The use of betaine as a novel senotherapy

By

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Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

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Abstract

Background: The global population demographic comprising those aged 65 years and over is increasing. Despite an increase in human life expectancy over the past 150 years, this has not been matched by a similar increase in health span (i.e. years of disease free living). Consequently, ageing populations present with more age-/lifestyle related diseases such as cardiovascular disease (CVD), chronic kidney disease (CKD) and cancer as part of a diseasome of ageing. Betaine is a key component of one-carbon metabolism required physiologically as an osmolyte, an antioxidant and a methyl donor for maintenance of the epigenetic landscape of ageing and mitochondrial function.

Objectives: The present study aims to assess how dysregulated ageing underpins the development of vascular ageing and the effects of betaine to mitigate this.

Methods: A series of experiments using real time cell analysis (RTCA), transcriptomics, immmunohistochemistry, immunocytochemistry and real-time PCR for a range of validated biomarkers of vascular ageing have been investigated in primary and induced pluripotent stem cell (iPSCs)-derived vascular smooth muscle cells (VSMCs) from human subjects. Betaine was then examined in *in vivo* models for its geroprotective effects on Drosophila melanogaster *(D. melanogaster)* and Caenorhabditis elegans (*C. elegans)*.

Results: Our data indicate that betaine is a potent senotherapeutic able to extend primary VSMCs life span and diminish expression of biomarkers of cellular senescence (p16, p21, Nrf2, SerpineB2, cytoplasmic chromatin fragments), and the senescence-associated pro-inflammatory secretome (IL1β, IL6), as well as biomarkers of VMSCs damage (FOXO4, LMNA). In iPSCs-induced VSMCs, betaine has also displayed potential geroprotective effects by downregulating vascular calcification, extracellular vesicles and oxidative damage. Additionally, it increased total mitochondria content while protecting mitochondria membrane potential against DMSO treatment. Our vivo models (*C. elegans*, *D. melanogaster*) exhibited up to 20% lifespan extension after supplementation with betaine.

Conclusion: Our data indicate that betaine may be a powerful naturally occurring senotherapy and suitable for safe future clinical development.

Publications associated with this research

1. Tran, N., Schurgers, L. and Shiels, P.G., 2023. Mitigating the effects of accelerated vascular ageing in hypertension. *Clinical Science, 137. (IF=6.7)*

2. Shiels, P., Tran, N., McCavitt, J., Neytchev, O. and Stenvinkel, P., 2023. Chronic kidney disease and the exposome of ageing. In *Biochemistry and Cell Biology of Ageing: Part IV, Clinical Science* (pp. 79-94). Cham: Springer International Publishing.

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Acronyms and abbreviations

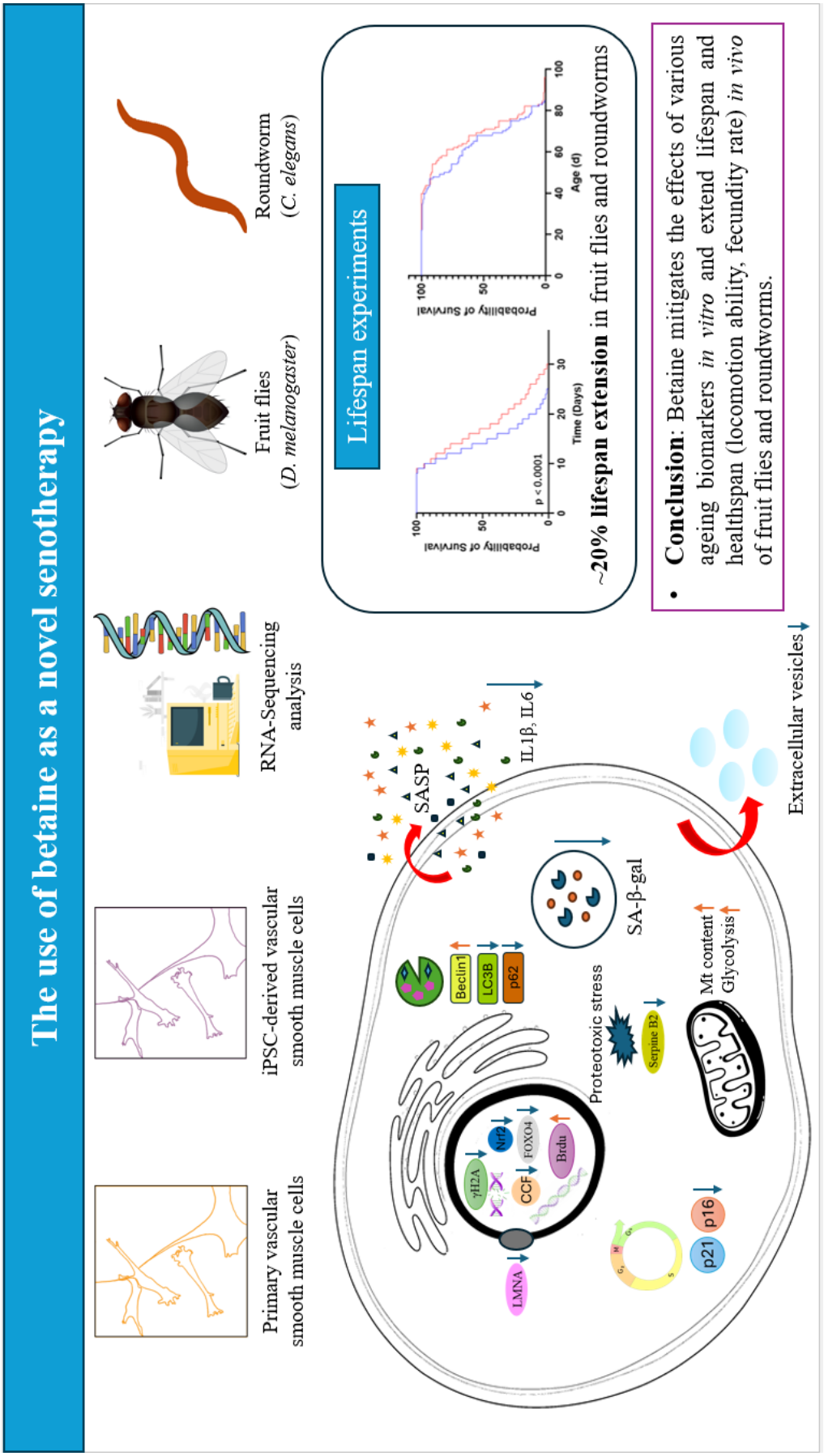
RNA	Ribonucleic acid
DNA	Deoxyribonucleic
VSMCs	Vascular smooth muscle cells
iPSCs	Induced pluripotent stem cells
SCs	Senescent cells
SAβG	Senescence-associated β-galactosidase
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
CKD	Chronic kidney disease
CVD	Cardiovascular disease
RT-PCR	Real-time Polymerase Chain Reaction
CCF	cytoplasmic chromatin fragment
SAM	S-adenosylmethionine
1C	One carbon
AGE	Advanced glycation end products
SAH	*S*-adenosylhomocysteine
PCA	Principal Component Analysis
GO	Gene Ontology
DO	Disease Ontology
ROS	Reactive Oxygen Species
WHO	World Health Organization
FGF-23	Fibroblast growth factor 23
SEP	Socioeconomic Position
Pi	Phosphate
SAGA	senescence-associated growth arrest
cGAS	cyclic GMP-AMP synthase
STING	stimulator of interferon genes
DNA-SCARS	DNA segments with chromatin alterations reinforcing senescence
DDR	DNA Damage Response
ATP	Adenosine Triphosphate
mtDNA	Mitochondrial DNA
OXPHOS	Oxidative phosphorylation

NO	Nitric Oxide
UV	ultraviolet radiation
Nrf2	Nuclear factor erythroid 2–related factor 2
ARE	Antioxidant response element
KEAP1	Kelch ECH associating protein 1
miRNAs	Micro RNAs
IL1β	Interleukin 1β
IL6	Interleukin 6
D+Q	Dasatinib and Quercetin
CFPWV	Carotid-Femoral Pulse-Wave Velocity
mTOR	Mammalian target of rapamycin
PI3K	Phosphatidylinositol 3-kinase
AKT	Protein kinase B
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
BCL-xL	B-cell lymphoma-extra large
AMPK	AMP-activated protein kinase
SIRT1	Sirtuin 1
SIRT6	Sirtuin 6
FOXO4	Forkhead box O4
FOXO5	Forkhead box O5
BCL-2	B-cell lymphoma 2
TMAO	Trimethylamine N-oxide
MAT2A	Methionine Adenosyltransferase 2A
SAM 1/2	SAM synthetase
MTs	methyltransferases
SAHH	SAH hydrolase
HMT	Histone methyltransferases
CpG	Cytosine-Phosphate-Guanine
TREM2	Triggering receptor expressed on myeloid cells 2
IGF2	Insulin-like Growth Factor 2
NDA	Dietetic Products, Nutrition and Allergies
BHMT	Betaine-homocysteine methyltransferase
γH2AX	Phosphorylation of H2AX

PBS	Phosphate-buffered saline
EDTA	Ethylenediamine tetraacetic acid
RTCA	Real time cell analysis
BrdU	Bromodeoxyuridine or 5-bromo-2'-deoxyuridine
ΔΔCT	Double delta cycle thresholds
OCR	Oxygen Consumption Rate
CO_2	Carbon dioxide
LMNA	Lamin A
ECAR	Extra Cellular Acidification Rate
mtROS	Mitochondria Reactive Oxygen Species
RC	Respiratory Capacity
EPC	Energy Production Capacity
TCA	Tricarboxylic acid
PGAM-1	Phosphoglycerate Mutase 1
HIF-1α	Hypoxia-inducible factor-1
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
TGF-β	Transforming growth factor β
TβRI	Recombinant transforming growth factor-β type I
TβRII	Recombinant transforming growth factor-β type II
MAPK	Mitogen-Activated Protein Kinase
RNA-Seq	RNA-Sequencing
LDL	Low-Density Lipoprotein
HDL	High-Density Lipoprotein
mtDNA-CN	Mitochondria copy number
iVSMCs	Induced pluripotent stem cells-derived vascular smooth muscle cells
SM22α	Smooth muscle 22 alpha
RUNX2	Runt-related transcription factor 2
EVs	Extracellular vesicles
MGP	Matrix Gla protein
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
P/S	Penicillin/Streptomycin
RFP	Red fluorescent protein

GFP	Green fluorescent protein
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
DCFDA	Dichlorofluorescein diacetate
CCP	Calcium-phosphate particles
MMP	Matrix metalloproteinases
H_2O_2	Hydrogen peroxide
C. elegans	Caenorhabditis elegans
D. melanogaster	Drosophila melanogaster
S. cerevisiae	Saccharomyces cerevisiae
w^{Dah}	White Dahomey
NGM	Nematode growth media
FUDR	5-fluoro-2'-deoxyuridine
MR	Methionine restriction
SOD	Superoxide dismutase
CAT	Catalase
GSH-Px	Glutathione peroxidase

Graphical abstract



Chapter 1: Introduction and literature review

1.1 The biology of ageing

1.1.1 Definition of ageing and life expectancy

Ageing is a process of segmental decline in physiological functions and capability which increases the risk of morbidity and mortality over time (Li et al., 2021). This age-related deterioration displays substantial inter-individual variation and affects the body's organs at a variety of rates (Fulop et al., 2010). In 1952, Peter Medawar first described the ageing process as an "unsolved problem in biology" (Medawar, 1952). Since then, many theories have been proposed as part of the ongoing process to elucidate this mysterious, yet default aspect of human lives. Three main theories of ageing, based on the targeted level of the ageing process, have been introduced to enhance our insight into the mechanisms of ageing. They consist of (i) evolutionary theories, (ii) systemic theories, (iii) molecular and cellular theories (see Table 1.1) (Lipsky and King, 2015).

Evolutionary theories	Systemic theories	Molecular and cellular theories
Mutation accumulation theory	Neuroendocrine theory	Error catastrophic theory
Disposable soma theory	Immunologic theory	Free radical theory
Antagonistic pleiotropy theory		Waste product theory

Table 1.1| Theories of ageing. *There are three main theories of ageing: (i) evolutionary theories, (ii) systemic theories, and (iii) molecular and cellular theories. Evolutionary theories consist of mutation accumulation theory, disposable soma theory and antagonistic pleiotropy theory. In systemic theories of ageing, neuroendocrine and immunologic theories have emerged as important theories of ageing based on hormones and the immune system. Molecular and cellular theories focus on the accumulation of time-dependent cellular damage, including error catastrophic theory, free radical theory and waste product theory.*

1.1.1.1 Evolutionary theories of ageing

Evolutionary theories define ageing as an outcome of declining natural selection pressure (Weinert and Timiras, 2003). According to these theories, natural selection strongly conserves genes which maximize the early growth and reproduction fitness of an individual, as a coping mechanism for potential environmental risks (i.e. predation, diseases, accidents, limited food sources). As the population is naturally controlled by these environmental factors, the majority of individuals within the population will not reach old age. This adverse situation leads to negligible pressure to maintain genetic characteristics that promote longevity. Evolutionary theories are further classified into smaller branches, including (a) mutation accumulation theory, (b) disposable soma theory and (c) antagonistic pleiotropy theory. Each theory provides potential explanations for the large degree of variation in lifespan within the same species or between species.

In mutation accumulation theory, Medawar has indicated that senescence and diseases occur from the accumulation of unfavourable underlying mutations in the population (Medawar, 1952). This theory also emphasizes that detrimental mutations, which provide no benefit in reproduction fitness, are undesirable for the natural selection process. Expression of detrimental genes can be detected early in life to prevent mutations from passing further. Therefore, genes with higher mutation rates are absent from future generations. One example of this theory is progeria. People with progeria syndrome are characterized by premature ageing with shorter lifespans, which makes it more challenging for them to pass their mutant genes to their future offspring.

Disposable soma theory focuses on the limited resources allocated to different cellular processes in the body (Kirkwood, 1977, Kirkwood, 1997, Kirkwood and Holliday, 1979). According to Thomas Kirkwood, ageing happens due to an evolutionary preference for growth and reproduction over DNA repair maintenance. This biological trade-off results in accelerated cellular damage, telomere attrition, accumulation of mutations, stem cell remains controversial.

Williams (1957) introduced antagonistic pleiotropy theory of ageing, which is currently widely accepted among gerontologists (Williams, 2001). This theory points out the opposite effects of the same so-called beneficial gene on the fitness of the organism, depending on specific stages of life. It is suggested that there is a strong connection between the ‘positive’ and ‘negative’ effects in one gene, allowing the compromise of natural selection to maintain the pleiotropic gene despite its potential detrimental effects.

Williams suggested that a variety of genes related to growth and reproductive fitness are selected for their advantageous impact early in life yet carry undesirable detrimental effects with advanced age. The ability to increase reproduction capacity comes with the cost of shortening lifespan. One example of antagonistic pleiotropy is the process of cell growth. In the early stage of life, cell growth is essential for the development, reproduction success and survival of individuals. Under the influence of internal and external stressors, cells grow uncontrollably and spread into surrounding tissues. This leads to an increase in cancer incidence among the aged population. Limited cell cycle has been used as an effective strategy of the body to minimize the development of cancer cells. However, this phenomenon contributes to the accumulation of senescent cells, followed by tissue dysfunction and chronic low-grade inflammatory processes that contribute to age related disease.

1.1.1.2 Systemic theories of ageing

Systemic theories of ageing indicate diminishing functions of essential organs as a major cause of the ageing process (Weinert and Timiras, 2003). Gerontologists describe these theories based on two main phenomena, known as the neuroendocrine theory and the immunologic theory.

The neuroendocrine theory proposes that neural and endocrine dysfunction are key factors that lead to ageing (Dil′man and Dean, 1992). As these systems are responsible for coordinating body responses (i.e. reflexes, muscle contraction by the nervous system/ metabolism and reproduction by the endocrine system) to the external environment, it is pivotal to maintain their functions as part of physiological homeostasis. The reduction of body responses to stress in older individuals due to an impaired hypothalamus-pituitary axis (HPA) is an important component in this theory. However, this theory has not provided sufficient evidence since various organisms with ageing phenotypes are absent of complex neuroendocrine systems. Changes in cellular structures and functions are suggested to exert fundamental changes to neuroendocrine systems, resulting in the accumulation of distinct ageing phenotypes. Chronic kidney disease (CKD) is a typical example of an age-related disease that is associated with repeated or chronic biological stressors (McGuinness et al., 2018). This is pertinent to the increased prevalence of CKD patients as a consequence of the accumulation of allostatic load (i.e. type 2 diabetes, obesity and a sedentary lifestyle), reflected in the association between ‘burden of lifestyle’ and renal dysfunction (Stengel et al., 2003). Allostatic (over)load refers to the cumulative

biological 'wear and tear' on the body over the life course (Shiels et al., 2021a). A higher incidence of delayed graft function (DGF) after renal allograft has been demonstrated with expanded criteria donor (age > 60, creatinine ≥1.5 with a history of high blood pressure) (Mallon et al., 2013, McGuinness et al., 2016, Menke et al., 2014). As a result, the allograft with increased biological age is less resilient to transplant-associated stresses, thus causing a delay in restoring physiological functions.

The immunologic theory was first introduced by Walford in 1969 (Walford, 1969). According to this theory, immune system functions deteriorate with age, as it loses the sensitivity and efficacy resulting in build-up of infectious and inflammatory factors in the body. It has been widely reported that the immune response reaches its peak during puberty and progressively declines over time (Simon et al., 2015). Age-related autoimmunity phenomena, which occurs when the body's immune response reacts to its own proteins, is the main evidence for this theory. Other examples include the reduced body capacity to respond to infectious diseases, as T-cells fail to properly detect mitogens.

1.1.1.3 Molecular and cellular theories

Molecular and cellular theories have revealed several underlying mechanisms which result in dysregulation of the ageing process (Weinert and Timiras, 2003). Among these, there are three main theories which have gained substantial attention from scientists, namely error catastrophic theory, free radical theory and waste product theory.

First suggested by Leslie Orgel in 1963, error catastrophic theory emphasizes the occurrence of faulty proteins as a consequence of errors in upstream molecular processes (i.e. transcription/ translation) (Orgel, 1963). The accumulation of faulty proteins over time causes a feedback loop of accelerated inaccuracy in protein synthesis. As a result, high mutation rates eventually exceed the body's 'error threshold' which leads to error catastrophe. The catastrophe often refers to the inability of the organism to produce sufficient numbers of viable offspring that can sustain the continuation of the species. However, a research study has identified that protein synthetic errors do not increase with age in human fibroblasts obtained from progeroid donors with accelerated ageing (Harley et al., 1980). Another paper has reported no changes in codon recognition by ribosomes at the first two positions with age (Mori et al., 1983). Therefore, Orgel's theory is currently rejected by the field.

In 1956, the free radical theory was proposed by Denham Harman (Harraan, 1955). This hypothesis has been continually modified before reaching its current form (Harman, 2009). It postulates that ageing is an outcome of free radical accumulation and correlates with oxidative damage. Cumulative intracellular oxidative stress caused by free radicals initiates cellular and tissue damage over time. According to this theory, reactive oxygen species (ROS) and other free radicals produced in mitochondria can target multiple cellular macromolecules such as lipids, proteins and DNA. A variety of model organisms, such as Saccharomyces cerevisiae (*S. cerevisiae)*, Drosophila melanogaster *(D. melanogaster)* or Caenorhabditis elegans *(C. elegans)*, have documented extended lifespan after antioxidant therapy (Varela-López et al., 2023). Recently, the theory has been widely used, not only in the ageing process but also in other age-related diseases. Typical examples of age-related diseases, such as Parkinson's disease, atherosclerosis, CKD, cardiovascular disease (CVD) and diabetes, have all been linked to free radical damage (Pizzino et al., 2017).

An further theory has been presented by V. Gladyshev, termed the waste product theory (Gladyshev, 2012, Gladyshev, 2013). The hypothesis that this theory is formulated around, explains ageing as the accumulation of damaged molecules and byproducts caused by oxidation, analogous to the free radical theory. These by-products include aged collagen, lipofuscin, advanced glycation end products (AGE), damaged enzymes or organelles. Depending on the biological pathways that produce these by-products, some of them can be removed from the body by excretion or degradation. Others are disintegrated into a variety of by-products under enzymatic procedures. Non-dividing cells, such as cardiac or nerve cells, are highly vulnerable since the presence of these by-products can negatively affect cellular functions (i.e. cell signalling, ion transport, etc). The build-up of biological waste leads to cellular damage before progressing into toxicity, ageing or eventually cell death. Actively dividing cells are protected from this biological waste due to their high turnover rate, which can dilute the amount of damage after the cell division process.

1.1.1.4 Life expectancy and healthy lifespan

Originally, ageing was defined by two main parameters – average and maximum lifespan (Jayanthi et al., 2010). The term 'average lifespan' or 'life expectancy' has been defined as the number of years at which 50% of the individuals in a particular population are surviving (Balcombe and Sinclair, 2001). Maximum lifespan, on the other hand, refers to the longest possible lifetime an individual in a given population can reach. Presently, the number of the old in the global population is increasing in almost all countries worldwide, but especially in developing regions (Organization, 2021). The increase in life expectancy has registered more than double in the last 150 years, from 32 years to 71 years of average lifespan (Finch, 2010, Saloni Dattani, 2023). Most people are now expected to live into their sixties or beyond due to improvements in the public health and medicine. By 2030, one in six adults is expected to be aged 60 years or older, equating to approximately 1.4 billion globally (Organization, 2021). This older population will continue to increase by ~ 67% and reach 2.1 billion by 2050.

The gap between increased lifespan globally and health span (years of healthy living) has grown. People are living longer, but they are also suffering from many age-related diseases, such as CKD, CVD, hypertension, type 2 diabetes, obesity, cancer, osteoporosis, frailty and neurodegenerative disease (Stenvinkel et al., 2019). These conditions comprise a 'diseasome of ageing', which brings with it significant social and economic burdens Ifor governments and public health systems globally. Before Covid, the healthcare sector was overwhelmed by the financial burden of age-related chronic diseases, which was estimated at $47 trillion from 2010-2030 (Chen et al., 2018). This financial burden has continued to increase in the post-Covid era, and its effects are amplified in areas of social deprivation, particularly prevalent in the low- and middle-income nations that can least afford to meet such a challenge. The 'diseasome of ageing' is commonly associated with low-level chronic inflammation (inflammageing), mitochondrial damage, high serum phosphate levels, suppression of nuclear factor erythroid 2–related factor 2 (Nrf2) expression and reduced gut microbiome diversity (Kooman et al., 2014).

In mammals, hyperphosphatemia is a key driver of the 'diseasome of ageing' (Kooman et al., 2014). The source of phosphate, which mostly comes from basic food consumption such as meat, fish and eggs, is naturally regulated by vitamin D, parathyroid hormone and fibroblast growth factor 23 (FGF-23)/klotho (Kooman et al., 2014). Dietary-induced hyperphosphatemia contributes to poorer health in the general population. This condition

is a result of over-frequent red meat consumption. To lower the risk of disease development, it may be beneficial to implement a shift to plant-based or non-red meat protein in the diet. This is in keeping with the World Health Organization (WHO) recommendations to move to a more Mediterranean-style diet. The benefits of such a diet are intuitive, but many are socially patterned (Craven et al., 2021a). In keeping with these observations, excessive red meat consumption has also been reported to affect adversely the composition of microbiota. Similarly, this displays a degree of social patterning, with the most biologically aged at lower socioeconomic position (SEP) exhibiting higher phosphate (Pi) levels, poorer renal function and more pathobionts. Conversely, those at higher SEP possessed lower Pi levels, better renal function, lower biological age and a more salutogenic microbiota.

1.1.1.5 Fecundity rate and ageing

The fecundity rate refers to the maximum reproductive output at which a population or individual can produce offspring over its lifetime (Bradshaw and McMahon, 2008). This concept is different from fertility, which is characterized by the actual performance of an individual in the process of reproduction (Sear et al., 2016). The fecundity rate is highly variable within individuals of the same or different species (Zhang et al., 2021). Important factors that influence the fecundity/fertility rate involve age, body size, population density, mate preference and environmental conditions (Bradshaw and McMahon, 2008). In the human population, 74% of women below 30 years of age have been found to conceive within 12 menstrual cycles, whereas this proportion respectively drops to 62% and 56% for women between 31-35 and 36-40 years (Menken and Larsen, 1986). The physiological mechanisms contributing to reduced fecundity/fertility rate are not fully elucidated. Nonetheless, some age-associated pathways were proposed including mitochondria dysfunction, DNA damage, genetic mutations and telomere shortening (Owen A, 2024). Mitochondria dysfunction plays a central role in decreasing oocyte and egg quality (Podolak et al., 2022). A study conducted on women of different age ranges (<38 and ≥38 years old, normal or diminished ovarian reserve) reported that advanced-age women had significantly lower numbers and quality of oocytes and mitochondria (Lu et al., 2022). Compared to young females, mitochondria morphology and structures of advanced-aged groups underwent considerable changes, especially with reduced mitochondria content (total mitochondria per cell) and mitochondria copy number (mtDNA-CN, number of mtDNA copies per mitochondrion) (Lu et al., 2022). In addition, exposome factors (i.e. toxins, radiation exposure, oxidative stress and certain medical

treatments) can all contribute to a lower fecundity rate due to DNA damage, potentially disrupting early embryo development (Musson et al., 2022). Currently, there are several senotherapeutic drugs that can improve female reproductive outcomes, such as metformin and the drug combination of dasatinib and quercetin (D+Q). Metformin treatment has led to positive impacts on spontaneous pregnancy success and reduced miscarriage rate while delaying ovarian ageing in patients with polycystic ovary syndrome (Joham et al., 2022). Together with D+Q, metformin is significantly more effective in removing senescent cells (SCs) to preserve the oestrous cycle (Du et al., 2022).

1.1.2 Hallmarks of ageing

Across taxa ageing shares common hallmarks; namely genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, disabled macroautophagy, chronic inflammation, and dysbiosis and altered intercellular communication (see Figure 1.1) (López-Otín et al., 2023). These hallmarks are affected by the exposome (total biotic and abiotic stresses over the life course), thus influencing the expression of the (epi)genome and contributing to inter-individual variation in ageing and health span (Shiels et al., 2021b). Even though exogenous stress significantly influences the rate of ageing, it remains unclear whether these factors interplay, either cumulatively, independently, or synergistically with the ageing process (Shiels et al., 2019). The hallmarks of ageing are directly associated with an increased burden of allostatic load over the life course. They act as a reliable measure of systemic burden at a cellular and molecular level, both in normative ageing and age-related pathologies (Shiels et al., 2021b).

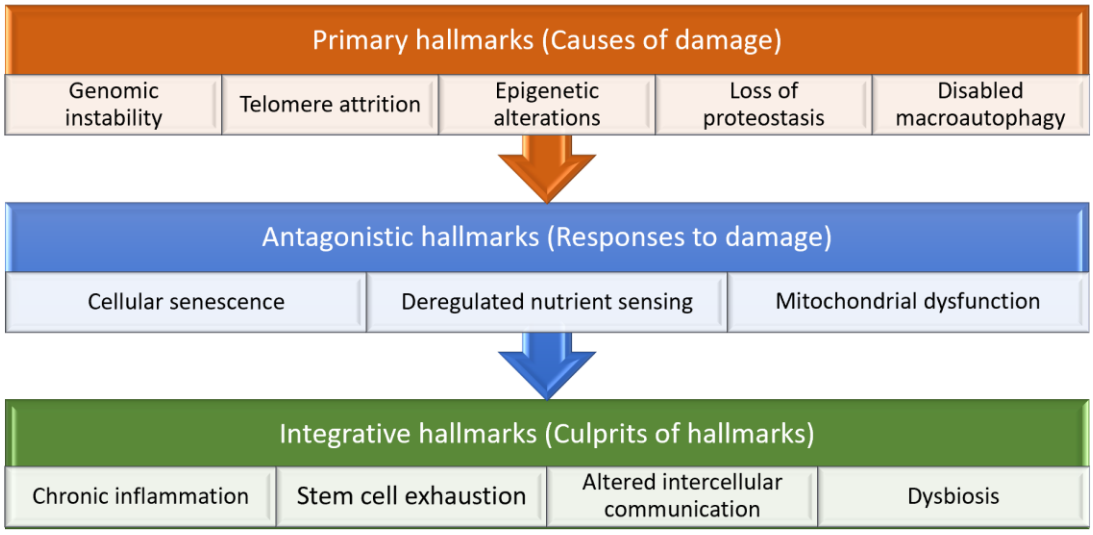


Figure 1.1| Hallmarks of ageing. *Ageing shares common hallmarks across taxa; namely genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dysregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, disabled macroautophagy, chronic inflammation, and dysbiosis and altered intercellular communication.*

1.1.2.1 Cellular senescence

During ageing, the process of 'cellular senescence' occurs when cells enter a state of permanent arrest of the cell cycle, known as senescence-associated growth arrest (SAGA). SCs are resistant to apoptosis and release catabolic factors such as the senescence-associated secretory phenotype (SASP). Excessive accumulation of SCs results in long-term inflammation and damage to surrounding cells and tissue dysfunction (Di Micco et al., 2021). This is due to a so-called 'bystander effect' when SASP negatively affect their local environment. SCs are important drivers of the ageing process with many distinct features. They possess broad and flattened morphology with vacuolation-senescence-associated β-galactosidase activity (SAβG), a characteristic pro-inflammatory SASP and senescence-associated heterochromatin foci (SAHF) (Banito and Lowe, 2013). Increased expression of cell cycle inhibitors p21, p53, and $p16^{INK4a}$ are responsible for maintaining the stable arrest of senescence. The process of cellular senescence is often followed by the secretion of a wide range of diverse inflammatory and SASP mediators including pro-inflammatory cytokines, chemokines, and growth factors (Gonzalez-Meljem et al., 2018). The burden of SCs and SASP increases over time and results in the onset of age-related pathologies. A variety of typical characteristics of senescent cells are summarized in the table as follows (see Table 1.2).

SASP can act both autonomously (autocrine) and non-autonomously (paracrine) to strengthen senescent phenotypes while simultaneously affecting other adjacent tissues. Interleukin 6 (IL6), a key SASP driver of the ageing process, is responsible for sustaining intracrine and paracrine senescence (Herbstein et al., 2024). Intracellular IL6 accumulation leads to the activation of NFkB and the buildup of cytosolic chromatin fragments (CCF) (Herbstein et al., 2024). CCF is a process of SCs-associated DNA fragments being released from the nuclear lamina to the cytoplasm (Dou et al., 2017). Together with the loss of Lamin B1 with age, the nuclear envelope loses its integrity which results in nuclear blebbing (Kamikawa et al., 2021, Wren et al., 2012, Bercht Pfleghaar et al., 2015). From that, CCF in the cytoplasm can activate cyclic GMP-AMP synthase (cGAS) – stimulator of interferon genes (STING) signalling pathways, initiating the scavenging process of damaged cells by promoting inflammation-associated immune response through cytokine signalling (Dou et al., 2017).

Intracellular IL6 can also induce the amplification and maintenance of SASP-associated non-autonomous effects of SCs (Herbstein et al., 2024). By inducing long term low-grade

inflammation, also known as 'inflammaging', SASP-associated pro-inflammatory molecules (i.e. cytokines, chemokines) promote paracrine effects on neighbouring tissues (Franceschi et al., 2018). This is reflected in the altered behaviours of adjacent cells, eventually leading to cellular and tissue damage. A relatively small number of SCs (5-15%) in the tissue can cause substantial damage to the cellular microenvironment, which causes other neighbouring cells to enter the same 'senescent state' (Wang et al., 2009). Accumulation of senescent cells and tissue dysfunction are causative factors of a range of non-communicable diseases in ageing global populations.

Typical characteristics of senescent cells
▪ Permanent cell cycle arrest, resistant to apoptosis
▪ Morphology: flat, larger, multiple with enlarged nuclei, cytoplasmic anomalies (vacuolization and granularity) and abnormal organelles.
▪ Overexpression of cell-cycle arrest genes-CDKN1A/p21 and CDKN2A/p16
▪ Upregulation of mitochondrial-stress-responsive molecule – GDF15
▪ Activation of DNA damage response (DDR) - γH2AX, p53BP1
▪ Accumulation of the endogenous lysosomal beta-galactosidase enzyme (SA-β-gal).
▪ Stimulation of senescence-associated secretory phenotype (SASP) – specifically proinflammatory cytokines (i.e. IL1β, IL6, IL8), growth factors and matrix metalloproteinases
▪ Senescence-associated heterochromatin foci (SAHF) at specific DNA loci
▪ DNA-SCARS with persistent DNA damage
▪ Loss of DNA methylation and other epigenetic marks
▪ Protein damage and carbonylation increases with age
▪ Loss of transcriptional regulation, resulting in transcriptional noise and drift
▪ Loss of Lamin B1
▪ Shortening of telomere length

Table 1.2| Typical characteristics of senescent cells. *Senescent cells are characterized by irreversible terminal growth arrest while remaining in the body system due to apoptosis resistance. They possess distinct flat, enlarged morphology with multiple abnormal nuclei and organelles. Multiple changes in gene expression have been recorded in senescent cells, including the upregulation of cell-cycle arrest genes (CDKN1A/p21 and CDKN2A/p16), mitochondria-stress-responsive gene (GDF15), DNA damage response (γH2AX, p53BP1), endogenous lysosomal beta-galactosidase enzyme (SA-β-gal), senescence-associated secretory phenotype (SASP), senescence-associated heterochromatin foci (SAHF) and DNA-SCAR. In addition, senescent cells acquire higher levels of protein damage and carbonylation with increased age. This leads to the loss of transcriptional regulation, resulting in transcriptional noise and drift. Significant loss of Lamin B1 and telomere attrition are also associated with cellular senescence.*

1.1.2.2 Telomere attrition

The ageing process is characterized by the occurrence of telomere attrition in different primary somatic cell types (Shammas, 2011). Telomeres are nucleoprotein complexes which consist of repetitive DNA sequences and proteins at the ends of linear chromosomes (O'Sullivan and Karlseder, 2010). The $(TTAGG)_n$ repeats of telomeric DNA are surrounded by proteins which protect genomic integrity and maintain the cell division process. Telomeres also prevent chromosomes from eroding or fusing. The typical length of human telomere ranges from 5 to 15 kb base pairs, with distinct variations depending on individuals, particularly genetics, age, lifestyle factors and health conditions (Pickett et al., 2011). During each cell division, the telomere loses its length by 30 to 50 base pairs due to the end replication problem (Huffman et al., 2000). When a telomere reaches its minimal length, the exposed DNA ends trigger p21/p16-associated cell cycle arrest as part of DDR signalling pathway (Ghadaouia et al., 2021). This persistent signal induces permanent proliferative arrest, followed by the initiation of cellular senescence. Accumulation of telomere dysfunction can also result in cellular apoptosis or autophagy in a variety of cell types (Rossiello et al., 2022). This does not happen in senescent cells which are resistant to apoptosis. Pertinent to this thesis, inflammation, stress-induced premature senescence as well as enhanced expression of mutated telomeric repeat-binding factor 2 protein has been reported to exacerbate VSMCs senescence in atherosclerosis (Uryga et al., 2021a). Specifically, oxidative damage plays a pivotal role in enhancing telomere shortening and reducing activities of telomerase enzymes in VSMCs. Even though telomere attrition is considered an important hallmark of ageing, it is insufficient to use this pathway to explain the ageing process in permanent, quiescent or terminally differentiated cells.

1.1.2.3 Genomic instability

Genomic instability closely interacts with other features of the ageing process (Li et al., 2023). It has been associated with increased DNA damage levels, DNA repair dysfunction and telomere attrition. Changes in genetic and epigenetic information (i.e. abnormal histone modification, reduced DNA methylation, disrupted chromatin organization) contribute to the occurrence of genomic instability (Putiri and Robertson, 2011). The genome is strongly vulnerable to external DNA damaging factors (Clancy, 2008). Typical examples of these contributing factors comprise UV light, genotoxins or ionizing radiation. As a result of errors in the DNA replication and repair process, mutations often occur during short-term or chronic exposure to those detrimental sources. DNA damage caused by mutations in the genome can be observed in various ways, such as aberrant DNA sequences, DNA breaks, gaps, nicks or dysfunctional intermediates that are required for successful transcription, translation and replication processes (Li, 2008). In VSMC senescence-associated vascular ageing, increased levels of DNA damage biomarkers and repair enzymes were identified in blood vessels of patients with atherosclerotic and coronary artery disease (Botto et al., 2002, Martinet et al., 2002).

1.1.2.4 Epigenetic alterations

Changes in epigenetic information during ageing are complex, with cell type-specific features observed (Soto-Palma et al., 2022). Modification of the epigenetic landscape is achieved through distinct alterations in DNA, RNA and chromatin associated proteins through post-translational modification, such as methylation, acetylation, phosphorylation and ubiquitination. Among these, methylation, often found on DNA and histone structures, has been the most extensively studied. Global DNA hypomethylation is a feature of accelerated ageing (Girling et al., 2024). Typically, the total level of 5-methylcytosine – a methylated form of the cytosine nucleotide in DNA – progressively reduces with age (Singhal et al., 1987, Wilson et al., 1987, Zin'Kovskaia et al., 1978), Global DNA hypomethylation has been reported in VSMCs as a feature of replicative ageing (Pogribny and Beland, 2009). Histone methylation, on the other hand, however, displays distinct patterns of alteration in different species, depending on an activating or repressive role in gene expression regulation. For instance, H3K27me3 – a methylated form of histone H3 – can display contradicting roles in regulating the ageing processes dependent on the species and cell type. In *C. elegans*, global loss of H3K27me3 has been detected (Jin et al., 2011, Scaffidi and Misteli, 2006, Shumaker et al., 2006). Conversely, increased H3K27me3 has been reported in in killifish with increasing age along with

mouse and other human cell types (Baumgart et al., 2014, Liu et al., 2013). An exception in humans may be with Hutchinson–Gilford progeroid syndrome, where H3K27me3 marks decrease with age.

1.1.2.5 Mitochondria dysfunction

With age, dysregulated mitochondrial functions have been linked to the development of multiple age-related chronic diseases (Srivastava, 2017). Mitochondria are organelles found in eukaryotic cells which produce adenosine triphosphate (ATP) – a primary source of cellular energy through a series of oxidative phosphorylation reactions. These processes continually generate ROS as a direct by-product of ATP production. As ROS accumulation increases over the life course, the detoxification mechanisms fail to eliminate these deleterious reactive agents from the body. This eventually leads to greater mitochondrial dysfunction. Ageing leads to mitochondria dysfunction which exacerbated loss of age-related physiological function in a vicious cycle (see Figure 1.2). The potential consequences include somatic mitochondria DNA (mtDNA) mutations, diminished oxidative phosphorylation (OXPHOS) activity, oxidative damage as well as lowered mitochondrial quality control (Tuppen et al., 2010). There is increasing evidence on the impact of mitochondrial dysfunction during vascular ageing and in age-related chronic diseases including CKD, CVD or neurodegenerative diseases. Correspondingly, in senescent VSMCs, enlarged mitochondria with higher levels of fusion/fission defects, mtDNA mutations and impaired membrane potential have been observed which then mitigates the production of ATP (Botto et al., 2002, Byrne et al., 2019, Ma et al., 2020). As a result, this deterioration of mitochondria activities has proven with a direct effect on the median lifespan of different species.

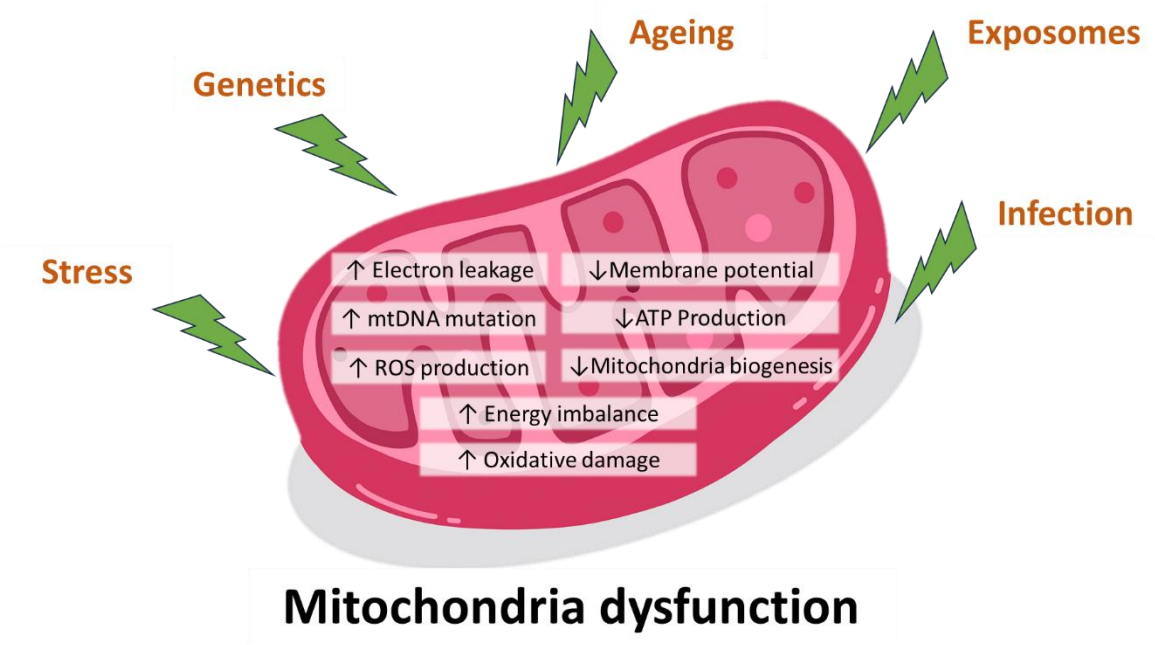


Figure 1.2| Internal and external factors which cause mitochondria dysfunction. *Mitochondria functions can be affected by many aspects of human lives, such as increased stress levels, genetic mutations, ageing, the exposome (i.e. sedentary lifestyle, age-related diseases) and other infections. Common characteristics of mitochondria dysfunction include electron leakage, mutations in mitochondria DNA, increased ROS production, energy imbalance or oxidative damage. In addition, impaired mitochondria biogenesis with reduced membrane potential and ATP production is directly associated with sustained mitochondria damage.*

1.1.2.6 Oxidative damage

Oxidative stress is considered a fundamental contributing factor in low-grade persistent inflammation in the ageing process (Maldonado et al., 2023). Potential molecular mechanisms that enhance oxidative damage include chronic build-up of ROS related macromolecular damage, decreased nitric oxide (NO) production and impaired Nrf2/ARE antioxidant defence system (Ngo and Duennwald, 2022). ROS are primarily produced by mitochondrial oxidative respiration. However, exposure to external exposome factors (i.e. food, smoking, UV radiation or pollution) can also stimulate ROS production. Typical examples of harmful ROS agents include peroxides, superoxide, hydroxyl radicals, and singlet oxygen. In vascular ageing, excessive amounts of these free radicals have been associated with cellular damage, vascular remodelling and endothelial dysfunction. Eventually, NO production, which plays a crucial role in maintaining homeostasis in vascular tone, is inhibited (Ngo and Duennwald, 2022).

Moreover, the Nrf2/ARE antioxidant defence pathway also significantly becomes dysregulated with age (Arefin et al., 2020). Nrf2 is a transcription factor regulating the activity of more than 500 cytoprotective genes with the ability to regulate cellular resistance to oxidative stress (see Figure 1.3). Upon exposure to unfavourable exposome factors, Kelch ECH associating protein 1 (Keap1) – an endogenous inhibitor of Nrf2 – dissociates from Nrf2 to facilitate Nrf2 translocation to the nucleus(Arefin et al., 2020). Nrf2 then triggers adaptive homeostasis by binding to antioxidant response elements (AREs) of its target cytoprotective genes to enhance redox homeostasis, restore proteostasis as well as mitigate the inflammatory burden. Importantly, the Nrf2-Keap1 system is modifiable and accessible to therapeutic approaches via drugs of both synthetic and natural origins. It has been shown that the expression of the Nrf2 gene reduces with age, which results in cellular dysfunction and the appearance of the hallmarks of ageing (McClelland et al., 2016).

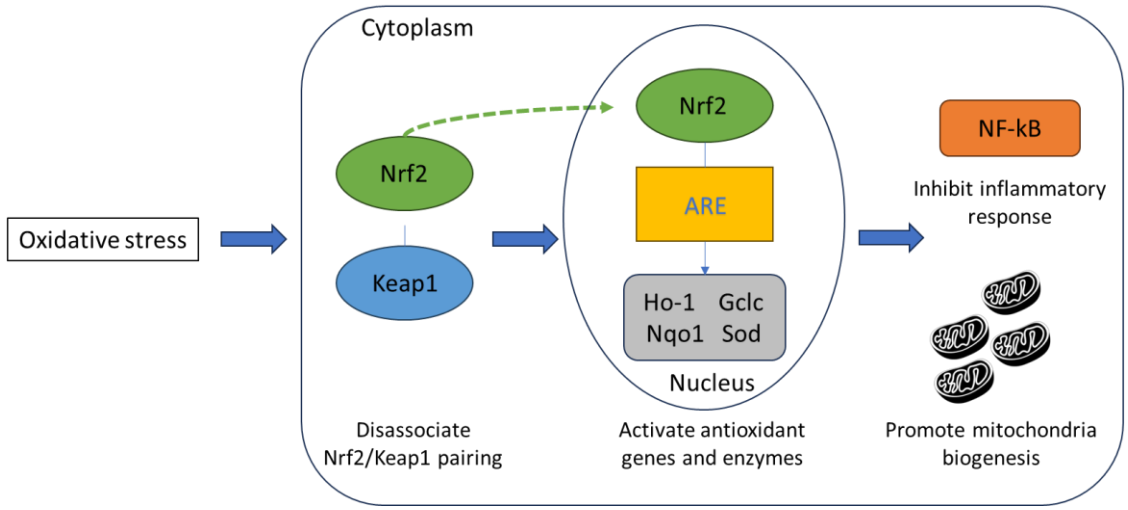


Figure 1.3| Nrf2-ARE antioxidant defence pathway. *Nuclear factor-erythroid factor 2-related factor 2 (Nrf2) is a transcription factor that initiates the metabolic switch of more than 500 cytoprotective genes. Upon exposure to unfavourable exposome factors, Keap1 – an endogenous inhibitor of Nrf2 – dissociates from Nrf2 to facilitate Nrf2 translocation to the nucleus. Nrf2 then triggers adaptive homeostasis by binding to antioxidant response elements (AREs) of its target cytoprotective genes to enhance redox homeostasis and restore proteostasis. Nrf2 also inhibits NF-kB-associated inflammatory response while promoting the biogenesis of mitochondria.*

Therefore, targeting Nrf2-modulated responses via nutritional interventions and supplementation to mitigate the effects of ageing and maintain an individual's physical and physiological capacity has been actively pursued. Consequently, natural sources of Nrf2 activating compounds, found in foodstuffs such as broccoli sprouts (sulforaphane), grapes (resveratrol), tea (flavonoids), tomatoes (lycopene/ cinnamaldehyde), turmeric (curcumin), other fresh vegetables and fruits (Shiels et al., 2021b) have been viewed as possible therapeutic agents within the concept of 'Food as Medicine' (Mafra et al., 2021a).

