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Chapter 8

General discussion

The aim of this thesis was to identify in the human blood transcriptome, relevant pathways and potential biomarker profiles that associate with chronological age and discriminate between ‘healthy agers’ from long-lived families and normative ageing controls. Such profiles may harbour determinants of the biological ageing rate.

We studied genome-wide gene expression profiles in blood of members of the Leiden Longevity Study (LLS) and replicated our findings by extended sampling within the unique LLS cohort. The findings of the exploratory analysis prompted us to investigate multiple genes in the *IL7R* and *MTOR* pathways for association with familial longevity. The results obtained by examining mRNA from blood samples brought us to study mTOR protein levels and signalling in primary skin fibroblasts from the corresponding donors in the LLS. Finally, to discover robust, biologically relevant gene networks as markers of chronological ageing in larger sample sizes, we performed an explorative network-based meta-analysis on large publicly available transcriptomic datasets.

Main findings

Chapter 3 describes the genome-wide explorative transcriptomic study performed within the Leiden Longevity Study cohort. The explorative study resulted in 360 probes (259 genes) to be associated with familial longevity, out of 2,953 probes (1,853 genes) differentially expressed in nonagenarians and middle-aged controls. We selected 22 genes from the 259 for follow up studies by using a text mining tool applying criteria that genes have known connections with cellular ageing. In Chapter 3 therefore we only investigated a fraction of the total number of potential transcriptomics biomarkers associated with healthy ageing, which will be described later in this discussion.

The microarray study showed that reduced expression of *ASF1A* and *IL7R* marked both chronological ageing and familial longevity in middle-age. The follow up experiments for *ASF1A* included RNAi experiments in *c. elegans* (David Gems, personal communication); no homologue gene is present in worms for *IL7R*. Two homologues of the *ASF1A* gene, *asf1-1* and *unc-85* were knocked down using RNAi in young adult worms and survival was measured in both the RNAi worms and the controls. Though downregulation of *asf1-1* resulted in a small significant increase in lifespan, the combination of knocking down both *asf1-1* and *unc-85* did not result in any change in lifespan. These results most likely illustrate random variation of nematode lifespan.

To then expand the study of the *IL7R* pathway in humans, we measured expression levels of six genes interacting with *IL7R* according to the STRING software and tested this network for association with familial longevity in the LLS (**Chapter 4**). We found several genes in the network to be associated with familial longevity in middle-age indeed, independent of the difference in blood cell proportion and disease prevalence between offspring of nonagenarians and their partners. Hence we conclude that expression levels of *IL7R* in blood may represent a marker of biological age (detecting differences between healthy and normative ageing). Subsequent measurement in the future on the complete LLS and additional cohort studies will enable an extensive survival analysis to be performed to indicate whether expression levels of these genes mark mortality risks and to test for the association with morbidity. The same analysis steps have revealed for example the leucocyte telomere length as a biomarker of ageing (1;2).

MTOR signalling has been implicated extensively in lifespan regulation in studies of animal models and in disease prevalence and progression in both animal models and humans. In our study, *MTOR* gene expression was part of the “normative ageing-signature” resulting from the explorative analysis (**Chapter 3**). Therefore we further investigated mTOR signalling in the Leiden Longevity Study (**Chapter 5**). Expression of genes belonging to both complexes mTORC1 and mTORC2 or downstream thereof showed association with chronological age in the LLS. The observed relation of decreased transcriptional activity of mTORC1 and lifespan extension described in the literature on animal models was in agreement with the low expression level in the human longevity families. The increased transcriptional activity of mTORC2 we observed with chronological ageing was not described previously. For two of the genes, *RPTOR* and *PRR5L*, belonging to respectively the mTORC1 and mTORC2 part of the pathway, we found decreased expression to be associated with familial longevity in middle-age, which association only for *RPTOR* was independent of differences between offspring and partners in cell proportions, glucose levels and prevalence of type 2 diabetes.

