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Review article

Epigenetic reprogramming as a key to reverse ageing and increase longevity

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ABSTRACT

The pursuit for the fountain of youth has long been a fascination amongst scientists and humanity. Ageing is broadly characterized by a cellular decline with increased susceptibility to age-related diseases, being intimately associated with epigenetic modifications. Recently, reprogramming-induced rejuvenation strategies have begun to greatly alter longevity research not only to tackle age-related defects but also to possibly reverse the cellular ageing process. Hence, in this review, we highlight the major epigenetic changes during ageing and the state-of-art of the current emerging epigenetic reprogramming strategies leveraging on transcription factors. Notably, partial reprogramming enables the resetting of the ageing clock without erasing cellular identity. Promising chemical-based rejuvenation strategies harnessing small molecules, including DNA methyltransferase and histone deacetylase inhibitors are also discussed. Moreover, in parallel to longevity interventions, the foundations of epigenetic clocks for accurate ageing assessment and evaluation of reprogramming approaches are briefly presented. Going further, with such scientific breakthroughs, we are witnessing a rise in the longevity biotech industry aiming to extend the health span and ideally achieve human rejuvenation one day. In this context, we overview the main scenarios proposed for the future of the socio-economic and ethical challenges associated with such an emerging field. Ultimately, this review aims to inspire future research on interventions that promote healthy ageing for all.

1. Introduction

1.1. Ageing

Ageing is a ubiquitous natural process marked by a progressive decline in cellular, tissue, and physiological functions across all organ systems (Fakouri et al., 2019; Guo et al., 2022). This process is accompanied by an exponential increase in mortality following the Gompertz law (da Silva and Schumacher, 2019). As cells divide and proliferate, telomeres progressively shorten until they stop the cell cycle, leading to death and cell senescence, a well-known ageing hallmark. Furthermore, over-proliferation of cells accelerates telomere shortening, potentially contributing to premature ageing and the development of age-related diseases, including cancer. Conversely, insufficient cellular proliferation can also contribute to ageing by leading to tissue atrophy and the inability to repair damage. Therefore, maintaining a balance in cellular proliferation is vital for healthy ageing (Vaiserman and Krasnienkov, 2021).

Moreover, ageing is a complex process that manifests itself through a combination of variable and predictable changes. For instance, thymic involution, presbyopia, and sarcopenia are well-documented and somewhat deterministic phenomena observed in most vertebrates. However, the rate and extent of those can vary greatly among individuals (Crooke et al., 2022; Larsson et al., 2019; Liang et al., 2022; Thomas et al., 2020). Therefore, despite certain predictable aspects, the overall process of ageing occurs in a non-linear or inconsistent manner (Laffon et al., 2021).

Several studies suggest that ageing should be considered a disease, emphasizing the plasticity of the ageing process, having a potential for treatment, rather than an inevitable process (Guo et al., 2022; Stallone et al., 2019). Notably, there have been many efforts to formally classify ageing as a disease, as such a step is fundamental to formally advance

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appropriate clinical diagnosis and longevity interventions (Calimport et al., 2019; Khaltourina et al., 2020). For instance, organ and tissue senescence-related disease codes have been proposed to be included in ageing classifications in the World Health Organization (WHO) International Classification of Diseases (ICD) (Calimport et al., 2019).

Although this field is replete with mechanistic theories exploring the underlying molecular and cellular alterations that might contribute to the ageing phenotype, it remains a constant topic of scientific inquiry. So, even though these rather complex processes do not provide a paradigmatic description of the causes of ageing, they are collectively referred to as hallmarks of ageing (Gems and de Magalhães, 2021; López-Otín et al., 2013; Tsurumi and Li, 2012).

1.2. Hallmarks of ageing

The original hallmarks proposed by López-Otín et al. (2013) include genomic instability, telomere attrition, epigenetic changes, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Laffon et al., 2021; Lidzbarsky et al., 2018; López-Otín et al., 2013; Toniolo et al., 2023).

They can be categorized into three groups: primary, antagonistic, and integrative hallmarks. Primary hallmarks consistently have negative effects and initiate damage accumulation; antagonistic hallmarks have dual effects depending on their intensity, so at high levels, they can exacerbate negative effects; and integrative hallmarks arise when tissue homeostasis mechanisms can no longer compensate for accumulated damage (Aunan et al., 2016). Additionally, these can also be subdivided into molecular, cellular, and systemic hallmarks, based on their impact at different levels (López-Otín et al., 2023) (Table 1). Furthermore, the ageing field is continuously expanding and proposing new hallmarks, including extracellular vesicles (Manni et al., 2023), compromised autophagy, microbiome disturbance, altered mechanical properties, splicing deregulation, and inflammation (López-Otín et al., 2023; Schmauck-Medina et al., 2022).

1.3. In the pursuit for the fountain of youth

Humans have long sought ways to prolong life and restore health, as depicted in the myths of the 'fountain of youth' through various cultures and times in history. The metaphor has served as an illustration of anything that potentially increases longevity (Aunan et al., 2016). As the average lifespan increases and the elderly population grows, age-related diseases including neurodegenerative, cardiovascular, and metabolic diseases have become more prevalent, resulting in significant social and economic consequences worldwide (Guo et al., 2022; Lidzbarsky et al., 2018; Toniolo et al., 2023). This escalating burden has placed ageing research at the center stage (Chakravarti et al., 2021), creating a huge academic and commercial industry interest (Aunan et al., 2016). The investigation in this field aims to identify interventions and treatments that can promote healthy ageing and prevent, delay, or even reverse age-related diseases (Aunan et al., 2016; Guo et al., 2022). Cutting-edge technologies such as omics and artificial intelligence show promise in the study of ageing mechanisms and treatment options (Guo et al., 2022). Lifestyle modifications such as caloric restriction (Flanagan et al., 2020), a Mediterranean-style diet (Shannon et al., 2021), and exercise (Rebelo-Marques et al., 2018) can reduce the incidence of age-related conditions (Guo et al., 2022). Moreover, stem cell transplantation, senolytics, elimination of senescent cells, and epigenetic reprogramming offer new directions for treating ageing-related diseases. However, identifying safe pharmaceutical targets for ageing improvement remains a challenge (Guo et al., 2022; López-Otín et al., 2013). Despite this and the endless unanswered questions, the pursuit of healthy longevity for humankind continues, until the fountain of youth is finally unlocked (Guo et al., 2022).

Considering the vast amount of information surrounding the complex multifaceted molecular processes of ageing and its potential therapeutic targets, it is impossible to cover all of them comprehensively within the scope of a paper's length. Hence, the authors have chosen to narrow their focus to strategies that specifically target epigenetic changes in ageing. Specifically, epigenetic reprogramming appears to be the most promising intervention at the moment, not only allowing it to slow down but also potentially reverse cellular ageing.