1.1.2.7 The Exposome

Endogenous 'wear and tear' across the bodies tissues and organs is reflective of total life course biotic and abiotic exposures, within what is termed the exposome (Shiels et al., 2021b). This exposure starts prenatal and comprises a range of environmental or nutritional/lifestyle sources. A range of biomarkers of ageing have been used to measure exposome effects, including DNA methylation clocks, telomere length, markers of cellular senescence (p16, p21, p53), senescence-associated beta-galactosidase (SA-β-gal) activity, and pro-inflammatory SASP products (Joosten et al., 2003, Kooman et al., 2017). More recently, the measurement of somatic mutation rate has also emerged as a putative marker of ageing that can be applied in this context (Cagan et al., 2021). However, as yet, no gold standard has emerged to measure ageing at a biological level. Expression of any such marker should reflect biological 'wear and tear' caused by the loss of adaptive responses due to exposure to exposome stressors. The impact of the exposome can be gauged from the fact that only three exposome factors, comprising air pollution, smoking and an unhealthy diet, account for ∼50% of total deaths worldwide (Lim et al., 2012). Typically, when applied to the diseasome of ageing they indicate that there has been an acceleration in the rate of ageing that correlates with disease progression (Shiels et al., 2021a). A variety of typical exposome factors have been shown in Figure 1.4 with an emphasis on their impacts on epigenome, the epigenome dynamics are reflective of responses to a change in environmental conditions.

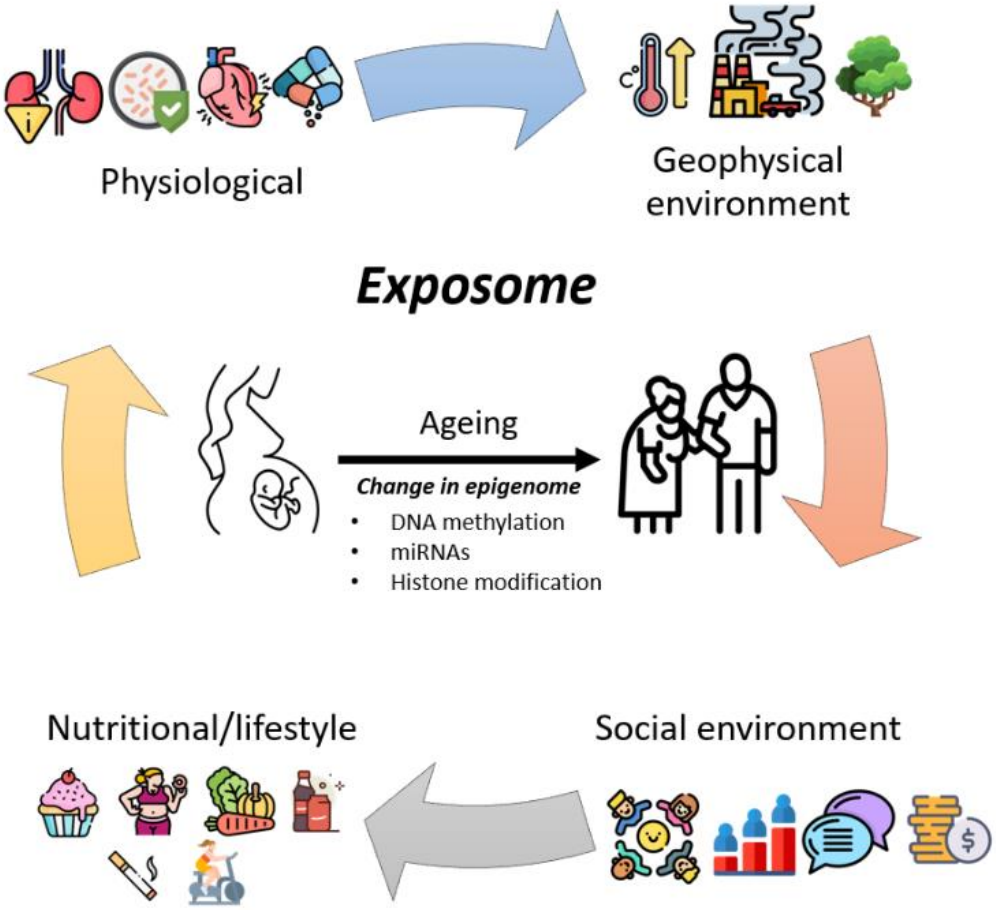


Figure 1.4| Roles of the exposome in ageing. *The process of ageing involves gradual changes in a variety of epigenome components such as DNA methylation, microRNAs (miRNAs) or histone modification. Internal and external exposomes which vary greatly among individuals, have major impacts on how people age. Common factors affecting the ageing process include physiological, geophysical environment, nutrition, lifestyle and socioeconomic status. The image has been obtained from a published article 'Ageing – Oxidative stress, PTMs and disease' in Molecular Aspects of Medicine (Ebert et al., 2022a).*

1.2 Vascular ageing

1.2.1 Definition and causes of vascular ageing

Ageing exerts a detrimental impact on the vasculature (Dai et al., 2020a). Changes in vascular structure and function with age have been termed 'vascular ageing', followed by the deterioration of different body organs (i.e. heart, kidney, etc.). Under the influence of harmful exposome factors, early vascular ageing may occur, characterized by cellular senescence, chronic inflammation, oxidative stress, DNA damage, cellular dysfunction and an imbalance between pro-ageing and anti-ageing defence. The increase of low-grade persistent inflammation and oxidative stress as part of the dysregulated ageing process compromises vascular function and eventually results in diseases such as CKD, CVD and hypertension (Dai et al., 2020a). These changes associated with vascular ageing can be identified in endothelial cells and vascular smooth muscle cells. Yet, effective intervention is required to minimize the possible disease progression. Typical alterations in micro- and macrovasculature during vascular ageing are summarized in Table 1.3.

Cellular changes	**Microvascular changes**	**Macrovascular changes**
Increased oxidative damage	Impaired endothelium-mediated vasodilation	Increased arterial stiffness
Increased inflammatory biomarkers (TNF-α, MCP-1, Interleukins, TGFβ1, NF-kB, IFN- γ)	Reduced angiogenic capacity	Increased collagen and elastin fragmentation
Elevated VSMCs senescence	Phenotypic alterations of microvascular cells (endothelial cells, VSMCs, pericytes)	Reduced vessel elasticity
Increased VSMCs migration/proliferation	Decreased NO bioavailability	Vessel wall destruction
VSMC remodelling	Microvascular wall thickening	Reduced ability to diminish pulsatile stress
Endothelial dysfunction	Lymphatic system alteration	Increased vascular calcification
Increased DNA damage		
Mitochondria dysfunction		

Table 1.3| Common characteristics of vascular ageing. *Vascular ageing is a complex and multifaceted phenomenon, comprising distinct changes in cellular, microvascular and macrovascular properties. Typical cellular changes in vascular smooth muscle cells (VSMCs) include elevated levels of oxidative damage, inflammatory and senescent biomarkers (TNF-α, MCP-1, Interleukins, TGFβ1, NF-kB, IFN- γ). VSMCs also undergo increased levels of cellular remodelling, migration or proliferation. This results in endothelial dysfunction due to excessive DNA damage and mitochondria dysfunction. Changes in microvascular functions are involved in impaired endothelium-mediated vasodilation, angiogenic capacity and reduced NO availability. Phenotypic alterations of microvascular cells, microvascular wall thickening and lymphatic system alterations are also important aspects of vascular ageing. In terms of macrovascular alterations, typical changes have been recorded including increased arterial stiffness, vascular calcification and vessel wall destruction together with collagen and elastin fragmentation. Reduced levels of vascular elasticity with inadequate ability to diminish pulsative stress have also been linked to the severity of vascular ageing.*

1.2.2 Potential treatment of vascular ageing

So far, several studies have demonstrated the use of senotherapeutic approaches to remove SCs to ameliorate aged vasculature. Clearance of $p16^{Ink4a+}$ SCs in an atherogenic mouse model with navitoclax - a potent anti-cancer drug that inhibits B-cell lymphoma 2 (Bcl-2) protein - slowed atherogenesis onset after nine-days treatment (Childs et al., 2016). From that, an improvement in vascular structure and function, such as fibrous cap stability, collagen deposition, reduced elastic fibre degradation has been reported. The outcomes confirmed the senolytic efficacy of navitoclax in selectively killing SCs and its potential role in the treatment of early vascular ageing. Another study has shown that a combination of D+Q (5 mg/kg + 10mg/kg) can eliminate SCs and reduce atherosclerosis progression in mice (Roos et al., 2016). This senolytic cocktail improves vasomotor function by eliminating SCs, reducing senescent phenotypes and plaque calcification even though plaque size remained unchanged.

Other drugs with senolytic activities have been used to reduce carotid-femoral pulse-wave velocity (CFPWV) – a golden standard to measure vascular stiffness and accelerated vascular ageing (Van Bortel et al., 2012). One example is rapamycin with consistent evidence for its ability to reduce arterial stiffness and CFPWV in aged mice (Lesniewski et al., 2017). However, due to its potential side effects (such as anaemia,

thrombocytopenia, diarrhoea, and interstitial pneumonitis), it is necessary to develop safer analogues of rapamycin to maximize their impacts in societal healthcare. Additionally, a sirtuin activator may also be useful in the treatment of vascular ageing. Resveratrol activates sirtuin 1 and prevents arterial wall inflammation and stiffening caused by high-fat and sugar diet in non-human primates (Mattison et al., 2014). In other animal models, resveratrol also ameliorates age-associated biomarkers and pathology (Elmadhun et al., 2013).

Collectively, these studies provide concrete evidence for using senotherapeutic drugs to mitigate the physiological impairments that come with ageing, thus hampering the progression of early vascular disease. At the same time, there are still missing pieces in the puzzle that need to be solved before any official senotherapeutic (geroscience based) intervention can be introduced. Considerations include the exact role of SCs in chronic disease predisposition, development and progression as well as limited clinical trials. An additional concern is the use of morbid lab-based animal models in drug development studies. These animal models are metabolically morbid with a 'humanized' microbiome as captured in an unnatural geophysical and social environment, thus extending the translational gap from animal research to human efficacy profile. This remains to be fully addressed and the study of vascular ageing in non-captive animals is being explored (Stenvinkel et al., 2018).

1.3 Senotherapy

1.3.1 Definition of senotherapy

Senotherapy is therapeutic intervention to selectively targeting physiological deficits in ageing. Classical examples of such senotherapeutics have involved use of naturally occurring bioactive substances, such as sulforaphane, fisetin, resveratrol and combinations of natural and synthetic agents such as D+Q. This age-targeted treatment particularly focuses on activating a range of biological pathways, such as SCs apoptosis promotors, cytoprotection for healthy/dividing cells and antioxidation to mitigate the effects of dysregulated ageing (Raffaele and Vinciguerra, 2022).

1.3.2 Different types of senotherapy

Among senotherapies, three main emerging approaches include use of senolytics, senomorphics and senostatics. Senolytic drugs specifically kill SCs by inducing apoptosis (Kang, 2019). Senescent cells are typically resistant to apoptosis. On the other hand, "senomorphics" or "senostatics" interfere with the senescence-associated signalling pathways by suppressing the effects of inflammasome and SASP, without causing apoptosis in SCs.

Several drug candidates have been demonstrated with senotherapeutic activities by specifically eliminating SCs, thus hampering the impacts of SCs-related inflammatory products. These agents have different tissue targets, molecular and cellular mechanisms that allows precision or personalized treatment (Kang, 2019). By removing SCs, in a range of pre-clinical models, senolytic drugs have been shown to mitigate the physiological decline within the ageing process, suppress the specific phenotypes of chronic ailments in various organs, enhance physical activities/strength and delay all determinants of mortality (Kang, 2019). In the cases of senomorphic and senostatic drugs, their impacts are mainly based on the restoration of cellular activity and viability, thus extending lifespan. Resveratrol is a senomorphic agent that has been identified as suppressing cellular senescence and improving lifespan in zebrafish, mice and invertebrates (Muhammad and Allam, 2018, Wang et al., 2019, Ye et al., 2010). Rapamycin, well known for its ability to inhibit mammalian target of rapamycin (mTOR), has been shown to extend the lifespan of male and female mice by approximately 8% and 11%, respectively (Miller et al., 2014). In *D. melanogaster*, rapamycin treatment has also been proven to significantly extend the lifespan (Regan et al., 2022). The outcome is female-specific, with a decreased level of age-related gut pathology and increased

autophagy. Commonly used senotherapeutic drugs were mentioned together with their mechanisms of actions and effects in Table 1.4.

Senotherapeutic agents	***Mechanisms of action***	***Effects***	***Reference***
Dasatinib & Quercetin (D+Q)	Src tyrosine kinase inhibitor Bcl-xL protein inhibitor	Senolytic – Induce apoptosis of SCs	(Novais et al., 2021)
Fisetin	PI3K/AKT and NF-κB pathway inhibitor, topoisomerase and pro-inflammatory cytokines TNFα, IL6 inhibitor, ROS scavenger	Senolytic and senomorphic – induce apoptosis of SCs and reduce SASP	(Yousefzadeh et al., 2018)
Navitoclax	Bcl-2, Bcl-xL and Bcl-W inhibitor	Senolytic – Induce apoptosis of SCs	(Mohamad Anuar et al., 2020)
Piperlongumine	Activation of ROS-dependent p38/JNK signaling pathways	Senolytic – Induce apoptosis of SCs	(Rawat et al., 2020)
Sulforaphane	ROS scavenger, NF-κB pathway inhibitor, Nrf2 activator	Senolytic – Induce apoptosis of SCs	(Mafra et al., 2021b)
Resveratrol	NF-κB/IκB inhibitor, AMPK and SIRT1 activator	Senolytic and senomorphic – induce apoptosis of SCs and reduce SASP	(Muhammad and Allam, 2018, Ye et al., 2010)
Forkhead box protein O4 (FOXO4)-related peptides	Forkhead box protein O5 (FOXO5)-p53 interaction inhibitor	Senolytic – Induce apoptosis of SCs	(Huang et al., 2021)
Bcl-2 family inhibitor	Bcl-2 inhibitor	Senolytic – Induce apoptosis of SCs	(Lafontaine et al., 2021)

Table 1.4| Commonly used senotherapeutic agents. *Senotherapeutic compounds are classified into two main categories, namely senolytics (inducing apoptosis of SCs), senomorphics or senostatics (interfering with bystander effects of SCs). Several examples of senolytic compounds include D+Q, navitoclax, piperlongumine, sulforaphane, FOXO4-related peptides and Bcl-2 family inhibitors. Other senotherapeutic agents can act as both senolytics and senomorphics, such as fisetin and resveratrol. Each agent mitigates the effects of cellular senescence in distinct mechanisms of action.*

1.3.3 Role of naturally derived senotherapy

As part of a senotherapeutic approach, naturally occurring nutritional substances are considered a very strong lever for enhancing human health within the strategy of Food as Medicine (Mafra et al., 2021a). According to the 'Food as Medicine' concept - first introduced by Hippocrates circa 400 B.C, human beings benefit from having a balanced diet (Mafra et al., 2021b, Witkamp and van Norren, 2018). Diets containing a variety of vegetables and fruits are preferred as they contain bioactive substances that promote the body's cytoprotection systems. Consequently, celebrated 'anti-ageing' foods, such as the strawberries, grapes, wine, bilberries, tea and other vegetables might have a direct role in protecting against age-related diseases (Bensalem et al., 2018, Kojima et al., 2020, Navarro-Hortal et al., 2021, Petersen and Smith, 2016). However, the success of conventional dietary studies is highly variable and depends on many factors, including non-standardised composition. Additionally, as the choice of food is mainly based on personal preference and culture, many patients tend to be non-compliant with dietary recommendations. Moreover, the surrounding environment, internal and external stressors play a crucial role in shaping their eating habits, resistance to diseases as well as biological age (Shiels et al., 2021b). As there is a diverse range of food composition in human diets based on specific cultures and locations, the overall impacts of different combinations of food components on gut microorganisms are very complex. For instance, the Western diet strongly favours the use of red meat and ultra-processed foods with limited vegetables and fruit usage and has been indicated with higher levels of proteolytic bacteria and dysbiosis (Garcia-Mantrana et al., 2018). People who consume these diets on a regular basis are more prone to develop further age-related complications (Bischoff, 2016). The standardisation of beneficial food components still needs to be amended to ensure overall health quality. By emphasizing the importance of the exposome for healthy ageing, senotherapeutics surpass typical concerns in conventional studies (such as morbid models, unclear long-term effect, unexpected external factors) and possibly creates a geroscience based new era for preventing the onset of chronic disease based on targeting specific ageing drivers.

1.3.4 Potential disadvantages of senotherapy

Despite their merits, there are some considerations before senotherapeutics can be used widely. There are lots of conceptual considerations for their employment and when in the life course they should be best used. Several adverse effects of senotherapy are discussed as follows:

1. Clearance of SCs using senolytics can cause damage in specific age-associated conditions. It has been shown that the removal of senescent β-cells led to type 2 diabetes in mice (Helman et al., 2016).
2. Senomorphic or senostatic drugs cannot eliminate SASP sources permanently, which requires repeated administration to ensure efficacy. This may result in other essential pathways suppression and disturbance in tissue homeostasis due to blocking of SASP. Another problem is the fate of SASP-suppressed SCs after the treatment as many of the immune cells are recruited by the SASP factors. Thereby, it is possible that senomorphic/senostatic drugs can give rise to non-functional tissues rather than improving the functions of these organs.
3. Another concern is the lack of information regarding the optimal time points of administration of senolytic or senomorphic agents within the life course. This requires an appreciation of antagonistic pleiotropy, since the drugs may exert either beneficial, neutral or negative impacts on different organs at a specific point of time (Williams, 1957). It remains unclear whether they provide protection and improve future health span when administering early in life or should only be administered in middle years and later stage.
4. Other cryptic side effects have also been identified while usings senotherapeutic drugs. For instance, sulforaphane significantly reduces the water intake in young mice (Bose et al., 2020). To minimize the risks, more studies should be performed to validate the side effects of various senolytic, senomorphic and senostatic drugs in a tissue-specific manner (Zhang et al., 2023).
5. Current studies of senotherapy have insufficiently demonstrated the effect of potential compounds in response to the external environment and lifestyle factors. Cumulative effects of the exposome, such as pollution, diet or socioeconomic position, result in ‘wear and tear’ damage to the body over time (Shiels et al., 2021b). With the increasing incidence of lifestyle diseases, *in vitro* and *in vivo* experiments should be designed to improve overall healthspan by tackling long-term exposure to external stressors.

6. Potential side effects on deteriorating fecundity rate due to the 'trade-off' between fertility and longevity. For instance, two senotherapeutic compounds have been associated with impaired fecundity rate in vivo, including rapamycin and navitoclax. Rapamycin, an mTOR-inhibitor and immunosuppressant, causes follicle cell invasion, and somatic oocyte destruction, thus leading to the drastic reduction in fecundity rate in in fruit flies (Thomson and Johnson, 2010, Alves et al., 2022, Buchanan et al., 2018). Navitoclax, a prominent anti-cancer drug that inhibits Bcl-2 with senolytic effects on SCs, has been shown to significantly deplete all stages of ovarian follicles in female mice (Xia et al., 2024).

1.3.5 Lessons from Nature

As human being coexists in nature, its impact on human physical and mental wellbeing is both crucial and undeniable. By using models and systems derived from the natural world, i.e. biomimetic approach to identify potential solutions to complex human health problems (Stenvinkel et al., 2021b). The main merit of this concept, compared with conventional lab models, is the recapitulation of the products of natural selection. Whereas lab-based animal models are metabolically morbid, they are not typically representative of the biology of their natural counterparts (Clayton et al., 2016, Stenvinkel et al., 2018). Their microbiomes are typically "humanised" with the unnatural geophysical and social environment which further prevent them from becoming an ideal model for a wide range of healthcare issues in the 21st century . For ageing research, biomimetics offers a variety of interesting animal models for study, including animals with negligible senescence and superior capacity to cope with extreme environmental stress (Stenvinkel et al., 2021b). For instance, bowhead whales (living >200 years), tubeworms (>300 years) and the longest living, cancer-free rodent – naked mole rate (~30 years) are known both for their long lifespan with natural protective mechanisms against many diseases. Other natural models are also useful in research on cancer (Elephant, Tasmanian devil, dog), metabolic disease (hibernating bears, lemurs), Covid-19 (bat, llama) or alcoholism (tree shrew) (Stenvinkel et al., 2021a).

Notably, in the animal kingdom, hibernating bears offer us with ingenious biomimetic insights, highlighting the physiological mechanisms by which they protect themselves against an excessive burden of lifestyle diseases. Due to their reduced metabolic activities, hibernating bears are resistant to insulin-resistance associated type-2 diabetes regardless of their profound weight gain by the end of summer (Stenvinkel et al., 2020). A recent study conducted on bears in different living conditions, has emphasized the activation of metabolic switch in free ranging bears, compared to captive bears. This switch facilitates a reduction in the production of the pro-inflammatory microbial metabolite Trimethylamine N-oxide (TMAO) and increased production of betaine from choline by gut microbes. The result is that the methylome is maintained in free ranging bears, instead of cardiovascular and kidney damage - associated with TMAO derived from TMA when processed in the liver (Ebert et al., 2020b). Correspondingly, in the wild, bears do not develop lifestyle related pathologies such as CKD, CVD or muscle loss due to prolonged period of inactivity and starvation (Stenvinkel et al., 2013). However, in captivity, the opposite occurs.

In contrast to hibernating bears, the study also identified the diminished levels of circulating betaine in captive felids, which are known for their profound susceptibility to CKD. Their betaine levels were found to reduce by 4-fold in comparison to hibernating bears, similar to that of CKD 3 patients. As CKD has substantially affected older domestic cats (up to 1/3), various contributing factors (i.e. ageing, high phosphorus, comorbidities due to high meat diet) have been elucidated (Brown et al., 2016). As such, the composition of the gut microbiota has significant impact on healthy ageing (Shiels et al., 2019). In the old, a reduction in the diversity of the intestinal microbiota has been reported, which inversely correlated with the incidence of the "diseasome of ageing". The composition of microbiota also changes with increasing biological age (Craven et al., 2021a). While a significantly larger number of pathogenic microbiotas have been observed in those at higher biological age, more salutogenic microbiota have been observed in individuals at lower biological age. This feature is also affected by SEP, which is associated with significantly lower betaine levels in those at lower SEP, and thus maintenance of the epigenetic landscape of ageing. Even though scientists have identified a correlation between gut microbiota and ageing, more studies are required to understand the underlying mechanisms to better prevent age-related chronic diseases. Understanding the underlying mechanisms of this beneficial metabolic switch observed in bears and the potential effects of betaine might offer us new strategies to intervene in age-related diseases in humans, which are directly linked by sedentary lifestyles and other environmental factors.

1.4 One carbon metabolism

1.4.1 One carbon metabolism pathway

One carbon (1C) metabolism consists of interconnected metabolic pathways that supply methyl groups for many essential cellular processes, especially methylation of DNA, RNA and related proteins (Ducker and Rabinowitz, 2017a). This metabolic process is characterized by two main cycles – methionine remethylation and folate metabolism. Found in the cytoplasm, nucleus and mitochondria, 1C metabolism is responsible for molecular biosynthesis, maintenance of genome integrity, epigenetic patterns as well as antioxidant defence (Stover, 2009, Stover and Field, 2011, Ducker and Rabinowitz, 2017b). During 1C metabolism, methionine is metabolized into adenosylmethionine (SAM) before being converted to *S*-adenosylhomocysteine (SAH) (see Figure 1.5) (Clare et al., 2019). This process continues as SAH undergoes hydrolysis, which eventually gives rise to homocysteine. The series of enzymatic reactions are catalysed by distinct enzymes comprising of methionine adenosyl transferase (MAT2A), SAM synthetase (SAM 1/2), methyltransferases (MTs), SAH hydrolase (SAHH), homocysteine methyltransferase (HMT) (Lauinger and Kaiser, 2021).

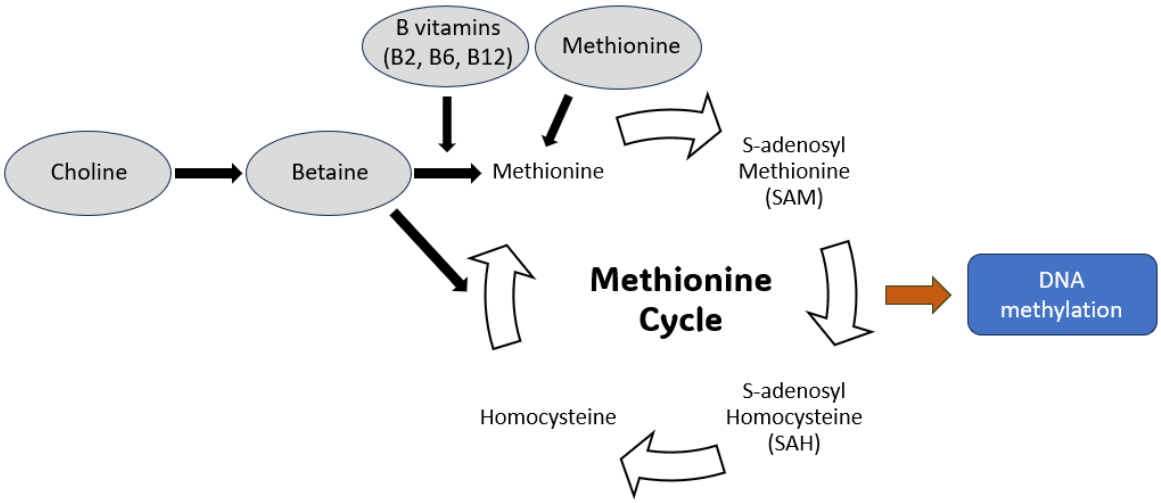


Figure 1.5| The methionine cycle in one-carbon metabolism, supplies methyl donor groups from nutritional sources. *Several dietary components that can increase intracellular methionine storage include betaine, choline and other B vitamins (B2, B6, B12). During 1C metabolism, methionine is metabolized into adenosylmethionine (SAM) before being converted to S-adenosylhomocysteine (SAH). This process continues as SAH undergoes hydrolysis, which eventually gives rise to homocysteine.*

Homocysteine, an intermediate molecule, can be recycled to initiate 1C metabolism. In order to convert homocysteine to methionine, it is requisite for specific dietary components to be available in the body system that act as methyl donors and enzymatic cofactors. Several dietary components have been shown to regulate 1C metabolism, including betaine, choline, methionine and other B vitamins (B2, B6 and B12) (Ducker and Rabinowitz, 2017b).

1.4.2 One carbon metabolism in ageing

During ageing, DNA methylation patterns change in association with altered 1C metabolism. Recent studies have reported reduced DNA methylation or hypomethylation in human, rats and mice (Singhal et al., 1987, Stone et al., 2021, Vanyushin et al., 1973). Abnormal methylation events have also been linked to different diseases within the diseasome of ageing. For instance, whole-blood global DNA hypomethylation with accelerated oxidative stress has been observed in CKD patients (Zinellu et al., 2017). A decrease in DNA methylation in three cytosine-phosphate-guanine (CpG) loci in the triggering receptor expressed on myeloid cells 2 (TREM2) gene has been reported to increase susceptibility to Alzheimer's diseases (Ozaki et al., 2017).

In addition, a dynamic switch in the epigenetic landscape is essential in helping organisms to adapt to the exposome changes (Schrey et al., 2016). As age increases, 1C metabolism-induced methylation functions as an adaptive response while influencing rate of health span and potential lifespan. During the Dutch Hunger Winter of 1944–45, a lower level of DNA methylation was reported in the insulin-like growth factor 2 (IGF2) gene to increase glucose storage for individuals conceived during the famine, compared to their unexposed siblings (Heijmans et al., 2008). This study has provided strong evidence supporting the hypothesis that exposure to environmental stressors early in life can result in lifelong epigenetic alterations. Together with DNA methylation, histone methylation as part of 1C metabolism also plays an important role in maintaining epigenetic landscapes. The imbalance between transcription-activating and repressing histone marks, SAM: SAH in histone methylation are main causal factors of accelerating ageing process (Han and Brunet, 2012, Parkhitko et al., 2019).

Another contributing factor to impaired DNA methylation via methionine metabolism is mitochondrial dysfunction. This can be explained by mtDNA depletion and lower metabolite levels, which cause abnormalities in nuclear DNA methylation (Lozoya et al., 2018). The main pathway for betaine production in human cells is in mitochondria, through the oxidation of choline. Mitochondrial dysregulation occurs with ageing; hence betaine levels can become depleted with a knock-on effect on the maintenance of the epigenetic landscape of ageing and the other hallmarks of ageing.

1.5 Betaine

1.5.1 Definition and sources of betaine

Betaine, also known as trimethylglycine, is a natural product derived from plants, animals and microorganisms (Arumugam et al., 2021). Common dietary sources of betaine include wheat bran (7200 µg/g), spinach (6000-6450 µg/g), beets (4800 µg/g), quinoa (3900 µg/g) and animal meats (i.e. turkey, beef) (Figure 1.6). Average daily intake of betaine varies across geographical and cultural diversity with the mean ranges from 70-300 mg/day (EFSA Panel on Dietetic Products et al., 2017). According to EFSA guidelines on Dietetic Products, Nutrition and Allergies (NDA), a maximum amount of 6 mg/kg of betaine of body weight per day is considered safe (EFSA Panel on Dietetic Products et al., 2017). This recommendation applies together with additional betaine intake from a person's daily diet. Effective daily dosage of betaine highly depends on the purpose of supplementation. For instance, 3-6 g betaine/day (patients) and 1.5-3 g (healthy participants) have been frequently used in research to reduce homocysteine levels (Ashtary-Larky et al., 2022b). To improve exercise resistance, a 2.5 g/day (divided into two 1.25 g) has been tested (Arazi et al., 2022). Within the body, the production of betaine mainly occurs in liver and kidney mitochondria (Willingham et al., 2020).

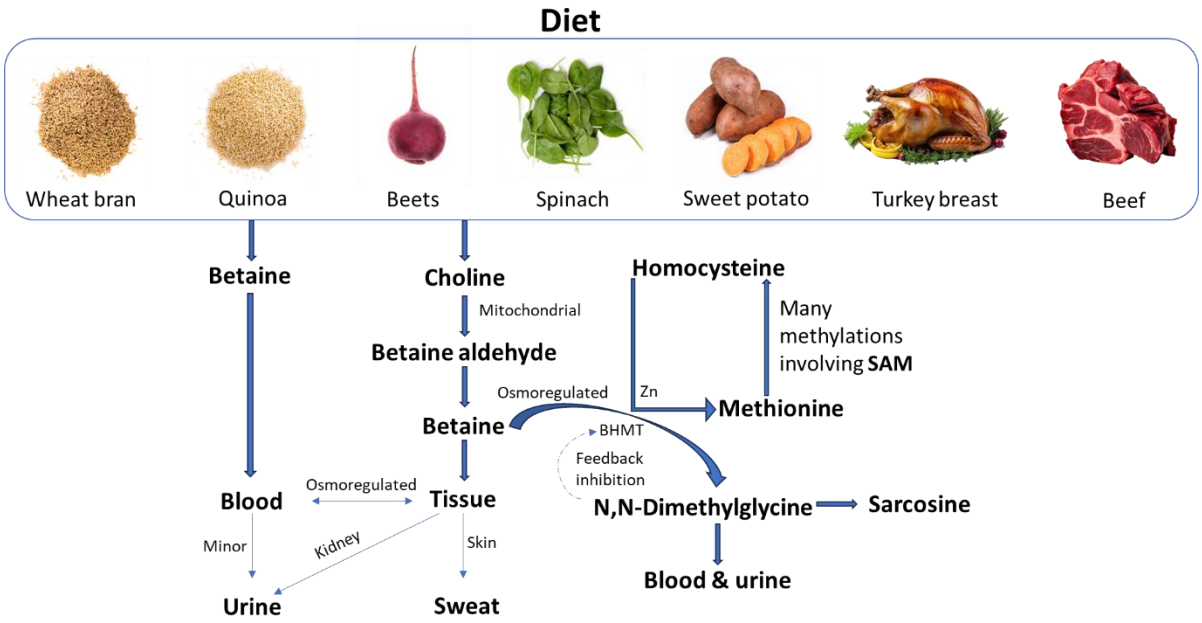


Figure 1.6| Sources and metabolic pathways of betaine. *Betaine is a byproduct of choline metabolism and is available in many dietary sources, including wheat bran, quinoa, beets, spinach, sweet potatoes and meat. In the kidney and liver, the process of*

producing betaine in the mitochondria starts with the oxidation of choline to betaine aldehyde. Following that, betaine aldehyde is converted to betaine by the enzyme choline dehydrogenase. Betaine, as an osmolyte, maintains higher concentrations in most tissues, compared to its concentration in blood. A minimal amount of betaine is eliminated by catabolism in urine and sweat. Betaine is converted to N-N- dimethylglycine and sarcosine via the enzyme homocysteine methyltransferase (BHMT). As part of the metabolic pathways of betaine, homocysteine – also known as a cardiovascular disease biomarker – is converted into methionine.

1.5.2 Betaine functions in human health

Betaine is widely known for its two main functions (i) as a potential methyl donor and (ii) as an osmolyte (Craig, 2004). Possessing three methyl- groups in its structure, betaine supports transmethylation reactions. As betaine is also generated via choline metabolism, it is converted firstly into dimethylglycine, followed by sarcosine (a.k.a. mono-methyl glycine) and eventually glycine, via different enzymatic reactions (Ueland, 2011). As glycine is further added into the amino acid pool, remaining methyl groups participate in the process of converting cardiovascular disease-associated risk factor homocysteine to essential amino acid methionine (Anioła et al., 2014, Craig, 2004). The process of donating methyl groups by betaine has been outlined above (see Figure 1.6). As a methyl donor, betaine supports crucial biochemical processes, particularly in maintaining healthy homocysteine levels, vital for cardiovascular well-being (Ashtary-Larky et al., 2022a).

In addition, betaine has been ascribed antioxidant effects in inflammation-associated pathologies, such as CKD, CVD. Several studies have reported reduced ROS production, NF-$_{\kappa}$B regulation and cytotoxicity after betaine supplementation (Lee et al., 2013). The inhibition of pro-inflammatory interleukin 1 β (IL1β) cytokine has also been illustrated after betaine treatment (Xia et al., 2018) consistent with ascribed anti-inflammatory and potentially senotherapeutic properties. Betaine also acts as an osmolyte to maintain normal cell volume. Notably, it provides cells and vessels with protection against cell shrinkage or swelling due to hyper- or hypo-osmosis (Lever and Slow, 2010). Betaine supplementation also contributes to protein synthesis, which can aid muscle development when combined with exercise (Cholewa et al., 2013). Some research suggests betaine might have protective properties for different body organs (i.e. kidney, gut, liver, adipose tissue, heart, nervous system, reduced fat loss, skin and muscle) (Arumugam et al., 2021).

Overall, betaine's diverse functions underscore its importance in maintaining various aspects of human health (see Table 1.5).

Typical functions of betaine in human health
▪ Regulate methionine metabolism
▪ Protect against ROS-associated oxidative damage
▪ Lower levels of homocysteine in blood
▪ Act as osmolyte agent to maintain normal cell volume
▪ Provide methyl groups to remethylate homocysteine
▪ Suppress the activation of pro-inflammatory cytokines – IL1β, IL11
▪ Improve muscle mass
▪ Protect different body organs, i.e. kidney, gut, liver, adipose tissue, heart, nervous system, reduced fat loss, skin and muscle.

Table 1.5| Functions of betaine in human health. *Betaine is a naturally occurring substance with various beneficial effects on human health. As a methyl donor, betaine is responsible for regulating methionine metabolism in the liver and kidneys as well as providing methyl groups to remethylate homocysteine. Elevated homocysteine levels have been associated with increased risk of cardiovascular diseases such as atherosclerosis, coronary artery disease or stroke. As such, betaine treatment has been demonstrated with potential efficacy in lowering homocysteine levels in blood. Betaine also acts as an osmolyte agent which provides cellular protection against internal and external stressors (i.e. high sodium level, temperature). The antioxidant effects of betaine have also been confirmed by suppressing the activation of pro-inflammatory cytokines (i.e. IL1β, IL11). Supplement with betaine has been associated with increased total muscle mass and different body organs protection, such as kidney, gut, liver and heart.*

1.6 Project aims

We have sought to determine if betaine can slow the progression of ageing and enhance health span in human cells and animal models, through a pragmatic series of experiments.

- **Hypothesis**: Based on a range of human *in vivo* studies in the Shiels lab, designed to investigate exposome factors that contribute to the progression of the ageing process, we have hypothesised that betaine is a key mediator of potential senotherapeutic effects in vivo.
- **Aim**: To modulate age related mitochondrial dysregulation and enhance cellular health span
- **Objective1**: To mitigate betaine loss with age *in vitro* and thus slow replicative ageing
- **Objective 2**: To ameliorate the effects of the hallmarks of ageing *in vitro*
- **Objective 3**: Extend healthspan and/or lifespan in pre-clinical models of normative ageing.
- **Objective 4**: To determine if any salutogenic effects are mediated through rejuvenation of metabolic reprogramming.
- **Objective 5**: To identify whether betaine exerts positive effects on fecundity rate while maintaining lifespan extension in vivo

Chapter 2: Effects of betaine on vascular smooth muscle cells

2.1 Introduction

Ageing is a segmental process of decline in physiological functions and capability which increases the risks of morbidity and mortality over time (Bareja et al., 2019). This age-related deterioration displays substantial inter-individual variation and affects different organs at different rates (Fulop et al., 2010). During ageing, blood vessels experience a range of morphological and physiological changes due to prolonged stress and hormonal dysfunction. Premature vascular ageing accounts for approximately 10-27% of the global population, depending on different modifiable (i.e. hypertension, and exposome factors such as obesity, smoking, alcoholism) and non-modifiable risk factors (i.e. age, genetics, family history) (Gómez-Sánchez et al., 2022). It has been hallmarked into six pathological features of ageing that are common in many age-related diseases (López-Otín et al., 2013). These hallmarks consist of arterial and capillary stiffness, endothelial dysfunction, oxidative stress, impairment in angiogenesis capacity, early onset of atherosclerosis, and low-grade chronic inflammation (Ghebre et al., 2016).

The development of vascular ageing remains complex and multifactorial. Many of signalling mechanisms of age-related changes in vasculature are shared by diseasome of ageing (González et al., 2023). Recent findings suggest that a range of diseases such as CKD, CVD are closely correlated with vascular smooth muscle cells (VSMC) senescence, focusing on their links with oxidative stress and inflammation (Ferrucci and Fabbri, 2018a, Laurent, 2012). VSMC senescence is mainly regulated by the p16- and p21-associated senescence pathways, increased oxidative stress, telomere damage, vascular calcification and chronic inflammation (Chi et al., 2019). Bystander effects generated by the SASP of senescent VSMCs, induce non-senescent VSMCs to grow and migrate. This results in vascular remodelling and occurrence of structural, functional and mechanical dysfunction in blood vessels (Harvey et al., 2015). Substantial increase in the levels of inflammatory factors, such as IL6 and IL1β, has also been reported in various age-related chronic diseases (Sesso et al., 2015, Rea et al., 2018). Moreover, increased vascular calcification is strongly linked to diseasome of ageing and poor prognosis of cardiovascular diseases. Several markers of VSMCs senescence, such as Lamin A (LMNA) or phospho-histone H2A, have been found in calcified VSMCs (Cobb et al., 2021, Ragnauth et al., 2010). These findings provide strong evidence for the connection between vascular ageing, VSMCs senescence and age-related chronic diseases.

Observations in general population cohorts have indicated that accelerated ageing correlates with a decrease in levels of betaine (Craven et al., 2021b). Betaine, also known as trimethylglycine, is derived from choline and L-carnitine metabolism and is a component of one-carbon metabolism that plays a physiological role in cytoprotection under stress. Additionally, it acts as a methyl donor inputting into the SAM cycle. A lower level of betaine is also an indicator of accelerated epigenetic ageing typified by decreased global DNA methylation levels (Dobrijević et al., 2023). It has also been shown that higher concentrations of serum betaine lower hypertension risk, compared to individuals with lower serum betaine (Huang et al., 2023). Studies have also suggested anti-inflammatory effects of betaine during ageing (Go et al., 2005b).

As vascular ageing accompanies many ageing-related diseases, targeting VSMCs senescence via senotherapy is considered a promising treatment strategy. A senotherapy can be defined as a therapeutic intervention to selectively target physiological deficits in ageing (Kim and Kim, 2019). Typical examples of senotherapies have involved the use of agents to unlock the capacity for SCs to undergo apoptosis. The exact proportion of senescent cells in living organisms is largely unknown, even though some potential evidence estimated that the number is at most 15% (de Magalhães and Passos, 2018, Biran et al., 2017). Senotherapy have involved using repurposed pharmacological agents, such as Dasatinib alongside a naturally occurring bioactive substance such as Quercetin, or use of non-pharmacological, nutritionally derived agents, such as sulforaphane, fisetin, resveratrol (Raffaele and Vinciguerra, 2022). Current senotherapeutic compounds also have a narrow spectrum cell specificity. To minimize the risks and enhance efficacy, more studies are needed to validate various drugs in a tissue and cell-specific and life-course-dependent manner. We have therefore investigated morphological and physiological changes in VSMCs after treatment with betaine, in order to provide more insight into the use of betaine as a novel senotherapeutic agent to target vascular ageing.

We have investigated the senotherapeutic effects for betaine using primary human VMSCs and assessed its effects on the expression of a series of well-established biomarkers of cellular ageing, including expression of p16 (*CDKN2A*) and p21 (*CDKN1A*), senescence β-galactosidase expression, SASP-associated IL6 and IL1β expression, cytoplasmic chromatin fragment events (γH2A.X staining), mitochondria functions as well as other biomarkers for VSMCs damage and cell stress. Our data demonstrated the significant improvements of these key biomarkers in VSMCs after long-

term supplementing with betaine. The expression of genes that are associated with cell cycle arrest (p16, p21), oxidative damage (IL6, IL1β), VSMCs senescence (FOXO4, LMNA) and cellular stress (Nrf2, SerpineB2) was found to be substantially downregulated, together with β-galactosidase expression and CCF events. Interestingly, there were no noticeable alterations in mtDNA-CN and telomere length after the treatment. We also observed the potential metabolic switch, initiated by betaine, from aerobic to anaerobic respiration, which might explain the underlying mechanisms in which betaine optimizes the proliferation capacity of VSMCs.

2.2 Materials and methods

Cell culture. Vascular smooth muscle cells (VSMCs) were obtained from healthy donors under full ethical consent from Prof. L Schurgers laboratory Maastricht University. VSMCs were cultured in Cascade Biologics Medium 231 (M-231-500) supplemented with penicillin (100 units/mL), streptomycin (0.1 mg/mL), amphotericin B (F, 0.25 µg/ml), and smooth muscle growth supplement. All the above reagents were obtained from Thermofisher Scientific, unless stated otherwise. The cells were cultured under typical conditions of 5% CO2 at 37ºC in a humidified incubator. Passage of VSMCs was performed using a split of 1:3 (before treatment) or 1:2 (during treatment) when the cell confluency reached ~80%. A standard protocol was followed using Dulbecco's phosphate buffered saline (DPBS 1X) to wash the cells after the medium was removed and trypsin-EDTA (0.05%) to detach the cells from the flasks (Merck, 2023).

Real-time cell analysis. The ACEA xCELLigence RTCA MP system was used to monitor cell growth on E-Plate 96 View 96-well plates. VSMCs - passage 6 (18 cell division) and passage 16 (48 cell division) were counted with a haemocytometer using 0.5% Trypan blue before seedling into 96-well RTCA plate. Plates were seeded with 2000 cells/well in 200 µl medium. Experiments with different betaine concentration (ranging from 10 µg/ml to 1 mg/ml) were carried out in quadruplicate and outlier wells were excluded from the analysis. Cells were left to settle for approximately 24 h, then treated and monitored for at least 72 h. The Cell Index was normalised with respect to the last timepoint before treatment. Slope analysis was carried out considering a period of 24 h after treatment. ACEA RTCA software version 1.2.1 was used.

5-Bromo-2′-Deoxyuridine (BrdU) Labelling. Cells were plated in an 8-well chamber slide (5,000 cells/well) for 24 hours in a normal culture medium. On the next day, the old medium was replaced by betaine-supplemented medium for another 24 hours before staining with BrdU labelling assay. 5-Bromo-2'-deoxy-uridine labelling and detection kit II (Catalogue no. 11299964001, Roche) was used following the manufacturer's instruction. The cells were aspirated and washed twice with PBS. The BrdU labelling medium was prepared beforehand (1:1000 dilution in VSMCs, filtered through a membrane with a pore size of 0.2 µm to ensure no aggregates remaining in the solution) and then added to the cells for 4 hours at 37°C with 5% CO_2. VSMCs were then washed three times with washing buffer which was previously diluted 1:10 in milli-Q water. During the fixation process, 0.2 mL of the ethanol fixative was added to each well for 10

minutes at −20 °C. The cells continued to be washed three times with a washing buffer. In the next step, VSMCs were incubated for 30 minutes in an anti-BrdU solution at 37°C, followed by three times washing with the washing buffer. Anti-mouse-Ig-AP solution was then added to the cells and incubated for 30 minutes at 37 °C. VSMCs were washed again with washing buffer thrice. Colour substrate buffer solution (100 mM Tris HCl, 100 mM NaCl, 50 mM MgCl2, at a pH of 9.5) was added to each well and incubated at room temperature for 15 minutes. Finally, the cells were washed two more times and added with 70% glycerol (Thermofisher Scientific) diluted in milli-Q water. Visualisation was achieved with Marianas SDC platform (Intelligent Imaging Innovations Ltd) at 10x objective, 0.45 NA. The BrdU area and total cell area were identified by SlideBook software (Slidebook2024.2).