The mTOR pathway has been extensively studied in cell lines before, and previous studies in the LLS cohort demonstrated different cellular responses to stress in skin fibroblasts of the offspring and control groups. We questioned whether primary fibroblasts from LLS donors would also reflect reduced transcriptional activity of the mTOR pathway, altered activity at the level of protein expression and whether such a cellular model would allow further studies to link mTOR pathway genes to the metabolic

characteristics of longevity families. Thus, as a functional follow-up of the conclusion that gene expression of mTOR pathway genes in blood seem to mark familial longevity, gene and protein expression of mTOR-related genes were investigated in cultured primary skin fibroblasts of donors from the LLS (**Chapter 6**). Though fibroblasts of LLS offspring of nonagenarians and controls did show differences in cellular senescence and apoptosis previously, we were not able to detect differential mTOR gene or protein expression levels between these groups, neither did we observe any correspondence of variation in expression of mTOR genes in blood and fibroblasts of the same subjects in a group. Our main conclusion was that mTOR signalling in blood and skin fibroblasts appear to be differentially regulated. We included an indirect oxidative stress challenge by adding rotenone for 3 days in the fibroblast experiments. This challenge did not enhance any potential mTOR gene or protein expression difference in the cells from offspring and controls. Such challenges may be tested more extensively using different concentration and incubation time of rotenone, as described in previous senescence and apoptosis experiments within the LLS study (3).

Potentially suitable next approaches for functional studies include the investigation of protein expression or downstream processes like autophagy directly in Peripheral Blood Mononuclear Cells. Challenge tests such as nutrient deprivation or stress induction in the fibroblast cell system might otherwise enhance differences between the groups. Another option would be to investigate the mTOR pathway expression and activity in other tissues such as fat and muscle in which mTOR signalling is active. In these tissues nutrient intake, glucose and insulin levels and exercise influence protein synthesis, energy metabolism and glucose uptake (4), all processes in which the mTOR pathway is involved and which play a role in biological ageing and health.

In **Chapter 7** we describe an integrative network-based approach to discover robust markers for normative ageing combining multiple large-scale expression studies in blood. The combination of co-expression of genes and protein-protein interaction networks resulted in five robust networks enriched for “*Translational elongation*”, “*Cytolysis*” and “*DNA metabolic process*” associated with normative ageing. One of the identified modules predominantly consisted of ribosomal proteins and translation elongation factors. This module was found to be down-regulated with chronological age and suggested age-associated limitation of protein synthesis. Another major player in protein synthesis and ageing is mTOR signalling. Not only does mTORC1 regulates protein synthesis, mTORC2

activity is influenced by ribosomes (5), although the exact way in which this happens is unclear at this moment. The next step would be to establish whether the five co-expressed PPI networks that may be considered as robust markers for normative ageing, are also markers for biological age. In this regard the network including *ASF1A* already showed an association with survival at old age, suggesting their gene expression to be indeed markers for biological ageing.

