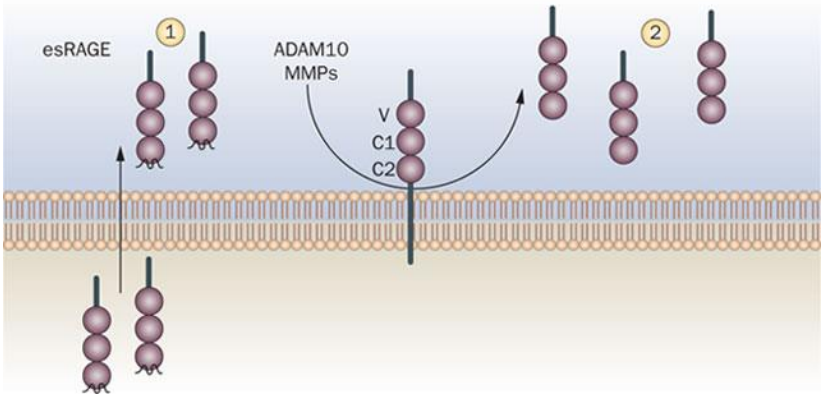

Fig7: Experimental evidence suggests that at least two forms of soluble RAGE exist. Endogenous secretory (es)RAGE derived from alternative splicing and (c)RAGE formed by the cleavage of extracellular RAGE from the cell surface by either ADAM10 or by MMPs. *(Vivette D'Agati & Ann Marie Schmidt - Nature Reviews Nephrology)*

Indeed, blocking fl-RAGE using sRAGE, as observed also in *Rage-/-* mice, interferes with the progression of chronic inflammation thereby limiting tissue injury. Of note, the effects of sRAGE are more marked than the RAGE gene deletion[222] suggesting that sRAGE might block the action that ligands exert via other receptors.

However, the levels of soluble RAGE remain lower compared with fl-RAGE[229], raising the question about how the low levels of soluble RAGE could act effectively on fl-RAGE. Moreover, soluble RAGE not only act as a simple competitor for RAGE ligands but it is also able to disturb fl-RAGE activation by unsettling the preassembly of the receptor on the cell membrane. It has also been shown that RAGE–RAGE binding occurs via the V and C1 domain, allowing sRAGE to interact with membrane-bound fl-RAGE. The heteromultimers that include also the soluble isoform are not signaling-competent,

since sRAGE lack the cytoplasmic domain. Moreover, recent studies demonstrated that sRAGE, by binding Mac-1, can induce by itself cytokines secretion and mediate macrophages and neutrophil migration, survival and differentiation[234,235].

Splice Variants of RAGE

As it was anticipated before**,** in addition to the full-length receptor of RAGE that has full signaling and ligand-binding potential, many truncated isoforms of RAGE have been discovered in both human and mice.

Malherbe et al. first identified in 1999 the presence of soluble splice variants of RAGE formed by alternative splicing of the RAGE gene. A number of soluble splice variants have then been reported and they have been named using different nomenclatures such as sRAGE1/2/3 and esRAGE[236,237]. One of them, the membrane-bound form, lacks the N-terminal domain and, it is not able to bind AGE. The other isoform is able to circulate and bind RAGE ligands, thereby protecting against the AGE-induced vascular injury.

Moreover, in 2008 Hudson et al.[229] went further in the investigation of human RAGE splice variants by the identification and classification of splice variants of RAGE according to the Human Gene Nomenclature Committee. Thirteen alternative splice variants of RAGE were revealed by the analysis of 100 samples of lung and 100 samples of Aortic Smooth Muscle Cells (AoSMCs).

Among these different isoforms only four were previously described (including the full-length form of RAGE) and nine novel variants were discovered.

Most of the soluble RAGE splice variants are degraded at mRNA level through Nonsense-Mediated mRNA-Decay pathway (NMD)[229]. The RAGE_v1 (also named as esRAGE) is the only splice variant that is secreted from cells as it is not degraded by the NMD pathway. It is also the second most abundant RAGE isoform after the fl-RAGE.

The regulation of alternative splicing of the RAGE gene is poorly understood. It has been shown that the relative expression ratios for the full length RAGE transcript to the sum of its splice-variants encoding truncated soluble variants differed greatly in different tissues[238]. It was therefore concluded that the pre-mRNA of RAGE must be subject to regulated alternative splicing through yet unidentified mechanism(s). Since the greatest part of the experiments performed to study RAGE functions are conducted in mouse models, it is crucial to pay attention to the different splicing variants present in mice and maybe not in human (Fig8).

Indeed, in 2009 Kalea[239] published a paper in which she described for the first time the different mice RAGE splicing variants in different tissues (Fig8).

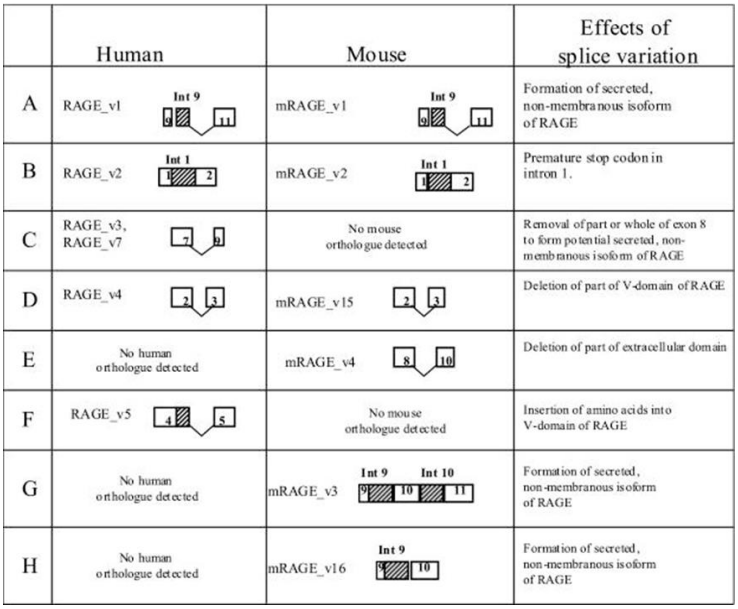

Fig8: Conservation and species-specific orthologues of human and mouse RAGE gene. Exon and intron splice variations for selected splice variants detected for humans and mice. (*Anastasia Z. Kalea – FASEB)*

In addition to the full length, the isoform most expressed in mouse tissues is mRAGE_v4. Interestingly, despite this isoform is highly expressed in mouse it is not present in human. It is described that mouse_v4 RAGE is an isoform that lacks the consensus aminoacidic motif crucial for the binding of MMP-9 and for the shedding performed by protease ADAM10, fundamental to produce the majority of soluble RAGE isoforms[240]. This means that this isoform cannot produce any form of soluble RAGE. In light of these data it is clear how careful has to be the interpretation of results when an animal model is used to recapitulate human condition.

Production of sRAGE via Proteolytic Cleavage of RAGE

In addition to alternative splicing variants, *in vitro* studies and *in vivo* experiments show that sRAGE can also be formed by

constitutively proteolytic cleavage of the membrane-bound fl-RAGE. A disintegrin and metalloproteinase (ADAM10) has been identified as the membrane protease that elicites the proteolytic cleavage of fl-RAGE in HEK293 and HeLa cells[231,232].

The proteolytic cleavage of fl-RAGE can be induced by the binding of HMGB1 and by cell stimulation with ionomycin and Phorbol 12-Myristate 13-Acetate (PMA) *in vitro*[241].

Despite the alternative splicing of RAGE gene can generate soluble isoforms, the proteolytic cleavage of membrane-bound RAGE is the major pathway for the production of sRAGE.

Modulation of sRAGE levels

Although the mechanisms of sRAGE (cRAGE and esRAGE) formation are quite well established, very little is known about their regulation and the few available *in vitro* studies are not sufficient to explain and understand the strong variability of sRAGE levels.

cRAGE (Clived RAGE) is derived from fl-RAGE at the direct cost of it while esRAGE is produced at the expense of a potential fl-RAGE mRNA[242]. Unfortunately little is known about the regulation of the mRNA splicing that produce esRAGE.

It has been observed that AGE member methylglyoxal induces RAGE and esRAGE expression in endothelial cells, while glyoxylic acid increases only the full-length form[243]. Also insulin can increase the production of esRAGE in cultured macrophages[244].

The cRAGE is produced by ADAM10 and other proteases on the extracellular domain of fl-RAGE. In fact, has been hypothesized that RAGE overexpression can be related with proteases activation that can in turn increase cRAGE.

RAGE expression is modulated by several external stimuli. TNF-α, β-estradiol and glycated albumin can upregulate fl-RAGE[245], while angiotensin-converting enzyme inhibitor (ACEi)[246], retinol (vitamin A)[247], metformin[248] and statins[249] can downregulate it.

As anticipated, ADAM10 is activated by calcium ionophore ionomycin and the PMA but it has never been demonstrated an increase in sRAGE formation by PMA-mediated activation[241]. Moreover ADAM10 is concentrated in lipid rafts and its activity is enhanced by lowering cholesterol levels[250] and its decrease could be one of the mechanism through which statins can rise sRAGE levels[251,252]. Another issue that makes cRAGE production more intricate is that activation of G Protein-Coupled Receptors (GPCRs) also induces RAGE shedding through ADAM10 and MMP9[253].

Since GPCRs are a huge receptors family, encoded by more than 800 genes[254], involved in hormone and metabolite signaling[255], these findings make very wide the scenario of RAGE cleavage regulation and highlight the need of further investigations before clinical conclusions on sRAGE levels.

Currently, data on cRAGE formation *in vitro* is limited. In cultured cells, sRAGE increases after treatment with ACEi[246], H2O2[256] and insulin[244].

In vivo, the difference in sRAGE levels may be mostly explained by the dissimilarities among population studies. Most of the studies, however, do not explicit information about ethnicity and RAGE polymorphisms, which are known to influence sRAGE levels[257]. Kidney dysfunction, associated with higher sRAGE levels, is not well distinct among the different studies leading to a bias.

In a pharmacokinetics study, Renard et al. showed that recombinant rat sRAGE has a longer half-life in diabetic rats than non-diabetic animals.

In fact the higher presence of AGEs in diabetes constitutes an additional source for the distribution of sRAGE[258]. In this case, AGEs present inside the tissues could sequester and decrease circulating sRAGE levels.

Different studies, probably due to different inclusion criteria and a diverse pathophysiology, describe that sRAGE levels are associated to different factors or have opposite trend regarding the same context.

An increase in soluble RAGE levels is often associated with acute inflammation but for example Achouiti et al. did not observe changes in sRAGE after experimental human endotoxemia (n=8) or during severe sepsis (n=51)[259].

Another important factor is the genetic background; indeed, a study conduct by Hudson et al. showed significative differences in sRAGE mean levels according to ethnicity, with non-Hispanic black subjects having lower sRAGE levels than non-Hispanic white and Hispanic ones[260]. In another study on type 2

diabetics, afro-Caribbean patients had lower sRAGE than white and south Asian ones[261].

RAGE ligands

AGEs

AGEs are a heterogeneous group of molecules deriving from the reaction of reducing sugars or other α-carbonylic compounds with amino groups, not only within proteins but also lipids and nucleic acids. In addition to the endogenous glycation biological processes, AGEs also derive from diet, in particular from high heat-processed food[262].

Glycation is a non-enzymatic process, firstly discovered by Louis Maillard who observed chemical modifications to heated amino acids in the presence of reducing sugars, a process referred to as the Maillard reaction. The last stages of glycation are irreversible and comprise different types of chemical modifications (Fig9).

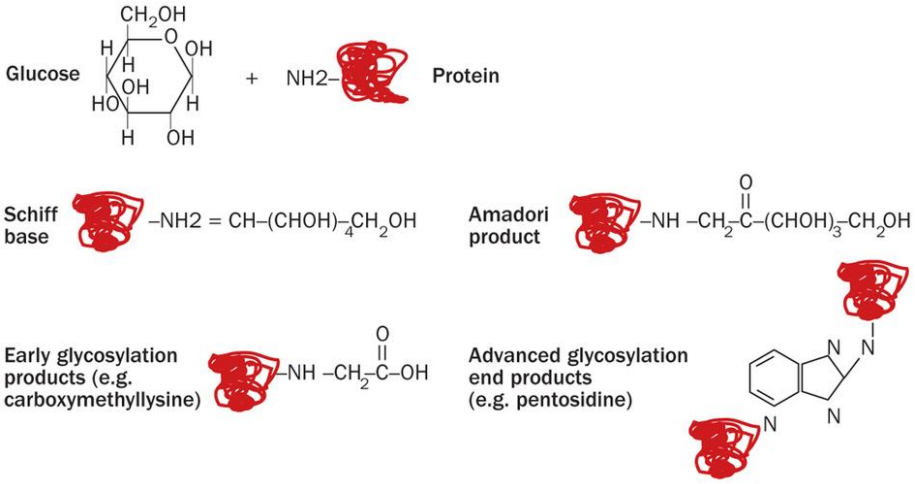

Fig9: Step-by-step mechanisms by which advanced glycation end products (AGEs) are formed In the presence of high levels of circulating monosaccharides and polysaccharides, as occurs in diabetes and metabolic syndrome, the aldehyde group of an aldose (shown here as glucose) can bind to a protein amino group. This non-enzymatic reaction generates a covalent bond (Schiff base). Over time, several chemical rearrangements occur, where the OH group, close to carbon-nitrogen bond binds the nitrogen forming a ketone. Over one hundred rearrangements, known as Amadori products, have been mapped. Subsequent chemical rearrangements, which include reduction, hydration and further oxidation, will generate early and advanced glycation end products. *(John A. Hardin, Neil Cobelli, Laura Santambrogio - Nature Reviews Rheumatology)*

Glycated molecules can accumulate within the tissues or circulate as soluble compounds but in the majority of the cases, glycation will result in the degradation of the molecule. The accumulation of glycated proteins could be caused either by the increase of AGEs formation (as it occurs in diabetes conditions, where protracted high blood glucose concentrations are present, and during aging) or by the impairment of AGE degradation. AGE deposits have been localized in the skin, lung, kidney, intestine, intervertebral discs, as well as in the heart and arteries of aged and diabetic patients[263]. The possibility that AGE modified proteins act as soluble factors was

mostly tested using modified albumin that was shown to activate macrophages/monocytes, cardiac fibroblasts, vascular smooth muscle cells and endothelial cells[264,265,266].

The principal mechanism by which protein glycation can induce aging and age related diseases consists in the binding of soluble AGEs to RAGE that stimulates many intracellular signaling pathways including the p21ras–MAP kinase pathway, the NADPH oxidase–ROS–NF-kB pathway and the Jak–Stat pathway[267]. AGEs are also able to enhance the production of inflammatory cytokines, such as IL-1, IL-6 or TNF-α and lead, via RAGE, to a persistent tissue inflammation[268]. AGE accumulation is one of the most important mechanism of aging and can be used as a marker of aging in humans.

Skin collagen glycation degrees can predict mortality in C57BL/6 mice. In diabetic patients AGE skin presence, analysed by skin autofluorescence, is a suitable predictor of fatal cardiac events within the next 5 years[269].

High Mobility Group (HMG) Box-1

HMGB1 is a highly conserved and ubiquitous chromatin-associated factor present in the nucleus of almost every cell type[270]. It belongs to the superfamily of HMG proteins, which consists of three families, HMGA, HMGB and HMGN. The protein structure of all these families share an acidic tail essential for DNA binding, but every family has a unique functional motif and participates in distinct cellular functions[271]. In mammals, there are three members of the HMGB family, HMGB1, HMGB2, and HMGB3.

In particular, HMGB1 is a 25 kDa protein composed of 215 amino acids organized in two positively charged DNA-binding structures, named A and B boxes and a negatively charged C-tail, composed of 30 glutamic and aspartic acids. The A and B boxes are helical structure, partly covered by the tail, which is folded over the protein. HMGB1 has two nuclear localization signals (NLS1 and NLS2) and two unusual Nuclear Export Signals (NESs) that imply the continuous shuttling between nucleus and cytoplasm (Fig10).

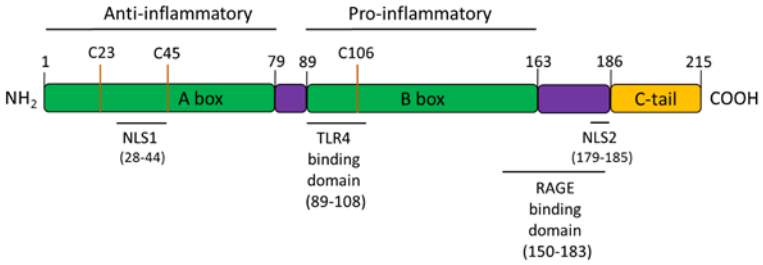

Fig10: The structure of HMGB1, denoting its two DNA binding domains (the A and B boxes) and the acidic C-terminal tail. A box has anti-inflammatory action, while B box has pro-inflammatory function. Also highlighted are the three cysteine (C23, C45 and C106) residues important for immunological functions. Intracellular shuttling of HMGB1 between the cytoplasm and nucleus is regulated by the acetylation of lysine residues in the nuclear localization signal (NLS) domain. HMGB1 residues 150–183 interact with receptor for advanced glycation end products (RAGE) to mediate chemotaxis, proliferation and differentiation.

In the nucleus HMGB1 binds and bends the minor groove of DNA in a no sequence-specific manner, regulating chromatin structure, gene expression and gene transcription[272]. An interesting peculiarity of HMGB1 is its high dynamism, in fact it is able to interact with a new nucleosome every second scanning the DNA in search for the right binding site[273].

In certain conditions HMGB1 can translocate from the nucleus to the cytosol to be secreted and the various functions played

by extracellular HMGB1, also via RAGE, are still matter of studies since the first evidence discovered in 1999[274].

Extracellular HMGB1 acts as Damage Associated Molecules Patterns molecule (DAMPs) and is directly involved in inflammation. In this context it is important the binding of HMGB1 to RAGE.