The recently proposed Information Theory of Aging (ITOA) posits that ageing results from the gradual loss of cellular information, primarily in the form of epigenetic information, leading to the erosion of cellular identity. In contrast to the somatic mutation theory (Morley, 1995), ITOA not only explains similar ageing patterns in individuals with distinct genomes but also justifies why identical mice and human twins can age at different rates (Lu et al., 2023). Notably, the observation that old cells and tissues can be epigenetically reprogrammed to a more youthful state to achieve lifespan extension without apparently reversing mutations reinforces the suspicion of a predominantly epigenetic basis for ageing (Lu et al., 2023). Therefore, by targeting the

Table 1
The nine original hallmarks, accompanied by a short definition of their contribution to ageing, the categories in which they suit in, and references to papers explaining these hallmarks in detail.

Hallmark	Contribution to ageing	Category	References
Genomic instability	Increased DNA damage, mutations, and genomic rearrangements that accumulate leading to cellular dysfunction	Primary; molecular	Aunan et al. (2016);Chen et al. (2023);da Silva and Schumacher (2019);Fakouri et al. (2019);Guo et al. (2022);Laffon et al. (2021);Lidzbarsky et al. (2018);López-Otín et al. (2013);Martins et al. (2020);Toniolo et al. (2023)
Deregulated Nutrient sensing	Dysregulation of nutrient-sensing pathways (e.g., mTOR) affecting metabolism and energy balance.	Antagonistic; cellular	Aunan et al. (2016);Bjedov and Rallis (2020);Guo et al. (2022); López-Otín et al., (2013, 2023);Stallone et al. (2019)
Telomere attrition	Gradual shortening of telomeres that limits cell division and regeneration capacity	Primary; molecular	Aunan et al. (2016);López-Otín et al., (2013, 2023);Rossiello et al. (2022)
Mitochondrial dysfunction	Impaired energy production and increased oxidative stress	Antagonistic; molecular	Aunan et al. (2016);Bekaert et al. (2005);López-Otín et al., (2013, 2023);Lulkiewicz et al. (2020);Turner et al. (2019)
Epigenetic alterations	Changes in DNA methylation, histone modifications, non-coding RNA regulation and heterochromatin, that cause changes in gene expression patterns that disrupt cellular function.	Primary; molecular	Aunan et al. (2016);Galow and Peleg (2022);Kane and Sinclair (2019); Li et al. (2022);López-Otín et al., (2013, 2023)
Loss of proteostasis	Impaired protein folding and degradation leading to the accumulation of misfolded proteins that causes cellular damage.	Primary; molecular	Aunan et al. (2016);López-Otín et al., (2013, 2023);Ruano (2021)
Cellular senescence	Permanent growth arrest and secretion of pro-inflammatory factors.	Antagonistic; cellular	Aunan et al. (2016);López-Otín et al., (2013, 2023);Prašnikar et al. (2021);Song et al. (2020)
Stem cell exhaustion	Decline in the regenerative capacity of stem cells affects tissue repair and renewal	Integrative; cellular	Aunan et al. (2016);Bogeska et al. (2022);López-Otín et al., (2013, 2023);Morganti and Ito (2021);Sameri et al. (2020)
Altered intercellular communication	Disruption of cellular signaling and communication between cells.	Integrative; systemic	Aunan et al. (2016);López-Otín et al., (2013, 2023);Teissier et al. (2022);Zhang et al. (2013)

epigenome it could be possible to go “to the root” of the ageing process and influence multiple hallmarks simultaneously.

1.4. Epigenetic dynamics in ageing: normal mechanisms and age-related changes

Epigenetic modifications, including DNA methylation, histone modifications, and non-coding RNA molecules dynamically regulate gene expression patterns and contribute to cellular identity and function. These mechanisms ensure proper development, tissue maintenance, and response to environmental cues throughout an organism’s lifespan (Kane and Sinclair, 2019).

However, with advancing age, the epigenome undergoes profound changes at all levels of chromatin and DNA organization. Epigenetic alterations in ageing encompass reduced global heterochromatin and DNA hypomethylation, site-specific DNA hypermethylation, altered histone modifications, and dysregulation of non-coding RNA (ncRNA) expression (Kane and Sinclair, 2019).

As organisms age, there is a reduction in global heterochromatin and DNA hypomethylation, leading to genomic instability and activation of transposable elements, which can disrupt gene regulation and genome integrity (Kane and Sinclair, 2019; Lee et al., 2020). Site-specific DNA hypermethylation affects specific genomic regions, including gene promoters, and can result in the silencing of critical genes involved in cellular processes such as DNA repair, immune response, and metabolism. Furthermore, alterations in histone modifications, such as changes in histone acetylation and methylation patterns, impact chromatin structure and gene accessibility. These modifications influence gene expression profiles and can contribute to age-associated phenotypes (Kabacik et al., 2022; Kane and Sinclair, 2019). Additionally, dysregulation of non-coding RNAs, including microRNAs, long non-coding RNAs, and circular RNAs disrupts gene regulatory networks and cellular homeostasis, further contributing to age-related changes (Kabacik et al., 2022; Wang et al., 2022). These epigenetic changes also contribute to various ageing hallmarks including genomic instability, telomere attrition, loss of proteostasis, cellular senescence, and mitochondrial dysfunction (López-Gil et al., 2023).

Understanding the dynamics of epigenetic modifications during ageing is crucial for unraveling the molecular basis of age-related diseases and identifying potential therapeutic interventions (Ji et al., 2023a).

2. Epigenetic reprogramming

Reprogramming-induced epigenetic rejuvenation is an emerging field of research focused on countering the ageing process through the modification of epigenetic marks and gene expression patterns. Reprogramming can be carried out in different ways, namely complete reprogramming and partial reprogramming (Basu and Tiwari, 2021; Simpson et al., 2021a).

2.1. Complete reprogramming and partial reprogramming

2.1.1. Complete reprogramming

Complete reprogramming refers to the process of converting somatic cells into induced pluripotent stem cells (iPSCs), which exhibit distinctive characteristics such as self-renewal ability and the potential to differentiate into various cell types. Pluripotency is supported by a complex network of signaling molecules and genes, particularly Oct4, Sox2, and Nanog (Al Abbar et al., 2020; Teshigawara et al., 2017), which are transcription factors that play a crucial role in maintaining this characteristic. The interplay between external signaling molecules and internal factors leads to the development of a specific gene expression pattern and the establishment of an epigenetic state characteristic of stem cells (Simpson et al., 2021b).

This method allows for the generation of patient-specific pluripotent stem cells with fewer ethical concerns compared to embryonic stem cells (ESCs), arising from the destruction of embryos during ESC isolation since these cells are derived from either the inner cell mass or epiblast of blastocysts (Al Abbar et al., 2020; Teshigawara et al., 2017; Zakrzewski et al., 2019). iPSCs closely resemble ESCs in terms of morphology, growth behavior, and responsiveness to growth factors and signaling molecules. Like ESCs, iPSCs can differentiate in vitro into cell types from all three primary germ layers (Puri and Wagner, 2023).

