In silico identification of genetic and pharmacological interventions to modulate ageing

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Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from experiments or analysis done with or by other researchers, I confirm that this has been indicated in the thesis.

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Abstract

As life expectancy increases and fertility rates decrease, the growing ageing population poses a significant challenge to the healthcare systems of developed countries. Ageing as the major risk factor for chronic diseases constitutes the primary target to reduce the burden of diseases and improve human health. However, ageing is a complex process and predicting potential interventions into it requires system-level approaches. In this thesis, I present the development of two computational methods using biological data to predict novel genetic and pharmacological interventions to ameliorate ageing.

My first study focused on identifying repurposable drugs to delay human ageing. Several computational drug-repurposing studies have been developed, but most of them focus on predicting geroprotectors using animal models data, even though certain aspects of ageing may be human-specific. Using drug-protein interaction information, I searched for drugs targeting a significant proportion of human ageing-related genes and pathways. The top-ranked drugs included a significant number of known geroprotectors, validating the capability of the method to discover drugs to modulate ageing. On the top of the list was tanespimycin, a heat shock protein inhibitor, whose geroprotective properties we validated experimentally.

My second study centres on determining the molecular mechanisms associated with healthy lifespan, and how to use this information to find new genetic interventions to delay ageing. In recent years, the number of transcriptomic studies of mouse models of ageing has increased dramatically, providing the opportunity to compare gene expression changes of long- and short-lived strains. I showed that differences in healthy lifespan are associated with expression changes in genes regulating mitochondrial metabolism. Using these gene sets as biomarkers of lifespan, I compared the mouse models of ageing against 51 genetically engineered mice and predicted candidate genetic and pharmacological interventions with the potential to delay ageing.

Through computational studies I predicted a narrowed down list of candidate genetic and pharmacological interventions to delay mouse and human ageing and validated

several predictions made by other researchers using different methods, confirming the robustness of computational methods to identify new anti-ageing interventions. With the discovery of tanespimycin as a new geroprotector, I revealed that a little proteostatic stress is good for longevity and that we can trigger this hormetic response pharmacologically. I exposed the complexity of ageing as I found multiple mechanisms to delay ageing, most of which were tissue-specific, and found evidence for new candidate hallmarks of ageing and novel biomarkers of lifespan.

Impact Statement

The rising ageing population is expected to become a major healthcare challenge of this century. As people live longer but not healthier lives, age-related diseases become increasingly prevalent in the elderly, causing multimorbidity and polypharmacy. The research I present in this thesis has the potential not only to contribute to our basic understanding of the ageing process, but also to show how to use this knowledge to identify new interventions to ameliorate it. Using different computational approaches, I identified candidate genetic and pharmacological interventions to delay ageing. I develop a method able to prioritise drugs with increased likelihood to affect human ageing, some of which are already approved for human use and with validated geroprotective properties in animal models. This study significantly reduces the number of drugs needed to be tested by proposing a narrowed down list of primary candidates. Among the candidate drugs, we experimentally validated the lifespan-extending properties of tanespimycin, a drug a mild side effects, currently in clinical trials for the treatment of cancer. I also made significant progress in deciphering the molecular basis of healthy lifespan by analysing transcriptomic data from well-established mouse models of ageing. Focusing on these molecular changes, I proposed gene knockouts likely to delay mouse ageing and potentially applicable to humans through pharmacological interventions.

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Publications arising from this thesis

- **Fuentealba, M.**, Dönertaş, H. M., Williams, R., Labbadia, J., Thornton, J. M., & Partridge, L. (2019). Using the drug-protein interactome to identify anti-ageing compounds for humans. *PLoS Computational Biology*, *15*(1), e1006639. Available at: https://doi.org/10.1371/journal.pcbi.1006639

- Dönertaş, H. M.(*), **Fuentealba, M.(*)**, Partridge, L., & Thornton, J. M. (2019). Identifying Potential Ageing-Modulating Drugs *In Silico*. In *Trends in Endocrinology and Metabolism* (Vol. 30, Issue 2, pp. 118–131). Elsevier Inc. (*) denotes equal contribution
  Available at: https://doi.org/10.1016/j.tem.2018.11.005

- Partridge, L., **Fuentealba, M.**, & Kennedy, B. K. (2020). The quest to slow ageing through drug discovery. *Nature Reviews Drug Discovery*, *19*(8), 513–532.
  Available at: https://doi.org/10.1038/s41573-020-0067-7

- **Fuentealba, M.**, Fabian, D. K., Dönertaş, H. M., Thornton, J. M., & Partridge, L. (2021). Transcriptomic profiling of long- and short-lived mutant mice implicates mitochondrial metabolism in ageing and shows signatures of normal ageing in progeroid mice. *Mechanisms of Ageing and Development*, *194*, 111437.
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Abbreviations

Akt1	Akt serine/threonine kinase 1
AMP	Adenosine monophosphate
AMPK	Amp-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BP	Biological processes
C57BL/6	C57 black 6
C57BL/6J	C57 black 6 from the Jackson laboratory
CC	Cellular components
CR	Caloric restriction
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Dietary restriction
ECGC	Epigallocatechin gallate
ER	Endoplasmic reticulum
Ercc1	Excision repair cross-complementing group 1
Ercc6	Excision repair cross-complementing group 6
FDA	Food and drug administration
Fgf21	Fibroblast growth factor 21
Ghr	Growth hormone receptor
GO	Gene ontology
GSEA	Gene set enrichment analysis
GTEx	Genotype-tissue expression
HGNC	Hugo gene nomenclature committee
HSF-1	Heat shock factor 1
HSP-90	Heat shock protein 90
IGF-1	Insulin-like growth factor 1
Igf1r	Insulin-like growth factor 1 receptor
IHME	Institute for health metrics and evaluation
Insr	Insulin receptor
IPTG	Isopropyl β-d-1-thiogalactopyranoside
Irs1	Insulin receptor substrate 1
ITP	Interventions testing program
KEGG	Kyoto encyclopedia of genes and genomes
LB	Lysogeny broth
Lmna	Lamin a/c
MANOVA	Multivariate analysis of variance
MF	Molecular functions
mRNA	Messenger ribonucleuc acid
mtDNA	Mitochondrial deoxyribonucleic acid

mTOR	Mammalian target of rapamycin
Myc	Myc proto-oncogene
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydride
NGM	Nematode growth media
NMR	Nuclear magnetic resonance
OR	Odds ratio
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinases
Polg	Dna polymerase subunit gamma
Pou1f1	Pou domain, class 1, transcription factor 1
PPI	Protein-protein interaction
Prop1	Prop paired-like homeobox 1
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
Rps6kb1	Ribosomal protein s6 kinase b1
Sirt6	Sirtuin 6
TAME	The targeting ageing with metformin
Terc	Telomerase rna component
Tert	Telomerase reverse transcriptase
UK	United Kingdom
UPR	Unfolded protein response
US	United States
WHO	World health organization
Xpa	Xeroderma pigmentosum group a-complementing protein

Chapter 1 General Introduction

1.1 Changes in life expectancy throughout human history

Anatomically modern humans have been living on earth for around 200 thousand years (Hammond et al., 2017), yet only recently in the span of human evolution, we started to live longer. Transitions from hunter-gatherer tribes to sedentary agricultural communities and then to urban societies shaped our lifestyle and set the basis for today's civilization, where humans are expected to live much longer and healthier lives.

In the upper Palaeolithic (50000 - 10000 BC), dying young was not as uncommon as nowadays. Based on human remains, it has been estimated that around 20% of infants died during the first year and that over 50% of all children did not survive puberty (Johnston & Snow, 1961). The high infant mortality limited life expectancy to around 35 years for males and 30 years for females (Dennell et al., 1986). As in the Palaeolithic, deaths due to violence were also common during the Mesolithic (10000 - 6000 BC), however, as populations became less nomadic, diseases like dysentery and malaria were common causes of death (Dennell et al., 1986). During the Neolithic (6000 - 2000 BC), the development of agriculture and domestication of animals caused a decrease in life expectancy, mainly because the new diet based on grains and vegetables lead to malnutrition (Dennell et al., 1986) and living in proximity to animals promoted zoonotic diseases (Fournié et al., 2017).

During the Bronze and Iron age (2000 - 500 BC) overcrowding was common in urban cities, water was usually contaminated, and diet was seasonal and unbalanced. Life expectancy was around 39 years for males and 32 years for females, but usually higher in the rulers, who were taller and rarely malnourished (Dennell et al., 1986). During classical Greece and Rome (500 BC - 500 AD), life expectancy remained at around 35 years, and diseases like tuberculosis, typhoid fever, smallpox and measles proliferated among dense urban populations (Cunha, 2004; Eddy, 2015; Papagrigorakis et al., 2006). At the start of the medieval period, life expectancy

increased. A prime example was Byzantine Constantinople with a life expectancy of 46 years for males and 37 years for females (Dennell et al., 1986). Unfortunately, this increase did not last, as poor weather during several years caused crop failure and consequent famine, and the bubonic plague killed an estimate of 30 to 50 million people (Durand, 1977).

The United Kingdom (UK) is the only country in the world with well-documented mortality data since the 16th century. However, before the 1800s there was no clear trend, as life expectancy usually fluctuated between 30 and 40 years **(Figure 1.1)**. Life expectancy did not increase because cities were overcrowded and dirty with no sewage system, making the perfect environment for the spread of infectious diseases. Beyond alcohol, medicine was mostly herbal, and surgery was performed with no anaesthetics, causing patients to often die from infections or septic shock. Even so, major health advances were made during this era, like the discovery of the first vaccine by Edward Jenner, an English physician who found that inoculating people with the mild cowpox virus conferred immunity to the smallpox virus (Jenson et al., 2016). Also, the link between poor health and living cramped was recognised, forcing industrialised countries to promote laws to improve the living and working conditions, including water supplies, sewers, streets and other sanitary matters (Fee & Brown, 2005). It was proven that diseases like cholera spread through water and that microorganisms lead to diseases (Pasteur, 1878). Based on Pasteur's germ theory of diseases, Robert Kohn could identify the bacteria responsible for diseases such as cholera and tuberculosis, leading to the development of several vaccines and saving millions of lives (Blevins & Bronze, 2010). All these health reforms and discoveries increased life expectancy in the UK to 45 years by the end of the 19th century and set the basis for much greater increases in the upcoming years.

Despite the increased life expectancy in developed countries, worldwide life expectancy remained at 32 years in 1900, reflecting the high inequality of health across the globe. However, improvements in health care, sanitation and living conditions in many countries and the discovery of antibiotics by Alexander Fleming (Flemming, 1929), boosted worldwide life expectancy to 66 years by the end of the 20th century. During the last decade, life expectancy has continued increasing in many countries despite all predictions. Only this time it is caused by a decrease in late-life

mortality instead of early-life mortality, mainly attributed to a reduction in tobacco use and deaths from cardiovascular diseases (Mathers et al., 2015). Nowadays, global life expectancy is around 72 years, but the inequalities remain. Countries like Central African Republic have a life expectancy of 53 years, whereas people living in Japan are expected to live on average 30 years more. Future projections show that there is a 50% chance that life expectancy will break the 90-year barrier in some developed countries by 2030 (Kontis et al., 2017).

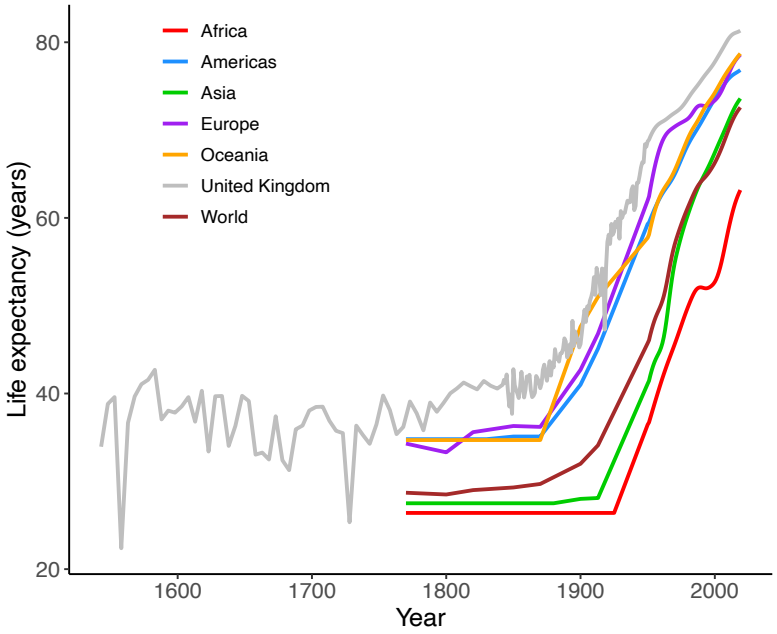


Figure 1.1. *Life expectancy in each continent, the world and the United Kingdom. Data obtained from Achey (2016).*

Co-occurring with the increase in life expectancy there has been a decrease in fertility rate, measured as the average number of children per women. Over the last 50 years, fertility rates halved, decreasing from an average of 5 children per woman to below 2.5 (Robbins, 2014). The increase in life expectancy and the decrease in fertility rates is causing an increase in the ageing population, a phenomenon sometimes, pejoratively, called “the silver tsunami”. The global median age in 1970 was 21 years, whereas nowadays is 30 years (Gliwicz, 1983). Now in the UK, 57% of the people are part of the non-working population (under the age of 15 and aged 65 and over) and this percentage is increasing rapidly. It is predicted that by the end of the century only 20% of the population will be working-age adults (15 to 64 years old). A high dependency ratio such as this is expected to cause serious problems to the economy

of the country as a higher proportion of the government expenditure will be on education for the youngest and healthcare for the oldest.

1.2 Living longer but less healthy lives

The increase in life expectancy is worth celebrating as a triumph of civilisation. However, the idea of interventions to increase human lifespan is unattractive for many people. One reason is that they think that living longer just means an extension of the moribund period at the end of life. This view may seem negative, but it is also at least partially true. An increase in life expectancy does not mean that those additional years would be in good health. Considering years lived in poor health as a combination of the prevalence of the conditions multiplied by the disability weight for each condition (obtained from surveys), we observed that the percentage of life lived in poor health is increasing in countries like the United States (US) and the UK **(Figure 1.2)**. Thus, while every day more people reach old ages, we also live more years with poor health and diseases.

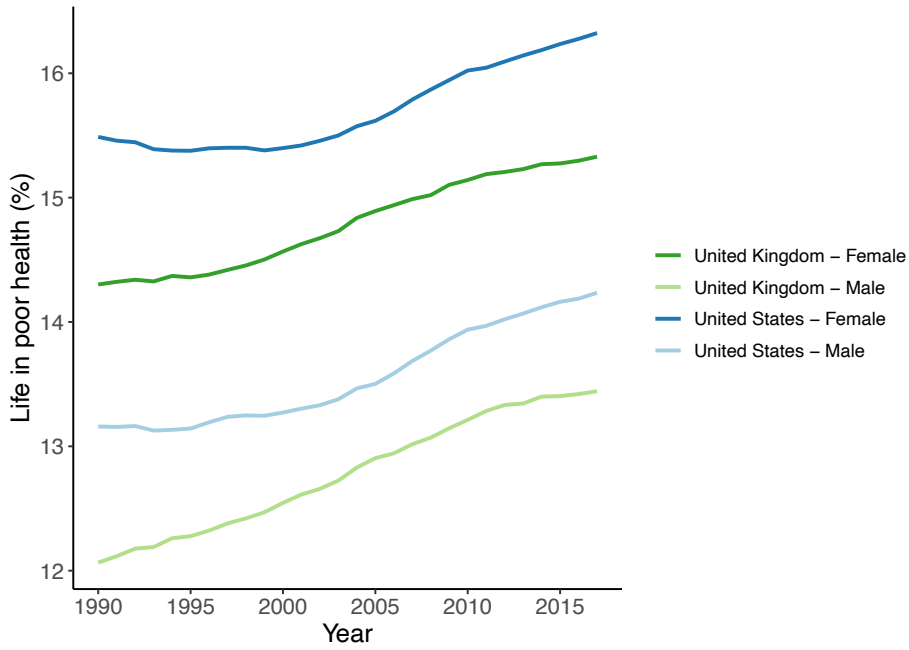


Figure 1.2. *Percentage of life in poor health in the US and the UK since 1990. Data obtained from the Institute for Health Metrics and Evaluation (IHME, 2017).*

One reason why our healthspan (i.e. years free from diseases) is not improving may be because traditional medicine is not preventive but reactive to diseases.

Unfortunately, studies suggest that the manifestation of the symptoms of a disease often indicates that the tissue damage started long ago and that it has reached an irreversible state (Fries, 2005). Thus, it is often not possible to completely cure diseases with current treatments. The lack of cures for many diseases is causing an even more dangerous phenomenon, which is the accumulation of 2 or more chronic diseases, also known as multimorbidity. People with multimorbidity represent a healthcare cost 5 times higher (Bähler et al., 2015) and die up to 20 years younger (Barnett et al., 2012) than patients with none or one chronic condition. Epidemiological studies show that by the age of 50 we have a 50% chance to have at least one chronic condition, whereas 15 years later we have the same chances to be multimorbid **(Figure 1.3)**.

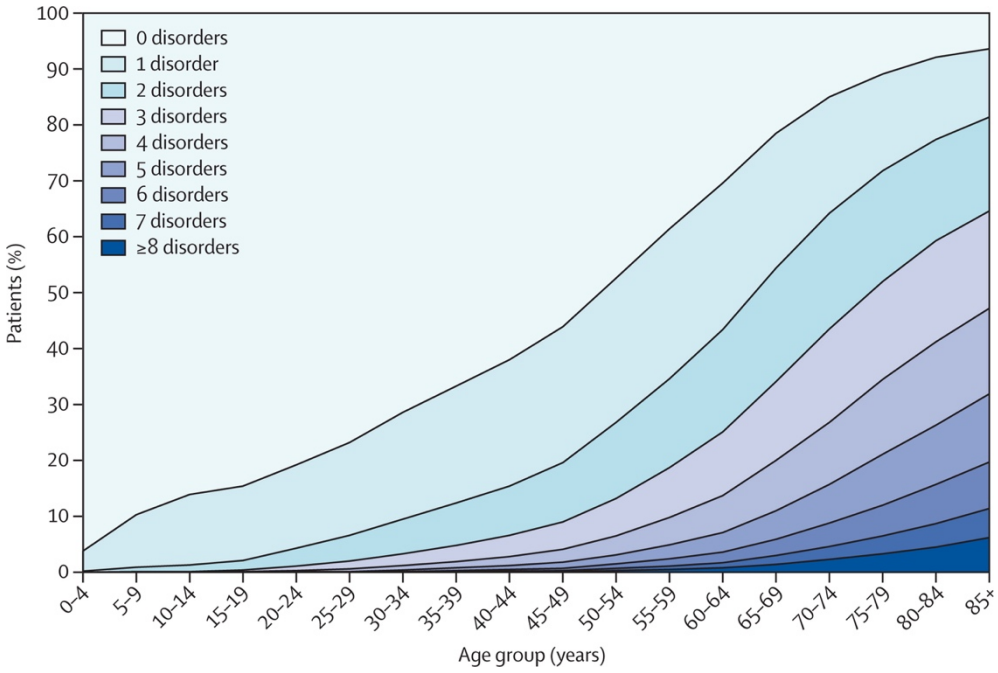


Figure 1.3. *Increase in multimorbidity with age. Figure extracted from Barnett et al. (2012).*

Moreover, given that people take medication for each disease diagnosed, it is not unexpected to observe that 20% of the general adult population (Guthrie et al., 2015) and 30% of the elderly (Bushardt et al., 2008) have concurrent use of five or more medications, or what it is known as polypharmacy. Polypharmacy also holds a substantial risk to health because it is well documented that drugs interact, causing severe side-effects. In fact, 12% of all hospital admissions of older patients are because of adverse drug reactions caused by commonly used nonsteroidal anti-

inflammatory drugs, antiplatelet agents, anticoagulants, hypoglycaemic and blood pressure-lowering drugs (Howard et al., 2007). Altogether, one of the primary objectives of biogerontology today is to develop treatments that can preventively allow people to live healthier for longer by reducing multimorbidity and polypharmacy.

1.3 Ageing is the major risk factor for chronic diseases

Along with the increase in life expectancy, the scientific discoveries and public health reforms during the 19th century contributed to changing the major causes of death from communicable diseases to non-communicable disease **(Figure 1.4)**. Whereas in 1990 the leading cause of death worldwide was respiratory infections, by 2000 it was cardiovascular diseases. In the future, the proportion of deaths due to non-communicable diseases will continue increasing from 71% in 2016 to 77% in 2030 (WHO Health statistics and information systems, 2018). Ischemic heart disease will remain as the main cause of death worldwide, accounting for 17% of all deaths, while Alzheimer's disease and other dementias will rise to become the 4th and 3rd most common cause of death in males and females, respectively.

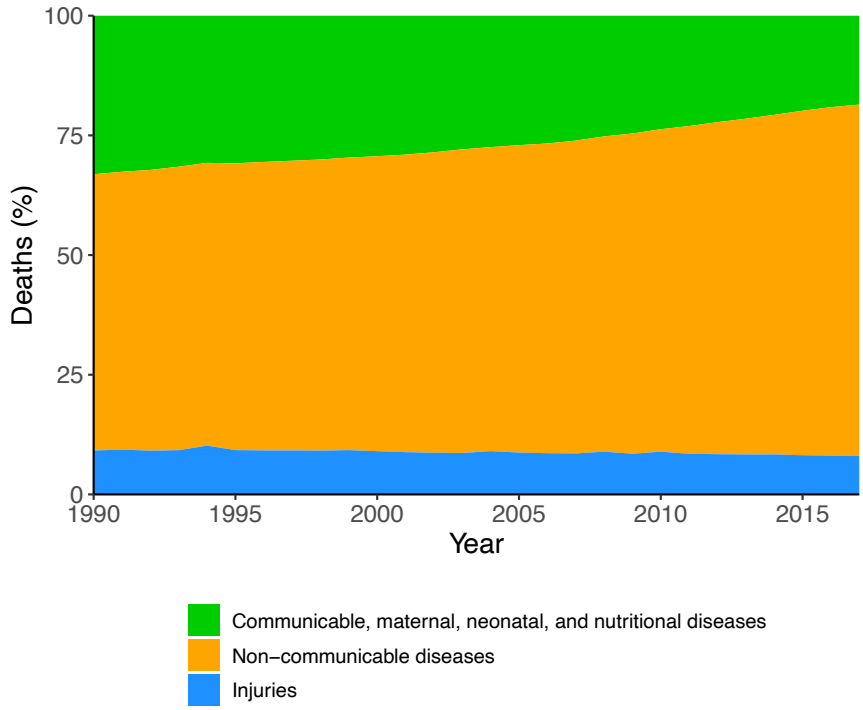


Figure 1.4. *Changes in the causes of death worldwide since 1990. Data obtained from the Institute for Health Metrics and Evaluation (IHME, 2017).*

Nowadays, 9 of the top 10 killer diseases in the UK are non-communicable and age-related. Thus, ageing constitutes a major risk factor for the deadliest diseases. For example, the risk of dying from ischemic heart disease, the leading cause of death worldwide, increases by 917-fold by the age of 80 **(Figure 1.5)**.

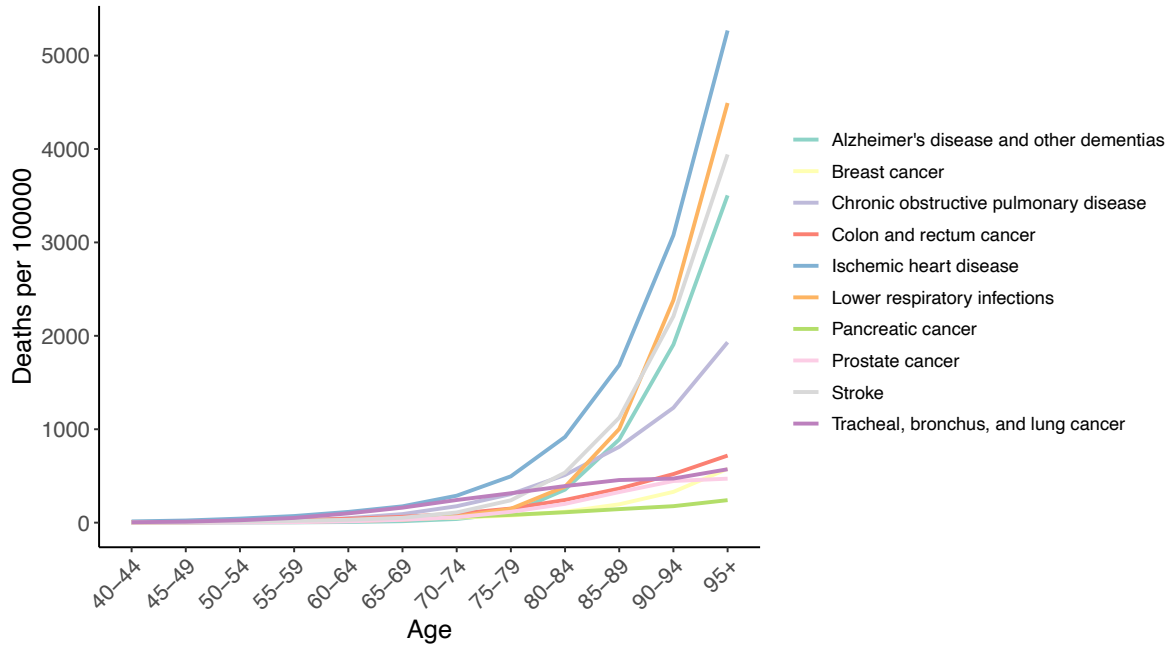


Figure 1.5. *Increase in the risk of death with age for the top 10 killer diseases in the UK. Data obtained from the Institute for Health Metrics and Evaluation (IHME, 2017).*

Ageing is the major risk factor for chronic diseases because it is the major driver of physiological decline. At the molecular level, this means that the mechanisms driving ageing overlap with the mechanisms of diseases. Thus, if we target the underlying mechanisms of ageing, we should also modulate the mechanisms of multiple diseases simultaneously. Supporting this concept, it has been found that genes associated with age-related diseases are involved in processes of ageing such as inflammation, cell cycle regulation and cholesterol/apolipoprotein metabolism (Johnson et al., 2015). More recently, in a study that I contributed, we showed that mutations that increase the predisposition for age-related diseases are enriched in genes associated with ageing (Dönertaş et al., 2020).

1.4 Theories of ageing

Although delaying ageing seems to be a promising and efficient strategy to reduce multimorbidity, to find new interventions to modulate ageing, we first need to

understand what ageing is. Over the years, dozens of theories of ageing have emerged. Following, I review some of the most popular divided in two groups: evolutionary and mechanistic theories of ageing.

1.4.1 Evolutionary theories of ageing

The firsts theories of the evolution of ageing derived from the observations of JBS Haldane, a geneticist who noticed that as individuals age, the force of natural selection decreases (Darlington, 1942). Haldane made this observation by wondering why the genes causing Huntington's disease were not removed by natural selection despite the prevalence of the disease, its dominant, monogenic inheritance, and its highly deleterious effect. Notably, he found that most of the symptoms were triggered after reproductive age, meaning that the disease-causing gene was passed into the next generation before the patient displayed any symptom. Thus, the disease-causing gene had only a minor effect on reproductive success and was therefore only weakly negatively selected.

Based on Haldane's finding, Peter Medawar proposed that mutations affecting traits that manifest at ages after the onset of reproduction will be subject to a progressively weaker force of natural selection because death from predation and other external hazards steadily reduce the number of mutant-bearers on which selection can act (Medawar, 1952). However, as humans learned to control the environment and treat diseases, the consequences of the accumulation of late-acting mutations became visible at older ages. Five years later, George Williams, an evolutionary biologist, proposed an additional evolutionary theory, also based on the decline in the force of natural selection with age. While Medawar thought that the mutations responsible for ageing were silent in early life and then had a bad effect later in life, Williams proposed that mutations could be favoured by evolution because of their contribution to reproductive fitness, even if they pose a disadvantage later in life (Williams, 1957).

