Universidade de Aveiro Departamento de Ciências Médicas

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Ana Filipa Seixas Fonte da Rocha

Metabolómica de linhas celulares: uma ferramenta para o estudo do envelhecimento

Metabolomics of cell lines: a tool for aging research

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Carla Alexandra Pina da Cruz Nunes, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro.

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palavras chave

Biomarcadores de envelhecimento, culturas de células para estudos de envelhecimento, envelhecimento, fibroblastos, FTIR, metabolómica

resumo

O mundo está a envelhecer e, torna-se fundamental perceber como é o desenvolvimento desse processo nos organismos, principalmente nos seres humanos. É sabido que este fenómeno não ocorre da mesma forma em todos os indivíduos e que existem alguns com idade cronológica de 80 anos e, ainda assim, apresentam uma condição de um indivíduo mais jovem. Assim, é essencial unir esforços para entender este processo. Atualmente, existem vários modelos para o estudo do envelhecimento, desde modelos animais a modelos computacionais ou celulares, sendo que este último apresenta mais vantagens. Modelos celulares, nomeadamente, modelos celulares de fibroblastos são cada vez mais utilizados em metabolómica para a investigação do envelhecimento, pois através destas técnicas é possível estudar indiretamente as vias desse processo, uma vez que fornece informações valiosas sobre a composição dos metabolitos presentes numa amostra. Uma abordagem combinatória dessas ferramentas, como FTIR, MS ou NMR, constitui uma mais valia, permitirá o estudo do envelhecimento a um nível molecular e, possivelmente, revelará novas descobertas.

key-words

Aging, aging biomarkers, cell cultures for aging studies, fibroblasts, FTIR, metabolomics

abstract

The world is aging and therefore it is essential to understand how the development of this process in organisms is, especially in human beings. Its knowing that this phenomenon does not occur in the same way in all individuals, and that there are some with a chronological age of 80 years and yet, they present a condition of a younger subject. Thus, it is essential to join efforts to understand this event. Currently, there are several models for the study of those process, from animal models, to computational or cellular models, with the latter having more advantages. Cell models, namely cell models of fibroblasts, are thus increasingly used for metabolomics research in the aging research since through these techniques we are able to indirectly study the pathways of this process, since this gives valuable insights into the composition of metabolites present in a sample. An approach that combines several of these tools, such as FTIR, MS or NMR, will be an asset and will allow for the study of aging at a molecular level and possible reveal new discoveries.

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ABBREVIATIONS

AFAR	American Federation for Aging Research
AKT	Protein Kinase B
Ala – Gln	Alanyl - Glutamine
AGEs	Advanced Glycation End products
AMP	Adenosine Monophosphate
AMPK	AMP – activated protein kinase
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
CRAMP	Cathelicidin-Related Antimicrobial Peptide
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
DPs	Population Duplicities
ECM	Extracellular Matrix
EF-1a	Elongation Factor 1-alpha
EGFR	Epidermal Growth Factor Receptor
EMC	Endoplasmic Reticulum Membrane Protein Complex
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
FOXO proteins	Forkhead box class O family member proteins
FTIR	Fourier Transform Infrared Spectroscopy
GH	Growth Hormone
GPC	L-a glycerophosphocholine
GC-MS	Gas Chromatography/Mass Spectrometry
HDFs	skin Human Diploid Fibroblasts
hMSC	human Mesenchymal Stem Cells

HSCs	Hematopoietic Stem Cells
H_2O_2	Hydrogen Peroxide
IGF-1	Insulin-like Growth Factor
IIS	Insulin/Insulin-like growth factor 1 (IGF-1) Signalling
IrrDBSsen	Irreparable Double Strand DNA Breaks
LC–MS	Liquid Chromatography/Mass Spectrometry
LMNA	Lamin A/C
MIR	Medium Infrared Range
MOAG-4	Modifier of Aggregation 4
mRNA	messenger Ribonucleic Acid
MS	Mass Spectrometry
mtDNA	mitochondrial Deoxyribonucleic Acid
mTOR	mammalian Target Of Rapamycin
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NK	Natural Killer
NMR	Nuclear Magnetic Resonance
NOXs	NADP-Oxidases
ODEs	Ordinary Differential Equations
PGE2	Prostaglandin E2
PEsen	Proliferative Exhaustion
PMLNBs	Promyelocytic Leukemia Protein Nuclear Bodies
PoMiCS	Post-Mitotic Cell Senescence
POT1	Protection of Telomeres protein 1
p-S6RP or pS6	phosphorylated S6 ribosomal protein
PSCs	Pluripotent Stem Cells
ROS	Reactive Oxygen Species
RS	Replicative Senescence
SA-βgal	Beta-Galactosidase Activity Associated with Senescence

SAHF	Senescence-Associated Heterochromatin Foci
SAM	Senescence-Accelerated Mouse
SAMP	Senescence-Accelerated Mouse Prone
SAMR	Senescence-Accelerated Mouse Resistant
SDFs	Foci of DNA Damage Associated with Senescence
SIPS	Stress-Induced Premature Senescence
SIRTs	Sirtuins
t-BHP	tert-Butyl Hydroperoxide
TCA	Tricarboxylic Acid Cycle
TRF2	Telomeric Repeat-binding Factor 2
UV	Ultraviolet light
UVB	Ultraviolet light B
WI-38	Human Diploid Lung Fibroblast Cell Line
γDNA polymerase	gamma Deoxyribonucleic Acid polymerase
γH2AX	phosphorylated form of histone H2AX

CHAPTER I: INTRODUCTION

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Aging is as a complex multifactorial process that, although universal, is not fully understood. It is believed that the impact of aging on health is influenced by sex, race, income, and education, and that age-related diseases are strongly associated the way people get old. The knowledge of biological aging and its comparison to the chronological age is a paramount contributor to predict metabolic decline and the onset of age-related diseases.

Therefore, there are several models for the study aging, from computational models to animals or even to cell cultures. As aging processes observed in whole human organism are somehow the reflexion of what happens in each cell type, it is possible to study aging process using cell lines, such as fibroblasts. These models have shown high potential for aging studies, as they are easier to handle, cheaper, do not require the same level of ethical consideration compared with animal and human studies and present little biological heterogeneity when grown under the same conditions and in the same context population. For aging studies, these aspects are a great advantage since cells have a huge variety of morphologic characteristics and markers that can be studied.

Interindividual variability in response to external factors and susceptibility to disease is a factor that is quite implicated in clinical practice. So, today, personalized medicine is highly valued, with the idea of prescribing the right medicine for the right patient. Thus, metabolomics has been increasingly used to predict disease susceptibility among the population in advance and consequently in the evaluation of therapeutic results, correlating the patients' baseline metabolic profiles with their responses. Metabolomics applied to cell lines gives inputs to personalized or integrative medicine; in fact, cell metabolomics is an emerging field that addresses fundamental biological and metabolic questions using modern analytical techniques, such as Fourier-Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR) or Mass Spectrometry (MS).

CHAPTER II: OBJECTIVES

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The study of aging biomarkers could help to understand the molecular and biochemical physiology of aging. Elderly individuals have age-associated pathologies that make it difficult to identify biomarkers of healthy aging, since they can be hidden by disease-related biomarkers. It is known that physiological and pathological conditions are associated with homeostatic changes in cells and tissues and that the identification of these changes can be captured by FTIR spectroscopy. This technique allows us to obtain different spectral patterns in the transmitted infrared light, allowing to identify signs related to aging at the molecular and biochemical level, helping in the identification of specific biomarkers.

Therefore, the objectives of this work are:

1. Gather information on what has been done at the metabolic level with metabolomic profiling techniques, mainly FTIR, in cell culture studies;

2. Recognize the usefulness of cell models for the study of aging and how they can offer us new insights into the aging process;

3. Establish experimental conditions for the acquisition of FTIR spectra of fibroblasts and characterize biomolecular composition of normal fibroblasts based on FTIR spectra.

Due to the current situation of Covid-19 pandemic, some of the initial objectives of this dissertation could not be fulfilled, namely:

4. Identify new aging biomarkers in cell models of human fibroblasts.

CHAPTER III: STUDY AGING BY FIBROBLASTS METABOLOME

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III. 1 Abstract

Aging is a complex multifactorial process that although universal is not fully understood. It is known that the impact of aging on health is influenced by multiple factors, such as sex, race, income, and education, and that age-related diseases are strongly associated to the way people get old. The knowledge of biological aging and its comparison to the chronological age is a paramount contributor to predict the metabolic decline and the onset of age-related diseases.

As aging processes observed in whole human organism are somehow the reflexion of what happens in each cell type, it is possible to study aging process using cell lines, such as fibroblasts.

Metabolomics analysis of cell lines, namely fibroblasts, gives inputs to personalized or integrative medicine; in fact cell metabolomics is an emerging field that addresses fundamental biological and metabolic questions using modern analytical techniques as FTIR, NMR or MS.

This paper revises the relevance of using fibroblasts as cell models to study the metabolome of aging.

Key words: Aging; Fibrobalsts; Metabolome; FTIR; MS; NMR

III.2 Aging

III.2. 1 What is aging?

Aging is a poorly defined process, highly variable between people (1). It is an inevitable result of life, characterized by a progressive decline in molecular fidelity after reaching sexual maturity, culminating in the loss of function and, finally, disease and death. In most organisms, the aging rate is inversely proportional to the average useful life (2), however as it is a heterogeneous process, both organizationally and molecularly, this premise cannot be generalized to all organisms (3, 4).

When assessing the consequences of an aging population, it is important to determine how strong is the link between chronological age and the decline in health. Although aging usually leads to increased morbidity, some people live the 70s, 80s, and 90s in good health, with active lifestyles, willingness and the ability to work (4).
Increasing studies indicate that the substantial variation in the impact of aging on health is influenced by multiple factors, such as sex, race, income, and education (5-7), suggesting that disease is not a necessary consequence of aging, so age is a very inaccurate predictor of health status (4).

Many people experience few or no health limitations throughout life and even old age; for them, chronological age is almost entirely irrelevant. Others experience substantial health limitations, even at a young age.

So, if it is not age that leads to these differences, what is it? There are molecular changes that are characteristic of aging, such as those that occur in nucleus (loss of histones, transcriptional changes, losses, and gains of heterochromatin, nuclear lamina breakage, global hypomethylation, focal hypermethylation and chromatin remodelling) (2). These changes are strongly dictated by environmental stimuli and the availability of nutrients, which in turn alter intracellular concentrations of metabolites (2). In other words, it seems that different life trajectories lead to dramatic differences in health and well-being, from genetic differences to socioeconomic and behavioural factors, such as exercise, nutrition, social involvement and support, stress levels, career experiences, and geographic location. These effects can be cumulative and determine the quality of life in old age (4).

III.2. 2 Aging data around the world

The world is aging. However, the pace of aging is not uniform. A distinct feature of the aging of the global population is its uneven speed across different regions of the world. (8).

The World Health Organization (WHO) defines an elderly person in developed countries as someone who is 65 or older. Or as an alternative definition someone who has exceeded the average life expectancy at birth.

Thus, the aged population (65 years and over) represents more than 7% of the total population in most developed countries, and the number is estimated to double by 2050 (1).

In Europe the same is true, constituting the region with the highest average of aging population in the world. The percentage of aged population in Europe increase every year, registering in 2010, 14%, and is expected to reach 25% in 2050 (9).

Similar to other countries, in Portugal, the aging population is also increasing, and it is expected to increase from 2.1 to 3.3 million people between 2015 and 2080 (10).

The aging of the population, although it is mainly due to lower birth rates, also reflects a history of human success with greater longevity. Today, living to 80s is no longer a rarity in many parts of the world, leading to new social and economic challenges for humanity (8).

III.2. 3 Alterations in metabolism with aging

Aging influences many biological processes. Changes in these processes are driven by physiological principles such as decreased flexibility, changes in circadian patterns; and a loss of physiological reserves that will lead to difficulties in the maintenance of homeostasis (11).

The progression of biological aging changes unevenly, meaning that organs age at different rates, being influenced by many factors such as genetics, lifestyle, environmental factors, metabolic rate, and

caloric intake (11). So, two individuals at the same chronological age could have different biological aging statues, leading to different individual risk profiles. Nevertheless, chronological age is one of the most important risk factors for adverse clinical outcomes.

It is now well established that biological aging correlates with the accumulation of oxidized biomolecules in tissues and biofluids and may result in changes in yield, specificity and lifetime of regulatory modifications such as phosphorylation, methylation and/or acetylation (12). Thus, it is expected that the diversity of these forms and by-products of damage of various metabolic processes will increase with advanced age (13). Therefore, the analysis of metabolic signatures associated with old age could provide a better understanding of the mechanisms of aging (14).

The concept of biological age considers the heterogeneity of the aging process in different individuals that results in differences in survival rates, so any measure of biological age must be better than chronological age in predicting mortality.

Thus, biological aging is undoubtedly the most universal contributor to the etiology of metabolic decline and related diseases. The aging process is metabolically characterized by insulin resistance, changes in body composition, physiological declines in growth hormone (GH), insulin-like growth factor-1 (IGF-1) and sexual steroids, as well as increased levels of ROS (15).

Research on the biology of aging can provide detailed information on the molecular mechanisms of age-related changes in organs, tissues and cells. Aging can be noticed and characterized in all proliferating cells and terminally differentiated cells. Thus, it is to be expected that the general aging processes observed in a liver, muscle or neuronal cell apply to other cells such as keratinocytes or dermal fibroblasts (16), which makes cells suitable models to study the overall conditions of the organism, including aging.

III.3 Fibroblasts for the study of aging

Aging is a somatic process that implies the progressive loss of function, resistance to stress, metabolic efficiency, and adaptive potential. Many signalling pathways, genes, and organellar functions become altered in the course of normal aging and/or have an impact on aging trajectories through genetic or pharmacological manipulation (17).

The aging of different types of cells, tissues, and organs is associated with different patterns of gene expression and altered tissue function (18-24).

It is generally accepted that aging has two main determinants: intrinsic factors, such as genetic makeup or somatic capacity, and extrinsic factors, such as lifestyle, nutrition or environmental influences (17).

Human skin is particularly suitable for discriminating between extrinsic and intrinsic aging processes because the entire organ is subjected to intrinsic aging, while extrinsic aging is restricted to places exposed to environmental factors (17).

Skin can be divided in two main parts: the upper part of the skin, the epidermis, is a stratified squamous epithelium, which provides an essential protective barrier. To maintain the integrity of the tissue, it regenerates continuously and plays an important role in healing. The epidermis is also capable of eliminating extrinsic macromolecular damage, thus preventing the accumulation of damage and becoming resistant to environmental stress (17); the lower part of the skin, the dermis, is a post-mitotic tissue that depends on adapting and repairing damage to homeostasis. It consists mainly of the extracellular matrix (ECM), which determines the structural and mechanical properties of the skin. The dermal ECM is controlled by fibroblasts, which hardly proliferate and, therefore, are much less capable of removing extrinsic damage (17). Thus, dermal fibroblasts constitute a long-term cell population, which undergoes continuous accumulation of damage and adaptation, two processes typically associated with extrinsic aging. Accordingly, most phenotypic changes in extrinsically aged skin, such as the formation of wrinkles, are linked to dysfunctions of dermal fibroblasts and the corresponding remodelling of the dermal ECM (25, 26). These characteristics have made dermal fibroblasts a preferred model for the study of extrinsic aging processes at the cellular level.

III.3.1 Hallmarks of dermal fibroblast aging

Dermal fibroblasts are long-lived cells that constantly suffer damage and adaptations, thus constituting a powerful indicator system of human aging. Thus, to study aging it will be important to study the markers that influence or lead to this condition.

DNA damage and genome instability

DNA damage and genomic instability are prominent markers of fibroblast aging as irreparable double-strand breaks and forced recombination are noted (27-30), as well as chromosomal aberrations (31). In the case of chromosomal aberrations, the evidence is still controversial, since in this case the skin location may be of particular importance, for instance, due to exposure to UV light (32). At the same time, there is also an accumulation of oxidative damage in DNA (33) and a decrease or alteration in the DNA repair systems (34, 35).

Telomere shortening and irreparable DNA damage at telomeres

Most of the current mechanistic understanding of the cellular senescence of skin fibroblasts was obtained in cells that acquired senescence due to telomere shortening induced by replicative senescence (36, 37).