In this approach, ageing and cellular differentiation are interconnected processes that are intricately linked and hence, cannot be separated. In other words, only by achieving a complete state of de-differentiation in cells and erasing their specific lineage identity, cells can suffer from epigenetic resetting and then be differentiated again. Although it has massive potential for regenerative medicine, this phenomenon does not seem to be appropriate for anti-ageing strategies, as it requires the loss of cellular identity and re-establishment of self-renewal capabilities (Al Abbar et al., 2020).

2.1.2. Partial reprogramming

On the other hand, partial reprogramming focuses on achieving epigenetic rejuvenation while retaining the original cell phenotype, rather than inducing pluripotency. To describe this type of rejuvenation accurately, the term "reprogramming-induced rejuvenation" (RIR) is more suitable, highlighting the nature of the process and the ultimate goal of the interventions. RIR holds potential as a safe anti-ageing treatment that can reverse ageing processes while preserving the identity of cells. This approach suggests that there is a safe time window for rejuvenation and full resetting of the epigenetic clock (Chen and Skutella, 2022; Chuang et al., 2017; Puri and Wagner, 2023; Simpson et al., 2021a; Talkhabi, 2019).

3. Reprogramming-induced epigenetic rejuvenation: emerging anti-ageing strategies

In this section, we briefly review the current emerging anti-ageing strategies, consisting of the well-known transcription factor-mediated reprogramming and other promising approaches, namely pharmacological interventions based on small molecules, in which DNA methyltransferase inhibitors and histone deacetylase inhibitors are included.

3.1. Genetically induced reprogramming mediated by Transcription Factors

One of the most remarkable and booming reprogramming strategies currently leverages on gene therapies mediated by the ectopic expression of transcription factors (TFs) (Fig. 1). The groundbreaking discovery of the Yamanaka factors, i.e., a cocktail of four reprogramming factors - Oct4, Sox2, Klf4, and cMyc (OSKM) - has revolutionized ageing research. The so-called partial reprogramming enables to reset of the epigenetic landscape of cells – DNA methylation patterns - rejuvenating cells and regenerating tissues, without reaching a pluripotency state, thus minimizing the risk of tumorigenesis (Galow and Peleg, 2022; Ji et al., 2023a; Puri and Wagner, 2023; Simpson et al., 2021a). Moreover, transient reprogramming influences major hallmarks of ageing at transcriptomic and cellular levels, such as autophagy levels and mitochondrial membrane potential (Sarkar et al., 2020).

This strategy has been able to dramatically reverse age-related phenotypes in many tissues in both cultured mammalian cells and rodent models. In one of the most cited studies to date (Lu et al., 2020), researchers showed that mammalian tissues retain a record of youthful epigenetic information that can be easily accessed to improve tissue function and regeneration in vivo. They were able to safely rejuvenate the age of neurons in retinal ganglion cells (RGCs) and, thus, reverse the vision loss in an aged mouse model of glaucoma. To achieve this, by adeno-associated virus (AAV) delivery, they expressed Oct4, Sox2, and Klf4 (OSK) TFs, excluding Myc, as it is an oncogene that reduces the

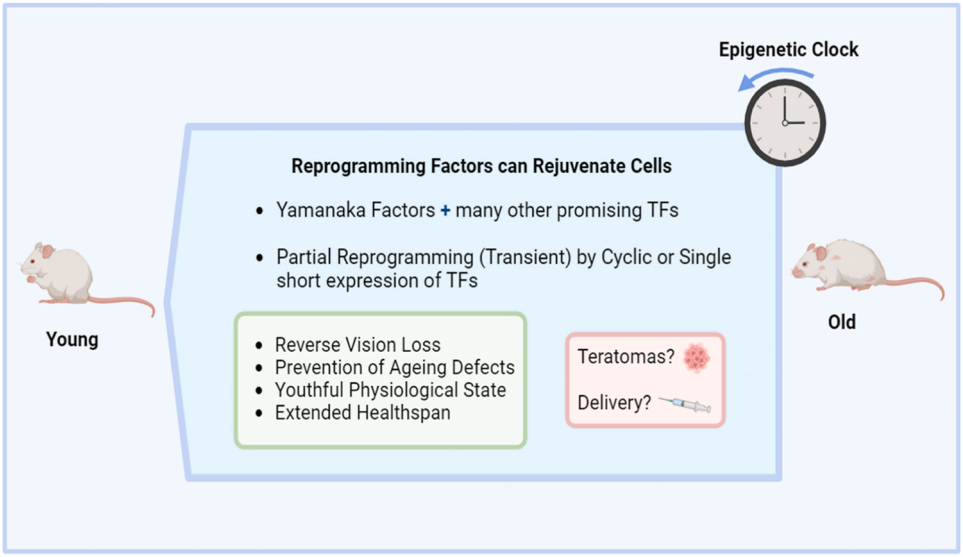

Fig. 1. Visual Representation of Epigenetic Reprogramming mediated by Transcription Factors (TFs) to reverse the epigenetic clock. Created with BioRender.com.

lifespan in mice. Reversal of DNA methylation of RGCs was intricately linked with axon regrowth and restoration of youthful vision, without oncogenicity or loss of identity (Lu et al., 2020). Indeed, the continuous expression of OSK in the RGCs of glaucomatous mice enabled a year-long significant improvement in the visual function without detrimental effects (Karg et al., 2023). In another study, the Yamanaka factors combined with two accessory factors (Lin28 and Nanog) were transiently expressed before the so-called Point of No Return (PNR), in which the cells return to the initiating somatic cell state (Sarkar et al., 2020). This way, the expression of the TFs in a short time (4 consecutive days) was enough to reprogram the cellular age while failing to erase the complete epigenetic signature, and the methylation age was reversed approximately by 5 years in human endothelial cells. This study was additionally extended to human-aged chondrocytes and murine skeletal muscle stem cells, also achieving partial reverse of gene expression, youthful physiological state, and enhanced regenerative potential (Sarkar et al., 2020). To decipher more closely the mechanisms involved in partial reprogramming, changes in the DNA methylome, transcriptome, and serum metabolites were recently studied in aged mice exposed to a single cycle of transient OSKM expression (Chondronasiou et al., 2022). Rejuvenation was achieved in the pancreas, liver, spleen, and blood in a systemic manner in vivo, even with low reprogramming, thus minimizing the risk of teratoma formation. Interestingly, this study showed that reprogrammed cells may influence the rejuvenation of non-reprogrammed cells, through secretion of soluble factors (Chondronasiou et al., 2022). However, to rejuvenate the transcriptome of senescent cells present in aged tissues, multiple cycles of OSKM expression may probably be necessary. Also, the authors discussed that the recovery period after OSKM expression is highly critical, as many rejuvenation changes occurred weeks after finishing the treatment (Chondronasiou et al., 2022).

