Healthy Ageing and Longevity 10
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Alexey Moskalev *Editor*

Biomarkers of Human Aging

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Healthy Ageing and Longevity

Volume 10

Series Editor

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Rapidly changing demographics worldwide towards increased proportion of the elderly in the population and increased life-expectancy have brought the issues, such as "why we grow old", "how we grow old", "how long can we live", "how to maintain health", "how to prevent and treat diseases in old age", "what are the future perspectives for healthy ageing and longevity" and so on, in the centre stage of scientific, social, political, and economic arena. Although the descriptive aspects of ageing are now well established at the level of species, populations, individuals, and within an individual at the tissue, cell and molecular levels, the implications of such detailed understanding with respect to the aim of achieving healthy ageing and longevity are ever-changing and challenging issues. This continuing success of gerontology, and especially of biogerontology, is attracting the attention of both the well established academicians and the younger generation of students and researchers in biology, medicine, bioinformatics, bioeconomy, sports science, and nutritional sciences, along with sociologists, psychologists, politicians, public health experts, and health-care industry including cosmeceutical-, food-, and lifestyle-industry. Books in this series will cover the topics related to the issues of healthy ageing and longevity. This series will provide not only the exhaustive reviews of the established body of knowledge, but also will give a critical evaluation of the ongoing research and development with respect to theoretical and evidence-based practical and ethical aspects of interventions towards maintaining, recovering and enhancing health and longevity.

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Biomarkers of Human Aging

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Chapter 1
Introduction

Alexey Moskalev

Abstract In laboratory studies, potential geroprotective interventions have been created: more than 400 geroprotectors, several gene and cell therapies. Their translation into medical practice is restricted, in part, due to the inability to assess clinical efficacy. Human biomarker panels are needed. Based on them, it will be able to predict the accelerated or delayed aging of an individual, track the effectiveness of procedures aimed at preventing aging, such as changing diets, lifestyles, increasing physical activity, geroprotective drugs. Aging biomarkers are an integrative qualitative and quantitative indicator of the functional state of a person and this is their key difference from the risk factors of specific age-related pathologies (type 2 diabetes, cardiovascular diseases, Alzheimer's or Parkinson's). In other words, aging biomarkers are indicators of a preclinical stage of father aging-related pathologies. Interventions should reverse these biomarkers to a younger state or slow down their changes with aging.

Keywords Aging biomarker · Biological age

Aging is the result of the destructive impact of metabolic errors and external stress factors on the individual development of the body, expressed in compensatory hyperfunction and failure of systems for maintaining homeostasis (from molecular to organismic levels) and increasing the likelihood of illness and death in life-compatible conditions.

The rate of aging in different people of the same age may distinguish significantly. They vary for different systems and organs within the same organism. The aging of one system causes changes in many others. For example, aging of the cardiovascular system may contribute to neurodegeneration and cognitive impairment, diseases of the liver and kidneys. Metabolic syndrome affects the aging of the immune system.

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Thus, in addition to the passport age (calendar, chronological), each person has a biological age, determined by the individual rate of aging. The rate of aging depends on the genetic characteristics of a person, and to a large extent, on the interaction of environmental factors with systems for maintaining homeostasis of the internal environment of the body, epigenetics.

When talking about the rate of aging, in classical biogerontology, as a rule, it means a change in the average and maximum lifespan of model animals. However, a person's life expectancy is so long that exploring the indicators of its longevity under the influence of lifestyle, diet, various drugs, gene and cell therapies is long and expensive. Therefore, the idea arose to identify the relationship with age of various physiological and metabolic changes, monitoring of which would help in assessing the effectiveness of anti-aging therapy.

The physiological norm adopted in modern medicine for many indicators changes with aging. This creates the prerequisites for observing and measuring age deviations. At the same time, the level of stochasticity (randomness) of deviations increases, which makes it difficult to interpret data on the rate of aging.

Biomarkers of aging are molecular, cellular and physiological parameters of the body that demonstrate reproducible quantitative or qualitative changes with age. Ideally, anti-aging interventions should reverse these biomarkers to a younger state or slow down the changes with aging.

Biomarkers of aging have great potential for early diagnosis and prognosis of the development of chronic age-related diseases, as well as monitoring the effectiveness of their prevention and treatment. Aging biomarkers are a common qualitative and quantitative indicator of a person's functional state and this is their key difference from the risk factors of specific age-dependent pathologies (type 2 diabetes, cardiovascular diseases, Alzheimer's or Parkinson's). In other words, aging biomarkers are indicators of preclinical stage of father aging-related diseases.

Many age-related pathologies develop for a long time in a latent form. In the early stages of the development of the disease, its clinical manifestations are nonspecific, that is, common with other age-related changes. In this case, the earlier such deviations from the norm associated with the risk of a specific disease are detected, the more effective prevention is, the more likely it is success in preventing life-threatening conditions.

The following main criteria for aging biomarker were proposed (Butler et al. 2004):

- Must change with age;
- Have to predict mortality better than chronological age;
- Allow foreseeing the early stages of a specific age-related disease;
- To be minimally invasive—do not require serious intervention or painful procedure.

We extended the list by additional criteria, that could increase their translation potential:

- To be sensitive to early signs of aging (as opposed to frailty and mortality, which are too late for prevention and geroprotection);

- Have predictability with collecting in the foreseeable time range;
- Have low analytical variability (robustness and reproducibility).

The most comprehensive online database of human aging biomarkers today is Digital Aging Atlas (http://ageing-map.org) (Craig et al. 2015).

Based on them, we can predict the accelerated or delayed aging of an individual, monitor the effectiveness of procedures aimed at preventing aging, such as changing diet, lifestyle, increasing physical activity, the effectiveness of geroprotective drugs.

The most generally accepted classification of existed biomarkers relies on the level of organization: molecular, cellular, physiological, psychological.

There are 3 different experimental approaches to develop new aging biomarkers:

1. *Empirical*. Search for significant correlations with age among a variety of physiological, clinical and biochemical parameters. The advantage of the approach is that the methods underlying it are already used in clinical practice. This approach has maximum translational potential and minimum cost.
2. *Aging mechanisms oriented*. Search for predictors of aging among changes associated with known aging mechanisms. Since the approach is based on one of the hypotheses about the causes of aging, it is difficult to confuse the cause with the effect or to base on the false correlation between parameter and age. However, there is always a chance that this is not the main reason of aging. In this case, the variability of the index will be great, and the predictive power is minimal.
3. *Omics*. Analysis of age-related correlates among the big data obtained from the analysis of various "omics": genome, epigenome, transcriptome, metabolome, proteome, microbiome. The main advantage of this approach is we can assume that not really know anything about the causes of aging nowadays and analyze all possible data of the person.

While there are no single definitive biomarkers for aging, that reflect of the all necessary criteria, a range of different measures have been proposed. In different organs and systems, aging processes occur at different times and at different speed. Thus, aging biomarker should be multimodal panel, based on different molecular and physiological parameters.

Analysis of the results of the study of the National Research Center for Preventive Medicine and the Russian Gerontological Scientific and Clinical Center, in which 303 people aged 23–91 was assessed according to 89 parameters, allowed us to make a rating of the predictive power of the parameters studied, including telomeres length levels of certain hormones, inflammation factors, blood metabolites, anthropometry and functional tests. However, the most important predictors of age were vascular health parameters (central arterial pressure, augmentation index, degree of stenosis, vessel wall thickness, pulse wave velocity) (Fedintsev et al. 2017). The biological age values obtained according to our model, as expected, were significantly higher in people with diabetes and hypertension than in healthy people.

We investigated more than 60,000 results of a general analysis of blood and extensive blood biochemistry using deep machine learning methods. The study created a universal model of biological age Aging.AI where most predictive parameters were

albumin and alkaline phosphatase (liver function), glucose (metabolic syndrome), erythrocytes (respiratory function) and urea (renal function) (Putin et al. 2016).

DNA methylation "epigenetic clock" is the most comprehensive predictor of total mortality today. It is also can predict mortality from cancer and cardiovascular diseases and vary in correlation with lifestyle and interventions (Quach et al. 2017).

In this book for the first time collected all advances in the area of human aging biomarkers. The accumulated data is quite enough to assess the rate of aging of the patient and individually monitor the effectiveness of therapies aimed at slowing aging. We need to do next big step, translating this knowledge into clinician practice.

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Part I
Biomarkers of Aging and Health

Chapter 2
Practical Detection of Biological Age: Why It Is not a Trivial Task

Boris Veytsman, Tiange Cui and Ancha Baranova

Abstract The determination of the "biological age" is one the most interesting problems in the biology of aging. The improvement of the biomarkers of aging is a very important problem. The necessity to use synthetic (i.e. holistic), rather than analytic (i.e. specific) measurements strongly contributes to a deeply complicated relationship between conventional biomedicine and a plethora of anti-aging interventions which are inferred from experimental studies of animals and observational studies of humans. Intrinsically holistic "omics" profiles, however, are subject to the "curse of dimensionality", discussed in this chapter. It is expected that an increase in the reliability of biomarkers of aging would be achieved by concerted efforts of biostatisticians, who would successfully combine data-driven and knowledge-based approaches, and the biologists who would be instrumental in critically evaluating insights generated in silico and ensure a discernible biological rationale for the metrics of biological age.

Keywords Biological age · Biomarkers · Curse of dimensionality · Omics · Holistic measures

One of the most interesting problems in the biology of aging is the determination of the "biological age", which might be a better predictor of the person's health state than the calendar age. Accordingly, biological age is often imagined as a holistic metrics describing the person as a whole rather than reflecting the functioning of the body

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through one or another representative parameter, or a combination of a relatively small number of such parameters. The goal to describe the process of aging as a whole firmly places the studies of aging into a systems biology realm (Yashin and Jazwinski 2015). On the other hand, the necessity to use the synthetic (i.e. holistic), rather than the analytic (i.e. specific) measurements strongly contributes to a deeply complicated relationship between conventional biomedicine and a plethora of anti-aging interventions which are inferred from experimental studies of animals and observational studies of humans.

To give some meaning to the concept of biological age let us imagine the process of aging as a trajectory in a multidimension space, each dimension being some measurable characteristics of the body's function or performance. These trajectories vary between individuals. Moreover, each individual follows the trajectory with a specific velocity, which may accelerate or decelerate depending on specific circumstances encountered along the trajectory. If we assume that each trajectory could be mapped in a set of natural coordinates known as a panel of biomarkers, we are able to compare the points on a given set of trajectories. Moreover, we are able to compare individuals and to check how far each of them is along the way; to understand why the velocity along the trajectory changes, and how is the trajectory itself determined.

It is further assumed that the biomarkers, or, to be specific, the levels of these biomarkers, which are used as indicators of the position along the trajectory, are objective. It is important to remember that objectivity here is no more than assumption which relies on one or another underlying theory of aging, or a combination of theories. One relevant example showing that the objectivity of biomarkers is relative is the famous aging-related measure of telomere length, commonly used as an integrative biomarker of stressful exposures and increased propensity to develop chronic disease or succumb to early mortality. Despite the telomere's popular designation as a mitotic clock, the relationship between telomere length and aging does not meet the requisite biomarker criteria (Mather et al. 2009). One of the four criteria for biomarker of aging proposed by the American Federation of Aging Research posits that a potential biomarker should be allow repeated testing without harming the subject (Simm et al. 2008). A blood test or an imaging test would satisfy this requirement. Accordingly, telomere length is most commonly profiled in peripheral blood mononuclear cells (PBMCs), that may be collected during routine venipuction. PBMCs, however, are far from being an uniform tissue; they represent a heterogeneous mixture of cell types of different physiological ages. Accordingly, these cells may have different telomere lengths (Lin et al. 2015, 2016). As the proportions of the cells in the mix may vary depending on state of the body (for example, reflecting underlying infection processes and stage of body recovery), average telomere length may fluctuate. Moreover, PBMCs composition may change with the process of aging itself (Aviv et al. 2006). Finally, the average telomere length in PBMCs may not correspond to telomere length in other tissues (Palmieri et al. 2014), may not be uniform in various human populations of the same age (Eisenberg et al. 2011), and may not accurately reflect regeneration potential of the cellular compartment (Böttcher et al. 2018). Taken together, these considerations

require multiple sampling for telomere length for different tissues, which makes a correct telomere length-based derivation of biological age unfeasible.

If we insist on the introduction of telomere length into the practice of the biological age detection, we would have to perform multiple measurement of telomere length in PBMCs of the same individual across many years and physiological states, then use this highly complex data matrix for deriving individual baselines and for determination of the trajectory of the aging curve along with defining the set of temporary conditions most significantly contributing to the increase of the measurements variability. This exercise may lead to conclusions being extremely valuable for the individual under study, but it would not be easy to transfer then to other individuals even within the same ethnicity, occupation or age group.

If we would like to expand our search for biomarkers of aging beyond mitotic clock, we would have to find some characteristics reflecting the state of various organs and tissues. For example, the concentration of a molecule that decreases or increases according to the extent of aging-related deterioration of body would be suitable as biomarker. The understanding that the measuring of this concentration might be a tall order comes from the ongoing efforts to find high-quality biomarkers for particular pathophysiological processes underlying human chronic diseases.

Indeed, an identification of a truly novel biomarker for a chronic disease with acceptable sensitivity and specificity levels is a rare event. The majority of clinically acceptable biomarkers are being around for quite some time, indicating that all low hanging fruits have been already picked. Ideally, we would like to use biomarkers which are inherently related to the pathogenic process, ones that function to either promote or prevent it. The functional involvement of a prospective biomarker implies that the pathophysiological process in question, along with all the molecular pathways connected to it, is shaped up by the adaptive selection. However, the properties of sensitivity and specificity are not inherent for biological molecules but are rather accidental. From the natural selection standpoint, these characteristics are irrelevant as they do not contribute to species' adaptation. When measuring aging, we could not expect that any particular molecular component of the living system would moonlight as an easily quantifiable counter of the system's decay, especially since the idea that aging is a purposeful, programmed series of events is being contested (Vijg and Kennedy 2016).

Another important problem which is pertinent to the discovery and validation of any biomarker, not necessarily an aging-related one, is the multitude of human population and the large variability between individuals. These factors make the establishment of reference intervals for biomarkers extremely difficult, thus precluding any generalization of the findings. Moreover, the entire concept of establishment of a reference interval for biomarkers of aging opens up a philosophical can of worms which start coming out with a question of whether aging should be considered a normal property of a living system at all.

2.1 Biomarker Panels

To overcome the problem of relatively low sensitivity and specificity of newly discovered biomarkers, researchers often combine them into multi-analyte biomarker panels. The logic under this approach is very straightforward. As aging influences many molecular networks, representing each of these networks with its own biomarker improves the robustness of the combined panel. In a typical high-throughput study of aging, panel-comprising biomarkers are mined in massive OMICS datasets obtained in the course of more or less precise quantitative measurement of as many same-class biomolecules as possible. The initial mining for biomarkers uses the so-called reductionist approach: each variable is tested for association with the phenotype of interest, such as age, or any aspect of aging, separately from all other potential biomarkers. In this approach, a large portion of the observed phenotypic variance remains, at best, unexplained or even ignored. An alternative approach relies on systems biology-guided integration of the data aimed at understanding the system as a whole rather than focusing on its individual components. A good example of aging-related studies of this kind are studies that demonstrated a modular decline in gene co-expression with age in mice (Southworth et al. 2009) and an age-associated disruption to the balance of alternatively expressed isoforms in certain human genes (Harries et al. 2011). It is important to note, however, that despite both these studies provided a set of important insights concerning the mechanistic underpinning of aging, a reduction of the results of these studies to the routine of biological age quantifying laboratories is impractical.

Therefore, we would have to get back to the biomarker panels comprised of the finite number of easily quantifiable molecules. It is important to note that predictive panels of biomarkers suffer from relatively low reproducibility of results when tested in independently collected sets of samples even when the respective disease is relatively well understood from mechanistic standpoint. This is especially true for the biomarkers identified using various OMICS platforms. Additionally, the ranked list of candidate biomarkers obtained by different research groups working with the same disease rarely match each other. One classical example of this kind is a mere two-gene overlap between two well-regarded prognostic signatures for breast carcinoma, 76-gene identifier described by Wang et al. (2005) and 70-gene set MammaPrint (van't Veer et al. 2002) (Fig. 2.1). Altogether, the robustness of the predictive gene signatures is low, and the membership in a prognostic list is not indicative of the involvement of analyte in pathophysiology of underlying disease (Ein-Dor et al. 2005).

For non-cancer biomarkers, the performance is even further away from desired marks. For example, in a number of recent studies, peripheral blood levels of the brain-derived neurotrophic factor BDNF, a commonly accepted biomarker of the aging and age-associated disorders of human brain (Levada et al. 2016), were found to be higher, lower, or unchanged in AD patients compared to healthy controls (Balietti et al. 2018). These strikingly varying results are being typically blamed on methodological issues, including the heterogeneity of patient cohorts due to the difference

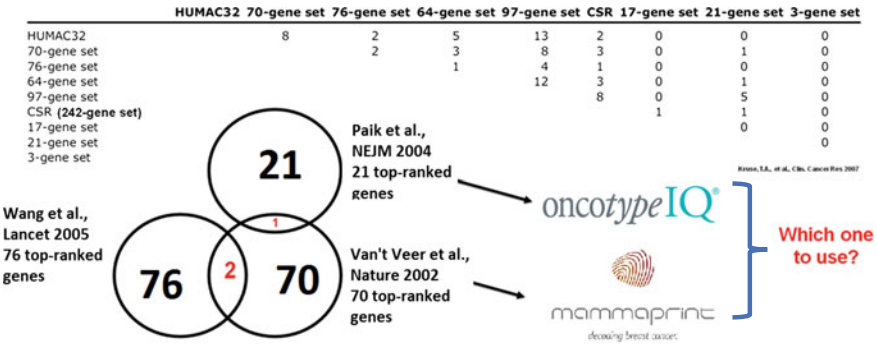

Fig. 2.1 The ranked lists of candidate biomarkers obtained by different research groups working with the same disease rarely return matching results. The table demonstrating that overlap between gene sets obtained by various research groups is minimal is from Thomassen et al. (2007), with changes. Two well-regarded, already commercialized prognostic signatures for breast carcinoma, 76-gene identifier described by Wang et al. (2005) and 70-gene set MammaPrint (van't Veer et al. 2002) have only two genes in common, thus, posing a difficult choice for a clinical practice

in recruitment criteria and the lack of control for dependent variables. It is even more important, however, that the observed contradicting results may be attributed to peculiar age related changes in serum BDNF levels which linearly increase to the age of 65, when the tendency markedly changes to rapid decrease in these levels (Neshatdoust et al. 2016). Some studies point that the levels of BDNF may reflect underlying response to the stress, thus indicating the degree of engagement of existing resources, or, ultimately, a point of "no return", where the resources are already exhausted (Balietti et al. 2017).

2.2 Combinatorial Approach to Biomarker Development: From Haphazard Elimination to Embracing a Curse of Dimensionality

To understand the roots of the problems that results from combinatorial approach to discovery and validation of biomarkers, let us consider first the standard framework for applying a diagnostic technique. We plan to measure some parameter p (say, the serum level of certain biomolecule) that is somehow related to the biological age A. Note that in this approach we implicitly assume that we know which biomarker to use for the determination of the effective age. In fact, the selection of the proper set of parameters to be input into the model (a set of candidate biomarkers) is a separate and very difficult problem. In some cases, our understanding of the process of aging may help: if we know that aging is accompanied by the shortening of telomeres, the length of telomeres is a good candidate. In other cases we may try data mining: we can make a panel of putative tests, attempt to validate them all and then select only

these most closely correlated with age. Obviously, the described approach suffers from the observation bias: researchers attempt to observe correlations daily, and only these that were actually observed ends up being published. A large volume of observations do not return reportable results, and, therefore, remain not published. Because of that, each individual reported correlation might reflect a statistical fluke resulting from the so-called multiple comparisons hurdle, which is known to plague biomarker research. On the other hand, if a biomarker is not selected as an input to the model, it is "lost" forever. The selection step is irreversible.

It is important to understand that the obvious solution to this problem—a brute force approach known as "let's input them all"—would not help. Attempts to analyze the data with the dimensionality (the number of variable features) higher than the amount of individual, specimen-specific measurements for each feature commonly ends up in the so-called "overfitting" of the model. Overfit models may perfectly describe the set of samples in the initial analysis, but turn out to be unable to classify independently collected sample sets, thus failing validation step. Recent works dealing with outcomes of high-throughput experimental workflows showed that when the number of variables is high enough, a good separation of the classes may be achieved even for sets of classifiers selected randomly (Venet et al. 2011).

This problem is widely known as "curse of dimensionality". A term introduced by Bellman to describe the result of the exponential increase in volume of Euclidean space associated with adding extra dimensions (Bellman 1957).

Typically, the problem of high dimensionality of biomarker space is solved by removing a majority of variables, in a process of a feature selection that increases the classification power (Mayer et al. 2011; Saeys et al. 2007). A proper feature selection is paramount for extracting potential biomarkers from high-throughput datasets where a researcher cannot grasp several thousand parameters being analyzed at once. There are several algorithms to identify and interpret the patterns within the data, for example, principal components analysis, clustering, multidimensional scaling, or regularization techniques. Nevertheless, erroneous inferences resulting from the haphazard feature selection are not that uncommon (Fig. 2.2).

The "curse of dimensionality" may be illustrated by evaluating the sparsity of data by comparing the size of the unit sphere with the unit cube as the dimension of the space increases.

Consider a panel of N_g biomarkers of aging. We "train" the test on N_p of patients at various stages of aging. The probability that the dataset describing a new, $(N_g + 1)$-st patient, closely resembles the dataset describing some other patient from our training cohort may be calculated using a model of "closeness". Suppose the level for each of our biomarkers varies between -1 and $+1$. A simple Euclidean distance in the N_g dimensional space would aid in classifying any two patients as "close resembling each other" if the distance between the corresponding points is less than 1. The model could be further simplified by assuming that these points are uniformly distributed in the N_g-dimensional hypercube with the volume of 1. If each point in the training set would be enveloped in a sphere of radius 1, then the total volume of these spheres, not counting overlaps, is $N_p V_s$, where V_s is the volume of a unit ball in the N_g dimensional sphere, equal to

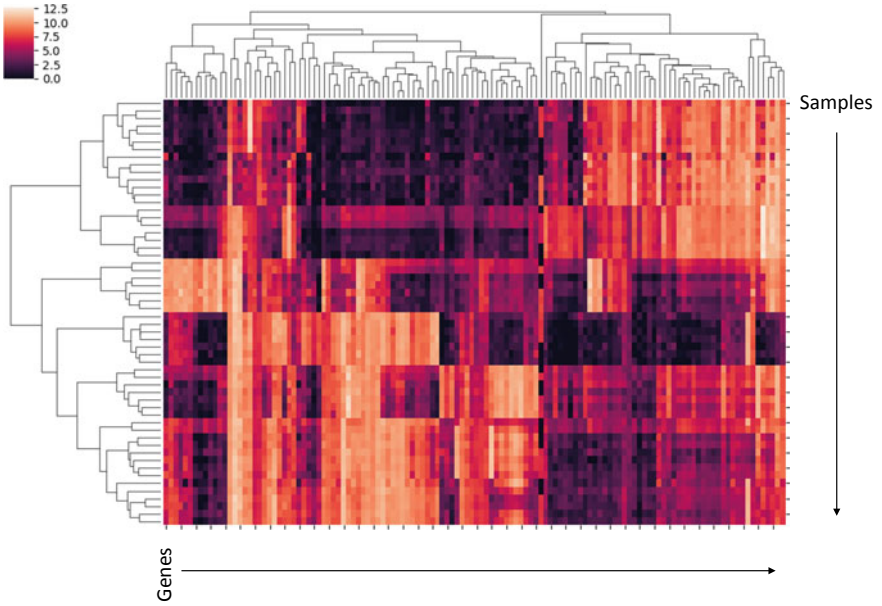

Fig. 2.2 A typical heatmap resulting from gene expression profile analysis. In this particular case, the clustering is hard to interpret. Functional analysis of the pathways may aid in gaining some insights, but are prone to erroneous inferences

$$V_s = \frac{\pi^{N_g/2}}{\Gamma\left(N_g/2+1\right)}$$

Γ being the Γ-function. Therefore, the probability is less than

$$P < N_p V_s$$

For a low dimensional space, the right hand side of this equation is usually above 1. Indeed, two random circles of radius 1 almost always cover a unit square. However, in highly dimensional spaces the situation changes dramatically. Gamma-function in the denominator of V_s grows much faster than the exponential function. In mere 20-dimensional space $V_s = 0.026$, with more than three dozen non-overlapping unit spheres necessary for covering the unit square. For 100-dimensional space $V_s = 2.4 \times 10^{-40}$, In other words the probability of two randomly selected points to resemble each other is approaching zero. When the number of biomarkers in the panel reaches the size that is typical for OMICS, the probability that a new patient would "resemble" any patient in the training set diminishes in a similar way. In other words, the amount of training data needed to cover a certain reasonable fraction of a feature range grows exponentially with the number of dimensions (Fig. 2.3).

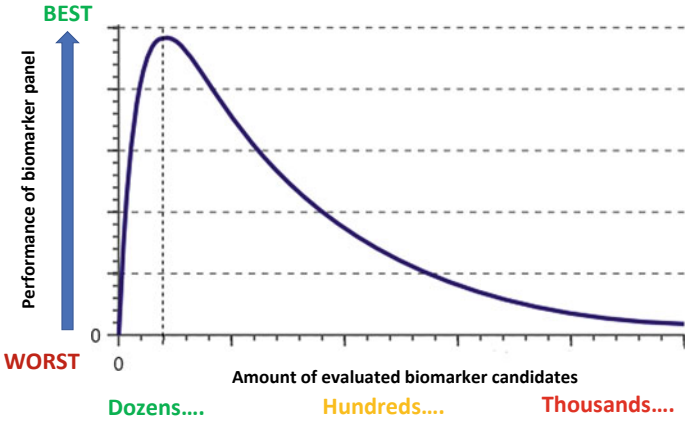

Fig. 2.3 With an increase of the dimensionality of analyzed dataset, the performance of classifiers improves until the optimal number of analyzed features is reached. Further increase in amount of analyzed features without increasing the number of specimens results in a decrease in classifier performance

2.3 Pascal Versus Bayes

The theory of probability and statistics has being developed by two prominent mathematicians: Blaise Pascal (1623–1662) and Thomas Bayes (1702–1761), who lived almost a century apart. The works of Pascal are particularly known for the use of gambling to illustrate the notion of expected value, and for the probabilistic argument (Pascal's Wager) to justify the belief in God and virtuous life. In Pascal's view, each throw of the dice is independent from a previous throw. The view of Bayes is substantially more advanced as it allows for prior assumption to change based on experience, thus admitting that the experience may accumulate, and this accumulation is applicable to statistics. In an individual, a combination of inherited gene variants forms a good prior assumption about the probability to live till fifth decade of life. However, genetics-derived assumption about the probability to live till sixth decade of life will be modified by the lifestyle and events of the previous decade. While Pascal's view tempts us with its straightforwardness and ease of calculations, Bayesian view seems to reflect the reality to substantially larger degree.

Therefore, a modern approach to the mining for biomarkers in high-dimensionality datasets is based on the Bayesian ideas. In that, we begin with defining the biological age as a parameter somewhat related to calendar age. In absence of any tests at all, the a priori probability for a patient to have the biological age between A and $A + dA$ is $P(A;a)dA$, where a is the calendar age, and $P(A;a)$ is a bell-shaped function of the difference between A and a. Suppose that the probability to measure a biomarker level b depends on the age A as $P(b;A)db$. Then a posteriori probability of the biological age is

$$P(A; a, b) = P(A; a)P(b; A) / \int P(A'; a)P\left(b; A'\right)dA'$$

What happens if instead of one parameter b we have n different parameters for each patient? Geometrically this means using an n-dimensional vector $\boldsymbol{b}$. How can we use this vector for the prediction?

The simplest idea is "flattening" the space. Let $f(\boldsymbol{b})$ be a scalar function of n variables. Then we can just pretend this is our new parameter and use the one-dimensional theory for making prediction. When the number of parameters is small, the selection of this function is relatively easy: in the simplest case we just combine parameters linearly then select only those which produce the best result for the group of patients with the known age. This is the *training* of our test. After the training stage we get the optimal combination of measurements to apply to independently enrolled cohort of patients for test validation.

2.4 Multiparametric Datasets Reduction Relies on Additional Information Context and Its Correctness

For a biomarker researcher, a sample is a patient, and dimensionality is the number of candidate genes. Extracting predictive signatures from the OMICs datasets comprising many data points, each quantitatively describing the state of some parameter, for example, the level of expression of individual mRNA is far from being easy.

Information theory tells us that the amount of information we could possibly extract from the results obtained on N_p patients is proportional to N_p. Any linear formula combining N_g gene expressions has the amount of information proportional to N_g. It means that to generate a reliable biomarker test *we need to initially profile many more patients than genes:* Np ≫ Ng. The calculation by Ein-Dor et al. (2006), who performed re-analysis of several published breast cancer data sets and the predictive lists of genes resulting from them, showed that to achieve a typical overlap of 50% between two predictive lists of genes, at discovery cohort of at least several thousand patients should be enrolled. At the first glance, in case of the prediction of biological age, the enrollment of extremely large cohort may seem feasible. When our major outcome is today's age, extensive diagnostic services are not needed. However, we should bear in mind that our task is far from being as simple as binary classification of having or not having a progressive type of cancer. In studies of aging, we essentially predict a "continuous" outcome, as rate of descending along the curve of ageing may change non-linearly. This requires either enrolling multiple cohorts of individuals, each cohort being within certain age bracket, or longitudinal profiling of individuals, with multiple sets of measurements collected along their ageing journey.

As discussed above, fundamental theory of information tells us that proper analysis requires that the amount of samples should substantially exceed the amount of the variables (the dimensionality) accessed for the system of study. No "creative" statistics technique can solve this problem. In thermodynamics, one can make very

sophisticated thermal machines, but their efficiency is set by the fundamental laws of this area of physics, and, therefore, cannot exceed the theoretical limit. In biological systems analysis, one can improve the performance of the model, but there is no way to extract meaningful conclusions about thousands of genes based on the data from hundreds of patients—fundamental laws of information theory explicitly preclude that.

A fundamental hypothesis on which many machine learning methods are based (Goodfellow et al. 2016) posits that in many problems most samples lie on a hyperplane in the parameter space with the dimension much smaller than number of parameters, and hopefully smaller than the number of samples. This allows one to circumvent the information theory limits and extract the low dimensional representation of the data using some regularization technique. However, the lack of success in the biomarkers of aging indicates that even if this hypothesis is correct, the dimension of the relevant subspace is still much larger than the number of available samples.

This means that to get robust predictions we cannot use data alone: we must add some assumptions about underlying biological processes and blend them with the data (McDermott et al. 2013). For example, we can measure the individual correlation of each candidate biomarker with the age of the subjects in the training set, and then shrink the biomarker list to include only those that have the highest correlations with age at every life stage of a given individual. In other words, we look at the correlations between the all candidate biomarkers, and for each group of highly correlated parameters select only one "typical representative". For example, one may remove the expression levels for individual parts of large protein machine (and leave the value for entire protein complex), or delete all but one among many alternatively spliced mRNA isoforms. These and others, even more sophisticated techniques are reviewed by McDermott et al. (2013). It should be noted that the quality of biological assumptions is an important issue. There is a significant hope that it will improve with an accumulation of biological data and its subsequent interpretations.

Let us suppose we successfully dealt with biomarkers discovery phase by reducing and then ranking the list of features according to likelihoods they could serve as viable inputs into predictive models. However, the "dimensionality curse" discussed above can produce another non-trivial problem. Suppose we selected two different training sets, both being drawn from the same set of patients profiled using some high-throughput biomarker discovery platform. If the set of discovered biomarkers is robust, we would expect the both training datasets would produce comparable results. An experiment of this kind was performed by Ein-Dor et al. (2005), using a single breast cancer dataset that was analyzed by a single method. However, the training datasets were randomly assembled and different in each analysis trial. The outcome of this study was most frustrating: the resultant sets of biomarkers were not unique; in fact, they were strongly influenced by the subset of patients used for training. In other words, if we start from different groups of patients, we get completely different results. In statistics this property of the models is called the lack of identifiability; a very low identifiability of models seems to be a characteristic of an entire biomarkers research field, which highlights the widespread low reliability of the conclusions drawn from these models.

There is a hope that the situation could be saved by hypothesizing that different sets of biomarkers are not "intrinsically different". Indeed, if two genes belong to the same pathway, then the changes in the expression levels for either of them could used as proxies for the information about the pathway. In other words, these genes are interchangeable as biomarkers: an anomaly in the expression of any of them signifies a problem with this pathway. This is akin to the typical representative method for highly correlated parameters: it does not matter which parameter from the group is chosen since the parameters in the group are highly correlated.

2.5 Theory-Driven and Data-Driven Approaches to Deal with Complex Systems

Speaking generally, complex system of any structure may be dealt with from either theory-driven or data-driven approach. If we start from theory, we select variables that are most relevant to the process we study have to rely on our understanding of underlying processes to select variables. In biological terms, that means that we attempt to discern suitable candidate biomarkers from non-robustly ranked lists of biological molecules by analyzing underlying biological pathways and selecting these most relevant to pathogenesis of the disease we study. Unfortunately, our knowledge of biological processes is far from being perfect, and what we consider non-overlapping pathways may turn out to be related or cross-talking. Thus we may miss suitable biomarker due to the incompleteness of our knowledge. Additionally, for some diseases we do not have any reliable information. A good example would be a genetic disease for which the causative gene has not been discovered yet. These considerations limit applications of the theory-driven approach (also known as knowledge-based approach) for biomarkers discovery. However, when the genetic defect is known, for example, in Hutchinson-Gilford progeria syndrome which shares many features with physiological aging, a leap from a rare disease phenotype to population-wide phenomenon of age-dependent deterioration of function may lead to biomarker breakthrough (Ragnauth et al. 2010).

Knowledge based approach is about limiting ourselves to certain kinds of relevant data, while excluding all that irrelevant. The power of this approach could be illustrated by comparison of ancient physician to the modern one. Along with observations of symptoms, one of them adds the astrological information into the patient' chart, while another knows that stars and planets are not relevant, and thus excludes astrological data from the heap of the data to analyze. As evident from above, both an ancient physician and a modern scientist apply the theory-driven approach, they simply use different theories, each with its own degree of veracity.

In the second, data-driven approach we start with as little preconceptions as possible. A purely data-driven approach would be to start with as much data as possible, including astronomical ephemerides, and let the correlations show that the latter are not relevant. At the first glance, this approach is a fallacy: why should we include

the data which we *know* as irrelevant. We should bear in mind that the analyses we perform are not without costs, even if these costs are purely computational in its nature. However, data-driven or hypothesis-free approaches are very powerful as they truly do not require any data concerning a network of intricate ropes comprising a regulatory underpinning of any biological system.

While the data-driven approach is indispensable in validating the theories, its applications for theory generation is not so straightforward. In one recent study, utility of hypothesis-free approach was demonstrated by multi-dimensional mining of global collections of high-throughput transcriptome data that integrated, independently correlated and ranked the levels of individual mRNAs derived from over 4000 wet-lab experiments comprising 25,000 signatures (Kupershmidt et al. 2010). After tracing the replication of observed correlations across multiple independent datasets, researchers generated a number of meaningful hypothesis concerning the development of brown adipose, a tissue compartment with high relevance to obesity, metabolic syndrome and other human pathologies. Typically, in data-driven approach, amount of correlations to be evaluated is of such magnitude that we have to resort to machine learning (ML), or even so-called deep learning (DL) approaches capable of automatic discovery of the features to be used for classification.

2.6 Holistic Measures as an Orthogonal Solution

Holistic measures that look at entire set of high-throughput omics measurements simultaneously may offer a new direction in this field. A holistic approach of this kind would, for example, include all the gene levels from the RNAseq experiment without any pre-selection or discrimination, then, instead of projecting samples into a low dimensional space, considers the data in a high dimensional space where the dimensionality equals the number of genes in the RNA panel (Veytsman et al. 2014). If one plots all the tissue specimens from the same RNA-Seq experiment in multidimensional space, the clustering of the specimens would be easily observable. In theory, same-tissue specimens collected from young individuals should cluster together, and continue as a group even if the profile of each sample undergo its own minor disturbances caused by intrinsic or extrinsic forces. But as aging progresses, the corresponding gene expression profiles should deviate more and more from the center of a multidimensional space occupied by young specimens. If one can develop a metrics to calculate the distances between each pair of specimen profiles, then by measuring the distance of each specimen to the center of young space, one can infer an elusive biological age by the degree of overall aberration in the tissue expression profile.

Importantly, proposed composite biomarker may be suitable for a dynamic description of the process of ageing, which clearly does not fit into the classical two-bin "healthy/diseased" model of the human wellbeing, but rather require a value for continuous parameter of choice, for example, remaining lifespan. For each sample,

the distance from "young" would quantitatively describe the degree of the departure of the body from homeostasis.

It would be interesting to see how these distance correlate with so-called allostatic load indices (ALIs) commonly used as estimates of whole-body dysregulation and predictors of life-long outcomes of cumulative exposures to common stressors experienced over the lifespan (Juster et al. 2010). Wear-and-tear estimates of allostatic loads are assessed using a panel of biomarkers representing neuroendocrine, cardiovascular, metabolic, and immune systems. Perhaps it is not incidental that many features of ALIs are shared with published lists of acknowledged biomarkers of ageing (Xia et al. 2017; Lara et al. 2015).

2.7 Conclusions

A nascent discovery of the next generation of biomarkers is widely considered to be dependent on the power of the high throughput analysis. Respectively, mining various "omics" profiles remain a predominant occupation of computational biologists aiming at improving our understanding of the biology of health and disease. However, the "curse of dimensionality" is a major trap in on the road to this bright and shiny future.

Even though an integration of the concept of biological age into the practice of personalized medicine became a reality, improving the performance of biomarkers developed for the specific category of intended ageing-related use remains a priority. Examples of such uses include early detection of pathophysiological processes, their monitoring, and, most importantly, evaluation of the effects of various anti-aging interventions. It is expected that an increase in reliability of biomarkers of ageing would be achieved in course of the concerted efforts of biostatisticians, who would successfully negotiate an agreement between data-driven and knowledge-based approaches, and the biologists who would be instrumental in critically evaluating insights generated in silico and ensure a discernible biological rationale under the metric of biological age.

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Chapter 3
Biological Age is a Universal Marker of Aging, Stress, and Frailty

Timothy V. Pyrkov and Peter O. Fedichev

Abstract We carried out a systematic investigation of supervised learning techniques for biological age modeling. The biological aging acceleration is associated with the remaining health- and life-span. Artificial Deep Neural Networks (DNN) could be used to reduce the error of chronological age predictors, though often at the expense of the ability to distinguish health conditions. Mortality and morbidity hazards models based on survival follow-up data showed the best performance. Alternatively, logistic regression trained to identify chronic diseases was shown to be a good approximation of hazards models when data on survival follow-up times were unavailable. In all models, the biological aging acceleration was associated with disease burden in persons with diagnosed chronic age-related conditions. For healthy individuals, the same quantity was associated with molecular markers of inflammation (such as C-reactive protein), smoking, current physical, and mental health (including sleeping troubles, feeling tired or little interest in doing things). The biological age thus emerged as a universal biomarker of age, frailty and stress for applications involving large scale studies of the effects of longevity drugs on risks of diseases and quality of life.

Keywords Biological age · Frailty index · Blood sample · Questionnaire · Self-reported · NHANES · Inflammation · Physical health · Mental health

3.1 Background

Aging in most species, including humans, manifests itself as a progressive functional decline leading to increasing prevalence (Mitnitski and Rockwood 2016; Yu et al. 2017) and incidence of the chronic age-related diseases, such as cancers, diabetes, car-

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diovascular diseases (Zenin et al. 2019; Podolskiy et al. 2016; Niccoli and Partridge 2012) or disease-specific mortality (Barzilai and Rennert 2012), all accelerating exponentially at a rate compatible with that of the Gompertz mortality law (Gompertz 1820; Makeham 1860). The physiological indices or physiological state variables, such as blood biochemistry or cell count markers change with age in a highly coordinated manner. This can be best seen from a Principal Component Analysis (PCA), which is a mathematical procedure aimed at the identification of the most correlated or collective features in multidimensional data (Ringnér 2008). The method is an almost always the first choice in biological data analysis and searches for aging signatures in a multitude of biological signals ranging from blood samples to locomotor activity patterns (Pyrkov et al. 2018b), anthropological and functional measures (Park et al. 2009), and microbiome composition (Odamaki et al. 2016). In most cases only a few, most often the first, PCA scores were associated with age and hence could be naturally interpreted as biomarkers of age (Nakamura et al. 1988; Nakamura and Miyao 2007), see Jia et al. (2016) for a recent review and references therein.

The dimensionality reduction revealed by the PCA suggested that the age-associated changes are effectively controlled by the very few variables, naturally associated with the organism-level, or "macroscopic", properties, such as responses to stresses, risks of diseases and therefore to the remaining health- and life-span (Podolskiy et al. 2015; Fedichev 2018). It is therefore expected that almost any method using organism state variables to predict chronological age, frailty, diseases or the remaining life expectancy should produce the more or less same predictors, albeit at a different signal to noise ratio. This is why biological age estimates are associated with both mortality (Marioni et al. 2015; Horvath et al. 2015b; Christiansen et al. 2016) and morbidity risks (Horvath and Levine 2015; Horvath et al. 2014a, b, 2015a), as well as with life-shortening lifestyles (Gao et al. 2016) or diseases (Zhang et al. 2016; Horvath and Levine 2015; Horvath et al. 2014a, 2015a).

We performed a systematic evaluation of popular biological age models in the same cohort of NHANES study participants. We used a range of machine-learning tools spanning from popular linear chronological age predictors or log-linear models of mortality or morbidity, on one hand, to their fancier deep learning counterparts, on the other. Since the biological age is expected to reflect on the organism's state rather than on the performance of any specific functional subsystem, we used two sources of the biological information. The first choice was the most commonly used form of clinical blood analysis. In an independent calculation, we used a brief questionnaire developed to gauge the self-reported health of an individual over the last 30 days.

Interestingly, the biological age marker built using blood samples did not dramatically outperform the one obtained from the survey questionnaire. We show that biological aging acceleration of both models is associated with disease burden in persons with diagnosed chronic age-related diseases. For healthy individuals, the same quantity was associated with lifestyles, such as smoking, or variations in the current physical health and mental health status. The biological age thus emerged as a universal biomarker of age, frailty and stress, perfectly suitable for applications involving large scale studies of effects of longevity drugs on risks of diseases and on quality of life.

3.2 The Limits of Deep Learning of Chronological Age

A good biological age predictor should be strongly associated with chronological age and predict the incidence of future diseases and death. We chose these criteria to compare the models using data on lifestyle and chronic health conditions from NHANES (12,734 female and 12,007 male participants aged 18–85). All the study participants were split into training and test cohorts of equal size of 12,370 and 12,371 subjects. The training set was used to build models and all the results reported below were produced using the test set samples only. We used the same set of log-scaled complete blood cell counts (CBC) and blood biochemistry markers, such as concentrations of C-reactive protein, albumin, alkaline phosphatase, gamma-glutamyl transferase, globulin and serum glucose.

We started by training the baseline biological age model, the "LIN-bioage", which was a linear regression of the blood features trained to predict the chronological age. The blood markers captured the age-dependence of the organism state fairly well (the Pearson's correlation of "LIN-bioage" predictor with its target, the chronological age, was $r = 0.52$ with the root mean squared error (RMSE) of 15.9 years). The biological aging acceleration (BAA), i.e. the difference between the predicted biological age of an individual and the mean biological age of their age- and sex-matched peers (Pyrkov et al. 2018a), was associated with the risks of death and hence the remaining life expectancy, see Table 3.1. The effect size and the significance of the association between the BAA and mortality was formally established with the help of the Cox proportional hazards model (Cox 1992) using the subject's age, sex, smoking and disease (diabetes) status as additional covariates.

Deep neural networks (DNN) can be used to improve age-predicting accuracy by accounting for possible non-linear associations among features. We introduced a "DNN-bioage" predictor, the result of the model trained to minimize MSE between predicted and actual chronological age. The DNN architecture comprised six fully-connected layers, each having from 4 to 12 "leaky ReLU" neurons. The respective "DNN-bioage" model produced a better correlation with age (Pearson's $r = 0.64$, $RMSE = 14.6$ years) and yet did not demonstrate any improvement of the association with all-cause mortality, see Table 3.1.

We expected the BAA to increase depending on the health status, i.e. in groups of individuals diagnosed with at least one chronic age-related health conditions. More specifically, we formed a list of diseases, including diabetes, high blood pressure, angina pectoris, coronary heart disease, heart attack, heart failure, stroke, arthritis, chronic bronchitis, emphysema and any kind of cancer. The incidence of all the selected health conditions had a strong association with age and increased exponentially with age in the NHANES population. The BAA was significantly associated with the prevalence of the chronic diseases (see Table 3.1). The effect was larger and statistically stronger in the "LIN-bioage" model (2.5 years, $p = 1.2\text{E}{-36}$) than in the "DNN-bioage" (0.3 years, $p = 2.4\text{E}{-19}$).

In healthy subjects (i.e. those with no diagnosed chronic diseases), both models demonstrated increased biological age in groups of current smokers vs. those who

Table 3.1 The association of biological aging acceleration with all-cause mortality, health status and smoking lifestyle in bioage models for the NHANES cohort

	All-cause mortality		Current/never smoker		Health status	
Covariate	HR (95% CI)	*p*-value	Δ Bioage	*p*-value	Δ Bioage	*p*-value
Age	1.09 (1.09–1.09)	7.3E–267				
Sex	1.57 (1.49–1.66)	1.3E–18				
Diabetes	1.76 (1.67–1.87)	5.6E–23				
Smoking	2.35 (2.21–2.50)	6.5E–43				
+ Aging acceleration						
LIN-bioage	1.04 (1.04–1.05)	9.0E–72	2.4	6.5E–07	2.5	1.2E–36
DNN-bioage	1.05 (1.04–1.05)	1.1E–27	1.8	1.4E–05	0.3	2.4E–19
MORBID-bioage	1.05 (1.04–1.05)	2.7E–72	2.1	1.8E–09	3.0	4.2E–55
MORTAL-bioage	1.08 (1.08–1.09)	4.5E–138	2.0	3.7E–10	0.8	2.4E–15
MORTAL-bioage with explicit age	1.06 (1.06–1.07)	1.2E–163	2.6	1.1E–13	1.8	6.4E–28
HAZARDS-survey	1.07 (1.06–1.07)	7.2E–77	2.6	7.0E–15	1.8	8.8E–32
HAZARDS-blood-survey	1.07 (1.07–1.08)	3.9E–194	4.1	1.0E–18	2.4	3.8E–37
Deep HAZARDS-blood-survey	1.10 (1.10–1.10)	5.5E–219	4.2	6.7E–19	2.4	2.9E–36

never smoked. Again, the bioage difference was larger and more statistically significant for the "LIN-bioage" model (2.4 years, $p = 6.5\text{E}{-}07$) than for "DNN-bioage" (1.8 years, $p = 1.4\text{E}{-}05$).

3.3 Morbidity and Mortality Models Produce the Most Accurate Biomarkers of Age

The association of BAA with mortality is the most desired property of a good biological age model. Hence, we turned to log-linear mortality models trained in the same blood markers. We used the NHANES linked death register data (1999–2010 surveys, 7244 female and 7156 male participants aged 40–85, 1517 recorded death events during follow-up till 2011). The first choice was the Cox proportional hazards model with blood markers as covariates only, with no explicit age or sex labels. The log-scaled hazards ratio is a linear combination of blood features and can be calibrated in years as outlined in (Liu et al. 2018; Pyrkov et al. 2018b) and was used as an alternative biological age predictor, the "MORTAL-bioage". Although the correlation of the predicted age with the chronological age was lower ($r = 0.35$ with $RMSE = 17.5$ years), its association with mortality was notably improved ($HR = 1.08$, $p = 4.5\text{E}{-}138$) relative to that of the models based on the chronological age only, see Table 3.1.

The Cox proportional hazards model relies on the availability of morbidity or mortality follow-up data, which is almost always limited because it requires a long time to collect. Fortunately, the log-hazard ratio of a risk model can be well approximated by a log-odds ratio of logistic regression trained to predict the outcome label whenever the number of the events is sufficiently small (Green and Symons 1983; Abbott 1985). Accordingly, we used the study participant's health status, i.e. the presence of at least one of the age-related diseases, as the binary morbidity label for the logistic regression model. The resulting log-odds ratio was re-calibrated in years of life and referred to as the "MORBID-bioage" (Pearson's correlation coefficient to chronological age $r = 0.48$, $RMSE = 16.2$ years). The BAA of both "MORBID-bioage" and "MORTAL-bioage" were significantly associated with all-cause mortality and outperformed the chronological age predictors, see Table 3.1.

The "MORTAL-bioage" model performance could, in principle, be further improved by training a proportional hazard model including the age and sex covariates explicitly. We produced such model and after re-scaling to units of years referred to its log-proportional hazard ratio as the biological age of the "MORTAL-bioage with explicit age" model. The BAA of "MORTAL-bioage" trained with and without explicit age were highly correlated across the samples (Pearson's $r = 0.90$). Nevertheless, "MORTAL-bioage with explicit age" yielded a slight improvement in its statistical power, see Table 3.1.

In healthy (i.e. chronic disease-free) subjects, the mortality (with and without explicit age) and morbidity models demonstrated an increased and more statistically significant acceleration of bioage for current smokers over non-smokers than that produced by the best chronological age predictor, see Table 3.1. The mortality model with explicit age performed best. Notably, the effect of smoking on the BAA was reversible: there were no significant differences in the bioage levels in groups of never-smokers and those who quit smoking earlier in life, see Fig. 3.1a.

3.4 Biological Aging Acceleration, Lifestyles, and Self-reported Physical and Mental Health

We reasoned that the biological age is an organism-level property, characterizing the overall health of the organism and its interaction with environmental factors. To reveal this connection in detail, we used the BAA of the "MORTAL-bioage with explicit age" model and screened it for associations with self-reported lifestyles and physical and mental health status from the NHANES questionnaire.

In healthy subjects, the BAA was associated with lifestyle characteristics, such as vigorous physical activity (reduced biological age by ≈3 years) and self-reported general health (increased biological age by ≈2 years when fair or poor), see Fig. 3.1b. Smoking had the largest negative effect on biological age which increased by ≈3 years when smoking more than a pack per day.

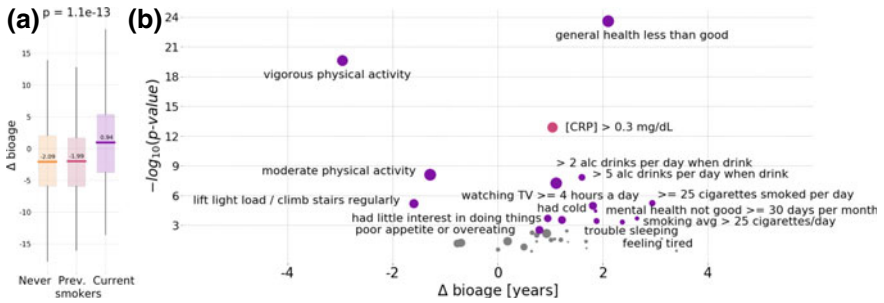

Fig. 3.1 Associations of biological aging acceleration with survey questionnaire responses. **a** BAA was elevated in groups of current smokers in age- and sex- matched cohorts in NHANES population. The effect of smoking was reversible according to all bioage models, since there were no significant differences between those who never smoked or quit smoking earlier in life (the box-plots here are produced with the help of the "MORTAL-bioage with explicit age" model). **b** Concentration of C-reactive protein was significantly associated with BAA of the survey-based risk model, the "HAZARDS-Survey" (the red dot). All the other data points (colored in magenta) represent the association effect (the horizontal axis) between the survey responses and the BAA of the "MORTAL-bioage with explicit age" risk model built using the blood analysis variables. Circle sizes correspond to relative number of persons in the smallest of the two compared groups, the largest size corresponds to 50%, i.e. to the groups of equal size

Most notably, we observed that several characteristics from the "Depression screener" category also showed association with increased biological age, including self-reported sleeping troubles and feeling tired or having little interest in doing things ($p \approx 3\text{E}{-4}$).

3.5 Biological Age Based on Self-reported Well-Being Questionnaire

So far, we focused on comparing the performance of biological age predictor's association with mortality and morbidity depending on various machine learning approaches involved in the training of the models and on different learning targets, such as chronological age or age at death. In this section we turn to investigation of the role of the source of biological signal. The use of clinical blood analysis variables is common, and yet requires an invasive medical procedure. Instead, we proposed using a brief questionnaire, including a dozen of questions related to overall physical and mental health of an individual over the one month prior to the assessment.

We used various data fields of the NHANES survey database with the best associations with all-cause mortality and characteristic of the current health status of a study participant on a relatively short period of less than a year, most during the last three months or less, see Table 3.2. The data-fields marked with a star ("*") were available for all studied NHANES cohorts (1999–2010) and were used as covariates in haz-

Table 3.2 NHANES survey data fields

NHANES variable code	NHANES variable name (as in 2005-2006 cohort)
HSD010	General health condition (*)
HSQ470	No. of days physical health was not good (*)
HSQ480	No. of days mental health was not good (*)
HSQ490	Inactive days due to phys./mental hlth (*)
HSQ500	SP have head cold or chest cold (*)
HSQ510	SP have stomach or intestinal illness? (*)
HSQ520	SP have flu, pneumonia, ear infection? (*)
ALQ130	Avg no. alcoholic drinks/day -past 12 mos (*)
WHQ070	Tried to lose weight in past year (*)
WHQ090	Tried not to gain weight in past year (*)
PAD020	Walked or bicycled over past 30 days (*)
PAQ180	Avg. level of physical activity each day (*)
PAD590	No. hours watch TV or videos past 30 days (*)
PAD200	Vigorous activity over past 30 days (*)
PAD320	Moderate activity over past 30 days (*)
PAD460	Activities to strengthen muscle (*)
RDQ031	Coughing most days - over 3 mo period (*)
RDQ050	Bring up phlegm most days - 3 mo period (*)
RDQ070	Wheezing or whistling in chest - past yr (*)
RDQ135	Limit usual activities due to wheezing (*)
RDQ140	Had dry cough at night in past year (*)
DPQ010	Have little interest in doing things
DPQ020	Feeling down, depressed, or hopeless
DPQ030	Trouble sleeping or sleeping too much
DPQ040	Feeling tired or having little energy
DPQ050	Poor appetite or overeating
DPQ060	Feeling bad about yourself
DPQ070	Trouble concentrating on things
DPQ080	Moving or speaking slowly or too fast
DPQ090	Thought you would be better off dead
DPQ100	Difficulty these problems have caused
SMD070	No. cigarettes smoked per day now
SMD641	No. days smoked cigs during past 30 days
SMD650	Avg no. cigarettes/day during past 30 days
RIDAGEYR	Age (*)
RIAGENDR	Gender (*)

ards models. We built another Cox proportional hazards model and transformed its log-hazard ratio into the "HAZARDS-Survey" bioage, using the selected set of questionnaire data fields including chronological age and sex as covariates. The resulting predictor demonstrated a good association of its BAA with all-cause mortality at the significance level of $p = 7.2\text{E}{-}77$, see Table 3.1. Somewhat surprisingly, the statistical power of the association of the BAA of the "HAZARDS-Survey" was poorer, although not dramatically worse than that of "MORTAL-bioage with explicit age" model, which was trained in the clinical blood markers and explicit age.

The BAA of the pure blood markers- and the survey-based only models were almost equally well associated with the presence of chronic diseases ($p = 6.4\text{E}{-}28$ and $p = 8.8\text{E}{-}32$, respectively). Both models were sensitive to smoking status and predicted 2.6 years of the bioage difference at the significance level of $p = 1.1\text{E}{-}13$ and $p = 7.0\text{E}{-}15$, respectively (Table 3.1), even though we intentionally excluded any data fields directly related to smoking from the Survey data fields. The questionnaire and the blood markers could be combined and used for training of a more powerful, the combined "HAZARDS-Blood-Survey" bioage model that yielded a minimally improved association with all-cause mortality ($p = 3.9\text{E}{-}194$, see Table 3.1). The combination of the two sources of biological data immediately yielded a significant improvement in association with the health status (2.4 years, $p = 3.8\text{E}{-}37$) and smoking (4.1 years, $p = 1.0\text{E}{-}18$).

The BAA of the risk models based on the questionnaire- and clinical blood analysis were significantly associated with the levels of C-reactive protein (CRP, see Fig. 3.1b).

3.6 Deep Learning of Proportional Hazards Models and Biomarkers of Aging Discovery

Finally, we used the blood markers and questionnaire features combined to train a DNN-based hazards model "Deep HAZARDS-Blood-Survey". The model was based on the same architecture as "DNN-bioage" with loss function replaced by negative logarithm of Cox-Gompertz likelihood adopted from Bender et al. (2005):

$$-\log LH = \sum_n \frac{M_0}{\Gamma} e^{\Gamma t_n + x_n} (e^{\Gamma \Delta t_n} - 1) - \sum_n \delta_n \left(\log M_0 + x_n + \Gamma t_n\right), \qquad (3.1)$$

where M_0 and Γ are the initial mortality rate and mortality doubling rate of the Gompertz mortality law, t_n is the age of n-th participant, δ_n and Δt_n are the event outcome and follow-up time till event or censorship, and x_n is the output of DNN. The parameters of the baseline Gompertz mortality model were fitted to NHANES follow-up data without the DNN output ($x_n = 0$). We obtained $M_0 = 5.7 \times 10^{-5}$ and $\Gamma = 0.084$ per year corresponding to the average lifespan of 79.9 years (see, e.g., Tarkhov et al. 2017 for the discussion of the Gompertz model). The model estimate

was in a good agreement with the life expectancy at birth of 79.3 years reported for the US (WHO 2016).

In limited cohorts of human subjects, the simultaneous determination of small corrections to the Gompertzian variables Γ and M_0 is a poorly defined mathematical problem (Tarkhov et al. 2017). We, therefore, held the baseline model parameters fixed in Eq. 3.1 and optimized the deep network to include the physiological-state dependent variables through the network output variable x_n (Pyrkov et al. 2018b). As for all other models above, the log-hazard ratio of the deep mortality model was re-scaled to years and referred to as the "Deep HAZARDS-Blood-Survey" bioage.

As expected, the model predictor demonstrated a better association with all-cause mortality. We did not, however, observe any significant improvement of association of the model BAA with the health status ($p = 2.9\text{E}{-36}$) and smoking status in the healthy individuals ($p = 6.7\text{E}{-19}$). Nevertheless, the calculation shows that deep biological age models based on Eq. 3.1 may be a good starting point for state-of-the-art biomarkers of aging discovery. The suggested procedure explicitly exploits the exponential nature of the mortality acceleration characteristic to aging process and deep learning architectures for automatic non-linear feature extraction.

3.7 Discussion

A good biomarker of aging should predict the remaining health- or lifespan and, at the very best, be causally associated with the underlying biology of aging. We presented a systematic investigation of biological age predictors trained in sets of various physiological indices (clinical blood biomarkers or a self-reported questionnaire). Using chronological age as the target for supervised training of biological age models is almost always a bad choice, since it implies minimization of the discrepancy between the predicted and chronological age (i.e. aging acceleration) and thus effectively destroys the model sensitivity to changes in health. This undesired effect was especially aggravated in combination with deep neural networks (DNN). At the same time, the exponential Gompertz mortality rate acceleration in human populations suggests that proportional mortality and morbidity hazards models can be used as powerful alternatives to obtain a novel biomarker of the organism's state, reflecting the levels of external and endogenous stresses.

Proportional hazards mortality models are increasingly common tool for biological age predictions. Most recent examples include the "PhenoAge" based on blood sample data (Liu et al. 2018). The "PhenoAge" prediction was further used to train a DNA-methylation-based aging marker DNAm PhenoAge (Levine et al. 2018). The number of samples in both studies was comparable to that in our present work. DNAm PhenoAge was reported to be associated with all-cause mortality at significance level $p = 9.9\text{E}{-47}$ in a meta-study comprising samples from the Framingham Heart Study, the Normative Aging Study, and the Jackson Heart Study ($\approx$9000 participants with $\approx$2000 death events observed in follow-up). The authors also mentioned that its performance was superior to epigenetic biomarkers of aging (see, e.g., the

value of $p = 1.7\text{E}{-}21$ reported in Levine et al. (2018) for Hannum model (Hannum et al. 2013) trained to predict chronological age. This result also seems consistent with with our findings here with clinical blood markers and earlier in analysis of human physical activity-based bioage models (Pyrkov et al. 2018b). The overall conclusion is that the most accurate chronological age predictors produced the poorest associations with all-cause mortality (see Table 3.1) and thus should be avoided whenever possible in favor of the explicit mortality or morbidity models.

Advanced machine learning tools are naturally called to improve the biological age predictions, see, e.g., a model trained from clinical blood markers (Putin et al. 2016) and facial photos (Bobrov et al. 2018). The examples presented here show that the full power of the deep learning architectures could be harnessed for feature extraction and non-linear models fitting of risks, rather than chronological age models (Pyrkov et al. 2018b). The risk models, however, require follow-up information involving the incidence of age-related diseases or death. The exponential nature of mortality and morbidity acceleration implies that a risk model could be approximated by a logistic regression (Green and Symons 1983; Abbott 1985; Pyrkov et al. 2018a) to health status. This is especially useful, since morbidity data is easier to collect. We established a high concordance and comparable statistical power of the models trained to predict mortality and morbidity risks or prevalence of chronic diseases.

Training a chronological age prediction model from biological signals is a common approach to produce a biological age model, such as e.g., gene expression (Peters et al. 2015), IgG glycosylation (Kristic et al. 2014), blood biochemical parameters (Levine 2013), gut microbiota composition (Odamaki et al. 2016), and cerebrospinal fluid proteome (Baird et al. 2012). Some physiological indices are better correlated to age. For example, the Pearson's correlation of our "LIN-bioage" biological age predictor with age was only $r = 0.52$, that is significantly lower than $r = 0.65 - 0.70$ reported for models based on IgG-glycosylation and $r \approx 0.90$ for proteome (Enroth et al. 2015) or DNA-methylation data (Horvath 2013; Hannum et al. 2013). The profound correlations of specific physiological indices with the chronological age may still be an important signature of the organism state dynamics not directly associated with stress, morbidity and mortality.

As far as we can judge from a comparison of methods for assessing mortality risks (Levine and Crimmins 2014), the bioage model produced in DNA-methylation data and modeling of mortality risks had a similar association with mortality and health risks as those built in the present work using blood sample and survey questionnaire. DNAm PhenoAge was reported as $\approx$2 years different in cohorts of smokers and non-smokers at the significance level $p = 0.0033$ in a group of 209 current, 701 former and 1097 never-smokers (Levine et al. 2018). Our models produced the bioage differences from 2 to 4.2 years in cohorts including 530 current and 1201 never-smokers in the test set at the significance level ranging from $p \approx 1.0\text{E}{-}5$ to $6.7\text{E}{-}19$, depending on the model. The biological aging acceleration in the DNAm PhenoAge and in all our models was smaller, than the expected lifespan difference between smokers and non-smokers (which was estimated to be as much as ten years Doll et al. 2004). The statistical power of, say, the "MORTAL-bioage" with explicit age

model would drop to comparable to that of DNAm PhenoAge if the number of samples would roughly be halved to match the cohort sizes of Levine et al. (2018).

In agreement with our findings here and in the technically similar analysis of physical activity data (Pyrkov et al. 2018a), the BAA of the DNAm Phenoage was associated with smoking and, in the same time, was not different in groups of never smokers and those who quit smoking early in life. We note, that the BAA remained significantly associated with smoking even if we restrict ourselves to the chronic disease-free subjects only and hence could not be simplified only to the excess of the disease burden inflicted by smoking. Another notable BAA association in the healthy cohorts was that with CRP. The latter is a molecular marker of inflammation caused by a wide variety of conditions, from infections to cancer (Heikkilä et al. 2007). We, therefore, argue that the BAA is a universal stress indicator, a signature of the organism-level stress response to a generic environmental or endogenous factors, including lifestyles and diseases.

Similar performance of all hazards-based bioage models regardless of the source of biological signal suggests that all of them extract the same underlying biological factor, which manifests itself on the organism level and could be measured with similar accuracy in biological signals of different kind. The biological aging acceleration of these models is associated with disease burden in persons with diagnosed chronic age-related diseases. For healthy individuals, the same quantity was associated with smoking, current physical and mental health. The biological age thus emerges as a universal biomarker of age, frailty and response to stress suitable for applications involving large scale studies of the effects of future anti-aging drugs and life-style interventions on risks of diseases and on quality of life.

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Chapter 4
Biomarkers of Health and Healthy Ageing from the Outside-In

Jonathan Sholl and Suresh I. S. Rattan

Abstract Understanding the phenomenon of health is crucial for ageing research since there is often an implicit view on what constitutes health and how to measure it. We provide some reflections on how we might better understand and measure health, discuss the basic biological principles of survival, ageing, age-related diseases and eventual death, and end by tying these ideas together to rethink the nature of and implications for healthy ageing. We defend a more positive view on health understood in terms of various phenotypic parameters, such as robustness and resilience, and show how it relates to the aim to support these parameters over time to achieve healthy ageing. Together, these ideas suggest that the most effective strategies for healthy ageing may come from the outside-in by altering those aspects of the environment that prevent robust and resilient phenotypic responses and by supporting those aspects which ensure such responses.

Keywords Lifespan · Healthspan · Stress · Longevity · Health · Robustness

4.1 Introduction

A progressively failing health is commonly seen as a universal sign of ageing and old age. The challenge, however, is to understand the phenomenon of health in conceptual and practical terms, and to identify any reliable biomarkers of health, which could be the basis for screening, testing and validating potential interventions for maintaining and/or improving health over time. This is crucial for ageing research since there is often an implicit view on what constitutes health and how to measure it. Is health merely the absence of disease, and is it achieved by removing pathological

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mechanisms? Is it an ideal of complete well-being? Is it even amenable to scientific investigation?

The aim of this article is to address these issues by: (1) providing some brief reflections on how we might better understand and measure health; (2) discussing the basic biological principles of survival, ageing, age-related diseases and eventual death; and (3) tying these ideas together to rethink the nature of and implications for healthy ageing. We defend a more positive view on health understood in terms of various phenotypic parameters, such as robustness and resilience, and show how it relates to the aim to support these parameters over time to achieve healthy ageing. This entails that we should transform our approach towards ageing interventions from being "anti-ageing" in the sense of reverting an older state of the body to some rejuvenated youthful state, to those focusing on maintaining health, supporting healthy ageing and extending the health-span.

4.2 Health, Health Parameters, and Biomarkers

There are various ways to understand the phenomenon of health, each with their benefits and drawbacks, but what is often underappreciated is how experimental practices can imply a certain perspective on health. For example, much of medical practice is guided by a rather old view of health understood in terms of the absence of disease (Jensen 1987). If the aim is to remove what is deemed to be pathological, then medical interventions will mainly seek to suppress symptoms or to revert a localized dysfunctional part back to a previous 'normal' functional level so as to postpone death. This view of health is problematic conceptually and practically. On the one hand, it requires a definition of 'normal function', which is very difficult to do whether philosophically or scientifically (Boorse 1997; Kingma 2014). On the other hand, it misses how reverting functioning to a previous level or merely suppressing symptoms can often do more harm than good, such as trying to revert multiple functions within a pathological system that have adapted to different functional levels back to previous levels (Sterling 2004; Truchetet and Pradeu 2018).

As is well known, the World Health Organization suggested a more idealistic definition of health understood as being more than the mere absence of disease, whereby the individual experiences a "state of complete physical, mental and social well-being". This definition has been heavily criticized, in part for being idealistic, and in part for implying that no one or very few individuals are actually healthy (Dubos 1965; Huber et al. 2011). A more pragmatic version of this definition could be developed by specifying *adequate* levels of well-being, understood in terms of relative independence in one's activities of daily living (Rattan 2013). While this is helpful, it is focused more on practical, personal or social considerations, rather than starting from an up-to-date empirical understanding of living systems and their dynamic regulation. Interestingly, by starting with the latter, we can still support the former considerations.

A third way to approach health, and one that aims to be empirically-driven, could be traced back to the many attempts to equate health with homeostasis (Cannon 1929). This approach starts from the idea that living systems, both young and old, have the intrinsic ability to respond, to counteract, and to adapt to external and internal sources of disturbance. This is what makes them different from inorganic and non-living systems. The traditional conceptual model to describe this ability is homeostasis, which, however, is not totally accurate. The main reason for this is the notion of "stability through constancy", which does not sufficiently take into account the dynamic nature of information and interaction networks that underlie the complexity of biological systems (Schulkin 2003). Instead of homeostasis, various concepts such as allostasis (McEwen and Wingfield 2003), robustness (Kitano 2004, 2007), or homeodynamics (Rattan 2007; Yates 1994) have been proposed to emphasize the fact that, unlike machines, the internal milieu of biological systems is not permanently fixed, is not at equilibrium, and is a dynamic regulation and interaction among various levels of organization. The challenge, then, is to translate these biological concepts into a clear and workable explanation of the phenomenon of health (Lemoine 2015).

We propose, then, that it is better to shift from thinking about what health *is* in terms of a state, classification, or category (e.g. conforming to 'normality'), to thinking about health in terms of what individual systems or organisms *are capable of* in the face of perturbations, disturbances, or stressors (*Lemoine and Sholl, in preparation*). This capacity can be tracked through various phenotypic parameters, which are the characteristics used to evaluate system performance, or the system's observable responses to specific perturbations. This is what we call a *parametric* view of health. This might sound abstract or vague, but this is precisely what is being done in research on: (1) how living systems maintain one or multiple set ranges for a given variable (homeostasis, allostasis), (2) how systems maintain or return to a previous functional level following perturbations (robustness, resilience), or (3) what are the various costs of maintaining states or functions over time (allostatic load). The first two parameters capture the phenotypic responses of a system to a specific perturbation or stressor at a given time, whereas the latter captures the cumulative costs or the 'wear and tear' of surviving and adapting to disturbances.

Take two concrete examples. When evaluating metabolism, this can be measured in terms of varying blood glucose levels (allostasis), the degree of insulin sensitivity (robustness/resilience), and the cumulative inflammatory or oxidative load (allostatic load). When evaluating stress-responses, these can be measured in terms of varying hormone levels such as corticosteroids (allostasis), the degree of stress tolerance of the sympathetic and parasympathetic nervous systems (robustness/resilience), and the cumulative inflammatory load resulting from chronic stress (allostatic load). In each instance, what is being specified is whether the system or organism is robust or resilient—what it is capable of—*with respect to* specified internal or external perturbations. Taken together, these parameters help to create a dynamic description of what characterizes an individual's overall healthy phenotype. Thus, health is not a mere state, but is a variable quantity marking the individual's capacity for tolerating and overcoming perturbations.

These parameters can then be broken down into more specific mechanistic *biomarkers*, which help to explain the specific phenotype being observed. It is, however, an open question as to which and how many biomarkers are to be used to explain these parameters as this will depend on the best available evidence and what is most relevant for a given individual. Some examples of the classical biomarkers include blood lipids, blood pressure, glucose sensitivity, or heart rate variability, and the newer ones include telomere length, epigenetic markers, mitochondrial metabolism, autophagy levels, and microbiome diversity (Sagner et al. 2017).

4.3 Homeodynamic Space and Heterogeneity

As we shift from evaluating health at a given time to the cumulative changes in health throughout a system's life, we can also track the wide range of maintenance and repair systems (MARS), which aim to evaluate how systems' capacities to resist perturbations change over time at various levels of organization (Rattan 1995a, 2015, 2016). Some of the main MARS are: nuclear and mitochondrial DNA repair; anti-oxidative enzymes and free radical scavengers; degradation of damaged DNA, RNA, proteins and other organelles; apoptosis; detoxification of harmful chemicals and metabolites; immune responses; wound healing and tissue regeneration, and other higher order processes such as thermal regulation, neuroendocrine balance, and circadian rhythms. Perhaps the three main biomarkers of the ability to maintain these phenotypic parameters through time are: stress response (how far a system can tolerate perturbations and stressors), molecular damage control (repair processes turning on after a system has been severely perturbed), and constant remodeling (the ability to compensate to avoid damages or following damages) (Rattan 2015, 2016).

It is important to remember that these biomarkers are tracking changes concerning parts or subsystems *within* an individual organism. This creates various challenges to understanding health. First, due to the fact that there can be many underlying causes for the same phenotypic response, and that one cause or biomarker can give rise to or explain multiple phenotypic responses, we cannot fully understand health except on the level of the whole organism. Second, insofar as these parameters are developed based on personalized data, the resulting descriptions will not reflect statistical norms but *individual* variability. This is of course the aim of personalized medicine, and yet many current guidelines are based on 'normal values', or ranges that describe what is normal for most people, most of the time. But we know that what is normal for one individual could be disastrous for another (inter-individual variations), what is normal at one time could be harmful at another (intra-individual variations), and even if normal functioning is one goal of medical practice, normality is not necessarily optimal performance. Third, there is the problem that a "resilient cell is not necessarily a healthy cell" (Smirnova et al. 2015), since resilience or robustness can also be found in pathologies, such as cancer or metabolic syndrome (Kitano 2004; Truchetet and Pradeu 2018). Consequently, to more adequately understand

health we need to have an approach that accounts for such variability on the level of the whole organism.

To that end, all the mechanisms and parameters mentioned above should be understood as involving hundreds of survival-assurance genes, complex metabolic pathways, and environmental inputs, whose products and their interactions give rise to a "homeodynamic space" (Rattan 2015, 2016). This space tracks all of what is currently known to constitute the abilities of the organism to survive and meet the demands of its environment.

Putting this together, *the mechanistic biomarkers are what are used to track the presence of the phenotypic parameters (markers of robustness, resilience, allostatic load,* etc.*), and together these parameters help to explain the size and changes to the homeodynamic space* (see Fig. 4.1). By tracking an individual's biomarkers experimentally and clinically, we can better differentiate phenotypic responses in healthy and pathological individuals. For example, perturbations resulting in increasing performance of parameters (e.g. being able to tolerate more or larger perturbations, or the same perturbation for longer time), versus those resulting in decreasing performance of parameters and increased vulnerability or fragility to future perturbations (Smirnova et al. 2015). In this view, ageing, age-related diseases and eventual death are the result of a progressive failure of homeodynamics. As we further discuss below, this is also reflected in the definition of ageing as the shrinkage of the homeodynamic space (Rattan 2013), implying a progressively failing health.

Thinking of health in this way has two immediate benefits. First, it allows for *quantitative comparisons*, making health amenable to scientific investigation. Since we are concerned with tracking changing parameters, we are not initially interested in whether a system's performance fits into a category (healthy vs. unhealthy), but in investigating the actual profile of how a system responds: whether it is more or less

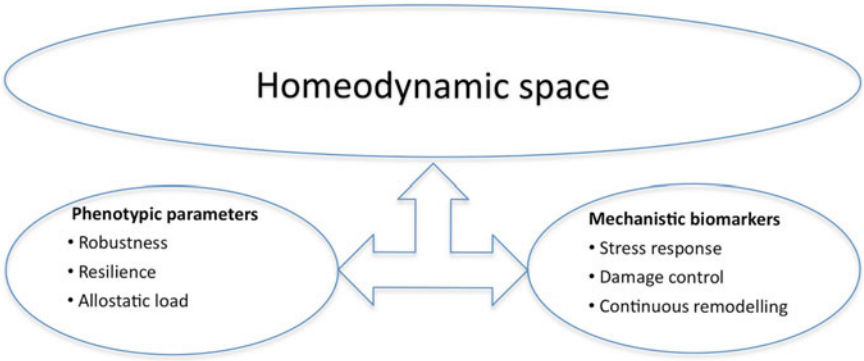

Fig. 4.1 Levels within a parametric view of health. The overall abilities of an individual are described by the homeodynamic space. This is in turn broken down into the phenotypic parameters that characterize the individual (or subsystem's) performance with respect to a specific perturbation. Finally, the mechanistic biomarkers are the measurable changes that help distinguish the different parameters and their contributions to the homeodynamic space

robust or resilient with respect to a specific perturbation. This allows us to clarify how systems can be healthier than one another, or even more importantly, healthier than themselves at different times (Schroeder 2013), and in different environments (Sholl 2016). Such comparisons need not assume health to be an ideal or perfect state, as in the WHO's aforementioned definition. Instead, they allow us to explore how to improve health markers relative to a given individual's starting point. Second, the study of these increasing/decreasing parameters and biomarkers of health provides an empirically-informed view of what *characterizes* healthy performance of living systems. As such, health is not best explained as the mere absence of disease, since health involves the *presence* of specific phenotypic responses. This positive view of health thus captures how being healthy is to be "more than normal" (Canguilhem 1991).

4.4 Recounting the Biology of Ageing

Before we can apply these ideas about health to healthy ageing, it will be helpful to recount the phenomenon of ageing (Rattan 2018). As understood through more than fifty years of modern biogerontological research, there is neither a rigid programme nor any real gerontogenes with the specific role of causing ageing and death of an individual. In the context of evolution, it is incorrect to assume that ageing and limited lifespan of an individual had some purpose or adaptive significance in terms of being advantageous for the species. In natural wild populations the probability of death by accidental causes, including disease and predation, is so high that there is never a significant number of long-lived individuals left that might require special mechanisms to terminate life for the sake of newly born individuals.

In contrast to the adaptive theories of the evolution of ageing and lifespan (Lenart and Bienertova-Vasku 2017), the non-adaptive theories state that ageing occurs either because natural selection is insufficient to prevent it, owing to its post-reproductive nature, or that senescence is a by-product of the expression of genes with early beneficial traits but deleterious and pleiotropic effects at later stages. Two major schools of thought (whose ideas are not mutually exclusive) in the non-adaptive theories of the evolution of ageing and lifespan are represented by the antagonistic pleiotropy theory, and the disposable soma theory based on Weismann's distinction between the soma and the germ line (Kirkwood 2008; Rose 1991). According to these theories, evolutionary forces have optimised conditions for efficient and successful reproduction either by (1) selecting for "good" early genes that later have "bad" effects, or (2) selecting for efficient maintenance and repair of the germ cells at the cost of somatic maintenance (Rattan 2006).

As regards the nature of genes involved in determining or regulating ageing and lifespan, a lot of effort has been put into discovering such genes, termed gerontogenes (Rattan 1995b). Although evolutionary theories of ageing and longevity discount the notions of an adaptive nature of ageing, and the diversity of the forms and variations in which age-related alterations are manifested suggest that the progression of ageing

is neither programmed nor deterministic, there appears to be a genetic component of some kind. The role of genes in ageing is indicated by: (1) an apparent limit to lifespan within a species; (2) some heritability of lifespan as evident from studies on twins; (3) human genetic mutants of premature ageing syndromes; and (4) some gene association with extreme longevity. The diversity of the genes associated with ageing and longevity of different organisms indicates that at the molecular level there are no universal pathways affecting ageing and longevity. Whereas the genes involved in repair and maintenance pathways may be important from an evolutionary point of view as the longevity assurance genes, each species may also have additional gerontogenic pathways which influence its ageing phenotype. Such genetic pathways have been termed as public and private pathways, respectively (Martin 2002). This notion of public and private gerontogenetic pathways may be applicable at the level of the individual as well.

The paradoxical situation of the genetic aspects of ageing and longevity on the one hand, and the stochastic nature of the progression of the ageing phenotype on the other, can be resolved by developing radically novel views about the nature of gerontogenes. The proposed term gerontogenes does not refer to any real genes, which have evolved specifically to cause ageing; and that is why the modified term "virtual gerontogenes" is more appropriate, as it reflects the altered state of other genes, giving the appearance of being the genes for ageing (Rattan 1995b).

It is also well established that the progression, rate and phenotype of ageing is different in different species, in individuals within a species, in organs and tissues within an organism, in cell types within a tissue, in sub-cellular compartments within a cell type, and in macromolecules within a cell. Individually no tissue, organ or system becomes functionally totally exhausted during ageing; and it is the dynamic interaction and interdependence at all biological levels that determine the quality and duration of life of an individual. Thus, biological ageing is an emergent, epigenetic and a meta-phenomenon, which is not controlled by a single mechanism or a central regulator (Rattan 1995a, 2015, 2016).

4.5 Healthy Ageing and Health Interventions from the Outside-In

Now, let us return to health to bring these insights together. With health as the capacity to tolerate and adapt to changing perturbations, and ageing as an emergent, decentralized multi-level phenomenon, healthy ageing would not be a return to a previous capacity as these capacities vary over time as our physiological needs and the demands of living change, sometimes randomly. While some see healthy ageing as fending off damage for as long as possible (de Grey 2006; Ryff and Singer 2009), this still assumes a negative view of health as the absence, delaying, or even removal of damage, and healthy ageing as primarily living longer.

Based on our positive view on health outlined above, we suggest that the aim to *maintain or support* an individual's physiological capacities to tolerate and adapt to perturbations and stressors should be central to any approach to ageing, be it individual, clinical, or social. Healthy ageing is not about remaining youthful but is what results when the parameters such as robustness, resilience, and allostatic load are supported such that one's overall homeodynamic space of *capacities* can be maintained for as long as needed. Increasing capacities, or what an individual is capable of doing, then, is not initially about living longer, but about increasing health-span: living well and thriving in the face of changing abilities and demands will often have longer life as a consequence.

Due to various practical limitations, it is understandable, albeit problematic, that the focus of ageing interventions tends to be on altering how internal systems respond to perturbations. This often entails altering biochemical and genetic pathways, immunological processes, or systemic regulatory mechanisms. However, since living systems are themselves embedded within larger systems and networks, many of which are shaped by their own niche-constructing actions (Odling-Smee 2003), understanding health in terms of robust- and resilient-responses implies that we also consider the broader environmental conditions that ultimately determine whether an organism is robust or not.

Taking these conditions into consideration forces us to ask a new question: how might we shape our environment so as to produce more robust and resilient individuals? *Since we are intimately linked to our environment through circadian, biochemical, metabolic, microbiotic, psychological, social, and ecological pathways, changing the environment is to change ourselves*. We thus argue that health, and healthy ageing, are facilitated by producing the most favorable conditions for increasing physiological capacities (increasing the overall homeodynamic space). Ageing interventions should thereby shift from trying to extend life by undoing internal damages, to that of producing an environment more conducive to increasing health-span.

4.6 Conclusions

We have argued here that a scientifically-informed approach to health and healthy ageing can come by first understanding how the phenomenon of health can be broken down into different aspects: the *phenotypic parameters* characterizing healthy responses, the underlying *mechanistic biomarkers* tracking these responses, and the overall *homeodynamic space* of an individual organism. With a more nuanced understanding of the complexities involved in ageing, with its lack of any underlying universal regulatory mechanisms and overarching adaptive functions, a more (w)holistic approach to healthy ageing is required.

These ideas suggest that the most effective strategies for healthy ageing may come from the outside-in by altering those aspects of the environment that are actually preventing robust and resilient phenotypic responses. The need for these various interventions is supported by the idea that if the most significant health problems

in many parts of the world have a social or environmental cause, then our medical treatments for diseases or ageing are undercut by returning individuals back to the same pathogenic social and environmental conditions that brought them to the clinic in the first place (Kresser 2017; Marmot 2015). Aiming to support or increase an individual's homeodynamic space through these various means would therefore be a more rational and wide-reaching approach to healthy ageing.

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Chapter 5
Epigenomic, Transcriptome and Image-Based Biomarkers of Aging

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Abstract The need to postpone age-associated decline and maintain late life healthspan is generally agreed, however, available tools and methods still lack accuracy. Indicators of biological age, or biomarkers of aging, therefore, have important roles in simplifying clinical diagnostics to allow healthcare to be tailored to individuals. Moreover, biomarkers of aging can alter current approaches to finding solutions to reduce biological age. Several families of biomarkers have emerged, though most of them are diseases-specific, some of them have great potentials as aging indicators. Here we review the current advances in biomarkers of aging. After describing the definition of aging biomarkers, we emphasize the importance of aging diagnostics, and discuss several basic considerations when modeling biological age. Finally, we highlight some biomarker candidates with the highest application potentials, including epigenome, microRNAs especially exosome microRNAs, and recently developed image-based phenome and microbiome markers.

Keywords Epigenetics · Transcriptome · Aging marker · Bioimage · DNA methylation · Chromatin structure · microRNA · Microbiome

5.1 Definition and Significance

Population aging is rapidly becoming a key concern worldwide as life expectancy is generally prolonged together with low birth rates. According to the Population Divi-

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sion of the United Nations the global number of centenarians should reach more than 25 million people in 2100 (Robine and Cubaynes 2017). The dramatic shift of median age of the population in many countries has emerged as an unavoidable problem. The WHO institutionalized the Global Burden of Disease (GBD) which has been measuring disability and death from a multitude of causes worldwide since 1990, as a critical resource for informed policymaking (http://www.healthdata.org/gbd). Early this year, WHO added an extension code for 'aging-related' via ICD-11 (http://www.who.int/classifications/en/), officially classified aging itself as a disease, and adding a code to a disease not yet recognized by ICD effectively allows for drugs targeting that disease to be clinically evaluated and approved, and for insurance companies to provide coverage for therapies targeting that disease. Despite the controversy about the broad science of Juvenescence involving ethical issues, an idealistic goal for aging research is to boost defenses and prolong healthspan, postponing the onset of morbidity, without necessarily extending lifespan (Timmons 2017; Hansen and Kennedy 2016). Therefore WHO proposed the need for an accurate measure of system stability, robustness or homeostasis, as course and outcome of deterioration in aging (Organization 2017). Whereas currently the vast majority of research that aims to identify novel anti-aging interventions uses lifespan measures as a primary readout to detect anti-aging effects.

Aging biomarkers are measures and criteria supposed to accurately define the biological responses to the 'passage of time', as a better replacement for chronological age in clinical implication when commenting on the health of an individual (Belsky et al. 2015). Distinguished from disease-specific biomarkers, aging biomarkers identify the aging process underlying diseases, with the capability to quantify aging degree on people with no clinical disease, thus making it possible to prevent an aging-related disease before its onset (Riera and Dillin 2015; Rae 2010).

It is critically important to pay attention to the discrepancy of biological age and chronological age. The fast-agers are those whose biological age markedly exceeds the chronological age (Chen et al. 2015). Since age is the greatest risk factor for most major chronic diseases (Kennedy et al. 2014), the diagnosis of 'early aging' may facilitate the pre-clinical diagnosis of aging-related diseases with a prolonged preclinical period. For an individual identified as fast-ager, more rigorous screening could be scheduled years earlier than his/her chronological age would otherwise indicate. Controversially, this could lead to personalized health surveillance plans as well as personalized pension plans and other nonmedical strategies (Scherbov and Sanderson 2016). Those fast-agers are likely to be regularly accessing services and thus may be more likely to undergo screening (or to have early signs and symptoms recognized), particularly where screening coverage rates are related to health service funding or to quality indicators that may encourage the screening of those with high risk indicated by their biological age (Sarfati et al. 2016; Rutherford et al. 2015; Gross et al. 2006). Conventional medical care is focused more on symptom relief with pain medication and surgical procedures (Riera and Dillin 2015), yet it is acknowledged that many clinical diseases can be highly manageable by therapeutic and lifestyle interventions, capable of drastically improving individual health and longevity (Lau et al. 2015; van der Stok et al. 2017; Mitchell et al. 2017). Screenings for specific

diseases are expensive, invasive, and restricted to specialist centers. Fast-aging may indicate an increased risk above a population average, which may be possible to facilitate the solution of the problem of population screening. The diagnostics of fast-agers can also alter the strategies of pharmaceutical industry from individual chronic diseases to targeting ageing underlying them, by altering biological age as a reliable read outs of reducing the prevalence of a number of age-correlated pathologies (Belsky et al. 2015; Crossland et al. 2017; Lamb et al. 2006; Hopkins 2008).

On the other hand, the diagnosis of slow-agers may pinpoint critical factors facilitating healthy and productive longevity. The extremes are the centenarians, who largely avoided or postponed most aging and chronic age-related diseases and geriatric syndromes and are characterized by decelerated aging. Taking advantage of the fact that centenarians are characterized by an extreme (healthy) phenotype versus patients suffering from age-related pathologies has possible therapeutic use (Armstrong et al. 2017; Santoro et al. 2018). In addition, a question that has long been of great interest in the field of aging research is that whether aging is a part of development process predetermined in genome (showing a certain predictable trajectory or 'homeorhesis'), or a result of the accumulative stochastic damage (de Magalhaes 2012; Gladyshev 2016). Whether an individual will follow a trajectory of fast- or slow-agers provide an insight into the nature of aging.

5.2 Considerations

Determining which biomarkers to measure is a remaining challenge. It is unlikely that a single array alone would be sensitive and specific enough when applied to the population (Chen et al. 2015). For example, the recent report of lifespan extension in mice treated with the FDA-approved mTOR inhibitor rapamycin represented the first demonstration of pharmacological extension of maximal lifespan in mammals (Harrison et al. 2009). Longevity effects of rapamycin may, however, be due to rapamycin's effects on specific life-limiting pathologies, such as cancers. While rapamycin did extend lifespan, it ameliorated few well-studied aging phenotypes (Neff et al. 2013), and exerted adverse effects on testicular degeneration and cataract severity (Wilkinson et al. 2012). A trial designed to measure a single readout of aging is only reflective of the changes in this biomarker, which may not be related the actual process of aging, thus reinforcing the necessity of surveying a wider array of clinical hallmarks of aging.

A critical variable in term of measuring aging biomarkers is the chronological age. An assay is likely to be of different sensitivity and specificity when applied at any chronological age. One possible reason is the onset of particular aging biomarkers may differ a lot. For example, defective mitochondrial energetics, and reduced muscle mass, often seen as the first sign of age, start at the third decade of life (Cartee et al. 2016), while the incidence of cardiovascular disease starts to progressively increase at 45–54 years of age (Shirakabe et al. 2016). Aging also imposes

a robust constraint on the onset of many neurological diseases, ranging from late onset neurodegenerative diseases, such as Alzheimer's (AD) and Parkinson's diseases (PD), to early onset psychiatric disorders, such as bipolar disorder (BPD) and schizophrenia (SCZ) (Glorioso and Sibille 2011). Another reason may contribute to the variation of biomarker performance is the inconsistent genetic background. For experiments in aging animal models, researchers tend to use the latest time point before population start to die as the observation of 'old' group when examining the genetic background. As centenarians are known to be characteristic of extremely health phenotypes (Robine and Cubaynes 2017), whereas patients suffering from age-related diseases may diminish in elder age groups, building a linear model using molecular and physiological data from adults aged 20–100 years and ignoring the lack of a consistent genetic background is not valid. An ideal biomarker assay should have optimal performance when applied at middle-age people, a key target group in whom the benefits outweigh the potential harms of screening (Schoenborn et al. 2018). Asymptomatic adults with a chronological age of 40–60 years may beat the top of picking order when recruited into normal aging remodeling (Timmons 2017), to avoid the complications of growth/maturation in the young group and the impact of population stratification in the older group. In addition, this could reduce the cost of clinical trials because the recruitment of older asymptomatic adults would yield clinical symptoms/events over the duration of a trial at an enhanced rated, making the trial size smaller and/or of shorter duration. In terms of economic consideration, conducting health screen and preventive drug trials on middle-aged could be more cost-effective.