Replicative senescence may not be a valid model for fibroblast aging *in vivo* since fibroblasts rarely proliferate and age-dependent loss of telomeres, measured on the skin of donors of different ages, is minimal (38). Accordingly, the replicative lifetime of the skin fibroblasts analysed *ex vivo* does not correlate with the chronological age, morbidity or mortality of the donor (39, 40). Since dermal fibroblasts are post-mitotic cells, it can be assumed that the replicative shortening of the telomer does not itself play a role in its aging (41). However, this does not completely exclude the role of telomeric damage in extrinsic skin aging. The extrinsic DNA breaks inflicted on the dermal fibroblast telomeres

can be irreparable, providing a persistent signal of DNA damage that induces the cell cycle to stop in a similar way to telomere shortening (17).

Disruption of post-transcriptional pre-mRNA processing

It has been known for some time that patterns of expression of alternative splicing mRNA (42-45) and protein isoforms (46-48) translate into changes during development and aging. Available data suggest that aberrant pre-mRNA splicing of the LMNA (Lamin A/C) gene could play a role in the photographic aging of the skin (49-51). Also, studies show that other pre-mRNAs could also be subjected to aberrant splicing in aged fibroblasts and that genes and mechanisms involved in post-transcriptional processing of pre-mRNA could be subjected to aberrant splicing in aged fibroblasts and may be important targets of extrinsic aging (50, 52, 53).

Epigenetic alterations

The available studies indicate that DNA methylation plays a role in the intrinsic and extrinsic aging of fibroblasts, with underlying divergent mechanisms, precluding a conclusive interpretation of global pattern changes (54, 55). Furthermore, it is not clear whether epigenetic changes are an epiphenomenon or play a causal role in chronological age and adaptation/maladaptation processes that contribute to the aging of the dermal fibroblast (55, 56).

Loss of proteostasis

Skin aging is associated with the accumulation of oxidized proteins, which need to be removed by the proteasome (57) and/or autophagy (58). The proteasome activity in dermal fibroblasts decreases during aging (57), as well as after exposure to UV irradiation, which has been attributed to decreased expression or inactivation of proteasome subunits and the accumulation of endogenous inhibitors (59-

61). So, age-related decline in proteasome activity plays a causal role in human fibroblast senescence and possibly skin aging (17).

Decreased autophagy

Autophagy is considered a protective factor in chronic degeneration of various organs (58, 62, 63). Three different forms of autophagy are distinguished - micro-autophagy, chaperone-assisted autophagy, and macro-autophagy (17). Chaperone-assisted autophagy is believed to clean up defective macromolecules (58) and macro-autophagy removes dysfunctional mitochondria that contribute to the aging process (64, 65). The decrease in age-related autophagy appears largely due to post-translational mechanisms, such as acetylation/deacetylation of autophagy proteins (66). If this process occurs also in dermal fibroblasts, autophagy could be a factor that influences the aging process and, therefore, can provide a potential target for anti-aging intervention (17).

Mitochondrial damage and dysfunction

Damage to mitochondria is a hallmark of aging in many tissues (67) and leads to decreased respiratory capacity, unbalanced ROS levels, altered mitochondrial biogenesis as well as altered fusion/fission balance (17).

Studies have shown that there is a significant decrease in the potential of the mitochondrial membrane, accompanied by a significant increase in ROS levels in samples of dermal fibroblasts from old donors (68). Oxidative stress also appears to play an important role in the aging process. This is defined as the imbalance between the production of reactive oxygen species (ROS) and their elimination (17). The generation of ROS by redox systems inside the mitochondria (by electron transport chain (ETC)) and in the external cell membrane (NADP-oxidases, NOXs) is balanced by antioxidant defense systems. The balance can be reduced as the ETC function becomes defective during aging in several model organisms (69). Thus, the levels of mitochondrial ROS increases in dermal fibroblasts from old donors (68).

At the same time, other studies have shown that mitochondrial content increases in fibroblasts subject to replicative senescence in culture (37, 70, 71). Mitochondrial mass and biogenesis also increase in primary cells recovered from elderly humans (72). Currently, it is believed that the increase in mitochondrial mass in senescent and aged cells is due to the retrograde triggering of the myth-biogenic stress response by increasing the production of ROS, decreased ATP synthesis or persistent signs of DNA damage caused by dysfunctional mitochondria (73, 74) that are not removed properly because autophagy is under-regulated (65).

For the mitochondrial changes referred to, mitochondria are highly dynamic organelles that constantly undergo fusion and fission events to adapt their shape and number to preserve cellular homeostasis (75, 76). Mitochondrial fusion and fission events help in the maintenance of the integrity of mtDNA, regulate cellular redox status, cooperate in the elimination of mitochondria damaged by autophagy (mitophagy) and are directly involved in the execution of the apoptotic program (77, 78). The deceleration of mitochondrial fusion and fission events associated with age is thought to serve to adapt to an increased burden of mitochondrial damage (79, 80). Proteins crucial for mitochondrial fusion and fission are highly conserved and expressed in dermal fibroblasts.

Cellular senescence

Cell senescence is a genetic program that limits cell proliferation. It is activated mainly by shortening of the telomeres (81), but also by non-telomeric damage to DNA and other stressors (81, 82). Senescence is characterized by an irreversible stop of the cell cycle triggered by pathways involving p53, pRB, p16INK4A (81) and p21 (CIP1) (83).

Alternatively, cell senescence can be induced in culture by oxidative stress (stress-induced premature senescence, SIPS), which gives rise to a distinct phenotype (84). This distinct phenotype consists of alterations in morphology, cells show flattened and enlarged cellular morphology (85, 86); senescence-associated β-galactosidase activity; cell cycle regulation; gene expression and telomere shortening (84).

Senescent cells accumulate in the body during normal aging. Although it is considered a beneficial mechanism for tumour suppression in young people (87, 88), cell senescence already contributes to aging *in vivo* (89-91).

Altered intracellular communication

Foreskin fibroblasts subjected to replicative senescence in culture become refractory to the paracrine growth impulses mediated by the EGF receptor (EGFR) due to the negative regulation of EGFR and positive regulation of tyrosine phosphatases (SHP-1 and PTPN6) (92, 93). The lack of response from EGF involves the negative regulation of endocytosis mediated by caveolin and clathrine (94, 95). The EGF response is attenuated by the overexpression of caveolin in juvenile fibroblasts and restored by caveolin knockdown in senescent fibroblasts (95).

It was proven that EGFR-dependent activation by oxidative stress can play a protective role in the adaptation of keratinocytes to UV light since it is known to increase cell survival (96). Paracrine epidermal/dermal crosstalk of epidermal growth factor (EGF)-signalling seems to play an important adaptive role in extrinsic aging since the estrogen-mediated release of epidermal growth factor from keratinocytes protects the dermal hyaluronan/versican matrix (97).

The EGF signalling in dermal fibroblasts acts protectively in remodelling processes of the dermal ECM during extrinsic aging (97). The loss of the EGF response capacity of dermal fibroblasts possibly promotes extrinsic skin aging processes (17).

Alterations of the cytoskeleton

The most obvious morphological changes that are associated with replicative senescence of fibroblasts *in vitro* and recapitulated to some extent in primary dermal fibroblasts isolated from elderly donors include increases in cell surface and cell volume, as well as changes in cell shape and mobility (98). As these properties largely depend on the function of the cytoskeleton, cytoskeletal changes are expected to occur during the aging of the dermal fibroblast. Dermal fibroblasts from old donors are less plastic

and less mobile than those from young donors (99). It has been suggested that these changes are due to structural changes in the intermediate filaments caused by increasing expression of vimentin. The other cytoskeletal target molecule relevant to the aging process appears to be actin. The actin content in dermal fibroblasts does not appear to change in the course of replicative senescence (100) or to differ between primary and young donor cell cultures (101), while the state of the actin filaments has been reported to have changed during replicative senescence as well as during aging *in situ* of human fibroblasts. However, the available studies reach contradictory results and it is still unclear whether, during aging, the actin filaments become thicker or thinner or just redistributed within the cell (17).

Disruption of circadian regulation, a decrease of NAD^+ and sirtuin activity

Fibroblasts comprise a molecular circadian oscillator that gives rhythmic diurnal expression to many genes (102). The local clock on the skin and other peripheral organs is under the general control of a light-sensitive lead pacemaker in the suprachiasmatic nucleus (103) and additionally receives input from a variety of extrinsic factors and cellular response systems, including redox status (104-109), energy levels (for example via AMPK) (110), DNA damage (111) and cellular stress response systems (112-114). Circadian regulation has been demonstrated for several pathways involved in the dermal fibroblast aging process (17). Genetic or environmental disorders of circadian regulation induce a variety of pathologies that are also known to increase with age (115). Thus, the susceptibility of the dermal fibroblast to extrinsic aging is likely to depend on its position in the circadian cycle.

Genome-wide alterations in gene expression networks

As mentioned earlier, mitochondrial dysfunction and deficient energy metabolism are common features of aged cells, while most other systematic age-related transcriptional changes are consistent with an adaptation to this condition, which may follow different strategies in different organs and cell types. In the case of dermal fibroblast aging, it is largely due to post-transcription / translation processes (66).

Recent developments in “omics” field enabled the measurement of metabolomic profile of cells. Cell metabolomic data can be extrapolated to the whole organism and, although under-explored, may become a relevant marker for the study of aging. Application of cell line metabolomics in aging will be detailed below.

III.4 Why use the metabolome to the aging study?

Understanding the hereditary and epidemiological effects of aging is very important as this information will allow improved management of health, disease and extendlife (116). With metabolomics it is possible to better understand the biology of aging, since it is the comprehensive, qualitative and quantitative study of all low molecular weight molecules or metabolites in a biofluid, cell, tissue, organ or organism (117, 118). Metabolites are the final downstream products of genomic, transcriptomic, and/or proteomic perturbations and can reveal biological changes that are a result of changes in metabolic pathways such as age-related modifications.

Metabolomics involves the identification of a large number of metabolites, a number much higher than what is currently covered by standard clinical laboratory techniques. Therefore, comprehensive monitoring of biological processes and metabolic pathways promises to be fundamental for personalized medicine (119).

Metabolomics techniques allow to visualize how physiology is linked to external events and conditions, as well as to measure response to disorders, such as those associated with disease or aging. Metabolites are thus described as proximal reporters of the disease because their abundance in biological specimens is generally directly related to pathogenic mechanisms (120).

Since the metabolome is sensitive to a vast number of factors from genetics (gender, epigenetics, and gene polymorphisms) (118) to environmental (diet, stress, xenobiotic use, lifestyle, age and disease) (118) the design of a metabolomic study requires some precautions. To do research using a metabolomics approach it is important to gather increased data sets in order to reduce inter-individual variation. The metabolic fingerprint (the use of a potentially recognizable chemical pattern) specific to an individual sample is another concept that helps to overcome the problem of inter-variation because it allows the identification and interpretation of metabolome variation within a given individual(121).

Reliable, sensitive and specific molecular markers can provide valuable physiological information about these age-related changes. These markers can enable new insights into the mechanisms of maturation and aging and increase the ability to accurately assess the anatomical status of an individual at any time. Such markers may also lead to the identification of innovative treatments to retard or reverse abnormal molecular changes (116).

To study the metabolome of aging it is impossible, or at least extremely difficult to perform longitudinal studies with humans, so the alternative is to use animal or cellular models (such as fibroblasts). It is common to use cohorts of elderly individuals, however in these cases it is difficult to select the control of the basal metabolome as the population has associated comorbidities and it is difficult to study isolate the metabolome of age (122).

As molecular changes precede histological modifications, metabolomics has an enormous potential for early diagnosis of diseases. This is possible with the identification of a profile or only one biomarker. Identification of an aging biomarker profile or a single biomarker that define and characterize a healthy aging is a challenging task (123) or indicate the presence or the chance to develop of an age-related disease (124). Biomarker research can be used to follow the individual response to a specific treatment or can be used to design a specific treatment to a specific patient in the field of personalized medicine. In the case of aging biomarkers, these can be used to design a specific treatment for a patient with an age-related disease and thus prescribe an individualized treatment for each patient with the same condition.

The study of the metabolome of aging can also be used to define or study the evolution of the biological age by comparing it to the real/chronological age. The study of this deviation will allow the identification of the metabolomic profile of healthy aging in cases where metabolic age is lower than physiological age. This information will be useful, for instance, to predict the health status of individuals. If, by contrast, the metabolic age is higher than the physiologic age it will be possible to extract the metabolic profile of individuals likely to develop age-related diseases and develop/recommend treatments or lifestyle interventions in order to decrease the probability of developing these diseases.

The choice of the analytical tool is another fundamental question to answer a scientific question or to reach the research objective since each of these tools can provide different answers, so it is important to always keep the experimental design in mind when carrying out experiments using metabolomics techniques. In this way, sampling methods, sample preparation and data mining tools are other key factors that scientists should be aware of and that are important (125).

III.4.1 Techniques to study the metabolome

The identification of the metabolomic profile is of great importance in the development of new diagnostic tools for personalized or integrative medicine. Recent technological advances in nuclear magnetic resonance and mass spectrometry are significantly improving our ability to obtain more data from biological samples. Consequently, fast and accurate statistical and bioinformatics tools are needed to deal with the complexity and volume of data generated in metabolomic studies (126).

So far, metabolomics is considered one of the main "omics" tools that will most contribute to challenging research objectives, such as personalizing treatments in medical practice. Metabolites are the intermediates or end products of multiple enzymatic reactions and, therefore, are the most informative proxies of an organism's biochemical activity (126). Thus, current technologies allow the study of hundreds of metabolites in complex biological samples (127). Currently, Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) (128) stand out in this sense, however despite the advantages of these two techniques, they are time consuming, hence another technique that allows the analysis of samples in a non-destructive way and with the ability to study the fingerprint of each sample, Fourier Transform Infrared Spectroscopy (FTIR), begins to emerge.

All these techniques allow to do non-targeted metabolomic studies (*Figure 1*). These are characterized by the simultaneous measurement of all metabolites from each sample (126). This strategy, known as a top-down strategy, avoids the need for a specific earlier hypothesis about a specific set of metabolites and instead looks at the overall metabolomics profile. Consequently, these studies are characterized by the generation of large amounts of complex data, which makes high-performance bioinformatics tools necessary (126).

Given the usefulness of these tools, several papers have been published on the diagnostic importance of different spectroscopic and imaging techniques in the aging field.

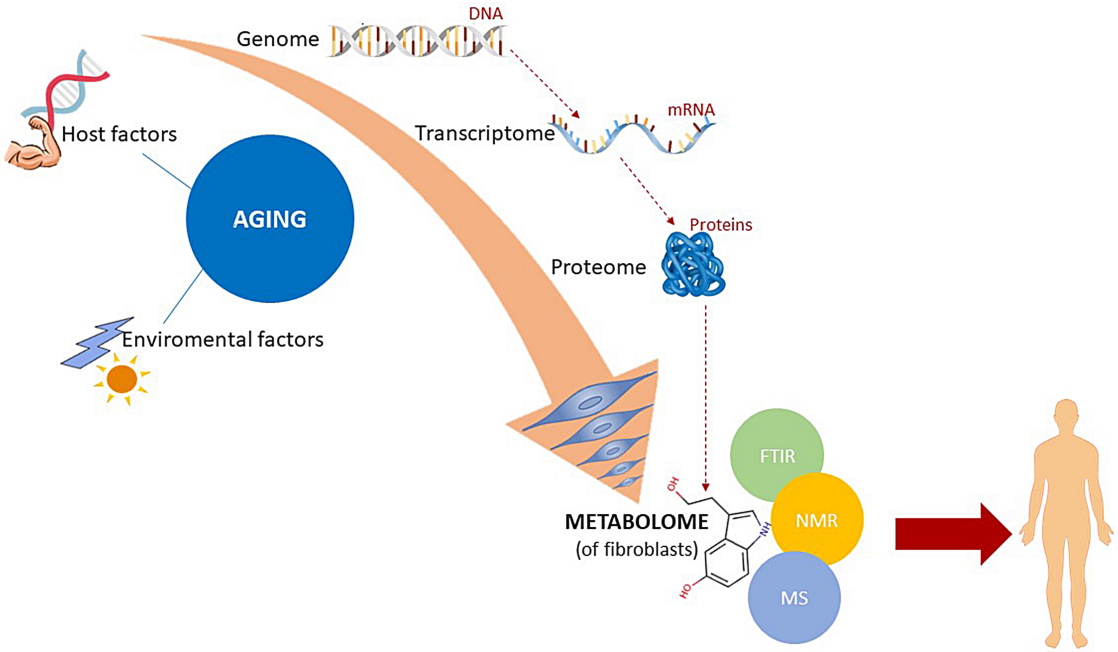

Figure 1| The aging process could be studied by the metabolome analysis with the metabolomics tools, namely FTIR, NMR and MS.

