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Proteomics and metabolomics in ageing research: from biomarkers to systems biology

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Abstract

Age is the single greatest risk factor for a wide range of diseases, and as the mean age of human populations grows steadily older, the impact of this risk factor grows as well. Laboratory studies on the basic biology of ageing have shed light on numerous genetic pathways that have strong effects on lifespan. However, we still do not know the degree to which the pathways that affect ageing in the lab also influence variation in rates of ageing and age-related disease in human populations. Similarly, despite considerable effort, we have yet to identify reliable and reproducible 'biomarkers', which are predictors of one's biological as opposed to chronological age. One challenge lies in the enormous mechanistic distance between genotype and downstream ageing phenotypes. Here, we consider the power of studying 'endophenotypes' in the context of ageing. Endophenotypes are the various molecular domains that exist at intermediate levels of organization between the genotype and phenotype. We focus our attention specifically on proteins and metabolites. Proteomic and metabolomic profiling has the potential to help identify the underlying causal mechanisms that link genotype to phenotype. We present a brief review of proteomics and metabolomics in ageing research with a focus on the potential of a systems biology and network-centric perspective in geroscience. While network analyses to study ageing utilizing proteomics and metabolomics are in their infancy, they may be the powerful model needed to discover underlying biological processes that influence natural variation in ageing, age-related disease, and longevity.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

All authors conceived of and developed the outline for this manuscript. J.M.H. and Y.L. wrote the first version of the manuscript, which was subsequently edited by all authors (J.M.H., Y.L., S.D.P. and D.E.L.P.).

Introduction

Age is the single greatest risk factor for - myriad = diseases. Individuals vary dramatically in longevity and risks of age-related disease, yet we still have just a rudimentary understanding of the underlying genetic variation and biological processes that influence variation in ageing (the breakdown of biological processes as individual grow older), age-related disease (those diseases that increase in frequency as individuals age), and longevity (the total time an individual lives). We face four main challenges if we are to more fully understand the biological factors that affect rates of ageing within natural populations. First, despite major advances in our understanding of the molecular basis of ageing [1], we are still far from a comprehensive understanding of the causal mechanisms, both molecular and environmental, which influence ageing, age-related disease, and longevity. The earliest molecular genetic studies of ageing focused on understanding individual genes that had significant effects on age-related diseases and longevity. Hundreds of individual genes and proteins [2] have been found to increase lifespan when either knocked down [3–6] or overexpressed [7]. However, while these genes extend lifespan in model organisms, many of these genes do not show significant variation in human populations, which suggests that they may not be contributing significantly to natural variation in ageing and longevity. This leads to the second challenge in understanding the molecular mechanisms that influence ageing and longevity. Compared with laboratory model organisms, humans are extremely genetically diverse. Few genes of large effect on longevity or age-related disease have been discovered compared with model organisms [8]. In humans, the most reproducible, significant contributor to lifespan appears to be the *APOE* gene, which is closely associated with risk of Alzheimer's disease [9]. The genetic determinants of variation in healthy ageing may well vary among populations, and what we learn from a study in one part of the world might not apply to another. Our failure to find causative alleles for traits that often have a high heritability is part of the larger 'missing heritability' problem in humans, where the sum of all the genetic effects associated with highly heritable traits is typically far less than the heritability measured (reviewed in [10]) (though some argue genetic [epistatic] interactions cause us to overestimate the heritability of many complex traits [11]). Third, the environment can have an enormous effect on age and age-related diseases [12,13], but determining the specific impact of slight changes in the environment, as well as gene-by-environment interactions, on ageing and longevity has proven difficult [14–16]. Last, despite extensive efforts to identify predictive factors, or 'biomarkers', of longevity and age-related diseases, until now our efforts have met with relatively little success [17–19].

