Title: Ageing, Skeletal Muscle and Epigenetics

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Summary:

We are living in an ageing society. In 2019, 1 billion individuals were already aged over 60. The number of people in this demographic is predicted to reach 1.4 billion by 2030 and 2.1 billion by 2050 (WHO). In the USA, individuals over 65 represent the fastest growing segment of the population (US census bureau). Similar trends are seen in the UK, with 16.2 million people already aged over 60, equivalent to 24% of the total population (Age UK; https://www.ageuk.org.uk/globalassets/age-uk/documents/reports-and-publications/later_life_uk_factsheet.pdf). Indeed, in the UK, people over the age of 60 outnumbered those under the age of 18, for the first time in 2008. This statistic still prevails today.

Because of medical and biopharmaceutical progress, lifespan is increasing rapidly, but healthspan is failing to keep up. If we are to increase healthy living, then we need to begin to understand the mechanisms of how we age across the lifecourse, so that relevant interventions may be developed to facilitate 'life in our years', not simply 'years in our life'.

It is reported that only 25% of ageing is genetically pre-determined. This fits with observations of some families ageing very quickly and poorly and others ageing slowly and well. If this is indeed the case and the rate of ageing is not fixed, then this knowledge provides a significant opportunity to manipulate the impact of environmental influencers of age. With that in mind, it begs the question of what are the mechanisms of ageing and is there potential to manipulate this process on an individual-by-individual basis?

The focus of this manuscript will be on the process of muscle wasting with ageing (sarcopenia) and the potential of exercise and its underlying mechanisms to reverse or delay sarcopenia. There will be a focus on epigenetics in muscle wasting and the capability of exercise to change our skeletal muscle epigenetic profile for the good. The manuscript ends with considerations relating to facial ageing, Botox treatment and gene editing as a tool for plastic surgeons in future.

Theories of ageing:

As we age, we see a progressive decline in the functioning of all systems of the body, including the brain, the nervous system, the heart, lungs and vascular systems and our skeletal muscle, the latter in the face of increased fat mass and therefore associated metabolic dysfunction (1). Because of increasing lifespan, but not necessarily healthspan, a need prevails to determine the underlying biology of ageing.

If we are to develop interventions to sustain health, then a need exists to determine the biomarkers of healthy ageing. Given the fluid nature of ageing, large scale, lifecourse studies are required to capture the inter-individual variability of the ageing process. This will facilitate the targeting of appropriate interventions, ultimately increasing health and reducing the cost of life-style related diseases of ageing. In the absence of understanding the biology of ageing, such studies are required immediately. To assume that good health is simply the opposite of poor health will set us up to fail an ever-increasing ageing population.

One way to approach this challenge is to develop biomarkers of health based on the nine hallmarks of ageing proposed by Lopez-Otin *et al* (2). In their manuscript, the authors suggest: "Each 'hallmark' should ideally fulfil the following criteria: (i) it should manifest during normal aging; (ii) its experimental aggravation should accelerate aging and (iii) its experimental amelioration should retard the normal aging process and, hence, increase healthy lifespan" (2). These hallmark criteria are met with varying success, with the third being the most difficult to achieve, given the compensatory role that many proteins play, when something is knocked down. The hallmarks identified, together with an indication of underlying evidence relating to their importance in the ageing process are detailed in Table 1.

Impact of Exercise and Nutrition on Muscle wasting with ageing – healthspan vs. lifespan:

The progressive loss of muscle mass and strength with age, which is referred to as sarcopenia impacts negatively on health. Muscle wasting is not restricted to old age, with declining muscle mass evident from our early 30s. While ageing correlates strongly with muscle wasting, so too do inactivity, gender and chronic catabolic disease (cachexia). Non-pharmacological interventions to delay the processes of muscle wasting include resistance exercise and a healthy diet, with nutritional strategies focussing on the inclusion of protein, timing of intake and additional micro-nutrient supplementation (3). The maintenance of muscle mass with age will enable maintained function, metabolism, reduced morbidity and increased health.

