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Phenome and genome based studies into human ageing and longevity: An overview☆

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ABSTRACT

Human ageing is an extremely personal process leading across the life course of individuals to large population heterogeneity in the decline of functional capacity, health and lifespan. The extremes of this process are witnessed by the healthy vital 100-year-olds on one end and the 60-year-olds suffering from multiple morbid conditions on the other end of the spectrum. Molecular studies into the basis of this heterogeneity have focused on a range of endpoints and methodological approaches. The phenotype definitions most prominently investigated in these studies are either lifespan-related or biomarker based indices of the biological ageing rate of individuals and their tissues. Unlike for many complex, age-related diseases, consensus on the ultimate set of multi-biomarker ageing or lifespan-related phenotypes for genetic and genomic studies has not been reached yet. Comparable to animal models, hallmarks of age-related disease risk, healthy ageing and longevity include immune and metabolic pathways. Potentially novel genomic regions and pathways have been identified among many (*epi*)genomic studies into chronological age and studies into human lifespan regulation, with *APOE* and *FOXO3A* representing yet the most robust loci. Functional analysis of a handful of genes in cell-based and animal models is ongoing. The way forward in human ageing and longevity studies seems through improvements in the interpretation of the biology of the genome, in application of computational and systems biology, integration with animal models and by harmonization of repeated phenotypic and omics measures in longitudinal and intervention studies. This article is part of a Special Issue entitled: Model Systems of Aging - edited by "Houtkooper Riekelt".

1. Phenotypes and endpoints of human ageing

Molecular, cellular and anatomical changes occur with advancing age at every level of human physiology. Often it is not all that clear which of such changes can be a phenotypic starting point for studies into their genetic and molecular determinants. Consequently, the question is to what extent physiological changes represent early marks of a decline of function in a continuum towards disease. For example, there is still a lot of debate with respect to what extent plaques and tangles in the ageing brain represent early phases of dementia in old age or another functional entity. It is also not that clear whether similar characteristics of early and late life onset disease share causal factors. The pathways identified in Mendelian disorders, such as early onset osteoarthritis and dementia, expressing ageing like phenotypes, are not necessarily key to the gradual degenerative changes and functional decline that increases the morbidity and mortality risk of ageing populations. In essence, phenotypes along the complete life course matter in ageing research. Early life factors, such as birth weight, childhood body composition and skeletal growth associate to late life complex health traits, such as cardiovascular disease (CVD) and osteoarthritis [1,2]. Mid-life factors, such as low levels of physical capability, also associate with late life conditions, such as higher rates of mortality [3]. Molecular observations accompany these studies at the phenotypic level. Persisting epigenetic effects in the genome assessed in mid-life, for example, accompany early prenatal adverse exposures such as during the Dutch Hunger winter [4], resulting in late life consequences. Hence, human studies into the molecular basis of ageing have focused on a range of age-related phenotypes and endpoints along the complete human life course [5,6]. Such endpoints include the loss of functional capacity as measured by repeated assessment of, for example, verbal memory for cognitive capacity, grip strength, gait speed, chair stand and balance tests for neuromuscular capacity, lung function (VO2 max), heart rate as read out of organ specific functioning and estimates of well-being based on questionnaires into activities of daily living and

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social environment. In addition, disease-related traits that change with age are used as endpoints (e.g. blood pressure, serum cholesterol, insulin resistance, bone mineral density). Epidemiological and clinical studies investigate diagnoses of age-related disease (e.g. dementia-, diabetes-, osteoporosis and cerebrovascular events) and disease-specific biomarkers as endpoints. In addition to the ageing-related phenotypes, lifespan-related phenotypes are studied as endpoints such as all cause, specific death cause, or event free mortality [7]. Finally, following the advanced field of animal studies into lifespan extension longevity was explored in humans using survival beyond a certain upper limit (longevity) as endpoint.

2. Biomarkers

Ageing is a highly personal process only partially driven by genetic determinants. Even inbred animals under controlled nutritional regimes differ largely in their physiological decline and age at death. A lot of effort in human ageing research is therefore focused on identifying biomarkers of the pace of the individual ageing process (biological as opposed to chronological age [8]). Such markers would theoretically predict relevant clinical endpoints for the individual and would allow to monitor the lowering of the pace of ageing and the improvement of health as the result of interventions (for example following medical and lifestyle interventions). The quantitative biomarkers of the ageing process include serum proteins and metabolites, genetic markers (e.g. accumulation of somatic mutations, telomere length changes), epigenetic markers (e.g. changes in DNA methylation, messenger- and micro-RNAs), and markers of physiological performance (e.g. cognition and handgrip strength tests). Single molecular biomarkers of clinical endpoints are for example serum glucose, free triiodothyronine (fT3) levels, CDKN2A (p16) gene expression, and leukocyte telomere length (LTL). There is especially an extensive literature on LTL associations with a range of health outcomes including mortality [[9], for an overview of the largest meta-analyses]. Other markers are focused on specific tissues, isolated for disease oriented studies, such as urine markers of bone turnover (uCTX-I) and degradation of cartilage (uCTX-I), markers used in osteoporosis and osteoarthritis research.