These challenges suggest that examining genes and genetic variation alone may not provide a complete picture of the molecular mechanisms that influence ageing and longevity. Genes shape phenotypes by working through a complex network of various molecular ('omic') domains that exist between genotype and phenotype. We hypothesize that investigating these functional molecular domains will help elucidate the causal mechanisms of ageing (Figure 1). While lab-based genetic studies have gone from success to success [3,5,6,20], the same is not true for genome-wide association studies (GWAS) designed to identify alleles that account for variation in lifespan in human populations [21,22]. The shortcomings of GWAS, not just for ageing but for all human traits, have been explored in many publications [23,24].

The overarching challenge has been that despite measures of heritability pointing to a strong genetic component for most complex traits in human populations, GWAS typically are able to explain only a small fraction of that heritability, leading to the aforementioned ‘missing heritability’ problem. One common hypothesis is that this shortfall is due to traits being shaped by many common alleles with effect size too small to detect and by large-effect alleles that are too rare to detect [25]. This has led some researchers to focus on differences among individuals in the transcriptome, searching for significant age-related differences in mRNA expression across genes that may explain the variance seen in ageing and longevity [26–29], and tissue-specific transcriptomic profiles have been well established in humans [30]. However, while transcriptomic profiles can capture some of the variance in the ageing phenotype [31], growing evidence suggests mRNA levels do not capture all variance in phenotypes, because they do not necessarily correlate with protein expression levels [32,33]. And although we now have a much greater appreciation for the functional importance of RNA molecules in general, it is proteins that make up the actual enzymes that catalyze different biological reactions, and metabolites that are the building blocks of structural elements and biochemical pathways. In addition, these ‘endophenotypes’, specifically metabolites, capture variation seen not only in genes but also in the environment, allowing us to understand the biological impact of the environment on ageing and age-related diseases. In this light, our review focuses on the study of proteins and their biological products, metabolites, via global proteomic and metabolomic profiling. We suggest here that this approach can provide a more robust method to discover causal mechanisms of ageing, age-related disease, and longevity.

Ageing arises from the failure of coordination of thousands of RNAs, proteins, and metabolites that form a biological network to modulate longevity across multiple tissues and cellular organelles [34]. In addition to changes in individual metabolites and proteins, the interaction between them per se is critical for regulating the functions that maintain survival and reproduction. By looking not only at changes in mean levels of proteins and metabolites but also in the way that abundances of these molecules correlate with one another in larger networks, we can generate new and potentially powerful hypotheses about ageing and age-related diseases. Networks consist of a set of ‘nodes’ (here, proteins or metabolites) connected to one another through ‘edges’. The edges in biological networks can be defined either by directional biochemical interactions (metabolite *a* is a known precursor of metabolite *b*), nondirectional biochemical interactions (protein *x* interacts physically with protein *y*) or more commonly, by expression correlations (e.g. concentrations of two metabolites are correlated across a set of tissue samples). In the latter case, two metabolites that are not directly connected in a metabolic pathway may nonetheless be connected in the network if their concentrations are correlated with each other. From a network perspective, the critical changes that occur with age may not be changes in concentrations (i.e. removing or adding a node in the network), but rather changes in edges connecting different nodes. For example, Laye et al. [35] provide an example where two specific metabolites show no change in mean value under high-yeast versus low-yeast conditions, but do show a change in correlation structure, with the two metabolites negatively correlated under high yeast, but positively correlated under low yeast. Similarly, one could imagine two metabolites that

have the same concentrations in young versus old animals, but whose correlation changes between ages, or between two genotypes that differ in lifespan [36].

Here, we present a short review of the potential of proteomic and metabolomic ageing studies, from individual biomarkers of age and age-related disease to metabolic pathway and finally to network analysis, which we hope will provide a deeper understanding of the biological processes that influence ageing and longevity. We then suggest the further need for integrating networks of multiple 'omics' data types to discover a complete picture of the molecular causes and consequences of ageing.