Resistance exercise culminates in *de novo* protein synthesis, as a result of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) signalling pathway being activated (4). Furthermore, the combination of resistance exercise with the addition of appropriate nutritional interventions (including e.g. essential amino acids such as leucine rich supplementation) will maximally promote protein synthesis and ultimately increase muscle mass. This is achieved by optimised dual activation of mTORC1 by a protein called Ras homolog enriched in brain (Rheb). Rheb binds to the N-terminal domain of mTORC1, resulting in a shape change and activation of mTORC1. Rheb's interaction with mTORC1 is maximal when the mechanical load experienced by the muscle, because of resistance exercise, is sensed via various mechano-tranducers culminating in the movement of TSC2 (an inhibitor of mTOR) away from Rheb (5). Meanwhile leucine-rich protein is sensed by Sestrin2 that ultimately results in the movement of mTORC1 towards Rheb (6), culminating in greater Rheb activation of mTORC1, protein synthesis and muscle hypertrophy than either stimulus alone (7).

Older individuals do have an adequate, albeit delayed response to higher volume resistance exercise-induced protein synthesis (8), however, display 'anabolic resistance' to protein feeding, requiring larger amounts of essential amino acids after exercise to evoke a similar protein synthetic response to that seen in young adults (9). Despite these acute increases in mTORC1 stimulating protein synthesis and the maintenance or hypertrophy of muscle mass

in older individuals, the chronic activation of mTORC1 has surprisingly been demonstrated to evoke atrophy via feedback inhibition of mTORC1 and subsequent increased expression of the E3 ubiquitin ligases (10), as well as increased autophagy (11), evoking protein breakdown. Indeed, chronic and inappropriate activation of mTORC1 is also observed in aged muscle, where inhibition of mTOR, using rapamycin, can prevent some, albeit not all, of the reduction of muscle mass with age (12). Indeed, these data, in aged skeletal muscle tissue, make sense given the first studies demonstrating that reductions in mTOR using rapamycin may be important in extending lifespan in nematode worms (13). Six years later, these studies were extended to genetically heterogenous murine models, where rapamycin extended median and maximal lifespan of both male and female mice when fed beginning at 600 days of age (14). Furthermore, caloric restriction, which compromises mTORC1 activity has also been associated with extend lifespan (reviewed in (15)).

Another compound to inhibit mTOR is Metformin, an insulin-sensitiser that has also gained attention as an anti-ageing agent. Metformin acts upstream of mTOR, to inhibit its activity (15, 16). Therefore, it has garnered interest for its potential to extend lifespan. Metformin is already in use in the treatment of type 2 diabetes, therefore there is extensive biotech interest in the repurposing of metformin to slow the ageing process (for review see (17)).

These compelling data, if taken alone depict mTOR and its signalling pathway in a bleak manner, regarding ageing. With both pharmacological and non-pharmacological interventions, which limit its activity, enabling extended lifespan. Taken in isolation, this inhibition may appear to be a beneficial and effective route in delaying ageing. However, in the context of specific tissues, such as skeletal muscle, while the inhibition of the chronic inappropriate activation of mTOR experienced in old muscle may prevent some of the muscle mass loss experienced with age, its blockade (e.g. by rapamycin or metformin) may also compromise the acute protein synthetic response to resistance exercise and nutrients. Therefore, perhaps preventing

a healthy long life that would also benefit from the improved functional performance (i.e strength gains) associated with exercise and a healthy diet. Overall, raising the question of whether long life or healthy life is the holy grail of such research.

With this caveat in mind, when undertaking age related research in model systems, it is important to be sure not only what the outcome measure is, but also the impact such an intervention may have on the functioning of other key tissues in the body and thereby on health. Ultimately the goal is healthy long life, rather than long life per sé.