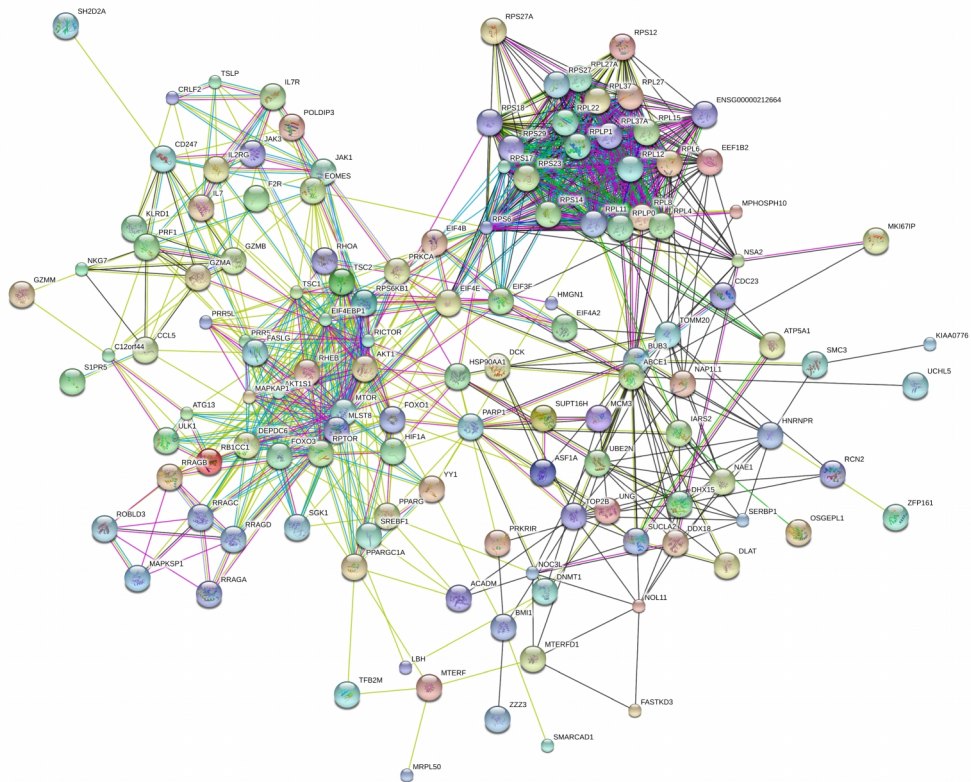


Figure 1. Total PPI network for the genes contributing to the mTOR pathway, IL7R network and the five robust co-expressed PPI modules

Connecting our findings and conclusions

We have identified five networks of which the gene expression levels in blood are robustly associated with chronological ageing, including genes involved in “*Translational elongation*”, “*Cytolysis*” and “*DNA metabolic process*”. In addition three major candidate genes were found for which the

transcription in blood differed between longevity families and controls, thus potentially marking the biological age and the rate of ageing: *ASF1A*, *IL7R* and *RPTOR*. Prospective studies relating the expression levels to morbidity and mortality will reveal the value of these levels as biomarkers of ageing.