Later, Thomas Kirkwood (Kirkwood, 1977), suggested a specific physiological version of the antagonistic pleiotropy idea. This theory suggests that organisms distribute their limited resources between reproduction and maintenance (e.g. DNA damage repair),

and because evolution prioritises reproduction, body maintenance could not be efficient, causing the accumulation of damage and ageing. However, this theory does not explain why organisms under caloric restriction (i.e. low resources), live longer and age slower than organisms with a normal diet (Section 1.5.1).

More recently, Mikhail Blagosklonny proposed that ageing is caused by the continuation of development (Blagosklonny, 2008). During development, genetic pathways involved in biosynthesis are switched on, however, as the organism ages, these programs are not switched off, causing organ damage and ageing. Thus, ageing is not programmed but quasi-programmed as it is not encoded in our genome but happens because there is no mechanism to switch off the developmental programmes. However, this theory does not consider hypertrophy as a manifestation of ageing, despite studies of protein expression levels in humans indicate that the changes during ageing represent not only extensions (hyperfunction) but also reversals (hypertrophy) of developmental programmes (Somel et al., 2010). Also, it does not explain how excessive gene activity can be maintained considering the inexorable accumulation of molecular damage with age (Bae et al., 2018; Franco et al., 2018; Lei Zhang et al., 2019).

1.4.2 Mechanistic theories of ageing

Medawar's and Williams' theories provide suitable explanations on why we age, but they do not give any information on what are the molecular mechanisms by which ageing occurs. A more mechanistic theory of ageing is the free radical theory of ageing (Harman, 1956), which proposes that reactive oxygen species (i.e. ROS), which are by-products of oxidative metabolism, react with cellular molecules causing random damage to cellular components. Over time, damage accumulates, causing tissue dysfunction and consequently ageing. The problem with this theory is that it is not clear why this type of damage is considered primary. Also, it has been observed that the generation of ROS correlates positively with the increase in lifespan in some animals (Bazopoulou et al., 2019; Sanz, 2016; Scialò et al., 2016) and that ROS even serve as critical signalling molecules in cells, mainly through covalent modification of specific cysteine residues in redox-sensitive proteins (reviewed in D'Autréaux & Toledano

2007 and Ray et al. 2012). Thus, it is evident that the role of ROS in ageing needs reconsideration and it is not a primary damage. In this sense, in a broader view, López-Otín et al., (2013), used experimental evidence from animal models to propose nine cellular and molecular hallmarks that contribute to the process of ageing. Briefly, a hallmark of ageing should (i) manifest during normal ageing, (ii) its experimental aggravation should accelerate ageing; and (iii) its experimental amelioration should retard the normal ageing process and, hence, increase healthy lifespan. Based on this definition the authors proposed 9 candidate hallmarks of ageing **(Figure 1.6)**:

Genomic instability

Changes in DNA integrity and stability caused by exogenous physical, chemical and biological agents and endogenous threats including DNA replication errors, spontaneous reactions and ROS. The resulting legions to the DNA include point mutations, translocations, chromosomal gains and losses, double-strand breaks and adduct formation.

Telomere attrition

Cumulative and progressive loss of telomere-protective sequences from chromosome ends. If telomeres are shortened to a critical length, cell division stops (i.e. replicative senescence). It occurs *in vitro* after 40 to 60 cell divisions (i.e. Hayflick limit).

Epigenetic alterations

Changes in DNA-methylation patterns, post-translational modification of histones and chromatin remodelling. Epigenetic alterations cause transcriptional noise, RNA processing aberrations, impaired DNA repair and chromosomal instability.

Loss of proteostasis

Accumulation of protein polypeptide chains caused by a failure in the mechanisms for the stabilisation of correctly folded proteins (i.e. heat shock proteins) or the recognition and degradation of unfolded proteins (i.e. proteasome and lysosome).

Deregulated nutrient sensing

Alterations to pathways regulated by nutrient levels. Examples include the Insulin- and IGF-1 signalling pathways for the sensing of glucose; the mTOR pathway, for the

sensing of amino acids; the AMPK complex, for sensing AMP levels; and the sirtuins for sensing NAD+ levels.

Mitochondrial dysfunction

Perturbation of the mitochondrial function by mtDNA mutations, destabilisation of the electron transport chain complexes, altered mitochondrial dynamics (i.e. fission and fusion) and defective quality control by mitophagy. Mitochondrial dysfunction increases ROS generation, reduces energy output and promotes cell death and inflammation.

Cellular senescence

Cell cycle arrest coupled to detrimental phenotypic changes. A primary characteristic is a dramatic change in the cell secretome, which becomes highly enriched in pro-inflammatory cytokines and matrix metalloproteinases.

Stem cell exhaustion

Decline in the regenerative capacity of tissues. It is mainly driven by the age-related reduction in the number of haematopoietic stem cells, mesenchymal stem cells, satellite cells and intestinal stem cells. However, an excessive proliferation of stem and progenitor cells can also be deleterious by accelerating the exhaustion of the stem cell niche.

Altered intercellular communication

Changes in the molecules involved in the cell-cell communication (e.g. endocrine, neuroendocrine and neuronal). A common alteration associated with intercellular communication is “inflammageing”, a low-level chronic inflammatory phenotype that occurs during ageing.

Despite the remarkable progress made in understanding the ageing process through the lens of these hallmarks, it remains largely unknown how alterations in one particular hallmark affect the others (interconnectedness), a question that I will explore during this thesis.

Figure 1.6. *Hallmarks of ageing. Figure extracted from López-Otín et al. (2013).*

1.5 The ageing process is malleable

The experiments that gave rise to the hallmarks of ageing proved that the ageing process is malleable mainly through dietary, genetic and pharmacological interventions. In the following sections, I review in more detail some of the most promising interventions into ageing and the history behind their discovery.

1.5.1 Dietary interventions

Even though ageing research is a relatively new field, the wish to prolong life is old. In the 17th century, Robert Boyle, one of the founders of modern chemistry, wrote a wish list of inventions for the future and on the top of the list were “the prolongation of life” and the “recovery of youth”. Unfortunately, it was not until 1935 that Clive McCay discovered the first life-extending intervention. He took white rats and split them into two groups, one with the normal amount of food and the other one with less amount

of food but enough nutrients. Surprisingly, he observed that rats on the low-calorie diet lived around 30% longer and had fewer age-related diseases such as cancer, atherosclerosis and cataracts (McCay et al., 1989). Since then, the positive effects of caloric restriction on lifespan and healthspan have been reported in multiple species, from single-cell yeast to mammals, including monkeys (Colman et al., 2014; Mattison et al., 2012). Recently, studies of humans on caloric restriction have shown that they display improved metabolic and hormonal factors implicated in the pathogenesis of age-related diseases (Most et al., 2017) and younger biological age than ad libitum-arm participants (Belsky et al., 2018).

Unfortunately, the use of caloric restriction to ameliorate ageing is not very suitable for humans, usually characterised by very low compliance to even mild (90%) dietary regimes (Racette et al., 2006). A more achievable dietary intervention for humans is intermittent fasting, an eating pattern that cycles between periods of fasting and eating normally. Studies as early as 1946 showed that rats fasting 1 in every 3 days, live on average up to 20% longer (Carlson & Hoelzel, 1946). Recently, studies from our lab showed that intermittent fasting also extends lifespan in flies (Catterson et al., 2018). In humans, intermittent fasting reduces body weight and improve biomarkers associated with diabetes, cardiovascular disease and cancer (Patterson et al., 2015).

1.5.2 Genetic interventions

In 1983, most scientists thought that ageing was too complex to be affected by single gene mutations. However, Michael Klass, a postdoc working in David Hirsh's laboratory at the University of Colorado (US) thought differently. Using chemical mutagenesis, he aimed to identify long-lived worm mutants (Klass, 1983). As many of the long-lived mutant worms he discovered showed reduced food intake, he concluded that their increased lifespan was driven by caloric restriction. After Klass' experiments were published, he moved to work in the industry, but his mutants were not forgotten. At the same time, another researcher from the same university was also exploring the genetic basis of ageing. Using Klass' approach, Tom Johnson isolated a strain that ate normally and lived up to 65% longer and he called this mutant *age-1*, hoping that

other mutants regulating ageing would be eventually discovered (Friedman & Johnson, 1988).

Years later, motivated by Johnson's work, Cynthia Kenyon aimed to discover novel long-lived mutants using a fresh approach. Because worms give birth to around 300 offspring, it is very difficult to find the parents among the offspring to know whether they lived longer. To solve this problem, Kenyon used *daf-2* mutant worms, a temperature sensitive-mutant that promotes the entrance to dauer (i.e. German word for "enduring"), a hibernation state characterised by reduced growth. She planned to treat adult worms at 20 ºC with a chemical that causes random mutations and then shift the mutants to 25 ºC so the offspring hatch as dauer and the parents would be easily spottable as they will have greater size. Surprisingly, as the experiment started, something unexpected happened, the control group with the *daf-2* mutation lived twice as long as wild-type worms, moved well and reproduced fairly normally (Kenyon et al., 1993). The discovery of this mutant shocked many scientists at that time. In the words of my supervisor Professor Linda Partridge, "It was the crucial mutagenesis, it knocked my socks off" (Speech at the 350th anniversary of the Royal Society).

Cloning of the *daf-2* gene revealed that it encodes a transmembrane receptor protein with homology with an insulin/insulin-like growth factor (IGF-1) receptor, whose function is to regulate growth and reproduction (Kimura et al., 1997). Briefly, when there is an abundant food supply, the *daf-2* receptor triggers a phosphorylation cascade that starts with *age-1* and ends with *daf-16*, a transcription factor. If *daf-16* is not phosphorylated, as in *daf-2* mutants, it migrates to the nucleus to express genes that repress reproduction and growth and prolong lifespan.

After the discovery of the *daf-2* mutant, alterations in the insulin/IGF-1 signalling pathway in other organisms also showed similar effects on ageing. The first was a mutant strain of *Drosophila* called *chico* (i.e. "small boy" in Spanish) because the homozygotes are around half the size of wild-type flies. Our laboratory showed that reduced expression of *chico* extends lifespan in *Drosophila* (Clancy et al., 2001). Moreover, *chico* limits lifespan via inhibition of the transcription factor homologue to *daf-16*, called *dFOXO* in *Drosophila*. Loss-of-function of the insulin/IGF-1 receptor in *Drosophila* also extended lifespan (Tatar et al., 2001). In worms and flies, there is a

single insulin/IGF-1 receptor, while in mammals there is an insulin receptor (*Insr*) and an IGF-1 receptor (*Igf1r*). Mice with heterozygous loss of *Igf1r* live on average 26% longer (Holzenberger et al., 2003), whereas deletion of *Insr* in adipose tissue, extends mean lifespan by 18% in male and female mice (Blüher et al., 2003).

Our best candidate interventions to target human ageing are obtained from experiments in mice, however, this is very expensive and time-consuming. The mean lifespan of the most commonly used strain of mouse (i.e. C57BL/6) is on average between 26 to 28 months and the mice can live up to 40 months without considering the effects on lifespan of the tested intervention. It has estimated that two lifespan experiments in mice may take up to 10 years and cost over 130000 USD (Robertson et al., 2011). Thus, it is highly convenient to study ageing using mice with accelerated ageing, which can provide important clues about ageing in a fraction of the cost and time. To our knowledge, the first mouse model of premature ageing was published by the Hoeijmakers' group in 1997. The authors found that disruption of *Ercc1*, an endonuclease necessary for nucleotide excision repair, lead to genomic instability and caused a significant lifespan reduction and growth retardation, while displaying ageing phenotypes such as sarcopenia, kyphosis, poor coordination, loss of visual acuity and ataxia (Weeda et al., 1997).

1.5.3 Pharmacological interventions

The first formal study testing the effect on lifespan of different molecules was done by Thomas Gardner in 1948. He thought that queen bees lived longer than her workers because of Royal jelly, a honeybee secretion that is used to feed the queen bee larvae. He fractionated the various components of the royal jelly and tested each one for life-extending properties using *Drosophila melanogaster* as a model. Flies fed with pantothenic acid (Vitamin B5), a compound representing 5% of royal jelly, lived 27% longer than controls (Gardner, 1948). Notably, other components of royal jelly did not increase lifespan significantly or decreased lifespan. Ten years later, the same compound was tested in mice (Pelton & Williams, 1958). Male and female mice with pantothenic acid supplement lived 18% and 20% longer, respectively. More recent studies have confirmed the positive effects of Royal jelly on mouse lifespan (Inoue et

al., 2003), but the major contribution of pantothenic acid to this lifespan extension remains unconfirmed. In humans, administration of Royal jelly to healthy volunteers improved indicators of physical and mental health (Morita et al., 2012).

In the 1970s, the first synthetic drug with geroprotective properties was found. Aspirin, a synthetic derivative of salicylic acid, showed to increase the lifespan of flies up to 40% (Hochschild, 1971), however, in subsequent experiments with female C57BL/6J mice, the same author could not find a significant increase in lifespan (Hochschild, 1973). More recently, the Intervention Testing Program (ITP) showed that aspirin increases lifespan in heterogeneous male mice by a modest 8% (Strong et al., 2008), yet these effects were not replicated at higher doses (Miller et al., 2019). Despite several clinical trials in humans have shown that aspirin treatment is associated with a reduction in cardiovascular events and stroke, these benefits may be outweighed by a significant increase in the risk of gastrointestinal bleeding and haemorrhagic stroke (Berger et al., 2006; McNeil et al., 2018).

Rapamycin

In 2009, Harrison et al. showed that rapamycin, a molecule isolated from *Streptomyces hygroscopicus*, a bacterium found in the 70s in Rapa Nui (Chile), significantly increased the lifespan of genetically heterogeneous mice (Harrison et al., 2009). This finding was relevant for two main reasons: i) most of the drugs or compounds shown to extend lifespan by then did not have a clear mechanism of action, while it was already established that the primary target of rapamycin was mTOR (Heitman et al., 1991), a protein involved in the regulation of cell growth and metabolism. Also, it was already known that mTOR regulated longevity, as genetic down-regulation of mTOR increased lifespan significantly in *C. elegans* (Vellai et al., 2003) and *Drosophila* (Kapahi et al., 2004). ii) the discovery of rapamycin as a potential geroprotector was also significant because it was a drug already approved for human use since 1999. Thus, the safe dosage and side effects in humans were already established. Today, the aim is to determine if rapamycin can prevent the ill effects of ageing in healthy individuals without major side effects. A recent short-term clinical trial of rapamycin in healthy elderly people showed no major adverse effects (Kraig et al., 2018). Also in elderly volunteers, a 6-weeks clinical trial of the mTOR

inhibitor everolimus, an analogue of rapamycin, was generally well tolerated and showed to enhance the response to the influenza vaccine by 20% (Mannick et al., 2014). A more recent study combining everolimus with BEZ235, a dual PI3K/mTOR inhibitor, found that 6 weeks of treatment substantially reduced the number of infections in the following year (Mannick et al., 2018).

Metformin

In 2010, another drug much commonly used in humans was associated with ageing. Metformin, a drug used to treat type 2 diabetes, showed to extend lifespan in *C. elegans* (Onken & Driscoll, 2010). Two years later, our lab showed that metformin did not extend lifespan in *Drosophila*, but it activated AMPK and reduced lipid storage (Slack et al., 2012). Later, the ITP reported modest effects in mouse lifespan by metformin, but they were not statistically significant (Strong et al., 2016). Considering that metformin is by far the most prescribed drug for type 2 diabetes, retrospective epidemiological studies allowed researchers to find associations between metformin usage and reduction of age-related diseases or mortality. A large epidemiological study found an increase of 18% in the median all-cause survival in patients under metformin compared to the general population, although higher morbidity was observed in the group taking the drug (Bannister et al., 2014). However, this study was conducted by comparing people with type 2 diabetes and treated with metformin against the rest of the population. Thus, it is not clear if metformin would have benefits in non-diabetic individuals. Moreover, metformin users may have greater health-seeking behaviours than the control population as they have had contact with a clinician. Despite these questions remain unanswered, similar findings in various epidemiological studies (reviewed in Campbell et al. 2017) motivated the scientific community to evaluate the geroprotective properties of metformin in clinical trials. The Targeting Ageing with Metformin (TAME) initiative is planned to study the effects of metformin in non-diabetic people aged 65 to 79 by assessing multiple markers of age-related health (Barzilai et al., 2016).

Lithium

In the mid-19th century, lithium was used to treat several diseases, including rheumatism, mania and depression. The stabilising mood effects of this drug were so

popular that in 1929 it was even part of the famous soft drink “7 Up” which originally was called “7 Up Lithiated Lemon Soda”. Lithium has shown to induce a dose-dependent extension of lifespan in yeast, *C. elegans*, and *Drosophila* (Castillo-Quan et al., 2016; McColl et al., 2008; Sofola-Adesakin et al., 2014; Tam et al., 2014; Zarse et al., 2011), however, its effects on mammalian lifespan have not yet been reported. In *C. elegans* and *Drosophila*, lithium maintains locomotor performance during ageing (Castillo-Quan et al., 2016; Tam et al., 2014). In animal models of age-related diseases like Alzheimer’s disease, Huntington’s disease and stroke, lithium is neuroprotective and ameliorates the pathology (Chiu & Chuang, 2010; Farina et al., 2017; X. Zhang et al., 2011). In humans, high levels of lithium in drinking water in regions of Japan have been associated with lower suicide rates (Ohgami et al., 2009) and reduced all-cause mortality (Zarse et al., 2011). Also, people with bipolar disorder taking lithium have longer leukocyte telomeres (Martinsson et al., 2013). Unfortunately, lithium is considered a “dirty drug” because frequently causes side effects, including nausea, hand tremors, lethargy, blurred thinking and weight gain. Thus, further safety studies in healthy individuals are required to determine which dosage reduces the risk of side effects but maintains the positive effects on ageing.

To determine which interventions may have a positive effect on human ageing is probably the most important question in ageing research today. In a recent review published with my supervisor Linda Partridge, we have examined the drugs considered the most promising to ameliorate human ageing (Partridge et al., 2020). Based on their robustness in preclinical and clinical trials, we grouped them into two tiers. The top tier includes the three drugs mentioned previously (i.e. rapamycin, metformin and lithium) plus senolytics, acarbose, spermidine and NAD+ enhancers, while the second-tier included non-steroidal anti-inflammatory drugs (e.g. aspirin), reverse transcriptase inhibitors, systemic circulating factors, the microbiome, glucosamine, glycine and 17α-estradiol.

1.6 Omics studies of interventions into ageing

In the previous section, I reviewed some of the most promising interventions to delay ageing. Over the years, researchers have used high-throughput technologies to

determine the molecular changes that these interventions induce at different biological levels in a hypothesis-free manner. In this section, I summarise the observations of a few of these omics studies in animal models.

1.6.1 Transcriptomics

During transcription, a gene's DNA is copied into RNA transcripts. The transcriptome is the set of all RNA transcripts in the cell. Through microarray and sequencing methods researchers can measure the abundance of these transcripts and estimate which genes are being expressed higher (up-regulated) or lower (down-regulated) than in the controls. Transcriptomic studies have been widely used to identify molecular mechanisms associated with mammalian ageing, for example by comparing different species (Fushan et al., 2015) or mouse strains (Houtkooper et al., 2013; Swindell, 2007) with different lifespans. Additionally, several studies in mice have revealed differentially expressed genes and pathways in response to genetic (Boylston et al., 2006; Rowland et al., 2005; Selman et al., 2009; Y. Zhang et al., 2012), pharmacological (Martin-Montalvo et al., 2013) and dietary (Rusli et al., 2015; Swindell, 2009) interventions that affect ageing. The transcriptomes of pairs of lifespan-extending interventions have also been compared, for instance, treatment with rapamycin and caloric restriction (CR) (Fok et al., 2014), long-lived Ames and Little dwarf mice (Amador-Noguez et al., 2004) and CR and Ames dwarf mice (Tsuchiya et al., 2004), revealing conserved changes in gene expression. However, comparison of multiple lifespan-extending interventions, including Snell, Ames, and Little dwarf mice together with CR and CR-mimetic compounds have shown discrepant findings with gene signatures enriched in steroid metabolism, cell proliferation, and cellular morphogenesis (Swindell, 2007) or oxidative phosphorylation, drug metabolism and immune response (Tyshkovskiy et al., 2019).

1.6.2 Proteomics

After transcription, RNA transcripts (mRNA) are translated into proteins by the ribosome. However, studies indicate that there is a poor correlation between mRNA and protein expression data (Ghazalpour et al. 2011; Pascal et al. 2008; Chen et al.

2002). Microarrays and mass spectrometry-based methods are the most commonly used technologies for the high-throughput study of the proteome. Interestingly, studies of the proteome of calorie-restricted mice or treated with rapamycin showed increased protein half-life compared to age-matched controls (Karunadharma et al., 2015). More recently, multi-tissue proteomic profiling of long-lived *Drosophila* insulin signalling mutants showed increased mitochondrial electron transport chain proteins in the fat body and proteasomal subunits in the gut (Tain et al., 2017). Similarly, proteomic profiling of *C. elegans daf-2* mutants showed an up-regulation of proteins in core metabolic pathways, including glycolysis, pentose phosphate pathway, citric acid cycle and electron transport chain complexes (Depuydt et al., 2014).

1.6.3 Epigenomics

As with proteins, DNA also undergoes modifications. The epigenome is the entire set of chemical changes to DNA and histones in the cell. DNA methylation is the most commonly studied epigenetic modification, and bisulfite genomic sequencing is considered the gold standard for its detection. Studies in mice have shown that age-associated DNA methylation changes are suppressed in Ames dwarf, calorie-restricted, and rapamycin-treated mice (Cole et al., 2017; Hahn et al., 2017; T. Wang et al., 2017). In contrast, evidence of DNA methylation in *C. elegans* and *D. melanogaster* has been elusive and controversial since the 1980s, with no robust proof of its location or function (Dunwell & Pfeifer, 2014).

1.6.4 Metabolomics

As proteins (i.e. enzymes) interact within metabolic networks, they produce metabolites that are used to produce energy and form the building block of the cell. The metabolome comprises the complete set of the metabolites within the cell. Metabolites are usually measured using NMR spectroscopy and mass spectrometry. Global metabolomic analysis in long-lived growth hormone-releasing hormone knockout mice has revealed differences in metabolites of mitochondrial metabolism, specifically the response to oxidative stress, serotonin degradation and nicotine degradation (Hoffman et al., 2020). Similarly, studies of mice under caloric restriction

revealed changes in metabolites related to energy status, but no metabolites significantly changed in mice treated with rapamycin (Fok et al., 2014). However, the combination of dietary restriction and rapamycin treatment showed greater effects on the metabolome than dietary restriction alone.

Despite the remarkable contribution of omics studies in long-lived models to understanding the molecular mechanisms of ageing, it remains largely unknown if these molecular changes are causal for the effects in lifespan and if opposite omics signatures are observed in models with accelerated ageing or during normal ageing, questions that I will explore during this thesis.

1.7 *In silico* prediction of interventions to target ageing

Even though we have made significant progress in finding new genetic and pharmacological interventions to modulate ageing, and to monitor the mechanisms underlying these changes, thousands of genetic interventions remain untested, especially in mammalian models. Moreover, high-throughput studies indicate that without prior knowledge, only 1 in 20 interventions tested prolong lifespan (Ye et al., 2014). In this regard, computational methods can speed up the identification of interventions with an increased likelihood of affecting ageing. In this section, I review the available computational methods for the prediction of geroprotectors and genes associated with ageing.

1.7.1 Computational drug repurposing for ageing

Over the years, several bioinformatic methods have been developed to identify potential geroprotective drugs using different strategies and sources of data. Two studies adopted methods based on the hypothesis that proteins or ligands with similar structures are likely to bind similar ligands or proteins, respectively, to predict drug-target interactions. The first of these studies aimed to identify novel drugs targeting three specific temperature-sensing proteins implicated in ageing in the rotifer *Brachionus manjavacas* (*TRP7*, *S6P*, *FhBC*) (Snell et al., 2016). The authors used a virtual screening software called FINDSITEcomb that combines protein modelling with

sophisticated threading approaches to model the target. The pockets in the model are then compared with the pockets of the crystallographic structures of proteins with ligands or modelled structures with known binders. The ligands of the top 100 ranked pockets are then compared with a library of screened ligands and ranked by ligand similarity. The authors screened 1347 FDA approved drugs *in silico* and tested four drugs for each target experimentally in the rotifers for their effects on lifespan and healthspan. Out of the 12 compounds tested, 5 significantly increased the rotifers' lifespan.

In a subsequent study by the same authors, the number of proteins analysed was expanded to include a set of ageing-related genes found in other animal models that have orthologous genes in rotifers (Snell et al., 2018). This time, 94 targets were screened *in silico* using the FINDSITEcomb software. The top 1% binding compounds for each target were further ranked by their cumulative lifespan extension achieved by genetic interventions into their targets as taken from experimental model organism data and filtered according to availability and previously predicted side effects (Zhou et al., 2015). From the 31 drugs experimentally tested in rotifers by two 10-day survival screens, seven drugs were further tested in two whole-life survival analyses, two of which resulted in a median lifespan extension of 13–42%.

Another *in silico* screening study was restricted to a single gene, AMP-activated protein kinase (AMPK), activation of which partially mediates the effects of DR on ageing (Mofidifar et al., 2018). To find new molecules to activate AMPK and theoretically mimic DR, the authors performed virtual screening using molecular docking of 1908 FDA approved drugs. The interaction between the top-ranked compounds and their targets was then checked by molecular dynamics. The study reported four compounds with predicted high affinity for AMPK, but these were not tested experimentally.

Using a priori information on known ageing-related genes, geroprotective drugs, or gene expression profiles, several studies have implemented a series of similarity-based approaches to identify novel geroprotectors. Given that drugs targeting ageing-related gene products are expected to affect the ageing process, Fernandes et al. (2016) focused on finding drugs that target the human orthologues of genes

associated with lifespan in animal models. For this calculation, inhibitory drugs interacting with anti-longevity genes and activators targeting pro-longevity genes were considered. In total, 376 drugs were scored, and 20 were statistically significant. Thirteen of these 20 drugs targeted histone deacetylases, and three were previously associated with lifespan extensions in animal models.

An alternative approach is to find drugs similar to known geroprotective drugs using machine learning, which is a strategy well-suited for prediction tasks. Liu et al. (2016) attempted to predict new geroprotectors for *C. elegans*. They adopted a semi-supervised algorithm trained with high-confidence geroprotectors derived from an experimental screen for *C. elegans* (Ye et al., 2014), together with their associated ageing-related genes curated from the literature and the GenAge database (De Magalhães & Toussaint, 2004). They produced a rank-ordered list of 785 drugs with a potential to increase lifespan in worms, with experimental validation for one drug in their list, using a lifespan assay. A separate machine learning approach was trained with chemical descriptors of known geroprotective drugs and functional annotation of their targets (Barardo et al. 2017). Using a supervised algorithm (i.e., random forest), they generated a ranked list of drugs predicted as lifespan-extending compounds, although no validation was performed.