RAGE-deficient mice are protected from endotoxemia, suggesting that HMGB1-induced lethality during sepsis is mediated through RAGE signaling[275]. HMGB1 levels are elevated in patients with mechanical trauma, strokes, acute myocardial infarction, acute respiratory distress, and liver transplantation[276,277,278,279,280]. In a model of hepatic ischemia-reperfusion injury, HMGB1 levels are increased as early as 1h after reperfusion and are sustained for at least 24h[280]. Administration of soluble RAGE or neutralizing anti-HMGB1 antibody decreased liver damage[280,281]. RAGE-deficient mice also exhibit less damage following ischemia reperfusion injury in the heart[282].

HMGB1 provokes a proinflammatory phenotype in endothelium not exclusively by enhancing cytokine secretion, but also by increasing RAGE expression. RAGE activation can trigger a positive feedback loop after ligand binding, increasing its own expression[245], through its, promoter, which encloses binding sites for NF-kB that enhance the up-regulation of this receptor. Thus treating endothelial cells with anti-RAGE antibodies blocked at least in part the proinflammatory power of HMGB1[283,284].

S100 Proteins and RAGE

Moore identified for the first time an unfractionated mixture of the S100B and S100A1 from the bovine brain and called it ‘S100’ because the mixture was soluble in 100% saturated solution of ammonium sulphate[285]. S100 proteins are small acidic proteins (10-12 kDa), found exclusively in vertebrates[286], that constitute the largest subfamily of calcium binding proteins of the EF-hand type. Among the 25 members (with a sequence homology of 25-65%) described until now, 21 members (A100A1-S100A18, trichohylin, filaggrin and repetin) are clustered at chromosome locus 1q21[287,288].

Structural analyses have revealed that the most of the S100 proteins exist in cells as anti-parallel homo/hetero dimers. The S100 protein monomer consists of two Ca2+ binding motifs of the EF-hand type separated by a flexible region, the hinge. In each Ca2+ binding motif of the EF-hand type, a Ca2+-binding loop is flanked by α-helices, resulting in a helix-loop-helix arrangement.

Thus, helices I and II flank loop 1 and helices III and IV flank loop 2[289]. In the case of S100 proteins, the first Ca2+-binding loop is unconventional, longer and rearranged, whereas the second Ca2+- binding loop is canonical. Hence, the two Ca2+-binding sites in an S100 protein bind Ca2+ with different affinities, a higher affinity for C-terminal site and a much lower affinity for the N-terminal site[290]. The second Ca2+-binding site is followed by a C-terminal extension.

The C-terminal extension and the hinge region have the highest sequence variability among the S100 proteins. Upon Ca2+ binding, the S100 dimer undergoes conformational changes due to the reorientation of helices III and IV and forms clefts like target protein binding sites[291,292] (Fig11).

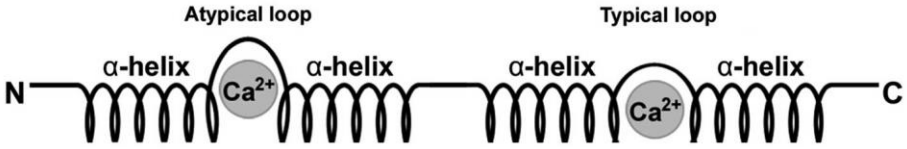

Fig11 - Schematic representation of the secondary structure of an S100 protein. *(Yi-Fei Ji, Hua Huang, Feng Jiang, Run-Zhou Ni, Ming-Bing Xiao - International Journal of Molecular Medicine)*

It is well documented that S100 proteins have a broad range of intracellular and extracellular functions[290,286]. Intracellular role consists in the control of protein phosphorylation and enzyme activity, calcium homeostasis, regulation of cytoskeletal components and regulation of transcriptional factors. When they are present in the extracellular compartment, they act like cytokines and perform their role activating RAGE[293]. In particular, some members of this family act as leukocyte chemoattractants, macrophage activators and modulators of cell proliferation[294]. These functions associate S100 proteins with a variety of pathologies such as inflammation and cardiac diseases, in which RAGE can play a central role. In particular the most important risk factors for CV diseases like diabetes mellitus, obesity, smoke and hyperlipidemia have been related to increase circulating levels of S100A8/A9.

RAGE, aging and heart

There are very few studies that investigate the role of membrane RAGE isoforms during aging. In human it has been described that RAGE mRNA levels in heart decrease during aging[295]. In a study on mice instead, the authors observed that mice that lack of RAGE spontaneously develop pulmonary fibrosis[219].

On the contrary RAGE is known to be usually abnormally up-regulated and plays crucial roles during the development of many human diseases such as diabetes and CVD. Depending on which ligands provoke RAGE activation, different pathways involved in the onset and progression of CVD are triggered.

Recent clinical evidences have suggested that AGEs-RAGE system plays an important role in the development and progression of heart failure in patients with or without diabetes. In the histopathological investigation of cardiac tissue, increased levels of AGEs were observed in heart failure patients with and without diabetes[296]. High circulating levels and cardiac deposition of AGEs could influence the progression of heart failure through its cross-linking to ECM and/or indirectly through the RAGE-mediated myocardial injury. In animal studies, it has been observed that male C57BL/6J mice receiving a high-fat diet developed cardiac hypertrophy, inflammation, mitochondrial-dependent superoxide production and cardiac AGEs accumulation, which were attenuated in RAGE KO mice fed a high-fat diet[297]. Another report from Petrova et al. showed the involvement of AGEs-RAGE pathway

in myocardial Ca2+ homeostasis. Systolic and diastolic intracellular calcium levels in cardiomyocytes were decreased in RAGE transgenic mice, highlighting the impairment of AGEs-induced calcium handling in diabetes-induced cardiac dysfunction[298]. AGEs-RAGE axis contributes to the pathogenesis of CAD. Eleveted serum AGEs concentrations correlated with the presence of CAD[299,300]. In an animal model of ischemic perfusion injury, the administration of sRAGE and the deletion of RAGE gene lead to a decreased injury in diabetic and non-diabetic hearts[301,282]. Furthermore, myocardial infarction by coronary artery ligation effects have been showed to significantly decrease in RAGE KO mice[302].

In the context of myocardial cardiac events, it has already been determined the fundamental role of HMGB1 and RAGE in the mediation of heart acute inflammation in ischemia reperfusion (I/R) injury[223]. During post-myocardial infarction remodeling the effect of hyperglycemia is essential[303]. In fact, incubation in a hyperglycemic setting of isolated cardiac fibroblasts, macrophages and cardiomyocytes caused a persistent activation of HMGB1 and its downstream effector, NF-kB in all cell types. This stimulation was dependent on HMGB1-RAGE axis, since the block of this pathway by RNA interference or gene KO decreased this inflammatory response. These data evidently demonstrated that the expression of HMGB1 is not limited to inflammatory cells and indicate that RAGE is the major functional receptor mediating the proinflammatory and pathologic effects of HMGB1 in cardiac tissue.

Among s100 proteins, increased circulating levels of S100A8/A9 have been related to the most important risk factors for CV diseases like diabetes mellitus, obesity, smoke and hyperlipidemia.

Atherosclerotic plaques show elevated levels of S100A8 and S100A9 both mice and humans[304]. By the activation of TLR4 and RAGE, S100A8/9 could be proatherogenic. In fact, plaque size decreased in TLR4 knock-out mice and the lack of RAGE is associated with delayed development of plaques and reduced vascular inflammation in ApoE−/− mice[284]. The diabetic ApoE−/− mice show high plasma S100A8/A9 levels and develop atherosclerotic injuries that are, as mentioned before, characterized by elevated content of S100A8/A9 and also display high levels of AGEs and activated NF-kB[305]. These characteristics are abolished when AGER gene is ablated[305], demonstrating that RAGE and its ligands have a central role in enhancing atherogenesis associated with diabetes.The binding of S100A8/A9 to RAGE on phagocytes was shown to activate NF-kB, enhance inflammatory cytokine secretion and immune cell recruitment into the myocardium in a mouse model of coronary occlusion demonstrating that S100A8/A9 can increase the local myocardial inflammation helping myocardial remodeling and the development of HF[306].

sRAGE, aging and heart

Since soluble forms of RAGE may be involved in the feedback regulation of the toxic effects of RAGE-mediated signaling,

recent research has been focused on investigating circulating sRAGE levels in human diseases. Multiple studies have investigated human sRAGE and esRAGE levels and their association with a variety of pathyphysiological conditions[307,308,309]. Soluble RAGE has been suggested to be a novel biomarker for either the disease and/or disease severity and may potentially be used to monitor the response to therapeutic interventions. Falcone et al. were the first group to report an inverse correlation between plasma sRAGE level and the severity of angiographically proven CAD in a case-control study of Italian non-diabetic men[310]. Another cross-sectional case-control study by Geroldi et al. showed that plasma sRAGE levels was decreased in patients with essential hypertension compared to normotensive controls and plasma sRAGE levels were inversely associated with pulse pressure[311]. Lowered plasma sRAGE also correlated with the increased incidence of lacunar and atherothrombotic strokes in a prospective study of acute stroke patients[312]. Furthermore, it has been suggested that plasma sRAGE is significantly lowered in hypercholesterolemic than normocholesterolemic patients and that plasma level of sRAGE is negatively associated with hypercholesterolemia[251]. These clinical studies imply that vascular disorders or vascular risk factors may be linked to the decrease in circulating sRAGE. Moreover sRAGE has been linked also to aging by Geroldi et al. in 2006[313] (Fig12).

Table 1. Plasma Soluble Receptor for Advanced Glycation End Products (sRAGE) Levels in the Three Study Groups

Plasma sRAGE, pg/mL	Healthy Centenarians (n = 80)	Healthy Young Controls (n = 110)	Precocious Patients with Acute Myocardial Infarction (n = 110)
Mean ± standard deviation	1,640 ± 728	1,200 ± 467	953 ± 660
Median	1,460	1,050	720
Range	432–2,999	520–2,870	142–2,940
<776, n (%)	7 (8.8)	24 (21.8)	57 (51.8)

Note: Plasma sRAGE concentrations were significantly different across the three study groups (Kruskal-Wallis test, $P < .001$). Post hoc Dunn's tests revealed significant differences between young controls and healthy centenarians ($P < .01$), as well as between precocious patients with acute myocardial infarction and age-matched controls ($P < .001$).

Fig12: High levels of soluble receptor for advanced glycation end products may be a marker of extreme longevity in humans. *(Geroldi D,Journal of American Geriatrics Society)*

In this paper the authors show that healthy centenarians have elevated levels of sRAGE compared to precocious myocardial infarction patients but also to forty healthy subjects, suggesting that sRAGE could be a good biomarker of healthy aging. However, opposite results have also been reported. Japanese patients with CAD and type 2 diabetes show a positive correlation between increased sRAGE concentration and inflammatory markers levels[314]. On the other hand, low circulating sRAGE levels in type 2 diabetic patients have been reported, highlighting also an inverse relation between glycemic control and S100A12 protein with plasma sRAGE levels[307]. sRAGE level is also increased in patients with impaired renal function, especially in those with end stage renal disease[315], suggesting that sRAGE level is influenced by renal function. In type 1 diabetes, it has been reported that circulating sRAGE levels were significantly elevated when compared to non-diabetic controls and those patients with retinopathy had the highest sRAGE levels[316]. Since most of the studies correlates aging or pathological conditions to low levels of sRAGE,

experiments in which this protein is injected in animal models is largely used to understand mechanisms at the basis of this processes. In diabetic mice deficient for ApoE, a model of atherosclerosis, the treatment with sRAGE by daily injections for two weeks, suppressed diabetic atherosclerosis in a glycemia and lipid independent manner[317]. This suggests that the interaction between AGE and RAGE is central in the development of accelerated atherosclerosis in diabetic context that is the major cause of their morbidity and mortality. Sinoaortic denervation in rats resulted in enhanced activity of aldose reductase, reduced activity of glyoxalase, accumulation of AGE, and upregulated expression of RAGE in aortas. In sinoaortic denervated rats, the treatment with sRAGE can attenuate the remodeling and the dysfunctions that derive from this condition, underling that the activation of RAGE is fundamental for aorta remodeling and endothelial dysfunctions[318]. The increase of sRAGE levels seems to ameliorate pathological conditions blocking the activation of RAGE and sequestering its ligands that can exert their role via other receptors. This could make sRAGE a new therapeutic target or therapeutic molecule in different pathological context.

Scope of the thesis

The progressive demographic shift towards higher proportions of older people within the population in many countries worldwide has enhanced the research focus on aging, an unavoidable condition that represents the major risk factor in the later life. Moreover, the prevalence of cardiovascular diseases increases with age, and intrinsic cardiac aging can strongly influence cardiac morbidity and mortality. However, there is no criterion reference for assessing healthy aging due to the complexity of the aging phenotype.

The identification of biomarkers in aging context could provide a dynamic and powerful approach to evaluate healthy status of the subjects with obvious applications in disease prevention, diagnosis, and disease management. An important feature of human aging is its sexual inequality. Thus, in studies aiming to find good indicator of healthy aging, the differentially evaluation of males and females plays a key role.

The Receptor for Advanced Glycation End-products (RAGE) is a cell surface multiligand receptor able to bind different molecules such as AGEs, S100 peptides and High Mobility Group Box-1 (HMGB1). The multi-ligand nature of RAGE places it in the midst of the onset and progression of many chronic inflammatory diseases that are mainly associated to aged people. Along with the membrane-bound RAGE (fl-RAGE), secreted isoforms of the receptor (sRAGE) have been described. sRAGE acts as a decoy receptor that neutralizes the

activity of circulating ligands involved in RAGE-mediated disorders. Indeed, blocking of fl-RAGE using sRAGE, as well as the use of *Rage-/-* mice, interferes with the progression of chronic inflammation thereby limiting tissue injury. Interestingly, reduced amount of circulating sRAGE has been found in many different chronic inflammatory diseases including cardiovascular pathologies. Moreover, healthy centenarians show significantly higher sRAGE levels than healthy subject of <40 years suggesting that elevated levels of the soluble receptor could be considered a biomarker of longevity.

The principal aim of the study is to determine for the first time whether sRAGE is a biomarker of aging and age-related cardiac remodeling, and whether there are gender-related differences. Furthermore, taking advantage of *Rage-/-* mice we aim to evaluate whether and how RAGE can influence the age-related cardiac remodeling.

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Chapter 2

Circulating levels of soluble Receptor for Advanced Glycation End Products (sRAGE) decrease with aging and may predict age-related cardiac remodelling

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(part of the data are included in a manuscript in preparation)

Abstract

Background: Aging is an unavoidable risk factor in later life that can influence the onset and progression of many diseases. In fact, the high incidence of cardiovascular diseases in the elderly is mainly attributable to cardiac remodelling associated to physiological intrinsic aging. RAGE is a multi-ligand receptor involved in many age-related disorders. Its soluble isoform (sRAGE) acts as a decoy receptor being able to block the activation of the membrane-bound receptor, and its circulation levels have been found altered in several chronic and acute pathologies. The role of RAGE isoforms in aging and, in particular, cardiac senescence has never been investigated. Moreover, the finding of reliable biomarkers able to assess individual health status of subjects has important applications in prevention, diagnosis, and disease management. In this context, the aim of this study was to ascertain whether sRAGE is a biomarker of aging and age-related cardiac remodelling, and evaluate the contribution of RAGE isoforms to cardiac aging.

Results: Serum of male and female from 20 to 92 years old healthy subjects was collected and sRAGE levels were evaluated by ELISA. We found a significant decrease of circulating sRAGE in males while only a trend in females. Accordingly, we observed a strong correlation of sRAGE with chronological age in male but not in female subjects. Male and female mice at different age (2.5-12-22-months, Young, Middle

Age (MA) and Old, respectively) undergone 2D-echocardiography to determine the left ventricle (LV) dimensions and function during aging. Serum sRAGE similarly declines from the Young to the MA group in both sexes, and inversely correlate with LV dimensions and function, preferentially in males. No detectable amount of RAGE protein was found in LV at all ages.

Rage-/- mice displayed a significant increase of LV volumes and diameters in diastole and systole, and a concomitant decrease in ejection fraction (EF) and fractional shortening (FS), compared to age-matched wt animals during aging with the strongest differences present between the MA groups. Moreover, MA *Rage-/-* mice exhibited higher deposition of collagen and expression of heart failure marker genes (BNP and Ankrd1) in respect to the wt counterpart. Conversely, no differences in cardiomyocytes size were observed at any age between the two genotypes. Finally, microarray functional annotation analysis based on the interaction between age-genotype revealed that the chronic lack of RAGE affected the expression of genes associated to contractile fibre function, antigen presenting process and adaptive immunity, insulin pathway, cell death and apoptosis. We also found a correlation between LV volumes and diameters in diastole and systole and differentially expressed genes involved in several processes like muscle contraction, fibrosis, wound healing and regulation of apoptosis.

Conclusions: Our results indicate that sRAGE is a serum biomarker of healthy aging and age-related cardiac remodeling, preferentially in males. The absence of RAGE in mice exacerbates adverse cardiac remodeling with age. We propose that, among RAGE isoforms, sRAGE may play a pivotal role in cardiac senescence.

Introduction

The average lifespan of humans is increasing, and, with it, the percentage of people entering the age of over 65 and older age group are growing rapidly. People are living longer, but that does not necessarily mean that they are living healthier. In fact, aging represent an unavoidable risk factor in the later life and can lead to many related pathology with cardiovascular diseases that remains the leading cause of death in the elderly[1,2].

A factor that further complicates the already intricate biological process of aging, is the well-known difference observed between males and females during aging. In developed nations, women tend to live longer than men and have notably lower death rates than men[3]. Also cardiac aging follow sex-specific patterns of remodelling that could differently affect men and women in terms of LV mass, hypertrophy and diastolic or systolic functions[4].

The main causes for the sex differences in longevity are sex hormones[5], and extrinsic factors such as lifestyle, health habits, exercise and diet. Nowadays, there is no a good model that explains adequately sex differences in elderly[6].