Going further, a single short reprogramming of OSKM (i.e., 2.5 weeks treatment) was also enough to prevent musculoskeletal deterioration and fibrosis in mice, when applied in early life, thus, extending healthy lifespan by 15% (Alle et al., 2022). On the other hand, cyclic induction of the OSK cocktail has been shown in a recent preprint to extend the median remaining lifespan by 109% and enhance several health parameters, when applied in later stages of life, i.e., in 24-week-old mice (~77 years in human age) (Cano Macip et al., 2023). These could be promising data for preclinical proof of low dosage and minimally invasive, safe, partial reprogramming potential usage for clinic therapeutic interventions to prevent and reverse ageing defects in both the young (Alle et al., 2022) and elderly individuals (Cano Macip et al., 2023). In addition to the traditional Yamanaka factors, there are many other promising candidates for cellular age reprogramming, **(**Table 2**).** For instance, cyclic expression of a truncated version of FOXM1 has been shown to delay senescence-associated progeroid and natural ageing phenotypes in mice (Ribeiro et al., 2022). Also, the highly conserved chromatin-modifying complexes, Polycomb Group (Pcg) proteins, such as Tet proteins, have been shown to be highly involved in the process of epigenetic reprogramming of cells (Singh and Zhakupova, 2022). Going further, to assess the safety and efficacy of the previously cited partial rejuvenation studies, larger animal models will be needed before advancing to human studies. Notably, very recently, visual function has been successfully restored in a nonhuman primate clinical model for the very first time using the OSK Yamanaka cocktail factors (Ksander et al., 2023). This is an unprecedented step forward for the first human clinical trials in the future, not only for this condition but also for many other age-related phenotypes. We firmly believe this cellular rejuvenation technology will lead to the future of longevity research. Moreover, to enhance complete cellular reprogramming, combinatorial approaches could be further explored. For instance, the additional silencing of the

Table 2
Summary of promising transcription factors (TFs) and respective key findings, in alternative to the classical Yamanaka factors for Epigenetic Reprogramming approaches.

Promising TFs	Key Findings	Reference
Msx1	Restores youthful gene expression when transiently expressed in aged myogenic cells in mice.	Roux et al. (2022)
FOXM1	Cyclic expression delays natural ageing and extends lifespan in mice.	Ribeiro et al. (2022)
Tet1, Tet2	*In vivo* reprogramming to reset epigenetic clock to regenerate retina is Tet-dependent in mice.	Lu et al. (2019)
ATOH1, Gf1	Co-expression regenerates mature mammalian cochlear hair cells.	Lee et al. (2020)
Top2a	Topoisomerase2 is required for partial reprogramming in vitro and in vivo, to enhance liver plasticity and regeneration in mice.	Hishida et al. (2022)
Ascl1, Brn2, Myt1l (BAM factors)	Can induce rapid changes in fibroblast transcriptome to a neuronal one to enable successful reprogramming.	Basu and Tiwari (2021)
bHLH	Generates neurons after reprogramming, successfully used for ischemic injury recovery in mice.	Basu and Tiwari (2021)

senescence-associated microRNA-195 and of the long non-coding RNA Zeb2-NAT has been shown to facilitate reprogramming into iPSCs (De Jesus et al., 2018; Kondo et al., 2016). Future studies should also focus on elucidating the mechanisms by which epigenetic reprogramming may rejuvenate cells and alleviate many ageing hallmarks. Moreover, the "point of no return", in which cells revert to a fully embryonic-like state is still quite unclear, as it can range from a week to only a few days in different studies, and even transient reprogramming may lead to iPSC formation and tumorigenesis. Finally, delivery of transcription factors based on viral vectors can be challenging, as these have shown potential safety concerns and off-target effects in gene therapies (Rilo-Alvarez et al., 2021; Yang et al., 2023).

3.2. Chemically induced reprogramming mediated by small molecules

The concerns regarding reprogramming methods reliant on transcription factors prompted researchers to explore alternative approaches (Kim et al., 2020). As a result, there has been a shift toward small-molecule-mediated reprogramming, as small molecules are cost-effective, easy to control, and can be administered orally, making them ideal for large-scale production (Kim et al., 2020). These compounds of less than 500 Da in size can target any portion of a molecule, including enzymes, receptors, and signaling pathways (Megino-Luque et al., 2020).

Many small molecules have emerged for chemical reprogramming, which can be broadly classified into three major categories: signaling, epigenetic, and metabolic modifiers (Knyazer et al., 2021). Given that the scope of this review is focused on epigenetic reprogramming, we will focus on small molecules that fit into the epigenetic modifiers category. Most inhibit either methyltransferases (see Section 3.2.1.) or histone deacetylases (see Section 3.2.2.), while others possess either dual activity or combined activities (Knyazer et al., 2021). Several combinations of small molecules have been tested so far for their ability to reprogram cells, and at least 10 of these chemical cocktails have been established including components such as 5-azacitidine, trichostatin A, DZNep, and valproic acid. Researchers have successfully reprogrammed somatic cells into iPSCs using chemical cocktails (Kim et al., 2020; Knyazer et al., 2021; Lu et al., 2023; Zhong et al., 2021). More importantly, they were able to induce partial cell reprogramming without transfection of stemness-related TFs, showing the ability to rejuvenate cells without altering their genomic identity (Yang et al., 2023), thereby offering a safer alternative to genetic manipulation methods.

3.2.1. DNA methyltransferase inhibitors

DNA methylation is an epigenetic modification that involves the addition of a methyl group to the C-5 position of cytosine residues, forming 5-methylcytosine (5mC). This process occurs mainly in cytosine-phosphate-guanine (CpG) dinucleotides, and it is catalyzed by DNA methyltransferases (DNMTs) to regulate gene expression and cell differentiation during the development (Xiao et al., 2019). Other forms of DNA methylation include 4-methylcytosine and 6-methyladenine (Xu et al., 2021).

The association between abnormal DNA methylation patterns and ageing has been extensively reported (Wilkinson et al., 2021; Xiao et al., 2019; Xu et al., 2021). Indeed, age-dependent alterations in DNA methylation, including global hypomethylation and site-specific hypermethylation, have been linked to various age-related diseases, including cancer, diabetes, neurodegenerative disorders, and cardiovascular diseases (Aguet et al., 2017; Al-Haddad et al., 2016; Jin et al., 2018; Locke et al., 2019; Luo et al., 2022; Mitsumori et al., 2020). Therefore, targeting DNMTs with specific inhibitors to delay or reverse these pathologies has emerged as a potential anti-ageing strategy.