The Connectivity Map Resource provides drug-induced expression profiles for 1309 compounds. Comparing these profiles with ageing-related gene expression signatures using a gene-set enrichment analysis can reveal drugs that generate changes in expression correlated (positively or negatively) to those seen in ageing (or any other biological process or disease). The first study of this kind used DR expression profiles in rats and rhesus monkeys to find DR mimetics (Calvert et al., 2016). They identified 11 drugs that might increase lifespan by mimicking DR. They experimentally tested several of the drugs in *C. elegans* and found that most extended lifespan. In a similar approach, in a study that I contributed, we used a meta-analysis of gene expression changes in the ageing human brain to identify robust gene expression changes in ageing and find drugs targeting those genes (Dönertaş et al. 2018). Using the Connectivity Map data, we identified 24 drugs and provided *in silico* validation by showing significant enrichment of known geroprotective drugs in their list. Recently, Yang et al. (2018) used a network-based method, called ANDRU (ageing network-

based drug discovery). Instead of relying on model organisms, this approach was driven by human transcriptome data (GTEx) from young and old adipose and artery tissues and signatures from the CREEDS database (Wang et al., 2016) to identify differentially expressed genes within the ageing-related networks and drugs reversing these changes. They report three distinct drugs ranking in the top five. Although none was previously reported as a lifespan modulator, these drugs target pathways that change in expression with age, such as metabolic enzymes and lipid metabolism.

1.7.2 Identification of ageing-related genes

As with drugs, several methods have been developed to identify genes with the potential to modulate ageing, most of which use machine learning algorithms (reviewed in Fabris et al. 2017). The first prediction of ageing-associated genes came with the creation of the GenAge database. Based on a network of genes associated with ageing and their interacting partners, the authors used a “guilty-by-association” strategy to identify new genes likely to affect ageing (De Magalhães & Toussaint, 2004). Similarly, Managbanag et al. (2008) used genes known to increase replicative lifespan in yeast from different databases and literature search to identify new longevity-associated genes. A shortest pathway analysis predicted 88 genes that were experimentally tested. Experimental deletion of 7 (8%) of these genes increased replicative yeast lifespan ($p = 0.02$).

Li et al. (2010) using 140 ageing-associated genes in humans with protein-protein interaction data constructed a network from which extracted topological features. The negative set of genes not associated with ageing used in the machine learning algorithm was composed of genes randomly selected from the Human genome. The study determined that proteins involved in ageing tend to have a higher number of interaction partners, interact with other ageing-related genes and are located at the centre of protein-protein interaction networks. The same research group repeated the analysis using longevity-associated genes in flies and mice an reached to similar conclusions (Feng et al., 2012; Song et al., 2012).

Huang et al. (2012) aimed to predict whether the deletion of a gene will decrease or increase in lifespan by using a two-layer model. The authors used a list of 533 gene deletions with known effects on yeast lifespan (i.e. increase, decrease or no effect). Gene expression data from 33 of these gene deletions was used to identify 442 genes discriminative for lifespan. The discriminative genes were merged with genes associated with ageing in the GenAge database to form a set of 525 lifespan-related genes. A machine learning model was built including biochemical and physicochemical features from the lifespan-related genes and features from an interaction network. They observed that the centrality of the deleted gene and the participation in chromatin silencing were important predictors of longevity.

In a more straightforward approach, using data from the GenAge database, Wan et al. (2015) constructed four datasets of genes associated with longevity in yeast, worms, flies and mice. Then the GO terms annotated to each gene were used as features for each gene. The authors found that the features that classified better genes into pro- or anti-longevity were the participation in biological processes associated with ageing, including autophagy, translation, DNA repair and telomere organisation. Fabris & Freitas (2016) used a set of genes associated with ageing or mortality phenotypes in mice, and gene features such as amino acid sequence, sequence motifs, protein-protein interactions and KEGG pathway annotations in a machine learning model to classify mouse genes into ageing-related or non-ageing related. They observed that features associated with KEGG pathways lead to the highest predictive accuracy (AUC = 0.71) and proteins involved in the Prolactin signalling pathway were likely ageing-related. More recently, Kerepesi et al. (2018) used 21000 proteins features to classify all proteins in the human genome into ageing-related or non-ageing related. The authors found that 36 protein features resulted in a model with a remarkable 98% accuracy. The feature with the best predictive power was “number of ageing-related interaction partners”, indicating that proteins interacting with known ageing related-targets are more likely to affect ageing.

1.8 Thesis objectives

In this chapter, I explained why further research on ageing is needed. Briefly, I described how human life expectancy is continuing to increase worldwide, and how although this trend is to be celebrated, the later years of life are still often spent in poor health and lowered quality of life. Luckily, these ill health effects are not inevitable, as they are directly caused by ageing, a process that is malleable in animal models through genetic or pharmacological interventions. Still, to find new geroprotective interventions and understand their mechanisms of action is challenging. In the following chapters, I will describe computational approaches aiming to address these challenges, in the attempt to develop future treatments that can allow people to live healthier for longer.

In section 1.5.3, I described several drugs with geroprotective properties in animal models. Unfortunately, there are still thousands of drugs and targets with geroprotective potential that have not been tested and we still do not know if these drugs will work against human ageing. Despite, ageing researchers have aimed to accelerate this translation through the development of computational methods to prioritise geroprotective drugs and targets (Section 1.7), only a few have focused on making predictions directly based on human ageing data. In chapter 2, I will introduce a drug repurposing approach based on finding which drugs target a significant proportion of human ageing-related genes and pathways. I will validate our findings by i) comparing them against drugs with known geroprotective properties in animal models and ii) testing experimentally the effect on *C. elegans* lifespan of the top-ranked compound. Finally, I will compare our results against all published drug repurposing studies to determine if our candidate drugs are also repeatedly predicted to affect ageing.

In section 1.6, I described how the use of omics technologies led us to achieve a better understanding of the mechanisms of ageing. However, comparisons of the various mechanisms of ageing are scares and it is still not yet clear how we can use this knowledge to find new targets and drugs to module ageing. In chapter 3, I will present a meta-analysis of transcriptomic data from 18 mouse models of ageing, aiming to

understand i) what are the similarities between interventions with similar effects on lifespan compared to interventions opposite effects on lifespan? ii) are the molecular mechanisms of ageing tissue-dependent?, iii) what are the most common mechanisms of ageing within intervention with similar lifespan effects? iv) are there some conserved changes with opposite directions between long- and short-lived mice? v) do the changes during normal ageing mimic the changes on short-lived models and reverse those of long-lived models? vi) which genetic interventions induce similar transcriptional changes to those observed in the mouse models of ageing?

In the final chapter (chapter 4), I will discuss our contributions to the ageing field and future directions of research.

Chapter 2 Identification of geroprotectors for humans

2.1 Introduction

Although pharmacological interventions have the potential to ameliorate ageing in humans, the development of drugs for this purpose would present major challenges. The trial of new drugs to modulate human ageing would require treating healthy individuals over long periods, which is unlikely to be allowed by drug agencies, particularly for drugs with unknown safety profiles. For this reason, it is more feasible to repurpose drugs already approved for specific diseases, or that passed the safety tests but failed against their original indication, than to target ageing itself with new drugs (Newman et al., 2016; Partridge, 2016). Moreover, even if we focus on drugs that passed the safety tests, thousands of drugs would require to be tested. A solution is to develop computational methods that can prioritise drugs for testing based on existing molecular data. As I described in Section 1.7.1, at least 11 computational methods have been developed to identify potential geroprotectors. However, more than 70% of the methods use animal models data to predict geroprotectors, despite the translation from non-mammalian species to humans is still a challenge, and certain aspects of ageing may be human-specific. In this regard, based on the empirical observation that proteins involved in ageing tend to interact with many other ageing-related proteins (Section 1.7.2), that the identification of drugs with multiple ageing-related targets represents a suitable strategy to find geroprotectors for humans.

In this chapter, I present a study aimed to use molecular data from humans to develop a drug-repurposing method capable of prioritising geroprotective drugs. Using publicly available databases, I rank-ordered drugs by their probability of affecting ageing, by measuring whether they targeted more genes related to human ageing than expected by chance. I further enhanced the power of the approach to predict ageing-modulating drugs by using other biological annotations such as pathways and ontology terms as the comparator. Finally, I integrated the ranked lists of drugs calculated from different data sources and experimentally validated the geroprotective properties of the top-ranked drug. Most of the work presented in this Chapter has been published as Fuentealba et al., (2019).

2.2 Results

2.2.1 Defining a dataset of drug-protein interactions and ageing-related genes

I inferred drug-ageing association by comparing drug-gene interactions with gene-ageing associations. **Figure 2.1** presents an overview of the procedure I used to prioritise compounds associated with ageing. First, a dataset containing the interactions between drugs and proteins was built based on data from the STITCH database (Szklarczyk et al., 2016). As I was focused on finding drugs applicable to humans, I kept only drugs targeting human proteins and successfully mapped to the DrugBank database (Law et al., 2014) using the UniChem resource (Chambers et al., 2013) **(Figure 2.1A)**. The resulting dataset was composed of 18393 interactions between 2495 drugs and 2991 proteins. More than half of the drugs (51.1%) in the dataset are approved for human use, 18.6% are in some phase of the approval process, and 28.4% have been shown to bind to disease targets in experiments.

The next step was to define a set of genes associated with human ageing. For this purpose, I used the Aging Clusters resource (Blankenburg et al., 2018), which contains information from 4 different sources including genes: i) changing expression with age ii) whose DNA methylation levels change with age iii) associated with age-related diseases and iv) included in manually curated databases of genes linked with longevity in genetic studies (Budovsky et al., 2013), associated with cellular senescence (Zhao et al., 2016) or showing evidence for a causative role in human ageing (Tacutu et al., 2013). To improve the confidence of the human ageing-related genes selected, I only used 1216 genes found in at least 2 of the 4 data sources.

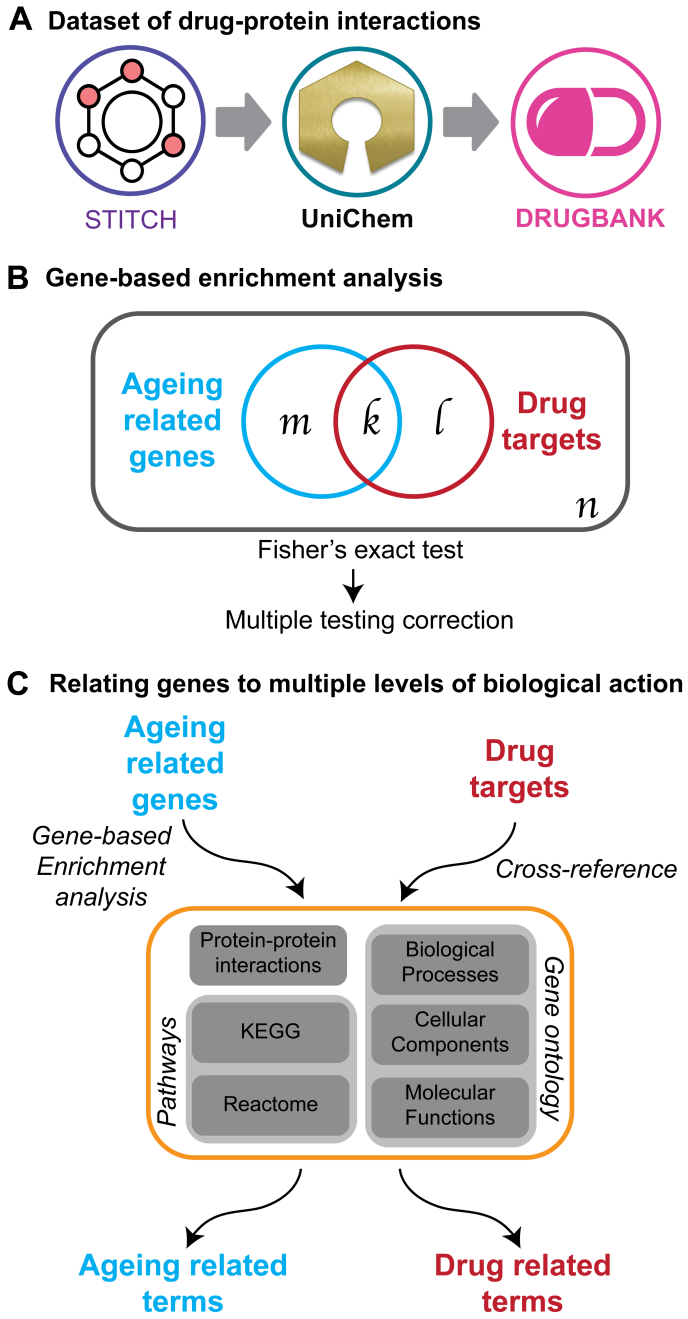


Figure 2.1. *Overview of the methods used in this study to prioritise compounds likely to ameliorate ageing in humans. A) STITCH chemicals were mapped into DrugBank drugs using the UniChem resource programmatically. B) The significance of the drug-ageing inference was calculated using Fisher's exact test, which calculates the probability that the overlap between two samples (ageing-related genes and drug targets) drawn from the same universe is due to chance. This comparison was made at different biological levels. C) Diagram of the procedure to expand the "gene" information into multiple biological levels. Ageing-related genes were mapped to other levels using an enrichment analysis, while the drugs' targets were cross-referenced with the list of genes defining each annotation.*

2.2.2 Gene-based inference of drug-ageing associations

Based on the premise that drugs targeting more ageing-related genes were more likely to affect the ageing process, I calculated the statistical significance of the overlap between the gene targets of each drug and the ageing-related genes **(Figure 2.1B)**. From the 1147 drugs analysed, 19 were statistically enriched for ageing-related targets after multiple testing correction **(Table 2.1, Supplementary Table 1)**. I performed an *in silico* validation of our method to prioritise ageing-related drugs, by comparing the obtained list with the DrugAge database (Barardo et al. 2017). Six out of the 19 drugs have already been reported to significantly extend the lifespan of at least one model organism **(labelled in black in Table 2.1)**, while only 1 was expected by chance. Using literature mining, I identified studies showing the association with ageing of other three drugs in the list, including cAMP analogues (Tong et al., 2007), selenium (Hao et al., 2016; Zhang et al., 2018) and tanespimycin (Fuhrmann-Stroissnigg et al., 2017; Fujikake et al., 2008). In contrast, I also found evidence for the DNA-mediated, pro-ageing (anti-longevity) effects of doxorubicin (Buttiglieri et al., 2011), cisplatin (Nonnekens & Hoeijmakers, 2017) and hydrogen peroxide (Lisanti et al., 2011). Altogether, 12 of the 19 (63%) candidate drugs showed experimental evidence to modulate ageing.

Because of limited data on which alterations in the ageing-related genes are beneficial or detrimental, I expected to obtain anti-ageing and pro-ageing drugs in our list. I performed an interaction-based similarity analysis to determine if drugs with similar effects on ageing had similar mechanisms of action. I found that pro-ageing compounds clustered separately from the other drugs, however, known geroprotectors showed different mechanisms of action **(Supplementary Figure 1)**. Similarities were found in the mechanism of action of sorafenib and regorafenib, bexarotene and GW-501516, and sirolimus and ECGC, in agreement with a previous study (Aliper et al., 2017).

Table 2.1. *Drugs significantly enriched for ageing-related targets. The names of the drugs previously shown to extend lifespan in animal models are in bold and genotoxic molecules are in italic. The columns k(l) and m(n) are consistent with the diagram in Figure 1B. OR stands for odd-ratios and adj.p-value is the p-value adjusted for multiple testing.*

Drug name	Status	OR	p-value	adj.p-value
Resveratrol	Investigational	2.52	2.09E-08	1.82E-04
Sunitinib	Approved	8.11	4.92E-08	2.15E-04
Genistein	Investigational	2.84	6.40E-07	1.86E-03
Simvastatin	Approved	2.80	1.53E-06	3.35E-03
Tanespimycin	Investigational	6.71	2.64E-06	4.62E-03
Regorafenib	Approved	9.16	4.43E-06	6.45E-03
Epigallocatechin gallate	Investigational	2.50	5.96E-06	7.44E-03
Doxorubicin	Approved	2.78	7.20E-06	7.87E-03
Selenium	Approved	6.25	9.44E-06	9.17E-03
Celecoxib	Approved	3.46	1.58E-05	1.38E-02
Indole-3-carbinol	Investigational	6.32	1.83E-05	1.46E-02
Hydrogen peroxide	Investigational	2.00	2.85E-05	2.07E-02
GW-501516	Investigational	9.56	6.23E-05	3.82E-02
Bexarotene	Approved	7.60	6.98E-05	3.82E-02
Dorsomorphin	Experimental	7.60	6.98E-05	3.82E-02
Sorafenib	Approved	3.03	7.25E-05	3.82E-02
Sirolimus	Approved	2.30	7.42E-05	3.82E-02
Cisplatin	Approved	2.38	8.39E-05	4.07E-02
cAMP	Experimental	2.29	1.00E-04	4.60E-02

2.2.3 Drug-ageing association based on pathways and ontologies

Given that geroprotective effects are likely to be mediated through altered pathway activity and cellular function, I investigated if I could enhance the prediction of geroprotectors using other biological annotations as comparators. I calculated the pathways and gene functions enriched in ageing-related genes. A total of 82 KEGG and 54 Reactome pathways were enriched in this set of genes, as well as 1177 biological processes (BP), 69 cellular components (CC) and 103 molecular functions (MF). Also, I calculated that 676 proteins interacted with the set of ageing-related genes. These terms were defined as the set of ageing-related terms **(Figure 2.1C, left side)**. Equivalently, drugs were then associated with these terms through association with their targets using the list of genes defining each term according to the DAVID

knowledge-base (Huang et al., 2007) and the biological database network (Mudunuri et al., 2009). This mapping procedure resulted in a set of terms from each data source related to each drug (drug-related terms) **(Figure 2.1C, right side)**. Analogously to the gene-based association analysis, I calculated for each biological annotation if the overlap between ageing-related terms and drug-related terms was statistically significant using a Fisher's exact test. This procedure generated 6 lists of ranked compounds (i.e. based on protein-protein interactions, biological processes, cellular components, molecular Functions, KEGG and Reactome pathways) in addition to the gene-based analysis **(Supplementary Table 2-7)**.

I evaluated the similarity between the ranking of compounds in the different lists and observed a moderate concordance (Kendall's W = 0.58, p-value = 1.02E-266). The highest correlations were observed between the results from biological processes and cellular components (Kendall's tau = 0.51, p-value < 2.2E-16) **(Supplementary Figure 2)**, while the lowest was observed between cellular components and genes (Kendall's tau = 0.16, p-value = 3.289E-11). An intrinsic limitation of our strategy is that it is based on genes known to be associated with ageing. To evaluate the possibility of research bias, I performed random permutations to simulate the enrichment of each drug for a random set of terms at each biological level. None of the top-ranked drugs in each list ranked higher than in the analysis in more than 1.7% of the simulations **(Supplementary Table 8)**.

Given that our method rank-orders the drugs by the significance of the association, it is possible to quantify the overall capability of the strategy to prioritise geroprotectors by calculating for each list the fraction of known geroprotectors (ranked by p-value) among the fraction of drugs considered in each analysis **(Figure 2.2A)**. The enrichment for geroprotectors was quantified by calculating the area under the curve (AUC) generated by plotting these two variables. The maximum AUC was obtained when biological processes (AUC = 0.69) were used as the comparator **(Supplementary Figure 3)**. The use of genes showed the lowest enrichment when all evaluated drugs were considered (AUC = 0.59), which suggests that the use of higher biological levels to calculate the inference improves the prediction capabilities and that the use of genes leads to a loss of power to rank drugs targeting a low proportion of ageing-related genes, which is observed in **Figure 2.2A** as a loss of enrichment after

25% of the drugs were ranked. I evaluated if the AUCs were statistically significant by calculating the AUC from the simulations generated to quantify the research bias. The p-value for each curve was calculated as the number of simulated results with an AUC equal or higher than the analysis. All lists showed an enrichment higher than expected by chance **(Supplementary Table 9)**. When I only considered the first 20 top-ranked drugs, I observed that using biological processes or cellular components to perform the comparison showed the highest proportion of geroprotectors (45%), while only 2 geroprotectors (10%) were found among the top 20 drugs when KEGG pathways were used. Although the number of drugs reported to decrease lifespan in animal models is smaller than the set of geroprotectors, I repeated the enrichment analysis using anti-longevity drugs **(Figure 2.2B)**. The enrichment for anti-longevity drugs was lower than for geroprotectors **(Supplementary Figure 3)**. The highest AUC was observed when cellular components were used (AUC = 0.63) while using genes showed the lowest enrichment for anti-longevity drugs (AUC = 0.54).

Because various cutoff values can be selected to define the dataset of drug-protein/protein-protein interactions and enriched GO terms/pathways, I repeated the analysis using different confidence scores in the STITCH and STRING databases and p-value cutoff in Gene Ontology (GO), KEGG and Reactome to explore its influence on the performance. When I measured enrichment for geroprotectors, I did not observe a major change in the AUC when higher or lower confidence scores were used **(Supplementary Figure 3)**. The selection of a lower p-value cutoff leads to the same (GO:CC and KEGG) or lower (GO:MF and Reactome) enrichment whereas the use of a higher p-value cutoff leads to a decrease (GO:MF and Reactome) or increase (GO:CC and KEGG) in the AUC. A similar lack of trend was observed in the enrichment for anti-longevity drugs. Overall, the cutoffs used initially (p-value of 0.05 and confidence score of 700) maximised the enrichment for geroprotectors when genes, Reactome pathways and molecular functions were used.

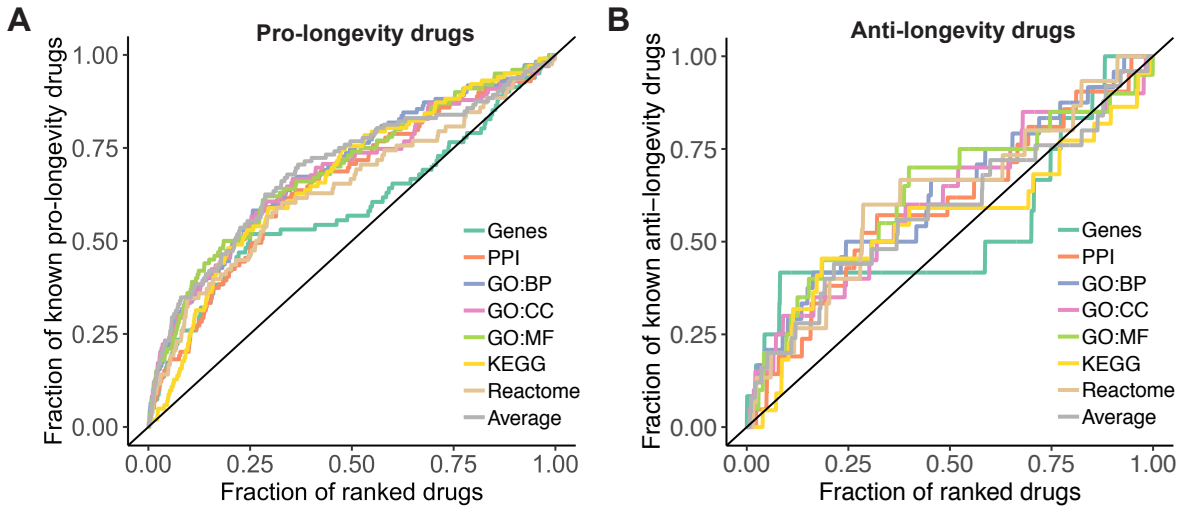


Figure 2.2. *Comparison of the results using different data sources. Enrichment curves for A) pro-longevity drugs and B) anti-longevity drugs. The results of each data source are displayed in lines with different colours. The enrichment expected by chance is shown as a diagonal line with AUC = 0.5.*

Even though all the lists of drugs showed significant enrichment for geroprotective drugs, they showed low overlap between the top-ranked compounds. Thus, I integrated the results into a single list accounting for the complexity of the multitiered effect of drugs by calculating their average ranking in the different analyses. The combination generated a list equally enriched as the maximum AUC obtained by the previous analysis (AUC = 0.69). Among the top 10 drugs with the best average ranking (**Table 2.2, Supplementary Table 10**), I found 2 drugs that have shown to extend lifespan in animal models (trichostatin A (Tao et al., 2004) and celecoxib (Ching et al., 2011)). Half of these 10 drugs are classified as kinase inhibitors, while 8 are anti-cancer drugs and 7 are approved for human use.

2.2.4 The *HSP-90* inhibitor tanespimycin as a novel geroprotector

Leading the joint ranking was tanespimycin, a well-characterised *HSP-90* inhibitor that has been shown to activate the transcription factor *HSF-1* and induce a heat shock response (Fujikake et al., 2008). As a proof-of-principle, I investigated whether tanespimycin could activate *HSF-1* and extend lifespan in the nematode worm *C. elegans*. To perform these experiments, I establish a collaboration with a research fellow from our Institute whose expertise is on protein homeostasis. The following

experiments were performed by Dr John Labbadia and his student Rhianna Williams at the Institute of Healthy Ageing.

Table 2.2. *Top-ranked compounds using multiple levels of biological action. The names of the drugs previously shown to extend lifespan in animal models are in bold. The numeric values represent the ranking of the drugs when different sources of data (columns) are used. The last column is the ranking average (Avg.) for each drug in the 7 ranked lists.*

Drug name	Status	Genes	PPI	Gene ontology			Pathways		Avg.
				BP	CC	MF	KEGG	Reactome	
Tanespimycin	Investigational	5	26	57	43	44	39	9	31.86
Imatinib	Approved	63	3	21	34	12	66	38	33.86
Sunitinib	Approved	2	1	59	31	31	56	63	34.71
Trichostatin	Experimental	83	41	19	54	13	41	52	43.29
Geldanamycin	Investigational	32	37	87	76	47	13	21	44.71
Sorafenib	Approved	16	68	11	15	8	155	42	45.00
Dasatinib	Approved	41	12	43	81	62	49	35	46.14
Erlotinib	Approved	27	6	93	85	71	64	7	50.43
Etoposide	Approved	23	11	20	90	32	120	67	51.86
Celecoxib	Approved	10	2	33	42	34	180	70	53.00

To test the efficacy of tanespimycin dosing in *C. elegans*, we grew worms expressing mCherry under the control of a *hsf-1* (*C. elegans* orthologue of *HSF-1*) responsive promoter (i.e. *hsp-16.2*) (Mendenhall et al., 2015) on solid media plates containing various doses of tanespimycin. Worms were exposed to tanespimycin continuously from the first larval stage (L1) of development, or the first day of adulthood. Worms grown continuously on tanespimycin plates exhibited a dose-dependent activation of the *hsf-1* transcriptional reporter, starting at 25 μM and peaking at 100 μM (**Figure 2.3A-B**). Similarly, exposure to tanespimycin plates only in adulthood resulted in significant activation of the *hsf-1* reporter at 50 and 100 μM concentrations. No markers of toxicity were observed in any treatment groups, except for the 100 μM larval group, which were developmentally delayed by 24 hours and had a significantly reduced brood size **(Supplementary Figure 4),** consistent with chronic *HSP-90* inhibition (Melo & Ruvkun, 2012). Together, these data demonstrate that tanespimycin activates *hsf-1* in *C. elegans* and that treatment during adulthood is not associated with overt toxicity.

We next sought to determine whether tanespimycin treatment could extend lifespan in *C. elegans*. To circumvent potential longevity effects arising from delayed development and reproduction, we exposed worms to 100 µM tanespimycin plates from the first day of adulthood. Tanespimycin treatment significantly extended median and maximal lifespan compared to vehicle-treated controls **(Figure 2.3C)**. To determine whether the effects of tanespimycin on lifespan require *hsp-90* (*C. elegans* orthologue of *HSP-90*), we also exposed worms to tanespimycin treatment in the presence of *hsp-90*(RNAi). Consistent with previous reports, *hsp-90* (RNAi) treatment significantly reduced *hsp-90* mRNA levels compared to empty vector treated controls **(Figure 2.3D)**, and significantly shortened *C. elegans* lifespan **(Figure 2.3C)** (Somogyvári et al., 2018). Furthermore, upon depletion of *hsp-90*, tanespimycin treatment no longer increased lifespan compared to vehicle controls **(Figure 2.3C)**. These data suggest that tanespimycin treatment extends lifespan in an *hsp-90* dependent manner, but that severe depletion of *hsp-90* is toxic to animals, despite the activation of protective stress responses.