Potentially more reliable than single-marker indicators for the individual biological age are those constructed by multi-biomarker algorithms. Examples are the original Frailty Index [10], followed by novel versions such as the Frailty Index 34 [11], the 10-biomarker US National Health and Nutrition Survey (NHANES)-based measure of “Biological Age” [12] and the multi-biomarker for ageing in younger adults (< 40 years) based on physiological deterioration across multiple organ systems (e.g., pulmonary, periodontal, cardiovascular, renal, hepatic, and immune function [6]. These multi-factorial markers are considered biological age indices outperforming chronological age in predicting mortality over variable follow-up times or the decline of functional capacity. Here we will discuss novel developments based on omics biomarkers, assays representing variation in the overall genome, epigenome, transcriptome or metabolome.

3. Omics biomarkers

In the past few years the omics technologies (transcriptomic, epigenomic, metabolomic and proteomic measures) have fueled the search for biological age indices. Candidate gene expression and methylation studies in smaller cohorts were followed by genome-wide omics analyses in large cohort studies, mainly using peripheral blood samples collected at a single time point. Age-associated changes in the transcriptome landscape [13,14] or DNA methylome landscape [15,16] were found to associate with health parameters. Some of the genes identified in the blood transcriptome were followed up successfully in animal models leading to a focus on the kynurenine pathway [17], for example. Here we will mainly discuss the developments concerning the epigenetic and metabolomic changes, both hallmarks of ageing across species [18].

3.1. Epigenetic markers

Epigenetic changes, reflecting gene expression regulation by DNA methylation, chromatin modification and non-coding RNA's, occur in the nuclear and mitochondrial genome with age [[19], for a review]. Genome-wide DNA methylation studies of peripheral blood revealed global hypo and gene-specific hyper methylation changes with age that generally associate poorly to expression of the corresponding genes [20]. Nevertheless, DNA methylation at many genomic loci closely tracks chronological age, which lead to the construction of biological age predictors based on cross sectional DNA methylation-age relations. In one of these studies, the DNA methylation age (DNAmAge) of biological samples from a diversity of tissues was calculated using the weighted average of DNA methylation levels at 353 CpG sites [16]. From this, a DNAmAge acceleration can be calculated per individual, by comparing the DNAmAge with the chronological age. Independent of age and classic risk factors this DNAmAge acceleration in blood was found to be predictive of many health conditions as well as mortality [21]. The marker associated to cognitive functioning and in brain tissues to neuronal ageing rate and Alzheimer's disease and was used as phenotype for genetic studies (see below for a description of these findings) [22,23]. A comparison of this to other molecular predictors such as transcriptome age [14], and LTL, indicated that DNAmAge was the best molecular predictor of health parameters and mortality thus far [[9], for a review]. The omics studies in ageing, did not yet measure the decline with age at multiple time points across the life course along with repeated measures of functional decline, although samples and measures are available in long-standing cohort studies.

The candidate or genome-wide DNA methylation studies do not provide a direct entry for mechanistic explorations, although epigenetic changes with age potentially represent stochastic and environmental influences on gene functions. DNA methylation changes at the clock loci or other loci at which methylation changes almost linearly with age (e.g. *ELOVL2*, [24]) are not understood in mechanistic terms although immune/inflammatory or apoptotic processes have been speculated to underlie these changes. Large-scale characterization of epigenetic divergence with age, however, revealed CpG loci especially at polycomb repressed regions in the human methylome to be associated with gene expression changes in trans with a key role in DNA repair, apoptosis and metabolic pathways [25].

3.2. Metabolomic markers

Another type of omics analysis is that of hundreds of related and unrelated metabolites in the human metabolome as biomarker of ageing and disease. Metabolomics profiles were demonstrated to predict various diseases such as cardiovascular disease [26,27] and type 2 diabetes. The mass spectrometry–based blood metabolome was shown to associate with familial longevity [28,29], while the urine metabolome was used to generate a biological age predictor [30]. In addition, by stepwise regression analysis, a profile of 4 out of 106 tested ^{1}H NMR-based metabolites was extracted that predicted mortality [31]. The metabolome has great potential as biomarker source also because of the biochemical interpretation (especially in the targeted analysis approach) of the relation to health parameters.

3.3. Genetic markers of ageing and longevity: somatic mutations

In the 1960s, it was suggested that aging is the outcome of accelerated accumulation of somatic DNA mutations [32] and hence accumulation of errors in the primary structure of proteins [33] with catastrophic effects for key functions of somatic maintenance. The whole genome level of detectable somatic variants that may occur in a lifetime was investigated in blood and other tissues in multiple middle aged cohorts, patient studies, and among the oldest old. The accumulation of somatic mutations differs per tissue, since environmental

exposure and self-renewal rate is different per tissue.