Several DNMT inhibitors (DNMTis) including 5-azacitidine (i.e., Vidaza®), decitabine (i.e., Dacogen®), and RG108 have been identified in clinical and pre-clinical stages as potential therapeutics to prevent age-related diseases. However, only 5-azacitidine and decitabine are FDA-approved as anti-tumor agents (Dhillon, 2020; Kaminskas et al., 2005).

5-azacitidine (5-AZA) and decitabine are cytidine analogs that inhibit DNA methylation by forming an irreversible covalent bond with DNMTs once incorporated into the DNA sequence. The resulting hypomethylation can reverse age-related changes in DNA methylation patterns and improve cellular function (Giri and Aittokallio, 2019; Zhang et al., 2022). This has been demonstrated in various cell types, including immune cells, stem cells, and neurons, and it is clinically used in the treatment of myelodysplastic syndrome and hematologic tumors (Daskalakis et al., 2002; Kantarjian et al., 2006; Schmelz et al., 2005). A study on human adipose-derived stem cells showed that 5-AZA was able to reverse their aged phenotype, revealing its therapeutic potential in slowing down and even reversing age-related changes in stem cells (Kornicka et al., 2017). In another in vitro experiment, decitabine has recently been shown to enhance the differentiation of human MSCs into insulin-secreting cells, thus potentially establishing a regenerative solution for patients with diabetes (Elsharkawi et al., 2020). Furthermore, comparative studies evaluating healthy human and Alzheimer's disease (AD) patients samples have indicated the role of DNA methylation in the progression of AD associated with ageing, making both 5-AZA and decitabine promising therapeutic options for targeting age-related neurodegenerative diseases (Altuna et al., 2019; Gu et al., 2021; Mitsumori et al., 2020).

RG108 is a small molecule DNMT inhibitor that has also shown promise as an anti-ageing agent in preclinical studies. RG108 was first identified as a compound that selectively inhibits the activity of DNMT1, the primary DNMT responsible for maintaining DNA methylation patterns during DNA replication. Unlike other DNMTis, which can also inhibit other DNMT isoforms, it has a high specificity for DNMT1, making it a potentially safer and more effective therapeutic agent (Zheng et al., 2021). This compound has been reported to alleviate cellular damage caused by oxidative stress by restoring the altered methylation pattern of senescence-associated genes in human bone marrow mesenchymal stromal cells (MSCs), which holds promise for improving treatment efficacy in ageing-related diseases (Li et al., 2020). In amyotrophic lateral sclerosis (ALS), RG108 was able to restore the function of MSCs isolated from ALS patients by inhibiting DNMTs (Assis et al., 2018). In another study using a mouse model of ALS, this agent improved motor function and extended the lifespan by 25% (Martin et al., 2022). These findings suggest that RG108 could potentially reverse the detrimental effects of age-related neurodegenerative disorders. Moreover, a recent study showed that RG108-treated macrophages improved the vascular function of aged mice by reducing DNA methylation and increasing the expression of genes associated with arteriogenesis, highlighting the potential of RG108 as a therapeutic intervention for age-related vasculopathies (Mantsounga et al., 2020; Xiao et al., 2019).

In the field of epigenetic reprogramming, both 5-AZA and RG108 have been shown to improve the reprogramming efficiency induced by different transcription factors. 5-AZA can enhance reprogramming by OSKM in a dose-dependent manner (Huangfu et al., 2008; Shi et al., 2008). Notably, 5-AZA treatment has also been observed to drive partially reprogrammed cells toward full reprogramming (Basu and Tiwari, 2021). Despite the potential exhibited by these DNMT inhibitors, there is still limited experimental evidence regarding their direct effects on age-related diseases.

3.2.2. Histone deacetylase inhibitors

Ageing is associated with changes in histone acetylation, particularly alterations in specific histone marks and in the expression of histone deacetylases (HDACs) (Lee et al., 2021). The acetylation of core histones is controlled by the opposing actions of histone acetyltransferases (HATs) and HDACs, whose activities are correlated with gene activation and gene silencing, respectively (Al-Mansour et al., 2023; Lee et al., 2021; McIntyre et al., 2019; Pasyukova and Vaiserman, 2017).

Acetylation neutralizes the electrostatic interaction between histones and DNA, leading to a more accessible chromatin conformation for transcriptional machinery. In contrast, HDACs remove acetyl groups from histone lysine residues and other regulatory proteins, resulting in a condensed and transcriptionally repressive chromatin (Al-Mansour et al., 2023; Lee et al., 2021; Pasyukova and Vaiserman, 2017). Mammals have 18 distinct HDACs categorized into four classes: Class I, subclass IIa, subclass IIb, Class III, and Class IV (Wilkinson et al., 2021; Xiao et al., 2019; Xu et al., 2021). Classes I, II, and IV HDACs employ zinc as a co-factor for catalytic activity, while class III HDACs (SIRTs) require NAD+ for enzymatic function (Al-Mansour et al., 2023; McIntyre et al., 2019). Sirtuins (SIRTs) are structurally and functionally different from the other classes of HDACs. Their dependency on NAD+ links sirtuins to cellular metabolism and energy status (Wang et al., 2022). By sensing NAD+ levels, sirtuins can respond to fluctuations in cellular energy and nutrient availability, allowing them to coordinate cellular responses to various stressors, including those associated with ageing. Since they are more related to other hallmarks, they wont be covered in this review. HDAC inhibitors (HDACi) are small molecules that target the active site of various HDAC enzymes, leading to inhibition of their activity (Ji et al., 2023a) and have shown promise in the treatment of age-related chronic disorders, targeting not only epigenetic changes but also, indirectly, the remaining hallmarks of ageing (McIntyre et al., 2019; Pasyukova and Vaiserman, 2017). In detail, these modulate gene expression patterns by reversing age-related changes in histone acetylation, directly activating pro-longevity genes, inducing protective gene activation, and modifying the acetylation state of non-histone proteins (Al-Mansour et al., 2023). Additionally, HDACi may impact several mechanisms, such as telomere lengthening, DNA repair, proteostasis, mitochondrial dysfunction, and cellular senescence. In preclinical studies, HDACi show promise in preventing obesity and insulin resistance; improve cardiac function (treating atherosclerosis) and muscle mass (combating sarcopenia); alleviate neurodegenerative phenotypes restoring memory deficits, reducing amyloid plaque deposition, and activating synaptic plasticity markers. In cancer, these small molecules display antitumor effects by reactivating tumor suppressor genes (e.g., p53) and repressing proto-oncogenes. Furthermore, HDACi have exhibited anti-inflammatory effects, suggesting their capability to treat age-related immunosenescence and inflammageing (Al-Mansour et al., 2023; McIntyre et al., 2019). Moreover, HDACis have shown potential therapeutic applications in premature ageing conditions like Hutchinson-Gilford progeria syndrome (HGPS) and Cockayne syndrome (CS), by reducing progerin levels, restoring heterochromatin organization, reorganizing gene expression patterns, improving autophagic function, and thus rescuing skin phenotypes (Al-Mansour et al., 2023). These findings suggest that HDAC inhibitors have the potential to counteract multiple aspects of ageing and offer possible strategies for promoting healthy ageing (Al-Mansour et al., 2023; McIntyre et al., 2019).