Skeletal Muscle and Sarcopenia – Beneficial Impact of Exercise and Nutrition:

To reiterate, as we age, we see a progressive loss in muscle mass, strength and function. This is not limited to old age, by the time we reach our 30's we have already lost ~5% of our peak mean muscle mass. This increases to ~15% in our middle age and ~30% in our 70s. Although difficult to prove, it is suggested that losses of ~40% of lean muscle mass are incompatible with life. This progressive decline in muscle mass and strength is worsened by acute/chronic illness, decreased physical activity and poor lifestyle choices (18). Furthermore, accelerated sarcopenia has negative implications for quality of life, with the disability threshold being reached at a younger age, with obvious implications to reduced independence (19). Sarcopenia is also associated with a worsened survival rate, regardless of the underlying cause (20). We would therefore argue, that maintaining muscle mass and function using exercise and nutritional strategies are fundamental to healthy ageing and something which, as discussed above, could be compromised by chronically inhibiting mTOR activity.

If lifestyle (e.g. exercise and nutrition) plays a key role in regulating muscle mass, then adaptability is key. If we can understand the mechanisms underpinning adaptation to lifestyle factors, then potentially we can influence not only lifespan, but also healthspan through sustained and healthy muscle mass. To challenge this hypothesis, our attention should focus

on environmental influencers of muscle mass regulation and therefore the role of epigenetics in skeletal muscle adaptation.

Epigenetics, muscle and ageing:

Extensive variability exists in the incidence and rates of sarcopenia. Only 25% of ageing is genetically pre-determined, suggesting significant potential to manipulate environmental influencers of ageing-related sarcopenia. It is therefore worth considering that rapid changes in gene expression, because of environmental influencers and not elicited via genetic variation/mutation, may be caused by epigenetic modifications in skeletal muscle.

Epigenetics enable the interaction between lifestyle/environmental factors and modifications to DNA and histones, without changes in the DNA sequence. These epigenetic 'tags' occurring because of lifecourse events can influence gene expression, which occurs as a result of: 1) histone tags, which open or condense chromatin. Examples include, histone acetylation, performed by Histone Acetyl Transferases (HATs) culminating in chromatin opening, thus facilitating target gene transcription. By contrast; 2) histone methylation, performed by Polycomb Repressive Complex 2, leads to chromatin condensation and repressed transcription. DNA methylation (hypermethylation) of cytosine–guanine (C–G) pairings, especially in CpG-rich regions called CpG islands and in important regulatory regions such as promoters, typically leads to chromatin condensation and repressed transcription (21, 22). Reduced methylation (hypomethylation) provides a more relaxed chromatin, enabling gene expression to occur (21, 22). A primary hallmark of ageing is an altered epigenetic landscape, with data from Zykovich et al, using DNA methylation arrays (covering 450,000 CpG sites) revealed that aged skeletal muscle (n=24 healthy aged males; age range: 68-89 years) was hypermethylated across the genome, compared to young (n=24 healthy young males; age range 18-27) muscle (23). Interestingly, the hypermethylation sites occurred predominantly within the bodies of the genes, rather than within the promotor regions and were reportedly

linked with axon guidance signalling, as evidenced using gene ontology mapping. Finally, the authors reported that they were able to classify young and old tissue, based on 500 of the CpG sites and as such were the first to identify an epigenetic signature of ageing muscle (23). Since then, it has been confirmed that the methylation status of approximately 200 CpG sites can accurately predict chronological age in skeletal muscle tissue (24, 25). We extended these findings using technology allowing greater coverage of 850,000 CpG sites in skeletal muscle tissue, as well as isolated skeletal muscle derived stem cells, from aged (mean 83 years) versus young adults (mean 27 years) (26). Indeed, we also demonstrated an accumulation of methylation (hypermethylation) in both aged muscle tissue and isolated muscle derived stem cells. With tissue displaying enriched hypermethylation in load/growth associated gene pathways such as focal adhesion and mTORC signalling and isolated cells displaying this enrichment in genes within the calcium signalling pathway. Like Zykovich *et al*. (23), we also determined that genes involved in axon-guidance signalling in both aged tissue and isolated muscle cells demonstrated enriched hypermethylation compared with their younger adult counterparts.