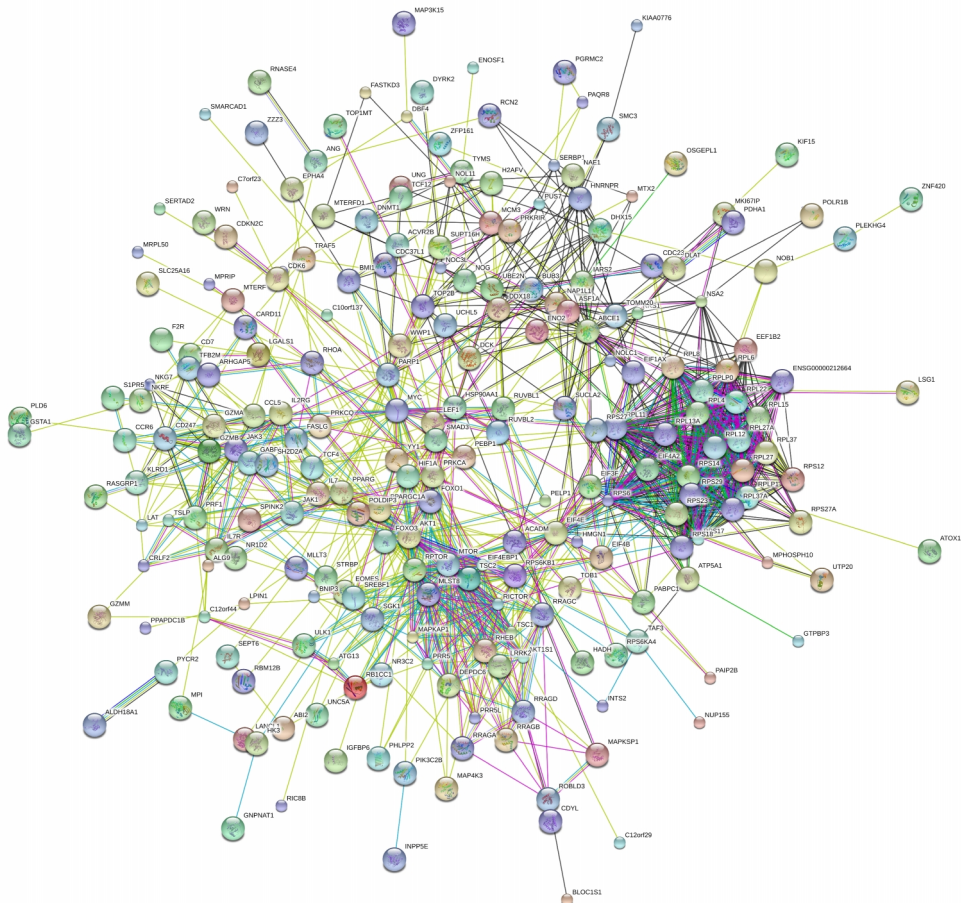


Figure 2. Total PPI network for the genes contributing to the mTOR pathway, IL7R network and the five robust co-expressed PPI modules and 99 potential marker genes

To prioritize the 236 uninvestigated potential marker genes that resulted from the explorative analyses in Chapter 3, we explored their relation with the interactive networks identified in Chapter 4, 5 and 7. By using STRING software we created a protein-protein interaction network for the genes contributing to the mTOR pathway, IL7R network and the five robust co-expressed PPI modules (Figure 1). Next we explored whether the 236

uninvestigated potential marker genes connected to the normative ageing networks. We found that 230 genes were recognized by STRING, 8 of which were already part of the mTOR or IL7R network or were part of a network found in the meta-analysis in chapter 7 (namely *MTOR*, *IL7R*, *ABCE1*, *ASF1A*, *DCK*, *LBH*, *MPHOSPH10* and *ZZZ3*) and 99 showed direct interactions with networks of normative ageing (Figure 2). Thus almost half of the genes that resulted as potential marker genes from chapter 3 showing association with chronological age and familial longevity connected to robust networks of normative chronological ageing resulting from the meta-analysis, plus the mTOR and IL7R networks. The majority of the connected genes showed at least two interactions enhancing the evidence that they connect to these networks. Table 1 shows the 99 genes prioritized for future replication studies. The other 123 genes do not show any interaction with the normative ageing network.

Remarkably, five of the interacting genes that have not yet been tested for replication in Chapter 3, *ABCE1*, *DCK*, *ASF1A*, *MPHOSPH10* (*MPP10*) and *ZZZ3*, are part of the robust age-associated co-expressed PPI module F in the meta-analysis (Chapter 7, Figure 2, module F). This module was not enriched for any biological process but seems to be related to the mitochondrion and cell cycle. Since these five genes have not only been associated with normative chronological ageing in Chapter 3 and the robust meta-analysis of Chapter 7, but also with familial longevity (Chapter 3), these genes may be the best next genes to be studied as potential markers of biological ageing and longevity.

Table 1. The 99 genes prioritized for future replication studies

Gene name							
ABI2	CCR6	GSTA1	LPIN1	NOLC1	PLD6	RRS1	TOP1MT
ACVR2B	CD7	GTPBP3	LRRK2	NR1D2	PLEKHG4	RUVBL1	TRAF5
ALDH18A1	CDC37L1	H2AFV	LSG1	NR3C2	POLR1B	RUVBL2	TYMS
ALG9	CDK6	HADH	MAP3K15	NUP155	PPAPDC1B	SEPT6	UNC5A
ANG	CDYL	HK3	MAP4K3	PABPC1	PRKCQ	SERTAD2	UTP20
ARHGAP5	DBF4	IGFBP6	MLLT3	PAIP2B	PUS7	SLC25A16	WRN
ATOX1	DYRK2	INPP5E	MPI	PAQR8	PYCR2	SMAD3	WWP1
BLOC1S1	EIF1AX	INTS2	MPRIP	PDHA1	RASGRP1	SPINK2	ZNF420
BNIP3	ENO2	KIF15	MTX2	PEBP1	RBM12B	STRBP	
C10orf137	ENOSF1	LANCL1	MYC	PELP1	RIC8B	TAF3	
C12orf29	EPHA4	LAT	NKRF	PGRMC2	RNASE4	TCF12	
C7orf23	GABPA	LEF1	NOB1	PHLPP2	RPL13A	TCF4	
CARD11	GNPNAT1	LGALS1	NOG	PIK3C2B	RPS6KA4	TOB1	