The Receptor for Advanced Glycation End-products (RAGE) is a cell surface receptor belonging to the immunoglobulin superfamily molecules, able to bind several peptides, including AGEs, S100 proteins and High Mobility Group Box-1 (HMGB1)[7]. The multi-ligand nature of RAGE places it in the midst of the onset and progression of many chronic

inflammatory diseases, such as diabetes, atherosclerosis, Alzheimer and cancer[7,8,9]. Binding of RAGE to its ligands activates several pathways including MAPKs, Src, NF-κB, and regulates inflammation, cell migration and adhesion[7,10,11]. Notably, RAGE ligands function through other receptors as well[12,13,14]. RAGE is ubiquitously expressed at extremely low level during homeostasis on most of cells and tissues and increases at sites of injury[7], where its ligands accumulate. An exception is the lung, which exhibits high basal amount of RAGE on alveolar epithelial type I cells[11].

Structurally the membrane-bound RAGE (fl-RAGE) is composed of an extracellular domain containing three Ig-like domains, responsible for the binding to the ligands, a single transmembrane helix and a short cytoplasmic tail necessary for the receptor activation[15]. Alternative splicing of RAGE transcripts generates both conserved and species-specific variants[15,16]. Recently, a rodent-specific variant, mRAGE_v4, has been characterized, that encodes for a membrane-bound protein (M-RAGE) missing 9 amino acids in the extracellular domain of the receptor, which are necessary for ADAM10 and MMP9 proteolysis[17]. M-RAGE is highly expressed in the murine lung and is resistant to shedding and does not contribute to soluble cRAGE generation[17].

Along with the above-described membrane-bound RAGEs (fl-RAGE and M-RAGE), secreted isoforms of the receptor exist, generally named (soluble RAGE, sRAGE)[10]. In particular, esRAGE derives from alternative splicing[18], while cleaved

RAGE (cRAGE) from the shedding of fl-RAGE by the proteases ADAM10 and MMP9[19] Both isoforms are found in human circulation, esRAGE representing only 20% of sRAGE pool[10], and they are functionally equivalent[10] acting as decoy receptors that neutralize circulating ligands involved in RAGE-mediated disorders[20]. Interestingly, blocking of fl-RAGE activation by the administration of sRAGE in animal models interferes with the progression of chronic inflammation thereby limiting tissue injury[7] . Similarly, RAGE knock-out mice (*Rage-/-*) are protected by the onset of several acute and chronic diseases[21]. Of note, in some cases the effects of sRAGE are more marked than the gene deletion[20] suggesting that sRAGE might block the activity that its ligands can exert *via* other receptors.

Reduced amount of circulating sRAGE has been found in diabetes[22], atherosclerosis[23], aortic valve stenosis[24] and in patients affected by acute myocardial infarction[25]. Interestingly, although levels of sRAGE correlate with the degree of advancing HF[26], recent studies have shown that sRAGE is increased by several cardiovascular drugs[27,28,29,30].

Since the old population is increasing constantly, the setting of a group of molecules able to reveal the healthy status and/or the intrinsic aging of organisms and of a given organ, differentially in males and females, is one of the main challenge within the biomarkers research field.

The role of RAGE during aging has been poorly studied so far. It has been observed that healthy centenarians have a significantly higher levels of sRAGE than healthy subject of <40

years suggesting that elevated concentrations of sRAGE could be a successful marker of healthy aging[25].

In the present study, we determined levels of circulating sRAGE in healthy subjects aged 20-92 years and in male and female Young, Middle-Age and Old mice and their relationship with cardiac aging. We found that sRAGE declines in humans with aging, preferentially in males more than in females and, inversely correlates with age-related cardiac remodelling in mice. Furthermore, we observed that *Rage-/-* mice develop a detrimental adverse cardiac remodelling with senescence.

Thus, our results suggest that sRAGE is a biomarker of healthy aging and may regulate proper cardiac aging.

Materials and Methods

Human study

Study population

Participants were recruited from the Italian National Research Center on Aging (INRCA), Ancona. 118 subjects gave their written informed consent to participate in the study, which was approved by INRCA's Ethics Committee. The age of participants are comprised between 20 and 92 years. The number of male and female is 45 and 73 respectively. The healthy status of the subjects was assessed using standardized questionnaires, laboratory assays and physical examination. Subjects were considered healthy if at the time of blood collection they did not have any major acute and/or chronic age-related disease such as myocardial infarction, chronic heart failure, Alzheimer's disease (AD), Type 2 diabetes or cancer. Subjects with a Cumulative Illness Rating Scale (CIRS) > 2, which indicates a comorbid state, were excluded[31]. Information collected included vital signs, anthropometric data, and medical history.

Human Elisa assays

Whole human peripheral blood was collected in a tube without anticoagulants. Once collected, the whole blood was left to clot undisturbed at room temperature for 15–30 minutes. The clot was removed by centrifuging at 2.000 g for 10 minutes in a refrigerated (4°C) centrifuge. The resulting supernatant was immediately transferred into a clean polypropylene tubes,

aliquoted and stored at -80°C, Elisa kits were used to test serum levels of sRAGE (DY1145, Human RAGE DuoSet ELISA, R&D Systems Inc., Minneapolis, MN), S100A8/A9 (EK-MRP8/14 Bühlmann Laboratories Ag, Switzerland), HMGB1 (ST51011, IBL International-Tecan, Switzerland) and AGEs (STA-518 Cell Biolabs, INC. San Diego, CA) following manufacturer's instruction

Statistical analysis

Data were analyzed with GraphPad Prism software version 5 (GraphPad Software, Inc, La Jolla, CA, USA). Healthy subjects were divided into three different groups depending on chronological age (≤ 45, 46-74, ≥75 years old) and analysed by One-way ANOVA with Bonferroni post-hoc test. The linear correlation of each variables with chronological age of the participants has been evaluated by Pearson test since the distribution of our data were estimated normal.

Animal study

Animals

C57/BL6 wild-type (wt) or *Rage-/-* (KO)[32,20] mice were housed in standard mouse cages on a 12:12 h light-dark cycle and fed a normal chow diet *ad libitum*. Ten weeks-, twelve or twenty-two month-old (Young, MA and Old respectively) animals were weighted and anesthetized with an intraperitoneal (i.p.) injection of ketamine-medetomidine cocktail (100 mg/Kg-10mg/Kg). Then mice were perfused with PBS or paraformaldehyde (PFA) 10% from the apex of the heart that were dissected out and

processed as described below. Length of tibia was assessed (TL).

Protocols complied with national and international law and policies (4D.L. N.116, G.U., supplement 40, 18-2-1992; EEC Council Directive 86/609, OJ L 358,1,12-12-1987; The Guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals, and with the US National Research Council 1996).

Echocardiography

Transthoracic echocardiography was performed using the Vevo 2100 high-resolution imaging system (Visual Sonic, Toronto, ON, Canada) and a 40-MHz linear transducer with simultaneous ECG recording. Analyses were performed on mice lightly anesthetized with 0.5-1% isoflurane/oxygen mix (>400 beats/min). Two-dimensional short axis M-mode was performed at the level of mid papillary muscle to measure the parameters summarized in Tables 4-5. Data and imaging were analyzed using the VisualSonics Cardiac Measurements Package by a blinded investigator.

Western Blotting (WB)

Hearts isolated from mice were separated in LV and RV and immediately frozen. LV were lysed (Ultra Turrax Homogenizer) in RIPA buffer (Tris-HCl pH7.4 10 mM, NaCl 150 mM, EDTA 5 mM, SDS 0.1%, Sodium Deoxycholate 1%, Triton X-100 1%) in presence of proteases (P8849, Sigma, St. Louis, MO, USA) and phosphatases inhibitors (Cat. 04906837001, Roche, Mannheim, Germany), incubated on ice for 30 min and centrifuged at

12000 g for 15 min at 4°C. Supernatants were collected and protein concentration was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein extracts (10-40 µg) were separated by SDS-PAGE and transferred to Amersham Hybond ECL Nitrocellulose membranes (GE Healthcare, Little Chalfont, United Kingdom). Membranes were blocked in PBS-0.1% Tween 20 (PBST) containing 5% powdered skimmed milk and probed with primary antibody against Tubulin (T-6199, Sigma-Aldrich, 1µg/ml) and RAGE (AF1145, R&D, 0,2 µg/ml) diluted in PBST with 5% powdered skimmed milk over night at 4°C. Secondary antibodies were incubated 1h at room temperature. Proteins were visualized by the ECL PRIME detection system (GE Healthcare, Buckinghamshire, UK).

Immunoistochemistry (IHC)

For IHC analysis, paraffin embedded heart sections were de-paraffinized, re-hydrated and boiled for 20 min in the antigen retrieval buffer (sodium citrate pH 6.0; #S2369, DAKO, Glostrup, Denmark; EDTA pH 8.0). After washing in PBS-0.1% Triton X-100 (PBS-T) slides were incubate in 3% H_2O_2 (H1009, Sigma-Aldrich Co., St. Louis, MO) for 10 min, to inactivate endogenous peroxidase, and then blocked in 5% goat serum in PBS-T for 1 hour at room temperature (RT). Primary antibodies against Collagen I (Ab292, Abcam, 5 µg/ml) and CD45 (Ab10558, Abcam, 2 µg/ml) were dissolved in antibody diluent (DAKO), and incubated overnight at 4°C in a humidified chamber. Sections were first incubated with biotin-conjugated

goat anti-rabbit antibody (Vector Laboratories, Burlingame,CA, USA,) and then with horseradish (HRP)-conjugated streptavidin (ABC kit, # PK-6100, Vector Laboratories) for 30 min at RT. Immunoreactions were revealed using 3,3 diaminobenzidine (ImmPACT™ DAB substrate,#SK-4105, Vector Laboratories) as chromogen and slides were counterstained with hematoxylin. Negative controls were performed with omission of the first antibody.

Images were taken by Axioskop II microscope (Zeiss, Germany) using a digital camera (AxioCam Color-Zeiss). Quantification of Collagen I deposition and of CD45 positive cells were determined on ten different fields from each section, using Axiovision Software™ Rel 4.8 (Zeiss).

Quantitative RT-PCR (qRT-PCR)

The total RNA from LV was extracted using TRIzol reagent (Invitrogen) following manufacturer's protocol. Tissue samples were homogenized with the TissueLyser (Qiagen) and RNA extracted by RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was treated with the TURBO DNA-free Kit (Invitrogen). cDNA was synthesized with the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Real-time PCR was performed on a Bio-Rad iCycler Thermal Cycler with iQ5 Multicolor Real-Time PCR Detection System using the iQ SYBR Green Supermix (Bio-Rad) and specific oligos (Table Oligonucleotides). The relative gene expression levels were determined using the $2^{-\Delta\Delta CT}$

method. Gene expression levels were normalized to the average of 4 house-keeping genes (Ppih; hprt; gusb; ldhA).

Oligonucleotides Table		
Gene	**Forward primer (5' -> 3')**	**Reverse primer (5' ->3')**
ppih	ctggagtcgccagtatttacc	ctttccatccagccaatcac
gusb	tatgggcatttggaggtgat	gctctccgaccacgtattct
hprt	ggagcggtagcacctcct	ccaaatcctcggcataatga
ldhA	agacaaactcaagggcgaga	cagcttgcagtgtggactgt
BNP	ccagatgattctgctcctgc	tgaactatgtgccatcttgg
Ankrd1	cggacctcaaggtcaagaac	tgaggctgtcgaatattgctt

Isolation of Mouse Cardiomyocytes

Cardiomyocytes (CMs) were isolated from dissociated hearts obtained from MA C57BL/6 wt or *Rage-/-* mice. Mice were anesthetized by intraperitoneal injection of a ketamine-medetomidine cocktail (100mg/Kg-10mg/Kg) and 100 U/ml heparin. The hearts were quickly removed via thoracotomy, cannulated via the aorta and attached to a Langendorff apparatus. All the solutions used in the apparatus were calibrated at 37°C and carbogen (5% CO2, 95% O2)-saturated. The heart was perfused with Perfusion Buffer (PB; 120 mM NaCl, 14.7 mM KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4*7H2O, 10 mM NaHEPES, 4.6 mM NaHCO3, 30 mM taurine, 10 mM 2,3-butanedione-monoxime, 5.5 mM

Glucose; pH 7.4) for 4 minutes and then switched to Digestion Buffer [DB; PB containing 600 U/ml collagenase II for 3 minutes. At this point, the perfusion was continued with DB supplemented with 0.12 mM CaCl2 (DB2) for 8 minutes. The ventricles were isolated, transferred to a 60mm dish containing DB2 and cut into small pieces. Then, the tissue was homogenized by gently pipetting after the addition of a Stopping Buffer (SB; PB with 10 %FBS and 0.12 mM CaCl2). Cell suspension was filtered through a nylon filter (pore size 210 µm) into a conical tube and let sediment by gravity for 10 minutes at 37°C. The cardiomyocytes pellet was resuspended in SB supplemented with 0.9 mM CaCl2 and let sediment for 14 minutes. The final pellet was resuspended and cells were plated for 2 hours with complete Minimun Essential Medium [MEM, with 10.000 U/ml Penicillin, 10.000 µg/ml Streptomycin (Sigma-Aldrich) and 20 mM L-Glutamine (Sigma-Aldrich)] containing 2.5% FBS. Then, the medium was replaced with complete MEM containing 1% FBS and cultured up to 48h. Images of CMs were taken with optical microscopy (Carl Zeiss, APOTOME) with 400X of magnification and cell area, the minor diameter and the major cell diameter were measured using AxioVision 4.8 software (Carl Zeiss).

Evaluation of CMs cross sectional area

Paraffin embedded heart sections were deparaffinized and rehydrated. Slides were incubated with orange-fluorescent tetramethylrhodamine conjugate Wheat Germ Agglutinin (WGA) (W7024 Invitrogen, 2µg/ml) for 10 min at room temperature and

then with Hoechst 33342 (Life technologies, 10 µg/ml) for nuclei staining. Slides were mounted with fluorescent mounting medium (DAKO S3023). Image acquisition was performed on optical fluorescent microscopy (Carl Zeiss, APOTOME) with 400X of magnification. Cardiomyocyte cross sectional area (approximately 160 cells/group) was measured using AxioVision 4.8 software (Carl Zeiss).

Mouse ELISA assay

Mice blood was collected by retrohorbital bleeding. Serum was obtained after an overnight incubation at 4°C and centrifugation at 10000g for 15 minutes. ELISA kit specific for mouse RAGE (MRG00 R&D Systems Inc., Minneapolis, MN) was used to test the presence of sRAGE in mice sera following manufacturer's instruction.

Statistical analysis

Data were analyzed with GraphPad Prism software version 5 (GraphPad Software, Inc, La Jolla, CA, USA). Comparison between groups of different age without any other variable was assessed by one-way ANOVA with Bonferroni post hoc test. Comparison between groups of different age and genotype was assessed by two-way ANOVA with Bonferroni post hoc test. The linear correlation of each variable with sRAGE has been evaluated by Spearman test since the distributions of our data were estimated not normal. A value of $P < 0.05$ was considered statistically significant; values are presented as means ± SE. Significance indicators are *$P<0.05$, ** $P<0.01$, *** $P<0.0001$.

Gene expression analysis

Tissue samples were homogenized with the TissueLyser (Qiagen) and total RNA was isolated using RNeasy Mini Kit (Qiagen) following the manufacturer‘s protocol. Extracted RNA was treated with the TURBO DNA-free Kit (Invitrogen). RNA from the four different mice groups LV, 2.5 months and 1 year old wt and Rage-/, were purified and prepared for SOLiD sequencing, according to the manufacturer‘s recommendations. RNA yield, chemical purity and protein contamination were measured using NanoQuant plate and M200Pro spectrophotometer (Tecan Group Ltd, Männedorf, Switzerland). RNA integrity number (RIN) was determined with Agilent RNA 6000 Pico Chips on 2100 Bioanalyzer instrument (Agilent Technologies, CA, United States). RNA was reverse transcribed, labeled, and linearly amplified using the Total Prep RNA Amplification Kit (Life Technologies, Carlsbad, CA), in order to be hybridized to HumanHT-12 v.4 Expression BeadChip microarrays (Illumina, San Diego, CA), according to manufacturers' instructions. To estimate technical variability, 20% of the samples were hybridized in duplicate. Arrays were read using the high resolution confocal scanner iScan (Illumina) and signal quantification and quality control were performed with the Genome Studio v. 1.9.0 software (Illumina).

Primary analyses were performed using BRB-ArrayTools v. 4.5.0 developed by Dr. Richard Simon and BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html). Data variance stabilizing transformation and

robust spline normalization were conducted with the *lumi* R package. Genes showing minimal variation across the set of arrays were filtered out under any of the following conditions: when the *P*-value of the log-ratio variation was greater than 0.01 and/or the 90th percentile of intensities was less than 120 (the background level corresponding to a detection *P*-value greater than 0.01, as calculated by Genome Studio). Multiple probes were reduced to one per gene symbol by using the most variable probe measured by interquartile range across arrays. Replicate arrays were averaged.

Given the balanced factorial design of the study, we used a two-factor ANOVA to determine which genes were mainly influenced by the class effect *per se* (genotype) and/or by the interaction effect of the two factors (genotype and age). Genes were considered statistically significant if the P-values (for either class or interaction), computed from the F-distribution and corrected for multiple testing with the false discovery rate (FDR), were less than 0.10 (corresponding to nominal P-values < 0.001). This was followed by pairwise comparisons to identify significantly different gene expression at alpha less than 0.05 (Student's 2-tailed t-test) between genotype classes at any ages.

We then performed a global test of whether the expression profiles differed between the groups by permuting the labels of which arrays corresponded to which sample. For each permutation, the *P*-values were re-computed and the number of genes significant at the 0.001 level was noted. The proportion

of the permutations that gave at least as many significant genes as with the actual data was the significance level of the global test. The test showed that the probability of getting the number of genes significant by chance at the 0.001 level if there were no real differences between the groups was less than 0.05.

The clustering algorithm CLICK (CLuster Identification via Connectivity Kernels), implemented in the java-based tool EXPANDER (EXPression ANalyzer and DisplayER) v. 7.0 was used to identify clusters of co-regulated genes with distinct mean expression patterns among the groups. CLICK makes use of graph-theoretic and statistical techniques to recognize tight groups of highly similar elements. The default homogeneity parameter 0.65 was used for a balanced intra-cluster similarity and inter-cluster separation. To test whether the group of genes in each cluster was enriched for a particular function, we performed gene set and pathway enrichment analyses using two tools. First, we used the TANGO (Tool for ANalysis of GO enrichments) algorithm implemented in EXPANDER, which performs hyper-geometric enrichment tests and corrects for multiple testing by bootstrapping (1000 bootstraps) and estimating the empirical *P*-value distribution for the evaluated sets. A GO biological process was considered significantly enriched in a cluster if its FDR corrected *P*-value was lower than 0.05.