Proteomics and metabolomics as ageing biomarkers

Over the past several decades, research in the biology of ageing has attempted to discover 'biomarkers' of ageing. Ideally, these biomarkers would be predictive not only of an individual's chronological age (years lived so far), but also of their biological age (which we assume is indicative of quality and quantity of years yet to be lived). As such, biomarkers could predict risk of future age-related morbidity and mortality. However, the search for these biomarkers has often failed [18,19,37]. For example, telomere length has been a highly studied 'biomarker' of ageing, as they shorten as individuals grow older, yet telomeres have failed as a predictor of mortality [18,19]. As suggested by Horvath's recent efforts to use methylation markers as a biological clock [38], we hypothesize that high-dimensional protein and/or metabolite profiles might emerge as ideal biomarkers of ageing, as they represent the 'endophenotypes' between phenotype and genotype.

The earliest (and most common) proteomic and metabolomic studies of ageing have sought potential biomarkers of age-related diseases (reviewed in [39,40]). Comparing quantifications of individual metabolites or proteins between diseased and control individuals allows researchers to determine if specific molecules are significantly different between the two groups. The search for metabolites and proteins as biomarkers has been particularly common in the area of neurodegenerative disease. While markers in cerebrospinal fluid for Alzheimer's [41] and Parkinson's diseases [42] exist, the tests are highly invasive and not always accurate predictors of the disease. Therefore, proteomic and metabolomic studies of blood plasma have the potential to discern accurate, less invasive biomarkers of neurodegenerative disease. For example, numerous cross-sectional studies have attempted to find levels of proteins and metabolites associated with Alzheimer's [43–46] and Parkinson's diseases [47–50]. In addition to potential biomarkers, these studies discovered individual molecules that may play a significant role in disease associated pathology. However, experimental or epidemiological validation of the role of these molecules in disease pathology is rare.

Cross-sectional biomarker studies over the last 5–10 years have attempted to discover predictors of other age-related diseases as well, including cardiovascular disease and Type 2 diabetes (T2D). Protein and metabolite biomarkers of coronary artery disease [51], heart failure [52,53], and atherosclerosis [54,55] have been identified that distinguish control and diseased individuals. In one recent study, plasma protein biomarkers were associated not only with future cardiovascular events, but also with future mortality from cardiovascular

disease within an 18-year follow-up time [56]. Taken together, these cardiovascular studies suggest that the abundance of differentially regulated inflammatory response proteins may be potential biomarkers of cardiovascular events. Similarly, biomarker work in T2D has found individual proteins and metabolites whose quantities differ in control subjects versus those with the disease [57–60]. Furthermore, individual metabolite levels are associated with complications from T2D [61], and the levels of certain proteins predict future diabetic neuropathy [62]. Finally, metabolomic and proteomic biomarkers of interactions between age-related morbidities exist. For example, metabolomic analysis has revealed significant lower levels of glycerophosopholipids in T2D patients compared with controls, with the lowest levels seen in those individuals with T2D and a concurrent diagnosis of cardiovascular disease [63]. However, while proteomics and metabolomics have the potential to lead to the development of new predictive biomarkers of age-related diseases, they are not the only biomarkers being developed. For example, in cardiovascular disease, small molecule low-density lipoprotein molecules [64,65] and vascular imaging [66] have both been proposed as biomarkers of the disease.

As a pre-requisite for identifying biomarkers of ageing and long lifespan, multiple studies have attempted to characterize how the metabolome and proteome changes with age in both humans and model systems. The abundances of individual metabolites change with age in several species, including worms [67,68], flies [35,69], mice [70–72], and humans [57,73,74]. In the same vein, differences in abundances of individual metabolites have been associated with long life across multiple species, including work on long-lived mutant worms [75], longevity selected flies [76], calorie restricted and long-lived mutant mice [77], and long-lived human populations [78–80]. Interestingly, fewer studies have attempted to find protein biomarkers of ageing or longevity, and those studies that have been carried out have found mixed results. Proteins related to circadian rhythm show decreased abundance in old compared with young worms [67]. Conversely, in a study of over 4000 proteins in mice, Walther et al. [81] found no effect of age on overall protein abundances. Similarly, aged mouse brains do not show significant changes in protein abundances compared with young mice, yet large differences were seen in metabolomic profiles between the two groups [82]. However, a study of the Twins U.K. cohort found 13 proteins differentially regulated between young and old individuals, 10 of which were replicated in an independent cohort [83]. Overall, it appears that significantly more metabolite associations with age are found across organisms as compared with differentially expressed proteins. This potentially suggests that changes in metabolite quantifications and flux may be more important biologically to the ageing phenotype than protein concentrations.