Some widely used HDACis include sodium butyrate, valproic acid, phenylbutyrate, and trichostatin A (Lu et al., 2023). Regarding the licensed sodium butyrate, this inhibitor induces hyperacetylation of histone H4, reducing senescent cells, extending lifespan, and improving various age-related phenotypes mentioned above. Panobinostat® also demonstrates a senolytic effect. In a previous study, the effects of the ketone body D-beta-hydroxybutyrate, a class I HDACi, were determined on *Caenorhabditis elegans*. The results showed that this compound was able to inhibit HDAC and activate stress response pathways, including DAF-16/FOXO, increasing the lifespan of *C. elegans* by about 20% (Edwards et al., 2014). In a recent preprint, a cocktail of Tranylcypromine and Repsox was able to strikingly extend the lifespan of *C. elegans* by 42.1% (Schoenfeldt et al., 2022).

Overall, HDACis show promising results in preclinical studies, exhibiting the ability to rejuvenate ageing cells and tissues, enhance cellular reprogramming, and activate pathways related to cellular metabolism and stress response (Wang et al., 2022). Nonetheless, further research is needed to explore their specific mechanisms of action and improve their specificity and safety for human clinical translation (McIntyre et al., 2019).

4. Current challenges of reprogramming-induced rejuvenation to achieve longevity

Despite the potential of epigenetic and cell reprogramming to reverse the aged phenotype and delay ageing, several challenges are hindering the advancement of such strategies. To summarize, some of these limitations include:

a) Incomplete understanding: The knowledge of the epigenetic and cellular mechanisms underlying ageing is still not fully understood. Although significant progress has been made, there is still much to learn about the intricate processes that regulate gene expression and cellular reprogramming (Pagiatakis et al., 2021; Wang et al., 2022).
b) Long-term effects: The stability of the rejuvenated state achieved through reprogramming is a significant concern. Sustaining youthful characteristics and preventing the reversion to an aged state over extended periods is a complex challenge that requires continuous monitoring and optimization of reprogramming techniques (Wang et al., 2022).
c) Viral vectors: Viral-based delivery of reprogramming factors e.g. by AAVs, can lead to pathological insertional mutagenesis and reactivation of reprogramming factors (Chen et al., 2023; Guan et al., 2022). Moreover, as most AAVs show an inherent liver tropism, full-body rejuvenation strategies may be more challenging (Lu et al., 2023).
d) Neoplastic development: Reprogramming factors like OSKM genes may induce neoplastic development in reprogrammed cells (Taguchi et al., 2021), emphasizing the necessity to explore alternative reprogramming methods to mitigate potential carcinogenic risks. In addition, genomic integrations facilitated by reprogramming viruses can also trigger the development of tumors (Wuputra et al., 2020).
e) Translation to human rejuvenation: While reprogramming has shown success in rejuvenating animal tissues, the application of these findings to humans may require the development of new technologies. Hopefully, since epigenetics is the primary regulator of ageing and cellular reprogramming can rejuvenate cells at various levels, it may be feasible to develop the necessary technologies for applying reprogramming in human rejuvenation therapies (de Magalhães and Ocampo, 2022).

To address these challenges and unlock the full therapeutic potential of epigenetic reprogramming, further research, technological advancements, and rigorous validation studies are required to optimize the selection and characterization of reprogrammed cells.

5. Epigenetic clocks in age prediction and reversion

Epigenetic reprogramming strategies are able to reset the epigenetic clock and rejuvenate cells, but what does this really mean? Chronological age and biological age are distinct concepts: the former refers to the actual years that a person lives, while the latter takes into consideration the genetic makeup, environmental factors, and physiological status. Epigenetic clocks, also known as DNA methylation clocks have become the most promising candidates for estimating biological age. These powerful tools enable us to measure the effectiveness of longevity interventions such as epigenetic reprogramming, allowing us to accurately predict the biological age of tissues and humans, **(**Fig. 2**)** (Duan et al., 2022; Noroozi et al., 2021). These have been used in many of the previously cited studies in this review, for instance, for determining the most robust time range of transient reprogramming to achieve cell rejuvenation and to evaluate the effects of in vivo reprogramming in cell physiology. The gathering of genome-wide DNA methylation data from

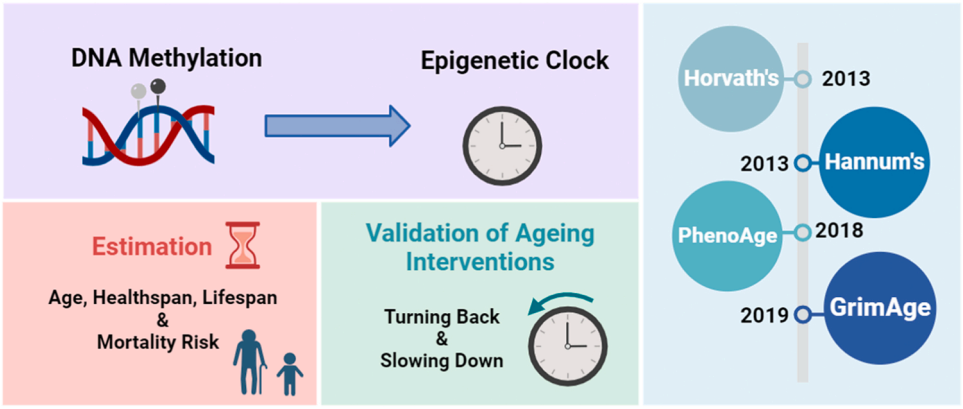

Fig. 2. Visual representation summarizing the contribution of Epigenetic Clocks to Ageing Research. Created with BioRender.com.

large datasets and high-throughput sequencing assays has enabled the development of these tools, based on mathematical models and machine-learning methods (Duan et al., 2022; Johnson et al., 2022; Noroozi et al., 2021). In essence, there are two types of epigenetic clocks. The first-generation clocks, including Horvath's, Hannum's, and Weidner's, consider chronological age as a surrogate measure of ageing and regress it on DNA methylation status of selected CpGs sites in the genome, converting it into units of years. On the other hand, the second-generation clocks, such as PhenoAge and GrimAge, derive biological age based on surrogate clinical biomarkers of organ functionality and physiological regulation, being more accurate predictors of lifespan, healthspan, mortality risk, and even epigenetic age acceleration. Horvath's model is the most widely used clock to strongly predict age, which includes 353 CpG sites from multiple cells, tissues, and organs, including blood, kidney, liver, etc (Duan et al., 2022; Johnson et al., 2022; Simpson et al., 2021a). However, recent research has shown that the GrimAge DNAm clock seems the most reliable and promising one, accurately predicting age-related clinical phenotypes such as frailty and cognitive function (McCrory et al., 2021). All in all, these epigenetic predictors have ushered a new era of molecular research in the field of ageing and will undoubtedly be the key to advancing and refining epigenetic reprogramming interventional studies. Further research is needed to better understand the limitations of existing epigenetic clocks and to develop new ones that are more specific to certain tissues and cell types.