FTIR for the study of senescence and aging

Fourier Transform Infrared Spectroscopy (FTIR) is a widely used technique for fingerprinting (129). FTIR identifies the composition and molecular structure of biological materials without the need of probe molecules (130). This technique uses sensitive, non-invasive and undamaged radiation (131-134) and requires minimal sample preparation (135).

Biological compounds absorb infrared energy in the medium infrared range (MIR, 4000-400 cm^{-1}), producing infrared spectra that are specific for each sample (129). All samples show obvious band differences across the analysed wavelength region: bands between 900 and 1200 cm^{-1} are attributed to carbohydrates, and nucleic acids, bands between 1300 and 1500 cm^{-1} are assigned to proteins and lipids and prominent amide I and II bands between 1500 and 1800 cm^{-1} are assigned to proteins (136). Additional vibrations due to functional ester groups in the lipids are observed at 1740 cm^{-1} and CH_3 and CH_2 groups are observed between 2848 and 2992 cm^{-1} (136).

In addition, this technique also provides molecular-level information, enabling investigation of functional groups, bonding types and molecular conformation (135).

Due to the aging of the population, the understanding of the underlying molecular mechanisms is increasingly important.

The study of fibroblast aging using metabolomics techniques is still little explored, however several studies report that senescent cells accumulate in a variety of human pathologies, including aging. Therefore, the study of senescence markers in fibroblasts can give us a perspective of the metabolome of aged fibroblasts (137).

Senescent cells, while metabolically active, stop dividing after a finite number of cell divisions. These cells are known to influence tissue structure and function due to secreted components (138).

FTIR can register biochemical fingerprints of cells and tissues, allowing a distinction between different cell states or between healthy tissue and cancerous tissue. Likewise, it has already been used successfully to distinguish senescent cells from non-senescent cells, as well as to investigate other molecular changes that occur at the level of cells and tissues during aging (136, 139). In this way it can offer a non-invasive method that allows the monitoring of the progression of senescence in real time *in vitro* and *in vivo*.

When aging, normal diploid fibroblasts irreversibly interrupt the cell cycle and become senescent due to the loss of proliferation potential (36). At the same time, biomolecular damage occurs, which in turn induces aging in most cells in the absence of repair mechanisms (140, 141).

To identify unknown molecular structures, in relation to the lipidome and metabolome, a 2018 study (139) used FTIR to study and compare the biomolecular composition of three cellular states of human fibroblasts: proliferation, quiescence and senescence. According to this study in senescent cells, the protein bands (amides I and II between 1500-1700 cm^{-1}) decreased and a lipid band (1740 cm^{-1}) increased. Comparing the proliferating cells with the senescent cells, the proliferative cells showed greater extensions of α helical structural proteins (1652 cm^{-1}) while in senescent cells greater amounts of bands associated with amino acids (1584 cm^{-1}) were observed, suggesting proteolysis. The bands associated with the collagen were not unambiguously identified. As for DNA / RNA content, senescent cells had the lowest ratio.

Another study (136) revealed the spectral changes observed in senescent cells reflect changes in the general biochemical composition associated with differences in the metabolism of young proliferating cells versus aging cells. This study detected changes in the CH elongation region (assigned to proteins and lipid composition) in the studied cell lines. The infrared spectra is sensitive to changes in the composition of lipids such as phospholipids (136). These molecules can be observed by spectral characteristics around 1750 cm^{-1}, associated with the C=O stretching vibrations of the carboxyl groups of the esters. In regions of shorter wavelengths, typical bands assigned to phosphate groups of DNA and RNA are observed, due to asymmetric vibrations of phosphodiester elongation (136).

Due to the relatively small amount of DNA or RNA per cell mass (± 10% w / w) compared to the largest amount of proteins (± 60% w / w), the most prominent band in FTIR spectra of biomolecules is located in the region around 1652 cm^{-1}, assigned to amide I band of proteins. This band is sensitive to the protein secondary structure and can give lots of information about changes in protein content of the analysed sample (136).

Thus, in summary, what has been pointed out by the literature is that in the transition to senescence, FTIR spectroscopy identified changes in biomolecular composition and modification but identified only small changes in molecular abundance. Further studies on this subject may be relevant to understand the potential of FTIR spectroscopy to evaluate senescence-associated changes in fibroblasts.

NMR for the study of senescence and aging

Nuclear Magnetic Resonance (NMR) Spectroscopy is a fast and highly reproducible spectroscopic technique, based on the absorption and re-emission of energy from atomic nuclei due to variations in an external magnetic field (142). In this field, different types of metabolomic data are generated. In the analysis of samples of biological origin, hydrogen is the most used nucleus ($_{1}$H-NMR), due to its natural abundance in biological samples. However, although less frequent, other atoms such as carbon ($_{13}$C-NMR) and phosphorus ($_{31}$P NMR) are also targeted by NMR, providing additional information on specific types of metabolites (143). The resulting spectral data on NMR allow the quantification of the concentration of metabolites as well as the provision of information on their chemical structure. The spectral areas of the peaks generated by each molecule are used as an indirect measure of the amount of the metabolite in the sample, while the spectral peak pattern that informs about the physical properties of the molecule is used to identify the type of metabolite (126).

NMR spectroscopy applications are not limited to liquid samples, as it can also be used on solid, gaseous or tissue samples. In addition to its main applications in molecular identification and structural elucidation, NMR spectroscopy can also be used to study the physical and chemical properties of molecules, such as electron density and molecular dynamics (144). Consequently, NMR has become the main tool for structural biology studies, as it allows the study of molecular structures and molecular dynamics under biological conditions.

In addition, NMR spectroscopy has been used in a wide range of research areas including structural biology, organic chemistry, inorganic chemistry, biochemistry, physics, biology and drug discovery.

NMR spectroscopy has been proposed as one of the most relevant methods in metabolomic applications, including clinical diagnosis (116, 144-153) since the high number of metabolites that can be detected simultaneously in a short period represents an additional advantage of this method (144).

NMR spectroscopy has been used for some time in the study of aging. One of the first studies in this remote area was made in 1996, when *Rutter et al.* (154), used this technique to monitor changes in cell chemistry associated with aging and to study the properties of human fibroblasts as they aged in culture. In this study, the main conclusion obtained focused on changes in lipid metabolism in which the lipid content increased in senescent fibroblasts, and on the increase in cholesterol in cells that were senescent.

More recently, studies have shown the use of NMR to assess senescence in dermal fibroblasts as well as in human lung embryonic fibroblasts.

Different studies with NMR and fibroblasts have shown that, during senescence, ATP levels decrease, while AMP levels increase, increasing the AMP: ATP ratio (155, 156). This increase in the AMP:ATP ratio leads to the activation of the AMP-activated kinase resulting in a senescent phenotype. AMP-activated kinase regulates different metabolic pathways, including lipid and glucose metabolism (157). By analysing senescent dermal fibroblasts, authors concluded that the metabolites belonging to the purine metabolism were also altered, showing lower ATP levels and higher inosine levels. At the same time, they concluded that L-α glycerophosphocholine (GPC) is elevated in senescent fibroblasts (158) while serine cannot be detected (159). Also, there was detected a decrease in the formation of monounsaturated fatty acids and reduced formation of phospholipids (159).

In embryonic human lung fibroblasts, results of a study showed that senescence is mainly marked by an increase in GPC, inosine, glutamine, and leucine, while UDP-GlcNAc, ADP + ATP, glutamate, phosphocreatine and phosphocholine (PC) are decreased (158). Lactate and acetate also show a tendency to decrease in senescent cells (158).

Regarding aging studies, one study investigated whether strains of skin fibroblasts derived from offspring of nonagenarian siblings show differences in cell metabolism *in vitro*. For this purpose, they measured the metabolites in cell culture supernatants of fibroblast strains (160).

In this study authors revealed that fibroblast strains derived from the offspring of nonagenarian siblings show different levels of metabolites in their cell culture supernatants when compared to those of their partners. The main differences were observed in the metabolites involved in energy metabolism (160). However, they also reported that the consumption of alanyl-glutamine (ala-gln) and glucose was higher in the fibroblast strains derived from the offspring (160). When available, glucose is the main source of energy for cells, explaining the decreasing levels of glucose in cell culture supernatants of fibroblast strains. Elevated glucose levels have also been shown to induce senescence in human fibroblasts (161).

In addition, it was found that the production of glutamine, alanine, lactate and pyroglutamic acid is greater in the fibroblast strains derived from the offspring while the essential amino acids valine, leucine, isoleucine, serine and lysine, showed reduced levels. In parallel, these strains of fibroblasts from the offspring show less lipid metabolism (160).

MS for the study of senescence and aging

In contrast with the other two above mentioned techniques, Mass Spectrometry (MS) does not involve the absorption of electromagnetic radiation. The basic principle of mass spectrometry is to generate ions from either inorganic or organic compounds and separate these ions by their mass-to-charge ratio (m/z), detecting them qualitatively and quantitatively by their respective m/z abundance (162). For the spectrometer to generate peak signals for each metabolite, it is necessary to first ionize the biological sample (126). The ionized compounds resulting from each molecule will generate different peak patterns that define the fingerprint of the original molecule. Currently, a wide range of instrumental and technical variants is available for MS spectrometry. These variants are mainly characterized by different methods of ionization and mass selection (163). In metabolomics, MS is usually preceded by a separation step, which reduces the high complexity of the biological sample and allows the analysis of MS of different sets of molecules at different times, such as a thousand candidate metabolites from tissues and biofluids (126). Finally, mass spectrometry assays are based on the chemical measurement of specific amino acid sequences, not biological antibody recognition, thus allowing proteins to be tested across species with absolute molecular specificity (164).

The study of changes in the metabolome of aged fibroblasts using mass spectrometry is still little explored, however there are several studies that study the changes in the proteome of aged fibroblasts through mass spectrometry (101, 165, 166), so this technique should be considered as an equally promising technique in the study of the aging metabolome. There is also another study, which investigated the extracellular metabolome, by liquid chromatography/mass spectrometry (LC–MS) and gas chromatography/mass spectrometry (GC-MS), of several lines of human fibroblasts with induced senescence (167), indicating that several serum metabolites are independent indicators of chronological age and some of them accumulate outside senescent fibroblasts regardless of cell cycle arrest. However, for the context of this review, this study is not so relevant.

Thus, despite the scarcity of the literature, there are studies that evaluate senescence markers in fibroblasts, so it can give us a perspective of the analysis of the metabolome of aged fibroblasts. One of the studies that used liquid chromatography/mass spectrometry (LC–MS) and gas chromatography/mass spectrometry (GC-MS) to study senescence in fibroblasts, established two cell lines of senescent fibroblasts, one for the proliferative exhaustion or replicative senescence (PEsen)

and the other for the failure to repair DSBs (IrrDBSsen). Through these cell lines they studied the metabolism of cells and what are their main cellular perturbations that lead to a changes during senescence (137).

For analysis of the cell metabolome, the study started by reporting changes in redox metabolism and nucleotide metabolism, revealing an increase in catabolic purine products (hypoxanthine, 7-methylguanine and urate) in PEsen and IrrDSBsen cells. This increased production of urate was accompanied by the production of hydrogen peroxide, which also remains elevated, suggesting that higher levels of nucleotide catabolism can withstand oxidative stress (137). At the same time, multiple dipeptides appear to be decreased in senescent cells, perhaps as a result of increased uptake, but also due to increased catabolism to provide carbon skeletons for the tricarboxylic acid (TCA) cycle (137).

Some lipids and their intermediates also increased in PEsen cells (137), namely, the essential fatty acids that are important for the large increase in membrane organelles that are important components of the senescent cellular phenotype, and there is evidence that this can be mediated by a regulatory element of sterol (168).

This same study revealed that PEsen cells show evidence of increased glycolysis metabolism and the pentose phosphate pathway. These data were consistent with energy metabolism being diverted from the TCA cycle in established PEsen cells, to prevent further damage to the cell and reduce oxidative damage (137).

Based on the study by *James et al.*, another study from 2016 studied the metabolism of young fibroblasts, by ultra-high performance liquid chromatography/mass spectroscopy and gas chromatography/mass spectroscopy, in fibroblasts in growth and in proliferative exhaustion, revealing which metabolites are specific for senescence (169). In this study, the authors confirmed the results obtained by *James et al.*, and concluded that NADPH is high in all conditions of cell cycle arrest. The levels of NAD^+ are maintained in the PEsen cells, but not the levels of NADH, suggesting that the levels of NAD^+ may contribute to the biology of PEsen by a different mechanism of energy production (169). NAD^+ is also a coenzyme that regulates the family of deacetylases known as sirtuins (SIRTs) that have multiple anti-senescence functions (170), and can mediate the anti-aging effects of calorie restriction (170). In this study, they also concluded that high levels of prostaglandin E2 (PGE2) in PEsen cells lead to senescence and promote the characteristics of this phenotype (169).

When comparing the results of the three techniques, one can conclude that changes in the lipid metabolism, by increase of the lipid content in the cells is a consistent result in all studies. In studies in which FTIR was used as a metabolic tool, it was also reported a decrease in the bands referring to proteins and an increase in the bands associated with amino acids. In NMR studies, the identified biomarkers essentially reveal a decrease in ATP levels and an increase in AMP levels, leading to an increase in the AMP/ATP ratio, an increase in L-α glycerophosphocholine (GPC) and a decrease in the formation of monounsaturated fatty acids and phospholipids. High levels of NADPH and prostaglandin E2 (PGE2) were detected through MS. Thus, as can be observed a study of aging that combines these three techniques can provide significant insights into this process, since each of the techniques can provide information on different groups of compounds.

III.4.2 Advantages and Challenges: FTIR, NMR and MS

All the metabolic tools presented have distinct strengths and weaknesses that can benefit or prejudice studies of the metabolome of aged fibroblasts. (*Figure 2*).

The most relevant advantages of NMR for metabolomics include a relatively high transfer rate, n simple methods for quantifying metabolites and redundant spectral information to improve the accuracy of metabolite identification (171). However, NMR is limited to detecting only the most abundant metabolites (≥ 1 µM) (171), while MS has a much higher sensitivity and readily measures concentrations in the femtomolar to attomolar range (172). On the other hand, MS detects only metabolites that ionize readily, and therefore about 40% of chemical compounds are not observable by MS (172). At the same time, given the complexity and heterogeneity of the samples, the presence of one metabolite can lead to the non-detection of others (173).In many cases, definitive identification of molecular structures is difficult using only MS and complementary techniques are needed. Saccharides, for example, play an important role in various biochemical processes associated with aging, such as the regulation of biochemical pathways and cellular interaction, however due to the singularly complex structural diversity they are difficult to characterize by MS (174) . Thus, for a clear identification of these compounds from complex samples, such as fibroblasts, complementary techniques such as NMR or FTIR are necessary.

Although it has been recognized that FTIR is not as specific and sensitive as MS (175) the speed and reproducibility of FTIR is undeniable. In addition, due to its holistic nature, FTIR has been recognized as a valuable tool for metabolic fingerprints, as it is able to analyze carbohydrates, amino acids, fatty acids, lipids, proteins, nucleic acids and polysaccharides quickly and simultaneously, non-destructively, non-invasive, with a minimum amount of sample and minimal sample preparation (175). One of the potential limitations of FTIR is that water absorption is very intense, however this problem can be overcome by dehydrating the samples, or by using attenuated total reflectance (ATR) as a sampling method (175).

Although both are based on the interaction between electromagnetic energy and a molecule of interest, FTIR identify functional groups within a molecule and can be used to quantitatively determine the concentrations of molecules within the sample. Contrarily NMR is used primarily to determine the

chemical structure of a molecule (175). For all these reasons, and given the pros and cons of all techniques, the possibility of constituting a combined approach makes its use extremely important for metabolome studies.