Ye et al. [34], for example, investigated monozygotic twin pairs of 40 and 100 years and detected just a handful of discordant somatic single base substitutions in the centenarians using a range of whole genome and follow up sequencing platforms, concluding that a century of life did not result in a large number of detectable somatic mutations in blood. Whole genome sequences of DNA from several tissues of a 115-yr-old Dutch woman were compared to the sequence of her brain [35]. The authors estimated that the blood tissue during 115-year lifespan accumulated 450 somatic mutations, but these did not seem to have shortened the life of this centenarian.

Whole genome DNA sequencing data of 218 subjects in the Leiden Longevity Study (LLS) at a mean age of 94 years (88–103) and 646 subjects from the Rotterdam study at a mean age of 84 years (80–105), revealed somatic mutations in genes previously linked to hematopoietic malignancies, especially in *DNMT3A* and *TET2* [36]. Somatic mutations in these genes were previously linked to clonal expansion of hematopoietic stem cells. Earlier studies in large scale sequencing data actually indicated a steep age associated increase in the mutation frequencies of these genes from middle age onwards (Fig. 1) [37–40]. Prospective analyses of the middle-aged showed an increased risk for all-cause mortality for carriers of such mutations as compared with noncarriers, suggesting a rapidly increasing vulnerability among the elderly for adverse health effects of clonally expanding hematopoietic stem cells [39,40]. In contrast, however, the 8- to 10-year survival of the nona- and centenarian carriers did not show any indication of compromised survival [36]. The somatic mutations in *DNMT3A* and *TET2* are quantitative genetic markers of the ageing process and clonal expansion. Their predictive capacity or contribution to health effects in elderly is still under investigation.

In addition to the observational studies described, markers of the biological ageing rate (especially the metabolome, epigenome and proteome) are also being investigated in experimental study designs such as intervention studies to improve health and slow down ageing by lifestyle changes (calorie restriction and physical activity) or medical interventions. The metabolome, for example, was shown to monitor the beneficial response of elderly to a mild lifestyle intervention [41]. Blood leucocyte DNA methylation profiles marked obese individuals undergoing behavioral weight loss interventions [42]. A recent study in mice showed that epigenetic signatures of the liver DNA methylome resembling the DNAmAge clock in humans, marked longevity-promoting interventions (calorie restriction and dietary rapamycin administration) [43].

Despite the ongoing biomarker studies, to date there is no consensus on a gold standard biomarker set indicating the individual biological ageing rate. Comparisons of previous and novel predictors are being performed such as a comparison of the frailty index and the DNAmAge [44], but a systematic study of all molecular and functional markers in large cohorts, has not yet been performed. The biomedical community is eager to find and agree upon biomarker profiles of the biological age to classify subjects and patients rather than remaining dependent on calendar age. Medication is usually developed and tested in younger patients because of the heterogeneity among the elderly, even though these represent most of the users. This heterogeneity, likely due to a loss of homeostasis, obscures the effect of interventions and treatments.

It also raises the question as to the molecular basis for the heterogeneity in the ageing rate of human individuals and variation in lifespan. As in animal models, many studies have focused on finding the genetic basis of human lifespan regulation, expecting that the genes discovered determine the rate of ageing. In animal models, a range of hallmarks of the ageing process was discovered [18], as reviewed by other papers in this special issue. For example, in a variety of species the most prominent key to longevity seemed to be the capacity to maintain an effective proteome over time [45,46]. The molecular basis

