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Undulating changes in human plasma proteome profiles across the lifespan

Benoit Lehallier^{1,2,3,*}, **David Gate**^{1,2,3,4}, **Nicholas Schaum**⁵, **Tibor Nanasi**^{1,2,3,6}, **Song Eun Lee**^{1,2,3,4}, **Hanadie Yousef**^{1,2,3,4}, **Patricia Moran Losada**^{1,2,3}, **Daniela Berdnik**^{1,2,3,4}, **Andreas Keller**⁷, **Joe Verghese**^{8,9}, **Sanish Sathyan**^{8,9}, **Claudio Franceschi**^{10,11}, **Sofiya Milman**^{8,12}, **Nir Barzilai**^{8,12}, **Tony Wyss-Coray**^{1,2,3,4,*}

¹Department of Neurology and Neurological Sciences, Stanford University, Stanford, California, USA

²Wu Tsai Neurosciences Institute, Stanford University, Stanford, California, USA

³Paul F. Glenn Center for the Biology of Aging, Stanford University, Stanford, California, USA

⁴Department of Veterans Affairs, VA Palo Alto Health Care System, Palo Alto, California, USA

⁵Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, California, USA

⁶Institute of Cognitive Neuroscience and Psychology, Hungarian Academy of Sciences Research Centre for Natural Sciences, Budapest, Hungary

⁷Clinical Bioinformatics, Saarland University, Saarbrücken, Germany

⁸Institute for Aging Research, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, USA

⁹Department of Neurology, Albert Einstein College of Medicine, Bronx, New York, USA

¹⁰University of Bologna, Bologna, Italy

¹¹National Research Lobachevsky State University of Nizhny Novgorod, Russia

¹²Department of Genetics, Albert Einstein College of Medicine, Bronx, New York, USA

Abstract

Aging is a predominant risk factor for numerous chronic diseases that limit healthspan¹.

Mechanisms of aging are thus increasingly recognized as potential therapeutic targets. Blood from young mice reverses aspects of aging and disease across multiple tissues^{2–10}, which supports a

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*Correspondence: twe@stanford.edu (T.W.-C.) and lehallib@stanford.edu (B.L.).

Author Contributions

B.L. and T.W.-C. planned the study, D.B., C.F., S.M., J.V., S.S. and N.B. provided human plasma samples, N. S., S.E.L. and H.Y. performed the mouse experiments, B.L. analyzed the data with contributions from T.N. and A.K., P.M.L. developed the shiny app, B.L., D.G. and T.W.-C. wrote the manuscript, A.K., C.F., S.M., J.V., S.S., N.B. and T.W.-C. supervised the study, all authors edited and reviewed the manuscript.

Competing Interests

The authors declare no competing financial interests.

hypothesis that age-related molecular changes in blood could provide novel insights into age-related disease biology. We measured 2,925 plasma proteins from 4,263 young adults to nonagenarians (18–95 years old) and developed a novel bioinformatics approach, which uncovered marked non-linear alterations in the human plasma proteome with age. Waves of changes in the proteome in the fourth, seventh, and eighth decades of life reflected distinct biological pathways and revealed differential associations with the genome and proteome of age-related diseases and phenotypic traits. This new approach to the study of aging led to the identification of unexpected signatures and pathways, which might offer potential targets for age-related diseases.

Aging underlies declining organ function and is the primary risk factor for numerous diseases¹. Thus, a deeper understanding of aging is likely to provide insight into mechanisms of disease and to facilitate the development of novel anti-aging therapeutics. A growing number of investigators have applied genomic, transcriptomic and proteomic assays (collectively referred to as omics) to studies of aging¹¹. Human genetic studies have uncovered relatively few modifiers of aging, yet other omics modalities, which measure more dynamic gene modifications or products, have provided valuable insight. For example, the transcriptome varies greatly during aging across tissues and organisms¹², pointing to evolutionarily conserved, fundamental roles of developmental and inflammatory pathways¹³. Protein composition of cells, bodily fluids, and tissues change similarly with age and provide insight into complex biological processes since proteins are often direct regulators of cellular pathways. In particular, blood, which contains proteins from nearly every cell and tissue, has been analyzed to discover biomarkers and gain insight into disease biology. Accordingly, organismal aging results in proteomic changes in blood that reflect aspects of aging of different cell types and tissues.

Perhaps the strongest evidence that blood can be used to study aging comes from experiments employing heterochronic parabiosis, a surgically induced state which connects the circulatory systems of young and old mice. These studies show that multiple tissues, including muscle, liver, heart, pancreas, kidney, bone, and brain can be rejuvenated in old mice^{2–10}. Plasma (the soluble fraction of blood) from old mice is sufficient to accelerate brain aging following infusion into young mice⁹ and young plasma can reverse aspects of brain aging^{10,14}. Together, these studies support the notion that the plasma proteome harbors key regulators of aging. Identifying such protein signatures may help understand mechanisms of organismal aging. However, plasma proteomic changes with age have not been thoroughly exploited and require novel tools to derive insight into the biology of aging. Here, we carried out a deep proteomic analysis of plasma from young adults to nonagenarians. Using novel analysis tools, we discovered changes in protein expression across the lifespan and link these changes to biological pathways and disease.

Results

Linear modeling links the plasma proteome to functional aging and identifies a conserved aging signature

We analyzed plasma isolated from ethylenediaminetetraacetic acid (EDTA)-treated blood acquired via venipuncture from 4,263 healthy individuals aged 18–95 years from the

INTERVAL₁₅ and LonGenity₁₆ cohorts (Fig. 1a, Extended Data 1. Currently, one of the most advanced tools for the measurement of plasma proteins is the single-stranded oligonucleotide known as aptamer_{17,18}, which binds its target with high affinity and specificity. To generate a proteomic dataset of the human lifespan, we used the SomaScan aptamer technology, capable of quantifying thousands of proteins (Supplementary Tables 1 and 2) with high precision within and between runs₁₉ (Supplementary Table 3). The INTERVAL and LonGenity datasets analyzed here can be interrogated with an interactive web interface (https://twc-stanford.shinyapps.io/aging_plasma_proteome/).

Since females have a longer average lifespan than males₂₀, we assessed whether sex and aging proteomes are interconnected (Fig 1b–1d). Proteins most strongly changed with sex included well-known follicle stimulating hormone (*CGA FSHB*), human chorionic gonadotropin (*CGA CGB*), and prostate-specific antigen (*KLK3*). With age, the most prominent overall changes with respect to fold change and statistical significance included sclerostin (*SOST*), ADP Ribosylation Factor Interacting Protein 2 (*ARFIP2*), and growth differentiation factor 15 (*GDF15*), in addition to several sex proteins such as *CGA FSHB*. The proteins most strongly associated with age also changed significantly with sex, (Fig. 1d); 895 proteins out of the 1,379 proteins altered with age were significantly different between sexes ($q < 0.05$, Supplementary Table 4). These results are aligned with a number of studies demonstrating that males and females age differently₂₁. To determine whether these findings are representative of the general population, we compared changes identified in this study with findings from 4 independent cohorts from the US and Europe ($n=171$, age range 21–107y, Extended Data 1d) and with an independent study₂₂. Although these independent cohorts used an older version of the SomaScan assay measuring only a subset of the current proteins (1,305 proteins; Supplementary Table 2), we observed high consistency of the aging and sex proteomes across cohorts (Extended Data 2).

To establish the biological relevance of these changes, we queried GO, KEGG, and Reactome databases and measured enrichment of proteins in pathways using a “Sliding Enrichment Pathway Analysis (SEPA)” (Supplementary Tables 5 and 6). The heatmaps produced by SEPA firstly illustrate the relationship between the top 100 proteins and the biological pathways they represent; secondly, the heatmaps emphasize how a restricted list of top aging proteins reveals biological pathways which would have escaped common pathway mining modalities (Fig. 1e). SEPA indicated that incremental lists of proteins are needed to determine biological functions of sex proteins, and pointed to expected differences in hormonal metabolism and activity. Conversely, an extensive list of aging proteins contained enrichment for blood-related pathways such as heparin and glycosaminoglycan binding, as recently reported₂₂.