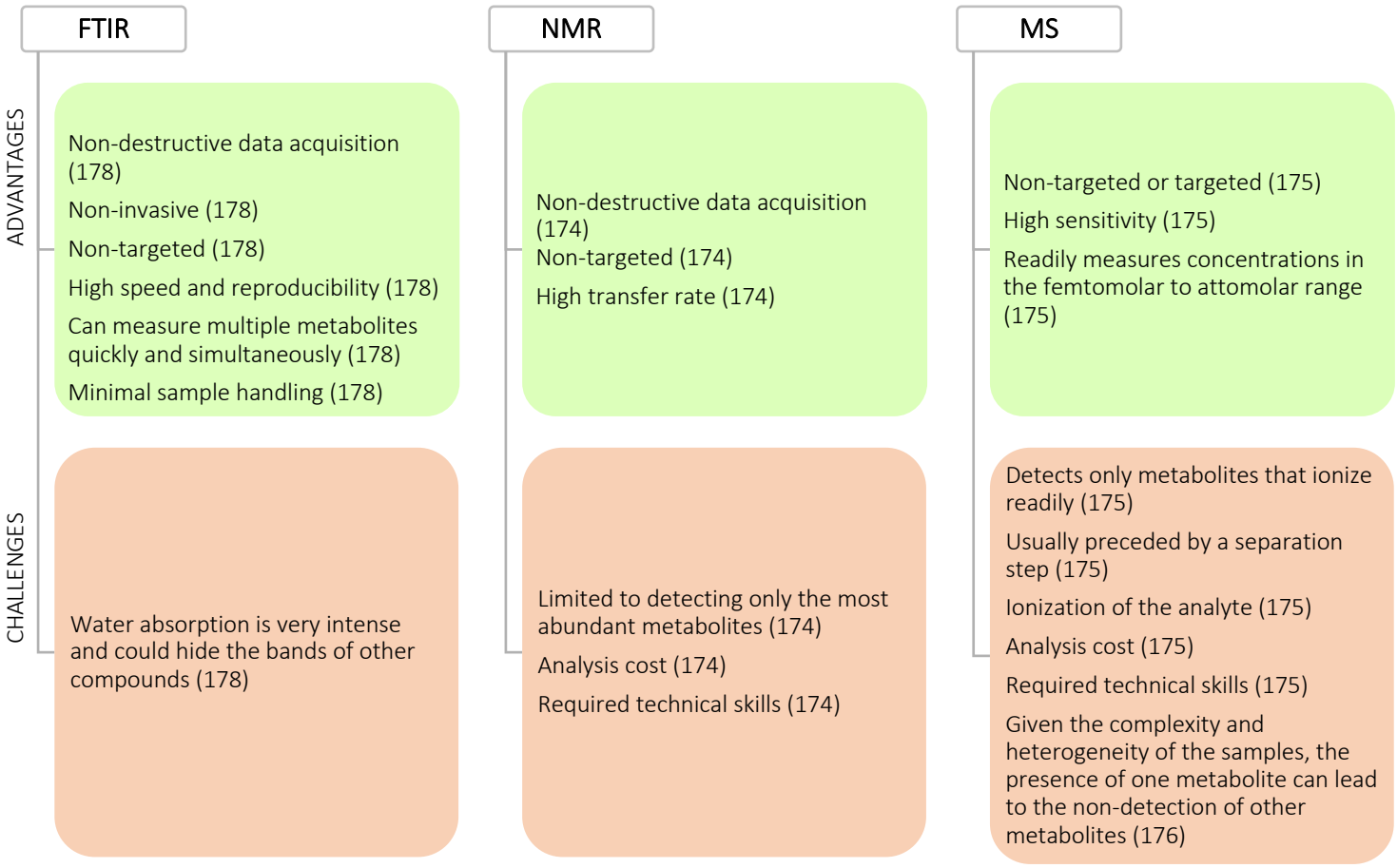

Figure 2| Principal advantages and challenges presented by the three metabolic tools used to study aging fibroblsts: FTIR, NMR and MS.

III.4.3 Established biomarkers of aging

The biomarkers of aging have the possibility to define a set of factors that characterize healthy aging and to predict useful life. Also, can help researchers to establish their research focus on a specific biological pathway to explain the biological process behind aging or aging-related diseases.

The American Federation for Aging Research (AFAR) has established some criteria to define a good aging biomarker: (1) it must predict the aging rate; (2) it must monitor a basic process underlying the aging process and not the effects of the disease; (3) it must be able to be tested repeatedly without harming the person; and (4) it must be something that is reproducible in humans and laboratory animals (176). Despite the constant and growing research in this area, it is unlikely that there will be biomarkers that meet all these criteria, so research focuses essentially on the first two criteria (176).

Some biomarkers of DNA changes resulting from the aging process are already established, such as serum DNA damage markers measured by ELISA immunoassay, including cathelicidin-related antimicrobial peptide (CRAMP), elongation factor 1-alpha (EF-1a), statin, N-acetyl-glucosaminidase and chitinase (177). In dermal fibroblasts from centenary donors, it has been revealed that these proved to be less sensitive to DNA damage induced by H_2O_2 than fibroblasts from young and elderly donors (178) and such experiments performed *ex vivo* can also be a potential biomarker of aging.

Nutrient signaling is another of the fundamental pathways studied in the aging process that is highly related to metabolism. Thus, the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway, which participates in the detection of glucose, is the best-known way to antagonize longevity. Paradoxically, serum IGF-1 levels when analyzed with microarray analysis decrease in wild type mice or mouse models with premature aging, while attenuating IIS activity by extending lifespan (179). These observations led to the possible inclusion of members of the IIS pathway, such as growth hormone and IGF-1, as biomarkers of aging (180). Target of rapamycin protein (mTOR) is also a potential biomarker of aging that can detect high concentrations of amino acids and its inhibition can prolong its useful life (181). Unlike the IIS pathway, mTOR activity increases with age in the epithelium of the ovarian surface of human ovaries, which contributes to pathological changes (182). The phosphorylated S6 ribosomal protein (p-S6RP or pS6) is a downstream target and therefore also a known marker for active mTOR signaling that could be measured by flow cytometry (182, 183). In contrast to the IIS and mTOR

function, 5′-adenosine monophosphate (AMP)–activated protein kinase (AMPK) and sirtuins detect nutrient shortages. While AMPK detects high levels of AMP, sirtuins are sensors of high levels of NAD^+ and both mark low energy states. AMPK is increased with age in skeletal muscles (184). Sirtuins could directly link cellular metabolic signaling to post-translational modifications of the protein through a chemical reaction (lysine deacetylation). During aging, NAD^+ is reduced (185) and sirtuins are negatively regulated (170). When evaluated by atomic force microscopy techniques the analysis of primary human dermal fibroblasts showed that SIRT1 and SIRT6 are downregulated (186), and simultaneously in human peripheral blood mononuclear cells, SIRT2 also decreases with age (187).

Protein carbamylation is one of the post-translational non-enzymatic changes that occur throughout the life of an organism, leading to the accumulation of carbamylated proteins in tissue (188). Therefore, is consider to be one of the hallmarks of molecular aging. Advanced glycation end products (AGEs) are a heterogeneous group of bioactive molecules formed by the non-enzymatic glycation of proteins, lipids and nucleic acids (189). These can be detected by high performance liquid chromatography, gas chromatography mass spectrometry and immunochemical techniques (190).

Regarding lipid metabolism, it was found that triglycerides increase with age and, therefore, can be a biomarker of aging (191). Studies of serum samples by lipidomics have identified phosphor / sphingolipids as putative markers and biological modulators of healthy aging and these could be detected using NMR spectroscopy (192).

Oxidative stress biomarkers have long been considered a class of aging biomarkers. Products of oxidative damage to proteins include o-tyrosine, 3-chlorotyrosine and 3-nitrotyrosine. 8-iso prostaglandin F2α is a biomarker for damage to phospholipids. 8-hydroxy-2′-deoxyguanosine and 8-hydroxyguanosine are produced by the oxidative damage of nucleic acids (193). The concentration of these biomarkers in body fluids can be detected by high performance liquid chromatography and mass spectrometry and constitute good aging biomarkers.

However, as expected from the complex nature of the aging process, aging biomarkers are several and multifaceted and consist of a dizzying variety of parameters, giving them a variable significance since they may not be equally useful and are therefore difficult to establish.

III.5 Conclusion

As mentioned throughout this review, the world is aging, and it is becoming increasingly important to find tools that allow the study of differences presented by individuals throughout aging.

In this sense, metabolomics begins to emerge and play an important role, since allows it the study in real time of metabolites, and subsequently of metabolic pathways. Spectroscopic tools such as FTIR, NMR or MS present a very high potential in this investigation, with an emphasis on FTIR since it allows the fingerprinting of the sample in a non-invasive manner and with minimal sample preparation.

Currently, the investigation of the fibroblast metabolome for aging studies is not a common, however there are several studies that analyse senescence in these cells, so the conclusions of these studies may be reproduced in fibroblasts from various donors aged different to compare the differences in their metabolome, thus giving new insights into biological aging and consequently to explain why individuals with the same age present so different health states.

CHAPTER IV: CELL CULTURE STUDIES: A PROMISING APPROACH TO THE STUDY OF HUMAN AGING

CHAPTER IV: CELL CULTURE STUDIES: A PROMISING APPROACH TO THE STUDY OF HUMAN AGING

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IV.1 Abstract

With the increasing aging of the world's population, a detailed study of the characteristics of aging, as well as the pathologies this process can lead to, is crucial. Therefore, there are several study models for aging, from computational models to animals or even to cell cultures. The latter have shown high potential for aging studies as they are easier to handle, cheaper, do not require the same level of ethical consideration required for animal and human studies and present little biological heterogeneity when grown under the same conditions and in the same context population. For aging studies, these characteristics are a great advantage since cells have a huge variety of morphologic characteristics and markers that can be studies. Thus, the aim of this review is to offer an overview of the models used in studies of aging, with focus on cell culture models, presenting the advantages and disadvantages of cell culture in the study of aging, of what information can we extract of these type of studies and how cell studies can be compared with the other models.

Keywords: Cell culture, Aging, *in vitro* models of aging, Cellular models of aging

IV.2 Biological aging

The population aging is increasing, and this fact brings many challenges to society. The increase of average life expectancy must be accompanied by an increase in healthy lifespan, instead of additional years with disabilities and illness (194). Also, older people present a huge variability of healthy status, meaning that there are individuals around 80 years old with the same physical and mental capacity as many 20-year-olds, while others experience a decline in physical and/or mental capacity sooner (194). However, the reasons for this variability are complex and are not yet fully understood.

In this way, biological aging can be understood as a challenging and multifactorial process, driven by genetic, environmental and stochastic factors that lead to the physiological decline of biological systems (195). All known organisms age and, although the maximum lifespan is different between organisms, it is remarkably consistent across species (196).

In humans, aging is characterized by declining tissue function and increased susceptibility to diseases like cancer, diabetes, cardiovascular disease, dementia, arthritis, sarcopenia and kidney dysfunction (197). Diseases and phenotypes associated with aging often occur at the tissue level, but have their roots in molecular and cellular damage (197). Thus, understanding the basic mechanisms of this process is essential for the development of effective therapeutic interventions against age-related diseases. These mechanisms have been extensively investigated in various model organisms and systems, however the results obtained must be analysed carefully when they are translated into human aging (195).

IV.2.1 Hallmarks of aging

Extending a healthy working life has become a challenge for present and future society and, at the same time, its major objective. Thus, the use of cellular models and model organisms in the research of aging has been essential to lead to a process of modulation of aging. Rather than being defined only as the passive and indefinite decline in physiological functions, aging is the result of a complex interconnection of genetic and biochemical mechanisms that were classified into nine molecular

characteristics: genomic instability, telomere attrition, epigenetic changes, loss of proteostasis, deregulated nutrient detection, mitochondrial dysfunction, cell senescence, stem cell exhaustion and communication altered intercellular system (197, 198) also know as the hallmarks of aging.

Genomic instability

Throughout life, DNA is vulnerable to a variety of exogenous mutagenic agents, like radiation or chemicals, and endogenous ones such as DNA replication errors, spontaneous hydrolytic reactions and reactive oxygen species (ROS) (34), which cause different types of DNA damage. The inability to completely repair all DNA damage leads to accumulation of somatic point mutations, deletions, integrations or even chromosomal rearrangements in the genome, which are inevitable over time, ending in an increased genomic instability with age (199).

Thus, to minimize these injuries, organisms seem to have developed a challenging network of DNA repair mechanisms that are capable of handling most of the damage to nuclear DNA (200). In addition, genomic stability systems also include specific mechanisms to maintain the length and function of telomeres, as well as to ensure the integrity of mitochondrial DNA (mtDNA) (201). However, in addition to these direct DNA lesions, defects in the nuclear architecture can also lead to genome instability and result in premature aging syndromes (202), such as Werner's syndrome and Bloom's syndrome (203).

Telomere attrition

Aging appears to affect the genome almost randomly by accumulation of DNA damage, but there are a few chromosomal regions, such as telomeres, that seem to be particularly susceptible to age-related deterioration (204). Telomeres are complex structures of tandem repetitions of hexanucleotides and linked proteins that protect the end of linear chromosomes. The length of telomeres decrease with each cell division throughout life (198) because telomerase (replicative DNA polymerase) does not have the capacity to completely replicate the terminal ends of linear DNA molecules. In parallel, the majority mammalian somatic cells cannot express telomerase and this can lead to the progressive and cumulative loss of protective telomeric sequences from the ends of the chromosomes (198). The

depletion of telomeres explains the limited proliferative capacity of some types of cells grown *in vitro*, the so-called Hayflick replicative senescence or limit (36, 205). It is also important to note that the shortening of telomeres is detected during usual aging process in humans and mice (206).

Telomeres can also be considered as DNA breaks that are invisible to DNA repair systems through the formation of a specialized complex of nucleoproteins, the shelterin (207). In this way, telomeres are not only shortened in the absence of telomerase, but also, the imposition of exogenous damage to the DNA of telomeres becomes invisible to DNA repair systems due to the presence of shelterins. Therefore, DNA damage in telomeres leads to a persistent type of DNA damage that ends in deleterious cellular effects, including senescence and/or apoptosis (208).

Epigenetic changes

Several epigenetic changes affect all cells and tissues during life (209). Epigenetic changes involve changes in DNA methylation patterns, post-translational modification of histones and chromatin remodelling (198). Increased acetylation of histone H4K16, trimethylation of H4K20 or trimethylation of H3K4, as well as decreased methylation of H3K9 or trimethylation of H3K27, are age-associated epigenetic marks (210). The multiple enzyme systems that ensure the generation and balance of epigenetic patterns include DNA methyltransferases, histone acetylases, deacetylases, methylases and demethylases, as well as protein complexes involved in chromatin remodelling. Variations in cellular inputs, such as signs of stress initiated by the aging process, can also alter the activity of some transcription factors responsible for regulating the expression or recruitment of chromatin modifiers, leading to epigenetic changes over time (211).

Loss of proteostasis

Aging and some diseases related to aging are related to changes in protein homeostasis or proteostasis. Some studies have showed the gradual accumulation of damaged / unfolded and aggregated proteins with age. Accordingly, a considerable number of age-related pathologies, like neurodegenerative diseases, are caused by a failure of the mechanism responsible for the

maintenance of proteostasis (212). This network of proteostasis is mainly composed of chaperones, which assist in folding, and the proteasome and lysosome-autophagy systems, which degrade proteins that can't pass quality control. In addition, the modifier of aggregation 4 (MOAG-4), which acts by an alternative route distinct from chaperones and molecular proteases, is believed to be a part of age-related proteotoxicity (213).

In this way, all of these systems work to preserve the structure of poorly folded polypeptides or to remove and completely degrade them, preventing the accumulation of damaged compounds and ensuring the continuous renewal of intracellular proteins (197). In this sense, many studies have shown that proteostasis is altered with aging (214). Additionally, the development of some age-related pathologies, such as Alzheimer's disease, Parkinson's disease and cataracts can be caused by chronic expression of unfolded or aggregated proteins (215).

Deregulated nutrient detection

Growth hormone (GH) and its secondary mediator, the insulin-like growth factor (IGF-1) (produced in response to GH by many types of cells), are a part of the somatotropic axis in mammals. The IGF-1 intracellular signalling pathway is similar to the insulin pathway, which informs cells of the presence of glucose. For this reason, IGF-1 and insulin signalling are known as the insulin and IGF-1 signalling (IIS) pathway. This is the most conserved way of controlling aging in evolution and among its various targets are the family of transcription factors, forkhead box class O family member proteins (FOXO proteins), and the mTOR complexes, which are also involved in aging and conserved by evolution (15). Polymorphisms or genetic mutations that decelerate the functions of GH, IGF-1 receptor, insulin receptor or its downstream intracellular effectors, like AKT, mTOR and FOXO proteins, have been associated with longevity, both in humans and in model organisms, illustrating the great impact of trophic and bioenergetic pathways on longevity (15). According with the relevance of the unregulated nutrient sensor as a hallmark of aging, dietary restriction increases the useful life or health of all eukaryotic species investigated, including unicellular and multicellular organisms from several different phyla, including non-human primates (216).