Metabolomic and proteomic studies can also be applied to non-model species. For example, age-related changes in protein concentrations are seen in mosquitoes [84], honeybees [85], sea urchins [86], and macaque hearts [87], and age-related longitudinal changes in metabolites have been measured in the common marmoset [88]. In addition, metabolite concentrations are correlated with longevity across 26 mammalian species [89], leading to new insights into how metabolite concentrations change across species with highly variable lifespans.

Taken together, these results suggest that both proteomic and metabolomic technologies could lead to the discovery of biomarkers of age and age-related morbidity. Moreover, these biomarkers could also elucidate the underlying causal mechanisms of ageing and age-related disease. However, there are numerous outstanding challenges. First, as has been shown in nearly all instances where biomarkers have been asserted, merely noting a reproducible change in the marker with age and establishing a correlation with health are not enough. True biomarkers must be predictive of the relevant outcome, whether that is current disease state, risk of future morbidity, or remaining lifespan, in cohorts that were not used for their initial characterization. Indeed, replication of individual biomarkers and predictive factors has been difficult, if not impossible. An Alzheimer's disease panel of metabolites had poor predictive ability [90]. Similarly, a protein panel developed in one cohort could not accurately group a different cohort's participants into correct age classes [91]. While these are just a few examples of lack of reproducibility and predictability in 'omics' biomarkers, the majority of proteomic and metabolomic biomarker studies have never been subjected to this level of scrutiny. For metabolomic and proteomic studies to have the potential to be useful predictors of age-related morbidities and mortality, we need better reproducibility and replication, as others have recently stated [92]. In addition, metabolomic and proteomic biomarker studies would do well to integrate analyses of both specificity (ruling out healthy individuals) and sensitivity (diagnosing unhealthy individuals) on biomarker predictions, similar to analyses done on other biomarkers [93]. Second, we need to incorporate long-term longitudinal measures of -omic profiles. Our ability to predict morbidity and mortality from -omic biomarkers may depend not on static measures at one time point, but rather on trajectories of these measures. Finally, metabolomic and proteomic studies are often constrained by lack of annotation. Proteins can only be easily identified in species with a well-annotated genome. Targeted metabolomic analyses can easily detect 200–300 well-defined metabolites that are found across species. Conversely, global metabolomic profiles have the potential to provide measures of thousands of chemical features. However, the majority of these features are defined only by a specific mass-to-charge ratio, or at best, chemical formula, but have unknown structure and are not matched to a known biochemical pathway.

Metabolic pathways associated with ageing

Much of the literature on biomarkers has focused on the search for individual molecules, or statistical combinations of molecules, which are predictive of age-related morbidity or mortality. However, many metabolomic studies have moved beyond individual biomarkers of ageing, focusing instead on metabolic pathway analysis, in which whole metabolic pathways are analyzed for significant changes. Deciphering entire pathways that are differentially regulated during ageing leads to new hypotheses about the underlying biological processes that may be influencing ageing and longevity. Work in *Drosophila* found significant down-regulation of fatty acid and sugar metabolism with age [69], and a study of metabolite pathway enrichment in dietary restricted flies showed a global decline in amino acid metabolism in response to dietary restriction [35]. Metabolomic profiles in aged mouse brains show significant changes in amino acid and nucleotide metabolism [82], and nucleotide metabolism is significantly altered with age longitudinally in the common

marmoset [88]. In addition, energy metabolism and amino acid metabolism are significantly altered in Alzheimer's patients compared with cognitively normal individuals [94]. Taken together, these results point to causal relationships between amino acid metabolism and nucleotide metabolism, and ageing and age-related morbidities, and potentially in response to lifespan extending interventions. However, the causal link is still unknown. Future studies with perturbations of these pathways are needed to understand if these pathways are themselves affecting the ageing phenotype, or just a by-product of ageing itself.