To identify genes whose expression was significantly related to LV volumes, we computed a statistical significance level for each gene using the Pearson's correlation test. These P-values

were then used in a multivariate permutation test in which the LV volumes were randomly permuted among arrays. We used the multivariate permutation test to provide 80% confidence that the FDR was less than 0.10.

Results

Circulating levels of sRAGE decrease in healthy subjects during aging

We evaluated circulating levels of sRAGE in healthy subjects at different age subdivided in young (≤45 years), elderly (46-74 years) and old (≥75 years) subgroups by means of ELISA assay. Table 1 reported the biochemical variables values of all enrolled subjects considering also their different sexes.

Table 1. Biochemical variables of 118 healthy subjects divided into three age-groups

Variables all subjects	<45 yrs n=26;	46-74 yrs n=74;	≥75 yrs n=18;	p value
BMI, *kg/m2*	25,60±4,791	26,81±3,938	26,10±3,274	0,4028
Total cholesterol, *mg/dL*	200,0±35,49	221,0±42,11	204,7±37,09	0,0442
HDL cholesterol, *mg/dL*	55,92±14,15	63,12±15,54	49,50±11,81§§	0,0012
Triglycerides, *mmol/L*	99,76±75,67	97,73±54,67	110,4±40,82	0,7089
Glucose, *mg/dL*	89,88±7,941	93,55±8,915	93,06±8,149	0,1746
HbA1c, %	5,488±0,3724	5,769±0,4229*	5,706±0,4221	0,0137
Insulin, *mcU/mL*	6,731±4,568	4,654±1,985*	6,683±4,246§	0,0033
WBC, *10^3/uL*	6,827±1,600	6,244±1,697	6,207±1,499	0,2761
PAI-1, *ng/mL*	18,44±8,317	18,01±8,751	26,89±14,21*,§§	0,0026
Hs-CRP, *mg/L*	2,468±4,332	2,604±3,212	3,088±3,115	0,8308
Creatinine, *mg/dL*	0,8038±0,2107	0,8176±0,1940	0,8722±0,2024	0,4993
ApoAI, *mg/dL*	173,0±32,96	183,6±33,50	168,0±24,77	0,1095
ApoB,*mg/dL*	101,5±43,27	106,7±29,05	104,4±30,92	0,7877
Telomere length *(T/S, a.u.)*	0,5762±0,2031	0,4707±0,1985	0,4088±0,1362*	0,0135

Variables Males subjects	<45 yrs n=11	46-74 yrs n=26	≥75 yrs n=8	p value
BMI, *kg/m2*	26,55±4,770	25,80±2,644	25,36±4,245	0,7564
Total cholesterol, *mg/dL*	198,5±21,56	203,8±39,62	189,0±45,93	0,6174
HDL cholesterol, *mg/dL*	50,09±11,49	54,69±13,81	46,13±7,754	0,2085
Triglycerides, *mmol/L*	116,0±69,50	112,3±64,54	114,0±44,91	0,9875
Glucose, *mg/dL*	91,91±7,204	93,35±7,869	91,50±6,094	0,7695
HbA1c, %	5,582±0,3488	5,754±0,4081	5,688±0,5276	0,5223
Insulin, *mcU/mL*	6,991±5,738	4,485±2,160	6,963±5,792	0,1342
WBC, 10^3*/uL*	6,794±1,569	6,794±1,879	6,810±1,739	0,9997
PAI-1, *ng/mL*	18,79±9,237	17,09±8,733	24,34±13,73	0,2028
Hs-CRP, *mg/L*	2,869±5,776	2,250±2,048	1,633±1,205	0,7162
Creatinine, *mg/dL*	0,9727±0,2005	0,9731±0,1801	0,9500±0,1852	0,9511
ApoAI, *mg/dL*	164,5±30,53	168,7±32,67	156,9±19,79	0,6253
ApoB,*mg/dL*	109,5±46,33	103,0±23,85	99,75±19,28	0,7571
Telomere length *(T/S, a.u.)*	0,5991±0,2066	0,4619±0,2060	0,4243±0,1345	0,1098

Variables Females subjects	<45 yrs n=15	46-74 yrs n=48	≥75 yrs n=10	p value
BMI, *kg/m2*	24,90±4,848	27,47±4,376	26,62±2,513	0,1346
Total cholesterol, *mg/dL*	201,1±43,75	231,2±40,82*	217,2±23,78	0,0396
HDL cholesterol, *mg/dL*	60,20±14,72	67,23±14,41	52,20±14,09§	0,0092
Triglycerides, *mmol/L*	88,93±79,98	91,26±46,82	107,5±39,47	0,6581
Glucose, *mg/dL*	88,40±8,365	93,74±9,600	94,30±9,627	0,1405
HbA1c, %	5,420±0,3858	5,791±0,4277**	5,720±0,3458	0,0124
Insulin, *mcU/mL*	6,540±3,695	4,772±1,913	6,460±2,801	0,0238
WBC, 10^3*/uL*	6,851±1,677	5,983±1,524	5,724±1,143	0,1095
PAI-1, *ng/mL*	18,18±7,900	18,71±8,797	28,93±14,99*,§	0,0099
Hs-CRP, *mg/L*	2,174±3,074	2,842±3,725	4,253±3,716	0,3671
Creatinine, *mg/dL*	0,6800±0,1082	0,7340±0,1449	0,8100±0,2025	0,1043
ApoAI, *mg/dL*	179,1±34,32	191,7±31,74	176,9±25,61	0,2284
ApoB,*mg/dL*	95,67±41,50	109,7±31,15	108,1±38,50	0,3925
Telomere length *(T/S, a.u.)*	0,5593±0,2060	0,4751±0,1984	0,3980±0,1436	0,1226

*elderly and Old versus Young- §old versus elderly (1way-ANOVA analysis) ; Values are means ± SD; *,§ P<0.05; **,§§, P <0.01; ***,§§§, P<0.0001.
BMI = body mass index; **WBC** = White blood cells; **PAI-1** = Plasminogen activator inhibitor-1; **ApoAI** = Apolipoprotein-AI; **ApoB** = Apolipoprotein B; **HbA1c** = glycated hemoglobin; **Hs-CRP** = High-Sensitivity C-Reactive Protein; **a.u.** =arbitrary units.

Serum sRAGE showed a significant and progressive age-related decrease from young to old subgroups (Figure 1A, left panel). Pearson's analysis revealed an inverse correlation between the chronological age of the subjects and circulating levels of sRAGE (Figure 1A, right panel). Interestingly, when males and females were analysed separately, we found that serum sRAGE concentration decreased in a progressive but not in a significant manner from young to old females (Figure 1B, left panel), while drastically declined among young and elderly or old males subgroups (Figure 1B, right panel). Accordingly, Pearson's analysis confirmed a strong inverse or no significant correlation among sRAGE and age in males and females, respectively (Figure 1C).

These data demonstrate that serum levels of sRAGE decrease during healthy aging in humans and, in particular, in male subjects.

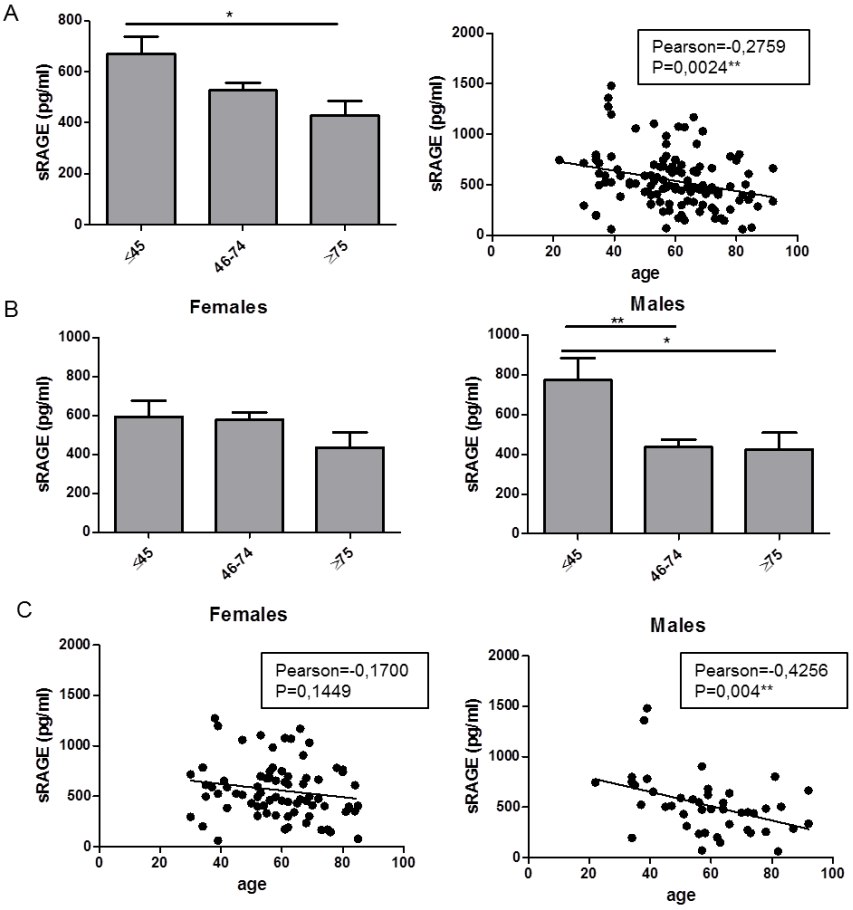

Figure1. sRAGE levels in healthy subjects. (A left panel) Circulating levels of sRAGE measured by ELISA in healthy subject divided in three different age groups. (**B**). The analysis was performed also by dividing the subjects in males and females. One-way ANOVA, values are means ± SE (≤45 n: M=11, F=15 ;46-74 n: M=26, F=48 ; ≥75 n: M=8, F=10 ; *, P<0.05; **, P <0.01; ***, P<0.0001). **(A right panel)** Correlation between sRAGE and the chronological age of the healthy subjects. (**C**)The analysis was performed also by dividing the subjects in males and females. Pearson correlation (n: M=45, F=73; *, P<0.05; **, P <0.01; ***, P<0.0001

RAGE ligands behavior during human healthy aging

Then, we assessed circulating levels of some RAGE ligands such as S100A8/A9, HMGB1 and AGEs in the same cohort of healthy subjects considering the differences between males and females. Serum levels of S100A8/A9 increase during aging in both genders (Table 2) but we observed a better correlation with chronological age in males than females (Table 3). HMGB1 progressively rises in males, while in females we observed only a slight augmentation in the old group in respect to the young one (Table 2). Accordingly, only males showed a significant positive correlation with chronological age (Table 3).

Finally, the circulating levels of AGEs showed only a slight increase in healthy aged subjects and a trend to correlate with the chronological age in both genders (Table 2-3).

In conclusion, we observed a slight increase of serum concentration of RAGE ligands during aging and a trend to positively correlate with the chronological age of the healthy subjects. The best correlations between RAGE ligands and age were found in males.

Table 2-RAGE ligands levels in healthy subjects

Males	≤45 yrs	46-74 yrs	≥75 yrs
AGEs *µg/ml*	2,596±0,1732 n=11	2,389±0,1409 n=26	2,850±0,2970 n=8
HMGB1 *ng/ml*	1,149±0,3460 n=9	1,752±0,3157 n=13	2,587±0,7759 n=6
S100A8/A9 *ng/l*	1,698±0,3072 n=11	2,114±0,3026 n=15	3,034±0,9853 n=7
Females	<45 yrs	46-74 yrs	≥75 yrs
AGEs *µg/ml*	2,720±0,3685 n=14	2,427±0,1696 n=36	3,471±0,3591[§] n=10
HMGB1 ***ng/ml***	1,492±0,3576 n=6	1,148±0,2302 n=20	1,620±0,7802 n=8
S100A8/A9 ng*/ml*	1,251±0,2831 n=11	1,696±0,1493 n=26	1,718±0,3187 n=10

Circulating levels of RAGE ligands measured by ELISA in male and female healthy subjects divided in three different groups depending on age. [#]elderly and Old versus Young; [§]Old versus MA (1way-ANOVA analysis); Values are means ± SE; [#,§] P<0.05; [##,§§], P <0.01; [###,§§§], P<0.0001.

Table 3-RAGE ligands correlations with chronological age

	Males	Pearson	p value	Females	Pearson	p value
AGEs *µg/ml*	n=45	0,069	0,6518	n=61	0,1681	0,1954
HMGB1 *ng/ml*	n=28	0,4023	**0,0338***	n=34	0,02399	0,8929
S100A8/A9 *ng/ml*	n=33	0,3119	0,0773	n=47	0,1794	0,2275

Correlation between circulating levels of RAGE ligands and chronological age of the male and female healthy subjects. Pearson correlation, *, P<0.05; **, P <0.01; ***, P<0.0001

Circulating levels of sRAGE decrease with aging in mice

We then ought to determine the variations of circulating concentration of sRAGE during aging in mice. We generated 2.5- (Young), 12- (Middle Age, MA) and 22-month- (Old) old

female and male C57BL/6 mice (wt). Soluble RAGE levels diminished significantly in the MA in respect to Young male and female groups (Figure 2A-B). No further decline but a slight increase of the soluble receptor was found in the Old compared to the MA mice for both genders (Figure 2A-B). Interestingly, Young females showed a higher amount of sRAGE compared to males mice that was maintained during aging (Figure 2A-B). Likewise human subjects, these data show that circulating levels of sRAGE decrease with aging in mice.

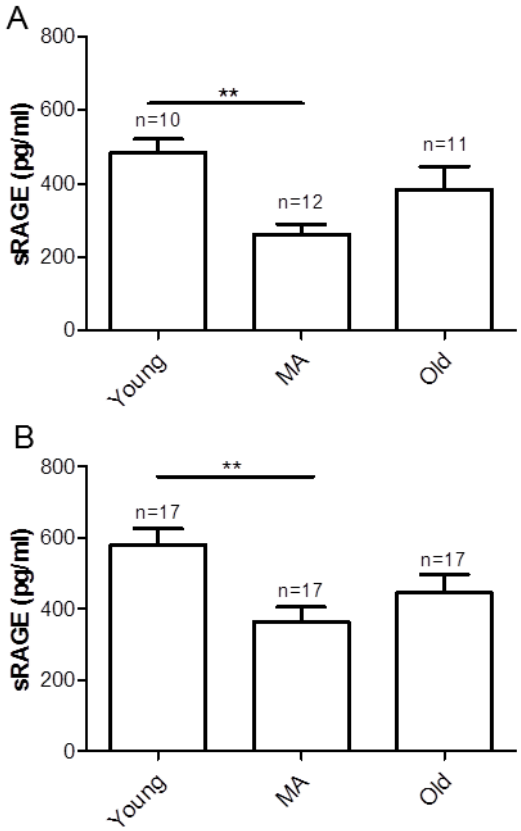

Figure 2.Circulating sRAGE levels in mice. Quantification of soluble RAGE in the serum of the indicated age groups of wt male(**A**) and female (**B**) mice by ELISA assay. Twenty-five µl of serum were tested. One-way ANOVA, data are mean ± SE (n: M=33; F=51; *, P<0.05; **, P < 0.01; ***, P < 0.0001).

Serum levels of sRAGE correlate with age-associated cardiac changes

Then we sought to investigate whether there is a relationship among circulating levels of sRAGE and age-associated heart remodeling in mice. Cardiac aging is characterized by an increase in LV dimensions, collagen deposition, cardiomyocytes hypertrophy and reduced functionality[33]. We first evaluated LV dimensions and function by 2-D echocardiography in Young, MA and Old male and female wt mice. The volumes and diameters in systole and diastole strongly increased from Young to MA mice while no further significant increase was observed between the Old and MA groups (Tables 4-5). Accordingly, these changes were accompanied by an impairment of LV function as revealed by the progressive reduction of EF and FS during aging that, however, reached significant values only in males (Tables 4-5). Mice also showed a steadily increase of LVAW in systole and diastole with age (Tables 4-5). The differences in LVAWd resulted significant between Young and Old groups of both genders, while LVAWs reached statistical significance only in females (Tables 4-5). Finally, LV mass increased in MA compared to Young mice and this increase was maintained in the Old group of both genders (Tables 4-5).

Table 4-Females LV functional parameters

		Young		MA		Old
	wt (n=15)	*Rage-/-* (n=15)	wt (n=19)	*Rage-/-* (n=21)	wt (n=14)	*Rage-/-* (n=16)
HR (bpm)	470,1±7,532	471,9±11,2	454,8±5,269	460,9±7,376	468,8±13,2	470±12,68
LVEDV (µL)	42,57±1,383	49,85±2,405	60,62±2,034$^{\#\#\#}$	74,72±2,609$^{***\ \#\#\#}$	63,09±2,519$^{\#\#\#}$	72,96±3,953$^{*\ \#\#\#}$
LVESV (µL)	6,698±0,5244	9,183±0,8279	11,29±0,7310$^{\#\#}$	16,92±1,164$^{**\ \#}$	12,16±1,441 $^{\#\#\#}$	18,00±2,756$^{*\ \#\#}$
LVEDD (mm)	3,243±0,04330	3,454±0,07157	3,755±0,05648$^{\#\#\#}$	4,098±0,06026$^{***\ \#\#\#}$	3,818±0,06434$^{\#\#\#}$	4,047±0,09721$^{\#\#\#}$
LVESD (mm)	1,531±0,04817	1,729±0,06296	1,872±0,05289$^{\#\#}$	2,208±0,06117$^{**\ \#}$	1,921±0,09190$^{\#\#\#}$	2,209±0,1300$^{*\ \#\#}$
EF (%)	84,53±1,070	81,89±1,081	81,61±0,8394	77,62±1,084	81,39±1,597	76,61±2,493
FS (%)	52,85±1,206	50,05±1,183	49,90±0,9119$^{\#}$	46,21±1,021	49,99±1,677	45,88±2,192
LVAWd (mm)	0,8764±0,02410	0,9651±0,02023	0,9477±0,04142	1,035±0,03688	1,016±0,03837$^{\#}$	1,125±0,04730$^{\#}$
LVAWs (mm)	1,506±0,04319	1,593±0,03966	1,601±0,05725	1,720±0,05191	1,684±0,05327$^{\#}$	1,763±0,05180
LV(mg)	76,66±2,061	93,33±3,301	116,7±4,556$^{\#\#\#}$	143,5±4,281$^{**\#\#\#}$	128,2±6,408$^{\#\#\#}$	161,6±11,7$^{**\#\#\#}$

**Rage-/-* versus aged-matched wt group (2way-ANOVA analysis); $^{\#}$ MA and Old versus Young of the same genotype-§Old versus MA of the same genotype (1way-ANOVA analysis); Values are means ± SE; $^{*,\#,\S}$ P<0.05; $^{**,\#\#,\S\S}$, P <0.01; $^{***,\#\#\#,\S\S\S}$, P<0.0001.