To determine whether the plasma proteome can predict biological age and serve as a “proteomic clock,” we used 2,817 randomly selected subjects to fine-tune a predictive model that was tested on the remaining 1,446 subjects (Fig. 1f). We identified a sex-independent plasma proteomic clock consisting of 373 proteins (Supplementary Table 7), which was highly accurate in predicting age in the discovery, validation, and the 4 independent cohorts ($r=0.93–0.97$, Fig. 1g and Extended Data 3a–b). Remarkably, subjects predicted younger than their chronological age performed better on cognitive and physical tests (Fig. 1h and

Supplementary Table 8). While a reduced model comprising only 9 proteins predicted age with good accuracy (Extended Data 3c and Supplementary Table 7), a combination of different sets of proteins may be required to model changes in a large set of clinical and functional parameters (Extended Data 3d).

Since most biological pathways that change with age are evolutionarily conserved²³, we next identified aging proteins conserved between mice and humans. We analyzed mouse plasma (n=110, aged 1 month to 30 months) using SomaScan, which reliably measures hundreds of non-human proteins²⁴ and has proven useful in mouse studies^{6,25} (Fig. 1i, Supplementary Table 9). In mice, 172 proteins changed with age (out of 1,305 measured, Supplementary Table 10, $q < 0.05$) and 46 proteins overlapped with human aging proteins (Fig. 1j).

Remarkably, many of these proteins were also modulated by heterochronic parabiosis: young mice exposed to old plasma (young-heterochronic mice) showed a relatively older plasma signature while aged mice exposed to young plasma (old-heterochronic mice) showed a younger signature (Fig. 1k). Altogether, standard linear modeling of the plasma proteome during the human lifespan revealed established aging pathways, possibly indicating accelerated and decelerated aging in humans and mice. Intriguingly, changes to the conserved aging proteins did not occur simultaneously (Fig. 1l). Thus, the chronology of aging in the plasma proteome requires further investigation.

Clustering protein trajectories reveals undulation of the aging plasma proteome

While standard linear modeling showed prominent changes in plasma protein composition, the undulating behavior of the 46 conserved proteins (Fig. 1l) and more globally by the 2,925 plasma proteins as a group when they were visualized as z-scored changes across the lifespan was striking (Fig. 2a–b). These undulating patterns were detected in independent human cohorts and in mice (Extended Data 4), suggesting they are robust and conserved.

To reduce complexity of the proteome, we grouped proteins with similar trajectories using unsupervised hierarchical clustering (Fig. 2c) and identified 8 clusters of protein trajectories changing with age, which ranged in size from 8 to 1,415 proteins (Supplementary Table 11). In addition to linear patterns (clusters 1 and 5), several non-linear trajectories were evident, including stepwise, logarithmic, and exponential (clusters 2, 3, 4, 6, 7 and 8) (Fig. 2d). Importantly, these cluster trajectories were similarly detectable in independent cohorts (Extended Data 5). Out of the 8 clusters analyzed, 6 were enriched for specific biological pathways ($q < 0.05$; Extended Data 6, Supplementary Table 12), suggesting distinct, yet orchestrated changes in biological processes during the lifespan. For example, proteins present in blood microparticles consistently decreased with age (cluster 5), other blood-related pathways such as heparin and glycosaminoglycan binding increased in a two-step manner (cluster 4); while levels of proteins involved in axon guidance and EPH-ephrin signaling remained constant until age 60 before rising exponentially (cluster 6) (Fig. 2d). Altogether, the majority of plasma proteome changes across the lifespan are non-linear.

Quantification of proteomic changes across the lifespan uncovers waves of aging proteins

To quantitatively understand the proteomic changes occurring throughout life, we developed the software tool Differential Expression - Sliding Window ANalysis (DE-SWAN) (Fig. 3a).

This algorithm analyzes protein levels within a window of 20 years and compares two groups in parcels of 10 years (e.g. 35–45y compared with 45–55y), while sliding the window in increments of 1 year from young to old. Using DE-SWAN, we detected changes at particular stages of life and determine the sequential effects of aging on the plasma proteome (while also controlling for the effect of confounding factors). This approach identified hundreds of proteins changing in waves throughout age (Fig. 3b). Summing the number of differentially expressed proteins at each age uncovered 3 crests at ages 34, 60, and 78 (Fig. 3c, Extended Data 7a and Supplementary Table 13). These crests disappeared when the ages of individuals were permuted (Extended Data 7b), but were still detectable using different statistical models (e.g. smaller/larger sliding windows) (Extended Data 7c, Supplementary Figure 1, Supplementary Table 13), indicating robustness of these age-related waves.

Intriguingly, the 3 age-related crests were largely comprised of different proteins (Fig. 3d, Supplementary Table 14), but a few proteins were among the top 10 differentially expressed in each crest, such as *GDF15*, consistent with its pronounced increase across the lifespan (Fig. 3a). Other proteins, like chordin-like protein 1 (*CHRDL1*) or Matrix Metalloproteinase 12 (*MMP12*), were significantly changed only at the last two crests, reflecting their exponential increase with age. Overlap between proteins changing at age 34, 60, and 78 was statistically significant ($p < 0.05$) but limited, (Fig. 3e) and most proteins changing in old age were not identified by linear modeling (Fig. 3f). This prompted us to use SEPA to determine whether these waves reflected distinct biological processes. Strikingly, we observed a prominent shift in multiple biological pathways with age (Fig. 3g). At young age (34 years), we observed a downregulation of proteins involved in structural pathways such as the extracellular matrix (ECM). These changes were reversed in middle and old age (60 and 78 years, respectively). At age 60, we found a prominent role of hormonal activity, binding functions, and blood pathways. At age 78, key processes still included blood pathways but also bone morphogenetic protein signaling, which is involved in numerous cellular functions²⁶. Pathways changing with age by linear modeling overlapped most strongly with crests at age 34 and 60 (Fig. 3g), indicating that dramatic changes occurring in the elderly might be masked in linear modelling by more subtle changes at earlier ages. Altogether, these results show that aging is a dynamic, non-linear process characterized by waves of changes in plasma proteins that reflect complex shifts in biological processes.

Proteins linked to age-related diseases are enriched in distinct waves of aging

The plasma proteome is sensitive to the physiological state of an individual but is also genetically influenced²⁷. To deconvolute complexity between genome, proteome, and physiology, we asked whether the top aging proteins change due to genetic polymorphisms or whether they are among the top predictors of disease, or phenotypic traits. More specifically, we sought to determine whether proteins that comprised the 3 waves of aging were uniquely linked to the genome or proteome of age-related diseases and traits (Fig. 4a). We used the ranked lists of the top proteins identified by DE-SWAN at each of the three crests (Fig. 3c and Supplementary Table 14) and summed the number of proteins linked to the genome and proteome of specific diseases and traits separately for each wave (i.e. the cumulative sum) (Fig. 4b–i and Extended Data 8). First, we mined the genomic atlas of the

human plasma proteome²⁷ (Fig. 4b) and discovered that the aging proteome is also genetically determined (Fig. 4c and Extended Data 8). However, the rank of proteins determined by trans-association appeared more random with aging (Fig. 4c), suggesting that other sources drive the aging plasma proteome. We then tested whether the waves of aging proteins were differentially linked with changes in cognitive and physical functions identified in Fig. 1h. Interestingly, the proteome associated with these traits overlapped with the proteome defining middle and old ages, when these functions decline the most (Fig. 4d and Fig. 4e). Finally, we used public datasets and summary statistics from SomaScan proteomic studies focused on age-related diseases, including Alzheimer's Disease (AD)²⁸, Down Syndrome (DS)²⁹, and cardiovascular disease (CVD)³⁰. A plasma proteomic study predicting body mass index (BMI)³¹ was used as a control since weight gain varies widely with age (Supplementary Figure 2). As expected, the proteome linked to BMI was not selectively enriched for proteins defining waves of aging (Fig. 4f). Conversely, CVD-associated proteins were strongly enriched in waves of proteins defining middle and old age compared to young age (Fig. 4g). This enrichment corresponded to an increased incidence of CVD after 55 years³². Finally, AD- and DS-associated proteins overlapped with the top proteins defining middle age and old age but not those defining young age (Fig. 4h–i). The fact that the proteome defining these two diseases also changed in old individuals of a separate disease-free cohort supports the notion of accelerated aging in DS and AD^{33,34}. Altogether, these results show waves of proteomic aging are differentially linked to the genome and proteomic traits of various diseases.