Wishing to extend understanding relating to the hypomethylating impact of aging in muscle cells *in vitro*, we utilised control unaged and replicatively aged C2C12 skeletal muscle cells (having undergone 30 population doublings). We identified that not only was the ability of these cells to form myotubes compromised as a result of the “ageing” process but that this capacity was worsened, if they were exposed to a single dose of the cytokine TNF-α, prior to the population doublings occurring (27). Furthermore, a second dose of TNF-α, later in the population doubling cycle, reduced the capacity still further, compared to those cells only receiving the cytokine later in life. These findings suggested a memory of the initial catabolic insult. Investigating this concept further, we determined that those myoblasts that experienced a single, early acute cytokine stress had significantly elevated myoD methylation after 30 population doublings, compared with “age-matched” controls. This is an important finding,

given that myoD is a key myogenic regulatory factor, whose methylation and therefore down-regulation would impact negatively on myoblast identity. These data therefore illustrate the retention of DNA methylation of an important myogenic regulatory gene throughout the proliferative lifespan of myoblasts that may influence atrophy in later life (27).

Exercise and Epigenetics: The beneficial impact on Muscle Adaptation:

Evidence now exists for hypermethylation with ageing to impact on genes important for myogenic potential. By contrast, lifelong physical activity is associated with a hypomethylated genome in the skeletal muscle of aged men (28) and younger individuals who undertake more physical activity (29). This contrasts with the hypermethylation observed in ageing muscle and questions whether exercise could be used as an effective intervention to reduce the hypermethylated methylome of ageing muscle and therefore epigenetically rejuvenate alterations in aged associated gene expression. We therefore first questioned, could exercise in human, like inflammation in mouse muscle cells, alter an epigenetic signature, but this time for good.

In research undertaken by Seaborne *et al* (30) eight previously untrained male participants (27.6 ± 2.4 yr, 82.5 ± 6.0 kg, 178.1 ± 2.8 cm, means ± SEM) completed an acute bout of resistance exercise (acute RE) followed by: 1) 7 weeks (3d/week) of resistance exercise (training), 2) 7 weeks exercise cessation (detraining) and finally 3) 7 weeks (3d/week) of resistance exercise (retraining) in a 21 week exercise study. Muscle mass and strength were analysed and revealed that following the first 7 weeks of training there was a significant increase in lean mass (analysed by DEXA) and strength. The seven weeks of detraining resulted in a return to baseline of both measures. Interestingly in the second bout of retraining the capacity to increase mass was not only significantly increased above starting baseline levels, but also above the increases observed following the first seven week training intervention. Skeletal muscle biopsies revealed that the first bout of training culminated in

approximately 8,000 CpG sites, which were hypomethylated and 9,000 CpG sites, which were hypermethylated. Similar numbers of CpG's were hypo- and hypermethylated following the detraining period. Interestingly, however, with retraining, now ~8,000 CpG sites were hypermethylated, but >18,000 CpG sites were hypomethylated, begging the question, whether this large increase in DNA hypomethylation could be involved in the increased mass and strength evident in the second later 7-week programme of retraining exercise (30).

Extending these findings at the gene expression level, it was identified that some CpG sites retained their hypomethylated and turned-on signature during detraining (even when lean mass was completely lost back to pre-exercise levels) following the first training period. This suggested that the DNA retained this methylated signature over a significant amount of time, suggestive of a molecular 'memory' at the epigenetic (DNA methylation) level. A phenomenon that has also been confirmed after weighted wheel running training in mice, where DNA methylation signatures were retained into detraining (31, 32). Furthermore, in the original human studies, a cluster of genes demonstrated hypomethylation and increased gene expression after training that were further hypomethylated with even larger enhancements in gene expression after later retraining, also demonstrating an epigenetic memory at the DNA level of the earlier training period, that led to enhanced gene expression following a later period of retraining. The authors also demonstrated that ~ 30% of genes that demonstrated hypomethylation across the methylome also had increased gene expression across the transcriptome after both acute resistance exercise and chronic training (33). With a larger predominance of these genes demonstrating hypomethylation and switched on gene expression profiles, specifically in promoter regions in response to resistance exercise (33). Overall, as suggested above, these data point to the interesting hypothesis that resistance exercise is a hypomethylating and gene switching on stimulus and that even following periods of inactivity where muscle returns to pre-exercise levels of mass and strength, undertaking training again in the future could have an even greater hypomethylating impact, resulting in

the hypothesis that periods of training throughout life could counteract the accumulation of methylation observed in ageing skeletal muscle.