The heterogeneity of aging is another major consideration to take into account for a comprehensive aging biomarker array. Across various aging model, when both genotype and environment are held constant, 'chance' variation in the lifespan of individuals in a population is still quite large (Rea et al. 2005; Liao et al. 2013; de Cabo et al. 2014). Though genetic perturbations and diet restriction have proven to increase longevity across species in well-defined experimental settings and isolated genetic backgrounds, exceptions found within heterogeneous mouse populations find that these treatments can also result in shortened lifespan (Liao et al. 2010), raising concern that clinical geroscience is premature. Preventative interventions into lifestyle aimed at slowing specific effects of aging have been successful, such as diet alteration (Diabetes Prevention Program Research Group 2015; Jenkins et al. 2018), exercise (Fan and Evans 2017), the two combined (Demark-Wahnefried et al. 2015), working memory training (Constantinidis and Klingberg 2016), caloric restriction (Kopeina et al. 2017), intermittent fasting (Thom and Lean 2017) and supplementation of vitamin D (Laslett et al. 2014). Nonetheless, the response to these interventions can show marked individual variation (Partridge et al. 2018). Ensuring classification and categorization of stereotypical human aging, clinical trials for aging biomarkers should include as many influencing factors as possible, covering a vast diversity of ages, ethnicities and sexes to achieve statistical relevance.

The aging biomarkers arisen from epidemiological cohort studies can possibly identify biomarkers of specific diseases but not per se. For example Type II diabetes is more prevalent in older people does not mean it follows that impaired glucose

tolerance is a useful biomarker for aging, as it can yield false-positive scores in young adults that have poor insulin resistance and glucose control (Timmons 2017; Dewey et al. 2014). Thus validation of the aging models must address a number of common-sense criteria about age-related diseases and health, universal aging. By contrast, distortion of depicting the 'rate of aging' can also stem from the over adjustment by removing the shared contribution to the variation between diseases and aging. For example, using whole blood RNA, a predictive model for aging was significantly correlated with impaired cognitive function and muscle strength, but this relationship was lost after adjusting the model for various clinical variables (Peters et al. 2015) and yet neuro-muscular decline is the most consistent functional phenotype of old age.

5.3 Epigenetic Aging Markers

Epigenetics encompass topics from gene-environment interactions and phenotypic plasticity, to centromere function, chromosomal imprinting, chromatin activity states, DNA methylation and so on (Deans and Maggert 2015). Although all of these topics are related to aging one way or another, here as aging biomarkers, we mainly focus on the change of chromatin structure, histone modification, and DNA methylation over the course of aging, which are considered to correlate with or even causally mediate the aging process.

5.3.1 Chromatin Structure

Chromatin is a complex of DNA, protein and RNAs. The dynamic and highly ordered three-dimensional structure of chromatin not only helps the compaction of the entire eukaryotic genome but also regulates the accessibility, recombination, repair and replication of genomic DNA (Venkatesh and Workman 2015). Although the structure of nucleosome—the smallest and repeating unit of chromatin—is stable, the higher-order structure including the interaction between distal genomic regions of chromatin as well as the interaction between the nuclear envelop and chromatin are very dynamic (Luger et al. 2012). Tightly packed genomic region and genomic regions associated with lamins are hardly accessible and not actively transcribed. Interestingly, significant chromatin structure changes occur during aging and senescence. These chromatin changes include loss of heterochromatin regions, global histone loss, chromatin spatial interaction changes, etc. (Sun et al. 2018).

Heterochromatin, originally recognized by the intense staining of DNA dyes, is characterized by condensed packaging of nucleosome, silenced transcription, H3K9me3 modification and binding of heterochromatin protein 1 (HP1) (Talbert and Henikoff 2017). Heterochromatin is inaccessible to the transcriptional machinery and could spread along chromosomes. Therefore, the condensation and relaxation of

heterochromatin play roles in regulating the expression of genes (Becker et al. 2016). Reduced levels of H3K9me3, H3K27me3, HP1 and the loss of heterochromatin are observed in premature aging diseases like Hutchinson-Gilford progeria syndrome (HGPS) and Werner syndrome (Shumaker et al. 2006; Zhang 2015). Large-scale change of heterochromatin and H3K9 methylation were also observed in C. elegans under mitochondrial stress, suggesting there could be a link between aging associated stress response, such as mitochondrial stress, and the formation of heterochromatin (Tian et al. 2016). In human and other invertebrates, the levels of HP1 and H3K9me3 also show a trend of decline during normal aging process (Scaffidi and Misteli 2006; Larson et al. 2012; Wood et al. 2010). Taken together, these reports indicate that the loss of heterochromatin could be a common marker of aging.

Advances in chromosome conformation capture (3C) methods and high-resolution nuclear microscopy greatly improved our understanding of higher-order chromatin organization. By analyzing the high resolution Hi-C data, chromatin segments were partitioned into different compartments (Lieberman-Aiden et al. 2009) and these chromatin compartments were further divided into smaller Topologically Associating Domains (TADs) (Dixon et al. 2012). Higher resolution 3C-based methods like ChIA-PET (Fang et al. 2016), HiChIP (Mumbach et al. 2016) and Capture Hi-C (Dryden et al. 2014) could even detect the chromatin loops inside TADs (Rao et al. 2014). Although there are only limited numbers of studies focus on the chromatin higher-order structure changes during cellular aging or more precisely cellular senescence, a general decline of the chromosome compartments and local chromosomal interactions were observed when comparing senescent cells and proliferating cells (Chandra et al. 2015; McCord et al. 2013). However, there is also reports observed unchanged pattern of chromatin compartmentalization and TAD boundaries accompanied with gain of short-range interactions and loss of long-range interactions during senescence (Criscione et al. 2016). These contradictory observations hint that the relationship between cellular aging and changes in chromatin higher-order architecture could be cell-type or tissue specific. More importantly, whether the changes observed for cellular senescence still hold true for organismal aging is still unknown.

Nucleosome is composed of a histone octamer coiled by 147 of DNA. Chromatin is a chain of these nucleosome structures linked by linker DNA. This complex of DNA and protein plays important roles in DNA repair, replication, transcription and chromatin remodeling (Bannister and Kouzarides 2011). The global occupancy of nucleosome is decreased during aging and senescence in many species, including yeast (Hu et al. 2014), worms (Ni et al. 2012) and mammals (O'Sullivan et al. 2010). Overexpressing histones or attenuates the decrease of histone could extends lifespan. Furthermore, the histone variant H3.3 and H2A.J also accumulate during aging (Contrepois 2017; Piazzesi 2016). Together, these reports emphasize the importance of nucleosomal components to the aging process, suggesting that the aging associated changes of histones can also be a potential aging marker.

5.3.2 Histone Modifications

In addition to histone variant, histone modifications also change during aging process (Benayoun et al. 2015). There are more than 100 modifications that was identified on different histone proteins, including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation, etc. (Zhao and Garcia 2015). Among these modifications, histone acetylation and methylation were the earliest discovered and most well-characterized (Wang et al. 2018). Although the exact molecular mechanisms between histone modifications and aging are obscure, there are many researches linking specific histone modification to aging or senescence. In this part, we review the potential of well-studied histone methylation and acetylation as aging markers. We also briefly introduce the role of other histone modifications in aging.

A wide variety of histone methylations were associated with aging in different organisms. H3K27me3 is a histone marker related to transcription silencing and heterochromatin. The demethylases of this modification are reported to modulate lifespan in worms, flies and mammals (Shumaker et al. 2006; Jin 2011; Maures 2011; Siebold et al. 2010). The age-associated changes in global H3K27me3 level are tissue dependent (Zhang 2015; Baumgart 2014). Moreover, when comparing the distribution of H3K27me3 in young versus old murine hematopoietic stem cells, both increase and decrease of H3K27me3 were observed at different genomic loci (Sun et al. 2014). In addition to H3K27me3, the age related changes of other types of histone methylation like H3K4me2/3, H3K36me3 and H3K9me3 are also well-studied. H3K9me3 is another repressive histone modification related to heterochromatin, which showed a global decrease during aging in Werner syndrome stem cell [what system?] (Zhang 2015). In contrast to H3K27me3 and H3K9me3, H3K4me2/3 is active transcription related histone marker and H3K36me3 is related to transcriptional elongation and splicing (Wood et al. 2010; Sen et al. 2015). In Drosophila, aging is accompanied with a gain of H3K4me3 and H3K36me3 (Wood et al. 2010). Whereas, levels of H3K4me2 were found to increase at the promoters and enhancers globally during aging in rhesus macaques (Han et al. 2012). Knocking down of the H3K4 methyltransferase SETD1A/SET-2 in *C. elegans* extends lifespan (Greer 2010). However, the effects of H3K4me3 on longevity are context dependent as both increase and decrease of lifespan were reported (Ni et al. 2012; McColl et al. 2008). Different from H3K4me3, lower level of H3K36me3 is linked to shorter lifespan in yeast (Sen et al. 2015). Other histone methylations like H4K20me3 were also involved in the aging process (Shumaker et al. 2006).

Acetylation is another crucial category of histone modifications that plays important role in longevity. As reported by in vitro and in vivo studies, histone acetylation deposited intensively in the actively transcribed chromatin compartment (Peleg et al. 2016). Histone acetyltransferases (HATs) and deacetylases (HDACs) are the enzymes responsible for maintaining the homeostasis of histone acetylation. Many HATs and HDACs including orthologues of Sir2, hda-3 and Rpd-2 can modulate the lifespan in yeast (Dang et al. 2009), worms (Tissenbaum and Guarente 2001) and flies (Rogina and Helfand 2004). The global levels of H3K56ac decline in yeast

during aging (Dang et al. 2009). H4K16ac, another histone modification associated with telomere maintenance, also exhibit a global loss in both physiological aging and HGPS model (Krishnan et al. 2011). H4K12ac was found to correlate with age-associated memory impairment (Peleg et al. 2010). Recently, a decrease of H3K27ac level was also found in human and mouse brain during aging in particular in the gene bodies of aging upregulated genes. This H3K27ac pattern can be used to predict aging-associated gene expression changes (Cheng et al. 2018).

Histone modifications other than methylation and acetylation were also found to be involved in the aging process. However, due to the limitation of detecting technique and a relatively weak association with aging, studies on these modifications are much fewer than methylation and acetylation. The phosphorylation of serine 10 in histone H3 (H3S10ph) was reported to increase in mice during aging (Kawakami et al. 2009). Conversely, H3S10ph show opposite trend during in vitro senescence of human fibroblasts (O'Sullivan et al. 2010). Histone ubiquitination was involved in the response to DNA damage, which can lead to premature neuron aging and mortality (Bergink and Jentsch 2009). The relationship between these histone modifications and aging are elusive, and further works are needed to clarify their potential as aging markers.

5.3.3 DNA Methylation

The major targets of DNA methylation are CpG dinucleotides, which are unevenly distributed throughout the genome. Clusters of CpG dinucleotides in the genome are called CpG islands which were often found at gene promoters (Schubeler 2015). Methylation of the cytosine in CpG dinucleotides generates 5-methylcytosine (5mC), which is the most abundant chemical modification of DNA in mammals. Most CpGs are methylated except CpG islands which were predominantly not methylated (Field et al. 2018; Deaton and Bird 2011). Methylation of CpGs at gene bodies promotes expression and suppresses cryptic transcription (Neri et al. 2017; Yang et al. 2014). Distal regulatory sequences including enhancers contain an intermediate level of DNA methylation, which would promote gene silencing (Elliott et al. 2015; Stadler et al. 2011). In normal cells, CpG islands are usually not methylated. CpG island methylation is associated gene silencing and is a feature of some cancer cells (Deaton and Bird 2011). The status of DNA methylation is not static. 5mC can be deposited by DNA methyl transferases (DNMTs) and removed by demethylases in the ten-eleven translocation (TET) family (Schubeler 2015; Wu and Zhang 2017). Among the approximately 28 million CpG sites in mammalian genome, many of them were reported to show age-associated changes (Fraga and Esteller 2007).

The availability of large cohort DNA methylation data facilitated researches on the relationship between DNA methylation and aging. Several groups have developed DNA methylation clocks that can accurately measure chronological age (Horvath and Raj 2018; Nevalainen et al. 2017; Wagner 2017). Furthermore, DNA methylation clocks predicting biological age are also established (Levine et al. 2018). All of these

clocks are derived by multiple linear regression algorithms. The methylation statuses of CpG dinucleotides from each donor with known chronological ages were used to train the linear models. A subset of CpGs combined with different weights were selected to predict the age of new samples (Field et al. 2018). This methodology can achieve great accuracy in predicting chronological age (with a correlation exceeds 0.95 between true age and predict age) (Horvath and Raj 2018). When using this regression-based method to estimate the relationship between DNA methylation and physiological dysregulation, even mortality and health span can be predicted (Levine et al. 2018). Although DNA methylation can produce very accurate prediction of age, there are still some questions not fully addressed. Due to the huge changes of DNA methylation status, these clocks cannot be applied to the development stages (Horvath 2013). Another question is about the mechanisms—why a small subset of CpG sites can accurately predict age is still unclear. When examine the CpG sites used in different clocks, the overlap are pretty low (Stubbs et al. 2017; Wang et al. 2017; Petkovich 2017). How aging and DNA methylation influence each other is also an open question, especially for those CpGs used in DNA methylation clock predictors.

5.4 Why RNA

The variation in human transcriptome, a 'reading output' of DNA, has proven an important reflection of human aging (Sood et al. 2015; Kang et al. 2011; Enge 2017). The abundance of RNA is influenced by genetic, epigenetic, and environmental factors and thus making it an ideal molecular entity to reflect complex physiological and pathophysiological process of aging (Timmons 2017). DNA methylation have achieved accurate age estimates for any tissue across the entire life course (Horvath and Raj 2018), but the age acceleration indicated by these 'epigenetic clocks' does not associate with subsequent fitness measures over the years follow up (Marioni et al. 2015). There have been several protein biomarkers claim to diagnose clinical status (Integrative Analysis of Lung Cancer et al. 2018; Wong 2017; Young et al. 2014; Keshishian et al. 2017; Chan et al. 2014), but they rarely shared common set of proteins across multiple studies, even when diagnosing the same aspect of aging (i.e. cognitive impairment). A reliable multiplex protein assay requires centralized laboratories to ensure technical performance, thus the sample collection and transport costs are high. A transcriptome-wide gene chip or RNA-seq can be globally unbiased, high-throughput and cheaply collected, transported and processed. Compared with targeted assays, a high-throughput/genome-wide assays can provide remaining data for assay optimization and serve additional models, including combined with specific disease biomarkers to estimate the risk for developing a certain disease or predicting the response to clinical interventions.

5.5 miRNAs as Aging Biomarker

MicroRNAs (miRNAs) have emerged as differently crucial post-transcriptional regulators during aging. miRNAs are short, noncoding RNAs that regulate the expression of mRNA targets in a sequence-specific manner by inducing mRNA degradation or translational repression (Bartel 2004).

Genome-wide miRNA profiles have identified differentially expressed miRNAs through aging process in a variety of organisms, including the whole body of *C. elegans* (de Lencastre 2010; Kato et al. 2011), the fruit fly *Drosophila melanogaster brain* (Liu et al. 2012), mouse brain (Li et al. 2011a), liver (Liang et al. 2011; Green et al. 2017) and human muscle cell (Singh et al. 2016; Drummond et al. 2011), oocyte (Battaglia et al. 2016), blood (Olivieri et al. 2017), and serum (Smith-Vikos et al. 2016). Many studies performed in *C. elegans* observed age-related changes in two highly conserved miRNA, let-7 and mir-34, both of which regulated conserved age-associated pathways. Let-7 regulates DNA damage checkpoint genes and mitochondrial respiration genes (Kato et al. 2011; Drummond et al. 2011). A target of let-7, daf-12, is reported to shorten lifespan when knocked down (Ewald et al. 2016; Fitzenberger 2014). Besides conventional miRNA functions in which miRNA inhibit transcription, let-7 can also act as an activator of RNA-sensing Toll-like receptor (TLR) 7 and induces neurodegeneration (Lehmann et al. 2012). And the function of mir-34 is involved in DNA damage response (Kato 2009), senescence (Tazawa et al. 2007) and cell death (He et al. 2007). Notably, though mir-34 is identified as age-associated by many studies, the directions of changes are not consistent (de Lencastre 2010; Kato et al. 2011; Ibanez-Ventoso et al. 2006; Li et al. 2011b, c; Khanna et al. 2011), and its effect on lifespan differ as well (Liu et al. 2012; Yang et al. 2013). Promotor hypermethylation of miR-34a was found in a various subsets of ovarian cancers, indicating its role in suppressing cancerous phenotype of the cells (Schmid et al. 2016). Moreover, the insulin/IGF-1 signaling (IIS) pathway acts as a central signal transductor of aging (Tatar et al. 2003). Many genes reported to extend lifespan in a manner dependent on this pathway. Each of the core genes in IIS pathway is predicted to be targeted by multiple aging-associated miRNAs (Inukai et al. 2012), suggesting the mechanism underlying miRNA as potential biomarker for aging.

Besides specific miRNAs identified as differently expressed during aging, there are global trends in miRNA expression. Many studies have observed a global decline with age in a number of organisms and tissues across taxa (Liu et al. 2012; Li et al. 2011a; Ibanez-Ventoso et al. 2006; Boehm and Slack 2005; Boulias and Horvitz 2012; Smith-Vikos et al. 2014; Noren Hooten et al. 2010; Bates et al. 2010; Maes et al. 2008; ElSharawy et al. 2012; Pandey et al. 2011). The decline is linked to downregulation of miRNA processing genes with age (Mori et al. 2012; Inukai et al. 2018), and could be prevented by life-extending interventions such as caloric restriction in both mice and *C. elegans* (Mori et al. 2012). But conversely in mouse liver (Green et al. 2017; Maes et al. 2008) and brain (Li et al. 2011a), miRNA exhibit predominant upregulation with age. Anyhow the global changes in miRNA expression are linked with aging.

Notably, across six different dietary and excise interventions, in mouse liver the majority of miRNA, similar to other non-coding RNAs, that show association with lifespan are negatively, instead of positively correlated with lifespan (Green et al. 2017).

Furthermore, miRNAs constitute promising extracellular biomarkers for aging. miRNAs can be released into the extracellular environment and travel between tissues (Hoy and Buck 2012) in protein-bound (Arroyo et al. 2011), membrane-bound (Hunter et al. 2008), or HDL-bound (Vickers et al. 2011) forms in the bloodstream. MiRNAs are relatively stable and abundant in the blood/blood component (serum/plasma) sample and can be quantified by high-throughput techniques such as qRT-PCR, microarray and miRNA-seq. Argonaute2 protein binds and protects circulating microRNAs from degradation, contributing a significant proportion of RNA in the blood (Huang et al. 2013). And miRNA can also be detected in various biofluids such as blood, saliva, tears, urine, amniotic fluid, colostrum, breast milk, bronchial secretions, cerebrospinal fluid (CSF), peritoneal fluid, pleural fluid, and seminal fluid (Machida et al. 2015; Weber et al. 2010). Thus extracellular miRNAs are expected to be a noninvasive biomarker for aging. Among all the sample types, exosomes isolated from serum seem to be more precise biomarkers compared with intracellular miRNA and non-exosomal miRNA (Cheng 2014) because exosomes has the ability to carry brain-originating miRNA across the endothelial cellular layers of the blood-brain barrier (Haqqani et al. 2013); and the exosome membrane protected RNA from RNase A degradation (Huang et al. 2013).

5.6 Microbiome as Aging Biomarker

Microbiome can be considered as an extra organ in the human body that does not age per se, but its composition in older people differs from that of young adults. What makes it an ideal biomarker for aging is that the alteration does not occur at a certain chronological threshold, instead it changes gradually with time (Mangiola et al. 2018). The composition of gut microbiota shifts towards a *Bacteroidetes*-predominated population with age (Claesson et al. 2011). However, the variations in the microbiota among the elderly are greater compared to young adults, so the trend of relative composition is less informative in terms of aging biomarkers (Claesson et al. 2012). The transition from young adults to elder people also include a decline in microbiota diversity, a decrease in saccharolytic bacteria and an increase in proteolytic ones (Bischoff 2016). Although gut microbiota diversity is not significantly associated with age, but associated with the general decline in the health status and aging-related frailty (Claesson et al. 2012; Jackson et al. 2016; Jeffery et al. 2016), thus it is an indicator of biological age rather than chronological age per se.

The differences between the intestinal microbiota in young and old adults can be explained by the aging-associated decline in immune system and gut physiology. A low-grade, long-term chronic inflammation is observed in a range of species (Clark et al. 2015; Li et al. 2016; Conley et al. 2016), accompanied by many age-

related diseases (Bischoff 2016; Konturek et al. 2015). Such inflammatory state might make the host more sensitive to gut bacteria, and vice versa, the alterations of intestinal microbiota are associated with various pathological conditions with age. Therefore, the host-microbe homeostasis is prognostic of aging-related risks. Besides, aging-associated breakdown in gut barrier has profound effects on the shift in elder intestinal microbiota. This barrier dysfunction shows up as microbe expanding into non-permissive area (Li et al. 2016; Franceschi and Campisi 2014). The cell-wall component of translocated intestinal microbe was found in plasma of old mice (Thevaranjan 2017), suggesting the potential for microbiota as signatures of the complex aging physiological changes interacting with microbial homeostasis.

5.7 Imaging-Based Aging Biomarker

The image capturing and processing methods have been developed and widely applied in the aging related studies (Xia 2017; Chen and Han 2015). These methods mainly focus on the aging phenotypes quantification and aging biomarker identification (Zhao et al. 2016). The perceived age from facial images has been shown to be a biomarker of aging (Gunn et al. 2008). The perceived facial age is also significantly associated with variants in genes (Liu et al. 2016). Some quantitative facial features increase with aging, such as the mouth width and nose width, while some facial features decrease with aging, such as the slope of eye corners (Chen et al. 2015). Facial pigmentation gradually appears with the increase of age. The analysis of facial image shows that the facial pigmentation is also highly associated with some biological conditions, such as the carotid atherosclerosis (Miyawaki et al. 2016).

In human brain, the analysis of high-resolution magnetic resonance images shows that the tissue densities in the frontal, temporal cortices and parietal significantly decline during aging (Colcombe et al. 2003). The regional brain volume declines with normal aging, such as the ventricular CSF and frontal gray matter regions (Driscoll et al. 2009). The whole-brain volume also significantly decreases with increasing age (Scahill et al. 2003). These quantifiable changes of brain are consistent with the decrease of memory during aging.

5.8 Conclusion

Aging is an inevitable phenomenon, but some intervention regimens have been successful in extending lifespan in model organisms, together with the trait displayed centenarians, compression of morbidity at high age, revealing the controllability of aging and its effects. The interventions to prevent ill-health with age require better measures of aging process and responses to interventions. Age-related changes at molecular levels as both cause and consequences of aging, occur in the initial step before the systemic and structural changes along aging process, giving the potential

for molecular signatures we mention above as biomarkers for predictive interventions of aging.

Although enormous progresses in aging biomarkers have been made in the past years, measures of aging per se have not entered into routine clinical practice anywhere. Many of the biomarker models identified so far are built using a specific set of training and testing samples from a single study, thus the molecules selected may reflect unknown features of that particular cohort and may include laboratory or platform biases. Whether they can be reliable used in a widespread population is unknown. To generate robust aging biomarkers, academic laboratories need to create large public biomarker database, so that researchers can use independent external validation to avoid building predictors on stochastic or accidental associations. Moreover, biological researchers need to better integrate practices from mathematical and computer sciences to avoid false-positive findings and increase diagnostic and therapeutic capabilities and deepen the understanding of aging.

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Chapter 6
An Overview of the Molecular and Cellular Biomarkers of Aging

I. A. Solovev, M. V. Shaposhnikov and Alexey Moskalev

Abstract The pace of physiological aging differs much in individuals, thus the tools to measure it precisely are to be found. Every quantitative trait of the organism, which is known to change with age, may be used as a biomarker of aging, but consequently not all biomarkers are informative and valuable for diagnostic purposes. The present chapter contains a review of basic molecular and cellular biomarkers of aging which are subdivided into groups named after the main target (it may be a molecule, a signaling cascade (or molecular complex), an intracellular structure or its functions) which significant changes are measured during aging. The process of biomarkers' evaluation is described in the review as well as the procedure of endophenotype evaluation. We also discussed the relationship between endophenotypes and biomarkers in the context of existing "omics" approach in biogerontology.

Keywords Biomarker · Aging · Biological age · Endophenotype · Omics

6.1 Introduction

Since 1950s the term "biological marker" had been known, but only in 1980s, the "biomarkers" became popular and widely spread. In context of aging the concept of biomarkers aroused interest after an emergence of two frequently cited works by López-Otín et al. (2013) and Engelfriet et al. (2013). First one suggested to introduce several groups of aging signs on molecular and cellular levels called afterwards

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"hallmarks of aging" by analogy with "hallmarks of cancer" and the second one represented basic human biochemical markers of aging appropriate for longitudinal studies. Consequently, the main idea of the biomarkers is a quantitative estimation of biological differences in health between people of the same age, do precise and exhaustive appraisal based on numerous analyses and tests' results often appropriate for predictive models' creation. The holistic view of the biomarkers in aging science may be mixed with the "biological age" which is less objective and shows the deficit between predicted individual's life expectancy and the idem marker measured for the cohort or population of the same age. Theoretically and practically many biomarkers may be used for biological age estimation, but the last is depersonalized at least, being connected to the average values known for population and not appropriate for precision medicine.

The biomarkers of aging are diverse and may be found in different levels of organisation, but we will focus on molecular and cellular one's in the present chapter.

6.1.1 Identification of Biomarkers

Criteria for biomarkers' identification were postulated by AFAR (The American Federation for Aging Research) as four statements, which every biomarker of aging must be fitted.

(1) The biomarker predicts the pace of aging
(2) The aging biomarker must reflect the fundamental process included in physiological aging and never in pathogenesis of a disease
(3) It must be reproducible and non-invasive, in other word harmless for a person tested multiple times
(4) It must be reproducible in model animals belonging to different taxa

It is no doubt that the biomarker, which is described above, is an ideal model. Nevertheless, at least the two first demands of AFAR have to be satisfied. When we discuss molecular mechanisms of aging we mean that they are quantifiable, predictive and informative in estimation of aging speed, as well as they must be related to some fundamental process which lays in the biological basis of aging.

Despite the fact that numerous biomarkers exist, most of them do not describe the state of the whole organism (even omics ones). The evaluation of aging biomarkers and composition of biomarker panels consisting of the most informative tests, which are targetable by novel interventions, is now a new challenge accepted by in silico biogerontology (Putin et al. 2016). A computational approach engaging deep machine-learning algorithms and neural networks in biomarker selection opens a new perspective for systemic analysis of populations' big data and for population specific biomarker panels' creation.

Unfortunately the redundancy of diverse data creates a great difficulty, so called "curse of multidimensionality", in analysis and interpretation of the results when a

complex study have been carrying out during which the thousands of dimensions (measurements of biomarkers' values) appear.

6.2 Molecular Biomarkers of Aging and Their Evaluation

The diversity of molecular biomarkers of aging process may be subdivided into several classes according to the level of genetic information transduction or in other words "central dogma of molecular biology", there is a one more classification of aging molecular biomarkers based on already mentioned concept called "hallmarks of aging", in the present chapter we use the first one.

In the basis of our hypothetical construct deoxyribonucleic acids stand including their modifications and complexes formed with DNA. The quality of the DNA molecules (genome, mitochondrial genome, epigenome) is probably the most obvious marker, which correlates with lesion appearance on other levels. The second stage of information transduction is transcription of information encoded in DNA into RNA, specific transcriptomic and post-transcriptional signatures may be found in aging, the genome-wide transcription profile is a significant biomarker as well as small profiles of non-coding RNAs in different substances and tissues. The translatome level is the next stair in the aging biomarkers' hierarchy, it consists of the whole proteome, peptidome and a set of their post-translational modifications (including chromatin modifications). The metabolome stands above and unites all the processes being a direct bond with the environment. Nowadays, the metabolome-wide barcode becomes a new instrument of aging rate measurements as well as whole genome methylome (the most predictive aging biomarker ever).

6.2.1 DNA-Associated Biomarkers of Aging

Telomere attrition is known as one of the first described molecular aging biomarkers (Bekaert et al. 2005). Telomeres are hexameric tandem-repeated complexes including ribonucleoproteins located at the chromosome ends, every DNA replication makes telomeres shorter due to the exonuclease activity of DNA-polymerase, but there is an enzyme called telomerase that can resynthesize the lost telomeric repeats. This enzyme is active mainly in immortalized and stem cells, but the activity in somatic cells is facultative. The length of telomeres in cells indicates replicative status of the tissue. It is possible to predict the life expectancy and aging rate having the tissue-specific dynamic profiles of telomere attrition in proliferating tissues. Telomeres change their length after various interventions in some way activating telomerase. Two properly studied biological markers of aging associated with telomeres exist: blood leukocyte telomere length and proteins induced by telomeric DNA damage response. Also a locus 3q26 (rs12696304) was identified as an aging accelerator. It encodes TERC, a ribonucleic component of human telomerase. Individuals carrying

the gene variant mentioned above have biological age differed from those who had normal variant, according to telomere length the difference was 3–4 equivalent years (Codd et al. 2010).

The next DNA-associated biomarker is a methylome also known as the epigenetic clock. Probably it is the most intensely studied biological marker of age due to its high precision, using epigenetic clock it becomes possible to estimate the biological age with 3.5 year accuracy; there are 353 CpG clock islands in total (Horvath 2013). The 193 sites are hypermethylated and positively correlate with aging. The 160 islands being hypomethylated negatively correlate with aging. The logarithmic curve of clock ticking (signals from 353 CpGs) is observed in growing individuals afterward the pace of "ticking" slows down, thus, the curve becomes linear when the adulthood starts.

DNA-methylation is not the only modification observed in aging genome, epigenetic biomarkers are diverse and associated with histone modifications e.g. senescence-associated heterochromatic foci or with histone loss, however the chromatin and histone biomarkers are poorly understood in the context of aging (Tang et al. 2011). However, aging alters the levels of CpG hydroxymethylation (Tammen et al. 2014), this process has not been studied as deeply as DNA-methylation and the biomarker has been being used only for hepatic bioptates (Tammen et al. 2014) and tested on human brain (Akbarian et al. 2013).

Mitochondrial DNA copy number have been evaluated as a biomarker of age (Barazzoni et al. 2000). The value of indirectly measured mitochondrial genomes (with the use of cytochrome c oxidase (COX) mRNA expression) decreases with age as wells as the quality of mtDNA (Barazzoni et al. 2000; Zhang et al. 2017). In addition, cell-free mitochondrial and genome DNA circulating in plasma is known to be a diagnostic marker for several diseases, but in nonagenarians (90+) its fragmentation degree, especially low molecular weight fraction, may be used for life expectancy measurements (Jylhävä et al. 2011).

6.2.2 RNA-Associated Biomarkers of Aging

The development of post-genomic tools determined new perspectives in aging biomarkers' selection, consequently Peters et al. (2015) showed a possibility of "transcriptomic age" measurements carried out on blood samples' transcriptional profiles for which a large cohort (more than 14,000 persons of European ancestry) dataset was used for calibration. Differentially expressed genes (1497 sequences in total) are chosen as indicators of "transcriptomic age".

There is no doubt that transcriptional profile is very diverse and some specific fractions of RNA may be utilised as biomarkers of aging. Non-coding RNA fraction is one of the novel identified groups of transcripts which levels correlate with age. Short sequences of miRNA group (less than 23 bases) circulating in plasma were first associated with murine aging (Li et al. 2011). The miR-21 group was associated with human and murine hearing loss as well as inflammation (Olivieri et al. 2012).

Circulating miRNAs were studied as biomarkers in context of healthy and unhealthy aging trajectories. Changes of miRNA levels were compared between groups of centenarians, their offsprings and people with a typical rate of aging (Olivieri et al. 2017). In centenarians (whole blood study) miR-106a, miR-126, miR-20a, miR-144, miR-18a were downregulated with age and miR-320d, miR-320b were upregulated (vs. old group) miRNAs (ElSharawy et al. 2012). Olivieri et al. (2012) detected miR-21 downregulation in old individuals' plasma and upregulation in people from the <65 years cohort. It was showed that miR-363, miR-1974, miR-223, miR-148a, miR-148a upregulation versus old group on EBV transformed immortalized B-cells (Gombar et al. 2012). Serna et al. (2012) tested mononuclear cells of centenarians versus old and young people and explored miR-21 and miR-130a upregulation.

In serum of old donors Hooten et al. (2010) found a decrease in miR-151a-3p, miR-181a-5p, miR-1248 levels. Meder et al. (2014) detected a positive correlation with age in levels of miR-34a-5p, miR-145-5p, miR-1284, miR-93-3p and miR-1262. Ameling et al. (2015) observed miR-126-3p, miR-21-5p, miR-30b-5p, miR-30c-5p, miR-142-3p, let-7a-5p age-dependent level increase and miR-93-5p downregulation.

Long noncoding (lnc)RNAs is another fraction of transcriptome which is described as strongly associated with aging. Every aging-associated process from the "hallmarks of aging" classification is known to be regulated by numerous long noncoding RNAs (Grammatikakis et al. 2014), thus lncRNAs determine gene expression patterns in age-related diseases, these sequences are known to be the modulators of energy metabolism and immune response, as well as neurodegeneration and proliferation (Kim et al. 2016). For example, *MIR31HG* or *Meg3* level increase is strongly associated with cell senescence in humans.

6.2.3 *Proteomic and Metabolomic Biomarkers of Aging. Endophenotypic Approach to Proteomic and Metabolomic Biomarkers*

Studying aging, we constantly face the unmeasurable distance between genetic information encoded in the DNA and the phenotype. The causal network which results in lifespan alteration may be informative not only in marginal, but also in premarginal regions which lead to invariant events. In other words speaking of proteomic and metabolomic biomarkers of aging, we cannot bypass the concept of the endophenotype. This term has been borrowed from psychopathology, where it is defined as phenomenon (often biological or physiological) known to be associated both with genetic traits and identifiable symptoms of a disease, somewhat intermediate (Gottesman and Gould 2003). Aging is a phenomenon of great complexity and it has several described phenotypes (if we consider aging a disease, e.g. accelerated or slowed down one), thus the diversity of aging endophenotypes has not been characterized fully yet, but it is potentially countable. Interestingly, the idea of a biomarker looks

like the concept of an endophenotype, but there are criterions which biomarker of aging must satisfy to become an endophenotype of aging as a disease: it must be associated with an age-related disease; it must be heritable; also it has to manifest in person independently from illness manifestation; in addition, in family anamnesis it must be associated with the disease (Hoffman et al. 2017).

Probably the criteria are too strict for the present day due to the lack of omics data, but they give a strong basis for evolutionary development of the "biomarker of aging" concept. The basic proteomic markers of aging were detected in the earliest works on protein mass-spectrometry. The comparison of individual proteomic and metabolic profiles between healthy controls and unhealthy experimental group observed great differences in aging-associated proteome and metabolome changes. There are several age-related pathologies like Alzheimer's and Parkinson's diseases, type II diabetes, atherosclerosis, heart failure, coronary artery disease, which have their own markers, due to a high penetration of the last four the search of healthy aging biomarkers is much complicated as a result of total metabolic network alteration (Hoffman et al. 2017; Gregory et al. 2015). Summarizing the data obtained in numerous metabolomic and proteomic experiments on worms, mice and humans, it becomes clear that metabolic profile is more flexible and has more vivid response to aging, conversely, the number of proteins differentially expressed in aged models is much lower (Hoffman et al. 2017).

6.2.4 Widely Used Metabolic Biomarkers of Aging

The metabolome itself is a biomarker of aging, but metabolomic approach is too complicated and expensive for everyday usage by e.g. general practitioners, physicians or geriatricians, although we sorted out several elementary biomarkers of aging which were identified also in metabolomic research.

Using liquid chromatography and mass spectrometry approaches Chaleckis et al. (2016) compared blood samples taken from young cohort with samples of aged individuals. In the study 126 metabolites were detected in total, a coefficient of variation every was measured for every compound in every individual. It was shown that 14 metabolites found in blood samples significantly change (increase or decrease) with age: 1,5-anhydroglucitol, dimethyl-guanosine, acetyl-carnosine, carnosine, ophthalmic acid, UDP-acetyl-glucosamine, N-acetyl-arginine, N6-acetyl-lysine, pantothenate, citrulline, leucine, isoleucine, NAD^+, and $NADP^+$ (Chaleckis et al. 2016). However, these are not the only metabolomics biomarkers of aging identified in numerous studies.

Lipid metabolism is mainly impaired in aging, thus different fractions of the lipidome change levels with age (Lewington et al. 2003). Triglycerides are shown to increase their level with age so this fraction may be considered as the biomarker of aging (Liu et al. 2017). Cholesterol (total), low-/high-density cholesterol (lipoprotein) are also predictors of morbidity and mortality according to meta-analyses (Prospective Studies Collaboration 2007; Briel et al. 2009; Sarwar et al. 2007). In

addition, phospho- and sphingolipids were identified as markers of healthy aging (Montoliu et al. 2014).

Protein metabolism has much products that may be utilized as biomarkers of aging. Carbamylated proteins accumulation is a result of non-enzymatic posttranslational modifications of polypeptides, mainly N-terminus of the protein is modified, and also it happens with the side chains containing Lys or Arg (Gorisse et al. 2016). Isocyanic acid obtained in urea decomposition reacts with N-termini and side chains of proteins and peptides. The most preferential targets in this case are matrix proteins, like type I collagen and elastin, having long half-life.

In the context of aging advanced glycation end products (AGEs) become a current issue; proteins, lipids, and nucleic acids are usually glycated during high-sugar solution exposure, this process takes place without enzymes' presence, their accumulation leads to the inflammation and apoptosis and is indicated in age-related obesity as well as in diabetes (Semba et al. 2010). There are also N-linked glycans, sugar chains in them are bonded to the amide group nitrogen only in asparagine, a vivid example is an inflammaging-associated modification of Asn297 in IgG-G0 (Sanada et al. 2018).

Different metabolic reactions have radicalized side products, often environmental impacts cause oxidative stress, as a result oxidative damage markers are widely spread in organism, their amounts usually grow with age as oxidative stress is one of the most studied phenomena being both a cause and a consequence of aging so discussed compounds fit properly on the role of aging biomarkers. Such compounds as 3-nitrotyrosine, 3-chlorotyrosine and o-tyrosine are strongly associated with protein damage in aging (Stadtman 2006). Malondialdehyde, 4-hydroxy-trans-hexenal, 4-hydroxy-trans-nonenal, 8-isoprostaglandin F2α and aldehydes C6–C12 are usually applied as biological markers of lipid oxidative damage during aging (Marcourakis et al. 2008). Such compounds as 8-hydroxy-2′-deoxyguanosine and 8-hydroxyguanosine appear when DNA is damaged under prooxidant treatment and oxidative stressor exposure, thus these residues may be used as indicators of age-related oxidative damage (Rübe et al. 2011; Mecocci et al. 1993). It is worth noting that the majority of free radicals are produced by mitochondria, the functional status of mitochondria is already from a class of cellular biomarkers, the paragraph below is dedicated to them.

6.3 Cellular Biomarkers of Aging

To system cellular biomarkers of aging, we divided them into classes named after hallmarks of aging. They are mitochondrial dysfunction markers, the markers of intercellular communication deregulation, the markers of cellular senescence and proteostasis loss. Mostly the cellular markers of aging are endophenotypes formed by cells or organelles and cell populations.

6.3.1 Mitochondrial Biomarkers of Aging

Mitochondrial hydrogen peroxide production was one of the earliest biomarkers it had been being measured by using α-glycerophosphate as a substrate in fluorimetric tests (Sohal 1991). Lon protease activity is decreasing and aconitase turnover is significantly changing with age, together they are indicating mitochondrial dysfunction (Bota et al. 2002). The leading mitochondrial enzyme (citrate synthase) level is reduced in aged skeletal muscles, mitochondrial capacity for oxidative phosphorylation is decreased with age as well as mitochondrial ATP production, also we have already mentioned above that the amounts of mitochondrial DNA and RNA are lower in cells of aged individuals (Short et al. 2005). The defective mitochondria cause the appearance of damage-associated molecular pattern molecules (DAMPs) which are closer to intercellular communication.

6.3.1.1 Biomarkers of Aging in Context of Defective Intercellular Communication

Circulating cellular components and organelles, free radicals, peroxidized lipids, a variety of metabolites e.g. extracellular ATP, ceramides, urate concrements, succinate, amyloid, etc. are associated with intercellular communication because specific sensor molecules, which consequently induce the response to cell danger (Franceschi and Campisi 2014), identify them. In addition, DAMPs which may be heat shock proteins, S100, high-mobility group box 1 or histones are candidate biomarkers of aging which indicate cellular death and immune reactions (Huang et al. 2015).

Nlrp3, also known as an inflammasome, activates pro-caspase-1 that is followed by IL-1β and IL-18 release (proinflammatory cytokines). As mitochondria is offspring of bacterial symbionts our innate immunity is targeted on their component, thus cardiolipin being an antigen (as well as other pathogen-like compounds) promotes endogenous inflammatory and immune response which is represented by a variety of signaling molecules activated primarily by Nlrp3 and other sensors or downstream cascade elements. C-reactive protein, interleukin-6, and alpha 1-antichymotrypsin are associated with accelerated brain aging and cognitive decline (Schram et al. 2007). Pro-inflammatory response to endogenous pathogene-like molecules is not the only variant of cell senescence promotion. It is also connected to senescent cells producing senescence-associated secretory phenotype (SASP). Chemokines, and growth factors, tumor necrosis factor-alpha, IGF binding proteins, matrix metalloproteinases, monocyte chemoattractant protein-1 and already mentioned IL-6 increase take place in aging associated with chronic inflammation (inflammaging) and cell senescence (Acosta et al. 2013).

6.3.2 Cellular Aging and Senescence Biomarkers

In tissues with high level of mitotic activity the number of senescent cells reflects the process of aging there are several biomarkers of cellular senescence. Protein β-galactosidase (SAβ-gal) activity increase, elevated expression of $p16^{INK4A}$ and hypophosporilation of RB are the most typical biomarkers of cell senescence, also there are two endophenotypes: cell cycle arrest and resistance to mitogens and oncogenic transformation in tested cells. These traits may be measured and are appropriate as biomarkers of aging processes connected to cell senescence as well as already mentioned SASP elements (Sharpless and Sherr 2015).

6.3.3 Markers of Impaired Proteostasis in Endoplasmic Reticulum May Be Biomarkers of Aging

Intercellular proteostasis is composed of three basic systems: ubiquitin proteasome system, autophagosome and chaperons. Two first systems are involved in protein degradation and the third assists in folding of proteins enhancing their stability. Several processes may be indicative for proteostasis estimation; quantitative traits of these processes one can use as specific biomarkers of aging (Labbadia and Morimoto 2015). Here we list leading processes indicating age-related proteostasis loss they are: increased chaperone depletion, protein aggregation and appearance of aggregates in cells, impairment is observed in macroautophagosome function, its activity is decreased, and also cargo recognition, phagophore induction and APG-LYS fusion are reduced. Chaperone mediated autophagy and L2A levels are known to be decreased in aged organisms. The same statement is correct for UPS activity, ubiquitination and subunit levels assembly, these markers are decreased, consequently all abovementioned processes being measured can act as biomarkers aging (Kaushik and Cuervo 2015).

6.4 Conclusion

Overall, the abundance of biomarkers of aging is high, they differ much in precision and reliability as well as in invasiveness, generally it is impossible to sort out the best biomarker, because only a panel of biomarkers (not only cellular and molecular ones) describing the group of processes within aging may be truly informative. It would be exact to note that “the more—the better” is leading principle of the field. In context of current and future predictive and personalized medicine, we have to identify the top list of trustworthy markers, which are connected to biological aging only which make accurate prognosis of individual aging trajectory the reality or even the routine.

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Part II
Proteomics and Glycomics Biomarkers of Aging

Chapter 7
IgG Glycans as a Biomarker of Biological Age

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Abstract Immunoglobulin G (IgG) has an important role in various processes of the immune response and its functions can be modulated by *N*-glycans attached to its Fab and Fc portions. The composition of IgG glycans can be deciphered using different analytical methods, based on liquid chromatography, mass spectrometry and capillary electrophoresis. IgG glycome composition is partly heritable, but is also under the influence of various environmental factors. It therefore represents an interface between genes and environment, as evidenced by IgG glycosylation pattern changes associated with chronological age, various diseases and lifestyle-related variables. IgG glycans are therefore considered an excellent biomarker of the general health state of a person, i.e. biological age.

Keywords IgG glycome · Aging · Diseases · Biological age · Biomarker · Inflammaging

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
CGE-LIF	Capillary gel electrophoresis with laser induced fluorescence detection
C_H	Constant heavy domain
C_L	Constant light domain
Fab	Fragment antigen binding
Fc	Fragment crystallizable

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FcγR	Fcγ receptor
IgG	Immunoglobulin G
IVIg	Intravenous immunoglobulin
LC-ESI-MS	Liquid chromatography electrospray ionization mass spectrometry
MALDI-TOF-MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
RA	Rheumatoid arthritis
TPNG	Total plasma *N*-glycome
UHPLC-FLR	Ultra-high-performance liquid chromatography with fluorescence detection
V_L	Variable light domain
V_H	Variable heavy domain

7.1 Immunoglobulin G Glycosylation

Immunoglobulin G (IgG) is the most abundant glycoprotein in the blood. It has an important role in various processes of the immune response: neutralization of microbes and toxins, complement activation, antibody-dependent cellular cytotoxicity (ADCC) and opsonization for phagocytosis by macrophages and neutrophils. It is secreted by plasma cells and its concentration in the serum is between 7 and 18 mg/mL in healthy adults (Gonzalez-Quintela et al. 2008). IgG is a highly stable antibody with a half-life of approximately 21 days (Spiegelberg and Fishkin 1972; Spiegelberg et al. 1968). Based on the amino acid sequence, IgG can be divided into four subclasses: IgG1, IgG2, IgG3 and IgG4 (named in the order of decreasing abundance) (PH 1988). All subclasses show over 90% homology in amino acid sequence, but differ to a certain extent in their biological activity (Vidarsson et al. 2014).

The IgG molecule is composed of two light and two heavy chains, with a total molecular mass of approximately 150 kDa (Arnold and Wormald 2007; Zauner et al. 2013). The light chain (25 kDa) consists of a variable (V_L) and a constant (C_L) domain while the heavy chain (50–70 kDa) contains four domains: one variable (V_H) followed by three constant heavy domains (C_H1, C_H2 and C_H3). The two heavy chains are mutually connected by disulfide bonds and each heavy chain is connected to one light chain by a disulfide bond, thus forming a Y-shaped structure. The light chains with the V_H and C_H1 domain of the heavy chains form the fragment antigen binding (Fab) portion, while the C_H2 and C_H3 domains of the two heavy chains form the fragment crystallizable (Fc) portion of the IgG molecule. The Fab and the Fc part of the molecule are connected by a flexible hinge region, which allows for individual movement of the two Fab arms (Arnold and Wormald 2007; Murphy 2011). The Fab region binds a specific antigen and the Fc region binds different ligands, such as its receptors on the surface of various immune cells.

Glycans are one of the fundamental components of the cell. They are complex oligosaccharides, whose biosynthesis is not driven by a genetic template but is a stochastic process involving coordinated actions of many enzymes and other proteins. The structural variability of glycans is thus enormous (Varki et al. 2015). Glycans represent an integral structural part of their corresponding glycoproteins, significantly influencing their folding, structure, stability and function (Raju and Scallon 2006; Russell et al. 2009; Ishino et al. 2013). This makes glycans important for nearly all physiological processes in the human body (Varki 2017). They play a role in the quality control of nascent proteins (Hebert et al. 2005) and modulate the interactions between cells and between molecules (Varki 1993; Hart and Copeland 2010), thus affecting, for instance, angiogenesis (Tei et al. 2002) and antibody effector functions (Albert et al. 2008; Nimmerjahn and Ravetch 2008).

The IgG molecule can be glycosylated at both the Fc and Fab portions of the molecule. In the heterogenous mixture of serum IgG glycans over 30 different individual glycan species can be found. The most complex human IgG glycan is a biantennary digalactosylated and disialylated structure with a bisecting *N*–acetylglucosamine and a core fucose, made up of 13 monosaccharide residues (Fig. 7.1). The remaining IgG glycans correspond to this tridecasaccharide with the lack of one or more monosaccharide residues.

Almost all IgG glycans are *N*-glycans due to their attachment to the protein part of glycoprotein through the amidic nitrogen atom of asparagine side chain. Each IgG heavy chain is *N*-glycosylated at a highly conserved Asn-297 of the Fc domain. The Fc glycans are thought to maintain the open conformation of the Fc part in the area of the two C_H2 domains, allowing for Fc ligand binding (Arnold et al. 2007; Subedi and Barb 2015). The type of glycan attached to the Fc domain significantly affects the IgG's folding, structure and stability (Krapp et al. 2003; Pricer et al. 1974; Mimura et al. 2001). It also affects IgG's effector functions through modulation of the binding affinity to its canonical and non-canonical receptors and other ligands, such as complement component C1q (Mimura et al. 2001; Mimura et al. 2001; Shields et al. 2002; Kao et al. 2015; Dekkers et al. 2017). A putative *N*-glycosylation site is positioned at Asn-322 of IgG3 although no glycans attached to it have yet been

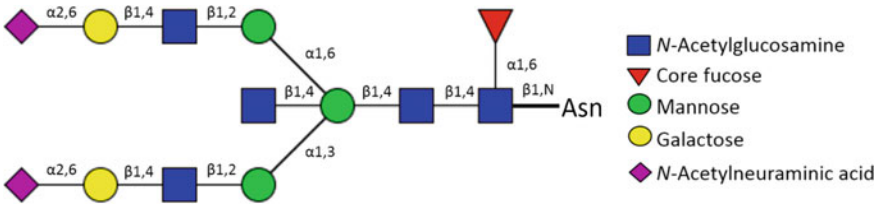

Fig. 7.1 The most complex human IgG *N*-glycan. In most cases, human IgG glycans are assembled by enzymatic addition of monosaccharide residues to the core composed of two *N*-acetylglucosamines, three mannoses and two antennary *N*-acetylglucosamines. More than thirty different glycan species are made by variable addition of core fucose, bisecting *N*-acetylglucosamine, antennary galactoses and antennary sialic acids (*N*-acetylneuraminic acids)

described (Maverakis et al. 2015). In addition, IgG3 is the only IgG subclass carrying *O*-glycans—attached to the oxygen atoms of three threonines within its elongated hinge region (Plomp et al. 2015).

The Fab domain is known to be *N*-glycosylated in 15–25% of IgG molecules but contains no conserved glycosylation sites due to the variation in the amino acid composition (Stadlmann et al. 2010). The existing *N*-glycosylation sites present in the Fab region are a result of somatic hypermutation during antigen-specific immune response (Dunn-Walters et al. 2000). Lack of conserved glycosylation sites makes the glycans attached to the Fab region more difficult to study, however they are known to be rather flexible and to affect the IgG's stability and half-life but also antigen binding and overall immune response (Arnold and Wormald 2007; Jefferis 2005; Rombouts et al. 2016; Holland et al. 2006; Bondt et al. 2014; Coloma et al. 1999; Wright et al. 1991; Leibiger et al. 1999; van de Bovenkamp et al. 2016; van de Bovenkamp et al. 2018). The glycan compositions of the Fab and the Fc domain differ (Anumula 2012; Böhm et al. 2012), the most pronounced differences being the increased levels of bisection (~10% for Fc vs. ~45% for Fab), galactosylation (~67% for Fc vs. ~94% for Fab) and sialylation (~19% for Fc vs. ~92% for Fab) but reduced levels of fucosylation in the Fab glycans (~94% for Fc vs. ~69% for Fab) (Zauner et al. 2013; Bondt et al. 2014; van de Bovenkamp et al. 2016). The cause of these differences is partly in the degree of accessibility for glycosyltransferases and glycosidases: the Fab glycosylation sites are more accessible compared to the conserved site in the Fc domain, which is located between the two heavy chains and therefore much less accessible to enzymes (van de Bovenkamp et al. 2016).

Variable monosaccharide composition, presence of two different glycans at the Fc region of a single IgG molecule (asymmetric glycosylation), appearance and/or disappearance of non-conserved glycosylation sites at the Fab region during somatic hypermutation and subclass-dependent variation in IgG glycoprofile all result in the extreme heterogeneity of the IgG glycome. This probably results in thousands of different IgG glycoproteins being present in an individual at any given time (Arnold et al. 2007; Seeling et al. 2017).

Differences in IgG glycosylation pattern represent a valuable tool for fine-tuning and modulation of its structure and consequently of its function (Gornik et al. 2012). For instance, particular changes in the glycosylation pattern, most notably core-fucosylation, are well known to modulate the efficacy of monoclonal antibodies (Arnold et al. 2007; Nose and Wigzell 1983; Kumpel et al. 1994). Certain changes in IgG glycosylation are also associated with age, sex hormones and disease status of an individual (Baković et al. 2013; Ruhaak et al. 2010; Gudelj et al. 2018; Parekh et al. 1988; Yamada et al. 1997; Shikata et al. 1998; Rook et al. 1991; Van de Geijn et al. 2009; Kapur et al. 2014; Wuhrer et al. 2009; Kapur et al. 2014). Consequently, population studies have reported very high levels of inter-individual variation in the composition of IgG glycome (Pučić et al. 2011).

Analysis of IgG glycosylation patterns is of increasing interest in many fields of research such as biomedical and biological research, pharmaceutical and healthcare industry and biotechnology. Due to the great complexity of IgG *N*-glycosylation, there is particularly a need for studies that will help identify regulatory pathways

that cause its alteration in aging and various diseases. Therefore, it is no wonder that a need arose for the development of even more sensitive, less labor-intensive and easily implementable technologies that will improve our knowledge on IgG glycosylation patterns and the regulation of IgG glycosylation.

7.2 Methods for the Analysis of IgG *N*-Glycans

Due to the biological and functional importance of glycans, the analysis of protein glycosylation is of interest in many areas of biomedical research. Glycan analysis is technically challenging due to their complexity and heterogeneity, as well as the possible existence of multiple glycosylation sites on a single glycoprotein. This is the reason glycomics and glycoproteomics were lagging behind genomics and proteomics regarding the amount of accumulated data in the last century—but have been rapidly catching up in the last 20 years.

When it comes to IgG, different studies concentrate either on total IgG glycome or on its specific subsets (Fab, Fc, subclass specific glycans)—depending on the research topic—and require an appropriate analytical approach. For detailed and most informative analysis, several complementary approaches are usually employed in combination.

There are several strategies to study IgG glycosylation (Huhn et al. 2009). The first IgG glycan analyses undertaken around the end of the previous century were low-throughput and were performed using lectin assays (Parekh et al. 1985; Flögel et al. 1998; Tsuchiya et al. 1998; Dalziel et al. 1999). Today, a large number (several hundred or even thousands) of samples are routinely analysed (Wahl et al. 2018; Menni et al. 2018; Šimurina et al. 2018; Lemmers et al. 2017; Theodoratou et al. 2016). For this, the development of sensitive, robust and affordable high-throughput methodologies for IgG glycosylation analysis was essential. Currently, several high-throughput approaches for IgG *N*-glycan and *N*-glycopeptide analysis are in use: ultra-high-performance liquid chromatography with fluorescence detection (UHPLC-FLR), liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS), capillary gel electrophoresis with laser induced fluorescence detection (CGE-LIF) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Trbojevic-Akmacic et al. 2016).

UHPLC-FLR is a robust and widely used analytical technique for high-resolution separation and quantification of released *N*-glycans, routinely labeled with 2-aminobenzamide (2-AB), 2-aminoacetic acid (2-AA) or procainamide (Baković et al. 2013; Bones et al. 2010), thus analysing the bulk of Fab and Fc glycans if applied on undigested IgG. It is very easy to use and highly reproducible with relatively low cost of equipment. This technique enables simultaneous analysis of both neutral and charged glycans and effectively separates glycan isomers, thus providing detailed (branch-specific) information about the glycan structure. The main disadvantage of this technique is its inability to analyse subclass-specific IgG glycosylation, because it provides no information about the original glycan attachment site. Additionally,

as is the case with CGE-LIF, glycan peak annotation is challenging and relies on the use of other methods: MS, exoglycosidase digestion, or the combination of these two approaches (Dell 2001; Mittermayr et al. 2011; Zhang et al. 2004).

CGE-LIF is a very sensitive and robust analytical method for IgG glycan separation and quantification. Released glycans are routinely labeled with 8-aminopyrene-1,3,6-trisulfonate (APTS). Similar to UHPLC-FLR, it reliably analyses neutral and sialylated released glycans, separating glycan isomers, and thus providing very detailed and quantitative information on glycan structure (including the differentiation between α-2,3 and α-2,6 linked sialic acids), but no information on the original glycan attachment site. This technique has the potential to develop into the fastest high-throughput methodology for glycan analysis since it can be multiplexed with up to 96 capillaries in parallel (Huffman et al. 2014). However, despite its advantages, it is still relatively rarely used for glycan analysis due to the lack of glycan standards, underdeveloped database for glycan peak annotation and complicated coupling to MS for peak identification which results in challenging and demanding peak annotation (Bunz et al. 2013).

MS-based methods are mostly used for analysis of glycopeptides, thus measuring subclass-specific Fc glycosylation with very high throughput and sensitivity, and also have the ability to elucidate glycan structures when used in tandem mode (MS/MS) (Baković et al. 2013; Huffman et al. 2014; Plomp et al. 2017). Recently capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS) has emerged as another sensitive method for glycopeptide analysis (Kammeijer et al. 2017). In addition to IgG glycopeptide analysis, MALDI-TOF-MS is also used for the analysis of released glycans (Komatsu et al. 2016; Harvey 1999). The main disadvantages of MS-based techniques are their complexity, high cost of equipment, and less reliable quantification—this refers particularly to sialylated glycoforms, wherefore different strategies for sialic acid stabilization have been developed (Huffman et al. 2014; Morelle and Michalski 2007; Desantos-Garcia et al. 2011; Shubhakar et al. 2014). Ethyl-esterification of free sialylated glycans and dimethylamidation of sialylated glycopeptides in turn enable the differentiation between α-2,3 and α-2,6 linked sialic acids using MALDI-TOF-MS.

7.3 IgG Glycans Modulate Immune Response Mediated by IgG

IgG is involved in multiple humoral processes: antigen neutralization, ADCC, antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), etc. Due to its dual binding capacity (it contains antigen-binding sites in its Fab region and binding sites for ligands involved in innate and adaptive immune response in its Fc region) IgG represents a link between adaptive and innate immunity. It can induce a pro-inflammatory response and recruit innate immune effector cells during infection with pathogenic microorganisms or as a result of an

autoimmune disease (Masuda et al. 2007). On the other hand, it can also induce an anti-inflammatory response, best exemplified through the use of intravenous infusion of high doses of immunoglobulin (IVIg) therapy as an efficient anti-inflammatory treatment for many autoimmune diseases (Schwab and Nimmerjahn 2013).

IgG glycans are considered to be important regulators of immune response mediated by IgG (Gornik et al. 2012). Their complete removal results in the loss of IgG pro- and anti-inflammatory activity (Arnold and Wormald 2007; Schwab and Nimmerjahn 2013). Moreover, the structural heterogeneity of the IgG Fc region arising from alternative glycosylation modulates its 3D conformation, which can result in a changed binding affinity to its ligands (Krapp et al. 2003).

There are two types of canonical (type I) Fc receptors for human IgG: the activating receptors (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA and FcγRIIIB) and the inhibitory receptor (FcγRIIB), which can activate or inhibit immune responses through their specific expression pattern on immune effector cells (macrophages, natural killer cells, various granulocytes etc.) and selective affinity of the IgG Fc for specific FcγR (Nimmerjahn and Ravetch 2008). IgG glycan modifications are known to modulate the immune response by changing the affinity of IgG for specific FcγRs. For example, IgG molecules containing Fc *N*-glycans without core fucose are up to 100 times more efficient in activation of ADCC compared to the IgG molecules containing core-fucosylated Fc N-glycans, due to the increased affinity for the FcγRIIIA (Masuda et al. 2007; Ferrara et al. 2011). Likewise, terminal galactosylation is indispensable for the efficient initiation of the anti-inflammatory signaling cascade by the FcγRIIB (Karsten et al. 2012), and terminal sialylation dampens the activation of innate immune cells expressing activating FcγRs on their surface (Seeling et al. 2017). In particular, terminally sialylated IgG is known to have a diminished affinity for the FcγRIIIA, resulting in a greatly reduced ADCC and enhanced anti-inflammatory effects (Maverakis et al. 2015; Raju 2008).

Non-canonical (type II) FcγRs are C-type lectin receptors, such as dendritic cell-specific intercellular adhesion molecule grabbing non-integrin (DC-SIGN), C-type lectin domain family 4 member A (CLEC4A) and B-cell receptor CD22, that bind the IgG at the C_H2–C_H3 interface when its Fc region is in the closed conformation. This is in marked contrast with the canonical FcγRs, which bind the IgG near the hinge-proximal surface when its Fc region is in the open conformation. The Fc region alternates between the open and closed conformation depending on the sialylation status of the molecule, thus making terminal sialic acids on IgG a switch between distinct receptor specificities resulting in different immunological outcomes (pro- vs. anti-inflammatory action) (Pincetic et al. 2014). For example, terminal sialic acids are considered responsible for the anti-inflammatory activity of IVIg preparations administered at high doses (Schwab and Nimmerjahn 2013). Molecular pathways underlying the resolution of inflammation triggered by binding of terminally sialylated IgG to non-canonical FcγRs are still not fully elucidated (Seeling et al. 2017).

IgG glycans can also modulate the immune response by affecting complement activation triggered by IgG: agalactosylated IgG is thought to act in a pro-inflammatory manner by the activation of complement system through the alternative pathway (Banda et al. 2008), and through the lectin pathway following the binding

to mannose-binding lectin (MBL) (Arnold et al. 2006; Ji et al. 2002; Nimmerjahn et al. 2007; Malhotra et al. 1995), although this remains controversial (van de Geijn et al. 2008; van de Geijn et al. 2011).

The discoveries on the consequences of IgG glycan variation on IgG effector functions have had a tremendous impact on the biopharma industry. Since seemingly slight differences in IgG glycosylation can have profound effect on monoclonal antibody safety and efficacy (Reusch and Tejada 2015), major efforts have been invested into the production of monoclonal antibodies (most of which are of IgG1 subclass) with desired glycosylation patterns. This includes antibody glycoengineering by chemoenzymatic synthesis and by manipulating cellular control of glycosylation, as well as the developments in subsequent quality control steps involving IgG glycan analysis (Cymer et al. 2018; Le et al. 2016; O'Flaherty et al. 2018). On the other hand, supplementing large epidemiological and clinical studies, as well as genome wide association studies (GWAS) with IgG glycomics and glycoproteomics data enables the creation of hypotheses on the functional relevance of IgG glycans and the discovery of novel IgG glycosylation related biomarkers for various diseases.

7.4 IgG Glycosylation Pattern Changes are Associated with Chronological Age

Changes in IgG glycosylation pattern associated with chronological aging were first reported over 30 years ago. In this study the level of agalactosylated structures decreased until 25 years of age and increased afterwards with advancing age, changing in a parabolic shape. At the same time, the abundance of digalactosylated structures changed inversely to agalactosylated (Parekh et al. 1988). This finding has in the meantime been replicated in multiple larger cohorts, which also offered a more detailed analysis of age-related changes in IgG glycosylation pattern.

The first study performed on children and adolescents reported that age-related IgG glycoprofile changes during childhood and adolescence were gender specific. In girls, a decrease in core fucosylation was observed while in boys the incidence of bisection in core fucosylated sialylated structures increased with age. However, the abundance of agalactosylated IgG structures changed in the same direction in both sexes: they decreased steadily and reached the minimum level in the early adulthood (Pučić et al. 2012). In later studies sex differences were not observed, but an increase of monogalactosylated structures and stable overall galactosylation levels during childhood were reported (Pezer et al. 2016; De Haan et al. 2016). Moreover, high level of galactosylation in newborns, which disappears in very young children, an increase in fucosylation and decrease in bisection after birth were also noticed (De Haan et al. 2016). However, changes in the levels of core-fucosylated and bisected IgG glycoforms were reported to be dependent on the IgG subclass (Pučić et al. 2012; Pezer et al. 2016; De Haan et al. 2016). Reports on age-related changes in IgG sialylation levels remain inconsistent: either no change, or a decrease in the

abundance of specific groups of sialylated glycans on particular IgG subclasses (IgG1 and IgG2) with aging in children were observed (Pučić et al. 2012; Pezer et al. 2016; De Haan et al. 2016).

Most studies examining age-related changes in IgG glycoprofile performed on adults reported the highest abundance of digalactosylated structures and the lowest level of agalactosylated structures in early adulthood, and a decrease in the amount of galactosylated and an increase in the amount of agalactosylated structures with aging (Baković et al. 2013; Parekh et al. 1988; Yamada et al. 1997; Pučić et al. 2011; Plomp et al. 2017; Yu et al. 2016; Krištić et al. 2014; Vanhooren et al. 2007). All the studies that examined the level of glycans structures bearing a bisecting *N*-acetylglucosamine reported an aging-associated increase (Ruhaak et al. 2010; Yamada et al. 1997; Pučić et al. 2011; Vanhooren et al. 2007). Changes in the level of core-fucosylated and terminally sialylated IgG glycans were also observed in association with aging, with no agreement on the direction of change for either of these two traits (Baković et al. 2013; Shikata et al. 1998; Pučić et al. 2011; Yu et al. 2016; Krištić et al. 2014). However, for sialylated structures a decrease was more frequently reported (Baković et al. 2013; Shikata et al. 1998; Pučić et al. 2011; Yu et al. 2016; Krištić et al. 2014).

Since IgG *N*-glycome makes a significant contribution to the total plasma N-glycome (TPNG), particularly when it comes to neutral (non-sialylated) glycans (Clerc et al. 2015), studies on TPNG can also be used (with appropriate caution) as a proxy to assess the age- and disease-associated changes in the IgG glycosylation pattern when it comes to the level of galactosylation. In accordance with studies performed on isolated IgG, total plasma and serum galactosylation patterns were also found to be associated with chronological age, with the level of agalactosylated glycan structures increasing and the level of digalactosylated glycan structures decreasing with aging (Vanhooren et al. 2007; Ruhaak et al. 2011; Knežević et al. 2009; Knežević et al. 2010).

7.5 IgG Glycosylation Pattern Changes are Associated with Different Diseases

The proportion of galactosylated structures is relatively stable within an individual (Novokmet et al. 2014) but it is the most variable IgG glycosylation trait within different populations (Gornik et al. 2012). An increase in the level of agalactosylated IgG is associated with different diseases with an inflammatory component, such as autoimmune diseases (rheumatoid arthritis, systematic lupus erythematosus, inflammatory bowel disease), infectious diseases (tuberculosis, HIV infection, hepatitis), metabolic diseases (galactosemia, type 2 diabetes) and cancers (colorectal carcinoma, gastric cancer, breast cancer, ovarian cancer), as well as with different markers of inflammation and cardiometabolic health (Gudelj et al. 2018). Likewise to humans, the increased abundance of agalactosylated IgG was find to be associated with a number of autoimmune diseases, cancers and inflammatory conditions in animal models

(Kuroda et al. 2001; Endo et al. 1993; Dall'Olio et al. 2013). The increased level of agalactosylated IgG in inflammatory conditions appears to be reversible and tightly associated with the disease status. For instance, a reduction in the level of agalactosylated IgG is visible in RA patients after the treatment with anti-TNF monoclonal antibody (Van Beneden et al. 2009; Croce et al. 2007; Pasek et al. 2006). Likewise, spontaneous remission seen in RA patients during pregnancy is associated with an increase and the postpartum flare with a decrease in the abundance of galactosylated IgG (Rook et al. 1991; Van de Geijn et al. 2009; Alavi et al. 2000; Pekelharing et al. 1988; Bondt et al. 2013).

Since glycans with terminal galactose are the substrate for sialyltransferases, IgG galactosylation and sialylation are associated to a certain degree—many diseases that are marked with a decreased level of galactosylated IgG *N*-glycans also display a decreased level of sialic acids (Gudelj et al. 2018). However, there is a difference: IgG galactosylation is associated with age, sex, and certain pathological processes, mainly those that involve inflammation, which makes this trait a sort of interface between physiological and pathological processes. Terminal sialic acids, on the other hand, mainly seem to serve as a switch between pro- and anti-inflammatory activity of IgG. This is best demonstrated on the example of IVIg, as already described.

Although the effect of core-fucose is very prominent regarding IgG function, as already mentioned in the context of ADCC, its role in different diseases is probably highly dependent on the exact pathological mechanisms involved in particular disease. This is evidenced by an inconsistency in the direction of changes for the level of core-fucosylated structures in different diseases (Gudelj et al. 2018).

The existence of different glycopatterns for total and antigen-specific IgG in some diseases suggests that IgG glycosylation is highly regulated during immune responses and that glycans present on antigen-specific IgG are involved in the disease pathogenesis. On the other hand, the glycans present on the bulk serum IgG reflect the health status of the entire organism. They therefore have the potential to serve as an add-on biomarker to improve the performance of the existing disease markers for disease predisposition, diagnosis, prognosis and response to therapy.

7.6 IgG Glycans Play a Role in Inflammaging

The associations between certain IgG glycosylation traits and chronological aging are well established. The one described most often is the decrease in galactosylation level accompanying aging, but the increase in the abundance of bisected IgG species, and the decrease in the abundance of sialylated IgG species are also mentioned, as already described above. Since the same pattern of changes is often also associated with an increased level of inflammation and immune activation (Gornik et al. 2012; Anthony and Nimmerjahn 2011; Franceschi et al. 2007), a question arises whether this dynamics of IgG glycosylation pattern changes is a mere consequence of aging and aging-associated processes or it has a functional relevance and is a contributor to the aging process.

A correct balance between pro-inflammatory and anti-inflammatory activity of IgG is crucial for successful aging. However, as we age, our immune system changes—various inflammatory stimuli provoke an imbalance between the pro-inflammatory and anti-inflammatory networks and lead to a low-grade systematic inflammation that characterizes the elderly, described as inflammaging (Franceschi 2007). The inflammatory stimuli supplying inflammaging can be of exogenous origin (e.g. viral infections, diet, lifestyle) (Sansoni et al. 2014), but also of endogenous origin (released by damaged and necrotic tissues) such as pro-inflammatory agalactosylated IgG (Dall'Olio et al. 2013) and pro-inflammatory circulating microRNA (Olivieri et al. 2013). The systematic inflammatory condition alters the glycosylation machinery of antibody-producing plasma cells resulting in the increased expression of agalactosylated and less sialylated IgG. Pathogenic IgG, whose glycosylation has been changed in this manner, can activate the immune system through different mechanisms: complement activation (Malhotra et al. 1995), interaction with lectin receptors of antigen-presenting cells (Dong et al. 1999; Yabe et al. 2010), phagocyte activation (Nakajima et al. 2011) or formation of autoantibody aggregates (Leader et al. 1996) and thus amplify the inflammatory signal. This indicates that agalactosylated IgG is not only a marker of inflammation and aging, but also a factor in the establishment of a self-amplifying inflammatory loop (Dall'Olio et al. 2013; De Martinis et al. 2005; Franceschi et al. 2006).

7.7 IgG Glycans Are a Powerful Biomarker of Biological Age

Biological age is a measure of a person's health, irrespective of their actual chronological age. For example, a chronological 80–year–old can have the biological health (age) of a 30–year–old, and vice versa. Biological age of an individual can, but does not have to, correspond to their chronological age. The difference between chronological and biological age of an individual is caused by various genetic and environmental factors (e.g. lifestyle). Since biological age represents a good indicator of the general health status, it can be used to monitor the aging processes and assess how healthy a person's aging process is.

It is a well-established fact that IgG glycans are highly correlated with age in both sexes, with a model using a particular combination of IgG glycan traits explaining up to 58% of variation in chronological age (Krištić et al. 2014). The heritability of IgG glycome is estimated at up to 77%, highly depending on the model and the glycan structure (Menni et al. 2013), but between 30 and 50% for most structures (Pučić et al. 2011). Since heritability refers to the % of variation in the IgG glycome composition explained by genes, the remaining variability in the IgG glycome composition must therefore be associated with the effects of age and environment, namely different disease- and lifestyle-related variables. Indeed, after correcting for chronological age, IgG glycosylation pattern is reported to correlate with traits such as

insulin, fibrinogen, hemoglobin A1c, body mass index, triglycerides, fasting plasma glucose, waist circumference, D-dimer, total cholesterol, low-density lipoprotein, high-density lipoprotein, urea, uric acid, systolic and diastolic blood pressure, white blood cell count, heart rate, alanine transaminase and aspartate aminotransferase—i.e. physiological parameters related to biological age (Yu et al. 2016; Krištić et al. 2014). IgG glycans therefore represent an interface between genes and environment, which makes them an excellent biomarker of biological age, if compared to the IgG glycosylation pattern of a healthy population of same age, sex, and ethnicity (Fig. 7.2). In addition, since the level of inflammation (which is known to be mirrored in the IgG glycome composition) and not telomere length, is a predictor of successful aging (Arai et al. 2015), IgG glycans are expected to be a better biomarker of biological age than the often cited telomere length. One of mechanisms by which environment influences IgG glycome composition is through epigenetic regulation of genes involved in IgG glycosylation (Klasić et al. 2018). The exact contribution of different environmental influences to the changes in biological age as measured by IgG glycans remain to be investigated.

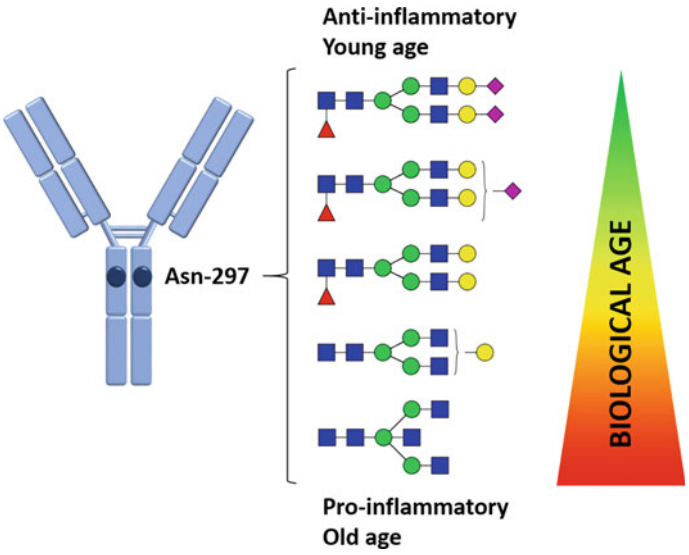

Fig. 7.2 IgG glycosylation pattern is a good biomarker of biological age. A representation of changes in IgG glycosylation pattern associated with aging and inflammation

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Chapter 8
Oxidatively Modified Proteins and Maintenance Systems as Biomarkers of Aging

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Abstract The accumulation of non-functional oxidized proteins is a hallmark of aging both in cells and in the body. This age-related build-up of proteins modified by oxidative processes results, at least in part, from an increase in reactive oxygen species and other toxic compounds from both cellular metabolism and external environmental factors. Failure of protein maintenance (i.e. oxidized protein degradation and repair) is another major contributor to the age-associated accumulation of damaged proteins. Oxidative damage to the cellular proteome, leading to the formation of carbonylated proteins derives from the direct oxidation of several amino acids side chains and also through protein adducts formation with lipid peroxidation products and dicarbonyl glycating compounds. Since the accumulation of oxidatively damaged proteins is believed to participate to the age-related decline in cellular function, their identification has been achieved in human or mammalian animal models of aging and age-related diseases as well as in human fibroblasts and myoblasts during cellular senescence and upon oxidative stress. Indeed, the identification of damaged protein targets is expected not only to define potential biomarkers of aging but also to give insight into the mechanisms by which these damaged proteins accumulate and may contribute to cellular dysfunction.

Keywords Protein oxidation · Protein glycoxidation · Oxidized protein degradation · Proteasome · Oxidized protein repair · Methionine sulfoxide reductases · Protein maintenance · Cytoskeleton · Energy metabolism

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8.1 Introduction

A characteristic of aging, both in cells and in the body, is the accumulation of non-functional oxidized proteins. This age-related accumulation of proteins modified by oxidative processes results, at least in part, from an increase in reactive oxygen species (ROS) and other toxic compounds from both cellular metabolism and external environmental factors (Stadtman 2006; Ugarte et al. 2010). Another major contributor to the accumulation of damaged proteins with age is the decreased efficiency of the intracellular elimination (degradation and repair) of oxidized proteins (Friguet 2006; Baraibar and Friguet 2012; Chondrogianni et al. 2014).

Inside the cell ROS are formed mainly by the mitochondrial electron transport chain during aerobic respiration resulting in the production of superoxide radical ($O_2^{\circ -}$). Superoxide is then converted into hydrogen peroxide (H_2O_2) by superoxide dismutase, which in the presence of traces of metals (Cu^+ or Fe^{++}) is itself converted by the Fenton reaction to hydroxyl radical (OH°), the most deleterious ROS. Peroxisomes, which are organelles involved in the oxidative metabolism of organic molecules, also contribute to the endogenous formation of ROS. External stresses of a physical nature (such as ultraviolet radiation) or chemical (toxin or xenobiotic) may also participate in the intracellular production of ROS. To trap these ROS, the cell has an arsenal of antioxidant defenses, both enzymatic (superoxide dismutase, catalase, glutathione peroxidase, peroxiredoxins, etc. …) and non-enzymatic (vitamin C, vitamin E, flavonoids, carotenoids, etc. …). However, when their production becomes too large to be completely trapped by antioxidant defenses, ROS are reacting with biological macromolecules (lipids, proteins and nucleic acids), causing irreversible damage (Berlett and Stadtman 1997; Stadtman and Levine 2003).

With the notable exception of the oxidation of cysteines and methionines for which specific systems of reduction have been demonstrated, in all other situations where the amino acid modification of the polypeptide chain is considered irreversible, the problem arises as to the removal of oxidized protein by degradation (Petropoulos and Friguet, 2005; Farout and Friguet 2006; Chondrogianni et al. 2014). At the intracellular level, the degradation of oxidized proteins is mainly ensured in the nucleus and in the cytoplasm by the proteasome, a high molecular weight proteolytic multicatalytic complex. The observed decrease in proteasome activity with age plays a crucial role in the accumulation of abnormal oxidized proteins that can be toxic to the cell. Thus, depending on the cell or tissue type, the decrease in proteasome activity with age can come from the combined action of: (i) the decrease of its expression, (ii) the existence of structural modifications of the proteasome subunits, (iii) the presence in aged cells of damaged proteasome inhibitory proteins (Shringarpure and Davies 2002; Farout and Friguet 2006; Breusing and Grune 2008; Baraibar and Friguet 2012). Other protein maintenance systems are also capable of repairing oxidatively damaged proteins. Concerning the methionine sulfoxide reductases system, which has the ability to reverse the oxidation of methionine in proteins, its activity and its expression are also altered during aging and cellular senescence (Petropoulos et al. 2001; Picot et al. 2004.)

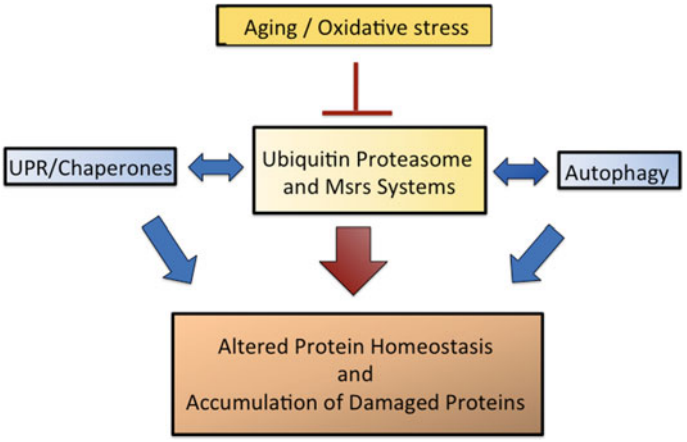

Fig. 8.1 Schematic representation of the alteration of the protein homeostasis network during aging and upon oxidative stress. Accumulation of proteins modified by oxidative processes with age results from an increase in reactive oxygen species and other toxic compounds from both cellular metabolism and environmental factors together with a decreased efficacy of the intracellular degradation and/or repair of oxidatively damaged proteins (Baraibar and Friguet 2012)

In this chapter, the different types of oxidative modifications of proteins, the incidence of which increases with age, are first described. Then, specific proteins that have been identified as increasingly carbonylated or modified by glycation/glycoxidation or conjugation with lipid peroxidation products in human or mammalian animal models of aging and age-related diseases and that may represent interesting biomarkers are presented. Intracellular degradation and repair systems for oxidized proteins, with a special attention devoted to the involvement of the proteasome and of the methionine sulfoxide reductases system (Fig. 8.1), and their impairment during the aging process are then discussed. Finally, since the accumulation of oxidatively damaged proteins is believed to participate to the age-related decline in cellular function, their previously reported identification in human fibroblasts and myoblasts during cellular senescence and upon oxidative stress is also presented. Indeed, the identification of damaged protein targets is expected not only to define potential biomarkers of aging but also to give insight into the mechanisms by which these damaged proteins accumulate and may contribute to cellular dysfunction.

8.2 Protein Modification by Oxidation and Related Pathways

Proteins constitute the main targets for ROS mediated damage that occurs directly or indirectly through their reaction with lipids and carbohydrates and the subsequent generation of oxidized products that can react with proteins. Oxidations of proteins

by ROS can be classified into those that oxidize the amino acid side chains and those that oxidize and cleave the peptide bond. Almost all amino acid side chains can react with the hydroxyl radical OH° but certain amino acids are more sensitive to oxidation with such ROS as hydrogen peroxide and superoxide. Indeed, sulfur-containing amino acids methionine and cysteine are readily oxidized by all sorts of ROS, while histidine and aromatic amino acids are also very sensitive to oxidation.

Oxidation of cysteine first results in either the formation of a disulfide bridge or a sulfenic acid. Sulfenic acid can be converted to disulfide or sulfenamide or further oxidized to sulfinic and then sulfonic acids. Both disulfide and sulfenic acid can be enzymatically reduced by different enzymatic systems such as the thioredoxin/thioredoxin reductase and the glutaredoxin/glutathione/glutathione reductase systems while sulfinic acid reduction has so far been limited to oxidized cysteines within the active site of peroxiredoxins (Biteau et al. 2003). Cysteine can also react with nitric oxide to produce S-nitrosothiol. Oxidation of methionine leads to the formation of methionine sulfoxide and further oxidation of methionine sulfoxide leads to the irreversible formation of methionine sulfone. Some oxidative modifications are rather specific in terms of oxidized residues and products generated such as the oxidation of phenylalanine to tyrosine, which can be further converted to di-tyrosine (Giulivi et al. 2003). Tyrosine residues also represent preferred targets for nitration by nitrogen dioxide and peroxynitrite and are converted to nitrotyrosine.

Oxidation of several amino acid residues such as lysine, arginine, proline and threonine results in the formation of carbonyl groups (Berlett and Stadtman 1997). Carbonyl derivatives can also originate from the fragmentation products of the peptide bond oxidative cleavage (Stadtman and Levine 2003). Aminoadipic and glutamic semi-aldehydes resulting from the oxidation of lysine and arginine, respectively, are quantitatively important products of the carbonylation reaction. Protein carbonylation has been considered as an indicator of severe oxidative damage as well as age- and disease-derived protein dysfunction since this modification often leads to a loss of protein function, as well as an increased thermosensitivity and hydrophobicity (Berlett and Stadtman 1997).

Protein carbonyls are the most commonly used marker of protein oxidation and different methods have been developed for the detection and quantification of carbonylated proteins. Most of these methods are based on immunochemical and/or spectrophotometric assays of protein carbonyls previously derivatized by 2-4-dinitrophenylhydrazine to form 2-4-dinitrophenylhydrazone protein adducts (Levine 2002). We have developed a novel application of the difference gel electrophoresis (DIGE) approach, but for the detection and quantification of carbonylated proteins, referred as to Oxi-DIGE (Baraibar et al. 2012a, 2013). In Oxi-DIGE, protein carbonyls derived from any biological sample are labeled with two spectrally resolvable fluorescent hydrazide probes that bind specifically to carbonyl groups in proteins (Fig. 8.2). The matched dyes have the same ionic and pH characteristics but absorb and/or emit light at different wavelengths, producing different color fluorescence. Recent studies by the group of J. Ros and E. Cabiscol have also demonstrated the usefulness of fluorescent hydrazides for analyzing protein carbonylation caused by oxidative stress and chronological aging in yeast (Tamarit et al. 2012). A central

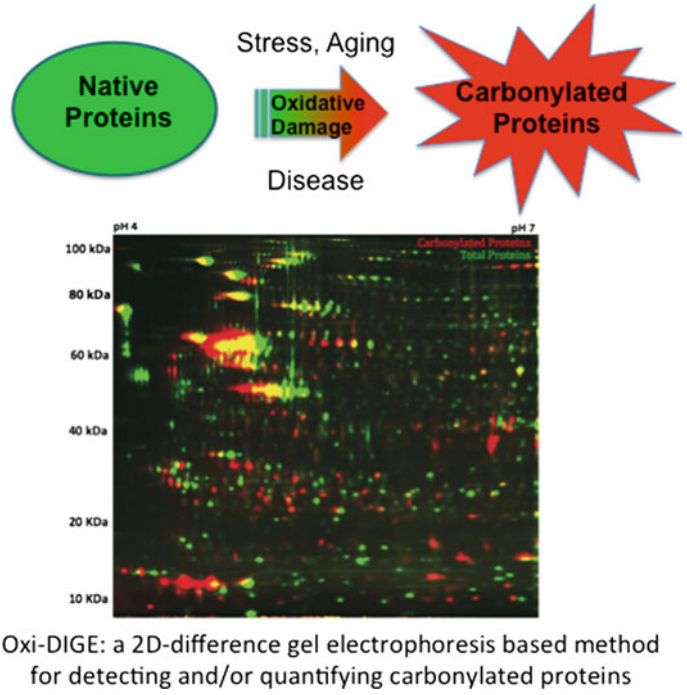

Fig. 8.2 Representative Oxi-DIGE analysis for the quantitative detection of carbonylated proteins. In Oxi-DIGE, protein carbonyls are labeled with two spectrally resolvable fluorescent hydrazide probes that bind specifically to carbonyl groups in proteins. Labeled carbonylated proteins from different groups of samples are then co-resolved on a single 2D gel for direct quantification. Oxi-DIGE analysis can be performed for the quantification and identification of carbonylated proteins in any biological sample (Baraibar et al. 2013)

advantage of the use of sensitive fluorescent probes is the detection of lower abundance carbonylated proteins. For spot excision from the gel to establish its identification, either total protein stain by NHS-ester cyanines or BodipyFL-Hz would be the method of choice (Tamarit et al. 2012). In carrying out Oxi-DIGE, labeled carbonylated proteins from different groups of samples are co-resolved on a single 2D gel for direct quantification. In addition, the Oxi-DIGE method provides a significant improvement in terms of reproducibility and statistical support of the data for proteomic analysis of carbonylated proteins, which is essential for the robust identification of this modification, and can be applied to the identification of carbonylated proteins in any biological sample.

In addition to direct oxidation of certain aminoacid side chains, protein carbonyl derivatives can originate from the conjugation on cysteine, lysine and histidine residues of such aldehydes as malondialdehyde, acrolein and 4-hydroxy-2-nonenal (HNE). Indeed, oxygen free radicals can attack cellular membranes and induce lipid peroxidation resulting in the production of these reactive aldehydes which are precursors of advanced lipid peroxidation end products (ALE) that have been found to

accumulate on proteins during aging and certain age-related diseases (Sayre et al. 1997; Szweda et al. 2003). Moreover, sugar aldehydes or ketones can also react with the amino groups of lysine and arginine through a Schiff base which is rearranged to form an Amadori product (e.g. fructosamine when the reacting sugar is glucose). These products are referred as to early stage glycation adducts that are further modified to form stable end-stage products also called advanced glycation end products (AGE) through either rearrangement, oxidation, dehydration, fragmentation and/or cyclization. Deleterious effect on protein function is observed when the modification affects critical amino acids within the protein and many proteins, including intracellular proteins, accumulate with age as AGE-modified in vivo (Horiuchi and Araki 1994).

8.3 Age-Associated Accumulation of Oxidatively Modified Proteins

Accumulation of damaged macromolecules, including oxidatively damaged proteins, is a hallmark of aging, both at the cellular and organismal level, which is resulting from increased oxidative stress and/or failure of protein repair and maintenance systems (Stadtman 2006; Ugarte et al. 2010). ROS are produced as byproducts of oxidative phosphorylation and aerobic metabolism. Moreover, ROS production and accumulation are usually increased during disease pathogenesis, in particular age-related diseases (Kregel and Zang 2007). Transient exposure to low concentration of ROS induces cell proliferation and regulates the activation of several signaling pathways (Apel and Hirt 2004) while excess of ROS causes oxidative damage to lipids, proteins, and nucleic acids (Mecocci et al. 1999; Avery 2011). Protein oxidation is particularly detrimental as the resulting damages to protein structures can render oxidized proteins inactive and/or prone to form protein aggregates, hence leading to cellular functional abnormalities (Picot et al. 2007; Baraibar et al. 2012b).

ROS can induce various types of protein oxidative modifications either directly or indirectly by reactions with secondary products of oxidative stress (Stadman and Levine 2003). The irreversible oxidation of residues other than cysteine and methionine most frequently leads to hydroxylated and carbonylated aminoacid side chain derivatives. The exponential accumulation of carbonylated proteins during life span both at the cellular and organismal level and their particular increase in organs affected by age-related diseases, imply that this “Oxi-proteome” (i.e. the restricted set of proteins targeted by oxidation) may be a potential molecular substratum for many of the associated cellular dysfunctions. Indeed, carbonylated proteins are generally less active, less thermostable and are exposing hydrophobic amino acids at their surface, making them prone to form protein aggregates. Since oxidative modifications that give rise to carbonyl groups generally cause loss of catalytic or structural alterations in the affected proteins, the increased level of oxidatively modified proteins

observed during aging and age-related disease has been proposed to have deleterious effects on cellular and organ function.

Increased levels of protein carbonyls have been observed in age-related diseases, such as neurodegenerative diseases (amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, and Huntington's diseases), cataractogenesis, systemic amyloidosis, muscular dystrophy, progeria, Werner's syndrome, rheumatoid arthritis, and respiratory distress syndrome (Berlett and Stadtman 1997; Dalle-Donne et al. 2003; Martinez et al. 2010). Elevated levels of proteins modified by lipid oxidation products reactive aldehydes are associated with neurodegenerative diseases, iron induced renal carcinogenesis, cardiovascular disease, as well as elevated levels of protein glycation/glycoxidation end products (AGE) are associated with diabetes mellitus, neurodegenerative diseases, atherosclerosis and Down's syndrome. Significant advances in the past recent years have been made towards the identification of proteins targeted by these modifications, although their possible causative role in the pathogenesis of these diseases has yet to be elucidated.