Discussion

Our analysis of the plasma proteome reveals complex, non-linear changes over the human lifespan. Although linear analysis provides information about the aging plasma proteome, modeling non-linear protein trajectories is necessary to fully appreciate these undulating changes.

It is well known that men and women age differently²¹, yet we were surprised that 2/3 of proteins changing with age also change with sex (895/1,379 proteins). This supports the National Institutes of Health (NIH) policy on the inclusion of women in clinical research and using sex as a biological variable in experiments. Nevertheless, a unique proteomic clock can be used to predict age in men and women, and deviations from this plasma proteomic clock are correlated with changes in clinical and functional parameters (Fig 1f–h). This panel of 373 proteins can be used to assess the relative health of an individual and to measure healthspan, analogous to epigenetic clocks based on DNA methylation patterns³⁵. More large-scale plasma proteomic studies are required to establish the validity and utility of this clock and whether specific protein subsets are more appropriate to reflect particular clinical and functional parameters.

Blood is a sensitive marker of functional aging that also plays an active role in aging. Numerous studies have shown that soluble factors from young mouse blood reverse aspects of aging^{2–10,36}. Here, we describe a 46-protein aging signature that is conserved in humans and mice, containing known aging proteins such *GDF15*³⁷ and *IGF1/INSR*³⁸ but also less investigated ones (Fig 1l). This conserved signature will allow deeper investigation of

translational aging interventions in mice, such as heterochronic parabiosis, which partially reverses age-related changes of these proteins (Fig 1k).

By deep mining the aging plasma proteome, we identify undulating changes during the human lifespan. These changes are the result of clusters of proteins moving in distinct patterns, culminating in the emergence of 3 waves of aging. Unexpectedly, we find these clusters are often part of shared biological pathways, particularly cellular signaling (Fig. 2). In addition, we provide biological relevance for the 3 main waves of aging proteins (Fig. 3), which are characterized by key biological pathways with little overlap. In comparison, linear modeling fails to identify changes occurring late in the 8th decade of life (Fig. 3g). We conclude that linear modeling of aging based on omics data does not capture the complexity of biological aging across the lifespan. Thus, DE-SWAN will be invaluable for analyzing longitudinal datasets with linear and non-linear, quantifiable changes, and for integrating non-linear changes in the analysis of omics datasets.

Sources of variation of the plasma proteome can be diverse and under genetic control^{27,39}. Intriguingly, we observed that the relative importance of trans-associations decreased with aging (Fig. 4c), which led us to investigate sources of variance with a focus on disease-associated proteomes. Proteins comprising the middle and old age waves differentially overlapped with proteins associated with cognitive and physical impairments. These proteins also discriminated patients from age-matched controls in Alzheimer's disease, Down's syndrome, and CVD (Fig. 4g-i), suggesting that the characteristic plasma proteins of aging are amplified in these age-related diseases. Using an disease-free cohort, we provide evidence of accelerated aging for these two diseases^{33,34}. Further investigation of these proteins is warranted to determine whether these associations indicate aging biomarkers and/or causal mechanisms of disease. Nonetheless, these results suggest that variance within the aging plasma proteome slowly transitions from hard coding factors (i.e. genomic) to soft coding factors (e.g. diseases, environmental factors, and resulting changes in cognitive and physiological functions).

The undulating nature of the aging plasma proteome and its interactions with diseases ought to be considered when developing proteomic signatures for diagnostic purposes. Indeed, disease proteomes overlap significantly with the waves of aging proteins (Supplementary Table 15). Taking into account heterogeneous and complex changes to the plasma proteome during life will likely improve the sensitivity and specificity of prognostics and diagnostics tests. Moreover, these results are pertinent when considering the use of blood or blood products to treat aging and age-related diseases⁴⁰. Specifically, identifying plasma proteins that promote or antagonize aging at different stages of life could lead to more targeted therapeutics and/or preventative therapies. Such reliable tests and treatments are urgently needed for numerous diseases and, in the future, we hope to describe plasma proteome changes that predict subjects transitioning to disease. Of particular interest are studies of Alzheimer's disease for which blood-based biomarkers are unavailable and clinical symptoms are believed to occur up to two decades after disease onset.

Methods

Plasma proteomics measurements

The SomaScan platform was used to quantify relative levels of protein involved in several processes such as intercellular signaling, extracellular proteolysis, and metabolism. This platform was established to identify biomarker signatures of diseases and conditions, including cardiovascular risk³⁰, cancer⁴¹ and neurodegenerative diseases²⁸. The SomaScan platform is based on modified single-stranded DNA aptamers (SOMAmer[®] reagents) binding to specific protein targets. Assay details have been previously described¹⁹. Different versions of the SomaScan assay were used in the LonGenity, INTERVAL and the 4 independent human cohorts. These versions contained 5,284, 4,034, and 1,305 aptamers, respectively.

Out of the 4,034 aptamers measured in the INTERVAL cohort, 3,283 were contained in the publicly available dataset (<https://ega-archive.org/studies/EGAS00001002555>). Our study focuses on 2,925 aptamers with identical SeqId and SeqIdVersion in both INTERVAL and LonGenity cohorts (Supplementary Table 1). Out of the 2,925 aptamers, 888 were measured in the 4 independent cohorts and in mice (Supplementary Table 2).

Human cohorts characteristics

INTERVAL cohort—Participants in the INTERVAL randomized controlled trial (ISRCTN24760606) were recruited with the active collaboration of the National Health Service Blood and Transplant England (www.nhsbt.nhs.uk), which has supported field work and other elements of the trial. DNA extraction and genotyping was co-funded by the National Institute for Health Research (NIHR), the NIHR BioResource (<http://bioresource.nihr.ac.uk/>) and the NIHR Cambridge Biomedical Research Centre at the Cambridge University Hospitals NHS Foundation Trust. The INTERVAL study was funded by NHSBT (11–01-GEN). The academic coordinating center for INTERVAL was supported by core funding from: NIHR Blood and Transplant Research Unit in Donor Health and Genomics (NIHR BTRU-2014–10024), UK Medical Research Council (MR/L003120/1), British Heart Foundation (RG/13/13/30194) and the NIHR Cambridge Biomedical Research Centre at the Cambridge University Hospitals NHS Foundation Trust. Proteomic assays were funded by the academic coordinating center for INTERVAL and MRL, Merck & Co., Inc. A complete list of the investigators and contributors to the INTERVAL trial has been previously reported¹⁵. The academic coordinating center would like to thank blood donor center staff and blood donors for participating in the INTERVAL trial. For more information, see the Life Sciences Reporting Summary.

Proteomics measurements from 3,301 human plasma samples (1,685 males and 1,616 females) from 2 different subcohorts were used for this study. Age ranged from 18 to 76 years with a median age of 45 (1st Quartile=31, 3rd Quartile=55). Sample selection, processing and preparation were detailed previously²⁷.