Cellular network stability and ageing

Work on individual pathways has led researchers to begin to look at global pathway and network structure of different biological phenomena, as increasing evidence suggests that the cross-talk between different pathways has a significant impact on ageing [35,95,96]. This systems biology approach allows us to explore the possibility that the process of ageing might be affected in fundamental ways by age-related changes in network structure. In this case, rather than looking at changes in concentrations of individual genes, proteins, and metabolites, the suggestion is that changes in cross-talk between biochemical building blocks play an equally or more important role [35,95,97–100]. This network-based framework allows us to understand a more complete picture of the biological mechanisms that influence ageing, age-related diseases, and longevity.

Although we have few studies on metabolomic networks and ageing, previous work on gene co-expression networks and protein–protein interaction (PPI) networks suggests a strong link between network stability and ageing. This provides us with a framework to seek common network behavior associated with the ageing process. In these networks, each node indicates an individual gene/protein/metabolite, while each edge indicates either the biochemical or physical interaction between the two nodes (e.g. in a PPI network) or a statistical correlation, such as between each of all possible pairs of transcripts measured in a large number of samples. In young animals, connections between nodes tend to be strong and numerous, ensuring the functional cellular signalings and biochemical reactions in this network, often reflective as network integrity. The integrity of the network can be measured by network connectivity (the degree to which any two nodes in this network are directly or indirectly linked). Interestingly, network connectivity often declines as animal's age. For example, gene expression correlations across different tissues decrease in aged mice compared with young mice, indicating the loss of genetic connection and network integrity [98]. In PPI networks, ageing-associated proteins are highly connected, indicating the critical role of maintaining network integrity [101]. Consistent with this, an investigation into the dynamics of metabolomic networks with age in flies found that correlations of metabolite concentrations decreased in old flies [35]. Together, these studies imply that network stability is compromised in old animals at the level of the transcriptome, proteome, and metabolome. We suggest that as the cellular networks in these animals break into small, non-communicated pieces, the process of cellular homeostasis is dramatically disrupted, and that leads to a series of ageing hallmarks such as mitochondrial dysfunction, genomic instability, and loss of proteostasis (homeostasis of the proteome) [1].

Most cellular networks, such as transcriptional networks and PPIs networks, are scale-free networks that are resistant to genetic and environmental perturbations [102]. In scale-free networks, a small number of 'hub' genes interact with many partners while the majority has very few partners. The integrity of hub gene edges is critical for overall network integrity [103]. Removal of hub genes significantly disrupts network connectivity and is considered a potential mechanism of ageing [99,103]. Evidence from yeast [97], *Drosophila* [95], and human brain studies [95,100] suggests that ageing associated genes are more likely to be 'hub' genes, and computational simulations show that network stability is severely reduced by removing nodes consisting of these ageing genes from the network. One caveat is that hub genes might be defined as hubs simply because they are well studied so that more interactions have been observed. However, a recent study indicates that after correcting the number of publications for each gene, ageing and ageing-related disease genes still have more connections than control genes [104].

In PPI networks of both *Drosophila* and human, the expression levels of genes associated with proteins in only a few functional modules (i.e. co-expressed gene clusters enriched for particular functions), mostly pertaining to cellular proliferation and differentiation, were changed during ageing. However, disturbing these 'core' modules significantly affects network integrity [95]. In the transcriptome of mice, the expression correlation of the targets of transcriptional factor NF-κB are significantly decreased in old animals, suggesting NF-κB is a central modulator of network integrity [98]. This finding calls for further investigation of the molecular nature of these hub genes, including the consequence for network structure of altering NF-κB signaling, and how this and other genes maintain network stability to potentially modulate longevity. Genes that maintain proteostasis, such as chaperones, are among the most important hub genes that affect ageing [105]. Impairment of proteostasis machinery is observed not only in old animals, but also in age-related diseases [105,106].