Long term cell treatment with betaine. VSMCs were cultured with standard medium and betaine 1 mg/ml (Sigma Aldrich)-supplemented media. Betaine was added directly to the VSMCs culturing medium in every passage from young to old cells. In every passage, DNA or RNA were extracted from the remaining cells for further downstream experiments.

Real-time cell doubling time. Cell doubling time is the time taken for a cell population to double in number. This number was recorded during the entire duration of cell growth.

RNA extraction. VSMCs were added with 1 ml of TRIzol reagent (Thermofisher Scientific) per flask. These cells were kept in -80ºC for at least 24 hours to improve the efficacy of the following RNA extraction step. The RNA extraction was performed with a Zymo RNA Clean & Concentrator^{TM}-5 kit according to the manufacturer's instructions. The RNA pellet was resuspended in 40 µl nuclease-free water. RNA quantity was identified using a NanoDrop 2000 spectrophotometer. RNA is stored at -80ºC for long term storage.

DNA extraction. Cells were collected and extracted genomic DNA in the Maxwell® 16 Cell DNA Purification Kit as stated in the manufacturer's protocol. DNA quantity was identified using a NanoDrop 2000 spectrophotometer. DNA is stored at -20ºC for long term storage.

Reverse transcription. 125 ng of each RNA sample was reverse transcribed into cDNA using the Invitrogen SuperScript II reverse transcriptase synthesis kit (Ref number: 18064-014) following the manufacturer's instruction. RNA was incubated with random

primers and dNTPs mix 10 nM at 65ºC for 5 minutes. In the next step, previous mix were combined with RNase® Out, 0.1 M DTT, 5X First strand buffer and SuperScriptTM II reverse transcriptase before running the recommended PCR cycle (12 minutes at 25ºC, 50 minutes at 42ºC, 15 minutes at 70ºC and 5 minutes at 4ºC). cDNA was diluted 4-fold with nuclease-free water before using 1 µl of the diluted sample per well for qPCR quantification.

Real-time Polymerase Chain Reaction (RT-PCR). The SCAPs plates (TaqMan Array 96-Well FAST Plate) were custom-made and prepared was carried out on the diluted cDNA using Taqman Universal MasterMix II no UNG and following the TaqMan Thermofisher Scientific protocol. The PCR reaction was performed in an Applied Biosystems 7500 Fast Real-time PCR system. The thermal condition was recommended by the manufacturer (2 minutes at 50ºC, 10 minutes at 95ºC, 15 seconds at 95ºC for 45 cycles and 1 minutes at 60ºC). HPRT1, GUSB and GAPDH were used as endogenous controls, chosen from a panel of four options. The experiment was run using Applied Biosystems 7500 software version 2.0.6 provided by the manufacturer. ExpressionSuite version 1.3, GraphPad 10.2.1 were used for data analysis and graphs demonstration.

Senescence β-Galactosidase Staining. Cells were plated in a 12-well plate (25,000 cells/well) for 24 hours before staining with SA β-gal. The Senescence Cells Histochemical Staining Kit (Merck, UK) was used following the manufacture's instruction. The cells were aspirated and washed twice with PBS. The fixation buffer was prepared beforehand and then added to the cells for 6-7 minutes at room temperature. During the fixation process, 0.5 mL of the staining mixture were added to each well (for 10 mL mixture: 8.5 mL milli-Q water, 1 mL staining solution, 125 µL Reagent B, 125 µL Reagent C, and 0.25 mL X-gal Solution, filtered through membrane with a pore size of 0.2 µm to ensure no aggregates remaining in the solution). The cells were incubated for 4 hours in the staining mixture at 37 °C without CO_2 until the cells are stained blue. The plate was sealed with parafilm to prevent drying out. Visualisation was achieved with brightfield microscopy, and pictures were taken using EVOS™ FL Auto 2 (Thermofisher Scientific). Total cell number and cells positive for SA β-gal were counted.

γH2A.X Staining. The cells were stained for γH2A.X and DAPI 72 h after plating. Cells were washed twice with PBS before being fixed with 100% methanol (Merck, UK) at room temperature for 5 minutes. The cells were then permeabilised with 0.1% Triton™ X-100 (Merck, UK) diluted in PBS and incubated at room temperature for another 5

minutes. The cells were added with blocking solution. Between each step, the cells were washed twice with PBS. The blocking solution consisted of 1% bovine serum albumin, 10% goat serum (Merck, UK), and 0.3 M glycine (Thermofisher Scientific) in 0.1% PBS-Tween® 20 (Merck, UK). The incubation took place at room temperature for 15 minutes. The primary rabbit antibody anti-γH2A.X (phospho S139) (cat. No. ab2893, Abcam, UK) was diluted 1:10,000 in blocking buffer before incubated at 4°C on a moving platform overnight. The cells were washed three times with PBS before adding secondary goat anti-rabbit Alexa Fluor® 488 antibody (1:1000; cat. no. ab150081, Abcam, UK). The incubation was at room temperature for 1 hour on a moving platform. After three more washes with PBS, the cells were stained with 1.43 μM DAPI (Thermofisher Scientific, UK), diluted in PBS at room temperature for 1 h on a moving platform. At this point, the plate was covered from light. Finally, the cells were washed thrice and added with 70% glycerol (Thermofisher Scientific) diluted in milli-Q water. Visualisation was achieved with fluorescence microscopy, and pictures were taken using EVOS™ FL Auto 2 (Thermofisher Scientific). Total cell number and γH2A.X -positive cells for CCF events were counted.

Mitochondria copy number. The relative level of mtDNA-CN was measured by real-time PCR using Applied Biosystems 7500 Fast Real-time PCR system. Two Taqman conserved mtDNA gene target - ND1 (Assay ID Hs02596873_s1) and 7S AB (Assay ID Hs02596861_s1) – were used to determine the expression of mitochondria gene. Conserved Taqman nDNA gene target - RPPH1 (Assay ID Hs03297761_s1) was used as an endogenous nucleus control gene. DNA (5 ng in 4.5 uL) were mixed with 0.5 uL Taqman gene target and 5 uL Taqman Universal MasterMix II no UNG in a 10 uL reaction. Each reaction was performed in triplicate wells on 96-well plates. The double-delta Ct method (ΔΔCT) was used for relative quantification of gene expression after removal of outlier wells, and the obtained quantification was transformed to exponential value 2-ΔΔCT. Applied Biosystems 7500 software version 2.0.6 was used to run the experiment and ExpressionSuite version 1.1 was used for data analysis. The relative mtDNA-CN was calculated based on mtDNA/nDNA. This can be done either by the classical double-delta Ct method (ΔΔCT) or by calculating the ratio between mtDNA molecules and nDNA molecules. Relative quantification data was exported to GraphPad 10.2.1 for further analysis and graph demonstration.

Telomere length assay. PCR reaction was set up by adding 7.5 µL of either Telomere or 36B4 (Single copy control) master mix into each well of a 96-well plate. Master Mixes were prepared according to the Telomere Assay Protocol with the use of QuantiNova 2x SYBR Green I Master Mix, Telo1/Telo2 Primers (300nM), 36B4d Primer (500nM) and 36B4u Primer (300nM). This was followed by adding 2.5 µL of Standards to create standard curve or 2.5 µL of sample DNA in triplicates, containing 5 ng of DNA diluted in nuclease free water, making final reaction volume 10 µL. Six standards were made using a ten-fold serial dilution with nuclease-free water.

Telomere and 36B4 plates were run on the Roche LightCycler 480. Running conditions for the Telomere plate were: 1 cycle of heat start at 95˚C for 2.5 minutes, 40 cycles of amplification at 95˚C for 5 seconds, 60˚C for 10 seconds and 68˚C for 2 minutes. Then followed by 1 cycle of Melt at 95˚C for 30 seconds, 59˚C for 30 seconds and 95˚C for 2 minutes and 1 cool cycle at the end at 40˚C for 10 seconds. Running conditions of the 36B4 plate were set by following QuantiNova Quick-Start Protocol cycling conditions that were pre-saved in Roche LightCycler 480 software (1 cycle of heat start at 95˚C for 2.5 minutes, 45 cycles of amplification at 95˚C for 5 seconds and 60˚C for 10 seconds. Then followed by 1 cycle of Melt at 95˚C for 30 seconds, 59˚C for 30 seconds and 95˚C for 2 minutes and 1 cool cycle at the end at 40˚C for 10 seconds). After running the plates absolute quantification was performed and 2nd derivative maximum results were used to perform further data analysis in Excel. Data was reported as the ratio of Telomere concentration to 36B4 concentration (T/S ratio).

Autophagy and Nrf2 target gene expression. RT-PCR was performed on the generated cDNA using TaqMan gene expression assays and following the TaqMan Thermofisher Scientific protocol. For autophagy genes, 1 ul of diluted cDNA of Beclin1 (Hs01007018_m1), LC3B (Hs00797944_s1), p62 (Hs01061917_g1) were tested. Three Nrf2 target genes were used, including Hmox1 (Hs01110250_m1), Nqo1 (Hs00168547_m1), Gclc (Hs00155249_m1). These sets of genes were run in parallel with the endogenous control HPRT1 (HS02800695) in Taqman Universal MasterMix II no UNG (Thermofisher Scientific) and performed on a 7500 FAST RT-PCR system (Applied Biosystems).

Oxygen consumption rate (OCR) measurements. VSMCs were seeded with 7,000 cells/well into a specialized Seahorse XF24 Cell Culture Microplate, Agilent Technologies. Four wells were left without cells as controls. Cells were incubated for 24

hours in 500 µL of normal VSMCs medium before being exchanged into medium with betaine 1 mg/ml. Treatment with betaine 1 mg/ml medium has been conducted in 48 hours. The Seahorse XFe24 sensor cartridge was prepared in advance by soaking it overnight in XF Calibrant solution (100840-000, Agilent). This hydration process took place at 37°C in an incubator without CO_2. One the assay day, old medium was replaced by 500 µL of XF DMEM medium (103575-100) supplemented with glucose (5 mM), pyruvate (1 mM) and L- glutamine (2 mM). The plate was then plated in the incubator (37^oC, without CO_2) for 1 hour. To stimulate the mitochondria metabolism inside the control and betaine-treated cells, oligomycin, FCCP and rotenone/antimycin A were prepared in the XF DMEM medium in 2.5 µM, 1 µM and 0.5 µM, respectively before loading into the injection ports A, B and C of the hydrated Seahorse XFe24 sensor cartridge. The prepared sensor cartridge was then placed into the Agilent Seahorse XFe24 Analyzer to calibrate the instrument. After the calibration process finished, the microplate holding the VSMCs was substituted for the calibration plate. Then, a particular protocol from the "Cell Mito Stress Test" assay was initiated. The data was collected from Seahorse XF24 machine and analysed in Wave 2.6.3 software.

Statistical analysis. All statistical analyses were performed using GraphPad Prism (version 10.2.1). Unless indicated otherwise, statistical significance was determined by two tailed t-test (not significant (NS) $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ or **** $P \leq 0.0001$). In all experiments, we used two biological replicates and three technical replicates, with the exception of RTCA (4 technical replicates) and OCR measurements (5 technical replicates). Whenever possible, individual datapoints are shown to clearly represent whether the data are normally distributed.

2.3 Results

2.3.1 Betaine increases vascular smooth muscle cell growth

We undertook the real-time cell analysis on young and old VSMCs treated with a range of betaine concentrations (10 µg/ml, 50 µg/ml, 500 µg/ml, 1 mg/ml and 5 mg/ml) to assess how betaine affected their growth rate (completed RTCA data can be found in Appendixes). Our data showed a significant increase in VSMCs growth treated with various doses of betaine, in particular, for betaine 1 mg/ml. Based on our initial observations, we conducted a long-term experiment using betaine 1 mg/ml treatment on every passage of primary VSMCs, from young to older stages. Therefore, the following results in this chapter were accomplished with the in-depth evaluation of various key biomarkers of vascular ageing between control and betaine-treated cells with our defined concentration of 1 mg/ml. The cell growth analysis (Figure 2.1a) for young VSMCs (passage 6) indicated that there were no significant differences in growth rate for three days post-treatment, between the betaine-treated cells and control cells. Old VSMCs (Figure 2.1b) were also tested with betaine 1 mg/ml to compare the growth rate after the treatment. A significant increase in growth rate was observed for betaine-treated old VMSC the first day after treatment ($p = 0.0077$) and over the remaining period of the experiment (day 2 with $p < 0.001$, day 3 with $p < 0.0001$), suggesting that betaine exerts a beneficial effect on vascular smooth muscle cell growth.

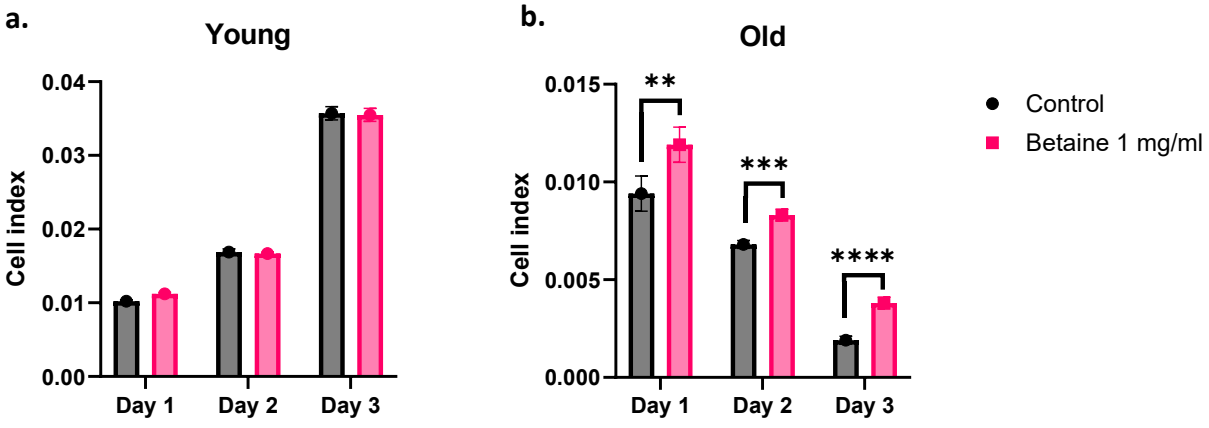


Figure 2.1| Vascular smooth muscle cell growth after betaine treatment. (a) *cell growth in betaine-treated young VSMCs and (b) cell growth in betaine-treated old VSMCs. No significance changes were observed in cell growth in young VSMCs over the first three days after treatment. In older VSMCs, betaine increases cell growth significantly compared to the control cells. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).*

2.3.2 Betaine promotes the proliferation rate of vascular smooth muscle cells

We continued to explore the changes in the proliferation rate of VSMCs following betaine supplementation with the utilization of BrdU labelling assay. By using anti-BrdU antibodies to detect DNA synthesis in newly dividing cells, we were able to quantify the number of 'newly born' VSMCs within 4-hour experiments. From that, the ratio of BrdU labelling area over total cell area has been calculated to determine the proliferation rate of VSMCs between control and betaine-treated cells. In both groups, our data showed no significant difference in BrdU-labelling cells in early passaged VSMCs (see Figure 2.2a). This is scientifically plausible since younger cells are still robust and healthy, allowing them to grow rapidly. Therefore, betaine supplementation does not have a noticeable impact when used on younger cells. However, we recorded a considerable increase in BrdU-labelling cells (up to 3-fold-change) in betaine-treated VSMCs, compared to untreated ones (p-value < 0.001) (see Figure 2.2b). Older control cells have demonstrated a variety of senescent morphology (i.e. enlarged nuclei, flattened) with exceptionally few BrdU-labelling cells. Betaine-treated VSMCs, on the other hand, possessed a substantially higher number of newly dividing cells, indicating that betaine can promote the proliferation capacity of VSMCs in later stages.

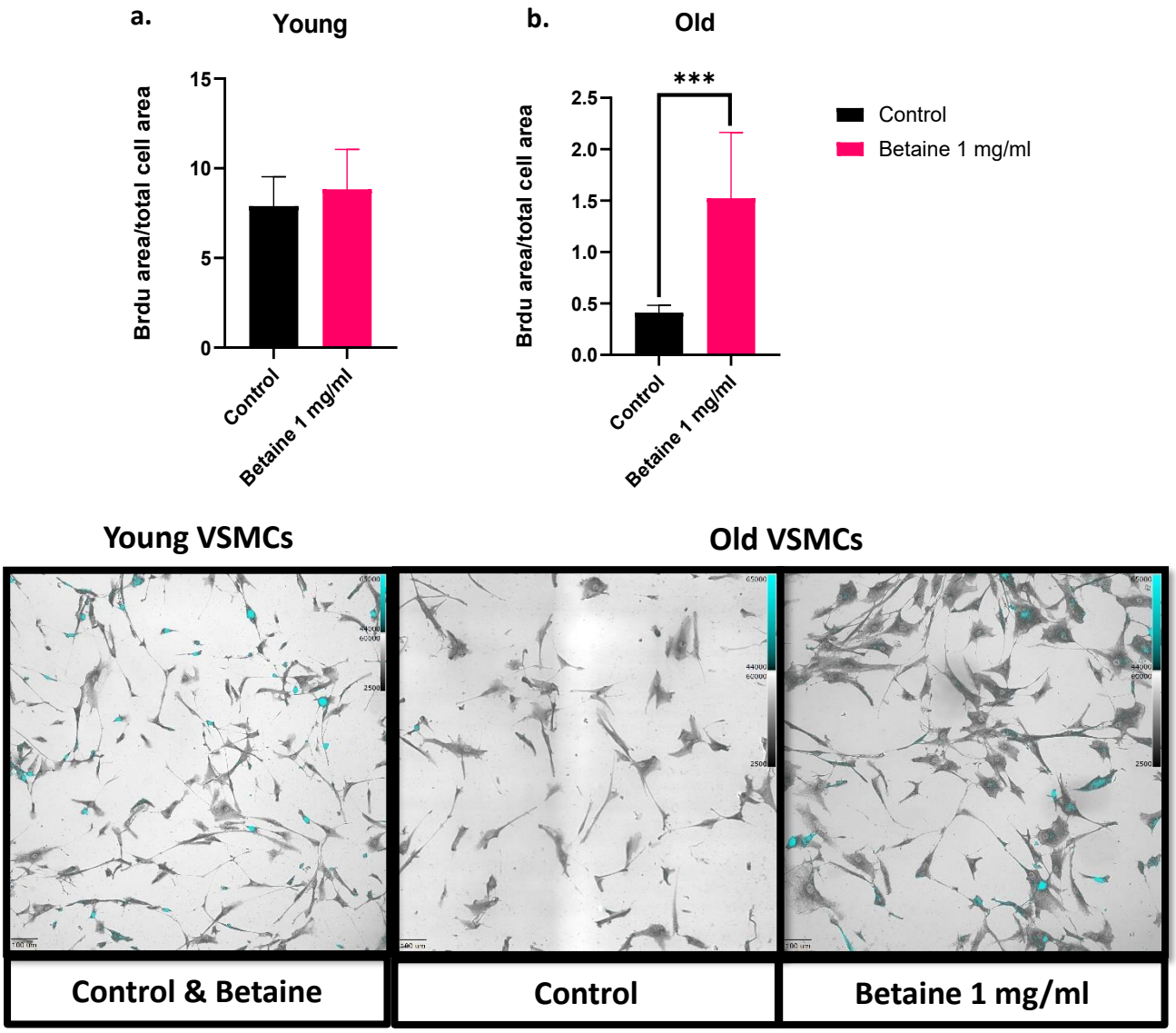


Figure 2.2| Vascular smooth muscle cell proliferation rate after betaine treatment. (a) *proliferation rate in betaine-treated young VSMCs and (b) proliferation rate in betaine-treated old VSMCs. No significant changes were observed in proliferation rate in young VSMCs after betaine treatment. In older VSMCs, betaine increases proliferation rate significantly compared to the control cells. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.3.3 Betaine extends vascular smooth muscle cells life span in vitro

During the culturing process, we assessed whether there were any differences in the doubling time between the control and treated VSMCs (see Figure 2.3). From passage 6 to passage 15 (18 to 45 cell divisions), VSMCs treated with betaine doubled at a similar rate to control cells. When the cells reached passage 15 to passage 22, we observed a slower growth rate in both control and treated cells. The number of days varied from 4-7 days, depending on the passage number of VSMCs. The cell growth rate gradually decreased over the course of time and passage. During the later stages of the experiment, we observed significant changes in VSMC morphology, consistent with cells entering senescence. Cells entering replicative senescence showed typical characteristics, including a 'broad flattened' appearance and enlarged nuclei with cytoplasmic granularities. At passage 23 (66 cell divisions), control VSMCs experienced a complete termination in cell growth. Betaine-treated cells continued to grow at a slow rate, equal to 20 days before reaching passage 24. We recorded passage 25, ~ 75 cell divisions, as the last passage of betaine-treated VSMCs.

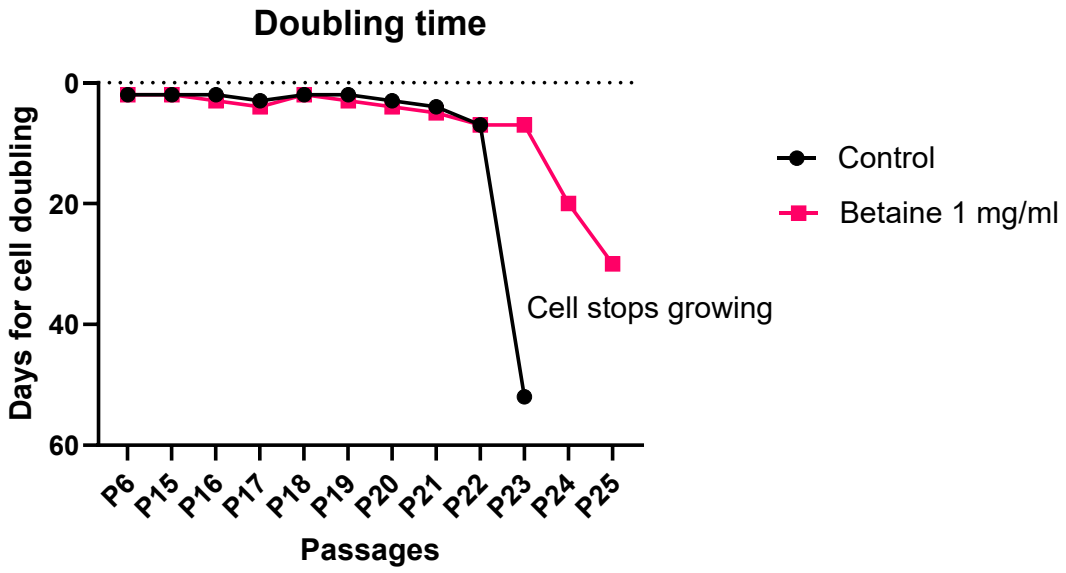


Figure 2.3 | Real-time cell doubling time showing number *of days the cells require to be confluent before splitting. Betaine-treated cells reached higher number of passages than the control cells.*

2.3.4 Betaine decreases appearance of hallmarks of cellular ageing

We then sought to determine whether betaine has beneficial effects reflected in the. expression of established biomarkers of cellular ageing, including the expression of CDKN1A and CDKN2A, SA-β-gal staining, CCF events and SASP markers.

2.3.4.1 Betaine prevents vascular smooth muscle cells from entering cell-cycle arrest.

To further investigate any possible impact of betaine on the cell cycle and cellular senescence, we measured transcriptional expression for the cell cycle inhibitors CDKN1A (p21) and CDKN2A (p16) (Figure 2.4). These two genes play a pivotal role in cell-cycle arrest in response to numerous stressors and replication stress. We observed a significant increase in expression of both genes in control VSMCs at two different cellular replication age points (young and older cells) ($p<0.05$). After treatment with betaine, we recorded a reduction in the expression of both CDKN1A and CDKN2A in treated VSMCs versus controls (Figure 2.4).

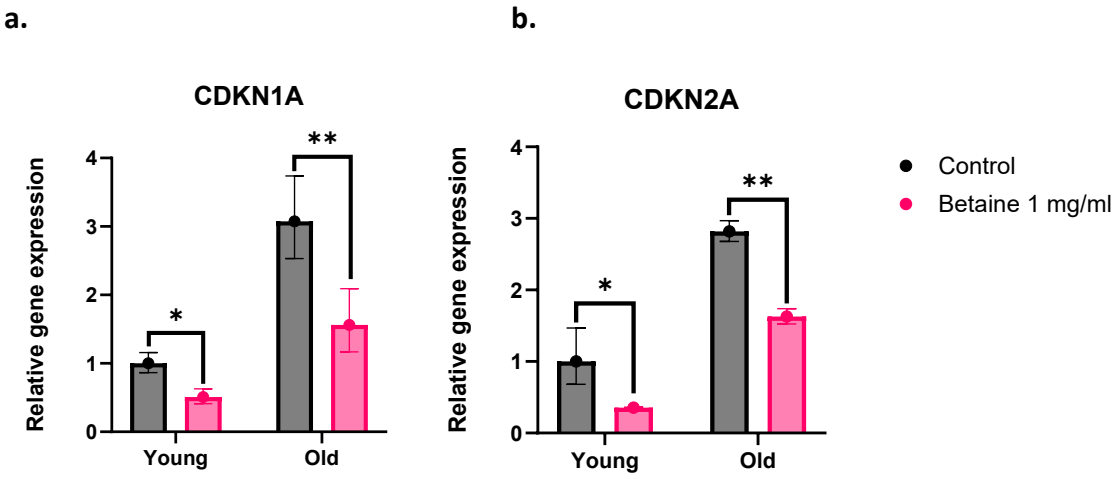


Figure 2.4| Senescent biomarkers gene expression. *CDKN1A (p21) and CDKN2A (p16) are two most widely used biomarkers for cellular senescence. Betaine reduces the expression of p21 and p16 in young and aged VSMCs. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).*

We then correlated these data with a determination of the proportion of senescent cells after treatment with betaine measured through SA-β-gal staining. This reveals the lysosomal accumulation of beta-galactosidase and is one of the most common biomarkers of cellular senescence (Figure 2.5). SA-β-gal staining indicated that SA-β-gal positive cells were significantly higher in aged-control cells (Figure 2.5a). On the other hand, very few positive signals of SA-β-gal were found in betaine-treated cells. The appearance of

control cells was also similar to typical morphology of senescent VSMCs, including 'flattened' appearance, enlarged or irregular nuclei shape and cytoplasmic granularities. Another characteristic of SA-β-gal positive control cells was that they were larger in size, which was associated with an increase in organelle abnormalities. Despite being at the same passage number, betaine-treated cells appeared more homogenous, with a typical spindle-shaped morphology. There were no signs of significant enlargement of cell nuclei or increased granulation in the cytoplasm. Statistical analysis also showed the difference was significant with $p<0.0001$ (Figure 2.5b).

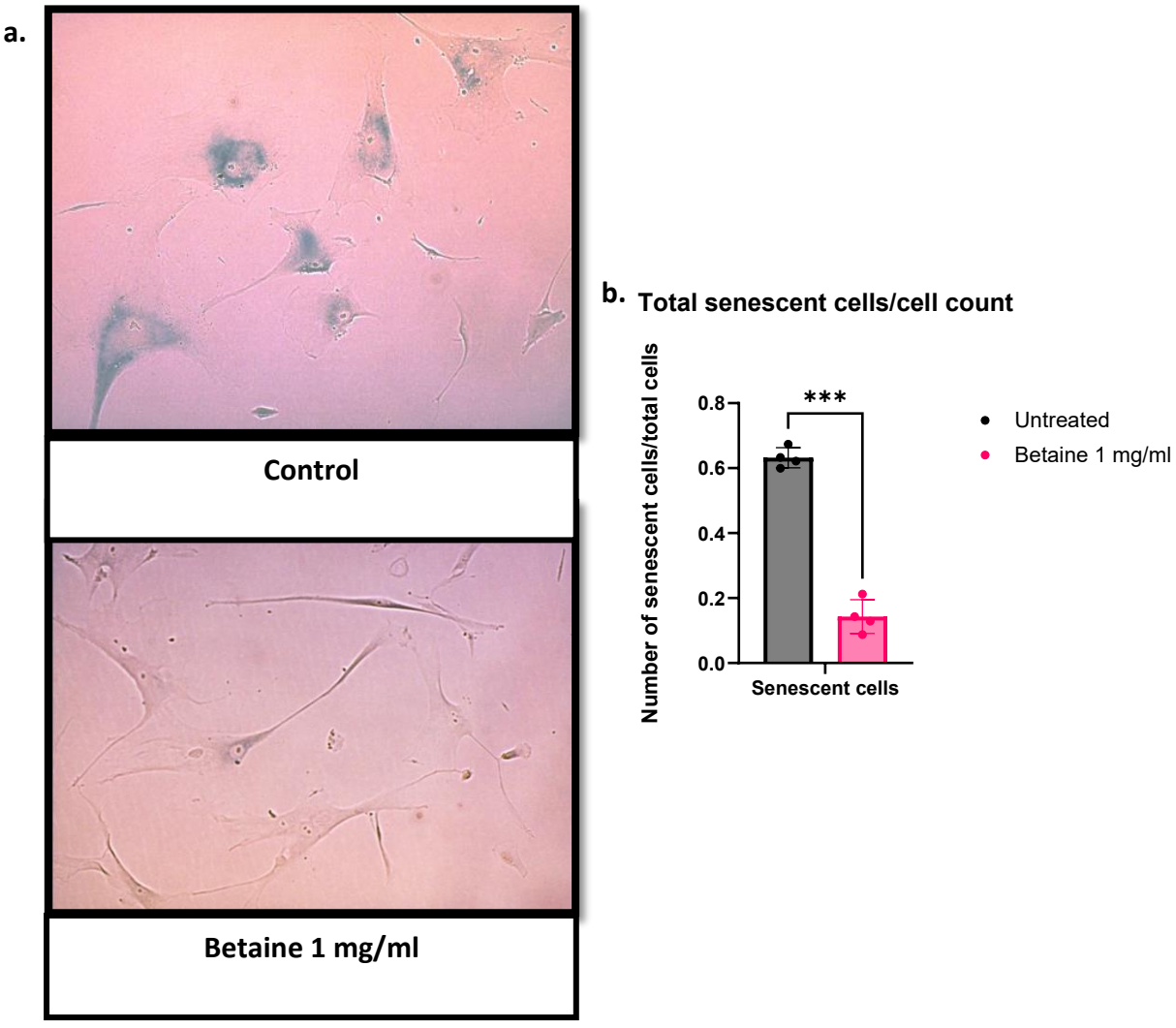


Figure 2.5| Senescent cells staining using SA β-gal. *SA β-gal-positive cells were found more in control aged cells, compared to betaine-treated cells. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.3.4.2 Betaine mitigates the effects of senescence-associated secretory phenotypes (SASP)

We then sought to determine if betaine could ameliorate the pro-inflammatory effect of the SASP, a feature accompanying cardio-renal disease. This was determined through measurement of the expression IL1β and IL6, two pro-inflammatory cytokines recognized as members of the SASP. Notably, IL6 has been shown to be associated with vascular remodelling and oxidative stress in age-related diseases (Wang et al., 2021a). The outcomes revealed a significant increase in IL1β and IL6 expression in VSMCs as part of replicative senescence (Figure 2.6). The expression of IL1β peaked in old cells at more than 100 times the level observed in young cells. This phenomenon was also observed for IL6, with more than 50 times increase in gene expression observed in aged VSMCs. Compared to young cells. Critically, cells treated with betaine showed a significant reduction in both pro-inflammatory factors – IL1β (~4.5-folds, $p<0.001$) and IL6 (~2.5-fold, $p<0.01$), indicative of betaine mitigating pro-inflammatory stress due to replicative senescence.

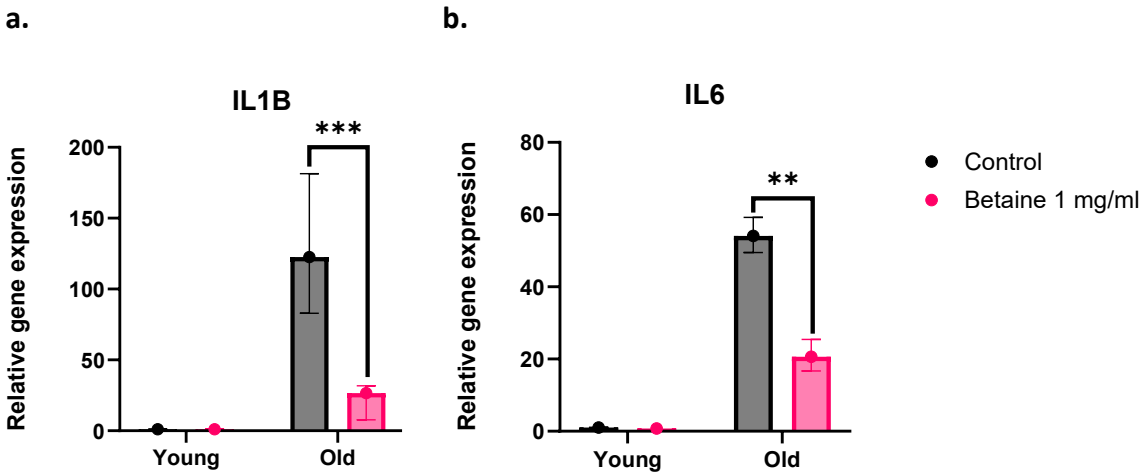


Figure 2.6| Senescence-associated secretory phenotypes. *IL1β and IL6 are two most prominent SASP-associated components that cause low-grade chronic inflammation in age-related diseases. Betaine significantly reduced IL1β and IL6 gene expression after long term treatment. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).*

2.3.4.3 Betaine mitigates the occurrence of cytoplasmic chromatin fragment (CCF) events.

To continue exploring the effects of betaine on VSMCs senescence, we evaluated its impact on the generation of CCF. It has been reported that SCs release these fragments of nuclear chromatin into the cytoplasm as part of the DNA damage triggering development of the SASP (Miller et al., 2021). The formation of CCF has been strongly related to VSMCs senescence, which increasingly accumulates during the life course (Uryga et al., 2021a). As cells approach a senescent state, the occurrence of CCF events can be stained with DAPI and γH2AX since they are important markers of nuclear DNA and DNA damage-associated blebs in SCs. In our study, we used γH2AX antibody to specifically target CCF. Senescent VSMCs with cytoplasmic DNA fragments, which showed positive for γH2AX stain, were significantly higher in control cells (Figure 2.7). Betaine-treated cells had lower γH2AX-positive cells.

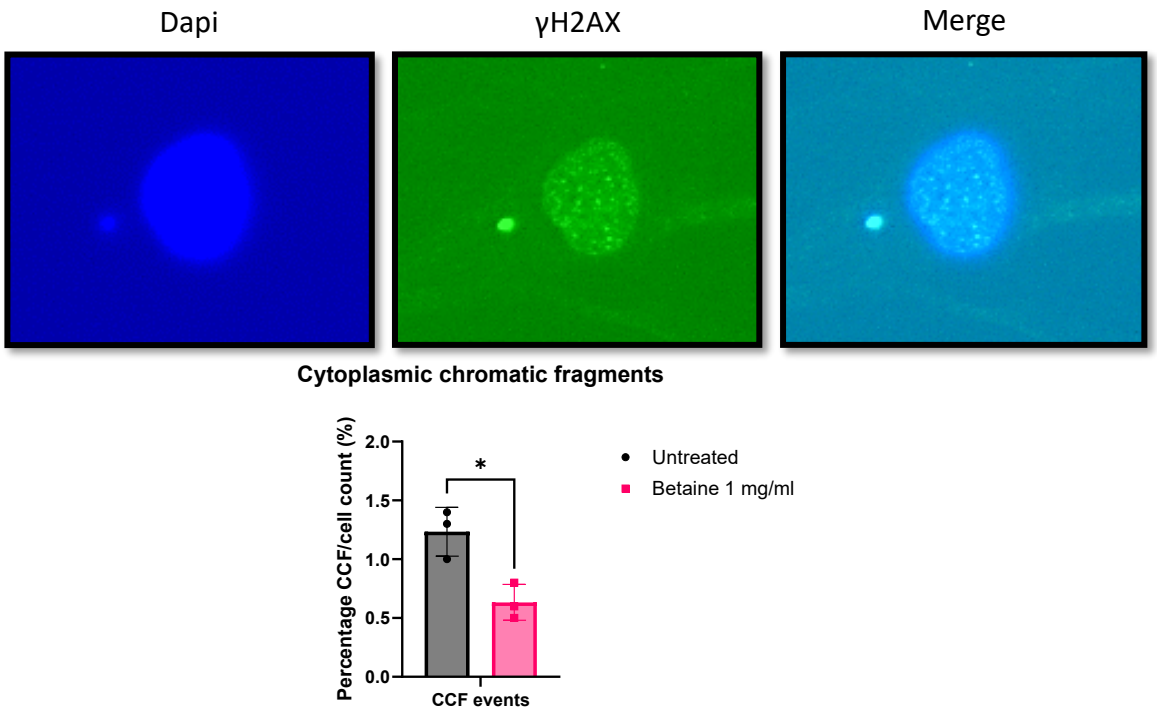


Figure 2.7| Cytoplasmic chromatin fragments events. *CCF has been widely used as a cellular senescence biomarker in age-related diseases. Betaine significantly reduced CCF after long term treatment. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.3.5 Betaine exerts cytoprotective effect in vascular smooth muscle cells.

We have also sought to evaluate whether betaine is geroprotective (i.e. protects old cells). To do so, we have evaluated its capacity to modulate the expression of FOXO4 and LMNA, two emerging biomarkers for senescent VSMCs (Figure 2.8). As FOXO4 is essential for the cellular response to oxidative stress, the increase in FOXO4 expression in young cells might be a part of cytoprotection engendered by betaine (p=0.04) (Figure 2.8a). In older cells, FOXO4 has been found to cause cell cycle arrest by activating a number of genes in the Go quiescence phase (Baar et al., 2017). FOXO4 has also been shown to maintain the viability of senescent cells. We observed a reduction in FOXO4 expression after betaine treatment which prevented cells from entering a senescent state (p=0.0027).

LMNA is a novel biomarker of human vascular ageing, that is upregulated in older cells. Overexpression of prelamin A, a protein that is encoded by the LMNA gene, accelerates VSMC senescence (Ragnauth et al., 2010). It disrupts mitosis and facilitates DNA damage in VSMCs, causing mitotic failure, genomic instability and senescence as cells age. Notably, betaine reduced expression of LMNA in aged VSMCs (p=0.048) (Figure 7b). The effects were only observed in old VSMCs, consistent with both being geroprotective. Based on our result, betaine can target VSMC senescence-associated LMNA as it does not affect the young VSMCs. Furthermore, the significant protection of only the older cultures may be a reflection of the younger VMSCs being more physiologically resilient.

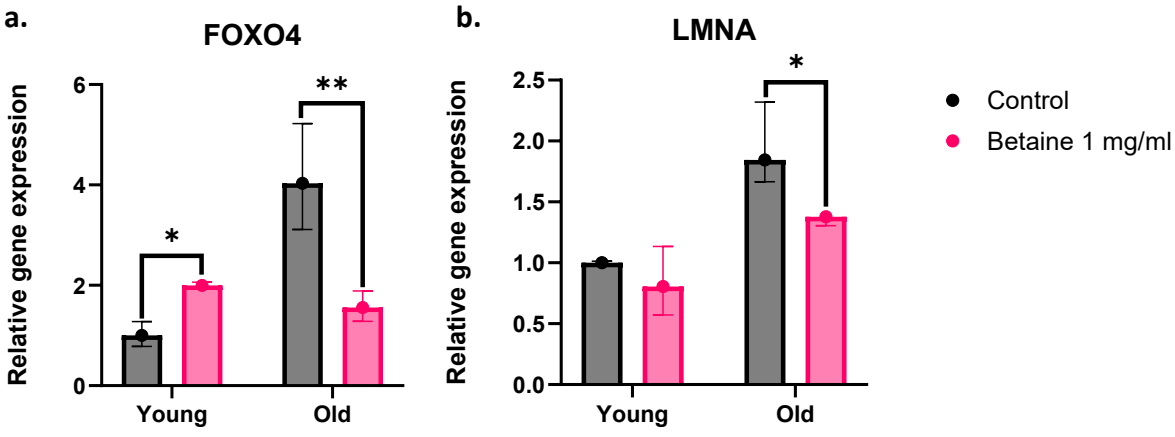


Figure 2.8| Vascular smooth muscle cell senescence biomarkers FOXO4 and LMNA. *FOXO4 and LMNA are two novel biomarkers of VSMCs senescence. Betaine reduces FOXO4 and LMNA expression in aged cells. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.3.6 Betaine mitigates cell stress in vascular smooth muscle cells with increasing age.

To learn more about the capacity of betaine to mitigate cell stress, we used Nrf2 and SerpineB2 gene expression as two important biomarkers of cellular responses to growth under stressful conditions. Nrf2 is a master regulator of cytoprotective responses, regulating ~2% of the genome. It is typically activated during cell stress and oxidative damage responses. The increase in Nrf2 expression also significantly suppresses osteogenic transition and apoptosis in VSMCs (Jin et al., 2022, Wei et al., 2019). We observed a significant reduction in Nrf2 expression after betaine treatment, both in young and old VSMCs (Figure 8a). In young VSMCs, the expression of Nrf2 significantly decreased after betaine treatment (p =0.01).

SerpinB2, a serine protease inhibitor, has been associated with SCs and cell stress. Elevated levels of SerpinB2 have been found in inflammation-induced cellular stress in various age-related diseases (Hsieh et al., 2017, Schroder et al., 2011). We observed upregulation of SerpineB2 with increasing cellular age in culture (p<=0.0001). After treatment with betaine, the expression of SerpineB2 was significantly reduced (p=0.025), which was comparable to SerpineB2 expression in young VSMCs (Figure 8b).

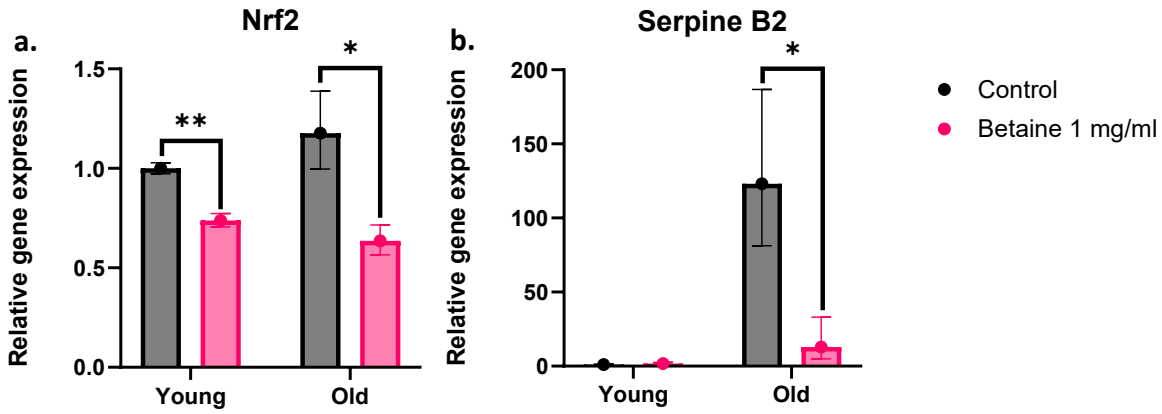


Figure 2.9| Cell stress gene expression of Nrf2 and SerpineB2. *Nrf2 is a master regulator of cytoprotective responses, while SerpineB2 often increases in gene expression during cell stress. Betaine reduces Nrf2 and SerpineB2 gene expression after long term betaine treatment. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.3.7 Betaine has no significant effects on mitochondrial copy number

To investigate possible impact of betaine on mtDNA-CN, gene expression was measured by quantitative PCR for a panel of young and old VSMCs, in treated cells and controls (see Figure 2.9). These included proven markers of mtDNA and nuclear DNA (nDNA) to identify the ratio. In all control and drug-treated samples in different age groups, no significant effect was observed on mtDNA-CN after treatment with betaine (see Figure 2.9). Our data also showed the increase of mitochondria DNA in older passage VSMCs. Changes in mtDNA-CN can lead to various cellular dysfunctions, so avoiding this reduces potential side effects (Clyde, 2022). The observation that there is no change in mtDNA-CN following betaine supplementation is important, as this indicates that betaine does not significantly interfere with mitochondrial biogenesis or dynamics. By maintaining mitochondrial homeostasis, it is less likely to cause systemic metabolic complications or cellular energy deficits. Since betaine treatment did not affect mtDNA-CN, it might be suitable for a wider range of pathological conditions.