Mitochondrial dysfunction

Many studies have shown that mitochondrial function decreases with age (217). Several models of progeroid rats reveal mitochondrial defects and some of their age-related phenotypes show changes when mitochondrial homeostasis is improved (218). Additionally, mice with mutated mtDNA develop early signs of aging, further supporting a causal role of mitochondrial dysfunction in the aging process. Finally, the stimulation of mitochondrial biogenesis through resistance exercise attenuated the age-related characteristics in these same mice (219). Thus, the exact mechanism by which mitochondrial dysfunction acts in the aging process remains uncertain. The free radical theory of aging assigns an important role to oxidative damage induced by the increase in the production of reactive oxygen species (ROS) over time, initiated by defective mitochondrial function (198).

However, dysfunctional mitochondria can contribute to aging regardless of the formation of ROS, as exemplified by studies with mice deficient in γDNA polymerase (220). These changes can happen through various mechanisms, for instance, mitochondrial deficiencies can affect apoptotic signalling, increasing the predisposition of mitochondria to permeabilize in response to stress (221) and trigger inflammatory reactions, favouring inflammatory reactions formation of mediated ROS and/or activation of facilitated permeabilization of inflammasomes (222). In addition, mitochondrial dysfunction can directly impact cell signalling and inter-organellar interference, affecting the membranes associated with mitochondria that constitute an interface between the external mitochondrial membrane and the endoplasmic reticulum (223).

Finally, another recent study has shown that mitochondrial dysfunction might also induce senescence with a distinct secretory profile, called senescence associated with mitochondrial dysfunction (224). This type of senescence has been seen *in vivo* in mtDNA mutant mice, however further investigation is need in other level of complexity to the role mitochondria in the aging process (198).

Cell senescence

Cell senescence can be described as a stable stop in the cell cycle coupled with stereotyped phenotypic changes (225). Senescence is induced by telomere attrition, DNA damage or other signs of stress that lead to the activation of antiproliferative pathways, like p16Ink4a-Rb and p19Arf-p53-p21, triggering to

growth arrest (226). In senescence induction, cells secrete numerous pro-inflammatory cytokines, growth factors and matrix remodelling enzymes that constitute the so-called secretory phenotype associated with senescence (227).

Initially the senescence process was originally described by Hayflick in human fibroblasts passed in series in culture (36). Currently it is know that the senescence observed by Hayflick seems to be caused by the shortening of telomeres (228), but there are other stimuli that apparently have a relation with aging and trigger senescence, regardless of this telomeric process. Most remarkably, non-telomeric DNA damage and repression of the INK4 / ARF locus, which occur progressively with chronological aging, are also capable of inducing senescence (229).

Senescent cells can play a role in wound healing processes, as well as in suppressing tumours since it appears to impair the spread of aberrant cells. However, with age and as a consequence of inefficient elimination or defective regenerative capacity of the tissue, senescent cells accumulate and lead to a chronic inflammatory state that impairs the proper function of the tissue (198). Therefore, its sustained presence over time can be harmful and boost the aging process.

Stem cell exhaustion

The decline in tissue regenerative potential is one of biggest marks of aging. Studies in elderly mice have revealed a general decrease in the activity of the cell cycle of hematopoietic stem cells (HSCs), with older HSCs undergoing less cell division than young ones (230), which is related to the accumulation of DNA damage (230) and overexpression of cell cycle inhibitory proteins, such as p16INK4a (231). These functional changes in stem cells have been found in virtually all compartments of adult stem cells, including the rat's anterior brain (232), bone (233) or muscle fibres (234).

Telomere shortening is another cause of stem cell decline with aging in multiple tissues (235). These examples prove that the decline in stem cells appears as the integrative consequence of various types of damage. However, deficient proliferation of stem cells and progenitors is obviously detrimental to the long-term balance of the organism, excessive proliferation of stem cells and progenitors can also be detrimental in accelerating the depletion of the stem cell niches (197).

Altered intercellular communication system

Aging can be seen as a global decline in the organism, so it is impossible to rule out the role that the extrinsic cellular environment plays in this complex process (198). In addition to autonomous cellular changes, aging also involves changes in the level of intercellular communication, be it endocrine, neuroendocrine or neuronal (236, 237). Neurohormonal signalling (e.g., renin-angiotensin, adrenergic, insulin-IGF1 signalling) tends to be deregulated in aging as inflammatory reactions increase, immunosecurity against pathogens and pre-malignant cells decreases and the composition of the environment peri and extracellular changes, affecting the functional and mechanical properties of all tissues (197).

After understanding the characteristics of aging, reflected in these genetic and biochemical mechanisms, it is possible to design, perform and interpret studies on aging based on models and expect that the obtained results will contribute with more knowledge to the biology of aging.

IV.3 Relationship between *in vivo* and *in vitro* aging

Aging is a complex process, and there is no consensus on how to define it. In addition, there is conflicting evidence about aging as a similar process in all organisms or particular to each species (238). Thus, organisms are extremely diverse in the rates at which they age, despite similarities in the level of individual cells. In this way, it is essential to understand how the behaviour of cells dissociated from the organism and maintained in culture can provide insights into aging (239).

Cell cultures have been used in biological research since 1912. Carrel (240) isolated and cultured chicken cells to study aging processes (240) and concluded that the single cell is immortal and aging and death are phenotypes related to a multicellular organism.

However, in 1961, Hayflick and Moorehead concluded that Carrel's research was wrong and that cells have limited capacity for proliferation in culture (36). In addition, they revealed that cells had an aged appearance after depleting their potential for replication. Thus, they concluded that the unicellular replicative senescence contributed to the aging of the organism (36), which promoted the use of cell cultures to study the aging processes (239) and *in vitro* cellular studies became the basis of all studies in human biology.

Currently, it is agreed that cells in culture behave differently due to the loss of connections between cells and the extracellular matrix of other regions of the body, such as the immune system or hormonal signals (241). It is also known that cells in culture have a high sensitivity to disturbances while *in vivo* they can maintain their function more or less stable throughout the life of the organism (for instance, cells in organisms are less sensitive to carcinogenesis than cells in culture) (242).

This characteristic of cells *in vivo* is due to the multicellularity in the organs and the metabolic cooperation between cells (243). If a specific biochemical pathway is defective in a cell, the surrounding cells can deliver the missing product through permeable membrane junctions (244) which is not the case in cell cultures. Also, it has been observed that in organisms stem cells can undergo thousands of divisions with a small reduction in growth rate or function during life, while decaying rapidly when dispersed into cell culture. And again, the answer seems to be in the organizational state of the tissue as scattered cells tend to rapidly lose their differentiated potential, while tissue fragments that contain many cells retain their potential (245).

However, despite the limitations of cells used in *in vitro* studies when compared to what happens *in vivo*, cell cultures can provide information and molecular identifications about the intrinsic sensitivity of cells to oxidation and other stresses, the ability to detect and deal with DNA damage, telomere dysfunction and the response to oncogenic stimuli, information that in turn, is related to the species' lifespan and consequently to the aging process (239).

IV.4 In vitro signs of aging

Cellular aging *in vitro* has distinct characteristics, namely in cell morphology and in the appearance of specific metabolites or even biomarkers of aging (246).

Aged cells have similar morphology to cells that exhibit replicative senescence, so by the morphology of senescent cells we can infer the morphology of aged cells. The morphological changes observed include an increase in cell size, nuclear size, nucleolar size, the number of multinucleated cells, the prominence of the Golgi complex, the increased number of vacuoles in the endoplasmic reticulum and cytoplasm, the increased number of cytoplasmic microfilaments and large lysosomal bodies (247). The increase in the intracellular content of RNA and proteins can cause the increase in cell and nuclear size and the number of inclusion bodies that may produce a reduction in protein degradation by proteasome-mediated pathways, decreased RNA renewal and uncoupling of cell growth from cell division. Aged cells also appear to exhibit increased sensitivity to cell contact (247), possibly as a result of changes in interactions with the extracellular matrix (ECM) or in the expression of secreted proteins (86), resulting in reduced densities (248).

In what concerns aging biomarkers, changes in small molecules are observed, such as changes in the levels of IGF-1, epidermal growth factor (EGF) and the c-fos proto-oncogene, or changes in the biological activity of β-galactosidase (SA-βgal) and the appearance of macromolecules such as senescence-associated heterochromatin foci (SAHF) and promyelocytic leukaemia protein nuclear bodies (PMLNBs) (249, 250). IGF-1 is known to be produced by many types of cells and plays an important role in regulating cell proliferation. IGF-1 mRNA production is reduced to an undetectable level in senescent cells, while IGF-1 receptor mRNA production remains at a detectable level (251). Epidermal growth factor (EGF) signalling impairs receptor binding downstream in non-proliferative senescent fibroblasts (252) and in WI-38 (human diploid lung fibroblasts) senescent cells, a loss of c-fos has also been reported, suggesting that the lack of proliferation in aging of cells is partly due to the selective repression of c-fos (253). For all that has been said, cells aged *in vitro* have unique metabolic characteristics that allow their clear distinction as well as their introduction in aging studies.

IV.5 Models of aging

In the past few decades, research on the underlying genetic and biological causes of aging has led to notable advances, not only in understanding the mechanisms of aging, but also in interventions that can increase life and health. Given the difficulty to carry out longitudinal studies of aging with humans there was the need to resort to the development, validation and use of models that mimic aging and that allow the transposition of the results to humans. Not only cellular models are used, other tools like computational and animal models and more recently organoids, are at the forefront of this research and produce, daily, a wealth of information, allowing us to find common points with human aging.

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IV.5.1 Computational models

As it will be mentioned and explained throughout this review, the increase in knowledge about aging was driven by the study of a wide variety of model organisms such as nematodes, yeasts, rodents, or primates. However, it remains essential to understand how the processes associated with aging interact with each other since they do not act isolated and their interdependence is fundamental in the deregulation of biological systems (254). Thus, given this complexity, computational models arise since they have the ability to integrate detailed information and complex dynamics associated with biological systems (255), and also to represent, manipulate and analyse the dynamics of complex biological processes in aging as this process is highlighted by its global nature and biological interactions that transcend the temporal and spatial scales (256).

In addition, there is a huge need for aging to be studied in an integrated manner, as no single mechanism supports it. The knowledge of the interactions between these processes is crucial to understand aging and improve health time, and in this way, it is becoming more and more common to combine computational modelling with laboratory experimentation. However, to evaluate how these models can be used for this purpose, it is essential to understand what a computational model is. Computer modelling is then defined as a process by which a system of interest is precisely defined

using mathematics (257). Thus, a mathematical model is inserted into a computer to simulate the dynamic behaviour of the biological system of interest. These models offer several advantages over traditional approaches as a means of exploring hypotheses, an alternative view of the mechanisms that underpin aging biology, and are still easily updated as knowledge of aging biology evolves and provides a cohesive structure to bring together the different biological elements that characterize this knowledge. A crucial aspect of the computational model creation process is to minimize the biological details necessary to display the phenomenon of interest, maintaining sufficient details for the model to retain predictive power. Thus, computational models for research on aging are generally developed according to a series of stages (258). The first step to follow will be to define a hypothesis, that is, the question the model will try to answer. This step has a fundamental importance since a well-defined hypothesis will dictate the contour points of the model. To establish this hypothesis, it is necessary to create a list of biological entities, which will form the model and after the identification of these entities and their interactions, the model will be summarized in a diagram. This diagram can after being used as a map to guide the assembly of the model on a computer. At this point, it will be necessary to select a software tool to assemble and simulate the dynamics of the model (258). Then, the model's initial parameters and conditions need to be entered into the software tool and the model can be simulated. Depending on the mathematical structure, it will be simulated in a deterministic or stochastic way. In summary, the main difference between a stochastic and a deterministic model is that, given the same set of conditions and initial parameters, a deterministic model will produce the same output; however, given the same conditions and initial parameters, a stochastic model will generate a different solution for each simulation.

After understanding how the models work, it becomes relevant to understand what types of models exist for researching the aging process. Hence, there is a wide spectrum of models that represent many aspects of aging. These models include telomere dynamics models (259-262), mitochondrial dynamics models (263-267) or DNA repair models (268-270), all models of intracellular processes. At the same time, models focused on cellular pathways were also developed as models to mimic the mTOR pathway and its interaction with other associated mechanisms of cell aging, such as cell senescence (271, 272), or models of folate metabolism and DNA methylation dynamics (273).

In short, given all these models, we can conclude that computational models can represent the intrinsic complexity associated with aging, improve understanding of the biology underlying aging, help generate new insights, highlight gaps in current knowledge and still lead to counter explanations

intuitive and unusual predictions about aging that would not otherwise be apparent if the systems were not studied in an integrated manner.

IV.5.2 Animals models

In vivo studies allow testing the effect of manipulation or treatment, targeted, or dispersed, on the entire organism. Several model organisms are used in the study of aging since they offer additional advantages over humans, namely, their basic biology and genomes are well documented and are easier to manipulate genetically, and have a much shorter life span than of humans, allowing longitudinal studies (241). However, despite these advantages, the use of animals in studies has limitations, such as the choice of animal model, that is generally made out of convenience and not by specific characteristics applicable to human aging (241), which is why extrapolation to the organism human is of utmost importance.

Invertebrates may have special properties that allow them to serve as a model for the study of physiological mechanisms, including those of aging. These organisms have benefits since they have a short lifespan, widely sequenced genomes, variations in senescence within or between closely related species and tissue regeneration in adults (274). Several of these studied invertebrate models are hydra, tunicata, rotifers, nematodes, fruit flies, water flea and sea urchin. The study of these types of invertebrates was important to identify new and potentially missing genes that modulate pathways related to longevity at the cellular, molecular and organism levels, to study mechanisms of stem cell biology to understand their response to injuries in model systems with and without regeneration capacity; and examine the cellular, molecular and physiological mechanisms of tissue aging and homeostasis of these organisms and consequently adapt them to humans (274).

Despite the use of this type of invertebrate, research on aging and longevity in model invertebrate systems began especially with the first genetically treatable systems, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the yeast *Saccharomyces cerevisiae*. *C. elegans* has a short adult life (approximately 2 weeks) and a well-documented anatomy that is visible using a microscope, allowing easy observations of changes in the aging of the entire organism, tissues, organs

or even at molecular and cellular levels (275-279). The main advantage of *D. melanogaster* is the ability to clarify how genes that have an established role in regulating the lifespan of the organism particularly influence cell and tissue function, and how their specific tissue functions may be linked (280, 281). However, *D. melanogaster* is far from being a good model for human aging, as it shares only 60% of the human genome. *S. cerevisiae*, as a single-celled organism, is easily cultivated, manipulated and observed; it has a well-studied genome, sharing characteristics with larger and more complex organisms constituting a convenient organism for the study of the aging phenotype (241).

A better similarity with humans is thus found in vertebrate organisms, as is the case of the mouse *Mus musculus*, which is the most used model in biological research for several reasons: mice are small, have a short generation time and an accelerated lifespan, their genome is well reported in literature and can be easily manipulated (241). In parallel, they are biologically similar to humans, exhibiting several of the same diseases and conditions. However, mice apparently do not develop several important age-related diseases, such as atherosclerosis and diabetes, which can limit their potential as an aging model (241). Given these difficulties in mimicking aging in mice, an accelerated senescence model (SAM) has emerged which is a model that was established by phenotypic selection of a common gene pool of the AKR/J mouse strain (282). In 1975 in a litter of this strain some mice became senile at an early age and had a shorter shelf life and thus were selected as progenitors of the mice prone to senescence acceleration (SAMP). In 1981, the SAM model was established, including nine main SAMP lines and three main senescence-accelerated mouse resistant (SAMR) lines, each of which exhibited characteristic disorders, for example, degenerative arthrosis (SAMP3), senile osteoporosis (SAMP6) , age-related cataracts (SAMP9) or normal aging with colitis (SAMR5), among others (283).

The rat *Rattus norvegicus* is also a model used in aging studies, mainly to investigate age-related changes in the cognitive and neural systems. There are sufficient similarities between rodents and humans in the anatomy of brain systems, particularly to examine age-related changes in the brain and behaviour in parallel (274).

Although all of these organisms are models used in research, they are all short-lived, which is one of the desired characteristics as model organisms. However, this may not be appropriate for the study of human aging. Thus, in last years, more studies have been carried out on long-lived organisms, such as mole rats, bats, primates or whales that might be more appropriate models for perceiving healthy human aging.