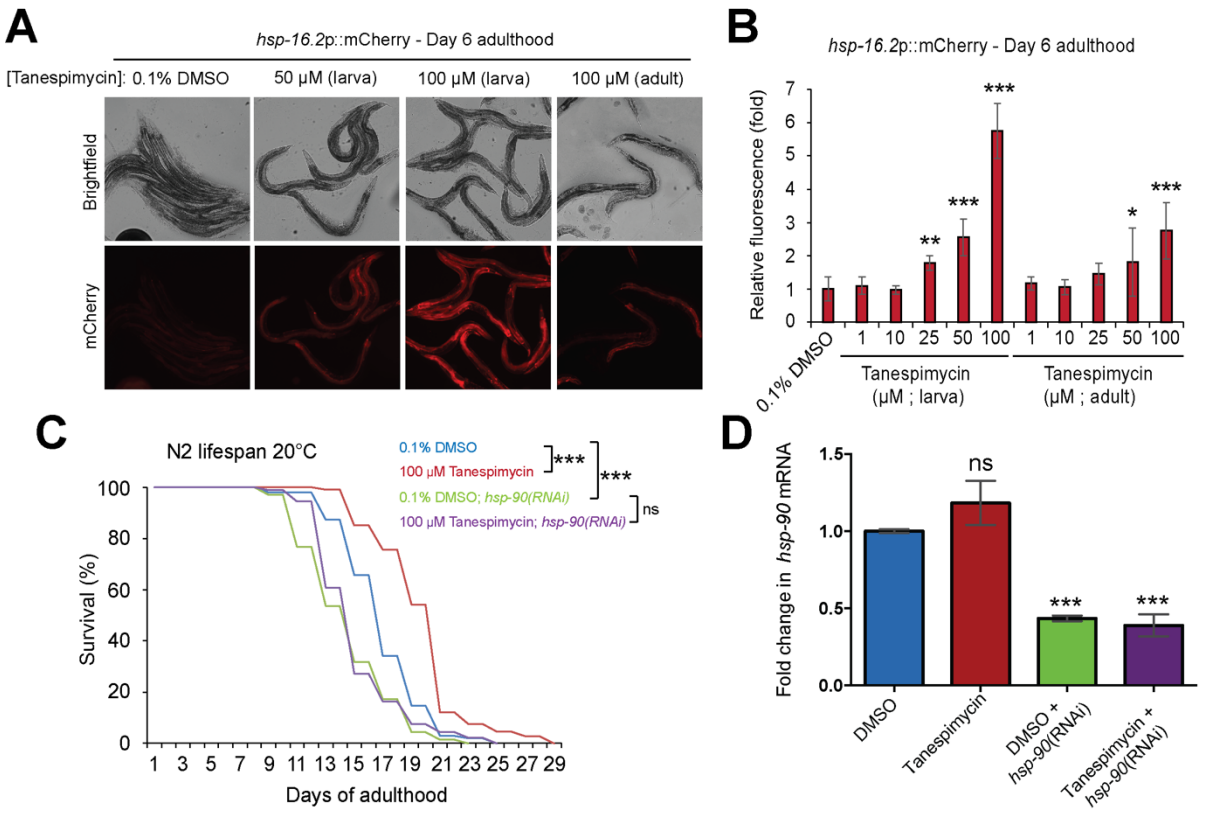


Figure 2.3. *Pro-longevity effect of tanespimycin in C. elegans. Representative fluorescent images of day 6 adult, hsp-16.2p::mCherry transcriptional reporter worms,*

*grown on plates containing 0.1% DMSO (vehicle) or different concentrations of tanespimycin continuously from the first larval stage, or the first day of adulthood onward. B) The relative fluorescent intensity of hsp-16.2p::mCherry worms grown on plates containing 0, 1, 10, 25, 50, or 100 μM tanespimycin continuously from the first larval stage or only from the first day of adulthood onward. Values plotted are the mean of at least 5 animals, and error bars represent the standard deviation from the mean. Statistical significance relative to the DMSO control group was calculated by one-way ANOVA with Tukey post-analysis pairwise comparison of groups. * = p < 0.05, ** = p < 0.01, *** = p < 0.001. C) Lifespan at 20 ºC of N2 worms grown on plates containing 0.1% DMSO or 100 μM tanespimycin from the first day of adulthood onward in the presence of empty vector control or hsp-90(RNAi). Statistical significance was calculated by Log-rank (Mantel-Cox) test. *** = p < 0.001. Treatment groups: 0.1% DMSO (n = 102, 14 censored, median lifespan = 17 days), 100 μM tanespimycin (n=107, 9 censored, median lifespan 21 days), 0.1% DMSO + hsp-90(RNAi) (n = 69, 30 censored, median lifespan = 15 days), 100 μM tanespimycin + hsp-90(RNAi) (n = 92, 22 censored, median lifespan = 15 days). D) Relative hsp-90 mRNA levels 48 hours following exposure to empty vector control or hsp-90(RNAi). Levels of hsp-90 mRNA were normalized to the geometric mean of three housekeeping genes (cdc-42, rpb-2, and pmp-3). Values plotted are the mean of 3 biological replicates and error bars represent standard deviation. Significance levels were calculated as in Figure 2.3B.*

2.2.5 Comparing the results with eleven *in silico* drug-repurposing studies

As I described in Chapter 1, the drug-repurposing methods to target ageing differ in their aims and data sources. This made me wonder how similar were the results obtained by the different methods, including ours. To facilitate their comparison, together with Melike Donertas, a PhD student working with Professor Janet Thornton, we collected and summarised each study in terms of i) the drugs identified, ii) the genes targeted by these drugs, and iii) all biological pathways (KEGG) known to be targeted by drugs **(Figure 2.4)**. Additionally, we compared the results with the manually curated databases of ageing-related genes (GenAge) and drugs (DrugAge).

Most of the work presented in this section has been published as Dönertaş et al., (2019)(co-first author).

Drugs

Overall, we observed that only 12% of all DrugAge drugs are prioritised by at least one study (41 of 346 drugs in DrugAge), with one in every four drugs discovered already present in DrugAge, reflecting the prioritisation process and the low number of drugs reported as significant by each study (15 drugs on average). Also, the 163 drugs identified usually differ between studies with 91% (149 drugs) of them identified just by one study. From the remaining 14 drugs present in more than one study, trichostatin, geldanamycin, tanespimycin and vorinostat were identified by three studies **(Figure 2.5A)**, all of which, excepting tanespimycin, were previously experimentally validated for geroprotective effects in animal models (McDonald et al. 2013; Barardo et al. 2017). Surprisingly, the method I presented was able to prioritise 3 of these drugs (trichostatin, geldanamycin and tanespimycin) among the top 10 candidates **(Table 2.2)**.

Genes

Overall, 103 genes (34%) of the GenAge human genes and 94 genes (10%) of the GenAge model organism genes were targeted by the candidate drugs in at least one study. This difference possibly reflects the different sizes of the datasets, with over three times the number of model organism genes in GenAge. Based on the DGIdb database (Barardo et al. 2017), 796 genes (27% of the druggable genome) are targeted by at least one drug identified in the computational studies **(Figure 2.5B)** and, while few genes were identified in multiple studies, some of them were present in the GenAge database (Tacutu et al., 2018). Two of these genes DDIT3 (DNA Damage Inducible Transcript 3) and ERBB2 (Erb-B2 Receptor Tyrosine Kinase 2) were targeted by the drugs prioritised in eight studies. However, nine studies identified drugs targeting BIRC5 (Baculoviral IAP Repeat Containing 5) and KRAS (KRAS Proto-Oncogene, GTPase), and ten studies predicted drugs modulating ABCB1 (ATP Binding Cassette Subfamily B Member 1), which have not previously been related to human ageing. Despite this, genes discovered by multiple studies do not necessarily suggest higher relevance to ageing and may instead reflect research bias (e.g. genes

targeted by many drugs because of a role in a prevalent disease such as cancer). We observed that 80% of known geroprotectors target at least one gene targeted by the candidate geroprotective drugs identified by these twelve computational studies.

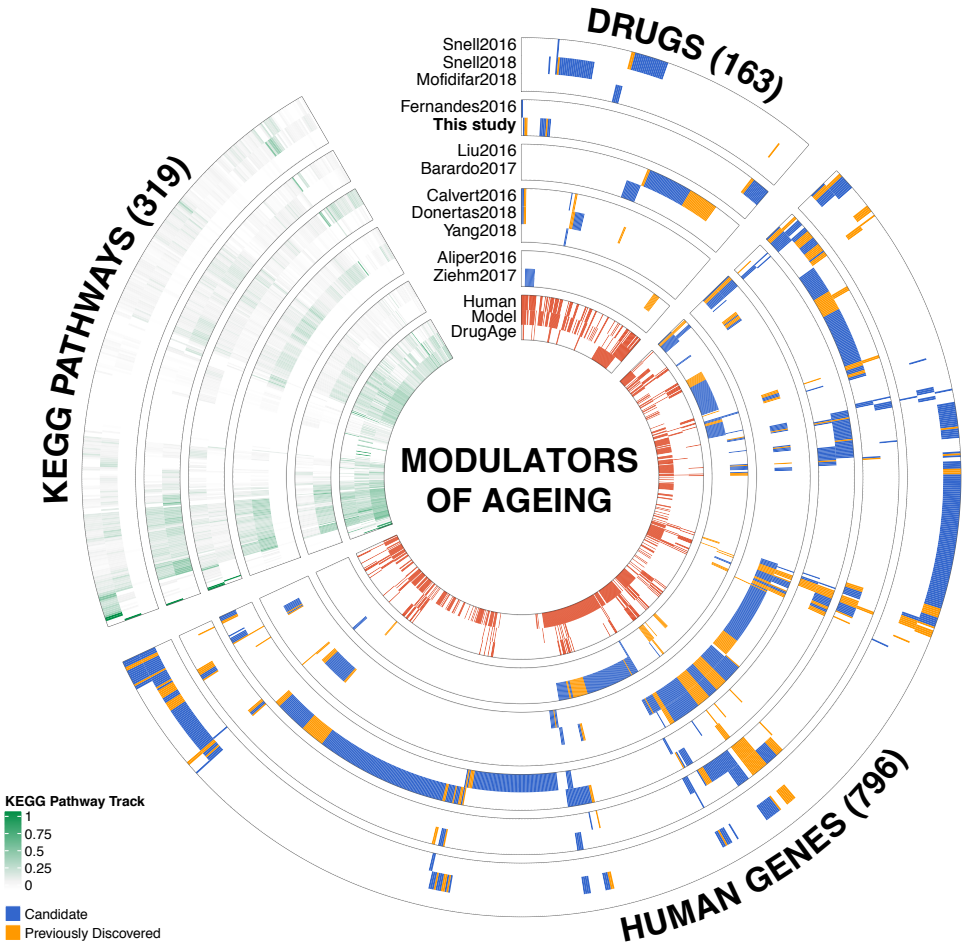


Figure 2.4. *Drugs, human genes and KEGG pathways discovered in the 12 studies. Circular heatmap of the drugs discovered by each of the 12 studies (drugs sector), genes targeted by these drugs (human genes sector), and the pathways including these genes (KEGG pathways sector). Drugs, genes and pathways are clustered independently to reflect discovery patterns from the studies. For the drugs and human genes sectors, the inner circle shows whether drugs or genes were previously associated with ageing, based on the DrugAge or GenAge database, respectively. If a drug was not present in DrugAge, it was classified as "candidate", and the cell was coloured blue, whereas if the drug was already in DrugAge, it was classified as "previously discovered" and the cell coloured in orange. An equivalent strategy using the GenAge databases instead of DrugAge was used for the human gene sector. In*

the inner wheel, we present the overlap with drugs targeting ageing-related genes (drug sector – GenAge Human/Model tracks) and for the human gene sector the overlap with genes targeted by the drugs in DrugAge (Human genes sector – DrugAge track). The KEGG pathways sector shows the proportion of genes on each pathway targeted by the drugs discovered by each study. The cells representing KEGG pathways were coloured using a continuous gradient from white to green, where white means that none of the genes in that pathway were targeted by the drugs identified. In the section closer to the centre of the heatmap, we show the proportion of ageing-related genes in these pathways and the coverage of genes targeted by drugs in the DrugAge database.

Pathways

Intriguingly, among the 319 druggable KEGG pathways, 92% include at least one gene targeted by the drugs identified in the studies. The same tendency was observed for genes in GenAge (83% Model GenAge & 74% Human GenAge), or genes targeted by the DrugAge drugs (88%). While this may suggest ageing is ubiquitous and affects all pathways, another possibility is that genes present in many pathways could be discovered repeatedly because they play a central role in diseases and regulatory mechanisms. Although this may not conclusively prove that ageing is systemic, the prioritised candidate drugs have a genome-wide effect.

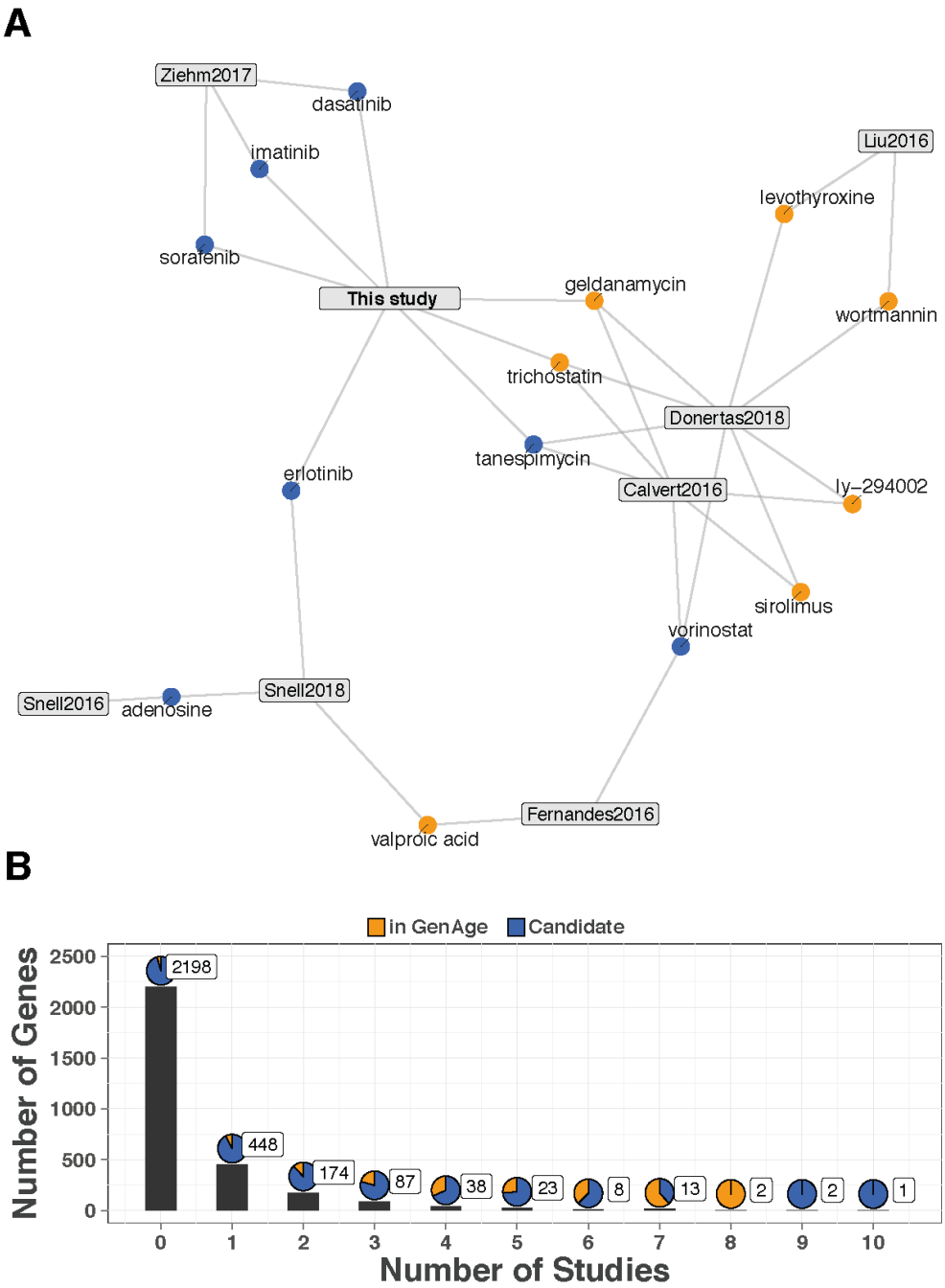


Figure 2.5. *Candidate drugs and genes from the druggable genome proposed by multiple studies. A) Network representation of candidate drugs discovered by multiple studies and the studies in which they were found. Orange nodes show drugs previously discovered to affect lifespan in animal models (DrugAge) and blue the novel candidates. The edges link drugs identified in the relevant study. B) Distribution of the number of genes targeted by the drugs identified with respect to the number of studies. The x- and y-axes show the number of studies and genes, respectively. The pie charts show the percentage of genes in GenAge (human database) for each category. The boxed numbers show the total number of genes in each category.*

2.3 Discussion

I designed this study to infer and rank drugs matched to ageing at multiple levels of biological activity using a simple statistical test. In an initial gene-centric analysis, 19 drugs were identified as candidates expected to modulate ageing in humans. A major finding was that 6 of the statistically significant drugs (resveratrol, genistein, simvastatin, epigallocatechin gallate, celecoxib and sirolimus) have already shown geroprotective properties in experimental studies in model organisms. This statistically significant enrichment suggests that, despite its simplicity, the method is robust to prioritise geroprotectors. Then, I expanded the analysis to higher levels of biological complexity and again found statistically significant enrichment for geroprotectors in all cases. Compounds ranked high on average included trichostatin and celecoxib, drugs known to prolong lifespan in animal models (Calvert et al., 2016; Ching et al., 2011; Tao et al., 2004). The compound ranked highest on average was tanespimycin, an *HSP-90* inhibitor, shown to acts as a senolytic agent by killing human senescent cells without affecting the viability of healthy cells (Fuhrmann-Stroissnigg et al., 2017) and to ameliorate disease phenotypes in *Drosophila* models of Huntington's disease and spinocerebellar ataxia (Fujikake et al., 2008). We found that tanespimycin treatment extended median (23%) and maximum (16%) lifespan in *C. elegans*, through its target *HSP-90* (*hsp-90* in *C. elegans*), possibly through the induction of cytoprotective pathways caused by mild stress. Thus, these positive effects on lifespan caused by tanespimycin may represent a hormetic response, a biphasic dose-response characterized by a low-dose increase of lifespan and a high-dose reduction of lifespan. If this is the case, even greater increases in lifespan may be achieved at even lower drug concentrations. To identify the maximum response within the hormetic zone a wider range of concentrations needs to be tested.

Evidence from the literature supports the senolytic action of other drugs that I identified as potentially geroprotective. Dasatinib, a kinase inhibitor ranked 7th on average, has been reported to induce apoptosis in senescent preadipocytes (Zhu et al., 2015). Combination of dasatinib and quercetin, which also inhibits *HSP-90*, induced apoptosis in senescent murine mesenchymal stem cells and mouse embryonic

fibroblasts *in vitro*, improved cardiovascular function in aged mice, and decreased bone loss and age-related symptoms in progeroid mice (Zhu et al., 2015).

Tanespimycin, geldanamycin and trichostatin among the top 10 compounds from the combined ranked list have been previously proposed as geroprotective for humans using computational analysis (Dönertaş et al. 2018). Also sorafenib, imatinib, dasatinib in our list overlap with the predictions from Ziehm et al. 2017; and erlotinib with the candidate geroprotectors from Snell et al. 2018, meaning that in total 7 of our top 10 drugs candidate drugs have been previously predicted to influence ageing by other drug-repurposing methods. Moreover, among all the drug-repurposing methods currently available for ageing, the method presented here identified 7 of the 14 candidate drugs prioritised by multiple studies.

2.3.1 Limitations

Despite the capability of the method to prioritise geroprotective drugs, I identified several limitations. For non-commonly studied drugs, the interactome is incomplete. This may explain why I observed many anti-cancer and well-known drugs in our results. However, I assessed the research bias using permutations and I found no significant effect on our results. Another limitation of our approach is that the drug-protein interaction data may include false positive interactions leading to unreliable results. However, we mitigated these effects by only including interactions with a confidence score that prioritize interactions found in multiple data sources. Also, the drug-protein interaction information completely neglects the tissue specificity of the proteome. Thus, further studies are required to determine which tissues these geroprotective drugs are affecting. Also, inferred associations do not provide information about the directionality of the effect, which in this case means that it is unknown if the drugs will delay or accelerate ageing. While I indirectly assessed this using an interaction-based similarity analysis between the drugs, resulting in clusters or pairs of drugs with a similar mechanism of action, experiments are required to determine the precise effects of each drug on ageing.

2.4 Methods

2.4.1 Data sources

Drug-protein interaction dataset

Chemical-protein interactions were extracted from the Search Tool for Interactions of Chemicals (STITCH) database 5.0 (Szklarczyk et al., 2016). I chose this resource because it acts as a probabilistic network, by collecting interactions from multiple sources, including experiments, databases and a text-mining algorithm. Individual scores for each source are combined into an overall confidence score using a naive Bayesian formula defined as $Score = 1 - \prod_i (1 - S_i)$, where S_i represents the confidence score for the source i. Later, because the Bayesian combination of scores can overestimate the effect of small individual contributions, the score is corrected for the probability of observing an interaction by chance. The overall confidence score ranges from 0 to 1, where a value of 0.4 or greater is medium confidence, and a score equal to or higher than 0.7 is high confidence. To get a reliable set of interactions, I removed all interactions with a confidence score lower than 0.7. The database also maps the direction of each interaction, i.e. whether the chemical acts on the protein or if the protein modifies the chemical (e.g. transformation of the chemical during a catalytic reaction). To confine the analysis to the actions of chemicals on proteins, only the cases where the chemical activates or inhibits a protein were kept. To focus on drugs in development or approved for human use, I filtered the chemicals in STITCH 5.0 by the drugs in DrugBank 5.0 (Law et al., 2014) using UniChem (downloaded on July 2017)(Chambers et al., 2013). The InChi key for each drug was retrieved from PubChem (http://pubchemdocs.ncbi.nlm.nih.gov/pug-rest) and used to obtain the DrugBank identifiers via UniChem. The names of the drugs were obtained from the DrugBank vocabulary file, and the development status was obtained using the structure external links file. Finally, I mapped the Ensembl identifiers for each protein into the HUGO Gene Nomenclature Committee (HGNC) approved gene names using Ensembl Biomart (version 91) (Durinck et al., 2009).

Drug-related terms

I mapped the targets of each drug in the drug-protein interaction dataset to multiple biological levels by using the information about the genes that define each level analysed. I downloaded (June 2018) the gene-centric definitions of GO terms and Reactome pathways from the DAVID knowledgebase (Huang et al., 2007). Genes on each KEGG pathways were obtained using the biological database network (https://biodbnet-abcc.ncifcrf.gov/db/db2db.php, version 20151204)(Mudunuri et al., 2009). Protein-protein interactions were mapped directly using the STRING database (version 10) (von Mering et al., 2005). Only proteins interacting with the set of ageing-related genes with a confidence equal of higher than 0.9 were considered.

Ageing-related genes

Genes present in manually curated databases are more susceptible to research and reporting bias than those found in objective searches. Instead of selecting a set of ageing-related genes from a particular study or database, I used genes linked with ageing from the Ageing Clusters resource (https://gemex.eurac.edu/bioinf/age/, downloaded on December 2017). This repository contains the results of a network-based meta-analysis of human ageing genes (Blankenburg et al., 2018) that considered 35 different datasets. The author classified the genes into the following 4 categories: i) curated ageing-related genes from databases such as GenAge (Build 17) (Tacutu et al., 2013), LongevityMap (Build 1) (Budovsky et al., 2013) and CSGene (Zhao et al., 2016); ii) genes differentially expressed with age, regimes of CR or healthy ageing; iii) age-related changes in the methylation of cytosine guanidine dinucleotides (CpGs) in the DNA; and iv) genes associated with age-related diseases from databases such as the Human Gene Mutation (version 2015.03) (Stenson et al., 2014) or the Human Phenotype Ontology (version 2016.01.13) (Köhler et al., 2014). To improve the reliability of the set of ageing-related genes and reduce research bias, I considered only the genes present in at least two categories.

Ageing-related terms

Using the set of ageing-related genes, I performed gene-based enrichment analysis to infer the function and pathways associated with ageing. Gene Ontology (BP, CC, MF) terms were calculated using the enrichGO function from the clusterProfiler

package (version 4) (Yu et al., 2012), using the Benjamini and Yekutieli method (Benjamini & Yekutieli, 2001) for adjustment, a conservative correction that does not rely on the assumption that the test statistics are independent. The adjusted p-value cutoff was set to 0.05 and for biological processes I consider the top 500 terms enriched. Enriched KEGG pathways were determined using the enrichKEGG function from the clusterProfiler package, using the same parameters used for the gene ontology enrichment. Reactome pathways were calculated using the function enrichPathway from the ReactomePA package (Yu & He, 2016). Protein-protein interactions were obtained using STRING (Version 10) (von Mering et al., 2005) database.

2.4.2 Statistical analysis to rank the drugs

Independently of the biological level, the drug-ageing associated was inferred by calculating the statistical significance of the drug-related terms and ageing-related terms using a Fisher's exact test. Drugs were associated with ageing at the following biological levels: gene, pathways (KEGG, Reactome), functions (GO:BP, GO:CC, GO:MF) and indirect protein interactions. The universe was defined as all the terms on each level associated with at least one drug. Thus, drugs with a lower p-value modulate a higher proportion of ageing-related terms than that expected by chance. To control for the false discovery rate, I used the Benjamini and Yekutieli adjustment (Benjamini & Yekutieli, 2001). A p-value lower than 0.05 after multiple testing correction was considered significant.

2.4.3 Measuring the impact of research bias

Some drugs have been more studied than others, which could bias the results towards drugs with a higher proportion of discovered targets. To evaluate the impact of this research bias, I randomly selected the same number of terms that were used as ageing-related terms 1000 times, and I repeated the statistical analysis. Then, I counted the times the statistically significant drugs appeared on the same or lower ranking. I expected that drugs associated with many terms would rank higher independently of the random set generated.

2.4.4 Enrichment for pro- and anti-longevity drugs

Each drug list generated was ranked by the p-values obtained from the statistical analysis. Then, I transformed the ranking of the drug into a value ranging from 0 to 1. A set of 142 pro-longevity drugs and 30 anti-longevity drugs present in the DrugAge (Build 1) and DrugBank 5.0 databases were used to determine the occurrence and ranking of pro- and anti-longevity compounds in the lists, respectively. The ranking was then scaled into a value between 0 to 1. The AUC between the variables describing the pro-longevity drugs and drugs analysed was calculated using the function AUC from the DescTools package (Version 0.99) (https://cran.r-project.org/package=DescTools). To measure its statistical significance, I calculated the AUC of the lists previously generated to measure the research bias, and I counted the number of simulations with an equal or higher AUC.

2.4.5 Experimental procedure

Worm husbandry and lifespan

N2 and TJ3002 (zSi3002[*hsp-16.2*p::mCherry::*unc-54*; Cbr-*unc-119*(+)]II)] hermaphrodite worms were maintained as previously described (Brenner, 1974) at 20°C on 60 mm NGM plates. Plates were seeded with *Escherichia coli* (OP50) grown overnight in LB media. RNAi was essentially performed as previously described (Kamath et al., 2003) with the slight modifications that bacterial cultures were induced with 5 mM IPTG for 3 hours following overnight growth in LB, and tetracycline was not included in plates or bacterial cultures.