LonGenity cohort—LonGenity is an ongoing longitudinal study initiated in 2008, designed to identify biological factors that contribute to healthy aging¹⁶. The LonGenity

study enrolls older adults of Ashkenazi Jewish descent, age 65–94 years at baseline. Approximately 50% of the cohort consists of offspring of parents with exceptional longevity (OPEL), defined by having at least one parent that survived to 95 years of age. The other half of the cohort includes offspring of parents with usual survival (OPUS), who do not have a parental history of exceptional longevity. Proteomics measurements from 1,030 human plasma samples (457 males and 573 females) collected at baseline in LonGenity participants were used for this study. Age ranged from 61 to 95 years with a median age of 74 (1st Quartile=69, 3rd Quartile=80). LonGenity subjects are thoroughly characterized demographically and phenotypically at annual visits that include collection of medical history and physical and neurocognitive assessments. Sixty-eight subjects without clinical and functional data were excluded from the analysis. The LonGenity study was approved by the Institutional Review Board (IRB) at the Albert Einstein College of Medicine.

Additional 4 independent cohorts—One hundred seventy-one human plasma samples (84 males and 87 females) were obtained from 4 different cohorts from the US and Europe (VASEattle, PRIN06, PRIN09, GEHA). Sample selection, processing and preparation of the VASEattle cohort were detailed previously⁴². Subjects from the PRIN06, PRIN09 and GEHA⁴³ cohorts were enrolled by multiple Italian study centers. Subjects were mainly Caucasians. Age ranged from 21 to 107 years with a median of 70 years (1st Quartile=58, 3rd Quartile=89). Written informed consent was obtained for each subject. The IRB has determined that our research does not meet the definition of human subject research per Stanford’s Human Research Protection Program policy and no IRB approval was required for this study.

For these cohorts, all samples were stored at –80 °C and 150µl aliquots of plasma were sent on dry ice to SomaLogic Inc. (Boulder, Colorado, US). Plasma samples were analyzed in three different batches, 24 samples in 2015, 70 in 2016 and 77 in 2017. In addition to these 171 plasma samples, 12 additional aliquots from 4 of these samples were measured in the different batches to estimate intra- and inter-assay variability (Supplementary Table 3). Data for 1,305 SOMAmer probes were obtained. No sample or probe data were excluded. HybNorm.plateScale.medNorm files provided by SomaLogic Inc. were bridged to data from the 1st batch of samples using calibrators.

Normalization of INTERVAL and LonGenity datasets

Relative Fluorescent Units (RFU) of each plasma protein were log₁₀-transformed. We normalized the levels of each protein within each subcohort based on the average of the subjects in the 60–70 years range. Supplementary Figure 3 shows representative normalization examples. Note that this normalization is needed when fitting aging trajectories (Fig. 2) but does not affect the results when “subcohort” is included as covariate in the modeling.

The data from the 4 independent cohorts were log₁₀-transformed and bridged together using SomaLogic procedure based on calibrators. However, the number of samples in the 60–70 years range was too small to reliably bridge this data to the INTERVAL and LonGenity cohorts.

Linear changes in the aging plasma proteome

To determine the effect of age and sex at the protein level, we used the following linear model:

$$\text{Protein level} \sim \alpha + \beta_1 \text{ Age} + \beta_2 \text{ Sex} + \beta_3 \text{ Subcohort} + \varepsilon$$

Type II sum of squares (SS) were calculated using the Anova function of the R car package⁴⁴. This SS type tests for each main effect after the other main effects. Q-values were estimated using Benjamini–Hochberg approach⁴⁵. It should be noted that the age range differs between cohorts. If the adjustment for cohort effect decreases the number of false positives, it could also alter the true positive rate. In the 4 independent cohorts, the “Subcohort” covariate also accounts for batch effect since samples from different cohorts were measured in different batches (except for PRIN06 and GEHA that were measured together).

To determine the relative proportion of variance explained by age and sex, we calculated the partial Eta2 as follows:

$$\text{Partial Eta2} = \frac{SS_{\text{effect}}}{(SS_{\text{effect}} + SS_{\text{error}})}$$

Validation of the aging proteins

To provide confidence in the reproducibility of the protein assays, we compared our findings with the associations with age reported by Tanaka et al.²². To this end, we merged our results with those from Tanaka et al. using “SomaId”. Of note, Tanaka et al. used the same version of the SomaScan platform that we used for the 4 independent cohorts (1,305 proteins).

Sliding Enrichment Pathway Analysis (SEPA)

To determine the biological meaning of group of plasma proteins, we ranked the top 100 proteins based on the product of $-\log_{10}(\text{p-values})$ and beta age (or beta sex) and queried three of the most comprehensive biological annotation and pathway databases: Gene ontology - GO⁴⁶, Kyoto Encyclopedia of Genes and Genomes – KEGG⁴⁷ and Reactome⁴⁸. Using these databases, we tested enrichment for pathways in the top 10 to top 100 proteins in increments of 1 protein. The 2,925 proteins measured in this study cover 90% of the human GOs, Reactome and KEGG terms containing more than 8 genes (Supplementary Figure 4).

To analyze each incremental list of proteins, we used the R topGO package⁴⁹ for GO analysis and the R clusterProfiler package⁵⁰ for KEGG and Reactome analyses. As input of SEPA, we used Gene Symbols provided by SomaLogic Inc. (Supplementary Table 1). The 2,925 proteins measured by SomaScan served as the background set of proteins against which to test for over-representation. Since several individual proteins (33 out of 2,925) were mapped to multiple Gene Symbols, we kept only the 1st Gene Symbol provided by Somalogic to prevent false positive enrichment. For KEGG and Reactome analysis, clusterProfiler requires EntrezID as input. Therefore, we mapped Gene Symbols to EntrezID

using the org.Hs.eg.db package⁵¹. Again, to avoid false positive enrichment, only the 1st EntrezID was used when Gene Symbols were mapped to multiple EntrezID. Q-values were estimated using Benjamini–Hochberg approach⁴⁵ for the different databases taken separately. For GO analysis, q-values were calculated for the three GOs classes (molecular function, cellular component, biological process) independently. To identify the most biologically meaningful terms and pathways, we reported only those with 20–500 proteins measured by the SomaScan assay. In addition, we focused on pathways consistently highly significant ($q < 0.05$ for at least 20 different incremental list of proteins) and kept the top ten pathways per condition (e.g. for each wave of aging proteins). Ranking was performed based on the minimum *fdr* across the incremental lists of proteins. SEPA can be viewed as an extension of the GSEA approach⁵², with more control for true and false positives.

Validation of the aging signature in mice

Male and virgin female C57BL/6JN mice were shipped from the National Institute on Aging colony at Charles River (housed at 67–73 °F) to the Veterinary Medical Unit (VMU; housed at 68–76 °F) at the VA Palo Alto (VA). At both locations, mice were housed on a 12-h light/dark cycle and provided food and water ad libitum. The diet at Charles River was NIH-31, and Teklad 2918 at the VA VMU. Littermates were not recorded or tracked. Mice 18-months-old and younger were housed at the VA VMU for no longer than 2 weeks before euthanasia, and mice older than 18-months were housed at the VA VMU until they reached the experimental age. After anaesthetization with 2.5% v/v Avertin, blood was drawn via cardiac puncture. All animal care and procedures were carried out in accordance with institutional guidelines approved by the VA Palo Alto Committee on Animal Research.