To further address the role of modified proteins, we have performed a bibliographical search for specific proteins identified as increasingly carbonylated or modified by AGE or HNE in human or mammalian animal models of aging and age-related diseases (neurodegenerative diseases, cancer, diabetes) in articles published in peer-reviewed journals (Baraibar et al. 2012c). A total of 183 modified proteins were identified in brain, cerebellum, spinal cord, skeletal muscle, liver, eye, and cerebrospinal and bronchoalveolar fluids. Due to the high number of studies addressing the importance of protein carbonylation in the pathogenesis of neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's diseases, most of the proteins belong from the brain (Butterfield et al. 2006). However, several proteins have been identified consistently modified in other organs such as liver and eye, indicating that the spectrum of proteins targeted by these modifications may be conserved. Among them, cytoplasmic proteins were predominant, followed by proteins from mitochondria, nucleus, endoplasmic reticulum and plasma membrane. However, since most of the studies were performed in total tissue soluble extracts, membrane and mitochondrial proteins are clearly underrepresented when compared to cytosolic proteins.

Functional grouping indicated that proteins were distributed within biological processes such as inflammatory response, cellular metabolism, free radical scavenging, protein synthesis and folding. Concerning proteins involved in the inflammatory response, inflammation is now accepted as a key factor in physiological aging, referred as to "inflamm-aging" (Franceschi et al. 2000), as well as in the development of several age-related pathologies including neurodegenerative and cardiovascular diseases. Interestingly, the inflammatory environment is highly oxidative, and increased protein oxidation has been described, generating a positive feedback process. Proteins involved in energy metabolism were also evidenced in the referenced modified proteins. The most represented canonical pathways across the entire dataset included: glycolysis/gluconeogenesis, citrate cycle, pyruvate metabolism, amino acids degradation, mitochondrial dysfunction, cell death, butanoate metabolism, nrf-2 oxidative stress response and cellular function and maintenance.

More recently, we have identified carbonylated proteins in human *rectus abdominis* muscle obtained from old and young healthy donors to better understand the mechanisms by which these damaged proteins build up and potentially affect muscular function (Lourenço dos Santos et al. 2015). Using a bi-dimensional gel electrophoresis-based proteomic approach coupled with the immunodetection of carbonylated proteins, 14 proteins were found to be increasingly carbonylated in biopsies from old donors compared to young counterparts. Interestingly, about half of them are already present in the above-mentioned list of 183 oxidatively modified proteins (Baraibar et al. 2012c). These proteins are involved in key cellular functional pathways such as cellular morphology and transport, muscle contraction and energy metabolism. Since, impairment of these pathways has been previously described in skeletal muscle during aging, the irreversible oxidation of these proteins, leading to their functional decline, may therefore contribute to the sarcopenic phenotype by negatively impacting on such pathways.

8.4 Age-Associated Impairment of Oxidized Proteins Elimination by Repair and Degradation

In contrast to DNA, for which many repair enzymes and pathways have been described for oxidative and other insults, oxidized protein repair is limited to the reduction of certain oxidation products of the sulfur-containing amino acids, cysteine and methionine. Indeed, damaged intracellular proteins are mainly eliminated be degradation by the proteasomal and the lysosomal pathways. Major systems that have been implicated in oxidized protein repair include thioredoxin/thioredoxin reductase and the glutaredoxin/glutathione/glutathione reductase systems for the reduction of sulfenic acid and disulfide bridges, the sulfiredoxin for the reduction of sulfinic acid when formed on the catalytic cysteine of peroxiredoxins, and the methionine sulfoxide reductases (Msr) for the reduction of methionine sulfoxide within proteins (Petropoulos and Friguet 2005; Lourenço dos Santos et al. 2018).

The Msr system is found in almost all organisms, from bacteria to mammals, and is composed of two enzyme families, MsrA and MsrB, that catalytically reverse the oxidation of the S-sulfoxide and R-sulfoxide diastereoisomeric forms of methionine sulfoxide, respectively (Boschi-Muller et al. 2008). Oxidized methionine sulfoxide reductases are then reduced by the thioredoxin/thioredoxin reductase system. Oxidation of methionine has been implicated in the impairment of protein structure and/or function while the reduction of methionine sulfoxide has been associated with the recovery of protein function. Hence, oxidation/reduction of methionine has been involved in redox regulation of protein-protein interactions and protein function. In combination with protein surface-exposed methionine residues, the Msr system has also been shown to be efficient as a built-in ROS scavenging system preventing further irreversible protein oxidation (Picot et al. 2005; Cabreiro et al. 2008). Since the Msr system can protect proteins from irreversible oxidation and that protein carbonyls

levels are usually referred as a marker of oxidative stress in pathophysiological conditions and during aging, the implication of Msr in diseases and in aging process would be expected.

Reduced MsrA activity was found in the brains of Alzheimer's disease patients (Gabbita et al. 1999) while it has been shown that oxidized proteins accumulate in tissues from patients exhibiting age-related diseases such as neurodegenerative diseases and cataracts (Gil-Mohapel et al. 2014; Swomley and Butterfield 2015). Amyloid ß-peptide (Aβ peptide) Met-35 oxidation is thought to be critical for its aggregation and neurotoxicity (Hou et al. 2002) and the absence of MsrA was shown to modify Aβ solubility properties and to cause mitochondrial dysfunction in a mouse model of Alzheimer's disease (Moskovitz et al. 2016). Oxidation of α-synuclein methionine residues in Parkinson's disease is thought to be the main reason of protein fibrillation, hence contributing to the pathology (Glaser et al. 2005). Interestingly, *MsrA* K.O. mice demonstrated behavioral abnormality (tip-toe walking) consistent with cerebellar dysfunction (Moskovitz et al. 2001), enhanced neurodegeneration with characteristic features of neurodegenerative diseases and increased light scattering, a common cataract symptom (Brennan et al. 2009).

Since the accumulation of oxidatively modified proteins during aging has been largely attributed to declining efficacy of the systems involved in protein homeostasis such as protein degradation and protein repair (Chondrogianni et al. 2014), impairment of the Msr system would be expected to play an important role in the aging phenotype. Indeed, our laboratory has shown that MsrA is down-regulated in aged rats (Petropoulos et al. 2001) and during replicative senescence of fibroblasts (Picot et al. 2004). Both cytosolic and mitochondrial Msr activities were found to decline upon replicative senescence (Ahmed et al. 2010) and increased MetO levels were also reported in membrane proteins of senescent erythrocytes (Brovelli et al. 1990).

The implication of the Msr system in regulating lifespan has been addressed in several studies but it remains controversial. This hypothesis has been originally tested using two different models: *MsrA* K.O. mice and *MsrA* overexpressing *Drosophila* (Moskovitz et al. 2001; Ruan et al. 2002). Overexpression of MsrA in *Drosophila* resulted in a 70% extension in their healthy lifespan (Ruan et al. 2002) while the knockout of the *MsrA* gene in mice reduced its lifespan by 40% (Moskovitz et al. 2001). In both studies, the MsrA-dependent lifespan modulation was related to its role in protection against oxidative stress but another study has since shown that the lack of MsrA in mice does not diminish lifespan although it does increase sensitivity to oxidative stress (Salmon et al. 2009).

Overall these studies indicate the importance of Msr system in aging and neurodegenerative diseases pointing out to its role as an antioxidant enzyme protecting cells and organisms from the deleterious effects of oxidative stress. However, the recent discovery that alternation between methionine oxidation and reduction could serve as regulator of protein function raises the interesting hypothesis that the role of the Msr system in aging and survival could also come from these intracellular signaling functions (Lourenço dos Santos et al. 2018).

Non-repairable alterations, which represent the majority of protein damage, are eliminated through protein degradation by the proteasomal or the lysosomal sys-

tems in the cytosol while oxidized proteins are degraded by the Lon protease in the mitochondrion (Ugarte et al. 2010; Hamon et al. 2015). These proteolytic systems have been documented to decline with age and during replicative senescence, hence implicating protein maintenance failure in the age-associated build-up of damaged proteins (Baraibar and Friguet 2012; Hamon et al. 2015).

The proteasomal and lysosomal pathways are the two main proteolytic cytosolic machineries by which intracellular proteins are degraded. Protein degradation by the proteasome is a key process for the maintenance of cellular protein homeostasis. In the cytosol and in the nucleus, the proteasome plays a key role in the removal of altered proteins since mildly oxidized proteins are good substrates of the proteasome in vitro and do not to require ubiquitin and ATP to be eliminated in vivo (Chondrogianni et al. 2014). However, some studies have shown that the ubiquitin-proteasome system could be implicated in the degradation of certain oxidized proteins. The increased susceptibility of oxidized proteins to degradation by the proteasome has been attributed to an increased exposure of hydrophobic amino acids at the protein surface and an increased flexibility of their C- and N-terminus extremity, making them more prone to degradation by either the 20S or 26S proteasomes (Grune et al. 2003). However, when proteins are highly oxidized or modified by glycation or conjugation by lipid peroxidation products, intra- and/or inter-molecular cross-links are formed that render these heavily modified proteins resistant to proteolysis by the proteasome (Friguet and Szweda 1997). Moreover, although proteins modified by either glycoxidation or conjugation with lipid peroxidation products, have been evidenced as ubiquitinated, suggesting that they might be substrates of the 26S proteasome, these modified proteins have also been found to be targeted to and degraded by the lysosomes (Bulteau et al. 2001; Marques et al. 2004).

Several studies have indicated that proteasome function is impaired during aging suggesting that its decreased functionality might be causally related to aging and age-associated diseases (Shringarpure and Davies 2002; Farout and Friguet 2006; Breusing and Grune 2008; Baraibar and Friguet 2012), although other studies have shown that this decline may not be universal (Cook et al. 2009; Altun et al. 2010). Pioneering studies from our group and that of Walter Ward showed that rat liver proteasome proteolytic activity is altered during aging (Conconi et al. 1996; Shibatani and Ward 1996; Shibatani et al. 1996). A decrease in proteasome peptidase activity has been since reported in aged tissues of other mammals (mouse, rat and bovine), like liver (Hayashi and Goto 1998), spinal cord (Keller et al. 2000), lens (Shang et al. 2001), heart (Bulteau et al. 2002) and retina (Louie et al. 2002). Furthermore, an age-related decline of proteasome activity has been also shown ex vivo in human lymphocytes (Ponnapan et al. 1999; Carrard et al. 2003), keratinocytes and fibroblasts (Petropoulos et al. 2000; Hwang et al. 2007) and in human primary cell cultures undergoing replicative senescence (Merker and Grune 2000; Sitte et al. 2000; Chondrogianni et al. 2003). Impairment of proteasomal activity has also been reported during aging in model organisms such as *Drosophila melanogaster* (Vernace et al. 2007; Tonoki et al. 2009) and *Caenorhabditis elegans* (Hamer et al. 2010).

Proteasome impairment has been reported at different levels, including decreased transcription of certain proteasomal subunits in mice (Huber et al. 2009), dissociation

of the proteasome complex in *Drosophila* (Vernace et al. 2007), and reduced proteasome proteolytic capacity in different aged mammalian tissues and organs (Chondrogianni and Gonos 2005; Farout and Friguet 2006). In contrast, centenarians who represent an interesting example of successful aging, and the long-lived naked mole rats were found to exhibit elevated proteasome levels and activity (Chondrogianni et al. 2000; Perez et al. 2009). The main emphasis has long been placed on preventing protein damage (i.e. interest in antioxidants for protecting against oxidative damage) rather than potentiating the mechanisms that normally handle these damaged products. However, several lines of evidence support the idea that the main problem is not the damage per se, but rather how the cell handles this damage. These includes the better understanding of the cellular mechanisms that contribute to protein quality control, the fact that some of the genes coding for the components of these systems have been implicated in lifespan extension, and the growing evidence supporting that failure of protein homeostasis represents an early event in aging (Lopez-Otin et al. 2013). In addition, recent studies have shown that the proteasome can be activated by genetic manipulations as well as by factors that affect either its conformation and stability or the expression of its subunits and the rate of proteasome assembly. Indeed, over expression of the 20S β5 subunit extended the replicative lifespan of cultured human fibroblasts (Chondrogianni et al. 2005) and both the lifespan and healthspan of wild type *Caenorhabditis elegans* (Chondrogianni et al. 2015). Over expression of 19S Rpn11 prolonged *Drosophila melanogaster* lifespan (Tonoki et al. 2009) while overexpression of the proteasome related transcription factors Rpn4 and/or Rpn6 enhanced the replicative lifespan and resistance to proteotoxic stress of *Saccharomyces cerevisiae* (Kruegel et al. 2011; Yao et al. 2015) and of *Caenorhabditis elegans*, respectively (Vilchez et al. 2012).

8.5 Oxidative Protein Damage is Restricted to Specific Protein Targets upon Oxidative Stress and During Cellular Senescence

Increased protein oxidative damage during aging is well documented and is believed to play an important role in cellular aging (Berlett and Stadtman 1997). However, the identification of the damaged protein targets has been usually performed approaching only a single type of modification (e.g. carbonylation or conjugation with HNE) in different aging model systems and tissues from aged animals (Kapphahn et al. 2006; Baraibar et al. 2012c; Lourenço dos Santos et al. 2015).

Our previous studies showed an increase in proteins modified with HNE, AGE and carbonylation in senescent WI-38 human embryonic fibroblasts (Ahmed et al. 2007). The identification of proteins targeted by these modifications showed that they represent a restricted set within the total cellular proteome that fall in key functional categories, such as protein quality control, energy metabolism and cellular morphology (Ahmed et al. 2010). Since impairment of these functional pathways has

been previously documented in senescent cells, the observed protein modifications may play a role in the development of the senescent phenotype.

The cytoskeletal proteins vimentin, actin and tubulin were found among the proteins identified as HNE-modified. Cytoskeletal proteins are involved in key cellular processes such as cell division, signal transduction, cell motility and protein synthesis. Follow up studies showed several structural changes of the intermediate filament protein vimentin during cellular senescence. Vimentin filaments form thick, long dense bundles in senescent cells while irregular and small fur-like networks in young or early-passage fibroblasts (Ahmed et al. 2010).

Cellular senescence is also accompanied by alterations in energy metabolism. Increased oxidative damage causes impairment of mitochondrial respiration affecting mainly the activity of complexes I, III and IV of the respiratory chain. Almost half of the modified proteins identified upon replicative senescence of human WI-38 fibroblasts were from mitochondria, which indicates a highly oxidative environment within this organelle during cellular aging (Ahmed et al. 2010). In senescent WI-38 fibroblasts, the iron-sulfur subunit of complex I and subunit α of ATP synthase, the subunit 1 of complex III, and FAD subunit of complex II have been identified as increasingly modified by HNE-, AGE-, and carbonylation, respectively. Among the modified proteins identified in senescent fibroblasts, the citric acid cycle enzymes malate dehydrogenase and 2-oxoglutarate dehydrogenase E1 component, glycerol-3-phosphate dehydrogenase, glycerol kinase and glutaminase appear to be specifically targeted by oxidation (Ahmed et al. 2010). These results suggest that modification of proteins responsible for energy metabolism may participate in the impairment of mitochondrial function observed in senescent cells.

Proteins directly linked with the regulation of protein homeostasis, such as protein folding and degradation were also identified as increasingly modified in senescent cells. Proteins with chaperone function, such as Hsc70, calreticulin, endoplasmic reticulum protein ERp29, as well as proteasome subunits linked to a decreased proteasome activity underscore this issue.

More recently, the occurrence of specific carbonylated proteins upon oxidative stress induced premature senescence of WI-38 human fibroblasts has been analyzed and their follow-up identification has been achieved (Le Boulch et al. 2018). Indeed, increased protein oxidative damage has been clearly associated to both cellular and organismal aging and accumulation of oxidatively damaged proteins has been also reported in HDFs upon both replicative and SIPS (Chondrogianni et al. 2003; Debacq-Chainiaux et al. 2005). Only carbonylated proteins were analyzed in SIPS fibroblasts and they were mainly cytosolic, either belonging to the cytoskeleton, involved in redox and energy metabolism or involved in protein maintenance. Interestingly, cytoskeleton, redox and energy metabolism as well as protein maintenance have been all reported to be impaired during cellular aging. Such carbonylated proteins accumulating in SIPS that were previously identified as increasingly modified for replicative senescence of WI-38 fibroblasts include: Actin, Vimentin, Glucose-6-phosphate dehydrogenase, Heat shock cognate 71 kDa protein, Heterogeneous nuclear ribonucleoprotein H, Tryptophanyl-tRNA synthetase and Tubulin. Other carbonylated proteins that were already found in dif-

ferent models of aging and age-related diseases (Baraibar et al. 2012c) include: Vimentin, Tubulin, Actin, Glyceraldehyde-3-phosphate dehydrogenase, Enolase, Pyruvate kinase, Protein disulfide-isomerase, Catalase and Heat shock cognate 71 kDa protein. From these comparisons, a similarity in the proteins targeted by oxidation emerges to a certain extent, suggesting that specific cellular functions might be affected by the build-up of this restricted set of oxidatively modified proteins due to their impaired functionality and/or altered regulation.

We have also previously characterized the proteome changes of adult human muscle stem cells (i.e. satellite cells or myoblasts) in response to oxidative stress (Baraibar et al. 2011). Using a dual proteomic approach, we intended to unravel the mechanism involved in human myoblasts dysfunction upon oxidative stress. Selective proteins either modulated at the expression level or those targeted by oxidation (carbonylated) were identified after a sub-toxic insult of hydrogen peroxide. For this purpose, a 2D gel electrophoresis-based proteomic approach coupled with immunodetection of carbonylated proteins, after their derivatization with DNPH, and identification of the spots of interest by mass spectrometry has been used. Twenty-one protein spots were evidenced, as increasingly carbonylated upon oxidative stress, indicating that only a restricted set of proteins is prone to accumulation upon oxidative stress. Most of the carbonylated proteins identified belong from the cytosol but also proteins from the nucleus, endoplasmic reticulum as well as the plasma membrane were identified. Major functional categories include energy metabolism, cellular assembly, protein synthesis, cell morphology and protein degradation. Modified proteins such as peroxiredoxins, GAPDH and alpha-enolase are involved in the antioxidant response and energy metabolism. Moreover, proteins involved either in protein degradation such as the proteasome regulatory subunit 10B, and in protein synthesis such as elongation factor 2 were found to be carbonylated. Such proteasome subunits carbonylation may explain, at least in part, the decreased proteasome activity observed, suggesting that oxidative stress do not only induce the modification of proteins but also compromise their degradation by affecting proteasome function.

Finally, we have also recently addressed the potential impact of oxidatively modified proteins on the altered metabolism of senescent human satellite cells (Baraibar et al. 2016). By using a modified 2D gels based proteomics approach, we have found that a restricted set of proteins is targeted by carbonylation and modification with advanced glycation/lipid peroxidation end products during the replicative senescence of satellite cells. 22 protein spots were shown to be increasingly carbonylated, 24 were increasingly glycated, and 8 were increasingly modified by HNE during replicative senescence (Baraibar et al. 2016). Each spot was excised from the gel and analyzed by MALDI-TOF/TOF-MS for protein identification. 28 proteins were identified as targets of one, two or the three modifications. Although similar proteomic approaches were used, this restricted sub-set of modified proteins in senescent myoblasts is different to those identified in senescent fibroblasts. Indeed, while an important number of mitochondrial proteins were found to be increasingly modified in senescent fibroblasts (Ahmed et al. 2010), this was not the case for myoblasts where most of the modified proteins are cytosolic. Nevertheless, modified proteins accumulating in senescent myoblasts that were previously identified as increasingly modified for

replicative senescence of WI-38 fibroblasts include: Vimentin, Glucose-6-phosphate dehydrogenase, T-complex protein 1 subunit zeta and Heat shock cognate 71 kDa protein. The identified proteins were then analyzed and grouped by metabolic pathways and cellular functions. Major biological functions include carbohydrate metabolism, cellular morphology, migration and proliferation, as well as protein quality control, protein degradation and free radical scavenging. Interestingly, 6 enzymes involved in glycolysis were found to be increasingly modified in the senescent myoblasts: aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate mutase, enolase and pyruvate kinase (Baraibar et al. 2016). The oxidative modification of these enzymes that are involved in the glycolytic pathway is concomitant with a decreased glucose oxidation and an increased NAD^+/NADH ratio, reflecting a decreased cellular reducing potential. Since the functionality of the mitochondrial respiratory chain was not affected in human myoblasts during replicative senescence, the decreased glucose oxidation we observed is most likely due to an impairment in glycolysis and/or TCA cycle (Baraibar et al. 2016). Oxidative modifications of enzymes involved in glycolysis and the TCA cycle have also been suggested to be an important pathophysiological factor in age-related diseases, such as neurodegenerative diseases (Martinez et al. 2010). Other studies have shown that the inhibition of glycolytic enzymes, such as GAPDH and PGAM by siRNA can induce premature senescence (Kondoh et al. 2005). Furthermore, metabolomic studies were indicative of a metabolic shift leading to an increased mobilization of non-carbohydrate substrates such as branched chain amino acids or long chain fatty acids. Taken together, these results are supportive of a link between oxidative protein modifications and the altered cellular metabolism associated with the senescent phenotype of human myoblasts (Fig. 8.3).

8.6 Summary—Conclusions

Oxidative damage to the cellular proteome, leading to the formation of carbonyl groups in proteins derives from direct oxidation of several amino acids side chains and through protein adducts formation with lipid peroxidation products and dicarbonyl glycating compounds. Failure of protein maintenance is a major contributor to the age-associated accumulation of damaged proteins that is believed to participate to the age-related decline in cellular function (Baraibar and Friguet 2012; Chondrogianni et al. 2014; Vanhooren et al. 2015). Indeed, all these damaging modifications have been implicated in cellular senescence, aging and age-related diseases (Baraibar and Friguet 2013). In most cases however, the proteins targeted by these deleterious modifications as well as their consequences have not been identified. In this context, quantitative proteomics approaches, including 2D-gel electrophoresis based methods, represent powerful tools for monitoring at the proteome level the extent of protein oxidative and related modifications and for identifying the targeted proteins. Based on 2D-Difference gel electrophoresis (2D-DIGE), we have developed a novel proteomic method called Oxi-DIGE for the detection, quantification and identifica-

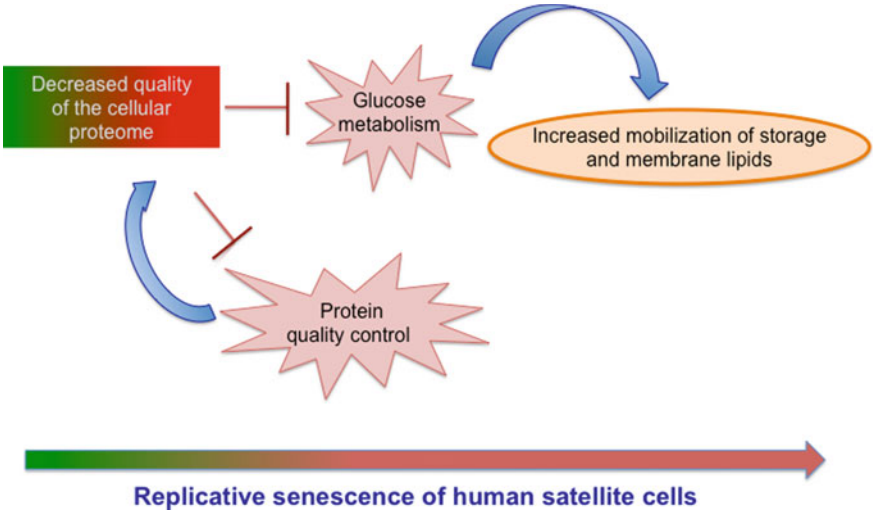

Fig. 8.3 Schematic representation of the impact of the decreased quality of the cellular proteome on protein quality control and energy metabolism during replicative senescence of human satellite cells. Both proteasome and Msr activities were decreased in senescent myoblasts. Moreover, six enzymes involved in glycolysis were found to be increasingly modified in the senescent myoblasts while metabolomic studies were indicative of a metabolic shift leading to an increased mobilization of non-carbohydrate substrates such as branched chain amino acids or long chain fatty acids (Baraibar et al. 2016)

tion of carbonylated proteins with the potential to be used in any protein containing samples (Baraibar et al. 2013). The identification of proteins targeted by carbonylation during cellular senescence, aging and age-related diseases showed that they represent a restricted set within the total cellular proteome that fall in key functional categories, such as energy metabolism, protein quality control and cellular morphology (Ahmed et al. 2010; Baraibar et al. 2012c; Lourenço dos Santos et al. 2015; Baraibar et al. 2016; Le Boulch et al. 2018). Interestingly, cross comparison of these data sets indicates an overlap in the proteins targeted by these modifications to a certain extent. An important outcome is that several enzymes that catalyze intermediate metabolism, have been found as increasingly modified during aging and upon cellular senescence and therefore may represent functionally accurate biomarkers of aging. Finally, these studies underscore the importance of performing proteomic analyses addressing different aspects, such as expression levels and modifications by carbonylation, conjugation with lipid peroxidation products or glycoxidation, to have a broader view of the age-related changes affecting the cellular proteome.

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Chapter 9
Is Impaired Proteodynamics a Key to Understand the Biomarkers of Human Cellular Aging?

Jacek M. Witkowski, Ewa Bryl and Tamas Fulop

Abstract Current understanding of the fates of cellular proteins during aging is fragmentary and far from complete. The attention of researchers in the field is on the process of proteostasis, aiming at detection and elimination of misfolded and aggregated proteins. In this chapter we discuss other aspects of the protein behavior during cellular aging, from translation to various posttranslational modifications to limited degradation, together defined as proteodynamics. We argue here that the quantitative and qualitative effects of changed proteodynamics in the aging cells may be at least the biomarkers of aging and the aging-related diseases including but not limited to chronic inflammation, cardiovascular, neurodegenerative, chronic kidney disease, type 2 diabetes mellitus and sarcopenia, or even constitute causative factors in both cellular aging and these ARDs. As an illustration we describe in detail the properties and roles of ubiquitous neutral cytoplasmic proteases—calpains, performing the limited proteolytic modification of multiple substrates and involved in the above mentioned pathologies and in cellular aging.

Keywords Cellular aging · Proteostasis · Proteodynamics · Posttranslational modification · Calpain-calpastatin system · Lymphocytes · Aging-related diseases

9.1 The Idea of Proteostasis and Proteodynamics

It is a truism that whatever happens in biological life at subcellular, cellular, tissue, organ or the whole body levels, cannot happen without the involvement/activity of proteins. It is the proteins that constitute the cellular and extracellular scaffolds maintaining shapes; proteinaceous transporters make possible the passage of water-soluble

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molecules across the membranes; enzymes—also proteins and their complexes—can modify each and every molecule that constitutes the cell; transfer of information (signal transduction) cannot happen without different proteins; proteins constitute the machinery of cellular development, growth, division and non-necrotic death …

Yet the proteins are not everlasting. They have to be synthesized or "translated" on the ribosomes, (also made of multiple, specific proteins) from the mRNA code itself "transcribed" from the DNA code, properly elaborated (matured) in the Golgi, work for a while and then be degraded to peptides to aminoacid "building bricks" (to be recycled into new proteins) by yet another broad group of proteinaceous enzymes.

Because the cellular proteins form such a complex, multilayer network of interacting molecules that constitute life (Engels), their fates in the cells (and outside) must be extremely precisely controlled. This control involves first the detection of the "need" for initiation of biosynthesis of any specific protein, control over the stages of peptide/protein production on the ribosomes (initiation, elongation and termination), as well as over the amount of the newly synthesized protein. Then, maturation of the protein making it functional, which may involve limited proteolysis (e.g., removal of the signal peptide, or cleaving a larger peptide into smaller, biologically active parts, as is the case for proopiomelanocortin (POMC) being cleaved to α-melanocyte stimulating hormone (MSH), ACTH, and β-endorphin). Other than that, newly formed, maturing proteins are chemically modified (post-translational modifications, PTMs). These modifying reactions may occur at any time after the peptide has been translated. Thus, their roles vary from enabling protein folding (resulting in the final, active, stable 3D structure), directing the protein to the specific site (organelle) inside the cell or allowing for it to be secreted, and making possible different interactions between various proteins and/or proteins with other molecules. Major PTMs include adding different sugar residues (glycosylation), lipids (e.g. palmitoylation), phosphorylation (the universal "switch" between inactive and active states of many proteins), acetyl or methyl residues and ubiquitilation. While most of the former are generally reversible which leads to change of protein status between active and non-active, the latter (ubiquitilation) is "tagging" the protein for final destruction in the proteasomes (itself being the multi-protein structures recognizing and then degrading ubiquitinylated proteins).

Overall, this control leading to stable amounts of all the necessary proteins being made when necessary, and eliminated and recycled when either not necessary for the cellular functions or damaged can be described as protein homeostasis in the cells, in brief—**proteostasis**. It is well recognized so far that proteostasis is impaired by cellular aging (Miller et al. 2014; Miller and Hamilton 2017; Martinez et al. 2017), leading to imbalance between different proteins, accumulation of "old" and dysfunctional proteins that cannot be metabolized [as the major recycling systems—proteasomes and lysosomes—are also malfunctioning in the aging cells (Cuervo and Dice 1998; Morimoto and Cuervo 2014)]. However, proteostasis is a relatively narrow term, defined as proteome maintenance by removal of misfolded proteins and toxic protein or peptide aggregates, with help of identifying chaperone proteins and degrading mechanisms [mainly proteasomal and lysosomal (Morimoto and Cuervo 2009), although for obvious reason of the maintenance of the proteome inside of

specific organelles also the mitochondrial and endoplasmic reticulum proteostasis are identified and shown to be impaired in the aging cells (Martinez et al. 2017; Hamilton and Miller 2017)].

However, apart from their recognition as misfolded and/or dangerously aggregated and their consequent elimination, fates of the proteins in the aging cells should also be considered from broader perspective, including the qualitative and quantitative efficiency of translation, as well as all facets of posttranslational modification and maturation. Thus, in our opinion **proteodynamics** is a better term describing all these processes and their changes occurring with advanced age, in cellular aging and senescence. As we highlighted in our recent paper, "Well-being of a cell depends on protein quantities, qualities and activities, all of which are affected by aging." and "Maintenance of adequate protein quality and its aging-dependent deterioration constitute a broader set of events called proteodynamics" (Witkowski et al. 2018). In this chapter we will mostly concentrate on multiple aspects of human cellular proteodynamics and its changes with aging, rather than on describing animal models.

9.2 Efficiency of Translation and Aging

Qualitatively and quantitatively efficient translation, eventually resulting in the adequate amounts of peptides with their aminoacid sequences directly corresponding to the DNA code in the relevant genes, depends on translational efficiency and capacity. Translational efficiency is described as the rate of protein synthesis on a ribosome, while translational capacity is determined by the number of ribosomes per unit of tissue (Chaillou et al. 2014). Initiation of translation is regulated by the translation initiation factors 2 (eIF2) and 4F (eIF4F), indirectly controlled by protein kinase mTORC1 (mammalian target of rapamycin complex 1) activated by protein kinase B (AKT). mTORC1 itself has recently became a target of potential pharmacological anti-aging [and anti-Age Related Diseases (ARD)] interventions after it was shown in the mouse and human cell models that its inhibition extends lifespan (Santulli and Totary-Jain 2013; Herranz et al. 2015; Wilkinson et al. 2012; Perkey et al. 2013).

Thus defined, translational efficiency does not take into account some quantitative aspects (like the amount of available mRNA for any specific protein, which is the consequence of the efficiency of transcriptional mechanisms as well as of the controlling activities of multiple noncoding microRNAs (miRNAs). Qualitative and quantitative changes in the latter associated with aging had been observed and described, as part of the epigenetic alterations associated with aging (Montesanto et al. 2012; Olivieri et al. 2017; Margolis et al. 2017).

The quality of the produced peptides leaving the ribosome depends e.g. on the lack of errors in their aminoacid sequence, which may depend on possible errors in transcription as well as errors in "reading" of the mRNA by the ribosome, pairing its code with that of appropriate tRNAs, and errors in binding of consecutive aminoacids brought in by relevant tRNAs in a stretch of peptide. To our knowledge, neither of

these aspects of translational efficiency was studied with respect to aging of human cells.

On the other hand, translational capacity depends on ribosome biogenesis. Unlike the translational efficiency, it seems to be at least partially shown that impaired ribosome biogenesis affects functions of different cell types. Thus, it has been reported that muscle mass is proportional to the intensity of ribosome biogenesis (Chaillou et al. 2014; Kirby et al. 2015). Also, impaired functioning of murine CD8+ T cells was associated with decreased ribosome biogenesis (Tan et al. 2017). Thus it is likely that reduced biogenesis of ribosomes in the muscle and in other cell types may be associated with aging and may lead to decreased translational capacity. Some data are emerging showing that this is true at least for human muscles, where impaired post-exercise ribosomal biogenesis was demonstrated for old individuals (Stec et al. 1985). Interestingly, recently the ribosome emerged as not only the provider of new peptide chains, but also the controller of their proper formation and early preventer of aggregation, which, as we mentioned earlier, are impaired with aging; thus the adequately working ribosome becomes a component of the proteostatic/proteodynamics network (Pechmann et al. 2013; Willmund et al. 2013; Brandman and Hegde 2016). Also, ribosomal proteins had recently been demonstrated to affect the gene expression, cell cycle, apoptosis, and DNA repair, all of which have been reported to be affected by aging (Wang et al. 2015). Still, the possibility of involvement of the impaired ribosomal function in the aging-associated changes in cellular proteomes has not been explored so far and requires thorough studies.

9.3 Posttranslational Modifications of Proteins and Aging

Recently the PTMs are more and more being recognized as potential biomarkers of diseases, including neurodegenerative, cardiovascular, diabetes and cancer(Mnatsakanyan et al. 2018; Thygesen et al. 2018; Zavialova et al. 2017; Abou-Abbass et al. 2016; Wende 2016). It so happens, that practically all of these diseases belong to the class "aging-related disease" i.e., with aging they become more and more prevalent, precipitating and characterizing the unsuccessful (or unhealthy) aging. Thus it is very likely, that at least some of the PTMs being currently considered the biomarkers of specific diseases are in fact biomarkers of aging. One prominent example might be the hyperphosphorylation of tau protein. This reaction, related to the activation of protein kinase cdk5 in the neurons by cleavage of its regulatory subunit by neutral cytoplasmic proteases called calpains (Lopatniuk and Witkowski 2011) (more about them below), is one of the two changes of the neuron (and generally brain) proteome considered pathognomonic in the development of Alzheimer disease (AD), where the other is the accumulation of beta-amyloid peptide (Aβ). Its consequence is the formation of characteristic neurofibrillary tangles (NFTs) in the neurons and glia of AD patients. However, tau hyperphosphorylation is observed in the brains of neurologically healthy middle-aged and older individuals as well (Alafuzoff 2018). Also, this process (when extensive and associated with clinical symp-

toms) is not limited to the AD, but occurs also in patients suffering from Parkinson disease, Lewy body dementia, Pick disease, progressive supranuclear palsy, corticobasal degeneration, and frontotemporal lobar degeneration which seems to make it rather the biomarker of aging and not necessarily of any specific, aging-related, neurological disease. On the other hand, appearance of pTau and aggregates of Aβ is supplemented by that of modified α-synuclein, sharing with Aβ the property of being/releasing antimicrobial peptides (Park et al. 2016) and hyperphosphorylated transactive response (Tar) DNA-binding protein 43 [pTDP43 (Park et al. 2016; Alafuzoff et al. 2008, 2009a, b, 2015)] not only in the neurological patients suffering from any of the abovementioned disease, but also in the neurologically healthy elderly, and the difference seems to be quantitative in nature, with the progression from young to (clinically) healthy elderly to manifest neurodegenerative disease. If this were proven to be true also for other intracellular proteins changes, we should expect many more potential biomarkers of cellular aging being their modified proteins. In fact, possible and already observed changes in the cellular proteolytic machinery occurring with aging have been acknowledged already at the end of last century; this work characterized the aging-associated changes in the lysosomal, proteasomal and cytosolic proteolytic systems (including calpains) mainly in the central nervous system, skeletal muscles, eye lens, liver, and cultured fibroblasts (Cuervo and Dice 1998, 2000).

We are proposing yet another, possibly ultimate PTM—proteolytic modification (not necessarily and not always degradative) executed by calpains (Witkowski et al. 2018). Unlike the proteasomes or lysosomal hydrolases (cathepsins and like), the calpains are mainly cytoplasmic, so they can have access to any intracellular protein. Some members of this 15-strong family of proteases also exist in mitochondria, which gives them the possibility to regulate intramitochondrial proteins as well. Given local conditions (including locally higher-than-average resting concentration of Ca^{2+}, necessary for activation of calpains) in the specific cytoplasmic domain surrounding the calpain molecule(s) they can either activate or inactivate intracellular proteins by modifying or removing their regulatory domains or ultimately degrade them (Lopatniuk and Witkowski 2011). The list of known calpain substrates includes cytoskeletal proteins, adhesion molecules, elements of signal transduction pathways (notably the [receptor] protein kinases), cation channels, growth factors and their receptors and many other (Witkowski et al. 2018; Lopatniuk and Witkowski 2011; Mikosik et al. 2016). Our own data show that constitutive calpain activity in resting human peripheral blood T lymphocytes is necessary for their activation and itself is sustained by constitutive transcription of relevant genes (CANP1 and CANP2) (Mikosik et al. 2016). In the same paper we show that resting calpain activity in the T cells maintains certain, presumably optimal for subsequent activation, levels of phosphorylated NFκB, p56Lck, and PLCγ (Mikosik et al. 2016); our new, yet unpublished observations suggest that the same is true also for the lymphocytic Zap70 kinase. Considering the broad (and so far not fully known) spectrum of their substrates, together with the possibility that, as we had shown for lymphocytes, some level of resting activity of calpains may occur also in other cell types modifying their proteins, uncontrolled calpain activity could be deleterious to the cell properties

and functions. Thus, the system has two safety valves built in; the first is the specific inhibitor called calpastatin, always present in the cytoplasm along with calpain molecules and activated by limited proteolysis by calpains themselves, while the second is calpain autoproteolysis (active calpain is a substrate for itself; its limited proteolysis renders it inactive) (Lopatniuk and Witkowski 2011).

Calpains are also passively and/or actively released/secreted by certain cell types, including the immune cells, and/or excreted in the exosomes, the formation of which they seem to facilitate (Taylor and Bebawy 2018; Taylor et al. 2017; Nishihara et al. 2001). We have seen differing calpain activities in the plasma samples from healthy individuals and rheumatoid arthritis patients (Witkowski et al., in prep). This implies that calpain may exert their modifying proteolytic activity against suitable extracellular proteins, although the list of such extracellular substrates of calpains is so far missing. On the other hand, calpains as cargo of the exosomes may be received by different other cell types and modify their intracellular proteins in turn (Potz et al. 2016).

Thus, in our opinion, these properties make the calpains an ultimate and universal system of protein modification, i.e. the important tool of maintaining the status of multiple cellular proteins in many cell types. Along this line, we have demonstrated the steady, aging-dependent decrease in the amounts and activities of calpains and calpastatin in various populations of human T and B lymphocytes; at the same time we saw the amounts of calpains and calpastatin in the cells from healthy centenarians to be maintained at the levels similar to these observed in the immune cells from healthy young individuals (Mikosik et al. 2013). Interestingly, in contrast to our observations, calpains were described to *increase* their activity in some other aging cell types, including the erythrocytes, where excessive calpain activity is responsible for the degradation of band 3 protein seen in old RBCs(Cuervo and Dice 1998). This makes the calpain-calpastatin system (CCS) another plausible, although requesting more detailed studies, biomarker of cellular aging itself in addition to improper activity of calpain being a potential biomarker of some aging-associated diseases, like the AD as mentioned above. The involvement of (usually excessive) calpain activities in multiple pathologies which are associated with aging (aging-related diseases, ARDs) is briefly characterized below.

9.4 Calpains and the ARDs

Calpains are also involved in the maintenance of protein status in the striated muscle and in some of its congenital (Duchenne dystrophy) and acquired pathologies. Thus, excessive activity of calpains (along with that of caspases and proteasomes, and excessive autophagy) is responsible for muscle weakness and cachexia in rheumatoid arthritis (RA) patients (Teixeira Vde et al. 2012). Interestingly, the mechanism of loss of muscular proteins due to excess calpain activity is apparently working via downstream effects on ubiquitin-proteasome pathway (UPP) and Akt phosphorylation (Huang and Zhu 2016). In addition, calpains are directly modifying many

cytoskeletal proteins of striated muscle, including myosin, titin, dystrophin, talin, troponin and spectrin (to mention the few), removing parts of the original protein and thus changing their abilities to bind and interact with each other in order to perform mechanical work of the muscle (Teixeira Vde et al. 2012). So, excessive activity of calpains has been proposed as one of the major mechanisms of muscle wasting and sarcopenia (Potz et al. 2016; Brule et al. 2010; Dargelos et al. 2007, 2008; Scicchitano et al. 2009; Vinciguerra et al. 2010; Timmer et al. 2018). Sarcopenia is among the key features of the frailty syndrome—a debilitating condition consisting *inter alia* also of immune and cognitive problems, to which succumb many of the aging individuals. A recent (2018) review characterizes multiple biomarkers of frailty; surprisingly enough, calpain is NOT among those (Cardoso et al. 2018).

Detrimental modification of multiple (including the above mentioned) proteins in cardiomyocytes by calpains (both cytoplasmic and mitochondrial) and other intracellular proteolytic systems is supposed to stand behind at least some forms of *heart failure* (Kunkel et al. 2015a, b). When considering this disease, we always think first of coronary vessels' atherosclerosis and related ischemia. However, the calpains may play a role also in the development of atherosclerosis. These proteases are active in endothelia, as well as in the infiltrating macrophages. Their excessive action in the first may lead to decreased nitric oxide production, weakening of the intercellular bonds and increased permeability, and apoptosis on contact with oxidized LDL, while in the second it facilitates their infiltration into the intima and induces degradation of cholesterol transporters [ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1)] leading to impaired cholesterol efflux and transformation of macrophages into foam cells (Miyazaki et al. 2013; Miyazaki and Miyazaki 2017, 2018).

It is well known that among the fastest deteriorating organs in aging are the kidneys. And here also the calpains (notably calpain-10) were found to be not only necessary for functions of different cells of the nephron, but for their survival; interestingly, amounts of active calpains in the aging kidneys drop significantly, which may possibly serve as another biomarker of aging-related *kidney failure* (Covington et al. 2008). Here, a link appears between calpain activities and decreasing with age activities of the "anti-aging hormone", Klotho (Manya et al. 2002, 2010). This protein with multiple activities including that of glucuronidase/glycosidase, made mostly (although by no means exclusively) in the kidneys, is known to control the calcium-phosphate balance (via affecting the metabolism of vitamin D3) and its decreased amounts are linked with kidney failure itself, as well as with osteoporosis, ectopic calcification, emphysema and respiratory failure, and atherosclerosis—again the aging-related conditions. Regarding the latter, activity of Klotho has been shown to reduce hyperactivation of calpain in human endothelia in vitro, which we have mentioned above as intimately involved in the appearance of the atherosclerotic lesions (Mencke et al. 2017).

Mentioned glycosidase activity of Klotho apparently serves to modify the N-glycans of channel and transporter proteins in the membranes, thus regulating transmembrane movement of several ions, including calcium (Akasaka-Manya et al. 2016). The latter may be the direct cause of effect exerted by Klotho on calpain activity. By the token of modifying the sugar residues of glycoproteins Klotho can

clearly be counted among the effectors of their PTM. On the other hand, Klotho acts also by suppressing the insulin/IGF1 signaling which is also prolonging the lifespan (Dermaku-Sopjani et al. 2013; Sopjani et al. 2015; Di Bona et al. 2014). Interestingly, in vitro treatment of myoblasts with IGF1 was shown to greatly increase the amount of calpain in these cells which led to their increased mobility (Leloup et al. 2006). Putting these facts together one cannot exclude that Klotho prevents muscle (and likely other tissues') aging also by decreasing available calpain activity and preventing it from adversely modifying the relevant proteins.

Our recent research had shown that decreased Klotho amounts (and glycosidase activities) are by no means limited to aging kidneys. In fact, we have shown that this activity of Klotho (and amounts of Klotho protein) are greatly reduced in the human peripheral blood T cells of healthy old people as well as in the same cells from RA patients (with RA being described as an aging-accelerating disease) (Soroczynska-Cybula et al. 2011; Witkowski et al. 2007). Interestingly, at the same time we have seen an increased activity of calpains in the RA patients' lymphocytes and other groups in the cartilage and synoviocytes (Mikosik et al. 2007; Szomor et al. 1999). Broadening and elaborating upon these data, calpain activity is more and more considered to be a stimulator of inflammatory processes. Thus, inhibition of calpain is decreasing the functions of neutrophils, especially the oxidative burst (Wiemer et al. 2010); overexpression of calpastatin (calpain inhibitor) in human Th lymphocytes and fibroblasts in vitro leads to decreased production of proinflammatory cytokines IL-6 and IL-17, via inhibition of IκB cleavage by calpain and consequent release of the active NFκB transcription factor (Iguchi-Hashimoto et al. 2011). Also, as we have demonstrated, inhibition of calpain activity in resting human T cells with tripeptide, membrane-permeant inhibitors decreases the production of multiple proinflammatory cytokines and practically abolishes proliferation upon stimulation in vitro (Mikosik et al. 2016). In this way, decreased calpain activities that we have seen in the lymphocytes of healthy old people may assist to the maintenance of low level of inflamm-aging [subclinical proinflammatory state mainly manifesting by increased concentrations of proinflammatory cytokines; the term coined by Franceschi (Franceschi and Campisi 2014; Franceschi et al. 2007, 2018)] which by itself may be beneficial for the health of elderly (Fulop et al. 2016a, b, 2017, 2018). In fact, critical role of calpain activities in the endothelia as well as immune/inflammatory and other cells for the development of inflammation has been proposed (Ji et al. 2016).

Finally, recent research suggested that calpains are implicated also in the development of type 2 *diabetes mellitus (T2DM)* and its complications, including the diabetic cardiomyopathy, nephropathy, and retinopathy. In most cases the underlying cause is impaired Ca^{2+} homeostasis in the cells of diabetics, leading to calpain hyperactivation (Wan et al. 2015; Panico et al. 2014).

9.5 Conclusions

So, most of the aging-related diseases, including the neurodegeneration, atherosclerosis and its cardiovascular complications, T2DM, and cancers seem to be at least in part the result of impaired proteodynamics, in a huge part related to disbalance in the calpain-calpastatin system. As this disbalance is seen also in the cells of healthy aged individuals, we can conceptualize the problem as continuity – from normal, healthy (young) proteodynamics to its impairment in healthy aging to its stronger impairment and defects in the aging-related diseases. Here, extremely long-living individuals (centenarians and supercentenarians) make a special group in whom changes in proteodynamics may be much slower than in those with shorter lifespans or even negligible. These considerations suggest that the numerous aspects of human

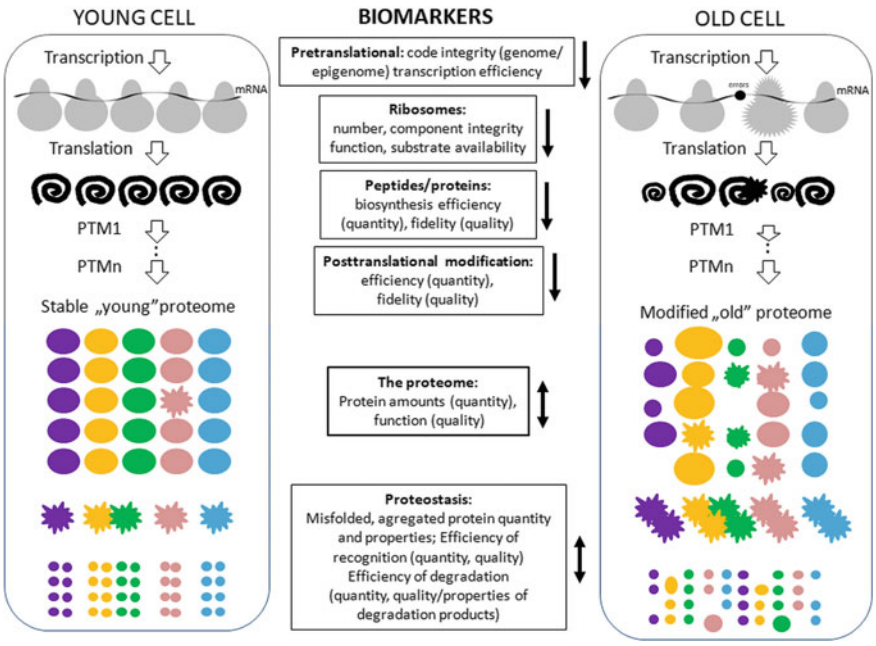

Fig. 9.1 Age-associated changes in proteodynamics and potential groups of quantitative and/or qualitative biomarkers. Left panel—young cell, right panel—old cell. The scheme assumes that in a young cell the elements and products of proteodynamic processes are in the quantitative and qualitative states adequate to the needs of an optimally performing cell and the proteome is relatively stable (in homeostasis). Aging affects all elements and products of proteodynamic processes, which may be considered as potential biomarkers, including the ribosomes (quantity and function), translated peptides (quantity and fidelity, i.e., aminoacid sequence reflecting that of mRNA), processes performing posttranslational modification (PTM1-PTMn) and proteostasis (recognition and degradation of misfolded and aggregated proteins, properties of the products of degradation). As a consequence, both the quantities and properties (functions) of proteome components may change. Potential pretranslational changes affecting the proteome are included for completeness. Arrows indicate possible change in the old versus young cells

cellular proteodynamics should be studied in more complex way, possibly leading to the definition of all changes occurring in the proteomes of aging human cells (including the effect of calpains and other proteolytic systems, which should be defined as a *lysoproteome*). These studies should then provide multiple new biomarkers of cellular aging in health and disease, possibly becoming future targets of interventions. These biomarkers may belong to both the protein-modifying enzymes executing the PTM and proteostasis AND to their substrates, differently modified in young and old cells. Groups of these and other potential biomarkers (including these associated with protein biosynthesis (translation) machinery and process), and differentiating the proteodynamics of young and old cells are proposed in the Fig. 9.1.

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Part III
Genetics and Epigenetics Aging Markers

Chapter 10
Genetic Markers of Extreme Human Longevity

Paola Sebastiani, Anastasia Gurinovich, Harold Bae and Thomas T. Perls

Abstract We review issues related to studying the genetics of extreme human longevity, including the definition of the phenotype, the choice of appropriate controls, the effect of population structure and assortative mating. We then discuss the important contributions to the genetics of extreme human longevity of the last few years that have arisen from genome-wide association studies and the work that is needed to discover additional longevity genes.

Keywords APOE · Assortative mating · Centenarians · FOXO3 · Genetic variants · Genome-wide principal components · Meta-analysis · Sequencing

10.1 Introduction

The word longevity indicates "long life" and, accordingly, most genetic studies of extreme human longevity have focused on subjects selected based on survival beyond some old age. The choice of the old age has posed challenges related to the use of chronological age and to the selection of a threshold that is sufficiently stringent to determine a highly heritable phenotype. In fact, the rarity of old ages can vary tremendously depending on birth cohort, environmental, cultural and lifestyle differ-

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ences. For example, over a 100-year period, the top 5th percentile for age of survival in Sweden increased by 10 years, from an average of 84 years for the 1800 birth cohort to 93 years for the 1900 birth cohort (both sexes combined). This marked advancement was likely due to a combination of improved infant and childhood survival, improvements in socioeconomic conditions including access to education—and, of course, public health and medical advancements (Brandstrom 1993). These demographic observations emphasized the need for researchers to be as specific as possible about the birth cohorts and percentiles of survival they are studying, in order to standardize the definition of longevity. In 2016, we proposed using a more standardized definition of longevity defined as survival beyond ages reached by less than a small proportion p of the population that can be determined using population and birth-year specific cohort tables. Using this standardized definition of longevity, we showed that the heritability of longevity begins to increase only when we consider ages reached by less than 1% of the population, for example 96 years for males and 100 years for females born in the USA in 1900 (Sebastiani et al. 2016). The findings were consistent with earlier work that suggested high heritability for living to 100 (Perls et al. 1998, 2002), and the low heritability of age at death that has been estimated to contribute to less than 25% of the total variability (Christensen et al. 2006). Unfortunately, the confusion between longevity, age at death, and life span continues to persist: for example, in their recent manuscript Ruby et al. (2018) concluded that the heritability of longevity is around 10% when they actually showed that the heritability of age at death is about 10%. The confusion between these two concepts continues to be a roadblock for genetic studies of extreme longevity, and is analogous to treating the heritability of glucose level and the heritability of Type 2 diabetes as the same phenotype.

In addition to selection of cases, genetic association studies of extreme human longevity require the selection of a referent group and this has proved to be a very challenging and still unresolved problem. Ideal controls to reduce the effect of unmeasured confounding would be individuals matched by birth year cohort, ethnicity, place of birth, place of living, and sex, who did not survive to extreme old ages (Sebastiani and Perls 2012; Giuliani et al. 2018a, b). This selection of controls is virtually impossible in studies conducted in the USA because of the various waves of migration (2018b), which is a possible reason for the lack of replication of many candidate gene association studies (Novelli et al. 2008). Investigators have used as controls individuals who died before reaching a fixed threshold age: for example, the studies included in the meta-analysis in Newman et al. (Newman et al. 2010) used as controls individuals who survived to an age between 50 and 80 years, while cases were defined as individuals who survived past age 90 years. We showed in reference (Sebastiani et al. 2017a) that using different age thresholds in the definition of cases and controls of extreme human longevity leads to biased estimates of the genetic effect. Since extreme human longevity is a rare trait in the population, simply using a random sample of individuals from the population rather than individuals who died before reaching a certain age may be a better choice of controls that is unlikely to include individuals who will leave to extreme ages reached by a very small portion of the population. However, this selection of controls is likely to introduce various

confounders that need to be accounted for in the statistical analyses (Giuliani et al. 2018b). In the next sections, we will describe techniques that have been used to address these problems.

10.2 Genetics

Defining extreme human longevity as survival past a certain threshold age limits the measures we can use to estimate the genetic heritability of this trait (Christensen et al. 2006). We and others have used sibling relative risks to estimate the increased chance of reaching extreme old ages in siblings of extreme long lived individuals (Sebastiani et al. 2016; Perls et al. 1998, 2002), and have shown that this measure of heritability increases only when we consider survival to ages reached by less than 1% of the population. These analyses and the familiality of patterns of extreme human longevity support the hypothesis that this trait has a large genetic component (Christensen et al. 2006; Perls et al. 2000). Until approximately 2005, genetic association studies of human longevity were based on candidate genes that were selected based on their role in the biology of aging. Gene variants were represented by the alleles of few single nucleotide polymorphisms (SNP), which are a single base variation in the DNA sequence (Sebastiani and Palmer 2017). Progress in genotyping/sequencing technology has created opportunities to search for genetic determinants of extreme human longevity genome-wide in an unbiased way (Visscher et al. 2017), and we will review some of the specifics of genome-wide association studies (GWAS) of extreme human longevity.

10.2.1 Effect of Population Structure

In GWAS it is important to adjust the analysis for population structure because a spurious association between the phenotype and a SNP could stem from ethnic-specific differences in allele and trait frequency (Solovieff et al. 2010). There are several strategies to control for population structure (Price et al. 2006; Epstein et al. 2007; Kimmel et al. 2007; Wang 2009). The most commonly used approach is to adjust the regression model by principal components calculated from the genome-wide genotype data (Sebastiani et al. 2017b). However, simply adjusting by population structure would not identify important variants specific to each of the group defined by different genetic composition, which are shaped through complex environmental influences and population dynamics (Giuliani et al. 2018a). An alternative solution is to design a matched study in which cases and controls are genetically matched (Solovieff et al. 2010; Hinds et al. 2004; Guan et al. 2009; Luca et al. 2008). For example, in our GWAS of extreme longevity in (Sebastiani et al. 2012a), we matched cases in the New England Centenarian Study (NECS) to controls selected from an Illumina supported repository (Sebastiani et al. 2012a). The matching was done by using only

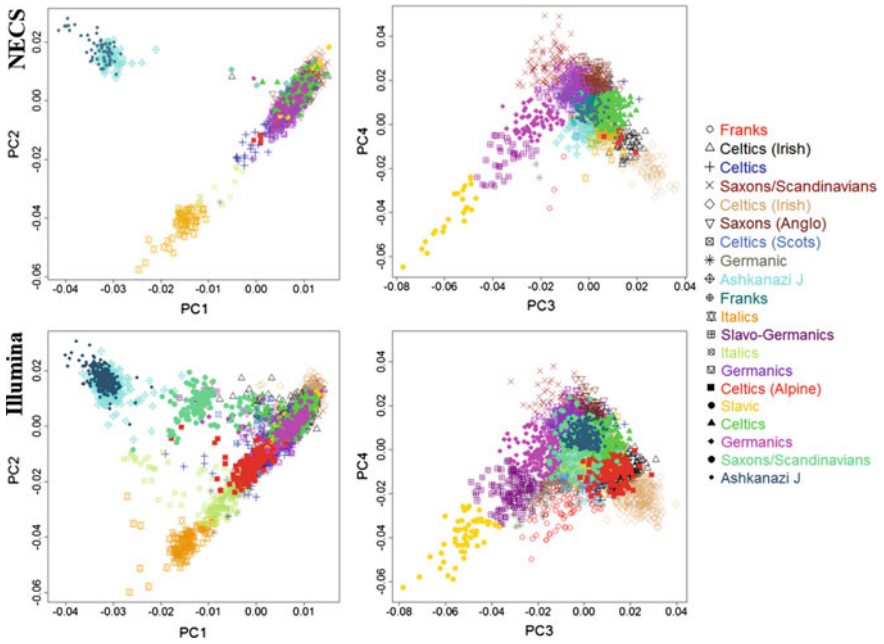

Fig. 10.1 Scatter plots of PC1–PC4 of the NECS subjects as compared to Illumina controls

controls that had a similar genetic architecture to the subjects in the NECS based on clusters of principal components (Solovieff et al. 2010). Figure 10.1 shows the scatter plots of the first 4 principal components that were computed using genome-wide genotype data using the EIGENSOFT software (Price et al. 2006, 2008) in NECS subjects (top panel) and Illumina controls (bottom panel). The plots show that some European ethnicities represented in the set of possible controls were not represented in NECS subjects. For example, the NECS did not include subjects of some Italics and Celtic Alpine ethnicities that are more prevalent in the geographic regions of North Italy, Switzerland, and South France, and controls with these ethnicities were not included in the matched set (Sebastiani et al. 2012a). Accounting for population structure is very important in studies of extreme human longevity conducted in the USA, in which the various waves of migrations in the last 2 centuries may have impacted the genetic structure of the older generations more substantially than in other regions.

10.2.2 Assortative Mating and Selection of Controls

Assortative mating is a pattern of sexual selection in which individuals with similar phenotype(s) and hence similar genotypes mate more frequently than randomly.

Analyses of spouse pairs in studies of EL suggest the presence of ancestry-based, assortative mating among the generation of centenarians that slightly decreases in younger generations (Sebastiani et al. 2017c). For example, we identified 155 spouse pairs in the proband generation and 735 spouse pairs from the offspring generation from the Long Life Family Study (LLFS)—a longitudinal family-based study of longevity and healthy aging—and 102 spouse pairs of centenarian's offspring from the NECS. These pairs included subjects with several European ethnicities. Next, we computed principal components (PCs) from genome-wide genotype data using the EIGENSOFT software (Price et al. 2006, 2008) and examined the genetic similarity between spouses in each pair using the top two principal components PC1 and PC2, which capture ethnic variability from East to West and North to South of Europe (Solovieff et al. 2010; Reich et al. 2008; Tian et al. 2009). PC1 and PC2 were significantly positively correlated between spouses with the following Pearson correlation coefficients: $r = 0.79$ for PC1 and $r = 0.7$ for PC2 in the proband generation from the LLFS, with median birth-year 1920; $r = 0.71$ for PC1 and $r = 0.4$ for PC2 in the offspring generation from the LLFS, with median birth-year 1945; $r = 0.67$ for PC1 and $r = 0.2$ for PC2 in offspring generation from the NECS, median birth-year 1932 (Sebastiani et al. 2017c). As an example, Fig. 10.2 depicts the scatter plot of PC1 and PC2 in the 102 NECS offspring spouse pairs. The analysis suggests a strong genetic similarity in spouse pairs of older generations and a decreasing trend of ancestry-based assortative mating in later generations. These results are consistent with the analyses conducted in the Framingham Heart Study (FHS) (Sebro et al. 2017).

The implications of this finding to genetic analyses are extensively discussed in Sebro et al. (2017). One of the main conclusions is that spouses of long lived individuals alone might not be ideal controls in genetic association studies of EL (especially in older, more endogamous generations) because of their ancestral similarity to cases (Sebastiani and Perls 2012; Sebastiani et al. 2012a, 2017b, c). For example, one of

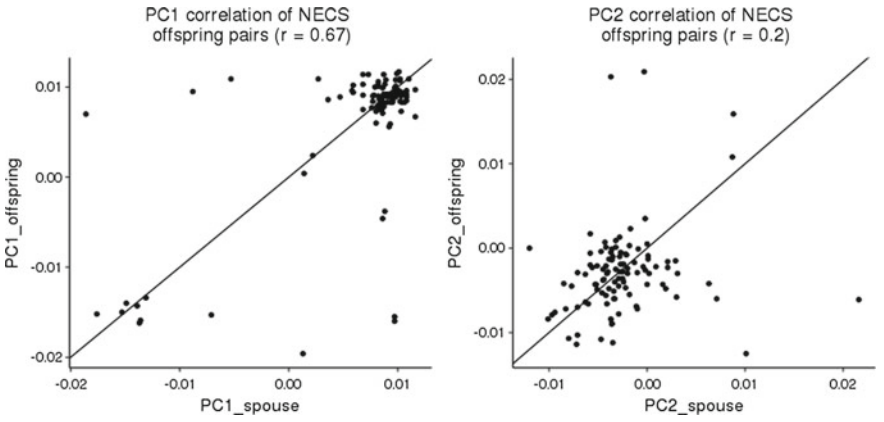

Fig. 10.2 Scatter plot of PC1 and PC2 in the 102 NECS offspring and their spouse pairs. Each point in the scatter plots represent a spouse pair, and the y-axis reports the values of PC1 and PC2 of offspring of centenarians, and the x-axis reports the values of PC1 and PC2 of their spouses

the surprising findings that emerged from analysis of various aging markers in the LLFS is that spouses of members of longevous families tend to be healthier than average (Sebastiani et al. 2013), and have substantially reduced mortality compared to a background population matched by birth year and sex (Pedersen et al. 2017).

10.2.3 GWAS of Extreme Longevity

GWAS typically aim to identify genetic variants across the human genome that associate with common, complex polygenic traits (Sebastiani et al. 2009). In a GWAS, the association between SNPs genotyped in an array and trait are examined individually, one at a time, and to correct for massive multiple testing, a genome-wide significant p-value of 5×10^{-8} has been widely adopted. In a GWAS of longevity and exceptional longevity, there are two modes of statistical analysis that tests the association between a single SNP and the trait. In a case-control study design, cases are defined as subjects who have attained a pre-defined age cutoff and controls are often general population controls. Using this study design, the genetic effect is the odds ratio for extreme longevity estimated typically via a logistic regression model, which compares the carriers and non-carriers of a specific allele of a SNP. A major limitation of this approach is the lack of controls matched by birth year (Newman and Murabito 2013), and the difficulty of appropriate control selection which can lead to bias in estimated genetic effects is well described above and in Sebastiani et al. (2017a). Alternatively, using the data from prospective cohort studies of extreme longevity, a time-to-event analysis can be performed to examine how genetic variants associate with age at death, conditionally on having attained an extreme age. In such a design, the Cox proportional hazards model can be used to estimate the hazard ratio for death. This approach can be particularly useful to better understand the degree of survival associated with genetic variants, conditional on having survived to an age cutoff.

To date, only a handful of GWAS of longevity (Deelen et al. 2011; Malovini et al. 2011; Nebel et al. 2011; Walter et al. 2011) and exceptional longevity (Sebastiani et al. 2012a; Zeng et al. 2016) have been published. Deelan et al. (2011) conducted a GWAS of longevity with 403 cases ($\geq$90 years) from the Leiden Longevity Study and 1670 younger controls from the population, and identified SNP rs2075650 as genome-wide significant hit. This SNP is located in the *TOMM40* gene, close to the apolipoprotein E (*APOE*) gene that has been a candidate gene in longevity since Schacter et al. showed that French centenarians have a very low frequency of the allele ε_4 that is associated with increased risk for Alzheimer's disease and vascular disease (Corder et al. 1993; Yip et al. 2005), and an increased frequency of the most uncommon allele ε_2 (Schachter et al. 1994). The *APOE*-longevity association was further confirmed in a study of 763 German long-lived individuals (mean age: 99.7 years, age range: 94–110) (Nebel et al. 2011). In a GWAS of southern Italians with 410 cases ($\geq$90 years) and 553 younger controls, the authors failed to identify genome-wide significant variants. The association between genetic markers and exceptional human

longevity was first studied in 801 centenarians (median age at death 104 years) and 914 genetically matched healthy controls from the New England Centenarian Study (Sebastiani et al. 2012a). The same SNP, rs2075650, in *APOE/TOMM40* reached genome-wide significance in this study, but although many variants reached extreme level of significance, they did not meet genome-wide significance. More recently, a GWAS was conducted in a sample of Han Chinese individuals including 2178 centenarians (mean age 102.7 ± 3.49 SD) and 2299 middle-age controls (mean age 48.4 ± 7.44 SD) (Zeng et al. 2016). This study replicated the association in the *APOE* locus, and found two additional genome-wide significant loci in *IL6* and *ANKRD20A9P*, but these variants remain to be replicated in ethnicities other than Han Chinese.

The lack of novel genome-wide significant findings in GWAS of longevity and exceptional longevity has been attributed to lack of statistical power to detect associations with genetic variants. It has been argued that significant loci discovered in many GWAS of common traits explain only a small fraction of the phenotypic variance, even for highly heritable traits (Manolio et al. 2009), and therefore very large sample sizes are needed to reach genome-wide levels of significance. Human extreme longevity is a rare trait and accruing sufficient number of very long lived individuals is extremely challenging (Sebastiani and Perls 2012), and the GWASs of exceptional longevity published so far may have been severely underpowered. Recently, investigators from the UK Biobank have conducted GWAS of human age at death using parental age at death as phenotype, and their offspring' genotype data (Joshi et al. 2016, 2017), arguing that the very large sample size of offspring generation should make up for the somewhat diluted phenotype. Their work showed replication of the genome-wide significant hits on chromosome 19, and additional variants that are associated with a premature age at death. Although the authors described their finding as relevant to the genetics of human longevity, they referred to longevity, age at death and human lifespan as if they were the same phenotype, but they are not.

10.2.4 Meta Analyses

Combining results from many smaller through a meta-analysis has become a common procedure in genetic association studies to overcome the limited sample size of individual studies (Evangelou and Ioannidis 2013). In GWAS of exceptional longevity, a weighted average of the estimated odds ratios (case-control study design) or hazard ratios (time-to-event analysis) from different studies is computed with weights corresponding to the precision of each study, although combining p-values or test statistics is also possible (Evangelou and Ioannidis 2013). A fixed effects meta-analysis assumes that all studies are estimating the same underlying population parameter (i.e. the genetic effect of an allele) and that different effect sizes from different studies are due to sampling error only. On the other hand, a random effects meta-analysis incorporates heterogeneity between studies by assuming that each study effect size is an estimate of study-specific population effect. Even though the

assumptions of the former method may not be appropriate for aggregating GWASs, fixed effects method is predominantly used to maximize power to discover novel variants, compared to random effects method (Evangelou and Ioannidis 2013).

The only meta-analysis of GWAS of exceptional human longevity has been published in Sebastiani et al. (2017b). The meta-analysis included four studies of extreme longevity—NECS (Sebastiani and Perls 2012), LLFS (Newman et al. 2011), Southern Italian Centenarian Study (Malovini et al. 2011), and Longevity Genes Project (Atzmon et al. 2004)—that contributed a combined total of 2070 cases who survived to the oldest one percentile of survival for the 1900 U.S. birth year cohort. The study confirmed the strong association between *APOE* and exceptional longevity, and further characterized that this association is likely due to selection for earlier mortality for Alzheimer's and vascular disease. Moreover, with a large set of extreme agers, several novel loci associated with exceptional longevity were discovered, including rare variants associated with increased odds for longevity in chromosomes 7 and 4. The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium have conducted few meta-analyses of longevity (Newman et al. 2010; Broer et al. 2015; Deelen et al. 2014), however, longevity was defined as survival past age $\geq$90 years in (Newman et al. 2010; Broer et al. 2015) and past age $\geq$85 years in (Deelen et al. 2014). The two larger meta-analysis studies found SNPs in the *APOE* locus to be genome-wide significant, but no other additional novel loci were identified.

10.2.5 Sequencing

A relatively small number of whole genome sequence studies of extreme human longevity have been published. We generated the first DNA sequences of two supercentenarians (aged >114 years) in Sebastiani et al. (2012b) and showed that their DNA sequences were enriched for longevity-associated variants discovered in (2012a), and that both sequences included a number of known disease-associated variants. Gierman et al. (2014) generated whole genome sequences of 17 supercentenarians, and although they could not identify novel mutations associated with EL, they confirmed the presence of deleterious variants that appeared to be neutral in supercentenarians' genomes. Presence of deleterious variants in centenarians' genomes was also confirmed in Freudenberg-Hua et al. (2014). These analyses are consistent with findings from GWAS s that have shown centenarians carry a significant number of disease-associated variants (Sebastiani et al. 2012a, 2015; Hapi Heart https://dsgweb.wustl.edu/progeni/studies/hapiheart.html) and suggest that there may be rare longevity promoting variants that buffer the effects of disease predisposing variants and extend lifespan.

10.3 Established Longevity Genes: *APOE* and *FOXO3*

After years of candidate and genome-wide association studies, only *APOE* and *FOXO3* have been found to be associated with extreme human longevity with definite replication.

The apolipoprotein E (*APOE*) gene has three well characterized alleles—e2, e3, and e4—that are defined by the combinations of the two SNPs genotypes: rs7412 and rs429358. There is overwhelming evidence that the e4 allele of *APOE* increases the risk for Alzheimer's disease and other aging-related diseases, while the evidence about the role of the e2 allele in longevity has been much less clear. This gene was first mentioned in relation to extreme longevity when Schachter et al. (1994) noticed that the e2 allele has an increased frequency in centenarians. GWASs have used tag SNPs in the APOE locus to investigate the association between variants of this gene and longevity. Several studies have shown an association, for example, between rs2075650 (a SNP within approximately 16 KB from rs7412) and a variety of aging-related diseases as well as longevity. On 12/31/2018, Pubmed listed 113 publications that referred to rs2075650, 12 of which mentioned longevity and one of these articles linked the SNP rs2075650 to the e4 allele of *APOE* (Schupf et al. 2013). Most of these references, however, missed the point that the uncommon allele of rs2075650 is associated with decreased odds for longevity, and that simply carrying the common allele of rs2075650 cannot be longevity promoting. An extended discussion about the precise role of rs2075650 and reduced chance for longevity is presented in Sebastiani et al. (2012a).

Establishing the role of the e2 allele with longevity has been challenging. Several studies have demonstrated conflicting results on the association between e2 and human extreme longevity in various ethnicities (Gerdes et al. 2000; Castro et al. 1999; Choi et al. 2003; Louhija et al. 1994; Feng et al. 2011; Garatachea et al. 2014; Blanche et al. 2001; Stakias et al. 2006; Panza et al. 1999). Additionally, a meta-analysis that pooled results from various studies found no significant association between e2 and EL (Garatachea et al. 2015). Some of the reasons for discordant results may have been inconsistent definition of cases and controls, and ethnic-specific association of *APOE* alleles with extreme longevity (Sebastiani et al. 2017a). We recently completed a large association study of *APOE* alleles and extreme longevity in a dataset comprising four centenarian studies (Sebastiani et al. 2018), and we showed that e4 is associated with decreased odds for EL, and an increased risk for death that persists even at the most extreme ages reached by less than 1% of the population. We also showed that carrying the genotypes e2e3 or e2e2 is associated with significantly increased odds to reach extreme longevity, compared to carrying the genotype e3e3. However, the protective, genetic effect of e2 decreases substantially at the tail of the survival distribution. This result was replicated in Danish and Japanese studies (Sebastiani et al. 2018).

Forkhead box O3 (*FOXO3*) is a transcription factor part of the "longevity pathway" that includes the insulin–IGF1 signaling (IIS) pathway (Morris et al. 2015). The latter is an evolutionarily conserved pathway with major impact on lifespan in several model organisms including nematodes (Kenyon et al. 1993), fruit flies (Clancy et al.

2001), and mice (Bartke 2008). *FOXO3* has emerged as a candidate gene associated with human exceptional longevity since the initial discovery by Wilcox et al. in a study of 213 long lived Japanase-American men (≥95 years) (Willcox et al. 2008). Multiple studies of various ethnicities, of mostly nonagenarians, Broer et al. (2015), Anselmi et al. (2009), Bao et al. (2014), Flachsbart et al. (2009), He et al. (2014), Li et al. (2009), Pawlikowska et al. (2009), Soerensen et al. (2010), Sun et al. (2015) replicated the *FOXO3*-longevity associations reporting a total of 17 SNPs in the gene. In Bae et al. (2018) we conducted a case-control meta-analysis of *FOXO3* SNPs using data from four studies of extreme longevity (Sebastiani et al. 2017b), and we confirmed the association of *FOXO3* and human extreme longevity. A careful examination of the genetic effect in each individual study however suggested that the effect sizes decreased with more extreme ages. To better understand if *FOXO3* alleles have an effect on survival to extreme old age, we performed a survival analysis among the cases who survived past the 1 percentile survival age of the 1900 birth year cohort. This follow-up analysis demonstrated that none of the 17 published SNPs was associated with survival beyond the oldest 1 percentile, suggesting that the common variants of FOXO3 may be associated with healthy aging and longevity but not extreme longevity.

10.4 Future Directions

Over the last decade, several studies have provided evidence that many centenarians and their offspring delay or escape aging-related diseases, such as cardiovascular and Alzheimer's diseases, and that more than 90% of people living to 100 are functionally independent at the mean age of 93 years and thus markedly delayed disability (Hitt et al. 1999; Terry et al. 2008). Many who live to 105 years and older, thus truly approaching the limit of human lifespan, also compress the age of onset of these diseases and disability towards the end of their very long lives (Andersen et al. 2016). We and other have conjectured that centenarians and their offspring share protective genetic and molecular profiles underlying biological function that can be leveraged to promote healthy aging and develop novel therapeutics for aging-related disease. However, genetic studies of extreme human longevity have produced limited findings, despite the strong heritability of this trait. Most of the published genetic association studies of extreme human longevity have searched for common variants, but the findings published so far point to rare variants, or rare recessive genotypes that associate with living to extreme old age, such as the *APOE* e2 allele. There have been limited effort in generating whole-genome sequences of centenarian genomes, and an important step to discover genetic variants of extreme human longevity will be to conduct whole-genome sequence studies of centenarians and family members.

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Chapter 11
Epigenetic Biomarkers of Aging

Morgan E. Levine

Abstract The development of valid and reliable biomarkers of aging has become a major initiative in Geroscience research. Our ability to distinguish biological from chronological age will enable identification of accelerated versus decelerated agers, and could also provide a valuable tool for assessing intervention efficacy. While various types of data can be used to quantify "biological age", perhaps the most successful applications have been using DNA methylation data. To date nearly a dozen different "epigenetic clocks" have been develop—most of which track age in a variety of tissues and cell types. Nevertheless, while they seem to share some characteristics, such as robust age prediction, their associations with age-related outcomes vary substantially. In moving forward, the utility of such measures will depend on our understanding of (1) why specific CpG dinucleotides exhibit such consistent DNAm changes over time, (2) what pathways or hallmarks are driven and/or reflected by alterations of DNAm, and (3) perhaps most importantly, whether these patterns of aging are amenable to intervention.

Keywords DNA methylation · Biomarkers of aging · Biological age · Epigenetics · Machine learning · Complexity

11.1 Introduction

11.1.1 Quantifying Biological Aging

While chronological age is arguably the strongest risk factor for death, disease, and disability, same-aged individuals remain heterogeneous in their susceptibilities to these various outcomes. One explanation is that chronological time, which we define as 'age', is an imperfect proxy of the degree of biological aging an individual has undergone. In response, quantifying the aging process, and in doing so, defining measurable estimates of 'biological aging' (in contrast to chronological aging) has

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become a major initiative in Geroscience research (Kennedy et al. 2014). Developing such biomarkers of aging may (1) help identify drivers of the aging process, (2) facilitate identification of at-risk individuals earlier in the life course, and (3) help to assess the efficacy of interventions aimed at slowing aging and extending healthspan.

Several key themes have been gleaned from the overlap observed in aging studies of model organisms, genetic epidemiology, and behavioral/pharmaceutical interventions. From these, seven interconnected "pillars of aging" have been proposed as the most promising targets for aging and longevity research (Kennedy et al. 2014). One of these critical areas of research is Epigenetics—and more specifically, the role of DNA methylation (DNAm) in aging. In this chapter, I will review recent applications in utilizing DNAm measures in an attempt to quantify biological aging. I will also discuss potential consideration for improving these tools, as well as the importance of considering changes in DNAm with age within the greater context of age related molecular alterations as a whole.

11.1.2 DNA Methylation and Aging

The importance placed on epigenetics as a mechanism of aging stems in part from the increasingly strong evidence showing that the methylome undergoes very precise alterations at specific CpG sites across the genome with age (Florath et al. 2013; Johansson et al. 2013; Rakyan et al. 2010; Teschendorff et al. 2010). A CpG dinucleotide denotes a site in which a cytosine is sequentially linked to a guanine via a phosphate in the linear 5′ → 3′ sequence (i.e. 5′—C—phosphate—G—3′). Cytosines specific to CpG dinucleotide have a propensity to become methylated to form 5-methylcytosine, particularly in regions called CpG islands (CGI)—areas that contain a high CpG density, often within the promoter region of a gene. Covalent attachments of methyl groups to cytosines in CpG dinucleotide is thought o to act by silencing local gene transcription either via inhibiting binding of activator proteins to enhancer elements, or binding Methyl-CpG-binding protein that recruit histone deacetylase, creating closed conformational chromatin structures. In mammals, it is estimated that over 70% of CpGs are methylated (Jabbari and Bernardi 2004), many of which play a role in cellular differentiation, and/or X-inactivation.

Interestingly, increasing age tends to be accompanied by CGI promotor-associated hypermethylation, and genome-wide hypomethylation. The specificity of predictable age-related hypo- and hyper-methylation changes at specific regions across the genome suggests that while aging itself may be stochastic, it manifests a distinctive molecular pattern. Given the specificity of DNA methylation (DNAm) with age, a number of "epigenetic clocks" have been developed as predictors of chronological age and/or age-related phenotypes. These clocks, which are typically measured in units of years, are meant to serve as estimates of biological age, or more accurately "epigenetic age". Epigenetic age estimates can be contrasted against individuals' chronological ages to capture between-person and/or between-tissue variably in the rate of aging. Samples with a higher epigenetic age, relative to the chronological age

of the donor, are characterized as exhibiting signs of accelerated aging. In contrast, a lower epigenetic age, relative to chronological age, signifies a decelerated rate of aging. That being said, it is important to point out that nearly all of the applications of the epigenetic clocks have been done in cross-sectional, rather than longitudinal data. Thus, the acceleration/deceleration terminology actually pertains to differences in the level of epigenetic relative to chronological age, not the rate of change. The presumption that differential levels reflect heterogeneity in the rates of aging only holds true if the levels have diverged over time—something that will be touched upon later.

11.2 Human Epigenetic Clocks in the Literature

11.2.1 First Generation Epigenetic Clocks

To date, numerous epigenetic clocks have been created for humans (Table 11.1). The first instance of which was the epigenetic age predictor by Bocklandt et al. (2011) that was developed in saliva using data from twin pairs. Using LASSO penalized regression and a leave-one-out approach, the authors were able to generate an age predictor that accounted for over 80% of the variance in chronological age and produced age estimates with an average absolute difference from observed age of just over 5 years. Two years later, two additional clocks were published—one by Hannum et al. (2013) and one by Horvath (2013)—which have since become two of the most recognized epigenetic aging clocks in the literature. Both the Hannum and the Horvath clocks were developed using penalized regression methods (elastic net)

Table 11.1 Epigenetic clocks in the literature

First author (clock name)	Year	No. of CpGs	Training tissue	Predictor outcome
Bocklandt	2011	2–3	Saliva	Chronological age
Hannum	2013	71	Whole blood	Chronological age
Horvath (pan-tissue)	2013	353	52 tissues/cells	Chronological age
Weidner	2014	3	Whole blood	Chronological age
Lin	2015	99	Whole blood	Chronological age
Vidal-Bralo	2016	8	Whole blood	Chronological age
Yang	2016	385	Whole blood	Chronological age
Zhang	2017	10	Whole blood	All-cause mortality
Levine	2018	513	Whole blood	Phenotypic age
Horvath (skin and blood)	2018	391	Buccal, whole blood, epithelium, fibroblasts	Chronological age

to train a predictor of chronological age based on DNAm levels measured on Illumina Infinium arrays covering tens to hundreds of thousands of CpGs. In the case of the Hannum clock, it was developed using 450 k DNAm data in whole blood from a wide age-range sample that included 656 individuals ages 19–101. The Horvath clock was developed using approximately 8000 samples, across 82 datasets (with either 27 or 450 k Illumina arrays) that collectively incorporated 51 non-cancer tissues and cell types. Given the diversity of samples, the Horvath clock is often referred to as the pan-tissue clock. Both clocks exhibit exceedingly high age correlations in full age range samples, often upwards of $r > 0.95$.

Other clocks in the literature have been developed using various alternatives to the methods used by Hannum and Horvath, including (1) quantitative and characteristic-based preselection of CpGs, and (2) by incorporating aging proxies other than chronological age. For instance, two clocks developed by Wolfgang Wagner's group—the 99 CpG model described in Lin and Wagner (2015), and the 3 CpG model by Weidner et al. (2014)—both used 450 k methylation data, but with pre-selected CpGs. Prior to employing a multivariate linear model, CpGs were considered if they exhibited correlations with absolute values above $|r| \geq 0.85$ in a dataset that included 575 pooled blood samples from four studies. The 3 CpGs in the measure by Weidner et al. were further selected based on recursive feature elimination, and conduciveness in a subsequent pyrosequencing analysis. A similar approach was employed by Vidal-Bralo et al. (2016), in which 102 CpGs were preselected given that they exhibited the strongest age correlations ($r > |0.85|$), at which point this was further reduced to just eight CpGs via forward stepwise linear regression.

11.2.2 Next Generation Epigenetic Clocks

A new clock by Horvath et al. also preselected CpGs before training an age predictor. In this case, CpGs with both the highest and the lowest absolute age correlations in the training data were selected. The training data in this case included samples from buccal cells, whole blood, epithelium, fibroblasts, skin, and cord blood—and thus this measure is referred to as the Horvath skin and blood clock.

Yang et al. (2016) developed what the authors refer to as a 'mitotic clock'. CpGs considered for this clock were restricted to those that are located in Polycomb group target genes that are hypomethylated in fetal tissue, but that show a trend toward hypermethylation with age. The mitotic clock is estimated as the average DNAm over the 385 CpGs that satisfied all inclusion criteria and is meant to capture the cellular turnover.

In contrast to the other clocks, two clocks, one by Levine et al. (2018) and one by Zhang et al. (2017) developed epigenetic aging measures by substituting in age-related outcomes as the predictor variable in place of chronological age. Based on replicated results from an epigenome-wide association study (EWAS) for all-cause mortality, Zhang et al. further selected CpGs using a LASSO penalized regression approach to predict mortality. Two measures were then proposed—one based on

continuous DNAm values in these CpGs, and one based on the sum of CpGs meeting a high-risk threshold (either the highest or lowest quartile value).

The Levine clock also incorporated mortality prediction, but through a two-step process that initially involved the incorporation of clinical multi-system biomarkers. In step 1, prior to examining DNAm, a measure of 'phenotypic age' was developed using a Cox proportional elastic net model to train a predictor of aging-related mortality risk based on traditional lab tests. This measure was then transformed using the Gompertz function to convert it to an age (in units of years). In step 2, the phenotypic age variable was then used as the outcome in training an epigenetic clock in whole blood—again by applying an elastic net penalized regression procedure. The resulting measure is based on DNAm at 513 CpGs linearly combined as a weighted sum.

11.2.3 Age Predictions Across Tissues and Developmental Stages

Interestingly, while many of these clocks were developed using blood samples, the majority track age in diverse tissues, suggesting that the epigenetic aging phenomena being captured by them is not specific to blood, and instead potentially represents universal aging changes.

When comparing these clocks, it is apparent that the original pan-tissue clock by Horvath produces the most consistent age predictions across tissue types ($r = 0.86–0.95$). This is unsurprising given that it was developed to be a multi-tissue age predictor. Nevertheless, the clocks by Hannum, Levine, Horvath (skin and blood), and Lin also exhibit robust age associations in diverse tissues and cell types. In general, the age correlations for these clocks remain moderate/high across the various tissues (Fig. 11.1). For instance, the clocks trained in blood appear to produce robust age estimates in buccal cells, colon, and skin. Additionally, the clocks by Hannum, Levine, and Lin all predict lower aging in dermis (red), compared to epidermis (pink). However, this is not true for either of the Horvath clocks—likely because they were trained to produce equivalent age estimates between these samples. Interestingly, region-specific comparisons can also be seen in brain tissue for all but the Horvath pan-tissue clock, which again produces equivalent estimates irrespective of sample type. On the other hand, the Hannum, Levine, Lin, and Horvath skin and blood clocks all show under estimation of age in glial cells from the occipital cortex (salmon). Moreover, as was originally reported for the Horvath pan-tissue clock, cerebellum (brown) appears to have a slower aging rate—indicated by a lower slope—for all five clocks.

Aside from the variations in tissues used to generate these clocks, they also vary in their age predictions in younger samples. Interestingly, the age-related methylation patterns captured by many of the clocks do not show linearity with age, rather there is evidence of a marked exponential increase in the rate of methylation changes

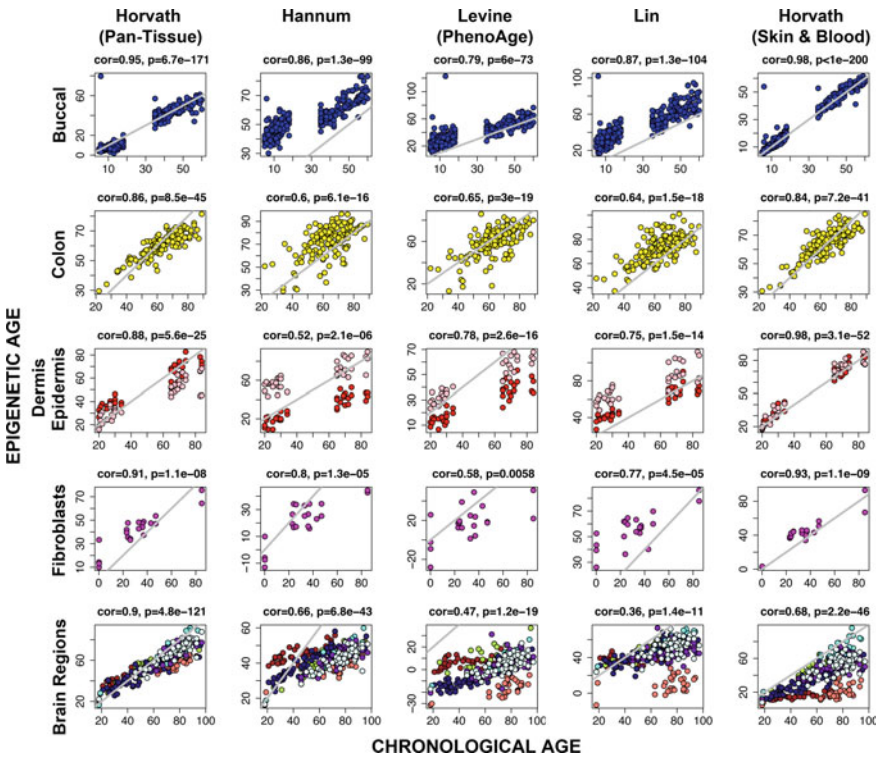

Fig. 11.1 Five epigenetic clock predictions in diverse tissues. Each row depicts a different tissue in which we test the age associations for various clocks (shown in separate columns). In the third row, dermis is represented by red dots, while epidermis is represented by pink dots. In the final row, the figure depicts the age predictions in cerebellum (brown), dorsal lateral prefrontal cortex (black), frontal cortex (purple), hippocampus (green yellow), glial cells in occipital cortex (salmon), neurons in occipital cortex (cyan), striatum (midnight blue), and temporal cortex (light cyan). Overall, the pan-tissue clock by Horvath produces the most accurate age predictions across tissues. Nevertheless, the clocks that were originally developed just in whole blood also show robust age associations regardless of tissue type

during development that levels off, becoming relatively linear typically after age 15–20 (Horvath 2013). In response, the two clocks by Horvath include a log function for samples under age 20 and therefore, produce accurate age estimates for young samples. Nevertheless, although the age predictions of other clock are not accurate in younger samples, there is evidence from some that they continue to correlate with age even during development (Levine et al. 2018).

11.2.4 Morbidity and Mortality Predictions

While accurate age prediction has exciting applications for forensics, perhaps the more important considerations for health and aging research is whether the various clocks differentiate lifespan and healthspan above and beyond what is captured by chronological age. In general, many of the clocks have been linked to diverse health outcomes. For instance, the clocks by Horvath, Hannum, Lin, Levine, and Zhang are all significant predictors of mortality risk to varying degrees (Levine et al. 2018; Zhang et al. 2017; Chen et al. 2016; Marioni et al. 2015a). After adjusting for potential confounders the Levine clock (in blood) has been shown to outperform the others in predicting mortality risk (Levine et al. 2018), as well as coronary heart disease (CHD), the number of co-existing diseases a person has been diagnosed with, and physical functioning. The Levine clock has also shown the strongest relationship to leukocyte telomere length (LTL).

A number of associations have also been found for cancers. In whole, blood, the Horvath pan-tissue, Levine, and Hannum clocks have all been linked to future cancer risk (Yang et al. 2016; Levine et al. 2015a, 2018; Ambatipudi et al. 2017; Zheng et al. 2016). Similarly, the Lin and Hannum clocks have been shown to differentiate tumor from normal tissue (Hannum et al. 2013; Lin and Wagner 2015), while the Horvath pan-tissue clock has been shown to be accelerated in breast samples from tumor adjacent tissue, compared to breast from normal controls (Hofstatter et al. 2018). Similarly, the Horvath pan-tissue and the Levine clocks have been shown to predict lung cancer risk among smokers, even after adjustment for pack-years (Levine et al. 2015a, 2018), suggesting they may reflect resilience to endogenous stressors, such as cigarette smoke. The links between epigenetic aging clocks and cancer are particularly interesting given the shared DNAm patterns in both aging and cancer (Ahuja and Issa 2000; Aunan et al. 2017; Klutstein et al. 2016)—suggesting they may actually be two sides of the same coin.