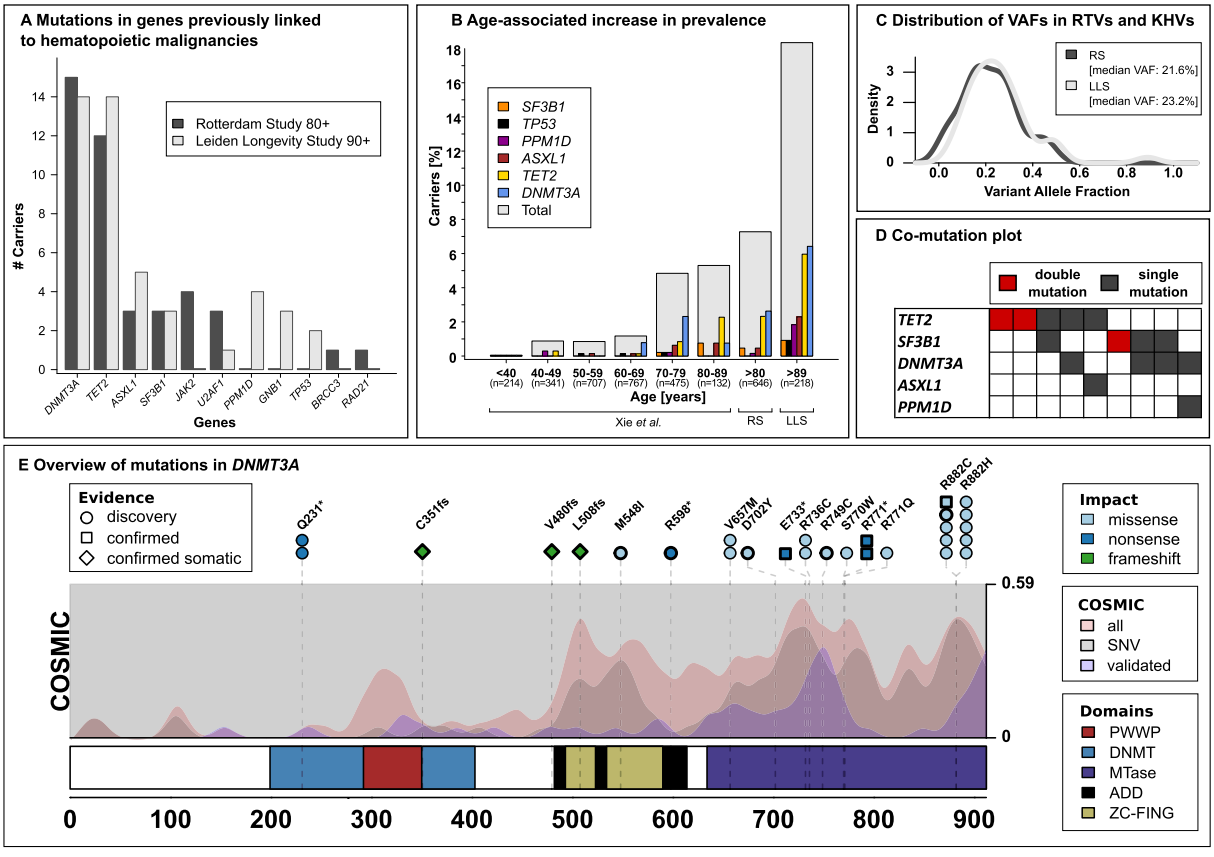

Fig. 1. Age-related increase in the frequency of somatic mutations in blood cancer related genes [36,38] detected by Next Gen Sequencing in subjects of different age groups.

for human longevity has not been revealed to such extend, however. We will now discuss the genetic component in human longevity and the phenotypic definitions and approaches used in studies to identify genetic determinants.

4. Lifespan and heritability

The human lifespan is influenced by environmental factors to quite an extreme level. During the past 200 years, life expectancy for women in Western societies increased from 45 years in 1840 to 85 years in 2015 [47]. In European countries such as the Netherlands, the oldest old started to show a pattern of extended life expectancy in the second half of the 20th century and the number of those over 65 will double before 2050 while those older than 85 will triple. Also, globally the life expectancy has increased. A child born in Brazil or Burma (Myanmar) today can expect to live 20 years longer than one born only 50 years ago [48].

Some of the elderly become extremely long-lived and show little or no signs of age-related disease [49]. A study of the health of super centenarians (aged 110–119), semi-super centenarians (aged 105–109), centenarians (aged 100–104), nonagenarians, and younger controls reported that the older the age group, the greater the delay in onset of major disease [50]. On average, first degree relatives of the long-lived also had an extremely long and healthy life as compared to relatives of average-aged persons [51].

Heritability estimates of the influence of genetic and environmental factors on lifespan are most robust in twin studies and further depend on the environmental context of the population and gender [[52], for a review]. Twin studies into lifespan determination showed that genetic influences account for maximally 27% of lifespan variation in current populations [53,54]. This may not be surprising given the fact that the increase in life expectancy of the last two centuries is mainly explained by improved nutrition, hygiene and medical care rather than genetic changes. The heritability of lifespan increases at higher ages [54]. Genealogical studies in pedigrees further revealed that lifespan tends to be predominantly transmitted from mothers to offspring with some preference for transmission to daughters [55], for a review]. Insights into the impact of the environment on longevity comes from the study of regions with a high prevalence of centenarians. In these historically and/or geographically isolated regions labelled ‘Blue Zones’, such as Okinawa and Sardinia, especially the nurturing factors of traditional lifestyle of high vegetable intake and physical activity, low stress, strong familial and community bonding and support for the elderly appears to contribute to the capacity to live to extreme ages [56].

Since the heritability estimates indicates the upper limit for the genetic component to be explained by genetic studies, the low heritability of lifespan even at higher ages is a problem. In the population at large, sporadic highly aged subjects as cases for genetic studies harbor large numbers of phenocopies [57]. Therefore genetic and molecular studies have focused on the clustering of survival to the highest ages (longevity) in families and partly revealed hallmarks of healthy ageing as previously found in animal models.

5. Phenotypic and molecular studies in longevous families

Clustering of longevity in families has been observed with the largest effects for the top 5% or less survivors in the population [51,58], suggesting selection of cases above this threshold to be best for large scale genetic studies [59]. Heritability of living to at least 100 years in longevous families was estimated at 0.33 in women and 0.48 in men [60]. Parents belonging to the top 1% survivors of their birth cohort have a recurrence risk of 2.3 to have children who also belong to the oldest 1% of their birth cohorts [58]. In addition to centenarian singletons, nonagenarian sib-pairs and their first degree relatives were found to live significantly longer than members of their birth cohorts [61] and show decreased morbidity in middle age [62,63].