Heterochronic parabiosis was conducted as previously described^{3,10,53} with 3- and 18-month-old mice. Briefly, incisions in the flank were made through the skin and peritoneal cavity of both mice, and adjacent peritoneal cavities were sutured together. Adjacent knee and elbow joints were then sutured together to facilitate coordinated locomotion. Skin was then stapled together using surgical autoclips (9-mm, Clay Adams), and mice were placed under heat lamps to recover from anesthesia. Each individual mouse was injected subcutaneously with Baytril antibiotic (5 µg per g) and buprenorphine (0.05–0.1 µg ml⁻¹) for pain management, and 0.9% (w/v) NaCl for hydration. Mice were monitored and administered drugs and saline over the next week as previously described.

EDTA-plasma was isolated by centrifugation at 1,000g for 10 min at 4 °C before aliquoting and storing at –80 °C. A total of 110 plasma samples were analyzed and aliquots of 150µl of plasma were sent on dry ice to SomaLogic Inc. (Boulder, Colorado, US). Samples were sent in two different batches, 29 samples in 2016 and 81 in 2018. Data for 1,305 SOMAmer probes were obtained and no sample or probe data were excluded. RFUs of each plasma protein were log₁₀-transformed.

The SomaScan assay has been developed and validated for human fluids but successfully used in mouse research^{6,25}. To understand how similar mouse and human sequences are, we downloaded all homologies between mouse and human along with sequence identifiers for each species (HOM_MouseHumanSequence.rpt) from MGI (<http://www.informatics.jax.org/>) as plain text files. Then, the protein reference sequences for both

organisms were extracted from UniProt (<https://www.uniprot.org/>). On these matched sequence pairs, for each protein we computed a global pairwise sequence alignment. The alignments have been calculated by using the R “Biostrings” library⁵⁴. The average identity was 0.85, supporting the use of the SomaScan assay with mouse plasma.

To determine the effect of age and sex at the protein level, we used the 81 samples from 1 month to 30 months. To this end, we fitted the following linear model:

$$\text{Protein level} \sim \alpha + \beta_1 \text{ Age} + \beta_2 \text{ Sex} + \varepsilon$$

Type II sum of squares (SS) were calculated and Q-values were estimated using Benjamini–Hochberg approach.

To characterize the effects of young and old blood on the aging plasma proteome, normed scaled Principal Component Analysis (PCA) was performed using the R `ade4`⁵⁵ package

Prediction of human biological age using the plasma proteome

To determine whether the plasma proteome could predict biological age, we used `glmnet`⁵⁶ and fitted a LASSO model (alpha=1, 100 lambda tested, “lamda.min” as the shrinkage variable estimated after 10-fold CV). Input variables consisted in Z-scaled log₁₀ RFUs and sex information. Two-thirds (n=2,817) of the INTERVAL and LonGenity samples were used for training the model and the remaining 1,446 samples were used as a validation. In addition, the 171 samples from the 4 independent cohorts were used to further assess the robustness of the predictive model.

To estimate whether a subset of the aging clock can provide similar predictive results, we used a two-step approach as we described previously⁵⁷. One hundred models (100 lambda) including 0 to 373 proteins were created in step 1 and we estimated the accuracy of each of these models on the discovery and validation datasets, separately. Broken stick regression was used to determine the best compromise between number of variables and prediction accuracy.

Associations between age and clinical and functions in old

We used the subjects from the LonGenity cohort to identify associations between deviations from the proteomic clock (age=predicted age-chronological age) and 334 clinical and functional variables (Supplementary Table 8). To this end, we tested the following linear model:

$$\text{VariableOfInterest} \sim \alpha + \beta_1 \text{ Age} + \beta_2 \Delta \text{Age} + \beta_3 \text{ Sex} + \varepsilon$$

For binary outcomes, logistic regression was used. This analysis was separately performed in the discovery (n=2,817) and validation cohorts (n=1,446). Type II sum of squares were calculated using the `Anova` function of the R `car` package⁴⁴.

Clustering of protein trajectories

To estimate protein trajectories during aging, plasma proteins levels were z-scored and LOESS (locally estimated scatterplot smoothing) regression was fitted for each plasma factor. To group proteins with similar trajectories, pairwise differences between LOESS estimates were calculated based on the Euclidian distance and hierarchical clustering was performed using the complete method. To understand the biological functions of each cluster, we queried Reactome, KEGG and GO databases, as described above.

Differential Expression - Sliding Window Analysis (DE-SWAN)

To identify and quantify linear and non-linear changes of the plasma proteome during aging, we developed a Differential Expression - Sliding Window ANalysis (DE-SWAN) approach. Considering a vector l of k unique ages, we iteratively used l_k as the center of a 20-year window and compared protein levels of subjects in parcels below and above l_k i.e. [$l_k - 10y$; l_k] vs [l_k ; $l_k + 10y$]. To test for differential expression, we used the following linear model:

$$\text{Protein level} \sim \alpha + \beta_1 \text{ Age}_{\text{Low/High}} + \beta_2 \text{ Sex} + \epsilon$$

Age being binarized according to the parcels. For each l_k , q-values were estimated using Benjamini–Hochberg correction. Type II sum of squares were calculated using the Anova function of the R car package⁴⁴.

To assess the robustness and relevance of DE-SWAN results, we tested multiple parcel widths (5, 10, 15 and 20 years). In addition, we used multiple q-values thresholds and compared these results with those obtained by chance. To this end, we randomly permuted the phenotypes of the subjects and applied DE-SWAN to this new dataset. To keep the data structure, age and sex were permuted together. In addition, we analyzed the INTERVAL and LonGenity cohorts separately (Supplementary Figure 1). Finally, we tested the same linear model when adjusting for subcohort. This led to a loss of statistical power when the age range of the INTERVAL and LonGenity cohorts were overlapping but the three waves of aging proteins remained and the ranks of the top proteins were nearly identical (Supplementary Figure 1). We used the model adjusted for subcohort when trying to understand the waves of aging proteins (Figure 3d–g and Figure 4). The significance levels of the intersections between aging plasma protein signatures identified by linear modeling and DE-SWAN at different ages were determined using the R SuperExactTest package⁵⁸.

Relationships between the aging waves and the genome and the proteome of disease and traits

To quantify the overlap between proteins changing with age at different stages of life and the genome and the proteome of diseases and traits, we ranked DE-SWAN results based on p-values and created a k-ranked list of aging proteins, L_k . To reflect the degree to which the genome/proteome are linked with the waves of aging plasma proteins, we walked down L_k and counted the number of proteins associated with the genome or specific proteome. When different versions of the SomaScan platform were used, we walked down L_k until reaching the top 100 proteins measured in both studies.

To identify specific genetic variants associated with the aging plasma proteome, we mined the summary statistics generated by Sun et al.²⁷, who found 1,927 associations with 1,104 plasma proteins. Qgraph⁵⁹ R package was used to create a network between the genome and the 2,925 proteins analyzed in this study.

To determine whether the aging proteome is associated with the proteome of clinical and functional variables, we used the subjects from the LonGenity cohort and tested the following linear model for the top variables identified in Supplementary Table 8:

$$\text{Protein level} \sim \alpha + \beta_1 \text{ Age} + \beta_2 \text{ Sex} + \beta_3 \text{ VariableOfInterest} + \epsilon$$

Type II sum of squares were calculated using the Anova function of the R car package⁴⁴.

To determine whether the aging proteome is associated with disease proteomes, we integrated data and results from previous proteomic studies using the SomaScan platform. We re-analyzed one Alzheimer's Disease - AD dataset publicly available by AddNeuroMed²⁸ and used summary statistics from published studies focused cardiovascular disease - CVD³⁰, Down Syndrome - DS²⁹ and body mass index - BMI³¹.

AddNeuroMed is a European multi-center study in which the AD proteome was quantified in plasma samples from 681 controls, Mild Cognitive Impairment (MCI), and AD subjects using a previous version of the SomaScan assay. Files used were downloaded from the Synapse portal in March 2016 (syn5367752) and included measurements of 1016 plasma proteins from 931 samples. We limited our analysis to the 645 samples available at visit 1 (191 control, 165 MCI and 289 AD). Raw data were log₁₀-transformed. Four samples (2 control and 2 AD) were considered as outliers based on visual inspection of the results of a Principal Component Analysis (PCA) and filtered out.