While not considered a normal part of the aging process, the Horvath pan-tissue, Hannum, and Levine clocks all relate to neuropathological hallmarks of Alzheimer's disease—such as neurofibrillary tangles and neuritic plaques (Levine et al. 2015b, 2018). Additionally, the Horvath pan-tissue clock and the Levine clocks have been shown to relate to cognitive functioning when assessed in blood. Unfortunately, all the findings surrounding cognition and Alzheimer's disease pathology reflect cross-sectional associations—the Horvath pan-tissue (in whole blood) was not found to predict future decline in cognitive functioning (Marioni et al. 2015b). In moving forward, it will be important to assess whether the same is true for the other clocks.

Differential epigenetic ages have also been observed for both pro-longevity and pro-aging phenotypes. For instance, individuals with Down syndrome (Horvath et al. 2015a), as well as those with HIV infection (Horvath and Levine 2015) have been shown to have faster aging rates in regards to both the Horvath pan-tissue and the Levine clocks (Levine et al. 2018). Accelerated aging has also been observed among those with Hutchinson Gilford Progeria Syndrome (but only for the Horvath skin and blood cock) (Horvath et al. 2018). Conversely, the Horvath pan-tissue and the

Levine clocks have found lower epigenetic ages among offspring of centenarians, relative to controls (Levine et al. 2018; Horvath et al. 2015b).

11.3 DNAm Biomarkers of Aging—A Single Node in a Larger Complex System

11.3.1 Popularity of Epigenetic Clocks

The increasing popularity of epigenetic biomarkers of aging—specifically based on DNAm—should not be interpreted as a stamp of importance, relative to other age-related alterations. Rather, it simply reflects the robustness of the technology. Compared to many of the other hallmarks of aging, DNAm (while only one form of epigenetic alteration), can be easily quantified both in vivo and in vitro using affordable and easily interpretable microarrays (Kurdyukov and Bullock 2016). For instance, the Infinium arrays from Illumina, which have become the preferred tool for exploring the role of the human methylome in disease, have led to an explosion of available DNAm data from diverse tissues and cells—most of which is publicly available through data repositories like Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA). Simultaneously, large observational studies, like the Framingham Heart Study, the Health and Retirement Study, the Women's Health Initiative, the Baltimore Longitudinal Study of Aging, and the Invecchiare in Chianti study have begun measuring DNAm in thousands of participants, enabling the merging of molecular measures with vast amounts of demographic, social, behavioral, and health information.

While measures capturing biological age and morbidity/mortality risk are not new to large epidemiological studies (Rockwood et al. 2002; Seeman et al. 2001; Kannel et al. 1976), the enthusiasm surrounding DNAm—and other molecular measures like telomere length, genetic variants, and gene expression (Jylhävä et al. 2017)—signals a desire to extend molecular explanations to population risk stratification for life expectancy and disease. However, results have not entirely lived up to the enthusiasm. While the age estimates of epigenetic clocks are often shockingly precise, the ability of such measures to capture aging heterogeneity, and thus differentiate morbidity and mortality risk among same aged individuals leaves a lot of room for improvement. The effect sizes for even the more predicative epigenetic clocks, pale in comparison to more traditional composite biomarker scores, based on traditional lab tests (e.g. glucose, albumin, cholesterol, etc.) (Levine et al. 2018; Jylhävä et al. 2017; Belsky et al. 2018).

11.3.2 Biological Levels of Organization and Modeling Complexity

One explanation may come down to biological levels of organization. It is likely that aging first manifests at the lowest levels of organization, working its way up the levels to produce the phenotypes we associate with growing old. Most of the proposed 'Hallmarks of Aging' reflect alterations at the molecular or cellular levels—a few degrees away from morbidity and mortality outcomes. Given the complexity at each level, as well as the stochasticity in aging changes, state transitions within higher levels (e.g. diagnosis of a disease) are more aptly predicted by measures capturing the level directly preceding it. This does no discredit the utility of molecular measures, rather it explains why clinical biological aging measures that reflect physiological functioning may be better indicators of health risks than measures that capture molecular alterations (Levine et al. 2018; Ferrucci et al. 2018).

Nevertheless, when it comes to interventions, our targets should be the lower levels of biological organization, given the temporal order in how dysregulation works its way up through the systems (Ferrucci et al. 2018). These changes will both be observable earlier and will be the most crucial if our goal is primary prevention. That being said, altered DNAm is only one example of a molecular change that creates instability at higher levels, and as with anything, one piece of the puzzle does not provide a good representation of the whole picture. If our goal is to estimate biological aging at the molecular, cellular, or even tissue level, we need to do a better job integrating all the hallmarks, and quantifying their interactions rather than studying each in isolation.

11.4 Unanswered Questions

11.4.1 Biological Underpinnings and Causality

Despite the relative success of the various epigenetic aging measures, there remains a lack of understanding of the underlying biology explaining why we observe specific DNAm patterning with age. Moreover, there is also a need to uncover the biological mechanisms linking epigenetic changes to other hallmarks and differences in lifespan and healthspan. While up to this point, most of the work involving development of DNAm clocks has taken an agnostic approach when it comes to the underlying biology, studies aimed at elucidating the dynamics, molecular causes, and downstream consequences of the aging-related DNAm changes captured by the clocks have the potential to uncover novel therapeutic targets for future aging interventions and may facilitate the development of more informed aging measures.

While we have observed connections between chronological time, DNAm, and morbidity and mortality (Chen et al. 2016; Marioni et al. 2015a; Levine et al. 2015b, 2016, 2018; Horvath et al. 2015a, b; Horvath 2013), we still do not have work-

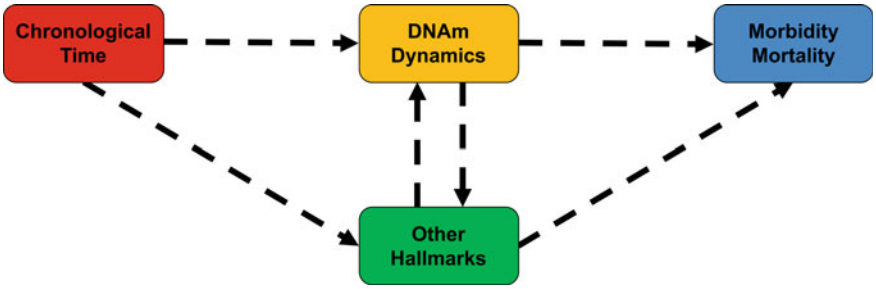

Fig. 11.2 Potential causal pathways between age, DNAm, hallmarks of aging, and morbidity/mortality. There is significant evidence that DNAm and many CpG sites changes as a function of age and also relate to differential risk of death and disease. However, it has yet to be determined whether DNAm plays a causal role in disease etiology, or whether it is simply a confounder for other age related changes on the causal pathway between age and outcomes

ing knowledge of why we observe such trends. This is an essential question when it comes to inferring causality, and in turn, informing intervention strategies. For instance, as illustrated in Fig. 11.2, it is possible that chronological time contributes to other molecular aging processes and it is through these alterations that differences in DNAm manifest. If this is the case, DNAm changes may not be a productive targets for intervention. Conversely, if chronological time has a direct effect on DNAm, which in turn diametrically contributes to the biological aging process, it suggests that therapeutics targeting such changes would be beneficial.

11.4.2 Distinguishing Innate, Developmental, Adaptive, or Stochastic DNAm Changes

In moving forward, emphasis should also be placed on linking observations of age-related DNAm changes to biological theories of aging. When it comes to theories of aging, there is a long-standing debate over whether aging manifests as the result of a genetic program or stochastic damage (Kowald and Kirkwood 2016; Kirkwood 1977). DNAm presents an exciting point from which to study this. It has been suggested that epigenetic clocks are evidence of programmed aging—a way in which the body regulates its own demise. However, the DNAm phenomena is just as easily accounted for from the perspective of stochastic damage accumulation, and/or entropy. According to the second law of thermodynamics (Hayflick 2007; Bortz 1986), there is a tendency for all things to go from highly ordered (low entropy) states to disperse/disordered (high entropy) states. Life is a product of a highly ordered and energetically expensive state. The precision needed to not only create, but also maintain life is so immense, it is astounding that it exists at all. Part of that precision is reflected in epigenetic regulation. DNAm patterns are exact, facilitating proper cellular functioning. However, as with all systems, over time the methylome

trends towards dysregulation due to laws of probability (Boltzmann 1878). Therefore, patterns of epigenetic drift observed with aging could be thought of as an exemplification of entropic dysregulation.

Unfortunately, there may be a problem in considering DNAm changes as a whole. There likely exists diverse types of DNAm changes that are pertinent to the aging process, each providing evidence for or against programmed versus stochastic aging theories. For instance, there are certainly low variability sites for which DNAm is set at birth to maintain homeostasis and/or appropriate biological functioning. Over time DNAm at some of these sites may become more variable, due to entropic forces (Jenkinson et al. 2017). Yet another type of DNAm may be in response to environmental perturbations, and thus may represent an adaptation to stressors (St-Cyr and McGowan 2017). There is good evidence that environmental conditions, such as deprivation, have an epigenetic impact (Chen et al. 2012; Kumsta et al. 2016; Skuladottir et al. 2016; Maegawa et al. 2017). Similarly, smoking has been shown to exhibit a robust DNAm signature (Zhang et al. 2017; Joehanes et al. 2016), and we have observed that epigenetic aging may have a bigger influence on death/disease risk among smokers (Levine et al. 2015a, 2018). A third type of age specific DNAm, may be programmed changes representing developmental switches. The role of DNAm in development is well documented, as a number of dynamically regulated changes have been shown to correspond with early and adolescent development, puberty, and puberty (Smith and Meissner 2013). This is particularly interesting given the links been development and aging (de Magalhães 2012); however, it remains to be seen whether DNAm changes that take place after development also unfold in a programmed fashion. Finally, individuals may exhibit innate differences in DNAm, that while remaining fairly fixed over the life course, may manifest as differences in other hallmarks of aging or susceptibility to death and disease. For instance, a number of CpGs in the Horvath pan-tissue clock and the Levine clock have surprisingly low age correlations (Horvath 2013; Levine et al. 2018). In the case of the Horvath clock, these CpGs may be proxies that differentiate tissues in aging, while in the Levine clock these CpGs may capture divergence of an individual's phenotypic from his/her chronological age.

11.5 Building Better Epigenetic Aging Measures

11.5.1 The Need for Longitudinal Studies of DNAm

Most of the age-related changes in DNAm described in the literature are from cross-sectional analysis. Thus, the phenomena captured by the clocks depicts differential DNAm across the age range, rather than changes in DNAm over time. Moreover, the residual between chronological and epigenetic age has been repeatedly referred to as "age acceleration"—Horvath has termed the phrases Intrinsic Epigenetic Age Acceleration (IEAA) and Extrinsic Epigenetic Age Acceleration (EEAA) to refer to

observed versus expected epigenetic ages for his pan-tissue clock and the Hannum clock, respectively (Horvath and Raj 2018). However, these are not in fact accelerations/decelerations, a term which implies a rate measurement. Rather, these measures simply capture the levels of epigenetic age, relative to chronological age. It is true that people may reach different levels as a result of differences in rate. However, the longitudinal dynamics of DNAm need to be further explored. For instance, it has yet to be shown whether (1) people start off at different levels and proceed at the same rate, (2) whether the rate of aging differs between individuals, which gets reflect in their levels when observed at a snapshot in time; or (3) whether rates differ during development, producing various levels in adulthood that then do not continue to diverge. Answering this question will have implications for the timing of interventions and also development of predictive models of risk. Finally, it will be important to track whether changes in the rate of DNAm are reflected in the rate of physiological and/or functional decline.

11.5.2 One Clock or Many?

While current epigenetic clocks have had some success in predicting outcomes of aging, as mentioned earlier, there remains of lack of understanding in terms of (1) what contributes to age-related DNAm changes, (2) whether DNAm changes are causal in aging, and (3) whether they are amenable to intervention. One potential obstacle in using the existing clocks to answer these questions is that they include a large number of CpGs with diverse age and tissue trends. Thus, it may be the case that the answers could vary depending upon which "type" of CpG one was considering.

For instance, Fig. 11.3 depicts tissue-specific age changes for four notable CpGs—the CpG in the first row is in the infamous *ELOVL2* gene (known to show strong age-related DNAm changes) (Weidner et al. 2014), while the other three CpGs were included in all three of the most widely known epigenetic clocks (Horvath and Raj 2018) by Horvath (2013), Hannum et al. (2013), and Levine et al. (2018). These CpGs exhibit drastically different age trends. For instance, cg16867657 (in a CGI that is 200–1500 bp upstream of the TSS for *ELOVL2*) exhibits a strong positive age trend in all six tissue types; however, this change may not represent epigenetic drift (or random error) given that in adult samples, DNAm levels tend to move away from 0.5, rather than towards it. This may suggest a compensatory/reactionary mechanism to other age changes. Conversely, cg2196230 (in CGI within the 5′ untranslated region, between the TSS and the ATG start site of *GREM1*) exhibits the hallmarks of what we consider epigenetic drift. Cg19722847 (downstream shore of CGI 200–1500 bp upstream of the TSS for *IPO8*) represents another CpG that may not exhibit drift. Instead it shows a moderate negative trend towards homogenous hypomethylation. Finally, cg05442902 (in the promotor associated south shore of *P2RX6*) shows negative non-drift related changes, but without the seemingly exponential decrease of DNAm during development.

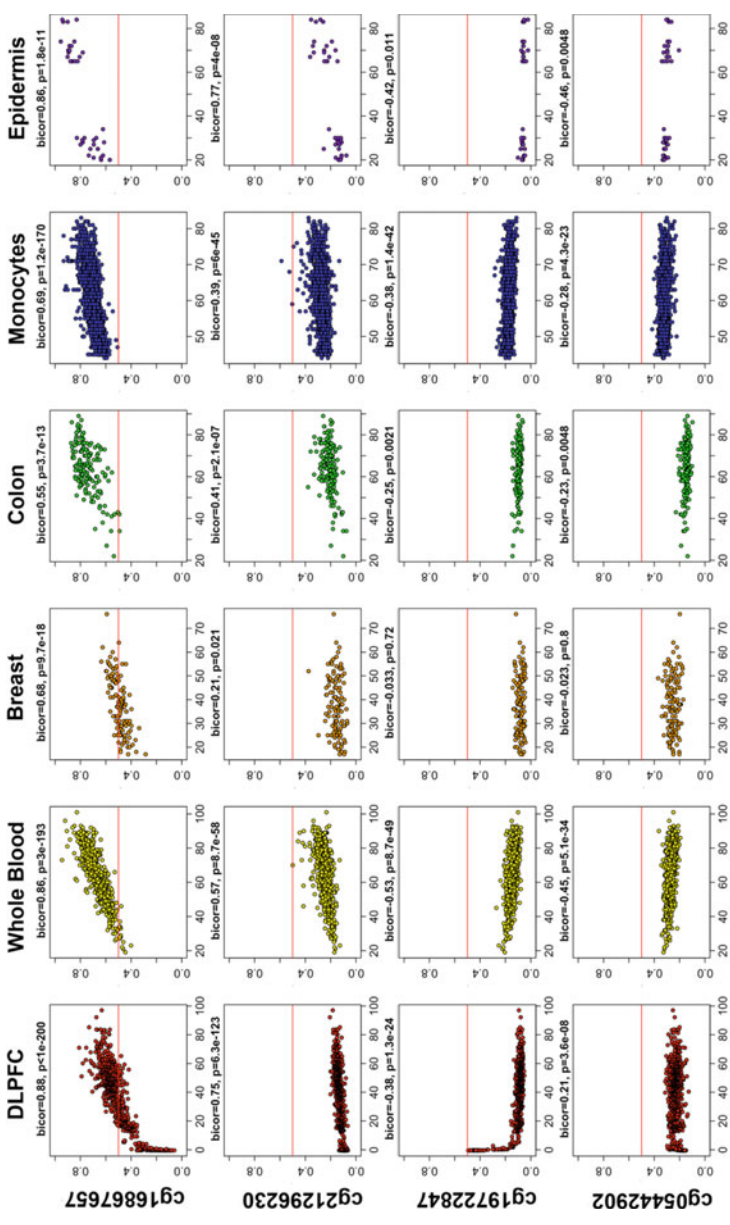

Fig. 11.3 Distinct age-related patterning in four CpGs across five tissues. Although the four CpGs all exhibit robust age associations across tissues, the directionality and magnitude of these changes vary. For instance, cg16867657 and cg19722847 both show accelerated change during development, but in the opposite direction. Moreover, both show divergence away from heterogeneous DNAm (0.5), which suggests a mechanism other than drift

When considering these diverse trends, it makes sense that there may be different mechanistic explanations and consequences for/of various types of epigenetic aging. Therefore, developing various clocks that represent higher-order DNAm dynamics and/or domains with aging may improve our understand of epigenetic aging and lead to the development of more robust epigenetic biomarkers of aging.

11.5.3 Multi-tissue or Single-Tissue?

Given that the majority of clocks trained using whole blood can be applied to a variety of diverse tissues and cell tissues, it is not clear whether training multi-tissue measures is advantageous. When the objective is to develop highly accurate age predictors—as in forensics or related applications—there is a distinct advantage to training multi-tissue clocks that produce unbiased age estimates regardless of sample type. However, when attempting to build proxies of biological age, adjusting for tissue differences may in fact be removing some of the very signal we are trying to capture.

There is no reason to believe that all tissues and cells age at the same rate. Tissues are highly variable in their susceptibility to degeneration and/or cancer (Tomasetti and Vogelstein 2015), and also display variations in many of the other hallmarks of aging (Frenk and Houseley 2018). These differences reflect disparities in cellular renewal rates, stem cell reserves, exposure to endogenous and exogenous stressors, and molecular maintenance systems. Thus, training epigenetic age measures that produce chronologically equivalent ages between tissues and cells may inadvertently remove some of the epigenetic aging phenomena that not only differentiate tissues, but also differentiate individuals with heterogenous aging rates. For instance, evidence suggests that expression of molecular hallmarks of senescence is highly tissue dependent, suggesting tissues vary in their accumulation of senescent cells (Hudgins et al. 2018). If this signal can be captured by DNAm, then a model aimed at producing unbiased tissue age estimates may actually treat this as a confounder, rather than part of the signal. Therefore, in moving forward, it may be more advantageous to identify CpGs for which there is tissue consensus in the direction of DNAm alterations, but allow for tissue-specific differences in the rate and levels of those changes.

11.6 Conclusion

Overall, methylation-based biomarkers of aging offer an exciting opportunity for the aging field and Geroscience research in general. Using such tools, researchers can theoretically assess the biological age of nearly any tissue or cell. We can conduct studies to determine how individualized patterning of epigenetic aging across methylation domains or across tissues and cells, gives way to personalized risk estimates—facilitating secondary prevention strategies. Additionally instead of relying

on long follow-ups for clinical trials, DNAm age measures could be applied as earlier read-outs when evaluating the efficacy of Geroprotective interventions.

Nevertheless, in order to improve the utility of these tools, we first need to determine why DNAm is altered so consistently with chronological time, and how various types of DNAm alterations relate to other hallmarks of aging. It will also need to be established whether age-related DNAm changes are causally linked to disease etiologies or instead serve as proxies for other drivers of pathogenesis. Finally, it is imperative to establish which of these age-related epigenetic changes are amenable to intervention, and if they are, which pathways contribute to heterogenous DNAm aging. Only then will epigenetic biomarkers prove to be useful tools for answering the questions—Why do we age? and What can we do about it?

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Chapter 12
DNA Methylation Markers to Assess Biological Age

Dmitriy I. Podolskiy and Vadim N. Gladyshev

Abstract Among the different biomarkers of aging based on omics and clinical data, DNA methylation clocks stand apart providing unmatched accuracy in assessing the biological age of both humans and animal models of aging. Here, we discuss robustness of DNA methylation clocks and bounds on their out-of-sample performance and review computational strategies for development of the clocks.

Keywords DNA methylation · Biomarker of aging · Epigenetic clocks · Elastic net regression

Discovery of a precise and robust molecular biomarker of age opens an important venue in aging research: the practical possibility to perform truly large-scale screens for dietary, pharmacological and genetic interventions extending lifespan of model animals and, potentially, of humans. Indeed, a critical limitation of any such screen is *time available* to do the experiment and determine its outcome. To be sure whether a given intervention truly extends the lifespan of the organism under consideration, a rule of thumb is to maintain control animals for at least a couple of mortality doubling times (~8 months for mouse) to be able to detect the effect of the intervention on the structure of the resulting survival curve. Thus, for long-lived organisms such as the naked mole rat (or, for that matter, humans with the mortality rate doubling time of ~8 years) the time of the experiment becomes large enough to make any such screening study impractical. Moreover, as sometimes interventions not influencing the mean lifespan modulate the maximal lifespan achievable within the cohort, it is ideal to wait for the whole cohort to die out to have access to the complete survival curve. This choice is even more limiting; for example, the maximal lifespan of C57Bl/6 mice routinely used as a control in lifespan screens is ~3 years, and some interventions (calorie restriction, growth hormone receptor knockout, etc.) can increase it to 4–4.5 years.

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While the lack of available time may be somewhat addressed by increasing the cohort size under study, the compensating effect is limited: recall that the survival curves of most organisms used as models to study human aging are largely determined by Gompertz law dictating that all-cause mortality increases exponentially with age, and as such, the corresponding survival distributions feature super-exponentially decaying tails at ages larger than the mean lifespan. Consequently, when one identifies parameters of these survival distributions using Kaplan-Meier analysis, the identification error depends on the cohort size sub-logarithmically weakly. Thus, we must conclude that the limitation of time available to perform a lifespan intervention screen and determine its results with statistical significance is of fundamental nature.

If developed, a good experimental biomarker of age would address this limitation, allowing to measure the biological age of an organism subjected to a lifespan-modulating intervention, and compare it to a control obtaining the estimate on effectiveness of the intervention right away. Early attempts to develop such a biomarker were based on modulation of gene expression levels with age (de Magalhães et al. 2009), change in somatic mutation counts with age (Dollé et al. 2000; Podolskiy et al. 2016; Podolskiy and Gladyshev 2016; Alexandrov et al. 2015), shortening of telomeres (Harley et al. 1990), and other approaches. Unfortunately, none of them proved to be sufficiently accurate to be used practically in large scale screens, but the situation finally may have changed with the discovery of DNA methylation clocks in humans (Hannum et al. 2013; Horvath 2013) and other mammals (Petkovich et al. 2017; Wang et al. 2017; Stubbs et al. 2017; Thompson et al. 2017; Polanowski et al. 2014).

Average DNA methylation across CpG sites present in the human genome or genomes of several animal models is known to systematically change with age (Field et al. 2018). For example, in both human and mouse the wave of increased methylation is strongly pronounced during the early period of organismal development (Hannum et al. 2013; Horvath 2013; Petkovich et al. 2017); it is then replaced by a relatively slow process of hypomethylation (on average across the genome) proceeding through the remaining lifespan of the organism (Fig. 12.1). Exploiting these systematic age-dependent processes allows to develop a potentially very precise biomarker of age in both human [with accuracy ~3–5 years in identifying the sample age (Horvath 2013)] and mouse [with reported accuracy as high as ~1 month for some DNA methylation clocks and mouse strains (Petkovich et al. 2017)].

As reviews and comparative analyses of already developed DNA methylation clocks are presented in other chapters of this book (see Chaps. 11 and 13), we devote this short chapter to the discussion of robustness of methylation clocks, their out-of-sample performance and the computational strategies for DNA methylation clock construction. We shall also discuss several possible pitfalls which researchers may encounter in the process of biomarker development; this discussion is largely applicable to any other biomarkers of aging based on a regularized linear or generalized regression of an arbitrary omics dataset to chronological ages of samples—including those constructed using deep learning and, generally, neural networks (see Chap. 18). We assume that the reader is familiar with the biology of the process of DNA methy-

lation, its mechanics, dynamics and significance in the regulation of gene expression (Field et al. 2018).

12.1 Building the Clock: The Meaning of Regularization Parameters and Number of CpG Sites Versus the Number of Samples

Canonically (Horvath 2013), once a dataset describing an age-dependent modulation of methylation levels on CpG sites across the genome is obtained, the CpG sites contributing to the potential clock are identified by performing elastic net regression of DNA methylation levels to the chronological age of organisms, from which the samples are collected.

Elastic net regression is a regularized regression of a superposition of stochastic variables X, in our case methylation levels $X \in [0, 1]$ on CpG sites in the genome numerated by the index j with its value getting as high as several million since there are ~30 million CpG sites in the mammalian genome (Field et al. 2018); in practice, $1 < j < n$ reaches values of ~2–4 million for samples obtained by reduced representation bisulfite sequencing (RRBS (Meissner et al. 2005) in what follows) and remains $\lesssim$1 million for samples assayed with Illumina chips. The regression process seeks to minimize the target function

$$\frac{1}{2N}\sum_{i=1}^{N}(Age_i - \beta_0 - \beta_i X_i)^2 + \lambda T_\alpha(\beta), \tag{12.1}$$

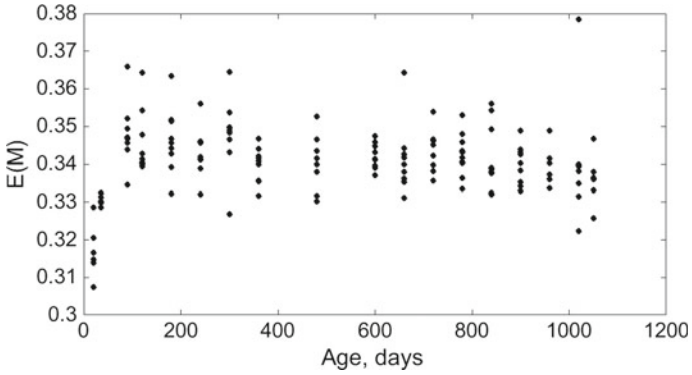

Fig. 12.1 Average methylation across ~2 million CpG sites in the genome of C57Bl/6 male mice, data from (Petkovich et al. 2017). The wave of increased methylation during development (1st month of life) is replaced by monotonous overall hypomethylation during the adulthood

with respect to the values of the intercept β_0 and the weights of individual predictors β; here

$$T_\alpha(\beta) = \alpha||\beta||_{L1} + \frac{1-\alpha}{2}||\beta||^2_{L2} = \sum_{j=1}^{n}\left(\alpha|\beta_j| + \frac{1-\alpha}{2}\beta_j^2\right), \tag{12.2}$$

N is a number of observations/samples with chronological ages Age_i, $||\beta||_{L1}$ and $||\beta||_{L2}$ are the standard L_1- and L_2-norms, respectively.

The purpose of extending the regularization norm (12.2) beyond a simple L_1-norm of the least absolute shrinkage and selection operator (LASSO) regression is to make the target function for the regression strictly convex (Zou and Hastie 2005), since the target function of LASSO regression is not guaranteed to possess this property. For the target function (12.1), (12.2) there exists a single global minimum, and, if the number of predictors n is not very large, there is hope that the global minimum will be reached within a small number of steps of a stochastic gradient descent process. Also, when the number of samples N is smaller than the number of predictors n (which is a typical situation in any omics-related problem and an especially saturated one in the case of the analysis of DNA methylation data characterized by the numbers of samples counted in hundreds and the numbers of observables in millions), the LASSO regression procedure based on L_1-norm typically chooses the number of clock sites $< N$ (number of samples). It is also easy to spot-check that virtually all DNA methylation clocks built using elastic net regression satisfy the very same property (Hannum et al. 2013; Horvath 2013; Petkovich et al. 2017; Wang et al. 2017; Stubbs et al. 2017), and thus elastic net regression inherits this drawback. As methylation levels on many CpG sites are strongly correlated between each other [this is the case for CpG islands and, more generally, for promoters of gene expression (Field et al. 2018)], both LASSO regression and its extension based on elastic net also tend to pick a limited number of CpG sites within the correlated subset and ignore the others.

The meaning of the regularization parameters in the target function (12.1), (12.2) is as follows. First, one expects that increasing the relative weight α of the L_1-norm makes the global minimum of the target function (12.1), (12.2) less pronounced, and lower α are therefore preferable. On the other hand, higher α means that vectors of weights β with larger L_2-norms $\sim |\beta|^2$ are penalized more strongly; this has a potential to decrease the number of CpG sites contributing to the constructed clock and therefore negatively influence its robustness. As for the overall weight λ of the regularizing part of the target function, it should be chosen according to the comparative performance of the clocks built with different values of λ; since the target function (12.1), (12.2) admits a single global minimum with respect to its parameters, we expect to capture its location on the optimization landscape of the problem by studying its cross-sections corresponding to varying λ. In a situation of a single global minimum of the target function one hopes that the drawbacks of LASSO regression discussed above are avoided. However, as we discuss below, this

raises another problem: the target function will possess a single global minimum even in the case of significant randomness built in the analyzed dataset.

12.2 Two Illustrative Examples. Notes on Robustness and Accuracy of Methylation Clocks Out-of-Sample

Generally, CpG sites in a mammalian genome are strongly non-equal biologically [some among them are deeply "buried" within the genome and virtually never accessible, while others are almost always accessible and their methylation levels promote or suppress the expression of genes (Field et al. 2018)], and elastic net regression of DNA methylomes to chronological age would in principle allow to identify important sites with methylation levels systematically changing with age. Let us, however, imagine that we are dealing with datasets characterized by very low signal-to-noise ratio (often datasets obtained by RRBS belong to exactly this category, as methylated and unmethylated read counts on individual CpG sites might fluctuate widely from sample to sample). Let us determine the answer, which a standard procedure might give in that case. Namely, we shall perform the following simple experiment: generate a number N of methylome snapshots covering n CpG sites, such as when the methylation level on each site is a random number uniformly distributed in the interval [0, 1]; each methylome should also be assigned a "chronological age", and we keep $n \gg N$ generating this dataset. To maximize the number of clock sites (robustness) and simultaneously ensure that the target function possesses a single global minimum, we choose $\alpha = 1/2$ providing the same weight to L_1- and L_2-measures.

It is easy to check that subjecting the resulting dataset to the procedure of elastic net regression with 20-fold cross-validation results in a rather "precise clock" based on ~200 CpG sites out of 60,000, see Figs. 12.2 and 12.3. Obviously, performance of this "clock" will be abysmal on a test set (which can be generated in the same fashion as the training set), but what have we really observed here? How was it even possible to construct a seemingly precise clock on a completely random data? The answer is that elastic net regression has very effectively performed noise fitting: when the number of observations is much smaller than the number of predictor variables (CpG sites), one will always find a subset of predictor variables which by a mere chance behave coherently as functions of chronological age, and the regularized regression will necessarily pick this subset to include it in the resulting clock.

While this experiment is illustrative, it does rarely correspond to the situation one faces when building the clock from the real DNA methylation datasets as its precision out-of-sample is negligible. We thus consider now a somewhat more advanced experiment: take the same number of samples N, assign them monotonously increasing chronological ages and assume that the methylation levels on every CpG site among n included in the simulated methylomes grow linearly as the functions of chronological age of samples. In addition, we shall also prescribe a uniformly distributed noise with the same amplitude,

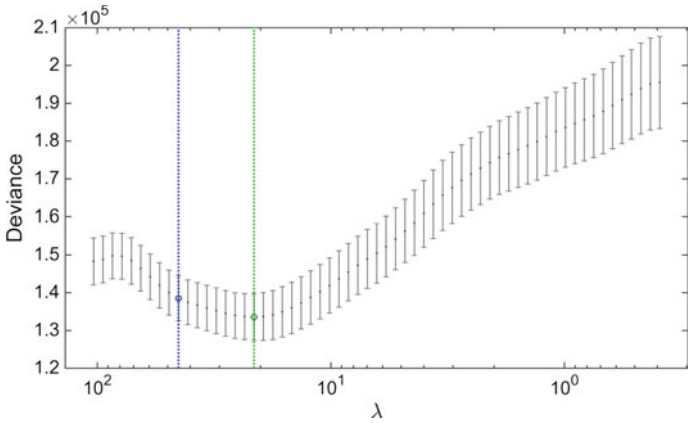

Fig. 12.2 Building a clock on a random dataset. We used 585 samples of random DNA methylomes containing 60,000 sites each. Behavior of the deviance error of the clock as a function of regularization parameter λ. The target function has a global minimum even on a completely random dataset. The green line corresponds to the minimum of the deviance function, and the blue line denotes a clock separated from the minimal deviance clock by 1 standard deviation

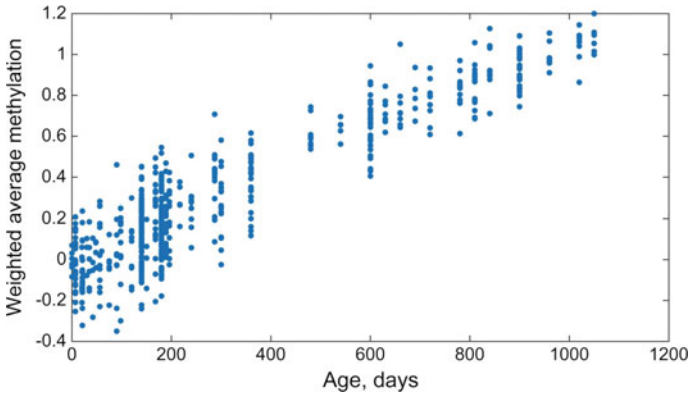

Fig. 12.3 Performance of a clock built on a random dataset. The weighted average methylation is clearly correlated with the "age" of samples. Performance of this clock on a test (random) dataset is negligible

$$X_i(Age) = (c \cdot Age + f(Age, i))/|c \cdot Age + f(Age, i)|,$$

to every CpG site in the methylome generated in the same fashion as considered in the previous numerical experiment and renormalize values of the methylation levels such that they remain defined in the interval [0, 1].

Subjecting the resulting dataset to the procedure of elastic net regression with $\alpha = 1/2$ and 20-fold cross-validation again reveals a rather precise clock with

~19 days on the training set and ~32 days on the test set of a similar size generated in a similar fashion, Figs. 12.4 and 12.5. Two important observations are now in order:

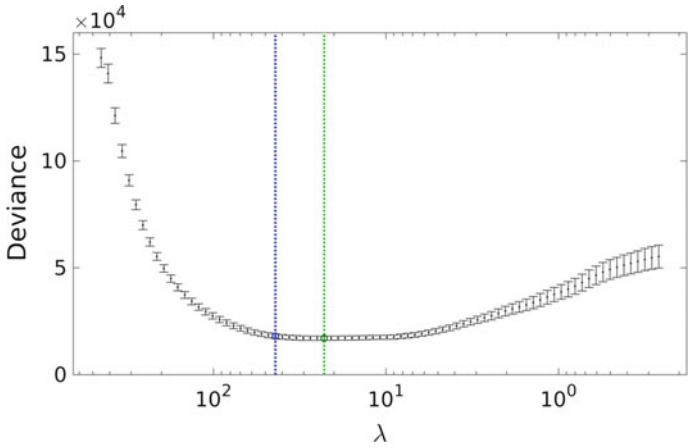

Fig. 12.4 Building a clock in a situation when every CpG site in the methylome is used as a clock site. Elastic net regression chooses 242 sites among 60,000 which produce the best clock. These sites are however chosen by fitting noise on top of the monotonous age-dependent signal present on every site; a particular realization of the random noise imprinted within the training set creates a perception that behavior of methylation levels on some sites with age is more systematic than the others. Again, the target function of the elastic net regression possesses a single global minimum as a function of optimization parameters

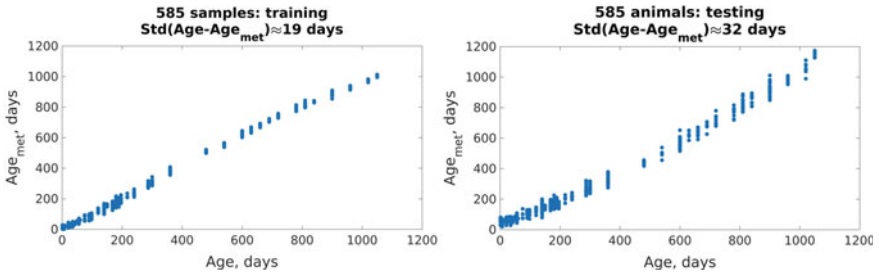

Fig. 12.5 Performance of the clock from Fig. 12.4. Shown is the performance on the training set (the standard deviation of the methylation age from the chronological age is ~19 days) and on the test (the same error is ~32 days). The error on a test sample is low because any CpG site among 60,000 covered can be used as a clock site. Similarly, any other clock built using an arbitrary linear combination of covered CpG sites would produce a similar performance out-of-sample

(1) There is a noticeable deterioration of clock performance on the test set. Obviously, it takes place because by construction any CpG site within simulated methylomes can be considered as a clock site. What the procedure of elastic net regression does in this case is again partly fitting noise because realizations of the random noise on some among n CpG sites will look like a consistent age-dependent signal, and the fitting procedure will pick them up. On the test set, there will be another combination of CpG sites, different from the constructed clock, characterized by the realization of the random noise looking the closest to a consistent age-dependent signal, etc.
(2) The performance reduction on the test set is only marginal since *any subset* of CpG sites included in the simulated methylomes will generate a clock with a comparable performance out-of-sample. Performance will also be relatively similar for clocks with any large (or small) number of the CpG sites contributing to them, but the clocks with larger numbers of clock sites will be characterized by more robust performance out-of-sample.

By construction, we did not consider here CpG sites with biological relevance and a robust age-dependent signal, but the take-away from the two examples presented here is that due to the existence of a global minimum of the target function on any training dataset and in a situation when the number of samples in the training set is much smaller than the number of predictor variables, one will often end up with a clock with a relatively low performance out-of-sample, as the elastic net regression will partially fit noise present in the dataset in this case. Let us now obtain an estimate for the upper bound on the clock performance out-of-sample.

12.3 Estimating an Upper Bound on Out-of-Sample Performance of Methylation Clocks

A general upper bound on the out-of-sample performance of a methylation clock can be obtained by performing a standard robustness analysis of the constructed clock. This analysis generally includes adding a very large number of realizations of a random noise to the training dataset and obtaining estimations of the biological age from the resulting dataset. Subsequently, the upper bound on the performance error out-of-sample is obtained by averaging over realizations of the random noise.

In the corresponding theoretical calculation, one considers a worst-case scenario, when the signal encoded in these counts is well below the level of noise ("maximal entropy" estimation). It is convenient to consider methylated and unmethylated reads count on every clock site as two uncorrelated Gaussian-distributed stochastic variables. Since the methylation level on a CpG site is defined as $M_i = \frac{Reads_{met}}{Reads_{unmet}+Reads_{met}}$, the denominator is also a Gaussian-distributed stochastic quantity with a mean $E(Reads) = E(Reads_{unmet}) + E(Reads_{met})$ and a variance $\sigma^2 = \sigma^2_{unmet} + \sigma^2_{met}$. Performing the Geary-Hinkley transformation (Geary 1930) on the stochastic variable M_i one finds that the stochastic variable

$$t \approx \frac{E(Reads)M_i - E(Reads_{met})}{\sqrt{\sigma^2 M_i^2 + \sigma_{met}^2 - 2\rho\sigma\sigma_{met}M_i}} \tag{12.3}$$

is normally distributed with zero mean and unit variance, and it is possible to obtain an approximate form for the distribution function of the methylation levels M_i. In particular, the expression for the error in the total weighted average methylation can now be bounded from above as

$$\approx \sum_{j=1}^{n} |\beta_j| E\left(\delta M_j^2\right) \lesssim \frac{1}{\sqrt{2}} ||\beta||_{L_1},$$

i.e., by the L_1-norm of the vector of clock weights. The error of the actual perceived methylation age is in turn given by

$$E\left(\delta Age^2\right) \lesssim \sqrt{2} f''\left(E\left(\beta_0 + \sum_{j=1}^{n} \beta_j M_j\right)\right) ||\beta||_{L_1}, \tag{12.4}$$

where the methylation age is related to the weighted average methylation $E(M)$ according to the functional dependence

$$Age = f(E(M)),$$

i.e., the upper bound on the clock performance out of sample is roughly determined by the convexity of the functional dependence $f(x)$ and the L_1-measure of the vector of clock weights. It follows immediately from the expression (12.4) that the clocks determined by a larger number of clock sites n will have a relative performance error lowered by a factor of $1/\sqrt{n}$ (according to the law of large numbers) at early chronological ages. As a very rough, "on the back of the envelope", estimate of the bound on the relative out-of-sample clock performance in this regime one can get as a formula

$$\frac{\sqrt{E\left(\delta Age^2\right)}}{E(Age)} \sim 0.6 \cdot \mathrm{E}\left(\left|Convexity_{\log f}\right|\right) \cdot \frac{1}{\sqrt{n \cdot E(\beta)}}, \tag{12.5}$$

where n is the number of CpG sites contributing to the clock, $E(\beta)$ is an average weight of a clock site in the expression for the weighted average methylation and $\mathrm{E}\left(\left|Convexity_{\log f}\right|\right)$ is a characteristic value of the second derivative of the logarithm of the clock function $f(x)$. We also note that the bound on the quantity $\sqrt{E\left(\delta Age^2\right)}$ grows with age monotonously [this is indeed what one sees most often in the analysis of methylation age on noisy datasets (Petkovich et al. 2017; Wang et al. 2017; Stubbs et al. 2017)].

12.4 Conclusion

Based on the analysis presented above, we would like to conclude that to build a DNA methylation clock using relatively noisy DNA methylation datasets such as those obtained by RRBS it might be beneficial to use a canonical LASSO regression, which corresponds to elastic net regression with $\alpha = 1$. LASSO regression does not excessively penalize clocks with larger numbers of CpG sites contributing to them. As such, it would generate a methylation clock with performance out-of-sample comparable to the clocks based on elastic net regression, and a LASSO-based clock will also have a higher robustness when applied to samples with several clock sites not covered. The drawback of LASSO on the other hand is that its target function is not guaranteed to possess a single global minimum.

The upper bound on the out-of-sample performance of elastic net regression clocks obtained here is uncomfortably high. For example, for the 90-site blood clock developed for mouse methylomes one finds that out of sample the error of age identification can go in probability as high as $\sqrt{E\left(\delta Age^2\right)} \sim 1.5 \cdot E(Age)$. Again, essentially the only parameter left for controlling this bound is the number of clock sites, and a clock including thousands of sites would lead to lowering this bound by an order of magnitude.

Recalling that both elastic net and LASSO regressions typically converge to a clock with a number of sites lower than the number of samples used to train it, we conclude that the increase in the number of sequenced samples will necessarily lead to the emergence of a new generation of DNA methylation clocks with significantly better performance accuracy and robustness.

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Chapter 13
Epigenetics of Brain Aging: Lessons from Chemo Brain and Tumor Brain

Anna Kovalchuk, Bryan Kolb and Olga Kovalchuk

Abstract The world's population is rapidly aging. Aging is a complex and multifaceted process of the loss of viability and increase in vulnerability that affects the entire organism, and encompasses a progressive decline on various levels—molecular, cellular, tissue, and organismal. Indeed, both internal and various external environmental factors—such as life style, diet, exercise, smoking, alcohol consumption, as well as exposures to physical, chemical factors, drugs, toxins, and pathogens can contribute to aging processes in the brain as a part of integrated genome and epigenome response orchestrated via altered expression of genes and pathways. It is also apparent that various environmental exposures which affect the brain may influence pathological processes implicated in aging. Moreover, various co-morbidities can impact the brain—such as age-related cardiovascular and other diseases, and cancer. Of the latter, cancer is of particular interest. Here, we discuss the effects of non-CNS tumor growth and chemotherapy on the brain and the effects of these phenomena on brain aging. We introduce the epigenetic theory of chemo and tumor brain, and the role of epigenetic mechanisms in tumor brain, chemo brain, neurodegeneration and aging.

Keywords Brain · Aging · Epigenetics · Chemo brain · Tumor brain

Abbreviations

4-HNE	4-hydroxynonenal
5hmC	5-hydroxymethylcytosine
5mC	5-methyl-cytosine
BDNF	Brain-derived neurotrophic factor
CA	Cornu Ammonis

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CNS	Central nervous system
CPP	Cyclophosphomide
DG	Dentate gyrus
DNMT	DNA methyltransferase
MBD	Methyl-binding domain
miRNA/miR	MicroRNA
MMC	Mitomycin C
NPAS4	Neuronal PAS domain protein 4
PDX	Patient derived xenograft
piRNA	Piwi-interacting RNA
PFC	Prefrontal cortex
PR + BC	Progesterone receptor positive breast cancer
siRNA	Small interfering RNA
TNBC	Triple negative breast cancer

13.1 Introduction

The world's population is rapidly aging. In 2017 approximately 13% of global population was over 60 years of age, and the current rate of growth of the aging population at about 3% per year. In Canada, one in seven Canadians is over 65 years old, and by 2020, four to five million Canadians will be over 80 years old.

Aging is a complex and multifaceted process of the loss of viability and increase in vulnerability that affects the entire organism, and encompasses a progressive organismal decline on various levels—molecular, cellular, tissue, and organismal. While aging is an integral part of natural life-span, aging-associated decline leads to predisposition to various major diseases such as cancer, diabetes, cardiovascular, autoimmune and auto-inflammatory, and neurological conditions. Whereas increased global longevity is positive, there still is alarming evidence that considerable numbers of people living significantly longer may suffer from age-related diseases including cardiovascular, diabetes, cancer, dementia, Parkinson's and Alzheimer's diseases. The clinical manifestations of abovementioned aging-related pathologies are preceded by long (10–20 years) asymptomatic periods of illness development. The major challenge of aging research is to distinguish the etiology and pathogenetic mechanisms of cellular and tissue aging from the myriad of changes that accompany it, and most importantly, to identify triggers that cause, facilitate, and promote it.

13.2 Brain Aging—Mechanisms and Underlying Factors

While aging impacts all organs and systems of the human body, brain aging is in a sense different. The brain is composed of highly differentiated cells (neurons) and

as post-mitotic cells they have a long lifespan that starts at birth and is comparable to the lifespan of the organism (reviewed in Isaev et al. 2018, 2019). Normal aging is linked to mitochondrial dysfunction, oxidative stress and DNA and cellular damage, reduction of dendritic branching and length, partial synapse loss, deregulation of neurotransmitters, and trophic factors (Isaev et al. 2018, 2019). Although it has been recently shown that there is no massive neuronal loss during the process of normal aging (cell loss is probably around 10%), some neurons acquire significant morphological changes and die, as reported in the CA1 hippocampal region, leading to cognitive deterioration (Isaev et al. 2018, 2019). In addition, loss of myelination and degenerative changes in axons in the white matter of frontal lobes contribute to brain aging and cognitive decline. These processes are compensated in normal aging by other brains regions involved in cognition, but aging processes can activate pathological pathways that underline the development of dementia, Alzheimer's disease, and Parkinson's disease (Isaev et al. 2018, 2019). These complex age-related neurological disorders are believed to be caused by alterations in various molecular pathways that are under environmental influences.

Indeed, both internal and various external environmental factors—such as life style, diet, exercise, smoking, alcohol consumption, as well as exposures to physical, chemical factors, drugs, toxins, and pathogens can contribute to aging processes in the brain as a part of an integrated genome and epigenome response orchestrated via altered expression of genes and pathways (Delgado-Morales et al. 2017). It is also apparent that various environmental exposures that affect the brain may influence pathological processes implicated in aging. Moreover, various co-morbidities can impact the brain—such as age-related cardiovascular and other diseases, and cancer. Of the latter, cancer is of particular interest.

13.3 Effects of Cancer and Cancer Therapy on the Brain

Cancer is an important age-related disease, and incidence of cancer are rapidly rising. By 2020, it is predicted that 1 in 2 Canadians will be affected by the disease. While modern cancer treatments have significantly increased survival rates, the treatments are known to cause profound side effects, which affect survivors. Chemotherapy is one of the main types of cancer treatment. It is based on the application of drugs that eradicate cancer cells (Hassan et al. 2010). In most cases, chemotherapy is systemic, administered intravenously through the bloodstream to cells all over the body, thus affecting not only cancer cells, but also healthy cells. Chemotherapy agents are designed to target rapidly dividing cells, and affect both tumor cells and healthy cells. As a result, they can cause numerous debilitating side effects. Cycling and dividing cells are found in hair follicles, skin and mucous membranes, bone marrow, and the gastrointestinal tract. Consequently, cytotoxic chemotherapy causes skin and mucous membrane lesions and sores, hair loss, dysfunction of the bone marrow, and gastro-intestinal syndromes (Group 2002; Raji 2005; Kayl and Meyers 2006). Currently, many people live with and beyond cancer, bringing the problem of

cancer survivorship to the forefront of healthcare. Cancer survivorship encompasses an array of physical, psychosocial, and economic issues, and focuses on efforts to increase the quality of life of cancer survivors. Whereas traditional side effects of chemotherapy, such as hair loss, nausea, vomiting and other gastro-intestinal manifestations, bleeding, and low white blood cell counts, have been known for some time, concerns about central nervous system (CNS) toxicity-associated side effects were first described in the mid-1970s (Silberfarb 1983), when Weiss and colleagues reported the neurotoxicity of cancer therapies (Weiss et al. 1974a, b).

Later, Silberfarb and colleagues outlined cognitive impairment in post-chemotherapy cancer patients (Silberfarb et al. 1980a; Silberfarb 1983) and proposed "*chemotherapy as a possible source of behavioral change and emotional distress in cancer patients*" (Silberfarb et al. 1980b). These studies, coupled with patient concerns, gave rise to the analysis of the CNS toxicity of chemotherapy regimens. From the patients' perspective, fatigue and cognitive dysfunction constitute a significant quality-of-life issue that persists over time. To draw attention to the cognitive impairment they experienced, cancer survivors coined the term "chemo brain," which is now widely used to describe post-chemotherapy CNS side effects (Downie et al. 2006). Chemo brain has been reported to manifest after chemotherapy in breast, lung, lymphoma, and gastrointestinal cancers, among others (reviewed in Olson et al. 2016; Vardy and Tannock 2007; Ahles 2012). Chemo brain manifestations can be persistent, with approximately 35% of patients reporting CNS side effects for months to years after treatment cessation (Ahles and Saykin 2002). Given all reports and studies outlining its occurrence, chemo brain is now accepted as an adverse side effect of chemotherapy.

13.4 Cancer-Associated Cognitive Change—The Tumor Brain

Recent longitudinal studies suggest that a degree of cognitive impairment may be present in some patients prior to receiving chemotherapy, and that this impairment worsens with chemotherapy (Vardy and Tannock 2007). Furthermore, a thorough pre-treatment assessment of patients revealed that, compared with their age groups, 20–30% of breast cancer patients exhibited decreased cognitive performance prior to treatments, and such manifestations were not linked to depression, anxiety, or fatigue (Ahles et al. 2008; Wefel et al. 2004). Later, Hurria and colleagues proposed the definition of "*cancer and cancer treatment-associated cognitive change*" (Hurria et al. 2007), or, as we recently termed it, 'tumor brain'. Until recently, the underlying molecular mechanisms of tumor brain remained unexplored.

13.5 Mechanism of Chemo Brain and Tumor Brain

An increasing amount of data has shown that chemotherapy imposes toxic effects on the CNS (Kaiser et al. 2014). As discovered in animal studies, chemotherapy exposure triggers oxidative stress and apoptosis, activates microglia, causes neuroinflammation and dysregulates myelination, suppresses neuronal proliferation and differentiation, affects levels of DNA methylation and hydroxymethylation, and alters histone modification and chromatin remodeling, causing aberrant gene expression of neurotrophic and neurogenic proteins in the brain (Christie et al. 2012; Seigers and Fardell 2011; Seigers et al. 2008, 2010a, b, 2013, 2015, 2016; Briones and Woods 2014; Kovalchuk et al. 2016c). These molecular changes account for the alterations in neurogenesis and exert a negative impact on memory and learning (Christie et al. 2012). The frequency and timing of chemo brain and its persistence suggest that the origins of chemo brain may be epigenetic and associated with aberrant global gene expression patterns (Wang et al. 2015).

One of our recent studies confirmed this notion. We analyzed epigenetic and gene expression changes in the hippocampus and prefrontal cortex of mice 24 h and three weeks after treatment with cytotoxic chemotherapy agents mitomycin C (MMC) and cyclophosphomade (CPP), which were previously reported to cause chemo brain. We found that MMC and CPP treatments led to persistent drug-, sex-, and brain region-specific changes in global gene expression profiles. We also observed that these chemotherapy agents caused changes in the global levels of DNA methylation and DNA hydroxymethylation, and led to increased levels of oxidative DNA damage (Kovalchuk et al. 2016c).

Unlike chemo brain, the mechanisms of tumor brain studies are not well known. In human studies, Ahles and Saykin suggested the possible roles of cancer-induced inflammatory response, as well as the potential contribution of genetic factors that underlie breast cancer formation, as potential mechanisms of tumor brain, albeit none of these mechanisms have been experimentally validated (Ahles and Saykin 2007; Ahles 2012). Nevertheless, based on the timing, frequency, and persistence of the cancer-associated cognitive impairment of tumor brain, it may well be associated with epigenetic and gene expression changes.

13.6 Epigenetics

Brain development is genetically predetermined; it is a stepwise and highly coordinated program that is executed via precise gene activity. While each cell of an organism carries the same amount and sequence of DNA, cells and tissues vary greatly in their structure and function. These structural and functional differences result from differential gene expression in various cell types. Gene expression underlies and determines organismal development, growth, functioning, and aging, as well as environmental interactions.

Epigenetic mechanisms set and maintain meiotically and mitotically heritable and stable patterns of gene expression and regulation. These occur without changing the DNA sequence. Epigenetic regulation controls gene expression, chromatin structure, and genome functioning through processes that include DNA methylation, histone modifications, chromatin remodeling, and noncoding RNAs (Sandoval and Esteller 2012; Jaenisch and Bird 2003).

13.6.1 DNA Methylation

Cytosine DNA methylation (or DNA methylation) is the covalent modification of DNA, whereby a methyl group (CH_3–) from S-adenosyl-L-methionine is added to the carbon 5 position of cytosine, yielding 5-methylcytosine (5mC). DNA methylation was the first epigenetic alteration identified. This process is crucial for the proper functioning of normal cells and tissues. In normal cells, DNA methylation governs the regulation of cell-type and tissue-specific gene expression, the silencing of parasitic and highly repetitive sequences, X-chromosome inactivation, the correct organization of active and inactive chromatin, and genomic imprinting (Esteller and Herman 2002; Jaenisch and Bird 2003).

In mammalian somatic cells, including brain cells, DNA methylation takes place mainly in the context of CpG dinucleotides (where a cytosine is followed by a guanine), which occur throughout the genome and are highly methylated to 70–90% (Pogribny and Beland 2012). These CpGs lie outside regulatory regions. In contrast, some CpGs are grouped in clusters called "CpG islands". They are located within the regulatory promoter regions of many genes and are usually unmethylated (Esteller and Herman 2002; Pogribny and Beland 2012). Promoter regions may gain or lose DNA methylation and thus become hyper- or hypo-methylated, leading to decreased or increased gene expression. While CpG methylation is central to the proper functioning of normal cells and tissues, non-CpG methylation is rare in mammals and has only been found in embryonic stem cells.

DNA methylation is carried out by DNA methyltransferase enzymes (DNMT1, DNMT3a, and DNMT3b), which work together to create and maintain methylation patterns. DNMT1 is involved in the maintenance of DNA methylation patterns after DNA replication, while DNMT3a and DNMT3b are de novo methyltransferases that target unmethylated and hemimethylated sites and establish new methylation patterns.

DNA hypermethylation is the gain of methylation at sites that are unmethylated under normal conditions, whereas DNA hypomethylation is the loss of methylation at sites that are methylated under normal conditions. Deregulation of the levels or activity of DNA methyltransferases may lead to altered methylation patterns (Pogribny and Beland 2012).

DNA methylation regulates gene expression. Hypermethylated gene promoters result in an "off" state of gene expression, while those unmethylated or undermethylated are deemed "on" (Jaenisch and Bird 2003). The association of DNA methylation

with the repression of gene expression is mediated by methyl CpG-binding domain (MBD) proteins, which selectively bind to and interact with methylated DNA. They then recruit additional proteins capable of modifying histones, conducting chromatin remodeling, and yielding compact and genetically-inactive heterochromatin. The presence of CH_3– groups may deter transcription factors from interacting with DNA, thus exerting an inhibitory effect on gene expression. Overall, DNA methylation is a known cause of inactive chromatin states and repressed gene expression, while the loss of DNA methylation correlates with elevated gene expression (Kovalchuk and Kovalchuk 2012).

DNMTs and MBDs are expressed and active throughout the developing brain. They support neuronal survival and plasticity, and assist in the regulation of learning and memory (Klose and Bird 2006; Ooi et al. 2007; Mehler 2008). The importance of DNA methylation extends far beyond the development of the nervous system. DNA methylation is implicated in many physiological neural functions, such as synaptic plasticity, the proper function of adult CNS neurons (Feng et al. 2010), and CNS repair mechanisms. In addition, it mediates responses to environmental stressors, such as exposure to ionizing radiation and chemotherapy agents (Kovalchuk et al. 2016a, b, c; Iskandar et al. 2010). For a long time, DNA methylation changes were thought to be permanent modifications that arose as a result of failure to maintain methylation patterns during and after cell division. However, it has become apparent that DNA methylation and DNA demethylation are dynamic processes, especially in the brain. In addition, DNA demethylation governs tissue-specific differentiation (Kovalchuk and Kovalchuk 2012).

Recent studies have found a second type of cytosine modification in mammalian DNA, 5-hydroxymethylcytosine (5-hmC). It was reported that 5-hmC is an oxidative derivative of 5-methylcytosine (5-mC) (Globisch et al. 2010), and a principal component of the DNA demethylation process. The existence of DNA demethylation was doubted until the discovery of DNA hydroxymethylation. The current model of DNA demethylation is as follows: in a set of reactions, TET (Ten-Eleven-Translocation) proteins oxidize 5-mC to 5-hmC, which can then be further modified, yielding unmethylated cytosine and thereby demethylating DNA (Guo et al. 2011; Wu and Zhang 2011).

In the genome, 5-hmC appears primarily within gene regions, including untranslated regions and exons. In contrast, its levels are depleted in introns and intergenic regions (reviewed in Sherwani and Khan 2015). The existence of 5-hmC was documented in various mammalian tissues and cell types, supporting its potential role in the maintenance of DNA methylation and demethylation balance, and in regulation of gene expression (Globisch et al. 2010). Brain tissues exhibit high levels of 5-hmC (Wen and Tang 2014); 5-hmC-mediated epigenetic changes are important for neurodevelopment and play a role in various neurological diseases (Chen et al. 2014; Szulwach et al. 2011). Several studies established the importance of DNA hydroxymethylation in Alzheimer's disease, Huntington's disease, Parkinson's disease, malignant gliomas, autism, and other neurological and psychiatric diseases (Sherwani and Khan 2015). Furthermore, exposure to environmental agents such as ascorbic acid, phenobarbital, diethylstilbestrol, and hydroquinone altered levels of

cellular 5-hmC and TET proteins (reviewed in Dao et al. 2014). DNA methylation levels are affected by radiation exposure (Koturbash et al. 2016). Our recent studies showed that exposure to chemotherapy agent mitomycin C affected the levels of both 5-mC and 5-hmC in the murine brain and led to decreased global DNA methylation and increased DNA hydroxymethylation in the prefrontal cortex tissues of female mice (Kovalchuk et al. 2016c).

13.6.2 Histone Modifications

DNA methylation is closely connected with the other components of chromatin structure, primarily, with histone modifications. The basic structure of chromatin is the nucleosome, where a strand of DNA is wrapped around an octamer of histone proteins (a tetramer of H3-H4 histone proteins with a H2A-H2B dimer situated on either side). Histones are small basic proteins that have a high affinity for DNA based on their positive charges and DNA's negative charge. The linker histone, H1, locks this structure and participates in the formation of higher-order chromatin packaging. In chromatic packaging, the small histone H1 is positioned outside the octamer and helps stabilize the structure. Octamers and DNA further fold to form a solenoid-like structure, which folds more to create radial loops. This happens with the help of non-histone scaffolding proteins. In the end, each chromosome is a small unit. Therefore, chromatin is very flexible; the tightness of the interaction between histones and DNA may change, allowing for the formation of loose, genetically active chromatin (euchromatin) and tightly-packaged, genetically inactive heterochromatin (Kovalchuk and Kovalchuk 2012). The connections between DNA and histone proteins within the nucleosome core and higher levels of chromatin packaging allow them to organize DNA, and therefore control gene expression, genome organization, and genome stability.

Histone proteins are composed of a high proportion of positively charged amino acids, such as lysine and arginine, giving them a positive charge and therefore the ability to interact electrostatically with negatively charged DNA. Histone proteins, especially their lysine and arginine-rich N-terminal tails, are often subject to chemical modifications that affect their interactions with the DNA strand, thus influencing chromatin packaging and gene expression. These alterations include acetylation, deacetylation, methylation, phosphorylation, SUMOylation, and ubiquitination (Jenuwein and Allis 2001; McGowan et al. 2008; Kovalchuk and Kovalchuk 2012).

Histone modification patterns, termed the 'histone code,' are cell and tissue-specific, and any histone modification changes can result in altered chromatin structure and gene activation or silencing (Jenuwein and Allis 2001; Kovalchuk and Kovalchuk 2012). Histone acetylation is linked to increased transcriptional activity, while histone deacetylation is linked with repression of transcription. Dynamic histone modifications and changes in chromatin structure occur not only during brain development, but also in mature neurons (Tsankova et al. 2007; Renthal and Nestler

2008; Kovalchuk and Kovalchuk 2012), whereby they help control expression of important genes.

13.6.3 Small RNAs

Small non-coding RNAs also partake in epigenetic control. Since the studies of Nobel Laureates, Fire and Mello, who first identified RNA-induced gene silencing in animals in 1998, knowledge of non-coding RNA-mediated regulation of gene expression has grown significantly. Around 98% of transcribed genomic DNA gives rise to non-coding RNAs (Yu 2008), including several groups of small RNAs, such as microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA) (Ghildiyal and Zamore 2009). These small RNAs are characterized by their small size (20–32 nucleotides), origins and production, interactions with members of the Argonaute family of proteins, and their roles in controlling gene expression (Ghildiyal and Zamore 2009).

The most studied are miRNAs, small (22–24 nucleotides long) single-stranded RNA molecules that regulate gene expression by altering mRNA stability and translational initiation. miRNAs are potent and universal regulators, and each miRNA can regulate thousands of different mRNA targets (Nelson et al. 2008). Similarly, each gene can be regulated by numerous individual miRNAs (Kovalchuk and Kovalchuk 2012).

Several hundred miRNAs are expressed at specific times during mammalian brain development (Nugent and McCarthy 2015). These miRNAs can lead to the down-regulation of gene expression through their interactions with complementary mRNA sequences, which can result in the degradation of mRNA or interfere with translation (Nugent and McCarthy 2015; Filipowicz et al. 2008). Because a single miRNA can regulate hundreds of mRNAs, miRNAs are critical for controlling the brain's gene networks. Furthermore, miRNAs can alter gene expression through interactions with other epigenetic mechanisms. For example, they can direct DNA methylation, alter post-translational modifications of chromatin, and even regulate translation in neuronal polyribosomes (Nugent and McCarthy 2015). While miRNAs are involved in several cellular processes, their expression can be regulated by other epigenetic changes, such as DNA methylation, histone modifications, and chromatin structure (Saito and Jones 2006). The expression of miRNAs is cell- and tissue-specific, and they are abundant in the brain (Kosik 2006). In the CNS, the expression of many miRNAs is regulated in a spatial and temporal manner in the CNS, suggesting that their regulation may be important in brain development and function (Barbato et al. 2009; Nugent and McCarthy 2015), as well as in the brain's responses to various environmental factors and stressors (Koturbash et al. 2011).

13.7 Tumor Brain and Chemo Brain—An Aging Connection

An in-depth understanding of the mechanisms of cancer and cancer treatment-related cognitive impairments, tumor brain and chemo brain, is essential for the future development of effective prevention and mitigation strategies and for understanding of their effects on the brain functioning and brain aging. In a series of our recent studies we proposed a new epigenetic theory of chemo brain and tumor brain in which the mechanisms behind the neurotoxic side effects of cancer and chemotherapy are epigenetically regulated, include alterations in the global transcriptome and the small RNAome, and are paralleled by changes in DNA methylation and the metabolome (Kovalchuk et al. 2017a, b, 2018a, b; Kovalchuk and Kolb 2017). Our analysis was focused on the hippocampus and the prefrontal cortex (PFC), in view of their roles in memory and learning and executive functions, as well as on previous data suggesting that both of these brain regions are affected in chemo brain (Kovalchuk et al. 2016c) and are important for brain aging (Delgado-Morales et al. 2017).

13.7.1 Hippocampus

The hippocampus is a bilateral limbic structure with a tube-like appearance that is positioned within the anterior medial region of the temporal lobe. It is comprised of two gyri, the Ammon's horn (Cornu Ammonis or "CA") and the dentate gyrus (DG), each of which contains distinctive types of cells. The cells of the Ammon's horn are pyramidal neurons. The Ammon's horn is subdivided into four regions: CA1, CA2, CA3, and CA4. Along with molecular and polymorphic layers, the dentate gyrus contains the most prominent layer of stellate granule cells (Kolb and Whishaw 2015; Dalley and Guillozet-Bongaarts 2008). The DG is extensively connected to the CA, resulting in virtually every pyramidal cell being connected with almost every granule cell. In case of structural hippocampal damage, this arrangement allows the DG to maintain some hippocampal functions (Kolb and Whishaw 2015).

There are massive connections from the hippocampus to other parts of the brain through several neuronal pathways. Of those, the perforant pathway, termed so because it "perforates" the hippocampus, links the hippocampus to the entorhinal cortex, which in turn has connections with most neocortical regions. The arch-fringe (fimbria/fornix) pathway arches along the edge of the hippocampus and links it with the anterior thalamus, prefrontal cortex, hypothalamus, and the basal ganglia. Hence, as suggested by Kolb and Whishaw (2015), the hippocampus may serve as "a way station" between the posterior neocortex on one end, and the frontal cortex, basal ganglia, and brainstem on the other. Within the hippocampus itself, input from the neocortex proceeds to the DG and further projects to the Ammon's horn (Kolb and Whishaw 2015; Dalley and Guillozet-Bongaarts 2008).

The hippocampus is one of the sites of adult neurogenesis, which takes place within the subgranular zone of the DG. This layer contains several types of cells, of which the most prominent are the neural stem cells, as well as the astrocytes, endothelial cells, and blood vessels that come together to create a nurturing environment for neuroblast proliferation, migration, and differentiation (Kolb and Whishaw 2015).

The hippocampus regulates several vital cognitive processes, such as spatial navigation and memory processing. It is also involved in the consolidation and storage of long-term memory, including declarative memory. The hippocampus is extremely vulnerable to stress, especially to the lack of oxygen, whereby even a short period of oxygen deprivation can result in damage (Kolb and Whishaw 2014). Several studies have reported that the hippocampus is a known target of chemo brain (Andres et al. 2014; Apple et al. 2017; Dietrich et al. 2015; Seigers et al. 2008, 2010a, b).

13.7.2 Prefrontal Cortex

The frontal lobe controls behaviour in time and space. The prefrontal cortex (PFC), as originally studied by Rose and Wolsey in 1948, is found in all terrestrial mammals. It is a large area of the frontal lobe, anterior to the motor, premotor, and cingulate cortex. The PFC receives projections from the dorsomedial thalamus and controls key executive functions such as planning, strategizing, emotional behaviours, and behavioural inhibition. It also governs temporal and working memory, and is responsible for abstract thinking and behavioural regulation (Kolb and Whishaw 2015).

In rodents, the PFC is subdivided into the medial PFC and the orbital frontal cortex, each of which has subregions. It receives dopaminergic inputs from the ventral tegmental area and projects to the nucleus accumbens. Most importantly, it projects to the other regions of the limbic system, such as the hippocampus and amygdala. The PFC undergoes prolonged development and is extensively interconnected with other cortical, subcortical, and brainstem sites (Kolb et al. 2012). The human dorsal lateral PFC, which is roughly equivalent to the medial PFC of rodents, is linked to brain regions involved in attention, cognition, and action, whereas the orbital PFC is connected to regions involved with emotion. As such, the PFC directs the control of cognitive actions, guiding the other regions of the brain to direct the flow of activities necessary to accomplish a certain task (Miller and Cohen 2001). Recent studies show that PFC is significantly affected by cancer treatments such as radiation therapy and cytotoxic chemotherapy and is a target of chemo brain (Kovalchuk et al. 2016a, b, c).

Patient tumor-derived xenograft (PDX) models provide excellent tools to model and study organismal effects of cancers, including the CNS side effects. We used PDX models to dissect how the growth of malignant non-CNS tumors causes molecular alterations in the PFC (Kovalchuk et al. 2017a, b). We noted that the growth of malignant non-CNS tumors negatively affected the PFC. Patient-derived xenograft (PDX) mice carrying triple negative breast cancer (TNBC) and progesterone-positive

breast cancer (PR + BC) PDXs exhibited altered gene expression in the PFC. The growth of TNBC and PR + BC tumors caused oxidative stress and aberrant DNA methylation. Chemotherapy treatments did not have any additional synergistic effects on the analyzed processes. The molecular changes observed in this study embody known signs of neurodegeneration and brain aging (Kovalchuk et al. 2017b).

We further conducted an in-depth analysis of microRNAome changes in the PFC tissues of chemotherapy-treated and untreated mouse TumorGraft™ models with triple negative and progesterone receptor positive breast cancer (Kovalchuk et al. 2017a). We showed that while miRNAome changes related to tumor growth were observed in the PFC tissues of both breast cancer groups, they were more pronounced in PR + BC animals than in TNBC ones. Both tumor growth and chemotherapy treatments led to the upregulation of the miR-200 family and the miR-183/96/182 cluster, as compared to controls. Deregulated miRNA changes were seen in parallel with decreased levels of the miRNA target, namely, the brain-derived neurotrophic factor (BDNF), which is an important regulator of neural development, survival, growth, differentiation, and plasticity, and furthermore, the miRNA changes were similar to those seen in neurodegenerative diseases and aging (Kovalchuk et al. 2017a).

We also dissected tumor brain's and chemo brain's manifestations in the hippocampal tissues of TumorGraft mice (Kovalchuk et al. 2018a). We reported that the growth of malignant non-CNS tumors has an impact on molecular processes in the murine hippocampus. We found that the growth of TNBC and PR + BC tumors significantly altered gene expression in the murine hippocampus. TNBC tumor growth caused oxidative stress that manifested in significantly elevated levels of 4-HNE. Tumor growth negatively affected the levels of neuronal transcription regulator NPAS4 and its target genes, among them BDNF, a member of the neurotrophin family of growth factors. Tumor growth was associated with a significant downregulation of PCNA, AKT 1, and ERK1/2, proteins that are important for controlling neuronal proliferation and survival. Again, all of the observed molecular changes strongly resembled those associated with neurodegenerative diseases and brain aging.

In addition to epigenome, we analysed the effects of malignant non-CNS tumors on the brain metabolome. We showed that the growth of pancreatic cancer, sarcoma, and lung cancer xenografts significantly affected the metabolic pathways in the brain. Tumor brain manifested as aberrant activity of protein synthesis pathways, sphingolipid pathways, and several other pathways that were previously shown to be affected in aging and neurodegenerative diseases (Kovalchuk et al. 2018b).

Our recent data provide solid evidence showing that the growth of malignant breast, lung, and pancreatic cancer, as well as sarcoma, negatively impacts the brain, even though the brain is not the site of the tumor cells. While some common patterns were observed between different tumor groups, several changes were persistent and specific to the type of tumor and affected brain region. All the observed molecular alterations strongly resembled those seen in neurodegenerative disorders and brain aging—such as DNA damage, oxidative stress, altered DNA methylation, deregulated global gene and miRNA expression, decreased levels of NPAS4 and

BDNF—key proteins involved in brain function and repair and brain aging. All of the aforementioned facts strongly indicate that growth of malignant non-CNS tumors and chemotherapy may in turn underlie and facilitate brain aging and neurodegeneration, bringing a new connection between two age-related conditions to the forefront of biomedical research (Kovalchuk and Kolb 2017).

13.8 Conclusions

In the future, Chemo brain and tumor brain must be analyzed in detail in the context of aging. Whereas cognitive impairments are important for adult cancer survivors, they are more critical for pediatric cancer patients and young adults. In Canada, 1500 new pediatric cancer cases are diagnosed yearly, and approximately 10,000 Canadian children today live with cancer. Leukemia is the predominant type of malignancy among pediatric cancers, constituting close to 1/3 of all cases (http://childhoodcancer.ca/education/facts_figures). With the development of new therapeutic regiments, 80–85% of pediatric leukemia patients survive. Nevertheless, many young cancer survivors suffer severe cognitive dysfunction (Follin et al. 2016; Hearps et al. 2016; Kunin-Batson et al. 2014). Because a developing brain is more sensitive to stress and to various toxic exposures, tumor brain manifestation may thus be more pronounced in young individuals. On the other hand, a developing brain is highly plastic and may harbor higher repair capacities. Cognitive impairment is a growing concern in cancer survivors older than 65 years of age (Mandelblatt et al. 2014). Hence, an in-depth analysis of tumor brain age-dependency will shed important light on the age-specificity of these phenomena. Overall, the majority of molecular changes in the PFC and hippocampus of tumor-bearing animals—such as altered gene expression and miRNA expression, decreased levels of NPAS4 and BDNF, deregulated levels of DNA methylation and hydroxymethylation, oxidative stress and oxidative DNA damage, as well as altered metabolome—were previously associated with aging and neurodegeneration (Fig. 13.1).

Preclinical animal model-based data can serve as a foundation for clinical research and for the development of cancer- and cancer therapy-related cognitive impairment and neurodegeneration biomarkers and lay a foundation for future translational and research approaches and for the development of novel research-based strategies and interventions that can help prevent and mitigate tumor brain and chemo brain, and may be further extended to mitigation of neurodegeneration and brain aging.

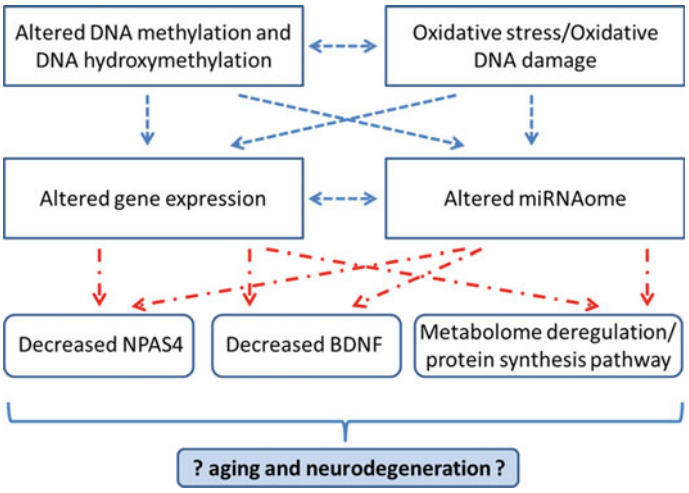

Fig. 13.1 Molecular changes in tumor brain and aging: schematic representation

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Chapter 14
Approaches and Methods for Variant Analysis in the Genome of a Single Cell

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Abstract Every cell in the human body has genomic variants that are either inherited or acquired during lifetime as a result of development, environmental exposure, and aging. Contrary to the former ones, the latter are present in a fraction of cells, which could be as small as a single cell, and are called mosaic variants. Therefore, studying single cells is the ultimate way of analyzing these variants. Analysis of a single cell genome is challenging due to low DNA amounts, and several strategies exist to amplify the DNA. The amplifications introduce errors and biases in the resulting material, which hinder the discovery of true variants. Furthermore, proper analytical considerations are important for both resolving introduced errors and comprehensive variant discovery. Thus, confident variant detection depends on combinations of the following four factors: (1) frequency and type of a mosaic variant; (2) strategy utilized for the discovery; (3) applied experimental and analytical method; and (4) funds and effort that can be invested into each experiment. As of now, none of the existing strategies and techniques are universally applicable to variants of all types, nor are they universally cost effective. Here, we will discuss strategies, experimental techniques, and analytical methods for discovery of a spectrum of mosaic variants from single cell analyses and approaches for validation of discovered variants.

Keywords Aging · Single cell genome · Genome variation · Mosaic variant · Somatic variant · Structural Variation (SV) · Whole-Genome Amplification (WGA) · Copy Number Variation (CNV) · Single Nucleotide Variation (SNV) · Mobile Element Insertion (MEI) · Indel · Lineage tracing

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14.1 Spectrum of Somatic Variants and Their Relation to Aging

The term genome variation (or simply 'variation' in the following) refers to a difference in the sequence of DNA between species, individuals of the same species (i.e., in a population), or cells of the same individual. There are different types of genome variations, spanning from simple Single Nucleotide Variation (SNV)—a difference in a nucleotide at a certain base pair—to complex Structural Variation (SV) involving multiple base pair rearrangements, deletions, duplications, inversions, and sequence differences (Box 1). Referring to variations between species typically implies differences between representative genomes, called reference genomes, of the discussed species, as species comprise multiple individuals, each one with a different genome (O'Leary et al. 2016). Referring to variations in a population, such as humans, typically implies differences between inherited genomes, or germline genomes, which are present in all cells of an individual (1000 Genomes Project Consortium et al. 2015). Genomic differences between the cells of an individual, most or all of which do not have identical genomes, are referred to as somatic variations. Therefore, only when referring to variations between cells it literally means differences between cells' genomes.

Box 1. Types of Genomic Variations

SNV—Single Nucleotide Variant, a difference in a nucleotide at a defined position, e.g., C to T.

SNP—Singe Nucleotide Polymorphism, an SNV frequently found in a population. The term is often used to denote inherited, germline variants and this is how we will use it here.

Indel—Short insertion (in-) or deletion (-del) in the genome. There is no common consensus about maximum indel size. An indel is commonly defined as an insertion or deletion smaller than 50 bp or 100 bp.

MEI—Mobile Element Insertion, an insertion of retrotransposon elements (endogenous retroviruses) into the genome. In humans four elements are present: ALU, LINE1, SVA, and HERV, but the latter one is believed to be inactive (Solyom and Kazazian 2012).

CNA—Copy Number Alteration, a region with fewer (deletion) or more (duplication) copies of DNA. CNAs typically refer to somatic alterations in the genome larger than indels.

CNV—Copy Number Variations, a CNA frequently found in a population. The term is often used to denote inherited, germline variants and this is how we will use it here.

LOH—Loss of Heterozygosity, a genomic region where all SNPs are homozygous either as a result of loss of one haplotype or replacement of one haplotype with the copy of another one. The term is used to describe acquired mutations.

Regions of LOH are typically large, as, in order to be detected, they must contain at least a few heterozygous SNPs. Deletion of one haplotype is also a CNA.
Inversion—Replacement of a sequence with its reverse complement.
Translocation—Rearrangement leading to covalent connection of DNA from two different chromosomes.
Chromosomal Aneuploidy—Change in the number of copies for a chromosome or arm(s) of a chromosome. Chromosomal aneuploidies are typically differentiated from CNAs for historical reasons and to stress likely different mutational mechanisms for the two types of variations.
Multiploidy—Global change in the genome ploidy (number of chromosomes).
SV—Structural Variant, a general term to denote large differences the in genome including CNAs, MEIs, inversions, translocations, chromosomal aneuploidies, multiploidies, and complex rearrangements that bare signatures of multiple aforementioned types. Highly complex and clustered SVs that sometimes involve multiple chromosomes are called chromotripsis.

The genomes of individual cells are not static and change (i.e., mutate) over time. Every time a cell divides, its two daughter cells have shorter telomeres and are likely to acquire mutations from replication errors that they will then pass on to their corresponding daughter cells at the next divisions (Kunkel 2004; Aubert and Lansdorp 2008). Additionally, unrepaired or incorrectly repaired DNA damage, either spontaneous, such as deamination caused by interaction with reactive oxygen species (Bacolla et al. 2014), or environmentally driven, such as UV damage (Ikehata and Ono 2011), also lead to mutations (Saini et al. 2016). It is therefore very likely that there are no two single cells with identical genomes in the human body. Moreover, as time passes and cells age, variations in each cell accumulate (Ramsey et al. 1995; Forsberg et al. 2012; Jacobs et al. 2012; Laurie et al. 2012; Blokzijl et al. 2016; Lodato et al. 2018).

Limited insight has been gained into the process of age-dependent variant accumulation and only for some variant types. The fundamental challenge is that in the context of aging, unlike for example in the context of organismal development, individual somatic genome variants are quite likely to exist only in a single cell in a given organism, making discovery technologically challenging, and experimental validation impossible in principle. The fundamental technological limitation lies is our very incomplete ability to comprehensively characterize the entire genome of a single cell. Some experimental techniques, such as FISH, provide only crude resolution, while others require single cell genome amplification, which, as discussed below, is a technical challenge. However, substantial knowledge about genome variation with age has been obtained from indirect studies, such as analyses of cancer genomes and studies on the occurrence of de novo variants in families.

It is now well established that typically there are 50–100 de novo SNVs present in each newborn human (Conrad et al. 2010; Michaelson et al. 2012; Rahbari et al. 2016;

Yuen et al. 2017; Maretty et al. 2017). Based on this knowledge, it was estimated that cells making up the germline acquired roughly 1.2 SNVs per cell division during development (Rahbari et al. 2016) and about 0.1–0.2 SNVs per cell division post-puberty (Rahbari et al. 2016; Milholland et al. 2017). Currently, there is not much data that would allow comparing post-zygotic per division mutation rates across tissues and cell types, albeit evidence exist that the rates are within the same order of magnitude (Bae et al. 2018). Moreover, it is not clear whether mutation rates per cell division during development are the same as during aging. Additionally, aging cells can also accumulate variants without cell division from endogenous and environment-specific mutation mechanisms.

An insight into post-zygotic mutation mechanisms has arisen from cancer, as almost all cancers are expanded from a single cell, i.e., have clonal origin. As such, the genome of the founder cell is in theory replicated in every cell of a cancer; in other words, the genome is amplified. In practice, cancer samples are often not perfectly clonal because of admixture of normal and immune cells. Furthermore, variant frequency in cancer samples cannot be used as an indication of time of origin because secondary mutations that occur in cancer cells can rise to high frequency due to subclonal expansion. However, multiple lines of evidence indicate that most variants in cancer originate prior to malignant transformation and can thus inform about mutagenesis in normal cells and its relationship to aging (Tomasetti et al. 2013; Milholland et al. 2015). Further indication that cancer mutations can inform about normal mutagenesis is provided by the analysis of the genome of over 7000 cancers of different types by decomposing the spectrum of SNVs into individual components, the so-called mutation signatures (Alexandrov et al. 2013; Lawrence et al. 2013). Most of these signatures are thought to represent individual mutational processes, and several of the signatures indeed match commonly occurring processes including errors in repairing methylated deaminated cytosine (signature #1), damage by UV light, DNA editing by the APOBEC enzyme, and compromised DNA repair mechanisms (Alexandrov et al. 2013; Lawrence et al. 2013). Contribution of the signatures varied with age, but only two signatures, #1 and #5, showed a monotonic increase with age in most cancer types, suggesting that they are the hallmarks of natural cell aging (Alexandrov et al. 2015). By considering those signatures as an aging-related intrinsic background supposedly without environmental contribution, the accumulation rate per cell was estimated to be from a few to a few dozen SNVs per genome per year for different tissues (Alexandrov et al. 2015). The same considerations but without spectrum decomposition into signatures estimated a somatic mutation rate as, roughly, 100 SNVs per cell per year in over 20 tissues (Milholland et al. 2015; Podolskiy et al. 2016).

The order of magnitude of these estimates roughly matches the estimates made from direct analyses of accumulation of mutations in small intestine, colon, liver, and skin fibroblasts (Blokzijl et al. 2016; Saini et al. 2016; Abyzov et al. 2017). Specifically, it was estimated that there are roughly one thousand SNVs per cell already present at birth in humans, and that new SNVs accumulate at a rate of 36 per year in somatic stem cells. Almost the same rate of mutation accumulation was ascertained by a single-cell study in neurons across the human life span (Lodato et al.

2018). At the same time, it was revealed that due to environmental influence, namely UV damage, mutational load can be different up to an order of magnitude (Saini et al. 2016). In contrast to the situation in somatic tissues, regression on parental age estimated that additional de novo variations in germ cells accumulate at a rate of 2–3 SNVs per year in fathers and 0.5–1 SNVs in mothers (Michaelson et al. 2012; Rahbari et al. 2016; Maretty et al. 2017). Other evidence based on comparison of somatic mutation rates in fibroblasts and germ cells also suggests that the germline has two orders of magnitude lower mutation rate than somatic tissues (Milholland et al. 2017).

For variant types other than SNVs, deciphering a possible association with aging from the analysis of cancer genomes is rendered particularly challenging by their overall less frequent occurrence as well as the difficulty in deriving proper descriptors for the mutational spectrum. Still, indirect insights were gained by a GWAS array analysis of DNA from blood from over 50,000 subjects (Jacobs et al. 2012; Laurie et al. 2012). Those studies revealed that CNAs larger than 2 Mbp are found in about 2% of cancer-free elderly people (older than 70 years) but are substantially less frequent (<0.5%) in individuals younger than 50 years. Additionally, somatic CNAs were observed in at least seven other human tissues (O'Huallachain et al. 2012). While these studies demonstrated that clonal mosaicism in blood increases with age and is present in other tissues, their analysis was limited to CNAs that are present in a large fraction of cells (>10%). Therefore, these studies could only inform about the most frequent and expanded cell clones, and were not sufficiently powered to reveal how many cells have different CNAs and how their frequency and spectrum change with age. Analysis of single skin fibroblast cells and neuronal nuclei estimated that about 30% of the cells carry CNAs at a variable frequency (<5) per cell, but were not powered enough to see a difference between children and adults (Abyzov et al. 2012; McConnell et al. 2013; Cai et al. 2014). Genome aneuploidies were observed to increase with age in liver hepatocytes and oocytes (Jones, 2008; Duncan et al. 2012). Additionally, it is known that aneuploidies as wells as somatic insertions of mobile elements such as L1 are present in brain cells, however, their frequency is debated and their association with age is unknown (Yurov et al. 2007; Baillie et al. 2011; Evrony et al. 2012, 2015; Erwin et al. 2016).

14.2 Strategies for Mosaic Variant Detection

Two major strategies for mosaic variants discovery are single cell and bulk tissue analyses (Fig. 14.1). In single cell analysis, the genome of only one individual cell is assayed in each experiment, and multiple individual cells can be analyzed simultaneously for the sake of statistically significant findings. The fundamental advantage here is that variants present in a given cell can be discovered, regardless of their frequency in the tissue. However, this also poses a fundamental challenge—discovered variants could be extremely infrequent in the primary tissue for validating them in the tissue or detecting them in multiple cells (Abyzov et al. 2012; Lodato et al. 2015; Blokzijl

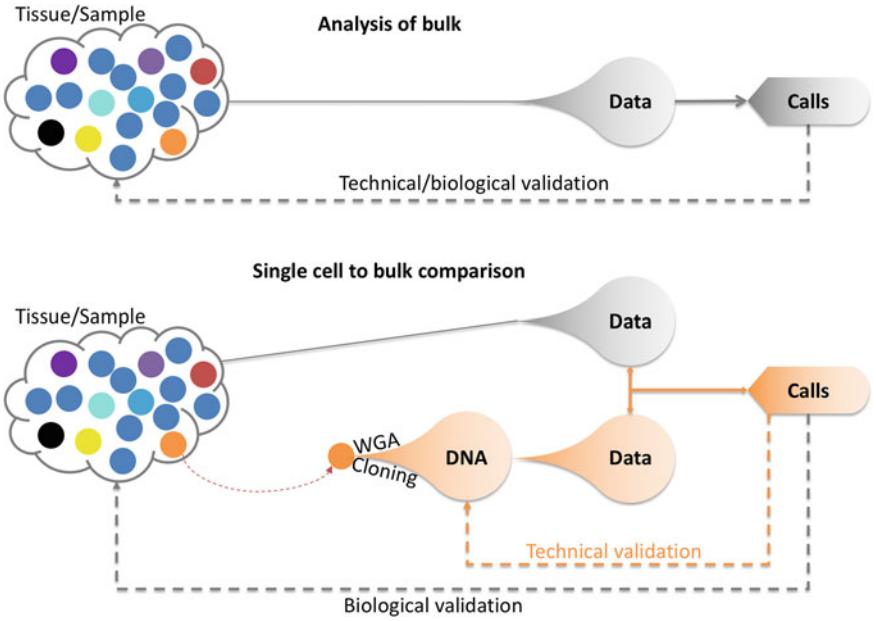

Fig. 14.1 Conceptual strategies for mosaic variants discovery. A sample can be analyzed in bulk (top), but in such strategy rare variants are unlikely to be found. At a standard 30× sequencing depth of coverage, variants below 20% variant allele frequency (VAF) are not detectable. However, technical validation is equivalent to biological validation. In the second strategy, individual cells are analyzed (bottom). Here amplification of a cell's DNA either by WGA or cloning is required. Because of this, technical validation of a call on amplified DNA is not the same as biological validation in the original sample. However, biological validation in the original sample is challenging for rare variants

et al. 2016; Abyzov et al. 2017). Therefore, one has to rely on robust experimental techniques and variant calling analytics to achieve confident variant detection. However, in order to apply certain experimental techniques, such as DNA sequencing, the genomes of single cells need to be amplified, either by whole genome amplification (WGA) or cell cloning. Because such amplifications can introduce DNA alterations, the discovery of variants in manipulated DNA does not guarantee their presence in the original tissue, and experimental validation of variant calls is required. Since it is essential that such validation is not confounded by artifacts of DNA preparation, we differentiate between biological validation, by which we mean validation of variants in cells from the original tissue sample, and technical validation, by which we mean validation of variants presence in manipulated DNA, e.g., DNA amplified with WGA (Fig. 14.1).

Another advantage of single cell analysis is that it enables an inherent control for variant calling. Except for haploid sex chromosomes in males and some regions of germline CNVs, there are two haplotypes in a cell for each allele, while mosaic variants are present on only one haplotype. This provides a mean of distinguishing

false positive calls (such as experimental artifacts) from true mosaic variants by variant allele frequency (VAF). For instance, when sequencing the genome of a cell, true mosaic SNVs should be present at roughly ~50% VAF, with strong or systematic deviation from this value being indicative of a given call or call set not representing true mosaic variants in the original cellular genome. Specifically, VAFs of false calls (because of amplification artifacts or sequencing errors) should be lower than 50%. Furthermore, the challenge of finding mosaic variants becomes equivalent to finding heterozygous germline variants already present in the cell. Consequently, germline variants of the sample where single cells were derived from can be used to estimate and optimize the sensitivity of the analytical methodology to call mosaic variants in each cell. However, special care needs to be taken to distinguish mosaic variants from germline heterozygous variants. Consequently, single cell analyses must compare the genome of a single cell to the genome of some reference tissue or other cells from the same individual. Lastly, single cell analysis allows determining the presence of multiple variants in the same cell, and their sharing across cells (Table 14.1), and this can inform about the order in which mosaic variants occurred over time, allowing for cell lineage tracing.