Phenotypic and molecular studies explored which mechanisms could contribute to familial longevity. Molecular studies of centenarians revealed the relative healthy or younger profiles of the oldest old in essentially all the above discussed omics profiles also including the metagenomic [64] and inflammatory [65] profiles. Long living families express favorable immune-metabolic health profiles that seem opposite to metabolic syndrome [66,67]. This was extensively demonstrated by investigating the offspring of nonagenarian sibling pairs (Leiden Longevity Study) and centenarians (Ashkenazi Jewish centenarians from the Longenity Project) in comparison to controls of similar age and body composition. The beneficial clinical hallmarks included low glucose, better maintenance of insulin sensitivity [68]; low free T3 [69] and TSH, large LDL and HDL lipoprotein particle size [70,71] and high adiponectin [72]. Members of longevous families show improved immune responses [73,74], glycosylation [75] and diminished immunosenescence as compared to controls [76]. Passtoors et al., [77] reported on the altered expression of genes in the mTOR pathway in longevity families, comparable to observations in lifespan extended animal models. In line with this pathway, the activation-induced autophagy in the CD4 + T-cell compartment was found to be significantly better maintained, which along with improved T-cell functional parameters, supports the notion that proteostatic and regulatory processes confer T-cells increased fitness in longevity families [78].

6. Genetic determinants of health span, lifespan and longevity

Various approaches were applied to identify lifespan regulating loci and loci involved in age-related traits and diseases. Common variants were tested originally in pioneering candidate gene studies, often based on insights from animal models (see other chapters in this special issue). Later hypothesis-free approaches were applied to find the most relevant genes in human lifespan regulation in linkage studies of longevous families and long-living sibling pairs, as for example in the genetics of healthy ageing (GEHA) study, [[79,80] for a review] and meta-analyses of unrelated cases and controls in genome wide association studies (GWAS) [[81–83], for reviews]. The candidate gene studies demonstrated the *APOE* locus (an apolipoprotein and the principal cholesterol carrier in the brain) [84,85]; and the *FOXO3A* locus (a transcription factor of the forkhead family linked to cell cycle arrest, autophagy regulation and lifespan extension in animal models) [86,87] to be the most robust longevity loci. The APOE locus had been selected as candidate at the time for its known association with coronary artery disease and Alzheimer's disease. Currently both genes are known to be involved in a range of clinical endpoints in human studies.

The various linkage studies ([79], for the largest) did not identify loci overlapping between independent studies other than the *APOE* locus at which gender differences in the linkage signal were observed, showing stronger effects in women. The effects at this locus are dominated by two common missense variants, rs429358 (Cys130Arg) and rs7412 (Arg176Cys) determining functional APOE alleles: ε2 (Cys130, Cys176), ε3 (Cys130, Arg176) and ε4 (Arg130, Arg176). The linkage signal among over 900 nonagenarian sib ships was driven more prominently by enrichment of the ε2 allele than depletion of the ε4 allele.

In the GWAS approach, the frequencies of common variants (minor allele frequency $>$ 1%) are studied in relation to quantitative traits or in case control designs of long-lived or disease-affected cases and population controls. Disease-oriented GWAS, especially the large meta-analyses, mapped considerable numbers of disease susceptibility and quantitative trait loci (http://www.genome.gov/gwastudies). The effects at multiple genes were later turned into polygenic risk scores, which represent a score per individual calculated, based on variation in multiple genetic loci and their associated weights in the major GWAS for the trait involved. The two largest and most robust GWAS for longevity, both in cases ≥ 90 years [88,89] versus middle aged controls (in the EU GWAS study, [88]) and those that died between 55 and 80 years (in the Cohorts for Heart and Aging Research in Genomic

Epidemiology, CHARGE study [89]), respectively, have only identified three loci associating with longevity; *APOE,* FOXO3A and an intergenic locus on chromosome 5q33.3. Also in GWAS on lifespan related traits, the most robust locus identified in Caucasian and Asian populations was the *APOE* locus confirming the mortality and frailty risk increasing effects of the ε4 allele [90] and longevity promoting effects of the ε2 allele. Despite environmental and ethnic differences the *APOE* and 5q33.3 loci were also found in a GWAS of Han Chinese centenarians (Chinese Longitudinal Healthy Longevity Survey (CLHLS); [91]). The *FOXO3A* locus was originally found in Japanese men (Hawaii Lifespan Study, HLS; [86]) and was soon after confirmed in a candidate gene study in individuals of Caucasian ethnicity [87] and in the GWAS study of Broer et al., [89].

We will now discuss the results on potentially novel longevity and lifespan loci for which replication of results were shown in some independent cohorts. Possibly due to (definition and environmental) heterogeneity, no locus other than *APOE* was identified in every GWAS. All the below mentioned novel longevity loci (often labelled by the gene closest to the top SNP) need replication in human cohort studies or animal models to consider them robust findings.

The rare allele of the top SNP at 5q33.3 that associated with survival ≥ 90 years in Deelen et al. [88], associates also with decreased all-cause and CVD mortality and lower diastolic and systolic blood pressure [88]. This chromosomal region harbours a lncRNA and the closest gene *EBF1* (a transcription factor essential for lineage specification in early B cell development) is especially interesting, since genetic variation in this gene has been associated with parental age at death (Health and Retirement Study, HRS; [92]), and with adipogenesis and CVD in interaction with chronic psychological stress [93].