To establish causative links among network stability and ageing, age-related disease, and longevity, one must examine the network changes in animals treated with lifespan extending interventions, including genetic, dietary, and pharmacological interventions. The comparison of network structure between dietary restricted (DR) animals and *ad libitum* (AL) fed animals indicates network connectivity is maintained in old animals under DR, suggesting that DR potentially extends lifespan through the maintenance of network integrity [35]. For example, on a DR diet, metabolite correlation networks are still largely intact in older flies, but not in flies on an AL diet [35] (see also Figure 2). A similar effect of DR on transcriptional network connectivity is also observed in worms [107] and mice [100]. Together, these studies indicate that lifespan extending interventions may have a significant influence on network stability. While these studies show great potential for using metabolomic and proteomic networks to understand ageing and longevity at the network level, few studies have investigated the effect of anti-ageing interventions on network integrity. We hypothesize that anti-ageing interventions, even though they extend lifespan through different molecular pathways [96], may all slow the pace of ageing by maintaining network integrity (Figure 2). To better understand mechanisms of ageing, future studies should place greater emphasis on how interventions that alter healthspan or lifespan affect proteomic or metabolomic network stability. In addition, studies on metabolomic network

stability, which are relatively rare at this time, will be particularly valuable to better understand how these basic building blocks (Figure 1) affect ageing.

Integrating multiple ‘omics’ into ageing research

Ageing studies using proteomics and metabolomics provide valuable insights into the process of ageing. Ultimately, our challenge will be to create a comprehensive multi-omic model of disease, one that combines not only the metabolomic and proteomic networks that lie between genotype and phenotype, as well as other domains, including the epigenome, proteome, microbiome, and the environmental factors that shape not only the downstream phenotypes, but also the various ‘omes’ that influence phenotype. And of course, even within domains there is enormous complexity. Only in recent years have we begun to appreciate the important regulatory roles played by various types of RNA, such as microRNAs and long non-coding RNAs. A wide array of different post-transcriptional regulatory mechanisms, such as microRNA-mediated degradation, leads to a lack of concordance between transcript levels and protein abundances [108]. Each ‘omic’ domain encodes unique information about genetic and biochemical processes and pathways that affect ageing. The complete understanding of the biology of ageing and ageing-related diseases thus requires a framework that integrates multiple omics approaches [109–113].

Computational methods that integrate multiple levels of omics data that can be applied to ageing and ageing-related disease. For example, using a general linear model, Padayachee et al. designed a statistical model to integrate metabolomic and transcriptomic data to understand the biological pathways leading to complex disease [114]. Using a network-based approach, PIUMet (prize-collecting Steiner forest algorithms for integrative analysis of untargeted metabolomics) combines untargeted metabolomic data and proteomic data to analyze molecular changes in disease [115]. Multivariate statistical analyses have given us the power to put multiple omics data into a statistical framework to understand the ageing process at a systems level. Moving forward, major challenges will be (1) to define and explain the statistical and functional connections across multiple domains; (2) to describe how these connections change with age, and how they response to perturbations that delay or attenuate the effects of age on morbidity and mortality, and (3) to test these connections as potential targets for therapies to ameliorate the effects of ageing.

Conclusions

Here, we have presented a brief review of proteomic and metabolomic analyses of ageing, age-related diseases, and longevity with a focus on integrating network-based approaches more broadly into the field. Ageing is a systematic, complex process involving cross-talk across large cohorts of molecules, pathways, organelles, cells, tissues, and organs, and investigating connectivity and robustness of this system may be the key to understanding the biological mechanisms of the ageing process. Studies of proteomic and metabolomic networks, although they are in their infancy, may be a key step in efforts to build a unified framework to better understand the mechanistic basis of ageing and age-related disease. It is obvious that systems biology and network analyses are poised to play a major role in

understanding the basic biology of ageing, and we are excited to see what research comes from their implementation.