When bulk tissue analysis is carried out, the genomes of many cells are assayed together. Its main advantage is that it avoids challenges that are inherent to single cells analysis. However, it has other challenges and disadvantages, the most fundamental of which is that it is limited in detecting mosaic variants that are present in numbers of cells considerably larger than one. Further discussion of this approach would go beyond the scope of this chapter.

Table 14.1 Comparative characteristics of bulk and single cell strategies for mosaic variants discovery

	Bulk analysis	Single cell analyses
Discovery of variants with low frequency in a tissue	Not possible	Possible
Data driven calibration of analytics based on germline variants	Not possible	Possible
Sample preparation and handling	Straightforward, relatively affordable	More complex, relatively expensive
Sequencing requirement	Deeper coverage, more expansive	Shallower coverage, less expensive
Technical validation	Possible for all variants	Possible for all variants
Biological validation	Possible for all variants	Possible for a subset of variants frequent in primary tissue
Resolving variant co-occurrence in a cell	Not possible	Possible
Cell lineage tracing	Not possible	Possible

14.3 Single Cell Analyses

14.3.1 Direct Observation of Mosaic Variants in Single Cells

It is possible to directly observe chromosomal aneuploidies under the microscope but only in dividing cells (O'Connor 2008). Therefore, such cytogenetic analysis is unlikely to provide an insight into the genomes of senescent cells and those that are terminally differentiated, such as neurons in the brain. It is also possible to see whole chromosome aneuploidies and large CNAs in single cells with Fluorescent In Situ Hybridization (FISH) (Langer-Safer et al. 1982), spectral karyotyping (Schröck et al. 1996), and their derivatives. Aneuploidies, particularly genome duplications, can be detected and quantified with flow cytometry (Dean and Jett 1974). These classical low-resolution techniques are often used as options for validation in conjunction with newer and/or higher resolution techniques.

14.3.2 Single Cell Whole-Genome Amplification (WGA)

Apart from microscopic examination, the amount of DNA extracted from a single cell is too small to suit the application of other experimental techniques. Therefore, the whole genome of a cell has to be amplified, and this amplification is the key step in the entire experiment, since the quality of the amplified DNA is a major determinant of the ability to detect mosaic variants in single cells. In vitro WGA is conducted using one of several enzymatic protocols that apply DNA polymerases and recently also RNA polymerases. However, errors and biases are inherent to all of these protocols, although typically different for different protocols and enzymes. Two characteristics determine the quality of amplification: uniformity of amplification across the genome and error rate in the amplified DNA. The uniformity is evaluated by variation in the coverage (i.e., variance in read-depth distribution for bins of a particular size across the genome) (Chen et al. 2017), median absolute pairwise difference (MAPD) measure (Cai et al. 2014), rate of allelic drop-outs (i.e., regions of the genome where none or only one out of two possible haplotypes is being amplified) (Evrony et al. 2012), and variance in the distribution of VAFs for heterozygous SNPs (Fig. 14.2). Metrics to measure the sequencing error rate are still in development, with the rate of chimeric sequences being one of the simplest yet informative measures (Picher et al. 2016).

The earliest WGA method is degenerate oligonucleotide-*primed* PCR (DOP-PCR) (Telenius et al. 1992). Even now, it results in one of the most uniform coverage (at larger than a few dozen kbp scale) across the genome and is best suited to discovering mosaic CNAs and other larger chromosomal aberrations (Navin et al. 2011; McConnell et al. 2013; Cai et al. 2014; Knouse et al. 2014; Gawad et al. 2016). Multiple displacement amplification (MDA), on the other hand, is an isothermal WGA technique which has the advantage of producing very long DNA fragments (up to

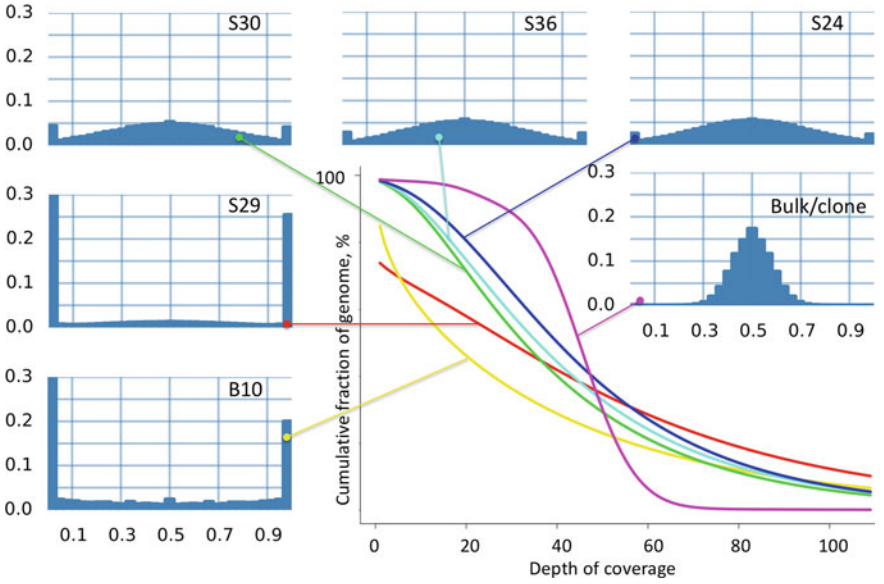

Fig. 14.2 Evenness of coverage for WGS of MDA-amplified cells suffers from MDA. The most even coverage is for cells S24, S30, and S36, still it is not as even as for bulk. For single cells, cumulative coverage plots across the genome (right bottom plot) do not have sigmoidal shape as for sequencing bulk, indicating non-even amplification. But for the mentioned cells the coverage profile is the closest to sigmoidal. For the other two cells S29 and B10 cumulative coverage starts from less than 100% indicating that there is a fraction of genome with no coverage. Measured variant allele frequency (VAF) distribution of heterozygous SNPs consistently pinpoints the same cells with most even amplification, i.e., with Gaussian (bell-shaped) distribution centered around 50% (0.5 mark on X-axis). Even for these cells, the distribution is much wider than that for bulk/clone, reflecting amplification bias of one allele over the other. Additionally, the distribution reveals regions with allelic drop-out; those contribute to bars at 0 and 1. Overall, drop-outs are below 10% of genome for each of the 3 cells with most even amplification. All samples were sequenced to about 40× depth of coverage with 100 bp reads

dozens of kbp long) and a much lower rate of error in the amplified DNA owing to high processivity and fidelity of the *ϕ29* polymerase (Dean et al. 2002). Given these properties and because of suffering from non-uniform coverage and allelic drop-outs, MDA is better suited for the discovery of SNVs, indels, and MEIs rather than for CNVs (Evrony et al. 2012; Lodato et al. 2015, 2018). However, there is currently no universal agreement in the field about the utility of WGA methods for the discovery of somatic mosaicism (Behjati et al. 2014; Blokzijl et al. 2016; Abyzov et al. 2017; Chen et al. 2017; Dong et al. 2017; Bae et al. 2018).

MDA is generally viewed as the most comprehensive genome amplification method since theoretically it can enable ascertainment of all variant types in a cell. The essential challenges it faces are the exponential nature of amplification, leading to errors and small differences in the first steps of amplification being exaggerated in the resulting DNA, as well as the inability to distinguish unrepaired DNA damage

from true variants. Thus, ongoing method developments are mostly based on MDA and attempt to mitigate its weaknesses.

Multiple annealing and looping-based amplification cycles (MALBAC) conducts a quasilinear amplification (Zong et al. 2012). From the combination of special primers and optimized temperatures for DNA amplification, melting, and looping the method is able to conduct up to 5 cycles of pre-amplification from the original cell's DNA, and only then enters the exponential amplification phase. Addition of the single-stranded DNA binding protein from Thermus thermophilus HB8 was reported to enhance the efficiency of amplification by MDA (Inoue et al. 2006). Similarly, the TruePrime method utilizes a DNA primase from Thermus thermophilus instead of random primers (Picher et al. 2016). This was claimed to improve MDA by resulting in a more uniform coverage with lesser allelic drop-outs and better SNV detection (Picher et al. 2016). Other strategies to improve amplification uniformity by MDA include addition of proper concentration of trehalose (Pan et al. 2008), and limiting reaction volume (Hutchison et al. 2005; Marcy et al. 2007; Gole et al. 2013; Fu et al. 2015). Recently, Linear Amplification via Transposon Insertion (LIANTI) conducts full linear amplification into RNA using T7 RNA polymerase with the amplified RNA being reverse transcribed into DNA at the last step of the protocol (Chen et al. 2017). The method also addressed the predominant amplification error of C-to-T that often arises from the artifact of cytosine deamination upon cell lysis. Deamination of C to U, which manifests itself in C-to-T during sequencing, is a natural process that occurs at low rate randomly in the genome (Shen et al. 1994). Treating DNA from lysed cells with uracil–DNA glycosylase (which eliminates uracil deaminated bases) was shown to improve SNV calling (Chen et al. 2017). Addressing the same issue of C-to-T errors, another method optimized lysis buffer prior to conducting MDA (Dong et al. 2017). Using this method, the spectrum of detected SNVs in WGA-amplified fibroblasts was shown to closely resemble the one obtained from clonal expansion experiments (Dong et al. 2017), potentially enabling reliable detection of SNVs and other variants in DNA amplified from single cells.

However, we caution the reader that many of the recently developed approaches for amplification of DNA from single cells have not yet been independently verified. Moreover, it is not clear whether the performances of individual methods may be different for different cell types, nuclei, and for different prior conditions, such as frozen versus freshly harvested cells or nuclei.

14.3.3 Clonal Expansion

Despite active development of methods and resulting improvements, single cell WGA is still far less reliable than *in vivo* genome duplication in dividing cells. Cells use a much more sophisticated molecular machinery, not recapitulated in WGA, to precisely duplicate their DNA and eliminate most replication errors by thorough proofreading and correction. The strategy of single cell cloning (Fig. 14.1) leverages the high fidelity DNA duplication in dividing cells to amplify the genome of the founder

cell of the clone and study its mosaic variants (Abyzov et al. 2012; Behjati et al. 2014; Blokzijl et al. 2016; Saini et al. 2016; Abyzov et al. 2017). Clones, if cultured for sufficiently long time, allow for extracting the amount of DNA necessary to apply an experimental technique of choice, e.g., whole genome sequencing (WGS), without WGA. Such an advantage is, at the same time, a fundamental limitation of clonal expansion as it can only be applied to culturable cells, e.g., stem cells, while terminally differentiated cells, e.g., neurons, are inaccessible to this strategy. Furthermore, the ability of a cell to proliferate may not only be determined by its differentiation state but also by its mosaic mutations. Consequently, deleterious mutations leading to cell senescence or proliferation arrest will not be discovered, and this represents the second fundamental bias inherent in the cloning strategy.

Additionally, this strategy has other less obvious challenges. It is essential to ensure clonality of cell colonies. Often clonality is deemed very likely (Abyzov et al. 2012) or verified post hoc (Blokzijl et al. 2016; Bae et al. 2018). When analyzing the data, variants generated during culturing have to be filtered out. If cells in a clonal colony divide at roughly the same pace and all of them survive, variants arising in such cells will have a small VAF in the colony. Specifically, each newly created variant during the first division of the founder cell will be on only one haplotype in only one daughter cell. As total number of haplotypes for two daughter cells and for diploid chromosomes is typically four, variant generated during the first division will have a VAF in the colony of 25%—significantly lower than the VAF of 50% for mutations present in the founder cells and heterozygous germline variants. For haploid chromosomes, such variants are expected to have a VAF of 50%, which is still half of that for mosaic variants in the founder cell and germline variants. Variants arising in later cell divisions will be present at even lower VAFs. Consequently, segregation of variants in a colony by VAF is an efficient way of sifting out culturing artifacts. Completely eliminating the artifacts may still not be possible, as individual cells may divide at a different pace, may be negatively or positively selected, enter senescence, or die; all of which may increase VAF for some variants in culture, making them indistinguishable by VAF from mosaic variants in the founder cell. The earlier the variants arise during clonal expansion, the more likely they are to be confused with the mosaic variants. Thus, it is desirable to monitor early stages of clonal expansions to ensure that there was no cell death and disparity in proliferation.

14.3.4 Other Strategies

A few alternative strategies have been described as options that would diminish the disadvantages of single cell amplification and of clonal expansion. Perhaps the most notable is an adaptation of clonal expansion for somatic cells, named Somatic Cell Nuclear Transfer (SCNT) (Hazen et al. 2016). In this strategy, the nucleus of a somatic cell is transferred to an enucleated oocyte that then proliferates and results in a clonal cell colony at low but appreciable rates. Moreover, such mouse oocytes, when transferred into the uterus, lead to a living animal, ensuring the harvesting

of plenty of DNA for experiments. Another strategy utilizes *in vivo* cell cycle S-phase arrest followed by WGA of the duplicated genome from the still undivided cell (Leung et al. 2015). This strategy has the advantage of starting WGA from a larger DNA amount than that of a single diploid cell and, theoretically, is useful for detecting mutations in cells with limited potential for proliferation. Finally, an elaborate technique involving cell mitosis in the presence of bromodeoxyuridine, selective degradation of the nascent strands from the daughter cells, and DNA barcoding from different cells (Falconer et al. 2012) leads to separation of reads by haplotypes, enabling discoveries of aneuploidies, large CNAs, and inversions. The protocol was adapted to the analysis of aneuploidies in nuclei from frozen brain (van den Bos et al. 2016).

14.4 Experimental Techniques for Mosaic Variant Detection in a Cell

Each strategy described above can be combined with multiple experimental techniques for data generation and analysis: comparative genome hybridization (aCGH) (Kallioniemi et al. 1992; Le Caignec et al. 2006; Fiegler et al. 2007; Vanneste et al. 2009), genotyping SNP arrays (aSNP) (Wang et al. 1998; McConnell et al. 2013), and DNA sequencing. The latter include whole genome sequencing (WGS) (Navin et al. 2011; Abyzov et al. 2012; McConnell et al. 2013; Behjati et al. 2014; Cai et al. 2014; Knouse et al. 2014; Lodato et al. 2015; Blokzijl et al. 2016; Saini et al. 2016; van den Bos et al. 2016; Abyzov et al. 2017; Milholland et al. 2017; Bae et al. 2018; Lodato et al. 2018), targeted capture and sequencing, such as exome sequencing, (Wang et al. 2014), targeted PCR amplification and sequencing (Martincorena et al. 2015), and L1-enrichment by PCR amplification and sequencing (Evrony et al. 2012; Erwin et al. 2016).

When designing a study to detect mosaic variants and choosing a strategy and an experimental technique, one has to consider characteristics of the variants to be found, (i.e., type and frequency in studied cells), expected location of variants, practical limitations of dealing with the sample (i.e., whether isolation or culturing of single cell/nuclei is possible), and budget restrictions. aCGH and aSNP, while being inexpensive, can only detect aneuploidies and CNAs. WGS on the other hand is the most comprehensive and least biased way to analyze genomes, as, theoretically, it can be used to discover all variants of all types. Practically, the power to detect variants depends on the depth of coverage after mapping the sequencing reads on the reference genome, read length, and insert size (the distance between paired reads). An average base coverage of about 40X is considered adequate to find germline SNPs, indels, MEIs, and most CNVs. For single cell analysis such coverage would be adequate to find mosaic SNVs, indels, MEIs, aneuploidies, and most CNAs (Zafar et al. 2016; Bae et al. 2018). However, WGS efficiency for single cell analyses depends on evenness and errors during whole-genome DNA amplification, which, as discussed above,

may be problematic for WGA. Hence, extensive technical and biological validations are necessary to gain confidence in the results.

The efficiency of applying WGS to SV detection depends on physical coverage, i.e., when counting un-sequenced bases between paired reads (Korbel et al. 2007, 2009). Thus, libraries with long span of 2–20 kbp between reads provide an efficient way of finding SVs even at extremely shallow 1X base coverage, as physical coverage can be folds larger. Complementary to finding SVs, WGS is very efficient to find CNVs and CNAs, particularly large ones. Sequenced reads cover cell's genomes proportionally to their copy number, i.e., higher or lower copy number of a particular genomic region is reflected, respectively, in higher or lower coverage depth. Biases in the coverage do exist but their main sources are known and can be corrected for (Abyzov et al. 2011). Even at moderate coverage of 1X–5X, the depth of coverage method is sensitive enough to find CNA of few kbp in size and larger in clonal colonies and single cells (Abyzov et al. 2012; McConnell et al. 2013). Combined with a special library preparation that separates reads by DNA strands, one can observe genomic rearrangements at extremely shallow coverage of less than 0.1X (Falconer et al. 2012; van den Bos et al. 2016).

The drawback of WGS is that it is a relatively expensive procedure. To reduce the cost, one can conduct targeted enrichment and sequencing of either specific genomic regions, genes, or genomic elements. For example, capturing and sequencing exomes is a widely utilized approach. Coding regions are most likely to harbor deleterious variants, while occupying only about 1% of human genome. Amplicon-seq is often used to enrich for the sequences of a group of genes, so-called panel sequencing. It is useful for the discovery of variants in genes related to a particular disease or cancer. PCR targeted for L1 mobile elements enables a cost-effective strategy to study "jumping" of mobile elements in a genome. The overall disadvantage of targeted sequencing is that it limits the score of variants that can be discovered, although, as discussed below, it is very appropriate for validation of discovered variants (see below). All the targeted approaches result in an uneven coverage across genome making the discovery of SVs and CNAs unlikely. Furthermore, capture, as it relies on oligonucleotide probes designed from the reference genome, is likely to miss sequences containing variants other than SNVs and a few bp long indels. And lastly, relative to WGS, all targeted approaches increase the chance of introducing artifacts stemming from the various additional experimental steps.

14.5 Analytical Methods for Variant Discovery

14.5.1 Leveraging Analytics from Cancer Genomics

Once the genome of a single cell is amplified, either through WGA or by cloning, and data for the genome analysis are generated, the next step is to find mosaic variants from the data. Straight analysis of the data in comparison with the reference

genome will result in a set of variants containing both germline and somatic variants. To separate the former from the latter it is common to compare the data from the cell to the data from some reference samples from the same individual the cell was derived from. As the cell and the reference sample have the same germline genome, the differences between the two are likely somatic variants (such comparison is pivotal in the aCGH approach). This is similar to the approach often conducted in cancer genomics, where one finds somatic variants present in the cancer sample by comparing its genome to the genome of some normal tissue from the same individual. Therefore, the wealth of analytics developed in cancer genomics for pairwise sample comparison can be applied to genomics of single cells (Abyzov et al. 2011; Koboldt et al. 2012; Cibulskis et al. 2013; Kim et al. 2018; Wala et al. 2018).

However, there are small but essential differences. First, in cancer genomics variants with VAF less than 50% for diploid chromosomes (and 100% for haploid) are of interest, as sample purity is hardly ever close to 100% and subclonal variants are frequent. In contrast, when analyzing variants in a cell, only variants at 50% VAF for diploid chromosomes (and 100% VAF for haploid ones) are of interest. Moreover, variants with smaller values of VAF should be filtered out as they likely represent culturing and WGA artifacts. Second, pairwise sample comparison is likely to miss variants with high VAF in the reference tissue (Fig. 14.3a), as such variants are hard to distinguish from true heterozygous variants. In the analysis of cancer, such variants are of less interest as the reference sample is normal tissue that is not expected to have variants related to cancer. Moreover, according to the main paradigm of clonal cancer etiology, variants leading to cancer should be extremely rare in normal tissues and only manifest themselves in the cancer samples. Contrary to that, mosaic variants with high frequency (HiF) in tissue may be of highest importance, as, due to their frequency, they are likely to exhibit the largest phenotypic effect on the tissue or organism. Additionally, they are more informative markers for lineage tracing than rare variants (Behjati et al. 2014; Evrony et al. 2015; Lodato et al. 2015; Bae et al. 2018). In turn, lineage tracing can inform about development (Bae et al. 2018) and lineage expansion in a tissue (Abyzov et al. 2017). Therefore, detection of HiF variants is a particularly important issue for mosaic variant analysis.

14.5.2 Detecting High Frequency Variants

Some HiF variants can be detected just from pairwise comparisons if found in multiple cells (Abyzov et al. 2012; Lodato et al. 2015). If conducting sequencing, increasing depth would allow for a better separation of HiF and germline variants, but the cost is often prohibitive. For example, at 80X whole genome coverage of both the cells and the reference tissue genomes, a variant with VAF of 20% can be distinguished from heterozygous germline variants with p-value of about 10^{-4}. But given the roughly 2 million heterozygous variants per individual genome (1000 Genomes Project Consortium et al. 2015), such level of confidence is not acceptable. For variants with higher frequencies, the separation is even more problematic. A compre-

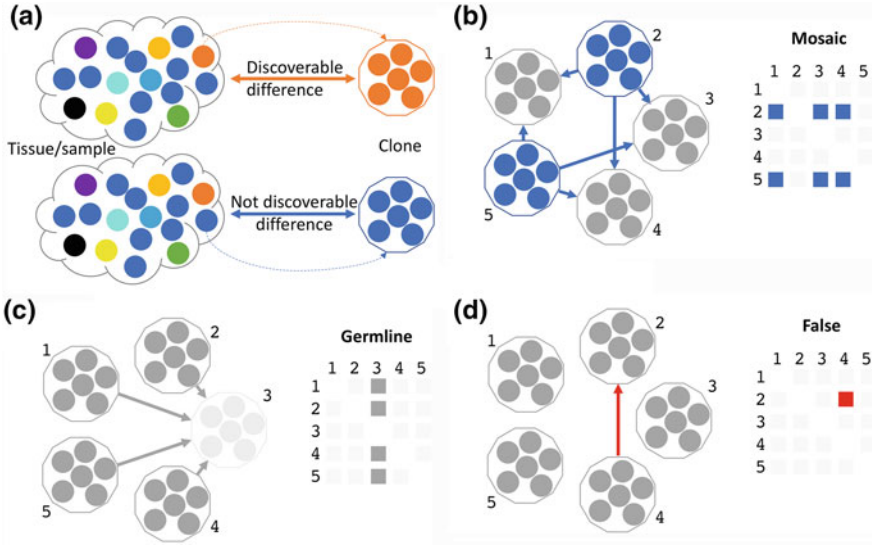

Fig. 14.3 The concept of discovering high frequency variants from clone-to-clone (or cell-to-cell) comparison. **a** Cells (circles) in a tissue/sample carry various mosaic variants represented by different colors. Rare variants (in orange) are easy to find from clone-to-tissue comparisons, but frequent variants (in blue) are not. **b** When comparing clones against each other the same frequent mosaic variants (in blue) are discovered multiple times for the following clone comparisons: 2 versus 1, 2 versus 3, 2 versus 4, 5 versus 1, 5 versus 3, and 5 versus 4. These calls line-up in rows in the table of pairwise clone comparisons (right). **c** Occasionally a germline variant (in gray) may not be present in a clone or founder cell of a clone (clone 3 in this example). In such case, the germline variants will be discovered multiple times for the following clone comparisons 1 versus 3, 2 versus 3, 4 versus 3, and 5 versus 3. These calls line-up in columns in the table of pairwise clone comparisons. **d** There could be also the case when a false positive (in red) is called when there is no real mosaic variant in a clone and it is not a missed (in a clone) germline variant. Such calls are sporadically distributed in the table of pairwise clone comparisons. For each call discovered from clone-to-clone comparisons one can construct a table of call distribution. From such a table, true mosaic variants, occasionally missed germline variants, and false positives can be easily distinguished. Thus, the comparison of multiples clones is an effective way to find mosaic variants including those frequent in the primary tissue/sample

hensive way for the detection of HiF somatic variants is to compare the genome of a cell to the genomes of the parents of the individual who the cell belonged to (Abyzov et al. 2017). Unfortunately, parental genomes are rarely available when conducting mosaic variant analysis. Therefore, the cell-to-cell (or clone-to clone) comparison is currently the most cost-effective and near comprehensive approach for finding HiF and other mosaic variants in a cell, as well as eliminating false positives (Bae et al. 2018).

The concept behind the approach is that for each HiF variant, the analysis of several cells from a tissue is likely to sample the variant in a subset of analyzed cells, while another complementary subset of cells will lack the variant. For example, when analyzing 10 cells from a tissue, a variant present in 40% of cells in that tissue

(i.e., with VAF of 20% on a diploid chromosome) is to be sampled in at least one analyzed cell with probability of 0.994. So, when comparing the genome of the cells from the two complementary subsets, the variant will be discovered multiple times (Fig. 14.3b). But note that one has to conduct $Nx(N-1)$ comparisons when comparing cells to each other rather than N comparisons when comparing N cells to the reference tissue. More comparisons would lead to more germline variants being incorrectly identified as mosaic (Fig. 14.3c), and more false positive calls. To address the issue, variant specific discovery matrices from cell-to-cell comparisons are constructed. For a true mosaic variant call, the variant will be arranged in rows of the corresponding matrix, for misinterpreted germline variants the call will be arranged in columns, and false positives will be sporadically distributed in the table (Fig. 14.3). It is a straightforward task to algorithmically separate the tree matrix patterns from each other. The simplest way is through calculation of the so-called "explanation score" (Bae et al. 2018).

Let n be the number of times the same variant was called from cell-to-cell comparison. A genuine mosaic variant could only be discovered from comparisons of a cell carrying the variant to a cell not carrying the variant. Therefore, in an ideal case and for the genuine mosaic variant this number will be $n = f(1-f)N^2$, where f is the fraction of cells with the variant and N is the number of cells being compared (cells are from the same person), allowing to derive f as $f = 0.5 - \sqrt{0.25 - n/N^2}$. Now the number of cells N_v with the variant is $N_v \approx fN$. From N and n we can calculate N_v, and if it is not a whole number round it to the nearest one. With the value of N_v we then ask a question: can we select that many cells (out of N in the experiment) to explain most of the observed variant calls n? In other words one has to maximize the number of calls for the variant in N_v cells $n_{max} = \sum_{i=1}^{N_v} n_i$, where n_i is the number of calls for the variant in cells i. Easy to see that n_{max} cannot exceed n and that for a real mosaic variant it should be close to n. Sporadic false positives calls will have very small n_{max} because of zero or very small N_v. Furthermore, if a germline variant is mistakenly called as mosaic variant due to coverage drop or allele drop-out in N_d cells, it will result in $n \approx N_d(N-N_d)$, $f \approx N_d/N$, $N_v \approx N_d$, and $n_{max} \approx N_d^2$. As N_d is expected to be much smaller than N, n_{max} for germline variants will also be much smaller than n. Therefore, one can use the ratio of n_{max} to n as a score to flag real mosaic variant, $S = n_{max}/n$. The score ranges between 0 and 1, with the values closer to the upper range indicating true mosaic variants. And in fact, when applied to real data, the score segregates almost exclusively around 0 and 1 (Bae et al. 2018). Then one can apply a conservative score cut off, for example 0.8, to select true mosaic calls from cell-to-cell comparisons. Experimental validation indeed confirmed that the approach finds HiF variants (Bae et al. 2018). Note that, as the meaning of the score is to evaluate how well calls for a variant are arranged in rows (Fig. 14.3), its generality and applicability is not affected by the variant discovery sensitivity.

Additionally, it was demonstrated that the validity of the approach can be judged from the analytical metric about the location of calls in the human genome. Such in silico validation requires minimal computational resources, is instant to calculate, and, thus, provides a quick way to perform validation of calls in future studies. The idea of the validation is based on the notion that not all bases in the genome

are equally accessible to current sequencing technologies. Particularly, some bases are prone to artifacts due to unreliable read mapping, typically because of being repeated in the human genome multiple times. Highly confident bases for calling SNVs were masked (as "P"—passed) by the 1000 Genomes Project (1000 Genomes Project Consortium et al. 2015). Therefore, after read mapping and analytical data processing, unbiased calling should result in roughly equally distributed variant calls between P and non-P bases. If additional calls made exclusively from the cell-to-cell comparison approach are also mostly located in P bases, this indicates that they are genuine.

The advantage of the cell-to-cell comparison approach has so far been demonstrated on data from clones but conceptually it should be applicable to data from WGA of single cells, perhaps less efficiently due to uneven coverage of WGA. It is also important to stress that the approach is not intended to distinguish genuine variants from amplification and culturing artifacts.

14.5.3 Resolving WGA Errors

As, discussed above, base substitution errors during WGA can be partially addressed by imposing a cut off on VAF in the data from a cell. For clones where the distribution of measured VAF is relatively tight, and deviations are determined by only stochastic coverage fluctuations, the cut off can efficiently separate mosaic and heterozygous variants from culturing artifacts (Fig. 14.2). However, for WGA data where the measured VAF is over dispersed due to unevenness of amplification, such considerations are not that effective. An alternative approach is to phase candidate mosaic variants to each haplotype of an individual (Fig. 14.4). A true mosaic variant is present on one and only one haplotype and on all of its copies, and, thus, will be perfectly *in-phase* with nearby heterozygous germline variants. Errors of WGA or culturing artifacts are also likely to be on only one haplotype, but on a fraction of its copies, which would result in poor phasing. Errors can also be present on both haplotypes and in such cases will be not "phasable". This approach allows for confident distinction between an amplification error and a mosaic variant even if the measured VAF of the error is higher than the measured VAF of the variant (Fig. 14.4).

The approach has been demonstrated to work for mosaic SNVs (Freed and Pevsner 2016; Ju et al. 2017; Lodato et al. 2018) but theoretically should be also applicable to indels as well as SVs, CNAs, and MEIs, if breakpoints of the latter are known with basepair resolution. The main limitation of the method is that the majority of variants cannot be phased to a haplotype with standard short read sequencing technologies. There are approximately 4 million germline variants per individual genome (1000 Genomes Project Consortium et al. 2015), with less than 3 million being heterozygous. Only heterozygous variants, which distinguish the two haplotypes, can be used for phasing. Therefore, each individual has on average one heterozygous variant per 1000–2000 bases, while modern and most commonly used sequencing technologies output paired reads with typical length of 100–150 bps. As a result,

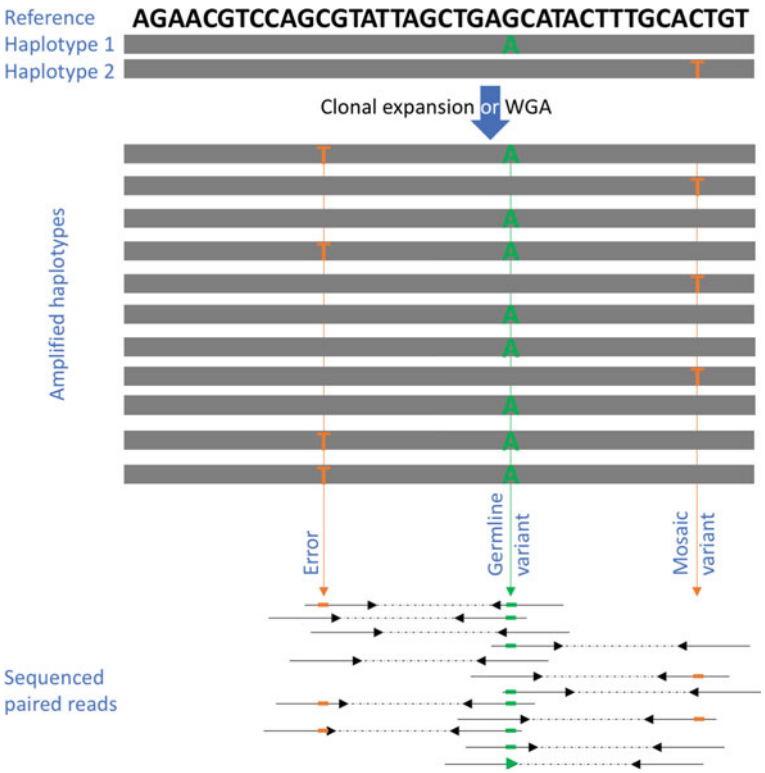

Fig. 14.4 Schematics of how phasing to a haplotype allows distinguishing mosaic variant from culturing artifacts or WGA errors. In this example, a cell has two haplotypes with two differences from the reference genome: a germline heterozygous SNP G > A and mosaic variant C > T. Two haplotypes in a cell can be unevenly amplified. However, the true mosaic variant will always be on one haplotype in phase with the corresponding allele of heterozygous SNPs and vice versa, i.e., the T of mosaic SNV and the reference G of the SNP will be always co-occurring in the data. An amplification error or culture artifact is also likely to propagate and be in phase with one of the haplotypes but the opposite is not true, i.e., error T (left) is co-occurring with the A of the SNP but the A may occur without the T. An error can be also present on two haplotypes (not shown). Note, that in the displayed example the measured VAFs for the error is higher than that for mosaic SNVs, still, phasing to haplotypes allows for confident distinction between the two calls

only a limited fraction of ~20% of candidate mosaic variants can be phased reliably to a haplotype (Lodato et al. 2018). Sequencing technologies with longer reads, such as MiSeq, IonTorrent, Oxford Nanopore, and PacBio, could be used to solve this problem, but they are 2–10 times more expensive per sequenced base, and some of them suffer from excessive sequencing error rate. The error rate can be dealt with by increasing depth of coverage, which further increases overall experimental cost. A promising technology is provided by 10X Genomics, which enables sequencing of linked-reads that can be used to phase variants into long germline haplotypes of millions of bases in length (Kitzman 2016). Thus, the 10X technology promises to

enable near complete phasing of mosaic variants, at least as long as these variants occur outside of segmental duplication regions of the human genome, where even 10X linked-reads cannot be brought to bear. However, the actual utility and efficiency of this technology with WGA DNA for finding mosaic variants has not yet been demonstrated.

14.6 Validation

It is crucial to establish validity of mosaic calls by confirming their existence in the original tissue or original DNA (Fig. 14.1) by using experimental methods that are orthogonal to those used for discovery. Methods for validation can differ significantly in required labor and cost, throughput, and sensitivity (Table 14.2). However, none of the available techniques is more sensitive than single cell or clonal expansion analysis itself. In extreme cases, a variant can be discovered in a cell that is the only carrier of the variant, and, after the discovery phase the variant could never be validated in the tissue, as it is not present there anymore—the only cell carrying the variant having been destroyed in the process of extracting the genomic DNA for discovery. Thus, biological validation in the original tissue can never be comprehensive, and not validating a call could be interpreted as either that the variant is too rare in the tissue to be validated or that the call is incorrect. Nevertheless, carrying out biological validation is essential as the validated calls inform about most frequent, and likely most functionally relevant, variants in the tissue and also estimate a "lower boundary" for counts of mosaic variants.

Technical validation in the DNA sample used for discovery is also necessary and essential, and can be conducted with the same techniques used for biological validation (Table 14.2). In fact, applying two or more orthogonal techniques to discover somatic variants and then taking the intersection of variant sets from the applied techniques is a form of technical validation. The fundamental limitation of technical validation is that seemingly validated calls may still be errors introduced in the DNA during preparation (e.g., during single cell WGA or clonal expansion culture) and thus be false positives. So, special considerations need to be taken in order not only to validate a call but also to demonstrate that it is not likely an introduced artifact, such as the expectation to find in single cells or clonally expanded populations at

Table 14.2 Comparison of validation methods

	Capture	Amplicon-seq	ddPCR
Sensitivity (%)	~0.5	~0.1	~0.01
Throughput	>1000	Hundreds	Dozens
Price per site	Low	Moderate	High
Labor	Little	Moderate	High
Turn around	Month	Weeks	Weeks

a mean 50% VAF for true mosaic variants on a diploid chromosome, as discussed above.

14.6.1 Validation by PCR/qPCR

A classical PCR technique combined with Sanger sequencing can be applied for validating SNVs, indels, and SVs, if breakpoints of the latter are predicted with near bp resolution. Validation of SNVs can only be done on a coarse-grained scale, i.e. validating presence or absence for HiF variants. Estimating the VAF or even defining whether a given SNV is present at low or high VAF is either subjective or impossible. Because of this, PCR, when applied in biological validations, is unlikely to confidently distinguish between HiF mosaic and heterozygous germline variants.

Contrary to that, when applied to validation of indels and SVs there can be a good degree of sensitivity and objectivity, due to the possibility of amplifying the likely unique sequence for indel/SV. For indels the sequence will result in a set of secondary peaks in the Sanger sequencing trace, while for an SV, PCR primers flanking its breakpoints will generate amplicon for haplotype with the SV and no amplicon or a different amplicon for the haplotype without the SV. Thus, presence of a dual band or just one band with the expected size validates the SV. Moreover, Sanger sequencing of the expected band allows fine, basepair-level resolution of SV breakpoints for ultimate validation. Indeed, it was shown that PCR can validate SVs down to a VAF of less than 1% (Abyzov et al. 2012).

qPCR, which stands for quantitative PCR, is a technique that monitors the amplification of a targeted DNA sequence in real time. For a given sample, it allows estimating the number of copies of the targeted region. Its application for validation of CNAs has the same limitations as the application of PCR to the validation of mosaic SNVs, as quantification is approximate.

14.6.2 Validation by ddPCR

Droplet Digital PCR (ddPCR) is a technique that allows for the precise quantification of a target allele in a given DNA sample (Hindson et al. 2011; Zhou et al. 2018). The target allele can be an SNV, indel, or the breakpoint sequence of an SV. It can be run simultaneously on multiple alleles, so alleles with known copy number provide a baseline to quantify the frequency of other regions. With large input DNA amounts and optimization, ddPCR can validate mosaic variants with VAF as low as 0.01% in the given sample (Abyzov et al. 2012; Lodato et al. 2015; Abyzov et al. 2017). Arguably, because of such superb sensitivity and complete orthogonality to sequencing, ddPCR is the gold standard technique for mosaic variant validation. Its major drawbacks are requirement of substantial labor and high per variant cost, limiting the application to few variants.

14.6.3 Validation by Re-Sequencing

Another approach for both technical and biological validation is in-depth reanalysis of candidate mosaic SNVs and indels with high depth-of-coverage sequencing. The central idea of the approach is to enrich DNA from the original tissue/sample by targeting the stretches of genomic sequences around candidate mosaic variants, so that sequenced reads would mostly carry the information relevant for analysis of the variants. This makes the approach cost-effective on a per site basis and with modern ways of enrichment it is also a high-throughput option. Enrichment can be accomplished with DNA capture or amplification of target sites. For example, 1000 sites with mosaic variants captured and sequenced to a depth of about 1000X are equivalent in terms of total reads to whole genome coverage at a sequencing depth of less than 0.5X.

For biological validation in the original sample, high depth-of-coverage is a prerequisite to acquire sufficient support for variants with low VAFs. At coverage above 1000X, which is relatively easy and inexpensive to obtain for thousands of variants, VAF down to 1% can be precisely quantified and the corresponding variants are validated. Some variants with lower VAFs (as low as a fraction of percent) could be validated if sequencing error background at their sites is small. For technical validation in WGA DNA of a single cell or from a clonal colony, high coverage is needed to precisely determine VAF for mosaic candidates. The VAF, as discussed above, can be used as strong evidence supporting genuine mosaic variants. At coverage of 1000X, the uncertainty in estimating VAF of mosaic SNV is only about 5%. Note that while WGA will shift the VAF of each mosaic variant due to uneven haplotype amplification the distribution for multiple variants will nevertheless be centered around 50% (Fig. 14.2).

As is the case for variant discovery, application of DNA capture and amplification suffers from differential efficiency of capture and amplification for alleles with and without mosaic variants, potentially leading to biased VAF estimates for indels. To mitigate this issue, one can design capture baits both from the sequence of the reference haplotypes and from the predicted haplotypes with mosaic variants.

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Part IV
Immunosenescence and Inflammaging Markers

Chapter 15
Are There Reliable Biomarkers for Immunosenescence and Inflammaging?

Tamas Fulop, Alan Cohen, Glenn Wong, Jacek M. Witkowski and Anis Larbi

Abstract Aging is accompanied by changes in the immune system, culminating in immunosenescence on one hand and inflammaging on the other. Science is striving for biomarkers to capture these heterogenous complex processes. However, when the basic interpretation does not capture correctly the underlying phenomenon, the biomarkers may not be accurate either. Thus, we tried to give a new interpretation of the immune changes with aging as mostly adaptative (and even partially beneficial) in physiological circumstances. So we need new biomarkers which could capture these novel concepts this new reality concomitantly to the old ones. Finding and applying them would in our opinion result in a better modulation of the immune changes in elderly subjects.

Keywords Inflammaging · Age related diseases · Innate immune system · Adaptive immune system · Trained innate immunity · Biomarkers · Immunadaptation

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15.1 Introduction

Aging is accompanied by several complex, mostly considered detrimental, biological changes (Lopez-Otin et al. 2013). The immune system is one of the ubiquitous systems of the organism which is strongly influenced by aging. The changes in aging occurring in the immune system are often referred as immunosenescence (in brief, reduced ability of mostly adaptative immunity to respond to antigenic challenges) (Goronzy and Weyand 2013; Pawelec 2012, 2018a; Yanes et al. 2017; Xu and Larbi 2017) and inflammaging (increased inflammatory readiness without overt symptoms of clinical inflammation, manifesting itself by e.g. increased levels of proinflammatory cytokines) (Franceschi et al. 2000; Pinti et al. 2016). An enormous effort has been deployed during the last three decades to define these changes and to understand the underlying mechanisms. The phenotypic and functional changes have been described, although many different and even divergent results have been reported in the domain (Fulop et al. 2018a; Larbi and Fulop 2014). It is always difficult to measure every single aspect of the immune response. Thus, there is an urgent need to better understand the biology of the immune changes with aging as well as their determinants. Towards this aim, a quest for biomarkers that would track the state of physiological immune changes with aging and ideally lent insights to the underlying mechanisms has been thoroughly researched (Saavedra et al. 2016; Pawelec 2018b; Torres et al. 2018). Indeed, a biomarker is anything that can be used as an indicator of a specific disease state or some other, also physiological, state of an organism. Thus, many markers were nominated as biomarkers of the immune aging process; however, until presently, none were implemented or confirmed by large human studies (Lara et al. 2015). In this chapter we will first review briefly the immune changes pertinent both to immunosenescence and inflammaging as presently conceptualised, and describe the immune biomarkers reflecting these changes as well as their practical usefulness. In the second part we will propose a different view (reinterpretation) of the immune changes with aging as an immunoadpatation and discuss whether this different perspective approach would help to generate better biomarkers of the immune system aging (Table 15.1).

15.2 The Concepts of Immunosenescence and Inflammaging

Both parts of the immune system, the innate and the adaptative, are affected by the aging process, however not in the same manner and at different rates. Also, the significance of these changes is presently considered as being part of the general detrimental processes of aging. However, a consensus seems to exist to consider that at least in part these changes result in the process called inflammaging.

Table 15.1 Putative present and future biomarkers in the reinterpretation of immunosenescence/inflammaging as immunoadaptation

Immune response	Biomarkers	Data
Innate response	Metabolic	–
	Epigenetic	–
	Exosomes	–
	Phenotypes	+
	Functions	+
	Senescence/differentiation	–
Adaptive		
T cells	Phenotype of differentiation	+
	Innate immune markers	+
	Senescence/exhaustion markers	+
	Functional	+
	Metabolic	–
	Epigenetic	+
B cells	Vaccination	+
	Phenotypes	+

15.2.1 Innate Immune System

The innate immune system is meant to protect the organism from any internal or external challenges (aggressions) as a first line of defense. This type of immunity is existing in various forms in the whole living kingdom of multicellular organisms from plants to humans (Muller et al. 2013). This is a very efficient, but not very specific, defense mechanism composed of several cells and soluble mediators.

The innate immune system is mainly composed from the NK cells, neutrophils, monocytes/macrophages and dendritic cells (DC) (Kolaczkowska and Kubes 2013; Vivier et al. 2008; Porcherey et al. 2005; Van den Bossche et al. 2017). All these cells have common functions, like phagocytosis and chemotaxis, as well as target-dependent functions that are specific for the given cell type, e.g. contact cytotoxicity (Tieri et al. 2010).

NK cells are powerful protectors from virus-infected, tumour and senescent cells (Gounder et al. 2018; Della-Chiesa et al. 2014; Raulet and Guerra 2009; Rodier and Campisi 2011; Caligiuri 2008). There are two major types of NK cells: the $CD56^{dim}CD16^{+}$ cytotoxic cells and $CD56^{bright}CD16^{-}$ producers of cytokines including IFNγ and TNFα. A fine balance between activation or inhibition of NK cell responses depends on the expression and communication between type I Ig superfamily activating receptors (NGK2D) and type II C-type lectin inhibitory receptors (NKG2A/CD94) (Long et al. 2013; Le Page et al. 2015). The cytolytic activity of NK cells is mediated through pathways involving release of granules containing granzymes and pore-forming perforin that allow delivery of granzymes to the target cell cytoplasm where they initiate apoptotic death pathways (Krzewski and Coligan

2012). The other pathway of NK-dependent cell killing involves members of the TNF superfamily of death receptors and their ligands (Chavez-Galan et al. 2009). Proportions and numbers of NK cell subpopulations are affected by aging (Solana et al. 2014; Camous et al. 2012). For example, the percentage of NK cells among peripheral blood lymphocytes is increased in healthy aging and centenarians but there is a decrease in the $CD56^{bright}CD16^{-}$ NK cell subset and an expansion of $CD56^{dim}CD16^{+}$ NK cells (Solana et al. 2012). Considering that $CD^{56bright}CD16^{-}$ NK cells have a high capacity to produce different cytokines and chemokines in response to cytokines released by other activated immune cells, their decreased proportion may be responsible for the defective overall production of cytokines and chemokines by NK cells stimulated with IL-2 or IL-12 observed in elderly individuals including nonagenarians. On the contrary, old individuals show an increased production of IFN by $CD56^{bright}$ cells, potentially representing compensatory mechanism to maintain the immunoregulatory role of these cells in older individuals (Solana et al. 2014). Furthermore, the effect of aging on the expression of NK cell receptors has been recently described (Tarazona et al. 2015). Although there was no alteration in CD16 expression or function in NK cells, expression of the activating natural cytotoxicity receptors NKp30 and NKp46 and of co-stimulatory molecule DNAX accessory molecule (DNAM)-1 were decreased. Analysis of NK cell inhibitory receptors showed an age-related increase in KIR expression and a decrease in CD94/NKG2A expression, although discrepancies could be found in different studies. As a functional consequence, NK cytotoxicity against classic NK cell targets was impaired but antibody-dependent NK cell cytotoxicity was not affected by aging.

Neutrophils are relatively very short-lived cells; however, their life span may be prolonged by pro-inflammatory cytokines (Fulop et al. 1997). They are very efficient to control various aggressions, mainly from bacterial and fungal origin. They may destroy microbes by phagocytosing them after being attracted by chemotaxis to the site of infection (Kolaczkowska and Kubes 2013). Once the microbes are phagocytosed, neutrophils kill them by using their intracellular lysosomal machinery. Most of these functions of neutrophils are mediated by receptors of different types, but the most important are the Fcγ, the complement and the pathogen recognition receptors (PRR) which elicit different signaling pathways to get the specific functional response (Prince et al. 2011; Thomas and Schroder 2013; Fukata et al. 2009; Turner et al. 2014). It has been demonstrated that neutrophils undergo with aging several changes, both phenotypical and functional (Fortin et al. 2008; Panda et al. 2009; Khanfer et al. 2012). The most important changes occur in their functions, as the chemotaxis, adhesion, intracellular killing and antibody-dependent cellular toxicity ADCC are all decreased with aging. The number of receptors generally does not change. However, the signaling elicited by these receptors is changing for the most important pathways such as the MAPK, PI3K, Jak-STAT and MyD88 pathways (Solana et al. 2012; Fulop et al. 2014). These changes altogether lead to a decrease in functions which may lead to decreased elimination of the pathogens (microbes), but nevertheless still leaves the individual capable to eliminate them in some way, as not all aged individuals die from infections each time when they are infected (Fulop et al. 2016; Fulop et al. 2018a, b). In contrast, these cells are activated at their basal, rest-

ing state as demonstrated by the increased activation/phosphorylation on tyrosine for most of the signaling molecules such as PI3K or Lyn (Fortin et al. 2006). This is also correlated to an increased production of pro-inflammatory mediators in the resting state by human neutrophils (Bandanarayake and Shaw 2016). Thus, these cells may contribute by this basal sustained activation to the low-grade inflammation occurring with aging, commonly called inflammaging. The trade off for this sustained basal activation is immunoparalysis for specific functions (Fulop et al. 2016). The reasons for this state will be discussed later in this chapter. In the mean time their capability to influence the fate of the adaptive system is also changed.

Monocytes/macrophages also play a very important role in the defense of the organism and the determination of the adaptive immune response. Monocytes are also subdivided in subpopulations based on the expression of CD14 and CD16, namely classical (CD14++CD16−), intermediate (CD14++CD16+) and non-classical (CD14+CD16++) (Baehl et al. 2016). These two latter subpopulations are more pro-inflammatory than the first one (Justo-Junior et al. 2018). After leaving the circulation they become tissue-specific macrophages. These macrophages may also differentiate into several subtypes, the most important being the M1 pro-inflammatory and M2 anti-inflammatory (Xu et al. 2016; Ginhoux and Jung 2014). However, these differentiations are plastic, so they can in some circumstances transform in each other back and forth. Monocytes and macrophages have similar functions to these of neutrophils, but extended in time. With aging these cells also undergo phenotypical and functional changes (Nyugen et al. 2010; Jackaman et al. 2017).

Although fewer data are available to support this contention (relative to NK cells and neutrophils), monocytes and macrophages undergo functional and phenotypical changes during aging. Mostly, their phenotype shifts towards an inflammatory phenotype with an increase in intermediate and non-classical subpopulations. They are able to secrete more pro-inflammatory cytokines mostly at basal state, while this secretory activity is decreased when the cells are specifically stimulated. The functions are differentially changing, and mostly controversial data concern the number of the surface PRR receptors, such as TLR1/2 and TLR4, reported either to decrease or to not change (Fulop et al. 2004; Shaw et al. 2011; Dunston and Griffiths 2010). So, the monocyte changes are heterogeneous but clearly shifted towards a pro-inflammatory phenotype with changed functions. Macrophages seem to be shifted to M2 differentiation stage, however keeping a capacity to secrete pro-inflammatory cytokines (Garg et al. 2014). The phenotypic changes in macrophages reflect a metabolic and epigenetic changes occurring with aging (Fang et al. 2018; Guzik and Cosentino 2018). Thus, these cells are considered at the center stage of the inflammaging concept (Franceschi et al. 2000).

Finally, yet another special innate immune cell type that has been shown also to behave differently in aging are the dendritic cells (DC). The antigen presentation to the naïve CD4 lymphocytes through the MCH-TCR pathway is altered mainly because of the altered processing of the antigens inside of the DC (Gardner et al. 2017). This precludes also a decrease in the adaptive compartment of the immune response with aging.

15.2.2 Adaptive Immune System

Once the innate immune response deals with the aggression using its tools, it handles the task of further defense to the adaptive immune system, composed of B and T lymphocytes. They react to specific antigenic stimulation in a ways controlled by multiple cytokines. B cells are responsible of the humoral immune response; they, and their differentiated progeny called plasmocytes are producing specific antibodies. T cells are responsible for the cellular immune response having various functions such as immunoregulation, helper and effector functions. T cells have been studied the most as the prevalent paradigm in immunosenescence is that T cells are the most affected by the age-related changes (Pawelec 2012, 2018a). After leaving the thymus as naïve T cells sometimes called the recent thymic emigrants (RTE), the peripheral T cells are differentiating through their encounters with antigens into various subpopulations. As with aging several antigenic encounters are made, the T cells which were primed by such encounters are increased (Larbi and Fulop 2014; Fulop et al. 2013a, 2005) and become central memory (CM), effector memory (EM) as well as the terminally differentiated T cells (TEMRA). Some of them create the inhibitory branch of the system, called the induced regulatory T cells (iTregs), which are also increased with age (Jagger et al. 2014). Concomitantly, the numbers of naïve T cells (RTEs) are decreasing (Pawelec 2018a; Jergovic et al. 2018; Ventura et al. 2017). These changes lead to the common assumption that the aging immune system is unable to react to new antigens and vaccination as the TCR diversity is diminished together with the loss of naïve cells and the primed (memory) cells increased (Appay and Sauce 2014). However, it remains debatable whether the remaining TCR reservoir is indeed restricted in its capacity to mount de novo responses as emerging evidence reveal cohort studies where elderly maintain similar numbers of influenza and EBV-specific T-cells despite the disproportionate expansion of CMV-specific cells during age-related CMV reactivation. In the context of vaccination, some studies have also described stronger vaccination responses in the presence of chronic CMV infection. The changes in T-cell function and distribution have also been linked to the additional burden of infectious diseases Together these changes are considered as a very detrimental situation. Furthermore, this is the base for the presumption that the age-related diseases (including malignancies) are increasing, as the aging organism is unable to efficiently combat them (Fulop et al. 2013b). In the mean time the infections are also increasing for the same reasons. It is not well settled whether the changes in the TEMRA cells are more due to their exhaustion or senescence (Appay et al. 2002). Many different markers of both conditions are expressed in these cells. The main difference is in their proliferative capacity. Nowadays it is also questioned whether the decrease of naïve T cells with aging is enough to explain this decreased reaction of old immune systems to new antigens (Goronzy et al. 2015). In any case phenotypic changes are always accompanied by functional changes. T cells from aged individuals are not able to proliferate as efficiently as those from young ones under specific cognate (or polyclonal) stimulation due to alterations in the signaling pathways activation (Fulop et al. 2014). The most involved signaling

molecule is the Lck (Le Page et al. 2014). It is of note that as in the case of innate cells the signaling pathways such as Lck, STAT are already activated in the quiescent state (Shen-Orr et al. 2016). This precludes the efficient activation of these T cells by antigens. However, when inhibitors of the negative control exerted by tyrosine phosphatase are relieving the inhibition, these old T cells may proliferate and produce IL-2 almost at the same level than T cells of young subjects (Le Page et al. 2014). So, this seems clear that the most of the functional alterations have a strong extracellular component which induces intrinsic alterations (Franceschi et al. 2017a, b). This extracellular stimulus is what was already referred above as inflammaging. As mentioned earlier, inflammaging is a subclinical, low grade inflammation characterized by a relative increase of the inflammatory mediators under chronic stress conditions (Franceschi et al. 2000). Thus, these mediators released during the inflammaging are modulating/stimulating also the T lymphocytes and maintain a basal activation state of the most important signaling molecules such as Lck or STAT which prevent their adequate activation (Le Page et al. 2014; Shen-Orr et al. 2016). Moreover, the dysregulated immune regulation is also contributing to the functional immune decrease (Douziech et al. 2002). As mentioned, in aging the Tregs were found to be increased, which suppress certain immune functions making it less efficient if it is not well targeted. In the mean time this inhibition by letting the underlying process to continue unstopped can also contribute to the inflammaging (Reynor et al. 2012). The differentiation of these T cells is heavily controlled and maintained by the immunometabolism characterising different T cell subsets (Klein Geltink et al. 2018). While the naïve T cells are using an anabolic pathway of OXPHOS, during their activation process the aerobic glycolysis may become prevalent (Almeida et al. 2016). There is presently no reliable data concerning the immunometabolism in T cells with aging. However, we can suppose that there is a disequilibrium in every aging T cell subset between the OXPHOS and aerobic glycolysis. In any case changing immunometabolism may skew the cells towards a certain phenotype which is the case in aging. The mTOR pathway, also shown to be dysregulated in aging, is also playing an important role in this differentiation (Johnson et al. 2015). Akbar's group found that the senescent CD45RA-expressing population engaged aerobic glycolysis to generate energy for effector functions (Henson et al. 2014). Furthermore, inhibition of p38 MAPK signaling in senescent CD8+T cells increased their proliferation, telomerase activity, mitochondrial biogenesis, and fitness; however, the extra energy required for these processes did not arise from increased glucose uptake or oxidative phosphorylation. Instead, p38 MAPK blockade in these senescent cells induced an increase in *autophagy* through enhanced interactions between p38 interacting protein (p38IP) and autophagy protein 9 (ATG9) in an mTOR-independent manner (Henson et al. 2014). Together, these findings describe fundamental metabolic requirements of senescent primary human CD8+T cells and demonstrate that p38 MAPK blockade reverses senescence via an mTOR-independent pathway. Such studies need to be performed for other, different T cell subpopulations. Finally, the question arises what are the main inducers of the aging-associated changes in the T cells? The most important and popular was the cytomegalovirus (CMV) (Pawelec 2014; Weltevrede et al. 2016). Until recently, the virus was considered as one of the most important

causes of this hyper-differentiation of the T cells. This was even considered once as the *primum movens* of the T cell aging as the contention of the reactivations of the CMV required so many committed T cells that during age they accumulate. However, recently it was shown that many aging-related attributes and functions such as longevity and response to vaccination were better under chronic CMV infection (Derhovanessian et al. 2013; McElhaney et al. 2015; Bajwa et al. 2017). Thus, with aging many very heterogenous changes occur in the T cell compartment, leading to altered phenotypes and functions of these cells.

While diminished immune functionality appears to be mostly universal across both the innate and adaptive arms of the immune system, they appear as losses because these modifications are often calibrated and compared against the younger self whose physiology is no longer relevant in the elderly setting. However, that life expectancy continues to increase for the elderly population—notwithstanding better healthcare—suggests that the elderly may preserve sufficient immune functionality to clear pathogens and tumour cells at a rate that sustains life. Although these immune adaptations, such as increased systemic inflammation, clearly impose a cost in terms of driving tissue and cellular attrition, their absence may come at a greater fitness cost. For example, the low-grade inflammation that characterises inflammaging may be necessary as trophic signals for aged immune cells or to promote the circulation and turnover of immune cells. Via deuterium labelling, it has been found that with the exception of naïve CD8 T-cells, the turnover rates of naïve and memory T- and B-lymphocytes are preserved during aging. The continuous emergence of populations that demonstrate preserved influenza- and VSV-specific immunity and high vaccination efficacy in the elderly—despite high CMV seroprevalence—also provides clear indication that the loss of vital immune functionality is not inevitable with age. If we define preserved immune functionality as advantageous and as a manifestation of successful aging, then it becomes important to define the immune correlates of maladaptive and successful aging so that we can design interventions to improve the odds of the latter. Since both intrinsic and extrinsic mechanisms are involved, comparisons involving both factors are required to account for the heterogeneous aging physiology that is observed across different populations. With the multitude of adaptations revealed by cellular mediators from both the innate and adaptive arms of the immune system, it becomes important to accurately capture specific patterns that emerge in different physiological contexts the discovery and accurate usage of immune aging biomarkers are important for defining these snapshots.

So, the question arises how we can capture the complexity of this extraordinarily diversified system? An obvious answer is that for this purpose we need biomarkers. So next we will discuss biomarkers related to the aging immune system and whether they are reliable.

15.3 Are There Reliable Biomarkers to Support the Present Concept of Immunosenescence?

15.3.1 What Is a Biomarker

According to the definition by the NIH, biomarkers are key molecular or cellular events that link a specific environmental exposure to a health outcome. Biomarkers play an important role in understanding the relationships between exposure to environmental chemicals, the development of chronic human diseases, and the identification of subgroups that are at increased risk for disease. Much progress has been made in identifying and validating new biomarkers that can be used in population-based studies of environmental disease.

Several types of biomarkers exist, including predictive, prognostic, clinical and physiological. Predictive biomarker is defined as a marker which can be used to identify subpopulations of patients who are most likely to respond to a given therapy (Biomarkers Definition Working Group 2001; Robb et al. 2016). Thus, predictive biomarkers are the basis for individualized or tailor-made treatment. Prognostic biomarker is a biomarker that provides information on the likely course of the disease in an untreated individual. Clinical biomarker is a biomarker that improves treatment an reduces health costs. Finally, physiological biomarker is a surrogate end-point for a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacologic responses to a therapeutic intervention.

15.3.2 Which Biomarkers are Considered for Immunosenescence and Inflammaging?

Considering the description of the present process of changes in the immune response with aging there were some biomarkers suggested to capture this process. The most important seem to concern the changes in the T cell compartment. It was largely accepted that the decreasing number of naïve T cells as well as the concomitant increase in memory cells (considered mostly as senescent) are very good biomarkers of immunosenescence (Pawelec 2018a; Effros et al. 2005; Ishikawa et al. 2016). This notion was sometimes extended to the functions of these cells including the altered proliferation and Il-2 production (Larbi et al. 2006). The telomere length was considered to some extent to complement these biomarkers of T cell aging (Kaszubowska 2008; Goronzy et al. 2006). There was a great hope that the immune risk profile (IRP) capturing these alterations and comprising inverted CD4/CD8 ratio, accumulation of CD8+CD28- late differentiated T cells, poor proliferative capacity, few B cells and CMV seropositivity associated with significantly increased 2-, 4, and 6-year mortality in the very old may be a generalised biomarker (Wikby et al.

2005). Later these biomarkers were extended to include the CD95 as it was shown to be predictive of successful aging (Hsu et al. 2006). This was recently extended by the inclusion of Tregs and special senescence markers such as CD57 or p16INK as biomarkers of immunosenescence (Larbi and Fulop 2014; Xu and Larbi 2017). Thus, the senescence associated secretory phenotype (SASP) applied to the late differentiated T cells is also considered as one of the most reliable biomarkers of immunosenescence (Callender et al. 2018).

As inflammaging is also part of the altered immune response with aging, inflammatory biomarkers—mainly pro-inflammatory cytokines including IL6, IL-1, and TNFalpha—were also considered as valuable biomarkers as they predicted frailty and also an overall mortality in elderly subjects (Sanada et al. 2018; Frasca et al. 2017). Activation of the inflammatory pathway was associated with functional decline and mortality. This was moreover considered also as a surrogate of the innate immune response measurement in aging (Franceschi et al. 2000).

Very recently the miRNA composition of the blood as well as the epigenetic clock in the PBMCs were considered as potential surrogate markers as they were more reflecting the general aging process (Olivieri et al. 2015). However, as they reflect somehow the biological aging, so they indirectly may capture from an other approach the better functioning of the immune system in contrast to the other always seeking for alterations (Olivieri et al. 2014; Jylhävä et al. 2017).

15.3.3 Are they Reliably Applicable?

Although it was a great hope to apply the IRP as a general biomarker of immunosenescence, besides the OCTO and NONA studies (Pawelec et al. 2009) it could not be reproduced in any other longitudinal or geriatric studies (Plonquet et al. 2011; Johnstone et al. 2014). This means that as good as they may reflect some changes observed in some human populations they are not capturing any of the fundamental changes in the immune response. What they capture is the tip of the iceberg rather than the underlying processes.

In a study published by the Cohen's group there was a tight link between the pro- and anti-inflammatory sides of the immune changes with aging (Morrissette-Thomas et al. 2014). This suggested that neither of these processes can exist without the other. So if we want to understand one of these processes we should study the other too.

The telomere length also was shown to be very versatile marker of the immune cell aging process, likely reflecting the history of challenges this system encountered (its immunobiography) (Franceschi et al. 2017a, b). Furthermore, many other newly discovered potential biomarkers such as the metabolic changes, exhaustion and microbiota are missing in the traditional concept of immunosenescence and inflammaging (Wherry and Kurachi 2015; Biagi et al. 2013; Thevaranjan et al. 2017; Klein Geltink et al. 2018; Henson et al. 2014).

Thus, considering either the concept or the biomarkers of the immune aging according to present paradigm(s) they may not reliably be applicable. So, we need

a reinterpretation of the immune changes with aging which will possibly generate more reliable biomarkers to be translationally applicable (Fulop et al. 2018b).

15.4 New Concept (Vision): Age-Associated Immune Changes as Immunoadaptation

What generated the need of reinterpretation of the present concept of immunosenescence and inflammaging? Recently, several studies appeared to indicate that the immune changes are not as detrimental as they were considered from the old-time studies (Pawelec 2012; Fulop et al. 2018b; Franceschi et al. 2017a, b; 2018).

First, more and more published experimental data seem to indicate that the CMV infection, originally considered evil, may altogether be a beneficial factor for survival and longevity. It was shown that the more the T cell (TCR) clones are diverse the more they assure a better immune response (Bajwa et al. 2017). It was also shown that elderly who have an extension of their CMV-devoted T cell clones live longer. Finally, patients having a chronic disease such as diabetes type 2 have a higher vaccination response if they are CMV seropositive in contrast to those seronegative (McElhaney et al. 2015). This is in line with the concept that a higher readiness (due to constant control over the CMV) assures a better response than shown by those who were not subjected to this hormetic training (Fulop et al. 2013a).

Other recent studies battle the very extensively accepted paradigm that elderly do not respond to vaccination. The first demonstration that this was false came from the studies of the SHINGRIX studies (Lal et al. 2015). This study tested a new vaccine containing the antigenic epitope of varicella zoster and an adjuvant stimulating the TLR4 (boosting the innate response). This vaccine since many years maintains a protective effect even in very old. Recently the group of Dr Larbi published the first report demonstrating that the elderly respond also to the influenza vaccination (Camous et al. 2018)). This is a breakthrough needing a reinterpretation of what we consider as immunosenescence.

Another study showed that among all the biomarkers studied the (controlled) inflammation was the most determinant factor for the longevity in semi-supercentenarians (Arai et al. 2015). This shows, as it was already demonstrated in centenarians, that aging is not characterized by the absence of inflammation but by the adequate control of this important protective process (Rubino et al. 2018; Sizzano et al. 2018, Sotgia et al. 2017). In line with this notion, these centenarians showed an increased anti-inflammatory response for controlling the inflammation.

It was also shown that cancer is decreasing in incidence after 90 years of age and centenarians are mostly spared from this disease (Salvioli et al. 2009). Once more this indicates that the ‘decreased immune response’ does not contribute to this typically immune regulated disease in the oldest old. Moreover, immune parameters may identify longer five-year survival in centenarians independent of their health

and functional status reinforcing further suggesting their independence (Bucci et al. 2014).

Together, these data indicate clearly that immune changes with aging are more adaptative than only detrimental. So, what we mean by adaptation and why it is necessary?

15.4.1 What Is Adaptation and Why It Is Necessary?

What the studies described above tell us on the immune changes with aging? They clearly show that the aging organism is adapting/remodelling itself by acquiring new characteristics to respond to the continuous challenges of life from the internal as well as from the external milieu.

What does it mean for the innate immune system? Recently we introduced in the aging field the notion of what has been already known as the trained innate immunity (Kleinnijenhuis et al. 2012; Netea and van den Meer 2017). When we performed a longitudinal study in the elderly hip fracture patients we have seen that even after 12 month the innate immune cells (monocytes and neutrophils) made more inflammatory cytokines upon stimulation, while their specific functions decreased (Baëhl et al. 2016). This led us to consider that in aging what we call inflammaging may be nothing else but a form of trained innate immunity. Thus, it emerges as a necessary state to be able to respond to the constant aggressions. However, in order to prevent its conversion to a clinical disease state, it should be controlled as in case of semi-supercentenarians. These persons have (and somewhat control) a low grade, clinically not perceptible inflammation (inflammaging) maintained by various chronic challenges throughout life (Arai et al. 2015). However, this state helps them to react more efficiently than those who do not have this controlled inflammation or have no means to control their inflammation (hyperinflammation). This also means that this reinterpretation of the inflammaging suggests the possibility to intervene in different way that we have thought before; namely not only the anti-inflammatory treatment can be damageable but it can also decrease the lifespan. This tells us also that we need a highly specific optimization of these processes through aging to make them act for the prolongation of the healthspan of the elderly patients.

Recently it was also demonstrated that live cells are releasing different forms of microvesicles which may contain various substances from genetic material via (mi)RNA to infectious material (Prattichizzo et al. 2017; Takasugi 2018). These vesicles became an important means of intercellular commutation. They play an important role in health but may also serve as mediators of diseases which affect aged people such as cancers, infectious diseases, cardiometabolic and neurodegenerative diseases (Shah et al. 2018). There is still very little knowledge on these vesicles in healthy aging and in relation to the immune changes, but quite a lot is already known the above-mentioned diseases. Still, as it was very recently shown by Storci et al. (2018) the DNA (cytoplasmic/cell free) included in these vesicles are able to modulate the inflammaging process. Thus, these cell-derived vesicles may be an

important player in the modulation of the adequate adaptation of the immune changes with aging.

In the aging adaptive immune system, the previously described changes can lead to better functioning in certain cases, e.g. in the case of already encountered antigens (memory). Thus, accumulation of memory cells may be beneficial for containing the already encountered antigens, at the expense of these newly met. At the end of life the immune system would retain its ability to respond mainly to these (cognate) antigens, however if new antigens arise they should also be successfully handled, or the organism would die which from an evolutionary perspective can be very beneficial for the species.

Moreover, the end-stage differentiated T cells in many cases acquire innate characteristics either in their phenotypes or functions, making them more close to the innate immune system than to the adaptive immune system such as the infection stimulated innate CD8+T cells (Kverneland et al. 2016; Wang et al. 2017; Muraille and Goriely 2017; Park et al. 2016; Biswas et al. 2014). It may be conceptualised that old subjects may need more robust innate immune response that adaptive one in some circumstances, like infections or cancer.

Thus together, the well functioning immune system with aging in a homeodynamic system is able to adapt and maintain efficient response to already encountered antigens, perhaps also to new ones as the incidence of cancer is decreasing over 90 years of age (Salvioli et al. 2009); it may also mount an efficient vaccine response as shown for varicella zoster and influenza (Lal et al. 2015; Camous et al. 2018). Moreover, the controlled anti-CMV response and the inflammaging may be the key for longevity. However, too much of a good thing may be a bad thing, which means that the dysregulation of this adapted, remodelled and reasonably finely tuned immune system of the aged may lead to the apparition of the clinically manifest age-related diseases.

15.5 Could we Find Reliable Biomarkers Based on this New Concept?

If we consider the reinterpretation of the immune changes with aging as adaptative, many suggested biomarkers (mentioned above) may not be used as real biomarkers. We should thus look elsewhere and differently for significant biomarkers which would be surrogate of the dysregulation in this adaptation.

So, what could be the future biomarkers of the immunoadaptation and inflammaging? From what we know presently the level of response to vaccination may be a robust biomarker of this adaptation. The acquisition of some treats of the innate immune cells by the adaptive cells may also be robust biomarkers of the well-functioning immune system with aging. The vesicles specifically derived from monocytes could also become robust biomarkers of the innate immune functions in aging. The balance between the pro- and anti-inflammatory markers may also show some promises in this field. Epigenetic changes may be also included to the

arsenal of the future biomarkers in immune cells (Horvath et al. 2015; Declerck and Van den Berghe 2018). One promising future biomarker for the immune aging are the metabolic changes in various cell types either from the signaling aspect such as the mTOR pathway or from the mitochondrial point of view (Ferrucci and Fabbri 2018). The already used phenotype markers may be revisited mainly from the exhaustion/senescence aspect.

However, it would be hard to conceive that one or two biomarkers would be able to capture the whole adaptative process in the aging immune system and concomitantly the dysregulation of this adaptation. Thus, most probably a cluster of biomarkers which will be able to act as a composite biomarker for changes of the immune system with aging can be only proposed when properly identified (Morrisette-Thomas et al. 2014; Monti et al. 2017). It is also conceivable that some of the old biomarkers may be recycled. The community should be opened to eventually repurpose certain old biomarkers and add them to the new ones already in the pipeline or to be discovered, in order to strengthen the functions of the proposed composite. The most important is that they should be the surrogates of real changes and not the interpretation that we are making of these changes. In this way we will move forward the field to use in clinical practice these future biomarkers to decide on interventions/modulation of the system.

15.6 Conclusion

Immunosenescence, as considered presently, is characterized by a high degree of heterogeneity which seriously hampers the possibility to find reliable biomarkers. This heterogeneity is the result of the chronic stimulation of the immune system (antigenic stress) occurring lifelong (immunobiography). This stimulation is the most important cause of immunosenescence/inflammaging, characterized by a reduction of the immunological space and a shrinkage of T cell repertoire, largely substituted by quiescent antigen-experienced cells and innate cells activation at already the quiescent state. Consequently, many biomarker candidates exist but none of them presently reliably represents immunosenescence.

In the light of the new data concerning immunosenescence/inflammaging these can be mostly considered when they are regulated and under control as lifelong adaptations to the chronic antigenic stresses. This view thus will necessitate finding other biomarkers which will capture efficiently this new concept. Maybe some of the old ones such as pro-inflammatory cytokines may be recycled and integrated into new cluster of biomarkers or not. In any case we should look for multiple biomarkers. The findings of these new biomarkers will help to find better ways to optimise the immune system function with aging leading to better healthspan.

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Chapter 16
Immune Parameters Associated with Mortality in Longitudinal Studies of Very Old People Can Be Markedly Dissimilar Even in Apparently Similar Populations

Graham Pawelec and David Goldeck

Abstract While challenging, only longitudinal studies can pinpoint biomarkers dynamically associated with a selected outcome. In human studies of ageing, this challenge is particularly onerous, both in terms of the time required and the outcome selected. While recognizing the limitations of our approach, taking the most robust and unequivocal outcome (all-cause mortality), and taking a very old population as the starting point, we have sought to define peripheral blood "immune signatures" predicting incipient mortality. Studies in overtly similar populations in Sweden, Belgium and The Netherlands reveal certain constellations of immune biomarkers associated with all-cause mortality in people >80 years of age. Unexpectedly, however, these "immune risk profiles" are different in the different populations. Thus, it is unlikely that it will be possible to identify "one-size-fits-all" biomarkers of ageing in different populations, at least when solely focusing on parameters of immunity.

Keywords Immunosenescence · Longitudinal studies · Immune signatures · Immune risk profile · Cytomegalovirus · Inflammaging · Biomarker of aging · Mortality

16.1 Introduction

Untold studies have unequivocally demonstrated by numerous different analytical approaches that the composition and functionality of essentially all aspects of immunity in humans is different in young and old people (see Nikolich-Zugich 2018) for a recent extensive review). Understandably, most studies have been cross-sectional,

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comparing younger with older individuals and assuming that the differences observed were due to changes over time as the person aged. This is obviously challenging to prove, given the long human lifespan, although some ongoing studies are attempting to enable such a long-term aim to be achieved by planning to follow younger populations over their lifespan [for example, the Berlin BASE II study (Bertram et al. 2014)]. Even such long-term commitments to the goal of demonstrating changes over time may generate data that are only relevant to the circumstances of the particular population studied, as we will seek to show from the material reviewed here.

16.2 Immunosenescence, Inflammaging and Disease

The theoretical framework for hypothesizing that changes to the human immune system contribute to ageing, disease and mortality is simple: the vertebrate immune system is believed to have evolved to protect the host against invasive pathogens, so any degradation of this capacity with ageing (loosely termed "immunosenescence") would be expected to cause more susceptibility to infectious disease and death in old people. Additionally, it is hypothesized that age-associated changes to the immune system also contribute to tissue damage via autoimmunity or exacerbated chronic inflammatory states. Thus, unbalanced and dysregulated immune function in older individuals reflects a greater degree of the usual immunopathological side effects that always accompany immune activation, but are better regulated in younger individuals, who also have more resilience for dealing with the "collateral damage" caused by immune "friendly fire". Hence, immune-mediated damage is more widespread; so-called "inflammaging" together with immunosenescence, is believed to contribute to many non-infectious diseases of the elderly, such as neurodegeneration, cardiovascular and autoimmune diseases and cancer (Fulop et al. 2014). However, in most cases these assumptions have remained untested hypotheses and direct evidence supporting such contentions is sparse. Even in experimental animals, usually mice, longitudinalFCD8 studies allowing age-associated changes in immunity to be correlated with clinical outcome are rare- and even when such studies have been performed, data from these short-lived animals would not be expected to be directly translatable to humans. Not only are most studies carried out on inbred, specific pathogen-free strains, but if done on wild animals, mice have evolved very different survival and reproductive strategies which affect longevity. So how can we approach this problem in people? Only species-specific data would be meaningful, preferably from longitudinal studies. The few such studies in humans thus far published are mostly limited to following people very old at baseline for a few years and taking the unequivocal clinical endpoint of mortality within a time frame which is logistically possible to study. Other important and informative clinical outcomes such as frailty development or vaccine responsiveness would be very desirable but are much more challenging to define, standardize and investigate. There is no arguing with mortality. It is to be hoped that at least some of the large-scale human surveys currently acquiring data will indeed be continued long-term and that their coordinators will recognize the importance of

measuring immune parameters in addition to the more commonly-collected data, as we are attempting to do in the Berlin BASE II study (Bertram et al. 2014). It is also to be hoped that more representative data from countries other than Western Educated Industrialized Rich Democratic (Weird) countries (e.g. Alam et al. 2013) will add to our knowledge base on the contribution of immune ageing to health and longevity in older adults. Given our experience summarized below on superficially quite similar populations nonetheless demonstrating markedly different immune risk profiles, the lesson will be that multiple (so-far undetermined) heterogeneous variables will most likely need to be integrated into a holistic picture of individual ageing. Again, this is an aim of the Berlin BASE II study, collating data on diverse fields of genetics, medicine, psychology, socio-economics, cognition—and immunity (Bertram et al. 2014).

16.3 The Swedish OCTO/NONA-Immune Longitudinal Studies

To the best of our knowledge, the first attempt to establish an immune signature associated with for all-cause mortality emerged from biobehavioural studies in Sweden. We were privileged to take part in some of the immunological analyses performed in these pioneering OCTO-Immune longitudinal studies set up by Anders Wikby and colleagues in Jönköping, southern Sweden many years ago (Wikby et al. 1994) in the context of the EU project "T-CIA" (2003–2005, see https://cordis.europa.eu/project/rcn/67245/factsheet/en). This established some simple immune parameters associated with 2-, 4-, and 6-year mortality in non-institutionalized subjects 85 years old at baseline. This was the origin of the concept of the "Immune Risk Profile" (IRP), which initially comprised a cluster of functional parameters (poor proliferative responses of peripheral blood mononuclear cells to T-cell mitogens), accumulations of CD8+ T-cells which resulted in CD4:8 ratio <1, lower B-cell counts, and infection with the common human ß-herpesvirus HHV5 (Cytomegalovirus, CMV) (Wikby et al. 2005; Pawelec et al. 2009). Unusually for this type of study, participants were re-examined and re-sampled every 2 years, which enabled dynamic changes in parameters to be associated with outcome, and not simply baseline parameters. This study showed that 16% of the subjects were in the IRP groups at baseline but a further 15% moved into over the 8-year follow-up (Olsson et al. 2000). Other interesting findings from this seminal pilot study were that a minority of individuals in the IRP group at baseline survived by reverting to a non-IRP phenotype, and that independent risk factors for mortality reflecting "inflammaging" (IL 6 in particular) as well as cognitive impairment were actually more closely correlated with mortality than the IRP—participants with both clusters of risk factors survived least well (Wikby et al. 2005). For more details on the OCTO studies (free-living older adults selected for exceptionally good health) and the NONA studies (representative free-living populations NOT selected

for very good health, but nonetheless with a similar IRP), please see a recent review (Pawelec 2018).

Our concept of the IRP has not been exactly validated by others' studies (or our own, see below), although several publications have appeared using an inverted CD4:8 ratio and IL 6 levels (together) as an IRP under several different circumstances and without knowledge of its relevance to survival. One study coming close to the original IRP definition (i.e. also including B-cells) showed that the IRP did correlate with survival in a UK cohort (Huppert et al. 2003), whereas a Spanish study reported that a CD4:8 ratio <1 at baseline was not associated with 3-year survival (Formiga et al. 2014). In other contexts, some associations with survival have been found under very different circumstances, presumably reflecting the importance of immune control and dysregulation not limited to ageing, for example surviving nosocomial lung infections (Plonquet et al. 2011). The IRP was also reported to be informative in HIV-infected patients (not due to CD4+ T cell loss) (Ndumbi et al. 2015). When adjusting the arbitrary cut-off value of > or < unity for the CD4:8 ratio, closer correlations may indeed be seen, as for example, in another UK study where a CD4:8 ratio <1.7 versus >4 informative for survival (Spyridopoulos et al. 2016). These finding to some extent reflect our own, where we attempted to establish immune signatures associated with survival in Dutch and Belgian populations, as well as the ongoing work with the BASE II study (the latter ongoing and to be reviewed elsewhere).

16.4 The Dutch Leiden 85-Plus Study and the Leiden Longevity Study

The Leiden 85-Plus study surveyed the majority of individuals reaching the age of 85 years in the small Dutch city of Leiden (Lagaay et al. 1992). Illustrating the importance of biobanking precious materials, thanks to participation in the EU project "Lifespan" (see http://www.lifespannetwork.nl/) we were able to access cryopreserved peripheral blood mononuclear cells from individuals 89 years of age at sampling and for whom 8-year follow-up mortality data were already available. At this advanced age, there had been no survivors in IRP+ subjects from the OCTO/NONA cohort, presumably due to selection (Strindhall et al. 2007), so it came as no surprise that we could not replicate the Swedish findings as there were no Leiden 85-Plus subjects in the IRP group at 89 years of age (i.e. none with an inverted CD4:8 ratio). However, immune parameters strongly correlating with remaining 9-year survival emerged from these studies, namely the accumulation of late-stage differentiated CD8+ T-cells (essential component of the Swedish IRP) was associated with better survival in this cohort, provided that these cells responded in a pro-inflammatory manner to CMV antigens, with no anti-inflammatory component (Derhovanessian et al. 2013). This emphasizes the over-riding necessity to maintain adequate surveillance against latent CMV, which was also seen in the Swedish studies (Hadrup et al.

2006). This may seem to go against the grain of inflammaging, but another interesting finding from the Leiden 85-Plus study was that higher levels of suppressive CD4+ T cells (known as regulatory T cells, or Tregs) were also correlated with better survival—perhaps by limiting the side effects of the essential immunosurveillance against CMV (Jagger et al. 2014). The ever-resurgent theme of CMV control was also prevalent in our studies of the remarkable cohort in the Leiden Longevity Study (LLS). Individuals in the F2 generation with long-lived parents and grandparents were less likely to become infected with CMV and less likely to over-react to its presence when then did become infected (Derhovanessian et al. 2010). Hence, amongst all the other putative factors influencing immunoageing and its contribution to mortality, the role of genetics and CMV would always need to be taken into account, as further illustrated by studies on heritability in twins (Goldeck et al. 2016).

16.5 The BELFRAIL Study

Finally, the BELFRAIL study in northern Belgium was set up to examine factors predicting frailty in later life, and to correlate development of frailty with mortality (Vaes et al. 2010). As with many such studies, it was not established primarily to look for immunological parameters, but in collaboration with Prof. Mathei, a substudy to phenotype immune cells in the blood was established. First results were indeed able to correlate immune parameters with the development of frailty (Adriaensen et al. 2015); thereafter, it became possible also to show determine correlations with 3-year mortality (Adriaensen et al. 2017). As with the Swedish studies, in this Belgian cohort, many subjects did have a CD4:8 ratio of <1 at baseline, but far fewer than in OCTO/NONA (7% rather than 16%). Another difference was the preponderance of individuals (over 30%) with a CD4:8 ratio >5, which was only seen in a few Swedish subjects. This was caused by a dominance of naïve CD4+ T cells, without any less CD8+ T cells. Correlations with survival in BELFRAIL were completely different from OCTO/NONA: in BELFRAIL, those with a CD4:8 ratio >5 were more frail and 3-year survival was lower—but only in women (Adriaensen et al. 2017)! There was absolutely no effect in men. To us, even more surprisingly, CMV-seropositive women with a CD4:8 ratio <1 (i.e. those who would have been in the Swedish IRP group with worse 2, 4 and 6-year survival) were the ones who survived best in BELFRAIL. This was in stark contrast to CMV-seronegative Belgian women with a CD4:8 ratio >5 who did worst (Adriaensen et al. 2017). These results are clearly at odds with those from OCTO/NONA. The resolution of this conundrum awaits further studies in the Belgian and other populations, but clearly indicates the heterogeneity and context-dependency of immune system-ageing-longevity trajectories in free-living people in the real world. We hope that our BASE-II study may shed further light on this matter, albeit with the proviso that such data may also only be relevant to people in Berlin now, and not necessarily for those elsewhere, or even in the same place in future.

16.6 Southern Italian Cohorts

To further contribute to knowledge about the impact of immune signatures on healthy ageing we studied a group of elderly from southern Italy which showed the common signs of immune ageing when compared to young individuals with 30% having a CD4:CD8 ratio ≤1 in contrast to 0% among the young. However, when we selected a group of age-matched centenarian offspring we found a higher CD4:CD8 ratio compared to the controls (due to higher frequencies of CD4+ T-cells and lower CD8+ T-cells) (Pellicano et al. 2014) Further, the frequencies of naïve T-cells were higher in the offspring compared to the controls and thereby closer to those of young individuals. Together with a fitter compartment of naïve B-cells (Colonna-Romano et al. 2010) this may reduce susceptibility to new infections in the offspring population and as a consequence an increased life span can be expected. This hypothesis awaits confirmation in follow-up studies. Age-related non-communicable diseases are also correlated with distinct immune signatures, such as increased frequencies of late-differentiated T-cells and a CD4:CD8 ratio <1, as observed in Alzheimer's disease patients compared to age matched controls (Pellicano et al. 2012).

16.7 CMV and the IRP in Other Ageing Studies

A recent study reported that serum IL-6 levels, but not CMV serology, were predictive of pre-frailty among the very old (Cao Dinh et al. 2018). This is consistent with earlier findings from the Newcastle 85+ study, where no correlation of frailty with the IRP (CD4:CD8 ratio <1) but only with inflammatory markers, such as IL-6 was found in a cohort of octogenarians. In contrast, reminiscent of the findings in BELFRAIL, memory/naïve T-cell and B-cell ratios showed the opposite to the results of the Swedish studies (Collerton et al. 2012). However, these studies had not taken mortality as the end point, but in later work on this cohort, Spyridopoulos et al. found that cardiovascular mortality was linked to CMV seropositivity and T-cell senescence (Spyridopoulos et al. 2016). These results are consistent with the earlier large National Health and Nutrition Examination Survey (NHANES) III which also determined that CRP and CMV seropositivity were risk factors for both all-cause and cardiovascular mortality (Simanek et al. 2011). An example that taking frailty or mortality as end-points can lead to opposite findings is also provided by the BELFRAIL study described above, where CMV seropositivity was negatively associated with frailty (Mathei et al. 2011). On the other hand, high anti-CMV titres, but not CMV serostatus per se, were associated with an increased risk for all-cause mortality (Mathei et al. 2015). The striking differences between the sexes in the BELFRAIL study also make clear that women and men need to be studies separately in this type of analysis. The Women's Health and Aging Studies (WHAS) I and II showed that IL-6 levels independently associated with prevalent frailty (Leng et al. 2007) and mortality at follow-up, currently under investigation.

16.8 Conclusions

Long-lived, free-living human populations are so heterogeneous in so many different ways that identifying robust immune biomarkers of ageing may well prove impossible. The same is likely to be true for any other biomarkers of ageing. Despite numerous attempts, little progress has been made in this respect ("While there are several candidates for biomarkers of aging, none have so far proven a true measure of the underlying aging process. A true biomarker of aging must meet certain criteria in order to be both accurate and useful …." see https://www.afar.org/infoaging/biology-of-aging/biomarkers-of-aging/), but the belief remains that collecting sufficient "big data" to cover large numbers of variables will eventually allow sufficient accuracy to be attained (Xia et al. 2017). As illustrated in this chapter, however, thus far, marked immune biomarker differences in several overtly similar European populations, suggest that establishing such biomarkers will remain a challenge for the foreseeable future. How and whether to intervene to modulate aged immune function in the elderly is therefore a crucial question, given this variability. A "one-size-fits-all" approach across different populations in different countries and of different ages is unlikely to be appropriate. Nonetheless, certain parameters do seem to be emerging as lowest common denominators in our and others' studies (Pawelec 2017) is that older people everywhere have low amounts of naïve CD8+ T cells and thus could have holes in their antigen recognition repertoires which might contribute to susceptibility to new pathogens. Developing technologies could theoretically identify such holes in the repertoire in advance and use receptor gene transfer to specifically compensate.

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Chapter 17
Gut Microbiota and Aging

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Abstract Gut microbiota is an up-to date theme that is widely studied. Now it is obvious that microbiota could be considered as one of the factors affecting aging process. Some reports suggested the potential mechanisms by which gut microbes may influence human health span. Aging-related conditions are accompanied by decline in immune system functioning and low-grade inflammation, and our microbes play a certain role in these processes. Gut microbiota composition changes during the aging process may indicate a more pronounced aging-related health loss.

Keywords Microbiome · Gut microbiota · Longevity · Butyrate · Aging-associated diseases · Short-chain fatty acids · Low-grade inflammation · Inflammaging · Lipopolysaccharide

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17.1 Introduction

The aging process is a reflection of the interaction between many factors including chronological ageing. Aging is a very individual process; it varies among the world population. This could be one of the causal factors contributing to the differences in the results of studies on microbiota in different age groups. Gut microbiota could be considered as one of the factors affecting the development of age-associated changes. Previously it was believed that the primary gut colonization occurs after birth, and the initial skin colonization—during the passage of the baby through the mother's birth canal. However, recent studies show that bacteria are present in meconium, amniotic fluid, placenta and cord blood (Jimenez et al. 2005; Moles et al. 2013; Aagaard et al. 2014) even in the absence of pathogenesis. There is a growing evidence that microbiota establishment starts in utero and largely depends on mother's nutrition, health (Canani et al. 2011; Almeida Morais et al. 2014) and stress level (Zijlmans et al. 2015). The "proper" infant intrauterine colonization is associated with further child's health (Scheepers et al. 2015). By 2–3 years, depending on the mode of delivery, nutrition and other factors, the so-called microbiota taxonomic "core" is established. The core (forming around 60–70% of the total microbial abundance) remains relatively stable throughout the rest of life (Bergstrom et al. 2014). The highest diversity is observed approximately in adolescence after puberty (Arumugam et al. 2011). The gut microbiota is a fairly stable ecosystem. However, as the body ages, gastrointestinal tract also changes: elderly people often suffer from constipation, difficulties with chewing and swallowing; taste and olfactory receptors sensitivity decreases, diet behavior and preferences change. At the same time, the microbiota composition undergoes some changes in the elderly. Delayed evacuation leads to an increased endotoxin level, accumulation of bacterial proteins and their fermentation products (Biagi et al. 2012). As it known, the low-grade inflammation and aging process are closely related via the so-called "inflammaging", which is the basis of non-communicable diseases. Thus, the gut microbiota should be considered as one of inflammation initiators.

According to some authors (Claesson et al. 2011; Tiihonen et al. 2008), the number of opportunistic pathogens in the gut increases with age. This phenomenon is also associated with the low-grade inflammation, senile asthenia or "frailty", increased intestinal mucosa permeability and decreased synthesis of short-chain fatty acids (SCFA). It is believed that the gut microbiota diversity is decreased in the elderly as well as the diversity of specific taxa linked to health benefits such as Bifidobacterium (Wang et al. 2015). There is evidence of a positive effect of probiotics in the elderly: certain strains of Bifidobacterium lactis improve granulocyte activity in elderly; Lactobacillus pentosus promotes the production of class A immunoglobulins and reduces the risk of respiratory infections (Shinkai et al. 2013). However, aging is an individual, multilevel and complex process. For example, according to a study conducted by Biagi et al. (2010), there is an increase in the levels of anti-inflammatory Eubacterium limosum bacteria among centenarians.