HR, Heart Rate**; LVEDV**, Left Ventricle End Diastolic Volume; **LVESV**, Left Ventricle End Sistolic Volume; **LVEDD**, Left Ventricle End Diastolic Diameter; **LVESD**, Left Ventricle End Sistolic Diameter; **EF**, Ejection Fraction; **FS**, Fractional shortening; **LVAWd**, Left Ventricle Anterior Wall in diastole; **LVAWs**, Left Ventricle Anterior Wall in systole; **LV,** Left Ventricle mass.

Table 5-Males LV functional parameters

	Young	MA	Old
	wt (n=10)	wt (n=16)	wt (n=15)
HR (bpm)	486±4,435	442,3±8,463	477,2±8,401
LVEDV (µL)	49,48±2.698	77,01±3,309$^{\#\#\#}$	75,11±3,606$^{\#\#\#}$
LVESV (µL)	8,644±1,031	17,92±1,411$^{\#\#}$	19,09±1,948 $^{\#\#\#}$
LVEDD (mm)	3,447±0,078	4,173±0,074$^{\#\#\#}$	4,114±0,078$^{\#\#\#}$
LVESD (mm)	1,686±0,0829	2,259±0,072$^{\#\#\#}$	2,299±0,104$^{\#\#\#}$
EF (%)	82,94±1,466	76,95±1,341	75,18±1,866$^{\#\#}$
FS (%)	51,28±1,621	45,63±0,266$^{\#}$	44,20±1,858$^{\#}$
LVAWd (mm)	0,9286±0,018	1,036±0,035	1,062±0,030$^{\#}$
LVAWs (mm)	1,579±0,049	1,678±0,054	1,677±0,045
LV(mg)	91,18±3,742	132,2±4,274$^{\#\#\#}$	141,1±5,607$^{\#\#\#}$

$^{\#}$ MA and Old versus Young -§Old versus MA (1way-ANOVA analysis) ; Values are means ± SE; $^{\#,\S}$ P<0.05; $^{\#\#,\S\S}$, P <0.01; $^{\#\#\#,\S\S\S}$, P<0.0001.

HR, Heart Rate**; LVEDV**, Left Ventricle End Diastolic Volume; **LVESV**, Left Ventricle End Sistolic Volume; **LVEDD**, Left Ventricle End Diastolic Diameter; **LVESD**, Left Ventricle End Sistolic Diameter; **EF**, Ejection Fraction; **FS**, Fractional shortening; **LVAWd**, Left Ventricle Anterior Wall in diastole; **LVAWs**, Left Ventricle Anterior Wall in sistole; **LV,** Left Ventricle Mass.

This analysis showed that aging influences LV dimensions and the main differences appeared between Young and MA mice. On the other hand, LV systolic function gradually decreased with age affecting mostly male animals.

To determine if the age-dependent decrease of circulating levels of sRAGE may reflect the cardiac changes associated to aging, we performed correlation analysis between serum concentration of sRAGE and echocardiographic parameters (Table 6). Spearman's analysis revealed a significant inverse correlation between sRAGE and LV end-diastolic dimensions (LVEDD and LVEDV) and LVAWs in females (Table 6).

Notably, sRAGE well correlated with all cardiac parameters in males (Table 6).

Table 6- Correlations between sRAGE and functional or morphological cardiac parameters

	Males		Females	
	Spearman	p-value	Spearman	p-value
LVEDV *(µL)*	-0,4208	0,0512	-0,4229	**0,0499***
LVESV *(µL)*	-0,4671	**0,0284***	-0,2336	0,2891
LVEDD *(mm)*	-0,4287	**0,0465***	-0,4268	0,0526
LVESD *(mm)*	-0,3981	0,0665	-0,071	0,7727
EF *(%)*	0,4338	**0,0437***	-0,1011	0,6545
FS *(%)*	0,4259	**0,0481***	-0,1338	0,5527
LVAWd *(mm)*	-0,5411	**0,0093****	-0,3502	0,1417
LVAWs *(mm)*	-0,4648	**0,0293***	-0,5716	**0,0106***
LV mass *(mg)*	-0,4869	**0,0216***	-0,3077	0,1635

Spearman correlations, *, P<0.05; **, P <0.01; ***, P<0.0001

Altogether, these results indicate that sRAGE is a potential biomarker of intrinsic cardiac aging and, in particular, for male mice.

Cardiac levels of RAGE isoforms during aging

To determine if the variation of circulating sRAGE reflects cardiac RAGE expression during aging we analyzed LV of Young, MA Old female and male wt mice by western blot. We have recently demonstrated that mouse lung expresses both two membrane-bound RAGE variants, fl-RAGE and M-RAGE, and the soluble cleaved cRAGE, which derives from the

shedding of fl-RAGE[10]. No detectable amount of any RAGE isoform was found at any age in the LV of both genders, while RAGE bands were clearly visible in the lung lysate (positive control; Figure 3 A-B). LV lysate from Young *Rage-/-* mice was loaded to show the specificity of the antibody.

Hence, these results indicate that RAGE is almost undetectable in LV and suggest that changes in circulating sRAGE cannot be directly associated to LV RAGE expression.

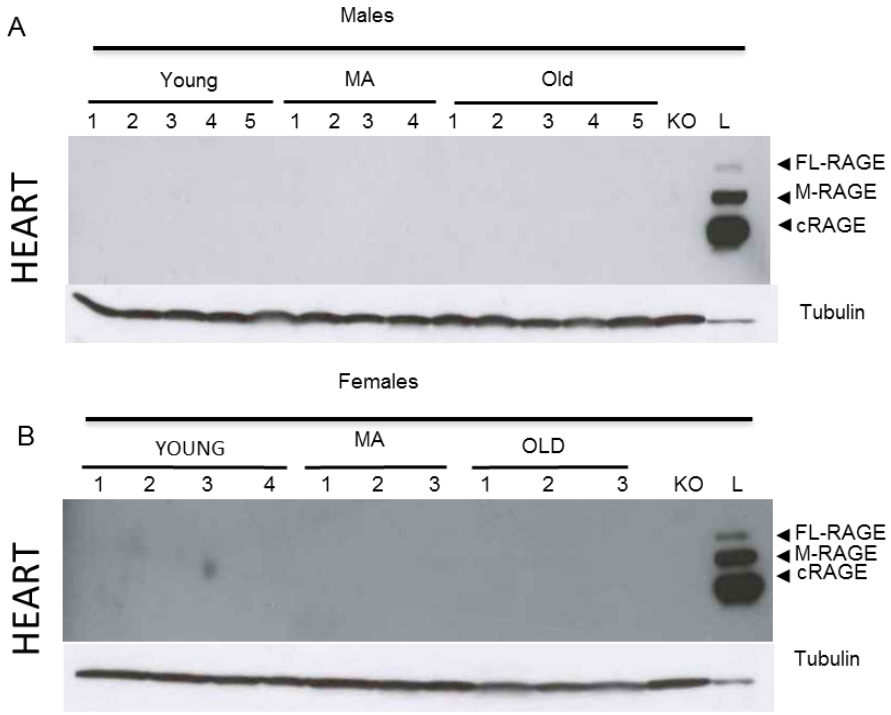

Figure 3. Cardiac RAGE isoforms expression. RAGE isoforms are not detectable in LV of male (**A**) and female (**B**) animals. Forty µg of protein lysate from LV of the indicated age groups of wt mice were probed with an antibody against the extracellular domain of RAGE. Forty µg of LV protein lysate from *Rage-/-* mice was used as negative control, and 10 µg of lung lysate of a Young wt mouse as a positive control for RAGE expression. β-Tubulin was used as loading control; (n=3-5/group)

Rage-/- mice show an increase of LV dimensions during aging

In order to better understand the role of RAGE in cardiac aging we decided to compare the cardiac phenotype of Young, MA and Old *Rage-/-* mice to corresponding wt groups. We generated *Rage-/-* mice both for males and females mice. Echocardiogrphic analysis of the wt and *Rage-/-* mice revealed a worsening in cardiac conditions of *Rage-/-* mice in both genders (Table 4, TableS1) but females showed higher statically significant differences between the two genotypes. In light of these data we further analyzed the heart of female wt and *Rage-/-* mice. LV mass of *Rage-/-* mice was higher than corresponding wt animals at any age but significantly different only between the Old groups (Tables 5-7) . Accordingly, *Rage-/-* exhibited a significant increase in LV/tibia length ratios by 1 year of age compared to age-matched wt mice (Table 7). Hearts of Young *Rage-/-* mice exhibited higher, but not significant LVEDV and LVESD dimensions in diastole and systole along with a reduction in EF and FS, respectively, compared to age-matched wt mice (Table 5). At 1 year of age, *Rage-/-* mice showed a further and significant increase in LVEDV and LVESD in diastole and systole, and incremental worsening in EF and FS in respect to MA wt mice (Table 5). These differences were attenuated when comparing the Old group of both genotypes (Table 5). In contrast, no differences in the LVAWs and LVAWd were observed among the genotypes with aging (Table 5).

Altogether, these data show that the lack of RAGE induces an exacerbation of LV dimensions associated with aging that occurs mainly within the first year of age.

Table 7- Female body and cardiac parameters

	Young		MA		Old	
	wt	Rage-/-	wt	Rage-/-	wt	Rage-/-
BW (g)	19,42±0,2618	20,83±0,3838	31,46±0,9168$^{\#\#\#}$	33,20±1,140$^{\#\#\#}$	29,89±0,6641$^{\#\#\#}$	36,44±1,403$^{***\#\#\#}$
TL (mm)	17,60±0,07071	17,09±0,02475***	18,41±0,1135$^{\#\#\#}$	18,91±0,1003$^{***\#\#\#}$	19,54±0,1439$^{\#\#\#\S\S\S}$	19,43±0,1373$^{\#\#\#\S\S}$
LV(mg)/BW(g)	4,021±0,09460	4,614±0,1439	3,736±0,1366	4,450±0,1952	4,054±0,3938	4,588±0,2944
LV(mg)/TL(mm)	4,331±0,1054	5,455±0,1955	6,346±0,2564$^{\#\#\#}$	7,592±0,2281$^{**\#\#\#}$	6,410±0,3141$^{\#\#\#}$	8,300±0,5792$^{***\#\#\#}$

*_Rage-/-_ versus aged-matched wt group (2way-ANOVA analysis); $^{\#}$ MA and Old versus Young of the same genotype-§Old versus MA of the same genotype (1way-ANOVA analysis) ; $^{*,\#,\S}$ $P<0.05$; $^{**,\#\#,\S\S}$, $P<0.01$; $^{***,\#\#\#,\S\S\S}$, $P<0.001$.

BW, Body weight; **TL**, Tibia length; **LV,** Left ventricle Mass.

Rage-/- mice show enhanced cardiac fibrosis but not hypertrophy with aging

In order to evaluate if the differences in LV dimensions between *Rage-/-* and wt mice is due to cardiac hypertrophy, we measured cardiomyocytes (CMs) size in all mice groups by means of WGA staining on heart sections. We observed a similar aging-dependent progressive increase of CMs size across Young, MA and Old wt and *Rage-/-* mice (Figure 4A). We confirmed these data using CMs isolated from hearts of MA *Rage-/-* and wt mice which did not show any difference in terms of cellular area, minor and major diameters (Figure 4B-C).

Then, we assessed whether augmentation of heart size in *Rage-/-* mice could be caused by the accumulation of collagen, a fibrotic process commonly associated with cardiac failure and aging. In particular, we evaluated interstitial and perivascular collagen I deposition by IHC on heart sections of all groups of animals. We found a gradually increase of collagen deposition in wt mice with aging (Figure 5A-B). Of note, MA *Rage-/-* mice had a higher amount of collagen I compared to the wt counterpart that did not increase further in the Old group (Figure 5A-B).

Thus, the age-associate alteration of LV dimensions of *Rage-/-* mice is mainly due to increase in fibrosis and not in cardiomyocytes hypertrophy.

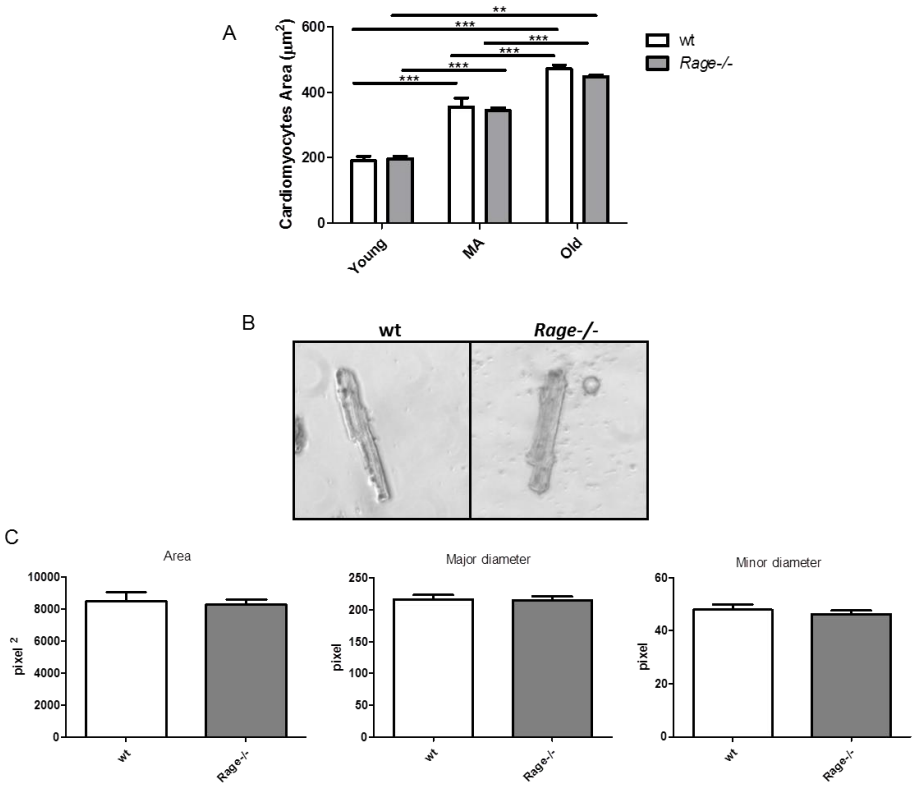

Figure 4. Cardiomyocytes size increase during aging without any difference between wt and *Rage-/-* mice. (A) Cross-sectional area of CMs evaluated by WGA staining during aging in wt and *Rage-/-* mice (One-Way ANOVA, Values are means ± SE n=160 cells/group; *, P<0.05; **, P <0.01; ***, P<0.0001); **(B)** Representative pictures of cardiomyocytes isolated from MA wt and *Rage-/-* mice. **(C)** Measurement of Minor Diameter, Major Diameter and Area of isolated CMs from MA mice.

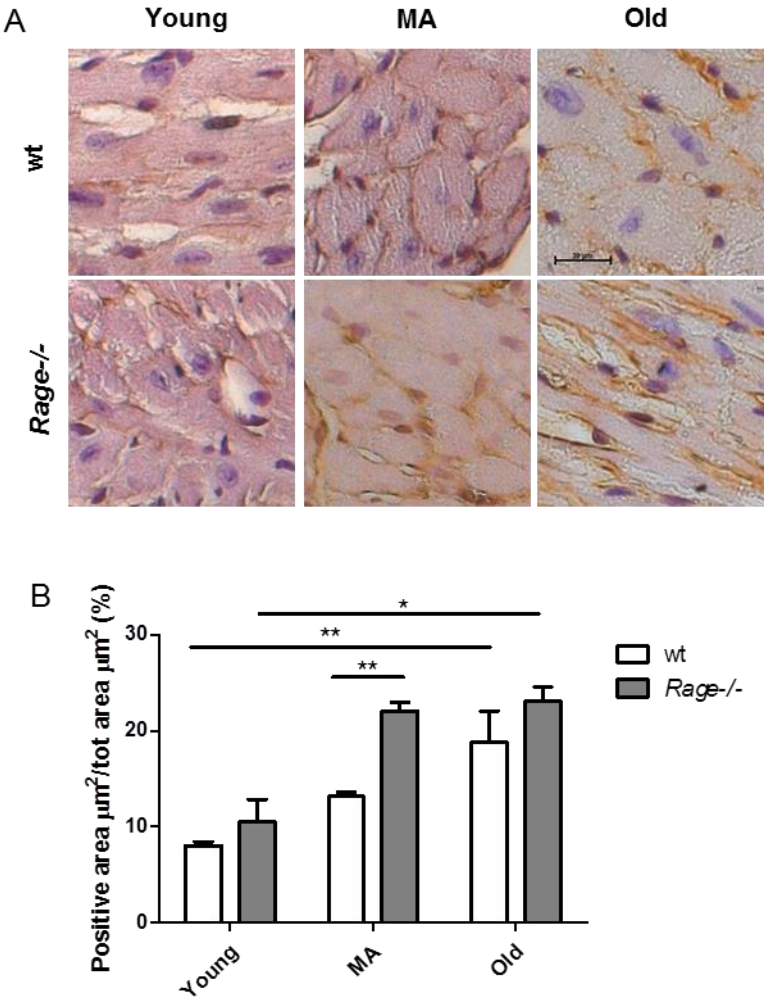

Figure 5. *Rage-/-* develop more fibrosis compared to wt mice during aging. (A) Representative images of LV mid-chamber sections stained for Collagen I (bar, 20 µm). **(B)** Quantification of Collagen I. One-way ANOVA for the analysis of mice of the same genotypes during aging; Two-way ANOVA for the comparison between the two genotypes during aging. Values are means ± SE (n= 4/group; *, P<0.05; **, P <0.01).