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Abbreviations

AL	*ad libitum*
APOE	Apolipoprotein E
DR	dietary restricted
GWAS	genome-wide association studies
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
PPI	protein–protein interaction
T2D	Type 2 diabetes

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Summary

- Metabolomics and proteomics have the potential to be useful predictors for ageing and ageing-related diseases.
- Metabolomic pathway analyses suggest amino acid, nucleotide, and sugar metabolism might be critical in modulating longevity across species.
- Network stability is an important ageing factor.
- An integrative model of multiple omics is required for a comprehensive understanding of ageing biology.

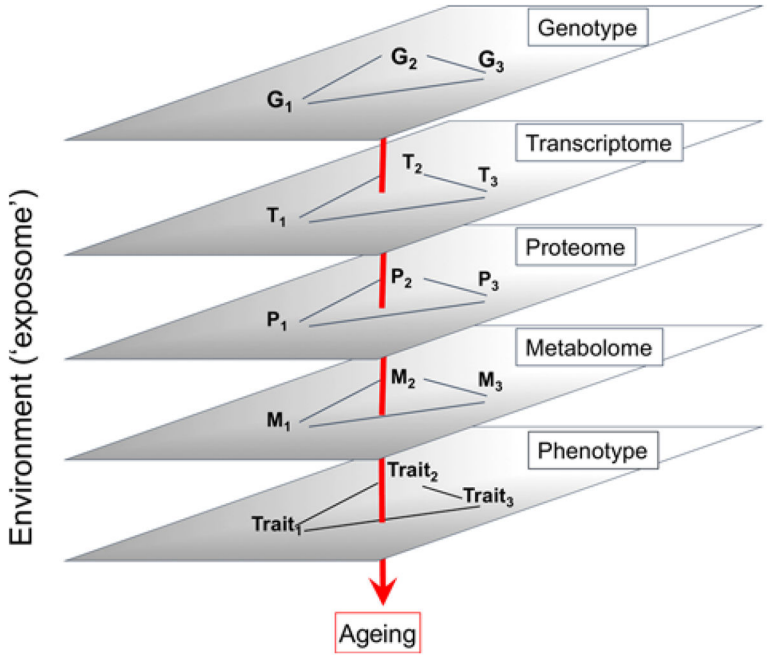

Figure 1. Genotype-phenotype pathway

Measurement of intermediate 'endophenotypes' allows us to develop a more complete understanding of the molecular mechanisms involved in ageing and longevity. The 'exposome' (environment) plays a large role across all levels of the pathway. Figure modified after Hoffman et al. [69], under Creative Commons CC BY 3.0.

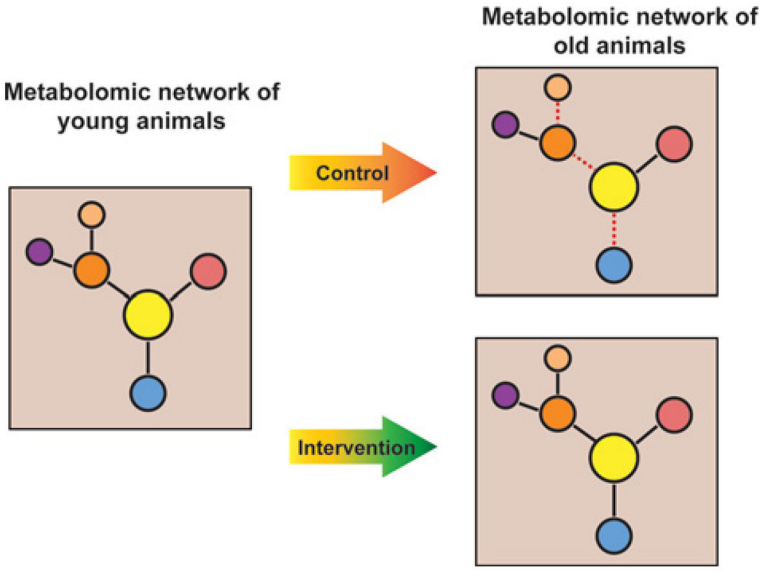

Figure 2. Lifespan extending interventions promote maintenance of biological networks into old age

Solid lines indicate intact connections, and dashed lines represent lost connections.