Recent studies suggested that the maintenance of the "youthful" or "healthy" gut microbiota composition during aging may delay or limit immunosenescence (Biagi et al. 2013). For instance, fecal microbiota transplantation from aged mice to germ-free younger ones promotes inflammation in the small intestine in these mice, enhanced leakage of the inflammatory bacterial components into the circulation, increased T cell activation, and therefore leads to "aging" of the immune system (Fransen et al. 2017).

17.2 Gut Microbiota and the Low-Grade Inflammation

Gut microbiota can influence our immune response by different ways including release of such molecules as lipopolysaccharide, peptidoglycans, lipoteichoic acids, and others. Lipopolysaccharide (LPS) is a component of gram-negative bacteria membrane that plays a role of endotoxin in the human body. It is constantly presented in the blood at low "physiological" concentration. LPS maintains the balance of the immune system (Liu and Redmon 2001). Excessive LPS concentration in the blood leads to endotoxemia, low-grade inflammation, endothelial dysfunction and other inflammatory conditions. TLR, in particular TLR4, are specific for LPS receptors. They are present in adipose tissue, membranes of monocytes, macrophages, myeloid, endothelial, mast cells, and intestinal epithelial cells (Fitzgerald et al. 2004). Membrane proteins (CD14, CD18, selectins, etc.), lipopolysaccharide binding proteins (LBP) are also sensitive to LPS.LBP, LPS and CD14 form a high affinity receptor complex. Depending on the form of CD14 (soluble or membrane-bound), the complex either binds to high-density lipoproteins (HDL) (soluble form), after which LPS is eliminated, or binds to the MD2 protein (membrane-bound), which increases the affinity and stability of the entire CD14/TLR4/MD2 complex (Fig. 17.1) (Tsukamoto et al. 2010; Gangloff et al. 1999). The signal passed through the complex to the cell initiates the synthesis of interleukins (IL), Il-1, IL-6, IL-18, tumor necrosis factor α (TNF-α), type I interferon, chemokines, and cytokines activation that induce differentiation of type I T-helpers and interleukins IL-12, IL-23, IL-27 (Gangloff et al. 1999; Kashtanova 2015). It should be noted that CD14 are also sensitive to the components of gram-positive bacteria—lipoteichoic acids (LTA) and peptidoglycans. They also serve as TLR type 2 and type 4 receptor ligands and can trigger a cascade of inflammatory reactions (Belkaid and Hand 2014).

17.2.1 Gut Bacteria and Serum Amyloid A

Serum amyloid protein (SAA) serves as a mediator of inflammation due to cytokine-like properties. SAA increase is associated with obesity, glucose metabolism impairment, and cardiovascular diseases (Faty et al. 2012). SAA increases cholesterol uptake from HDL cholesterol by macrophages, leading to the transformation of these

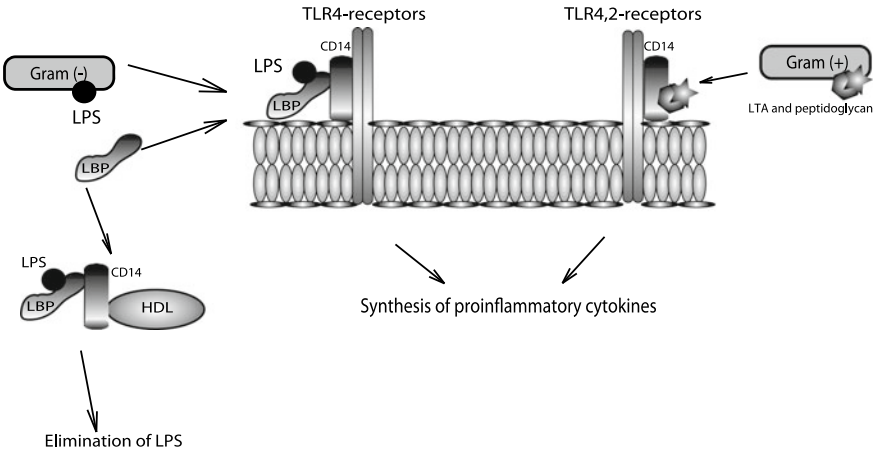

Fig. 17.1 Participation of lipopolysaccharides, peptidoglycans and lipoteichoic acids in the inflammation induction. *Note* Gram (−)—Gram-negative bacteria; Gram (+)—Gram-positive bacteria; LPS—lipopolysaccharide; LBP—LPS-binding protein; TLR—Toll-like receptors, LTA—lipoteichoic acid

lipoproteins into pro-atherogenic (Simons et al. 2013; Tsun et al. 2013). Expression of SAA (in particular SAA3) is also partially regulated via TLR signaling pathways and can be a response to high concentrations of LPS. It is noted that SAA expression is much lower in sterile mice than in wild (Migita et al. 1996). Probably, gut microbiota may initiate inflammation via this mechanism too.

17.2.2 Gut Microbiota and Suppression of Low-Grade Inflammation

Representation of certain bacterial taxa in the gut microbiota has an inverse correlation with proinflammatory cytokines levels. For example, some bacteria in Clostridium XIVa cluster are in inverse correlation with serum IL-6 and IL-8 (Biagi et al. 2010; Collins et al. 1994), and Faecalibacterium prausnitzii and Lactobacillus paracasei—with IL-8 and the IL-10/IL-12 ratio (Sokol et al. 2008).

17.2.3 T-Regulatory Cells and Gut Microbiota

T-regulatory cells inhibit immunocompetent cells activity (effector T cells, dendritic cells, macrophages, neutrophils) and control the immune response. In addition, they have an anti-atherogenic effect (Sanchez and Yang 2011; Ma et al. 2010). T-regulatory cells also control the immune response against viruses, bacteria, fungi, and par-

asites, prevent abnormal reactions of the immune system to commensal bacteria (Danikowski et al. 2017; Piccirillo and Shevach 2004). There is evidence that the use of probiotics increases the number of receptors to T-regulatory cells and decreases proinflammatory cytokines level (Pronio et al. 2008; Lorea Baroja et al. 2007).

17.2.4 Short-Chain Fatty Acids and Gut Microbiota

SCFA (acetic, propionic, butyric, isobutyric, isovaleric acids) are produced mainly by anaerobic bacteria. Butyric acid levels are the reflection of the gut well-being. They help to suppress putrefactive processes, maintain the microbiota balance, participate in motor activity regulation, and perform a detoxification function (Kumari et al. 2013). SCFA also have an anti-inflammatory activity, they inhibit the NF-kB (nuclear factor κB) activity, reduce proinflammatory interleukins synthesis. Some of Firmicutes, Eubacterium rectale/Roseburia and Faecalibacterium prausnitzii and others produce SCFA (Sokol et al. 2008; Kumari et al. 2013). SCFA consumption as well as high-fiber diet decrease the level of proinflammatory cytokines. Studies show that in the absence of GPR43 (G-protein-coupled receptors), chronic inflammatory processes are purely resolved in mice (Vernia et al. 1995; Harig et al. 1989).

Disruption of inflammation processes regulation was also noted in sterile mice with normal GPR43 quantity but lacking SCFA-producing bacteria (Maslowski et al. 2009). A proinflammatory dysbiosis, together with the decreased butyrate production in the gut, has also been linked to an increased risk of colorectal cancer (Ou et al. 2012; Candela et al. 2011). Also, lower butyrate levels may impact insulin sensitivity and energy expenditure by affecting mitochondrial activity (Gao et al. 2009). Further studies of the gut microbiota composition and metabolic activity is important for understanding the immune system functioning and the involvement of inflammation in the development of age-associated diseases.

17.2.5 Gut Permeability

Aging is associated with profound changes in gut health including increased intestinal permeability (Man et al. 2015; Nicoletti 2015). Some basic science and pre-clinical studies have previously suggested that gut permeability and bacterial translocation, coupled with altered microbiota profiles (Clark et al. 2015; Rera et al. 2012; Thevaranjan et al. 2017), may drive age-related increases in systemic low-grade inflammation. Thevaranjan et al. reported the increased permeability in a part of the gut containing the highest number of microbes, suggesting that greater intestinal permeability could allow bacteria or bacterial products into circulation (Thevaranjan et al. 2017). Recently microbiota profiles in serum between healthy young and older adults had been evaluated by T. Buford et al. Researchers found that serum of older adults contained fewer species representing a lower diversity than that of young adults as

well as some other features of serum microbiota in the elderly (Buford et al. 2018). Although the jury is still out on whether these microbes are viable.

17.3 Frailty and Gut Microbiota

Frailty syndrome is the most problematic result of ageing that is rapidly growing worldwide. Frailty is a geriatric state of reduced physiological reserve and increased vulnerability to stress that occurs when physiological systems are interrupted due to the accumulation of cellular damage and exceeded homeostatic reserve. It was shown that frail patients have impaired motility, compromised gallbladder function, and fasting or postprandial alterations in cholecystokinin and insulin release (Serra-Prat et al. 2009, 2013). Gut equilibrium consisting of the gut barrier, immune system, enteric nervous system, microbiota and its metabolites, and digestive system as well plays a pivotal role in the inflammageing and immunosenescence processes and in further development of functional deficits. Qi et al. recently showed a correlation between frailty and high serum levels of zonulin, a single chain protein corresponding to pre-haptoglobin 2, which opens tight junctions and is correlated with increased intestinal permeability (Qi et al. 2017). High intestinal permeability may explain the susceptibility to severe infections including sepsis in frail elderly patients. Also it was reported that both apoptosis and proliferation are increased in the elderly (Ciccocioppo et al. 2002).

Study comparing community-dwelling and long-term residential care elderly subjects showed a substantial impairment of the gut microbiota community-dwelling elderly along with a diet high in fat and low in fibers (Claesson et al. 2012). Gut microbiota composition can also be affected by medications intake, in particular, proton pump inhibitors, which are very commonly prescribed in the frail elderly. Proton pump inhibitors are associated with higher abundance of Streptococcaceae and lower microbial diversity as well as C. difficile infection (Jackson et al. 2016; Leffler and Lamont 2015).

There is a link between gut microbiota and cognitive decline [e.g., memory is impaired in germ-free mice, in contrast to conventionally reared mice (Gareau et al. 2011)] and neurodegenerative disorders including Alzheimer's and Parkinson's diseases. For instance, α-synuclein is presented in plexuses of the enteric nervous system prior to their appearance in the brain (Felice et al. 2016), and Parkinson's disease progression is associated with previous gut dysbiosis (Minato et al. 2017). LPS injections in mice cause an increase of amyloid-β in the hippocampus, along with cognitive deficits (Kahn et al. 2012). Recent study showed that amyloid-positive patients had higher proinflammatory cytokines levels along with a lower anti-inflammatory cytokines levels, and lower E. rectale levels, higher abundance of Escherichia/Shigella genera (Cattaneo et al. 2017). Notably, centenarians, outstanding examples of healthy aging, were shown to carry an increased fraction of health-associated gut bacteria, thus suggesting a possible role of the microbiota in healthy ageing (Santoro et al. 2018). Specific compositional gut microbiota peculiarities of

naked mole-rat (another examples of healthy aging or even negligible senescence) were shared with human gut microbial ecosystems of centenarians, considered as models of a healthy gut microbiome. Naked mole-rat fecal samples are enriched with short-chain fatty acids and other carbohydrate degradation products compared to human samples (Debebe et al. 2017).

17.3.1 Aging-Associated Conditions Treatment and Microbiota: Pro/Prebiotics

Due to the remarkable influence of the gut microbiota on the process of immunosenescence and inflammaging, it can be supposed that the gut microbiota may affect well-being and human lifespan and even be a target for anti-aging intervention (Ottaviani et al. 2011). There is evidence that probiotics may promote longevity in mice, possibly through low-grade inflammation suppression in the colon (Matsumoto et al. 2011). Probiotics showed efficacy in the reduction of common respiratory tract and gastrointestinal infections duration in elderly population in a multicenter placebo-controlled double-blinded study (Guillemard et al. 2010). Remarkable results of studies and meta-analyses have been shown for probiotics using in the prevention and treatment of Clostridium difficile-associated diarrhea in the elderly (Hickson et al. 2007; Kee 2012). In the elderly, pro- and prebiotics also have been suggested to mitigate constipation, which is also associated with inflammation, elderly delirium, and some other conditions (An et al. 2010; Carlsson et al. 2009; Pitkala et al. 2007). Published by Matsumoto et al. study (Matsumoto et al. 2011) in which mice were supplemented with Bifidobacterium probiotics during 11 months, showed an increased longevity in these mice compared to control group. Scientists link this effect to the lower levels of inflammation, especially in the gut. Although data are limited, the gut microbiota modulation by pro- and prebiotics may serve to reduce age-associated conditions. It has been even hypothesized that probiotic strains isolated from healthy elderly or centenarians may become the potential candidates for probiotic therapy intended for healthier aging (Yang et al. 2009; Shen et al. 2011). Moreover, there are a lot of insights in understanding the microbiota composition. Probably new bacteria that is difficult to cultivate such as Akkermansia (Zhang et al. 2018) would be used as probiotics to treat age-associated disorders.

17.3.2 Aging-Associated Conditions Treatment and Microbiota: Fecal Microbiota Transplantation

In line with pre- and probiotics, a novel approach called fecal microbiota transplantation (FMT) is widely discussed nowadays. FMT is also known as a stool transplant, is the process of transplantation of fecal bacteria from a healthy individual into a recipi-

ent gut. FMT showed an incredible efficacy against a Clostridium difficile-associated infection (CDI) (Moayyedi et al. 2017; Chen et al. 2018) and is actively examined as an inflammatory bowel diseases treatment (Verstockt et al. 2018; Costello et al. 2017). FMT applicability in CDI treatment is extensively studied in randomized controlled trials. Frozen capsulized fecal microbiota transplantation also seems to be shortly implemented in daily clinical practice in elderly patients with CDI (Tacke et al. 2015).

Recent data indicate a therapeutic FMT potential in metabolic disorders management. E.g., mice inoculated with the microbiota from the obese twin had increased adiposity and significantly decreased bacterial diversity compared to mice transplanted with the microbiota from a lean twin (Ridaura et al. 2013). Recently it was showed that FMT from healthy lean donors to obese type 2 diabetes patients resulted insignificant increase in insulin sensitivity, gut microbiota diversity in the allogenic versus autologous group (Vrieze et al. 2012). There is an evidence that gut microbial dysbiosis may also play an important role in neurological disorders, and several case reports have suggested a beneficial role of FMT in treating constipation and non-GI symptoms in patients with neurological disorders, which are very common in elderly (Aroniadis and Brandt 2013). FMT may protect mice with Parkinson's disease by reducing the microglia activation in substantia nigra, and reducing expression of TLR4/TNF-α signaling pathway components in gut and brain (Sun et al. 2018). Despite this, FMT should be treated with caution in the elderly due to the high risk of disease transmission.

17.4 Association Between the Gut Microbiota Composition and Aging Biomarkers

Recently we had studied an apparently healthy cohort and reveal that vascular changes may serve as a sign of the so-called biological age (Fedintsev et al. 2017). In part of this cohort we also evaluated the gut microbiota composition. Patients from Moscow and Moscow Region (Caucasians) aged from 25 to 76 years old who had passed a preventive outpatient examination in the FGBI National Research Center for Preventive Medicine (Moscow) were included in the cross-sectional study (Kashtanova et al. 2017). The study included 92 subjects aged 25–76 years. The average age of the participants was 57 ± 34 years. The proportion of men was 29% (n = 28), women—71% (n = 69).

For stool samples collected from the subjects, sequencing of the variable V3-V4 16S rRNA gene regions had been performed with and MiSeq sequencer according to the manufacturer's recommendations. Quality filtering of reads and taxonomic classification were performed using QIIME Software v 1.7 (Caporaso et al. 2010). Taxonomic composition of the samples was evaluated using reference-based approach according to the Greengenes v. 13.5 database (http://greengenes.secondgenome.com/downloads/database/13_50) using RDP Classifier. As a result of the classification,

operational taxonomic units (OTUs) relative abundance levels were determined. All statistical analyses were performed in R programming language (version 3.1.0). Statistical comparison of the groups of samples was performed using Mann-Whitney tests and generalized linear models (sex and age adjusted) (Zhang et al. 2011).

The phyla dominant in the samples were Bacteroidetes (12.7 ± 9.86% of total microbial abundance) and Firmicutes (57.09 ± 13.6%). About 50% of the total microbial abundance was represented by five genera: Blautia, Bacteroides, Prevotella, Faecalibacterium, and Clostridium. Blautia was the most dominant genus.

Subclinical atherosclerosis assessment was carried out by using the Q-LAB special application program for duplex scanning of extracranial brachiocephalic arteries in B-mode with parallel ECG recording. The standards proposed by the experts of the European Society of Hypertension and the European Society of Cardiology were used to assess common carotid artery (CCA) intima-media thickness (IMT). IMT <0.9 mm was considered normal; increased thickness was 0.9–1.3 mm. IMT thickening of CCA >1.3 mm or a local increase in IMT of 0.5 mm or a 50% increase in nearby IMT was defined as atherosclerosis. Local IMT thickening >1 mm, which caused stenosis of the lumen but did not affect its internal anatomy, was considered as a plaque (Stein et al. 2008).

The higher Blautia (GLM, $p = 0.004$) and Serratia ($p = 0.009$) abundance levels were, the greater the intima-media thickness was. The average value of the stenosis was 20 ± 20.12%. The carotid artery stenosis was directly associated with high abundance of Serratia.

PWV measurement was performed by using the SphygmoCor device. Pulse waves were recorded consistently with high precision applanation tonometer, which was superimposed on the proximal (carotid) artery and then, with a short interval, on the distal (femoral) artery. ECG was simultaneously recorded. PWV value of 10 m/s and higher was considered as increased (Wilkinson et al. 2010). The average value of PWV was 10.9 ± 2.6 m/s. PWV of more than 10 m/s was observed in 55 participants. PWV was associated with age, SBP, levels of urea, glucose, and glycated hemoglobin. There were no significant differences found at the first examination in patients with different PWV. After the separation of patients and studying the relationship between PWV and microbiota composition in subjects without type 2 diabetes, it has been found that the representation of Bacteroides was significantly higher in patients with PWV ≥ 10 m/s ($p = 0.0001$).

In this study we have shown the differences in the composition of the gut microbiota not only in patients with atherosclerotic vascular lesions but also with vascular wall rigidity.

Similar results were obtained in the studies of world populations (Klimenko et al. 2018). The gut microbiota diversity index was quite high. Shannon index takes into account not only species richness but also the uniformity (equitability of species according to their abundance). High diversity of the gut microbiota shows its stability as an ecosystem. The examined patients did not have severe disorders or diseases. However, we did not find any differences in the diversity between the groups with subclinical atherosclerosis or rigid vessels and with intact vascular wall.

The most represented genera in the samples were Blautia, Bacteroides, Prevotella, Faecalibacterium, and Clostridium. Blautia genus (Blautia coccoides in particular) was the most abundant one.

Establishing whether any structural or functional configurations of the gut microbiota are causally related to a given physiologic or disease phenotype is challenging. We attempted to recruit a homogeneous group of healthy individuals to analyze the interrelations between the gut microbiota composition and early vascular changes.

In this study Blautia was associated with IMC thickening. Blautia coccoides belongs to a class of gram-positive anaerobic bacteria (Clostridia, Firmicutes phylum). Recently, after a phylogenetic analysis of these bacteria, Clostridium coccoides were renamed to Blautia coccoides gen. nov (Liu et al. 2008). According to the Tuovinen et al. study, representatives of Blautia coccoides genus can activate TNFa and cytokines secretion in human gut, and interleukin-8 secretion to an even greater extent than the lipopolysaccharide of Gram-negative bacteria (Tuovinen et al. 2013). Also we had shown in the previous study that these bacterial taxa were associated with glucose intolerance and type 2 diabetes mellitus (Egshatyan et al. 2016).

The relative abundance of Serratia was higher in patients with higher CRP levels, IMC thickening, and stenosis of the arteries. Serratia is a genus of Gram-negative, facultatively anaerobic, opportunistic pathogens of the Enterobacteriaceae family. These bacteria contain lipopolysaccharide (LPS) also known as endotoxin. Its release occurs during the physiological microorganisms' destruction and membrane components synthesis. LPS is presented in blood at physiological concentrations required to maintain the normal functioning of the immune system and increase nonspecific resistance against infections and tumors (Liu and Redmon 2001). Increased concentration of LPS triggers the low-grade inflammation and this leads to endothelial dysfunction and other inflammation related conditions.

Bacteroides genus (belonging to Bacteroidetes phylum) was more abundant in donors with high PWV. Moreover, in this analysis, we excluded patients with initial stage of type 2 diabetes, and thus eliminated the influence of other factors. Interestingly, the fraction of these bacteria in the gut has been laid by some researchers as the "drivers"—the basis for determining the enterotypes or "fecotypes". The "Bacteroides fecotype" was associated with the so-called "Western diet" rich in animal proteins and fats (Ou et al. 2013; Wu et al. 2011). Bacteroides is also a genus of Gram-negative bacteria and some species of this genus are opportunistic human pathogens. According to the recent study, Bacteroides have genes, which trigger an inflammatory response (Tyakht et al. 2018). The results suggest potential links between the gut microbiota, inflammation and vascular walls. This concept requires further clarification.

The world of our small inhabitants seems to be a whole universe. We know not so much about them but their potential to affect our health also seems to be huge. Microbiota may modulate aging-associated changes in immunity and play a certain role in sarcopaenia development, and cognitive function, all of which are elements of frailty and aging process itself. The "number one" challenge for medicine now is to promote health span and reduce the duration and severity of aging-related diseases. The current preventive and therapeutic management, centered on the control of clas-

sical risk factors, is obviously insufficient to stop the pandemic of ageing diseases. Therefore, the search for new biomarkers is a priority in the modern scientific world. Microbiota may become a novel target to prevent early aging or even to manage some age-related disorders—as well as provide valuable features for predicting individual ageing trajectory.

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Part V
Systems Biology of Aging, Biological Age and Mortality Markers

Chapter 18
Deep Integrated Biomarkers of Aging

Polina Mamoshina and Alex Zhavoronkov

Abstract Recent advances in deep learning (DL) and other machine learning methods (ML) and the availability of large population data sets annotated with age and other features led to the development of the many predictors of chronological age frequently referred to as the deep aging clocks (DACs). Many of these aging clocks were pioneered by Insilico Medicine and are available for testing *online* via www.young.ai, www.aging.ai, and other resources. Several of the DACs demonstrate biological relevance and can be used for a variety of applications ranging from aging and disease target identification, identifying the age-related features implicated in diseases, the inference of causal graphs, analyze the population-specificity of the data type and analyze the mechanism of drug response and many others. The generative adversarial networks (GANs) can be used to synthesize new data of patients of specified ages in large volume providing for novel target identification techniques, as well as for anonymization and patient privacy methods. In this chapter we provide a brief overview of the DACs, their advantages, and disadvantages as well as the commentary on the challenges in this emerging field.

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18.1 Introduction

In recent years deep learning (DL) systems outperformed humans in multiple tasks including image and text recognition as well as in the game of Go. The advantage of these systems is in their ability to learn and generalize from a large number of examples LeCun (2015). DL methods rapidly propagated into the many biomedical applications starting primarily with the imaging, text and genomic data Mamoshina (2016), Zhavoronkov (2018). Recently, the DL technologies started propagating into biogerontology and converging with the bioinformatics of aging. Some of the flagship industry conferences including the 6th annual Aging Research for Drug Discovery in 2018 included many presentations of the applications of DL in aging research with the many biomarkers developed using the transcriptomic and imaging data types presented for the first time Bakula (2018). One of the main areas where DL is impacting aging research are aging biomarkers and geroprotector discovery Zhavoronkov (2019). The availability of large volume of data and new algorithms made it possible to use DL to start making predictions about the activity and pharmacological properties of small molecules Aliper (2016), identify mimetics of the known geroprotectors Aliper (2017) and discover new ones Zhavoronkov (2014), Aliper et al. (2016). The new techniques in DL converging with the advances in chemoinformatics enable the creation of completely novel molecular structures with the desired properties for the protein targets of interest in record time Kadurin (2017b), Putin (2018a), Polykovskiy (2018), Putin (2018b). Many efforts are on the way to use DL for predicting the outcomes of clinical trials Miotto (2016), Artemov et al. (2016). When integrated into an end-to-end drug discovery pipeline, these tools to enable rapid target identification for aging research (Fig. 18.1).

The main strategy for testing the effectiveness of an intervention in aging and longevity is to perform lifespan experiments in model organisms such as mice or perform a longitudinal study in humans. These studies are generally lengthy and do not always capture the effects of the intervention on a specific system, organ or data type. The development of highly accurate biomarkers of aging will help accelerate these studies and eliminate the need to wait until the end of life to measure the effectiveness of the intervention. Since 2013 the field of biomarkers of aging has exploded with the many predictors of the human chronological age developed using different data types demonstrated spectacular accuracy. The term “aging clock” is commonly used to describe such predictors. While the biological relevance of these clocks is being established some of these predictors can be used to predict the disease status and mortality with the subjects predicted to be substantially older than their chronological age having a higher probability of a health problem or mortality than the subjects predicted to be younger or close to their actual age.

The first aging clock was introduced by Steve Horvath in 2013. In his work, he used DNA methylation data and a simple linear regression model with ElasticNet regularization to predict the age of individuals with a mean absolute error of 2.7 years. Since then, a number of aging clocks has been proposed Zhavoronkov (2018). However, most of those clocks were developed using conventional statistical and

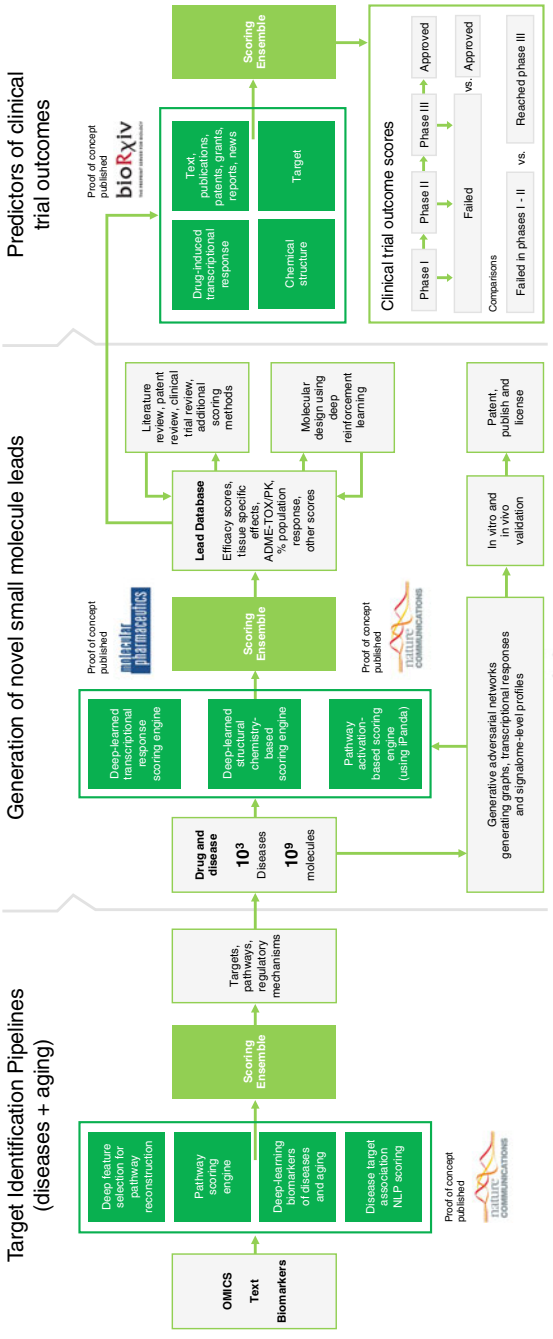

Fig. 18.1 Example of the end-to-end drug discovery pipeline for aging research using DL methods

ML methods such as simple linear regression and principal component analysis fitted to a limited number of data sets. Although ML methods are now routinely used in various research fields, more powerful approaches are needed to capture high-level dependencies between biomedical data and age. While there are many promising next-generation data analysis techniques, DL is gaining popularity in biomedicine. In the next sections, we cover the key principals of DL and summarize the studies describing the various applications of DL for age prediction and development of the biomarkers of aging.

18.2 Basic Concepts of DL

The renaissance in the field of ML was mainly driven by the increased computer processing power, availability of large data sets, and advances in algorithmic technologies and frameworks. This section will provide a brief overview of the main architectures used in biomarker development and basic principles. One of the most commonly used architectures in data analysis, feedforward neural networks, is a model that receives a vector of features on the input layer and returns an output vector. The deep neural network has multiple hidden layers between the input and output layers. Each layer is consisting of units with activation functions that receive information, combine it with certain weights and pass it to units in the next layers. Activation functions determine whether the input signal is going to influence the output of the network. Multilinearity of deep neural networks combined with nonlinear activation functions provides them with exceptional ability to extract complex dependencies in the data and automatically extract features most relevant to predictions. When networks are trained, unit weights are updated for the network to predict target values as close to the actual output as possible. In the case of age predictions, networks are trained using biological data as input to predict the age as accurate as possible. Giving their architecture, networks can be trained to predict age on multiple data types simultaneously. This can be achieved by training a set of networks on individual data types and combining them into an ensemble of models or by training a network on the input vector of multiple data types (Fig. 18.2). This multi-modal aging clocks trained on the multiple data types can help evaluate the importance of the individual data type as well as the features within the data type and across the data types paving the way for a new field of data economics.

Together with age prediction as a single output, a neural network can be used to produce a vector of multiple outputs, classes for example, or even the vector equivalent to the input. The second architecture, also known as an autoencoder, can be adapted to learn some low-dimensional representation of the data or to produce synthetic data similar to the dataset or given a certain condition. For instance, one can generate an artificial profile of a subject given the desired age or disease state. (Figure 18.3). Generated data later can be compared to the original one to explore a set of features that change upon the condition. Generative Adversarial Networks, tandems of a generator network and a discriminator network, provide another method

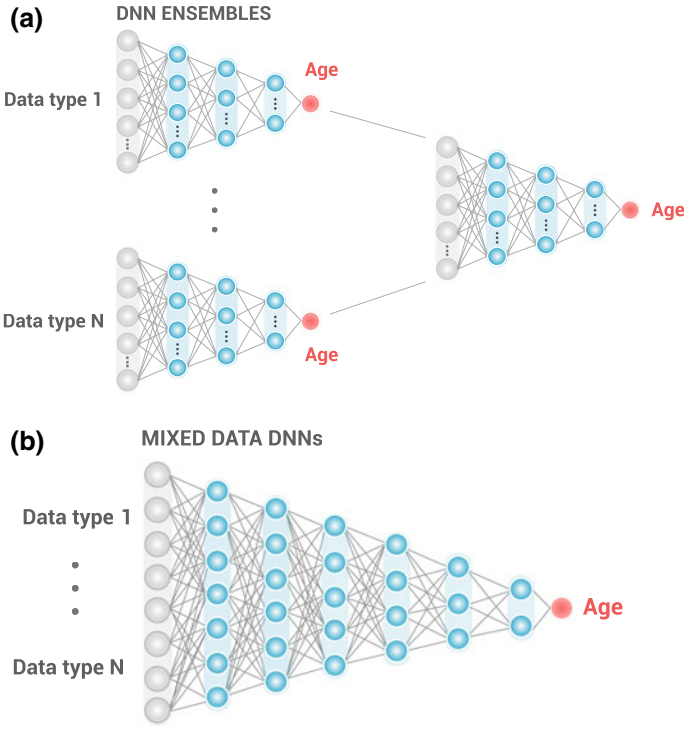

Fig. 18.2 Multi-modal biomarkers of aging. **a** Ensemble approach with multiple deep neural networks trained on different data types **b** One deep neural networks trained on multiple data types

for data generation. A generator produces a candidate vector of synthetic data and a discriminator network validates this vector identifying whether it is fake or real. Such data generation has been extensively explored for tasks including the creating of novel structures of drugs Kadurin (2017a), Kadurin (2017b), Putin (2018a), Polykovskiy (2018), Putin (2018b) or personalized rejuvenation or aging of facial images Song et al. (2017).

18.3 Deep Biomarkers of Human Aging

The field of DL in aging research took off in the early 2016 with a seminal work by Zhavoronkov's computational lab. In this section we outline applications of DL for aging biomarkers development and summarize them in Table 18.1. We have organized this section according to data types used to construct the deep clocks.

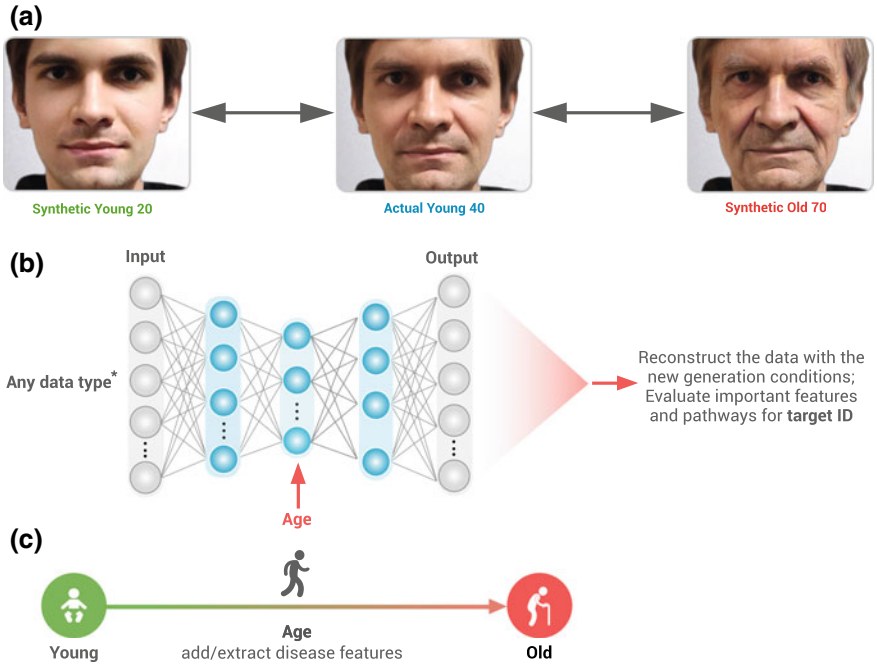

Fig. 18.3 The application of Generative Adversarial Networks (GANs) to aging research and biomarker development. **a** a graphical example of the generation of synthetic data using GANs using age as a generation condition; **b** basic adversarial autoencoder architecture with age as a generation condition for synthetic data generation and target identification; **c** a graphical representation of using GANs to develop the "digital twins" or "synthetic avatars" of the individual in time

18.3.1 Clinical Blood Test Markers

The first biomarker of aging utilizing the power of DL was built on clinical blood tests and validated on a large dataset of blood tests coming from routine screening tests of Eastern Europeans 20–90 years of age Putin (2016). An ElasticNet regularized ensemble of twenty-one feedforward DNNs with different architectures (with two to three hidden layers) was trained on the values of 41 blood markers from blood test of over 52,000 patents. Each network in the ensemble was optimized to predict the age of individuals as accurate as possible. Once the best parameters of those models were identified, a whole ensemble was tested on over 10,000 blood test samples of the previously unseen individuals. This approach achieved a mean absolute error of 5.5 years explaining over 80% of the variability in the data (R^2 of 0.82) in age prediction, significantly outperforming shallow ML model including linear regression with ElasticNet regularization (R^2 of 0.5 only) used to construct epigenetic clocks and blood transcriptomic clocks proposed by Peters et al. In fact, deep blood test clocks showed to be more accurate in age prediction compared to Peters et al model.

Table 18.1 A list of deep predictors of age

Data type	Accuracy	References
Blood biochemistry and cell count	$r = 0.80-0.91$ $R^2 = 0.65-0.82$ $MAE = 5.04-6.19$	Putin (2016), Mamoshina (2018a), Mamoshina (2018b)
Tissue-specific gene expression	$r = 0.91$ $R^2 = 0.83$ $MAE = 6.19$	Mamoshina (2018c)
Microbiome	$R^2 = 0.81$ $MAE = 3.94$	Galkin et al. (2018)
Brain MRI	$r = 0.96$ $MAE = 4.16$	Cole (2017)
Bone X-ray MRI	$Accuracy = 98-99\%$ within 2 years	Lee (2017)
Facial images	$r = 0.95$ $MAE = 2.3$	Bobrov et al. (2018)
Physical activity	$r = 0.75$ $RMSE = 14.0$	Pyrkov (2018)

r is for Pearson correlation coefficient, which shows the strength of a linear association between predicted and actual age
R^2 is for coefficient of determination, which shows the percentage of variance explained by the regression between predicted and actual age
MAE for mean absolute error, which demonstrates average absolute disagreement between the chronological age and the predicted age
RMSE is for root mean squared error, which demonstrates root squared disagreement between the chronological age and the predicted age.

Accessibility of this data, ease of assessment and availability of standardized assays, making deep blood test clocks an attractive alternative to epigenetic or transcriptomic clocks.

This work was further extended on population-specific datasets of blood test data of Canadians, Eastern Europeans, and South Koreans Mamoshina (2018a). Deep learned age predictors were compared by the predictive accuracy first when trained using population-specific data, and then when using a combined and ethnically diverse dataset that includes patients from all three patient populations. Models trained on individual populations predict other population with greater error. Moreover, the model trained on E. Europeans, on Koreans, Koreans are predicted younger their chronological age and vice versa. The model trained on a combined dataset of all three populations is equally accurate in age prediction of all of them. Validation on the publicly available The National Health and Nutrition Examination Survey (NHANES) dataset showed similar results on population-specificity, with the best-combined model predicting age. In line with those differences in simple blood tests, the top of the most important features for age prediction is also varied between populations. However, individuals that were found to have an older blood-age than their chronological age had increased risk of dying and vice versa regardless of the population. Importantly, the showed predicted age by DL blood predictor association with mortality was higher than the one with chronological age, demonstrating that developed deep clock is indeed an aging clock.

It was later confirmed by the ability of the DL blood clocks to show the effects of lifestyle choices such as smoking Mamoshina (2018b). Deep learned model showed

an acceleration of aging in smokers, by predicting smokers significantly older their chronological age compared to non-smokers regardless of their sex, lipid ratio or glucose level. Interestingly, a deep learned predictor trained on blood test prove to distinguish smokers from non-smokers with an accuracy of over 70%.

18.3.2 Molecular Markers

A first deep transcriptomic clock was proposed as a part of a panel of ML markers early in 2018 by Mamoshina et al. Mamoshina (2018c). A deep neural network was the most accurate age predictor showing the accuracy of 0.91 in terms of Pearson correlation and mean absolute error of 6.14 years. Further validation on the external GTEx dataset showed the accuracy of 0.80 with respect to the actual age bin prediction. Another promising finding was that the list of the features most relevant to age prediction identified by deep neural network is the closest results to the final consensus ranking produced by other ML age predictors suggesting the superior generalization abilities.

The most recent studies illustrating the power of deep neural networks for age prediction on other biomedical data type, microbiome profiles Galkin et al. (2018). Deep learned age predictor was built on 3,663 microbiome samples of over a thousand healthy patients from several populations. The best model predicts the age with a mean absolute error of 3.94 years. Another promising finding was that the deep microbiome clock can identify a set of microbial taxa that promote or slow down the aging process.

18.3.3 Imaging Markers

Most DL algorithms require a lot of data to train and test the system. To address this issue, a data transfer learning technique is commonly used in image recognition. Convolution neural networks allow transferring fitted parameters or pretraining from one domain to another. A large collection of images such ImageNet used to fine-tune a network first making possible to train age predictors on biomedical datasets limited in size. Another popular approach is to produce a set of synthetic images by augmenting the dataset in various ways.

Hence a large number of existing studies in the broader literature have examined the utility of imaging data for human age prediction. For example, convolutional neural networks trained on magnetic resonance imaging of the brain of over 2000 patients can predict the age of individuals with a mean absolute error of 4.16 years Cole (2017). Pretreained on an ImageNet bone x-ray image based age predictor showed to be not less accurate assigning ages with an error less than 10% within 2 years of time Lee (2017).

A good graphical example of an imaging clock is the PhotoAgeClock developed by the DL company Haut. AI specializing in skin imaging analysis in collaboration with a large skincare conglomerate Bobrov et al. (2018). The PhotoAgeClock uses the eye corners around the eye to predict the age of the individual with 1.9 year MAE accuracy. While seemingly useless from the biological perspective, the clock presents the many DL concepts applicable to the more biologically-relevant data types. It shows that it is now possible to identify the most important features that contribute most to the accuracy of the predictor. The resolution of the images used for training the deep neural networks may substantially impact accuracy and the most important features. High-resolution imaging of the skin may provide more biologically-relevant results.

18.3.4 Other Data Types

Physical activity data collected from accelerometer was another data type explored in the ability to predict human age using neural networks Pyrkov (2018). A convolutional neural network trained on an activity matrices achieved a relatively good accuracy of 0.72 in terms of Pearson correlation between predicted and actual chronological age, showing, however, a lower association with mortality compared to a less accurate in age prediction models. It must be pointed out thought that selected age acceleration metric is hard to interpret with regards to mortality analysis. Individuals predicted within the expected error of the model and close to their actual age will have higher values of the proposed acceleration metric compared to individuals predicted younger, but biologically we would expect them to age within the 'normal' range. To address this lack of mortality association and interpretability issues, authors proposed a convolutional neural network-based mortality predictor.

18.4 Conclusion and Future Perspectives

The field of aging biomarkers is rapidly evolving. The age predictors commonly referred to as the aging clocks are gaining popularity and the many new aging clocks are being developed by the research teams worldwide using a variety of data types from facial images and blood tests to cell-free DNA and microbiome. As more national biobanks like the UK BioBank become available, the field of aging clocks will continue to advance. Deep neural networks tend to outperform the other techniques in accuracy and allow for new techniques of feature importance and causality analysis and target identification. Since age is among the most abundant and universal features, experiments with age prediction also help advance the DL methods and help make DNNs more interpretable.

Authors believe that the applications of generative adversarial networks in combination with reinforcement learning to the synthesis of the new individual data using

age as a condition for generation is among the most promising areas in both aging research and DL. This technique allows to simulate the aging process of the individual in time and allows to analyze the features that change during this process.

The aging clocks developed using different data types often do not correlate and measure different processes on the system, organ, tissue, and cellular levels. When selecting the aging clock for a study is important to collect and analyze as many data types as possible. Many data types are highly variable and require more frequent testing to achieve a stable longitudinal signal.

Certain types of aging biomarkers can be used for the identification of new molecular targets or other areas for intervention. One of the most abundant data types and among the most valuable for DACs is tissue-specific transcriptomic data. This data allows for the development of highly accurate aging clocks and enables target identification hypotheses.

The main challenges of developing aging biomarkers include the data availability, data privacy, population coverage, and lack of interest from the industry and the government. There are multiple disconnected longitudinal data sets with a limited number of national biobanks. However, data privacy laws are making it increasingly difficult for academic and more so for industry researchers to access this data for training.

Lack of standards and lack of the basic rules of data economics is another industry challenge. While the industry is putting the major emphasis on genomic data, other data types of data types are often overlooked including the photos, video, voice, nail and hair composition, and many others. The timing for the collection of data is usually ignored. It is important to collect the many data types at the same time.

Despite the many challenges the convergence of DL and aging research is expected to continue with the many aging clocks going mainstream in the near future.

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Conflict of Interest AZ and PM work for Insilico Medicine, a for-profit longevity biotechnology company developing the end-to-end target identification and drug discovery pipeline for a broad spectrum of age-related diseases. The company applied for multiple patents covering the various methods for development and applications of the aging clocks. The company may have commercial interests in this publication.

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Chapter 19
Quantification of the Biological Age of the Brain Using Neuroimaging

James H. Cole, Katja Franke and Nicolas Cherbuin

Abstract The cosmetic and behavioural aspects of ageing become increasingly apparent with the passing years. The individual variability in physical ageing can be immediately observed in people's face, posture, voice and gait. In contrast, the pace at which our brains age is less obvious, only becoming apparent once substantial neurodegeneration manifests through cognitive decline and dementia. Therefore, a more timely and precise assessment of brain ageing is needed so its determinants and mechanisms can be more effectively identified and ultimately optimised. This chapter describes new approaches aimed at quantifying the biological age of the brain, so-called 'brain age'; reviews how brain age can be contrasted to chronological age to index risk of premature brain ageing; and explores how brain age can be used to investigate genetic, environmental, health, and lifestyle factors contributing to accelerated ageing. Particular attention is given to the statistical approaches underpinning brain age, evaluating their validity and limitations. The developing brain-age literature covering diverse populations, all stages of life, health and psychopathology, humans and animals, is critically and comprehensively presented. Finally, gaps in our knowledge and unresolved methodological issues are summarised, alongside proposing future directions and highlighting opportunities for further research in this promising and exciting field.

Keywords Brain ageing · Neuroimaging · MRI · Machine learning · Neuroscience

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19.1 The Brain During Ageing

> "I have reached an age when, if someone tells me to wear socks, I don't have to". Albert Einstein (1879–1955)

Across both time and cultures, old age has been associated with greater wisdom and judgement, as exemplified by the *Gerousia*, the council of elders who presided over the city-state of Sparta in the seventh century BC. The accumulation of experience and knowledge that conveys this wisdom is in fact the exception to the norm. The majority of other cognitive functions decline from the point of maturity (i.e., young adulthood) across the rest of the lifespan (Salthouse 2010; Deary et al. 2009; Park 2009). This functional decline at the behaviour level is underpinned by changes to the structural and functional composition of the brain. Given the numerous biological consequences ageing has for the human body (Lopez-Otin et al. 2013), it should be no surprise that the brain is profoundly affected by the ageing process.

Perhaps the most striking change to the brain associated with ageing is the fact that the brain shrinks. The organ weighs less (Dekaban 1978), and the cerebrospinal fluid (CSF) spaces (e.g., ventricles, sulcal spaces) are enlarged (Hubbard and Anderson 1981). All the major structures of the brain lose volume and the cortical mantle becomes thinner (Kemper 1994). These macroscopic observations made in vivo, using magnetic resonance imaging (MRI), and *post-mortem,* in histological studies, have been accompanied by research into the molecular and cellular make-up of ageing neural cells and brain tissue, characterising an array of changes associated with brain ageing.

19.1.1 Molecular and Cellular Brain Ageing

The complex biochemical and cellular milieu making up the human brain undergoes comprehensive alterations during the ageing process. As reviewed by Mattson and Arumugam (Mattson and Arumugam 2018), the biological mechanisms underlying these changes include: oxidative damage, inflammation, impaired DNA repair, protein mis-folding and impaired clearance, impaired lysosome and proteasome function, mitochondrial dysfunction, dysregulation of calcium homeostasis, reduced neurogenesis, dysfunction of the adaptive stress response, and disrupted neural network activity. To this, one can add evidence of epigenetic changes in DNA methylation (Hannum et al. 2013; Horvath 2013), disrupted neurotransmitter activity (Bäckman et al. 2010), altered gene expression (Soreq et al. 2017; Rodríguez et al. 2012) and cerebrovascular changes (Wardlaw et al. 2013).

From this list, two key observations can be made. Firstly, that brain ageing is essentially ubiquitous, affecting all neurobiological processes. Secondly, the highly inter-dependent and interactive nature of these signalling pathways and regulatory processes mean that none of the neurobiological changes associated with ageing are occurring in isolation. For Mattson and Arumugam, a potent driver of brain ageing

is 'metabolic complacency', whereby there is a chronic positive energy balance in the brain, perhaps as the result of a sedentary and over-indulgent lifestyle. While this remains to be determined, what is clear is that there are myriad potential biological factors that could, theoretically, index the extent to which ageing has affected the brain. However, a major limitation of directly targeting these biological hallmarks of brain ageing is accessibility. As the blood-brain barrier isolates the neurobiological environment, accessing neural material in vivo is highly invasive and is only possible under rare circumstances (e.g., during neurosurgery). This motivates the use of non-invasive approaches for measuring the ageing brain, and a forerunner of such techniques is neuroimaging.

19.1.2 Neuroimaging the Ageing Brain

Neuroimaging encompasses a wide range of specific methods that can be used to provide insights in the living human brain (Table 19.1). Chief amongst these is magnetic resonance imaging (MRI), which itself is highly versatile, encompassing multiple different modalities that relate to different aspects of neurobiology. Positron emission tomography (PET) is another important method, which enables interrogation of the molecular properties of the brain in vivo. In addition, electro- and magneto-encephalography (EEG, MEG) are neurophysiological techniques commonly used in ageing neuroscience. Alongside those, computed tomography (CT), near-infrared spectroscopy (NIRS), ultrasound and electrocorticography (ECoG) have also been employed to study the brain ageing, though less commonly. In this chapter we will focus on MRI research, alongside mention of PET and EEG studies, to characterise and quantify the brain ageing process.

T1-weighted MRI provides strong evidence for age-related decreases in grey matter (GM) and white matter (WM) volume, cortical thinning, sulcal widening and morphological changes to sub-cortical nuclei (Fjell et al. 2014, 2013; Raz et al. 2005; Walhovd et al. 2011). T2-weighted MRI shows an age-associated increase in the prevalence and magnitude of so-called 'white-matter hyperintensities' (Wardlaw et al. 2013; Habes et al. 2016), bright-spots on an image which are thought to reflect cerebrovascular abnormalities, also referred to as white-matter lesions or leukoaraiosis. Diffusion-weighted MRI, particularly diffusion tensor imaging (DTI), has demonstrated how ageing affects the microstructure of the white matter; characteristic alterations to microstructural indices such as decreased fractional anisotropy and increased mean diffusivity, thought to reflect demyelination or axonal degeneration (Song et al. 2005; Sun et al. 2005). Diffusion-weighted MRI has also been used to study the brain's network of connections, (i.e., connectomics), indicating that structural connectivity decreases with increasing age (Cox et al. 2016). In line with this, functional MRI (fMRI), in particular resting-state fMRI research supports the idea of decreased functional connectivity accompanying these age-related changes in structural connectivity (Sala-Llonch et al. 2015; Marstaller et al. 2015).

Table 19.1 Overview of neuroimage modalities, derived parameters, and resulting measures for brain age prediction

Tool	Modality	Parameters	Age-associated findings
MRI (magnetic resonance imaging)	T1-weigthed	Grey matter (GM), white matter (WM), subcortical volumes, cortical thickness, cortical surface area	Decreases in brain tissue volume, cortical thinning, sulcal widening and morphological changes to sub-cortical nuclei
	T2-weighted	Signal intensities	Increase in the prevalence and magnitude of white-matter lesions
	DTI (diffusion tensor imaging)	Fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), radial diffusivity (RD)	Alterations of the WM microstructure, reflecting demyelination or axonal degeneration
	DWI (diffusion weighted imaging)	Apparent diffusion coefficient (ADC); Structural connectivity: correlations, graph theoretic measures	Decreasing structural connectivity, reduced network integration
	fMRI (functional MRI)	Resting-state fMRI (rs-fMRI) functional connectivity: correlations, graph theoretic measures	Decreased functional connectivity accompanying changes in structural connectivity
	ASL (arterial spin labelling)	Cerebral blood flow	Reduced cerebral perfusion
	MRS (magnetic resonance spectroscopy)	Cerebral metabolites levels	Abnormalities in levels of key cerebral metabolites (e.g., *N*-acetyl-aspartate, myo-inositol and creatine)
	DCE (dynamic contrast-enhanced MRI)	Integrity of the blood-brain barrier	Reduced integrity of the blood-brain barrier
PET (positron emission tomography)	FDG ([^{18}F]fludeoxyglucose)	Cerebral glucose metabolism	Decreased levels of cerebral glucose metabolism

(continued)

Table 19.1 (continued)

Tool	Modality	Parameters	Age-associated findings
	[^{15}O]H_2O PET	Cerebral blood flow	Decreases in regional cerebral blood flow
	PiB ([^{11}C]Pittsburgh compound B)	Cerebral levels of fibrillar amyloid-beta	Increases in levels of fibrillar amyloid-beta
	[^{18}F]florbetapir PET	Cerebral levels of fibrillar amyloid-beta	Increases in levels of fibrillar amyloid-beta
	[^{11}C]SCH23390 PET	Levels of dopamine transmission	Reduction in D1 receptor levels
	[^{18}F]fallypride PET	Levels of dopamine transmission	Reduction in D2/3 receptor levels
	[^{11}C]PK1195 PET	TSPO-binding	Increases in microglia activation, suggesting increased neuroinflammation

Reduced cerebral perfusion has been associated with ageing (Lee et al. 2009), using the MRI technique known as arterial spin labelling (ASL), where the flow of magnetically 'labelled' blood can be mapped in the brain (Chappell and Macintosh 2017). The ageing brain is also associated with abnormal levels of cerebral metabolites, such as *N*-acetyl-aspartate, myo-inositol and creatine (Haga et al. 2009; Reyngoudt et al. 2012), assessed using magnetic resonance spectroscopy (MRS). Finally, the integrity of the blood-brain barrier is thought to be reduced in ageing (Montagne et al. 2015), with preliminary investigations of this coming from the use of gadolinium injections in dynamic contrast-enhanced (DCE) MRI.

While in-depth coverage of these findings is beyond the scope of this chapter, readers are directed to several reviews on the structure (Fraser et al. 2015; Hedman et al. 2012; Lockhart and DeCarli 2014), function (Eyler et al. 2011) and connectivity (Sala-Llonch et al. 2015; Leal and Yassa 2013) of the ageing brain.

As mentioned, other neuroimaging modalities beside MRI have provided valuable insights into the ageing brain. Myriad different radio-labelled tracers are available for PET imaging, targeting neurobiological processes including metabolism, perfusion, neuroinflammation, neurotransmission and proteostasis. Research using [^{18}F]fludeoxyglucose (FDG) found that older adults have decreased levels of glucose metabolism in the brain (Knopman et al. 2014; Yoshizawa et al. 2014; Murphy et al. 1996). Age-associated decreases in regional cerebral blood flow have also been reported, using [^{15}O]H_2O PET (Bentourkia et al. 2000). Meanwhile, studies using either [^{11}C]Pittsburgh compound B (PiB) or [^{18}F]florbetapir have associated increasing age with increasing levels of fibrillar amyloid-beta (Fripp et al. 2008; Fleisher et al. 2013). There is also evidence of an age-associated decline of dopamine signalling, with reduced D1 (Backman et al. 2011) and D2/3 (Dang et al. 2016) receptor levels, detected using [^{11}C]SCH23390 and [^{18}F]fallypride PET respectively. Finally,

TSPO-binding, measured using [^{11}C]PK1195 and thought to reflect microglia activation, has been shown to increase with age, suggesting that neuroinflammation is greater in older adults (Schuitemaker et al. 2012). Evidently, PET is uniquely powerful in providing information in vivo on the neurobiology of ageing. Taken together, PET research supports the idea that the ageing brain undergoes complex molecular alterations, which likely drive the characteristic macroscopic and phenotypic changes associated with older age. However, the cost and logistics of conducting PET studies alongside health concerns related to its radio-active nature preclude its use at scale. Cheaper, faster and more portable techniques are needed to study the ageing brain at a population level.

One of the chief advantages of EEG is its ease of use, low costs, mobility and low-levels of contraindications. MEG still requires complex expensive equipment; MEG machines are less ubiquitous than MRI scanners currently. Both techniques measure the electrical activity of the brain through the skull, using either electrodes or magnetometers. The application of EEG has been widespread, and older age has been associated with reduced activity in all four frequency bands: alpha, beta, delta and theta (Breslau et al. 1989) and reduced interhemispheric coherence (Kikuchi et al. 2000), though more recent research has seen increased beta band activity with ageing (Ponomareva et al. 2017). EEG data have also been used to generate predictions of chronological age (Al Zoubi et al. 2018), discussed further below.

Neuroimaging techniques are increasingly used to study ageing, thanks to the spread of technological infrastructure and expertise and the common availability of MRI scanners or EEG equipment. With the further development of data storage capacity and a move towards generating 'biobanks' containing large numbers of deeply-phenotyped participants, it is anticipated that neuroimaging will go on to provide still greater insights into the ageing brain as we seek to understand the causes of cognitive decline, neurodegeneration and dementia.

19.1.3 Consequences of Brain Ageing

This plethora of molecular, cellular, microscopic and macroscopic changes associated with age have a profound impact on human behaviour, physiology and psychology. This includes the characteristic decline in cognitive function associated with increasing age, or 'cognitive ageing'. Cognitive ageing particularly affects domains such as information processing speed, executive function, motor function and certain aspects of memory (Salthouse 2010; Deary et al. 2009).

This pattern of cognitive decline can generally be seen as driving the decreased functional capacity seen in older adults, which leads to reductions in employability and participation in hobbies and social activities. Eventually, activities of daily living are compromised, and levels of independence and self-care are affected (Eramudugolla et al. 2013; Johansson et al. 2015; Cameron et al. 2010; Greiner et al. 1996). This reduction in functional capacity is accompanied by aberrant sleep regulation and disrupted circadian rhythms, with insomnia and disordered sleep com-

monly reported (Desforges et al. 1990). Well-being is further impacted by an age-associated increase in negative affect, with low mood and other depressive symptoms often observed (Fiske et al. 2003; Chui et al. 2015). Whether these mood alterations are a direct result of changes to the brain or whether they arise in response to the decreases in functional-capacity and well-being remains an open question. Finally, the ageing brain is also increasingly susceptible to neurodegenerative diseases, such as Alzheimer's and Parkinson's (Qiu et al. 2009; Reeve et al. 2014), as well as stroke (Hollander et al. 2003). The morbidity and mortality associated with these pathological consequences of brain ageing are substantial (Prince et al. 2015). Given the projections for the ageing of the global population (Lutz et al. 2008), there is a growing need to reduce the burden these conditions place on individuals and society. Despite the well-described consequences of brain ageing, a key characteristic of brain ageing is the variability seen between different people. While some people remain cognitively healthy well into their tenth decade, others experience cognitive decline and age-associated brain diseases at much younger ages. This variability presents both a challenge; for those tasked with treating age-associated diseases, and an opportunity; it provides a biological target that can be measured and may better describe an individual's risk of positive or negative brain ageing compared to chronological age alone.

19.2 Measuring the Brain's Biological Age

Under the 'ageing biomarker' paradigm (Sprott 2010), researchers have explored whether it is possible to quantify the brain's 'biological age' (Cole and Franke 2017). The theory is that biological properties of the brain may be better predictors of health, capacity and residual lifespan, than chronological age itself. By measuring 'brain age', we may have a 'truer' (or at least more useful) representation of an individual's risk of cognitive decline, neurodegenerative disease and residual period of healthy cognitive function and lifespan.

As outlined above, neuroimaging is the preferred method for deriving brain-ageing biomarkers for a number of reasons. Firstly, it is non-invasive and generally well-tolerated, so can be measured repeatedly on the same individuals. Secondly, the wealth of different neuroimaging modalities available allow detailed structural and functional information about many of the brain's biological properties to be measured reliably and accurately. While other approaches to inferring neurobiology are available (e.g., retinal imaging, nasal biopsy, lumbar puncture to obtain CSF), these are less direct and potentially more invasive.

Aspects of brain structure or function have generally been related to ageing using conventional statistical models. For example, the brain measure of interest is the outcome (i.e., dependent) variable and age is a predictor (i.e., independent) variable in a linear regression model, or a straightforward bivariate correlation between the brain measure and age is used. An alternative approach is to employ multivariate statistics, enabling researchers to take multiple brain measures together and establish

whether they can be used to predict age as the outcome measure. Machine learning is particularly powerful in this case, as it lends itself to the analysis of high-dimensional (i.e., many predictor variables) datasets, such as those commonly obtained from neuroimaging studies (often comprising hundreds of thousands of measurements at voxels [volume elements, analogous to three-dimensional pixels] across the brain). Over the last decade, a growing number of studies have used neuroimaging data to predict chronological age; here, we review the state-of-the-art in the field. Technical research into brain age algorithm development tends to use used T1-weighted MRI alone (Table 19.2) to assess brain volume, morphology or cortical thickness. Other studies have employed diffusion-MRI, fMRI or EEG, while others have been multi-modal (Table 19.3). An overview of more technical studies is presented, before consideration of brain age applied in the context of neurological and psychiatric diseases (Table 19.4) and in physiological diseases and normally-ageing cohorts (Table 19.5). Studies that have assessed how genetic or environmental factors my influence brain age are also reviewed, as are animal studies (Table 19.6).

19.2.1 T1-Weighted MRI

Of the studies that have used T1-weighted MRI to predict age, some have used chronological age as a valid and reliable target value to evaluate the accuracy of a new algorithm or statistical approach (Ashburner 2007; Konukoglu et al. 2013). Meanwhile, others have more explicitly adhered to the ageing biomarker paradigm and considered how brain age performs in clinical contexts (Cole et al. 2015; Franke et al. 2010). Both types of studies are reviewed in Table 19.2. Where studies evaluated multiple approaches (e.g., different algorithms, different features), the lowest reported mean absolute error (MAE) has been included. Accuracy metrics were usually generated using in-sample cross-validation (often 5 or 10-fold), though some studies used either hold-out test sets or completely independent samples for accuracy assessment (Cole et al. 2017a; Lancaster et al. 2018; Varikuti et al. 2018). The majority of studies include voxelwise GM as the input predictor variables (i.e., features), though cortical thickness and related measures are also commonly used, occasionally in combination (Gutierrez Becker et al. 2018; Valizadeh et al. 2017). Where studies have used multiple modalities, the results from their T1-weighted MRI analysis have been included here, with multi-modal results in Table 19.3. Image pre-processing methods varied, though spatial normalisation was common. Some studies included explicit methods for feature selection or data reduction, such as randomly sampling 10,000 voxels (Konukoglu et al. 2013) or by employing principal components analysis (Franke et al. 2010), while others incorporated feature selection more directly into the model optimisation through the use of LASSO (least absolute shrinkage and selection operator) (Varikuti et al. 2018; Bagarinao et al. 2018; Khundrakpam et al. 2015). Conversely, two studies were able to remove the reliance on pre-processing or feature selection altogether by analysing raw T1-weighted MRI data using 'deep' convolutional neural networks (Cole et al. 2017a; Huang et al. 2017). Both studies

Table 19.2 Studies modelling brain-age using T1-weighted MRI

Study	N	Age, years (mean ± SD, range)	MRI features	Algorithm	Correlation between age and predicted age (r)	Mean absolute error (years)	Weighted mean absolute error (MAE/age range)
Ashburner (2007)	471	38.1,17–79	GM	RVR	0.86	–	–
Franke et al. (2010)	550	48.2 ± 16.6, 20–86	GM	RVR	0.94	4.61	0.070
Wang and Pham (2011)	20	50–86	GM + WM + CSF	HMM	–	6.2	0.172
Brown et al. (2012)	885	3–20	Cortical thickness, subcortical volume	MVNL-R	0.91	1.71	0.101
Franke et al. (2012)	394	10.7 ± 3.8, 5–19	GM + WM	RVR	0.93	1.2	0.086
Konukoglu et al. (2013)	414	18–86	T1 intensity values	NAF	0.94	–	–
Koutsouleris et al. (2013)	800	36.8 ± 11.1, 18–65	GM	SVR	–	4.6	0.098
Su et al. (2013)	290	43.3 ± 23.0, 18–91	GM	RVR	–	5.7	0.078
Wang et al. (2014)	360	47.0 ± 16.2, 20–82	Cortical thickness, surface area, curvature	RVR	4.57	0.94	0.074
Khundrakpam et al. (2015)	308	12.9 ± 3.8, 5–21	Cortical thickness	ENet	0.84	1.68	0.105

(continued)

Table 19.2 (continued)

Study	N	Age, years (mean ± SD, range)	MRI features	Algorithm	Correlation between age and predicted age (r)	Mean absolute error (years)	Weighted mean absolute error (MAE/age range)
Kondo et al. (2015)	1146	45.6 ± 15.7, 20–75	GM + WM + CSF	RVR	0.94	4.50	0.082
Cole et al. (2015)	1537	41.8 ± 20.0, 18–90	GM; WM	GPR	0.92	6.2	0.086
Fujimoto et al. (2016)	1099	20–80	GM + WM + CSF	RVR	0.93	4.48	0.075
Cole et al. (2017a)	2001	37.0 ± 18.1, 18–90	GM + WM	GPR	0.94	5.01	0.070
Cole et al. (2017a)	2001	37.0 ± 18.1, 18–90	Raw T1-MRI	CNN	0.94	4.65	0.065
Valizadeh et al. (2017)	3144 (total)	8–18; 18–65; 65–96	Cortical thickness, surface area, volumes	NN	0.91	1.23; 4.50; 4.97	0.123; 0.096; 0.160
Huang et al. (2017)	1099	20–80	Raw T1-MRI	CNN	–	4.0	0.067
Liem et al. (2017)	2354	58.7 ± 15.2, 19–82	Cortical thickness, surface area, subcortical volumes	SVR	–	4.83	0.077
Guggenmos et al. (2017)	97	43.7 ± 10.8, 21–65	Cerebral ROI volume	Ridge regression	0.54	6.9	0.157
Bagarinao et al. (2018)	293	21–86	GM	LASSO	0.86	7.18	0.110

(continued)

Table 19.2 (continued)

Study	N	Age, years (mean ± SD, range)	MRI features	Algorithm	Correlation between age and predicted age (r)	Mean absolute error (years)	Weighted mean absolute error (MAE/age range)
Lancaster et al. (2018)	2003	36.5 ± 18.5, 16–90	GM	SVR	0.94	5.08	0.069
Gutierrez Becker et al. (2018)	1563	6–92	GM + cortical thickness + volume	GPR	0.96	3.86	0.045
Pardoe and Kuzniecky (2018)	2367	24.8 ± 14.6, 8–69	Cortical thickness	RVR	–	7.2	0.118
Madan and Kensinger (2018)	1056	18–94	Cortical thickness gyrification + subcortical volume + fractal dimensionality	RVR	0.89	6.1†	0.080†
Monté-Rubio et al. (2018)	562	46.85 ± 16.40 (20–86)	GM; WM; Jacobians; Divergence, Scalar momenta	GPR	–	5.0	0.075
Eavani et al. (2018)	400	72.5 ± 9.4, 50–96	GM	SVR	0.80	4.41	0.096
Varikuti et al. (2018)	693; 1084	55–75; 18–81	GM	LASSO	0.69; 0.91	3.4; 4.9	0.170; 0.078
Aycheh et al. (2018)	2705	49–91	Cortical thickness	Sparse group LASSO + GPR	–	4.05	0.088

Age mean (± standard deviation) and range reported where available. CNN = Convolutional Neural Network; ENet = Elastic Net; GM = Grey matter; GPR = Gaussian Process Regression; HMM = Hidden Markov model; LASSO = Least Absolute Shrinkage and Selection Operator; MVNL-R = Multivariate Non-Linear Regression; NAF = Neighbourhood approximation forests; NN = Neural network; RVR = Relevance Vector Regression; SVR = Support Vector Regression; WM = White matter. † = Study reported median, not mean absolute error

Table 19.3 Studies modelling brain-age using diffusion-MRI, functional neuroimaging or multiple modalities

Study	N	Age, years (mean ± SD, range)	Features	Algorithm	*r*	MAE	Weighted MAE
Dosenbach et al. (2010)	238	7–30	Resting-state fMRI: functional connectivity	SVR	–	–	–
Brown et al. (2012)	885	3–20	T1-MRI + T2-MRI + diffusion-MRI	MVNL-R	0.96	1.03	0.061
Wang et al. (2012)	170	8–79	Resting-state fMRI: functional connectivity	Locally-adjusted SVR	–	7.5	0.106
Groves et al. (2012)	484	8–85	GM + cortical thickness + cortical area + FA + MD + MO	Smoothing spline	0.97	–	–
Mwangi et al. (2013)	188	4–85	FA, MD, AD, RD	RVR	0.90	6.94	0.086
Han et al. (2014)	201	4–85	DTI: structural connectivity	CBR	0.86	7.66	0.095
Irimia et al. (2014)	136	33.3 ± 11.6, 18–61	Cortical thickness, FA, structural connectivity	MLR	–	–	–
Erus et al. (2015)	621	15.1 ± 3.3, 8–22	GM, WM, ventricle volume, FA, ADC	SVR	0.89	1.22	0.087
Lin et al. (2016)	112	65.5 ± 7.2, 50–79	DTI: structural connectivity	ANN	0.80	4.29	0.148
Cherubini et al. (2016)	140	42 ± 14, 20–74	GM + R2* + FA + MD	MLR	0.96	–	–
Tsvetanov et al. (2016)	635	18–88	Resting-state fMRI	MLR	0.44	–	–
Liem et al. (2017)	2354	58.68 ± 15.2, 19–82	Cortical thickness + cortical area + Resting-state fMRI	SVR + RF	–	4.29	0.068

(continued)

Table 19.3 (continued)

Study	N	Age, years (mean ± SD, range)	Features	Algorithm	*r*	MAE	Weighted MAE
Li et al. (2018)	983	8–22	Resting-state fMRI: functional connectivity	CNN	0.61	2.15	0.154
Al Zoubi et al. (2018)	500	18–58	EEG	Ensemble (SVR + RF + GPR + ENet + XgbTree)	0.6	7.01	0.175
Eavani et al. (2018)	400	72.5 ± 9.4, 50–96	Resting-state fMRI	SVR	0.68	5.54	0.120

Age mean (± standard deviation) and range reported where available. ANN = Artificial Neural Network; AD = Axial Diffusivity; CNN = Convolutional Neural Network; CBR = Correlation-based regression; ENet = Elastic Net; FA = Fractional Anisotropy; GM = Grey matter; GPR = Gaussian Processes Regression; MD = Mean Diffusivity; MO = Mode of Anisotropy; MLR = Multiple Linear Regression; MVNL = Multivariate Non-Linear Regression; RD = Radial Diffusivity; RF = Random Forests; RVR = Relevance Vector Regression; SVR = Support Vector Regression; XgbTree = Extreme Gradient Boosting Tree

Table 19.4 Studies of brain-age in neurological or psychiatric diseases

Study	Clinical group	N	Age, years (mean ± SD)	MRI features	Algorithm	*Brain-PAD* (mean, years)
Psychiatric disorders						
Franke et al. (2010)	Alzheimer's disease	102	76 ± 8	GM	RVR	10.0
Franke and Gaser (2012)	MCI—stable	36	77 ± 6	GM	RVR	BL: −0.5 FU (3 years): −0.4
	MCI—progressive	112	74 ± 7	GM	RVR	BL: 6.2 FU (3 years): 9.0
	Alzheimer's disease	150	75 ± 8	GM		BL: 6.7 FU (2 years): 9.0
Koutsouleris et al. (2013)	High psychosis risk	89	25 ± 6	GM	SVR	1.7
	Schizophrenia	141	28 ± 12	GM	SVR	5.5
	Major depression	104	42 ± 8	GM	SVR	4.0
Gaser et al. (2013)	MCI—progressive (early/late)	58/75	74 ± 7/75 ± 7	GM	RVR	8.7/5.6
Schnack et al. (2016)	Schizophrenia	341	34 ± 12	GM	SVR	BL: 3.4 FU (4 years): 4.3
Löwe et al. (2016)	Alzheimer's disease (APOE ε4 carriers/non-carriers)	101/49	74 ± 7/76 ± 9	GM	RVR	BL: 5.8/6.2 FU (2 years): 8.3/7.7
	MCI—progressive (APOE ε4 carriers/non-carriers)	78/34	74 ± 6/75 ± 9	GM	RVR	BL: 5.8/5.5 FU (3 years): 8.7/7.3
	MCI—stable (APOE ε4 carriers/non-carriers)	14/22	77 ± 6/77 ± 6	GM	RVR	BL: −0.9/−0.9 FU (3 years): 0.0/−0.6
Nenadic et al. (2017)	Bipolar disorder	22	38 ± 11	GM	SVR	−1.3
	Borderline personality disorder	57	26 ± 7	GM	SVR	3.1
	Schizophrenia	45	34 ± 10	GM	SVR	2.6
Li et al. (2017)	Alzheimer's disease	411	75 ± 7	Hippocampal volume	SVR	7.0
Guggenmos et al. (2017)	Alcohol dependence	119	45 ± 11	GM	Ridge regression	4.0

(continued)

Table 19.4 (continued)

Study	Clinical group	N	Age, years (mean ± SD)	MRI features	Algorithm	*Brain-PAD* (mean, years)
Hajek et al. (2017)	Bipolar disorder	48	23.1 ± 4.5	GM	RVR	−1.0
	Schizophrenia (first episode)	43	27.1 ± 4.9	GM	RVR	2.6
Kolenic et al. (2018)	Psychosis (first episode)	120	27.0 ± 4.9	GM	RVR	2.6
	Psychosis + obesity	38		GM	RVR	3.9
Neurological disorders						
Cole et al. (2015)	Traumatic brain injury	99	38 ± 12	GM/WM	GPR	4.7/6.0
Cole et al. (2017b)	HIV	162	57 ± 8	Whole brain	GPR	2.2
Cole et al. (2017c)	Down's syndrome	46	42 ± 9	Whole brain	GPR	2.5
Pardoe et al. (2017)	Epilepsy (medically-refractory/newly-diagnosed)	94/42	32 ± 14/31 ± 11	Whole brain	GPR	4.5/0.9
Liem et al. (2017)	Objective cognitive impairment (mild/major)	632/251	58 ± 15/58 ± 16	Whole brain	SVR/RF	0.7/1.7
Savjani et al. 2017	Military traumatic brain injury	92	29 ± 7	Cortical thickness	LR/GPR/RF/SVR	Not reported

Age mean (± standard deviation) and range reported where available. BL = baseline, FU = follow-up, GM = grey matter, GPR = Gaussian process regression, LR = Linear regression; MCI = mild cognitive impairment, NLME = non-linear mixed effects model, RF = Random Forests, RVR = Relevance Vector Regression, SVR = Support Vector Regression, WM = white matter

Table 19.5 Studies of brain-age in physiological diseases and normal ageing

Study	Study sample	N	Age, years (mean ± SD)	MRI features	Algorithm	*Brain-PAD* (mean, years)
Normal ageing						
Franke et al. (2014)	Cognitively unimpaired older males	118	76 ± 5.3	GM	RVR	0.0
	Cognitively unimpaired older females	110	76 ± 1.1	GM	RVR	0.0
Cole et al. (2018b)	General population	667	73 ± 0.3	GM + WM	GPR	1.7
Kwak et al. (2018)	Subjectively younger	29	70.9 ± 6	GM	PLS	2.3
	Subjectively same age	19	69.6 ± 6	GM	PLS	5.5
	Subjectively older	20	73.8 ± 7	GM	PLS	3.4
Physiological disorders						
Franke et al. (2013)	Diabetes (type 2)	98	65 ± 8	GM	RVR	4.6
	Diabetes (type 2)—longitudinal	12	63 ± 7	GM	RVR	BL: 5.1 FU (4 years): 5.9
Ronan et al. (2016)	Obesity	227	58 ± 17	WM	NLME	10.0
Franke et al. (2018)	Gestational nutrient restriction (female/male)	22/19	67 ± 0.2/67 ± 0.1	GM	RVR	0.9/2.5

BL = baseline; FU = Follow-up; GM = Grey matter; GPR = Gaussian Process model; NLME = Non-Linear Mixed Effects models; PLS = Partial Least Squares; RVR = Relevance Vector Regression; WM = White matter

Table 19.6 Studies modelling brain-age in mammals

Study	Species	N (No. of scans)	Age (mean ± SD, range)	MRI features	Algorithm	*r*	MAE	Weighted MAE
Franke et al. (2016)	Rat	24 (273)	734 days ± 110,97–846	Whole brain	RVR	0.95	49 days	0.065 days
Franke et al. (2017)	Baboon	29 (29)	9.5 years ± 4.9, 4–22	GM	RVR	0.80	2.1 years	0.117 years

GM = Grey matter; RVR = Relevance Vector Regression; WM = White matter

achieved relatively high accuracy, supporting the promise of the application of deep learning to predict age from raw T1-weighted MRI data.

To better compare different studies, we computed a new accuracy metric, the *weighted MAE*. This is the MAE as a ratio of the age range of the sample tested, with a lower weighted MAE reflecting greater accuracy. This is important as the range of possible outcome values has a strong bearing on prediction accuracy, with a wider range of outcomes being more challenging. This is intuitive in the context of classification, as distinguishing two classes is simpler than three; and accuracy of 50% is at chance for two-class classification, whereas 50% for three-class classification is well above chance (which is 33%). Using the weighted MAE enabled comparison across studies that have used validation samples with different age ranges. In this light, the recent study of Gutierrez Becker et al. (2018) has the best performing age prediction model, which combined voxelwise GM data with cortical thickness and regional volume data using Gaussian processes regression. The weighted MAE also sheds new light on the results of Varikuti et al. (2018), who used two independent training samples to assess model performance. While the original MAE of their sample of older adults (MAE = 3.4 years) was lower than that of their lifespan sample (MAE = 4.9 years), when taking the age range into account, it seems the latter model was more accurate. This is backed up by the correlations between age and predicted age ($r = 0.69$ vs. $r = 0.91$) and is perhaps driven by the larger size of the lifespan sample ($n = 693$ versus $n = 1084$). The weighted MAE also explains why studies in younger groups seemingly performed better than adult-lifespan studies; the range of possible outcomes in child-adolescent groups is lower, so MAE naturally lower. Thus it would be incorrect to conclude that the developing brain can be more accurately 'aged', and thus is more homogeneous, than the adult brain, based on this evidence alone.

Regression algorithm choice varied considerably, with relevance vector regression (RVR) being most popular. Overall, the type of algorithm did not appear to have a strong bearing on model accuracy, though disentangling this from the influence of sample size (Franke et al. 2010) is not possible in the current literature. Combining multiple feature sources (e.g., voxelwise GM with cortical thickness) does appear to have a stronger bearing on model accuracy, as seen in studies that have evaluated this explicitly (Gutierrez Becker et al. 2018; Valizadeh et al. 2017; Madan and Kensinger 2018; Wang et al. 2014). This suggests that complementary orthogonal information relating to age can be gained by pre-processing T1-weighted MRI in different ways, thus combining feature sources appears to be a promising avenue for future research.

A number of studies in Table 19.2 evaluated feature importance in an attempt to determine the spatial patterns that drive the association between brain structure and age. There are multiple approaches to determining feature importance, and qualitative evaluation of the reported feature maps indicates that contrasting spatial patterns can emerge (Konukoglu et al. 2013; Fujimoto et al. 2016; Su et al. 2013). The most comprehensive assessment of feature importance was conducted by Varikuti et al. (2018), who performed 100 replications of each model. While generally they found that the regions that show the strongest linear correlation with age are commonly included as contributing features for age prediction, they also showed that the exact

pattern of regions varied depending on the composition of the training dataset. That, combined with the heterogeneous picture seen in other studies, suggests that there is no single canonical pattern of ageing, with potentially multiple analytical solutions to the prediction of age. This is perhaps unsurprising given the multivariate nature of brain imaging data and the highly-parameterised models used to predict age, as well as the heterogeneity observed more generally in the brain ageing process (Fjell et al. 2013; Storsve et al. 2014).

19.2.2 Other Modalities and Multi-modal Studies

Given that age-associated changes to the brain have been reported using various neuroimaging modalities, it is natural that researchers should attempt to leverage these techniques to generate predictions of brain age (Table 19.3). Diffusion-MRI and resting-state fMRI have been most commonly used to index structural and functional connectivity respectively. Diffusion-weighted MRI studies used either DTI metrics such as FA, mean diffusivity (MD), axial diffusivity (AD) and radial diffusivity (RD), or calculated indices of structural connectivity. This was generally done using tractography or by correlating FA values in different regions; these processes result in a connectivity matrix that reflects patterns of axonal 'wiring' between different parts of the brain, that can be conceptualised in a graph theoretic manner (Lin et al. 2016). Functional studies have derived connectivity matrices using the temporal correlations in BOLD activity across different regions, tapping into the brain's intrinsic connectivity networks (Smith et al. 2009).

When assessing the performance of these diffusion-MRI or fMRI brain-age models, the weighted MAE is comparable to those obtained using T1-weighted MRI. Nevertheless, the top performing single-modality models tend to be T1-MRI based, as demonstrated specifically by Liem et al. (2017), who compared cortical thickness with functional connectivity. Interestingly, Liem and colleagues also combined data sources, creating a 'stacked' multi-modal model. This multi-modal model outperformed any single data source in terms of having a lower MAE. This pattern can also be discerned across other studies, with higher performance levels achieved in studies that incorporate T1-MRI data alongside either diffusion-MRI or fMRI (Brown et al. 2012; Liem et al. 2017; Erus et al. 2015; Groves et al. 2012). This suggests that different imaging modalities overlap partially, but not completely in explaining age-related variance, thus account for important complementary variance. This motivates future research aimed at improving brain-age model performance to go beyond the current number of modalities included; three: T1-MRI, T2* relaxometry and diffusion-MRI (Cherubini et al. 2016), to include four or more sources of age-related signal.

Some studies did not directly attempt to model brain age in the ageing biomarker paradigm. Instead they conducted broader investigations of the relationship between the brain and chronological age and aimed at testing the strength of the brain-to-age relationship for a given feature or set of features. For example, Groves and

colleagues used linked Independent Components Analysis to fuse T1-weighted and diffusion-weighted MRI data, finding a broad multimodal component that related ageing to changes in cortical thickness, volume and DTI measures of white matter microstructure (Groves et al. 2012). Irimia et al. (2014) also looked at cortical thickness, comparing the relative predictive strength of different cortical regions to estimate age, as an indicator of how much age influenced structure in different local areas.

Finally, a single study has used EEG data to predict brain age (Al Zoubi 2018). This involved a range of different EEG-derived features (EEG amplitude, range, spectral, fractal, connectivity) and combined multiple machine-learning algorithms in an ensemble approach to generate age predictions. Performance of EEG model was poorer than the state-of-the-art approaches from MRI data. This may largely be a factor of sample size, as it is clear from both Tables 19.2 and 19.3 that performance improves with larger training sets. Therefore, research using larger samples than the n = 500 Al Zoubi et al. (2018) had available, is likely to yield improved performance. EEG is a particularly attractive proposition for brain-age studies, as EEG has much greater potential to scale-up data collection and be used in resource-scarce settings compared to MRI or PET. PET studies have yet to be used in the brain-age framework, likely due to the lack of sufficiently large training datasets being available.

19.3 Brain Age in Disease

19.3.1 Brain Diseases

A number of brain diseases have been investigated under the brain-age paradigm (Table 19.4). The rationale here is that these conditions have been previously associated with some age-associated changes in either physiology or behaviour, raising the possibility that these diseases contribute to an acceleration of the normal biological ageing process. Given that neurological and psychiatric conditions primarily influence the brain, this leads to the logical hypothesis that if these conditions are indeed influencing rates of biological ageing, then this should also manifest in the brain. Thus, brain-age presents an intuitive way to test such hypotheses.

Using the broad categories of psychiatric and neurological disease, we review the published literature. First, psychiatric diseases will be considered. In schizophrenia and psychosis, reports suggest that not only is greater brain ageing observed (Koutsouleris et al. 2013; Nenadic et al. 2017; Hajek et al. 2017), but that its effect becomes more prominent over time (Schnack et al. 2016). Conversely, in bipolar disorder and at-risk mental states evidence suggests that increased brain ageing may not be occurring (Koutsouleris et al. 2013; Nenadic et al. 2017; Hajek et al. 2017). Whether this distinction is due to differing disease severity or reflects diverse underlying aetiologies remains to be established. Interestingly, Kolenic and colleagues found that both psychosis and obesity contributed independently to added brain ageing, and that

when both were found in combination, added brain ageing was still greater; around 3.9 years (Kolenic et al. 2018). This suggests that non-brain health conditions, such obesity, can influence the brain's health, alongside central nervous system disorders like psychosis. Accordingly, alcohol dependence has also been shown to contribute to brain ageing (Guggenmos et al. 2017), a condition that likely involves damage to both the brain and other physiological systems.

Koutsourleris and colleagues' in-depth study of psychiatric brain ageing also included major depressive disorder (MDD) patients, reporting a mean added brain ageing of 4.0 years (Koutsouleris et al. 2013). Having borderline personality disorder was associated with a mean added brain ageing of 3.1 years. These initial studies in psychiatric disorders present a mixed picture based on the nature of psychiatric symptoms and how they influence brain ageing. Further studies looking at brain ageing and longitudinal outcomes or treatment response in psychiatric disorders offer a promising avenue for further research.

Mild cognitive impairment (MCI) and Alzheimer's disease are common pathological consequences of brain ageing. Alzheimer's disease patients have consistently been shown to have greater apparent brain ageing (Franke et al. 2010; Franke and Gaser 2012; Li et al. 2017; Löwe et al. 2016). In people diagnosed with MCI, brain age was a significant predictor of progression to dementia within three years (Franke and Gaser 2012; Löwe et al. 2016; Gaser et al. 2013). This suggests that brain age is sensitive to latent neuropathological processes that precede the overt manifestation of disease. In gradually progressive neurodegenerative conditions like Alzheimer's, ways of identifying those at higher risk of future deterioration and dementia will be invaluable for clinical practice and for clinical trial design, either to enrich trial enrolment or as a surrogate outcome measure.

Brain age has also been used in neurological settings. Increased brain ageing has been observed in survivors of a moderate-severe traumatic brain injury, both in civilian (Cole et al. 2015) and military (Savjani et al. 2017) samples. Treatment-resistant epilepsy, but not recently-diagnosed epilepsy was also associated with brain ageing (Pardoe et al. 2017). Adults with successfully treated HIV, potentially at risk of HIV-associated neurocognitive disorder, showed greater brain ageing compared to people without HIV cross-sectionally (Cole et al. 2017b). Nevertheless, longitudinal analysis indicated that rates of brain ageing were not influenced by having HIV (Cole et al. 2018a). While neurological diseases have distinct aetiologies, when these conditions are chronic, the downstream consequences and long-term outcomes are highly heterogenous within a single disease. Potentially, common underlying mechanisms in response to the initial pathology, such as neuroinflammation, may influence brain health over the longer-term in people with different chronic neurological conditions, resulting in brain atrophy that resembles that normally seen as a result of ageing. As with psychiatric conditions, brain age offers a way of indexing an individual's general brain health, relative to healthy people of a similar age, which may provide useful prognostic information for clinicians treating people with brain diseases.

19.3.2 Normal Ageing, Health and Physiological Diseases

Quantitative measurement of brain age may help to estimate the extent to which developmental factors, lifestyle, lifelong conditions, and chronic disease contribute to brain ageing. For example, Franke and colleagues showed that male offspring of mothers who were malnourished during pregnancy (Dutch famine 1944) had on average a significantly older brain age, by 4.3 years, when they reached their mid-sixties (Franke et al. 2018). These findings in humans are also consistent with those of an animal study indicating that under-nutrition during foetal development was associated with an older brain age, by 2.7 years, in young adult female baboons (Franke et al. 2017). In contrast, Cole and colleagues reported that early developmental factors in participants from the Lothian Birth Cohort (Scotland 1936) including paternal social class, childhood deprivation, and years of education were not predictive of brain age in individuals in their early 70 s (Cole et al. 2018b). This suggests that brain age is sensitive to some, but perhaps not all, developmental exposures likely to contribute to ageing processes.

The impact of chronic conditions that typically develop in middle-age after a protracted pre-clinical stage have also been investigated under the brain-age paradigm. Ronan and colleagues showed that obesity was associated with a significantly older brain age—corresponding to cerebral structure appearing 10 years older—from middle-age and into old age, in cross-sectional analyses of adults aged 20–87 years (Ronan et al. 2016). Mechanistically related to obesity, type 2 diabetes has also been found to be associated with premature ageing. In individuals in their mid-50 s, Franke et al. (2013) found that those with type 2 diabetes had a brain age on average 4.6 years older than those without. Importantly, longitudinal analyses in the same sample over four-year follow-up showed that every additional year with diabetes was associated with a 1.2-year increase in brain age. It is also worth noting that other lifestyle and health factors including blood glucose levels, depression scores, smoking duration and greater alcohol intake were also associated with higher brain age (range 3–6 years) across all participants in the same sample. In addition, in the Dutch famine cohort, heart rate, anti-hypertensive medication and alcohol intake were predictive of brain age but in males only (Franke et al. 2018). Furthermore, in a sample of cognitively unimpaired older adults (aged 60–90 years), Franke et al. (2014) reported that blood and body markers of metabolic syndrome and of liver and kidney functions were related to increased brain age (range 5.6–7.5 years) in males, whereas blood markers of liver and kidney functions as well as levels of vitamin B12 were significantly predictive of brain age in females (range 3.1–6.1 years). These effects of health and lifestyle markers on brain age were also shown to be additive, resulting in a difference of 15 and 5 years between males and females, respectively, with all ‘healthy’ versus all ‘unhealthy’ blood and body markers.

However, contradictory results are also available. For example, Cole and colleagues reported that brain age was not predicted by self-reported diabetes, cardiovascular disease and stroke (Cole et al. 2018b) and Franke and colleagues did not find an association between hypertension and brain age in the diabetes sample (Franke

et al. 2013), but did in a sample of cognitively unimpaired older males (Franke et al. 2014).

Interestingly, emerging evidence suggests that alongside associations of brain age with health and lifestyle parameters, brain age is also related to objective and subjective assessments of how old one appears. Biological ageing can be assessed by various objective measures that assess wear-and-tear, also referred to as allostatic load. Cole and colleagues found that those individuals with higher allostatic load had on average a higher brain age (Cole et al. 2018b). Consistent with these findings, subjective self-report of how old one feels have also shown that the older one feels the older their brain appears when assessed with the brain age measure (Kwak et al. 2018).

Finally, it appears that brain age is also predictive of mortality, perhaps the most compelling measure of cumulative morbidity. In the Lothian Birth Cohort 1936, every additional year in brain age was associated with a 6% higher risk of premature death. Importantly, brain age explained some of the variance in mortality above and beyond several health and volumetric brain measures (Cole et al. 2018b).

19.3.3 Genetics Influences on Brain Age

Currently only studies on the Apolipoprotein E (APOE) gene have been published in association with brain age. Cross-sectionally, neither APOE ε4-status, nor specific allelic isoforms had a significant effect on brain age in older healthy adults, MCI or Alzheimer's (Löwe et al. 2016). However, individual brain ageing accelerated significantly faster over 36-months follow-up in APOE ε4-carriers compared to APOE ε4-non-carriers both in people diagnosed with Alzheimer's at baseline (AD) or in those diagnosed with MCI at baseline who were subsequently diagnosed with Alzheimer's during follow-up (progressive MCI: *pMCI*). Moreover, in pMCI ε4-carriers older brain ages of about 6 years were observed at baseline, and brain ageing further accelerated during follow-up at the rate of 1.1 additional year per follow-up year. However, pMCI ε4-non-carriers were only found to have an older brain age by 0.6 years at baseline, while an increase of 1.7 additional years per follow-up year was found in AD ε4-carriers and of 0.9 years in AD ε4-non-carriers. In comparison, healthy controls as well as people diagnosed with MCI at baseline and at all time points during the follow-up did not show deviations from normal brain ageing trajectories (Löwe et al. 2016). Similarly, a cross-sectional *APOE* effect was not observed in people with Down's syndrome nor in a general population sample (Cole et al. 2017c; Cole et al. 2018b). Interestingly, Scheller et al. (2018) found an interaction between brain age and APOE genotype in a task fMRI study of working memory. Here, ε4-carriers with higher brain age recruited additional frontal regions in order to maintain working memory performance, suggesting neural compensation mechanisms at work to counter the neurodegenerative effects indicated by an increased brain age. While preliminary, these data suggest that APOE genotype may have a complex interactive relationship with the brain ageing process. While neuroimaging

measures of brain ageing appear to be heritable (Cole et al. 2017a), unsurprising given the demonstrated heritability of brain volume (Batouli et al. 2014), more research is needed to identify the specific genetic factors that affect brain ageing.