The naked mole rat *Heterocephalus glaber* is a non-model organism very important in studies of cancer and aging. This highly socialized rodent is the size of a mouse and has the ability to live up to five times longer than expected based on its small body size, living from 4 to 17 years in the wild and around 30 years in captivity (284). In fact, over the age of 24, *H. glaber* shows aging signs consistent with humans, like retinal degeneration and osteoarthritis (284), but exhibit insignificant senescence, no increase in related mortality and high fertility until death.

Bats are the second largest mammal order after rodents, and there are bats with longevity limits, namely *Myotis myotis* with an average longevity of 37.1 years and *Myotis brandti*, with average longevity of 41 years. However, longevity is generally high in all bat strains, which makes them an interesting model in study of aging (241).

Non-human primates are perhaps the most appropriate model for study aging and age-related diseases. Rhesus macaques *Macaca mulatta* has been the main focus of research on aging (285). This specie is frequently used in biomedical research due to its similarity to humans in genetics, endocrinology, physiology, neuroanatomy and cognitive function. However, there are disadvantages in the use of these monkeys in research such as their weight, strength, and the need for sophisticated equipment. In addition, strict social hierarchies and the potential for aggressive behaviour could mean that, to keep them in the laboratory, these animals need special consideration and environmental enrichment. Monkeys can also transmit many dangerous pathogens and costs and ethical concerns limit their contribution to research in aging (285).

Another of the non-model organisms for research in aging is the white-headed whale *Balaena mysticetus*, which is the longest-lived mammal, reaching the age of approximately 200 years (286). These whales are highly resistant to cancer and age-related diseases and, although research on these animals is very complicated, their study in the context of longevity can ameliorate our understanding of the molecular mechanisms of healthy aging (286).

For several years fish were kept away from research on aging due to some claims that they do not age (287). However those claims were countered (288) and fish emerged as a model system interesting in general biology and aging research. The zebrafish *Danio rerio* has a useful life of approximately two to three years and, therefore, may not be particularly advantageous for useful life studies compared to rodents. However, it has remarkable regenerative capacities that may be of interest to research in the field of tissue repair and, therefore, longevity (289). *Nothobranchius furzeri*, the turquoise killifishi, seems to be another specie that could be a promise model for longevity (285). Domestic species,

such as dogs and cats, can also represent interesting model systems for cross-sectional studies of aging, since they spontaneously develop many age-related phenotypes (290). However, their average lifespan of 10 to 14 years discourages longitudinal longevity studies.

Given all that has been presented, animal models form the basis of research on aging, as ethical issues, long useful lives, genetic heterogeneity, and several other limiting factors complicate the use of human beings in research on aging. Although these model organisms have shorter useful life, they somehow mimic some aspects of human beings, highlighting the universality of the aging process (285). Summarizing, a multifaceted approach using different model organisms seems to be the key to better understanding human aging and age-related diseases.

IV.5.3 Cellular models

Aging of postmitotic cells

Cells can be, in a very simplified way, classified into two fundamental types: mitotic and post-mitotic cells. Mitotic cells are those that maintain the ability to proliferate, regardless of their differentiation and have the ability to perform specialized functions (291). Regardless of their proliferative state, all mitotic cells could go through a complete cell cycle when properly stimulated. Post-mitotic cells, on the other hand, originate from mitotic stem cells and during the differentiation process they irreversibly lose their ability to proliferate (291). Despite their inability to start a cell cycle, post-mitotic cells can persist and function for long periods in the body. Unlike mitotic cells, post-mitotic cells never undergo tumorigenic transformation. Despite these differentiating characteristics between these cell types, they also have common characteristics such as dependence on extracellular components for their viability and phenotype (291). Due to their contrasting proliferative potential, it is possible that mitotic and post-mitotic cells suffer aging by different mechanisms. Also, it is possible that they differ in their sensitivity to stimuli that contribute to aging and/or age-related changes.

Aging is characterized by a loss or malfunction of post-mitotic cells (291). For example, neuronal synapses are commonly lost during aging, although neuronal cell bodies can persist. Results of cell

cultures suggest that agents, especially oxidants, that damage macromolecules often induce post-mitotic cell death. Up to now, it seems to be no way to replace the lost or dysfunctional post-mitotic cells, and the death or malfunction of the cells can severely compromise the tissues composed largely or entirely of these cells (291).

Some investigations have suggested that post-mitotic cells are also capable of entering a state of senescence and therefore constitute a model in the study of aging. In tissues made up mainly of post-mitotic cells, it is evolutionarily advantageous to preserve cell integrity and the cellular senescence of post-mitotic cells can prevent tissue degeneration induced by stress agents and promote tissue repair. Paradoxically, the generation of inflammatory mediators, called the secretory phenotype associated with senescence can lead to post-mitotic cell senescence (PoMiCS) that can contribute to disease progression (292-294).

There are several studies that suggest that PoMiCS occurs in subspecific circumstances, namely an age-dependent accumulation of lipofuscin (295) in post-mitotic or terminally differentiated cell types, such as neurons and cardiomyocytes (296). The increased rate of lipofuscin formation and accumulation (297) has been seen in these cells and has been confirmed as a marker of stress-induced and replicative senescence (298), and the staining of granules containing lipofuscin is now considered a robust biomarker for senescent cells *in vitro* and *in vivo*.

Post-mitotic cells have thus shown a high potential for the study of aging, particularly in the brain, because age-related functional decline in the brain is often attributed to a failure in proteostasis, mainly in post-mitotic neurons, which, therefore, is a distinct process of senescence. However, it is possible that proteostasis failure and cellular senescence have overlapping molecular mechanisms. It was identified that post-mitotic cell senescence is an adaptive response to stress and proteostasis failure (299). Neurons of the rat hippocampus in primary cultures showed that molecular changes indicative of senescence (β-galactosidase associated with senescence (SA-βgal), p16 and loss of lamin B1) and failure of proteostasis are relevant to Alzheimer's disease. In addition, senescent neurons exhibited resistance to stress (299).

Although the use of post-mitotic cell models is not very recurrent in the study of aging, there is increasing evidence that can be a study tool, especially in the study of pathologies of the nervous system that are relate to aging.

Cellular models of senescence

Aging is known as a progressive functional decline, that may lead to an increased risk of developing major human pathologies, such a cardiovascular disorders, cancer, diabetes and neurodegenerative diseases (300). Although these links with human disease, knowledge of the aging process remains limited. Despite its biological causes remain largely unknown, in the last decades a study have identified nine common cellular and molecular characteristics associated this phenomenon, including cell senescence (197). Although it plays physiological roles during normal development and is necessary for tissue homeostasis, senescence is a response to stress triggered by insults associated with aging, such as genomic instability or attrition of telomeres, which are the main characteristics of aging. Also, there is an intimate link between senescence and other antagonistic characteristics of aging (217) (*Figure 3*). At the same time, senescence also influences the integrative characteristics of aging. Somatic multipotent stem cells facilitate tissue homeostasis and the exhaustion of these cells occurs with age, leading to the decline in their functionality and in their capacity for renewal leads to tissue deterioration (300).

Thus, after the discovery of cell senescence, studies were carried out to characterize the senescent phenotype and to identify specific biomarkers. These biomarkers are typical morphology (301), irreversible growth arrest with overexpression of p21WAF-1 and p16INK-4a (302, 303), SA-βgal (304), altered gene expression (305), foci of heterochromatin associated with senescence (SAHFs) and foci of DNA damage associated with senescence (SDFs) (306) and loss of lamina B1 (307). Using some of these biomarkers, such as overexpression of SA-βgal and p16INK-4a, senescent cells have been shown to accumulate in tissues with age (304, 308). Therefore, senescence is an essential tool for the study of aging. A study have shown that several ways can lead to senescence or, at least, a "senescence-like" phenotype (309).

Hence, senescence inducing stimuli are innumerable. In cell cultures, it is known that one of the reasons for limited growth is the erosion of telomeres, however, senescence can also be activated by various types of stress, such as the aberrant activation of various oncogenes (310), damage to the structure chromatin (311), genomic damage in non-telomeric sites (312) and oxidative stress (313).

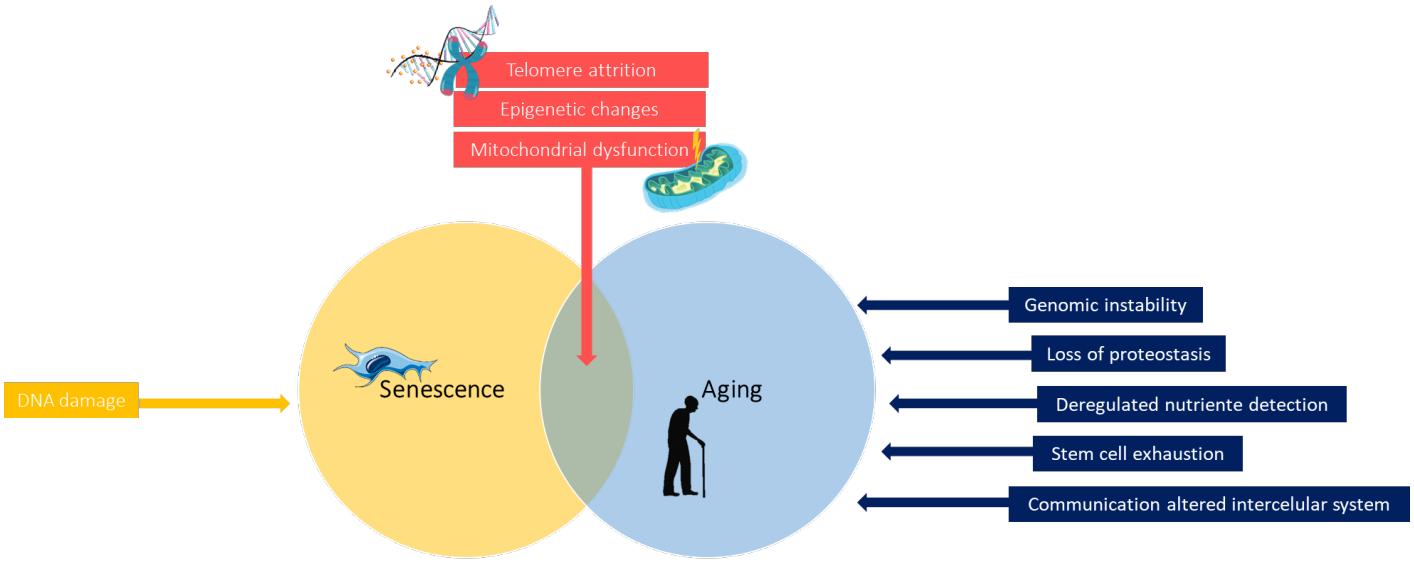

Figure 3 | Senescence and Aging. Senescence is one of the main hallmarks of aging and many of the mechanisms that lead to a senescent state correspond to those that lead to an aged state, so that senescence models can contribute to the study of aging.

Telomere-dependent or replicative senescence

As already mentioned, cellular senescence can occur *in vitro* through a variety of mechanisms such as shortening and wear of telomeres (309). Thus the replicative senescence or proliferative exhaustion (PEsen) is known as continuous culture of human cells until they stop proliferating after a variable number of divisions (309).

Telomeres are repetitive DNA sequences located at the ends of chromosomes and are associated within specific protein complex of telomeres, also known as shelterin/telosome (207). Cells that do not have shelterin components, such as protection of telomeres protein 1 (POT1) or telomeric repeat-binding factor 2 (TRF2), also undergo an aberrant response to DNA damage and premature senescence induction (314). However, it is known that the length of the telomeres is reduced after each cell division and this is inherently linked to the activity of DNA polymerase, which is not able to fully replicate a DNA strand (315). In this way, many senescence mechanisms involve the production of irreparable double strand DNA breaks (IrrDSBs), which can result from the disruption of telomeres (306) or the generation of oxidative damage to DNA and replication forks paralyzed in the phase S1

(224). The first events in the establishment of senescence are transiently reversible (268, 316), but the failure to repair DSBs (IrrDSBs) eventually leads to senescence and to the production of a matrix of secreted proteins called secretory phenotype associated with senescence (224, 317). Accordingly, irrDSBs in telomeres can contribute to senescence and aging in dividing and non-dividing tissues.

Similarly, it has been proven that as an organism ages, cells accumulate more divisions resulting in increased telomere erosion and senescence (300), apparently making this a good model for studying human aging. Changes in telomeres using T, B and Natural Killer (NK) cells from donors aged 20 to 25 and donors aged 60 to 65 were studied and it was proved that treatment with concanavalin A (a mitogen of T cells) caused an increase in the length of telomeres in samples from young donors, but it did not improve samples from older donors, who exhibited loss of parts of the telomeres, decreased length and potential for proliferation (318). Also, an *in vitro* study in human mesenchymal stem cells (hMSC) of the bone marrow also proved the existence of age-related changes in telomeres (319), revealing higher levels of damage in cells from older donors (320). In the study of $CD34^-$ and $CD34^+$ cells isolated from healthy donors of different ages have shown similar results (321).

Thus, aging studies that use this cell model, namely studies of metabolism, present promising results since they allow the identification of new biomarkers for senescence and aging. These studies also allow to prove that human serum metabolites associated with aging seem to be present in PEsen fibroblasts (137, 169).

Stress-induced premature senescence

Senescence can also arise after exposure of normal cells, immortalized, or transformed to the stress of physical or chemical agents, inducing oxidative stress and/or damage to DNA. This process was called stress-induced premature senescence or SIPS (322). The term "premature" refers to the onset of senescence in early population duplicities (DPs) compared to the maximum number of DPs in which senescence generally occurs and emphasizes the accelerated nature of the senescence process (323).

In vivo, cells are continually exposed to different types of stress and depending on the nature of the stress, its intensity and the type of cell, cells can: repair the damage or enter in a state of apoptosis or senescence. Thus, if the intensity of the stress is low, the cell can repair the damage and, after a transient interruption of the cell cycle, it will resume in its growth (309). If the stress is intense,

apoptosis will be initiated (309). Therefore, there is a zone of "moderate" stress and still in the subcytotoxic range, in which cells will not be able to repair all the damage caused by stress, their cell cycle will be irreversibly interrupted and they will enter into premature senescence (324). Potentially, almost any chemical or physical stress can induce cells to undergo senescence (309). Stress-induced premature senescence (SIPS) can be induced by the treatment of young primary cells, immortalized cells or cells transformed with a concentration of an infinite number of stressors. This treatment is usually repeated daily or non-consecutive days, leading to chronic oxidative stress, which seems to be one of the main triggers for premature aging (323).

Cellular senescence is accompanied and partially conducted by a persistent DNA damage response (DDR), leading to foci of DNA damage associated with senescence of telomeric and non-telomeric origin. In cells exposed to genotoxic stress, SIPS is considered to be generated by extensive damage to non-telomeric DNA (325). Regardless of the source, foci of DNA damage exhibit persistent DDR, in which factors of DDR, such as γH2AX (phosphorylated form of histone H2AX) and ATM (ataxia telangiectasia mutated) are accumulated. Thus, while replicative senescence (RS) results from the shortening of telomeres and/or the loss of their function (326) and affects almost all types of proliferating somatic cells in the body, SIPS is generally independent of telomeric erosion and caused by oxidative stress, genotoxic and oncogenic stimuli (323).

The types of stress that can induce senescence seem to be infinite and there are many models of SIPS that can be studied. Among the types of stressors one can highlight ultraviolet light (UVB), ethanol, tert-butyl hydroperoxide (t-BHP), hydrogen peroxide (H_2O_2, the most used agent), dicarbonyls, mitomycin C, doxorubicin, hyperoxia, irradiation, homocysteine, hydroxyurea and paraquat (323).

Cell senescence can be induced by chronic or acute stress protocols in different types of cells, such as lung and skin human diploid fibroblasts (HDFs), human melanocytes, endothelial cells, human retinal pigment epithelial cells or human erythroleukemia cells (327). Both protocols use sublethal concentrations of stressors, according to theoretical studies based on the stability of cellular systems (328). Chronic stress protocols consist on treating different types of human cells with prolonged exposure (several weeks) to a stressor such as mild hyperoxia in HDFs (with partial oxygen concentration around 40%) (329), chronic exposure of endothelial cells to homocysteine (330), prolonged culture of human umbilical vein endothelial cells in glycated collagen (331) and chronic exposure of colon fibroblasts to selenite (332).

For all these reasons, this model becomes an essential tool to study the impact of the formation of age-related aggregates and changes in proteolysis. The accelerated formation of aging characteristics reduces the time to obtain an aging phenotype and simplifies studies on aggregate formation and proteolysis in the aging process (323).