To identify plasma proteins associated with AD, we used linear models with diagnosis, age, sex and center as covariates:

$$\text{Protein level} \sim \alpha + \beta_1 \text{ Diagnosis} + \beta_2 \text{ Age} + \beta_3 \text{ Sex} + \beta_4 \text{ Center} + \epsilon$$

Type II sum of squares were calculated using the Anova function of the R car package⁴⁴.

To determine whether aging plasma proteins are involved in other disease signatures, we identified three studies using the SomaScan platform in large human cohorts providing detailed summary statistics. Carayol et al. mined the plasma proteome to obtain new insights into the molecular mechanisms of obesity. Out of 1,129 proteins measured, they identified 192 plasma proteins significantly associated with BMI ($p < 0.05$ after Bonferroni correction). Summary statistics we used were obtained from their "Supplementary Data 1"³¹. Ganz et al. derived and validated a 9 protein risk-score to predict risk of cardiovascular outcomes³⁰. In addition to these 9 proteins, 191 other proteins were significantly associated with cardiovascular risk ($p < 0.05$ after Bonferroni correction). Summary statistics for these 200 proteins are available in their eTable 4 and the 1,130 proteins measured of this study are listed in their eTable 1. Finally, Sullivan et al. used an extended version of the SomaScan

platform to study DS and identified a large number of dysregulated proteins²⁹. We used the results for the Discovery cohort (sheet A of the “Supplementary File 1”) in which, 258 proteins out of 3,586 proteins are reported to be associated with DS ($p < 0.05$ after Bonferroni correction).

Since detailed protein information was not available for all studies, we used either gene symbols or uniprotID to merge disease proteomes characterized in published studies with the aging proteome identified in this study. When multiple p-values were reported for the same gene symbol (or a combination of gene symbols), only the most significant p-value was retained.

Code availability

An R package for DE-SWAN is available in github: [lehallib.github.io/DEswan/](https://github.com/lehallib/DEswan/)

Data availability

We created a searchable web interface to mine the human INTERVAL and LonGenity datasets (https://twc-stanford.shinyapps.io/aging_plasma_proteome/)

The Human independent cohorts and mouse protein data are available in Supplementary Tables 16 and 17. The INTERVAL data is available through the European Genome-Phenome Archive (<https://ega-archive.org/studies/EGAS00001002555>).

Extended Data

a

Interval - Subcohort 1 (n=2,481 18-76y)

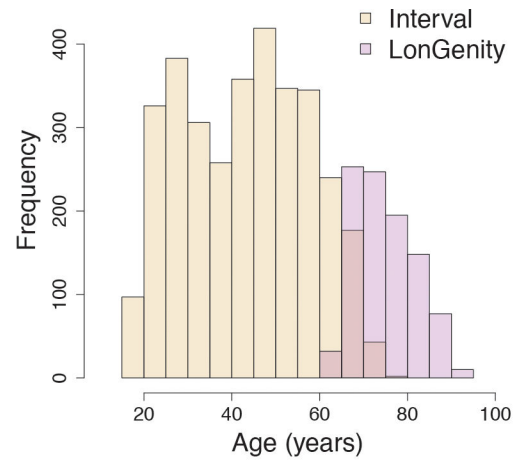
Interval - Subcohort 2 (n=820 18-74y)



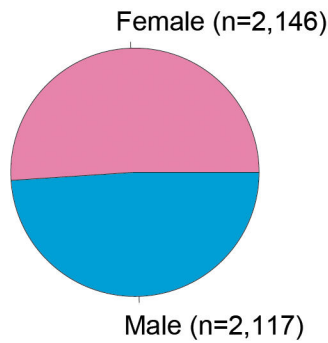
LonGenity - Subcohort 1
(n=457 61-94y)

LonGenity - Subcohort 2
(n=505 64-95y)

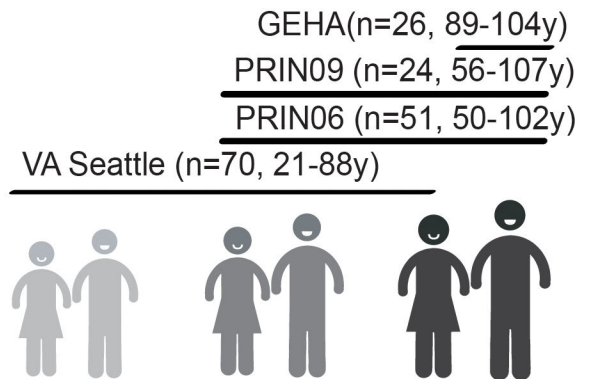
b



c

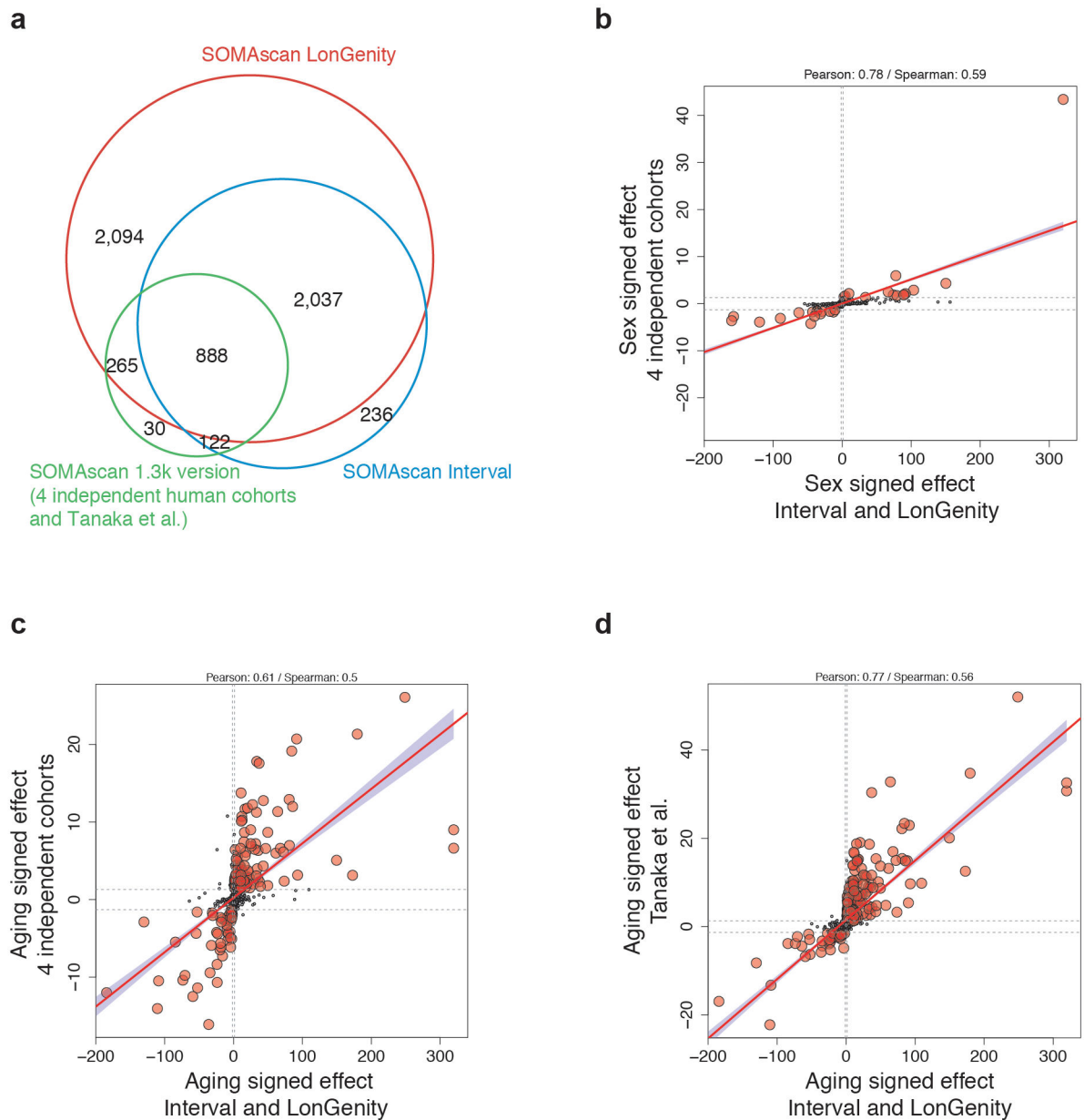


d



Extended Data Fig. 1. Sample demographics

Age (a, b), cohort (a, b) and sex distributions (c) of the 4,263 subjects from the INTERVAL and LonGenity cohorts. (d) Age and cohort distributions of the 171 subjects from the 4 independent cohorts.