Rage-/- mice show higher expression of heart failure markers

Finally, we evaluated the mRNA levels of heart failure (HF) markers like Brain Natiuretic Peptide (BNP) and ankyrin repeat domain 1 (ANKRD1). BNP expression progressively increased with aging in wt mice, while reached the highest levels already in the MA *Rage-/-* animals (Figure 6A). ANKRD1 did not change among wt groups, but increased in MA *Rage-/-* mice in comparison to Young *Rage-/-* animals, with no further variation in the Old *Rage-/-* group (Figure 6B).

Hence, these results suggest that the absence of RAGE induces HF markers associated with cardiac aging.

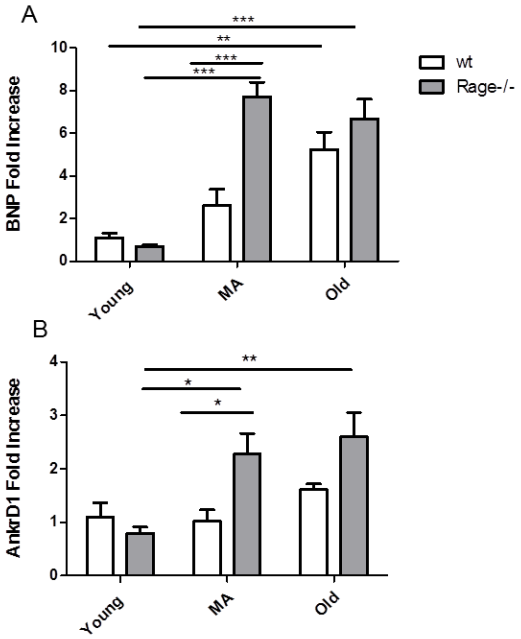

Figure 6. MA *Rage-/-* mice show an increase in HF markers compared to wt counterpart. HF marker genes BNP (**A**) and AnkrD1 (**B**) expression normalized to the average of 4 house-keeping genes (Ppih; hprt; gusb; ldhA). One-way ANOVA for the analysis of mice of the same genotypes during aging; Two-way ANOVA for the comparison between the two genotypes during aging. Values are means ± SE (n= 4/group; *, P<0.05; **, P <0.01; ***, P <0.0001).

RAGE and aging influence LV genes expression

To investigate how the lack of RAGE affects LV gene expression during aging, we performed a genome-wide gene expression analysis comparing Young and MA wt and *Rage-/-* mice, the two groups of animals in which we observed the greatest differences in the cardiac phenotype. The number of significant genes, by setting the proportion of false discoveries rate, at a FDR≤0.1 in a multivariate permutation F test ($p<0.001$), was 250. Within the 250 genes, we found 97 genes differentially expressed due to the effect of genotype, and 153 due to aging-genotype interaction.

The CLICK algorithm identified 5 clusters of genes (Figure 7):

1) 85 genes that were significantly upregulated in Young wt mice in comparison with the other groups.
2) 60 genes that had a strong increase in MA *Rage-/-* mice during aging.
3) 46 genes downregulated in *Rage-/-* groups compared to wt ones regardless of age.
4) 24 genes that gradually increased from Young wt to MA *Rage-/-* group.
5) 21 genes that increased from Young wt mice to Young *Rage-/-* and MA wt groups. The expression of these genes strongly decreased in MA *Rage-/-* group.

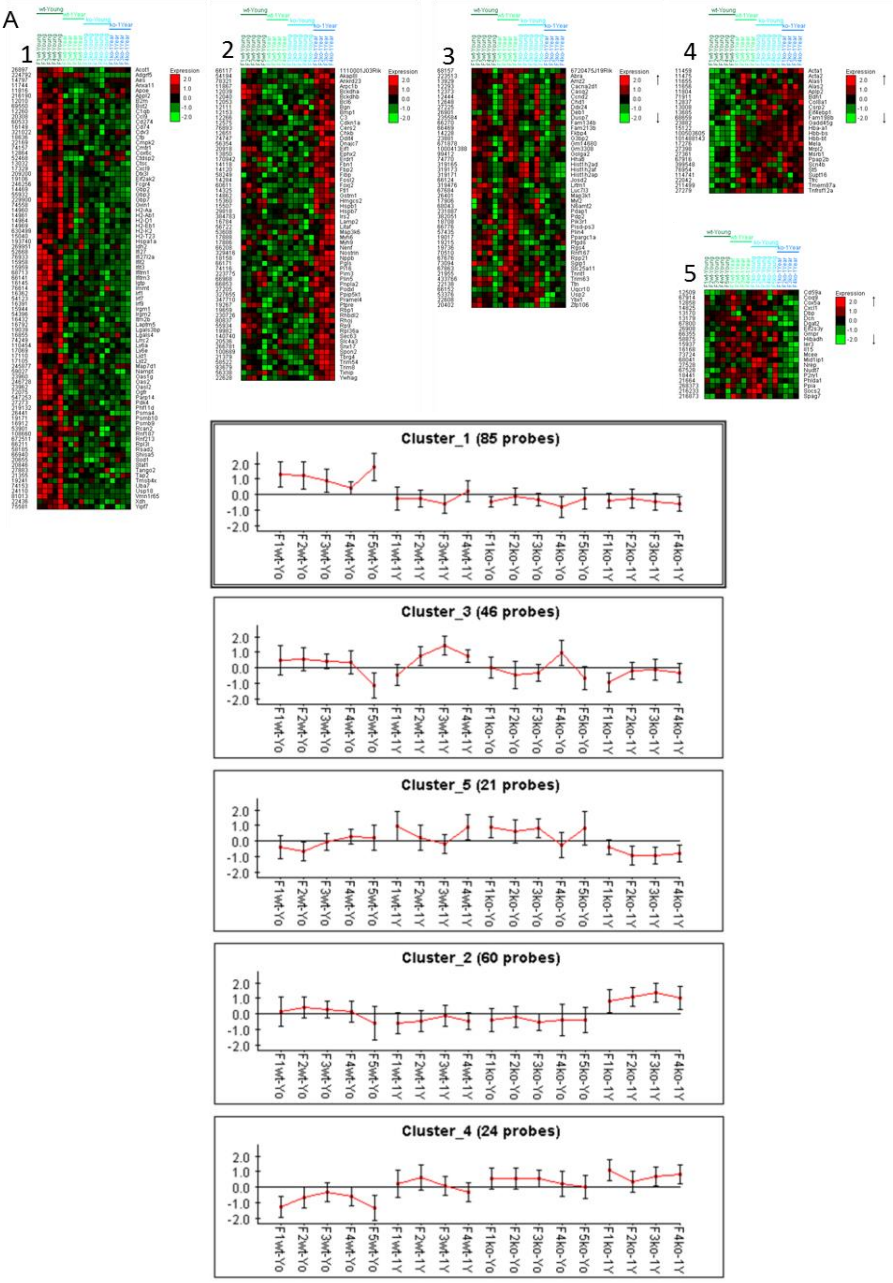

Figure 7.Clusters of genes significantly modulated by the genotype and the interaction between age and genotype. (A) Heatmap of clusters of CLICK analysis. Normalized expression values are mean-centered and represented with a green, black, and red color scale (green indicates below, black equal to, and red above the mean **(B)** Mean expression pattern of clusters. In the graphs **B**, each cluster is represented by the mean expression pattern over all the genes assigned to it for each sample; error bars denote ± 1 SD (n = 4/5 group).

Gene set enrichment analysis of the five clusters indicated that the absence of RAGE expression significantly modulates a number of biological processes during aging. In particular, cluster 1 was significantly enriched with genes belonging to the GO categories related to immune system according to the strong role of RAGE in the immunobiology of organisms[34], and cluster 3 which was significantly enriched with genes belonging to the GO categories related to muscle contraction like Titin, a molecule known to be involved in different form of cardiomyophathy[35]. The complete gene set enrichment analysis is reported in supplementary TableS2.

Thus, these data indicate the chronic lack of RAGE leads to a variation in the expression of genes involved in adverse LV remodeling.

Differentially expressed genes correlates with cardiac mice phenotype

In order to determine genes that are specifically involved in age-dependent LV remodeling, we performed a correlation analysis between the mRNA levels of genes belonging to the 5 clusters and LV functional parameters (LVESV, LVEDV, LVESD, LVEDD). Considering a FDR≤ 0.1, we found that, 7 genes correlate directly or inversely with LVSV, 8 genes with LVEDV, 17 genes with LVESD and 7 genes with LVEDD. The complete analysis is reported in Table S3. Figure 8 reports genes such as ACTA1, ZYX and serpin3n that positively correlate with all LV parameters. ACTA1 is involved in the formation of sarcomere and influences muscle contraction[36]

while ZYX is a focal adhesion protein with mechano-sensor properties[37]. Serpin3n is a serine protease inhibitor involved in wound healing processes[38] and its levels increase consistently with the cardiac fibrosis observed in MA *Rage-/-* mice. Furthermore, Siva 1, one of the genes that showed the strongest inverse correlation with LV diastolic volume and diameter, is known to be involved in the apoptotic process, usually impaired during aging[39]. Interestingly, the groups of mice spontaneously arrange themselves along the straight line from the Young wt to MA *Rage-/-* group (Figure 8), confirming that the cardiac phenotype changes associated to aging in wt mice are exacerbated in *Rage-/-* animals.

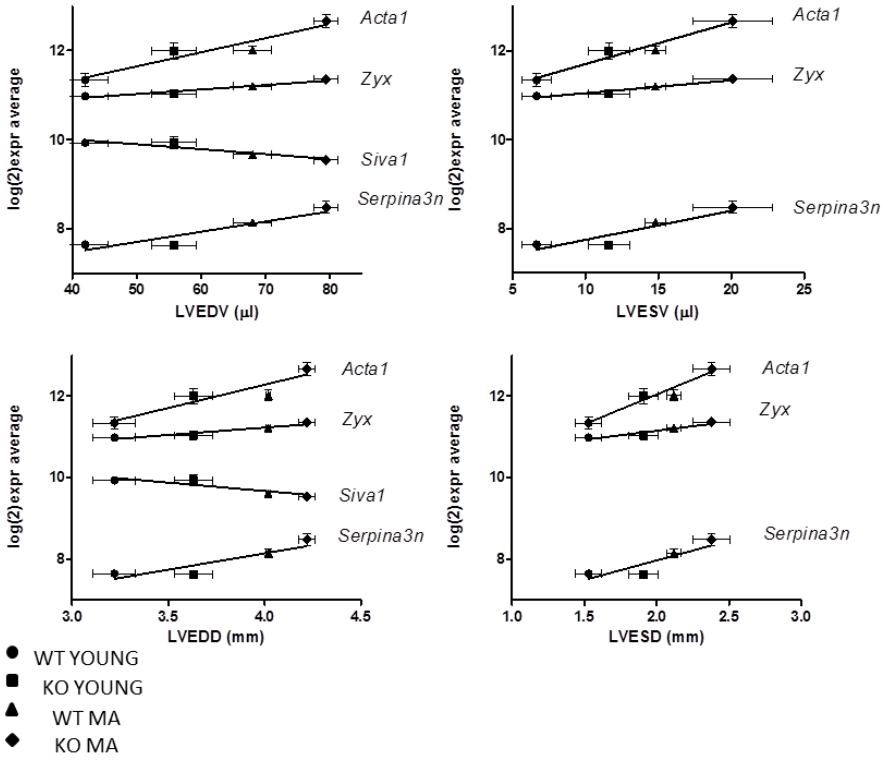

Figure 8. Differentially expressed genes correlate with echocardiographic values. Correlation analysis between echocardiographic parameters of LV volumes and diameters and the levels of differentially expressed genes. Values are means ± SE (n= 3/5 group). All genes shown have FDR ≤ 0,1.

Discussion

It has been previously shown that circulating concentrations of sRAGE decrease in different pathologic context such as diabetes, atherosclerosis and myocardial infarction[40,41,25]. Of note, Geroldi et al. published that healthy centenarians exhibit higher levels of sRAGE compared to 40-year-old people and proposed sRAGE as marker of longevity[25]. To our knowledge, none of these studies have investigated the sex differences.

Enrolled subjects were evaluated healthy after the assessment of standardized questionnaires, laboratory assays (reported in Table1) and physical examination. Despite some biochemical variables showed a significant difference during aging, these values, reported in Table1, are absolutely compatible with a healthy conditions, in line with healthy aging and overall not associated to a clear pathological condition. Moreover, Old people were subjected to in-depth examinations where were evaluated for all organs the healthy status and risk factors and also the presence of comorbidities that can affect the health of the subjects (CIRS)[31]. Almost all variables between males and females follow the same trend and the different statistical significance observed is probably due to the minor number of enrolled males compared to females. The power of the population evaluated in this study is that is composed by healthy people of different ages and that aged subjects showed a physiological decline in line with healthy aging.

In this population, we show for the first time that circulating sRAGE declines during healthy aging in humans and its levels inversely correlate with the chronological age of the subjects. Interestingly, we observed a different behaviour of sRAGE between males and females. Indeed, males showed a drastic drop of sRAGE levels from the young to the elderly group, while no further significant changes in the old subjects. Nevertheless, sRAGE circulating levels well correlate with the age of the participants (Figure 1). Differently, in females, we observed only a tendency to decrease of sRAGE in the old group without any decline in the elderly compared to the young group (Figure 1). Consistently, we did not found any significant correlation with chronological age of the enrolled females (Figure 1). In light of these data we propose sRAGE as a healthy and biological aging biomarker preferentially in males.

It is well known that an important feature of human aging is its sexual inequality. Life expectancy has increased with improvements in living conditions during the last century and has consistently revealed a survival difference between the sexes, with women living longer[42]. This could match with the sex-dependent different values of sRAGE. In fact, males show higher levels of sRAGE in the young group compared females, which, however, maintain higher values in older groups undergoing a slight decrease during aging compared to age-matched males (Figure 1). This behaviour could be associated to different aging processes occurring between the sexes.

Despite the abundance of studies performed to understand the

gender differences during aging and diseases, it is a field that still need deep investigations. It has been described that the longevity differences between sexes are derived from the genetic process of sex determination, as well as gender differences in lifestyles like smoking, alcohol consumption and exposure to risks such as occupational hazards and violence[43]. Moreover, also pregnancy and menopause change the lifespan and the healthy condition during aging[44]. In fact, the principal factors that influence the different phenotype from the born to the elderly between the two genders is the hormonal distinction. Females have more favourable innate biological health compared with males[42], consistently with the positive role of estrogens as antioxidant[45,46] and the influence that have on sexually dimorphic human immunocompetence. In general, estrogens are considered humoral immunity enhancers, whereas androgens and progesterone are considered natural immunosuppressant[47]. Interestingly, RAGE have a strong role in the immunobiology of the organism[48]. In fact, RAGE is expressed in many immune cell types including neutrophils, monocytes-macrophages, lymphocytes, antigen-presenting cells, as well as regulatory T cells[49] that are recruited in response to chronic inflammatory diseases[50]

The cause of the sex-dependent trend of sRAGE during aging will need further investigation.

sRAGE acts as decoy receptor neutralizing the binding of ligands to the membrane bound fl-RAGE and thereby attenuating its activity[20,51]. Moreover, the decoy effect of

sRAGE might block the action that RAGE ligands can exert also via other receptors rather than fl-RAGE[14,13]. The mechanism of action of sRAGE has never been investigated in the aging context. We found that, during aging, S100A8/A9, HMGB1 and AGEs, that are all involved in different ways in aging and in the pathogenesis of diseases that affect in particular CV apparatus[52,53,54,55,56,57,58], do not change or slightly increase, following an inverse trend compared to sRAGE and positively correlate with chronological age of the subjects (Table 2-3). Hence, it is plausible that during aging these molecules are free to bind and activate RAGE-dependent and/or -independent pro-inflammatory pathways worsening the organism conditions and make it more susceptible to different damages, a condition typical of senescence.

Notably, males show a better correlation between RAGE ligands and chronological age compared to females (Table 3), corroborating the hypothesis that in male sRAGE is a stronger indicator of the healthy status of the subjects.

We have generated Young, MA and Old male and female mice, and importantly, we found that the variation of circulating sRAGE with age in these animals is similar to human male subjects. Levels of sRAGE strongly decrease in the MA compared to the Young group. Male and female Old groups still show decreased sRAGE levels compared to Young animals but we observed also a slight and not significant increase compared to MA mice (Figure 2). RAGE expression is markedly higher in affected cells during normal development, diabetes,

cardiovascular disease, tumours and inflammation[59] The mechanisms that underlie the sRAGE production and cleavage are still to be totally clarified. Thus we can only hypothesize that age-related chronic inflammatory state or the possible presence of little solid tumours that usually occur in aged mice, would stimulate the generation and would allow the release of RAGE isoforms from the damaged cells of Old mice groups. This could explain the unexpected slight augmentation of sRAGE in Old mice.

Moreover, the absolute values of the soluble receptor are lower in female mice. Our findings also indicate that our animal model is a reliable one to study the effects of sRAGE variations in organs and tissues senescence as well as aging-associated diseases. Nevertheless, cardiac aging in mice well recapitulate human cardiac aging features, which include cardiac hypertrophy, fibrosis, diastolic dysfunction, reduced functional reserve and adaptive capacity to stress[60,61]. Echocardiography performed on the three age groups of mice confirms that aging influences LV dimensions and the major changes take place between Young and MA mice with no significant further alterations in Old animals in both sexes. On the other hand, LV systolic functions gradually decreases with age affecting mostly male animals, while LVAW in diastole and systole preferentially increase in females, consistently with human data[62,63]. Considering that cardiac changes associated with age are *per se* a marker of aging, we found that sRAGE is a biomarker of aging also in mice since its circulating levels well correlate with

most of functional and dimensional LV parameters in male, while with LVEDD, LVEDV and LVAWs in female animals (Table 6).