19.3.4 Environmental Influences on Brain Age

Environmental exposures have also been shown to have an impact on brain age. For example, Luders et al. (2016) showed that the brains of long-term meditators were estimated to be 7.5 years younger in their mid-50 s than brains of non-meditating controls. In addition, there was a main effect of sex, indicating that female brains were estimated to be 3.4 years younger than male brains. Follow-up analyses revealed that brain age did not vary as a function of chronological age in non-meditating controls, but significant associations between brain age and chronological age were detected in meditators. More specifically, in meditators there was a significant decrease of about 1 month in the brain age for every one-year-increase in chronological age over 50 years of age (Luders et al. 2016).

Rogenmoser et al. (2018) investigated the impact of music-playing on brain ageing, including non-musicians, amateur musicians (i.e., playing a musical instrument regularly but the main profession or education being outside the field of music), and professional musicians (i.e., performing artists, full-time music teachers, or full-time conservatory students), aged 25 $\pm$ 4 years. Participants in the three groups were closely matched with regard to age, sex, education, and amount of other leisure activities such as sports. The "musician status" had a strong effect on the brain age (non-musicians: -0.5 ± 6.8 years; amateur musicians: -4.5 ± 5.6 years; professional musicians: -3.7 ± 6.6 years), suggesting a general age-decelerating effect of music-playing on the brain.

In addition, a retrospective study on brain ageing in older individuals concluded that greater years of education and higher physical exercise levels (specifically, stair climbing) were associated with protective effects (Steffener et al. 2016). The use of the non-steroidal anti-inflammatory ibuprofen has also been associated with a transient decrease in brain age (Le et al. 2018) in a prospective trial, suggesting that some of the signal contributing to brain age predictions comes from persistent neuroinflammation. Although tentative at this stage, these studies highlight the possibility that intervention programs, either behavioural or pharmacological, may be able to moderate brain age, with the longer-term hope that these result in improved brain health during ageing.

19.4 Comparative Brain Ageing

19.4.1 Modelling Brain Ageing in Mammals

Brain ageing studies in human samples are almost always retrospective and tend to be confounded by a variety of lifestyle and environmental factors. Moreover, these studies do not readily allow for causal relationships to be determined. Therefore, animal experiments that introduce exposures to controlled factors are required to better quantify and understand the causal relationships between specific factors (e.g., perinatal nutrient delivery) and life-long effects on brain maturation and ageing.

Developmental programming studies have been mainly conducted in polytocous, altricial rodents, i.e., species with substantially different trajectories of foetal and neonatal brain development from monotocous, precocial mammals, including humans (Ganu et al. 2012; Fontana and Partridge 2015). Non-human primates have many similarities in physiology, neuroanatomy, reproduction, development, cognition, and social complexity to humans (Phillips et al. 2014; VandeBerg et al. 2009). The closest available species to relate to human programming in terms of reproduction, developmental physiology, gene function, or brain structure is the baboon (VandeBerg et al. 2009; Atkinson et al. 2015).

Species-specific MRI-based biomarkers for brain ageing are needed to model brain ageing and to indicate individual deviations from healthy brain ageing trajectories in experimental studies with mammals. Two species-specific adaptations of brain age have been recently developed for non-human primates (baboons) and rodents (rats) (Table 19.6), including novel species-specific pre-processing pipelines for anatomical MRI data (Franke et al. 2017; Franke et al. 2016).

An in vivo, non-invasive baboon-specific brain age equivalent, based on anatomical MRI, was applied to study the effects of moderate foetal undernutrition on brain ageing (Franke et al. 2017). By capturing individual deviations in the offspring, the goal was to translate findings to be comparable to humans. To enable comparative analysis of the effects of prenatal malnutrition, a baboon model of 30% reduction in global maternal nutrition during pregnancy was developed, while controlling for all other psychosocial stressors. In the maternal nutrient restriction (MNR) foetus, an altered trajectory of brain development during the foetal stage has been shown previously (Antonow-Schlorke et al. 2011). Subsequently, the adolescent MNR baboon offspring showed altered postnatal cognitive and behavioural performance at 3.3 years of age, equivalent to approximately age 11.5 years in humans (Keenan et al. 2013; Rodriguez et al. 2012). When applying brain age, premature brain ageing of about 2.7 years was demonstrated in the young adult MNR female participants (4–7 years; human equivalent 14–24 years (Franke et al. 2017)). The translational significance of this study is substantiated by a parallel MRI study in the Dutch famine birth cohort on exposure to foetal undernutrition during early gestation, in which decreased total brain volume in late adulthood was already shown in those who had been undernourished prenatally (de Rooij et al. 2016) as well as premature brain ageing by 4.3 years in older males (Franke et al. 2018).

19.5 Mechanisms of Brain Ageing

The evidence reviewed above clearly indicates that variability in cerebral senescence can be estimated using the brain age paradigm. This provides information beyond that offered by chronological age alone and shows promise in identifying/characterising genetic, health and lifestyle factors contributing to poorer brain health outcomes in later life. As noted previously several processes are implicated in biological ageing. What is less clear is which of these processes contribute most to the observed effects summarised in this review. Limited findings suggest that oxidative stress and pro-inflammatory mechanisms may be involved. Indeed, Franke and colleagues found that higher levels of tumour necrosis factor alpha (TNF-α), a cytokine involved in the pro-inflammatory cascade, were associated with higher brain age (Franke et al. 2013). Moreover, obesity and diabetes which have been found to be associated with brain age, are known to promote chronic low-level systemic inflammation. Converging evidence is also available from a study which investigated the associations between the menstrual cycle and brain age and found that higher oestrogen levels were associated with lower brain age estimates (Franke et al. 2015). Given oestrogen are known to have substantial anti-inflammatory properties it is likely that these properties underlie the observed effect. Moreover, in another study, Le and colleagues demonstrated that individual taking a common anti-inflammatory had a reduced brain age (Le et al. 2018). However, no association was found between telomere length and brain age (Cole et al. 2018b). Since, a major contributor to telomere shortening is oxidative stress and inflammation, these findings may suggest a more complex picture.

Thus, it is likely that other biological mechanisms also contribute to brain age variability. For example, cerebral blood flow and metabolism are known to be reduced in ageing and more so in those with type 2 diabetes and cardio-vascular disease and therefore would be expected to contribute to higher brain ages. Unfortunately, no research is available on this topic. It is also likely that lower neurogenesis and/or synaptogenesis may be implicated as they significantly contribute to brain structure, but again no evidence is available to assess their involvement.

Independently, or in combination with the mechanisms discussed above, it would appear that protein mis-folding and aggregation, such as amyloid-beta plaques, and the associated neurodegenerative changes observed in those most affected (e.g., individuals with dementia), clearly contribute to a greater brain age. Indeed, there are now several studies which demonstrate that populations with higher mis-folded protein loads have brain ages 5–8 years older than those not/less affected.

However, a number of issues remain currently unresolved. Beyond the implication of some low-level biological mechanisms it is unclear the extent to which genetic, environmental, social, and lifestyle factors contribute to increased brain age relative to each other. These factors tend to cluster and reinforce each other through feedback loops and it is therefore difficult to determine which contributes most or earliest to the underlying pathological processes. This is clearly illustrated by Franke et al. (2013) who concurrently showed an effect of type 2 diabetes, blood glucose levels, depression scores, smoking duration and greater alcohol intake in the same individuals.

Consequently, it will be important for new studies in this field to carefully consider the issue of shared variance, shared pathological pathways and co-morbidity as well as to develop research designs suited to assessing individual contributions of different mechanisms and risk factors for brain ageing.

19.6 Future Directions

As research into biomarkers of brain ageing matures, there are a number of further developments on the horizon. It is hoped that these developments will: improve our understanding of the neurobiological mechanisms underlying models of brain ageing; reduce potential confounds and improve model performance; and increase the clinical and societal impact of brain ageing research. These potential avenues can be grouped in more technical and conceptual developments, and into future applications and will be considered in turn.

19.6.1 Technical and Conceptual Developments

First and foremost, one major confound with any statistical modelling approach is the generalisability of the model to new data. The simplest way to deal with this is to increase the size of the training sample, thus increasing variability and making the training data more representative of the target population where the model could be applied. Neuroimaging has seen rapid adoption of data-sharing policies over the last decade and with the advent of projects such as the Human Connectome Project and UK Biobank Imaging Study, where data-sharing is the chief motivation, unprecedented amounts of neuroimaging data are becoming publicly-available. This means that future brain ageing research will benefit from access to much larger training datasets and to wider and more diverse validation datasets, where the predictions of brain age biomarker models can be tested to better evaluate generalisability.

Another important development comes from research into feature importance or 'interpretability' in multivariate models. In other words, the hope is to use brain age models to derive insights into which parts of the brain are most relevant for the ageing process. Many methods exist for assessing feature importance in classical and machine-learning models: standardised beta coefficients, LASSO approaches, model fit comparison, length-scales, impurity or entropy measures, non-negative matrix factorisation or even feature permutation. The limitation of these approaches is that they derive single 'modelwise' sets of feature importance metrics. While this may be highly informative, it overlooks the fact that there may be multiple different solutions to the same problem, making an assumption of homogeneity between individuals that is unlikely to be valid. By mapping feature importance at the individual level, it will be possible to discern different ways to arrive at the same classification label or regression value. Research into so-called 'saliency' or 'attention' maps is

very active in the machine-learning community, due to the recent rise of deep learning and the common criticism of deep learning as a 'black box'. By applying the principles of 'interpretable' machine learning to brain ageing models, it is hoped that individualised ageing maps can be generated. This opens the way to mapping the trajectories of spatial patterns of brain ageing within the individual, which could have great clinical utility. Individualised maps may also generate new hypotheses, uncovering latent subgroups within clinical samples or the general population who show similar patterns of brain ageing and may be more at risk of progression or age-associated diseases.

Other technical developments focus on models of disease-related deviation from patterns of healthy brain ageing (Gutierrez Becker et al. 2018; Li et al. 2017). Rather than assuming that diseases represent an acceleration of normal brain ageing, these models aim to incorporate both normal and pathological ageing into a single model, that can then be used to generate a biomarker of age-associated disease risk or decline, in conditions such as Alzheimer's. This direction will be important to explore further, as the evidence seems to suggest that chronic brain diseases result in both age-related and disease-specific pathological changes to brain structure and function. Thus, statistically learning different brain ageing models, both pathological and healthy, then combining them, should improve the accuracy and validity of such models for detecting specific diseases.

As highlighted above, a growing number of studies are incorporating multiple neuroimaging modalities into brain age models. This trend will likely continue, particularly with the ongoing release of the UK Biobank data (Miller et al. 2016). This will enable more accurate determination of a 'global' brain age, as well as modality specific 'volumetric age', 'connectomic age' or 'perfusion age'. This will lead to interesting investigations of the interrelatedness of ageing between different neural compartments and establish whether multi-modal brain age models are more informative about health outcomes than single-modality models. Given the exceedingly high-dimensional nature of multi-modality neuroimaging, this research will require the further evolution of methods for integrating data from different sources, potentially using techniques such as multiple kernel learning.

A final notable technical development is research aimed at reducing or eliminating the influence of site or scanner system effects on predictions of brain age. To be truly generalisable, a model's prediction should not be influenced by the location or the type of scanner used. While the current approach of using 'dirty' training sets from many different scanners and sites seems to give reasonable between-scanner reliability (Cole et al. 2017a), more sophisticated approaches are likely to be necessary if brain age is to be used as a biomarker in clinical practice. This may involve developing vendor or field-strength specific methods, or statistically learning the idiosyncrasies of a given scanner, so they can be eliminated during post-processing. Alternatively, efforts to harmonise acquisitions across different sites could be enhanced, though would be logistically challenging.

19.6.2 Future Applications

As the technical and conceptual side to brain age research develops, and models become more comprehensive, accurate and interpretable, it is important to promote research that applies these models for clinical and societal benefit and to answer scientific questions.

One major clinical application of brain age, and ageing biomarkers in general, is the ability to extrapolate from a single point measurement into an individual's future. Intuitively, if one appears biologically older, then one has a greater risk of age-associated deterioration and morbidity occurring earlier. However, what we cannot tell from a single point measurement is the rate of brain ageing. Potentially, increased brain age could reflect a static insult or a dynamic ongoing process, where the rate of ageing is accelerating, suggestive of a progressive pathological process. The prognostic implications of accelerated compared to added ageing are pronounced; clearly the long-term health outcomes would be less favourable if brain ageing is progressively accelerating away from the healthy 'norm'. Currently, few studies have conducted longitudinal neuroimaging assessments to establish whether brain age is static or accelerating (Schnack et al. 2016; Cole et al. 2018a). Further clinical studies with multiple longitudinal neuroimaging assessments will allow better modelling of brain ageing rates. This will in turn enable more accurate prognostic predictions to be made in chronic, progressive neurological and psychiatric diseases. Importantly, some studies use the term 'accelerating' when only cross-sectional data are available, potentially taking an inferential step too far. The terms 'added', 'premature' or 'accentuated' brain-ageing would be more appropriate in these cases.

Applications of brain age that further reinforce the validity of the framework will also be important for the field in general. In other words, demonstrating that models of brain age can accurately track a healthy individual's brain ageing over time, again using longitudinal neuroimaging data, will support its continued use in disease groups and hopefully its eventual clinical application.

Another important application is in brain and body ageing (Cole et al. 2019). The volume containing this this chapter is testament to the breadth of research into biological ageing across many different systems, organs, tissues and cells. Better integration of neuroscientific research, and particularly neuroimaging, with other spheres of biogerontology will be necessary to gain a comprehensive understanding of the biology of ageing. Hence, data from all available sources needs to be considered, whether brain, blood or biopsy.

Finally, brain age has great potential to be applied in clinical settings. Research using brain age in clinical screening, diagnostics and prognostics as well as clinical trial design is needed to showcase its potential to a wider audience of scientists, clinicians, policy makers and the public. While the prospect of mass-screening of the general population remains unfeasible using MRI, research into minimising data acquisition time, silent scanning and the advent of 'head-only' MRI scanners means that the costs and comfort associated with MRI data will improve over the coming years. EEG also presents an attractive alternative, for reasons of comfort, cost

and portability, were large-scale screenings to be conducted. More likely, studies showing that cross-sectional brain-age predictions at an early stage of a disease can predict outcome or even treatment response, could be pursued as an avenue offering great potential benefit to patients. The prospects for positive results in such studies is promising, given evidence that brain-age can help predict the development of Alzheimer's in people diagnosed with MCI (Gaser et al. 2013) and mortality risk in the general population (Cole et al. 2018b). Many clinical trials of interventions aimed at reducing the progression of neurodegenerative diseases and dementia risk, as well as other age-related morbidities, are underway. These include pharmacotherapies, exercise and diet programs. Brain age has the potential to be used to stratify trial enrolment, enriching samples with participants who have older-appearing brains and are thus more likely to experience rapidly progressing disease. Alternatively, brain age could be used as a surrogate outcome measure, as an indirect index of the efficacy of treatments aimed at slowing brain atrophy rates; allowing studies in asymptomatic or early disease stages to be conducted in much shorter time-frames than would be needed for overt clinical symptoms to manifest.

19.7 Summary

The increasing age of the global population has led to an increase in the prevalence of cognitive decline and neurodegenerative diseases, particularly dementia. Hence, there is a pressing need to measure the brain ageing process, understand its underlying mechanisms and develop diagnostic and prognostic tools and effective treatments to help people age with healthier brains. The combination of neuroimaging, particularly MRI, with machine learning analysis applied to ever larger datasets has enabled this new field of brain ageing research to develop. Already, the technical considerations for deriving more reliable and valid brain age measurements have matured considerably, and the accelerating interest and investment in artificial intelligence and data science are likely to further augment efforts to improve brain age methods. Philosophical and conceptual questions remain about what it means to measure age biologically and whether or not an 'old brain' is an 'ill brain' will be the subject of ongoing debate. Nevertheless, the practical and clinical applications of brain age research hold the potential to improve diagnostic and prognostic approaches in patients suffering from the adverse effects of brain ageing. In the long-term, quantification of the brain's biological age may help to understand and alleviate the causes of poor brain health in the ageing population, allowing more people to be cognitive healthy as well as wise in old age.

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Chapter 20
Arterial Aging: The Role of Hormonal and Metabolic Status and Telomere Biology

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Abstract Age-associated arterial wall changes create an enzymatically and metabolically favorable environment for cardiovascular diseases development. Understanding the essence of the processes underlying the development of CVD can open up new opportunities for more effective preventive actions. Age, gender, blood pressure, lipid levels, type 2 diabetes mellitus are traditional cardiovascular risk factors used in the most popular models, such as Framingham and SCORE. However, these risk stratification systems have a number of limitations and explain no more than 50% reduction in mortality from cardiovascular diseases. There is not enough information on the association between signs of arterial wall aging and factors that determine the aging processes in general and in their interaction with cardiovascular risk factors. The aim of the work was to study the relationship between parameters of arterial wall aging, traditional risk factors, hormonal status, leukocyte telomere length. A total of 303 ambulatory participants (104 males and 199 females) were recruited. The subjects ranged between 23 and 91 years of age, with a mean age of 51.8 ± 13.3 years. Based on the results obtained, the following conclusions can be drawn: the value of traditional cardiovascular risk factors for the vascular wall changes is reduced in older age; carbohydrate metabolism disorders and hypertension are "universal" risk factors most closely associated with the arterial wall condition in both younger and older group. To predict changes in the vascular wall, it is advisable to study insulin resistance, growth hormone, insulin-like growth factor-1 activity and leukocyte telomere length along with conventional cardiovascular risk factors.

Keywords Arterial aging · Cardiovascular risk factors · Insulin resistance · Growth hormone · Insulin-like growth factor-1 · Leukocyte telomere length · Telomerase activity

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20.1 Introduction

The fact that cardiovascular diseases (CVD) are still the leading cause of death worldwide (Fuster et al. 2011), determines the great importance of their primary prevention. Results of prospective studies, such as the Framingham, helped to identify the key factors of cardiovascular risk (CVRF) and were used to develop population-based strategies for the prevention of coronary artery disease (CAD) (Kannel 2002). However, these approaches have some limitations and explain no more than 50% reduction in mortality from 1980 to 1990 (Hunink et al. 1997). One of the most important CVRF is age. The frequency of atherothrombotic conditions increases with age. In the US, octogenarians account for 5% of the population; 20% of hospitalized with myocardial infarction are octogenarians, and among those who died from myocardial infarction octogenarians make up 30% (Bureau 2010). Nevertheless, the main efforts are aimed at reducing modifiable risk factors such as hypertension, hypercholesterolemia, smoking, etc., while age is considered as an unmodifiable, and therefore not preventable and "not treatable" risk factor. Until recently, it has been assumed that its role in the development of CVD is primarily associated with a longer exposure of other CVRF, while the specific effect of aging itself was not well enough understood. In fact, age-associated arterial wall changes create an enzymatically and metabolically favorable environment for CVD development and have important prognostic implications for the development of cardiovascular events (Vlachopoulos et al. 2010; Tziomalos et al. 2010; Green et al. 2011). Understanding the essence of the processes underlying the development of CVD can open up new opportunities for more effective preventive actions.

The vascular wall consists of three complex layers: intima, media and adventitia. The inner part of the intima is represented by a monolayer of specialized endothelial cells followed by a subendothelial space and a basal membrane. Smooth muscle cells (SMC) of the media are surrounded by a matrix connective tissue consisting mainly of elastin and collagen. Adventitia, the outer vessel layer, consists of fibrous connective tissue, blood vessel network that feed the vascular wall and nerve fibers. With age each of these layers undergoes complex changes leading to two main outcomes: wall thickening and stiffening. The condition of arterial wall depends to the greatest extent on the state of the endothelium. Endothelial cells, due to a variety of receptors, perceive mechanical signals such as blood pressure (BP) and blood flow velocity, chemical signals, for example, oxygen tension, levels of hormones, mediators, metabolites, and changes in the body's internal environment such as temperature. In response to these signals endothelial cells secrete cytokines and chemokines, growth factors and other substances that regulate structure and function of the arteries. The main sign of endothelium aging is the development of its dysfunction and increased permeability. A key role in maintaining the endothelium integrity belongs to nitric oxide (NO) that ensures the maintenance of the arteries' elasticity, stimulates the processes of vasodilation and relaxation and prevent adhesion of platelets and leukocytes. Advanced glycation end products (AGEs), which are accumulated with age, play a significant role in endothelial dysfunction. Glycation is the main

reason for spontaneous disruption of the structure of intracellular and extracellular proteins in various physiological systems. This process is intensified against the background of diabetes mellitus (DM). Endothelial cells express receptors to AGEs, which binding with AGEs initiate the expression of adhesion molecules and inflammatory cells transmigration, platelet aggregation, increase in endothelial permeability and, most importantly, a decrease in the NO bioavailability (Wautier and Schmidt 2004). Normally, endothelium damage, as a result of chronic inflammation and oxidative stress should be accompanied by its restoration. This function is mainly based on endothelial progenitor cells (EPC). They recover damaged or old vessels using the endogenous regeneration mechanism. There is conflicting evidence as to whether the number of EPC decreases with aging (Shimada et al. 2004) or their function suffers (Spyridopoulos et al. 2002). In any case, neoangiogenesis and endothelial repair are interrupted with aging. The assessment of endothelium-dependent vasodilation based on the change in the diameter of the superficial brachial artery in response to the reactive hyperemia is one of the most widely used methods for evaluation of the endothelial function (Celermajer et al. 1992). Aging of the media is characterized by structural and functional disorders of elastin and collagen along with migration of SMC from media into the intima. This process is stimulated by angiotensin II (AT II), whose destructive effect is not limited by intima, but plays a great role in age-associated changes in the media. As the number of collagen molecules increases, they become bound with glucose molecules and form cross-links by AGEs, which significantly increase collagen rigidity and disrupt normal processes of its transformation (Zieman et al. 2005). Elastin may also undergo the glycation process (Konova et al. 2004). The most important aspect of age-related changes in the vascular wall is its calcination. The fact that there is a clear inverse relationship between the degree of calcification of the vessels and the level of bone mineralization was labeled the calcification paradox (Persy and D'Haese 2009). Decreasing of the elasticity of the arteries is considered to be the most important sign of vascular aging, while the evaluation of the carotid-femoral pulse wave velocity (PWV) is the most widely used method to assess arterial stiffness nowadays (Laurent et al. 2006). Increased PWV (reference value $\leq$10 m/s) is a reliable independent predictor of unfavourable cardiovascular events and overall mortality (Van Bortel et al. 2012). The second main sign of vascular aging is the diffuse intima thickening, which occurs as a result of a variety of factors such as the accumulation of extracellular matrix proteins, collagen, glycosaminoglycans, SMC migrated from the media, increased adhesion molecules expression, and as a consequence increased adhesion of monocytes to the endothelial surface (Orlandi et al. 2000). Vascular wall thickness is assessed by means of duplex scanning of the extracranial part of the brachiocephalic arteries by measuring the intima-media thickness (IMT). IMT measurement is performed along the posterior wall in the distal third of the common carotid artery 1 cm from the bifurcation. The thickened wall is considered when IMT value $\geq$0.9 mm (European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). Atherosclerotic plaque (AP) is defined as the focal thickening of the vessel wall by more than 50% compared to surrounding areas or as a focal thickening of the intima-media complex by more than 1.5 mm protruding into the vessel lumen (Stein et al. 2008). It is known

that IMT correlates with high risk of myocardial infarction and stroke (O'Leary et al. 1999) and is considered as a marker of atherosclerosis. On the other hand, given that IMT increases linearly with age even in individuals without AP, intima-media thickening is believed to be a consequence of physiological remodeling of the artery wall during aging and of age-related inflammation, but not atherosclerosis (Nagai et al. 1998). Atherosclerosis and remodeling of the arteries are closely related and are caused by the same biochemical, inflammatory, and metabolic factors (Najjar et al. 2005). Increased IMT and AP do not always develop simultaneously. Plaques may reflect a later stage or other changes not associated with wall thickening. Although arteriosclerosis (arterial stiffness) and atherosclerosis have common features (enzymatic, metabolic, inflammatory, and cellular changes), common risk factors and outcomes (heart failure, stroke), and despite the fact that they often coexist and accelerate each other, they are considered as different processes (O'Rourke et al. 2010).

Lesions of target organs, including left ventricular hypertrophy, microalbuminuria, etc., increased stiffness and thickening of arteries can be considered as an intermediate stage between risk factors and CVD development. These markers along with traditional risk factors, especially in people with borderline values of traditional risk factors or with a family history of CVD, may increase the predictive value of classical CVD risk assessment. Arterial stiffness for example reflects existing arterial wall damage, while BP, glucose, and lipid levels have temporary fluctuations, and their values may not correspond to those for a longer or shorter period. In the Expert Consensus Document on Arterial Stiffness it was reported that PWV measurement is of greater predictive value than traditional CVRF (Laurent et al. 2006). The predictive value of PWV for the CAD development was the highest in patients from the low-risk group according to Framingham scale (Boutouyrie et al. 2002). Intima thickening serves as a favorable basis for further development of atherosclerosis. In a large study of healthy volunteers, it was shown that in those with the highest IMT CVD developed 4 times more often in the next 7 years than in those with the lowest IMT (Fleg et al. 1995), while in the prospective ARIC study (Atherosclerosis Risk in Communities Study, 1985–2016), the incidence of CAD was increased by 4.3 times in males and 19.5 times in females with IMT increasing from 0.6 to 1 mm (Heiss et al. 1991).

The concept of early vascular aging (the so-called EVA syndrome) was recently developed (Nilsson et al. 2009). Authors consider that cardiovascular risk depends not only on CVRF influence but also on the programming in the intrauterine period. Vessel structure and function are programmed in a certain way during early periods of life. Thus, the developmental disability of the foetus is associated with a decrease in the density of the capillary network, endothelial dysfunction, and smaller diameter of the arteries compared with children with normal fetal development.

20.2 Arterial Aging and Cardiovascular Risk Factors

Arterial wall changes frequently occur in individuals free of clinical CVD. Among participants of the Cardiovascular Heart Study (healthy elderly people) subclinical artery changes were found in 49% of women and 62% of men (Kuller et al. 1994). Results of the Guimaraes/Vizela Study, which was conducted in northern Portugal since 2010, showed that vascular wall changing started already at a young age (Cunha et al. 2015). The results of this study demonstrated that the prevalence of early vascular aging (high PWV under the age of 40 years) was 12.5%. At the same time, the most impressive results were obtained in people under 30 years of age. In 26.1% of them PWV exceeded 97.5 percentile of the mean values for this age group. In 34% of people under the age of 40 PWV exceeded 90 percentile of the expected values for this age. The value of PWV exceeded 10 m/s in 18.7% of people over 40 years and in 14.1% at the age of 40–50. It is important that these alarming data were obtained in a country with low CVD risk. The question of the association between PWV and IMT also has an ambiguous solution. Van Popele et al. (2001) and Kobayashi et al. (2004) demonstrated the association between IMT and PWV, while Zureik et al. (2002) showed that PWV is associated with AP but not IMT. Koivistoinen et al. (2012) revealed the relationship between PWV and IMT in the group of 46–76 years, but did not find it in people younger than 45 years. Thus, it should be recognized that until now the relationship between atherosclerosis and arteriosclerosis has definitely not been established.

Age, gender, blood pressure, lipid levels, type 2 diabetes mellitus (DM2) are traditional CVRF used in the most popular models, such as Framingham (D'Agostino et al. 2008) and SCORE (Conroy et al. 2003). However, these risk stratification systems have a number of limitations. The prognostic role of some risk factors has been demonstrated in studies involving mostly middle-aged people. It can be assumed that these risk factors would be less significant in those who did not suffer CVD till the older age. The association between traditional CVRF and the condition of the arterial wall in individuals without clinical CVD is also poorly understood. According to the CHS and ARIC studies, there is weakening of the association between traditional CVRF and subclinical changes in arteries in the older age (Howard et al. 1997). Lipids level and BMI in the elderly were less associated with CVD risk than in young people (Psaty et al. 1999), obesity and hyperlipidemia in the older age group were recognized as unimportant CVRF. It was suggested that elderly people with CVRF but without clinical CVD are resistant to the CVRF effects. Hypertension and impaired glucose metabolism were considered as the most significant risk factors of arterial aging. Psaty et al. showed that in the elderly the 5-year risk of myocardial infarction and stroke increases by 24 and 34% respectively for every 21 mm hg systolic BP (SBP). It is important that not only SBP higher than 140 mmHg but also subthreshold values are associated with increased cardiovascular risk (Psaty et al. 2001).

It was considered that the cardiovascular risk continuously increased with the BP increasing throughout the whole range of its values. According to modern concepts,

hypertension is associated not only with the atherosclerotic process, but also with arteriosclerosis. In this case, the cause-effect relationship is twofold. Increased BP has a significant hemodynamic load on the vessel wall, leading to its damage and remodeling. On the other hand, increase of arterial stiffness can develop against the background of normal BP, being a predictor of further development of hypertension (Nilsson et al. 2013).

Impaired glucose metabolism was recognized as another continuous risk factor in older people. Increase in fasting glucose (FG) level by 1 mmol/l was associated with an increased risk of myocardial infarction by 1.12 times, and the presence of DM2 by 1.9 times increased the risk of developing CAD (Psaty et al. 1999). Chronic hyperlipidemia and hyperinsulinemia promote SMC hypertrophy, fibrosis, endothelial dysfunction, and further disruption of the vascular wall properties, increasing its stiffness through the activation of the renin-angiotensin-aldosterone system and AGE deposition. It is important that arterial rigidity is not the result of a long-standing DM2 but develops as a result of hormonal and metabolic changes that occur even with the initial manifestations of insulin resistance (IR).

In a population of the Malmo Diet and Cancer study the authors studied the relationship between PWV and traditional CVRF corrected for mean BP and heart rate (Gottsater et al. 2015). On the one hand, the results demonstrated the relationship between hyperglycemia, abdominal obesity and dyslipidemia with arterial stiffness, but on the other, they did not reveal the relationship between PWV and smoking, glomerular filtration rate, low-density lipoprotein cholesterol. These results served as the basis for conclusions about the existence of clusters of different risk factors of atherosclerosis and arteriosclerosis. In the Caerphilly Prospective Study (McEniery et al. 2010) all CVRF except for hypertension had a very weak effect on arterial stiffness. In the Whitehall II Cohort study, central obesity (often adjacent to inflammation) was a reliable predictor of aortic stiffness along with heart rate in men and adiponectin level in women (Brunner et al. 2015).

There is even less information on the association between signs of arterial wall aging and factors that determine the aging processes in general and in their interaction with CVRF. Factors that influence the aging process include IR, growth hormone (GH) activity, insulin-like growth factor-1 (IGF-1), thyroid function and leukocyte telomere length (LTL).

20.3 Insulin Resistance and Arterial Wall

IR is a decrease of sensitivity of insulin-sensitive tissues (adipose tissue, liver, striated muscles) to insulin at the background of its sufficient concentration. IR leads to a decrease in triglyceride synthesis in the liver and increased lipolysis, which is accompanied by the free fatty acids release into the bloodstream and ectopic fat accumulation (in the liver, muscles, pancreas). This in turn violates the uptake of glucose in the muscles and liver, increases glucose production in the liver which leads to hyperglycemia. Hyperglycemia stimulates insulin production. Hyperinsu-

linemia along with hyperglycemia and hyperlipidemia cause metabolic disorders that ultimately lead to metabolic syndrome and DM2. Insulin stimulation by a local renin-angiotensin system in blood vessels causes an increase in NADPH-oxidase activity, a decrease in the NO bioavailability, as well as an increase in the reactive oxygen species (ROS) production (Wang et al. 2007). AT II, oxidative stress, endothelial dysfunction, pro-inflammatory cytokines and adhesion molecules activate matrix metalloproteinases (MMPs) that cause elastin molecules fragmentation and collagen stiffness (Jacob 2003). IR and hyperinsulinemia can lead to increased arterial stiffness even before the development of impaired glucose tolerance or DM2, as was shown in elderly patients with hypertension (Sengstock et al. 2005). It was established that IR and compensatory hyperinsulinemia affect a number of BP regulation mechanisms. Hyperinsulinemia contributes to the increase in the reabsorption of sodium and water by the kidneys, stimulates sympathetic nervous system and Na^+/H^+ metabolism activation in the vessels SMC, which causes accumulation of Na^+ and Ca^+ ions and an increase in the sensitivity to catecholamines and ATII pressor effects. Sympatho-adrenal system activation under the influence of hyperinsulinemia and elevated levels of free fatty acids leads to a violation of the circadian rhythm of BP with its insufficient decrease during night hours, i.e. to the development of nighttime hypertension. IR is characterized by the development of dyslipidemia, which is an increase in triglycerides and LDL, as well as a decrease in the level of high density lipoproteins. LDL is mainly represented by the subfraction of highly atherogenic dense particles. Their ability to bind to LDL receptors is reduced, so they circulate for a long time in the bloodstream, become oxidized, and are actively captured by macrophages. By secreting growth factors and cytokines macrophages cause a thickening of the vessel wall and contribute to the plaque development and destabilization (Ford et al. 2002). It was shown that hyperinsulinemia caused hyperfibrinogenemia and an increase in the activity of plasminogen activator inhibitor-1, which led to fibrinolysis failure. Violations in the fibrinolysis system contribute to the progression of atherothrombosis (Choi et al. 2007). The mechanism underlying the relationship between IR and arterial stiffness is unknown. Some studies found a relationship between IR and arterial stiffness in both patients with diabetes and healthy young individuals (Giltay et al. 1999). As a factor influencing arterial stiffness, the effect of insulin per se is of potential importance and is not definitively established. Unresponsiveness of endothelium-mediated vasodilation associated with IR could explain the link to arterial stiffness (Hu et al. 1997). Insulin has also demonstrated the potential to induce vascular SMC proliferation and migration in cell culture (Indolfi et al. 2001). These factors may contribute to increased arterial stiffness before the development of impaired glucose tolerance or diabetes (Scuteri et al. 2004). Additional studies are also needed to assess the effect of reversibility, i.e. improved insulin sensitivity as a way to decrease arterial stiffness.

20.4 Growth Hormone, Insulin-like Growth Factor-1 and Arterial Wall

GH is one of the main regulators of metabolic processes having both a direct effect on various tissues and organs which is mediated by the peripheral "mediator" IGF-1. The most of IGF-1 is synthesized in liver cells under the action of GH. However, there are other types of cells that produce IGF-1 and have receptors for it, in particular cardiomyocytes, SMC and endotheliocytes (Chisalita et al. 2009). These hormones are important anabolics that stimulate cell growth, proliferation, and tissue repair. The levels of GH and IGF-1 decrease with age in both laboratory animals and humans (Carter et al. 2002). People with GH deficiency and low circulating IGF-1 levels been documented to have an increased risk of developing CVD (Laughlin et al. 2004). The role of IGF-1 in cardiovascular pathology was firstly observed in patients with pituitary diseases. Epidemiological studies have shown that in patients with a long period of IGF-1 deficiency and somatotropic insufficiency, cardiovascular mortality was two times higher than in the general population (Higashi et al. 2012). GH/IGF-1 deficiency leads to physiological age-related changes in the cardiovascular system, such as a decrease in the cardiomyocyte number, fibrosis, collagen accumulation, and decrease in the synthesis of proteins, including contractile actin and myosin (LeRoith et al. 1995). Rosen and Bengtsson were the first to demonstrate a reduction in the quality of life in patients with hypopituitarism (HP) (Rosen and Bengtsson 1990). They analyzed data from 333 patients with HP who visited endocrinology clinics between 1956 and 1987. During the follow-up period 104 patients died, which corresponded to a significantly higher mortality rate compared to the general population. The excess mortality was related to deaths from CVD, and the most common causes were myocardial infarction, CAD, congestive heart failure, and cerebrovascular disease. Subsequently, the relationship between the level of GH/IGF-1 and the prognosis of CVD was revealed in non-HP patients. In a cross-sectional study, Spallarossa et al. showed that a low level of IGF-1 was associated with angiographically confirmed CAD (Spallarossa et al. 1996). A prospective case-control study (observed 600 participants for 15 years) showed that the level of IGF-1 below the median increased the risk of CAD (Juul et al. 2002). The level of IGF-1 predicted death from CAD in 1185 people of both sexes in a study conducted by Laughlin et al. (Laughlin et al. 2004). A low level of IGF-1 was associated with a worse prognosis in the early period of myocardial infarction (Conti et al. 2001). The explanation for these relationships may lie in the association of the hormone levels with the arterial wall, even at the stage of subclinical changes. Thus, flow-dependent vasodilatation of peripheral arteries was impaired in patients with GH deficiency (Smith et al. 2002). Galderisi et al. revealed a positive relationship between IGF-1 and coronary reserve (Galderisi et al. 2002). A cross-sectional study on 400 elderly men conducted by van Den Beld et al. revealed an inverse correlation between IGF-1 and IMT (van den Beld et al. 2003). These results become clear when considering that the cardiovascular system is the target of GH and IGF-1. Most clinical studies support the idea that normal levels of GH and IGF-1 are necessary to maintain endothelial health. IGF-1 is involved in the

synthesis of NO in endothelial cells, causing additional vasodilation of the arteries (Chisalita and Arnqvist 2004). IGF-1 is a powerful mitogenic, anti-apoptotic, and promigrant factor for both endotheliocytes and SMC (Arnqvist 2008). That is, IGF-1 can be pro-atherogenic by its ability to stimulate the migration and proliferation of SMC and macrophage migration (Renier et al. 1996), to promote the expression of adhesion molecules (Li et al. 2009), decrease in the level of IGF-1 can cause plaque destabilization (Libby and Sasiela 2006).

20.5 Thyroid Function and Arterial Wall

Thyroid dysfunction has a significant effect on the cardiovascular system, both on the development of atherosclerosis and arteriosclerosis. The increased thyroid function causes endothelial damage, hemostasis disorders, thrombosis, and hemodynamic changes. Thyroid hormones (TH) increase leads to the ROS formation, which induce expression of the adhesion molecules on endothelial cells (De Sibio et al. 2013). Hyperthyroidism is associated with an increase in the level of endothelial dysfunction markers such as E-selectin, intracellular adhesion molecule-1, and vascular cell adhesion molecules (Wenisch et al. 1995). Elevated freeT4 level is associated with increased concentrations of various procoagulant proteins, von Willebrand factor, fibrinogen, VIII and IX factors, which can contribute to plaques destabilization (Shih et al. 2004; Stuijver et al. 2012). High TH levels increase myocardial contractility, heart rate, pulse pressure and PWV (Delitala et al. 2015). On the other hand, TH deficiency is associated with worsening of traditional CVRF, including increased LDL, homocysteine (Christ-Crain et al. 2003) and procoagulant state development (Chadarevian et al. 2001).

Major epidemiological studies have assessed the relationship between thyroid function and clinical manifestations of atherosclerosis, such as CAD or stroke. The results were diverging. Thus, in some studies (Asvold et al. 2015), the relationship between thyroid function and CVD outcomes was not found, others reported an increased risk of cardiovascular events at both low (Rodondi et al. 2010) and elevated thyroid function (Chaker et al. 2016). The discrepancies between the studies may be partly due to differences in the observation period and age of participants and different approaches to atherosclerosis assessment.

There are fewer studies devoted to the association between thyroid function and subclinical arterial changes, which can be considered as signs of arterial aging. Recent cross-sectional studies, which evaluated the relationship between thyroid-stimulating hormone (TSH) with IMT and AP in general population, have come to different conclusions (Jorde et al. 2008; Volzke et al. 2004). The SardiNIA study analyzed data from 5815 people (aged 14–102 years) who did not have overt hyperthyroidism or hypothyroidism and who did not take drugs that affect thyroid function. Evaluated were serum TSH, free T4, IMT, and the amount of AP in the carotid arteries (Deli-

tala et al. 2015). There was no association between IMT and AP with subclinical thyroid dysfunction, which confirmed the conclusion that the acceleration of arterial remodeling and the development of atherosclerosis do not depend on minor changes in TH levels. The lack of a significant association between IMT and TSH has been shown in some papers (Jorde et al. 2008). On the contrary, other studies showed that the decrease in thyroid function within reference values is associated with IMT (Takamura et al. 2009). The data on the association between the TH and the AP are also questionable. Chiche et al. showed that the values of TSH and freeT4 were not related to the prevalence of carotid AP in both patients with clinical and subclinical hypothyroidism (Chiche et al. 2009). Conversely, Dörr et al. reported a relationship between subclinical hyperthyroidism and carotid AP and stroke development with an OR of 1.67 and 1.98, respectively (Dorr et al. 2008). A large-scale Rotterdam study examined the relationship between thyroid function and AP at various stages from subclinical to clinical manifestations (Bano et al. 2017). A total of 9420 participants were included in the study (mean age 64.8 ± 9.7 years). The median follow-up was 8.8 years (interquartile interval 4.5–11.8 years). Free T4 level was positively associated with a high rate of coronary calcium (odds ratio 2.28, 95% CI 1.30–4.02) and cardiovascular mortality (hazard ratio 1.87). This relationship was independent of traditional CVRF, it was observed in patients with hyper- and euthyroidism. There were no links between TSH and atherosclerosis. Both hyperthyroidism and long-term therapy with levothyroxine may disrupt vascular elasticity and lead to arterial stiffness even in subjects with normal BP values (Palmieri et al. 2004). Some studies reported an increase in arterial stiffness in patients with hypothyroidism (Obuobie et al. 2002). After normalization of thyroid function or against substitution therapy with levothyroxine a decrease in arterial stiffness was described in patients with hypothyroidism (Nagasaki et al. 2005). In the SardiNIA study, the relationship between PWV and free T4 was linear, direct, and independent of other factors (Delitala et al. 2015). The contribution of T4 to the variability of PWV was comparable to the contribution of other known cardiovascular risk factors, such as dyslipidemia and diabetes, and as a whole they accounted for 55% of the variability. Thus, we can assume a U-shaped relationship between the arterial wall parameters and thyroid hormones with deterioration in hypothyroidism as well as hyperthyroidism. Minor fluctuations in the thyroid function within subclinical changes do not play a significant role in the remodeling (aging) of the arterial wall.

20.6 Telomeres and Arterial Wall

Telomeres, as the TTAGGG tandem repeats at the ends of chromosomes, progressively shorten with each replication of cultured human somatic cells. Telomerase plays the key role in maintaining telomere length. The loss of telomere length is accelerated by chronic inflammation and oxidative stress (Serra et al. 2003). Leukocytes telomeres length (LTL) reflects both an individual's telomere length at birth

and the telomere attrition during the life course, demonstrating replicative history and cumulative oxidative burden (Aviv 2004).

Numerous studies have shown the association of LTL with CVD, DM2, and dementia (Haycock et al. 2014), which was relevant both for younger people (Fitzpatrick et al. 2007) and for the elderly (Epel et al. 2008). Associations between LTL and heart failure (van der Harst et al. 2007) and cardiovascular events prognosis (Willeit et al. 2010) were demonstrated.

Cardiovascular Health Study showed that every DNA reduction per 1000 nucleotides increases the risk of myocardial infarction and stroke by 3 times (Fitzpatrick et al. 2007). In a recent meta-analysis, a decrease in LTL by an amount equal to 1 standard deviation was associated with a 21, 24 and 37% increase in the risk of stroke, myocardial infarction, DM2, respectively (D'Mello et al. 2015). In patients with early manifestations of CAD (myocardial infarction at a young age), the average LTL was shorter in comparison with healthy people of the same sex and age. Moreover, the rate of telomere shortening in these patients was higher (Samani et al. 2001). In a large Danish study, which included 19,838 participants with the 19 years duration, multifactor analysis revealed an increased risk of myocardial infarction, CAD, and death (RR 1.24, 1.24, 1.49, respectively) in the group with the lowest decile of LTL as compared to the group with the highest decile (Weischer et al. 2012). The West of Scotland Primary Prevention Study also demonstrated that LTL may be a predictor of the adverse cardiovascular events development in the group of middle-aged men and high cardiovascular risk. In patients receiving statins (pravastatin), the increased risk due to shorter telomeres was eliminated (Brouilette et al. 2007).

20.7 Original Results

The aim of our work was to study the relationship between parameters of arterial wall aging, traditional risk factors, hormonal status, LTL. A total of 303 ambulatory participants (104 males and 199 females) were recruited. The subjects ranged between 23 and 91 years of age, with a mean age of 51.8 ± 13.3 years. We excluded subjects with previous history of drug medication for DM, hypertension or hyperlipidaemia; a history of stroke, CAD, peripheral arterial disease, arrhythmia, congestive heart failure, or valvular heart disease; hepatic or kidney failure, as well as cancer. We assumed that the nature of the associations between the studied indicators and the artery wall parameters may differ between younger and older ages, which is why separate analyses were conducted for these groups. The younger group included men of 45 years of age and under and women of 55 years of age and under, while the older subjects formed the older group. PWV measurement was carried out using a SphygmoCor device (AtCorMedical, Australia). Ultrasound examination of carotid arteries for the IMT and AP assessment was performed in B mode by a linear high-resolution 17–5 MHz sensor (PHILIPS iU22, Netherlands). Endothelium-dependent

vasodilation evaluation was performed using a test with reactive hyperemia. LTL was determined according to the method described by Cawthon (2002).

The clinical characteristics of patients are presented in Table 20.1.

The comparative characteristics of the arterial wall parameters is presented in Table 20.2.

We discovered a relatively high prevalence of arterial wall changes in the younger group.

To assess the associations between different arterial wall parameters logistic regression analysis was carried out. Only increased IMT (OR 2.34, CI 1.16–4.33, $p = 0.017$) and older age (OR 3.92, CI 2.13–7.19, $p < 0.0001$), but not the presence of AP and endothelial dysfunction, increased the likelihood of having stiff arteries.

The level of SBP and FG in most cases was associated with the arterial wall in both younger and older groups. Indicators such as gender, BMI, cholesterol played an important role in younger people. The association between CVRF and arterial wall parameters at a younger age was stronger than in older group. This is supported by data from multifactoral logistic regression analysis. In the younger group high FG increased the probability of PWV > 10 m/s by 5.75 times ($p = 0.003$, CI: 1.19–27.81), IMT $\geq$ 0.9 mm by 6.75 times ($p = 0.022$, CI: 1.32–34.56), in the older group it was associated with increased risk of having IMT $\geq$ 0.9 mm by 3.06 times ($p = 0.007$, CI: 1, 36–6.89) and was not associated with an increased risk of having stiff arteries. SPB >140 mmHg was associated with an increase in the probability of IMT $\geq$ 0.9 mm by 9.34 times ($p = 0.029$, CI: 1.26–69.59), decreased endothelial vasodilation <10% by 4.25 times ($p = 0.016$, CI: 1.32–13.75) in the younger group, increase in the probability of IMT $\geq$ 0.9 mm by 2.39 times ($p = 0.044$, CI: 1, 02–5.56), endothelial dysfunction by 2.19 times ($p = 0.05$, CI: 1.00–4.76) in the older group.

TSH did not demonstrate any associations with arterial wall parameters.

To identify the independent relationships of GH, IGF-1, HOMA, LTL with PWV, IMT, AP number, linear regression models were compiled using the arterial wall indicators as a dependent variable. In the general group, only PWV as a dependent variable was negatively associated with GH ($p = 0.018$), IGF-1($p = 0.013$), and LTL ($p = 0.053$) (with adjustment for CVRF). IMT and the plaques number did not demonstrate this relationship.

In the younger group, HOMA, GH, and IGF-1 demonstrated an independent relationship with all the studied arterial wall parameters, but LTL correlated only with PWV. The highest coefficient of $R^2 = 0.45$ was in the IMT variability model. Logistic regression analysis showed that the probability of having AP in younger group was increased in the first quartile of distribution of LTL (OR = 16,98; 95% CI 2,10-137,01; p=0,008). IGF-1 greater than the median (>140 ng/ml) was associated with a five-fold lower probability of AP being present (OR = 0.174; 95% CI 0.04–0.73; $p = 0.017$).

In contrast, there were no independent significant associations of GH/IGF-1, HOMA, LTL and arterial wall characteristics obtained through multiple linear regression analysis in the older group. The results indicate that the level of hormones (GH, IGF-1) and HOMA index were significantly related to the vascular wall parameters in the general and younger groups but not the older group. These data once again

Table 20.1 Characteristics of the patients included in the study (n = 303)

Parameter	All patients (n = 303)	Younger group (n = 144)	Older group (n = 159)	*p*
Age (years), M ± SD	51.5 ± 13.3	40.9 ± 8.7	61.1 ± 8.5	<0.001
Men (%)	34	26.4	41.5	<0.001
Smoking (%)	19.1	20.8	17.6	0.478
Arterial hypertension (%)	25.4	17.4	32.0	0.003
Hypercholesteremia (%)	66.3	61.1	71.1	0.068
Fasting hyperglycaemia (%)	22.4	10.4	33.3	<0.001
T2DM (%)	16.5	5.6	26.4	<0.001
Obesity (%)	24.4	23.6	25.2	0.754
Family history of CVD (%)	21.0	24.3	18.0	0182
TC (mmol/l), M ± SD	5.7 ± 1.2	5.4 ± 1.0	5.9 ± 1.2	<0.001
LDL-C (mmol/l), M ± SD	3.9 ± 1.1	3.7 ± 0.9	4.0 ± 1.4	0.008
HDL-C (mmol/l), M ± SD	1.2 ± 1.3	1.2 ± 0.3	1.2 ± 0.3	0.208
TG (mmol/l), Med (LQ − UQ)	1.04 (0.76–1.51)	0.92 (0.63–1.37)	1.2 (0.86–1.67)	<0.001
Fasting glucose (mmol/l)	5.8 ± 1.4	5.3 ± 1.0	6.1 ± 1.6	<0.001
HOMA, Med (LQ − UQ)	1.83 (1.31–2.93)	1.7 (1.19–2.52)	2.03 (1.41–3.22)	0.01
TSH (uU/ml), M ± SD	1.95 ± 1.16	1.88 ± 1.20	2.01 ± 1.11	0.344
GH, ng/ml, Med (LQ − UQ)	0.49 (0.12–2.0)	0.67 (0.11–2.84)	0.39 (0.12–1.06)	0.006
IGF-1, ng/ml, M ± SD	150.8 ± 58.6	161.9 ± 63.2	141 ± 52.6	0.004
LTL, M ± SD	9.77 ± 0.50	9.93 ± 0.48	9.63 ± 0.46	<0.001

Note: *CRP* high sensitive C-reactive protein, *CVD* cardiovascular diseases, *FG* fasting glucose, *GH* growth hormone, *HDL-C* cholesterol of high-density lipoproteins, *HOMA* insulin resistance index, *IGF-1* insulin-like growth factor 1, *LDL-C* cholesterol of low-density lipoproteins, *LTL* leukocytes telomere length, *TC* total cholesterol, *TG* triglycerides, *TSH* thyroid stimulating hormone, *T2DM* diabetes mellitus type 2, *p p*-value between younger and older groups

Table 20.2 Characteristics of the vascular wall parameters (n = 303)

	All patients (n = 303)	Younger group (n = 144)	Older group (n = 159)	*p*
PWV (m/s), M ± SD	8.8 ± 2.1	7.9 ± 1.7	9.6 ± 2.1	<0.001
PWV > 10 m/s (n, %)	88 (31.2)	20(14.9)	68 (46)	<0.001
IMT (mm), M ± SD	0.75 ± 0.19	0.64 ± 0.15	0.86 ± 0.17	<0.001
IMT ≥ 0.9 mm (n, %)	67 (23.5)	11 (8.1)	56 (37.3)	<0.001
AP (n), M ± m	1.09 ± 0.09	0.35 ± 0.07	1.77 ± 0.13	<0.001
Presence of AP (n, %)	138 (48.3)	30 (22.1)	108 (72)	<0.001
EDV (%),M ± SD	10.8 ± 3.5	11.6 ± 3.4	10.0 ± 3.4	<0.001
EDV < 10% (n, %)	105 (37.5)	35 (26.1)	70 (48.0)	<0.001

Note: *AP* atherosclerotic plaques, *PWV* pulse wave velocity, *IMT* intima-media thickness, *EDV* endothelium-dependent vasodilation, *p* *p*-value between younger and older groups

confirm the idea of a decreased role of certain factors in the older group in comparison with the younger one. Differences between older and younger people can be explained by the fact that people whose risk factors led to subclinical changes at a younger age after reaching old age had clinical manifestations of CVD and could not become participants in our study. In our study, IGF-1 apparently played a protective role in plaque formation, arterial wall thickening, and increasing stiffness. These effects can be associated with pronounced anti-inflammatory and antioxidant properties of IGF-1 and its ability to enhance reparative mechanisms (Sukhanov et al. 2007), primarily in the endothelium. Our studies are consistent with others indicating that patients with GH deficiency and low circulating IGF-1 had an increased risk of CVD (Vasan et al. 2003).

We have shown that the presence of short telomeres with a length corresponding to the first quartile of the distribution was associated with 17 times higher probability of the presence of AP in the younger group. These data are consistent with the results of the latest meta-analyses, which proved that short telomeres were associated with atherosclerotic CVD (D'Mello et al. 2015). Moreover, it is now widely acknowledged that LTL is not a passive marker but an active determinant of atherosclerosis development, since it determines the replicative and reparative abilities of tissue in response to the influence of risk factors (Calado and Young 2009). The important result of this work is the detection of the independent inverse relationship of LTL with both increased arterial stiffness and atherosclerosis. In other words, shorter telomeres are associated with stiff arteries. It is well-known that the process of vascular aging is characterized, even in apparently healthy subjects, by a number of deleterious changes within the vascular wall that are involved both in atherosclerosis and arterial stiffening. Aging is associated with endothelial dysfunction, decreased

bioavailability of NO, increased bioavailability of ROS as well as with low-grade inflammation. Age also induces degradation and fragmentation of elastic fibers and a non-enzymatic cross-linkage between collagen fibers. The functional capacity of stem and progenitor cells play a key role in these processes. These cells participate in damage repair and tissue differentiation processes, and they have an important role in maintaining tissue homeostasis, including the vessel wall. In clinical practice, the length of telomeres is determined in leukocytes and reflects the length of telomeres in stem cells and progenitor cells. There is increasing evidence that low telomerase activity and telomere shortening are key components of the reduction in stem cell reserves, age-associated tissue degeneration, and increased vascular stiffness (Sharpless and DePinho 2007).

Based on the results obtained, the following conclusions can be drawn: in the older age group, compared with the younger, the value of traditional CVRF for the vascular wall changes is reduced; carbohydrate metabolism disorders and hypertension are a "universal" CVRF most closely associated with the arterial wall condition in both younger and older age. The arterial walls should be evaluated at a young age, even in the absence of clinical manifestations of CVD and primarily in people with CVRF. To predict changes in the vascular wall, it is advisable to study not only conventional risk factors, but also indicators such as GH/IGF-1, LTL, and HOMA. GH/IGF-1 along with IR and LTL play important roles in the development of arterial aging. The negative relationship between GH/IGF-1 and arterial wall characteristics attests to the protective role of these hormones in arterial wall changes.

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Chapter 21
Circulating Biomarkers of Aging

Hongxia Zhang, Brian Wang and Kunlin Jin

Abstract A major goal of geroscience is to achieve the ability to predict the rate of aging using biomarkers for the purpose of extending lifespan and healthspan. Chronological aging is not a suitable marker as it does not capture the true status of age-related changes. So far, there is no biomarker or combination of biomarkers that has emerged even though some have shown promise. In this chapter, we summarize novel cellular, protein, and DNA-related biomarkers for biological aging that can be found specifically in the circulation "*for the life of every living thing is in the blood*".

Keywords Aging · Biomarkers · Blood · Lifespan · Circulation · MiRNA · Protein · Extracellular vesicles

Abbreviation

AD	Alzheimer's disease
APP	Amyloid precursor protein
AMAR	Apparent methylomic aging rate
AFR	Ascorbate free radical
B2M	B2 microglobulin
BoA	Biomarker of aging
BBB	Blood-brain barrier

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CR	Caloric restriction
CNS	Central nervous system
CSF	Cerebrospinal fluid
CAD	Coronary artery disease
DNA methylation	DNAm
DHA	Docosahexaenoic acid
ECs	Endothelial cells
EVs	Extracellular vesicles
FGFR	Fibroblast growth factor receptor
GDF	Growth differentiation factor
IDO	Indoleamine 2,3-dioxygenase enzyme
LTL	Leukocyte telomere length
OPCs	Oligodendrocyte progenitor cells
PBMCs	Peripheral blood mononuclear cells
ROS	Reactive oxygen species
RBCs	Red blood cells
rLTL	Relative leukocyte telomere length
rTL	Relative telomere length
SAA	Serum amyloid A
SORL1	Sortilin related receptor 1
SOD	Superoxide dismutase
TL	Telomere length
VCAM1	Vascular cell adhesion molecule 1

21.1 Introduction

Aging is characterized by the progressive loss of physiological integrity, which leads to an increased risk of functional damage and eventual death. As aging advances, structural and functional decline of the body can elevate the risk of major human diseases such as cancer, diabetes, cardiovascular and neurodegenerative diseases. However, chronological aging is a poor indicator of these age-related changes since one individual can age vastly differently from another. To properly determine biological aging, a considerable amount of resources have been devoted to identifying biomarkers of aging (BoA). A true BoA as proposed by the American Federation for Aging Research (AFAR; taken from *Infoaging Guide to Biomarkers of Aging*, 2016 edition) should meet the following criteria: (i) It must predict future onset of age-related conditions and diseases independent of chronological age; (ii) It must be easy to test accurately and reproducibly without the need for specialized equipment or techniques and cause little or no pain and stress to test subjects; (iii) It should work in laboratory animals and humans since preliminary testing is always performed in non-human subjects. Considering that aging is a combination of declining physiological processes, research has thus evolved from identifying single biomarker

candidates to developing panels of biomarkers (Lara et al. 2015) that reveal certain aging signatures in humans (Burkle et al. 2015; Lu et al. 2018; Sebastiani et al. 2017). Some biomarkers are derived from physical capability and anthropometry (Wagner et al. 2016) while others can be easily derived from the circulation from a simple and routine blood draw, which will be the particular focus of this chapter. Here, we review emerging biomarkers for biological aging that can be found specifically in the circulation i.e., blood.

21.2 Cellular Biomarkers of Aging in Blood

21.2.1 Red Blood Cells

Human red blood cells (RBCs) or erythrocytes have been and are still being studied to understand cellular senescence (Kaestner and Minetti 2017; Kumar and Rizvi 2014), which is a hallmark of aging. RBCs have an average lifespan of 115 days and those in the same age cohort are cleared by the reticuloendothelial system (Franco 2012). This limited period of ~115 days is a starting point of the aging process and its easy accessibility could potentially make RBCs a model for studying aging (Kaestner and Minetti 2017). Known markers of RBCs for studying aging include measuring the activity of plasma membrane redox (Rizvi et al. 2006), cysteine influx (Rizvi and Maurya 2008; Xiong et al. 2017), and enzymatic activity (Kumar and Rizvi 2014). Recently, other aging biomarkers have been identified from RBCs that can be added to the existing panel to determine their efficacies.

The protein band 4.1a/4.1b ratio correlates with the average lifespan of RBCs in circulating blood among different animal species, which can be used to determine exact age but cannot quite yet explain any mechanisms of aging (Inaba and Maede 1988; Kaestner and Minetti 2017). There has only been one study comparing young and aged RBCs derived from a mouse where the authors found an increase in the band 4.1a/4.1b ratio with age (Mueller et al. 1987). More studies with current single-cell technology using human RBCs may be able to shed more light on the utility of this ratio in the aging process.

Quercetin has been shown to significantly increase the activities of the plasma membrane redox system and ascorbate free radical (AFR) reductase thereby helping RBCs to combat oxidative stress effectively (Rizvi and Pandey 2010). Spectroscopic analysis demonstrated that intracellular uptake of quercetin by RBCs declined as a function of age, implicating it as a possible BoA (Kumar et al. 2018).

Docosahexaenoic acid (DHA), the most abundant omega-3 fatty acid constituent found in neuronal and retinal cells (Mohanty et al. 2016), can also be found in the circulation. Using 3-month-old and 13-month-old male Wistar rats, plasma DHA content was shown to increase with age while erythrocytic membrane DHA content was unchanged in the old versus young group (Letondor et al. 2014). In parallel, serum DHA content in humans was shown to increase with age (Crowe et al. 2008;

Dewailly et al. 2002, 2001) and had no sex-specific differences (Crowe et al. 2008), making DHA content a suitable universal BoA. Indeed, a study from Italy analyzed possible differences in the lipid composition of RBC membranes from nonagenarian offspring compared with controls matched in terms of age, diet, sex, smoking behavior, and medical history (Puca et al. 2008). The authors found that erythrocytic membranes from nonagenarian offspring had decreased lipid peroxidation and increased membrane integrity compared with controls, reinforcing the usefulness of RBC lipid composition and possibly DHA content in blood as biomarkers of aging.

21.2.2 Leukocytes

Peripheral blood leukocytes consist of a variety of white blood cells including granulocytes, lymphocytes, and monocytes.

The sortilin related receptor 1 (*SORL1*) belongs to a family of neuronal sorting receptors that is a critical player in Alzheimer's disease related events such as determining amyloid-beta levels and trafficking the amyloid precursor protein (APP) (Cuccaro et al. 2016). *SORL1* and its variants have been implicated in AD risk (Andersen et al. 2016; Felsky et al. 2014; Yin et al. 2016) and more recently, cognitive aging (Lin et al. 2017; Reynolds et al. 2013). Expression of the *SORL1* gene in peripheral blood leukocytes showed about a twofold increase in healthy elderly and AD patient samples compared with healthy young subjects, which is the first evidence suggestive of *SORL1*'s utility as a biomarker of aging (Furuya et al. 2012).

Average telomeric length that is routinely measured from leukocytes (LTL) has long been argued to be a BoA (von Zglinicki and Martin-Ruiz 2005; Xia et al. 2017) as it is an indicator for two hallmarks of aging namely, cellular senescence and oxidative stress, where telomeric length shortens with each cell division and increasing oxidative stress, respectively (Fossel 2000; Sanders and Newman 2013). However, there has been some controversy as to whether the average LTL value is indeed a useful BoA (Chilton et al. 2017; Sanders and Newman 2013; Simons 2015) since telomere length and dynamics can vary between tissues (Daniali et al. 2013; Gramatges and Bertuch 2010). For example, telomere length (TL) in nine elderly patients (age range: 73–95) found that TL was the shortest in leukocytes followed by skin then synovial tissue (Friedrich et al. 2000). This agreed with a recent cross-sectional study (age range: 19–77 years) looking at average TL in four different tissues where TL was the shortest in leukocytes followed by fat, skin, and skeletal muscle (Daniali et al. 2013). Since average TL may not be a good measure of biological aging, some studies have turned to analyzing relative telomere length (rTL) instead, which can be obtained by calculating the ratio of telomere repeats to a single-copy gene (T/S) (Ehrlenbach et al. 2009; Friedrich et al. 2000; Genot et al. 1989). Friedrich and colleagues measured rTL in peripheral leukocytes and found that it does not correlate well with rTLs from the tongue mucosa, liver, heart, brain, skeletal muscle, subcutaneous and abdominal fat, skin, skeletal muscle, and triceps (Genot et al. 1989). It must be noted however, that the tissues were obtained

from 12 human cadavers less than 12 h after death with an age range of 4 months to 88 years, all factors that may have affected rTL values. One study reported that although TL varied in different tissues, what was intriguing was that TL had similar rates of attrition with age, which raises the possibility of also using telomere attrition rate as a biomarker for aging (Daniali et al. 2013). Indeed, a report demonstrated that across 19 bird species that were studied, the rate of telomere loss correlated strongly with maximum lifespan (Tricola et al. 2018). What is certain amidst this controversy is that TL is unequivocally a BoA where it has been shown to be most predictive of lifespan at an early age; possessing a long TL early in life equates to having a longer lifespan (Heidinger et al. 2012).

T cells are generally in a state of quiescence when unstimulated. These unstimulated T cells make up about 60% of peripheral blood mononuclear cells (PBMCs). Through antigen-specific activation, T cells become highly proliferative and display a vast array of changes in gene expression (Zhao et al. 2014). As we age, T cell responses upon antigen presentation/stimulation become increasingly impaired but previous reports have only studied the behavior of unstimulated PBMCs with age (Beekman et al. 2013; Sebastiani and Perls 2012). Therefore, Tedone et al. studied the function of stimulated T cells across a group of 114 individuals (age range: 23 to 100+) and sought to identify potential biomarkers from their gene expression changes with age (2018). Interestingly, they found that in high-performing centenarians, the telomere length was longer and telomerase activity after T cell activation was greater thereby implicating these characteristics as potential BoA. Increasing age has also been associated with the loss of naïve CD8 T cells (Czesnikiewicz-Guzik et al. 2008). A subset of 157 participants from the Berlin Aging Study II (BASE-II) who are representative of a German urban population was analyzed and the authors found that naïve CD8 T cells did indeed decrease with age but only in older versus young men (Di Benedetto et al. 2015), citing a sex-specific difference in male versus female immunity (Caruso et al. 2013). A newly identified population of CD8+CCR7+ Treg cells (Suzuki et al. 2012) has been likened to memory T cells with multipotent and enhanced self-renewal properties (Gattinoni et al. 2011). Suzuki et al. (2012) reported that the number of these CD8+CCR7+T cells decreased as a function of age with more than 90% loss in those over 65 years of age (range: 18–85 years). There was a strong correlation between age and the induction of these Treg cells ($R^2 = 0.669$), which could play a critical role in immunosenescence, another hallmark of aging.

21.3 Protein Biomarkers of Aging in Blood

While many of these age-related changes may be the consequences of cell intrinsic and tissue-localized mechanisms of aging, there is much interest in identifying whether changes in secreted signaling proteins contribute to tissue aging and functional impairment. It has been shown that circulating systemic environmental factors can be exchanged in a "heterochronic parabiosis" model, resulting in the mutual influence via blood-borne factors (Conboy et al. 2005). Indeed, such changes in

plasma or cerebrospinal fluid (CSF) proteomes are not only abundant with aging and disease (Britschgi et al. 2011; Villeda et al. 2011), but factors in young blood or plasma from mice or humans have been shown to be sufficient for rejuvenation e.g., to increase brain function in the hippocampus (Castellano et al. 2017; Villeda et al. 2011; Villeda et al. 2014) and the subventricular zone (SVZ) (Katsimpardi et al. 2014). In addition to identifying pro-youthful factors in young blood that might boost rejuvenation, an emerging body of work has identified aged blood plasma and the factors within it, which may promote pro-aging processes in aging and age-related diseases (Smith et al. 2018). Of course, even though a single factor is identified as a specific rejuvenation or pro-aging factor, we also need to consider other circulating factors that it interacts with to cause the supposed effect.

21.3.1 Extracellular Vesicle Changes

Extracellular vesicles (EVs) are nano-sized (30–400 nm) membranous vesicles, which are thought to be released into the circulation and numerous biological fluids not only by one type of cell, but by cells of diverse origins such as platelets, erythrocytes and endothelial cells (Arraud et al. 2014), neurons, adipocytes and several other cells types (Santiago-Dieppa et al. 2014; Turpin et al. 2016). There are at least three types of EVs in the blood: (i) exosomes, which are small, 30–120 nm diameter membrane micro-vesicles, released by the fusion of multivesicular bodies with the plasma membrane; (ii) microvesicles released by budding of small segments of the plasma membrane; (iii) apoptotic bodies released from dying cells (Colombo et al. 2014; Yanez-Mo et al. 2015). Due to overlap in the size, density, and cargo of these different types of EVs, it is difficult to determine the type of EVs isolated from cell cultures and body fluid samples (Lotvall et al. 2014).

Exosomes have the potential for directional homing to specific target cells, dependent on the physical properties of their membranes (Liang et al. 2007). It was confirmed that exosomes represent a specific subcellular compartment and do not contain a random array of intracellular proteins, but are enriched with specific mRNA, miRNA and proteins that remain bioactive upon uptake by the recipient cell and thus play an important role in intercellular communication (Bobrie et al. 2011). Fortunately, this cargo is protected from degradation by proteases and RNases while the vesicle is in the interstitial space, and retains bioactivity once taken up by a recipient cell (Pusic and Kraig 2014). In this way, exosomes facilitate the transfer of interactive signaling and enzymatic activities.

EVs contain a multitude of bioactive substances that play a critical role in physiological and pathological conditions such as immune responses, neurobiology, stem cell processes, vascularization, tumor biology, as well as a variety of age-related chronic diseases including neurodegenerative diseases (e.g., Alzheimer's disease), metabolic diseases, and cardiovascular disease (Smith et al. 2015a), which led us to examine whether EVs can change with age in humans and animals.

21.3.1.1 Protein Changes in EVs with Age

The majority of research focuses on identifying differences in EVs when comparing disease states and matched controls. Increased concentrations of EVs has also been reported in cancer patients (Gercel-Taylor et al. 2012; King et al. 2012; Rodriguez et al. 2014). Eitan et al. found that EV concentration decreases with advancing age due to enhanced EV internalization by both activated monocytes and B cells in humans (Eitan et al. 2017).

Due to the transmission of bio-information through EVs between origin and target cells, EVs can be utilized for diagnostic purposes termed a 'liquid biopsy' (Santiago-Dieppa et al. 2014), which gives rise to the hypothesis where vesicle proteins may account for the age-related differences in concentration based on internalization and activation. Eitan et al. then tested this idea and found significant changes in protein levels both in EV surface and cytosolic proteins from lysed EVs (Eitan et al. 2017). Levels of several apoptotic markers in EVs decrease with age, including p53, cleaved PARP and cleaved Caspase-3. Three other proteins were significantly increased with age including CD151, a tetraspanin that enhances cellular processes involved in tumorigenesis and metastasis, MUCIN16 (also known as CA-125), a well-established circulating marker of ovarian cancer and possibly other types of cancers, and CD9, which also belongs to the tetraspanin family, commonly used as an exosomal biomarker that regulates cell adhesion, motility, activation and proliferation (Bari et al. 2011). CD14, MUC1 and NY-ESO1 are immune-related antigens, and levels of MUC1, MUCIN16, CD151 and NY-ESO1 are all reported to be higher in cancer cells (Chauhan et al. 2006; Kumari et al. 2015; Soo et al. 2012). In a recent study, MUC1 levels were observed to decrease with age in plasma EVs in a small group of white males (Baek et al. 2016). This difference further highlights the need for large clinical studies of EV alterations with age. In addition, the reduction in EV concentration may result from the autonomous proteostasis malfunction which can impair clearance mechanisms within aged cells (Eitan et al. 2017) for example, the presence of many misfolded proteins in EVs have been found in neurodegenerative diseases (Janas et al. 2016).

21.3.1.2 miRNA Changes in EVs with Age

MicroRNAs (miRNAs) are small non-coding RNAs of ~25 nucleotides long that regulate gene expression post-transcriptionally by binding to complementary sequences in the 3′-UTR of multiple target mRNAs (Ambros 2001). Hunter et al. found 104 and 75 miRNAs that were significantly expressed in micro-vesicle and PBMC samples, respectively, in which 71 miRNAs were co-expressed among each sample group, suggesting PBMCs could be one source of miRNAs that are present in the micro-vesicles or exosomes (Hunter et al. 2008). In addition, different miRNAs may be from different sources. Circulating miRNAs in micro-vesicles or exosomes can also be released by glioblastomas (Balaj et al. 2011) and mast cells (Valadi et al. 2007). The recent observations showed that miRNAs can be released extracellularly by

cultured cells either as free miRNAs or encapsulated into exosomes or into larger secreted membrane vesicles (Wang et al. 2010). In addition, miRNAs are present in biological fluids either as complexes with the protein Ago2 or inside microvesicles (Arroyo et al. 2011), suggesting a selectivity of miRNA targeting to vesicles. miRNAs were reported to regulate neurodegenerative diseases (Sheinerman and Umansky 2013), age-related diseases (cardiovascular and neurodegenerative diseases) (Olivieri et al. 2013b), and inflammatory disease (Olivieri et al. 2013a) to mediate epigenetic exchange during aging (Berdasco and Esteller 2012). Therefore, miRNAs and/or exosomes may be part of circulating systemic environmental factors and regulate systemic milieu directly or indirectly.

Recent studies reported distinct miRNA expression patterns in the stroke pathogenic process, including hyperlipidemia, hypertension and plaque rupture, and atherosclerosis (Cipollone et al. 2011). Specific stroke-induced miRNA expression profiles have been reported in the blood and brain in both animal models and patients (Dharap et al. 2009; Jeyaseelan et al. 2008; Tan et al. 2009). Altered inflammation-related miRNA profiles following intracerebral hemorrhage have been reported in the plasma (Guo et al. 2013). In addition, miRNA expression patterns have been used to predict subtypes of stroke (Tan et al. 2009). Circulating miRNAs have been widely investigated as biomarkers to distinguish disease from non-disease cases (Chen et al. 2008), especially in cardiovascular disease, where specific miRNA signatures have been shown to distinguish stable documented CAD (coronary artery disease) patients (Fichtlscherer et al. 2010) or peripheral artery disease patients (Li et al. 2011) from controls. Selvamani et al. investigated the effects of age and sex on miRNA expression patterns post-stroke, either using brain or circulating miRNA. Of the 168 circulating miRNAs examined 5 days post-stroke, 21 and 78 miRNAs were significantly upregulated at 2 days and 5 days post-stroke, respectively (Selvamani et al. 2014). Furthermore, a small cohort of five miRNAs (miR-15a, miR-19b, miR-32, miR-136 and miR-199a-3p) were found to be highly expressed exclusively in adult females who exhibited significantly lesser cortical and striatal damage as well as the least amount of sensory-motor deficit, compared with middle-aged females, adult males and middle-aged males (Selvamani et al. 2014). These five miRNAs cohort should be investigated further as a collective biomarker signature for stroke outcome.

21.3.1.3 EVs in Age-Related Diseases

The central nervous system (CNS) utilizes rejuvenation by re-myelination to restore nerve conduction and prevent axonal degeneration (Duncan et al. 2009; Edgar and Nave 2009). Efficiency of CNS re-myelination decreases with age (Gilson and Blakemore 1993), one reason being that there is age-related impairment in the recruitment and differentiation of oligodendrocyte progenitors (Sim et al. 2002). Ruckh et al. found that despite intrinsic alterations in oligodendrocyte progenitor cells, exogenous signals introduced by the young systemic milieu through parabiosis were capable of providing differentiation signals and retaining efficient repair (Ruckh et al. 2012).

Ruckh et al. described the ability of the youthful systemic milieu to increase neurogenesis in the brain and enhance re-myelination in aged animals (Ruckh et al. 2012). Based on this finding, Pusic and Kraig identified peripheral exosomes as potential circulating factors that are able to mimic the effects of the youthful milieu in enhancing the number of OPCs, their differentiation and ultimately, remyelination, not only from the serum of the young rat but also from aged rats exposed to an enriched environment (Pusic and Kraig 2014). Furthermore, the authors utilized a miRNA expression array and identified miR-219, a microRNA required for the production of myelinating oligodendrocytes, as the key cargo being transported by 'rejuvenating' exosomes following exposure to the enriched environment and as well as being present in young animals. Further, young exosomes were found to enhance central nervous system (CNS) myelination in vivo in aged rats (Pusic and Kraig 2014).

Taken together, these results show that the promyelinating effects of the youthful systemic milieu on the aged brain involve peripheral exosome-mediated delivery of miRNAs, providing a basis for future studies to determine the predictive role of peripheral blood miRNA signatures in human disease.

21.3.2 GDF11

Growth differentiation factor (GDF) is a member of the TGF-β superfamily, a conserved family of growth factors that govern a multitude of cellular processes (Chang et al. 2001). GDF11 was decreased in the serum and spleen of aged mice (Loffredo et al. 2013) due partly to epigenetic modifications (Berdasco and Esteller 2012; Fraga and Esteller 2007). Further reports identified GDF11 as a systemic rejuvenating factor that can exert positive effects in aging animals. Chang et al. demonstrated a more youthful expression of GDF11 in plasma derived from old heterochronic parabionts following heterochronic parabiosis, which makes GDF11 a candidate factor that may drive the regression in cardiac hypertrophy (Loffredo 2013). In vitro study of the effect of GDF11 on neonatal cardiomyocytes revealed the presence of GDF11 at the intercalated discs between adjacent cardiomyocytes, a key region for ligands/receptors known to affect hypertrophic signaling (Gustafson-Wagner et al. 2007; Johnston et al. 2009). Moreover, intraperitoneal injection of GDF11 into aged mice (23 months) resulted in significantly smaller cardiomyocytes and marked reductions in molecular markers associated with cardiac hypertrophy when compared to saline-injected age-matched controls (Loffredo et al. 2013). It was also reported that GDF11 declined in the muscle of aged mice and daily injections of recombinant GDF11 increased the number of satellite cells with intact DNA when compared to vehicle (Sinha et al. 2014). GDF11 supplementation increased the size of the neuromuscular junction, but did not alter gross anatomy, fat mass or muscle mass in aged muscle in the absence of injury. However, it was reported that there is a trend of increased GDF11 levels in the serum of aged rats and humans as well as increased GDF11 mRNA in the aged rat muscle (Egerman et al. 2015).

While GDF11's effect on cardiac and skeletal muscle rejuvenation has been shown, more work is warranted to identify systemic rejuvenating factors that can promote neurogenesis in the aged brain. Katsimpardi et al. showed that GDF11 is a potential pro-youthful factor that boosts neurogenesis in the subventricular zone (SVZ) and cerebral blood flow in aged mice when administered systemically (Katsimpardi et al. 2014). It is possible that increased blood flow might result in increased neurogenesis and function, which opens new therapeutic strategies for treating age-related neurodegenerative diseases. However, GDF11 may be not the sole protein constituting the CSF. For neurogenic rejuvenation in the mouse, daily administration of recombinant GDF11 (rGDF11) (0.1 mg/kg) for 4 weeks is required to obtain a 50% increase in the volume of brain blood vessels (Katsimpardi et al. 2014). However, administering a total of 8 injections of whole blood plasma (100 ul per injection) in 24 days can significantly improve the cognitive function of aged mice, which was partly mediated by the cyclic AMP response element binding protein (CREB) activated by exposure to young blood factors (Villeda et al. 2014).

Interestingly, the level of peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1α), an important regulator of mitochondrial biogenesis, was increased in the muscle derived from aged mice treated with GDF11 (Murphy and Thuret 2015), suggesting that GDF11 influences mitochondrial dynamics. GDF11 supplementation to aged mice also increased basal levels of autophagy, providing evidence for another mechanism likely underlying the cellular remodeling of muscle fibers (Sinha et al. 2014). Crucially, the cellular and ultra-structural changes following GDF11 supplementation translated to functional improvements as evidenced by an increase in exercise endurance and enhanced grip-strength (Sinha et al. 2014). Collectively, these studies demonstrate that the circulating mitogen GDF11 exerts profound effects on multiple tissue-specific stem and non-stem cells throughout the body via preservation of genomic integrity, enhanced mitochondrial function and increased autophagy.

21.3.3 IGF-1

IGF-1 is long believed to have neurotrophic properties and as a key growth-promoting factor, it regulates neurogenesis and synaptogenesis from embryonic to adult stages of the brain (Cao et al. 2011; Sonntag et al. 2013; Vicario-Abejon et al. 2003). The level of IGF-1 has been shown to decrease with age. Restoring it to a level similar to the younger environment can upregulate neurogenesis and improve learning (Lichtenwalner et al. 2001). Moreover, the aging choroid plexus conditioned media showed that decreased levels of BMP5 and IGF-1 promoted neurogenesis in the SVZ (Silva-Vargas et al. 2016). An in vivo study also found that short- and long-term peripheral infusion of IGF-1 can significantly increase the number of newborn neurons without inducing an increase in the number of astrocytes (Aberg et al. 2000), suggesting the selective effect of IGF-1 on neurogenesis in the adult brain.

On the other hand, reports have demonstrated that deficiency in circulating IGF-1 has been linked to lifespan extension in mice (Rojanathammanee et al. 2014), *Drosophila* (Tatar et al. 2001), and *C. elegans* (Kimura et al. 1997). Interestingly, lifespan extension is sexually divergent according to Ashpole et al. who demonstrated that early IGF-1 deficiency increased lifespan in female but not male mice (Ashpole et al. 2017). Due to the disparity of findings, the question remains as to whether a decreased IGF-1 level can directly affect longevity and whether exogenous replenishment of IGF-1 can decrease age-related diseases thus increasing healthspan.

21.3.4 Haptoglobin

Haptoglobin emerged as a strong candidate as it was increased in the blood of both old rats and aged human subjects (Pan et al. 2017). We confirmed that the circulation level of haptoglobin was significantly increased in both the aged rat and human plasma in 40 and 80 years old versus 20 years old (Pan et al. 2017), consistent with previous studies that levels of haptoglobin in the blood (Ding and Kopchick 2011; Hansson et al. 1983), liver, CSF and hippocampus increase with age in humans and rodents (Spagnuolo et al. 2014). Haptoglobin is also likely to play an important role in suppressing inflammatory responses as it binds free hemoglobin released from ruptured red cells, and its plasma level increases in response to inflammatory stimuli such as infection and autoimmune reaction (Ratanasopa et al. 2013). Furthermore, haptoglobin levels increase in various pathological conditions both in human and animals and may be a sensitive marker of blood-brain barrier dysfunction (Chamoun et al. 2001). Some studies showed increased haptoglobin levels in the CSF from patients with AD (Johnson et al. 1992), Parkinson's disease (PD) and Huntington's disease (HD) (Arguelles et al. 2010; Huang et al. 2011). Although the role of haptoglobin in these diseases is not yet fully understood, haptoglobin may be involved in pathogenesis or serve as a diagnostic marker. For example, elevated haptoglobin is associated with an increased risk for acute myocardial infarction, stroke, and heart failure (Holme et al. 2009). Pan et al. investigated its possible role in functional recovery in our ischemic stroke model (Pan et al. 2017). Young rats that were given haptoglobin had larger infarct volumes and more severe neurological deficits at 1 and 3 days for the corner test and at 3 days for the beam balance test and elevated body swing test. However, haptoglobin had little effect in aged ischemic rats. The administration of haptoglobin reproduced the effect of aged plasma in worsening outcome in young ischemic rats. The poorer functional outcome elicited by exposure to aged plasma may be mediated, at least in part, by increased haptoglobin in the aged blood.

21.3.5 Inflammatory Cytokines

Inflammation plays a key role in promoting cellular senescence and aging (Childs et al. 2015). However, the inflammatory mechanisms in aging are poorly understood. The inflammatory disequilibrium theory may explain how aging is a natural process of the disequilibrium between inflammation and anti-inflammation (Hunter 2012) but chronic gradual increase in proinflammatory status termed "Inflamm-Aging" has now been acknowledged as a main feature of aging (Franceschi et al. 2007; Xia et al. 2016). Here, we present inflammatory cytokines found in the circulation that have been implicated in the aging process.

21.3.5.1 CCL and B2M

Mice exposed to old blood had showed reduced neurogenesis in the hippocampus (Rebo et al. 2016; Villeda et al. 2011). Some of the inhibitory effects can be attributed to the chemokine CCL11 and the MHC class I associated molecule β2 microglobulin (B2M), which have been shown to be increased in the blood of aged healthy humans (Smith et al. 2015b; Targowski et al. 2005; Villeda et al. 2011). Similarly, using heterochronic parabiosis, Villeda et al. identified six factors (B2M, CCL11, CCL12, CCL19, CCL2, and Haptoglobin) that were elevated in the plasma of old unpaired and young heterochronic parabionts, and whose blood concentrations inversely correlate with the age-related decline in neurogenesis (Villeda et al. 2011).

Increasing peripheral chemokine levels were found to impair hippocampal dependent learning and memory (Smith et al. 2015b; Villeda et al. 2011). Specifically, increasing either B2M or CCL11 mimics an aged systemic environment leading to suppressed levels of hippocampal neurogenesis in young mice (Smith et al. 2015b; Villeda et al. 2011). Furthermore, CCL2 knockout mice exhibited increased neurogenesis after cranial irradiation (Lee et al. 2013), implying that systemic increase of CCL2 is a pro-aging factor.

21.3.5.2 TNF-α, IL-1ß, IL-4 and TGF-β

In addition to the factors identified through parabiosis, a number of other potential pro-aging factors have been reported, such as TGF-β, IL-6, IL-4 IL-1ß, and TNF-α (Smith et al. 2018). Circulating cytokines and chemokines with detrimental effects on the brain increase in the blood with advanced age (Villeda et al. 2011) including TNF-α, can induce the expression of endothelial VCAM1 through NF-κB signaling (Hosokawa et al. 2006; Zhang et al. 2011). Blood IL-6 and TNF-α in humans have been found to increase with age (Bruunsgaard 2006; Valiathan et al. 2016) and could suppress mouse neural progenitor cell function in vitro (Ben-Hur et al. 2003; Monje et al. 2003), which could indicate their contribution in the age-related decline of neurogenesis. Additionally, the high levels of TGF-β1 found in the aged brain and muscle

led to increased B2M levels, indicating a non-traditional role for TGF-β1 wherein it promotes inflammation rather than suppresses immune responses. To this end, Yousef et al. systemically administered Alk5 once daily for 11 days to inhibit TGF-β receptor 1, resulting in increased hippocampal neurogenesis and muscle regeneration in aged mice, confirming that TGF-β1 signaling inhibition could normalize B2M to young levels in the brain and muscle (Yousef et al. 2015). More on the dual-action of TGF-β, Hauri-Hohl and colleagues demonstrated a negative role of TGF-β signaling where ablation of this signaling pathway in medullary thymic epithelial cells augmented the size and function of the thymic medulla thus reducing autoreactivity by peripheral T cells (Hauri-Hohl et al. 2014). Conversely, using a similar mouse model, Ouyang et al. demonstrated a positive role of TGF-β signaling in regulating peripheral tolerance by aiding in the survival of natural regulatory T cells. The absence of TGF-β signaling led to widespread activation of T cells and diabetes development (Ouyang et al. 2010). Therefore, it is necessary to know whether supplementation of rGDF-11 (GDF11, a TGF-β family member) would be beneficial in reversing age-related immune system defects.

21.3.5.3 VCAM1

The blood-brain barrier (BBB) tightly monitors the entry of molecules from the periphery into the CNS (Engelhardt and Liebner 2014) and how youthful or aging factors in the circulation can cross into the brain to regulate brain function is still unknown (Wyss-Coray 2016). In the case of the well-known BBB protein that mediates interactions between vascular and immune cells, Yousef and colleagues found that levels of VCAM1 in the circulation (soluble VCAM1, sVCAM1) and those bound to the membrane of the BBB (membrane bound VCAM1, mbVCAM1) increased with age in mice and humans. Interestingly, only mbVCAM1 was responsible for reduced hippocampal neurogenesis and increased microglial reactivity in young adult mice, suggesting that it is important in mediating the effects of factors found in aged plasma (Yousef et al. 2018).

21.4 DNA-Related Biomarkers of Aging in Blood

21.4.1 DNA Methylation

The concept of using DNA methylation (DNAm) as a biomarker of aging is relatively new. Steve Horvath of UCLA demonstrated that it is possible to estimate biological age through the methylation status of cytosine-5 within CpG sites, which gave rise to Horvath's clock, a multi-tissue DNAm age estimator (Horvath 2013; Horvath and Raj 2018) that is freely available at the following website (https://dnamage.genetics.ucla.edu/home). Horvath showed that DNAm age can be measured from

the DNA of 51 healthy tissues and cell types (excluding the sperm) and can be applied to all stages of life (from prenatal samples to centenarians), which has been validated in several data sets (Marioni et al. 2016, 2015, 2019; Morin et al. 2018; Thompson et al. 2018; Verschoor et al. 2017). Using whole blood from 656 subjects (age range: 19–101), Hannum et al. devised a quantitative model of aging that showed differences in an individual's methylome age, which is otherwise known as Hannum's clock (Hannum et al. 2013). The *apparent methylomic aging rate* (AMAR; ratio of "predicted methylomic age" to "chronological age") is specifically influenced by sex where men were shown to be more prone to age faster than women by 4%. Moreover, this model was validated in other tissues such as kidney, breast, lung, and skin and had a high predictive power for chronological age ($R = 0.72$), indicating that AMAR measured changes specific to the methylome and not changes in whole blood composition with age. Recently, Levine et al. (2018) developed a new epigenetic BoA called DNAm PhenoAge that analyzes DNAm with clinical measures of phenotypic age such as albumin, creatinine, and glucose values. Although DNAm PhenoAge uses samples derived from the blood, it still associates strongly with chronological age ($r = 0.71$) and tissue/cell-specific aging e.g., liver ($r = 0.80$), colon ($r = 0.88$), and heart ($r = 0.66$). Furthermore, DNAm PhenoAge in the dorsolateral prefrontal cortex correlates positively with classical factors of Alzheimer's disease after adjusting for age e.g., neurofibrillary tangles (aggregates of hyperphosphorylated tau protein, $r = 0.1$, $p = 0.0073$), amyloid load ($r = 0.094$, $p = 0.012$), and neuritic plaques ($r = 0.11$, $p = 0.0032$). Further studies should help shed light on efficacy of this biomarker of aging in cases of clinical aging interventions.

21.4.2 Cell-Free DNA

Another DNA-related BoA in the blood is cell-free DNA (cfDNA), which are present in plasma and their levels increase remarkably in cases of cancer, stroke, and sepsis (Puszyk et al. 2009; Tsang and Lo 2007). Thus, they are thought to come from apoptotic cells (Langford et al. 2007), which reflects the magnitude of cellular damage that is known to increase with age. However, how age affects cfDNA and its utility as a BoA were unclear until recently. In a subset of participants from the Vitality 90+ study (Goebeler et al. 2003) conducted in Tampere, Finland, Jylhava et al. (2011) compared the quantity and quality of cfDNA from the plasma of 11 healthy women controls (age range: 22–73 years) and 12 nonagenarian women (born 1917). They reported that plasma cfDNA levels were higher in nonagenarians versus controls using all three methods of detection: Phenol method, Quant-iT assay, and β-Globin qPCR. Qualitatively, the method for extracting cfDNA played a critical role in determining whether certain fragments could be detected. In a different subset of participants from the Vitality 90+ study, that included 258 nonagenarians, Jylhava et al. (2012) evaluated the correlation between the level of plasma cfDNA and (i) inflammatory markers; (ii) 4-year mortality. They found that plasma cfDNA was a suitable biomarker for systemic inflammation due to the significant positive correlations (all

$p < 0.02$) with plasma C-reactive protein ($r = 0.294$), IL-1ra ($r = 0.153$), serum amyloid A (SAA, $r = 0.187$), and indoleamine 2,3-dioxygenase enzyme (IDO, $r = 0.208$) and negative correlation with HDL cholesterol ($r = -0.196$, $p = 0.002$). In addition, the Kaplan-Meier survival curve demonstrated that 4-year all-cause mortality was 56.2% (145 out of 258 participants) while the adjusted Cox regression analysis revealed that cfDNA is an independent covariate, further highlighting the utility of cfDNA as a possible BoA. A recent study by Neretti's group out of Brown University analyzed plasma cfDNA levels using whole-genome sequencing but found no significant differences in young (25 years, $n = 3$) versus old (70 years, $n = 3$) versus centenarians (101 years, $n = 6$), possibly due to the small sample size and high inter-individual variability within groups, which is commonly seen in BoA (Teo et al. 2018). Taken together, longitudinal studies and larger cohorts would be needed to definitively ascertain cfDNA's promising status as a BoA.