Other potential longevity loci identified were *RAD50/IL13*; IL6;ANKRD*20A9P*; SLC*22A4*; USP*42* and *TMTC2* [94,91 and 95]. The *RAD50/IL13/IL5* region on chromosome 5q31.1 (involved in DNA repair and inflammation) was found in an immuno-chip based comparison of in total about 3000 centenarians including German subjects of a mean age of 99 years and middle aged controls, and replicated in a French (mean age 102 years) and Danish cohorts (mean age 96 years). The SNP at IL-6, identified as a longevity locus in the Chinese GWAS, corresponded to longevity associations of genetic and serum protein level in previous candidate gene reports in Caucasians [96,97].

A GWAS in four studies (the New England Centenarian Study (NECS), the Long Life Family Study (LLFS), the Southern Italian Centenarian Study (SICS), and the Longenity Gene Project (LGP)) comparing about 2000 long-lived cases belonging to the 1% survivors of the 1900 U.S. birth cohort (≥ 96 years for men; ≥ 100 years for women) identified new rare variants on chromosomes 7 and 12 that were also associated with reduced risk for cardiovascular and Alzheimer's disease [95]. The variant on chromosome 7 is located in *USP42*, a possible transcription regulator, and is an eQTL for this gene in a variety of tissues. The more extreme phenotypes lead to relatively small numbers of cases for GWAS. They represent, however, the cases with the expected highest heritability for longevity. An even larger number of the 1% longest-lived survivors is currently being studied in a worldwide GWAS collaboration to circumvent the low power issue (personal communication).

Since parental lifespan is predictive of the health- and lifespan of offspring, recent GWAS studies into lifespan regulation focused on parental age at death. A GWAS in the UK Biobank (N = 75,000) reported significant GWAS signals with age- and gender-dependent effects for *APOE* and *CHRNA3/5* at 15q24, which was confirmed in three independent studies in the paper [98]but was not replicated in parental-age studies within the Framingham or Health and Retirement Studies [91]. The locus near *CHRNA3/5* differentially affected paternal (younger aged) lifespan. This *CHRNA3/5* locus encodes nicotinic acetyl choline receptor subunits, ion channels that mediate fast signal transduction at synapses and the identified variants were known to influence smoking-related phenotypes, lung cancer, chronic obstructive pulmonary disease, peripheral arterial disease, alcohol dependence and schizophrenia. Another observation in the UK Biobank study was that offspring of at least one parent passing the age of 1% survival in the population (≥ 98 years and ≥ 95 years, for women and men, respectively) had more protective alleles in polygenic risk scores for coronary artery disease, systolic blood pressure, body mass index, cholesterol and triglyceride levels and Alzheimer's disease [99].

The study of Tanaka et al., in the Health and Retirement Study [89], compared 51–61 year old subjects from African and European ancestry with at least one long-lived parent (mothers > 91 years, fathers > 87 years) and those without any long-lived parent. A genome-wide significant association for the *SMAD7* locus on chromosome 18 (a member of a family of TGF-β effector proteins that serves as an inhibitor of TGFβ signalling) was found, however the result was not replicated in two independent studies.

The first study investigating the prevalence of GWAS-identified risk alleles of disease endpoints and related traits among highly-aged subjects (95 years on average) were performed by Beekman et al. [100]. Making use of the same principle, a GWAS was based on the overlap between GWAS-identified loci from studies into longevity and those into disease endpoints and related traits [101]. Significant hits of this 'informed GWAS' (iGWAS) were replicated in independent studies, finding *CDKN2B/ANRIL* (implicated in the regulation of cellular senescence), *ABO* (tags the O blood group), and *SH2B3/ATXN2* (a signalling gene that extends lifespan in Drosophila and a gene involved in neurological disease) as potentially novel longevity loci. In the Wellderly study cohort, whole genome sequencing was performed in over 1300 cases of median age 84 years without chronic diseases or use of chronic disease medication and population controls [102]. GWAS for common or rare variants did not identify novel significant loci. Except for depletion of the frailty allele *APOE ε4* and a well-known coronary artery disease locus on chromosome 9, neither rare or common variants previously found in candidate longevity studies associated with the phenotype of this healthy ageing cohort, and neither did GWAS-identified common disease risk variants (as also observed in [100,103,101] nor rare pathogenic variants. In the Long Life Family Study (LLFS) a GWAS of a health endophenotype (multi-biomarker) construct, predominantly comprised of pulmonary and physical function domains, was performed and replicated in the Framingham study, revealing a signal on chromosome 1p13 near the *NBPF6* locus (a member of the neuroblastoma breakpoint family (NBPF) genes abundantly expressed in breast and liver tissues). A genome-wide significant association on chromosome 10p15 observed for the LLFS cohort was not replicated in an independent study [104].