Our data also suggest that RAGE is directly involved in the aging-dependent cardiac remodelling. Indeed, *Rage-/-* female mice display dilation of LV chamber in diastole and systole, accompanied with a slight decrease of systolic function compared to age-matched animals with the strongest differences present between the MA groups (Table 4). Moreover, MA *Rage-/-* mice exhibit higher level of fibrosis and HF marker genes while no differences in cardiomyocytes hypertrophy in respect to MA wt animals (Figures 4-5-6).

Inflammatory cells recruitment can influence cardiac phenotype in particular during pathologic conditions as MI[64]. The remodeling showed by our aged mice is not influenced by inflammatory cells recruitment, probably because of the lack of a real injury that strongly activates the inflammatory processes. In fact, we observed very few inflammatory cells in cardiac tissues and no differences in the presence of CD45 positive cells between Young and MA or between MA wt and *Rage-/-* mice that are the groups in which we saw the major differences regarding cardiac remodeling (Figure S1).

In the close future we will characterize *Rage-/-* male mice cardiac phenotype.

Genome-wide expression profiling on the LV of Young and MA wt and *Rage-/-* mice revealed changes in expression of genes that are significantly modulated by both aging and the absence

of RAGE (Figure 7), and that contributes eventually to the cardiac phenotype. We decided to analyse these groups of animals because of the major differences observed in LV dimensions, function and fibrosis between the two genotypes (Tables 4-7 and Figures 5-6). In particular, CLICK algorithm identified 5 clusters of genes differentially expressed due to the lack of RAGE and the interaction between age and genotype that were afterwards investigated with gene set enrichment analysis. The first cluster shows 85 genes significantly overexpressed in Young wt mice in comparison with the other groups. As expected, since RAGE has a strong role in the immunobiology of the organism, we found a significant enrichment of many classes of gene related to adaptive and innate immune response, antigen processing and presentation and regulation of T cells and cytokines[48]. Among them, CD74 is present in 13 of 20 subgroups of genes in Cluster 1 and declines in *Rage-/-* mice during aging. It has been reported that CD74 is protective against fibrosis and this is consistent with the increase of collagen deposition and fibrosis observed in MA *Rage-/-* mice[65]. The second cluster is composed by 60 genes that strongly increase during aging in *Rage-/-* mice. Gene set enrichment analysis revealed 4 classes of genes involved in metabolic processes and enzyme activity. Ankrd23 and BNP belong to this cluster and are known to be upregulated during HF or high systolic blood pressure, respectively[66,67,68].

The third cluster is composed by 46 genes downregulated in *Rage-/-* groups regardless the age and are related to muscle

contraction and macromolecules modification processes. Titin, a gene of this cluster, is one of the most important player in muscle contraction by interacting with sarcomere structure[35]. Loss of function mutations or truncation of this protein are involved in HF and have been associated to different form of cardiomyophathy[69]. Trim63, another gene of this cluster, binds Titin on sarcomere and is involved in the impairment of cardiac function and in the regulation of cardiac energetics *in vivo*[70,71].

The fourth cluster presents 24 genes whose levels gradually increase from Young wt group to MA *Rage-/-* mice. From the enrichment analysis we found 3 differentially expressed classes of genes related to regulation of biological quality, system development and hemopoiesis. Alas2, gene that encodes for the mitochondrial enzyme which catalyzes the rate-limiting step in heme (iron-protoporphyrin) biosynthesis, is present in all classes and it has been demonstrated to increase in human failing heart[72]. ACTA1 is also present in this cluster. This protein forms the core of the thin filament of the sarcomere where it interacts with a variety of other proteins to produce the force for muscle contraction[36]. However, elevated levels of ACTA1 are found in animal model of dilated cardiomyopathy[73]. The fifth cluster includes 21 genes whose expression increases from Young wt mice to MA wt and Young *Rage-/-* group and is strongly decreased in MA *Rage-/-* mice. Gene enrichment analysis highlights only one class of genes associated to the carbohydrate derivative metabolic process.

Further correlation analysis have highlighted specific genes such as ACTA1, ZYX and SERPIN3n that positively correlate with LV dimensions (Figure 8). Thus, an augmentation of their expression correlates with LV dilation that increase with aging and in particular in *Rage-/-* mice. ZYX is a focal adhesion protein that has been implicated in the modulation of cell adhesion and motility and acts as a sensor in integrin-mediated responses to mechanical force[37]. Serpina3n is a serine protease inhibitor involved in wound healing processes[38] and administration of recombinant Serpina3n in myocarditis and dilated cardiomyopathy animal models has been shown to enhance cardiac fibrosis[74]. Yet, the apoptosis-inducing factor Siva-1 inversely correlates with diastolic diameters and volumes. A decrease of Siva-1 levels is known to protect CMs and fibroblasts from apoptosis[75,76] one of the most important mechanism induced by genomic instability occurring during aging[39].

Confirming previous published findings[48], we could not detect any of all RAGE isoforms in LV protein extracts of wt mice at any age (Figure 3). It is plausible that circulating sRAGE is the receptor isoform directly affecting the process of LV aging, and the cardiac phenotype of *Rage-/-* mice depends on the lack of circulating sRAGE. The best way to address this hypothesis is to administer recombinant sRAGE in aged wt and/or *Rage-/-* mice, since the generation of a sRAGE KO mouse is not doable because the predominant part of sRAGE derives from the

shedding of membrane-bound fl-RAGE[10]. We are currently setting this experiment.

In conclusion, in this study we demonstrate that sRAGE is a biomarker of healthy aging and an indicator of age-related cardiac remodelling. Furthermore, we propose that sRAGE exerts cardioprotective activity by counteracting the deleterious processes associated to aging.

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Supplementary Information

Table S1-Males LV functional parameters

	Young		MA		Old	
	wt (n=10)	*Rage-/-* (n=8)	wt (n=16)	*Rage-/-* (n=18)	wt (n=15)	*Rage-/-* (n=14)
HR (bpm)	486±4,435	461,8±8,420	442,3±8,463	448,3±7,663	477,2±8,401	472,6±8,817
LVEDV (µL)	49,48±2.698	61,33±4,490	77,01±3,309$^{###}$	73,29±3,515	75,11±3,606$^{###}$	83,36±3,304$^{###}$
LVESV (µL)	8,644±1,031	12,80±1,408	17,92±1,411$^{##}$	15,95±1,358	19,09±1,948$^{###}$	25,80±2,904*$^{##\,§§}$
LVEDD (mm)	3,447±0,078	3,766±0,1157	4,173±0,074$^{###}$	4,03±0,081	4,114±0,078$^{###}$	4,294±0,071$^{##}$
LVESD (mm)	1,686±0,0829	1,978±0,090	2,259±0,072$^{###}$	2,149±0,076	2,299±0,104$^{###}$	2,574±0,103$^{##\,§§}$
EF (%)	82,94±1,466	79,35±1,418	76,95±1,341	78,32±1,680	75,18±1,866$^{##}$	69,65±2,614$^{#\,§§}$
FS (%)	51,28±1,621	47,56±1,466	45,63±0,266$^{#}$	47,07±1,519	44,20±1,858$^{#}$	39,64±0,781$^{#\,§§}$
LVAWd (mm)	0,9286±0,018	0,9394±0,031	1,036±0,035	1,110±0,032$^{#}$	1,062±0,030$^{#}$	1,135±0,042$^{##}$
LVAWs (mm)	1,579±0,049	1,647±0,053	1,678±0,054	1,782±0,052	1,677±0,045	1,649±0,043
LV(mg)	91,18±3,742	112,4±8,608	132,2±4,274$^{###}$	143,9±3,972	141,1±5,607$^{###}$	166,0±9,953*$^{###}$

*_Rage-/-_ versus aged-matched wt group (2way-ANOVA analysis); $^{\#}$MA and Old versus Young of the same genotype-§Old versus MA of the same genotype (1way-ANOVA analysis) ; Values are means ± SE; $^{*,\#,§}$ P<0.05; $^{**,\#\#,§§}$, P <0.01; $^{***,\#\#\#,§§§}$, P<0.0001.

HR, Heart Rate; **LVEDV**, Left Ventricle End Diastolic Volume; **LVESV**, Left Ventricle End Sistolic Volume; **LVEDD**, Left Ventricle End Diastolic Diameter; **LVESD**, Left Ventricle End Sistolic Diameter; **EF**, Ejection Fraction; **FS**, Fractional shortening; **LVAWd**, Left Ventricle Anterior Wall in diastole; **LVAWs**, Left Ventricle Anterior Wall in sistole; **LV,** Left Ventricle Mass.

Table S2-GO enrichment analysis of differentially expressed gene clusters.

Set	Enriched with	#genes	p-Value	Gene List
Cluster_1	response to other organism - GO:0051707	23	0,001	Lyz1, Lyz2, H2-Eb1, Gbp7, Fcgr4, Eif2ak2, Tap2, Ifitm1, Cmpk2, Ifitm3, Oas1g, Bst2, Cxcl9, Ifi27l2a, Oas2, Irf1, Rsad2, Irgm2, Stat1, Gbp2, B2m, Ifit2, Gbp3
Cluster_1	innate immune response - GO:0045087	14	0,001	C1qb, Gbp7, Cfp, H2-Aa, Ifitm3, Bst2, Irf1, Irf7, Irgm1, Irgm2, Stat1, Gbp2, Gbp3, H2-Ab1
Cluster_1	immune effector process - GO:0002252	14	0,001	C1qb, Ctsc, Cd74, H2-Eb1, Ifitm1, Cfp, Ifitm3, Oas1g, Bst2, Cxcl9, Irf1, Rsad2, Irf7, H2-Ab1
Cluster_1	antigen processing and presentation - GO:0019882	9	0,001	H2-T23, Cd74, H2-Eb1, Tap2, Psmb9, B2m, H2-Aa, H2-D1, H2-Ab1
Cluster_1	response to interferon-gamma - GO:0034341	8	0,001	Gbp7, Irf1, Irgm2, Stat1, Gbp2, H2-Aa, Gbp3, H2-Ab1
Cluster_1	response to organic substance - GO:0010033	23	0,001	Ctsc, Cd74, Gbp7, Fcgr4, Eif2ak2, Tap2, H2-Aa, Cmpk2, Sod1, Aes, Ifitm3, Oas1g, Irf1, Igtp, Irgm2, Stat1, Apoe, Gbp2, B2m, Ifit2, Ifit3, Gbp3, H2-Ab1
Cluster_1	regulation of immune response - GO:0050776	12	0,001	C1qb, H2-T23, Cd74, H2-Eb1, Irf1, Rsad2, Irf7, Tap2, Cfp, B2m, H2-D1, H2-Ab1
Cluster_1	positive regulation of adaptive immune response - GO:0002821	7	0,001	H2-T23, Cd74, Rsad2, Tap2, B2m, H2-D1, H2-Ab1
Cluster_1	catabolic process - GO:0009056	20	0,001	Rnf213, Lyz1, Psma4, Lyz2, Idh2, Tap2, Uba7, Rnf187, Sod1, Oas2, Igtp, Irgm1, Irgm2, Apoe, Usp18, Psmb10, Gbp2, Psmb9, Xdh, Gbp3
Cluster_1	peptide antigen binding - GO:0042605	4	0,001	H2-Eb1, Tap2, H2-Aa, H2-Ab1
Cluster_1	negative regulation of cell activation - GO:0050866	7	0,001	Cd274, Cd74, H2-Eb1, Irf1, Apoe, H2-Aa, H2-Ab1
Cluster_1	cellular response to interferon-beta - GO:0035458	4	0,003	Igtp, Gbp2, Ifit3, Gbp3
Cluster_1	small molecule metabolic process - GO:0044281	18	0,003	Rnf213, Cd74, Nampt, Idh2, Tap2, Acot1, Cmpk2, Sod1, Oas1g, Oas2, Ly6e, Igtp, Pdk4, Irgm2, Apoe, Gbp2, Xdh, Gbp3
Cluster_1	cellular macromolecule catabolic process - GO:0044265	9	0,005	Psma4, Oas2, Apoe, Usp18, Uba7, Rnf187, Psmb10, Psmb9, Sod1
Cluster_1	regulation of cell proliferation - GO:0042127	13	0,012	Cd74, H2-Eb1, Nampt, Eif2ak2, Rnf187, H2-Aa, Ifitm3, Cd274, Irf1, Stat1, Apoe, Xdh, H2-Ab1
Cluster_1	T cell activation - GO:0042110	6	0,018	Cd74, Fcgr4, Irf1, Rsad2, Psmb10, B2m
Cluster_1	protein metabolic process - GO:0019538	21	0,04	Rnf213, C1qb, Psma4, Ctsc, Cd74, Ctdsp2, Eif2ak2, Uba7, Rnf187, Cfp, Sod1, Oas2, Dtx3l, Irf1, Irf7, Pdk4, Usp18, Psmb10, Psmb9, B2m, Irf9

Set	Enriched with	#genes	p-Value	Gene List
Cluster_1	induction of programmed cell death - GO:0012502	6	0,082	Ctsc, Irf1, Itm2b, Apoe, Stat1, Shisa5
Cluster_1	positive regulation of cytokine production - GO:0001819	5	0,121	Cd74, Irf1, Rsad2, Irf7, Sod1
Cluster_1	positive regulation of T cell activation - GO:0050870	4	0,186	Cd74, Irf1, H2-Aa, H2-Ab1
Cluster_2	small molecule metabolic process - GO:0044281	14	0,006	Hmgcs2, Nppb, Bckdhb, Ankrd23, Gstm1, Bckdha, Rhoj, Ephx2, Rbp1, Ppip5k1, Myh9, Pgls, Chkb, Myh6
Cluster_2	phosphate-containing compound metabolic process - GO:0006796	10	0,134	Akap8l, Ptpre, Ephx2, Ppip5k1, Pim3, Tbrg4, Cdkn1a, Chkb, Map3k6, Fbp2
Cluster_2	cellular lipid metabolic process - GO:0044255	7	0,162	Cers2, Ankrd23, Ephx2, Rbp1, Chkb, Hmgcs2, Pnpla2
Cluster_2	enzyme inhibitor activity - GO:0004857	5	0,194	C3, Txnip, Hspb1, Pi16, Cdkn1a
Cluster_3	muscle contraction - GO:0006936	4	0,027	Trim63, Tnnt1, Myl2, Ttn
Cluster_3	protein modification by small protein conjugation or removal - GO:0070647	5	0,166	Trim63, Rnf167, Map3k1, Usp2, Josd2
Cluster_3	macromolecule modification - GO:0043412	10	0,198	Trim63, Rnf167, Map3k1, Pdp2, Usp2, Josd2, Dusp7, Pik3r1, Fkbp4, Ttn
Cluster_4	regulation of biological quality - GO:0065008	8	0,044	Acta2, Alas2, Tfrc, Ppap2b, Aplp2, Hba-a1, Tnfrsf12a, Scn4b
Cluster_4	system development - GO:0048731	9	0,06	Acta1, Gadd45g, Alas2, Tfrc, Ppap2b, Aplp2, Hba-a1, Col8a1, Tnfrsf12a
Cluster_4	hemopoiesis - GO:0030097	4	0,118	Gadd45g, Alas2, Tfrc, Hba-a1
Cluster_5	carbohydrate derivative metabolic process - GO:1901135	5	0,189	Nudt7, Il15, Eif2s3y, Dcn, Phlda1

GO: Gene Ontology

Table S3- Gene expression levels and LV parameters correlations

Diastolic Volume				Systolic Volume			
Symbol	**r**	**p-value**	**FDR**	**Symbol**	**r**	**p-value**	**FDR**
Acta1	0,902	< 1e-07	< 1e-07	Slc47a1	-0,868	< 1e-07	< 1e-07
Ciapin1	0,863	< 1e-07	< 1e-07	Acta1	0,931	< 1e-07	< 1e-07
Serpina3n	0,85	< 1e-07	< 1e-07	Ak3	-0,846	< 1e-07	< 1e-07
Siva1	-0,855	< 1e-07	< 1e-07	Ciapin1	0,85	< 1e-07	< 1e-07
Zyx	0,922	< 1e-07	< 1e-07	Nr1d2	-0,85	< 1e-07	< 1e-07
Desi1	-0,841	5,50E-06	0,0155	Tlr2	-0,848	< 1e-07	< 1e-07
Bdh1	0,833	2,18E-05	0,0527	Zyx	0,924	< 1e-07	< 1e-07
Mical3	0,831	2,83E-05	0,0598	Hsp90b1	-0,841	5,50E-06	0,0103
				Serpina3n	0,841	5,50E-06	0,0103
				Dennd4b	0,833	2,18E-05	0,0307
				Ifi27l2a	-0,833	2,18E-05	0,0307
				Samhd1	-0,833	2,18E-05	0,0307
				Atp2a3	0,831	2,83E-05	0,0319
				Trim30a	-0,831	2,83E-05	0,0319
				Usp18	-0,831	2,83E-05	0,0319
				Bst2	-0,828	3,53E-05	0,0351
				Dnajb2	0,828	3,53E-05	0,0351
				Mical3	0,826	4,29E-05	0,0403
				Bdh1	0,824	5,12E-05	0,0456
				Stk16	0,821	6,00E-05	0,0508
				Gbp3	-0,819	6,96E-05	0,0561
				Cdca2	0,809	0,000115	0,0849
				Cdc37	-0,809	0,000115	0,0849
				Gucd1	-0,806	0,000129	0,0909