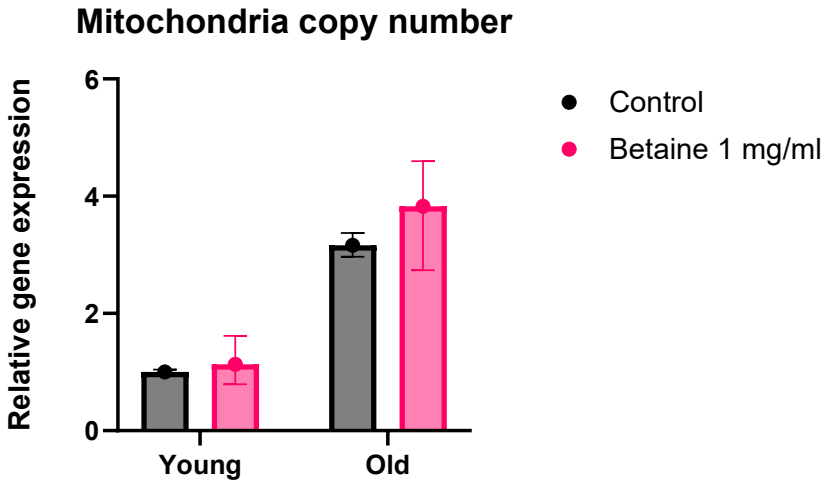


Figure 2.10| Mitochondria copy number in vascular smooth muscle cells after betaine treatment. *There was no significant difference between mtDNA-CN between control and betaine-treated cells. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).*

2.3.8 Betaine has no significant effects on telomere length

We performed an analysis to determine the relative telomere length between the untreated and betaine groups (see Figure 2.11). Telomere attrition is a hallmark of age-related diseases. The shortening of telomere length with age eventually induces cellular senescence (Uryga et al., 2021b). Therefore, telomere length has been widely recognized as a biomarker of ageing. Our data indicate that young VSMCs treated with betaine obtained no significant difference in telomere length, compared to non-treated cells. A similar result was also observed in older VSMCs. With each cell division, a small portion of the telomere is lost due to the inability of DNA polymerase to fully replicate the ends of chromosomes. This has also been reflected in our data, as telomere length was significantly reduced in later-passage VSMCs.

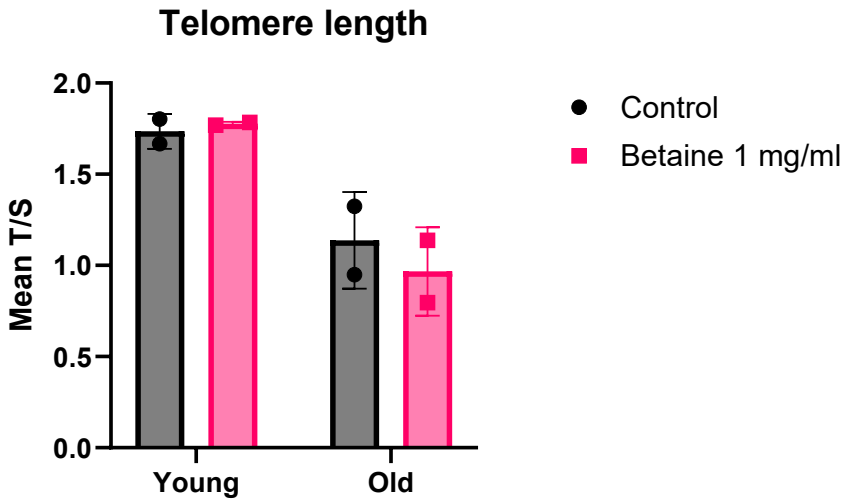


Figure 2.11| Telomere length in vascular smooth muscle cells after betaine treatment. *There was no significant difference between telomere length between control and betaine-treated cells. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.3.9 Betaine reduces expression of Nrf2 target genes in vascular smooth muscle cells

To further investigate the possible impact of betaine on downstream effects of Nrf2-associated antioxidation and cellular senescence, gene expression for three Nrf2 target genes Hmox, Gclc, and Nqo1 was assessed in different stages of control and drug-treated VSMCs through qPCR (see Figure 2.12). Hmox was significantly downregulated after treatment with betaine in older VSMCs. The same trend was also observed in the expression of Gclc with a statistically significant effect. As betaine reduces Nrf2 expression in older cells, it is consistent with reducing Hmox1 gene expression. Hmox and Gclc expression is triggered by Nrf2 activation due to cell stress as a protective mechanism in response to a variety of endogenous and exogenous stimuli (i.e. heat, UV irradiation, oxidative stress, hypoxia). In addition, NQO1 – a potential inhibitory mediator for vascular calcification – did not experience any significant changes in gene expression in VSMCs following betaine supplementation. Overall, these outcomes indicate that the betaine-treated cells were not under oxidative or inflammatory stress conditions. Long-term supplements with betaine might potentially mitigate the effects of cellular stress in older cells.

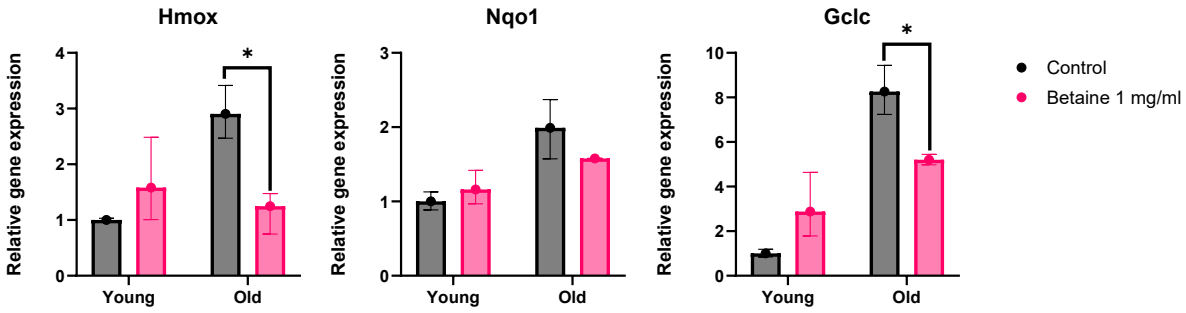


Figure 2.12| Nrf2 target gene expression of Hmox, Gclc and Nqo1. *Hmox and Gclc expression was significantly downregulated in older vascular smooth muscle cells. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.3.10 Betaine promotes autophagy in vascular smooth muscle cells

To explore the effect of betaine on autophagy processes has been explored as we examined the expression of three established autophagy genes, including Beclin1, LC3B and p62 (see Figure 2.13). We observed the upregulation in the expression of Beclin1– which plays a major role in autophagy activation in young VSMCs after betaine 1 mg/ml treatment (p-value<0.05) (Kang et al., 2011). This confirms that betaine can promote autophagic influx in young VSMCs. According to a previous study, a mice model with a premature ageing process demonstrates diminished autophagy activity with higher expression levels of p62 and LC3 genes (Chang et al., 2019). Our findings emphasize similar results as a significant increase of LC3B and p62 expression was recorded in aged VSMCs (see Figure 2.13). Notably, long-term supplementation of betaine on young and old VSMCs has significantly reduced the expression of both p62 and LC3 genes (p-value <0.05). From that, betaine can promote healthspan and lifespan via an autophagy-dependent mechanism.

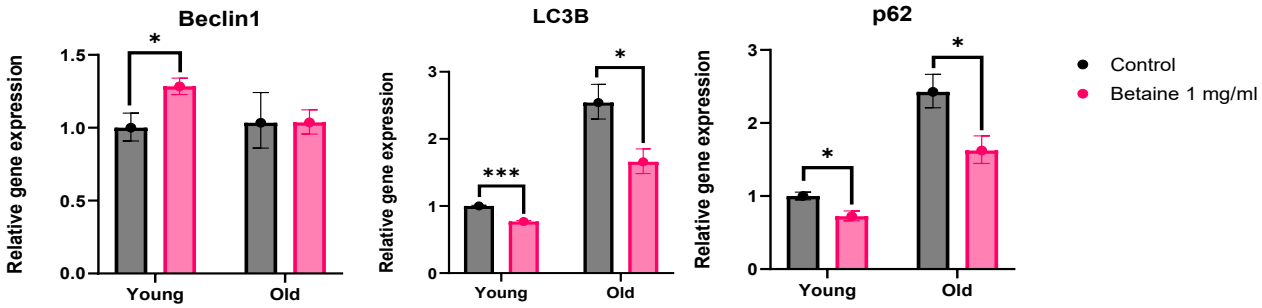


Figure 2.13| The gene expression of autophagy genes (Beclin1, LC3B, p62). *LC3B and p62 expression were significantly downregulated in young and older vascular smooth muscle cells. Beclin1 gene has been identified to be significantly increased in young VSMCs. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.3.11 Betaine activates a metabolic switch from aerobic to anaerobic respiration

Loss of betaine metabolism has been associated with increased mitochondrial dysfunction with age. To explore the effects of betaine on mitochondria functions, a comprehensive approach was conducted on VSMCs using the Seahorse Cell Mito stress test together with gene expression analysis. Our data showed a reduction in spare respiratory capacity (RC) and ATP production (see Figure 2.14) (p-value <=0.01). Interestingly, the extracellular acidification rate (ECAR) kinetics illustrated the consistent increase in the glycolysis level of betaine-treated cells throughout the experiment. Betaine was shown to reduce oxidative phosphorylation while activating glycolysis, as the spare RC and ATP production were significantly lower after drug treatment. To further clarify this result, we determined expression of Sirtuin 1 and Sirtuin 6, two important glycolysis inhibitors. Our gene expression data showed a significant reduction of Sirtuin 1 and 6 after betaine treatment in aged VSMCs. This tendency has also been seen in young cells in the case of Sirtuin 1. Therefore, it is plausible that betaine activates the metabolic switch from oxidative phosphorylation to glycolysis by inhibiting the Sirtuin 1 gene, allowing the shift of RC from aerobic to anaerobic respiration.

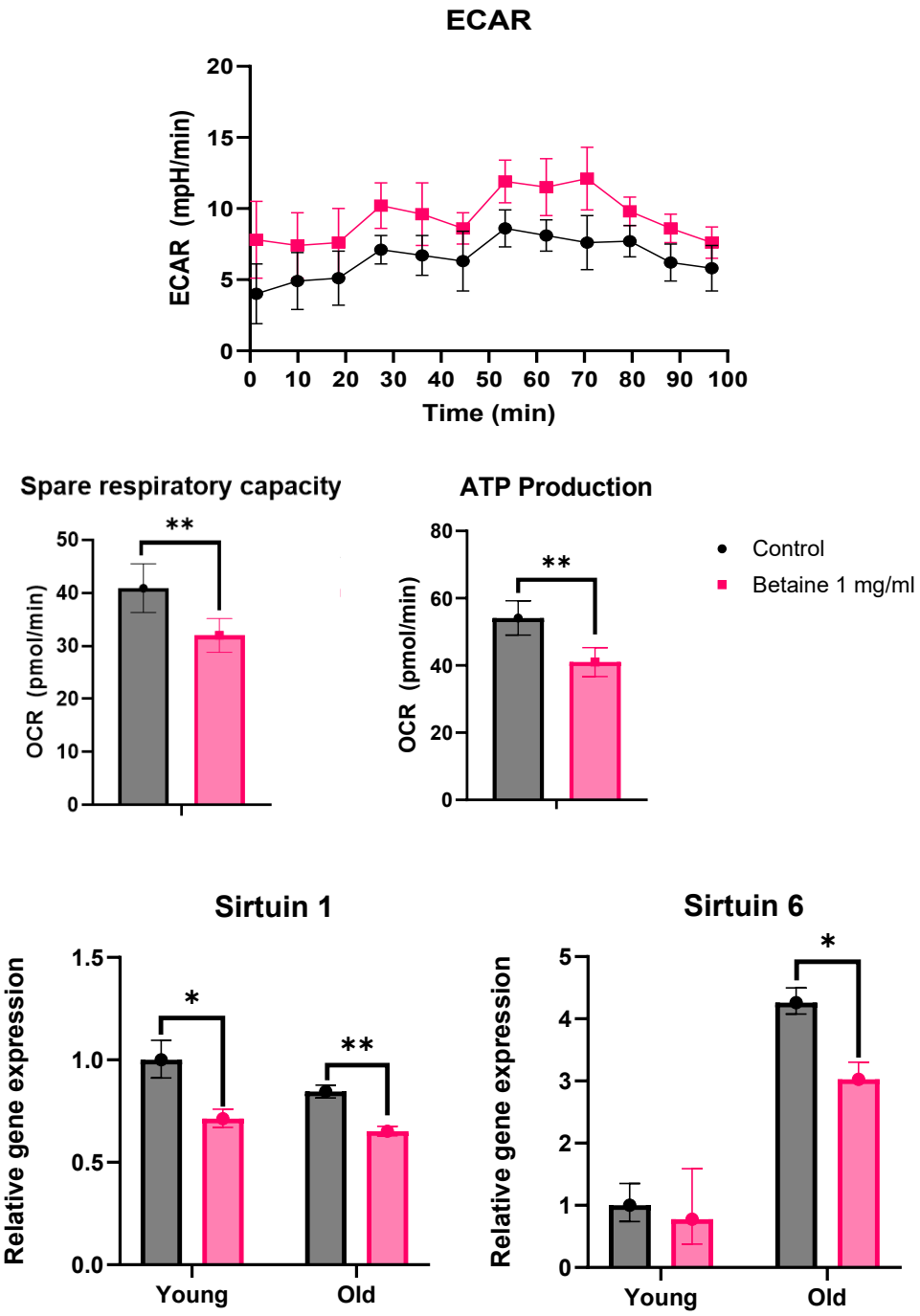


Figure 2.14| Aerobic and anaerobic respiration of vascular smooth muscle cells after betaine treatment. *The extracellular acidification rate (ECAR) significantly increased after betaine treatment in vascular smooth muscle cells. Similar results were observed in spare respiratory capacity (RC) and ATP production. The expression of Sirtuin 1 and 6 also reduced significantly in response to long-term supplement with betaine. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

2.4 Discussion

We report that long term treatment with betaine during a cellular replicative life course can rescue VSMCs from entering cellular senescence, as evidenced by a variety of biomarkers of ageing. Betaine-treated cells displayed an extension in life span with two passages more compared to the control, supporting the observation that betaine has the ability to delay VSMCs entering a senescence state (Ueland et al., 2005). Moreover, the morphology of aged betaine-treated cells is distinctive, overall size and shape appeared to be similar to younger VSMCs, which explains their superior growth *in vitro* both short term and long term (Figure 2.1, 2.2 and 2.5). Biomarkers of cellular ageing, SASP-associated agents, cytoprotective and cell stress responses were all ameliorated by betaine treatment. This suggests that betaine can be used as a potential senotherapy for vascular ageing

Differences between the control and betaine groups were strongly evident in *in vitro* life span analysis. Cells supplemented with betaine have longer lifespan while maintaining the youthful cell appearance, whereas control experienced a sudden stop in cell growth at passage 23 (Figure 2.3). This is directly in keeping with the recent findings on enhancing longevity in *Caenorhabditis elegans* (Lan et al., 2023b), that states three different senolytic drugs (i.e. metformin, quercetin, and minocycline) increased betaine levels. This high level of betaine is reported to be similar to younger nematode. The study concluded that betaine at suitable concentration prolongs lifespan, which has been confirmed in our study on VSMCs. Hence, we continued to conduct further experiments on VSMCs to elucidate the pathways underlying betaine effects on longevity. Telomere length has been used to compare betaine-treated and control group (Figure 2.11). To our knowledge, there has been no direct research on the effects of betaine on telomere length. However, as we used betaine as methyl donor, several studies have found some evidence about the link between DNA methylation and telomere length. Elevated level of DNA methylation has been linked to longer telomeres (Dong et al., 2017, Gadalla et al., 2012). Notably, it has been shown that reduced global DNA methylation also plays a role in shorter telomere length in adolescents (Dong et al., 2017). This causes genomic instability, which in turn accelerates risks of age-associated chronic diseases and overall mortality rate. As DNA methylation is negative affected by a variety of risk factors (i.e. diet, lifestyle, environment, social economic status) (McGuinness et al., 2012b), betaine can assist in maintaining global DNA methylation as a methyl donor. Even though we did not observe the significant changes in telomere length following betaine treatment, it is

possible that betaine still plays major role in supporting genomic integrity as a methyl donor.

Our data also showed the reduction in gene expression of various important senescence biomarkers. It has been widely known that CDKN2A (p16) and CDKN1A (p21) - two major genes for cell cycle arrest, are highly upregulated with age (Wagner and Wagner, 2022). We also observed similar phenomenon with these two senescence biomarkers increased up to four times as we continued culturing VSMCs. Interestingly, treatment with betaine was able to counteract the overexpression of p16 and p21 genes in both young and old VSMCs (Figure 2.4). Similar outcome has been previously reported in rat liver cancer model, in which supplement with betaine has reduced tumour suppressor gene p16 expression (Du et al., 2009). This intuition provides us the supporting evidence for betaine can also reduce the expression of p21 gene, which was observed after our gene expression assays. In keeping with this scenario of decreased senescence biomarkers in betaine-treated VSMCs, we also observed significant decreased levels of SASP-associated IL1β and IL6 treated groups (Figure 2.6). IL1β and IL6 are known to significantly elevate in vascular ageing and the development of age-related chronic diseases (Ferrucci and Fabbri, 2018b). The antioxidant effects of betaine have been widely examined, focusing on its protective capabilities against a variety of oxidative stress inducers (i.e. inflammation, reactive oxygen species). It is suggested that betaine provides nonenzymatic antioxidant effects by activating methionine-homocysteine cycle, thus creating a protective barrier around cells (Zhang et al., 2016). This supports the results from the SASP gene expression, suggesting that betaine data in our study may be further strengthen the effect of betaine as a powerful antioxidant. The outcome was also informative and identified specific pathways that betaine uses in protecting VSMCs from entering senescence state.

When analysing cytoplasmic chromatin fragments in each group after betaine treatment, we found that betaine reduced the occurrence of CCF events in VSMCs (Figure 2.7). CCF has been reported in a variety of human SCs caused by DNA damage or replicative exhaustion (Miller et al., 2021). It is also the main driver for SASP production as part of nuclear-cytoplasmic membrane detachment process (Vizioli et al., 2020). Targeting CCF-SASP pathway is considered a promising therapy in treatment of age-related chronic diseases. Several advances have been used to mitigate the formation of CCF and SASP in SCs, focusing on upstream and downstream formation of CCF as well as modulation

of mitochondrial ROS (mtROS) production. Potential drug candidates that target CCF-SASP mechanisms include trichostatin A or SAHA (preventing mtROS formation) SP600125 (blocking JNK1/2 activation of mtROS) (Vizioli et al., 2020). As betaine has shown positive effects on mitigating SASP production, there might be a causative link between reduced CCF events and the lower level of SASP in aged VSMCs. Notably, using betaine as a therapeutic compound to control CCF production might provide protection for DNA damage, nuclear structure instability or dysfunctional mitochondria.

In addition, our results also confirmed potential role of betaine in cytoprotection and mitigating cell stress. Betaine-treated VSMCs showed reduced expression of major VSMCs senescence biomarkers such as FOXO4 and LMNA (Figure 2.8). Our control cells have the significant upregulation of these two genes with age, revealed that non-treated cells experienced high level of cellular senescence due to replicative stressor. The upregulation of these FOXO4 and LMNA is strongly associated with the acceleration of vascular ageing (Bautista-Niño et al., 2016, Ya and Bayraktutan, 2023). As FOXO4 induces inflammation and senescence in VSMCs, it has been recently recognized as a target for senotherapeutic drugs (Tripathi et al., 2021). Blocking FOXO4/p53 signalling pathway by betaine can be crucial for controlling vascular inflammation caused by extracellular histones. This is consistent with the notion that betaine supplementation provides antioxidant protection for VSMCs that inflict cellular senescence. LMNA is responsible for the synthesis of Lamin A and is produced in most cells of the body (Gonzalo et al., 2017). The mutations in LMNA results in Hutchinson-Gilford progeria syndrome of premature ageing by interfering nuclear Lamin A processing, thus leading to the accumulation of prelamin A. Cells treated with betaine showed the downregulation of this gene, which is in keeping with our previous results regarding betaine as an effective senotherapy on vascular ageing. In keeping with these observations, two stress-related genes – Nrf2 and SerpineB2 – were significantly downregulated with betaine treatment (Figure 2.9). Further experiments on Nrf2 target genes also illustrated a significant decrease of Hmox and Gclc expression, as a result of Nrf2 downregulation after betaine supplementation (Figure 2.12). This is also consistent with our previous data, which showed that betaine provides beneficial impacts in improving cell stress, cytoprotection, extend life span and other biomarkers of cellular senescence. Future studies are needed to identify the specific molecular pathways in which betaine is acting on. This will provide a better insight into the potential use of this compound as a novel senotherapy for vascular ageing.

As we continued to explore the potential effects of betaine on mitochondria functions, we observed no significant difference in mtDNA-CN (a.k.a mitochondrial genomes per cell) between control and drug-treated-VSMCs (Figure 2.10). According to the 'biochemical threshold' theory, only when there is a 60-80% loss of mtDNA-CN, RC and energy production capacity (EPC) diminish (Boulet et al., 1992, Rossignol et al., 2003). Depletion of mtDNA occurs rarely and has been reported with no direct impacts on diseased organs (Basel, 2020, Grünewald et al., 2016). Previous studies have shown that only 20-40% of mtDNA-CN can already sufficiently maintain RC. MtDNA-CN can remain unchanged in some cases due to the upregulation of transcription and translation from pre-existing mtDNA copies. This phenomenon has been reported in human skeletal muscle during exercise, where mitochondria content (a.k.a. number of mitochondria per cell) and RC enhanced without a change in mtDNA-CN (Egan et al., 2013, Puente-Maestu et al., 2011). Our study provides the first detailed investigation into betaine's effects on mitochondrial function, particularly its impact on mtDNA-CN. While betaine does not exert a significant change in mtDNA-CN, we believe this helps to preserve normal cellular biogenesis while lowering the potential risks of mitochondria dysfunction. From that, advantageous impacts of betaine such as antioxidation and anti-inflammation can be prioritized to protect against other ageing-associated contributing factors.

Notably, we have successfully identified the potential pathways in which betaine promotes the proliferation rate of VSMCs. By activating a metabolic switch from aerobic to anaerobic respiration, betaine can enhance glycolytic flux – an important bioenergetic shift that happens during VSMCs proliferation and migration. Previous studies have reported that increased glycolysis is directly associated with VSMC proliferative and migratory behaviours, facilitated by platelet-derived growth factor (Heiss et al., 2016, Perez et al., 2010). As a result, depletion of the glycolysis process can substantially inhibit the growth and migration rate (Perez et al., 2010, Kim et al., 2017, Nef et al., 2008). Additionally, supplementing 1% betaine in mice fed with a high-fat diet has confirmed the significant upregulation of protein expression that is linked to the tricarboxylic acid (TCA) cycle and glycolysis process (Fan et al., 2022). By examining sirtuin1 (SIRT1) and sirtuin 6 (SIRT6) expression - two key regulators of the glycolysis process, we were able to further confirm our outcomes. SIRT1 functions as a critical regulator in the balance between aerobic oxidation and glycolysis (Koronowski et al., 2017). It inhibits glycolysis through the repression of the glycolytic enzyme phosphoglycerate mutase-1 (PGAM-1) (Hallows et al., 2012). SIRT6, another nuclear sirtuin, has been reported to

suppress glycolysis by acting as a co-repressor for hypoxia-inducible factor 1α (HIF-1α) (Zhong et al., 2010). The transcriptional activation of SIRT6 by SIRT1 suggests a coordinated regulatory mechanism by sirtuins in determining glycolytic flux in VSMCs. As long-term treatment with betaine effectively downregulated SIRT1 and SIRT6 expression, it is scientifically plausible to conclude that betaine is a potential bioenergetic switch that can improve the proliferation capacity of VSMCs.

As we investigated potential pathways in which betaine extends lifespan *in vitro*, we observed the significant upregulation of transcription of important autophagic biomarkers (i.e. Beclin1, LC3B and p62) following betaine supplementation. Our data is complementary to the previously published data, indicating the role of autophagy in betaine-associated cellular homeostasis and organ protection. Autophagy – a natural cellular degradation process to recycle damaged components – declines with age (Abdellatif et al., 2018). This leads to the excessive accumulation of damaged organelles (i.e. mitochondria) and aggregated proteins. According to previous studies, reduced autophagy activity in aged cells is contributed by impaired mitochondria function and accumulated oxidative stress (Salminen and Kaarniranta, 2012, Rambold and Lippincott-Schwartz, 2011, Lee et al., 2012). Our data have confirmed that betaine reduces oxidative stress (i.e. IL6, IL1β) and activates glycolysis-associated mitochondria homeostasis (more data can be found in Chapter 3) in VSMCs. There have been evidence that glycolytic-dependent cells (i.e. VSCMs) can significantly activate mitochondria biogenesis, resulting in autophagy upregulation (Hardie et al., 2012, Shi et al., 2020, Wu and Zou, 2020).

Overall, our data are consistent with a thesis where betaine provides a holistic approach to protect VSMCs against vascular ageing. These results strengthen the potential link between lower betaine level and the occurrence of various age-related chronic diseases, indicating that betaine supplementation may provide lots of beneficial health effects by different molecular pathways.

Chapter 3: Metabolic reprogramming occurs following betaine treatment

3.1 Introduction

RNA sequencing and transcriptomics are emerging techniques to explore molecular aspects of vascular ageing (Kwok et al., 2024). These techniques allow researchers to identify a variety of gene expression patterns, which are associated with biological pathways and age-related diseases. Physiological changes occurring with ageing are reflected in the transcriptome profiles of VSMCs at different cellular ages (Rosati et al., 2024). Key molecular pathways have been elucidated for vascular senescence (i.e. inflammation, oxidative stress, extracellular matrix modelling) (Stefens et al., 2024, Gao et al., 2020a). This advanced technique, frequently used in combination with other omics approaches, such as proteomics and epigenomics, provides a more holistic understanding of the multifaceted mechanisms driving vascular ageing.

A complex network of molecular pathways in vascular ageing have been identified by high-throughput RNA sequencing studies (Ma et al., 2021). Key findings from such studies include the identification of overexpression of cytokines and interferon-associated inflammation cascades, together with downregulation of antioxidant enzyme transcriptional expression (Gao et al., 2020a). These contribute to low-grade chronic inflammation and the expression of a SASP, prominent features in vascular ageing (Stojanović et al., 2020). Additionally, a variety of dysfunctions in DNA damage responses, repair mechanisms and epigenetic regulators also appear to contribute to the occurrence of structural and functional changes in VSMCs during ageing (Jiang et al., 2021).

Consequently, several promising therapeutic targets have been identified by RNA-sequencing (RNA-Seq) techniques to tackle vascular ageing. Three widely known such interventions include use of Nrf2 activators, senolytic agents and epigenetic modifiers. Nrf2 activation have emerged as potential strategy, as RNA-Seq data indicates reduced Nrf2 signalling in aged vasculature (Angulo et al., 2019, He et al., 2024). Enhancing Nrf2 activity could therefore boost antioxidant defences and general cyto-protection, crucial for maintaining vascular health. Additionally, senolytic agents represent another exciting avenue of therapeutic intervention. By targeting senescence markers identified through transcriptomics, senescent cells can be eliminated and the impact of the associated SASP minimised (Liu et al., 2024b, Troiani et al., 2022b). Recently, epigenetic modifiers have

also gained more attention, due to their ability to protect genome stability (Adelman et al., 2017). Genome instability is a feature of aged cells. Enabling all these mechanisms could potentially restore youthful gene expression patterns and rescue DNA damage responses in aged VSMCs.

In our previous chapter, we discussed a range of potential senotherapeutic activities for betaine, including SCs elimination, anti-oxidation, and cellular stress reduction while enhancing VSMCs proliferation via glycolysis-inducing pathways. To address whether betaine rejuvenated the VMSC and that this was reflected in the transcriptome in old cells, or whether it corrected metabolic reprogramming in cells as they entered replicative senescence, we undertook RNA sequencing (RNA-Seq) of control and betaine-treated VSMCs across their replicative life course. This chapter presents a more thorough collection of data that supports the potential use of betaine as an innovative senotherapeutic for both preventing and treating age-related vascular changes. To our knowledge, such transcriptomic analyses have yet to be explored in human VSMCs with replicative senescence. Bioinformatic analyses enabled characterisation of distinct changes in the transcriptomic landscapes as cells aged. Overall, we observed the significant impact of betaine on cellular membrane protection in younger cells. This is followed by the beneficial effects on VSMCs proliferation and angiogenesis during the middle-aged stage. As VSMCs become older, betaine has demonstrated protective effects on vascular ageing against cell cycle arrest and DNA damage.

3.2 Materials and methods

Cell culture. Vascular smooth muscle cells (VSMC) were obtained from healthy donors from the laboratory of Prof. L Schurgers Maastricht University. Primary cells were cultured in Cascade Biologics Medium 231 (M-231-500) supplemented with penicillin (100 units/mL), streptomycin (0.1 mg/mL), amphotericin B (F, 0.25 µg/ml), and smooth muscle growth supplement. All the above reagents were obtained from Thermofisher Scientific, unless stated otherwise. The cells were cultured under typical conditions of 5% CO2 at 37^{o}C in a humidified incubator. Passage of VSMC cells was conducted when the confluency reached more than 80% by 1:3 (before treatment) or 1:2 (during treatment) split. Standard protocol was followed using Dulbecco's phosphate buffered saline (DPBS 1X) to wash the cells after the medium was removed and trypsin-EDTA (0.05%) to detach the cells from the flasks.

Long term cell treatment with betaine. VSMCs were cultured with standard medium and betaine 1 mg/ml (Sigma Aldrich)-supplemented media. Betaine was added directly to the VSMCs culturing medium in every passage from young to old cells. In every passage, DNA or RNA were extracted from the remaining cells for further downstream experiments.

RNA extraction. VSMCs were added with 1 ml of TRIzol reagent (Thermofisher Scientific) per flask. These cells were kept in -80^{o}C for at least 24 hours to improve the efficacy of the following RNA extraction step. The RNA extraction was performed with a Zymo RNA Clean & Concentrator^{TM}-5 kit according to the manufacturer's instructions. The RNA pellet was resuspended in 40 µl nuclease-free water. RNA quantity was identified using a NanoDrop 2000 spectrophotometer. RNA is stored at -80^{o}C for long term storage.

Library construction and RNA Sequencing. RNA sequencing was performed via Illumina platforms by Novogene. 15 µl of 24 total RNA samples from control and treated VSMCs were sent to Novogene UK processing laboratory (≥ 20 million read pairs per sample ,150 base pair reads). The samples were tested for quality control (≥ 200 ng with RNA Integrity Number ≥ 4.0, A260/280 = 1.8-2.2, A260/230 ≥ 1.8) before proceeding to further stages. Among 24 samples, one was excluded from the library construction and RNA-Seq due to low quality. Total RNA samples were then purified into mRNA using poly-T oligo-attached magnetic beads. This allows the synthesis of first strand cDNA using random hexamer primers, followed by second strand cDNA. A library of cDNA

fragments was constructed with adapter ligation before continuing with PCR amplification. The prepared library was assessed with Qubit, RT-PCR techniques for its concentration. To determine the range of fragment sizes, a bioanalyzer was employed. Based on the effective concentration of each library and the desired data output, multiple libraries were combined. These pooled samples were then processed for sequencing using Illumina technology.

Bioinformatics analysis. All bioinformatics analyses in this chapter were conducted by the data scientist team in Novogene. Raw data from RNA-Seq was firstly processed through fastp software to obtain clean data. The cleaning process eliminated reads that contained adapters, those with poly-N sequences, and any low-quality reads. The software then calculated the Q20 and Q30 quality scores, as well as the GC content of the clean data. All subsequent analyses were conducted using only this refined, high-quality clean data. Hisat2 (version 2.0.5) mapping program has been used for reference genome based on the gene model annotation file. To quantify gene expression levels, we used featureCounts (version 1.5.0-p3) software to measure reads number per gene, which give rise to the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value. FPKM cluster analysis was also generated using the log2(FPKM+1) value.

Differential gene expression. Each sequenced library was normalized by read counts using the single scaling factor in edgeR R package (3.22.5). The comparison of gene expression between two conditions was also determined using the edgeR R package. Differential expression analysis between control and betaine-treated groups (four biological replicates per condition in one time point – young, middle, old) were assessed using the DESeq2Rpackage (version 1.20.0), with genes showing an adjusted P-value of 0.05 or less being classified as differentially expressed. This package was also used to generate Principal Component Analysis (PCA) plot. All statistical p-value was adjusted based on the Benjamini and Hochberg's method to minimize false discovery rate.

Enrichment analysis of differentially expressed genes. The clusterProfiler R package was used to perform Gene Ontology (GO) enrichment analysis on the differentially expressed genes. This analysis accounted for potential biases due to gene length. GO terms were deemed significantly enriched if they had a corrected P-value below 0.05. The Disease Ontology (DO) database, which combines the functions of human genes and their associations with diseases, was also utilized. DO pathways were considered significantly enriched if they showed a corrected P-value less than 0.05. To assess the statistical

enrichment of differentially expressed genes within DO pathways, the clusterProfiler software was also employed. This analysis helped identify which disease-related pathways were overrepresented among the differentially expressed genes.

3.3 Results

3.3.1 Gene expression patterns of vascular smooth muscle are distinct among different age groups

The degree of similarity in gene expression patterns across samples is crucial for assessing data reliability and determining which samples to include in further analysis. To do so, a PCA plot was generated as a reduced dimensional method to visualize gene expression correlation between different samples, in betaine-treated cells and controls (see Figure 3.1). This analysis allows the capture of most variation in PC1 (a.k.a linear combination that accounts for maximum variation), where PC2 (second most variation) or PC3 (third most variation) can explain fewer percentages of the data. The outcomes reported 33.47% of variations displayed similarities in PC1 (see Figure 3.1A). The number of data that can be explained by PC2 reduced to 12.26% while PC3 only depicted 9.43% variations of the total data (see Figure 3.1B). In total, 55.16% of the variations can be described by the first three dimensions in PCA plot. Even though more than 50% of our data can be explained in 3D PCA plot, the gene expression patterns of VSMCs in betaine-treated cells and controls across the lifecourse (young, middle-aged and old) are so distinct that more PCs are required to explain the whole data set. Notably, our results showed clear separation between different groups of samples depending on age (see markings in Figure 3.1). However, we were not able to separate between control and treatment groups. The samples belonging to the same age group, both treatment and control, were clustered closely together. These outcomes strengthen the reliability of our samples to continue for further analysis.

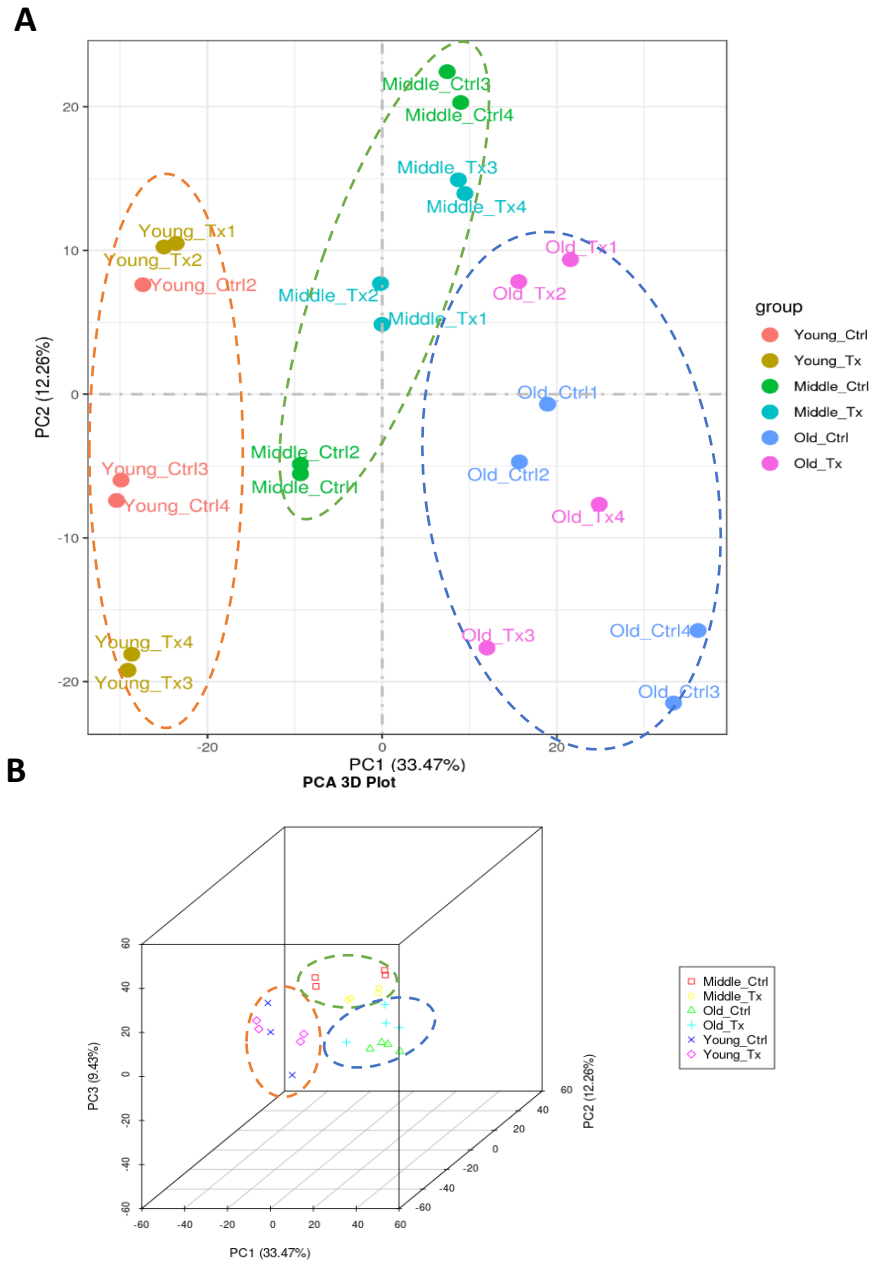


Figure 3.1| Principal component analysis (PCA) between control and betaine-treated RNA samples in young, middle and old vascular smooth muscle cells. A. 2D PCA plot, B. 3D PCA plot. *There was a clear separation between different groups of samples (young, middle and old) depending on age. Notably, control and betaine-treated groups were not distinctly separated and clustered together.*

3.3.2 Co-expression gene patterns of vascular smooth muscle after betaine treatment

To aid our understanding of the co-expression gene patterns in VSMCs after betaine treatment, we have sought to determine the number of genes that were uniquely or co-expressed between controls and drug-treated cells in different age groups (see Figure 3.2). 10712 genes in young VSMCs were co-expressed between control and treatment groups; with 292 and 469 genes were uniquely expressed in control and treated cells, respectively (Figure 3.2A). In middle-aged VSMCs, 347 genes were uniquely expressed in control while 323 genes were found exclusively in treatment group (Figure 3.2B). A total of 10658 genes were co-expressed between both groups. Older VSMCs registered 10316 co-expressed genes, together with 485 and 562 uniquely expressed genes in either control or treatment groups (Figure 3.2C). Further analysis has also been conducted to compare co-expression gene patterns between young VSMCs and middle-aged or older cells. Specific numbers of genes that were uniquely expressed and co-expressed between these groups have been summarized in Figure 3.2D and E.

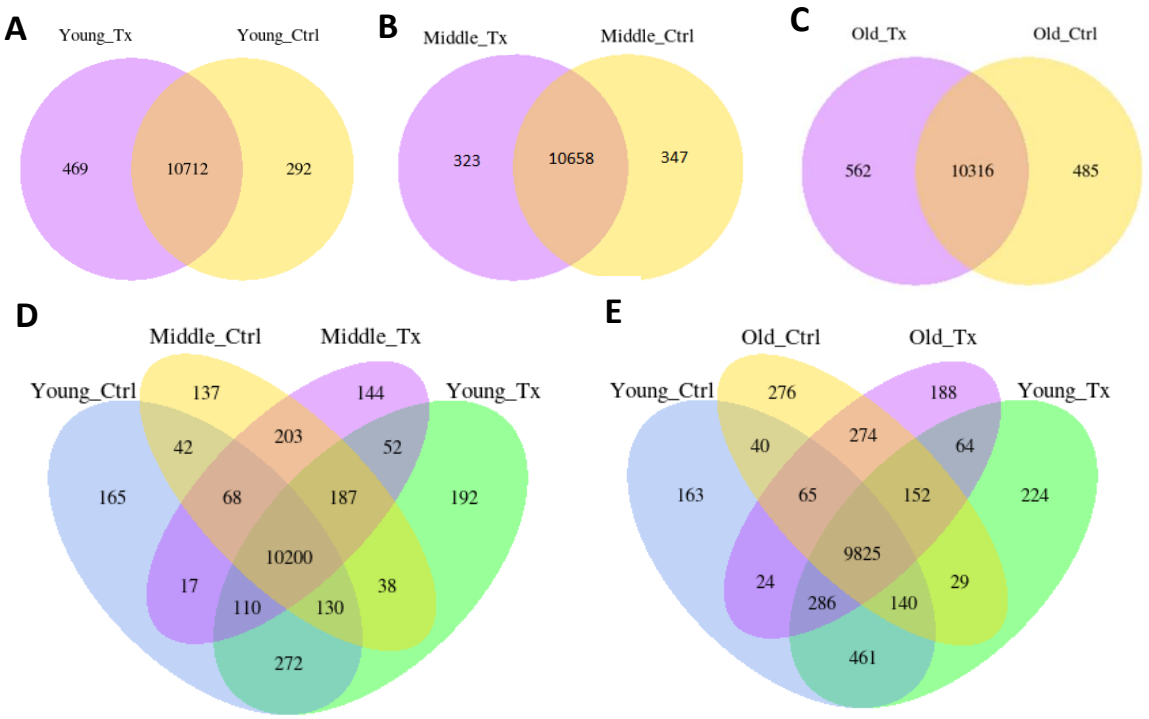


Figure 3.2| Co-expression Venn diagram showing number of genes that are uniquely expressed or co-expressed between two or more groups. *A. Young cells, B. Middle-aged cells, C. Old cells, D. Young versus middle-aged vascular smooth cells, E. Young versus old vascular smooth cells.*

3.3.3 Differential gene expression patterns of vascular smooth muscle after betaine treatment

We then sought to evaluate if VSMCs acquire differential gene expression patterns after betaine treatment. These data are shown in the using volcano plots below (see Figure 3.3). These display significantly up regulated and downregulated gene expression changes, in the respective transcriptomes, (see Figure 3.3). In young VSMCs, the majority of genes (24,045) showed no significant change in expression following betaine treatment, while 998 genes were upregulated and 1,114 were downregulated. Middle-aged VSMCs exhibited a similar pattern, with 23,992 genes remaining unchanged, 1,354 genes upregulated, and 1,160 genes downregulated. The most pronounced effect was observed in old VSMCs, where 23,190 genes showed no significant change, but there was a notable increase in both upregulated (1,551) and downregulated (2,138) genes compared to the younger age groups. This suggests that the betaine's impact on gene expression becomes more pronounced with increasing age in VSMCs.

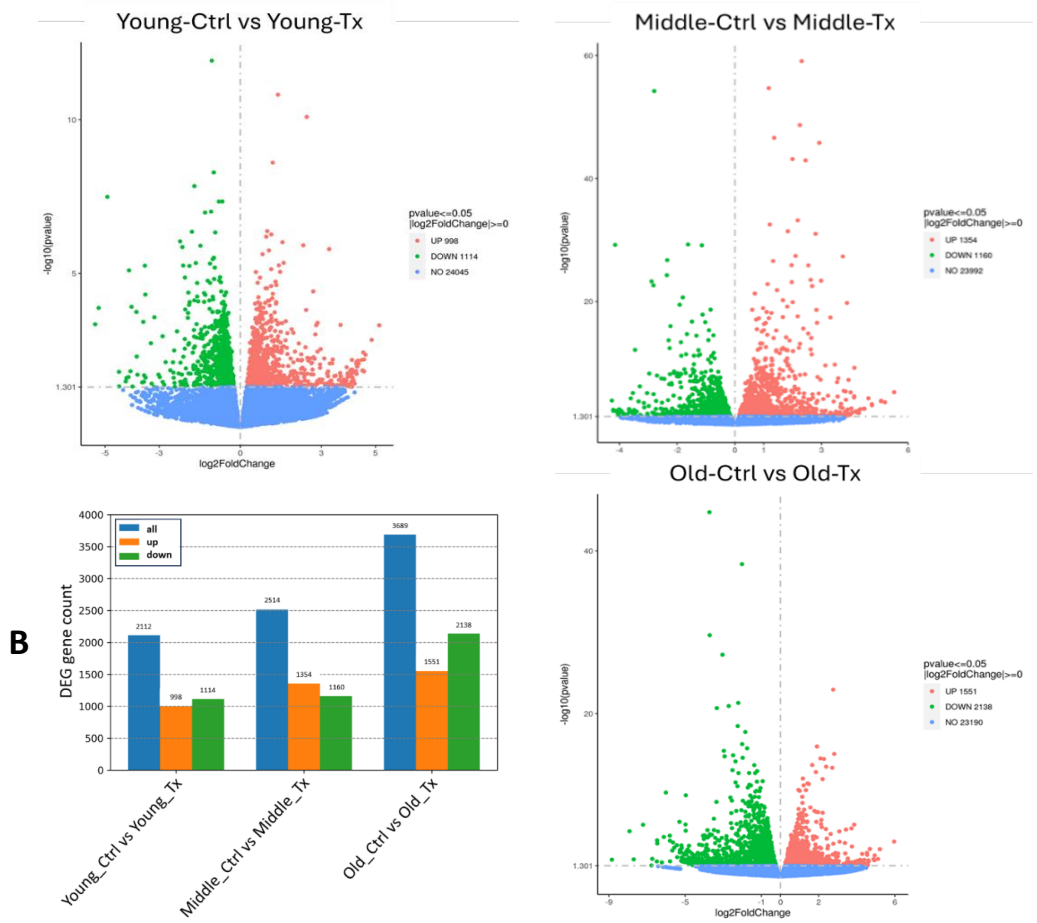


Figure 3.3| A. Differential gene volcano map between control and betaine-treated RNA samples in young, middle and old vascular smooth muscle cells. B. Differential Gene Number Statistics Histogram combining all data.

3.3.4 Betaine slows down the effects of vascular ageing

Gene expression data from human vascular smooth muscle RNA samples (n=23) were subjected to hierarchical clustering analysis to elucidate potential genetic pattern alterations during vascular ageing (see Figure 3.4). The analysis, which involved clustering FPKM values and row-wise Z-score normalization, revealed gradual changes in gene expression profiles from young to middle-aged control VSMCs. Betaine-treated samples in young clusters exhibited considerable similarity in gene expression patterns to control samples, suggesting minimal perturbation of biological characteristics. Similarly, middle-aged control and betaine-treated clusters demonstrated substantial gene expression similarities. Specific changes in gene expression patterns in middle-aged clusters after betaine treatment might potentially alter the structural and functional aspects of vasculature. Notably, distinct gene expression changes were observed between young and old control VSMCs, characterized by an almost complete reversal of green and red patterns. Our data indicate that betaine treatment may slow down the divergence in gene expression patterns between young and old vascular smooth muscle during ageing. Transcriptomic analyses revealed no significant difference between young VMSC and controls. However, changes were notable between untreated control cultures as a function of age. Such changes were minimised by betaine treatment (see Figure 3.4). These findings corroborate our previous observations regarding the youthful appearance and increased cell passages in betaine-treated VSMCs.