Tanespimycin dose-response test

Tanespimycin (Fisher Scientific) was solubilized in DMSO to stock concentrations of 1, 10, 25, 50, and 100 mM. 1 ml of DMSO or tanespimycin solutions were added to each litre of NGM media just before plate pouring to reach final concentrations of 1, 10, 25, 50, and 100 µM in plates. Plates were kept away from light, stored at 4°C, and used within 2 weeks of pouring. TJ3002 reporter worms were synchronised by bleaching and added to 0.1% DMSO or tanespimycin plates as L1s or as day 1 adults. Worms were transferred to fresh plates every day and then imaged on day 6 of

adulthood using a Zeiss Apotome fluorescent microscope and Hamamatsu Orca Flash 4.0 camera. Brightness and contrast were adjusted linearly, and equally, for all images, using Adobe Photoshop CS6. Fluorescence intensity was measured under different conditions using ImageJ. Significance testing of differences in fluorescence intensity was calculated by one-way ANOVA with Tukey pairwise comparison of groups using GraphPad Prism.

2.4.6 Lifespan assays

Gravid N2 adults were bleached to release eggs, and L1 larvae were allowed to hatch overnight in M9 buffer without food. L1 worms were then added to plates seeded with bacteria expressing an RNAi control vector (L4440) and containing 0.1% DMSO. Worms were added to plates at a density of approximately 50 worms per plate. On the first day of adulthood (50h post-plating L1s), worms were transferred to new 0.1% DMSO plates or 100 µM tanespimycin plates, seeded with L4440 or bacteria expressing dsRNA against *hsp-90* (*hsp-90*(RNAi)). Worms were transferred to fresh plates every day during the first 7 days of adulthood and every other day thereafter. Worms were scored for survival every two days by gently prodding animals repeatedly with a platinum wire. Animals that failed to exhibit signs of movement or pharyngeal pumping were scored as dead. Animals that displayed internal hatching of progeny ("bagging") or prolapse of intestine through the vulva ("rupturing") were censored from our analysis. Median lifespans and significance testing between lifespans of different treatment groups were performed in GraphPad Prism using a Log-rank (Mantel-Cox) test.

2.4.7 Real-time quantitative PCR in *C. elegans*

Approximately 50 worms per treatment group were collected and snap-frozen in 20 µl of M9 buffer, 48 hours after exposure to empty vector control or *hsp-90*(RNAi). Worms were lysed in Trizol reagent and RNA was extracted using a Qiagen RNeasy micro-kit. 1 µg of RNA was used to generate cDNA using BioRad iScript supermix, and real-time quantitative PCR of resulting cDNA (diluted 1:10 with nuclease-free water) was performed using BioRad SsoAdvanced Universal SYBR green supermix and a BioRad

CFX96 Real-time quantitative PCR system. Quantification of relative mRNA levels was performed using the standard curve method and *hsp-90* levels were normalized to the geometric mean of three housekeeping genes (*cdc-42*, *rpb-2*, and *pmp-3*). All kits and master mixes were used as per the manufacturer's instructions. The primers used were as follows:

hsp-90 forward – GACCAGAAACCCAGACGATATC
hsp-90 reverse – GAAGAGCACGGAATTCAAGTTG
cdc-42 forward – TGTCGGTAAAACTTGTCTCCTG
cdc-42 reverse – ATCCTAATGTGTATGGCTCGC
rpb-2 forward – AACTGGTATTGTGGATCAGGTG
rpb-2 reverse – TTTGACCGTGTCGAGATGC
pmp-3 forward – GTTCCCGTGTTCATCACTCAT
pmp-3 reverse – ACACCGTCGAGAAGCTGTAGA

Chapter 3 Prediction of genetic interventions to prolong healthy lifespan

3.1 Introduction

Ageing is a complex and pleiotropic process probably influenced by hundreds of genes. However, experiments in animal models have shown that it can be modulated even by single-gene mutations (Bartke, 2011; Folgueras et al., 2018; Kenyon, 2005; Partridge, 2018; Uno & Nishida, 2016). As almost all drugs are protein inhibitors, these gene mutations serve as a robust starting point to identify new pharmacological interventions to ameliorate ageing. Unfortunately, an assessment of the GenAge database indicates that the number of ageing-associated genes seems to have plateaued (de Magalhães, 2021), despite many still remain untested, especially in mammalian models. Thus, the development of computational methods to detect genetic interventions to prolong healthy lifespan is highly desirable.

In this chapter, I present the result of a study aimed at identifying molecular mechanisms of healthy lifespan and using this information to identifying novel genetic interventions with the potential to modulate ageing. I analysed and compared transcriptomic data from long- and short-lived mutant mice to identify molecular mechanisms associated with lengthening and shortening of healthy lifespan. By further comparing the transcriptomes of mouse models of ageing to the transcriptomic changes observed during normal ageing, I determined which interventions resemble or reverse normal ageing-related changes. Finally, I identified novel genetic interventions with the potential to ameliorate ageing, by comparing all transcriptomic data publicly available from genetic interventions in mice against the changes in gene expression observed in mice with delayed (long-lived), accelerated (short-lived) and normal ageing (ageing-related changes). Most of the work reported in this Chapter has been published as Fuentealba et al., (2021)

3.2 Results

3.2.1 Comparison of the transcriptome of long- and short-lived mutant mice

I first asked if genetic interventions that lengthen or shorten healthy lifespan show characteristic transcriptomic changes, by comparing publicly available microarray and RNA-seq data from 10 long-lived and 8 short-lived mouse models of ageing **(Figure 3.1)**. The data came from 26 independent studies and included samples from adipose, brain, liver and muscle. To avoid potential batch effects, I derived 57 independent datasets using each study, genotype, tissue, sex and age, and performed differential expression analysis **(Supplementary Table 11)**. Then, I calculated Spearman's rank correlation coefficient (r_s) between the gene expression fold changes **(Supplementary Figure 5)**. Given that the transcriptomes from several mutants were measured in multiple studies and within each study in multiple datasets, I averaged the correlations for the same genetic interventions. Since thousands of genes were used to calculate these correlations, even small correlations were statistically significant. To better estimate a threshold of significance for transcriptome-wide correlations, I analysed and correlated 65 transcriptomic datasets covering 51 genetic interventions in mice not previously associated with ageing **(Supplementary Table 12)**. I observed that 5% of the comparisons between the various genetic interventions had an absolute correlation coefficient higher than 0.15 **(Supplementary Figure 6)**. Thus, transcriptome-wide correlations above this value were considered statistically significant for the mutants affecting lifespan **(black squares in Figure 3.2A, C-E).**

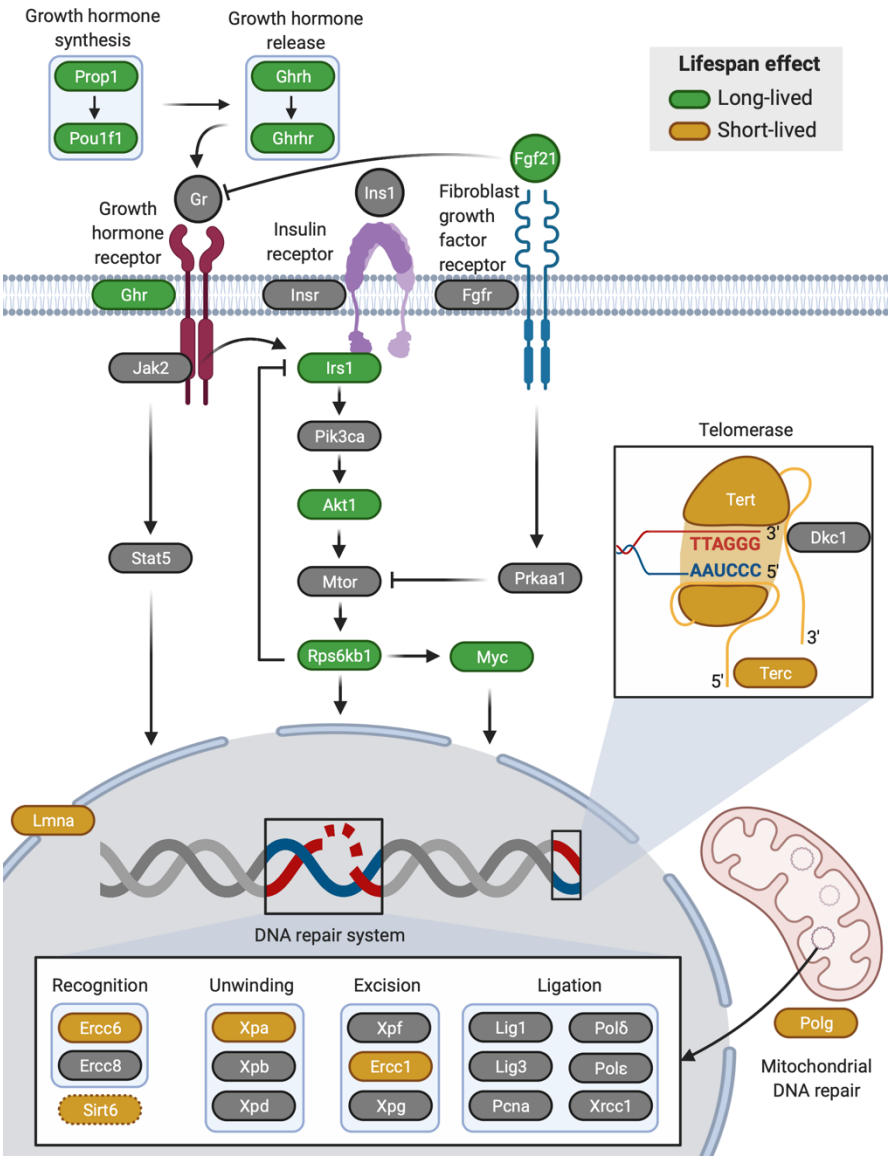


Figure 3.1. *Pathways and processes modulated by the mouse models of ageing with transcriptomic data available. Genes whose mutation lengthen or shorten lifespan are coloured in green and yellow, respectively. Figure created with BioRender.com.*

In the liver, 65% of correlations between interventions with equivalent effects on lifespan (i.e. long-lived vs long-lived and short-lived vs short-lived), were positive, reaching similarities as high as r_s = 0.64 (*Ghr*$^{-/-}$ vs *Pou1f1*$^{dw/dw}$) within long-lived mice and r_s = 0.47 (*Ercc6*$^{m/m}$*Xpa*$^{-/-}$ vs *Ercc1*$^{-/-}$) within short-lived mice **(Figure 3.2A)**. In long-lived mice, mutations in genes controlling the synthesis and release of growth hormone and the growth hormone receptor **(Figure 3.1, top left)** induced remarkably similar transcriptomic changes (mean r_s = 0.33). *Fgf21* over-expression induced a similar transcriptome to the growth-hormone-related mutants, probably due to its well-

known role in inhibiting growth hormone signalling (Inagaki et al., 2008). There were also positive correlations between interventions in the insulin signalling pathway, particularly between *Irs1*$^{-/-}$ and *Rps6kb1*$^{-/-}$ mutants, which is interesting considering that *Rps6kb1* directly phosphorylates and inhibits *Irs1* (Zhang et al., 2008). Although it is well known that growth hormone activates the insulin signalling pathway via *Irs1*, I did not detect any significant correlation between *Irs1*$^{-/-}$ and the growth-hormone-related mutants, possibly because of effects of growth hormone signalling on additional downstream targets to insulin signalling. Heterozygous mutation of *Akt1*, which is involved in insulin signalling, did not induce a similar expression pattern to other long-lived mice, possibly reflecting the pleiotropic effects of *Akt1* function and hence alternative mechanisms to prolong lifespan. Among short-lived models, the highest correlation was observed between interventions in the DNA repair system **(Figure 3.1, bottom)**, including *Ercc6*$^{m/m}$*Xpa*$^{-/-}$, *Ercc1*$^{-/-}$ and *Ercc1*$^{-/d7}$ (mean r_s = 0.24). *Sirt6*$^{-/-}$ showed similarity with *Ercc1*$^{-/d7}$ and *Ercc6*$^{m/m}$*Xpa*$^{-/-}$, which may be explained by the recently discovered function of *Sirt6* as a DNA strand break sensor and activator of the DNA damage response (Onn et al., 2020). The only statistically significant negative correlation I found was between *Tert* and *Sirt6* knockout mice. With some exceptions, these results suggest that mutations in genes whose product participate in the same process or directly interact within the same signalling pathway are more likely to induce similar transcriptomic changes.

Surprisingly, 78% of the correlations between interventions leading to opposite effects on lifespan (i.e. long-lived vs short-lived) were positive, but smaller than interventions with similar lifespan effects. The maximum correlation observed of this type was an unreported one between *Lmna*$^{G609/G609G}$ and *Rps6kb1*$^{-/-}$ (r_s = 0.23). I observed a correlation between interventions in the DNA repair system (i.e. *Ercc6*$^{m/m}$*Xpa*$^{-/-}$, *Ercc1*$^{-/-}$ and *Ercc1*$^{-/d7}$) and mutants of genes regulating the synthesis of growth hormone (i.e. *Pou1f1*$^{dw/dw}$ and *Prop1*$^{df/df}$), which has been previously attributed to similar gene expression patterns on the somatotropic axis and anti-oxidant responses (Schumacher et al., 2008). In summary, although many correlations were positive even when the effects on lifespan were opposite, correlations between interventions with equivalent effects on lifespan were more likely to reach the threshold of significance **(Figure 3.2B)**.

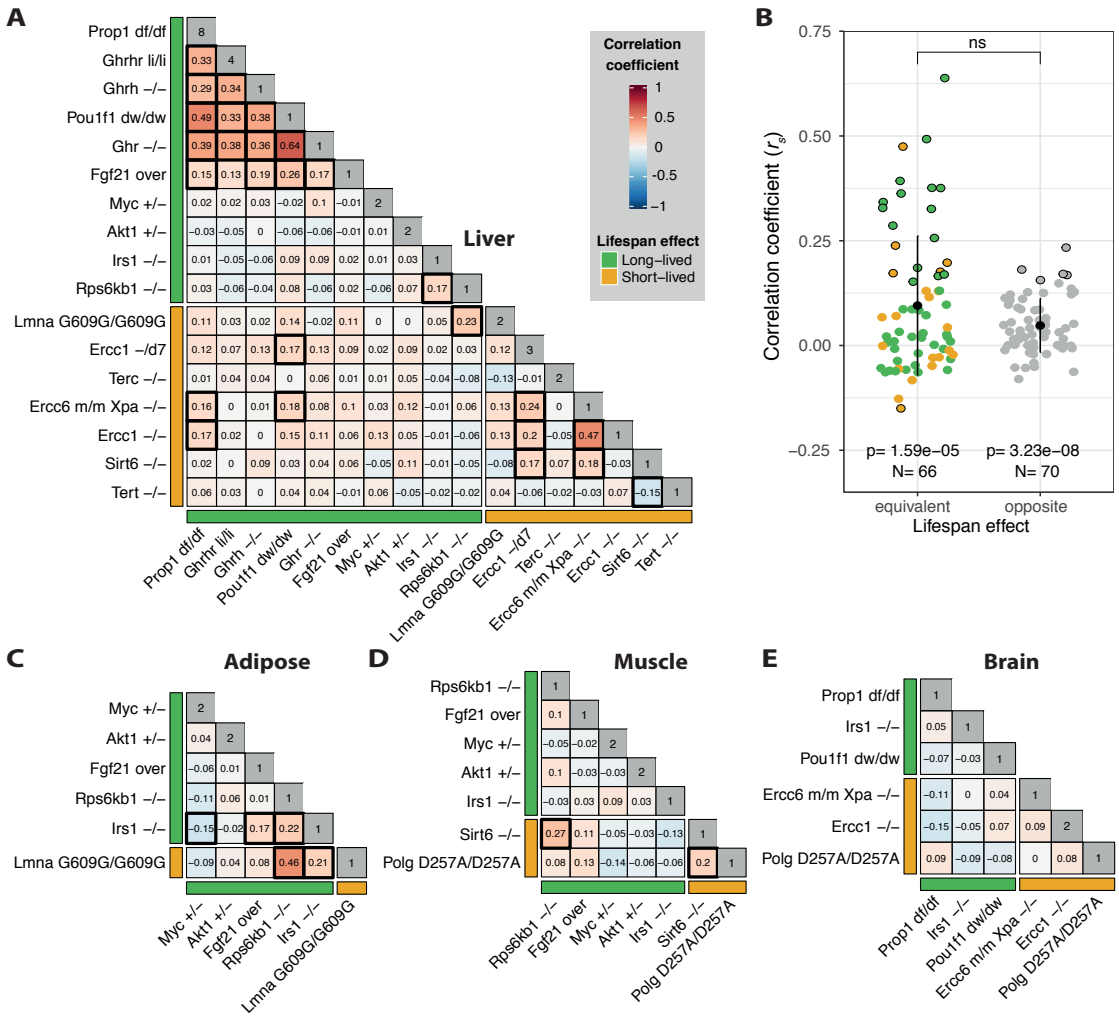


Figure 3.2. *Correlation analysis between the mouse models of ageing. (A) Spearman's rank correlation coefficients between the liver transcriptome of the mouse models of ageing. The intensity of the colours represents the magnitude of the correlations. Bars next to the heatmaps show the effect on lifespan from each intervention. Diagonals show the number of datasets associated with each intervention. Black squares mark statistically significant correlations (i.e.* $|r_s| > 0.15$*). (B) Pairwise correlations between the liver transcriptomes of interventions with equivalent or opposite impact on lifespan. Error bars represent one standard deviation from the mean. P-values below were calculated using a t-test with a population mean equal to zero as the null hypothesis. Statistical significance at the top is for the difference between the groups calculated using unpaired, two-samples Wilcoxon-test. Points circled in black represent statistically significant correlations. Transcriptome correlations between mouse models of ageing in the (C) adipose, (D) muscle and (E) brain. Heatmaps follow the same scheme used in panel A.*

I next assessed if the correlations I observed in the liver were present in other tissues. From the 51 pairwise correlations in the other tissues, 22 (43%) followed the same direction as in the liver, but 18 (35%) were in the opposite direction. The remaining 11 pairwise correlations corresponded to comparisons with the mutant *Polg*$^{D257A/D257A}$, for which there was no data available from the liver. Among the positive and significant correlations observed in the liver (i.e. $r_s > 0.15$) only the comparisons between *Lmna*$^{G609G/G609G}$ vs *Rps6kb1*$^{-/-}$ and *Irs1*$^{-/-}$ vs *Rps6kb1*$^{-/-}$ were significant in other tissues (**Figure 3.2C**, $r_s = 0.46$ and 0.22, respectively). The only negatively correlated interventions in the liver involved *Tert*$^{-/-}$, which was not measured in other tissues. I found tissue-specific correlations that were not significant in the liver, including *Rps6kb1*$^{-/-}$ vs *Sirt6*$^{-/-}$ in the muscle ($r_s = 0.27$) **(Figure 3.2D)** and *Irs1*$^{-/-}$ vs *Lmna*$^{G609G/G609G}$ in the adipose tissue ($r_s = 0.21$). Likewise, some correlations found in the liver were not detected in the brain such as *Pou1f1*$^{dw/dw}$ vs *Prop1*$^{df/df}$ and *Ercc1*$^{-/-}$ vs *Ercc6*$^{m/m}$*Xpa*$^{-/-}$ **(Figure 3.2E)**. This analysis indicates that interventions into ageing may induce tissue-specific effects.

3.2.2 Functional analysis of the transcriptional changes in the mouse models of ageing

I next sought to identify pathways enriched in the gene expression changes observed in the mutant mouse models of ageing. I only employed the liver data for this analysis, as it was the only tissue with enough interventions to identify statistically significant trends. I performed functional enrichment analysis on each dataset using Gene Ontology (GO) terms and a Gene Set Enrichment Analysis (GSEA). To identify gene sets (i.e. GO terms) with consist changes, I calculated their median rank based on enrichment scores across interventions and compared it against a random distribution of median ranks from the same number of interventions.

After multiple testing correction, I identified 470 gene sets in long-lived mice **(Supplementary Table 13)**, and 99 gene sets in short-lived mice showing consistent changes **(Supplementary Table 14)**. Remarkably, 93% of the gene sets found in short-lived mice were down-regulated, whereas 57% of the gene sets identified in long-lived mice were up-regulated. Surprisingly, 58 gene sets showed consistent

changes in both groups of mice **(Figure 3.3A)**, of which 55 were up-regulated in long-lived mice and down-regulated in short-lived mice. These gene sets included 36 biological processes, 10 of which were linked with energy metabolism and 6 with lipid metabolism. I observed the same trends in several processes associated with the metabolism of drugs, nucleic acids, amino acids and carboxylic acid. Consistent with the alteration in energy metabolism, I found similar gene expression patterns in genes forming the mitochondrial membrane and the electron transport chain, as well as genes coding for proteins with NADH dehydrogenase activity. Overall, these transcriptomic changes match well with previous studies in long-lived mice reporting an increase in protein levels and activity of several components of the electron transport system, and increased physiological markers of mitochondrial metabolism (Anderson et al., 2009; Brown-Borg et al., 2012; Westbrook et al., 2009). Also, the increased expression of genes controlling lipid metabolism is biologically meaningful, considering that previous studies have found that long-lived mice use fat as an energy source, instead of carbohydrates (Westbrook et al., 2009).

Based on a leading-edge analysis, I asked which genes contributed the most to the changes in gene expression observed in energy and lipid metabolism, and if some genes were acting as hubs between both sets of processes. As a filter, I selected genes causing the enrichment of more than one process in at least half of the mice. Interestingly, I observed that *Ppargc1a* (Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha), a transcriptional coactivator, was frequently involved in the up-regulation of energy and lipid metabolism in long-lived mice **(Figure 3.3B, red labels)**. Given that cells ectopically expressing *Ppargc1a* display resistance to oxidative stress (St-Pierre et al., 2006; Valle et al., 2005), activation of *Ppargc1a* in long-lived mice may explain why these mice maintain a high activity of the electron transport chain without causing oxidative damage. Remarkably, overexpression of *Ppargc1a* in skeletal muscle extend the lifespan of sedentary mice and led to molecular changes that resemble the patterns observed in skeletal muscle from younger mice (Garcia et al., 2018). Also, tissue-specific overexpression of the *Drosophila* orthologue (*dPGC-1/spargel*) in progenitor and stem cells within the digestive tract extends lifespan and delay the onset of ageing-related pathologies in the intestine (Rera et al., 2011).

Also involved in the up-regulation of energy and lipid metabolism I found *Mif* (Macrophage migration inhibitory factor), a cytokine whose increased expression has been noticed not only in long-lived dwarf mice but also in caloric and methionine restricted mice (Miller et al. 2002; Miller et al. 2005). In short-lived mice, *Aldh5a1* (aldehyde dehydrogenase family 5) and *Idh1* (isocitrate dehydrogenase 1) were down-regulated and involved in several processes associated with energy and lipid metabolism **(Figure 3.3C, red labels)**. Consistently, mice with mutations in these genes display premature death and increased oxidative stress (Hogema et al., 2001; Itsumi et al., 2015; Latini et al., 2007).

I further compared the genes leading the regulation of energy and lipid metabolism in long- and short-lived mice **(Figure 3.3B-C)** and I identified 5 genes in common: cytochrome c oxidase subunit 5A (*Cox5a*), cytochrome c-1 (*Cyc1*), NADH:ubiquinone oxidoreductase subunit B10 (*Ndufb10*), NADH:ubiquinone oxidoreductase core subunit V2 (*Ndufv2*) and ubiquinol-cytochrome c reductase (*Uqcrfs1*). I directly compared their normalised fold change in long- and short-lived mice using an unpaired two-sample Wilcoxon test. The expression of all 5 genes was regulated in opposite directions between both groups of mice and the differences were statistically significant ($p < 0.05$) **(Supplementary Figure 7)**.

In short-lived mice, most of the differentially expressed processes were down-regulated and linked with the mitochondria, which may indicate mtDNA damage. To probe this idea, I analysed the gene sets enriched in polymerase γ mutant mice (*Polg*$^{D257A/D257A}$), which display a 2500-fold increase in mtDNA mutations compared to wild-type mice (Khrapko & Vijg, 2007). Indeed, I observed that in muscle and brain, the gene sets in Figure 2A were strongly down-regulated, showing that short-lived mice induce a transcriptomic signature matching that of mutant mice with exacerbated mtDNA mutations **(Supplementary Figure 8)**.

I identified gene sets changing expression specifically in long- or short-lived mice. In long-lived mice, the top down-regulated process was B-cell mediated immunity, while the most up-regulated process was carboxylic acid catabolism **(Supplementary Table 13)**. Also, in long-lived mice, consistent with the up-regulation of the electron transport chain, I observed an up-regulation of processes linked with ATP synthesis

(Supplementary Figure 9). This result is in line with a previous study reporting an increase in ATPase activity in long-lived mice (Choksi et al., 2011). Similarly, I observed up-regulation of expression of genes involved in thermogenesis, another process activated by *Ppargc1a* in response to cold exposure (Gill & La Merrill, 2017) (Puigserver et al., 1998). Thus, *Ppargc1a* may be activated by the reduced core body temperature typical of long-lived dwarf mice due to a higher body surface to body mass ratio (Ferguson et al., 2007; Hauck et al., 2001; Hunter et al., 1999). Another well-recognised stimulator of oxidative metabolism and ATP production is calcium (Glancy et al., 2013; Griffiths & Rutter, 2009; Tarasov et al., 2012). Consistently, I observed an up-regulation of several genes involved in calcium homeostasis. Unfortunately, there is currently no evidence of the effect on lifespan of calcium treatment in mammals. Among the down-regulated gene sets in long-lived mice, I observed several linked with the response to endoplasmic reticulum (ER) stress, including the unfolded protein response (UPR) **(Supplementary Figure 10)**. This down-regulation may reflect lower levels of ER stress, as observed in long-lived *daf-2(e1370)* worms and under caloric restriction (Henis-Korenblit et al., 2010; Matai et al., 2019). In short-lived mice, the most down-regulated process was carboxylic acid catabolism, and the most up-regulated process was immune response activating signal transduction **(Supplementary Table 14)**. Overall, I only identified two additional groups of gene sets down-regulated, and they were related to nucleic acid metabolism and biosynthesis **(Supplementary Figure 11)**. In line with this observation, previous studies have shown a correlation between mitochondrial dysfunction and aberrant biosynthesis of nucleotides (Desler et al., 2007).