Altogether, these findings indicate that genetic studies into phenotypes such as top percent survivors, (highest) parental age at death, healthy old, low biological age (based on lifespan-related endophenotypes and multi-biomarker indices), despite the replication reported in the original publications, usually provide non-overlapping loci as compared to other publications, further illustrating the complexity of the phenotypes related to biological ageing in humans. In addition to the genetic studies, interesting results that need replication have been obtained by molecular studies into ageing including those into variation of the mitochondrial genome, pathway analyses and micro-RNAs ([83], for a review).

In contrast to the lifespan-oriented studies, the location of numerous robust loci was identified in GWAS-analyses of age-related traits and disease endpoints. As yet, most disease-oriented GWAS-studies focus on single point disease or functional decline endpoints rather than on repeated measures of functional decline during the life course, which would provide additionally interesting loci for studies into ageing. Here we touch on developments in the neurobiology field as illustration of the progress being made by disease-oriented studies into genetic determinants and epigenetic markers. GWASs of dementias and cognitive functioning traits revealed 19 susceptibility loci for Late Onset Alzheimer's disease (LOAD, [105]) accounting for 47% of the

population attributable risk and 3 loci for general cognitive functioning [106]. A recent GWAS focused on characteristics of the ageing brain using the DNAmAge acceleration score and neuronal proportions of different brain regions in post mortem material as phenotypes. The GWAS of brain DNAmAge acceleration demonstrated among others, a locus on 17q11.2 across multiple brain regions of which subsequent transcriptome analysis revealed association of the top SNP with expression of the *EFCAB2* gene, involved in Ca2 + signalling, synaptogenesis, dendritic arborization and cell survival [22,23]. The gene sets identified by the GWAS of DNAmAge acceleration were significantly enriched for genes previously associated with cognitive decline, dementia and Alzheimer's disease.

7. Towards functional analysis of interesting genetic loci

Usually, the effect of GWAS-identified variants marking the relevant genomic position is small and the position is often inter-genic. Hence, functional genomic studies are required in humans and animal models, including re-sequencing carriers of identified (susceptibility) alleles, to answer which specific genetic variation at an identified position influences the age-related trait. Transcriptomic studies allowed prioritization of genes near genome-wide significant SNPs, as indicated for some studies discussed above. Open source human databases of omics-data measured in blood and tissues are vital for functional analyses. Resequencing candidate hits and in silico analysis of flanking regions will predict potentially functional variants based on amino acid changes, motifs for transcription factor binding, micro RNA binding, or splicing will help to further prioritize candidate variants for follow up analysis by integrated in vivo models (i.e. animal models and cell models, including iPSC) and in silico assays (see Fig. 2). The progress in this field depends, among other things, on open source data, collaborations harmonizing omics-data in cohorts, applying intervention studies and experimental challenges to cells and subjects, thus creating pipelines of source material that allow for computational and systems biology to be applied.

A nice illustration of functional analyses performed in longevity research is provided by studies into the insulin/IGF1 signalling pathway, involved in lifespan extension in animal models and explored as a candidate pathway in human studies [107,108]. Following sequencing analysis, two mutations at the insulin receptor locus *IGF1R* (Ala-37-Thr and Arg-407-His) enriched in Ashkenazi Jewish centenarians as compared to controls, were found to be associated with reduced activity of the IGF1 receptor in immortalized lymphocytes and attenuated IGF1 signalling in mouse embryonic fibroblasts (MEFs) in a mouse Igf1r null background [109]. The results indicated that MEFs expressing the human longevity-associated IGF1R mutations attenuated IGF1 signalling and dysfunctional physiological response to a cell proliferation signal. These results demonstrate that the human longevity-associated IGF1R variants are function-reducing mutations, implying that dampening of IGF1 signalling may be a longevity mechanism in humans. Mouse models will help in the functional analyses of genes mapped in human longevity studies, as demonstrated by Murabito et al. [82]. Loci found in longevity GWAS and transcriptome studies [17] of the CHARGE consortium were mapped to the mouse chromosomal map. Eight of the ten top human genetic associations were located within a previously reported mouse life-span IGF-1 quantitative trait locus.

8. Conclusions and future perspectives

Despite the efforts to identify human longevity loci in parallel with research in animal models, not many genetic loci can be functionally followed up in elaborate cell and animal model studies as yet, except for mutations detected in candidate genes. Still, the many suggestive GWAS-loci identified in studies of various lifespan-related phenotypic definitions or the loci identified in epigenome and trancriptome studies may represent overlapping novel pathways that may also overlap with interesting QTLs mapped in animal models of ageing. The functional interpretation of identified sets of intergenic regions associated to lifespan regulation requires more insights in genome and systems biology and approaches in computational biology. Burden analysis in whole genome sequencing studies to identify loci among the oldest old enriched for mutations, require increased power and selection of informative subjects from families in which longevity clusters, preferably across multiple generations.