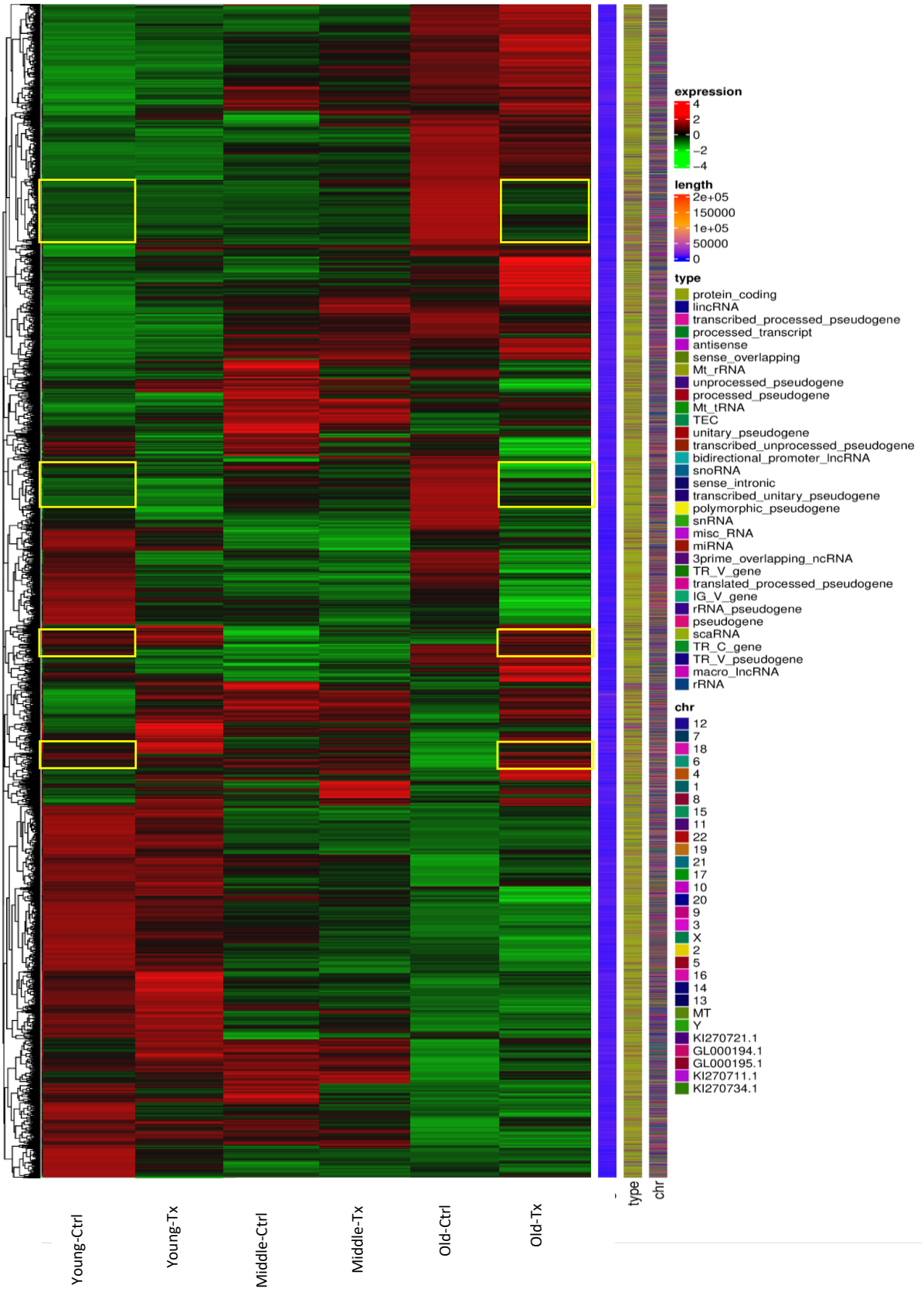


Figure 3.4| Differential expression gene clustering heatmap between control and betaine-treated RNA samples in young, middle and old vascular smooth muscle cells. *The heatmap represents the changes in gene expression (red – upregulation, green - downregulation) in VSMCs following betaine supplementation across the life course.*

With advanced age, the divergence in gene expression patterns can be diminished in older betaine-treated cells. This rejuvenating effect in gene expression can be visualized in specific genes (yellow boxes), where betaine maintained similar expression patterns as younger cells.

3.3.5 Betaine regulates different biological pathways in vascular smooth muscle cells, dependent upon cellular age.

3.3.5.1 In young vascular smooth muscle cells, betaine protects cell membranes and improves cellular locomotion.

To elucidate the biological functions and pathways significantly associated with differentially expressed genes following betaine treatment, we conducted an enrichment analysis using GO classification (http://www.geneontology.org/). GO enrichment analysis, a bioinformatic method that can classify overrepresented GO terms within a specific gene set, was employed to categorize gene clusters into biological processes and cellular components. In young VSMCs, betaine upregulated genes involved in cellular locomotion (40 genes), sterol biochemistry (20 genes), cholesterol biochemistry (14 genes), secondary alcohol biosynthesis and metabolism (14 genes), as well as steroid biosynthesis (13 genes) (see Figure 3.5). These findings suggest that betaine may maintain membrane integrity and composition by regulating essential components crucial for maintaining membrane rigidity, fluidity, and permeability. Conversely, betaine significantly downregulated genes associated with transmembrane receptor protein serine/threonine pathways (41 genes), TGF-β signalling (31 genes), and collagen metabolic processes (19 genes). Transforming growth factor β (TGF-β) signalling, initiated by ligand binding to type II (TβRII) and type I (TβRI) serine/threonine kinase receptors, acts as a tumour suppressor by inhibiting cell cycle progression in late G1 phase and stimulating apoptosis in various cell types. Consequently, betaine may enhance VSMC proliferation by downregulating cell cycle arrest via serine/threonine protein receptor-induced TGF-β signalling and reducing collagen breakdown.

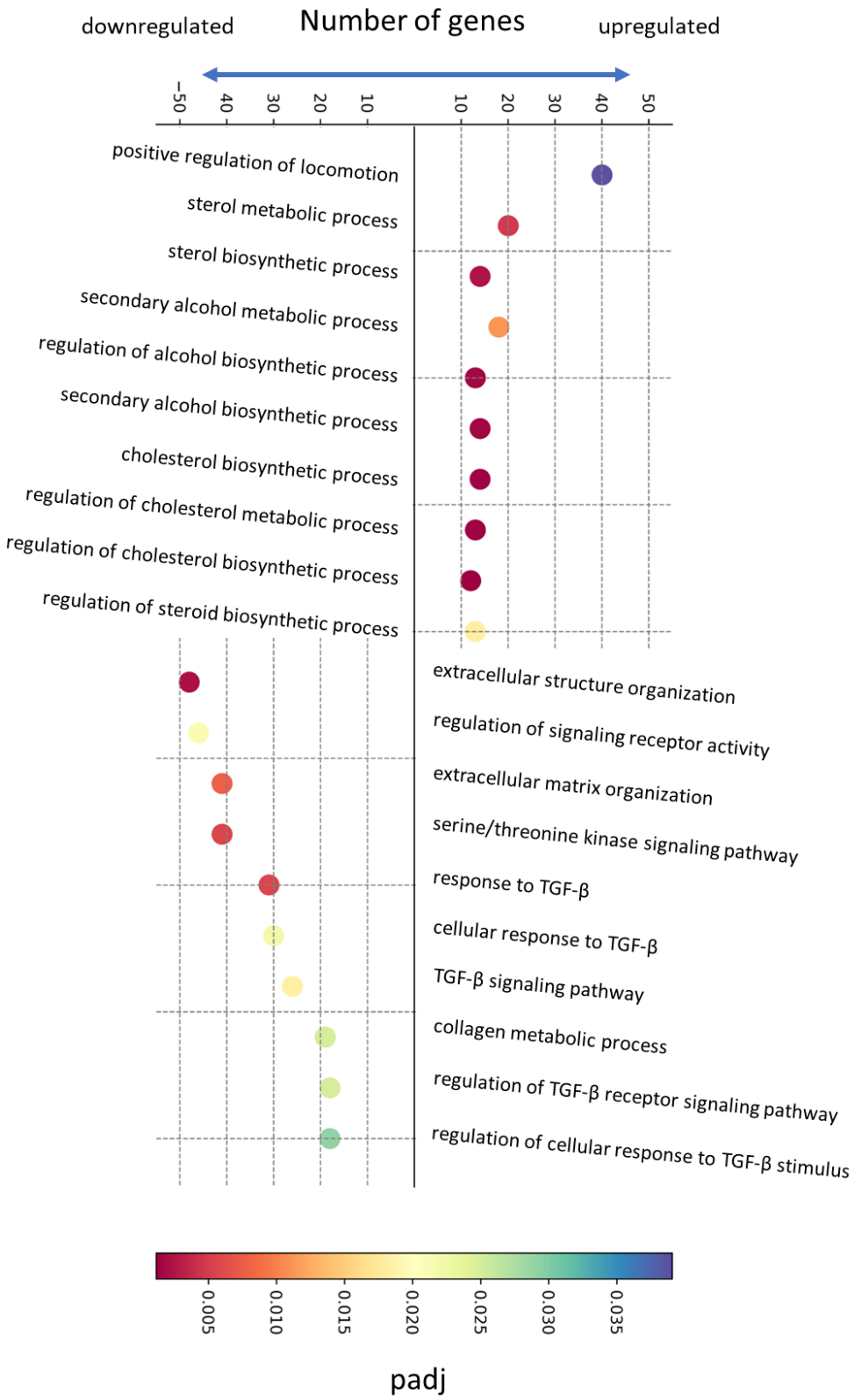


Figure 3.5| GO enrichment analysis scatter plot for biological pathways, compared between control and betaine-treated RNA samples in young vascular smooth muscle cell.

3.3.5.2 In middle-aged cells, betaine enhances vascular smooth muscle cell proliferation and development by improving homeostasis and inflammatory defence response.

In middle-aged VSMCs, betaine significantly upregulated gene clusters associated with cellular ion homeostasis (70 genes), angiogenesis (66 genes), positive regulation of cell development and the mitogen-activated protein kinase (MAPK) cascade (64 genes), regulation of cellular morphogenesis (57 genes), cell growth (51 genes), cellular defence response against inflammation (34 genes), and positive regulation of vasculature development (24 genes) (see Figure 3.6). Notably, the enhancement of smooth muscle cell proliferation (19 genes) contributes to the promotion of vasculature development. These outcomes confirm that betaine can increase VSMC proliferation when administered during the middle-age stage of cellular lifespan. Conversely, betaine significantly downregulated gene clusters involved in chemotaxis (55 genes), cell locomotion (48 genes) and migration (45 genes), muscle cell differentiation (41 genes), cell adhesion (40 genes), protein dephosphorylation (33 genes), regulation of cell shape (29 genes), lipid metabolic processes (18 genes), necrotic cell death (10 genes), cellular immune response (7 genes), and cholesterol storage (6 genes) in middle-aged VSMCs. These data suggests that betaine can suppress various cellular processes (adhesion, migration, differentiation) to prioritize VSMC proliferation, aligning with the understanding that these processes cannot occur simultaneously with cellular proliferation. This evidence indicates that betaine might activate a switch in middle-aged VSMCs, redirecting cellular resources from diverse processes towards proliferation.

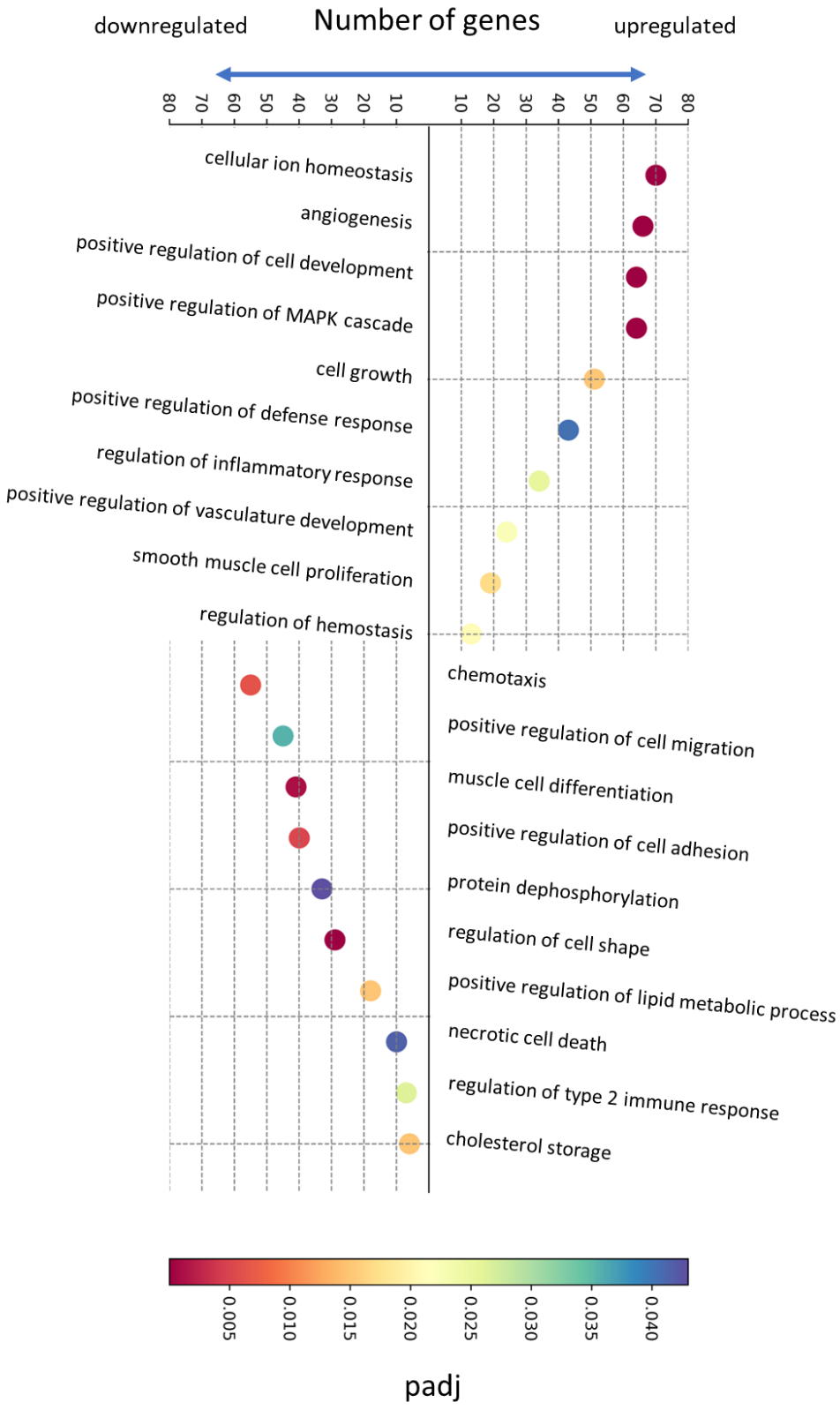


Figure 3.6| GO enrichment analysis scatter plot for biological pathways, compared between control and betaine-treated RNA samples in middle-aged vascular smooth muscle cells.

3.3.5.3 In old cells, betaine provides stimulates mitosis, DNA damage repair and methylation process for proteins and histones.

In aged VSMCs, betaine exhibited a multifaceted effect on gene expression, modulating several key cellular processes (see Figure 3.7). Betaine treatment in old VMSC had a diverse and subtle range of effects. Notably, the data revealed modest stimulatory effects on mitotic processes, particularly in upregulating sister chromatid segregation (36 genes) and cohesion (24 genes), as well as nuclear division (31 genes). Additionally, betaine appeared to influence double-strand break repair mechanisms (27 genes), protein and histone methylation processes (21 and 17 genes), the regulation of DNA recombination (12 genes), damage response (9 genes), and replication fork processing (7 genes). Conversely, the drug reduced the expression of gene clusters associated with the inhibition of intracellular signal transduction (95 genes) and enhancement of cellular responsiveness to lipids (85 genes). Furthermore, betaine appeared to stimulate phosphorylation processes (83 genes) and protein kinase activity (50 genes) through the decreased expression of their negative regulators. The compound demonstrates anti-inflammatory properties by suppressing NF-κB signalling (54 genes), type I interferon production (26 genes), and cytokine biosynthesis (21 genes). It also promotes apoptosis in senescent cells (51 genes), as evidenced by the downregulation of genes involved in negative regulation of apoptotic pathways. Additionally, we demonstrated cytoprotective effects for betaine in older VSMCs by reducing the expression of genes associated with organelle and mitochondrial disassembly (26 and 22 genes).

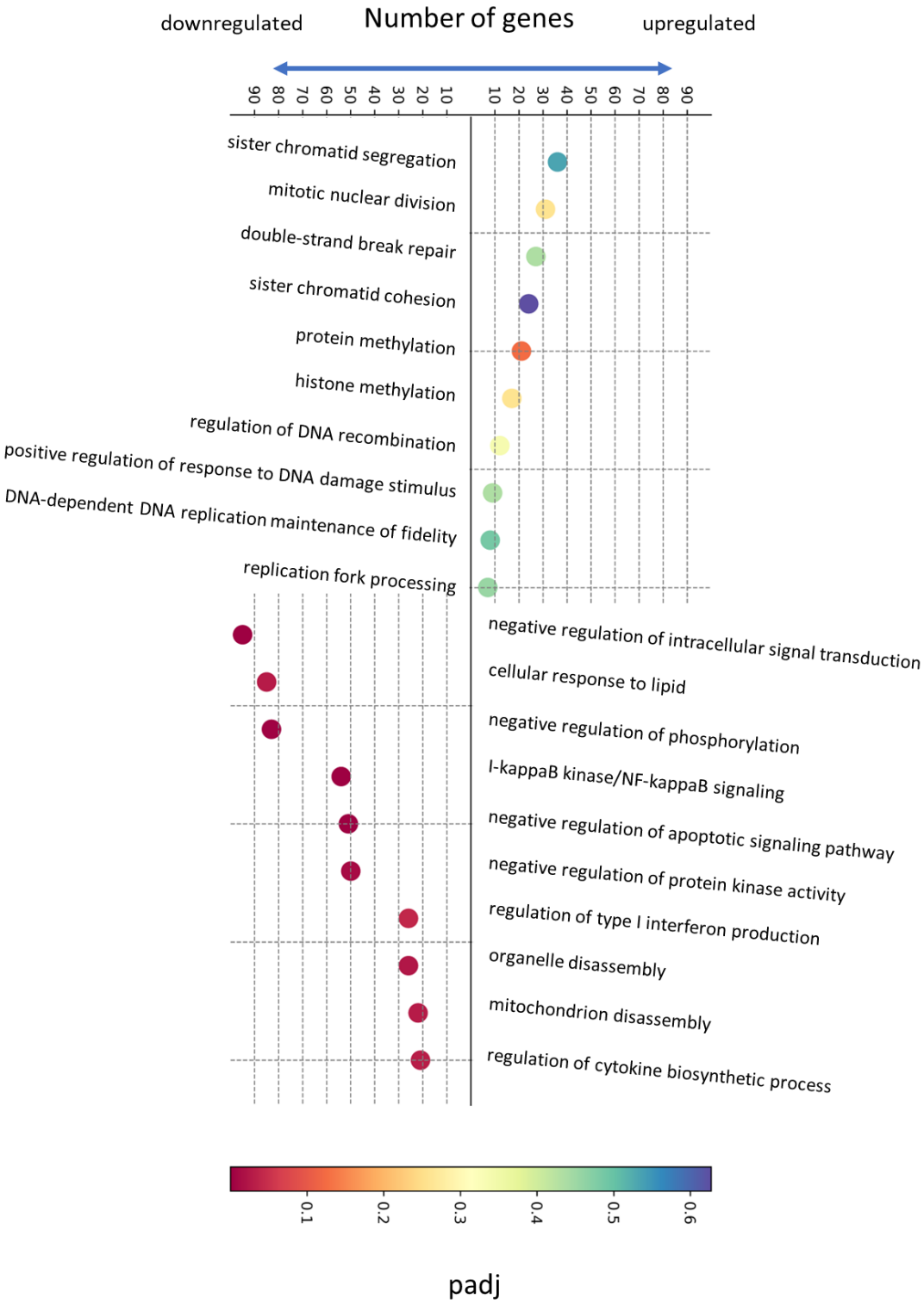


Figure 3.7| GO enrichment analysis scatter plot for biological pathways, compared between control and betaine-treated RNA samples in old vascular smooth muscle cells.

3.3.6 Betaine affects expression of known disease risk genes

3.3.6.1 In young vascular smooth muscle cells, betaine reduces expression of genes associated with vascular disease

To further elucidate the potential therapeutic implications of betaine, we employed DO analysis on our RNA-Seq data derived from control and betaine-treated VSMCs samples. The Human Disease Ontology (accessible at http://www.disease-ontology.org), provides a comprehensive framework for integrating pathomechanistic features of disease with gene function data. This analysis allows for the identification of significant associations between differentially expressed genes and human diseases, with a threshold of adjusted p-value (padj) < 0.05 indicating statistically significant enrichment. By assessing disease similarity based on functional gene associations, we were able to correlate the observed differential gene expression patterns with potential human pathologies. Interestingly, our results revealed that in young VSMCs, which inherently possess robust health and functionality, the administration of betaine did not elicit extensive protective effects (see Figure 3.8).

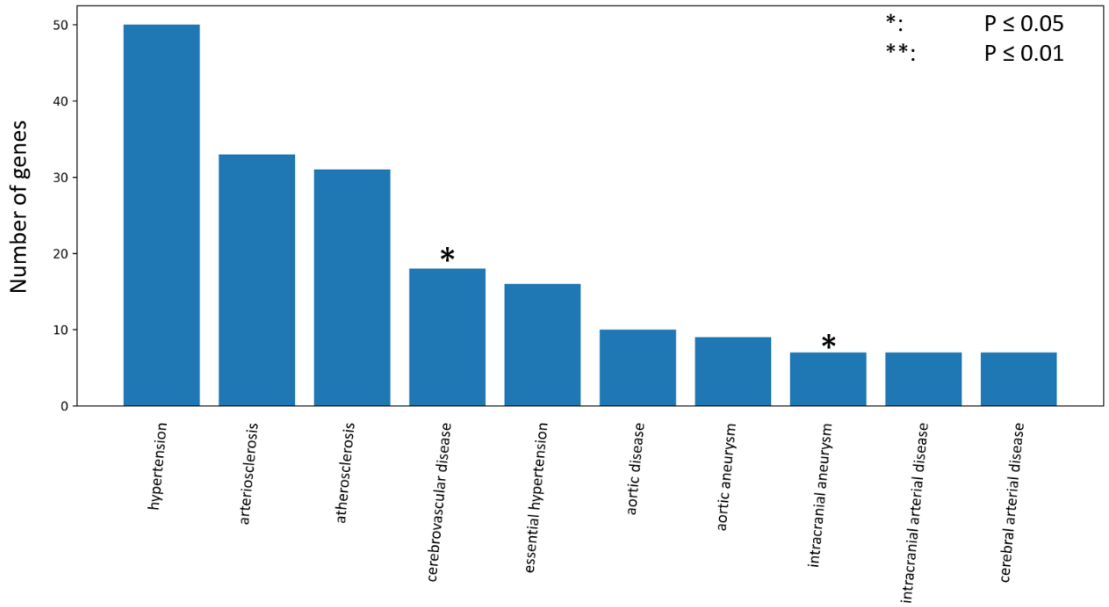


Figure 3.8| DO enrichment analysis histogram comparing between control and betaine-treated RNA samples in young vascular smooth muscle cells. *Two vascular dysfunction-associated diseases, including cerebrovascular disease and intracranial aneurysm, have been shown to significantly reduce risk following betaine treatment. All statistical p-values were adjusted based on Benjamini and Hochberg's method to minimize the false discovery rate.*

However, a notable finding was the significant reduction in the risk associated with two specific vascular diseases: cerebrovascular disease (18 genes) and intracranial aneurysm (7 genes). This observation suggests that betaine may confer targeted protection against vascular pathologies, even in relatively healthy cellular environments.

3.3.6.2 In middle-aged cells, betaine reduces cardiovascular disease risk

In middle-aged VSMCs, we observed a significant reduction in gene expression associated with heart disease (55 genes) (see Figure 3.9). Betaine exhibited effects on a range of other diseases include muscular disease (43 genes), myopathy (41 genes), muscle tissue disease (41 genes), congestive heart failure (25 genes), chronic obstructive pulmonary disease (23 genes), intrinsic cardiomyopathy (19 genes), cardiomyopathy (19 genes), cerebrovascular disease (17 genes), hypertrophic cardiomyopathy (10 genes). These findings collectively suggest that betaine may exert its effects through modulation of gene expression patterns common to a spectrum of vascular diseases, with particularly pronounced impacts on heart health.

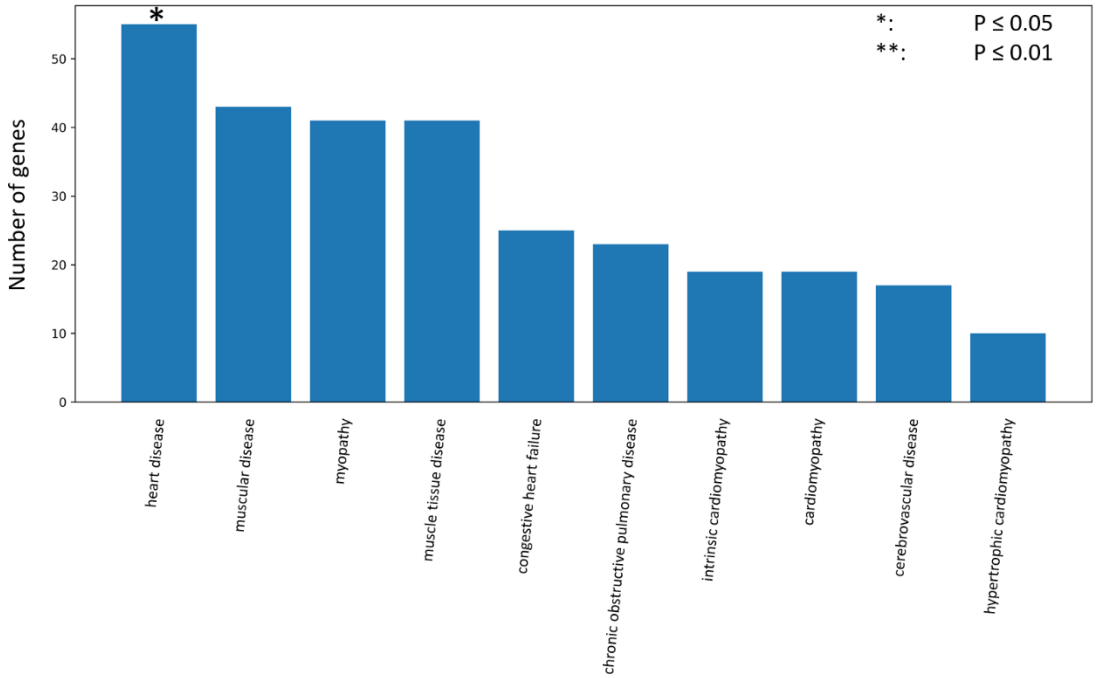


Figure 3.9| DO enrichment analysis histogram comparing between control and betaine-treated RNA samples in middle-aged vascular smooth muscle cells. *After long term supplementation with betaine, VSMCs has been identified with significantly reduced risk of heart disease. All statistical p-value was adjusted based on the Benjamini and Hochberg's method to minimize false discovery rate.*

3.3.6.3 In old cells, betaine reduces risks of various diseasome of ageing

Our comprehensive analysis of RNA-Seq data using Disease Ontology revealed that betaine exhibits a remarkable capacity to attenuate a broad spectrum of age-related pathologies in VSMCs, that comprise the 'diseasome of ageing' (see Figure 3.10). This effect is evidenced by the significant downregulation of genes associated with various age-related conditions. Specifically, the analysis identified substantial gene expression changes across multiple pathological conditions: rheumatoid arthritis (79 genes), lung disease (75 genes), arteriosclerosis (60 genes), atherosclerosis (59 genes), colon cancer (52 genes), head and neck cancer (46 genes), Parkinson's disease (38 genes), type 2 diabetes mellitus (36 genes), dementia (31 genes), and inflammatory bowel disease (19 genes). This broad-spectrum attenuation of disease-related gene expression suggests that betaine may possess pleiotropic effects on cellular ageing processes within VSMCs. The diverse range of affected conditions, spanning from inflammatory disorders to neurodegenerative diseases and various cancers, underscores the potential of betaine as a promising therapeutic agent for addressing the multifaceted nature of age-related pathologies. Moreover, the substantial number of genes modulated in each disease category implies a robust and systemic impact of betaine on cellular pathways involved in ageing and disease progression.

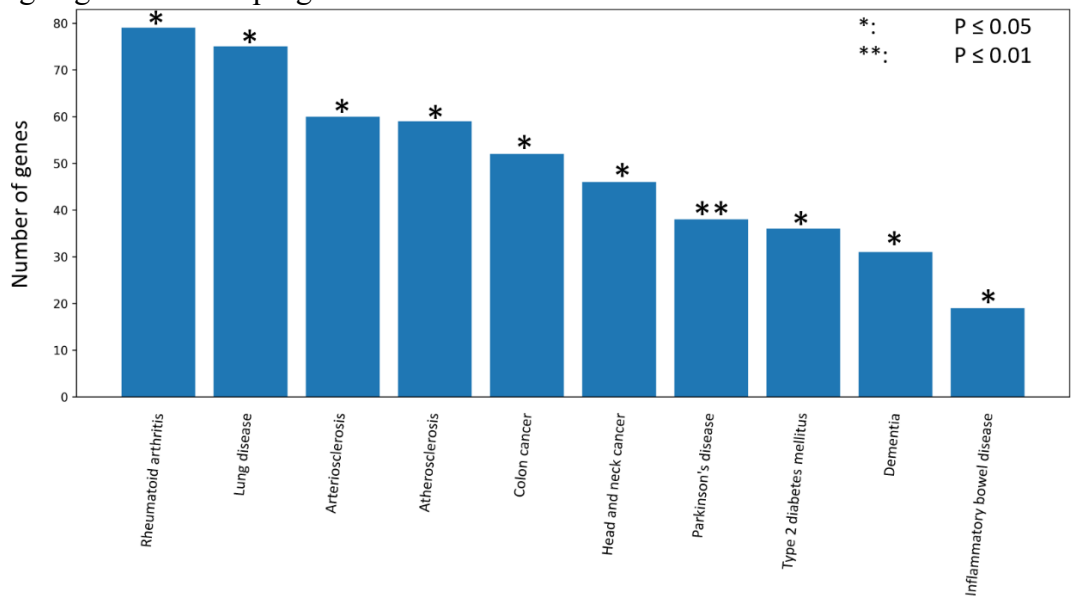


Figure 3.10| DO enrichment analysis histogram comparing between control and betaine-treated RNA samples in old vascular smooth muscle cells. *A series of diseasome of ageing, such as rheumatoid arthritis, atherosclerosis, Parkinson's disease, type 2 diabetes and dementia, have been shown with considerable risk reduction as VSMCs treated with betaine. All statistical p-values were adjusted based on Benjamini and Hochberg's method to minimize the false discovery rate.*

3.4 Discussion

Biological ageing (senescence) of VSMCs is relevant to the development of macro- and microvascular dysfunction (Ungvari et al., 2018). Therapeutic pathways that modulate VSMC phenotype have not yet been identified. Furthermore, the VSMC transcriptomic landscape in physiological ageing of human blood vessels remains uncharacterized. This chapter investigated the transcriptomic profiles of VSMCs across different age groups (young, middle, and old) and assessed the impact of betaine treatment using RNA sequencing. The analysis encompassed PCA, co-expression Venn diagram analysis, differential gene expression analysis, cluster analysis, functional analysis using GO terms and DO enrichment analysis. To the best of our knowledge, this chapter represents the first application of RNA-Sequencing methodology to human VSMCs during the ageing process.

PCA revealed distinct clustering for different age groups and between control and betaine-treated samples. Strong correlations were observed within each age group, with the three age groups forming distinct clusters. More than 50% of the data variance was explained by the first three principal components (see Figure 3.1), indicating substantial differences in expression patterns among VSMCs of different ages. This distinct separation of clustering patterns based on age groups has been recorded in other studies focusing on gene expression profiles to identify age-associated changes in biological functions (i.e. oocyte fertilization, cognitive function). (Mok et al., 2016, Sani et al., 2021). Co-expression analysis showed a decrease in co-expressed genes between age groups as VSMCs aged, with older VSMCs exhibiting a higher number of uniquely expressed genes. This pattern was consistent in both control and betaine-treated VSMCs, suggesting that while betaine may alter gene expression, it does not fundamentally change the trend of transcriptomic divergence with age. Differential gene expression analysis provided insights into age-related changes and the effects of betaine treatment. The number of differentially expressed genes between control and betaine-treated VSMCs increased with age, with the oldest cells showing the most substantial changes. This age-dependent response to betaine aligns with the observed increase in morphological and physiological changes of VSMCs during vascular ageing, suggesting that older cells may be more susceptible to therapeutic intervention (Xu et al., 2017).

Cluster analysis in human VSMCs revealed distinct patterns of gene expression between control and betaine-treated cells across all age groups. We observed a clear reversal of expression patterns was observed, with up- and down-regulated genes showing opposite trends between young and old cells. Our outcomes are similar to previous studies identified in other *in vivo* models, such as rodents, *D. melanogaster* (Sun et al., 2022, Bordet et al., 2021, Gao et al., 2020b). Intriguingly, the gene expression patterns of betaine-treated old VSMCs showed similarities to those of young control cells only in certain clusters. Changes in gene expression patterns due to senotherapeutic interventions have been reported for several senotherapeutic agents, such as resveratrol and quercetin. For instance, 18- and 27-month-old mice treated with resveratrol demonstrated resembled gene expression profiles in the liver and muscle of 9-month younger mice. The analysis of 10-month-old aged mice supplemented with quercetin revealed significant similarities with 8-week-old mice (Su et al., 2024). Therefore, our observation supports the hypothesis that betaine may induce a partial rejuvenation effect at the transcriptomic level, corroborating previous observations of youthful appearance and increased proliferative capacity in betaine-treated VSMCs.

Functional analysis of differentially expressed genes was conducted to elucidate the biological functions and pathways significantly associated with the transcriptomic changes induced by betaine in VSMCs of different ages. GO terms were utilized as a bioinformatic tool to classify gene clusters into biological processes and cellular components. In young VSMCs, betaine exhibited a protective effect on cell membranes and enhanced cell locomotion. The upregulation of genes involved in the biosynthesis and metabolism of sterol, cholesterol, and secondary alcohols suggests that betaine may play a crucial role in maintaining membrane integrity, including rigidity, fluidity, and permeability. These lipidic natural products are known membrane reinforcers, potentially mitigating stress-induced damage to cell membranes (Dufourc, 2008). This observation aligns with previous studies emphasizing the interaction between betaine and phospholipids in cell membranes, potentially increasing membrane stability (Corradi et al., 2019, Güler et al., 2016). Betaine is also known as an organic osmolyte which helps maintaining cell volume (Dobrijević et al., 2023). Concurrently, betaine promoted VSMC proliferation by downregulating gene clusters associated with TGF-β signalling, transmembrane receptor protein serine/threonine pathways, and collagen metabolic processes. Based on previous studies, TGF-β is responsible for inhibiting VSMCs

proliferation while contributing to a range of cardiovascular diseases (i.e. atherosclerosis, cardiac fibrosis) (Sakakibara et al., 2003, Singh and Ramji, 2006, Ruiz-Ortega et al., 2007). Despite no direct evidence indicating the relationship between betaine and TGF-β, betaine supplementation has been shown to diminish atherosclerotic lesion area in mice by 37% after 14 weeks. The anti-atherogenic effect has been confirmed by inhibiting excessive TNF-α-mediated aortic inflammatory response. In addition, we discovered another interesting finding betaine reduces the collagen metabolic process in young VSMCs. As collagen is a glycine-rich protein, betaine has been indicated with its potential effects on stimulating collagen production inside the body (Viennet et al., 2002).

The effects of betaine on middle-aged VSMCs were particularly pronounced in promoting cell growth. This was evidenced by the upregulation of genes involved in the MAPK cascade, anti-inflammatory responses, and cellular ion homeostasis. MAPK has been extensively studied as an important factor for different cellular process in VSMCs, including cellular proliferation, stress response and inflammation (Mii et al., 1996, Jiang et al., 1996, Moens et al., 2013). Despite its prominent role in VSMCs proliferation, MAPK is also involved in enhancing inflammatory mediators (i.e. IL1 β, IL6), followed by elevated stress response during this proliferation process (Kaminska, 2005). Interestingly, our data has identified that betaine - widely known for its antioxidant and cytoprotective effects - can activate simultaneously MAPK and anti-inflammatory pathways which further reinforce the maintenance of cellular homeostasis (Arumugam et al., 2021). A temporary switch in cellular processes was also observed, suggesting that betaine prioritizes cell proliferation over other biological processes such as adhesion, migration, and differentiation in this age group. As all these cellular processes required lots of ATP derived from mitochondria, we hypothesized that betaine might initiate the biological switch to promote VSMCs proliferation without causing excessive mitochondrial stress response. As a result, mitochondria homeostasis can be achieved throughout the life course of VSMCs.

In older VSMCs, the effects of betaine were more subtle. While no significant upregulation of gene clusters was observed, modest positive effects were noted in cell-repairing processes, cell division, protein/histone methylation, and DNA damage response pathways. Notably, betaine reduced the expression of gene clusters associated with age-related cellular activities, including signal transduction imbalance, impaired lipid metabolism, hyperphosphorylation and impaired apoptotic pathways. As cells age,

the ability to effectively transduce signals declines due to detrimental changes in receptor activities, mitochondria function and increased ROS-associated oxidative damage (Uryga and Bennett, 2016). This leads to altered intercellular communication, which is one of the well-known characteristics within the 'hallmarks of ageing', In addition, impaired lipid metabolism in SCs is central to age-related pathologies (Sharma and Diwan, 2023). This is pertinent to excessive levels of circulatory lipid components (i.e. triglycerides, low-density lipoproteins (LDL), and high-density lipoproteins (HDL)), leading to premature senescence *in vitro* and *in vivo*. Lifestyle-associated diseases such as obesity and type 2 diabetes, have a compelling association with increased SCs burden (Révész et al., 2018). In animal models subject to a high-fat die, accelerated cellular senescence occurs prematurely in different body tissues (e.g. kidney, liver, brain) (Kim et al., 2019, Zhang et al., 2018b, Ou et al., 2022). In vascular ageing, alterations in phosphorylation activities of specific proteins (i.e. NFκB, SIRT1) are responsible for impaired vasomotor function (Lesniewski et al., 2011, Bai et al., 2014). This age-related hyperphosphorylation process results in the abnormal activation of NFκB and SIRT1, thus enhancing pro-inflammatory arterial phenotypes and oxidative stress while blocking the longevity-mediated activity of SIRT1. The cytoprotective effects of betaine in older VSMCs were further emphasized by the downregulation of genes involved in organelle and mitochondrion disassembly, inflammation-associated NF-κB signalling, type I interferon production, and cytokine biosynthesis. Previous studies mainly focus on effects of betaine on cardiovascular and liver diseases (Ashtary-Larky et al., 2022b, Wang et al., 2021b). According to our knowledge, betaine's ability to influence intracellular signalling, lipid metabolism, phosphorylation and apoptosis pathways is related to its ability to protect VSMCs from cell cycle arrest, anti-oxidation and potentially interact with longevity-specific receptors.

Our DO enrichment analysis also reported the diverse effects of betaine in mitigating gene expression patterns that are associated to specific diseases. Even though young and middle-aged VSMCs are still healthy and robust, treatment with betaine provides substantial protection for vascular and cardiovascular diseases. Interestingly, treatment with betaine in older VSMCs significantly reduces the risk of different 'diseasome of ageing'. This indicates that long term treatment with betaine can provide protection against age-related chronic diseases. According to the previous studies, betaine has also been shown to exert potential protective effects in multiple age-associated pathologies, such as atherosclerosis, CKD and fatty liver disease (Liu et al., 2024a, Lv et al., 2009,

Alvarenga et al., 2022b). Recent evidence also suggests betaine supplementation might mitigate age-related impacts by improving muscle mass, autophagy activities and anti-inflammation (Chen et al., 2024a, Go et al., 2005a). We believe this can be explained by our previous data, indicating that betaine prevents mitochondrial dysfunction and oxidative damage (i.e. IL1β, IL6) while activating autophagy (i.e. Beclin1, LC3B, p63). This significantly decreases the occurrence of SCs and senescence-associated SASP, thus mitigating the dysfunction of a range of body organs, such as kidney and liver.

This comprehensive transcriptomic analysis provides valuable insights into the age-related changes in VSMCs and the potential rejuvenating effects of betaine. The distinct age-related transcriptomic signatures and the differential response to betaine treatment across age groups highlight the complex interplay between ageing and drug responsiveness in VSMCs. Notably, the partial restoration of a 'young' transcriptomic profile in older cells treated with betaine suggests promising therapeutic potential for addressing age-related vascular pathologies. The observed effects in this chapter indicate the capacity of betaine to modulate key cellular mechanisms associated with senescence. Overall, this preliminary evidence opens avenues for future research, particularly in exploring combinatorial approaches where betaine could be synergistically paired with other compounds to enhance senotherapeutic efficacy in aged VSMCs. Further research is warranted to elucidate the specific molecular pathways involved in this apparent rejuvenation effect and to explore the clinical implications of these findings.

Chapter 4: Effects of betaine on iPSCs-induced vascular smooth muscle cells

4.1 Introduction

Induced pluripotent stem cell (iPSCs)-derived VSMCs or iVSMCs have increasingly gained attention as a potential *in vitro* model for studying vascular ageing (Stephenson et al., 2020). By using patient-specific iPSCs, researchers can differentiate them into VSMCs that recapitulate the genetic and representative epigenetic landscapes of individual donors. This unique aspect allows iVSMCs to be produced in large quantities while possessing clinically relevant phenotypes and expressing specific bio-markers (i.e. smooth muscle 22 alpha, SM22α) (Dash et al., 2015). Importantly, iVSMCs derived from older donors often display age-related changes in phenotypic and functional characteristics. These alterations include expression of markers of cellular senescence and diminished proliferation capacity, as well as diminished responses to mechanical stress. iPSCs-derived cells are also suitable for long-term experiments for studying ageing processes, which often require cell culture for extended periods (Chehelgerdi et al., 2023). Consequently, iVSMCs can be utilized to further explore several common features that contribute to vascular ageing, including vascular calcification, extracellular vesicle secretion, oxidative damage and mitochondria dysfunction.

Vascular calcification is a pathological condition which is characterized by increased levels of calcium deposition in the vascular wall (Schlieper et al., 2016). Specifically, calcium and phosphate in hydroxylapatite form, are two major components that promote the mineralization of VSMCs (Schlieper et al., 2010a). This abnormal bio-mineralization process may occur in the medial and intimal layers of the vasculature, at varying degrees throughout the vascular system. There are two distinct types of vascular calcification, comprising arterial media calcification and accelerated calcification of intimal plaque (Ketteler et al., 2006). The overall prevalence of vascular calcification is highly dependent on age-related factors, and rises from <5% to >12% for individuals between 50 years to 80 years of age (Leopold, 2013). The impaired blood flow due to calcified deposits contributes to increased pulse wave velocity, endothelial dysfunction and thrombosis, eventually leading to multiple organ damage (Demer and Tintut, 2008). Numerous disease modalities within the 'diseasome of ageing', such as CKD, CVD and atherosclerosis, share common underlying mechanisms that affect vascular ageing, coincident with significant incidence of vascular calcification (Lanzer et al., 2014).

During vascular calcification, VSMCs experience a functionally deleterious switch from a contractile (migratory/ proliferative) to an osteochondrogenic phenotype (Owens et al., 2004, Speer et al., 2009). Long-term exposure to harmful exposome factors, leads to cellular stress that induces upregulation of vascular calcification-associated transcription factors (i.e. Runx2, Sox9), while downregulating VSMCs contractile proteins (i.e. SM22-α and SM α-actin) (Boström et al., 2011, Byon et al., 2008, Steitz et al., 2001). Consistent with this, a previous study has reported that Runt-related transcription factor 2 (Runx2) deficiency in VSMCs inhibits the development of vascular calcification in mice (Sun et al., 2012).

In addition, a direct link between extracellular vesicles (EVs) – also known as circulatory membrane structures with diverse properties – and an accelerated ageing process has recently gained more attention. EVs are membrane-encapsulated structures with a diameter from 30 nm to 5 μm, secreted by almost all types of cells inside the body (Yáñez-Mó et al., 2015). EVs are used as a general term to describe a collection of various forms of vesicles, such as exosomes (30–100 nm), microvesicles (100–350 nm), ectosomes (100 nm–1 μm) and apoptotic bodies (1–5 μm) (Mas-Bargues and Alique, 2023). These particles, produced by both living and apoptotic cells, have been found in CKD patients with vascular calcification (György et al., 2011, Schlieper et al., 2010b). Depending on the ratio between Ca^{2+} and phosphate levels in the surrounding environment, EVs with distinct components and calcifying properties have been reported in VSMCs *in vitro* (Kapustin et al., 2011, Reynolds et al., 2004). EVs are emerging for their role as a key biomarker of vascular ageing mediated by enhancing VSMCs-associated calcification (Kapustin et al., 2011, Shroff et al., 2013).