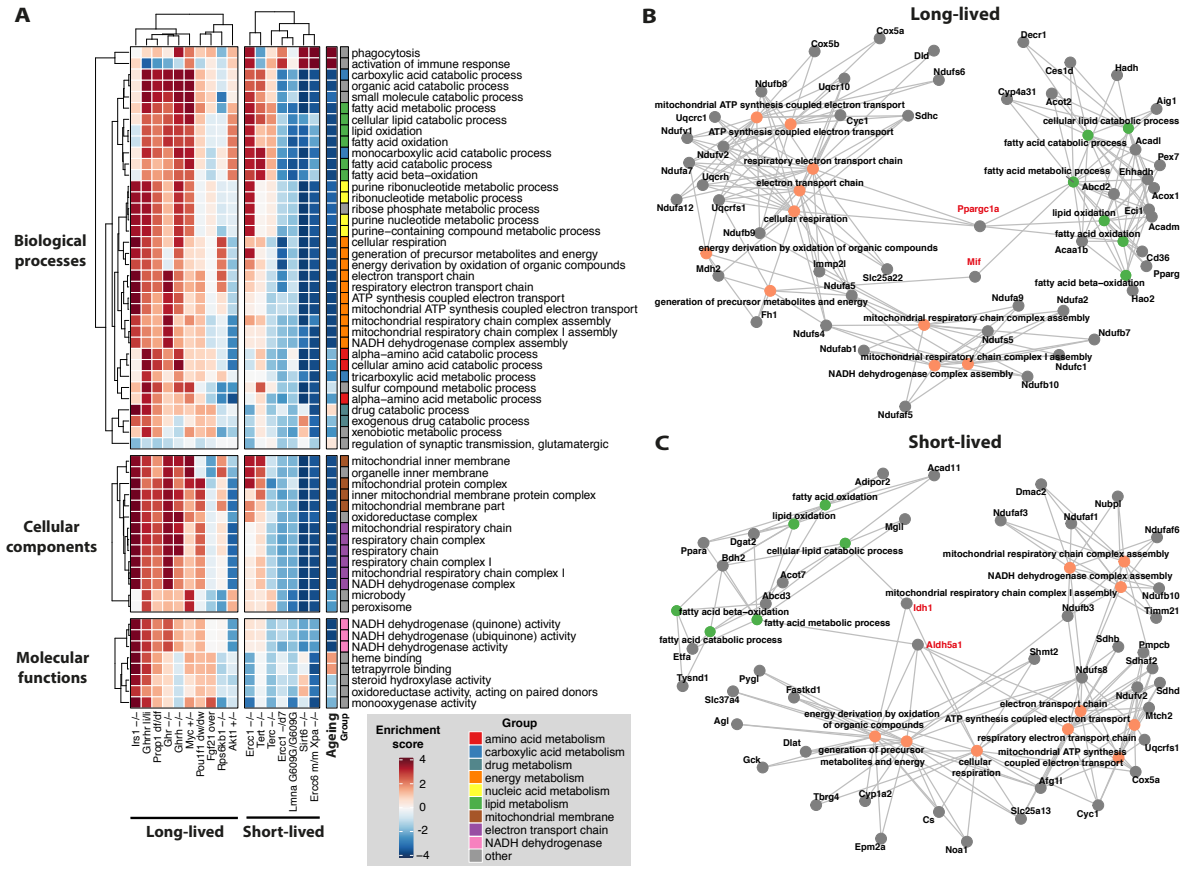


Figure 3.3. *Gene expression trends conserved in the mouse models of ageing.* (A) *Gene sets showing consistent changes in long- and short-lived mice (FDR < 0.05). The heatmap colours represent the statistical significance of the enrichment and the direction of the change. The 'Ageing' column represents the enrichment scores associated with the transcriptomic changes during ageing. The 'Group' column indicates different groups of gene sets with similar function. Genes regulating biological processes linked with energy and lipid metabolism in (B) long-lived and (C) short-lived mice. Labelled in red are genes involved in both sets of processes. Nodes representing genes are coloured in grey, and nodes from biological processes are coloured based on the groups in panel A.*

3.2.3 Interventions that shorten lifespan resemble the ageing transcriptome

I next compared the transcript profiles of the long-and short-lived mutant mice with profiles characteristic of normal ageing in multiple tissues. I performed functional enrichment analysis using age-related genes from wild-type mice. Changes during normal ageing resembled mostly the transcriptomes of the short-lived mouse models

(Figure 3.3A, 'Ageing' column). To test if these similarities existed at the gene level, I calculated the transcriptome-wide correlations between each mouse model of ageing and the ageing transcriptome. On average, I observed a positive and statistically significant correlation between the transcriptomes of short-lived mice and the changes during ageing (**Figure 3.4, left panel)**, while interventions that lengthened life displayed a correlation close to zero (mean r_s = 0.005). I further analysed the correlations at the pathway level using enrichment scores and obtained a similar result **(Figure 3.4, right panel)**. Overall, this analysis supports the hypothesis that accelerated ageing models reproduced partially the molecular changes observed during normal ageing.

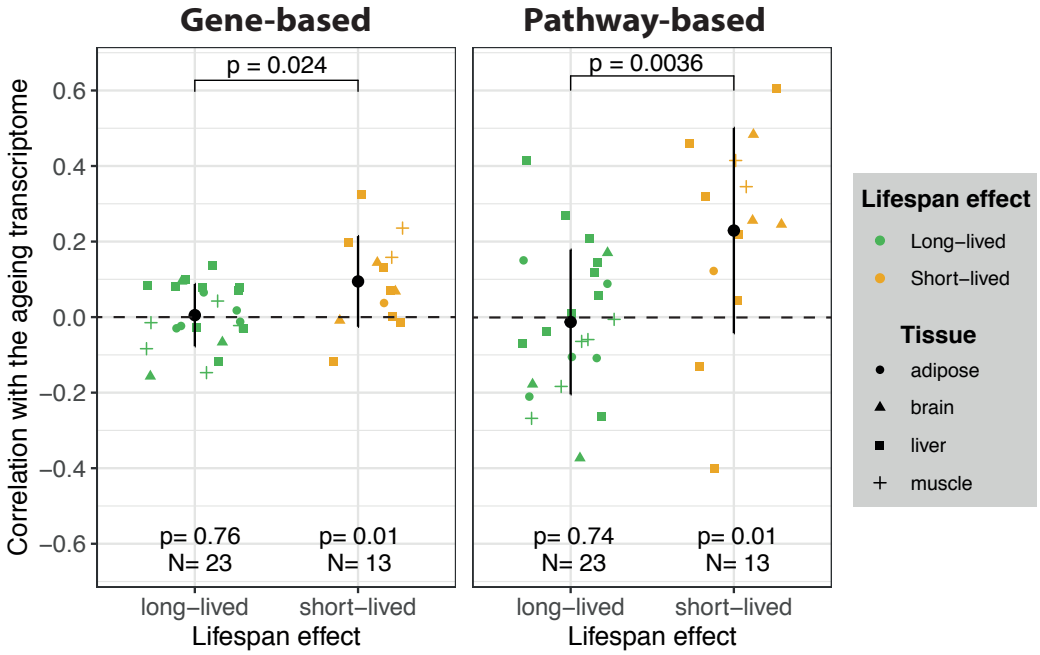


Figure 3.4. *Gene and pathway-based correlations between the transcriptome of ageing-related interventions and that induced by ageing. Each point represents one intervention, and the shapes indicate the tissue from which the transcriptome was derived. Transcriptomic changes in the mouse models of ageing were compared with the changes during ageing on the same tissue. Error bars show one standard deviation from the mean. P-values below were calculated using a t-test with a population mean equal to zero as the null hypothesis. P-values at the top are for the difference between the groups using an unpaired, two-samples Wilcoxon-test.*

3.2.4 Identification of genetic interventions affecting lifespan

I next investigated the use of gene sets consistently associated with lifespan **(Figure 3.3A)** to identify other genetic interventions that could affect ageing. Using publicly available datasets for other mouse mutants I examined their correlation with the transcriptomes of long-lived mice, short-lived mice and normal ageing **(Figure 3.5)**. I predict that genetic interventions more positively correlated with long-lived mice will lengthen lifespan, whereas mutants more strongly correlated with short-lived mice and ageing will shorten lifespan. From the 51 gene mutants analysed, 23 showed a higher correlation with long-lived mice and 28 a more positive correlation with short-lived mice. Among the 27 mutants with a positive correlation with the ageing transcriptome, 21 (77%) were positively correlated with short-lived mice, and 18 (66%) were negatively correlated with long-lived mice. Similarly, from the 24 mutants negatively correlated with ageing, 14 (58%) showed the same trends in short-lived mice, and 21 (87%) displayed the opposite trend in long-lived mice.

To evaluate the robustness of our correlation approach, I investigated whether the mutants were previously associated with changes in lifespan in the literature and the GenAge database (De Magalhães & Toussaint, 2004). I also determined if mutations in the orthologues of these genes in *C. elegans* and *D. melanogaster* showed effects on lifespan. Among the 51 gene mutants analysed, I found experimental evidence matching our predictions in 9 cases (Fisher's exact test $p = 0.017$). All genetic interventions with experimental evidence to lengthen lifespan showed a more strongly positive correlation of changes in gene expression with those seen in long-lived mice. For instance, the transcriptome of *Jak2* knockout mice showed an average $r_s = 0.18$ with long-lived mice, but a correlation close to zero against short-lived mice ($r_s = 0.002$). Consistently, fruit flies with a loss of function mutations in the *hop* gene (orthologue of *Jak2*) live on average 17% longer than wild-type flies (Larson et al., 2012). Similarly, transcriptomic changes induced by *Keap1* knockout in mice were positively correlated with long-lived mice ($r_s = 0.17$) and negatively correlated with short-lived mice ($r_s = -0.13$). As expected, *keap1* loss of function mutations extends lifespan of fruit flies by 8-10% (Sykiotis & Bohmann, 2008). The transcriptome of *Ahr* and *Dbi* knockout mice displayed a positive correlation with long-lived mice and a negative correlation with short-lived mice and ageing. Confirming our predictions, *C.*

elegans carrying a loss of function allele of *ahr-1* (*Ahr* in mice), display extended lifespan and increase motility and stress resistance (Eckers et al., 2016). Similarly in worms, knockdown of either *acpb-1* or *acbp-3* (*Dbi* in mice), extends lifespan (Shamalnasab et al., 2017). Among the interventions with a more positive correlation with short-lived mice and ageing, I found evidence of premature death in 6 cases, including *Sirt7*, *Dicer1*, *Pdss2*, *Rb1* and *Sgpl1* knockout mice (Frezzetti et al., 2011; Lin et al., 2011; Lyon & Hulse, 1971; Schmahl et al., 2007; Vakhrusheva et al., 2008). The negative effects on lifespan of *Dicer1* and *Sgpl1* have been also shown in *C. elegans*, as loss of *dcr-1* (*Dicer1* in mice) shorten maximum lifespan by 20% (Mori et al., 2012) and RNAi knockdown of *spl-1* (*Sgpl1* in mice), reduces median lifespan by 22% (Samuelson et al., 2007). Overall, the experimental evidence matching with our predictions supports the use of gene sets describing mitochondrial metabolism to predict the effects of genetic interventions on lifespan.

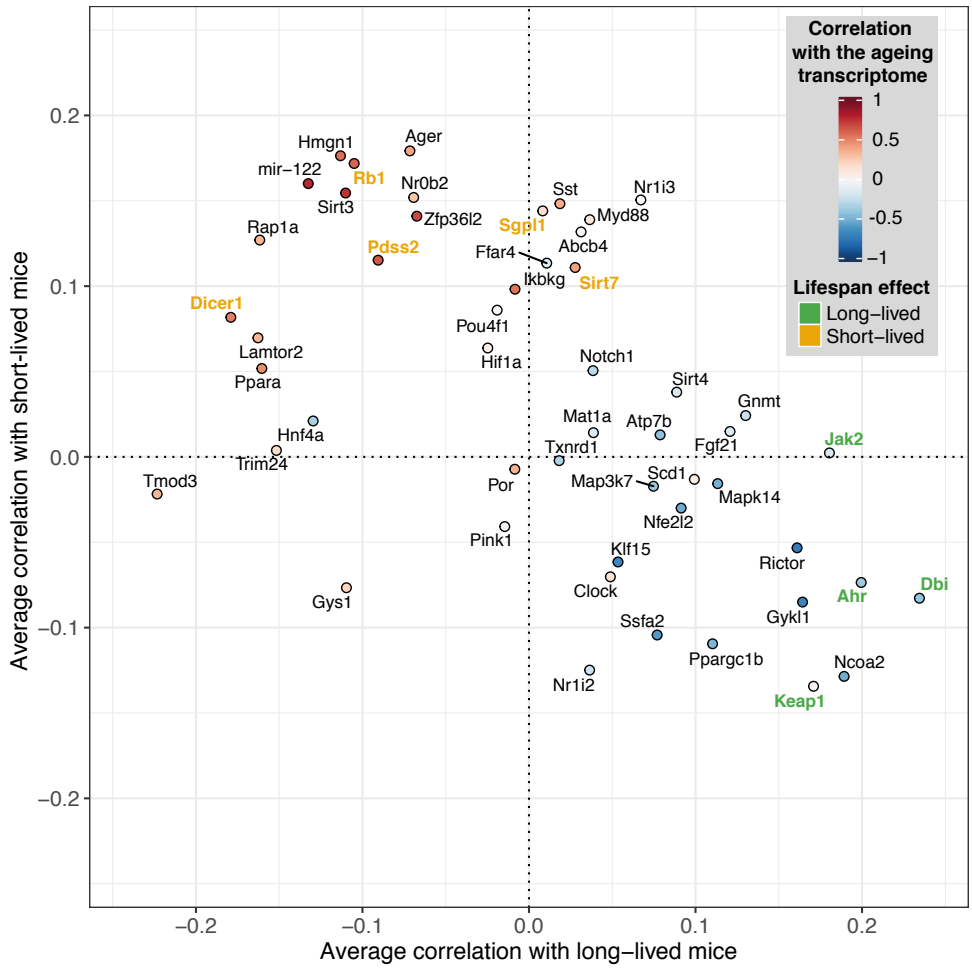


Figure 3.5. *Correlation between the transcriptomes of long- and short-lived mice and that induced by 51 different gene knockouts. Correlations were calculated using*

enrichments scores of the gene sets in Figure 3A. The colours of the dots represent the correlations with the ageing transcriptome. Labels represent the name of the gene knocked out. Gene knockouts known to lengthen or shorten lifespan in animal models are coloured in green and yellow, respectively.

Finally, using the DrugBank database (Law et al., 2014), I searched for drug targeting the human orthologues of the 16 gene knockouts with a positive correlation with long-lived mice and negative correlation with short-lived mice. As most drugs are protein inhibitors, these may represent potential geroprotectors that mimic the effects of knocking out the same target. I found 31 compounds associated with 7 knocked out genes **(Table 3.1).** From these compounds, 5 have been associated with lifespan extension in animal models (Fisher's exact test p = 0.01), including spermidine, vitamin E, estradiol, rifampicin and genistein. Notably, 2 of these compounds have already proven to be geroprotective in mice. Spermidine-treated wild-type mice live up to 25% longer and display delayed age-related decline in cardiovascular function (Eisenberg et al., 2016; Yue et al., 2017), while the median lifespan of vitamin E supplemented mice is 15% greater than of controls (Selman et al., 2008).

Table 3.1. *Drugs targeting the genes involved in the candidate genetic interventions affecting lifespan. Human orthologues from the mouse genes were obtained using WORMHOLE (http://wormhole.jax.org). Drugs labelled in bold are known geroprotectors.*

Mouse gene	Human gene	Drugs
Ahr	*AHR*	Mexiletine, Nimodipine, Flutamide, Atorvastatin, Leflunomide, Ginseng
Dbi	*DBI*	Coenzyme A, Hexadecanal
Keap1	*KEAP1*	Dimethyl fumarate
Mapk14	*MAPK14*	KC706, SCIO-469, VX-702
Ncoa2	*NCOA2*	**Genistein**, Methyltrienolone
Nr1i2	*NR1I2*	**Vitamin E**, **Rifampicin**, **Estradiol**, Erlotinib, Estradiol, Ethinyl Rifaximin, Paclitaxel, Docetaxel, Prasterone, Hyperforin, SR12813, Rilpivirine
Txnrd1	*TXNRD1*	**Spermidine**, Flavin adenine dinucleotide, Motexafin gadolinium, Arsenic trioxide, PX-12

3.3 Discussion

In this study, I collected and analysed publicly available microarray and RNA-seq data of 18 interventions that affect ageing and cause changes in lifespan, together with transcript profiles of normal ageing. The transcriptomes were more similar between interventions with the equivalent effects on lifespan, especially if they targeted the same pathway. I detected positive, but weaker, correlations between interventions with opposite effects on lifespan, in line with previous studies (Schumacher et al., 2008). The biggest correlation found in this case was an undiscovered one between *Lmna*$^{G609G/G609G}$ and *Rps6kb1*$^{-/-}$, in the liver and adipose tissue. Interventions like *Akt1*$^{+/-}$ (long-lived), *Myc*$^{+/-}$ (long-lived) and *Terc*$^{-/-}$ (short-lived) did not produce changes in gene expression that correlated with those from other interventions, suggesting the existence of different mechanisms to lengthen and shorten lifespan.

Based on functional enrichment analysis, I identified 58 gene sets (i.e. GO terms), which behaved consistently and showed opposite changes in gene expression in long- and short-lived mouse models. The data implicated mitochondrial metabolism as a key determinant of healthy lifespan. As in short-lived mice, I detected a transcriptomic down-regulation of mitochondrial metabolism with age in wild-type mice, confirming its relevance for normal ageing and supporting the hypothesis that models of accelerated ageing approximate normal ageing at the molecular level, as has been previously proposed (Burtner & Kennedy, 2010; C. Y. Liao & Kennedy, 2014).

Finally, comparing the gene sets associated with lifespan and ageing with those changing expression in mouse mutants with no known association with ageing, I found 16 gene knockouts that were positively correlated with expression changes in long-lived mice and negatively correlated with expression changes in short-lived mice. Our predictions, therefore, encourage future ageing studies on these mutant mice, specially *Keap1*, *Ahr* and *Dbi* knockouts, which already showed experimental evidence in *C. elegans* and *D. melanogaster* (Eckers et al., 2016; Shamalnasab et al., 2017; Sykiotis & Bohmann, 2008), as well as experiments with the drugs that prolong lifespan in invertebrate models and target the knocked out genes.

3.3.1 Limitations

An inherent limitation of the study is that is biased towards interventions in the nutrient-sensing pathways (long-lived mice) or the DNA repair system (short-lived mice), which have been more extensively studied. However, this allowed us to determine that genomic instability leads to mitochondrial dysfunction, and that deregulation of the nutrient sensing pathways improves mitochondrial function. To develop a complete picture of the interconnections between the hallmarks of ageing, transcriptomic data from interventions targeting other hallmarks of ageing are required. Most of our observations are extracted from the liver transcriptome because it was the only tissue with enough transcriptome data to identify robust trends across interventions. However, as I reported in Section 3.2.1, genetic interventions may have tissue-specific effects, meaning that the comparison of transcriptomic profiles is likely to be useful only when is done in the same tissue or cell type. Thus, further research in other tissues is necessary to determine the level of conservation of the mechanisms I found associated with lifespan. Finally, our analysis suggests the mitochondria is likely to have a primary role in determining lifespan, however, changes in mitochondrial genes at the expression level do not necessarily allow us to infer changes at the level of mitochondrial function nor establishing that these transcriptomic changes are causal for changes in lifespan.

3.4 Methods

3.4.1 Transcriptomic dataset collection

I obtained a list of short-lived and long-lived mouse models of ageing from Folgueras et al. (2018). Then, using the gene symbol of each mutant I searched in the meta-databases OmicsDI 1.0 (Perez-Riverol et al., 2017) and All of gene expression (Bono, 2020) for microarray and RNA-seq datasets from mice. Using the Mouse Genome Database (version 6.17) (Bult et al., 2019), I only included studies with raw data available where the genotype of the wild-type matched the genotype of the mouse model of ageing and where multiple samples per condition were available. I focused on datasets from liver, adipose, muscle and brain as these were the most common tissues among the datasets I found. The samples in each study were grouped into

datasets with the same sex, age and tissue, resulting in 57 datasets from 26 studies that were processed separately **(Supplementary Table 11)**. To investigate the gene expression changes during ageing, I used the dataset with the largest sample size for each tissue **(Supplementary Table 15)**.

3.4.2 Differential expression analysis

For microarrays, I obtained the raw data from the Gene Expression Omnibus (GEO) and Array Express database using the R packages GEOquery (version 3.13) (Sean & Meltzer, 2007) and ArrayExpress (version 1.52) (Kauffmann et al., 2009), respectively. Affymetrix array data were processed using the RMA algorithm from the package oligo (Carvalho & Irizarry, 2010) to perform background correction, quantile normalisation and summarization. I carry out the summarization using the core genes for exon and gene arrays. For Illumina and Agilent microarrays, I employed background correction and quantile normalisation using the limma package (version 3.4) (Ritchie et al., 2015). I removed probes mapping to multiple genes and kept the probe with the highest average expression across samples if multiple probes mapped to one gene. Then, I fitted a linear model of gene expression versus genotype for each gene and calculated the summary statistics using empirical Bayes. P-values were adjusted for multiple testing using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995) to obtain the false discovery rate. I calculated the gene expression changes of age in the same way, with the difference that chronological age was used as the independent variable instead of genotype.

RNA-seq reads were obtained from the Sequence Read Archive (Leinonen et al., 2011). I mapped raw reads to the mouse genome sequence (GRCm38.p6) using STAR (version 2.7) (Dobin et al., 2013) and counted mapped reads using featureCounts from the package Rsubread (version 2.6) (Liao et al., 2019). I removed genes with less than 10 counts on average across samples to reduce memory usage and increase data processing speed as recommended by DESeq2 manual (Love et al., 2017). Later evaluation of the influence of this pre-filtering showed no major effect on the results **(Supplementary Figure 12-13)**. Differential expression analysis was

then performed using DESeq2 (version 1.32) (Love et al., 2014) controlling the false discovery rate using the Benjamini-Hochberg method.

I further performed a principal component analysis on each dataset using the normalised gene expression values **(Supplementary Figure 14)** and did not observe any sample with outlier levels of expression sufficient to warrant exclusion from the analysis. I examine which experimental variables (i.e. study, genotype, tissue, sex, age, lifespan effect, technology) accounted for the transcriptional differences between the datasets. I performed a principal component analysis of the quantile normalised fold changes of 4074 genes detected across all 57 datasets **(Supplementary Figure 15)** and then tested which variables explained the differences observed in the first two principal components, applying a multivariate analysis of variance (MANOVA). As expected, datasets from the same study, genotype and tissue correlated more than expected by chance **(Supplementary Table 16)**.

To obtain gene expression data from genetic interventions not previously associated with ageing, I downloaded the metadata from all single gene perturbation signatures for mice from the CREEDS database (version 1.0) (http://amp.pharm.mssm.edu/CREEDS/#downloads)(Z. Wang et al., 2016). I manually removed interventions not coming from mouse liver or already included in our study. The remaining interventions were processed with GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/).

3.4.3 Transcriptome-wide correlation analysis

Using the log2 fold changes from the differential expression analysis, I calculated the correlation between the datasets using Spearman's rank method (Spearman, 1904) considering genes in common between the pair of datasets. On interventions with multiple datasets, I averaged the correlations first between datasets from the same study and intervention, and then with the same intervention across different studies. Heatmaps visualising correlations were created using ComplexHeatmap (version 2.7) (Gu et al., 2016).

3.4.4 Functional enrichment and consistency analysis

I performed functional enrichment analysis of each dataset separately using the function gseGO from the package ClusterProfiler (version 4.0) (Yu et al., 2012) on genes ranked by the sign of the log2 fold changes multiplied by the logarithm of the p-values from the differential expression analysis. Based on this rank-ordered list of genes, I further assessed if genes in each gene ontology term were more likely to be up or down-regulated than what is expected by chance based on 10000 permutations. I calculated an enrichment score for each pathway and each intervention by multiplying the log10(p-value) by 1 or -1 depending on the direction of the change. To identify GO terms with consistent changes, I calculated for each GO term the median rank of the enrichment score across short-lived or long-lived mice and I compare it against a random distribution of median ranks with the same number of interventions. P-values were obtained by dividing the observed median rank by the total number of random median ranks generated (i.e. $1e^6$). I adjusted the p-values for multiple testing using the Benjamini-Hochberg method.

As with the gene expression values**, I** tested which experimental variables explained the differences between the datasets using enrichment scores. In contrast to gene expression values, variation in enrichment scores was best explained by the effect on lifespan of the interventions **(Supplementary Table 17)**.

To visualise gene ontology terms specific to long- or short-lived mice, I calculated the similarity between the consistently varying terms using the overlap coefficient (oc). I constructed a network connecting the terms (nodes) where edges represent pairs of terms with an oc > 0.4. Clusters with less than 5 nodes were excluded.

$oc = \frac{|A \cap B|}{\min(|A|,|B|)}$, where A and B represent gene ontology term

Chapter 4 General discussion and concluding remarks

In this thesis, I aimed to identify genetic and pharmacological interventions to target ageing through computational studies. In this last chapter, I discuss the major findings of the studies in the context of ageing research and give a perspective on how to move the field forward considering our results.

Several methods designed to identify ageing-related genes have observed that one particular property of ageing-genes is to target other genes linked with ageing (Feng et al., 2012; Kerepesi et al., 2018; Li et al., 2010; Song et al., 2012). Taking this feature into account I evaluated if drugs that also target multiple ageing-related genes were also more likely to affect ageing, and more importantly if we could use this strategy to find drugs to target human ageing. Indeed, I discovered that this strategy is suitable to find geroprotectors as drugs targeting a significant proportion of human ageing-genes were more likely to have geroprotective properties than what is expected by chance.

Previous drug repurposing studies on Rheumatoid arthritis, Parkinson's disease and Alzheimer's disease have shown that the performance of this type of strategy can be greatly improved when the comparisons are made at higher levels of biological action (Issa et al., 2016; Tung, 2015). I tested and showed that this was also the case of ageing, displaying a greater enrichment for geroprotectors, particularly when biological processes are used as a comparator. That the effects on ageing are driven at the pathway level rather than the gene level is meaningful as genes within the same pathway tend to interact more often and ageing-genes interact with other ageing-genes. I also confirmed this in chapter 3, as genetic interventions within the same induce remarkable similar transcriptomic changes. Thus, genetic and pharmacological interventions in genes within ageing pathways are likely to affect ageing.

I also evaluated if the mechanisms of the candidate drugs were similar pointing to a central mechanism to regulate ageing. However, as the hallmarks of ageing suggest, I found different mechanisms to modulate ageing when I used drug-protein information and also gene expression as a comparator. This suggests that multiple pathways will be required to be targeted simultaneously to substantially ameliorate ageing,

especially in the case of pharmacological interventions which produce smaller effects in lifespan and healthspan than genetic interventions. Actually, recent studies have shown that the use of drugs targeting distinct subsets of the ageing-related pathways, complement one another and delay ageing with a higher potential compared to single-drug treatments (Admasu et al., 2018; Castillo-Quan et al., 2019).

One caveat of the drug repurposing methods for ageing developed so far is that they only evaluate the top candidates (reviewed in Dönertaş et al., 2019), however, it is possible that despite finding geroprotectors among the top candidates, most geroprotectors are negatively selected by the method. Considering this possibility, I measured the enrichment of our ranked list of compounds with respect to all known geroprotectors. This simple analysis can be used by other researchers to compare the performance of the drug repurposing methods for ageing.

In addition to validate our candidate drugs theoretically based on previous evidence, I also select the top-ranked compound tanespimycin to perform experimental testing in *C. elegans*. For the first time, we showed that mild inhibition of *HSP-90* with tanespimycin induce a stress response that triggers cytoprotective mechanisms and that stronger inhibition using RNA interference causes a reduced lifespan in agreement with previous studies (Somogyvári et al., 2018). This confirms the previous idea that a little stress may be good for cellular health and longevity (Labbadia et al., 2017), and more importantly, that we can trigger this hormetic response using drugs. Interestingly, we also observed a dose-dependent activation of *hsp-16.2*, a gene whose expression has been proposed as a biomarker of lifespan and thermotolerance (Mendenhall et al., 2012). Thus, considering that we observed a maximum activation at the concentration that the lifespan was performed, it is possible that higher concentrations could cause a higher lifespan extension.

Remarkably, three months after our publication, another drug-repurposing study based on human gene expression data, proposed tanespimycin as a candidate geroprotector and confirmed its effect on *C. elegans* lifespan (Janssens et al., 2019). Shortly after, a post-doctoral fellow working in the Partridge Laboratory at Max-Plank Institute for Biology of Ageing, tested the effect of tanespimycin in *D. melanogaster* and observed that it prolongs lifespan but only in female flies **(Supplementary Figure**

16) (Unpublished). Interestingly, in the mouse models of ageing with lengthened lifespan, I observed a common down-regulation of biological processes linked with the heat shock response. Given that I observed that activation of these processes causes an extension lifespan, it is likely that the changes observed in long-lived mice are the consequence of lower proteostatic stress levels than normal mice.