Interestingly, altered methylation of HOX family of genes was also identified in aged vs. young muscle tissue and primary human skeletal muscle-derived cells (29). Further, the impact of physical activity (n=30 endurance trained men) on HOX methylation was assessed, and revealed that highly active men displayed hypomethylated HOXB1, HOXA3, HOXD12 and HOXC4 compared to less active men. However, the opposite trend (to active males) was evident in aged muscle and cells, particularly for HOXB1 and HOXA3 which were hypermethylated in aged tissue and cells and displayed reduced gene expression. Finally, increasing levels of physical activity were associated with reductions in methylation in these HOX genes (29). Also suggesting increased physical activity may prevent the age-related epigenetic changes observed in the HOX family of genes.

Indeed, work by Thomis *et al* questioned whether increased physical activity in the form of resistance exercise could prevent age-related epigenetic changes (34). Similar DNA methylome studies to those in young adult humans described above via training were performed, but also included cast immobilisation and later retraining in elderly participants. The authors confirmed that aged muscle tissue was hypermethylated compared with young adult muscle at baseline and also confirmed that some genes demonstrated retained methylation signatures from earlier training into detraining and retraining, suggestive of an epigenetic memory of exercise in aged muscle. They also suggested that training was able to return 73% of CpGs in elderly muscle (hypermethylated) back towards levels seen in the young adults at baseline (hypomethylated), and that retraining enhanced this effect further (34). Therefore, suggesting that exercise could rejuvenate the aged skeletal muscle methylome toward signatures observed in younger adults. Finally, we also demonstrated this rejuvenation effect of exercise on the DNA methylome in the mitochondria of aged skeletal

muscle (35). Where in aged muscle, mitochondrial DNA (mtDNA) was observed to be hypermethylated. Importantly, resistance training almost completely reversed this hypermethylated signature in mtDNA towards the hypomethylated profiles seen in young previously trained individuals (35).

In **summary**, muscle wasting and increased hypermethylation, occur with the ageing process. Increasing exercise levels can alter the methylation profile resulting in methylome signatures, more like those evident in young people. The concept that skeletal muscle remembers prior epigenetic adaptations in response to e.g. increased physical activity and exercise can help prevent aged related epigenetic changes and may enable a retention of muscle mass into later life.

The question remains whether the Hox genes and their methylation states play a potential role in rejuvenation. It is known that exercise benefits function, metabolism, quality of life and health and that it alters the epigenome. It is also known that exercise impacts on collagen expression, blood flow, mitochondrial function, muscle tone and therefore also influences skin health. Finally, although exercise contributes to healthier looking skin, the role of the HOX genes in skin health remains to be determined. Importantly, however, what is becoming evident is the role of epigenetic regulation, more generally, of skin cells in natural and accelerated ageing (36).

Future Considerations for Facial Ageing: Impact of Botox

As we age, we see changes in the appearance of our face. These changes result from a multitude of adaptations, including: facial skeletal remodelling, fat pad atrophy, reductions in muscle tone and mass and weakening and thinning of the skin (for a full and recent review, which may be useful in the interpretation of the facial aging process see (37)). While it is beyond the scope of this manuscript to investigate the impact of facial muscle wasting during ageing, it does

warrant consideration, particularly from the perspective of facial aesthetics. A recent systematic review (38), examining the impact of Botulinum Toxin Type A (BONT-A) on muscle atrophy revealed that BONT-A injections caused muscle wasting in both human and animal models. Indeed, in 9 out of 10 animal studies, muscle loss following a single injection of BONT-A was significant, with reductions of between 18 and 60% reported (38). Although early human studies examining the impact of repeated botulinum toxin injections on orbicularis oculi muscle revealed denervation to be induced, authors suggest that the denervation changes and the negative impact on fibre size appear correlate with he time since the last injection and may be reversible (39). This is in contrast to a more recent human study which revealed not only neurogenic atrophy, of the gastrocnemius muscle, following BONT-A injections, but also a lack of full recovery up to 12 months following a single injection (confirmed by MRI and histochemistry) into the lateral head of the gastrocnemius muscle of two young, healthy male volunteers (40).