During normal conditions, EVs produced by VSMCs carry a large number of calcification inhibitors such as carboxylated Matrix Gla protein (MGP) and fetuin-A to inhibit the excessive mineralization deposition in the vasculature system. As vascular homeostasis is disrupted due to elevated Ca^{2+}, phosphate, SASP or ROS production, VSMCs undergo phenotypic switching and generate more EVs that contain pro-calcifying factors (i.e. phosphatidylserine and annexins) (Kapustin et al., 2011). A recent study on CKD patients has identified that Ca^{2+} and phosphate-derived mineral stress led to a significant decline in calcification inhibitors released by exosomes (Kapustin et al., 2015). This phenotypic alteration has also been observed in VSMCs during apoptotic or secondary necrotic processes (Proudfoot et al., 2000). From that, apoptotic bodies, together with pro-

calcification properties, have been detected during chronic VSMCs apoptosis in atherosclerosis *in vivo* (Clarke et al., 2008).

Vascular ageing is a complex and multi-level phenomenon, with various underlying mechanisms. To fully understand those contributing pathways, it is essential to combine different approaches and experimental models. The utilization of both pluripotent stem cells and primary VSMC cultures as *in vitro* models for drug screening offers a powerful tool to study this complicated process. While iVSMCs are robust, proliferative and can be easily genetically modified (Chehelgerdi et al., 2023), primary VSMCs directly derived from blood vessels, maintain many *in vivo* characteristics and offer an excellent fully differentiated cell phenotype for experimental testing systems. Primary VSMCs can be used immediately without the need for reprogramming and differentiation. They also retain age-associated epigenetic modifications that might be lost in iPSCs reprogramming. While primary VSMCs provide snapshots of different stages of vascular ageing (young versus old), iVSMCs allow us to study early developmental aspects and the progression of the ageing process from a 'young' starting point. Together, they offer a more complete picture of the ageing process from development to late-stage ageing. By using both iVSMCs and primary VSMCs as *in vitro* models in vascular ageing research, our findings can be reinforced by a reliable validation platform. The results observed in primary VSMCs can be confirmed in iVSMCs and vice versa, thus increasing confidence in the final outcomes. Especially in the drug development process, it is also crucial to combine various models as it can improve the translatability of findings to *in vivo* situations.

In Chapter 2, we established the optimal concentration dosing of betaine for delaying VMSC ageing in vitro using RTCA. We have successfully demonstrated that this treatment mitigates a range of hallmarks of ageing, including the accumulation of senescent cells, inflammatory burden, replicative senescence and mitochondrial dysregulation. We have demonstrated that betaine treatment mitigates the expression of cellular biomarkers of ageing and the SASP, including CDKN1A, CDKN2A, IL6, IL1β and Sa β-gal. In this chapter, the senotherapeutic effects of betaine on iPSCs-derived VMSC ageing were assessed based on other important characteristics of vascular ageing, including vascular calcification, total mitochondria content and membrane potential, IL6-induced inflammation, extracellular vesicles and ROS-induced oxidative stress.

4.2 Aims and objectives

We sought to explore the potential impact of betaine on iPSCs-derived VSMCs in the context of age-related vascular calcification. By identifying the functional and morphological changes of mitochondria in different medium conditions (i.e. calcified medium with high Ca^{2+}, inflammatory medium with increased levels of IL6, ROS or dimethyl sulfoxide-DMSO), we sought to gain insight into the mechanistic pathways in which betaine acts on iVSMCs to prevent accumulation of vascular calcification during ageing. Our objectives are listed as follows:

- **Objective1**: To establish whether betaine can protect iVSMCs against vascular calcification
- **Objective 2**: To evaluate mitochondria functions under the effects of betaine supplementation in different pathological conditions
- **Objective 3**: To determine if any salutogenic effects are mediated through removal of deleterious extracellular vesicles.

4.3 Materials and methods

Cell culture. Human iPSCs-derived vascular smooth muscle cells from a healthy donor of Turkish/Middle Eastern origin in early 30 years of age – passage 9 – were obtained in Prof. Leon Schurgers' lab in Maastricht University. The cells were cultured in DMEM + Glutamax + pyruvate (31966021) supplemented with 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin (P/S). All the above reagents were obtained from Thermofisher Scientific, unless stated otherwise. All flasks were coated for 1h at room temperature with 0.1% gelatine (Sigma, G-6144) and then overnight with DMEM supplemented with 10% FBS. iVSMCs were cultured under typical conditions of 5% CO_2 at 37ºC in a humidified incubator. Cells were routinely tested for mycoplasma. Passage of iVSMCs cells was conducted when the confluency reached 100% or more (cells forming layers on top of each other) by 1:3 (before treatment) or 1:2 (during treatment) split. Standard protocol was followed using phosphate buffered saline (PBS) (Presenius Kabi) to wash the cells after the medium was removed and TrypLE (Thermofisher Scientific) to detach the cells from the flasks.

Long term cell treatment with betaine. iVSMCs were cultured with standard medium and betaine 1 mg/ml (Sigma Aldrich)-supplemented media. Betaine was added directly to the iVSMCs culturing medium in every passage from young to old cells. In every passage, DNA, RNA or cells were extracted from the remaining cells for further downstream experiments.

Real-time cell analysis. The ACEA xCELLigence RTCA system was used to monitor cell growth on E-Plate 96 View 96-well plates. iVSMCs – passage 15– were counted with a haemocytometer using 0.5% Trypan blue before seedling into a 96-well RTCA plate. Plates were seeded with 4000 cells/well in 200 µl medium. Experiments with different betaine concentrations (ranging from 10 µg/ml to 1 mg/ml) were carried out in quadruplicate and outlier wells were excluded from the analysis. Cells were left to settle for approximately 24 h, then treated with either normal medium or calcification medium (DMEM + GlutaMax + pyruvate, 5% FBS, 1% P/S and 3.6 mM Ca^{2+}) and monitored for at least 72 h. The Cell Index was normalised with respect to the last time point before treatment. Slope analysis was carried out considering a period of 24 hours after treatment. ACEA RTCA software version 1.2.1 was used.

Vascular calcification assay. The calcification experiment was conducted based on BioHybrid assay designed by Jaminon, Armand MG, et al (Jaminon et al., 2021). Human iVSMCs of control and betaine-treated cells were seeded in 24 gelatin-coated internal wells in the 48-well plates at a density of 10,000 cells/well. The 24 wells at the edge were filled with PBS. After 24 hours, iVSMCs were cultured in calcification medium (DMEM + GlutaMax + pyruvate, 5% FBS, 1% P/S and 3.6 mM Ca^{2+}) with or without betaine for up to 10 days. As mentioned in the BioHybrid assay, cells were supplemented at the start of the experiment with fetuin-A-AlexaFluor®-546 (1–3 µg/mL; prepared in-house) and Hoechst 33,342 (1 µg/mL; Invitrogen). Images were taken daily with red fluorescent protein (RFP), DAPI channel (cell count) and brightfield (calcified area) for up to 10 days. The progression of calcification areas was followed by imaging the same area over the entire experiment. The Cytation 3 system (BioSPX) was used for imaging. Further analysis was performed using Gen5 software v.2.9 (BioTek). Normalization was done by readout RFP confluence per well per cell count.

Total mitochondria content and membrane potential assay. Human iVSMCs of control and betaine-treated cells were seeded in 24 gelatin-coated internal wells in the 48-well plates at a density of 10,000 cells/well. The 24 wells at the edge were filled with PBS. After 24 hours, old medium was replaced by new medium supplemented with MitoTracker® Red CMXRos (50 nM; M7512; Thermofisher Scientific) or JC1 probe (2 µM; M34152; Thermofisher Scientific) for labelling total mitochondria content and mitochondria membrane potential, respectively. Hoechst 33,342 (1 µg/mL; Invitrogen) was used to visualize cell nucleus. Cells were imaged after 30 minutes of incubation. The Cytation 3 system (BioSPX) was used for imaging. Further analysis was performed using Gen5 software v.2.9 (BioTek). Total mitochondria content was measured by normalizing readout RFP confluence per well per cell count. For mitochondria membrane potential experiment, the ratio of RFP (high-membrane potential)/GFP (low-membrane potential) fluorescence was measured to indicate the level of mitochondria depolarization process.

Mitochondria membrane potential assay after CCCP treatment. Human iVSMCs of control and betaine-treated cells were seeded in 24 gelatin-coated internal wells in the 48-well plates at a density of 10,000 cells/well. The 24 wells at the edge were filled with PBS. After 24 hours, the old medium was replaced by a new medium supplemented with carbonyl cyanide m-chlorophenylhydrazone (CCCP) (50 µM, Thermofisher Scientific) dissolved in DMSO to induce disruption of mitochondrial membrane potential. DMSO

was used as a negative control. Cells were imaged after 15 minutes of incubation with JC1 probe (2 µM, M34152; Thermofisher Scientific) and Hoechst 33,342 (1 µg/mL; Invitrogen). The Cytation 3 system (BioSPX) was used for imaging. Further analysis was performed using Gen5 software v.2.9 (BioTek). The ratio of RFP (high-membrane potential)/GFP (low-membrane potential) fluorescence was measured to indicate the level of the mitochondria depolarization process.

Inflammation assay. Human iVSMCs of control and betaine-treated cells were seeded in 24 gelatin-coated internal wells in the 48-well plates at a density of 10,000 cells/well. The 24 wells at the edge were filled with PBS. After 24 hours, iVSMCs were cultured in an inflammation medium (DMEM + GlutaMax + pyruvate, 5% FBS, 1% P/S and 50 ng/ml IL6) with or without betaine for up to 10 days. Cells were supplemented at the start of the experiment with a JC1 probe (2 µM, M34152; Thermofisher Scientific) and Hoechst 33,342 (1 µg/mL; Invitrogen). Images were taken daily with fluorescent protein (RFP, GFP) and DAPI channel (cell count) for up to 10 days. The Cytation 3 system (BioSPX) was used for imaging. Further analysis was performed using Gen5 software v.2.9 (BioTek). The progression of inflammation areas was followed by imaging the same area over the entire experiment. The ratio of RFP (high-membrane potential)/GFP (low-membrane potential) fluorescence was measured to indicate the level of mitochondria depolarization process. Normalization of high-potential mitochondria was achieved by readout RFP confluence per well per cell count.

Extracellular vesicles assay. Human iVSMCs of control and betaine-treated cells were seeded in a 12 gelatin-coated well plate at a density of 60000 cells/well. After 24 hours, the old medium was changed into a 0.5% FBS – extracellular vesicle (EV) free medium. EV-free medium was generated by ultra-centrifugating the FBS for 1 hour at 100.000g. EV-media and protein from the cells were collected on ice on the following day. The culture medium was centrifuged at 3000g for 10 minutes at 4ºC and stored at -20ºC. The remaining cells were washed with PBS before adding 100 µl of RIPA buffer (89901, Pierce) supplemented with MS SAFE (MSSAFE-5vl, Sigma) to each well. iVSMCs were scraped off the wells and collected in Eppendorf tubes, followed by 10 seconds of vortex. Cells were left on ice for another 2-3 minutes before centrifuging at 12000 rpm for 20 minutes at 4ºC. Supernatant with protein was collected into new tubes and stored at -20ºC. Next, samples were diluted 1:40 in milliQ and a minimum of 1ml was injected into the ZetaView particle tracking analyser (NTA) to measure extracellular vesicle (EV)

content. Settings were optimized to detect particles with a median size of 100nm, sensitivity was set to 75, and frame rates at 30 and 11 positions were measured. The exact number of extracellular vesicles per sample was achieved by normalizing EV number to the total protein quantified with the Micro BCA Protein Assay Kit (23235, Thermofisher Scientific).

Oxidative stress assay. Human iVSMCs of control and betaine-treated cells were seeded in a 96-well black-wall plate (Corning, NY, USA) overnight at a density of 7000 cells/well in DMEM (31966021, Gibco) with 10% FBS (10270106, Gibco). After 24 hours, the new medium was replaced by the old culture medium with only 2.5% FBS for one day. On the following day, cells were washed once with PBS. Cells were then incubated with 2',7' –dichlorofluorescin diacetate (DCFDA, 20 µM, D-2935, Molecular Probe) and Hoechst 33342 (1 µg/mL, B-2261, Sigma) diluted in PBS for 30 minutes in 5% CO_2 at 37ºC. Wells without cells or DCFDA were used as negative controls. Next, cells were washed once with Krebs–Ringer phosphate glucose buffer (KRPG, 145 mM NaCl, 5.7 mM NaH_2PO_4, 4.86 mM KCl, 1.22 mM $MgSO_4$, 5.5mM glucose, pH 7.4). iVSMCs were then treated with different concentrations of hydrogen peroxide (H_2O_2, 25 µM, 100 µM, 200 µM and 500 µM) in KRPG buffer (100 µL/well). Cells were imaged every 5 minutes for one hour (excitation 485 nm/emission 529 nm) using a Cytation 3 system (BioSPX). Intracellular ROS levels were determined by analysing the area under curve normalized by cell count.

Statistical analysis. All statistical analyses were performed using GraphPad Prism (version 10.2.1). Unless indicated otherwise, statistical significance was determined by two tailed t-test (not significant (NS) $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ or **** $P \leq 0.0001$). In all experiments, we used two biological replicates and four technical replicates, with the exception of oxidative stress assay (three technical replicates). Whenever possible, individual datapoints are shown to clearly represent whether the data are normally distributed.

4.4 Results

4.4.1 Betaine increases iPSCs-induced vascular smooth muscle cell growth

We have sought to evaluate by real-time cell analysis the impact of iVSMCs treated with 1 mg/ml betaine under standard growth conditions and in calcification medium, to assess how betaine affected their growth rate (see Figure 4.1). Under standard growth conditions (normal growth medium), betaine supplementation increased the growth rate of iVSMCs in culture, compared to controls (p-value < 0.001). Our cell growth analysis for iVSMCs in calcification medium indicated that there were no significant differences in growth rate for two days post-treatment, between the betaine-treated cells and control cells. However, betaine treatment resulted in an increase in growth rate by the third day after treatment. These outcomes suggest that betaine exerts a beneficial effect on vascular smooth muscle cell growth.

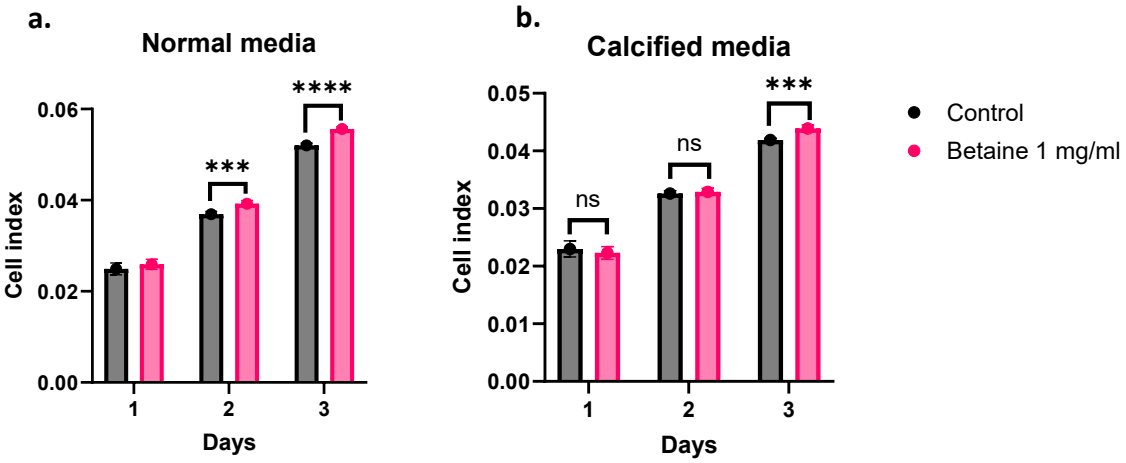


Figure 4.1| iPSCs induced-vascular smooth muscle cell growth in normal and calcification media after betaine treatment. (a) *cell growth in betaine-treated iVSMCs in normal media and (b) cell growth in betaine-treated iVSMCs in calcification media. In normal culture media, no significance change was observed in cell growth in iVSMCs on the first day after treatment. iVSMCs registered a significant growth in the next two days after betaine supplementation. In calcification medium, betaine increases cell growth significantly compared to the control cells on the third day of treatment. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

4.4.2 Betaine protects iPSCs-induced vascular smooth muscle from vascular calcification

Vascular calcification, characterized by excessive deposition of calcium-phosphate particles (CCP) in blood vessels, contributes to increased morbidity and mortality in the elderly population (Demer and Tintut, 2008). To aid our understanding of the potential geroprotective effects of betaine, we have sought to determine if betaine can protect iVSMCs from vascular calcification and if so, how is this achieved. The level of calcified area per cell count was measured in both control and drug-treated cells in high Ca^{2+} medium for 10 consecutive days, to identify phenotypic and structural changes of iVSMCs over this period of time. We observed that betaine treatment significantly decreased the number of calcified areas by 50% (see Figure 4.2). After 5 days of high Ca^{2+} medium treatment, all cells in control samples were calcified, whereas only half of the betaine-treated iVSMCs remained non-calcified (p-value < 0.01). The fluorescent and brightfield imaging also depicted the elevated CCP deposit in control cells, in contrast to betaine-treated cells. This suggests the promising capacity of betaine in mitigating the development of vascular calcification in iVSMCs *in vitro*, providing a potential platform for further studies that focus more on betaine and vascular calcification.

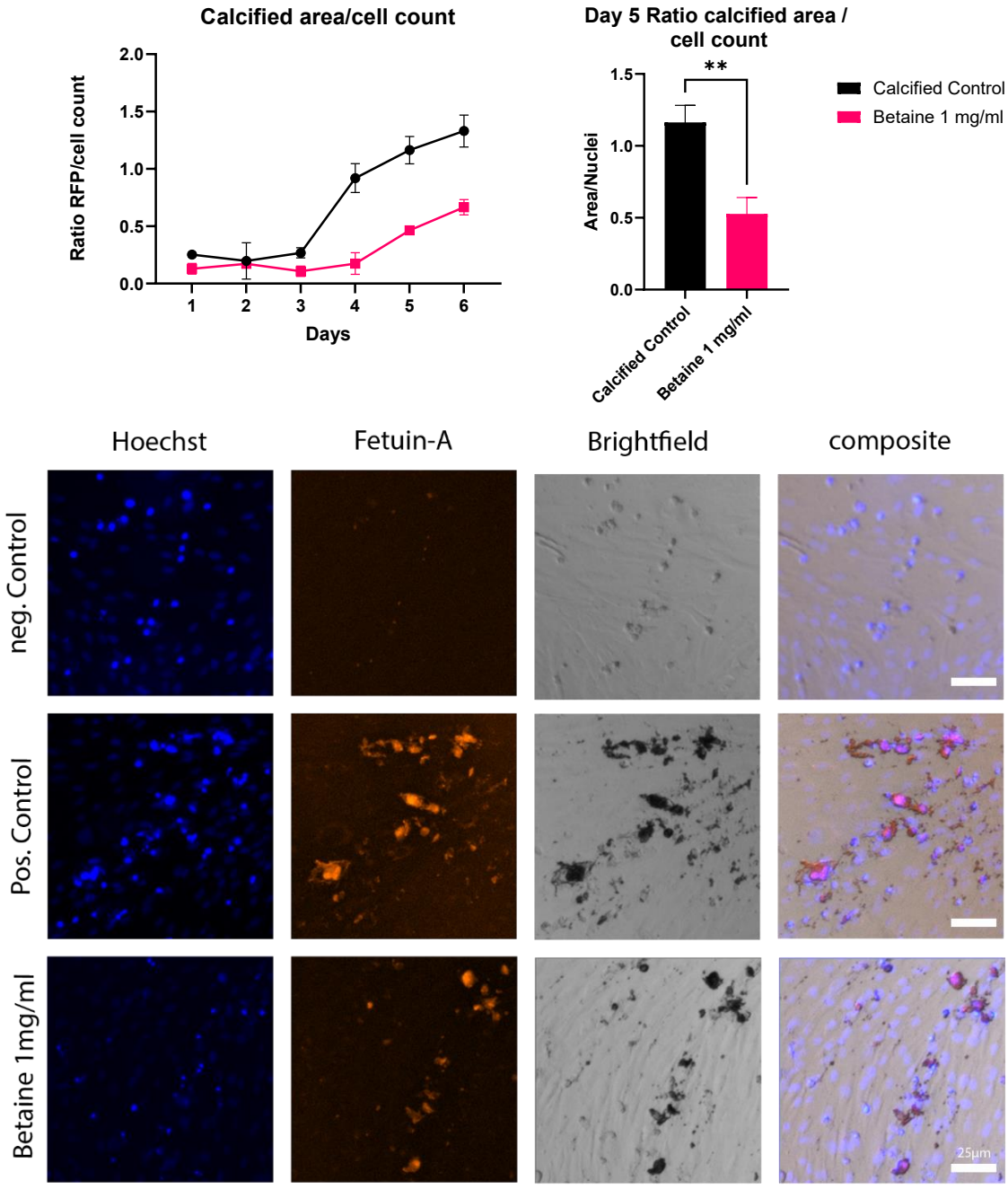


Figure 4.2| Vascular calcification level in iPSCs induced-vascular smooth muscle cells after betaine treatment. *Long term supplement with betaine significantly reduced vascular calcification in iVSMCs. The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

4.4.3 Betaine provides beneficial effects on mitochondria in iPSCs-induced vascular smooth muscle cells

4.4.3.1 Betaine increases total mitochondria content, yet has no significant effects on mitochondrial membrane potential

Mitochondria are essential components for energy production and redox signalling in vascular health. With advanced age, accelerated mitochondria dysfunction is characterized by a gradual decline in mitochondrial density and an increase in mitochondrial depolarization. We have explored if betaine could mitigate the effects of mitochondria dysfunction induced by ageing, by labelling mitochondria with Mitotracker (total mitochondria content) and JC-1 (mitochondria depolarization) dyes. The outcomes revealed a significant increase in total mitochondrial content in iVSMCs after long-term betaine treatment (see Figure 4.3). The mitochondrial density in betaine-treated cells was almost double, compared to control samples (p-value < 0.001). Interestingly, we did not observe changes in the ratio of high potential/low potential mitochondria. This indicates that betaine might preserve mitochondrial density in iVSMCs yet has no significant effect on mitochondria membrane potential (MMP). iVSMCs-supplemented with betaine might obtain higher mitochondria content without considerable alterations in the mitochondria depolarization process.

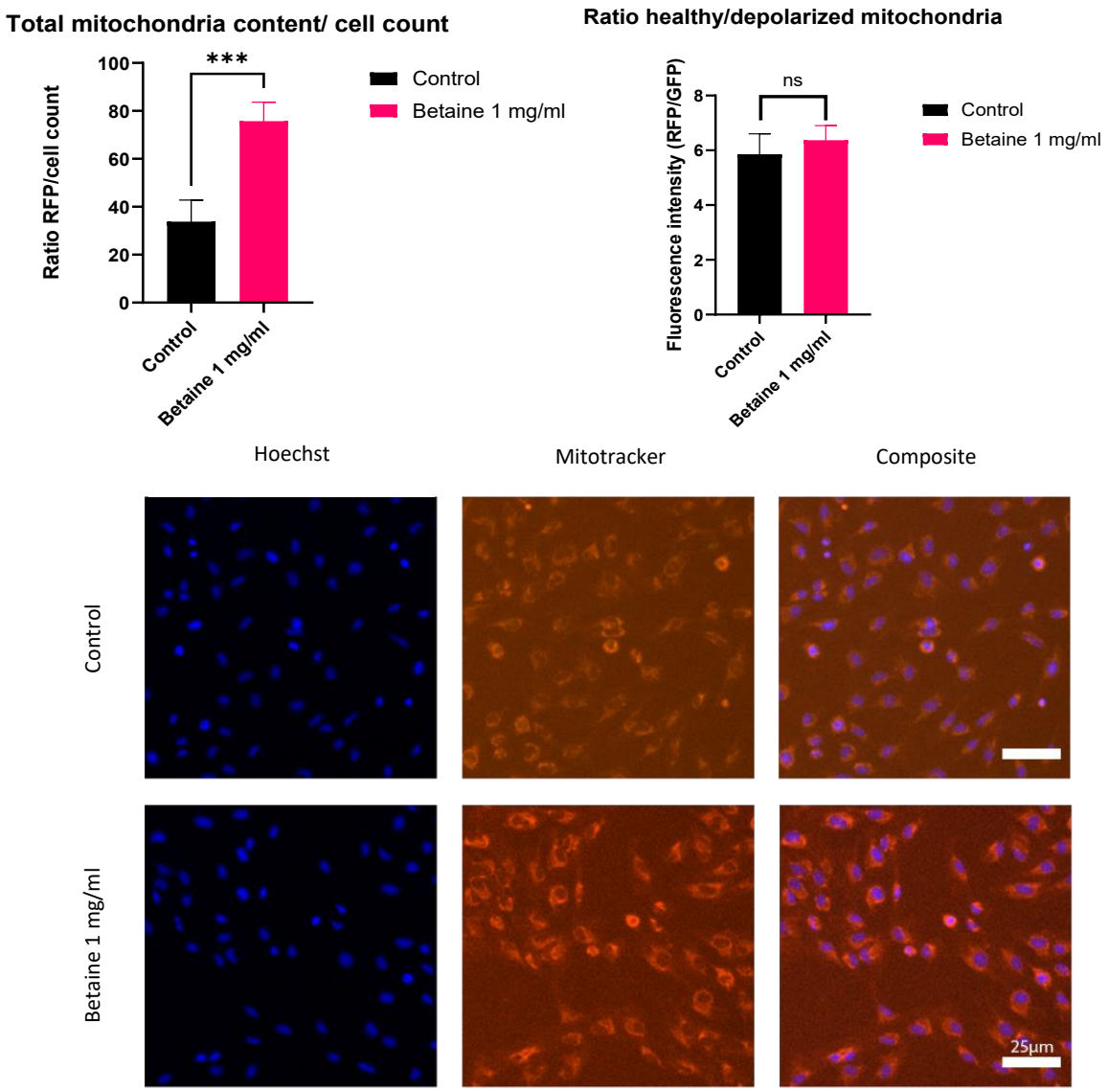


Figure 4.3| Total mitochondria content and mitochondria membrane potential in iPSCs induced-vascular smooth muscle cells after betaine treatment. *Betaine significantly increased total mitochondrial content, yet has no effect on mitochondrial membrane potential in iVSMCs. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).*

4.4.3.2 Betaine protects mitochondria membrane potential from DMSO

The effect of betaine in protecting MMP against DMSO-associated mitochondrial dysfunction was evaluated through a JC-1 membrane potential labelling assay. iVSMCs were also treated with CCCP, a mitochondrial oxidative phosphorylation uncoupler, as a positive control to ensure the sensitivity of JC-1 to changes in membrane potential. DMSO is an organic solvent with a prominent effect on cryoprotection (Marcantonini et al., 2022). However, DMSO has been linked with a variety of harmful impacts on mitochondrial functions by promoting mitochondrial membrane damage, oxidative stress and disrupted MMP (Święciło et al., 2024, Dludla et al., 2021). DMSO-associated mitochondrial dysfunction has been reported in different cell types, such as astrocytes and adipocytes (Yuan et al., 2014, Dludla et al., 2018). This intensive damage to mitochondrial structure and integrity can eventually result in cell death. By adding 0.02% DMSO to iVSMCs, we observed a relatively low fluorescent intensity for the ratio of RFP (high potential mitochondria)/GFP (low potential mitochondria) in control cells (see Figure 4.4). In contrast, iVSMCs treated long-term with betaine possessed a significantly superior level of RFP/GFP, up to 5 times higher than controls, indicating that betaine protects against DMSO-associated mitochondrial dysfunction. Low levels of DMSO have been shown to significantly increase the mitochondrial depolarization process in iPSCs-induced VSMCs. Microscopic imaging has also indicated the adverse effects of DMSO on mitochondrial distribution in iVSMCs. As CCCP and JC-1 are commonly combined to examine MMP, we reported the high sensitivity for JC-1, as CCCP induced a permanent mitochondrial membrane disruptor in both groups (see Figure 4.4).

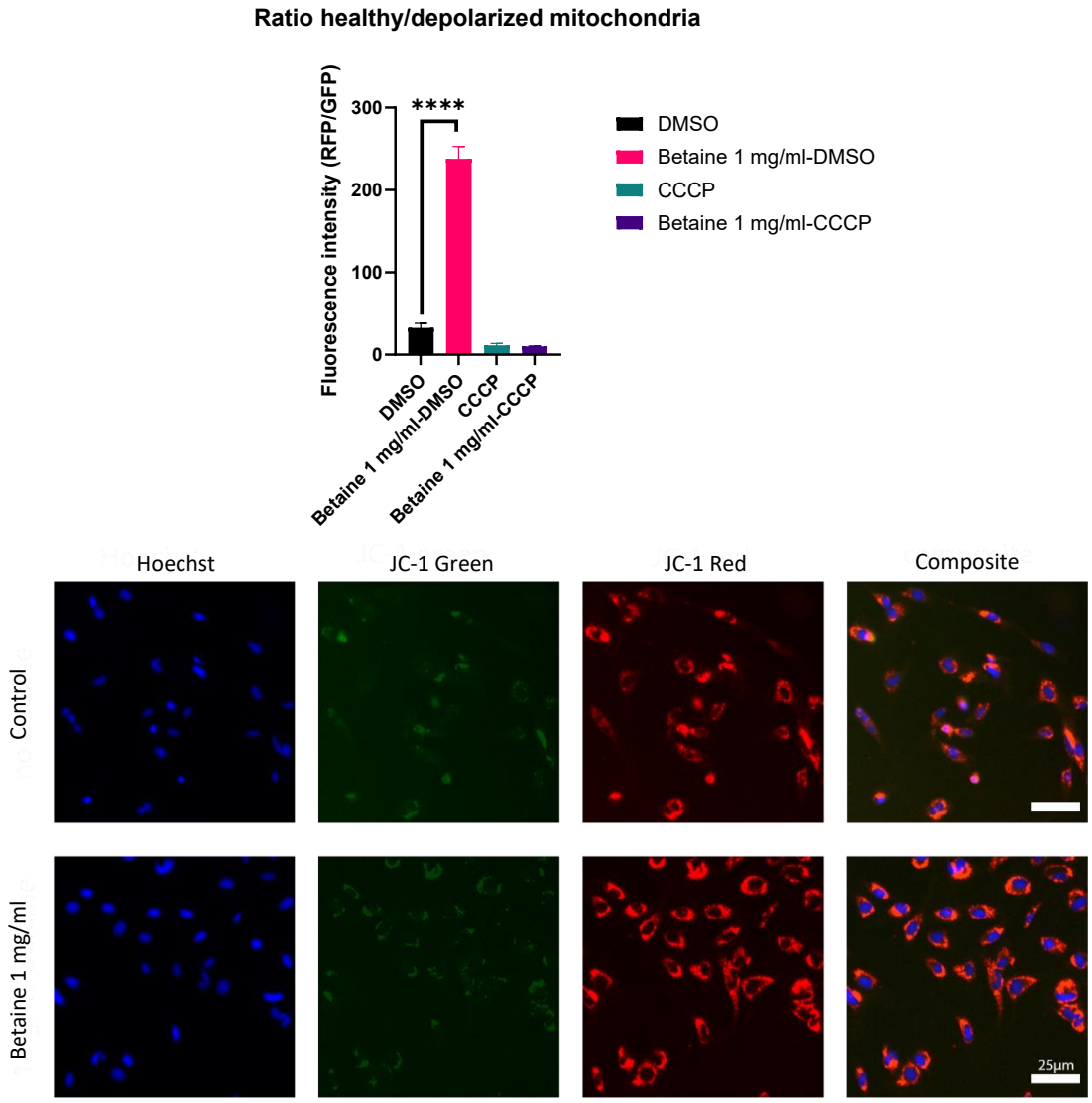


Figure 4.4| Mitochondria membrane potential in iPSCs induced-vascular smooth muscle cells after betaine treatment. *Betaine has provided a considerable protection against DMSO-associated mitochondrial dysfunction in iVSMCs. Drug-treated cells have showed a significantly higher level of RFP (high potential)/ GFP (low potential) in mitochondria, compared to the control cells. Fluorescent images also demonstrated the adverse effects of DMSO on the mitochondria depolarization process. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).*

4.4.3.3 Betaine does not protect mitochondria membrane potential against IL6-induced inflammation

We continued to explore the cytoprotective properties of betaine to mediate SASP-associated inflammation in this iVSMCs model, incorporating a JC1 labelling assay *in vitro*. The changes in JC1 fluorescent intensity were recorded in iVSMCs for 7 consecutive days, are shown in Figure 4.5. For this investigation, IL6 – a major component of an acute inflammatory response mediated by the SASP was evaluated for the effect of senescence associated inflammatory processes on the MMP (Herbstein et al., 2024). Autocrine and paracrine senescence associated process have been widely investigated, for their ability to engender adverse bystander effects on neighbouring cells (Kuilman et al., 2008, Sapochnik et al., 2016). By supplementing normal culture media with 50 ng/ml IL6, we sought to identify the potential effects of IL6 on MMP, and whether betaine can provide sufficient protection on the mitochondria polarization process. Throughout the seven days of the experiment, the RFP /GFP ratio of both groups, reflecting the changes in MMP activity, reached a peak by the second day (see Figure 4.5). This might be explained by the robust nature of iVSMCs, allowing early cell growth while maintaining mitochondrial function during the first day after being exposed to IL6-containing medium. Despite fluorescent intensity gradually declined over the next few days of the experiment, there were no significant changes in MMP in both the control and treatment groups. The total area of RFP (high potential mitochondria) over cell counts in both groups gradually enhanced in five days with a substantial decline from the sixth day. Overall, betaine showed no significant effects on the MMP against IL6-induced inflammation.

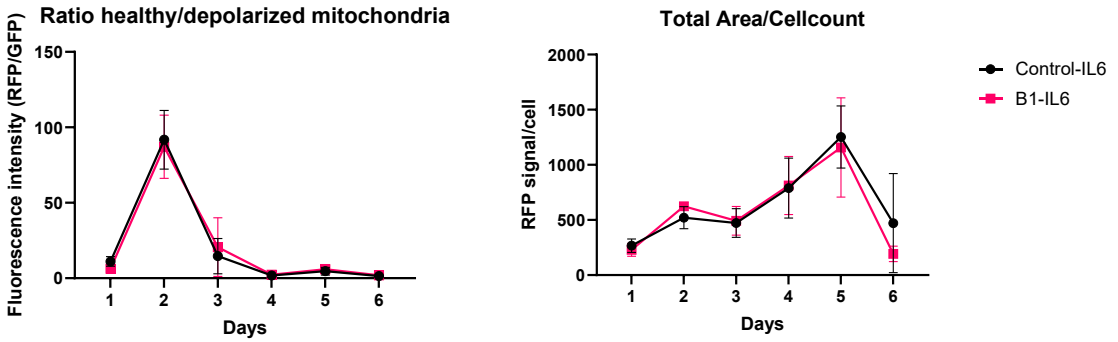


Figure 4.5| Mitochondria membrane potential in iPSCs induced-vascular smooth muscle cells after betaine treatment in IL6-induced inflammation medium. *Betaine exerted no significant effects on the mitochondrial depolarization process in iVSMCs in IL6 containing medium. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).*

4.4.4 Betaine mitigates the effects of extracellular vesicles during ageing

To determine how EVs in iVSMCs were affected by long-term betaine treatment, we employed a particle tracking analyser to measure total EV content. By normalizing the total EV count to total protein in each sample, we then sought to investigate whether betaine could mitigate the production of age-related EV, with a view to determining to what extent observed changes in the quantity and particle size of EVs after the treatment. During ageing, EVs produced by aged VSMCs are characterized by increased inflammatory proteins and pro-senescence markers (Manni et al., 2023). Our data clearly indicated that betaine-treated cells released fewer EVs, compared to the control group (p-value <0.05) (see Figure 4.6). To further confirm the biological properties of these EVs, we performed the particle size assessment in our samples from both groups. Interestingly, there was a considerable variance between EV sizes in the two groups. Control cells, in contrast to betaine-treated cells, generated smaller EVs size varying from 125 to 136 nm.

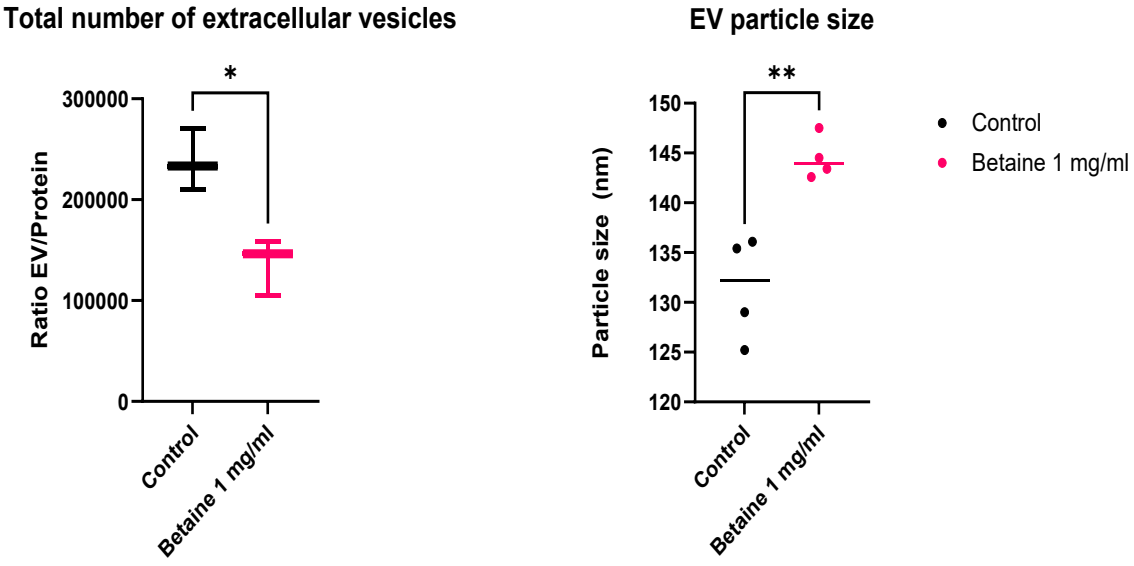


Figure 4.6| Extracellular vesicles in iPSCs induced-vascular smooth muscle cells after betaine treatment. *Long term treatment with betaine has significantly reduced the total number of extracellular vesicles (EV) released by iVSMCs. The extracellular vesicle particle size was also different between two groups. Betaine-treated iVSMCs produced extracellular vesicles with greater size ranging from 142 – 148 nm, compared to the control group (125 – 136 nm). The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

EVs derived from the drug-treated group were greater in size, between 142 and 148 nm. Previous studies on EVs have revealed that small EVs are potential mediators of systemic ageing (McIlvenna and Whitham, 2023). Following the treatment with small EVs from senescent cells in healthy cells, there was a decline in cell proliferation rate (BrdU assay), elevated levels of cell cycle inhibitors (CDKN1A, CDKN2A) or DNA damage assay (CCF) (Borghesan et al., 2019). As a result, our outcomes have provided concrete evidence of the important role of betaine supplementation in mitigating the secretion of ageing-associated EVs.

4.4.5 Betaine mitigates the effects of ROS-induced oxidative stress during ageing

We have previously reported that oxidative stress is a key characteristic of accelerated ageing and disease, correlated with diminished detectable betaine levels (Ebert et al., 2022b, Craven et al., 2021b). We, therefore, used DCFDA - Cellular ROS Assay (see Figure 4.7) to determine if supplementing iVSMCs with betaine could mitigate the effects of ROS-induced oxidative stress during ageing. Hydrogen peroxide (H_2O_2) with a range of numerous concentrations (25 µM, 100 µM, 200 µM and 500 µM) was utilized as a major source of ROS to trigger the potential oxidative damage in iVSMCs. As expected, the levels of ROS produced by iVSMCs showed a significant positive correlation with increased concentration of H_2O_2. Notably, betaine treatment significantly decreased the production of ROS in iVSMCs treated with H_2O_2 at lower concentrations (25 µM and 100 µM (p-value < 0.05)). This beneficial impact of betaine was observed over the entire course of the experiment. However, we did not observe any significant alteration in ROS levels after betaine treatment at higher dose of H_2O_2 (200 µM and 500 µM). These two extreme doses are potentially lethal with the ability to promote cellular senescence in VSMCs. Our data showed that the effects of betaine on protecting iVSMCs against ROS-induced oxidative damage might vary, depending on the changes in H_2O_2 concentration.

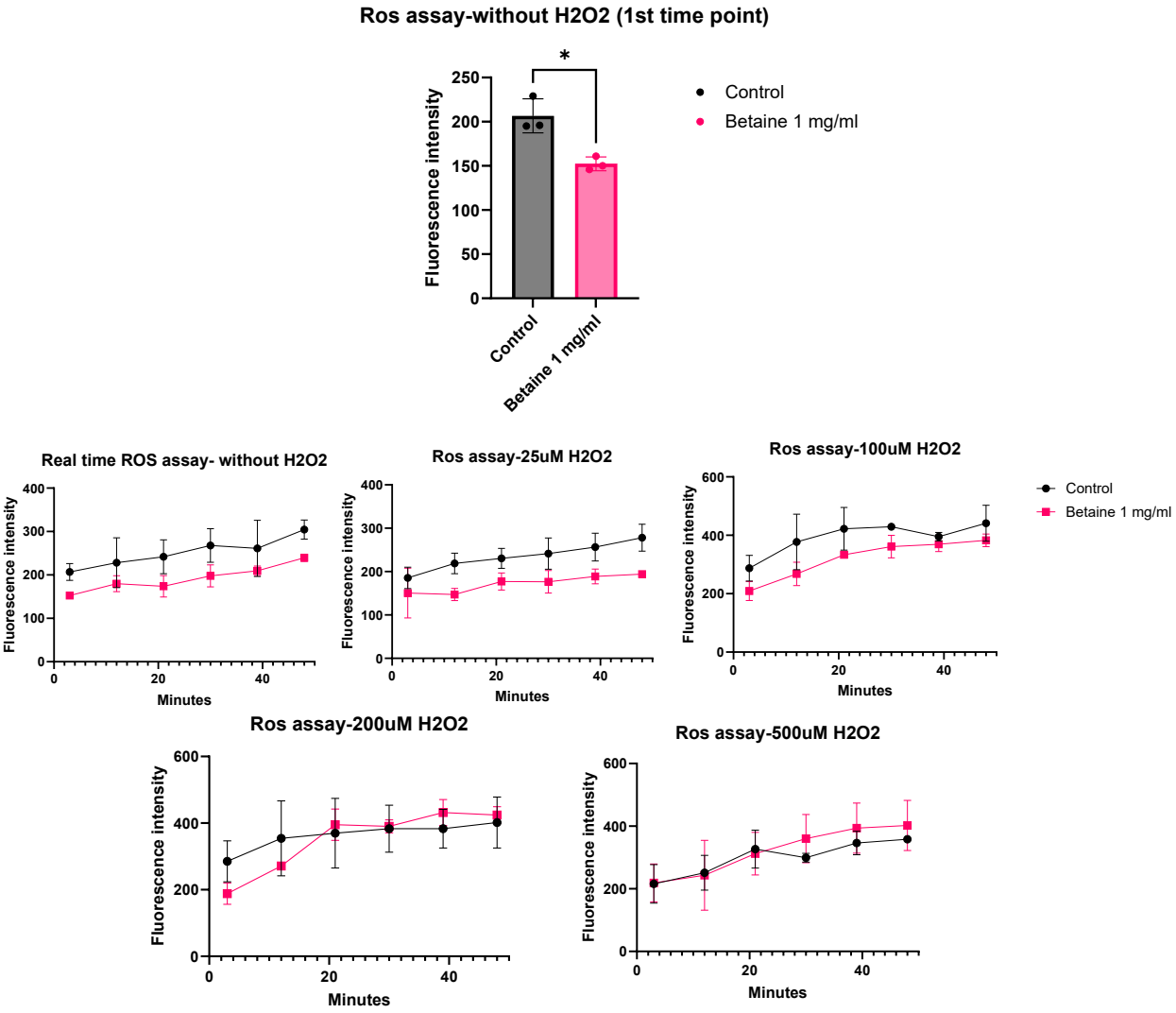


Figure 4.7| ROS-induced oxidative stress in iPSCs induced-vascular smooth muscle cells after betaine treatment. *Long-term treatment with betaine has various effects on ROS-associated oxidative damage in iVSMCs, depending on the concentration of H_2O_2. At lower concentrations of H_2O_2 such as 25 µM and 100 µM, betaine has efficiently reduced the levels of ROS released by iVSMCs. However, there was no significant change in ROS production in response to higher dosage of H_2O_2 including 200 µM and 500 µM. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).*

4.5 Discussion

In this chapter, we have demonstrated the potential role of betaine in mitigating the effects of vascular ageing in iPSCs-induced VSMCs. The beneficial effects of betaine were identified using various well-established features of vascular ageing, including vascular calcification, measurement of total mitochondrial content and membrane potential, IL6-induced inflammation, changes in extracellular vesicle production and ROS-induced oxidative stress. Previous studies have demonstrated a significant correlation between these features with accelerated biological age (Schlieper et al., 2016, Li et al., 2022b, Shiels and Ritzau-Reid, 2015, Panagiotou et al., 2018, Ebert et al., 2022b). Our data indicated a reduction in vascular calcification, a change in extracellular vesicle size, characteristics and decreased ROS-associated-oxidative damage at sub lethal levels of oxidative challenge by H_2O_2. Betaine-treated iVSMCs showed upregulation of total mitochondria content, yet no significant impact was observed on mitochondria membrane potential. Even though betaine has been recorded as having trivial effects on iVSMCs in IL6-related inflammation, its cytoprotective effects have been confirmed in oxidative damage caused by DMSO and H_2O_2 challenge.