Molecular and physiological age predictors, reflecting different aspects of ageing biology, may be used in genetic studies to map loci involved in biological ageing of tissues and systems. To agree on an ultimate set of biological age predictors for genetic studies, classification and risk prediction of metabolically vulnerable elderly and monitoring the response to interventions (lifestyle, medication, therapy), more consistent data collection is vital. Increasingly clinical studies into age-related organ failure adopt molecular insights in the biology of ageing by focusing on senescent cell markers, markers of metabolism, telomere length and epigenetic age changes to define molecular and phenotypic hallmarks of kidney, lung, heart and brain ageing. To dissect heterogeneity in the rate and nature of intrinsic physiological decline with ageing takes a consistent comparison of data on age predictors, harmonizing repeated molecular and phenotypic measures along the life course in longitudinal studies. If heterogeneity is based on variation in homeostatic (loss of) control, on immune and metabolic shifts, we should also measure biomarkers more dynamically (repeated measures of change over different time scales (short challenges and longer exposure periods) to be able to link molecular vulnerability or resilience to functional and clinical resilience. Recent developments in 'Physiomics' such as molecular imaging, e-tracking by wearables and biosensors, will fuel the dynamics of molecular ageing research (Fig. 3) and insights in the personal ageing process of the individual.

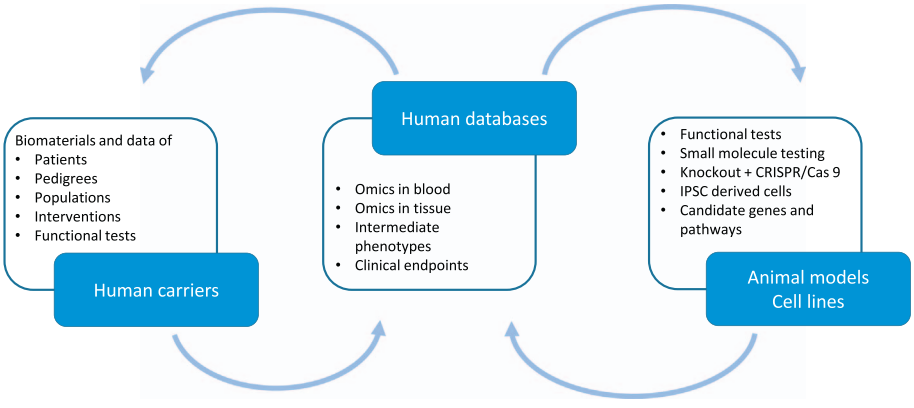

Fig. 2. Schematic presentation of cross-species pipeline of functional genomic studies.

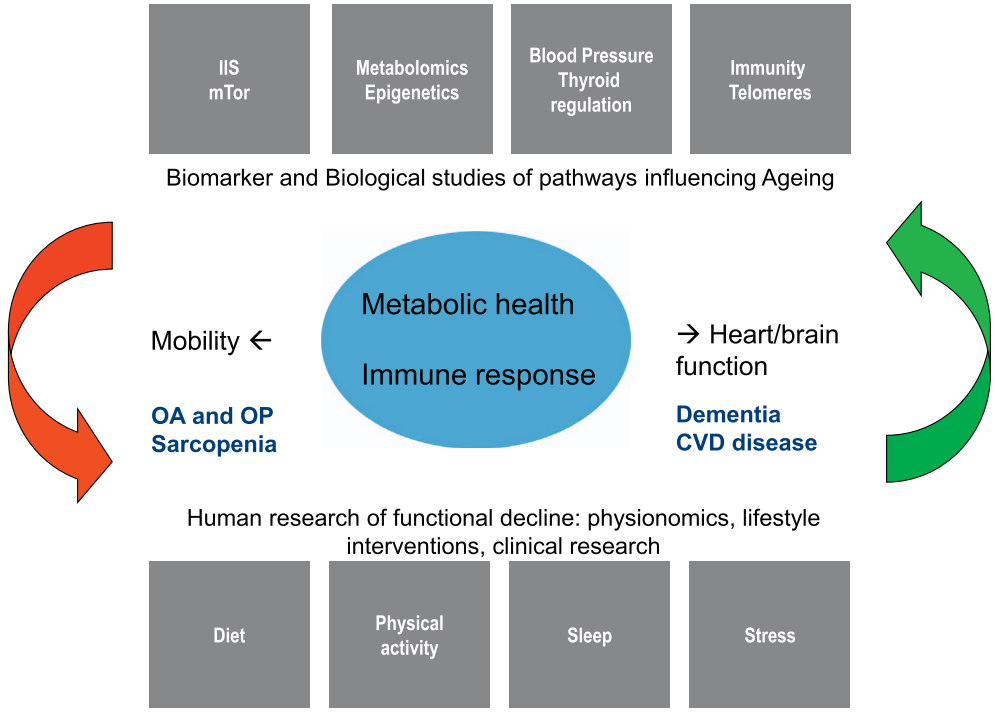

Fig. 3. Schematic presentation integrating studies of the hallmarks of ageing as defined by biological studies across species with phenomics- and disease-oriented studies in humans. Abbreviations: OA = osteoarthritis; OP = osteoporosis; CVD = cardiovascular disease.

Transparency document

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