Fibroblast cultures derived from old and young human donors

Cultures of skin fibroblasts have been extensively studied in human genetic disorders. The first view of the mechanisms of various inherited metabolic disorders was obtained in biochemical and histochemical studies conducted in cultures of skin fibroblasts derived from patients and their families (333). Thus, fibroblast cultures to study aging at the cellular and molecular level, seem to have great potential. The initial studies were focused on comparisons between fibroblast cultures of the human fetal lung in the early and late stages of their *in vitro* life (36, 334). However, the differences observed in human fetal lung cells due to serial *in vitro* passage may not accurately reflect human cell aging *in vivo* (335). Hence, skin fibroblast cultures were used to confirm these observations and they made these cells as the main resource in aging studies, indicating an inverse relationship between the total number of duplications of the cell population ("life *in vitro*") and the age of the donor of the cell culture (334, 336, 337). This relationship has led to the use of human cells of young and old people in numerous studies on cell aging (337).

Over time it has been proven that normal human embryonic fibroblasts undergo only a fixed number of reproductive cycles *in vitro* and that this manifestation could be a manifestation of aging at the cellular level (338). The finite proliferative capacity of these cells is approximately 50 population duplications, however, when the proliferative capacity for normal human adult fibroblasts in culture was evaluated, it was proved that this capacity decreased to about 14 to 29 duplications (334). Thus, it is believed that there is an inverse relationship between the age of a human donor and the proliferative capacity *in vitro* (336, 337, 339).

Given this limited number in cell proliferative capacity, it is important that in a natural aging cell model, such as in dermal fibroblasts recovered from donors of different ages, the number of cell passages is limited to avoid replicative aging *in vitro* and thus this is a reliable model for the study (340). In parallel, in order to increase the robustness of the correlations that can be linked to aging and as there are

inter-individual differences in the rate at which a person ages, it is important to include donors of different chronological ages (340).

These cell cultures may allow, in the future, to evaluate the effects of aging *in vivo* on a variety of cellular and molecular parameters in genetically homogeneous cultures, since they can be obtained from the same individuals at different times in their useful life *in vivo* (333).

IV.5.4 Organoids

The culture of human cells provides an alternative for the study of aging, however many functional signs of this process occur at tissues and not in cells and therefore, are not easily apparent in traditional cell culture models. Thus, organoids are presented as a model capable of circumventing these difficulties of cell culture because they are sufficiently complex to present relevant signs of aging at the molecular, cellular and tissue levels and are increasingly presented as an alternative to animal studies (341).

Organoids can be obtained from many sources, mainly primary cells and tissue or pluripotent stem cells (PSCs). Cell lines are less suitable sources for most organoid applications in aging research, due to immortalization, avoidance of cell senescence or the origin of tumours. Contrary, tissues from donors of different age range are a more suitable model for the study of aging phenotypes (342).

By providing the organotypic structure in an *in vitro* model, organoids allow the study of tissue aging in a more productive and experimentally accessible way, allowing the study of emerging signs of aging at the tissue level and paving the way for future human therapies.

Several signs of aging, particularly those that manifest at the level of tissues and organs, can be better represented by a more complex system. These signs include a breakdown in tissue homeostasis, stem cell dysfunction and susceptibility to specific tissue diseases and cancer (197).

In this way, organoids have emerged as a potent tool for the study of aging, however they are only in their initial stages. Some molecular signs of aging, such as protein aggregation, oxidation and glycation, can be analysed by non-destructive and high-performance microscopy (343, 344), being organoids much more accessible to images of living cells than whole organisms. Given this advantage, high-resolution live images of the development, stem cell dynamics and tumour cell invasion have been demonstrated in several organoid systems (345, 346).

Epigenetically, aged cells acquire hyper and hypomethylation, as well as greater variation in epigenetic marks (347). The intrinsic aspects of aging cells are preserved in primary cells and tissue-derived organoids (348). The main molecular damages in proteins during aging are glycation, oxidation and unfolding, namely endoplasmic reticulum membrane protein complex (EMC) proteins, which are very susceptible to non-enzymatic glycation, leading to their hardening (349). As the matrix is an important component of organoid culture, organoids are a natural model for the effects of aged ECM on tissue

function and organoid assays can allow the experimental study of changes associated with aging in the structure of EMC and the functional consequences.

Chemical damage can also lead to the breakdown and aggregation of proteins and other biomolecules, such as lipids. While lipid peroxidation compromises the membranes and leads to the accumulation of oxidized fatty acids, the damage to proteins accumulates in the form of carbonylation, fragmentation and cross-linking (341). Along with the breakdown, protein oxidation associated with age results in aggregates resistant to proteolysis, intra- and extracellularly. One of the major signs of aging is the accumulation of lipid-protein aggregates of intracellular lipofuscin (344), and although their role in pathogenesis is discussed, they are prominently associated with dysfunction of the central nervous system (350). These manifestations of neurodegenerative pathology might be more apparent in 3D culture than in traditional cell culture, where the secreted proteins diffuse freely in the culture media instead of accumulating near the cells.

For all these reasons, organoids could be, in the future, a powerful emerging tool for the study of aging as they offer the practical advantages of simpler organisms and traditional cell culture, as well as the complexity necessary to model the phenotypes at the tissue level that accompany aging (341). This system has the potential to shape the human body with incomparable fidelity.

As demonstrated throughout this section, the mechanisms of aging have been increasingly investigated in different models. Cellular and animal models are widely used to study age and although much of the knowledge is generated through this approach, the results of the studies must be carefully compared and cautiously translated into human aging.

IV.6 Aging cell culture

Cells *in vitro* have unique characteristics that give them unique properties for the study of aging. Thus, despite an increasingly in-depth knowledge of cell behaviour and signalling in this process, cell cultures as a study tool still present some challenges. However, these challenges are often overcome in view of the numerous advantages presented.

IV.6.1 Major advantages of study aging in cell culture

The study of aging through *in vitro* models can bring numerous advantages (*Figure 4*). One of the main advantages that can be highlighted is the possibility of studying cultures of different organisms and of different ages of donors, allowing the comparison between many types of cells from stem cells, to fibroblasts or endothelial cells, allowing its use in the study of genetics and biology of aging (241). Another benefit is the ability to study the responses isolated from the original environment and easily perform manipulations and treatments directly on the cells, since there is no connection between the cells and the extracellular matrix of other regions of the body, such as the immune or hormonal system, and thus it is possible to eliminate signalling noise from the pathway in the background (241). In addition to these advantages, the use of *in vitro* cultures allows the consistency and reproducibility of the results that can be obtained, as well as the performance of studies with human cells that otherwise could not be performed *in vivo* (351). Finally, *in vitro* cultures are economic models allowing to reduce spending on animal models (351).

IV.6.2 Challenges of aging cell culture

Cell culture for the study of aging presents some challenges (*Figure 4)* since it is not possible to fully mimic the environment *in vivo*. Thus, the culture conditions can be one of the biggest challenges to the study. One of the main differences between cell growth *in vitro* and *in vivo* is exposure to direct light when cultured *in vitro*, since fluorescent light can cause double-strand DNA breaks (352) and damage the photoactive components of the cell culture (353). Other culture conditions, such as hydrogen peroxide (354), tert-butyl hydroperoxide (305), ultraviolet radiation (355) and gamma radiation (356), can cause cells to show a premature senescent phenotype. At the same time trypsinization can also cause cellular stress (357).

Timeline is another of the most critical points to take into account because aging studies that try to recreate a senescent phenotype are usually time-consuming, as numerous population duplications will be necessary for cells to acquire shorter telomeres (senescence inducing factor) (246). Thus, the ideal seems to be to study a sample in culture at different ages than to examine different samples at different periods.

Another factor that constitutes an important issue is the relationship between replicative senescence and senescence *in vivo*, since a decline in the useful life of skin fibroblasts in culture has been observed due to the age of the donor (336, 337, 358), however, there are no studies that prove that there is an inverse relationship between donor age and proliferative capacity (358).

However, despite the challenges presented, what will stand out the most will be the lack of connection between cells and extracellular matrix from other regions of the body, such as the immune or hormonal system, and this constitute a great difficulty when trying to translate the effect of a manipulation or treatment for the whole organism (241).

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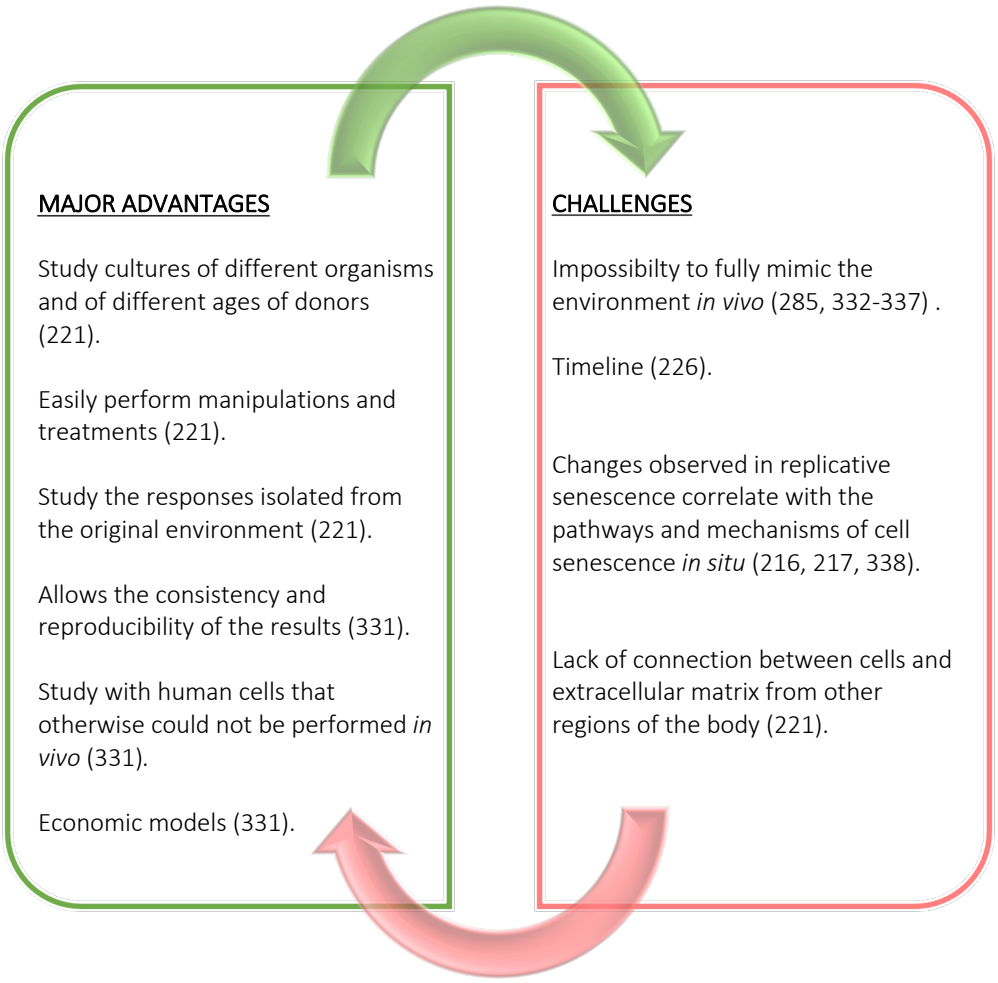

Figure 4| Major advantages and challenges of aging cell cultures.

IV.6.3 Metabolomics: an approach for cell culture analysis

As mentioned above, cell culture in the context of study of aging can present some challenges, however some of these challenges can be circumvented, such as the establishment of culture conditions favourable to cell development using metabolomics techniques and mimicking the *in vivo* environment as closely as possible.

The exometabolome, meaning, all metabolites in the surrounding extracellular environment, presents itself as a potential for study. This metabolome is the result of the metabolite exchange between cells and the culture medium and therefore its composition will reflect the metabolic activity of the cells

(359). Throughout cell growth and development, exometabolome can vary in response to experimental disturbances and thus its analysis using metabolic tools will allow monitoring of metabolic changes over time in the culture, optimizing conditions for cell growth, analysis of drug effects and even the identification of biomarkers present in the culture (359).

The combined use of different types of metabolomic approaches, such as Nuclear Magnetic Resonance (NMR) or Mass Spectrometry (MS), can lead to the determination of approximately 1000–2000 metabolites (360) and, although this number is not the total number of metabolites found in the system, it can provide a large set of information, as their concentrations will represent sensitive markers of genomic and phenotypic changes of cells growing in that medium (361).

Thus, profiling technology allows the rapid evaluation of extracellular metabolites and can be applied to the development of cell culture media that mimic the environment *in vitro*, which is of fundamental importance in the study of aging, since changes in culture conditions can compromise the characteristic phenotype of aging or senescence.

Some studies present methodologies based on the analysis of the use of metabolites, for the development of cell culture media for specific applications (362, 363). Others reported that serum variations can lead to contamination with exogenous metabolites, which consequently will lead to changes in endogenous cell metabolism (364). So, is essential to quantify the nutrients present in the environment and the depletion of the essential nutrients for the culture (363) and thus provide a more biologically relevant culture medium for the study of aging in cell culture.

IV.7 Concluding remarks and future perspectives

Aging is a multifactorial process and that is why studies in the area present increased difficulties, requiring creative and innovative approaches to understand this process. Three major categories of models can be highlighted that allow the study of aging, animal, computational and cellular models but none can answer all the questions. Also, contradictory results *in vivo* have been obtained regarding aging, mainly due to the existence of physiological and genetic differences between model organisms, such as mice, rats or bats, and humans and the differences in possible research methodologies between *in vitro* and *in vivo* studies. Despite some disadvantages pointed out in this review, *in vitro* cell culture studies of aging gain prominence. However, since in many cases age-related phenotypes researched and studied *in vitro* are not visible *in vivo* or are not relevant to the model, analysing data obtained from aging cell culture requires proper tools and with extreme caution is of paramount importance to understand which changes can be translated into the human processes of aging.

Thus, although this is an expanding field and much progress has already been made, a complete understanding of the mechanisms associated with age still has a long way to go and some questions remain unanswered: what are the main causal factors and what are the secondary factors or just the consequence of aging? What are the interconnections and combinatorial effects on aging? To conclude, it seems that the best approach for the study of aging may be the use of several models to answer these questions and thus achieve a more detailed approach to the process. In this way, cell culture models, due to the possibility of simultaneously study of different organisms with different ages, its easiness of manipulation and low associated costs, seem to be one of the best models to start these type of studies, to give insights into the aging process before the use of more complex models be planned.

CHAPTER V: METHODS

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V.1 FTIR Method Overview

Infrared radiation (IR) is a type of non-invasive and non-destructive radiation that leads to the vibration of the covalent bonds of molecules when absorbed by cells. Thus, Fourier Transformed Infrared Spectroscopy (FTIR) is one of the preferred methods of infrared spectroscopy due to its speed and sensitivity. Within the medium infrared range (MIR, 4000-400 cm^{-1}), all molecules have specific vibrational frequencies corresponding to discrete levels of vibrational energy, which characterize the infrared spectrum of each compound (129). Through this technique obtained peaks correspond to the vibration frequencies between the bonds of the atoms that make up the sample (365). As different functional groups absorb characteristic frequencies, the resulting spectrum will represent the molecular fingerprint of sample (366).

V.1.1 FTIR- ATR

The presence of water in the cells can cause some problems in the spectral analysis due to their absorptions in 3285 cm^{-1}, ~2100 cm^{-1} and 1640 cm^{-1} which overlap the bands of other components (367-369). In this perspective, the *Total Attenuated Reflection* (ATR) appears, which is one of the approaches for FTIR sampling, and which is considered a good approach for the study of hydrated biological samples, such as cells. In this technique it is possible to dry the water present in the sample and put the sample in total exposure with the ATR element (Ge, ZnSe, ZnS or diamond) reducing the length of the infrared light path within the sample and, thus, the absorbance of the bands of water are not received by the detector (129). Therefore, the infrared light reaches the sample and enters the ATR element generating a critical angle. Due to internal reflection, the beam is reflected several times inside the crystal, creating an evanescent wave that extends beyond the ATR element. Because the sample is in close contact with the ATR element, this evanescent wave loses energy at frequencies

identical to the sample's absorbance and the resulting beam is used to generate the sample's absorption spectrum (129). This way, the most important advantages of FTIR include independent measurements of the sample thickness and the ability to analyze living cells in aqueous systems.