Vascular calcification, recognized as a hallmark of vascular ageing, is directly linked to the increased risk of morbidity and mortality in the older population (Hobson et al., 2023). This deterioration of vascular structure and function has been reported across the diseasome of ageing, including CKD, CVD or type 2 diabetes (Dai et al., 2020b, Rennenberg et al., 2009, Harper et al., 2016). However, there is currently limited research focusing on the underlying metabolomic pathways of calcified vasculature. A recent study conducted on a patient with chronic ischemic colitis has shown a significant reduction in betaine expression as a consequence of severe venous calcification (Deng et al.). Even though the potential correlation between betaine deficiency and pathogenic venous calcification has not been confirmed, it is biologically plausible that betaine plays an essential role in protecting vascular health against calcification. Our data indicates that betaine is an effective naturally derived bioactive compound that can mitigate the effects of vascular calcification. The level of iVSMCs calcification was diminished up to 50% following betaine treatment. Betaine is known for its antioxidant properties together with reducing plasma homocysteine levels (Arumugam et al., 2021). As vascular calcification is characterized by the excessive accumulation of inflammatory biomarkers and plasma homocysteine, this finding is thus consistent with previous studies regarding the

protective role of betaine against vascular calcification (Demer and Tintut, 2014, Jung et al., 2022).

Our data revealed that betaine significantly upregulated mitochondria content, with approximately a 2-fold increase compared to control cells. This cytoprotective effect on mitochondria functions has confirmed our initial hypothesis that betaine can mitigate mitochondria dysfunction in vascular ageing. Notably, we proposed that betaine provides exercise mimetic effects, illustrated by the improvements in mitochondria content without changing mtDNA-CN or MMP. Mitochondrial dysfunction plays a substantial role in promoting age-related vascular damage (Witkamp and van Norren, 2018). Changes in mitochondrial functions can be reflected in total mitochondrial content and MMP (Miettinen and Björklund, 2016, Kuznetsov and Margreiter, 2009). With advanced age, mitochondrial function and MMP in different cell types (i.e. endothelial cells, skeletal cells) are found to reduce progressively (Miller et al., 2012, Sugrue and Tatton, 2001, Gouspillou et al., 2014b, Tracy et al., 2021, Seo et al., 2016). This phenomenon has been reported in various *in vivo* in humans, rodents, primates and *C. elegans* (Pandya et al., 2015, Duicu et al., 2013, Gouspillou et al., 2014a, Rottenberg, 2023). In particular, the positive effects of exercise and endurance training on mitochondria content have been extensively studied (Granata et al., 2018, Hoppeler et al., 1985, Montero and Lundby, 2017, Meinild Lundby et al., 2018). Young healthy participants, recruited in a 6–8-week training intervention, demonstrated 1.1 to 1.6-fold increased mitochondria content (a.k.a. mitochondrial density volume) following the experimental period. This leads to the increased activation of the kinase signalling pathway, followed by 25-35% increases in mitochondria content after 6-7 training sessions (Gollnick et al., 1974, MacInnis and Gibala, 2017). In addition, studies in different models (i.e. humans, mice, *C. elegans*) have proven no significant correlation between exercise and mtDNA-CN (Yang et al., 2022, Maclaine et al., 2020, Hartman et al., 2018). Notably, the direct relationship between mtDNA-CN and MMP has been discussed, reflecting variations in mtDNA-CN resulting in relevant changes in MMP levels, oxidative damage and energy reserves (Guha and Avadhani, 2013). For instance, previous evidence indicates that a reduction in mtDNA-CN results in the diminishing of MMP in yeast, which negatively interferes with the proliferation rate due to genomic instability (Veatch et al., 2009). According to our data, we did not observe any changes in mtDNA-CN in primary VSMCs, which makes it biologically plausible that no significant alteration has occurred in MMP. Our finding aligned with recent studies which have also utilized betaine as a potential treatment to

enhance mitochondrial functions and cellular survival (Jung Kim, 2018b, Zhang et al., 2019, Chen et al., 2024b).

Interestingly, there was no substantial alteration in MMP in drug-treated iVSMCs, both in normal and IL6-induced inflammation medium. We previously observed in a general population cohort that well-known ageing biomarkers (i.e. SASP) only account for less than 10% of the ageing-associated inflammation burden (Shiels et al., 2011, McGuinness et al., 2012a). A much greater proportion of the inflammatory process derives from pro-inflammatory microbial metabolites (i.e.TMA). Therefore, it is possible that betaine-induced mitochondria protection is more significant in the microbial inflammatory process, compared to IL6-induced inflammation. In addition, the effects of IL6 on MMP might be distinct among cell types. In CD4+ immune cells, IL6 has been reported to facilitate the formation of mitochondrial respiratory chain supercomplexes, which can sustain a high MMP (Yang et al., 2015). Another study also pointed out the cardio-protection effects of IL6 by inhibiting abnormal mitochondria depolarization process (Smart et al., 2006).

As such, the cytoprotective effect of betaine on the MMP of iVSMCs was observed when DMSO was included in the culture medium. DMSO is responsible for mitochondria dysfunction and apoptosis due to the accumulation of oxidative damage (Yuan et al., 2014). By exerting significant protective effects on DMSO-associated oxidative damage, betaine can effectively regulate MMP in iVSMCs. The positive impact of betaine on MMP has been recorded in multiple cell types *in vitro* (Lee, 2015a, Jung Kim, 2018b). Despite our relatively low dose of DMSO (0.02%) on iVSMCs, it has been shown that iPSCs-derived VSMCs, as well as other iPSCs cells, are highly sensitive to DMSO. This is pertinent to drastic changes in phenotypic, transcriptomic, methylomic and proteomic landscapes of iPSCs cells following exposure to 0.1% DMSO (Verheijen et al., 2019). In iVSMCs, we have observed the deleterious impact of 0.02% DMSO, whereas betaine-treated cells remained to have a substantially high RFP signal (~ 6-fold higher than control). This indicates the potent protective effects of betaine on MMP against DMSO-associated oxidative damage and mitochondria dysfunction.

Extracellular vesicles are gaining more attention as a novel biomarker for vascular ageing (Manni et al., 2023, Mas-Bargues and Alique, 2023). The potential link between extracellular vesicles, vascular calcification and premature vascular damage has been proposed, emphasizing the upregulation of phenotypic switching of VSMCs during the

ageing process (Schurgers et al., 2018). According to our observations, there is a remarkable variation between the EV's size in control and betaine-treated cells. While control iVSMCs released EVs with relatively smaller sizes (125-136 nm), we observed larger-size EVs (142-148 nm) secreted in betaine-treated samples. Indeed, there are currently no studies which explored whether distinct sizes of EVs play a role in vascular ageing. Even though we observed the differences in EV sizes, they are still classified as microvesicles (Alique et al., 2017). We seek to utilize different isolation methods to preferentially collect specific size ranges of EVs to analyse their composition. This will elucidate the role of EV sizes and their contribution to vascular ageing. In our experiment, we also reported that betaine significantly reduced EVs secretion in iVSMCs. The mechanism underlying this effect can be due to the potential correlation between 1C metabolism and EVs production. In a study of microalgae ageing, researchers have identified the increased secretion of EVs as a damage removal mechanism in older cells (Deng et al., 2024). Up to 30% of aged cells released EVs, which contain ROS and other detrimental components, to rejuvenate from the debris of cellular damage. As aged cells were supplemented with betaine, the proliferation rate of microalgae was restored with substantially lower EV production. In addition, the antioxidant properties of betaine have also been confirmed in our experiments on iVSMCs with different concentrations of H_2O_2. Other *in vitro* and *in vivo* studies have also proposed similar roles of betaine in preventing ROS-associated oxidative damage while improving cellular viability (Alirezaei et al., 2015, Kim et al., 2014, Li et al., 2022a). Overall, our findings are relevant to studies of interventions across the diseasome of ageing. Betaine has a diverse range of therapeutic impacts *in vitro* which can be applied to other age-related pathologies. These promising results have encouraged us to expand our research further to *in vivo* models of ageing. In particular, we examined different concentrations of betaine in *C. elegans* and *D. melanogaster* which will be presented in the following chapter.

Chapter 5: Effects of betaine on different *in vivo* models (*D. melanogaster and C. elegans*)

5.1 Introduction

Multiple *in vivo* models have been utilized in research on ageing, allowing the exploration of physiological and structural alterations during both normative and dysregulated ageing processes (Mitchell et al., 2015). Among them, fruit flies (*D. melanogaster*) and nematode worms (*C. elegans*) are two key models to study the complexity of this naturally occurring process (Mack et al., 2018, Piper and Partridge, 2018). These two models are relatively short-lived, lasting only around 70-90 days for *D. melanogaster* and 18-20 days for *C. elegans* (Koliada et al., 2020, Zhang et al., 2020). These model systems are thus ideal to exploit for studying ageing, or the diseasome of ageing, due to their quick generation, turnover and capacity for rapid data generation. *D. melanogaster* and *C. elegans* also share significant genetic homology with humans. Notably, approximately 60 – 80% of *D. melanogaster* and *C. elegans* genes have homologs in humans (Igboin et al., 2012, Lai et al., 2000, Consortium, 1998). Both *in vivo* models are small, inexpensive and easily maintained in laboratory settings. By using various molecular and genetic modifying techniques, such as transgenics and gene knockouts, scientists have been able to adjust the genome profiles to suit specific requirements for individual studies.

During ageing, *D. melanogaster* demonstrates a significant deterioration in physiology and cellular functions (Piper and Partridge, 2018). The degree of population survival over the life course indicates the progression of ageing under the influence of internal and external factors (i.e. poor genetic profiles, temperature and diet) (Pearl and Parker, 1921). Important biomarkers of physiological decline in *D. melanogaster* have been identified, focusing on changes in metabolism, behaviour, fertility rate and physical activity. For instance, aged flies often experience reduced protein and fat synthesis, feeding levels, flying, walking, egg laying and impaired negative geotaxis (Piper and Partridge, 2018). These 'ageing' features of *D. melanogaster* can assist in examining potential lifespan-modifying interventions, healthspan or exposome effects on ageing. In *D. melanogaster*, oxidative damage and cellular senescence are major drivers of ageing (Ito and Igaki, 2016, Landis and Tower, 2005). Several studies have emphasized the correlation between excessive SASP accumulation, mitochondria dysfunction and the dysregulation of the ageing process (Ito and Igaki, 2016, Wang et al., 2003).

C. elegans is another powerful model for studying cellular and molecular pathways of the ageing process (Tissenbaum, 2015). It is characterized by a small and transparent appearance, with moderately simple anatomy. *C. elegans* can produce offspring either by self-fertilization or mating with the opposite sex (Corsi et al., 2015). Due to its short lifespan (2-3 weeks), even trivial effects on the longevity of *C. elegans* can be elucidated. A range of genetic modifications can be easily performed on *C. elegans* such as genetic mutations, gene knockout or transgenesis. The dysregulation of the ageing process can be observed at the morphological, functional and behavioural levels. For instance, aged *C. elegans* have progressively thicker cuticles, and thinner hypodermal cylinders together with the disorganized muscle sarcomeres (Herndon et al., 2017). Multiple behavioural characteristics decline with age, especially locomotion, food-seeking ability and chemotaxis (Hosono, 1978, Glenn et al., 2004). During ageing, it has been proved that *C. elegans* experiences a significantly higher degree of DNA damage, insoluble protein accumulation and mitochondria dysfunction (Klass et al., 1983, David et al., 2010, Gaffney et al., 2018). Even though cellular senescence is one of the major hallmarks of the ageing process, its role in *C. elegans* remains unclear.

In this chapter, we sought to explore the potential effects of betaine on healthspan and longevity on two key *in vivo* models of ageing – *D. melanogaster* and *C. elegans*. To do so, multiple behavioural and functional assays have been conducted, ranging from a lifespan, fecundity and climbing assays between betaine-treated and control animals. Betaine was prepared directly in fly food or mixed in the water before treatment. Our outcomes showed that lower doses of betaine can effectively extend the female lifespan in both *D. melanogaster* and *C. elegans*. No significant changes in lifespan were observed in male flies. In addition, we observed the positive effects of betaine on both climbing height and fecundity rate throughout the lifetime of female flies. The changes in climbing height in male D. melanogaster were inconsistent with considerable improvements reported at various time points.

5.2 Materials and methods

Fly stock and husbandry. White Dahomey (w^{Dah}) flies, which were used in the following experiments, were maintained on a 12h:12h light:dark cycle with the controlled temperature at 25°C and 60% humidity. The wildtype of Dahomey (wt^{Dah}) was first found in Dahomey (or Benin) in West Africa in 1970 (Dobson et al., 2023). Large populations of wt^{Dah} stock were kept in large cages with different generations. By backcrossing the w1118 mutation into outbred Dahomey flies, w^{Dah} stock was generated. Parental w^{Dah} flies were crossed in several cages containing juice agar plates and yeast smeared on the side. The w^{Dah} eggs were discarded after 24 hours and new juice agar paste were replaced for the old ones. After 18 hours, eggs were collected and moved to fresh food for 11 days. After that, newly emerged adult flies were transferred to fresh food to allow 24-hours mating. Flies were then separated into single-sex vials for further experiments. Fly food was prepared every two weeks, containing 10% brewer's yeast, 5% sucrose and 1.5% agar. Food was also added with 3% nipagin and 0.3% propionic acid as preservatives while keeping in the cool environment at 4°C.

Addition of betaine or rapamycin to fly food. Three conditions of betaine (10 µg/ml, 100 µg/ml and 1 mg/ml) and rapamycin 200 µM were examined in the following healthspan and lifespan assays of w^{Dah} flies. For all experiments, betaine (Sigma Aldrich, 200 mM stock in distilled water) and rapamycin (LC Laboratories, R-5000, Lot number ASW-149, 50 mM stock in ethanol) were added to the fly food while it was still liquid around 50-60^{o}C. Control food was prepared by adding a similar amount of distilled water without any drug into the separate fly food. The stock solution was mixed well in fly food before being distributed into individual fly vials (approximately 6-7 ml of food per vial). Fly food was cooled down at room temperature overnight before storage at 4°C. During the development process of w^{Dah} flies, they were kept on controlled food without any drug addition. On the experiment day, fly food was allowed to warm up at room temperature for at least 15 minutes before being used.

Lifespan assay in *D. melanogaster*. The lifespan experiments were conducted separately in mated female and male flies. Female and male flies were collected and divided into groups of 15 flies. Each treatment condition contained 10 vials with 15 flies per vial. In all experiments, ten vials were secured together by DrosoFlippers (drosoflipper.com) to allow easy transfer of flies between two vials. Flies were transferred to fresh food every two to three days on Monday, Wednesday and Friday. During the transfer, numbers of

dead and censored flies were documented until no flies remained. Survival curve analysis was calculated using GraphPad Prism 10.3.1.

Egg laying assay. The mated female flies were housed in separate vials at a density of 5 individuals per vial. For each condition, 15 vials of flies were prepared to calculate the number of eggs. Flies were transferred into fresh food on Monday, Thursday and Friday. Food vials were stored every Friday to count the number of eggs on each vial after 24 hours. All the vials were photographed before being processed via ImageJ software.

Climbing (negative geotaxis) analysis. The climbing experiments were conducted separately in mated female and male flies. Two vials of fly food from each treatment (control, betaine 10 μg/ml, 100 μg/ml, 1 μg/ml, rapamycin 200 μM), each containing 15 flies, were placed next to each other in a single DrosoFlipper. The flies followed the same protocol as lifespan assay while being recorded for climbing assays every once a week. By transferring the flies to empty vials on both sides of the DrosoFlipper, an upright column of 20 cm in height was created for each fly set. Flies were then tapped to the bottom and allowed to climb upwards for 15 seconds. After that, the images were taken as a video using an iPhone 15 Pro and still images were taken from the video 15 seconds after the flies were tapped to the bottom of the tube. The conversion between each height in pixels to height in cm was achieved by using manual multi-point selection in Fiji software (Schindelin et al., 2012).

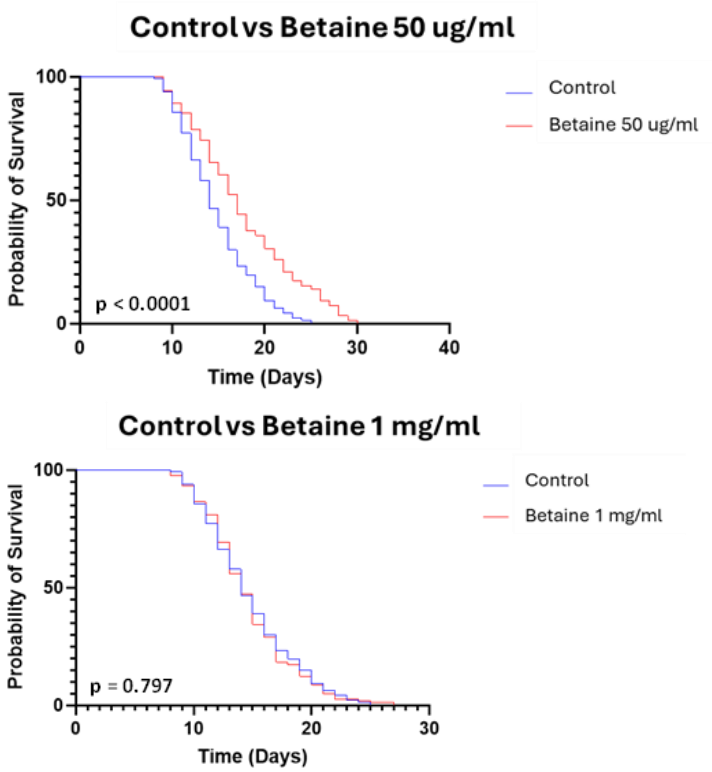
Survival assay in *C. elegans.* This experiment was performed in Prof. Christian Riedel's lab at Karolinska Institute. Wild-type C. elegans (N2) animals were grown according to standard methods (Stiernagle, 2006). First, animals were synchronized by bleaching and then hedging overnight in M9. Resulting L1 larvae were then placed on nematode growth media (NGM) plates seeded with OP-50 E. coli and grown at 20°C until the late L4 stage. Next, animals were transferred to NGM plates containing 5-fluoro-2'-deoxyuridine (FUDR, used to prevent progeny production) and heat-killed OP-50 E. coli (Heat-killing prevents potential degradation of administered drugs by the bacterial metabolism.). Betaine was added to the plates to a final concentration of 50 μg/ml (0.5 mM) and 1 mg/ml (10 mM), and the animals were then grown at 20°C. Animal survival was monitored as previously described (Hamilton et al., 2005).

5.3 Results

5.3.1 Lower doses of betaine increased lifespan of female *C. elegans*

To confirm the translational aspects of our *in vitro* results in *in vivo* models, we undertook a lifespan assay to identify the effects of betaine treatment on *C. elegans*. Two different doses of betaine (50 μg/ml and 1 mg/ml) were given to female worms to examine their potential effect on extending lifespan (see Figure 5.1). These doses are selected based on our previous outcomes from RTCA experiments on VSMCs, in which 50 μg/ml (lower dose) and 1 mg/ml (higher dose) showed the most significant increase in growth rate. Our data confirmed that betaine is a potent mediator of longevity in *C. elegans*, adding up to 21.4% of median lifespan. By using a dose of betaine at 50 μg/ml (0.47mM) we observed a significant increase in mean lifespan in *C. elegans* from 14.76±0.26 to 17.61±0.38 days of mean lifespan (p-value <0.05 and a ~20%

Importantly, the beneficial effect of betaine on longevity appeared to be dose dependent. At a higher dose of betaine (1 mg/ml; 8.5mM)) on *C. elegans*, we did not detect any improvement in lifespan. This data allows us to explore the role of betaine further in other *in vivo* models, such as fruit flies or rodents.



Treatment	N	Median Lifespan	Mean lifespan	Range	Shortest-lived 10%	Longest-lived 10%
Control	228	14	14.76±0.26	8-25	9.30±0.04	22.30±0.10
Betaine 50 µg/ml	224	17	17.61±0.38	9-30	9.40±0.03	28.14±0.07
Betaine 1 mg/ml	163	14	14.64±0.30	8-27	9.06±0.06	22.31±0.17

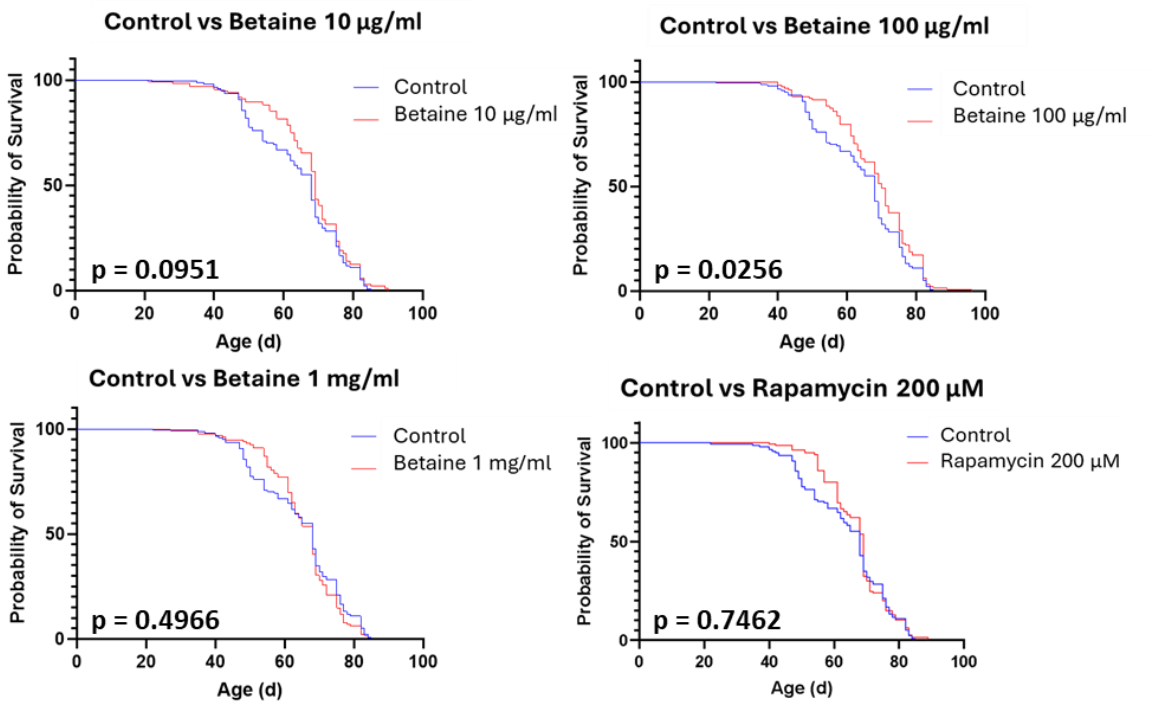
Figure 5.1| Lifespan assay of female *C. elegans* after betaine treatment. *Betaine has dose-dependent impact on C. elegans lifespan. A lower dose of betaine (50* µg/ml*) significantly increased the lifespan of C. elegans by ~ 20%. However, a similar effect was not observed with betaine used at 1 mg/ml. There was no significant difference between C. elegans treated with control and betaine 1 mg/ml. The differences are calculated by using one-way analysis of variance (ANOVA). The lifespan graph was produced based on the data measured in Prof. Christian Riedel's lab at Karolinska Institute.*

5.3.2 Lower dose of betaine increased lifespan of female *D. melanogaster*

We sought to determine whether betaine could extend lifespan in *D. melanogaster*, and if so, which dose provided the most impact on the longevity. Thus, the lifespan of *D. melanogaster* (n=150 female flies from w^{Dah} stock) was assessed for a range of betaine doses (10 µg/ml, 100 µg/ml and 1 mg/ml), rapamycin 200 µM and controls (see Figure 5.1).

D. melanogaster treated with lower doses of betaine (10 µg/ml, 100 µg/ml) displayed positive changes in overall lifespan. Betaine 10 µg/ml increased mean lifespan to 67.43±1.04 days, compared to 64.23±1.10 days for controls. Median lifespan was 69 days and maximum lifespan 95 days compared to control flies which only reached 68 days median lifespan and 90 days maximum lifespan (table/Fig 5.1). Betaine given at 100 µg/ml, substantially increased the lifespan of w^{Dah} flies (p-value = 0.008), with 71 days median lifespan and 68.33±1.01 days in mean lifespan. Interestingly, the last fly from this group survived for 96 days – 6 days longer than the expected lifespan of *D. melanogaster*. The total improvement in lifespan in *D. melanogaster* was ~ 20%, compared to control flies.

We did not see any significant changes in fly longevity after treatment with 1 mg/ml betaine or rapamycin positive control used in female flies



Treatment	Sample size	Median Lifespan	Mean lifespan	Range	Shortest-lived 10%	Longest-lived 10%
Control	138	68	64.23±1.10	22-85	41.36±1.84	82.78±0.26
Betaine 10 µg/ml	137	69	67.43±1.04	21-90	40.57±2.33	83.78±0.70
Betaine 100 µg/ml	128	71	68.33±1.01	40-96	45.31±1.30	84.31±1.07
Betaine 1 mg/ml	133	68	65.05±0.91	27-85	43.15±1.96	80.77±0.75
Rapamycin 200 µM	148	69	66.78±0.74	40-89	50.67±1.34	81.33±0.82

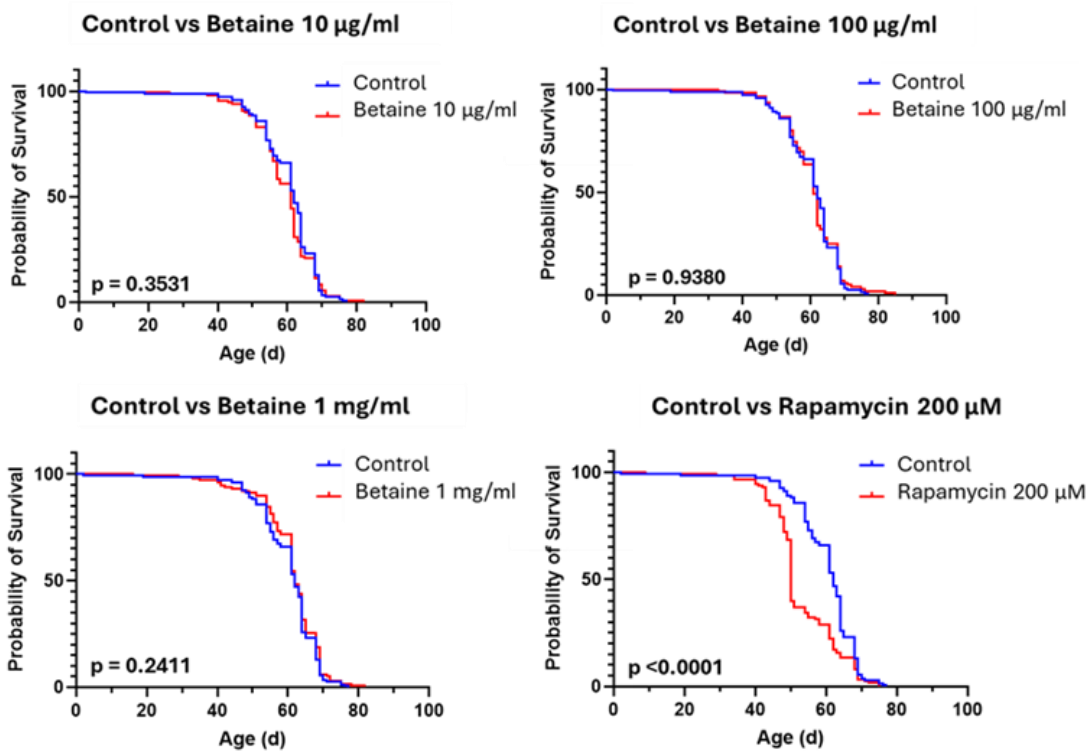
Figure 5.2| Lifespan assay of female *D. melanogaster* after betaine or rapamycin treatment. *The effects of betaine on the female fly's lifespan were dose dependent. Lower dose of betaine (*100 µg/ml*) significantly improved the lifespan of D. melanogaster by up to 20%. However, there were no significant changes in fly lifespan after long-term treatment with 1 mg/ml betaine or rapamycin. The analyses were performed by using Kaplan-Meier curves with statistical analysis by log-rank (Mantel-Cox) test.*

5.3.3 Betaine does not increase lifespan of male *D. melanogaster*

The effect of betaine supplementation on male w^{Dah} flies was then evaluated through an expanded lifespan assay using a similar experimental setting as for female *D. melanogaster*. No significant changes were observed in male lifespan between control and betaine-treated groups (p-value > 0.05) (see Figure 5.3). The median lifespan of male w^{Dah} flies ranges from 61-62 days, and the longest-lived fly was recorded in betaine 100 µg/ml with 85 days.

Betaine 10 µg/ml had no significant impact on mean lifespan with 58.7±0.9 days (61 days in median lifespan), compared to 60.1±0.77 days (62 days in median lifespan) for controls (p-value=0.3531). Maximum lifespan was 82 days compared to control flies which reached 77 days (table/Fig 5.3). Betaine given at 100 µg/ml, registered with 61 days median lifespan and 59.7±1.0 days in mean lifespan. The lifespan of w^{Dah} flies treated with betaine 1 mg/ml was similar with 60.5±0.8 days in mean lifespan and 62 days in median lifespan.

Interestingly, we observed a significant reduction (up to 20%) in male fly lifespan in our positive control senotherapeutic compound – rapamycin. Flies supplemented with rapamycin (200 µM) registered a considerable decline in median lifespan (50 days) and mean lifespan (52.6±0.81 days), compared to respective untreated control (62 and 60.1±0.77 days; (p-value <0.0001). The longest-lived fly in the rapamycin group reached 75 days, while in the control this was 77 days. These outcomes confirmed the noticeable discrepancies between female and male w^{Dah} flies in response to the same treatment. This requires us to understand more about sex-specific genetic behaviours and traits (i.e. sleep patterns and stress resistance) in flies, to identify an effective therapeutic approach to extend lifespan in *D. melanogaster*.

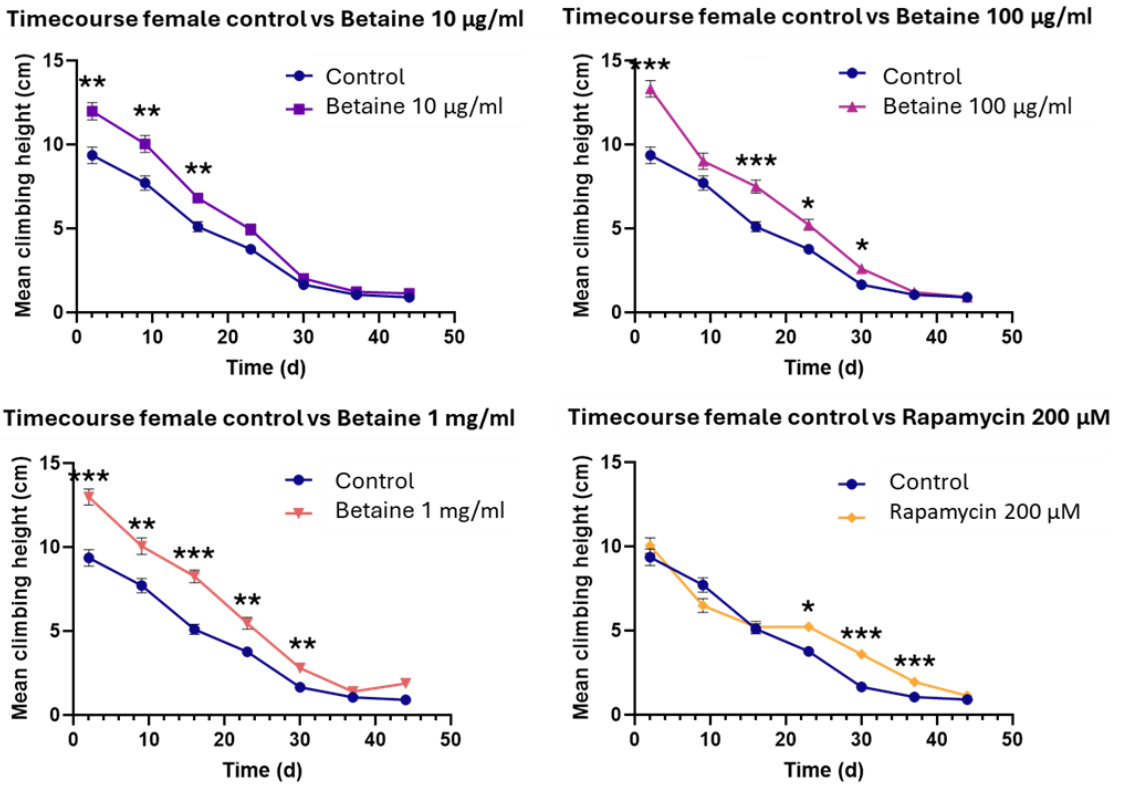


Treatment	Sample size	Median Lifespan	Mean lifespan	Range	Shortest-lived 10%	Longest-lived 10%
Control	147	62	60.1±0.77	2-77	41.2±3.5	71.1±0.79
Betaine 10 µg/ml	133	61	58.7±0.9	2-82	38.2±3.4	72.2±1.0
Betaine 100 µg/ml	104	61	59.7±1.0	1-85	39.4±4.6	73.5±1.9
Betaine 1 mg/ml	140	62	60.5±0.8	16-82	39.6±2.4	71.5±1.1
Rapamycin 200 µM	140	50	52.6±0.81	9-75	36.8±2.4	69.6±0.64

Figure 5.3| Lifespan assay of male *D. melanogaster* after betaine or rapamycin treatment. *There were no significant changes in the lifespan of male flies treated with or without betaine. Interestingly, rapamycin significantly reduces male lifespan by 20% in the median lifespan. The differences are calculated by using Kaplan-Meier curves with statistical analysis by log-rank (Mantel-Cox) test.*

5.3.4 Betaine supplementation effectively enhances negative geotaxis ability of female D. melanogaster

To determine the negative geotaxis ability of female w^{Dah} flies' stock following the treatment, a climbing assay was conducted. Negative geotaxis is an innate escape response in flies where they instinctively move upward against gravity when startled. In their natural environment, this behaviour helps flies escape predators. In research, negative geotaxis analysis is commonly used as a measure of locomotor ability, ageing-related decline in mobility or overall health quality. By measuring the average height climbed by the flies in 15 seconds, we were able to compare the potential changes in control and treated flies. According to our data, betaine increased climbing ability in female flies in a dose-dependent manner. All betaine concentrations demonstrated a beneficial dose-dependent impact on climbing height. All three concentrations of betaine (10 µg/ml, 100 µg/ml, and 1 mg/ml) consistently enhanced climbing performance compared to control across all age groups, with improvements ranging from 14-69% (see Figure 5.4). The highest concentration of betaine showed the strongest effect, particularly in older flies, reaching a 69% improvement at day 30. Lower doses of betaine also improved the climbing height of female flies, yet the duration is shorter with milder effects. Rapamycin, on the other hand, displayed a more age-dependent effect (see Figure 5.4). In young flies (days 2-16), rapamycin had minimal impact on climbing ability, showing performance levels relatively close to control. However, its effect became more pronounced in older flies, with dramatic improvements observed at days 30 and 37, reaching 117.4% and 86.1% of control values respectively. This suggests that while betaine provides consistent enhancement of climbing ability across ages, rapamycin's benefits are more specifically targeted toward maintaining climbing performance in aged flies. In each treatment, the percentage of climbing height changes, in comparison to control height for a specific time point, has been summarized in Figure 5.4.

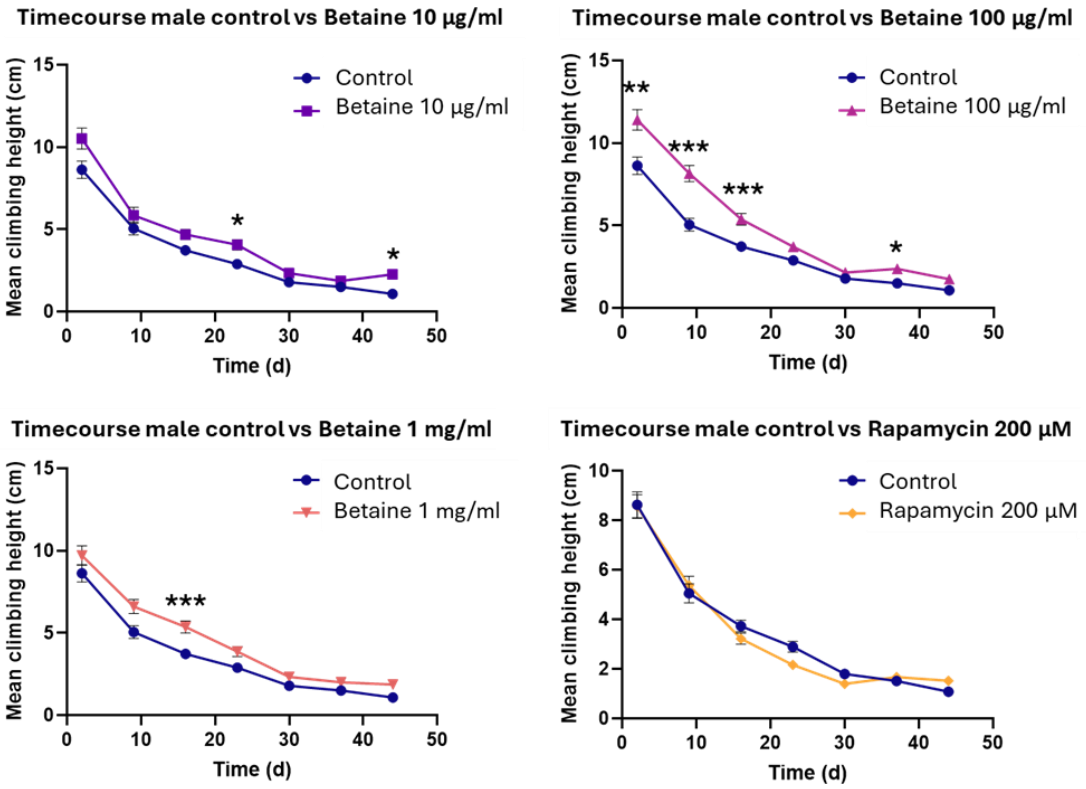


	Changes in climbing height as percentage of control height for a specific timepoint in female flies (% of control)				
Age (d)	**Control**	**Betaine 10 µg/ml**	**Betaine 100 µg/ml**	**Betaine 1 mg/ml**	**Rapamycin 200 µM**
2	100	127.9	142.3	138.6	107.4
9	100	130.9	116.9	130.5	82.7
16	100	133.7	147.3	162.0	102.0
23	100	131.4	138.7	145.1	138.9
30	100	122.1	157.5	169.0	217.4
37	100	116.3	114.3	131.8	186.1
44	100	126.6	101.5	97.2	124.4

Figure 5.4| Climbing assay of female *D. melanogaster* after betaine or rapamycin treatment. *While betaine demonstrated a dose-dependent effect on climbing height, rapamycin specifically displayed a more age-dependent effect in female flies. All three concentrations of betaine have significantly enhanced the climbing performance of a variety of age groups. Rapamycin, on the other hand, showed minimal impacts on younger flies yet profound improvements in older ones. The differences are calculated by using one-way analysis of variance (ANOVA).*

5.3.5 Betaine supplementation exerted inconsistent effects on negative geotaxis ability of male D. melanogaster

We sought to identify the negative geotaxis ability of male w^{Dah} stock, under the long-term supplementation of similar betaine and rapamycin treatment to female *D. melanogaster* (control, betaine 10 μg/ml, 100 μg/ml and 1 mg/ml and rapamycin 200 uM) (see Figure 5.5). In all control and treatment groups, we observed a natural decline in climbing ability with accelerated ageing. The outcomes were not consistent between betaine treatments, with only betaine 100 μg/ml having a positive effect early in the experiment from weeks 1-3. This reached a maximum 61.5% improvement in climbing height at day 9 (p-value <0.05). Notably, this effect disappeared at weeks 4 and 5 but recovered later in week 6. The other two concentrations (10 μg/ml and 1 mg/ml) did not show any significant influence on the climbing ability of male *D. melanogaster* during the first two or three weeks. While flies treated with betaine 10 μg/ml were recorded with increased climbing height after 3 weeks with a 40.9% improvement, the highest dose of betaine induced beneficial effects of 44% improvement 2 weeks following treatment. The final time point of the experiment registered a significant increase in climbing height in flies treated with betaine 10 μg/ml and 1 mg/ml. Notably, rapamycin did not have any effect on the negative geotaxis ability of male flies at any time points measured. Data are summarized in Figure 5.5.

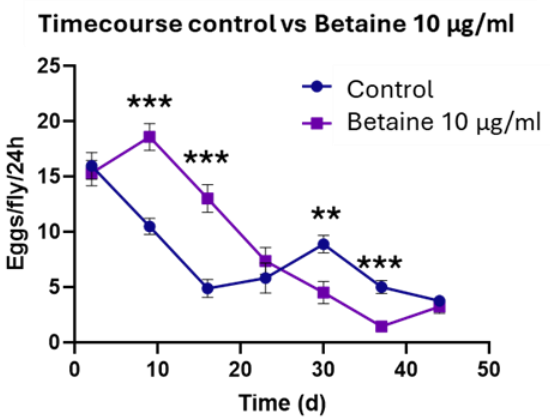


	Changes in climbing height as percentage of control height for a specific timepoint in male flies (% of control)				
Age (d)	**Control**	**Betaine 10 µg/ml**	**Betaine 100 µg/ml**	**Betaine 1 mg/ml**	**Rapamycin 200 µM**
2	100	122.0	132.3	112.5	99.2
9	100	115.9	161.5	130.9	106.4
16	100	125.9	144.3	144.1	86.5
23	100	140.9	128.4	133.9	74.7
30	100	130.5	120.2	129.7	77.8
37	100	123.5	157.8	132.8	110.9
44	100	210.2	162.3	172.6	140.8

Figure 5.5| Climbing assay of male *D. melanogaster* after betaine or rapamycin treatment. *The outcomes of betaine treatment were not consistent in male flies, with only betaine 100 µg/ml exerted significantly positive effects early in the experiment (weeks 1-3). Other concentrations of betaine (10 µg/ml and 1 mg/ml) substantially increased climbing height at a specific time point in the middle (week 2 or 3) and final stage (week 7) of the experiment. Rapamycin has no noticeable effect on the negative geotaxis ability of male flies at any time points measured. The differences are calculated by using a one-way analysis of variance (ANOVA).*

5.3.6 Betaine increases fecundity rate in female flies

To further investigate the potential effects of betaine on the healthspan in Drosophila, we conducted a fecundity assay on female flies. The fecundity assay is a valuable measurement of healthspan due to its role in indicating metabolic health, reproduction system functions and stress response. As the rate of decline in egg number is directly correlated with ageing, novel interventions that can effectively maintain fecundity may lead to enhanced healthspan. The effects of betaine on egg-laying showed markedly distinct patterns in older flies (see Figure 5.6). All betaine treatment initially enhanced fecundity rate, with dramatic increases between days 9-16 (p-value <0.05). The biggest effect was observed at day 16, where betaine treatments increased egg-laying by 67-97% higher than control flies. Following that, the lowest dose of betaine (10 μg/ml) experienced a sudden drop in egg numbers between days 30-37, with up to 70% reduction compared to control flies. As the flies aged over time (from day 23 onwards), there was no significant change in fecundity rate in female flies treated with betaine 100 μg/ml and 1 mg/ml. In contrast, rapamycin treatment dramatically suppressed egg-laying throughout the entire experimental period, reducing it to as low as 3.2-6.3% of control levels between days 9-30. Overall, betaine has demonstrated positive effects on fecundity rate in younger flies but not in older ones. Rapamycin consistently and severely inhibited fecundity compared to both control and betaine treatments.