21.5 Conclusions

The phrase *"for the life of every living thing is in the blood"* is particularly apt for this chapter, especially when we described novel biomarkers such as telomere length, extracellular vesicles and cell-free DNA. These predictors of biological aging hold great promise and represent a step forward in the race toward predicting not only mortality and lifespan but also healthspan. It seems almost certain that these biomarkers will have to be used in combination to increase their predictive power while further longitudinal instead of cross-sectional studies will be needed to establish causality. The future for identifying markers of biological age can only get more interesting because of the number of increasingly grey societies around the world seeking to reduce the burden of healthcare. We hope this chapter has piqued your interest and given you some insight into the latest trends in circulatory biomarkers of aging.

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Chapter 22
Molecular Signature of Aging Driven by Wnt Signaling Pathway: Lessons from Nematodes

Marco Lezzerini and Yelena V. Budovskaya

Abstract Aging is a universal biological process that afflicts every creature on this planet. To date, we have an inferior understanding of what causes this degeneration. A commonly held view is that aging is the result of damage accumulation over a lifetime. However, research has shown that aging is not only the result of wear and tear in the organism but also of genetic programs involved in organismal development that go awry as selective pressure is released. With this in mind, we have investigated the Wnt signaling pathway, as a significant and highly conserved developmental pathway that guides many essential events during embryonic and larval development. We also discuss how these genetic programs orchestrate changes in the organism that could cause aging and open a new research direction on the role of Wnt signaling in aging and age-related diseases.

Keywords Aging · Wnt signaling · Antagonistic pleiotropy · Caenorhabditis elegans · Developmental programs · GATA transcriptional circuit · Gene expression · RNA sequencing · Reproduction · Metabolism

22.1 Introduction

22.1.1 Studies of the Natural Aging Process

Before diving into the investigation of genetic mutations that affect aging, it is vital to understand the basic biology of the natural aging process. What we call aging, refers to a gradual deterioration of an organism that occurs over time. In human, this process characterized by the decline of several body functions, most notably, a reduced mass and contraction power of the muscle (known as sarcopenia), decreased

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bone density, alterations of the cardiovascular system, decline in cognitive functions and a general pro-inflammatory state of the whole organism. This decline represents the primary risk factor for most, if not all, human age-related diseases like cancer, type 2 diabetes, osteoporosis, cardiovascular and neurodegenerative conditions. For a long time, aging has been considered a passive stochastic process that is not subject to any regulation. We now know that this is not the case. Many studies have identified signaling pathways that can affect the aging process. Interestingly, none of these genetic pathways primarily works in age regulation. Instead, they naturally participate in processes such as development, nutrient sensing and metabolism, mitochondrial function and stress response (Pitt and Kaeberlein 2015).

In the attempt to define aging at the molecular level, nine hallmarks have been currently proposed to describe the cellular aging (López-Otín et al. 2013). These are genomic instability (Moskalev et al. 2012), telomeres attrition (Blackburn et al. 2006; Blasco 2007), epigenetic alterations (Talens et al. 2012), loss of proteostasis (Koga et al. 2011; Zhang and Cuervo 2008; Hutt et al. 2009), deregulated nutrient sensing (Barzilai et al. 2012), mitochondrial dysfunction (Kujoth et al. 2005; Vermulst et al. 2007; Trifunovic et al. 2004; Vermulst et al. 2008), cellular senescence (Rando et al 2012), stem cell exhaustion (Rando and Chang 2012), and altered intracellular communication (Conboy et al. 2005; Villeda et al. 2011; Loffredo et al. 2013). However, it is important to remember that it is currently unclear if these processes are causes or consequences of the aging process. In other words, we do not know the primary causes of aging. For example, although we know that random somatic mutations accumulate in the genome over time, in *Drosophila melanogaster*, it has been shown that calorie restriction (CR), the most robust intervention that extends the lifespan in all the species, prolongs the life without affecting the manifestation of these mutations (Edman et al. 2009). As aging is a multifactorial phenotype, one can assume that multiple biological factors participate in changing the entire process. Whether these hallmarks represent primary or secondary causes of aging, they provide a framework to study this process with a systematic approach, and they constitute the background of knowledge we can build on to uncover the mechanisms of aging.

Several model organisms have been used to study the aging process, including yeast, fruit flies, nematodes, and mice (Tissenbaum and Guarente 2002). Of these, the nematode *Caenorhabditis elegans* (*C. elegans*) is one of the most attractive and useful model organisms because it has a relatively short lifespan of approximately two weeks, enabling one to quickly assess the effects of mutations and various experimental treatments on lifespan and age-related fitness. Additionally, there is no somatic cell division in the adult worm, and cells that are lost during adult life are consequently not replaced (Sulston and White 1980). Therefore, aging in *C. elegans* is entirely post-mitotic, reflecting a gradual loss of function in somatic cells as they grow old, and the analysis of aging in worms is not complicated or masked by the process of regeneration. Most importantly, 83% of the worm's proteome has human orthologs (Lai 2000), which makes discoveries very relevant to a better understanding of the aging process in humans.

The physiological aging process in worms was thoroughly characterized. Old worms move slowly, have a lower pharyngeal pumping rate, and are flaccid

(Bolanowski et al. 1981). The appearance of tissues and organs in old worms reveals that the pharynx becomes packed with bacteria and the age-related pigment lipofuscin accumulates in the intestines (Gerstbrein et al. 2005; Herndon et al. 2002). The subcellular architecture of various cell types shows degeneration of muscle and intestinal cells (Herndon et al. 2002; McGee et al. 2011), and under more detailed examination, individual neurons display a gradual decline in branching and electrical activity which likely leads to reduced mobility and sensory perception (Pan et al. 2011; Tank et al. 2011).

The vast majority of molecular-aging research in *C. elegans* build upon finding and characterizing conditions or mutations that affect longevity. Elegant genetic studies have identified several hundred genes that alter lifespan. Remarkably, over 90% of these genes were found to function in growth and development pathways in all eukaryotes (Smith et al. 2008). Further analysis of genetic data revealed several signaling cascades that modulate lifespan in *C. elegans* and higher organisms. For instance, insulin/IGF1 signaling, rapamycin (TOR) signaling, Wnt signaling, sirtuins, protein translation, mitochondrial signaling, microRNAs, autophagy, metabolism, development, reproduction, stress resistance, and reproduction are all vital molecular processes that have been shown to regulate longevity (Houtkooper et al. 2010; Houtkooper et al. 2012; Mukhopadhyay and Tissenbaum 2007; Parker et al. 2012; Wolff and Dillin 2006). Therefore, their alteration in the hope of attaining beneficial effects on longevity often comes with a "price." For example, mutations in one of the best-studied signaling cascades involved in increased longevity, namely the Insulin/IGF-1 signaling pathway, causes a delay in development, a smaller size of the mature animal, and changes in metabolism (Tatar et al. 2003). Indeed, most of the aging genes that have been identified have very pleiotropic functions, and their involvement in the regulation of longevity could be assigned to a side effect of other, more essential determinants like development regulation, growth, or metabolism (Curran and Ruvkun 2007; Tacutu 2012). Therefore, the question then arises whether aging genes even exist. To answer this question, one has to investigate beyond the effects of mutations on lifespan and consider the function and regulation of developmental genes in aging.

22.1.2 Theories of Aging

Many hypotheses have been proposed to explain why animals age and dies, but at a very general level, one can divide them into two major groups. What distinguishes these two groups is the factor they point at a fundamental cause of aging. The damage theories represent the first group. According to these theories, the random accumulation of molecular and cellular damage is the primary force driving aging. The damage derives from different sources such as free radicals produced by cellular metabolism, exposure to chemicals, radiation, pathogens, toxins, errors in replication and translation, etc. The organisms react by activating defense and repair responses. These mechanisms, however, fail to repair the damage entirely. Hence

molecular and cellular damage accumulates over time until it reaches critical levels, causing pathologies and ultimately death. For many years the aging research has been dominated by one of these theories: the free radical theory of aging (Harman 1956; Medawar 1952). It postulates that the aging process is in large part driven by free radicals and other reactive oxygen species (ROS) endogenously formed from normal metabolic reactions. These reactive molecules are in turn responsible for oxidative damage in macromolecules inside the cell, resulting in a progressive loss of function and development of the aging phenotype. Undeniably, damage accumulation does explain some of the molecular changes observed with age, such as increased levels of protein oxidation and age pigment accumulation (Herndon et al. 2002; Heidler et al. 2010). Furthermore, genetic studies have revealed that most mutants were showing extended lifespans either have increased resistance to stress (e.g., *daf-2*) (Honda and Honda 1999) or decreased metabolic rates and the concomitant decrease in the production of free radicals (e.g., *clk-1*) (Ewbank et al. 1997).

However, recent results in worms and mice have led researchers to question the oxidative damage theory as the primary or exclusive cause of aging. To name a few examples: both loss-of-function and overexpression of superoxide dismutase (*sod*; genes responsible for the removal of ROS) in both worms and mice do not consistently affect lifespan (Perez et al. 2009; Van Raamsdonk and Hekimi 2012). Also, increased levels of protein oxidation in *sod-2* mutants or peroxide treatment do not affect worm longevity (Doonan et al. 2008; Yang et al. 2007), and even in some cases lead to lifespan extension (Van Raamsdonk and Hekimi 2012). Indeed, treating worms with low concentrations of paraquat (Van Raamsdonk and Hekimi 2012), or juglone (Heidler et al. 2010) causes increased levels of ROS and lead to lifespan extension, which is counter-intuitive to the oxidative damage theory. Additionally, *C. elegans* mutants displaying a decreased energy metabolism and prolonged lifespan, do not show reduced oxidative damage (Van Raamsdonk et al. 2010). Collectively, these and many other findings put the oxidative damage theory in question and propose that aging may involve additional mechanisms leading to accumulation of deleterious and irreversible changes.

The second group of theories instead, proposes that the aging process has its genetic determinants. Collectively, they suggest the existence of genetic programs provoking aging either as a purposeful effect or as an unwanted side effect of genetic pathways working in other biological processes. In either case, these signaling pathways drive the molecular and physiological changes that result in aging and, ultimately, in death. The antagonistic pleiotropy theory of aging is one of the most known among the genetic approaches—this theory based on the idea that the age-related decay governed by developmental pathways that go awry later in life. This phenomenon referred to as developmental drift. Pleiotropy refers to the fact that genes involved mediate multiple, or pleiotropic, functions; antagonistic because these functions are beneficial early in life, and detrimental in later stages. It was first proposed by Williams (1957) and further developed by Dilman (1971) and Dilman and Young (1994). In essence, this theory suggests that since a single gene can affect multiple traits, some genes that control development and increase fitness early in life when the force of natural selection is high (i.e., during development and reproductive period),

may have harmful, unselected effects later in life when natural selection is weak (i.e., beyond a reproductive age). An example of this can be found in the human eye lens, which continues to grow throughout life, but eventually leads to presbyopia or farsightedness in old age (Harding 2002).

22.1.3 Antagonistic Pleiotropy Theory of Aging in C. elegans

Until recently, aging studies in *C. elegans* have shown very few clear examples of antagonistic pleiotropy. This fact was mainly because by engaging in discoveries of mutations that affect lifespan; researchers forgot to ask questions about the natural function of the "aging gene" in longevity. One of the most famous and best-studied examples in *C. elegans* is the insulin-like signaling pathway. Mutations in this pathway that affect longevity include *daf-2* (which encodes an insulin-like receptor), *age-1* (which encodes a PI3 kinase), and *daf-16* (which encodes a Forkhead transcription factor). Loss-of-function mutations in *daf-2* and *age-1* both lead to significant (~100%) lifespan extension (Johnson 1987; Kenyon et al. 1993; Morris et al. 1996), while additional loss-of-function mutations in *daf-16* suppress lifespan extension in the *age-1* and *daf-2* mutant backgrounds (Kenyon et al. 1993; Tissenbaum and Ruvkun 1998). However, before conclusions can be made on these finding, we first need to know whether the insulin signaling pathway is a part of the normal aging process.

Elegant genetic studies from the Kenyon lab have shown that this signaling cascade is active in young adult worms and by post-developmentally inactivating insulin signaling, lifespan can be extended (Dillin et al. 2002). However, we still do not know if the insulin-signaling pathway is age-regulated. Does its activity decrease, increase, or remain constant throughout the life of the animal? More research needs to be committed to investigating the role of this and many other developmental pathways that play a role in *C. elegans* longevity. It remains a possibility that molecular changes associated with age in *C. elegans* orchestrated by intrinsic factors, like developmental programs, which are not appropriately modulated later in life.

22.1.4 Studies of the Natural Molecular Aging

Together, damage accumulation and developmental drift theories provide a baseline for current thinking about molecular mechanisms of the aging process. The relative involvement of each type of aging mechanism can be addressed by conducting an unbiased experiment that focuses on the whole aging process rather than a few signaling pathways and mutations. This challenge was solved by using full-genome DNA microarrays to profile gene expression changes associated with natural aging. This global transcriptional profile gives us a unique starting point in understanding the molecular mechanisms of aging.

Several laboratories performed genomic analysis of natural worm aging on wild-type and various mutants showing an altered lifespan (Budovskaya et al. 2008; Lund et al. 2002; Hill et al. 2000; Golden and Melov 2004). DNA microarray experiments and other genetic studies reveal that expression differences in old worms are caused by three GATA transcription factors: namely ELT-3, ELT-5, and ELT-6. In old age, ELT-5 and ELT-6 expression increases and expression of ELT-3 decreases, resulting in activation changes in a multitude of genes with GATA regulatory sites. Notably, this is the first molecular pathway that describes the molecular basis for aging in *C. elegans*. We examined age-regulation of the *elt-3* transcriptional aging circuit and were surprised to find no evidence that cellular damage or environmental stresses cause its regulation. Instead, we found that *elt-3* expression in the adult controlled by increased expression of the repressors *elt-5* and *elt-6*, which also guide *elt-3* expression during development (Budovskaya et al. 2008).

Since the publication of these results in 2008, several groups have found GATA transcription factors to be necessary for guiding the aging process in *C. elegans* (Samuelson et al. 2007; Xu and Kim 2012; Zhang et al. 2013). GATA transcription factors not only play essential roles in development and aging of *C. elegans* but are also found to be instrumental in higher organisms. In humans, GATA transcription factors play a vital role in the development of almost all tissues (Bresnick et al. 2012; Lango Allen et al. 2012). Therefore, the fact that mutations in GATA factors associated with various types of age-related human pathologies, including cancer, is not surprising (Zheng and Blobel 2010). The functions of these transcription factors are likely tightly regulated during normal development. We, therefore, asked the question whether the genetic drift of the GATA transcriptional circuit observed during aging is an isolated event, or rather a result of coordinated processes where a critical regulatory pathway continues to function post-developmentally, thus causing a drift within this particular transcriptional circuit.

During normal hypodermal development in the worm, both *elt-5* and *elt-6* are regulated by Wnt signaling (Cassata et al. 2005; Gorrepati et al. 2013; Koh et al. 2002), suggesting that age-related changes in Wnt signaling could account for differences in the *elt-5/elt-6/elt-3* GATA transcriptional circuit (Fig. 22.1). Thus, our findings indicate the possibility that molecular changes associated with age in *C. elegans* orchestrated by intrinsic factors, like developmental programs, which are not appropriately modulated later in life. Based on this previous knowledge, we set out this project to characterize how Wnt pathway intervenes in the aging process at a molecular level and to define to what extent this can be considered an example of developmental drift in the context of aging.

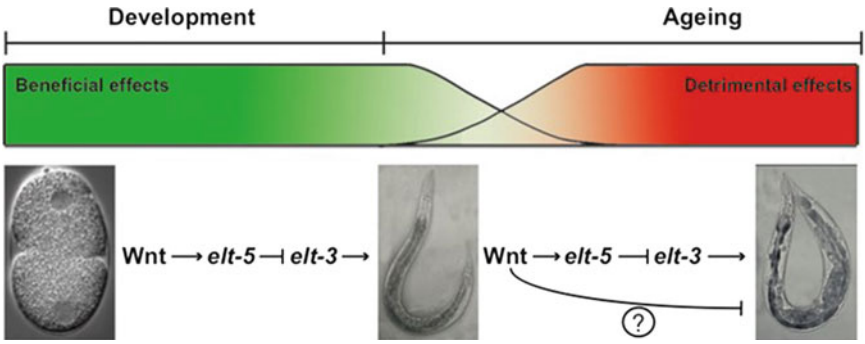

Fig. 22.1 Model of antagonistic pleiotropic effects mediated by Wnt in *Caenorhabditis elegans*. During development, Wnt signalling guides the changes in the expression of *elt-5* and *elt-3* GATA transcription factors involved in hypodermal development. In aging, Wnt signalling may drive some of the changes that characterize the aging process, possibly acting through the same genes involved in developmental processes. Figure adapted from Lezzerini et al. (2013)

22.2 Wnt Signaling Pathway in Development and Aging

22.2.1 Wnt Signaling Pathway Mechanics During Development and Adulthood

Wnt signaling is one of the major developmental pathways, evolutionarily conserved in all animals (Logan and Nusse 2004). Genomes of almost all metazoans contain several Wnt genes. In humans, 19 Wnt genes encode Wnt ligands. Throughout the animal kingdom, most of these genes are structurally and functionally highly conserved, suggesting that Wnt signaling played an essential role in the evolution of multicellular organisms (Clevers and Nusse 2012). The Wnt signaling pathway participates in a multitude of developmental processes as well as tissue homeostasis and control of stem cell proliferation and differentiation in adulthood (Clevers and Nusse 2012). It is therefore not surprising that deregulation of Wnt signaling pathway linked to many developmental defects in addition to many age-related diseases including osteoporosis and cancer (Clevers and Nusse 2012).

Mutagenesis experiments on Wnt genes suggest that their primary function is transcriptional regulation through the canonical/β-catenin signaling pathway (Logan and Nusse 2004). In the canonical Wnt/β-catenin signaling pathway, in the absence of the Wnt signal, β-catenin activity is inhibited by a cytosolic destruction complex consisting of Axin, GSK-3β (glycogen synthase kinase 3β), APC (adenomatous polyposis coli) and CK1 (casein kinase 1). This destruction complex enables GSK-3β-dependent phosphorylation and subsequent proteosomal degradation of the β-catenin protein. This continual elimination prevents the β-catenin from migrating into the nucleus and activating the expression of target genes. When Wnt binds the co-receptors, frizzled (Fz) and low- density lipoprotein (LDL) receptor-related protein

(LRP)5/6, disheveled (Dvl) is activated and inhibits the GSK-3β kinase, which in turn, leads to the stabilization of cytosolic β-catenin, which can then accumulate and translocate to the nucleus. In the nucleus β-catenin interacts with DNA-bound T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, leading to the activation of specific genes that control a wide array of processes during embryonic development (Logan and Nusse 2004; MacDonald et al. 2009) (Fig. 22.2).

In addition to their canonical signaling pathway, Wnts can act via noncanonical Wnt pathways that signal independently of β-catenins (Veeman et al. 2003). In these cases, Wnt signaling is transduced either via calcium flux, c-Jun N-terminal kinase

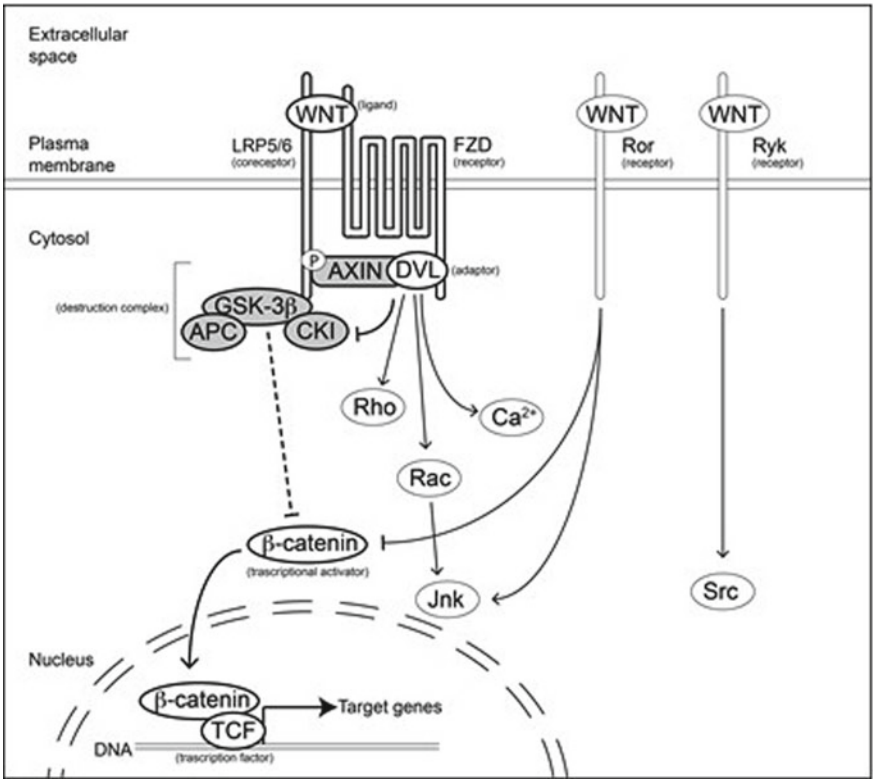

Fig. 22.2 Schematic representation of the canonical Wnt signalling pathway in human. In bold, the canonical pathway with its core components is depicted. Following the binding of Wnt ligand (WNT) to Frizzled receptor (FZD), the adaptor protein Dishevelled (DVL) is recruited at the membrane, and in turn, it represses the activity of the destruction complex (4 proteins in gray). The β-catenin, generally directed to proteasome degradation by the destruction complex, starts to accumulate and migrates into the nucleus where it binds to the TCF transcription factor, activating the expression of the target genes. Note that in *C. elegans*, also a Wnt/β-catenin asymmetry pathway exists (also known as non-canonical pathway), which is not included in this scheme [for a review see (Phillips and Kimble 2009)]. Also, Wnt ligands have also been shown to bind to other receptors such as the receptor tyrosine kinase Ror and Ryk. Figure adapted from Lezzerini et al. (2013)

(JNK), protein kinase C, or G proteins. A wide array of genes that are regulated by noncanonical Wnt signaling pathways have been implicated in the control of gastrulation, as well as in the induction of the heart tissue, dorsoventral patterning, tissue development, and neuronal migration (Veeman et al. 2003).

In adulthood, Wnt signaling primary is known for its function in maintaining stem cell function. For example, Wnt signals prove to be particularly essential to sustain self-renewal of intestinal stem cells, skin, and hematopoietic system (Korinek et al. 1998; DasGupta and Fuchs 1999; Gat et al. 1998; Reya et al. 2003). In line with this function, misregulation of Wnt signaling has implicated in various cancers, most notably of those tissues where it plays a primary role in stem cells maintenance. The best-understood case concerns colorectal cancer, where the vast majority of clinical trials characterized by mutations in APC (the component of the destruction complex) resulting in aberrant activation of this signaling (Korinek et al. 1997). Germline mutations of the same gene cause a hereditary cancer syndrome called familial adenomatous polyposis (FAP), in which patients display the early development of intestinal polyps that at later stages proceed into malignancy (Kinzler et al. 1991; Nishisho et al. 1991).

Along with colorectal cancer, other kinds of cancer like melanoma, hepatocellular carcinomas and several types of solid tumors connected to this pathway (Rubinfeld et al. 1997; Reya and Clevers 2005). Dysfunctions of Wnt signaling have also been linked to various age-related diseases like type II diabetes, underlining a connection between Wnt signaling and metabolism (Grant et al. 2006; Christodoulides et al. 2006; Van Camp et al. 2013). To date, several groups have investigated how this developmental pathway intervenes in the regulation of the aging process. It is not yet clear whether Wnt activity delays or accelerates aging. Evidence that supports both views can found in the scientific literature.

In several studies, Wnt activity was shown to lead to beneficial effects. For example, Wnt activation has been shown to positively influence adult stem cell maintenance (Kuhnert et al. 2004; Hoffman et al. 2004). More recently, Miranda et al. provided evidence that neuronal progenitors decrease following the attenuation of Wnt signaling from astrocytes in the aging brain (Miranda et al. 2012). Also, senescence seems to be delayed by Wnt activity. In their study, Ye and colleagues showed that cellular senescence in cultured human fibroblasts displayed a parallel decrease in canonical Wnt signaling by reduced expression of Wnt2 (a ligand that activates canonical signaling), and the addition of Wnt3a enhanced cellular proliferation and delayed cellular senescence (Ye et al. 2007).

Although these experiments indicate a protective role of Wnt signaling in aging, several other studies suggest the contrary. Brack and colleagues have shown that when young mice perfused with serum deriving from older mice, myogenic-to-fibrogenic fate conversion increases in young muscle progenitors, a process that characterizes aged muscles. They showed that this effect depends on Wnt3A activity and can be suppressed by injections of Dickkopf-1 (DKK-1), a Wnt antagonist that can enhance muscle regeneration in old mice as well (Brack 2007). Results from the Komuro group 117 corroborated the data. They reported that Wnt signaling activity increases with age in serum and multiple tissues of wild-type mice, promoting the age-associated

decline in tissue regeneration. In another study by Liu et al. (2007), the accelerated aging observed in the Klotho mouse model has been ascribed to chronic Wnt stimulation (due to the absence of the Klotho protein, another Wnt antagonist similar to DKK-1) contributing to stem cell depletion and aging. Also, several reports have shown that mutations that lead to over-activation of the Wnt signaling pathway contribute to the development of many age-related diseases, such as cancer, osteoporosis, and metabolic dysfunction (MacDonald et al. 2009).

Different reasons can be hypothesized to explain these seemingly contradictory observations. First of all, it should be kept in mind that there are multiple Wnt ligands (19 in human and 7 in mice) and these proteins can bind to a set of receptors, some of them also involved in other signaling pathways (Clevers and Nusse 2012; Phillips and Kimble 2009). This diversity may to some extent explain how the same Wnt ligand can produce different effects. Second, an age-dependent (or condition-dependent) effect of Wnt signaling can also play a part: downstream Wnt target genes may change with age, such that Wnt signaling promotes beneficial effects at an early age but becomes detrimental at an older age. In such a scenario, also the importance of co-factors should be considered. For example, in addition to binding to TCF proteins, Essers et al. reported that β-catenin could also functionally interact with the FOXO transcription factor when the cells exposed to oxidative stress (Essers et al. 2005). The crosstalk between different pathways and the context/tissue-dependent association of β-catenin with various transcription factors could account for the differences observed in aging studies that addressed the Wnt pathway function. These are just a set of possible explanations to try to reconcile the opposing roles of Wnt signaling in aging, and apparently, more work is needed to test these hypotheses further. We choose to investigate the effect of Wnt signaling pathway on aging using *C. elegans* as a model system.

22.2.2 Wnt Signaling Pathway in C. elegans

Like humans, the *C. elegans* genome encodes multiple genes for Wnt ligands (Wnts). However, there are only five genes (compared to 19 in humans) that encode Wnt ligands in *C. elegans*; *lin-44, egl-20*, *mom-2, cwn-1* and *cwn-2* (Eisenmann 2005) (Table 22.1). *C. elegans* utilizes these distinct ligands to control the activity of the sole TCF/LEF-1-like transcription factor, POP-1, which regulates expression of specific Wnt target genes in over a dozen *C. elegans* developmental processes (Lezzerini and Budovskaya 2013).

By analyzing the levels of expression for each Wnt ligand using GFP transcriptional reporters (Gleason et al. 2006), we found that all five Wnts continue to express after development completed. Additionally, we were able to measure the general activity of the Wnt signaling pathway in vivo throughout the natural aging process in *C. elegans* by using the worm homolog of the TOPFLASH reporter, POPTOP (Green et al. 2008). We discovered that Wnt activity is up-regulated as worms age (Lezzerini and Budovskaya 2013), which is consistent with observations of general Wnt sig-

Table 22.1 List of the Wnt pathway components in *C. elegans* and their closest homologs found in human

Pathway component	*C. elegans* gene	Component function	Closest human orthologs (Pitt and Kaeberlein 2015)
Wnt ligand	*egl-20*	Signalling ligand	*WNT7A, WNT7B*
	cwn-1		*WNT4*
	cwn-2		*WNT5*
	*mom-2**		*WNT9*
	*lin-44**		*WNT7A, WNT7B*
Frizzled receptor	*mig-1*	Receptor	*FZD10*
	cfz-2		*FZD8*
	*mom-5**		*FZD1*
	*lin-17**		*FZD10*
LRP/Arrow	*lrp-1*	Co-receptor	*LRP2*
Dishevelled-family	*dsh-1*	Adaptor protein	*DVL3*
	*dsh-2**		*DVL2*
	*mig-5**		*DVL3*
Destruction complex	*gsk-3*	Post-translational degradation of β-catenin	*GSK3A, GSK3B*
	kin-19		*CSNK1A1*
	apr-1		*APC*
	pry-1		*AXIN1, AXIN2*
β-catenin	*bar-1*	Transcriptional co-activator/TCF nuclear export	*CTNNB1, JUP*
	*wrm-1**		*CTNNB1*
	*sys-1**		*CTNNB1*
	hmp-2#		*CTNNB1*
MAP kinase	*lit-1**	TCF nuclear export	*NLK*
TCF/LEF	*pop-1*	DNA binding protein	*TCF7, LEF1*

Table lists the core proteins of the Wnt pathway in *C. elegans*. 1 = closest orthologs as identified in (Shaye and Greenwald 2011); * = components participating in the Wnt/β-catenin asymmetry pathway. Note that both pathways use *pop-1* as a transcription factor. # = *hmp-2* is the C. elegans β-catenin specialized in cell adhesion (Korswagen et al. 2000). In humans, a single β-catenin regulates both adhesion and Wnt signalling. Table adapted from Lezzerini et al. (2013)

naling activity in old mice and aged human tissues (Brack 2007; Liu et al. 2005). Interestingly, all five Wnts act as activators of *elt-5* GATA expression and down-regulation of any of these Wnts causes down-regulation of *elt-5* and subsequent up-regulation of *elt-3* GATA transcription factors. These results suggest redundant negative function of Wnt signaling during aging. Nevertheless, this is not the case.

We have recently discovered that the function of all five Wnts is only redundant in the regulation of the *elt-3/elt-5* transcriptional circuit and not in longevity as a whole (Lezzerini and Budovskaya 2013). For example, in *mom-2*/Wnt and *cwn-*

2/Wnt knockout/loss-of-function mutants *elt-3* expression (a biomarker of young age) is increased, and worms respectively live ~5 and ~18% longer compared to wild-type controls. In contrast, in *lin-44*/Wnt and *egl-20*/Wnt knockout/loss-of-function mutants, the *elt-3* expression is also increased, but worms respectively live ~30 and ~25% shorter than their wild-type controls. The *elt-3/elt-5* transcriptional circuit is not the only genetic circuit regulated by Wnt.

As a clear example of antagonistic pleiotropy, it seems that continued activity of *mom-2*/Wnt and *cwn-2*/Wnt in adult worms is detrimental for longevity, while on the other hand *lin-44*/Wnt and *egl-20*/Wnt are probably coordinating other genetic programs that are beneficial for worm longevity, which is an example of pleiotropy. That is then why the mutation in *lin-44*/Wnt and *egl-20*/Wnt decreases the lifespan of the worms despite its advantageous effect on the *elt-3/elt-5* transcriptional circuit. To better understand the dual role of the Wnt signaling pathway in age regulation, we performed an extensive analysis of transcriptional outcomes of *mom-2* and *lin-44* Wnts signaling using genetics, and Next-Generation Sequencing approaches.

22.2.3 *The Asymmetry Wnt Signaling Pathway Drives Molecular Aging in* C. elegans

Many asymmetric divisions during *C. elegans* embryonic and larval development use the Wnt/β-catenin asymmetry pathway, also known as non-canonical Wnt signaling pathway (Eisenmann 2005; Mizumoto and Sawa 2007). As in the canonical Wnt signaling pathway, β-catenin is playing the most crucial function in this pathway as well. In *C. elegans*, the non-canonical pathway utilizes MOM-2 and LIN-44 Wnt ligands, WRM-1 and SYS-1 β-catenins, and components of a MAP kinase cascade. Several main components of the Wnt/β-catenin asymmetry pathway, such as the Frizzled receptor, are asymmetrically localized in two neighboring cells that utilize this pathway (Mizumoto and Sawa 2007). In the daughter cell that does not receive the Wnt signal, β-catenins are targeted for proteosomal degradation by the destruction complex, much in the same way as in the inactive canonical Wnt signaling pathway (Fig. 22.3a). In the second daughter, upon ligand binding, two subsequent events occur. First, the MAP kinase cascade activates MOM-4/TAK1 (a MAPKKK-related protein) that phosphorylates and activates LIT-1 (Nemo-like kinase) to form a complex with WRM-1/β-catenin. This LIT-1/WRM-1 complex translocates into the nucleus and phosphorylates the POP-1/TCF not bound to the DNA, facilitating its nuclear export. Second, the SYS-1/β-catenin accumulates and translocates into the nucleus (Fig. 22.3a). Together, this results in lower levels of the unbound POP-1/TCF and high levels of SYS-1/β-catenin inside the nucleus. The resulting drop in the POP-1 to SYS-1 ratio allows the formation of the POP-1/SYS-1 complex that converts POP-1 bound to the DNA from a transcriptional repressor into a complex that acts as a transcriptional activator and leads to the up-regulation of the Wnt target genes (Fig. 22.3a).

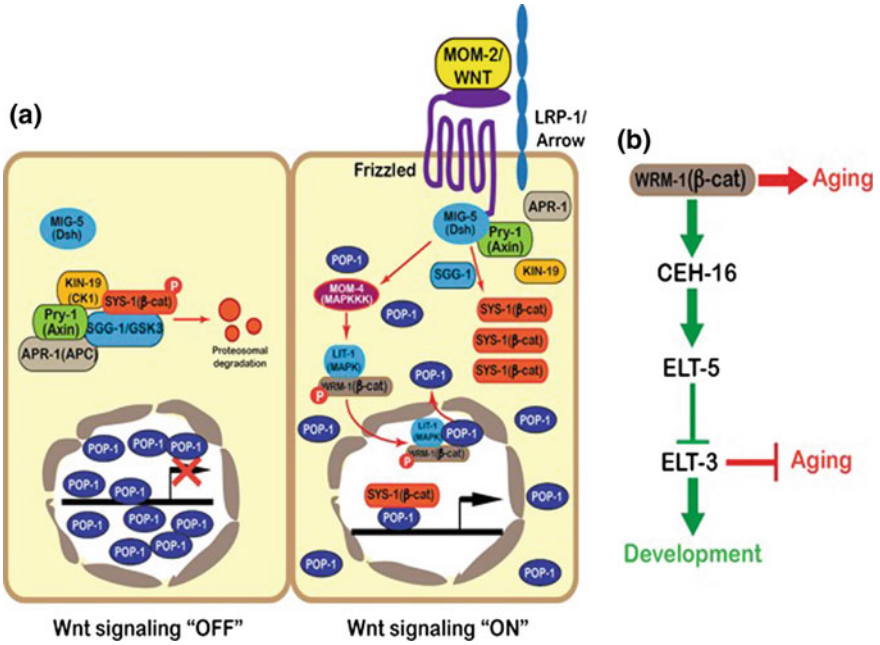

Fig. 22.3 Wnt signalling asymmetry pathway. **a** Schematic representation of the current model of the Wnt/β-catenin asymmetry pathway with its major components. See the text for more details. **b** WRM-1/β-catenin participates in epidermal developmental regulating the downstream genes *ceh-16*, *elt-5* and *elt-3*. In red are shown the effects on aging as predicted by our model

Previous studies have shown that the activity of the Wnt/β-catenin asymmetry pathway is essential for the asymmetric cell division of seam cells during embryonic and larval development to produce self-renewing seam cells, differentiated hypodermal cells, as well as neuronal and neuronal support cells (Banerjee et al. 2010; Gleason and Eisenmann 2010; Sulston et al. 1983). Also, previous studies show that the Wnt/β-catenin asymmetry pathway acts through the ELT-5 GATA transcription factor to control hypodermal morphogenesis (Fig. 22.3b) (Cassata et al. 2005; King et al. 2009).

We investigated the role of the Wnt/β-catenin asymmetry pathway in aging *C. elegans* and showed that WRM-1 expressed throughout the life of the worms and it still regulates the *elt-5/6/3* GATA transcriptional circuit similarly as it does during development (Lezzerini 2015). In the *wrm-1(ne1982)* mutant background the expression of the repressors *elt-5* and *elt-6* diminishes, and *elt-3*is found up-regulated relative to the wild-type, the expression pattern that was previously shown to be associated with prolonged longevity (Budovskaya et al. 2008; Lezzerini and Budovskaya 2013). We found this to be the case for the Wnt/β-catenin asymmetry pathway. We revealed that *wrm-1* mutation, along with all the other components of the MAP kinase cascade that participate in its regulation, prolong the lifespan of the worms. These results suggest an overall post-development detrimental effect of this path-

way in age-regulation. However, this lifespan effect is only partly mediated by *elt-3*, underscoring the complexity of longevity as phenotype, where a multitude of factors participates in determining the outcome.

To better understand the molecular mechanism underlining the effect on longevity mediated by Wnt asymmetry pathway, we performed a gene expression analysis on the *mom-2(or77)* and *wrm-1(ne1982)* mutants by RNA-seq. Wnt/β-catenin asymmetry pathway is known to be one of the major developmental pathways, so we sought to investigate how it influences the gene expression on the transition from developmental stages to adulthood and aging. For this reason, we chose the L4 developmental time point and day 2 (3 for *wrm-1(ne1982)*) in early adulthood (Lezzerini 2015).

We hypothesized that Wnt/β-catenin asymmetry pathway regulates the same genes or pathways during development and aging. However, when we compared the gene expression profile of the *mom-2(or77)* and N2 animals in the L4 larval stage, we found no significant change (Fig. 22.4a). In adulthood (day 2), we identified only 46 genes differentially expressed between *mom-2(or77)* and wild-type worms (Fig. 22.4b). These genes are involved in general metabolic processes, such as oxidation and drug metabolism, rather than organismal morphological development. Although surprising, this is consistent with our previous observations. The *mom-2 (or77)* mutation is a weak allele, and do not affect timing or physiology of the worm's development. This result also suggests that the longevity phenotype is probably not due to compensating mechanisms taking place during development.

In contrast, by comparing the gene expression in the temperature-sensitive *wrm-1(ne1982)* mutant to the wild-type animals, we observed more profound effects, both in development and adulthood. We found 193 differentially expressed genes in the *wrm-1(ne1982)* mutants compared to the wild-type at the L4 larval stage (Fig. 22.4a). These genes are mostly down-regulated, suggesting that *wrm-1*/β-catenin is acting as an activator during development. Gene ontology (GO) analysis of the targets revealed that *wrm-1*/β-catenin regulates many aspects of development and metabolism (Lezzerini 2015).

In adulthood, we identified 391 differentially expressed genes (Fig. 22.4b). Interestingly, these genes are almost evenly distributed between being up and down-regulated. This result suggests that in adult worms Wnt signaling functions both as an activator and as a repressor. GO analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) database search shown that most of the *wrm-1* down-regulated genes are involved in body morphogenesis (mainly cuticle development), fat metabolism, age-regulation, and chromatin remodeling, which possibly indicates secondary responses involving activation/repression of the transcription.

Additional analysis of both, developmental and adult, datasets revealed that many *wrm-1(ne1982)* regulated genes were previously identified as age-regulated genes (ARG—Age-Regulated genes) (Fig. 22.4c). GO analysis of all the targets in adulthood revealed that most of the ARG intervene in body morphogenesis and regulation of aging, as well as amino acids and fatty acid metabolism.

When we compared the developmental and adult datasets, we found 35 genes in common. Surprisingly, most of these genes display an opposite direction passing from L4 to adulthood, with 27 out of 35 genes being down-regulated in L4, and

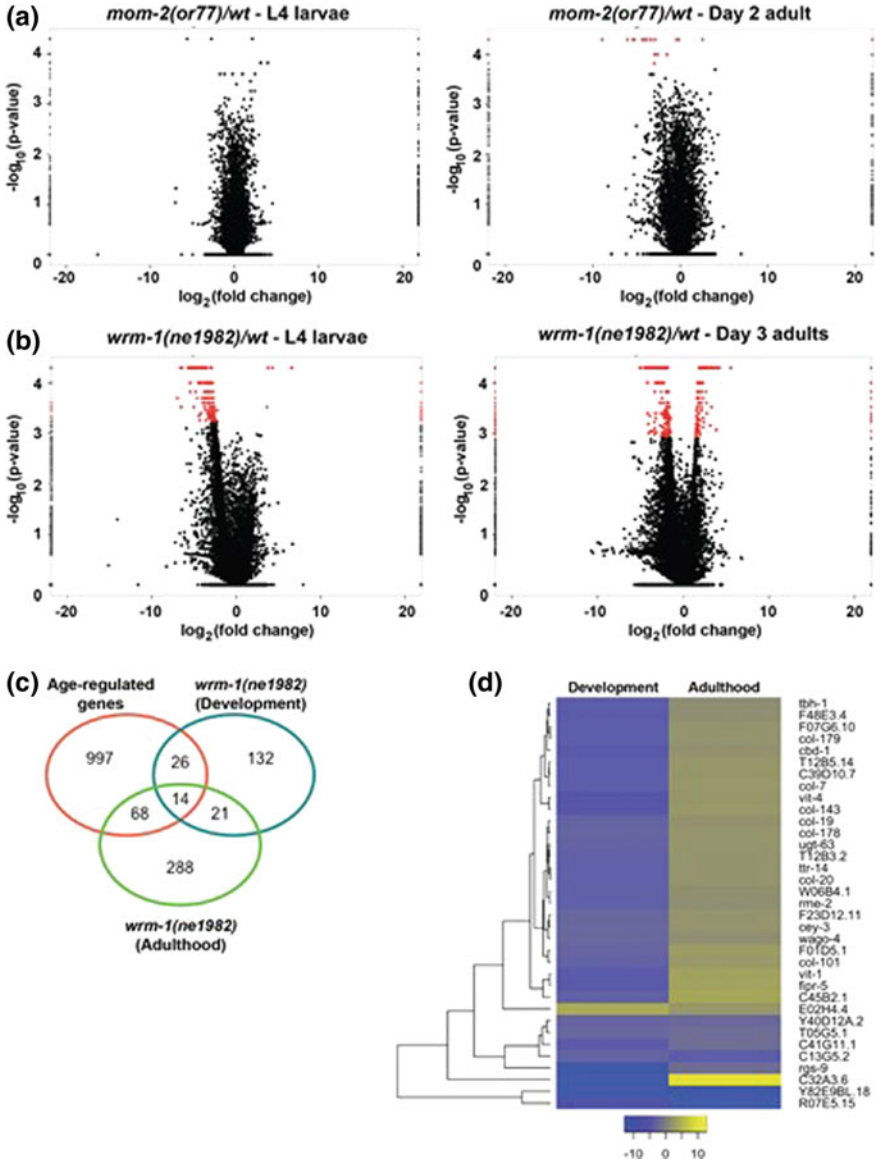

Fig. 22.4 Transcriptome analysis of the *mom-2(or77)* and *wrm-1(ne1982)* mutants. **a** Volcano plots are demons trating the gene expression changes in the *mom-2(or77)* mutant, on the L4 larval stage and day 2 of adulthood. **b** Volcano plots show the gene expression changes in the *wrm-1(ne1982)* mutant, on the L4 larval stage and day 2 of adulthood. **c** Venn diagram illustrating the role of *wrm-1*/β-catenin in the regulation of previously identified age-regulated genes in both development and adulthood. **d** Heat map of the 35 genes in common between L4 and day 3 in the *wrm-1(ne1982)*. Blue/yellow indicate a decrease/increase in gene expression relative to the wild-type

up-regulated on day 3 (Fig. 22.4d). This expression profile suggests a change in the transcriptional activity mediated by *wrm-1*, which switches from activator in L4 to repressor of the same target genes in early adulthood.

The gene set showing a switch in expression from development to adulthood contains several transcripts of unknown function, annotated as non-coding RNAs (10 out of 27). Among the annotated genes, the *col*-type genes are the most represented (*col-179*, *-7*, *-143*, *-19*, *178*, *-20*, *-101*), followed by the vitellogenin genes (*vit-4* and *vit-1*). Also, *tbh-1*, *rgs-9,* and *wago-9* were also found. The *tbh-1* (tyramine beta-hydroxylase) is orthologous to human DBH whose activity is required for the biosynthesis of the octopamine neurotransmitter. *rgs-9* is an ortholog of several human RGS proteins (regulators of G-protein signaling RGS1, 13, 16, 18 and 21). Lastly, *wago-4* is an ortholog of multiple human AGO components (argonaute RISC catalytic component 1, 2, 3, and 4). We validated most expression changes using transcriptional GFP reporter constructs.

Reasoning that this event may be mediated by other factors/pathways intervening during this transition, we identified the germline as a possible tissue affecting the Wnt activity in adulthood. We showed that in the absence of the germline *mom-2* expression is drastically reduced, possibly indicating a general Wnt activity parallel reduction. However, we observed no drastic changes in the germline function of Wnt signaling pathway mutants. An exception is a *pop-1* mutant that showed a little but significant reduction in the number of eggs laid. This effect may be ascribed to embryo early development defects, but more work is needed to dissect this aspect. However, overall our results suggest that the germline affects Wnt signaling starting from early adulthood and not the other way around.

As previously reported, germline plays an essential role in the aging process. Worms with functional gonads but lacking the germline, are sterile and live longer (Hsin and Kenyon 2009; Arantes-Oliveira et al. 2002). The absence of the germline results in the activation of several effectors, which prolong the longevity. In such condition, for example, DAF-16/FOXO3a and PHA-4/FOXOA1, two transcription factors are known to promote longevity, are both activated in the intestinal cells (Hsin and Kenyon 2009; Wang et al. 2008; Lapierre et al. 2011; Ghazi et al. 2009). The gene expression changes mediated by these transcription factors, not only render the animals more resistant to stresses but also enhance autophagy and fat metabolism (Lapierre et al. 2011; Ghazi et al. 2009; Goudeau and Aguilaniu 2010). In these examples, the absence of the germline activates these proteins. In our study instead, we found that Wnt signaling may be attenuated rather than enabled by the lack of the germline, as indicated by mom-2 expression levels in the *glp-1(e2141)* mutant (Lezzerini 2015).

Interestingly, DAF-16 and PHA-4 were found activated in the intestine, the same tissue we also found to be essential for longevity in the Wnt mutants. These observations indicate a direct link between the intestine and the germline, with the former reacting to signals coming from the latter. Another important observation is that, except *pop-1*, the Wnt mutants live longer without displaying a compromised reproduction, the condition that very often characterizes the long-lived mutants. It remains

to understand the precise nature of the signal coming from the germline, and the exact mechanism by which it affects Wnt (and possibly others) signaling pathways.

In summary, we used *C. elegans* to analyze the function of the Wnt/β-catenin asymmetry pathway in age-regulation. We showed that the pathway is active in adulthood and is detrimental to longevity. By examining the gene expression changes in the *wrm-1(ne1982)* mutants we discovered a functional switch of this Wnt component from the activator to the inhibitor of a small set of genes, suggesting a possible cross-talk with other transcription/regulatory factors. Also, we found that WRM-1 goes from regulating mainly organismal growth in development, to regulating oxidative phosphorylation and fat metabolism pathways during aging. We believe that the Wnt signaling targets presented here can be used as biomarkers in future screens. This study should help to characterize further the effects on longevity mediated by Wnt signaling, a developmental pathway whose functions in aging are still to be fully elucidated.

22.2.4 Molecular Aging is Driven by the Canonical Wnt Signaling Pathway: The Role of the BAR-1/β-Catenin in Aging and Development

In *C. elegans*, the canonical Wnt signaling is the most similar to the well-described Wnt/β-catenin signaling pathway present in vertebrates. In the nematodes, it utilizes the BAR-1/β-catenin, and its architecture is very well conserved. Similarly, as in vertebrates, when the signal is absent, the BAR-1/β-catenin is continuously degraded after being targeted for proteosomal degradation by the destruction complex. The signaling cascade gets activated when a Wnt ligand binds to the Frizzled receptor and the LRP co-receptor. The binding results in the inhibition of the destruction complex and stabilization of BAR-1/β-catenin in the cytoplasm. Subsequently, BAR-1 migrates into the nucleus where it binds to the POP-1/TCF factor bound on the promoter regions, and together they activate the transcription of the target genes (Fig. 22.5) (Eisenmann 2005).

We analyzed the impact of the Wnt canonical pathway in aging, investigating the effects mediated by BAR-1/β-catenin. We used a weak allele mutant of *bar-1* and showed that these animals live longer. Conversely, by over-expressing this β-catenin on the first day of adulthood, the animals show a decreased lifespan. Also, we showed that BAR-1 regulates the *elt-5/3* GATA transcriptional circuit during aging in a similar fashion as WRM-1 does during development. In the *bar-1(mu63)* mutant background the expression of the repressor *elt-5* diminishes, and *elt-3* is found up-regulated relative to the wild type. This expression pattern that was shown to be associated with increased lifespan (Budovskaya et al. 2008; Lezzerini and Budovskaya 2014). These results suggest an overall detrimental effect of this pathway in age-regulation.

To dissect the additional molecular mechanisms underlying the observed aging phenotype, we analyzed the transcriptome changes driven by the Wnt asymmetry

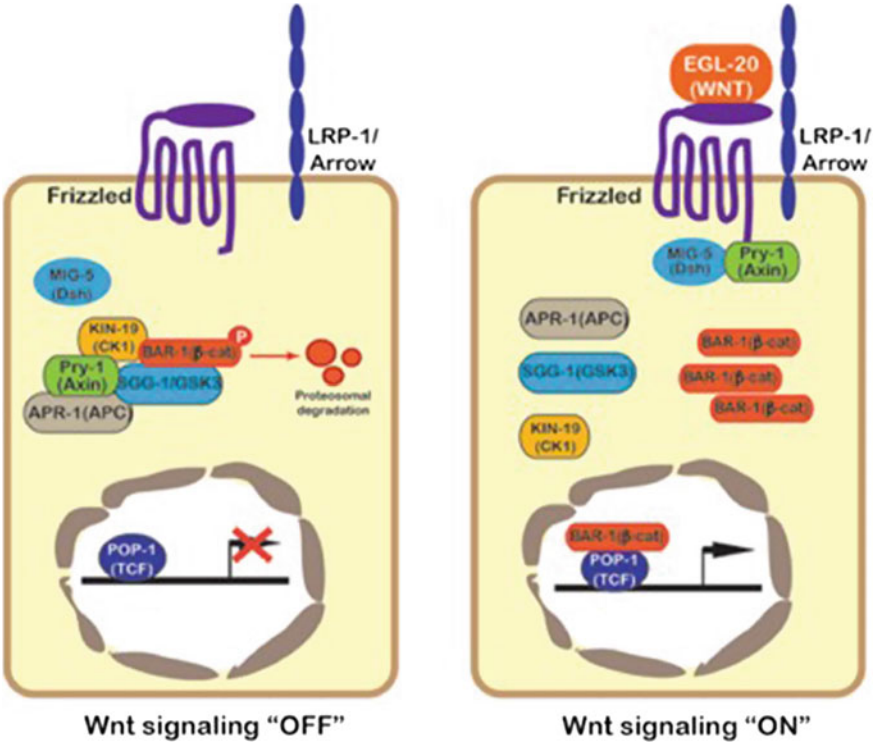

Fig. 22.5 Wnt canonical pathway in *C. elegans*. Schematic representation of the canonical Wnt pathway with its major components. See the text for more details

signaling cascade using the *bar-1(mu63)* mutants. The analysis reported 2865 genes that are differentially expressed during aging, 218 genes and 1537 genes in L2 and L4 larval stages respectively. These datasets are enriched for genes that regulate many aspects of *C. elegans* development (Fig. 22.6).

When we compared the development datasets with the adulthood dataset, we found a group of 35 genes in common for which *bar-1* switches its function from early development to adulthood. Specifically, it acts mainly as an inhibitor during early stages of development, and as both an inhibitor and an activator in late development and early adulthood (Fig. 22.7). This suggests a qualitative change in the BAR-1 function, as the animals complete their development and enter into adulthood. These changes are similar to the ones we observed in the analysis of the WRM-1 regulated genes.

We have reported that the germline plays a vital role in both the aging processes and the Wnt/β-catenin asymmetry signaling pathway activity during early adulthood. This relation may be relevant for the canonical Wnt signaling pathway as well. We did not address this hypothesis directly yet. However, the germline function was found as an enriched biological process regulated in the *bar-1(mu63)* mutant. Therefore, it

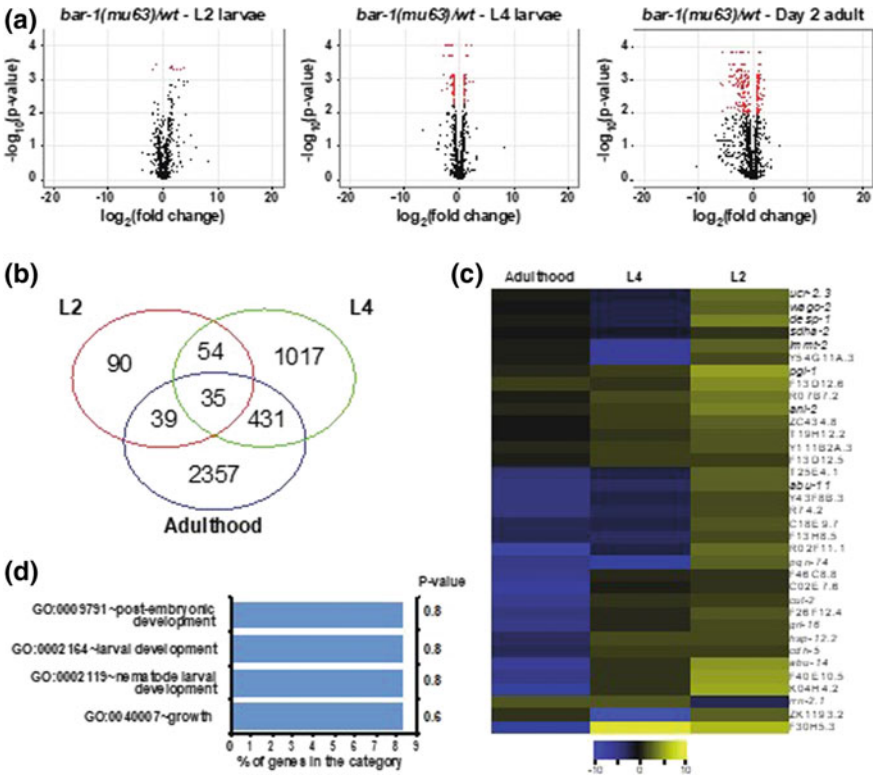

Fig. 22.6 Transcriptome analysis of the *bar-1(mu63)* mutant. **a** Volcano plots demonstrating the gene expression changes in the *bar-1(mu63)* mutant, on L2, L4 larval stages, and day 2 of adulthood. **b** Venn diagram for the three datasets **c**. Heat map and **d** Functional annotation analysis of the 35 genes in common

is possible that a connection between the germline and the canonical Wnt pathway exists.

Having analyzed the global gene expression changes in five different Wnt signalling mutants, gave us in the unique possibility to perform multiple comparisons among these mutants displaying a different lifespan. We compared the gene expression changes in the long-lived mutants *mom-2(or77)*, *wrm-1(ne1982)* and *bar-1(mu63)* mutants. We discovered that both the canonical and the Wnt/asymmetry signaling pathways regulate the same set of genes that are responsible for the epigenetic and metabolic changes that contribute to the aging process. In contrast, *lin-44*/Wnt mediated signaling might engage the fatty acid metabolism to slow down the aging process and promote the survival (Fig. 22.7). These data also raise the critical question: do we have a clear separation between the canonical and the asymmetry Wnt signaling cascades during aging? The considerable overlap between the *wrm-1* and *bar-1* regulated genes indicates that both pathways, although governing different aspects of development, might converge on the same functions during aging. This

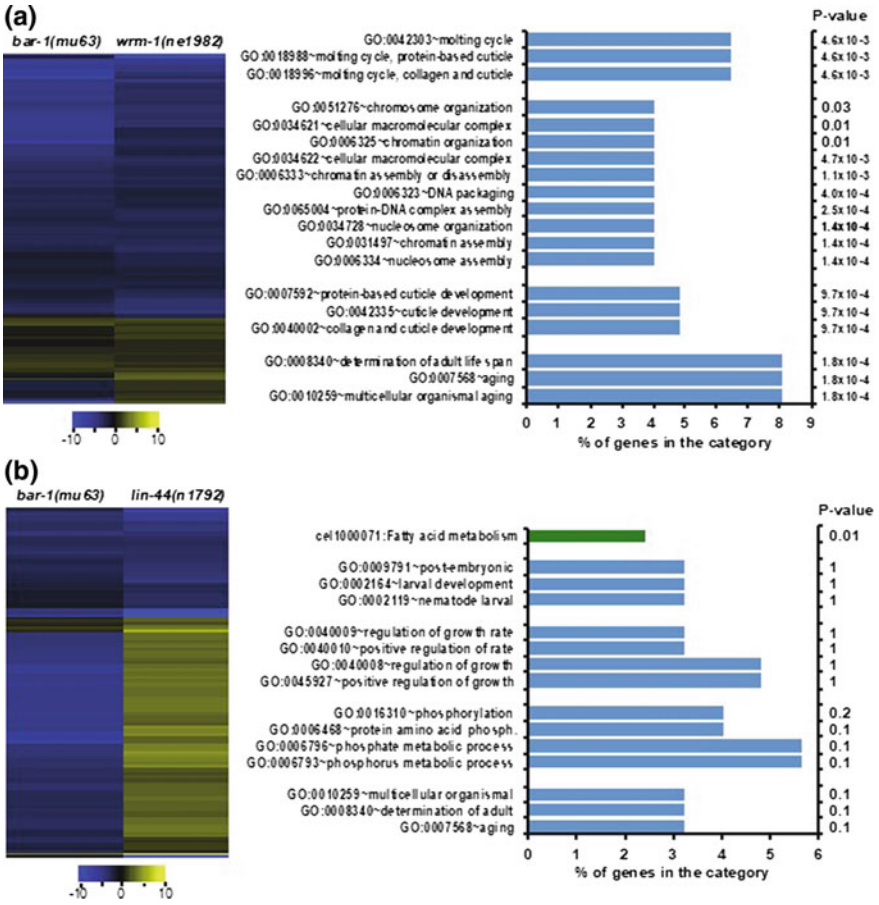

Fig. 22.7 Common differentially expressed genes in the *bar-1(mu63)*, *wrm-1(ne1982)* and *lin-44(n1972)* mutants. **a** Left panel—Heat map indicating the $\log_2$ fold change of the 148 genes in common in the long-lived Wnt mutants. Right panel—biological processes affected by these genes **b** Left panel—Heat map indicating the $\log_2$ fold change of the 135 genes in common between the *bar-1(mu63)* and *lin-44(n1972)* mutants. Right panel—biological processes affected by these genes

study provides a basis for a further detailed investigation into the role that the canonical Wnt signaling governed developmental pathway, and its downstream targets may play in the progression of aging.

22.3 Prospective

Studies in *C. elegans* have revealed an essential role of developmental pathways in the aging process. First, they provide a new way to approach the question of

how molecular pathways regulate aging. For example, in our studies, we found that activity of one of the significant developmental cascades, the Wnt signaling pathway, increases as worms grow old and, at least in part, guides the aging process in *C. elegans*. This phenomenon is a classic example of antagonistic pleiotropy (Dilman 1971; Dilman and Young 1994; Williams 1957) because it describes how a trait that is useful early on in life can become detrimental to the animal in old age. Thus, our work on the Wnt signaling pathway helps unite evolutionary and genetic studies of aging.

During development, the function of Wnt ligands is under natural selective pressure as they play an essential role in building a fully functional organism (Fig. 22.1). However, in old worms that are beyond reproductive potential, there is little or no advantage to maintaining proper Wnt ligand expression and therefore selective pressure on the Wnt ligands declines. This possibly shortens the worm's longevity as is observed in the controlled environment of the laboratory but has an insignificant effect on population survival in the wild where old worms are extremely rare (Fig. 22.1). Thus, Wnt signaling, and as a consequence, the *elt-3/elt-5/elt-6* hierarchy, is a developmental program that may drift during aging, just because later on in life the natural selective pressure no longer maintains it. It is harder, however, to explain the results concerning the function of *lin-44* and *egl-20* Wnt ligands during aging. In this case, it is evident that the continuation of developmental programs in aging is not always wholly antagonistic.

By developing *C. elegans* as a model system to study molecular mechanisms that are regulated by the Wnt signaling cascade during aging, we can make the first attempt to answer the prominent question of which developmental forces continue to work as the animals grow old and thus contribute to aging and progression towards death. If we understand this aspect, we can potentially apply this knowledge in higher organisms to develop therapies with the goal to delay aging, and therefore prevent the occurrence of many age-related diseases.

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Chapter 23
The Problem of Integrating of Biological and Clinical Markers of Aging

Arnold Mitnitski and Kenneth Rockwood

Abstract Our goal is to address human health as a *whole*, with an ultimate goal of understanding how health changes with age. Here we discuss the need to integrate multidimensional information about health (and not only human) that is available in many biological and clinical databases. Reviewing several means of such integration (frailty index, dysregulation score, and some indices of biological age) we pay particular attention to the frailty index, which allows such an integration in a simple but an effective way. Most importantly, it makes it possible to apply mathematical modeling techniques and computer simulations to gain insight into the complexity of human health. By applying such modeling methodology (complex dynamical networks) we can take advantage of one of the most essential characteristics of biological systems—the interdependence of multiple biological and clinical characteristics of such systems. That also explains the effectiveness in many practical applications of using the frailty index based on the multiple clinical and biological traits instead of selecting only those that survive iterative p value testing.

Keywords Aging · Complexity · Biomarkers · Frailty · Deficits accumulation · Frailty index · Mortality · Biological versus chronological age · Complex networks

23.1 Introduction

Biomedical systems are complex. Their complexity often challenges the application of quantitative methods, including mathematical modeling to investigate such systems. But what does complexity mean? The context is always unavoidable. For example, a mathematical approach to physiologic 'complexity' was addressed in the nineties by Lipsitz and Goldberger (1992) in relation to nonlinear irregular dynamics. They argued that loss of complexity was a defining feature of aging. Here we present a complementary view of the problem of complexity related to data analysis

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and mathematical modeling of multiple health attributes commonly available in large epidemiological and clinical databases. We start from a statement attributed (perhaps not exactly) to Aristotle: "The whole is greater than the sum of its parts" (Barnes 1991). Whatever its provenance, this statement is directly related to many biomedical systems, and to our health as an example of such a system. The reason for that is the *interactions* between multiple *parts* and organs that make up the *whole* from its parts. Still, the phrase is not fully appreciated by the community of data analysts and mathematical modellers who are often guided by the other ancient principle of "divide and conquer". How can changes across multiple organ systems be captured? How are their many biological and clinical characteristics best understood? Let us grant that we see the human organism as a complex network of interacting parts with "health" obliging that these parts depend on each other. Understanding how health attributes interact is not necessarily made any easier by having more information available, such as from new biological tests and from densely populated electronic medical records. The number of interactions dramatically increases with the number of health attributes and that often imposes limitations on the application of mathematical modeling and computer simulations [the so-called "curse of dimensionality" by Bellman (1957)]. Instead, to advance our understanding of human health and the aging process we need to create mathematical models that can help to unify a large amount of observational data. For this, we need integrative measures that define the overall health states of individuals in order to find ways of improving health and thereby addressing the aging process. Here, we discuss some directions in how we approach multidimensional information about the health of individuals. Our method aims to integrate this information and to use mathematical modeling and computer simulation to deal with complexity. Our goal is thereby to address human health as a *whole,* with an ultimate goal of understanding how health changes with age.

23.2 Personal Health Monitoring and Evaluation

As defined in Wikipedia "Personalized medicine, also termed precision medicine, is a medical procedure that separates patients into different groups—with medical decisions, practices, interventions and/or products being tailored to the individual patient based on their predicted response or risk of disease." The PubMed database shows that the number of yearly peer-reviewed publications related to personalized medicine increased during the last decade from 662 (2007) to 7167 (2017); the number of publications related to precision medicine increased from 389 (2007) to 4427 (2017). Even so, the problem is not new. Hippocrates urged that physicians must "treat the patient, not the disease". Even though so-called "evidence based medicine" emphasizes the need to take into account the body of empirical facts related to the *population*, this dictum remains powerful. But how can we use the unprecedented access to large amounts of information about many aspects of human health to assess, evaluate, and monitor personal health?

Personalisation in health implies the use of information from as many as aspects of patient health as possible, including diagnoses, blood and urine tests, diagnostic images, genetic information, pharmacy, demographics, and more. We argue that although much effort goes into making such characteristics available, not enough has been done to understand the need for integration or to develop integrative measures. For this, it is useful to ask "What is health?" Although the question might seem rhetorical, it loses none of its force. Rattan (2013) presented a good account of why we need both objective and subjective measures of health, using the example of hemodynamics and aging. In the context of our inquiry, we consider three major characteristics of health: (i) multidimensionality (multiplicity of aspects of health), (ii) dynamics (changes over time of its attributes/dimensions), (iii) interdependence of different attributes/aspects of health that influence each other).

Various aspects of health include: symptoms and signs of illnesses and impairments, functional limitations, and biological measurements, including images (X-ray, ultrasonic, etc.), genes, metabolites, and microbiota. Symptoms and signs of illnesses and impairments are usually identified by health professionals and stored in databases. To this we can add other information—including genetics and "omics". Simply listing all actual and potential data sources that are relevant to the health status of a patient presents an impressive challenge. This challenge increases by orders of magnitude if we add temporal aspects of data/repeated measures. We now are in the very beginning of such data collection—tracking some aspects of health is now routine (e.g., blood glucose, arterial blood pressure). Wearable tracking devices will be used to personalize important aspects of health (e.g. related to the cardiovascular system). Still, what can be done with all this?

The obstacles to progress in personalized medicine are many and non-trivial: (i) technological/technical, such as insufficient capacity of modern computers and computer networks—that will be solved with the rapid progress in the development of the computer and IT technologies; (ii) privacy issues—the concerns are serious (possible misuse of privacy); (iii) security issues (related to privacy)—individual health records are protected by the laws in many countries and how to guarantee that individual health records will not be misused is crucially important. The development of peer-to-peer technologies (e.g., block chain-like technologies) and cloud computing will likely afford access to personal information.

23.3 Biomarkers of Aging

23.3.1 Biomarkers (Definition)

Changes in health can be assessed by multiple biological traits called "biomarkers of aging". Will such biomarkers be quantitative characteristics of individuals that can indicate not only the presence of illnesses but also aging processes? Can they tell us about lifespan? Contrasting life span and health span is often misleading: health span

and life span must be highly correlated—sick people don't usually live as long as those who are healthy. An emphasis on individual biomarkers, however, has obscured the development of mathematical models that integrate across biomarkers. Models are essential for any theoretical understanding of aging.

According to Miller (2001): "Outside of gerontology, biomarkers play an important, acknowledged role as surrogate measures of processes or traits that are themselves difficult to measure." He suggested the following definition of a biomarker of aging. To be considered as a biomarker, a trait would need to meet three criteria "(i) it should predict the outcome of a wide range of age-sensitive tests in multiple physiological domains, in an age-coherent way, and do so better than chronological age; (ii) it should predict remaining longevity at an age at which 90% of the population is still alive; and (iii) its measurement should not alter either life expectancy or the outcome of subsequent tests of other age-sensitive traits" (Miller 2001). He concluded that "The successful identification of biomarkers would constitute a landmark in our understanding of the aging process and provide invaluable tools for screening potential anti-aging interventions that could be of major benefit to preventive medicine and public health" (Miller 2001). Since then, the study of biomarkers of aging has intensified with over 1500 publications in PubMed. Even so, almost two decades after Miller's paper the hope of finding biomarkers that help to comprehensively understand the aging process remains elusive.

23.3.2 Biological and Clinical Markers—The High Dimensionality Challenge

It is important to underscore that biomarkers of aging can also be biomarkers of some illnesses. That speaks to the difficulties with distinguishing aging of diseases (Gavrilov and Gavrilova 2006) and explains why we do not favor the idea of *healthy aging*—"the notion of health aging is an oxymoron, like a healthy dying or a healthy disease" (Gavrilov and Gavrilova 2006). It has been suggested to use "successful aging, aging well, postponed aging, slowing aging … and hopefully aging reversal" (Gavrilov and Gavrilova 2006). Indeed, an organism's age-associated susceptibility to stress makes it easier to acquire age-associated illnesses not to mention greater age-associated lethality of some common infections like influenza. That is why we favour extending the definition of biomarkers to include not just "purely" biological traits (like those obtained from blood and urine tests, immune cells, etc.) but also clinical signs and symptoms that usually characterize functional changes, or illnesses. In short, we favour using all available characteristics of health that are age-associated. This approach is an alternative to the common strategy of carefully selecting traits to be considered as biomarkers. Instead, we say "use them all!" including those from different "omics" (genomics, proteomics or metabolomics, and epigenomics). The latter is particularly important, in that the substantially lower cost of their evaluation

can help efforts to understand aging using DNA methylation processes (Horvath 2013).

23.3.3 Dynamical Aspect of Time Changes of Biological and Clinical Markers

Aging biomarkers typically change over time. The characteristics of the time scales of such changes vary greatly between biological and clinical markers, in ways that we suggest are informative. Slow (secular, long-term) trends are particularly important to understand aging and should be distinguished from the relatively rapid fluctuations caused by the countless influences of the environment. In our view, it is particularly important—and generally better reflects what happens with aging—to integrate a large amount of biological and clinical markers in a relatively small number of *unified* measures of the aging process rather than to carefully select "the best" biomarkers. In or view, the latter is an information-losing strategy, as our network studies (below) suggest. In our experiences, *ALL* health related information yields better evaluation of individual health. Before outlining our approach we mention briefly an alternative view that until recently dominated the research in the applications in epidemiology and biostatistics.

23.3.4 The Biomarkers Selection Strategy

Following conventional parsimony, many researchers try to define the "best biomarkers" using standard statistical/psychometric techniques to produce a relatively small set of the most useful biomarkers. Allostatic load is one example of such an approach where the combination of blood/urinary biomarkers and blood pressure is used to build an index of allostatic load (McEwen and Stellar 1993). In other cases, generally, biomarker selection is based on the correlation with age; this assumes that the higher the correlation with age, the more that biomarker tells us about the aging process. The situation is not so simple, however. Let's consider the so-called "biomarker paradox". Because the biomarkers are typically weakly (even if significantly) correlated with chronological age, the temptation to use the biomarker with highest age correlation makes sense. But consider a biomarker that is perfectly correlated with age. Why bother with it? Why not just use age? (which will work in all but forensic applications). Likewise, the rings in a tree trunk will allow us to estimate the age of the tree but not the state of its "health". As nicely put by Gavrilov and Gavrilova, "the information of regular seasonal tree rings tells us everything about tree age but little about tree aging" (Gavrilov and Gavrilova 2006). We find that analogy quite instructive in light of spreading of some means of estimating biological age based

on maximizing of the correlation of biomarkers with age, such as DNA methylation age may be an example (Horvath 2013) (as considered below).

23.3.5 Biomarkers and Reference Values

The deviation of health from "normality" can be evaluated by comparing the values of the biomarkers in individuals with reference values that represent the "norm", but this definition of a norm may not be as straightforward as it might seem. For example, for many blood tests the reference values of biomarkers could be found from the "normal" population (i.e. the population without known major diseases). Such values are often age- and sex-dependent, further complicating their interpretation as aging biomarkers. For many novel biomarkers (e.g. cellular biomarkers of immunosenescence) values are not well standardized. Further, the identification of what is normal for age may be influenced by interactions between the biological and clinical markers. As noted above, the number of such interactions increases exponentially with the number of biomarkers to be considered. That itself significantly contributes to the complexity of the problem of evaluating health of people comprehensibly.

23.3.6 How to Integrate Multiple Biological and Clinical Markers?

Integration is conventionally understood as "the act or process of combining two or more things so that they work together" (Oxford English Dictionary); Biological and clinical markers of aging have primarily been intergrated in three ways: as *frailty indices*, as *indices of biological age*, and as the *statistical distance ('dysregulation') score*.

23.3.7 Frailty Index

The frailty index (FI) was introduced in 2001 (Mitnitski et al. 2001) as a means of quantifying general health in older adults and was later extended to adulthood (Rockwood et al. 2011). The FI was defined as a ratio of the number of health deficits that individuals accumulated to the total number of deficits available in the database or the study (Mitnitski et al. 2001; Mitnitski and Rockwood 2015). We also demonstrated that the frailty index is generally less dependent on the nature of the items that make it up, but from the number of items (Mitnitski and Rockwood 2015). Empirically, using approximately 40 (or more) health characteristics allows us to robustly predict adverse outcomes (Mitnitski and Rockwood 2015; Mitnitski et al. 2005).

Earlier estimates using cross-sectional analyses suggested that deficits accumulated exponentially, at an annual rate of 3.5% (Mitnitski et al. 2005). Using longitudinal data, the rate of deficit accumulation from the Canadian National Population Health Survey was estimated as 4.5% (Mitnitski and Rockwood 2016). This estimate was notably stable during the adult life span. The corresponding average doubling time in the number of deficits was 15.4 years, roughly 30% less than we had reported from the cross-sectional analysis (Mitnitski and Rockwood 2016). A more recent report using longitudinal data suggests that the average doubling time in the number of deficits was 12.6 years, and this was similar in those aged 65–74 years and those aged 75+ (Hoogendijk et al. 2018).

23.3.8 Frailty Index in Biological Data and Animal Studies

The FI approach has also been shown to work with cellular biomarkers of inflammation, cellular senescence, and oxidative stress, and genetic markers (Mitnitski et al. 2015). As many biological processes are implicated in aging, the systemic effects of these processes can be elucidated using the FI approach—recently it has been shown that subclinical deficits, when combined together, increase the risk of death. The idea of amplifying weak-effect measurement was demonstrated by Yashin and colleagues, who found that in a study of joint influences, small-effects genes influenced longevity. They found that significant influence such taking individually small-effects is substantial and significant—genetic variation can be described by a relatively simple "genetic dose—phenotypic response" relationship (Yashin et al. 2010). The other important development of the FI started by Howlett et al. in introducing the FI from laboratory tests—the area of increasing interest of researchers (Howlett et al. 2014; Blodgett et al. 2017).

During the last few years, the FI methodology has become a widely used technique to study aging in mice (Whitehead et al. 2014; Kane et al. 2017; Antoch et al. 2017; Justice et al. 2019). An FI composed from routinely available blood tests produced exactly the same properties (Whitehead et al. 2014) as the FI previously reported in epidemiological and clinical investigations. Moreover, the mouse model demonstrated that the FI applied to animal models not only shares the same properties as was established in humans, but also scales in the same way with respect to the lifespan of animals and humans. The estimation of similar rates of deficit accumulation across the life course encourages the use of this approach in translation (Whitehead et al. 2014; Rockwood et al. 2017; Feridooni et al. 2017; Kane et al. 2018, 2019).

23.3.9 The Components of the FI Are Equally Weighted

Note that this approach to health deficits is silent on the nature of the deficit (e.g. disease, disability, symptoms, sign, laboratory or imaging or electrodiagnostic abnor-

mality). Interestingly, this has proven to be surprisingly controversial, especially for clinicians. Clinical training traditionally emphasizes diagnostic parsimony. A single cause for a large number of abnormalities is more likely to be correct than an explanation that invokes multiple causes. (It is certainly more psychologically satisfying to the person who recognizes it). Clinical training also emphasizes diagnostic precision; what works for condition A might be harmful for similarly looking condition B. (Confusion in a diabetic is a trivial example: giving insulin will help the patient whose confusion is due to blood sugar being too high, but be harmful to the diabetic in whom confusion reflects blood sugar being too low.) The discipline of precision and parsimony is not readily overcome, for good reason. Even so, it is less well suited to the reality of the nature of aging, especially as the number of deficits increases. Attempts to 'disentangle disability and comorbidity from frailty' are rooted in this approach.

A second reason that the nature of the deficit less important than the number of deficits has proven controversial is that it seems counter-intuitive. How can a skin problem and a heart attack be equivalent, in the sense that each is simply being a single deficit? But the truth is that they can be. Not every heart attack is fatal. Not every rash is benign. To the extent that they impair function or induce a spiral of other diagnoses, they will add to the deficit count, and in that way the frailty index will capture their unequal nature in relation to prognosis.

Considering deficits in relation to age and not just diseases—i.e. adopting a systems perspective—also appear to have implications for understanding the epidemiology of late life illness. An interesting report in this regard was published in Neurology in 2011, in the same issue as two other papers that reported 'novel risk factors for Alzheimer's disease'. Instead, the third paper (Song et al. 2011) combined 19 so-called "non-traditional dementia risk factors" in an index variable. The index variable (composed of items such as a history of diarrhea, dentures or foot problems) was a stronger risk for predicting all causes of late-life cognitive decline than was any traditional risk factor. The perspective offered here is that deficit accumulation reflects impaired recovery time, and that age-related recovery processes in the brain will not be unrelated to those in other parts of the body. It gives some broad insight into why deficits are so powerful, even if they are not known to be specific risk factors for the disease in question. Recent work by our group has replicated this observation in another cohort, and for both heart disease and osteoporosis (Kennedy et al. 2014; Wallace et al. 2014). We have also shown that non-traditional risk factors not only increase the risk of dementia, but mediate the relationship between neuropathology (Alzheimer disease) and its clinical expression as Alzheimer dementia (Wallace et al. 2019).

23.3.10 Indices of "Biological Age" (BA)

Another increasingly popular approach to assessing health in individuals is based on the indices of biological age (Hochschild 1994; Levine 2013; Kimura et al. 2012).

Such indices have also been calculated using DNA methylation data (DNAMeth Age) age measures (Horvath 2013; Hannum et al. 2013), or similar indices obtained from protein profiling (Enroth et al. 2015) and locomotor activity (Pyrkov et al. 2018). We can also mention an index of BA suggested a while ago by our group that is based on the FI (Mitnitski et al. 2002; Mitnitski and Rockwood 2014). Indices of biological age were known for at least two decades but recently they were applied to the epidemiological data showing reasonable performance (Levine 2013; Belsky et al. 2015). A few year ago, an index of biological age was obtained from using a deep learning neural networks approach to biomarkers (Mamoshina et al. 2018; Putin et al. 2016). DNA methylation age, suggested by Horvath, and now further developed by calibrating not only by CA but also morbidity and mortality to assess *phenotypic age* (Liu et al. 2018), which represents a major achievement in assessing individual biological age. Or course, each of these means of assessing health status during aging are the measures of heterogeneity of people's health at the same age, so that the term "biological age" betokens such heterogeneity (Mitnitski et al. 2017a). Head-to-head comparisons have been performed for some of these measures, revealing that their performances were roughly comparable. DNA methylation age is usually so highly correlated with chronological age that is not clear how much uncertainly its addition explains (see the "biomarkers paradox" below). Understanding the heterogeneity in health of older adults is a compelling question in the biology of aging (Mitnitski et al. 2017a). In most cases, indices of BA are calculated using regression models, in which the dependent variable was chronological age and independent predictors were biomarkers. Machine learning has also been applied in order to calculation index biological age from the list of biomarkers. The performance of such indices was assessed by comparing the prediction error [e.g., the difference between the individual's chronological age and estimated "biological age", called delta age or age acceleration (Horvath 2013)]. Such error being relatively small (several years) and it was argued that it supports algorithms of biological age estimation. During recent years the different algorithms based on the machine learning techniques were applied to large samples of biomarkers. One problem with using multiple biomarkers to estimate BA is that different measures of BA give different estimates, and that these estimates are rather weakly correlated (Belsky et al. 2018). In contrast to the relatively small number of biomarkers used to calculate indices of BA, DNA methylation age has been calculated using many items (several hundred!) (Horvath 2013; Liu et al. 2018) with a procedure for variable selection (elastic nets) that also can be related to the field of machine learning rather than "classical regression", although the boundaries between the field are not so clear. In any event, until now, all these means remain rather empirical tools, and the problem of understanding their origins needs further investigation.

23.3.11 The Biomarkers Paradox and the Klemera-Doubal Algorithm of Estimating BA from Chronological Age

Chronological age (CA) is an essential factor for predicting health outcomes (e.g. mortality) at a population level. It is often used in different clinical scales from cardiology to dementia. In the biomarker selection strategy, the correlation of a candidate biomarker with CA is a primary criterion for its selection. Blindly following such logic we can get to the so-called "paradox of biomarkers" which implies that such a "perfect" biomarker that can be replaced by CA should be insensitive to differences in individuals. The paradox emerges from the fact that such a biomarker is not informative in assessing health (and therefore BA that is health related) in individuals although would be useful in for example forensic studies. An attempt to incorporate CA in BA prediction algorithm was presented by Klemera and Doubal (the KD algorithm) in their interesting paper in 2006 (Klemera and Doubal 2006) and gained some popularity (Levine 2013; Belsky et al. 2015, 2018) despite its detailed criticism (Mitnitski et al. 2017a). The origin of the "effectiveness" of the KD algorithm was demonstrated and discussed by Mitnitski et al. (2017a). An attractive feature of the KD algorithm is that the estimate of BA looks more "realistic"—otherwise due to the generally weak correlation between the biomarkers age and the result of the estimation of BA shows unrealistic estimated for some individuals (baby age for some with "normal" biomarkers values and Methuselah-like estimate for those with high deviation of the biomarkers values from the norm). It is also clear that adding CA to the battery of biomarkers drags down the estimates of BA closer to CA preventing the estimates of BA being too high (e.g., >120 years). Whereas this might be considered by some as an advantage of the KD algorithm this happens, however, at the expense of its clarity.

23.3.12 Statistical Distance (SD) as a Measure of Physiological Dysregulation

While the FI is based on the dichotomized biomarkers defined as *deficits* (that requires often judgement to make such dichotomization) the *dysregulation metrics* (scores) approach proposed by AA Cohen and colleagues suggested to incorporate biomarkers in their natural scales (Cohen et al. 2014). The sum of square differences of the values of the biomarker from its referent values was defined as a measure of physiological dysregulation. Such measure is known in statistics as multivariate (or Mahalanobis) distance and is considered as such a measure of dysregulation because its increase might reflect pathological changes in the functioning of the organism. The dysregulation score was found to be a promising measure of robustness and resilience in aging studies and a new indicator of preclinical disease (Arbeev et al. 2018). We have found no face-to-face comparison of the dysregulation score with the FI but would make two points. Both measures calculate the difference between the current state

of the individual and the reference state in multidimensional n-dimensional space defined by the biomarkers values. Mathematically, both the FI and SD calculate the distance in multidimensional space between the reference and the current states. The difference is that the SD calculates the Cartesian metrics (distance) whereas the FI is related to the so-called Manhattan distance. A small FI should generally correspond to the small SD although the large deviation from the norm in even one component of SD may be attenuated in the FI. Both measures are topologically equivalent. Even so, counting deficits seems much easier than calculating the sum of squares of the differences for the set of biomarkers. One problem common to both the FI and SD is the uncertainty with the reference values of biomarkers. In many cases, such values are unknown and also could be population specific—that is not yet an investigated area. The challenge of the reference values for biomarker remains in both methodologies. More intensive investigation should shed light on the problem in the future.

23.3.13 Frailty Index and Recovery Time

Quantification of the aging process in terms of the number of deficits accumulated by individuals when they age allows us to apply more advanced mathematical models than those commonly employed in current aging studies. One such model is based on the analogy between the process of stochastic dynamic of changes in the number of deficits (FI) and the changes of the length of queue (so-called "queuing theory") (Mitnitski and Rockwood 2015). In that way, the number of accumulated deficits (the length of the queue) can be considered as the product of the intensity of environmental stresses (arrival at the queue) by the average net recovery (processing) time. This approach explains not only the fact of possible decrease in the number of deficits at a shorter time scale but also explains the patterns of the increase of the FI over a longer period, which will be proportional to the recovery time (Mamoshina et al. 2018). The three fold increase of the FI from 20 to 90 years can be seen as the result of the similar increase of the recovery time (Mitnitski and Rockwood 2015). The increase of the average net recovery time is an essential empirical characteristic of aging—it is well known that the recovery from many conditions (even those not considered as age-related, like influenza) takes much longer for older people than for younger adults. The average net recovery time can be assessable in individuals and as such presents an opportunity for studying the factors that could speed it up (Mitnitski and Rockwood 2015).

23.3.14 The Complex Networks Model of Aging and Mortality. Why Do We Age?

The reason that the FI works so well in many settings lies in the fact that deficits used for its operationalization are inter-connected: when a sufficient number of such deficits is considered, together they capture a systemic effect on health. It is their interconnectedness which explains why "the problems of old age come as a package" (Fontana et al. 2014).

We developed a computational model in which possible health attributes are represented by the nodes of a complex network that allows us to explore the mechanistic relationships between aging, frailty and mortality (Taneja et al. 2016; Farrell et al. 2016, 2018; Mitnitski et al. 2017b; Rutenberg et al. 2017). Nodes of the network correspond to generic health attributes, and are not explicitly identified. Each node can be either damaged (thereby representing a deficit) or undamaged. Damage of connected nodes facilitates local damage and makes local recovery more difficult (Taneja et al. 2016; Mitnitski et al. 2017b). The connections between nodes represent significant correlative relationships between health attributes, that can be causal or not. A relatively small number of nodes ("hubs") are well connected whereas most nodes are not. Such asymmetry is represented by a scale-free distribution of the number of connections (the connectivity degree) for each node. The two most connected nodes are mortality nodes; the next most connected nodes which are not mortality nodes are frailty nodes (Taneja et al. 2016). Frailty nodes broadly correspond to clinically or biologically significant health characteristics. Most nodes have few connections. During simulations, nodes are damaged randomly reflecting environmental influences intrinsic features and their interaction—such as in inflammation. Even so, the rate of damage of an individual node increases as more of its connected neighbors are damaged (Liu et al. 2018; Mitnitski et al. 2017a). An example of the results obtained using this model is shown in Fig. 23.1 for three model individuals at age 60 years. The individual on the right panel (c) has greater damage than do the two others (a, b). The first one (a) has the longest lifespan and healthspan corresponding to the lowest degree of damage.

The model explains not only the known patterns of not only mortality (the celebrated Gompertz law) but also how health status (indicated by the FI) gives rise to increasing vulnerability of people when they age (Mitnitski et al. 2017b). An important feature of the model is that its parameters do not depend on time. In other words, we did not impose any age-dependent changes in the model; even so the age-related patterns of damage propagation however emerge. This speaks strongly against the idea of an aging program. One can say that aging is initiated by the environmental stresses that occur locally in many parts and late propagate due to interdependence of multiple health attributes and thus becomes a global phenomenon including entire organism.

The complex network model allows us to characterize the effects both global and local damage to individual networks. Given that highly connected nodes are the major contributor to the risk of death, our model allows us to study how local damage to

these hub nodes changes the rates of deficit accumulation and patterns of mortality. This will make it possible to investigate how interventions to repair individual nodes might postpone damage propagation. By comparing the longitudinal behavior of the model with clinical data, we will be able to assess the signatures of successful clinical intervention in people with complex needs are the major contributor to the risk are the major contributor to the risk of death, our model allows us to study how local damage to these hub nodes changes the rates of deficit accumulation and patterns of mortality.

Our network model of interconnected nodes offers solid theoretical support for the FI methodology. It shows how variability in deficit accumulation gives rise to variability in the risk of death for people of the same age, which is the basis of frailty in both its statistical and clinical senses. With the model, however, we are now able to ask then in a way that allows explicit quantitative approaches to an area still often in thrall to semantics.

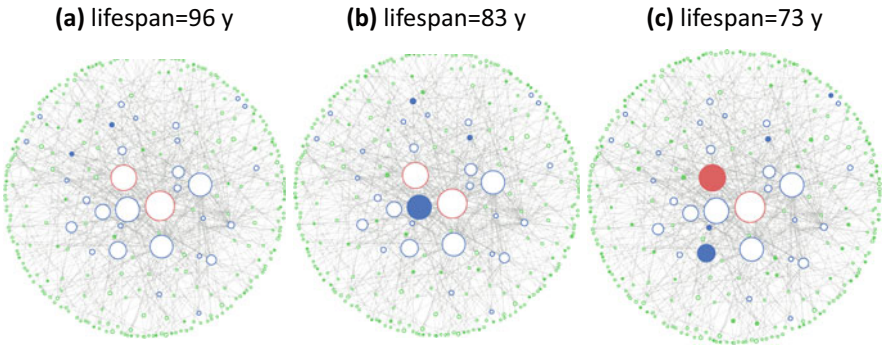

Fig. 23.1 Connectivity networks of the model individuals with different damage level at age 60. Nodes represent potential health attributes (deficits) that can be damaged (filled) or undamaged (empty). There are three types of nodes: mortality (red), frailty (blue) and the others (green). Individuals die when both mortality nodes (red circles, being the two most connected nodes) are damaged. Also shown are 30 frailty nodes (blue circles) and 268 other nodes (green circles). Circle size is proportional to node connectivity. **a** The lifespan of this individual was 96 y.o. At year 60 the low damage was obvious. 10 frailty nodes and 39 other nodes were damaged. This individual at age of 60 y.o. had the lowest damage of those three. **b** This individual died at age 83. At age 60 neither mortality node was damaged, 12 frailty nodes and 55 other nodes were damaged. **c** This individual died at age 75 y.o. At age 60 one mortality node was damaged, 14 frailty nodes (of 30), and 73 (of 268) other nodes were damaged. Of note, the damage of non frailty nodes correlated with the damage of frailty nodes, and with the lifespan indicating the importance of subclinical damage (that is how non frailty nods can be interpreted). We thank Spencer Farrell for providing the simulations used to plot the figures

23.3.15 Conclusion

Biological and clinical markers lie in the basis of the assessment of health of individuals. Such markers can be used to build aging sensitive metrics. Moreover, the markers can form the basis of assessing of the process of aging itself. The problem of integrating a large amount of biomarkers is being addressed by using the indices of frailty, biological age and dysregulation. Of these means, the FI is most robust, reliable and simple. It can be created from any set of health indicators including biological and clinical markers of aging and health, used in the electronic records and allows monitoring of health and its changes over time. The FI is not only a useful health utility measure but importantly has a strong theoretical support in the complex dynamical network theory of aging that is currently under development. The theory explains why the interdependence of variables (representing the attributes of health) is essential for understanding of the basic properties both of the FI and the aging process. Further progress in the filed will go hand-by-hand with the development of new technologies that allow more data to be collected and interpreted. Any future interventions to modify the aging process will be monitored using the approach considered here as a means of evaluating such interventions.

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