V.1.2 FTIR-ATR spectometer

A common FTIR spectrometer consists of a source, interferometer, ATR-crystal, detector, amplifier, analog-to-digital converter and a computer (370). The source generates infrared radiation that passes the sample through the interferometer and reaches the detector. Then, the signal will be amplified and converted into a digital signal by the amplifier and analog-digital converter, respectively, where the signal is subsequently transferred to a computer in which the Fourier Transformation is performed and finally the sample spectrum will be obtained (370). In an IR spectrum the abscissa axis corresponds to the wavelength, wavenumber or frequency and the ordinate axis to the absorption intensity or to the percentage of transmittance. For the acquisition of a spectrum the main goal is determine a signal (S), which represents the amount of radiation absorbed, emitted or reflected by the sample.

V.2 *Fibroblast cultures derived from old and young human donors.*

Dermal fibroblasts constitute a cell population that suffers a continuous accumulation of damage and adaptations, and that present microanatomical and phenotypic differences that give fibroblast subpopulations different attributes in therapeutic applications (371). The interest in the use of fibroblasts for cell culture stems from their favorable features, such as the ease of obtaining them from skin biopsy samples from various parts of the body. In addition, they exhibit rapid and continuous proliferation in the presence of serum, unlike other types of cells, such as keratinocytes, which require additional growth factors (372). Furthermore, fibroblasts *in vitro* have specific characteristics, namely in their morphology and in the presence of specific metabolites or even biomarkers of aging (246).

Thus, given these characteristics, fibroblasts have become the main resource in aging studies, indicating an inverse relationship between the total number of duplications of the cell population ("life *in vitro*") and the age of the donor of the cell culture (334, 336, 337), and this relationship leads to the use of human cells of young and old people in numerous studies on cell aging (337).

For all these reasons, and taking into account all existing models for the study of aging, fibroblasts from donors of different ages seem to provide valuable insights into aging at the cellular level and furthermore they are a model that is easy to obtain and manipulate.

This way, in order to perform this technique on fibroblasts, cells from donors of different ages were cultured for subsequent FTIR analysis. The obtained spectra and appropriate multivariate analysis tools will reveal the differences in the biological aging process.

V.3 Cell lines.

In this study were used human fibroblast cells from donors with different ages from Coriell Institute (see *Appendix 1* for detailed information about cell lines): AG10803, from abdomen of a 22 years old male; AG02222, from abdomen of a 49 years old male and AG16102, from arm of a 69 years old male.

V.3.1 Fibroblast cultures.

Fibroblasts were grown in DMEM: F-12 (1: 1) medium (Thermo Fisher Scientific 11320074) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific 10270106), in a T75 cell culture flask (Thermo Fisher Scientific 130190). Fibroblasts were incubated at 37ºC with 5% CO2 and 100% humidity. They were grown to > 90% confluence until they were sub cultivated. For that, cells were washed once with 1× phosphate buffered saline (PBS, pH 7.4) and detached by adding 0.05% Trypsin-EDTA (Thermo Fisher Scientific 25300062). Fibroblasts were sub cultivated in the ratio of 1:2 and therefore counted. All cultures that are used in the present study were grown under predefined and highly standardized conditions (*Figure 5*).

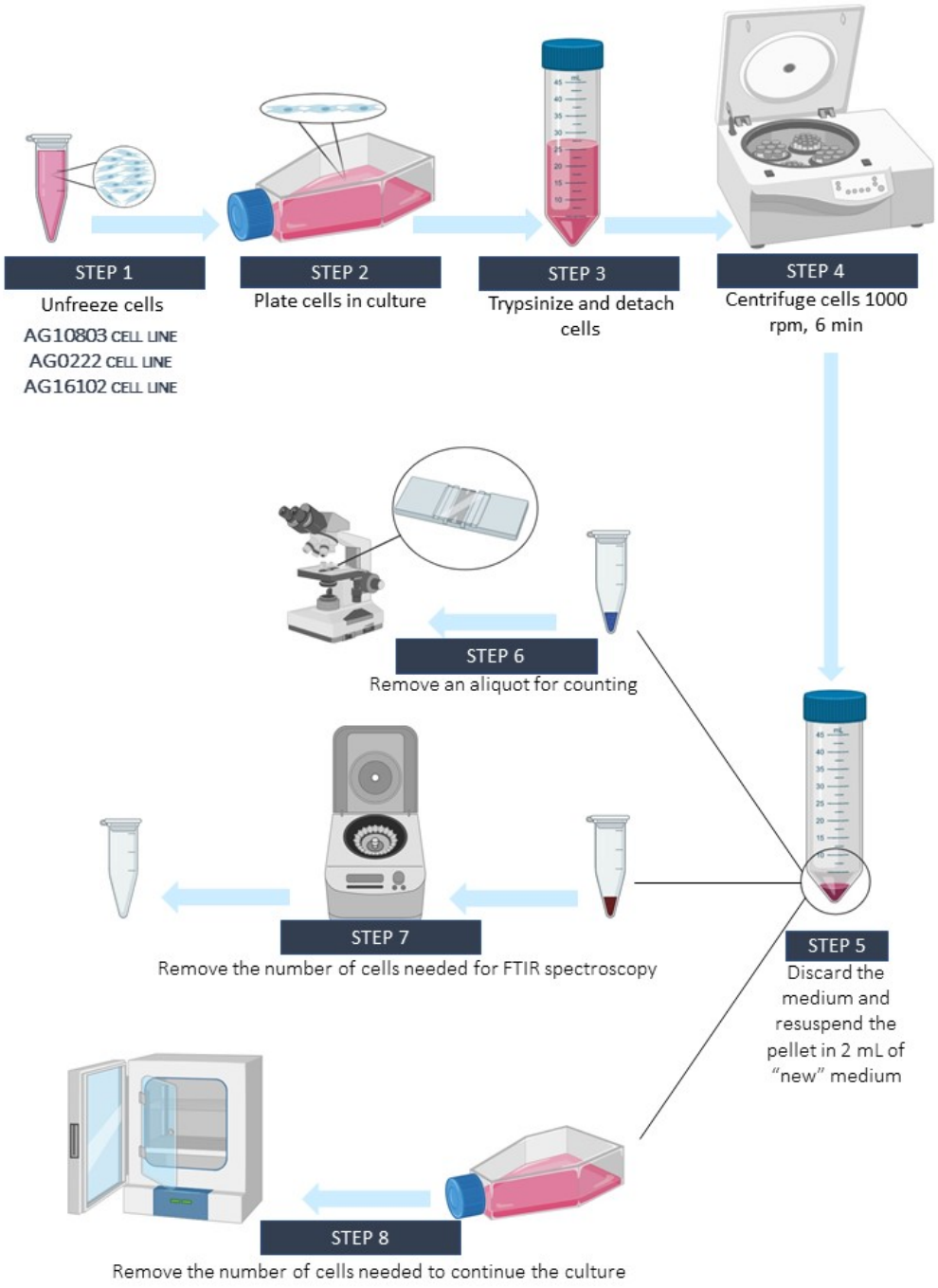

Figure 5| Schematic representation of the cell culture process. After the cells are cultured, they will be used for counting, for carrying out FTIR spectroscopy and finally for continuing the culture (*Images by BioRender*).

After the growth of cell lines, these were stored for later FTIR analysis. However, given the current situation of Covid-19 pandemic, the experiment had to stop at this point, and it was not possible to obtain the acquisition of the spectra of the different cell lines. Nevertheless, the procedure was established and below is a representation of a spectrum of fibroblasts (*Figure 6*). This figure allows to demonstrate what would be expected to be observed.

V.4 *FTIR spectroscopy experimental conditions for cell culture analysis.*

FTIR measurements of cells in an aqueous environment result in very weak cellular signals dominated by water spectra. Thus, for FTIR analysis, cells were dried to avoid water bands. In this way, prior to spectroscopic analysis, PBS was removed from cell samples that were then stored at 80ºC. A Bruker Alpha spectrometer was used to test the experimental conditions to acquire spectra. A spectral range of 4000-600 cm^{-1} and an 8 cm^{-1} resolution with 64 co-added scans was used. The room temperature and humidity during all process were maintained at ± 23ºC and ± 35%, respectively and the spectra were only obtained when these room conditions were fulfilled. The acquisition of background single beam was performed against air (empty crystal).

As previously mentioned, although this procedure could not be performed when analyzing fibroblasts spectra, there are key spectral regions that need to be considered. A well-marked peak initially appears, in the ~ 3280 cm^{-1} region associated with the amide A band that is a band referring to proteins. The 3000 - 2800 cm^{-1} region is mainly associated to fatty acids while the 2800 - 1700 cm^{-1} region is associated with lipids. Proteins occupy the region of 1700-1300 cm^{-1} where two bands stand out, mainly amide I (between 1700 - 1600 cm^{-1}) and amide II (~ 1540 cm^{-1}). The 1300 - 1100 cm^{-1} region is associated with nucleic acids and finally the 1100 - 1000 cm^{-1} region associated with polysaccharides. Thus, any change in the intensity, shape of the bands or change to lower or higher frequencies may indicate expected changes at the cellular level and therefore reveal detailed information about the aging process.

A typical IR spectrum of fibroblasts in spectral range of 4000-600 cm^{-1} is presented in *Figure 6* and correspondent peak assignments are present in *Table 1*.

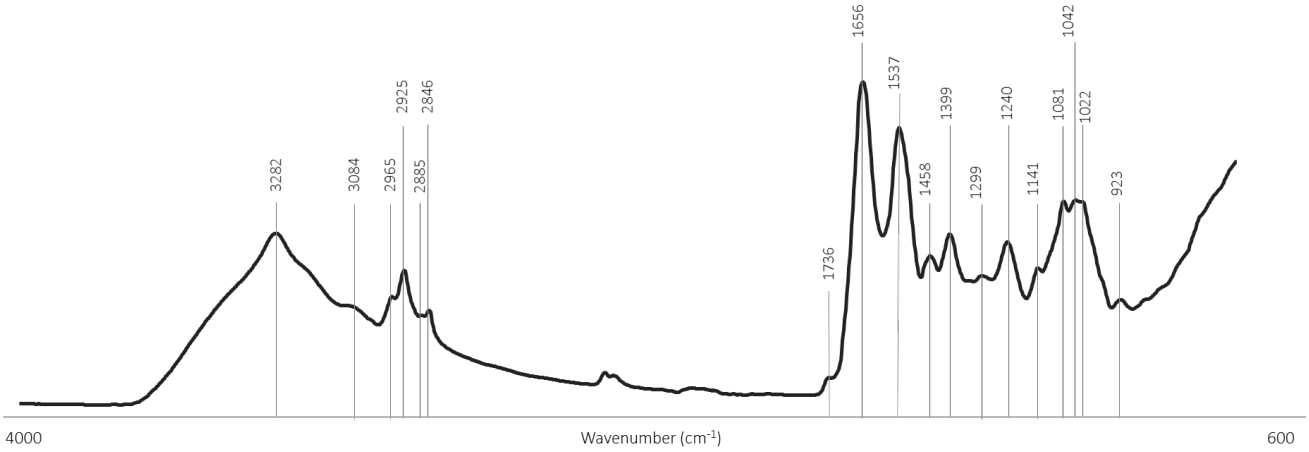

Figure 6| IR spectrum of fibroblasts in the 4000-600 cm^{-1} region with the main spectroscopic signals. X-axis: wavenumber (cm-1); Y-axis: Arbitrary Units (AU).

Table 1|Major vibrational modes and corresponding assignments found in fibroblasts.

WAVENUMBER (cm^{-1})	VIBRATIONAL MODE	MOLECULAR COMPONENT	REFERENCES
3282	N–H stretching of Amide A	Proteins	(366)
3084	C-H ring	Fatty acids	(373)
2965	C-H asymmetric stretching of -CH_3	Fatty acids	(366, 373)
2925	C-H asymmetric stretching of >CH_2	Fatty acids	(374)
2885	Streching C-H	Fatty acids	(375)
2846	Symmetric stretching of CH_3O	Lipids	(375)
1736	C=O stretching	Lipids	(376)
1656	Amide I band	Proteins	(377, 378)
1537	Amide II band	Proteins	(366, 373)

1458	CH_3 of collagen	Proteins	(379)
1399	Symmetric CH_3	Proteins	(380)
1299	Deformation N-H cytosine	Nucleic acids	(373)
1240	P=O asymmetric stretching of phosphodiesters	Phospholipids and nucleic acids	(381)
1141	C–O stretching	Carbohydrates/glycogen, nucleic acids	(366)
1081	Symmetric stretching P-O-C	Polysaccharides	(373)
1042	C–O stretching	Polysaccharides	(366)
1022	Glycogen	Polysaccharides	(382)
923	Left-handed hélix DNA (Z form)	Sugar-phosphate	(373)

After this insights obtained by a fibroblasts spectra, the objective will be to evaluate the spectrum obtained with cell lines from donors of different ages realized by FTIR and compare, with appropriate multivariate analysis tolls, the possible existing changes, and therefore find specific spectroscopic profile that could allow the identification of biomarkers that reflect this process.

The studies carried out so far with this technique do not compare cells with different donor ages, but senescent cells and normal cells, however the conclusions expected in this research will be in the same line of reasoning and therefore considering the results presented, it is to be expected that there are changes in biomolecular composition and modification, mainly in proteins and lipids, but also in nucleic acids and carbohydrates.

Thus, this investigation becomes interesting since it reflects aging as a whole and not only in senescent cells.

CHAPTER VI: CONCLUSIONS

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As mentioned throughout this work, the world population is aging and, in this way, more and more individuals are looking to extend and improve their useful life. The interest in the study of aging processes using metabolomic tools, such as FTIR, is growing, since it allows the identification of specific biomarkers and, consequently, the identification of potential therapeutic targets.

Cell lines can present several advantages in aging studies, since they require less ethical issues, greater ease of manipulation, and still allow the mimicking of different aging pathways, which allows a detailed study of this process. Taking these models into account, fibroblasts seem to have a high potential in this context, namely dermal fibroblasts, since they reflect the aging process not only in an intrinsic way, but can also reflect the changes induced by external factors that can lead to an aged state. In this way, this dissertation allowed to make a survey of the main cellular models in the study of aging and thus to understand which may be the most suitable for metabolomics studies. At the same time, FTIR experimental conditions were established and it was proved that the metabolic techniques present a high potential tool, namely the FTIR that allows obtaining the fingerprinting of a sample at low costs and in a non-destructive way.

However, it would be important to complete the experimental plan initially defined for this dissertation, with the identification of specific biomarkers of aging, taking into account previous research, it is expected that there will be changes in the biomolecular composition and modification, mainly in proteins and lipids, but also in nucleic acids and carbohydrates. The findings would be important to provide relevant insights not only for the study of aging but also for the development of possible therapies for diseases related to the aging process.

It would also be relevant to carry out this study not only using FTIR but also other metabolic tools, like MS and NMR, since these techniques, when used together, can provide complementary information and confirm and validate the data obtained by FTIR.

Thus, the key will be to conduct further, more detailed research to reveal the ideas that aging at the molecular level can translate at the level of the organism.

CHAPTER VII: REFERENCES

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CHAPTER VIII: APPENDIX

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Appendix 1| Cell lines information.

Cell lines	Repository
AG10803 cell line	NIA Aging Cell Culture Repository, Coriell Institute
AG0222 cell line	NIA Aging Cell Culture Repository, Coriell Institute
AG16102 cell line	NIA Aging Cell Culture Repository, Coriell Institute

Appendix 2| List of material used to perform the techniques described.

Material	Brand / Catalog number	Use
DMEM: F-12	Thermo Fisher Scientific / 71132007	Cell culture
10% Fetal Bovine Serum (FBS)	Thermo Fisher Scientific / 10270106	Cell culture
0.05% Trypsin-EDTA	Thermo Fisher Scientific / 25300062	Cell culture
Trypan Blue Solution 0.4%	VWR / SV30084.01	Cell culture
Dimethyl Sulfoxide (DMSO)	VWR / A3672.0050	Cell culture
T75 Cell Culture Flask	Thermo Fisher Scientific / 130190	Cell culture
Serological Pipettes 10 mL	Thermo Fisher Scientific / 11879181	Cell culture
Tubos Falcon 50 mL	VWR / 734-0448	Cell culture
Tubos Falcon 15 mL	VWR / 734-0451	Cell culture
Microcentrifuge Tubes, 1.5mL	VWR / 525-0990	Cell culture

Appendix 3 | *Solutions prepared in lab to perform the techniques described.*

Solution	Preparation	Use
Phosphate buffered saline (PBS)	- 800 ml distilled H_2O - 8 g NaCl - 0.2 g KCl - 1.44 g Na_2HPO_4 - 0.24 g KH_2PO_4 - adjust the pH to 7.4 and autoclave	Cell Culture