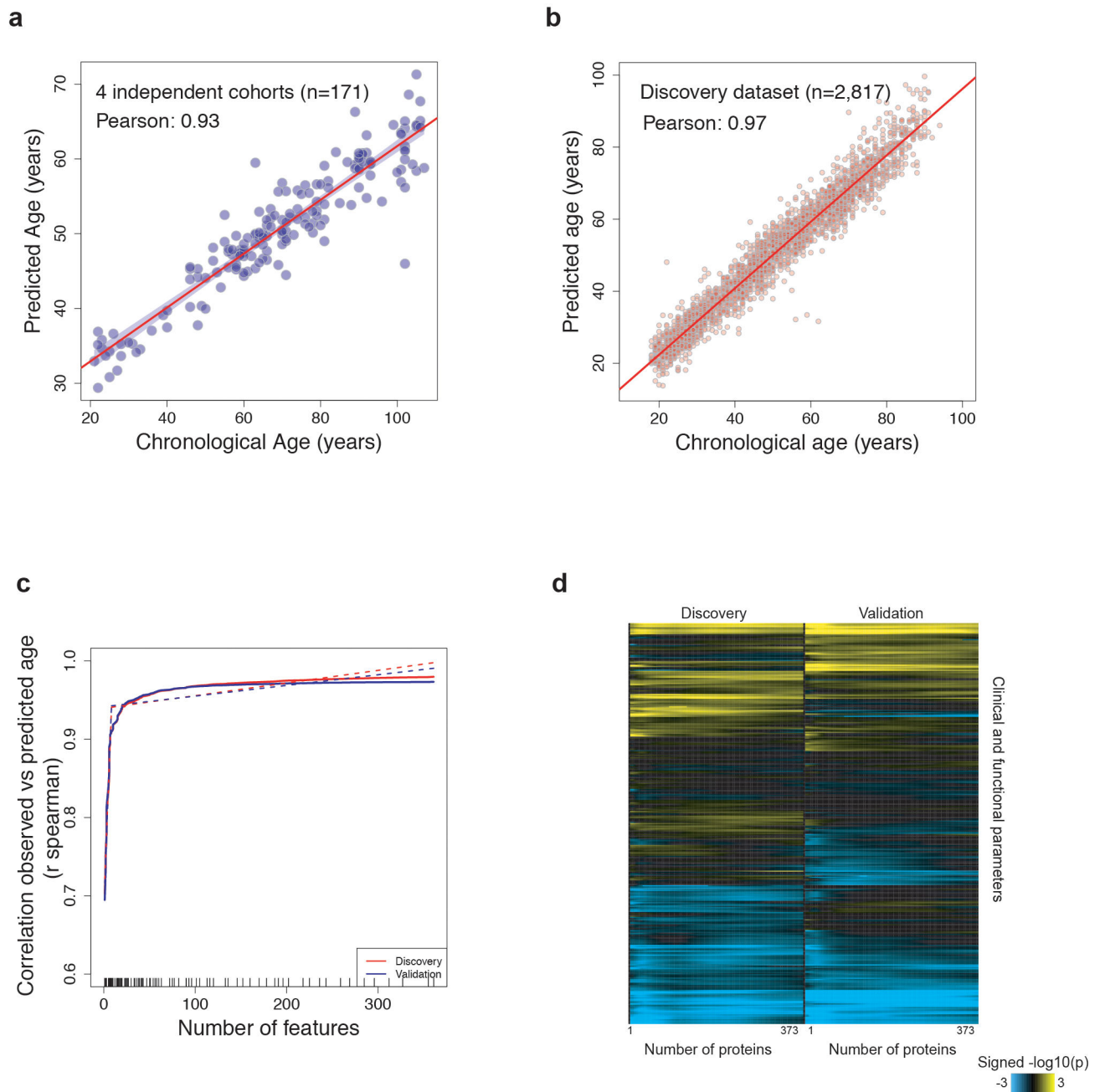
Extended Data Fig. 2. Comparing age and sex effects in independent cohorts

(a) Age and sex effects in the INTERVAL and LonGenity studies (n=4,263) were compared to age and sex effects in 4 independent cohorts analyzed together (n=171) and to age effect from Tanaka et al. (n=240, 2018)

The aging plasma proteome was measured with the SomaScan assay in these cohorts and 888 proteins were measured in all studies

(b) Scatter plot representing the signed $-\log_{10}(q \text{ value})$ of the sex effect in the INTERVAL/ LonGenity cohorts (x axis, n=4,263) vs the 4 independent cohorts (y-axis, n=171).

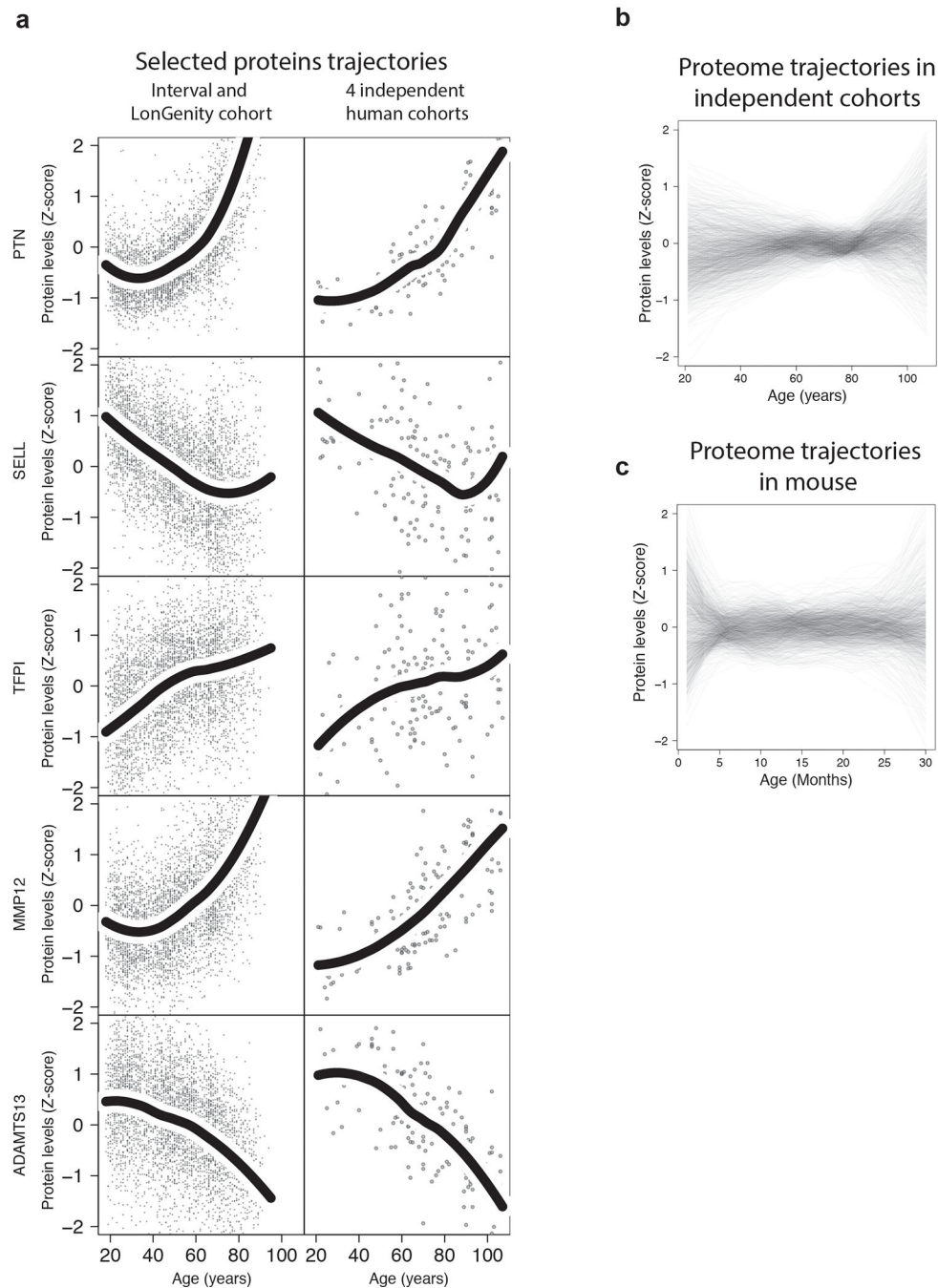
Similar analysis for the age effect in the 4 independent cohorts (c, n=171) and in Tanaka et al study (d, n=240)