6. The rise of longevity biotech industry

Not only is cellular reprogramming thriving in academic research, but it is also propelling a novel field in Biotech industry. Numerous recent companies have been currently invested billions of dollars, not only to treat age-related diseases, but especially to rejuvenate tissues, organs, and even whole humans, (Table 3). Although quite ambitious, the pursuit of human lifespan extension is undoubtedly an exciting desire, and in the foreseeable future, the longevity industry is expected to expand substantially (de Magalhães et al., 2017; de Magalhães and Ocampo, 2022).

7. Economical gains and ethical issues

What are the benefits of targeting ageing itself compared to treating individual age-related diseases? There could be colossal economic gains by increasing the healthspan and slowing down ageing, as people will contribute to the economy for a longer period. Notably, it is estimated that slowing down ageing by 1 year is worth US$38 trillion, and by 10 years could be even US$367 trillion (Scott et al., 2021). Longevity research could lead to a virtuous circle, where delaying the aging process leads to an increased average age within society. This could enhance the overall quality of life, potentially benefiting individuals from further anti-ageing therapies. In contrast, current traditional treatments that succeed in eradicating individual age-related diseases only lead to short-term gains in society (Fig. 3) (Scott et al., 2021).

The rise of ageing research also opens the door for several ethical and social concerns that should be discussed, (Fig. 4). What does it really mean to "reverse" or "delay" ageing? Is ageing a disease? Scientific research is still in its infancy and ageing interventions should be accurately validated before clinical translation, regarding the optimal period, frequency, and suitability of interventions. Furthermore, metaphysical concerns are raised as humans seem to be trying to play Gods. Is it morally acceptable to extend human life? If ageing is an integral part of the human experience, intervening in this process may be viewed as altering the fundamental nature of what it means to be human. Cultural, social, and religious factors lead to conflicting opinions, and resolving these philosophical issues will not be easy. Furthermore, in a time of such animal welfare concerns, the use of animal models in longevity research is a major obstacle. Also, demographic, economic, and sociocultural implications could be also raised. Without the proper regulations, longevity interventions could exacerbate the existing social inequalities, with only the wealthy and privileged being able to afford them. On the other hand, if these interventions are made accessible to everyone equally, these could lead to a growing overpopulation with devastating impacts on the environment, resource depletion, and strains on infrastructures. Furthermore, if ageing is seen as a disease, could it lead to prejudice and stigmatization of older individuals? To advance ageing research, there must be a widely acceptable redefinition of ageing. Moreover, there should be a responsible governance in its regulatory structure and processes, as well as appropriate legislation and regulations to ensure ethical, safe, and effective longevity research and interventions, specifically the promising epigenetic interventions. To finish, instead of a privilege, the pursuit of healthy longevity must be regarded as a fundamental humanitarian right to improve the quality of life, accessible to all members of society (Peng et al., 2023; Woo et al., 2019).

8. Outstanding questions

In ageing research, numerous intriguing questions remain unanswered. Here, we delve into key questions surrounding the molecular complexities of epigenetic dysfunction, the translational relevance of animal models, the prospects of rejuvenation interventions, the potential of epigenetic reprogramming as a routine procedure, the reliability of epigenetic clocks, and the ethical considerations surrounding anti-ageing advancements.

Table 3
Summary of Rejuvenation Biotech companies through cellular reprogramming.

Name & Launching Year	Aim	Approach	URL
AgeX Therapeutics (2017)	Unlock cellular immortality and regenerative capacity to reverse age-related changes in the body	Using pluripotent stem cells plus partial reprogramming to induce tissue regeneration	https://www.agexinc.com/
Altos Labs (2022)	Reversing disease to transform medicine	Cellular rejuvenation programming to restore cell health and resilience	https://altoslabs.com/
Calico (2013)	Understand the biology of ageing and age-related diseases	Various, including the use of transient reprogramming	https://www.calicolabs.com/
Gameto (2020)	Solve the problem of accelerated ovarian ageing	Applying cellular reprogramming to create human reprogrammed cells of the ovary	https://gametogen.com/
Iduna Therapeutics (Life Biosciences) (2017)	Develop epigenetic reprogramming therapies that allow the rejuvenation and replacement of tissues	Proprietary gene therapy that expresses OSK to reprogram the epigenome back to a younger state	https://www.lifebiosciences.com/
NewLimit (2022)	Radically extending human healthspan	Using epigenetic reprogramming therapies	https://www.newlimit.com/
Retro Bio (2021)	Develop therapies for diseases driven by the biology of ageing	Pursuing multiple programs, including cellular reprogramming approaches	https://retro.bio/
Shift Bioscience (2017)	Develop drugs that safely reset cells and tissues to a youthful state	Employing machine learning to better understand the causes of cellular rejuvenation	https://www.shiftbioscience.com/
Turn Biotechnologies (2018)	Develop mRNA medicines, focused on reprogramming the epigenome to restore capabilities that are often lost with age	Using mRNA cocktails for the delivery of reprogramming factors	https://www.turn.bio/
YouthBio Therapeutics (2021)	Develop gene therapies aimed at restoring more youthful epigenetic profiles	Employing partial reprogramming	https://youthbiotx.com/

Source:Adapted from (de Magalhães and Ocampo, 2022) with permission.

1. What are the specific molecular mechanisms behind epigenetic dysfunction that contribute to the ageing process and how do these correlate with the different hallmarks of ageing?
2. To what extent can the current in vivo aged animal models be translatable to the human ageing process in its entirety? Will emerging humanized in vitro 3D models such as organoids accelerate longevity research?
3. Realistically, how far are we from reprogramming-induced epigenetic rejuvenation interventions in human clinical trials? Will these rejuvenate organs and even the entire human body? Could these prevent and eradicate ageing-related diseases safely?
4. In the future, could epigenetic reprogramming be a routine medical procedure to reverse the biological age and extend human healthspan? Would these interventions be effective in both young and elderly individuals? How far could we go?
5. How reliable could epigenetic clocks be in research and clinical settings for developing and prescribing novel healthspan-prolonging interventions?
6. Will legislative and policy frameworks be able to keep pace with the scientific breakthroughs in the young science of anti-ageing treatments? How will bioethicists, society, and medical professionals perceive these emerging findings?