Given this controversy, although the authors of the systematic review (38) suggested the potential of muscular architecture reprogramming, as a result of muscle loss following BONT-A injections and because of the limited data available (regarding the long-term impact of the injections on muscle wasting), caution should be exerted until full understanding of the impact on BONT-A on facial muscle atrophy is gained. This is particularly important in the face of muscle wasting already evident with the ageing process.

Future Considerations: Gene Transfer and Plastic/Reconstructive Surgery:

Going forward and with advancing technology, it is possible we will move beyond BONT-A as a primary treatment intervention. This is particularly the case in plastic and reconstructive surgery, where the clustered regularly interspaced short palindromic repeats (CRISPR) system of genome editing is gaining interest in the treatment of human diseases, including those

relevant to the plastic surgeon, including: oncology, wound healing, immunology, and craniofacial malformations. While still in early days, wound healing of the skin provides an important environment for targeted gene therapy, as such wounds have high cell turnover rates, are easily accessible to transfect and to monitor, providing optimal conditions for gene transfer technologies (41).

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Table 1 Hallmarks of the ageing process

Hallmark	Evidence
Genomic instability	Accumulation of genetic damage occurs throughout life, with artificial induction revealing features of accelerated ageing and protection against aneuploidy extending lifespan (42)
Telomere attrition	Accumulation of DNA damage in chromosomal regions, including telomeres, which are more vulnerable to age-related deterioration, with telomere dysfunction accelerating ageing in murine and human models and telomerase activation delaying ageing in mouse models (43)
Epigenetic alterations	Lifestyle-related/disease mediated alterations in epigenetic tags e.g. methylation of DNA and methylaton / acetylaton of chromatin which influences gene expression. Where epigenetic deregulation of e.g. Lamina-associated domains underpin disease-specific alterations in progeroid gene expression (44)
Loss of proteostasis	Impaired protein proteostasis (stabilisation of correctly folded proteins) and quality control, results in aggregated proteins with age, accelerating the ageing process. The potential for therapeutic interventions to improve proteostasis and thereby delay the onset of age-related diseases is of interest (45)
Deregulated nutrient-sensing	Much research has focussed on impaired insulin, insulin-like growth factor, mTOR, AMPK and sirtuin activity in relation to deregulated nutrient sensing and ageing, with

	biomedical (rapamycin) or dietary (caloric restriction) interventions improving health in many model organisms (46)
Mitochondrial dysfunction	Extensive evidence, across many models reveals that mitochondrial dysfunction contributes not only to pathologies, but also the ageing process, with a recent study in human participants suggesting that dysregulated mitochondrial metabolism is a potential underlying cause of age-related frailty (47)
Cellular senescence	The growth arrest of cells as a result of e.g. telomere shortening, DNA damage and increased INK4 activation with age are indicators of cellular senescence, which may be protective in ageing or deleterious to lifespan, with the senescence-inducing tumour suppressor pathways capable of extending lifespan, but in contrast, removal of senescent cells is also capable of reducing age-related pathologies (2)
Stem cell exhaustion	A decline in tissue-specific stem cells with age, is associated with a decline in tissue function and regeneration and underpinned by both endocrine and local adaptation, with the development of pharmacological strategies to skew growth and differentiation pathways providing potential interventions for ageing/rejuvenation in non-human models (48)
Altered intercellular communication	Altered cellular behaviour, as a result of dysregulated cell:cell communication via e.g. inflammageing, the senescence-associated secretory phenotype and

	extracellular vesicles, contributes to the ageing process, with interventions targeting receptor binding, downstream signalling, senescent cell removal, all being investigated as strategies to delay the ageing process (49)