Another way that could indicate that our candidate drugs are meaningful is to determine if they have been predicted previously by other methods. Indeed, we found that our method was the best re-discovering drugs prioritized by multiple methods, with 7 of these drugs among the top 10 candidates. Tanespimycin is one of the few drugs predicted to delay ageing by multiple drug-repurposing studies. Thus, its experimental validation shows the robustness of the various methods to predict geroprotectors. However, no major focus has been attempted by the scientific community in testing the candidate drugs proposed by multiple studies, which is surprising considering the exponential development of drugs to target ageing (de Magalhães, 2021).

I revealed different mechanisms of ageing even within interventions with similar effects in lifespan, and I also found that the molecular changes that these interventions cause are different depending on the tissue. This complexity suggests that while some interventions may have beneficial effects in delaying ageing in some tissues, they may be detrimental for others.

Typically, studies aiming to identify the molecular mechanism of ageing search for molecular changes in long-lived or short-lived animal models that are not visible in normal strains. However, most researchers assume that these changes are beneficial (in long-lived models) or detrimental (in short-lived models), neglecting the possibility that they might be just the cellular response to the perturbation, a common phenomenon observed across a variety of biological conditions (Powers et al., 2018). A more robust strategy is to compare mouse models with different rates of ageing, for example searching the molecular mechanism observed during normal ageing and also in accelerated ageing mice but reversed in mice with delayed ageing. By doing this, I found that metabolic pathways associated with the mitochondria were the only ones with these properties. This result opens the possibility to use the expression of

mitochondrial genes as biomarkers of lifespan, which can contribute to significantly reduce time and cost associated with the identification of new geroprotectors.

Since the discovery of mouse models with accelerated ageing phenotypes (Weeda et al., 1997), researchers have wondered if these mice truly reproduce the effects of ageing (Burtner & Kennedy, 2010; C. Y. Liao & Kennedy, 2014). Confirming this idea, I found that as during normal ageing, mouse models with accelerated ageing are likely to display mitochondrial dysfunction, a hallmark of ageing. In contrast, enhanced mitochondrial maintenance was not a unique characteristic of long-lived strains, as they also showed changes in proteostasis and the immune response. However, this result needs to be taken with caution, as the higher number of processes associated with long-lived could be due a higher number of datasets involved, which increases the power to detect conserved changes. In recent years, researchers have claimed that the hallmarks of ageing require revision, as new studies have implicated other processes in ageing (Bhadra et al., 2020; Knupp & Miura, 2018). In our study, I found support for the relevance on ageing of processes such as ion transport, glycosylation, gene silencing and thermogenesis.

Besides the transcriptomic data from mouse models of ageing, there are several datasets of gene knockouts in mice. I used these datasets to determine which other genetic interventions resemble the transcriptomic changes of long-lived mice and could have the potential to prolong lifespan. Remarkably, I discovered that 6 of the gene knockouts that resemble short-lived models and ageing display premature death. Unfortunately, gene knockouts similar to long-lived mice have not been tested experimentally in lifespan assays, but down-regulation of several of the orthologues in animal models have shown to be geroprotective. Also, several of the drugs that target these genes have shown geroprotective properties, among which I found erlotinib, a drug also proposed by our first drug-repurposing analysis.

4.1 Future perspectives

Nowadays the drug-protein interactome is a large data source but far from completion. Thus, further research is needed to increase the drug-protein interaction data using high-throughput technologies like those currently available from kinases (Sorgenfrei et al., 2018). This will not only improve the robustness of the first method presented here but also promote the development of machine learning methods that are more suitable for prediction tasks. In this regard, it is also essential to encourage ageing researchers and scientific journals to publish results from drugs causing negative or no effect on lifespan, in order to have a balanced positive and negative set of drugs to evaluate the repurposing methods and construct better machine learning based methods. The method can also be improved by increasing the reliability of the drug-protein interaction database, yet this requires the complex task of manually identify the sources prone to include false positives and recalculate the confidence scores. It may be also possible to improve the enrichment for geroprotectors by using a different method to calculate the similarity between gene sets such as the Jaccard index or the Pearson's correlation which have produced meaningful results in other contexts (Bass et al., 2013). Also, the method could be potentially improved by using a different strategy to combine the results from the enrichment analysis, such as validated rank-based meta-analysis (Kolde et al., 2012), which may be less biased by the number of datasets involved.

So far, there is experimental evidence validating the geroprotective properties of tanespimycin in worms and flies. Thus, the next step is to test if tanespimycin prolongs survival in healthy mice. If this is the case, there is solid evidence to perform a short safety trial in humans. Based on previous clinical trials with tanespimycin, it is known that it only causes mild side effects at biologically active doses and that the side-effects are associated with the vehicle (i.e dimethyl sulfoxide *alias* DMSO) rather than off-target effects of the drug (Erlichman, 2009). Thus, it may be appropriate to investigate the possibility to reduce the side effects even further by using alternative delivery methods, as micellar nanocarriers previously developed for the delivery of tanespimycin (Katragadda et al., 2013; Xiong et al., 2009).

Also, the analysis presented in this thesis strongly encourage the experimental testing in *C. elegans* and *D. melanogaster* of the kinase inhibitors sorafenib, dasatinib, imatinib and erlotinib, and compounds like adenosine and vorinostat. Similarly, drugs that have already shown lifespan-extending properties in invertebrates like trichostatin A, valproic acid, wortmannin, LY-294002 and levothyroxine need to be tested in vertebrate models of ageing as killifish or mice.

Importantly, considering that lifespan experiments are time-consuming and expensive, researchers should consider the use of a different surrogate to measure the effects on ageing in laboratory animals. The most promising alternatives are transcriptomic and epigenetic clocks developed for animal models, which allows calculating biological age with a small error (Meyer & Schumacher, 2020; Thompson et al., 2018). The development of new drug repurposing methods and the evaluation of their biological age using omics clocks within the same study is expected to greatly accelerate the discovery of geroprotectors.

From the 80 mouse models of ageing described by Folgueras et al. 2018, only 19 have transcriptomic data available, with very few having proteomic, epigenomic or metabolomic data. Also, most of the transcriptomic data available come from liver samples, however, I confirmed that mutations affect tissues differently at the molecular level. Thus, further omics studies at different biological levels in different tissues are needed in the mouse models of ageing to provide a better characterisation of the molecular changes associated with ageing interventions. This, in turn, will help to understand the interconnections between the hallmarks of ageing and create more accurate biological clocks that can be used to quantify the effects on ageing of the interventions in a fraction of the time required for a lifespan experiment.

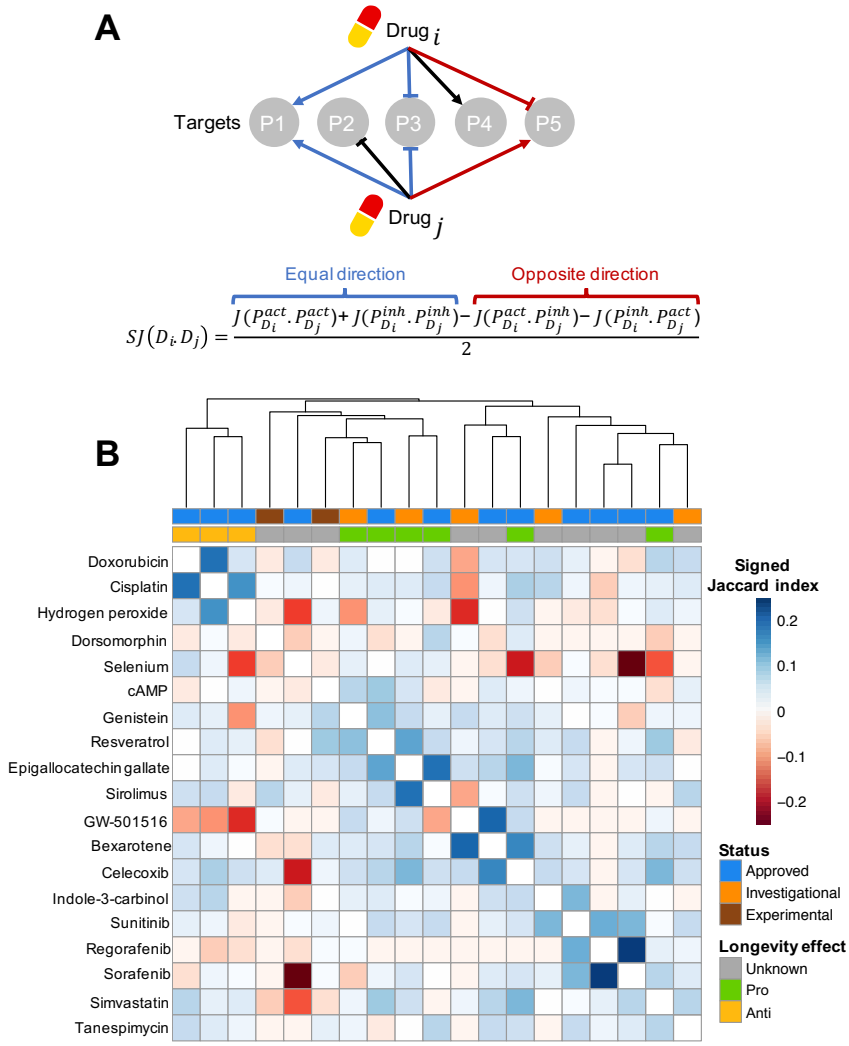
I found that lifespan extension was strongly associated with an increased expression of *Ppargc1a*. Given it has been observed that over-expression of *Ppargc1a* in the skeletal muscle is sufficient to prolong mouse lifespan (Garcia et al., 2018), the next step is to test if the same intervention in the liver also causes lifespan extension. Also, considering the relevance of *Ppargc1a* for mitochondrial biogenesis and thermogenesis, it would be interesting to determine if there is a difference in

mitochondrial copy number in long-lived mice and if transferring long-lived mice to higher temperatures at different ages normalises their lifespan.

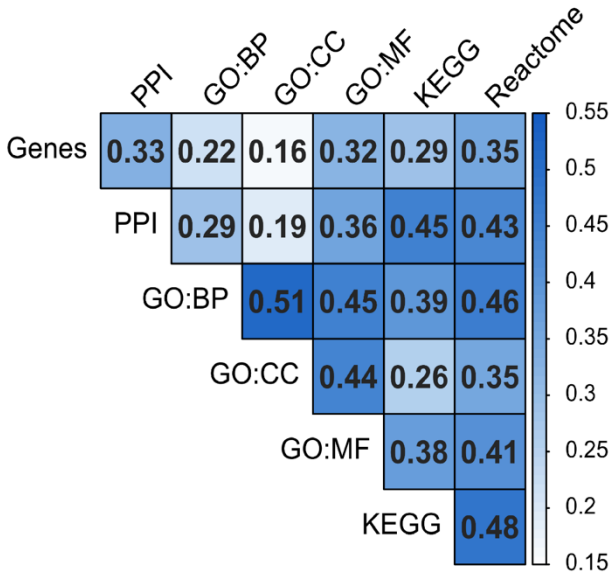
I discovered 16 gene knockouts positively correlated with expression changes in long-lived mice and negatively correlated with expression changes in short-lived mice. Our predictions, therefore, encourage the calculation of the biological age using the mouse epigenetic clock (Thompson et al., 2018) and lifespan experiments on these mutant mice and the drugs targeting the mutated genes.

Appendix - Supplementary material

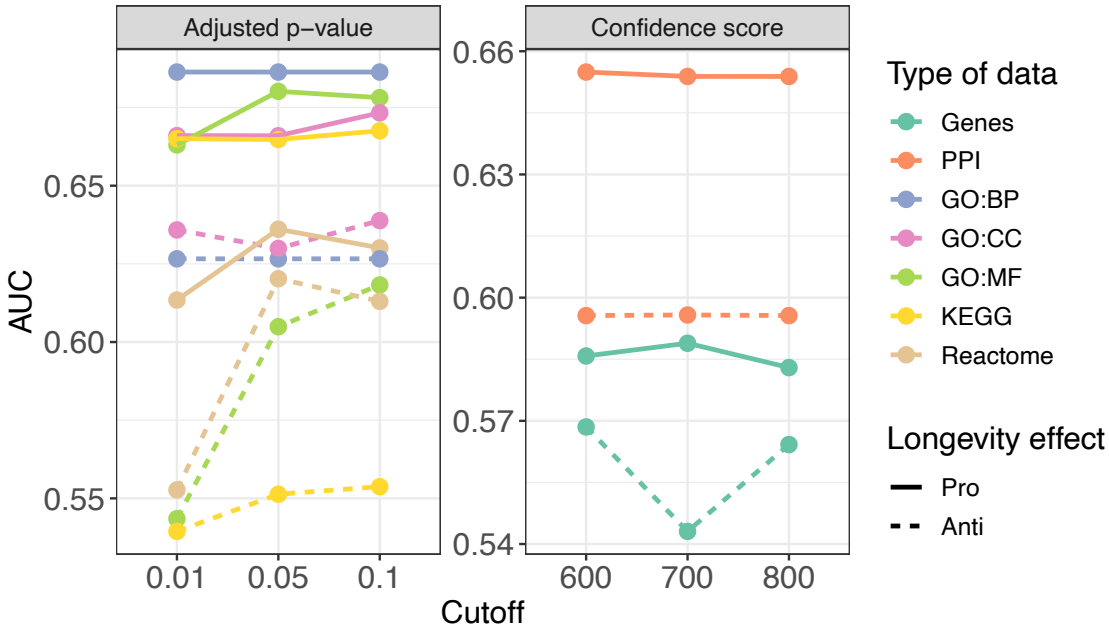
Supplementary Figures



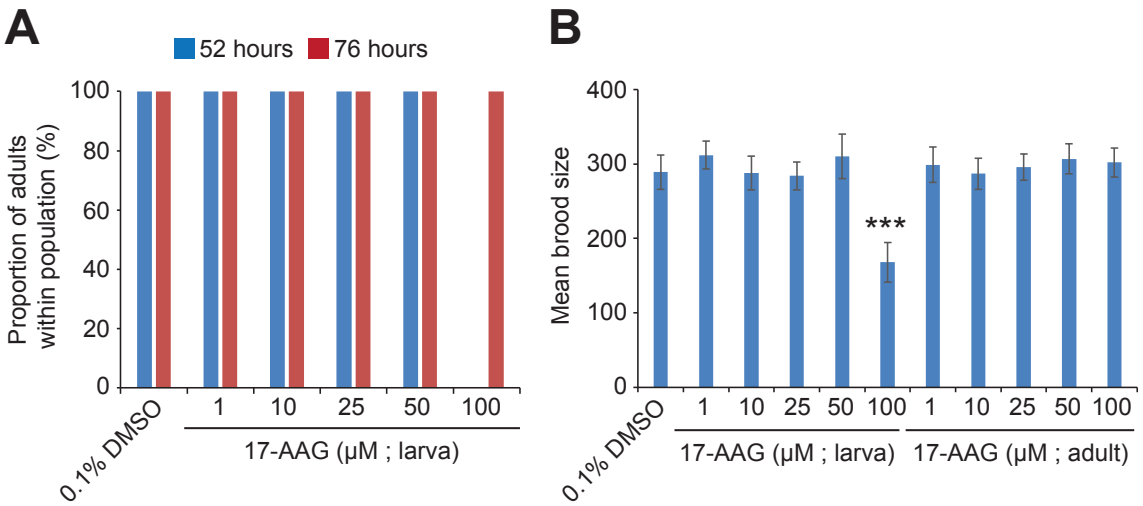
Supplementary Figure 1. *Interaction-similarities between the top-ranked compounds. A) Scheme of the interactions similarity between two drugs. Interactions with both drugs in the same direction are shown as blue lines while opposite interactions are displayed in red. B) A positive value (blue) represents a similar interaction pattern, a negative value (red) opposite interaction pattern. The values from the diagonal were removed for better display of the indexes between different compounds. Annotations were added in the upper part of the heatmap to indicate if the drugs were in the DrugAge database and their current development status. Row and columns were clustered by similarity values, and the calculated hierarchical tree is shown at the top.*



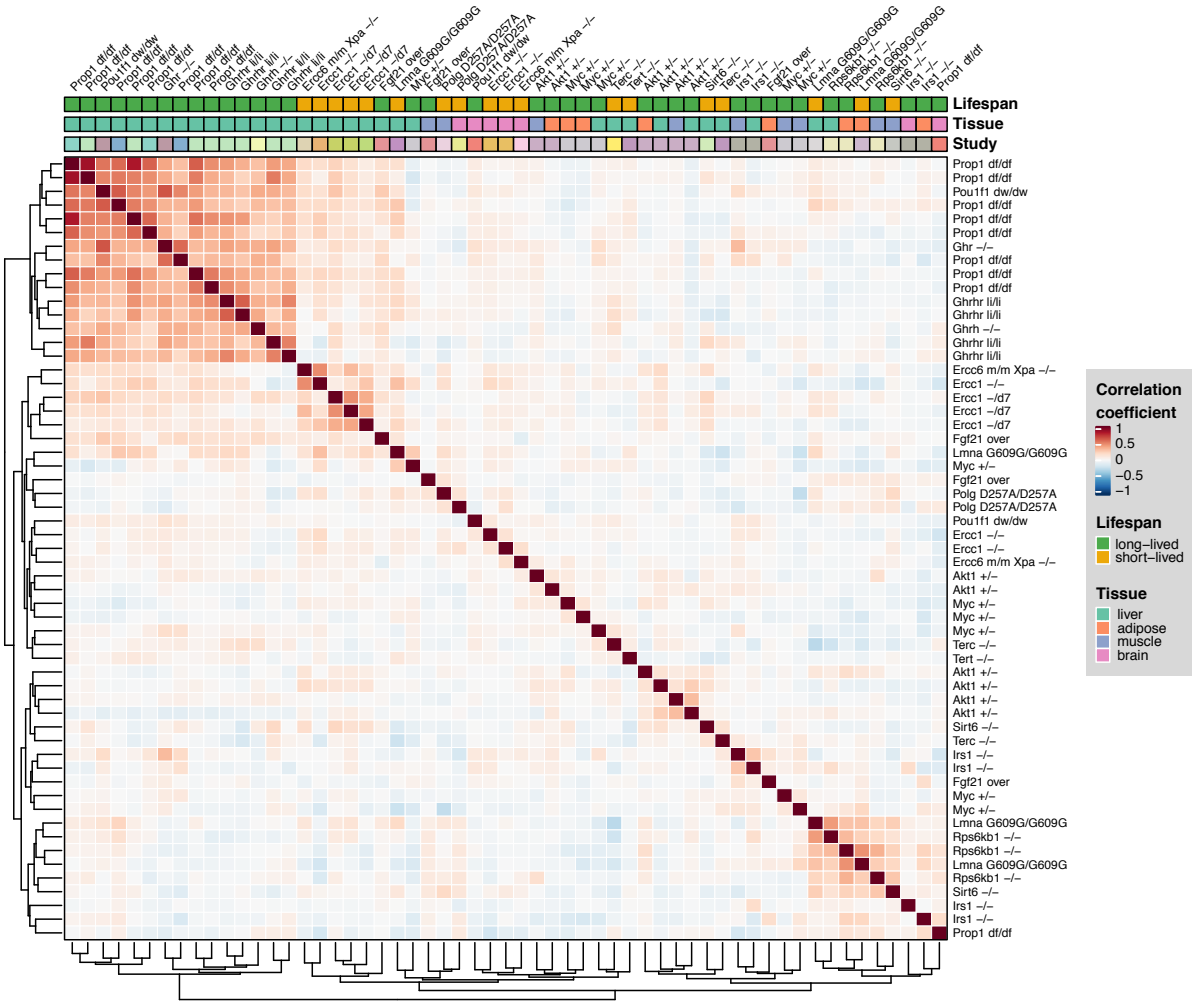
Supplementary Figure 2. *Correlation between the ranked list of compounds. Boxes are coloured by Kendall's correlation coefficient.*



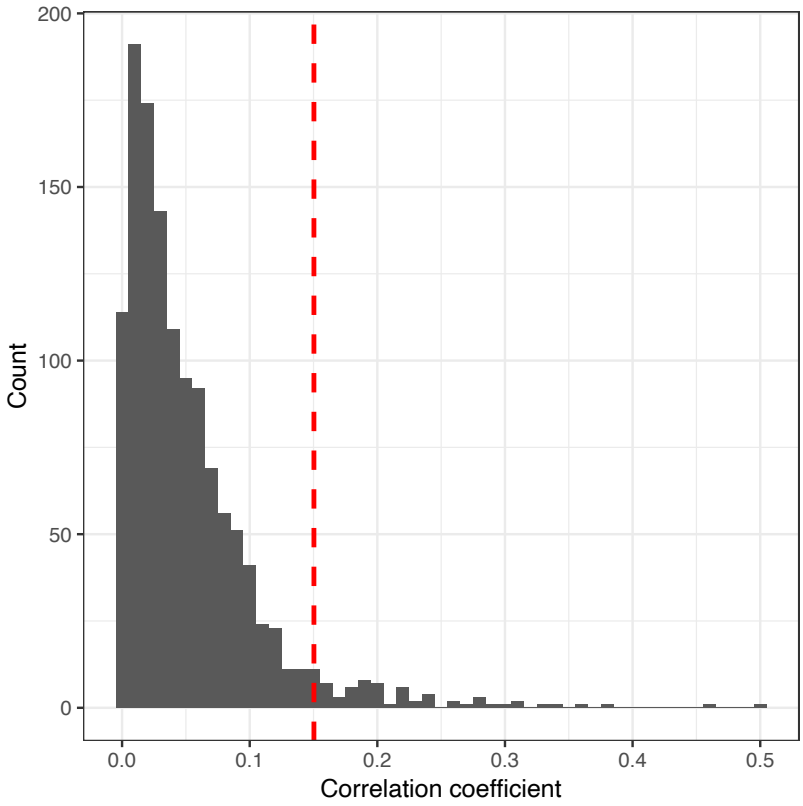
Supplementary Figure 3. *Area under the enrichment curves calculated using different cutoff values. The solid lines represent the enrichment for pro-longevity drugs and the dashed lines for anti-longevity drugs. I analysed 3 cutoffs for the confidence score (600, 700, 800) and the adjusted p-value (0.01, 0.05, 0.1). The colour of the lines and dot represent the use of different types of data as the comparator.*



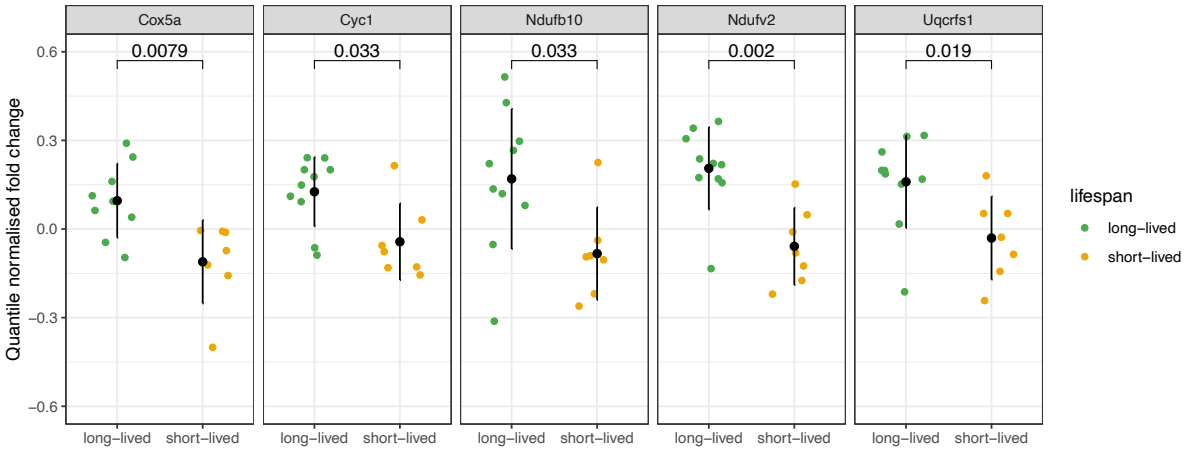
Supplementary Figure 4. *Exposure to high doses of 17-AAG early in life can delay development and reduce brood size. A) Proportion of the population that had reached adulthood 52 and 76 hours post-seeding of L1 worms to plates containing 0.1% DMSO or increasing concentrations of 17-AAG. B) Total number of progeny produced by worms exposed to 0.1% DMSO or increasing concentrations of 17-AAG from the first larval stage (L1) onward or from the first day of adulthood. The number of progeny produced by individual worms on days 1 to 5 of adulthood was counted and combined. 10 worms were scored per treatment group and the values plotted are the mean. Error bars denote standard deviation. Statistical significance was calculated by one-way ANOVA with Tukey pairwise comparison of groups. *** = $p < 0.001$.*



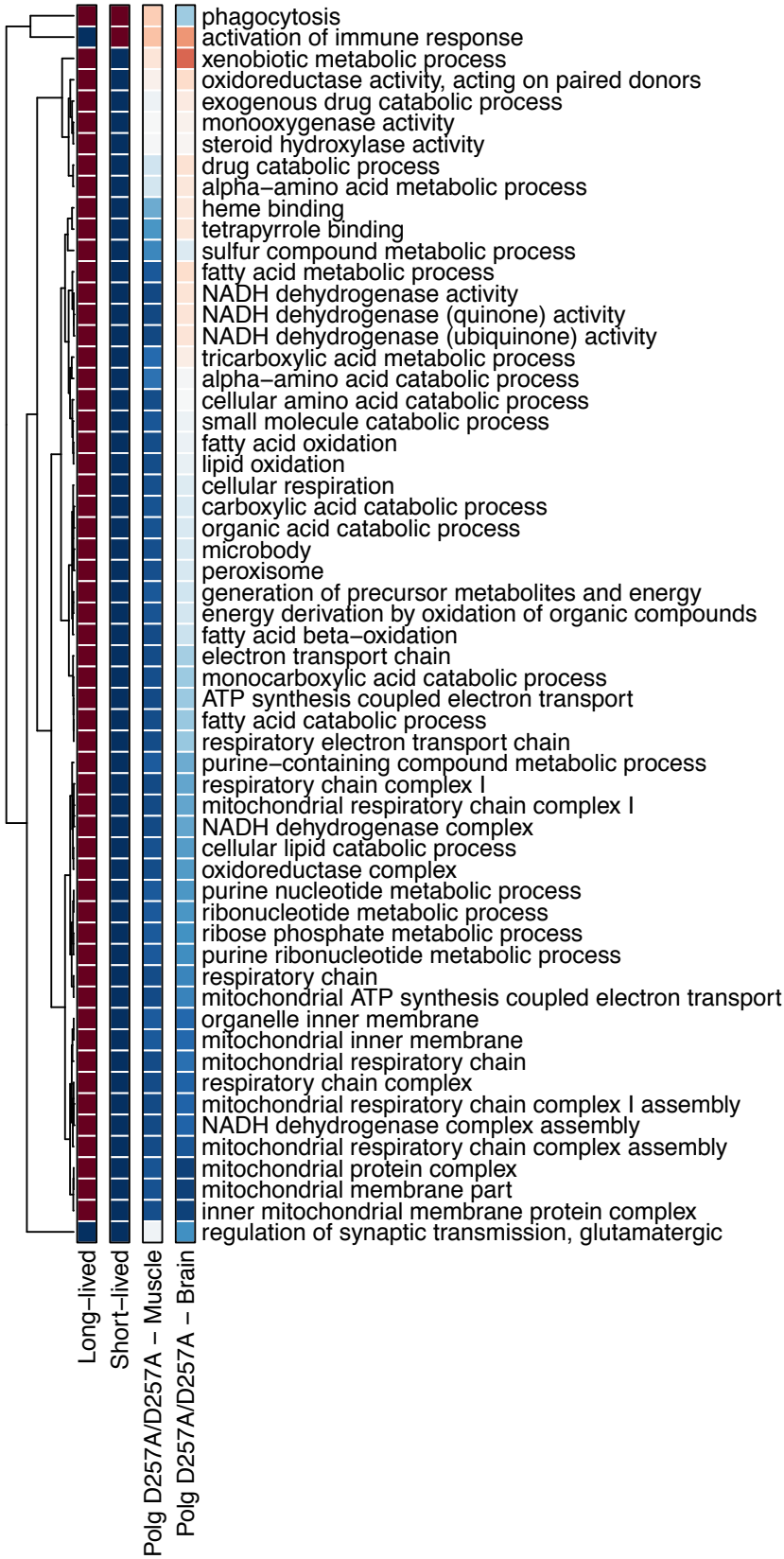
Supplementary Figure 5. *Spearman's rank correlation coefficients between the transcriptome of mouse models of ageing.*