9. Future perspectives and conclusions

Overall, epigenetic reprogramming is currently the most promising strategy to be harnessed for age reversal and human rejuvenation. Notably, according to the ITOA, the loss of epigenetic information throughout someone's life is driven by DNA breakage and repair-induced changes and this is a reversible cause of ageing (Lu et al., 2023; Yang et al., 2023). In other words, ageing in mammals is just now beginning to be perceived as a software problem in the system, which can be easily rebooted to restore the corrupted epigenetic information from an existing backup copy (de Magalhães, 2023; Yang et al., 2023). With such groundbreaking hypotheses, the manifestation of age-related diseases may one day be prevented and even reversed, which may lead to a revolutionary paradigm shift in traditional medicine. The plasticity and modulation of the epigenetic landscape play a pivotal role in the ageing process, nevertheless, such complexity is yet to be fully deciphered. Moreover, the ageing field is still in its infancy, and translating rejuvenation strategies to humans should be cautious, facing many hurdles, from long-term stability to safety and efficacy aspects to ethical and legislative considerations. Currently, the vast majority of epigenetic reprogramming research leverages on the well-acclaimed Yamanaka factors. Nonetheless, as gene therapies face many hurdles regarding their safe and efficacious delivery, alternative reprogramming strategies such as the use of small molecules for chemical-based cell rejuvenation is a highly promising alternative. Notably, in the future, artificial intelligence may further speed the screening of novel compounds for mammalian age reversal (Yang et al., 2023). Other alternative rejuvenation approaches using young blood plasma perfusion or parabiosis have also shown favorable effects against various aged-associated diseases in mice. Notably, numerous screened blood factors can restore youthful traits, revitalize organ function, and reduce the DNA methylation age (Hosseini et al., 2023; Ji et al., 2023b; Lu et al., 2023). Moreover, exploring the power of synthetic biology may be an interesting route to take in the future of longevity biotech, for instance, CRISPR/dCas9 has been recently employed for safer cellular reprogramming-based rejuvenation strategies in progeria mouse models (Hu et al., 2023; Kim et al., 2023). Furthermore, with the advancement of cutting-edge epigenetic clocks, the efficacy evaluation of reprogramming interventions and the discovery of novel ageing biomarkers will be widely useful in the field of longevity. More reproducible and widely established clocks will be necessary to identify the ideal timing of reprograming interventions (Lu et al., 2023). With these promising prospects, we stand optimistic to unlock new horizons in the pursuit of youth and extend healthspan for all.

CRediT authorship contribution statement

Beatriz Pereira: conceptualization, writing – original draft. **Francisca Correia:** conceptualization, writing – original draft. **Inês A. Alves:** conceptualization, writing – original draft. **Margarida Costa:** conceptualization, writing – original draft. **Mariana Gameiro:** conceptualization, writing – original draft. **Ana P. Martins:** supervision, writing - reviewing and editing. **Jorge A. Saraiva**: supervision, conceptualization, writing - reviewing and editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

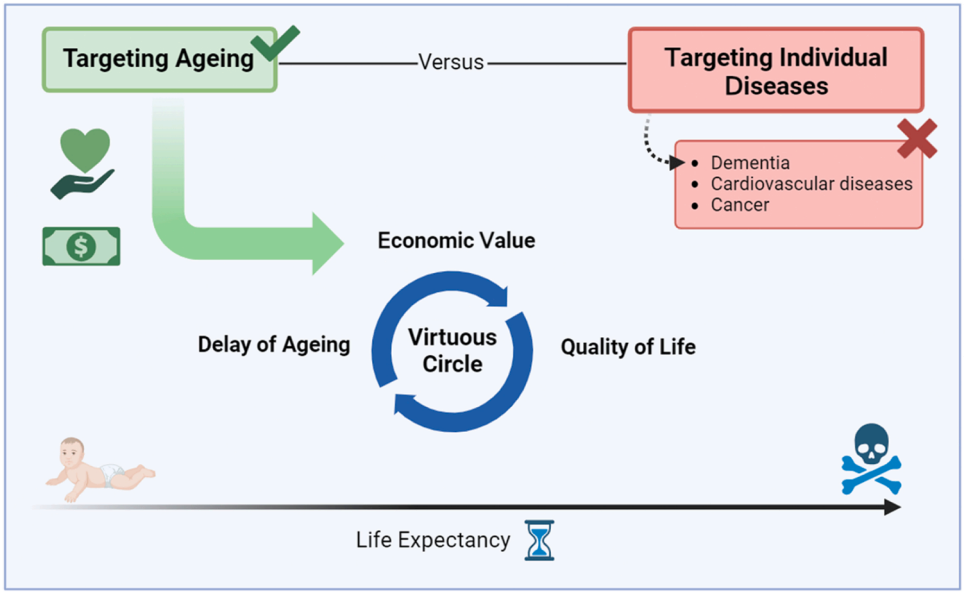

Fig. 3. Visual Representation of the economic gains of targeting ageing, in comparison with the traditional medicine. Created with BioRender.com.

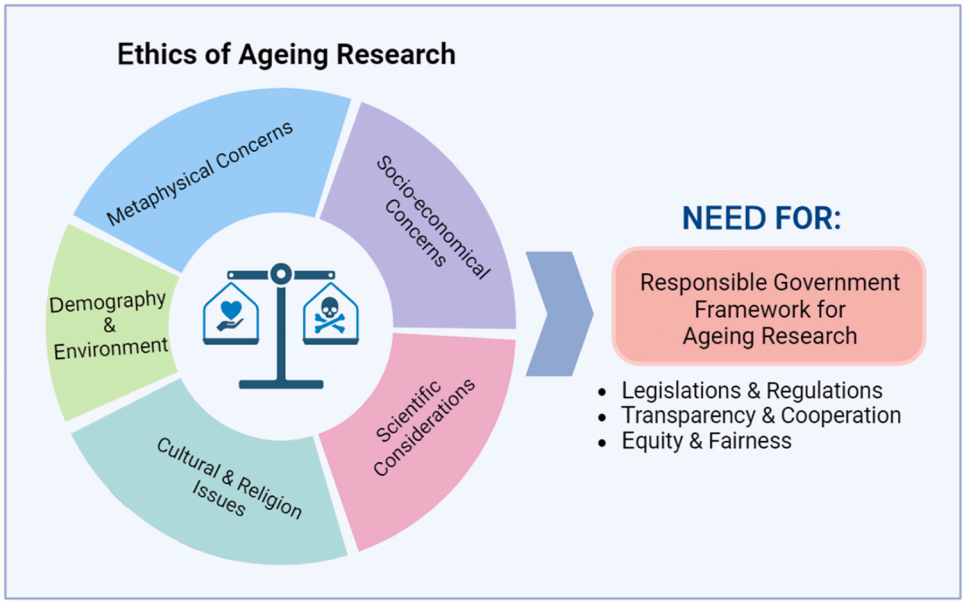

Fig. 4. Visual Representation of the main ethical concerns of targeting ageing and the necessary means to further advance the longevity field. Created with BioRender.com.

Data availability

No data was used for the research described in the article.

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