Timecourse control vs Betaine 10 µg/ml
Control
Betaine 10 µg/ml
Eggs/fly/24h
Time (d)
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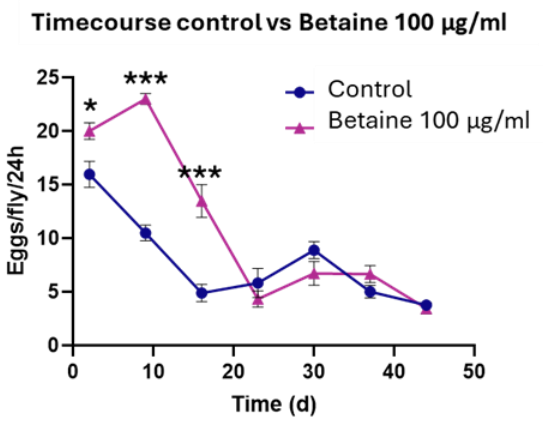
Timecourse control vs Betaine 100 µg/ml
Control
Betaine 100 µg/ml
Eggs/fly/24h
Time (d)
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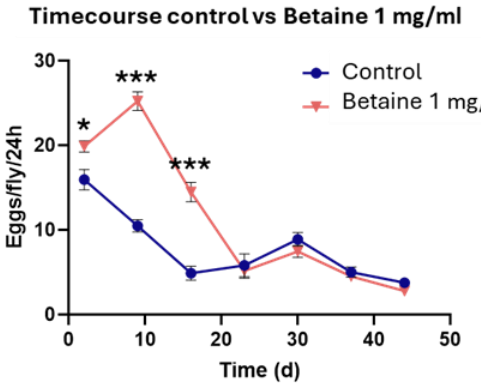
Timecourse control vs Betaine 1 mg/ml
Control
Betaine 1 mg/ml
Eggs/fly/24h
Time (d)
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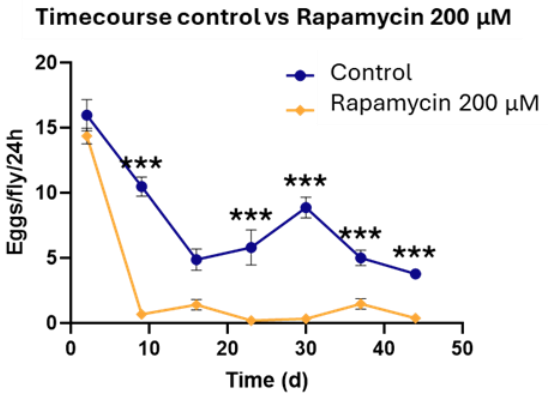
Timecourse control vs Rapamycin 200 µM
Control
Rapamycin 200 µM
Eggs/fly/24h
Time (d)
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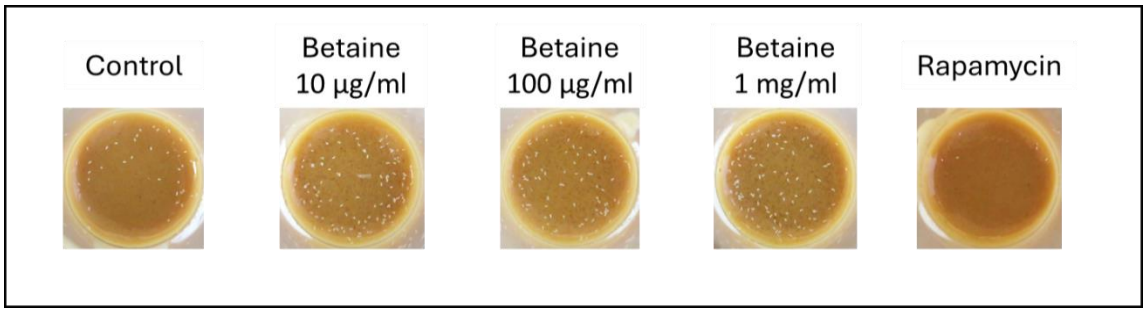
Control
Betaine 10 µg/ml
Betaine 100 µg/ml
Betaine 1 mg/ml
Rapamycin

	Changes in egg numbers as percentage of control eggs for a specific timepoint in female flies (% of control)				
Age (d)	**Control**	**Betaine 10 µg/ml**	**Betaine 100 µg/ml**	**Betaine 1 mg/ml**	**Rapamycin 200 µM**
2	100	95.9	123.1	124.5	90.0
9	100	177.3	214.9	240.5	6.3
16	100	267.2	276.4	296.7	28.7
23	100	126.8	74.3	89.0	3.2
30	100	50.9	75.6	84.1	3.6
37	100	29.0	132.7	89.9	29.4
44	100	85.7	91.3	74.3	9.9

Figure 5.6| Fecundity assay of female *D. melanogaster* after betaine treatment. *All the concentrations significantly improved the egg numbers of young female flies after treatment. From days 23 onwards, older flies did not show any alterations in fecundity rate following betaine 100 µg/ml and 1 mg/ml treatment. Surprisingly, betaine 10 µg/ml substantially reduced egg numbers in the later stage of the experiment. Rapamycin has a consistent detrimental effect on fecundity rate over the life course of female Drosophila. The differences are calculated by using one-way analysis of variance (ANOVA).*

5.4 Discussion

The use of *in vivo* models (*C. elegans* and *D. melanogaster*) has been widely implemented in ageing research due to their short lifespan, genetic conservation and minimal ethical considerations (Zhang et al., 2020, Piper and Partridge, 2018). Despite their simple anatomy and physiology, many fundamental biological pathways related to ageing (i.e. mitochondrial biology, stress response mechanisms and DNA repair pathways) are evolutionarily conserved between these organisms and humans. Previous chapters in this thesis have revealed that betaine supplementation can successfully mitigate a range of aspects of cellular ageing. Therefore, we sought to extend our studies through *in vivo* experiments to validate and reinforce our hypothesis. In this chapter, we assessed a variety of important healthspan and lifespan parameters in *C. elegans* and *D. melanogaster*, including lifespan, climbing and fecundity assays. In keeping with our previous observations, betaine is a potent senotherapeutic compound that can significantly extend the lifespan of both organisms by up to 20%. The effects are sex-specific, as increased longevity was only identified in female individuals. Interestingly, we observed no fecundity-longevity trade-off in betaine-treated flies, which poses concerns about whether it is essential to acquire this trade-off to achieve increasing lifespan. Compared to control groups, female flies treated with betaine showed significant improvements in healthspan with elevated negative geotaxis ability and egg numbers. This finding has also posed a remarkable twist on the potential effects of rapamycin in different *in vivo* models. Depending on the experimental settings and laboratory conditions, rapamycin might not be always effective in extending the lifespan of *D. melanogaster*.

According to published data, betaine demonstrates remarkable potential in enhancing age-related changes in various animal models. In young and old mice treated with betaine, anti-ageing effects on muscle mass, strength and running distance were reported with alleviated mitochondria dysfunction (Chen et al., 2024c). In addition, the protective roles of betaine on mitochondrial function has been highlighted in other body organs, such as liver and vasculature (Lee, 2015b, Heidari et al., 2018). Additionally, a research study in zebrafish has identified that betaine can rescue impaired mitochondrial dynamics by positively regulating mitochondria fusion and fission factors (i.e. MFN2 and DRP1), thus promoting cell viability (Jung Kim, 2018a). Notably, a direct relationship between betaine and longevity has been suggested by a recent study in *C. elegans*. Following the treatment with three anti-ageing drugs, including metformin, quercetin, and minocycline, betaine

levels were consistently higher and comparable to younger worms (Lan et al., 2023a). These findings provide solid evidence that the betaine metabolic pathway plays a key role in senotherapeutic potentials of many drugs. Our promising outcomes on the lifespan of C. *elegans* and *D. melanogaster* following betaine supplementation collectively highlight betaine's therapeutic potential in addressing multiple hallmarks of ageing, from mitochondria dysfunction to cellular senescence and chronic inflammation.

Climbing speed decreases with age in Drosophila, which is similar to age-related decline in walking speed in humans (Rhodenizer et al., 2008). In younger flies, both sexes demonstrate robust negative geotaxis responses with slightly faster climbing speeds in males. With age, climbing height has been proven to be linked with neurodegenerative disease and cognitive deficits (Aggarwal et al., 2019). The locomotor decline is significantly influenced by the deterioration of mitochondrial, muscular, and stress response systems (Hyatt and Powers, 2021). As the flies age, the progressive weakening of sarcomere organization and neuromuscular junctions leads to impaired muscle performance and coordination (Chechenova et al., 2024). Recent studies in aged flies have demonstrated that these pathways are interconnected – mitochondrial dysfunction triggers impaired stress responses that accelerate both mitochondrial and muscular deterioration (Cho et al., 2011). This leads to a detrimental cycle that progressively worsens locomotor function. Our data indicated a major increase in climbing height, with up to 69% improvement in both males and females. Based on our evidence in previous chapters, this can be explained by the positive effects of betaine on mitochondria functions and anti-oxidation capacity that enhance locomotor functions of Drosophila. In addition, betaine has been widely recognized for its role in improving muscle mass, strength and power, allowing better maintenance of negative geotaxis ability in aged fruit flies (Chen et al., 2024c). This is pertinent to betaine's role as a methyl donor; methionine serves as an amino acid for cellular energy production (i.e. creatine, carnitine) and protein turnover (Pekala et al., 2011, Brosnan et al., 2011, Robinson et al., 2016). As betaine provides more methionine, these processes are reinforced to increase muscle growth and buildup strength (Robinson et al., 2016). In addition, betaine has been reported to activate the insulin/IGF1 pathway, growth hormone (GH) and muscle protein kinase B (Akt) while reducing stress-associated cortisol levels (Apicella et al., 2013). In fruit flies, the insulin/IGF1 pathway plays a major role in regulating growth and body size (Weinkove and Leevers, 2000). Our findings, therefore, suggested that betaine is effective in

promoting protein synthesis potentially through the insulin/IGF-1 pathway, thus maintaining the locomotion ability across the life course of *D. melanogaster*.

Fecundity rate varies in Drosophila, depending on numerous contributing factors such as genetics, age, nutrition or environmental conditions (Collins et al., 2023). Notably, the trade-off theory between longevity and fecundity in *D. melanogaster* was proposed by G. C. Williams in 1957 (Williams, 1957). It is displayed that increased longevity negatively correlates with early fecundity (Flatt, 2011, Maklakov and Immler, 2016). This is due to the limited resources favouring reproduction and somatic maintenance over longevity in many organisms (Stearns, 1998). This phenomenon has been observed in rapamycin, widely recognized as a senotherapeutic agent that can extend the lifespan in female flies, which has been reported with significantly reduced fecundity within three days of treatment (Yu et al., 2024). Our data has validated this observation with up to 97% reduction in egg numbers after rapamycin treatment. Interestingly, recent studies have been challenging this trade-off theory between longevity and fecundity in *D. melanogaster* (De Araujo et al., 2023, Wit et al., 2013). No systematic changes were reported in several reproduction parameters such as egg laying, offspring survival and lifespan in the dispenser and resident *D. melanogaster* (De Araujo et al., 2023). In addition, another study did not observe such a trade-off between longevity-selected lines and control flies (Wit et al., 2013). This remarkable phenomenon has been demonstrated in betaine supplementation in Drosophila, as we observed no opposing effects between increased longevity and fecundity rate. Female flies treated with betaine have shown significant improvements in both lifespan and egg numbers throughout the experiment. The outcomes suggest that it is not a prerequisite to expect longevity and fecundity trade-offs in *D. melanogaster*, which offers scientists an opportunity to elucidate lifespan-modifying interventions without introducing harmful impacts to the reproduction outputs in humans.

Rapamycin, one of the most promising and extensively studied compounds in ageing research, has been used as a positive control in our animal study. This compound has emerged as a leading candidate for extending healthspan together with its original role as an immunosuppressant and antifungal agent (Ehninger et al., 2014, Bastidas et al., 2012, Dumont and Su, 1996). The positive effects of rapamycin have been relatively consistent across different species (i.e. mice, fruit flies, worms, etc) (Sharp and Strong, 2023, Regan et al., 2022, Zhang et al., 2024). To our surprise, we did not observe an increase in lifespan

or health span in fruit flies following rapamycin treatment. Long-term treatment with rapamycin did not exert lifespan-extending effects in female fruit flies, while negatively affected male lifespan. This variation might be explained by the male and female response to rapamycin's influence on autophagy (Regan et al., 2022). Recent studies also suggested that the timing and dosing of rapamycin treatment can influence its effectiveness. In mice, rapamycin has demonstrated positive effects on lifespan extension when administered later in life (Harrison et al., 2009). According to previous data, a single dose of rapamycin is considered sufficient and safe to improve lifespan and healthspan in rodent models (Johnson et al., 2013, Hebert et al., 2014). Notably, an intermittent schedule of rapamycin supplementation can improve stem cell regeneration while avoiding potential side effects (Blagosklonny, 2008). It is important to maintain the constant total dose of rapamycin while ensuring it reaches a high peak blood level (Kaeberlein, 2014). Therefore, a weekly supplementation of a higher dose has been shown to induce a maximum life extension effect. A consecutive drug-free period is also recommended to mitigate the undesirable effects. This might explain why we observed significant detrimental effects of rapamycin on fecundity rate without the extending lifespan in fruit flies. Interestingly, a lower dosage of rapamycin daily has been proven to be safe in the elderly (1 mg/day for several weeks) (Kraig et al., 2018). The administration of the compound has to be adjusted to ensure personalized life extension effects on individual patients.

In conclusion, this chapter provides us with concrete evidence regarding the senotherapeutic effects of betaine on two different species, *C. elegans* and *D. melanogaster*. Further studies on more complex animals (i.e. rodents) and human models are required to better understand the effects of betaine in the normative and accelerated ageing process.

Chapter 6: General discussion

This thesis has exploited a variety of *in vitro* and *in vivo* approaches to elucidate the potential roles of betaine in mitigating the effects of vascular ageing. Previously, it has been suggested that betaine concentration declines with age, reflected in multiple diseasome of ageing such as CVD, CKD or type 2 diabetes (Chen et al., 2013, Lan et al., 2024, Alvarenga et al., 2022a). Previous studies on bears have identified their natural resistance against lifestyle-associated diseases (i.e. kidney failure, osteoporosis, or atherosclerosis) during the hibernation period (Ebert et al., 2020a). Using a systematic biomimetic approach, we have identified a remarkable variation between betaine levels in free-ranging bears and captive brown bears. There was an increase of 422% in betaine levels in free-ranging bears which enables metabolic reprogramming that is essential to enable physiological adaptation during the hibernation periods. We hypothesized that metabolic reprogramming could be exploited in a range of other biological systems (i.e. human cell culture and model organisms) through betaine supplementation. As such, youthful metabolic characteristics could be restored via multiple mechanisms including mitochondrial regeneration, enhanced stress resistance, and activation of longevity-associated cellular pathways. Throughout a series of experiments, we have achieved and exceeded our primary objectives which will be further discussed in this chapter.

We have established optimal concentration dosing of betaine for delaying human VSMCs ageing *in vitro*, which has been translated into *D. melanogaster* and *C. elegans* models *in vivo*. Betaine demonstrates varied dose-dependent effects across different cellular contexts. Previous *in vitro* studies on other cell types (i.e. embryo cells, skeletal muscle cells) have used betaine concentrations between 10 μg/ml – 50 mg/ml (Zhang et al., 2018a, Du et al., 2018, Yi and Kim, 2012). At low concentrations (e.g. 50 μg/ml), it effectively reduces ROS in embryo cells, while 1 mg/ml can significantly influence myoblast proliferation and differentiation (Zhang et al., 2018a, Du et al., 2018). In endothelial cells, betaine suppresses NF-κB phosphorylation and Akt activation in a dose-dependent manner ranging from 10 to 100 μg/ml (Yi and Kim, 2012). Our findings confirmed that betaine 1 mg/ml is effective in mitigating VSMCs senescence and promoting cellular proliferation *in vitro*. Interestingly, when translating our data *in vivo* in *D. melanogaster* and *C. elegans,* we observed a remarkable extension in the lifespan of both species with lower doses of betaine (50-100 μg/ml). The higher dose of betaine 1

mg/ml did not show any significant impact on lifespan, yet no visible side effects were recorded during the lifespan assay.

There are several factors that might contribute to the dosage variations between in vitro and in vivo models of different species, including cell/tissue types, absorption rate, bioavailability and species-specific cellular responses. As betaine is mainly derived from dietary sources, it has excellent safety profiles that can be used long-term without any detrimental effects. Specifically, betaine has been suggested with 3 grams/twice/day as a starting dose, with a maximum dose of 20 grams (Schwahn et al., 2003, Lever et al., 1994, Turck et al., 2017). Excessive amounts of betaine are mainly secreted in the sweat (~50-270 mg/d) or urine (~10 mg/d), thus preventing the accumulation of residues inside the body (Lever et al., 2004, Craig et al., 2010, Lever et al., 2007, Sawka and Montain, 2000). Our outcomes underscore the importance of carefully selecting betaine dosage based on specific cellular environments and research objectives. It is crucial to examine a variety of betaine dosage to ensure the optimal impact on healthspan and lifespan in humans.

We have successfully demonstrated that this treatment ameliorates a range of hallmarks of ageing such as SCs accumulation (i.e. SA β-gal), inflammatory burden SASP (IL6, IL1β), replicative senescence and mitochondrial dysregulation (mtDNA-CN, mitochondria content, MMP), oxidative damage (FOXO4, LMNA, H_2O_2, DMSO), vascular calcification and extracellular vesicles. These markers are non-specific for vascular ageing, suggesting a broader therapeutic potential of betaine for the dysregulation processes of various diseasome of ageing. By targeting multiple interconnected ageing mechanisms simultaneously, betaine emerges as a promising molecular intervention capable of mitigating structural and functional decline across diverse biological systems. This novel intervention can be applied to a diversity of non-communicable diseases such as CKD, CVD or type 2 diabetes. In rodent models with CKD, betaine has been found to alleviate inflammatory response by downregulating different cytokines production (i.e. IL1β, IL18, IL6, and TNF-α) (Ghartavol et al., 2019, Fan et al., 2014, Hagar and Al Malki, 2014). Betaine is also known for its important role in blood pressure regulation in dialysis CKD patients, reflected by the strong correlation between elevated diastolic and systolic blood pressure and lower plasma betaine levels (Wang et al., 2018). In animal models with type 2 diabetes, betaine supplementation has positively influenced gene expression and anti-inflammation while mitigating excessive oxidative damage in vivo. The potential impacts of betaine have also been documented

in other age-associated diseases such as Parkinson's disease, atherosclerosis and non-alcoholic fatty liver disease (Alirezaei, 2015, Kathirvel et al., 2010, Lv et al., 2009). These supporting evidences provide us with confidence that betaine can be applied further in clinical trials, to protect our well-being against a series of age-related deterioration.

We have successfully, for the first time to our knowledge, delayed ageing in primary human VSMCs using manipulation of one-carbon metabolism. As it becomes increasingly dysregulated with age, one-carbon metabolism contributes to accumulated oxidative stress, mitochondrial dysfunction, and epigenetic alterations (Lionaki et al., 2022). By supplementing VSMCs long-term with betaine, we observed the downregulation of a series of well-established biomarkers of cellular ageing, including expression of p16 (CDKN2A) and p21 (CDKN1A), senescence β-galactosidase expression, SASP-associated IL6 and IL1β expression, cytoplasmic chromatin fragment events (γH2A.X staining), as well as other biomarkers for VSMCs damage and cell stress. While we have illustrated that one-carbon metabolism holds great promise for lifespan extension, scientists also advocate an opposite viewpoint on using methionine restriction (MR) as an alternative approach to enhance longevity. MR diet has been formulated by reducing the amount of methionine consumed from 0.86% to 0.17% (1.7 g/kg diet) to achieve up to 30% extension in longevity (Orentreich et al., 1993). The restriction of dietary methionine has shown several other health benefits, including improved metabolic health, anti-ageing and anti-inflammatory effects. However, long-term usage of the MR diet has been suggested with potential adverse effects, ranging from hyperhomocysteinemia, metabolic disorders and liver/kidney injuries (Tamanna et al., 2018). As methionine is an essential amino acid with various physiological and metabolic roles, we argue that betaine supplementation during MR might improve the overall healthspan and lifespan while minimizing the potential downsides. This involves the reduction of homocysteine levels which have been recognized as a key biomarker of cardiovascular diseases (McRae, 2013). Nevertheless, excessive betaine supplementation might counteract MR effects, so the optimal balance between the two interventions is sought to be determined. This holistic balance of methionine level in MR diet can be achieved with long-term supplementation of lower levels of betaine doses (i.e. 50 μg/ml, 100 μg/ml).

We have expanded our studies to pre-clinical models in *Drosophila melanogaster* and *C. elegans* to successfully demonstrate an extension in health span *in vivo*. By examining

different concentrations of betaine on two animal models, we were able to identify that a lower dose of betaine (50 – 100 μg/ml) is more beneficial, compared to a higher dose (1 mg/ml), in enhancing longevity. Despite low methionine intake, long-term supplementation with betaine in lower doses can maintain the methylation reactions that are responsible for normal cellular functions. The main pathways in which MR diets enhance longevity involve activating cellular stress response and autophagy (Kitada et al., 2021). As hyperhomocysteinemia has been reported in rats treated with MR diets, this can lead to toxicity at high levels (Tamanna et al., 2018). Betaine acts via the enzyme BHMT to convert homocysteine back into methionine, maintaining a balance in the methionine cycle (Ji et al., 2007). During the MR process, an alternative approach to regenerate methionine is required to compensate for the limited intake of methionine. In addition, restriction of methionine levels has been associated with the reduced activities of antioxidant enzymes (SOD, CAT, GSH-Px, and GSH), eventually leading to mitochondria dysfunction (Song et al., 2021). According to our data, we have determined that betaine is a positive regulator of mitochondria functions by improving the total mitochondria content and MMP under oxidative damage. Therefore, betaine supports methionine regeneration, decreased homocysteine levels and enhanced mitochondria functions, potentially mitigating other adverse effects of MR while maintaining its lifespan-extending benefits.

Additionally, we have successfully developed iPSCs-derived VSMCs and for the first time demonstrated that our betaine treatment can ameliorate and protect against oxidative damage and calcification. One recent study has suggested that betaine levels were significantly reduced in the intestinal specimens of patients with severe venous calcification (Deng et al.). From that, we have considered that betaine might be a potential intervention for tackling age-related vascular calcification. Interestingly, our data has shown that long-term supplementing iVSMCs with betaine promotes its protective effects on vascular calcification. Another contributing factor for calcified plagues is homocysteine, an amino acid whose regulation is centrally involved in one-carbon metabolism. The positive correlation between elevated homocysteine levels and vascular calcification has been extensively studied (Jung et al., 2022, Karger et al., 2020, Zhu et al., 2019a). As hyperhomocysteinemia can trigger oxidative stress in vascular ageing, it promotes the osteogenic transformation of VSMCs, excessive upregulation of calcification-related genes (i.e. Runx2, NOX4) and disrupt mineral homeostasis (Zhu et al., 2019b, Lai and Kan, 2015, Van Campenhout et al., 2009, De Martinis et al., 2020).

Betaine is recognised for its role in regulating homocysteine levels, thus allowing optimal protection against age-related vascular calcification. During ageing, the dysregulation in cellular and molecular functions activates a vicious cycle between hyperhomocysteinemia, DNA methylation dysfunction and oxidative stress. Notably, impaired methylation can lead to abnormal changes in VSMCs phenotypes and gene expression related to calcification. For instance, hypomethylation of the LncRNA H19 promoter is associated with vascular calcification in CKD patients (Wang et al., 2024). As a result, our proposed mechanisms involving betaine activities in vascular calcification include alleviating hyperhomocysteinemia, inflammation responses and donating methyl groups. This will now be tested in a murine pre-clinical model of hypertension and vascular ageing.

Furthermore, our data indicate that we have a potential therapy to combat the effects of the otherwise intractable laminopathy Hutchinson Guildford's progeria. Hutchinson-Gilford Progeria Syndrome is characterized by a de novo mutation in the LMNA gene, which produces an aberrant protein called progerin (Gonzalo et al., 2017). This protein fundamentally disrupts nuclear architecture, causing misshapen nuclear membranes and compromised cellular infrastructure that triggers a catastrophic chain of molecular dysfunction. Progerin accumulation accelerates cellular senescence through multiple interconnected mechanisms: it induces chronic DNA damage, impairs mitochondrial function, generates excessive ROS, and dysregulates epigenetic processes (Kristiani and Kim, 2023). At the cellular level, these molecular perturbations display reduced proliferative capacity, increased apoptosis, and systemic breakdown of normal cellular maintenance mechanisms (Cisneros et al., 2023). The progressive nuclear lamina destabilization creates a cascading effect that fundamentally undermines cellular integrity (Almendáriz-Palacios et al., 2020). This leads to rapid organismal ageing and the characteristic devastating clinical manifestations of progeria, including accelerated cardiovascular degeneration, skeletal abnormalities, and dramatically shortened lifespan (Ullrich and Gordon, 2015). As betaine has shown outstanding effects on mitigating cellular senescence, accumulated inflammation responses and mitochondria dysfunctions, we believe our outcomes in primary and iPSCs-derived VSMCs can be translated into the context of laminopathy Hutchinson Guildford's progeria. We are currently testing this *in vitro* using clinically derived primary cell isolates. If successful, this simple approach can be further utilized in clinical trials, offering a novel platform for improving the life quality of patients with this genetic condition.

To our best knowledge, we have developed a prototype non-drug approach to extend lifespan and health span without experiencing the trade-off between longevity and fecundity. The trade-off between longevity and fecundity is a fundamental biological concept that describes the inverse relationship between an organism's reproductive output and its potential lifespan (Djawdan et al., 1996). This is based on an evolutionary theory known as 'Antagonistic Pleiotropy', proposed by George Williams in 1957 (Williams, 1957). Several senotherapeutic agents have been identified with this trade-off including rapamycin, metformin and resveratrol. These compounds have been reported with potential longevity-enhancing effects yet significantly suppressing reproductive capacity. Specifically, our data has shown that rapamycin treatment on *D. melanogaster* resulted in up to 96% reduction in egg numbers per fly. By inhibiting mTOR pathway, rapamycin supports survival over reproduction by downregulating follicular and oocyte development (Alves et al., 2022). It is a potent immunosuppressive drug primarily used in organ transplantation to prevent acute and chronic graft rejection by inhibiting immune proliferation (Saunders et al., 2001). While effective in extending lifespan, rapamycin comes with significant side effects including increased susceptibility to infections, metabolic disruptions and compromised fertility (Li et al., 2014). It is essential to use rapamycin with careful medical supervision to balance its therapeutic benefits against potential adverse reactions. Interestingly, our data on *D. melanogaster* has indicated that betaine is more effective and safer than rapamycin in enhancing healthspan and lifespan of flies. There was no trade-off between longevity and fecundity, despite its remarkable efficacy. In female flies, we observed a significant increase in lifespan, egg numbers and negative geotaxis behaviour with a range of different betaine concentrations. Compared to control flies, betaine-treated flies have been reported with up to 15% increase in lifespan treatment, 20-70% improvement in egg numbers and motor coordination. On the other hand, we did not observe any changes in the lifespan of fruit flies treated with rapamycin. In addition, rapamycin rapidly diminished egg count within two days of initial treatment, with minimal impact on motor strength. Our findings suggest betaine surpasses rapamycin in safety and efficacy as a senotherapeutic agent. From that, we intend to develop these in the future for assessment of their suitability in other complex models such as rodents and humans.

Overall, we have achieved and exceeded our primary objectives. We have developed a novel mechanistic insight into vascular ageing that is applicable to all non-communicable diseases of ageing. Ageing is a complex and multifactorial phenomenon that needs to be

addressed in a systematic way. In this comprehensive study, we investigated the potential of betaine as a novel senotherapeutic agent by employing a multi-faceted research approach. We initially utilized both primary and iPSCs-derived VSMCs to elucidate the cellular and molecular mechanisms through which betaine exerts its effects. Then, we extended our research to two distinct *in vivo* model organisms: *D. melanogaster* and *C. elegans*. The outcomes from our *in vitro* and *in vivo* experiments provided a robust and clear understanding of betaine's role in tackling the dysregulation of the ageing process. Given the global trend of an increasingly ageing population, our research suggests that betaine could emerge as a promising, affordable, and safe intervention strategy to address the complex array of age-related diseases. To our current knowledge, betaine is the best senotherapeutic approach for the treatment of vascular ageing, with the possibility to extend its beneficial impacts within the diseasome of ageing. Compared to other widely accepted senotherapies (i.e. rapamycin), betaine provides safe, natural and multi-functional effects without any serious adverse effects. Notably, the supplementation with a higher dose of betaine (1 mg/ml) does not pose any negative effects on two different taxa (*D. melanogaster* and *C. elegans*). Together with the life extension effects, betaine also significantly improves fecundity rate which overthrows the 'trade-off' theory between longevity and fecundity, leading to its success in taking over rapamycin as the most effective senotherapeutic agent. This approach offers a potential breakthrough in managing the molecular and physiological challenges associated with ageing, presenting betaine as a significant candidate in the emerging field of senotherapeutics.

Chapter 7: Future work

Based on our current success in exploring the role of betaine in tackling vascular ageing, we aim to expand our *in vitro* and *in vivo* experiments to understand its therapeutic potential across age-related diseases. We will deepen our knowledge of betaine's molecular mechanisms, particularly its impact on alleviating mitochondria dysfunction and cellular protection. Betaine is a potent senotherapeutic compound with a variety of molecular pathways, allowing us to extend our experiments to other cell types such as human diploid fibroblasts and renal epithelial cells. Our upcoming studies also aim to explore betaine's efficacy in mitigating DNA damage, specifically in progeria cell models and laminopathy contexts. Notably, the preliminary outcomes in iPSCs-derived VSCMs have demonstrated that betaine treatment can ameliorate and protect against oxidative damage and calcification. This will now be tested in a murine pre-clinical model of hypertension and vascular ageing. Furthermore, our data indicate that we have a potential therapy to combat the effects of the otherwise intractable laminopathy Hutchinson Guildford's progeria. We are currently testing this *in vitro* testing using clinically derived primary cell isolates. We also intend to develop these in the future for assessment of their suitability to treat intractable laminopathies. Due to the safety and efficacy profiles of betaine, we are confident to explore our future work on other clinical trials to validate the human translations in age-related diseases such as hypertension, cardio-renal disease, haemodialysis, etc. As betaine is a naturally occurring compound with relatively high safety profiles, we are recruiting participants for the clinical trials using betaine as a supplement to tackle age-related pathologies. These participants will be assigned with specific dosage of betaine for an extended period, allowing us to characterise the potential improvements in physiology and body functions. We will also focus further on the epigenetic impacts of betaine by using longitudinal epigenomic profiling methods (i.e. whole genome bisulfite sequencing) to track betaine levels over the life course. By applying the biomimetic approach, the extensive role of betaine in activating metabolic reprogramming will be examined in a diversity of animal species. Overall, these multifaceted investigations seek to validate betaine as an efficient therapeutic intervention in the diseasome of ageing. From that, betaine can be widely recognized as a novel senotherapy to rejuvenate metabolic profiles, thus slowing down the effects of accelerated ageing in human populations.

Chapter 8: Conclusion

The use of betaine as a novel senotherapeutic to combat vascular ageing is quickly emerging. The main pathway for betaine production in human cells is in mitochondria, through the oxidation of choline. Mitochondrial dysregulation occurs with ageing; hence betaine levels can become depleted with a knock-on effect on the maintenance of the epigenetic landscape of ageing and the other hallmarks of ageing. We have achieved and exceeded our primary objectives. We have developed a novel mechanistic insight into vascular ageing that is applicable to all non-communicable diseases of ageing. This prototype non-drug approach has been successfully developed to extend lifespan and health span and tested this in two different *in vivo* models. As betaine is accessible and affordable, further studies should keep on exploring other pathways to achieve better treatment for diseasome of ageing.

Chapter 9: References

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Chapter 10: Appendices

10.1 Changes in growth rate of VSMCs after treatment with betaine

We conducted RTCA experiments on VSMCs using different betaine concentrations, ranging from 50 µg/ml, 500 µg/l, 1 mg/ml, 5 mg/ml and 10 mg/ml. In young VSMCs, there were no significant changes in growth rate between control and betaine-treated cells (see Figure 10.1). Older VMSCs experienced distinct alterations in growth rate, especially in 1 mg/ ml treatment. During the three days of the experiment, treatment with lower doses of betaine (50 µg/ml) did not show any beneficial effects on VSMCs growth rate. Notably, VSCMs treated with higher doses of betaine (500 µg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml) were observed with significantly higher growth rates from the second day of treatment (p-value < 0.05).

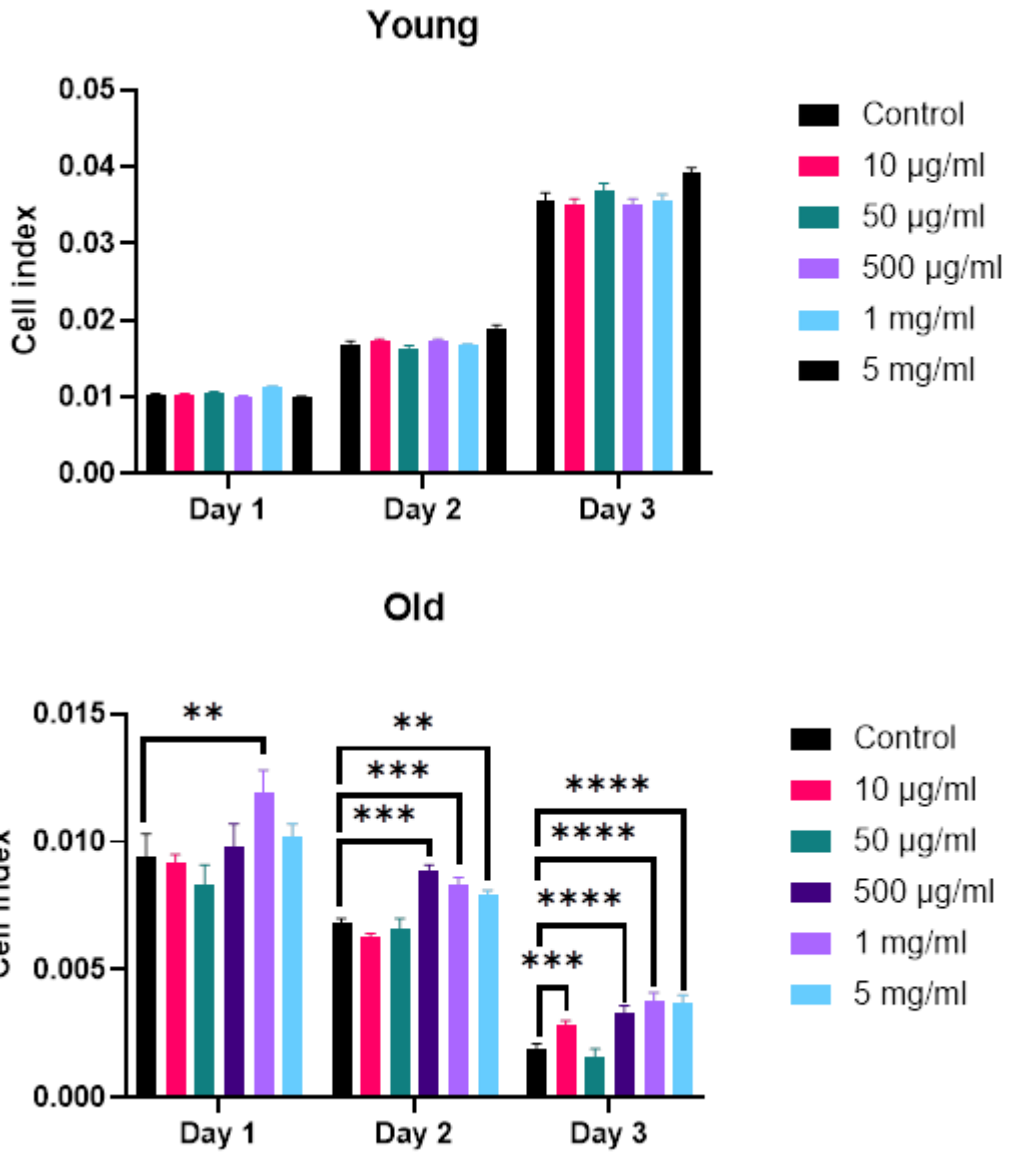

Figure 10.1| Changes in growth rate of VSMCs following betaine supplementation. *During the three days of the experiment, treatment with lower doses of betaine (50 µg/ml) did not show any beneficial effects on VSMCs growth rate. Notably, VSCMs treated with higher doses of betaine (500 µg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml) were observed with significantly higher growth rates from the second day of treatment (p-value < 0.05). The differences are significant (two tailed t test) with * (P < 0.05), ** (P < 0.01), *** (p < 0.001) or **** (p < 0.0001).*

10.2 Changes in gene expression in VSMCs during replicative senescence

- BAX: As VSMCs enter the senescence state, BAX- a widely known pro-apoptotic gene was found to be significantly upregulated. The expression of BAX progressively increases with age, as it doubles in gene expression in older cells. These findings suggest a cumulative cellular stress response, evidenced by an increased apoptotic signalling pathway activation in vascular ageing.

- CASPASE 3: Caspase-3 is a protease that plays a central role in apoptosis or programmed cell death. It has shown a dramatic increase in expression, from a 2- 4-fold difference in middle and old cells as compared to young VSMCs. This substantial rise indicates potential increased apoptotic activity during replicative senescence.
- CDKN1A: In VSMCs, there was a gradual increase in CDKN1A expression with age. As CDKN1A is a key marker of cellular senescence, increased expression of CDKN1A is associated with reduced cell cycle progression, accumulation of DNA damage and SCs. There was no significant change in CDKN1A expression between young and middle-aged VSMCs. However, as cells continued to grow, we observed the intense upregulation of this gene with up to 3 times increase in gene expression.
- CDKN2A: CDKN2A – another crucial biomarker of premature ageing- also exhibits similar patterns with CDKN1A. Gene expression did not drastically change in middle-aged cells, compared to younger VSMCs. A significant increase in CDKN2A expression was found in older cells, with up to 3 times higher. This indicates the exposure of cells to various stressors (i.e. oxidative stress, DNA damage), causing them to enter a state of permanent cell cycle arrest called senescence.
- EFNB1: During replicative senescence, we observed a remarkable increase in gene expression of EFNB1. By binding to EpH receptor kinases, EFNB1 plays a major role in cell adhesion and communication. So far, there has been no direct correlation

between EFNB1 and the ageing process. As EFNB1 increased more than 5 times in old VSMCs, we proposed that EFNB1 might play a protective role in promoting intra- and intercellular signalling with age.

- FOXO4: As FOXO4 is essential for cell response to oxidative stress, the consistent increase in FOXO4 expression from young to old VSMCs might be a part of cytoprotection. In older cells, FOXO4 has been found to cause cell cycle arrest by activating a number of genes in the Go quiescence phase (Baar et al., 2017). FOXO4 is also shown to elevate senescent cells and maintain their viability. Our data has illustrated this trend, as the expression of FOXO4 increased by 4-fold with age.
- LMNA: LMNA, a novel biomarker of vascular ageing, has been identified to be upregulated in VSMCs senescence. This leads to mitosis disruption and DNA damage, followed by genomic instability (Ragnauth et al., 2010). According to our data, there was also a significant increase in the LMNA gene (up to 2 times) as the cells grew older. Even though the expression of LMNA was similar between young and middle-aged VSMCs, the accumulation of LMNA initiates a permanent stress response in older cells, which accelerates the ageing process.
- LMNB1: Lamin B1 loss is a marker of cellular senescence, which indicates abnormalities in nuclear structure and functions. The decline in LMNB1 expression has been observed in our data, with up to 80- 90% loss as the cells progressed further to their middle and older stages. This contributes to the occurrence of many age-related tissue dysfunctions, especially vascular ageing.
- MCL1: Mcl-1 is considered an anti-apoptotic factor within Bcl-2 gene family. We observed a substantial increase in MCL1 expression (more than 2 times) in aged VSMCs, potentially related to SCs survival mechanisms. MCL1 overexpression has been suggested as a potential pathway for SCs to escape the apoptosis process (Troiani et al., 2022a). Inhibiting MCL1 expression might be an effective therapeutic strategy for inducing apoptosis in senescent VSMCs.
- NRF2: Nrf2 is a master regulator of cytoprotective responses, regulating ~2% of the genome. It is typically diminished in expression with increasing age and in the diseasome of ageing**.** In-vitro studies have shown that the depletion of Nrf2 activity induces uraemia-associated vascular calcification at high Pi levels (Laget et al., 2023). As the accumulation of ROS increases with age in VSMCs, Nrf2 is translocated outside the nucleus. This leads to the activation of the KEAP1/NRF2/P62 antioxidative pathway as part of cytoprotection and stress

response. Our data has reported a slight decrease in Nrf2 expression in middle-aged VSMCs, yet no changes were observed in old cells. From that, senescent VSMCs become more sensitive to exposome factors such as oxidative stress and DNA damage.

- NOXA: NOXA, a recognized member of the Bcl-2 family, which promotes p53-dependent activation of caspases and apoptosis, has a strong correlation with cellular senescence. Notably, NOXA is upregulated with age, together with other pro-apoptotic genes (Edwards et al., 2007). Our data indicates that the NOXA gene is three-fold overexpressed in senescent VSMCs. This might be due to the accumulation of DNA damage, SASP and other internal stressors.

- RUNX2: Surprisingly, the expression of RUNX2 gene – well-known for its role in vascular calcification – significantly reduces VSMCs replicative senescence. We observed a sharp decrease in RUNX2 expression, with up to 10-fold reduction in old cells. While previous studies have demonstrated a correlation between RUNX2 gene overexpression and advancing age, our experimental data suggest that this relationship is not generally applicable across all cell types and culturing conditions.
- SIRT2: SIRT2 gene is cytoplasmic deacetylase with a protective role for VSMCs against DNA damage and oxidative stress. As VSMCs grow older, analysis of age-related changes revealed a two-fold upregulation of SIRT2 gene expression in senescent VSMCs, compared to younger cells. This might be an intrinsic cellular defence mechanism of VSMCs that are activated during stressful conditions (i.e. ROS accumulation, exposomes).
- SIRT6: Sirtuin 6 (SIRT6) is a protein that maintains various body processes such as DNA repair, genomic stability and metabolic balance. Our data revealed the significant 4-fold upregulation of SIRT6 expression with age. This might suggest that senescent VSMCs are stimulated by multiple age-associated stressors, leading to the increased expression of SIRT6 as a cellular defence mechanism.
- IL1β: Our data revealed a prominent age-dependent increase in IL1β gene expression. Compared to young VSMCs, middle-aged cells experienced a moderate increase with nearly 6 times upregulation, followed by a dramatic elevation of 122-fold change in older cells. This exponential increase in IL1β expression aligns with the inflammatory ageing theory, as IL1β is a key pro-inflammatory cytokine that contributes to chronic low-grade inflammation characteristic of vascular ageing. The

substantial elevation in aged cells likely results from accumulated cellular damage, SASP, and dysregulation of inflammatory pathways that occur with advancing age.

- IL6: Similar to IL1β, we recorded a comparable expression pattern of IL6 gene with dramatic overexpression in both middle-aged and old stages. As VSMCs continue to grow, IL6 expression strikingly increases by 27 to 54-fold with age. This age-related IL6 overexpression is highly linked to vascular dysfunction, resulting in the development of numerous pathological conditions.
- SERPINB2: SerpinB2, a serine protease inhibitor, has been associated with SCs and cell stress. Elevated levels of SerpinB2 have been found in inflammation-induced cellular stress in various age-related diseases (Sen et al., 2020, Sánchez-Navarro et al., 2021). We observed upregulation of SerpineB2 with increasing cellular age in culture ($p<=0.0001$).
- SERPINE1: Overexpression of Serpine 1 has been linked to accelerated vascular dysfunction in multiple cardiovascular diseases, such as arteriosclerosis, thrombosis, and perivascular fibrosis. Serpine 1 is widely used as a biomarker to predict the potential onset of future cardiovascular disease-associated mortality (Samarakoon and Higgins, 2008). Serpine 1, a recognised hub of SASP activity, was extensively upregulated in VSMCs during replicative senescence. There was a 123-fold change between young and old VSMCs, indicating that this gene can be used as a novel biomarker for ageing.

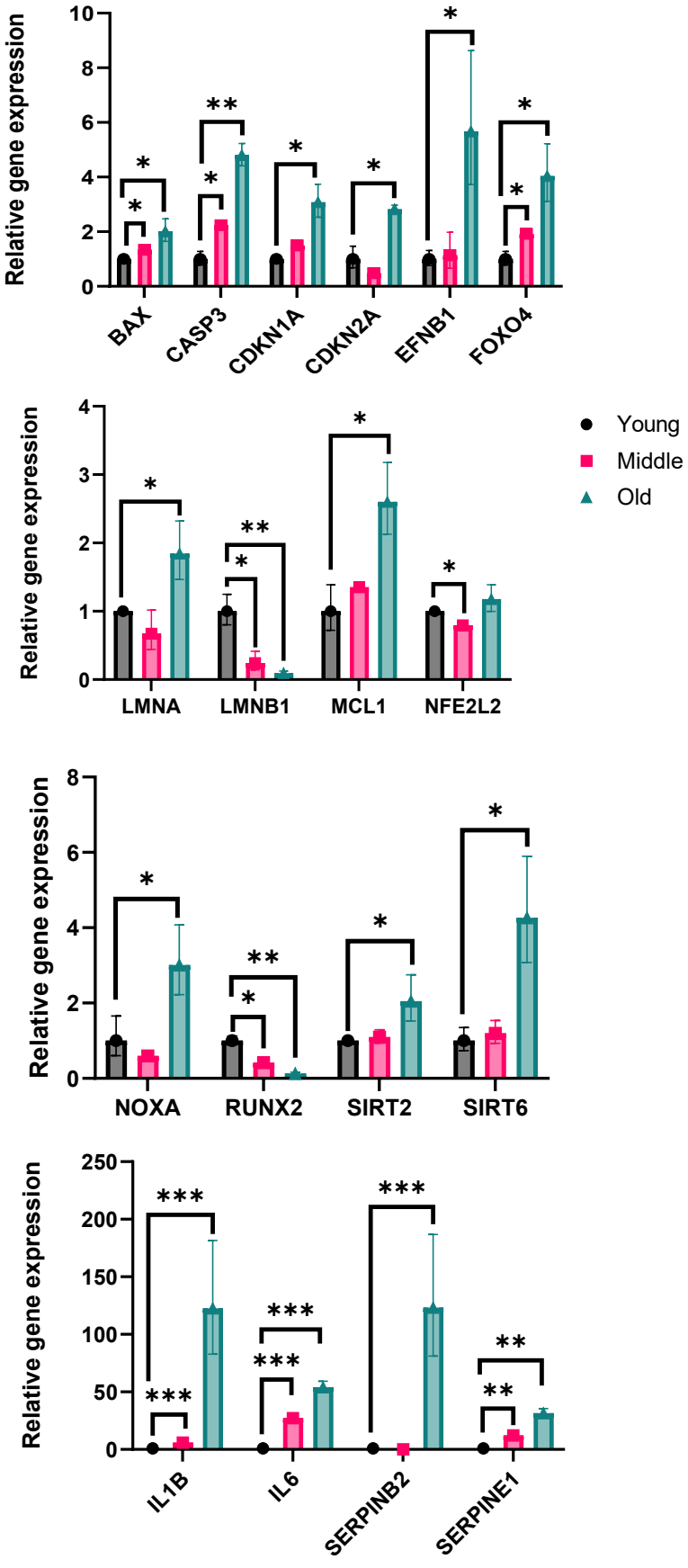
Relative gene expression
BAX
CASP3
CDKN1A
CDKN2A
EFNB1
FOXO4
LMNA
LMNB1
MCL1
NFE2L2
NOXA
RUNX2
SIRT2
SIRT6
IL1B
IL6
SERPINB2
SERPINE1
Young
Middle
Old

Figure 10.2| Changes of gene expression in VSMCs during replicative senescence. *TaqMan low-density array was used to evaluate the potential alteration of a series of genes associated with apoptosis (BAX, CASP3, MCL1, NOXA), inflammation (IL1, IL6, SERPINEB2, SERPINE1), cellular structures (LMNA, LMNB1, EFNB1), cell cycle progression (CDKN1A, CDKN2A, RUNX2), cytoprotection (NRF2, SIRT2, SIRT6, FOXO4). All of the apoptotic and inflammatory genes are found to be substantially upregulated, especially IL1β, IL6 and SerpineB2 experienced 50 to more than 100-fold change in gene expression in older cells. With advanced age, genes associated with cellular structures such as LMNA and EFNB1 were found to be significantly overexpressed, except for LMNB1. As CDKN1A and CDKN2A mainly contribute to the impaired cell cycle progression, their expression has been progressively over the life course. Interestingly, there was a considerable downregulation of RUNX2 gene - a transcription factor that is highly linked to increased risk of vascular calcification – as VSMCs age. As external and internal stressors accumulate with time, cytoprotective genes were found to be greatly activated in older cells. The differences are significant (two tailed t test) with * ($P < 0.05$), ** ($P < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$*