Diastolic Diameter				Systolic Diameter			
Symbol	**r**	**p-value**	**FDR**	**Symbol**	**r**	**p-value**	**FDR**
Acta1	0,897	< 1e-07	< 1e-07	Slc47a1	-0,871	< 1e-07	< 1e-07
Ciapin1	0,862	< 1e-07	< 1e-07	Acta1	0,95	< 1e-07	< 1e-07
Noxa1	-0,853	< 1e-07	< 1e-07	Ak3	-0,862	< 1e-07	< 1e-07
Siva1	-0,853	< 1e-07	< 1e-07	Dennd4b	0,862	< 1e-07	< 1e-07
Zyx	0,912	< 1e-07	< 1e-07	Ddc	-0,856	< 1e-07	< 1e-07
Slc47a1	-0,847	1,02E-05	0,0288	Tlr2	-0,871	< 1e-07	< 1e-07
Serpina3n	0,844	1,93E-05	0,0466	Zyx	0,924	< 1e-07	< 1e-07
				Trim30a	-0,85	2,00E-06	0,00423
				Hsp90b1	-0,847	1,02E-05	0,0173
				Samhd1	-0,847	1,02E-05	0,0173
				Ifi27l2a	-0,844	1,93E-05	0,0297
				Nr1d2	-0,835	5,24E-05	0,0739
				Ciapin1	0,829	8,00E-05	0,0951
				Rai2	0,829	8,00E-05	0,0951
				Atp2a3	0,826	9,56E-05	0,0951
				Siva1	-0,826	9,56E-05	0,0951
				Usp18	-0,826	9,56E-05	0,0951
				Serpina3n	0,824	0,000113	0,1
				Tro	0,824	0,000113	0,1

Correlation analysis between the expression levels of differentially expressed genes and echocardiographic values of LV volumes and diameters. Values are means ± SE (n= 3/5 group). Threshold setted to FDR ≤ 0,1

Fig S1 CD45 Positive cells in cardiac tissue

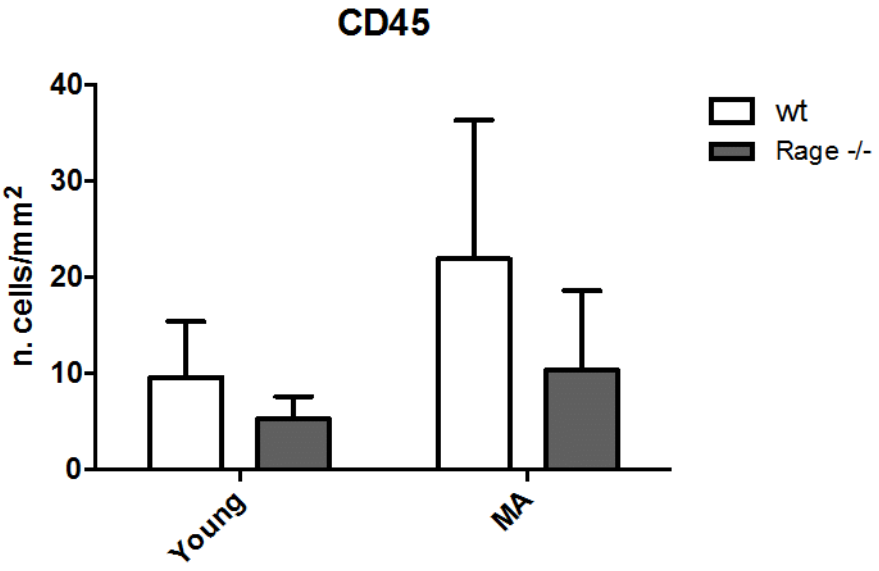

Figure S1. Inflammatory cells recruitment in cardiac tissue. Quantification of number of CD45 positive cells on tissues Area. Area was expressed as mm^2 due to the very low number of cells for Area. T-test for the analysis of mice of the same genotypes during aging; Two-way ANOVA for the comparison between the two genotypes during aging. Values are means ± SE (n= 4/5 group; *, $P<0.05$; **, $P<0.01$).

Chapter 3

Summary

Aging is an unavoidable risk factor in later life that can influence the onset and progression of many diseases. In fact, the high incidence of cardiovascular diseases in the elderly is mainly attributable to cardiac remodelling associated to physiological intrinsic aging. RAGE is a multi-ligand receptor involved in many age-related disorders. Its soluble isoform (sRAGE) acts as a decoy receptor being able to block the activation of the membrane-bound receptor, and its circulation levels have been found altered in several chronic and acute pathologies. The role of RAGE isoforms in aging and, in particular, cardiac senescence has never been investigated. Moreover, the finding of reliable biomarkers able to assess individual health status of subjects has important applications in prevention, diagnosis, and disease management. In this context, the aim of this study was to ascertain whether sRAGE is a biomarker of aging and age-related cardiac remodelling, and evaluate the contribution of RAGE isoforms to cardiac aging.

The principal result of this study is the assessment of sRAGE as a biomarker of healthy aging and a potential indicator of age-related cardiac remodelling preferentially in males, while in females the power of sRAGE as a biomarker is less reliable.

We evaluated by ELISA, in human serum of healthy subjects at different age (22-92 years old), the levels of sRAGE and we found a strong decrement during aging and a good inverse correlation with chronological age of the subjects in males. Females showed a similar trend but not significant values. RAGE ligands such as S100, HMGB1 and AGEs, involved in the onset and progression of age related

chronic inflammatory diseases, showed a trend to increase or, at least, to not decrease during aging in both sexes. The correlations between circulating levels of these molecules with the age of subjects are stronger in males compared to females, corroborating what proposed above. Taking advantage of a mouse model that well recapitulated human cardiac aging, we observed that sRAGE decrease during aging in male and female mice following the same trend while cardiac levels of RAGE resulted undetectable in both sexes, suggesting that soluble isoform play the main role in age-related cardiac remodelling. Male and female mice at different age (2,5-Young; 12-MA; 22-Old month old) were subjected to 2-D echocardiography and the results were correlated with their serum sRAGE levels evaluated by ELISA. Interestingly also this analysis revealed sRAGE as a stronger biomarker of cardiac remodelling during aging preferentially in males compared to females.

We wanted also investigated, taking advantage of a total *Rage-/-* model, if soluble isoform among the others, can play the principal role in directly influencing cardiac remodeling. *Rage-/-* mice of both of sexes showed a worsening in cardiac conditions evaluated by 2-D echocardiography. We further investigated the heart of female mice wt and *Rage-/-.* Consistently with echocardiographic values we did not find any increase in CM size between wt and *Rage*-/-. On the contrary we observed a significant increase of collagen deposition and of heart failure markers in *Rage-/-* mice compared to wt counterpart, in particular in MA groups. Moreover, we performed a gene expression analysis in order to evaluate how the lack of RAGE during aging can influence the gene expression scenario. We performed this analysis only on Young and MA female mice, gender and age where we saw the main differences. Microarray functional annotation analysis based on the interaction between age-genotype

revealed that the chronic lack of RAGE affected the expression of genes associated to heart development, contractile fibre function, antigen presenting process and adaptive immunity, insulin pathway, cell death and apoptosis. Moreover we found a correlation between echocardiographic parameters and differentially expressed genes involved in different process like muscle contraction (ACTA1), fibrosis and wound healing (SerpinA3n) or regulation of apoptosis (SIVA1).
Our results suggest that sRAGE is a serum biomarker of aging and age-related cardiac remodeling preferentially in males. Moreover, we demonstrated that that the lack of RAGE during aging lead to an adverse cardiac remodeling in *Rage-/-* mice.

Translational significance and future perspectives

The principal result of my thesis is the identification of sRAGE as a biomarker of healthy aging, preferentially in males, and as a potential indicator of age-related cardiac remodelling. In the recent years, increasing efforts have been made to identify molecules able to predict the individual health conditions during aging. Usually, the classical quantitative assessment of "aging rate" is based on the analysis of the mortality curves (Gompertz function) of populations. In other words, individuals must be followed until the end of their lives to determine their "biological age". A strategy to solve this problem is to identify age-related changes in physical functions or the presence of molecules that could measure "biological" age and the frailty of the subjects, predict the future onset of age-related diseases, and evaluate the expectation of life more accurately than “chronological” age. The final target is to find a molecular-based definition of aging that uses information from multiple circulating biomarkers to generate a set of molecules associated with different mortality and morbidity

age-associated risk. The discovering of new molecules could help to design efficiently this set in order to create a potent investigative, diagnostic, and prognostic tool.

Our study proposed sRAGE as a possible member of this group of molecules.

Indeed, sRAGE has the characteristics of a good marker of healthy aging according to the guidelines of the American Federation for Aging Research described below[1].

- •Must predict the rate of aging predicting better life expectancy than chronological age.
- •Must Control processes that are at the basis of aging, such as inflammation and tissue damage.
- •Must be able to repeatedly be tested without damaging the people.
- •Must be something that can be tested in laboratory animals before being validated in humans.

Our study revealed that the biomarker capacity of sRAGE is stronger in males than in females. In fact, senescence affects both men and women, but in different ways: a sexual dimorphism of different pathways in the setting of organism response to aging is normally present[2]. Therefore, the identification of biomarkers specific for each gender is fundamental.

In this study we also observed that the cardiac functions of mice during aging correlate with sRAGE levels allowing us to suggest sRAGE as a predictor of age-related cardiac remodelling. To confirm this hypothesis is necessary evaluate the behaviour of sRAGE also during human cardiac aging.

In fact, in the next future we have set a clinical study to evaluate, in healthy subjects at different ages, the correlation between sRAGE

levels and cardiac parameters estimated by cardiac magnetic resonance. In this way we will characterize, also in human, whether sRAGE is a specific biomarker of cardiac healthy age-related remodelling.

In the attempt to understand whether sRAGE has a role in determining the changes associated to cardiac aging, we will investigate its direct role in this process. Indeed, since RAGE isoforms are undetectable in the LV, we suggest that sRAGE can play the main role in influencing cardiac aging and the adverse remodelling observed. In light of this, the detrimental effect of the lack of RAGE observed in *Rage-/-* mice during aging is mainly related to the absence of sRAGE. To definitively prove that sRAGE is directly involved in the cardiac aging process, we want to set a rescue experiment in which sRAGE will be injected in aged wt and *Rage-/-* mice. The progressive understanding of sRAGE involvement in aging and age-related CVD will allow to suggest it as therapeutic molecule.

Recombinant sRAGE could be used for the treatment or prevention of diabetic complications, as well as in many RAGE-mediated disorders present in normoglycemic patients.

Since this protein is involved in many different pathways the main concern about recombinant sRAGE in clinics regards the tolerability and the potentially adverse effects in humans.

We also have to take in account that the proteolytic cleavage of the receptor plays a pivotal role regulating the balance between the detrimental RAGE signalling and protective effect of sRAGE. Intriguingly current available CV drugs like statins, ACE inhibitors, thiazolidinediones and Angiotensin II Type 1 Receptor Blockers, already have the ability to increase soluble RAGE levels[3,4,5,6,7,3,8].

In conclusion from the translational point of view, the understanding of the nature and the role of RAGE in aging and in age-related CVD,

can help in different phases of health management. In fact on one hand the levels of sRAGE, gender-specific biomarker of healthy aging and cardiac remodelling, can support physicians to choose the better therapy or interventions based on the healthy status of the subject and on the other hand sRAGE could be itself the possible therapeutic target to counteract RAGE-mediated disorders, in particular CVD.

Publications

1-The receptor for advanced glycation end-products (RAGE) is only present in mammals, and belongs to a family of cell adhesion molecules (CAMs).

Luca Sessa[1], Elena Gatti[1], **Filippo Zeni[2]**, Antonella Antonelli[1], Alessandro Catucci[1], Michael Koch[3], Giulio Pompilio[2], Gunter Fritz[3], Angela Raucci[2]*, Marco E. Bianchi[1]*.

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Abstract

The human receptor for advanced glycation endproducts (RAGE) is a multiligand cell surface protein belonging to the immunoglobulin superfamily, and is involved in inflammatory and immune responses. Most importantly, RAGE is considered a receptor for HMGB1 and several S100 proteins, which are

Damage-Associated Molecular Pattern molecules (DAMPs) released during tissue damage. In this study we show that the Ager gene coding for RAGE first appeared in mammals, and is closely related to other genes coding for cell adhesion molecules (CAMs) such as ALCAM, BCAM and MCAM that appeared earlier during metazoan evolution. RAGE is expressed at very low levels in most cells, but when expressed at high levels, it mediates cell adhesion to extracellular matrix components and to other cells through homophilic interactions. Our results suggest that RAGE evolved from a family of CAMs, and might still act as an adhesion molecule, in particular in the lung where it is highly expressed or under pathological conditions characterized by an increase of its protein levels.

2-MicroRNA-34a Induces Vascular Smooth Muscle Cells Senescence by SIRT1 Downregulation and Promotes the Expression of Age-Associated Pro-inflammatory Secretory Factors.

Ileana Badi[1,2], Ilaria Burba[1,3], Clarissa Ruggeri[1,2], **Filippo Zeni[1,2],** Matteo Bertolotti[1,2], Alessandro Scopece[1], Giulio Pompilio[1], and Angela Raucci[1,2].

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Abstract

Arterial aging is a major risk factor for the occurrence of cardiovascular diseases. The aged artery is characterized by endothelial dysfunction and vascular smooth muscle cells altered physiology together with low-grade chronic inflammation. MicroRNA-34a (miR-34a) has been recently implicated in cardiac, endothelial, and endothelial progenitor cell senescence; however, its contribution to aging-associated vascular smooth muscle cells phenotype has not been explored so far. We found that miR-34a was highly expressed in

aortas isolated from old mice. Moreover, its well-known target, the longevity-associated protein SIRT1, was significantly downregulated during aging in both endothelial cells and vascular smooth muscle cells. Increased miR-34a as well as decreased SIRT1 expression was also observed in replicative-senescent human aortic smooth muscle cells. miR-34a overexpression in proliferative human aortic smooth muscle cells caused cell cycle arrest along with enhanced p21 protein levels and evidence of cell senescence. Furthermore, miR-34a ectopic expression induced pro-inflammatory senescence-associated secretory phenotype molecules. Finally, SIRT1 protein significantly decreased upon miR-34a overexpression and restoration of its levels rescued miR-34a-dependent human aortic smooth muscle cells senescence, but not senescence-associated secretory phenotype factors upregulation. Taken together, our findings suggest that aging-associated increase of miR-34a expression levels, by promoting vascular smooth muscle cells senescence and inflammation through SIRT1 downregulation and senescence-associated secretory phenotype factors induction, respectively, may lead to arterial dysfunctions.

Abstract publication

Role of the receptor for advanced glycation end-products (RAGE) in age dependent left ventricle dysfunction

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Abstract

Background: Aging is the major risk factor for cardiovascular (CV) diseases: on one hand, it prolongs exposure to other CV

risks, on the other, intrinsic cardiac aging predisposes the heart to stress and increases mortality and morbidity in the elderly. The Receptor for Advanced Glycation End-Products (RAGE) is a multi-ligand receptor involved in many age-related disorders. Nevertheless, its role in intrinsic cardiac aging remains unexploited.

Purpose: To evaluate the role of RAGE in cardiac aging.

Methods: We compared 2 groups of female *Rage-/-* and C57BL/6 (wt) mice of different ages: 2.5- (Young) and 12-month (1 Year) old mice. Left ventricular (LV) parameters and function were evaluated by 2-D transthoracic echocardiography. Fibrosis was assessed by evaluating collagen I deposition on heart sections. Phosphorylation of AKT and GSK-3 β was determined on LV protein extracts. Microarray gene expression analysis was performed on RNAs from LV.

Results: Hearts of *Rage-/-* mice exhibited an increase in LV/tibia length ratio already at 2,5 months of age compared to age-matched wt mice, and this was accompanied with a slight but not significant increase of EDV and ESV (End-Diastolic and End-Systolic Volume) or EDD and ESD (End-Diastolic and End-Systolic Diameter). At 1 year of age, *Rage-/-* mice showed a further significant increase of EDV and ESV or EDD and ESD along with a reduction in the ejection fraction (EF) and fractional shortening (FS). In contrast, no significant differences were observed in the LV wall thickness between wt and *Rage-/-* mice at any age. Expression of heart failure marker genes (BNP, β-MHC and Ankrd1) did not differ between Young wt and *Rage-/-*

mice, but was induced in the 1 Year *Rage-/-* group.Compared with wt mice, 12-month old *Rage-/-* mice exhibited an increase in perivascular and interstitial fibrosis, and consistently a higher expression of TGF-β2 and TGF-β3 transcripts and activation of AKT. GSK-3β phosphorylation, known to regulate cardiac fibrosis, increased with age in wt mice, but was already induced at high levels in Young *Rage-/-* mice. Microarray functional annotation analysis based on the interaction between age-genotype revealed that the chronic lack of RAGE affected the expression of genes associated to heart development, contractile fiber function, antigen presenting process and adaptive immunity, insulin pathway, cell death and apoptosis.

Conclusions: Our results suggest that the absence of RAGE causes gene expression changes leading to age-dependent LV dilatation, fibrosis and heart failure.

Proceedings:

1-Badi, I. Burba, C. Ruggeri, **F. Zeni**, M. Bertolotti, A. Scopece, M. C. Caporossi, G. Pompilio, A. Raucci.

"MicroRNA-34a induces vascular smooth muscle cells senescence by SIRT1 downregulation and promotes the expression of age-associated pro-inflammatory secretory factors". WWW.Ricercamonzino.it "Who we are, What we do, Where are we going". Centro Cardiologico Monzino, Milan, Italy, February 27-28, 2015.

2-I. Badi#, **F. Zeni#**, I. Burba, M. Bertolotti, D. Ferri, F. Taccia, A. Scopece, M.C. Capogrossi, G. Pompilio and A. Raucci. *"MicroRNA-34a induces Vascular Smooth Muscle Cells Senescence and contributes to arterial inflammaging".* 6th Annual World Congress of Molecular & Cell Biology CMCB-2016. Dalian (China), April 25-28, 2016.

Poster Presentation

"Role of the receptor for advanced glycation end-products (RAGE) in age dependent left ventricle dysfunction".
Frontiers in Cardiovascular Biology 2016 Florence 8th-10th July Bienniel Congress of the ESC Council on Basic Cardiovascular Science.
Funded by DIMET

Oral Presentation

“Role of the receptor for advanced glycation end-products (RAGE) in age dependent left ventricle dysfunction”.
ICGEB Course “US Imaging" in collaboration with Fujifilm VisualSonics (8 - 10 February 2017, Trieste, Italy).
Selected for ICGEB travel grant

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