Supplementary Figure 6. *Distribution of pairwise correlation between the transcriptomes of mutants not previously associated with ageing.*

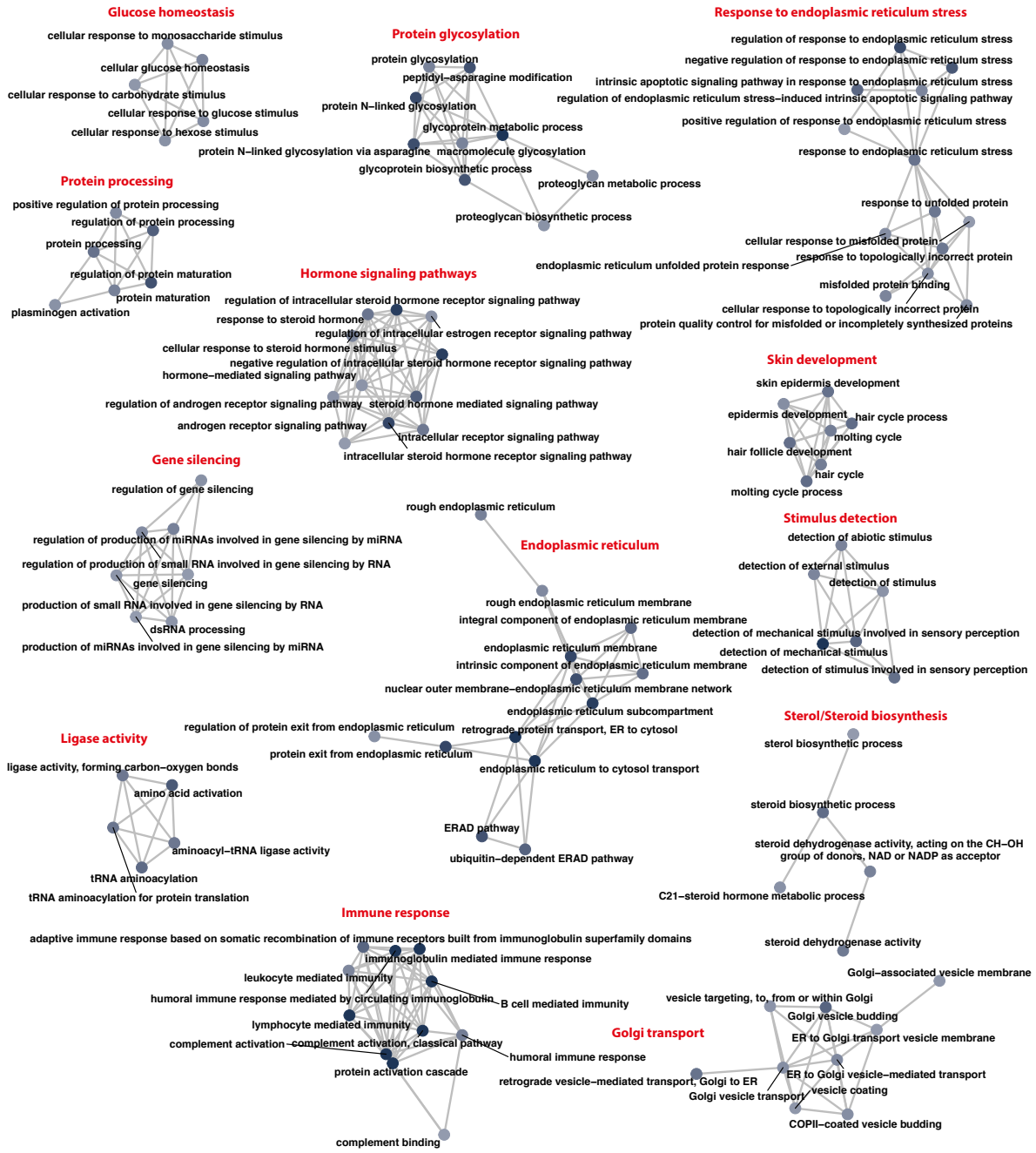


Supplementary Figure 7. *Expression of genes leading the regulation of energy and lipid metabolism in long- and short-lived mice.*

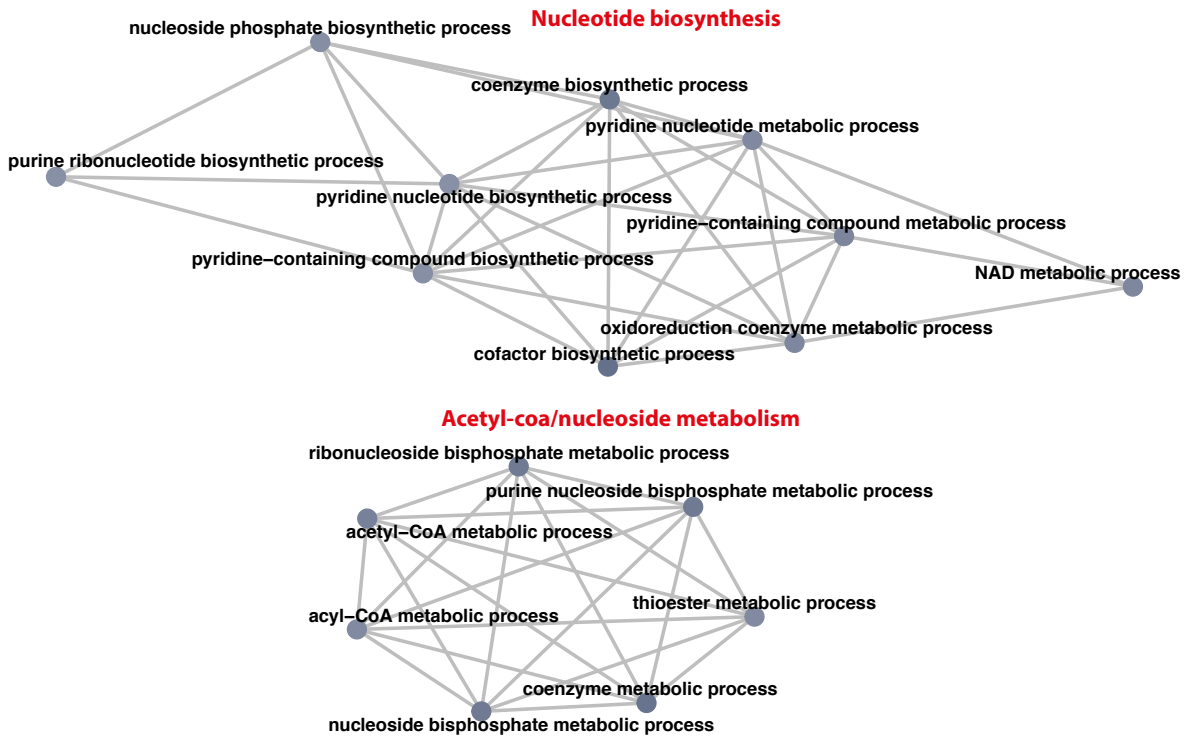


Supplementary Figure 8. *Transcriptional changes in Polg$^{D257A/\ D257A}$ mutant mice in gene sets associated with lifespan and ageing.*

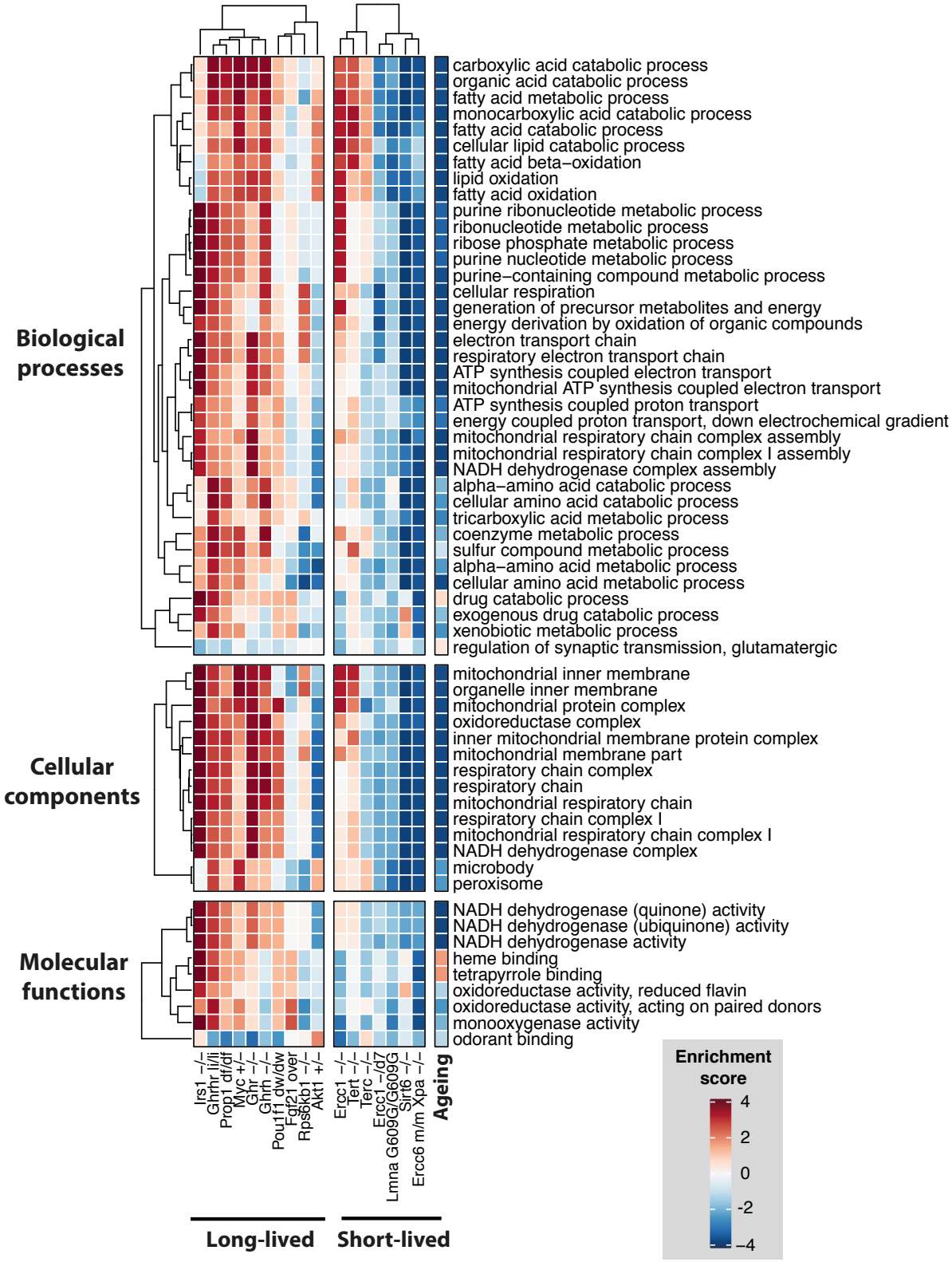
Supplementary Figure 9. *Gene set up-regulated in long-lived mice. Nodes represent GO terms and edges indicate shared genes between the terms. The colour of the circles denotes the significance of the consistency across mutant mice.*



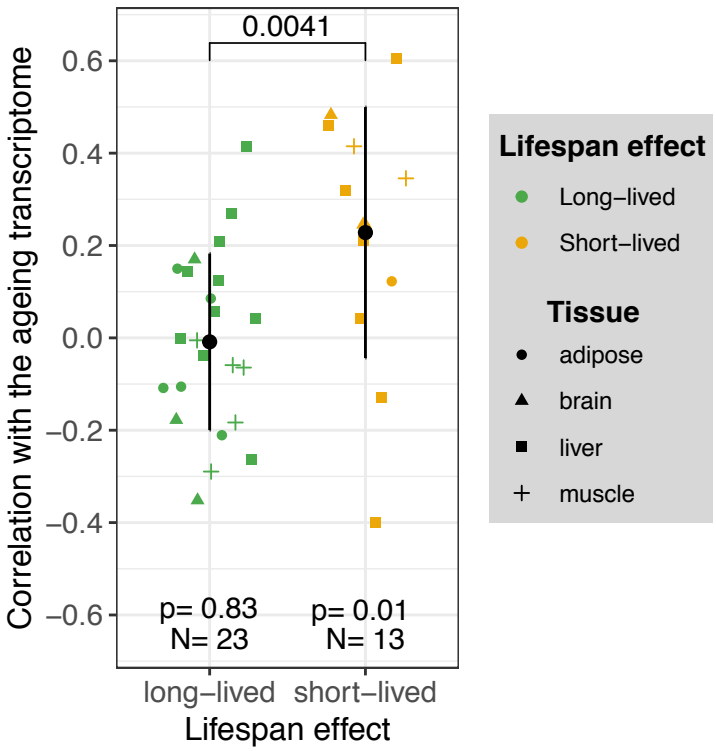
Supplementary Figure 10. *Gene set down-regulated in long-lived mice. Nodes represent GO terms and edges indicate shared genes between the terms. The colour of the circles denotes the significance of the consistency across mutant mice*



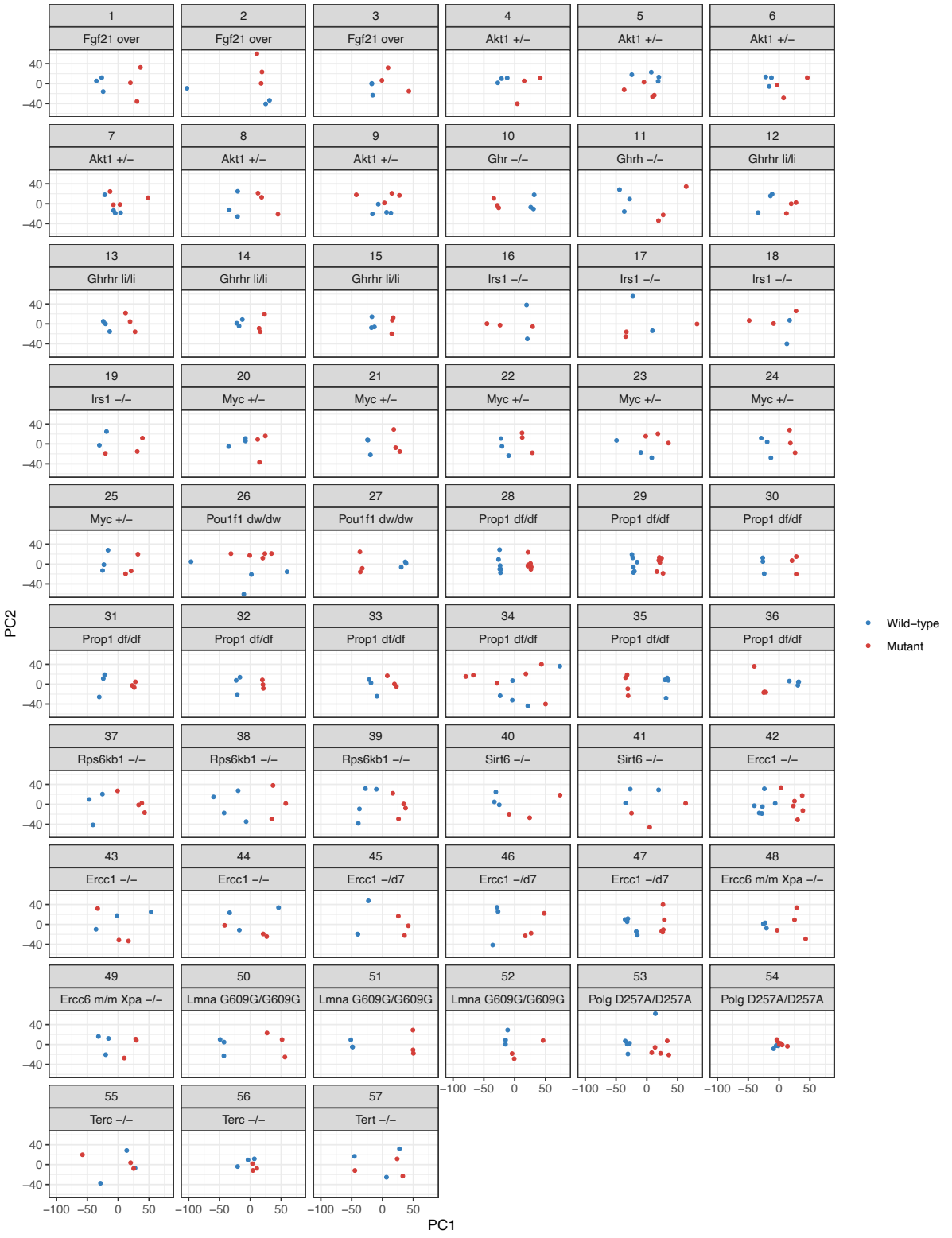
Supplementary Figure 11. *Gene set down-regulated in short-lived mice. Nodes represent GO terms and edges indicate shared genes between the terms. The colour of the circles denotes the significance of the consistency across mutant mice.*



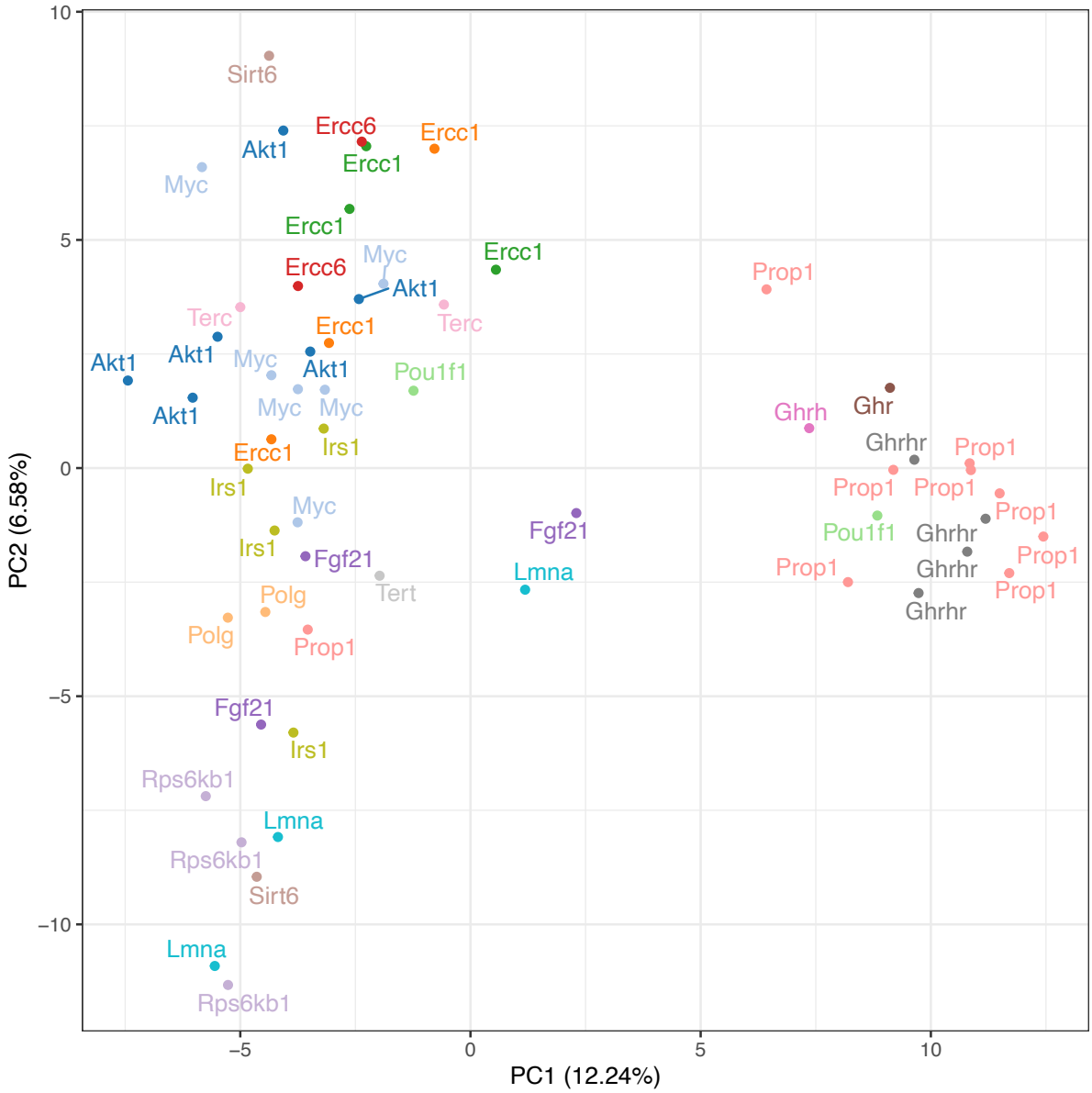
Supplementary Figure 12. *Gene sets showing consistent changes in long- and short-lived mice (FDR < 0.05). The heatmap colours represent the statistical significance of the enrichment and the direction of the change. The 'Ageing' column represents the enrichment scores associated with the transcriptomic changes during ageing.*



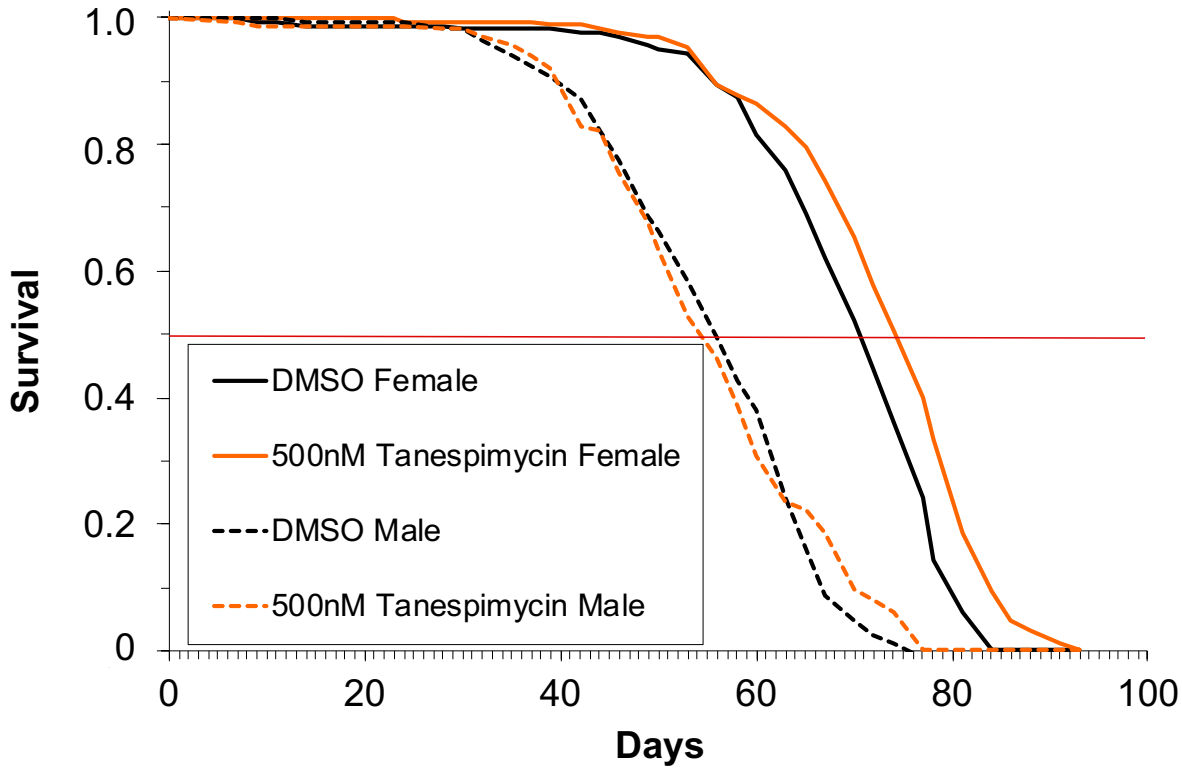
Supplementary Figure 13. *Correlations between the transcriptome of ageing-related interventions and that induced by ageing. Each point represents one intervention, and the shapes indicate the tissue from which the transcriptome was derived. Transcriptomic changes in the mouse models of ageing were compared with the changes during ageing on the same tissue. Error bars show one standard deviation from the mean. P-values below were calculated using a t-test with a population mean equal to zero as the null hypothesis. P-values at the top are for the difference between the groups using an unpaired, two-samples Wilcoxon-test.*



Supplementary Figure 14. *Principal component analysis of the datasets based on expression levels. The samples are coloured by the genotype.*



Supplementary Figure 15. *Principal component analysis of the interventions based on the normalised fold changes from the differential expression analysis. The labels and colour indicate the genes altered. In parenthesis on the axis is the percentage of the variance explained by each principal component.*



Supplementary Figure 16. *Survival of Drosophila melanogaster upon chronic treatment with Tanespimycin. Continuous lines represent female flies and dotted lines represent male flies. Black lines represent wild-type flies and orange lines represent flies fed with tanespimycin. Only female flies fed with 500nM Tanespimycin live significantly longer (log-rank test p-value = 1.26E-5).*

Supplementary Tables

Supplementary tables are available at https://github.com/msfuentealba/phd_thesis Comma separated files are named using the corresponding table names used in the text.

Table legends

Supplementary Table 1. *Enrichment analysis of drugs targeting ageing-related genes.*

Supplementary Table 2. *Enrichment analysis of drugs targeting genes interacting with ageing-related genes.*

Supplementary Table 3. *Enrichment analysis of drugs targeting genes involved in biological processes associated with ageing.*

Supplementary Table 4. *Enrichment analysis of drugs targeting genes involved in cellular components associated with ageing.*

Supplementary Table 5. *Enrichment analysis of drugs targeting genes involved in molecular functions associated with ageing.*

Supplementary Table 6. *Enrichment analysis of drugs targeting genes involved in pathways (KEGG) associated with ageing.*

Supplementary Table 7. *Enrichment analysis of drugs targeting genes involved in pathways (Reactome) associated with ageing.*

Supplementary Table 8. *Evaluation of the research bias for the top-ranked compounds on each list. The first column represents the type of data used as the comparator between ageing and drug targets. The p-value on the second column corresponds to the higher p-value obtained for the 20 compounds on the top of each list, and it represents how many of the 1000 permutations showed a higher rank than in the analysis.*

Supplementary Table 9. *Evaluation of the statistical significance of the enrichment for pro-longevity compounds. The first column represents the type of data used as the comparator between ageing and drug targets. The second columns show the p-value, which represents how many of the 1000 permutations used to evaluate the research bias, showed an AUC equal or higher than the analysis.*

Supplementary Table 10. *Average ranking of drugs for targeting ageing at different levels of biological actions.*

Supplementary Table 11. *Transcriptomic datasets of the mouse models of ageing.*

Supplementary Table 12. *Transcriptomic datasets of genetic interventions not previously associated with ageing. The last two columns represent the sample identifiers of controls and mutant mice.*

Supplementary Table 13. *Gene sets with consistent transcriptomic changes in long-lived mice.*

Supplementary Table 14. *Gene sets with consistent transcriptomic changes in short-lived mice.*

Supplementary Table 15. *Transcriptomic datasets of mice at different ages.*

Supplementary Table 16. *Experimental variables influencing the transcriptomic differences between the datasets based on gene expression values.*

Supplementary Table 17. *Experimental variables influencing the transcriptomic differences between the datasets based on enrichment scores*

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