Implications and future research

One of the main pathways resulting from animal research and from this thesis to mark normative ageing is the mTOR signaling pathway, for which we identified the gene expression of *RPTOR* in blood to be a potential biomarker for biological ageing in middle-age. We observed that the decreased transcriptional activity of the MTOR pathway in members of long-lived families corresponds to the lower prevalence of cardiovascular disease and type 2 diabetes (6), and increased sensitivity to insulin (7). As compared to their partners, matched for age and social and lifestyle factors, the longevity families further display a beneficial lipid profile (8) and their cells are better resistant to stress (3). This pattern of phenotypic characteristics resembles the characteristics of caloric restricted mice (9), including the lower mTOR signaling and the longer lifespan. The interesting difference is that in the human studies the longevity family members and partners do not differ in BMI or in calorie intake. We hypothesized that members of long-lived families have an endogenously beneficial setting of their metabolism and hence protection from diseases. The question rises whether the controls (representing normative ageing subjects) are able to adjust the settings of their nutrient sensing pathways by behavioral changes.

Growing Old Together

Based on the finding that nutrient sensing and mTOR signaling as in animal models is relevant for human ageing, we initiated intervention studies within the LLS cohort. To investigate whether the partners of the offspring may be able to improve their metabolic health by diet intervention and physical activity, we founded the “Growing Old Together” Study. Middle-aged couples of the Leiden Longevity Study; offspring of nonagenarian siblings and their partners, are subjected to an intervention of dietary restriction and physical exercise for 13 weeks, a 25% lowered energy expenditure in total. By examining the potential biomarkers of biological ageing identified in this thesis before and after the intervention, we may be able to determine whether improvement of metabolism is acquired by the intervention, for which participants this is the case and whether the genes identified in this thesis contribute to such improvement.

Since blood may not be the best tissue to investigate mTOR signaling, in the “Growing Old Together” study also abdominal fat and skeletal muscle tissue are being collected. Since skeletal muscle is the major site of glucose disposal in response to food intake and insulin, and since an impairment of glucose uptake in this tissue contributes to type 2 diabetes,

we will investigate whether expression differences in mTOR genes between offspring and controls are more pronounced in muscle tissue. Also, we will investigate which candidate genes for biomarkers for the rate of ageing are the best in marking metabolic health.

Future indications

In general, altered gene expression levels in the longevity family members may be the consequence of ageing, or merely a trait shared by the long-lived families or may truly contribute to human longevity. To investigate whether changes in potential biomarkers causally contribute to ageing, gene expression of specific genes should be measured quantitatively in blood of a large, long-term population-based longitudinal study from the age of 40 years onwards. Now that we have a number of candidate genes such studies may indeed be performed to establish the association with prospective morbidity and mortality. Ideally, longitudinal measurements should be performed for example every five years, including collection of information about cell type proportions in the samples and information regarding the subjects' health. Such analyses could be done in large ongoing cohort studies such as the Rotterdam Study cohort (10) and, since the LLS study was initiated over 10 years ago, includes highly and middle-aged subjects, also in the LLS study. The future longitudinal study of genomic biomarkers may include also quantitative measures of epigenetic variation (such as DNA methylation) and should go hand in hand with measures of novel physiological parameters of biological ageing, as reflected by proteomics and metabolomic measures (11). Novel markers of biological ageing will stimulate studies into identification of determinants and mechanistic factors causally involved in biological ageing. Potentially causal factors will need to be investigated in animal models in which all tissues can be studied and not as in healthy humans, just blood, skin, muscle and fat tissue. In future studies of human longevity families, next generation sequencing of both DNA and RNA can provide further insight into the genetic variation underlying the gene expression differences we observed and test whether this could form the basis of the familial longevity.

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