Extended Data Fig. 3. Deeper investigation of the aging proteomic clock

a) Prediction of age in the 4 independent cohorts (n=171) using the proteomic clock. Only 141 proteins out of the 373 constituting the clock were measured in these samples. (b) Prediction of age in the discovery cohort (n=2,817) using the 373 plasma markers. (c) Feature reduction of the aging model in the Discovery and Validation cohorts to estimate whether a subset of the aging signature can provide similar results to the 373 aging proteins. Dashed lines represent a broken stick model and indicate the best compromise between number of variables and prediction accuracy.

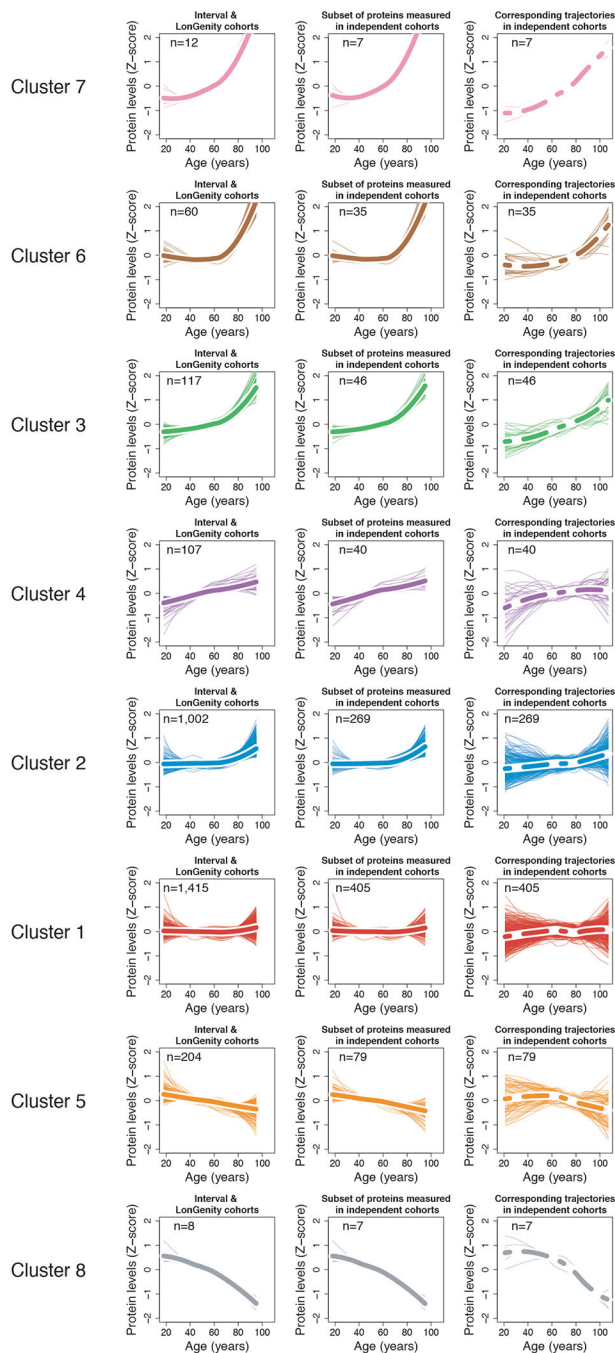
(d) Heatmap representing the associations between delta age and 334 clinical and functional variables. For quantitative traits, linear models adjusted for delta age, age and sex were used and significance was tested using F-test. For binary outcomes, binomial generalized linear models adjusted for delta age, age and sex were used and significance was tested using likelihood ratio chi-square test. As in (c) the analysis was performed for the top 2 to top 373 variables predicting age. The non-uniformity in the heatmaps suggests that specific subsets of proteins may best predict certain clinical and functional parameters



Extended Data Fig. 4. Proteins and proteome undulations in independent human cohorts and in mouse

(a) Trajectories of 5 selected proteins based on the INTERVAL and LonGenity cohorts (n=4,263, left) and 4 independent human cohorts (n=171, right). Trajectories were estimated using LOESS regression.

Undulation of the 1,305 plasma proteins measured in 4 independent cohorts (b, n=171) and in mouse (c, n=81). Plasma proteins levels were z-scored and LOESS regression was fitted for each plasma factor.



Extended Data Fig. 5. Cluster trajectories in independent cohorts

Protein trajectories for the 8 clusters identified in the INTERVAL and LonGenity cohorts (left column). Thicker lines represent the average trajectory for each cluster. Cluster trajectories for the subset of proteins measured in the 4 independent cohorts (middle column). Corresponding cluster trajectories in 4 independent cohorts (right column).

| | | | | |
|-----------|---|--|---|--|
| Cluster 1 | nuclear part (319) | cytosol (437) | nucleoplasm (250) | nuclear lumen (286) |
| Cluster 2 | Human cytomegalovirus infection (31) | Chronic myeloid leukemia (16) | Cytokine–cytokine receptor interaction (79) | FoxO signaling pathway (23) |
| Cluster 3 | extracellular region (80) | extracellular region part (69) | extracellular space (44) | proteinaceous extracellular matrix (16) |
| Cluster 4 | Chondroitin sulfate biosynthesis (4) | Heparan sulfate/heparin (HS–GAG) metabolism (5) | Chondroitin sulfate/dermatan sulfate metabolism (5) | Regulation of IFNG signaling (3) |
| Cluster 5 | extracellular space (62) | blood microparticle (10) | fibrillar collagen trimer (3) | banded collagen fibril (3) |
| Cluster 6 | EPH–ephrin mediated repulsion of cells (7) | EPH–Ephrin signaling (7) | ephrin receptor signaling pathway (8) | extracellular region (45) |
| Cluster 7 | extracellular space (8) | fibronectin binding (2) | extracellular region (10) | extracellular region part (9) |
| Cluster 8 | transmembrane receptor protein tyrosine kinase activity (3) | signal transducer, downstream of receptor, with protein tyrosine kinase activity (2) | integrin binding (3) | transmembrane receptor protein kinase activity (3) |

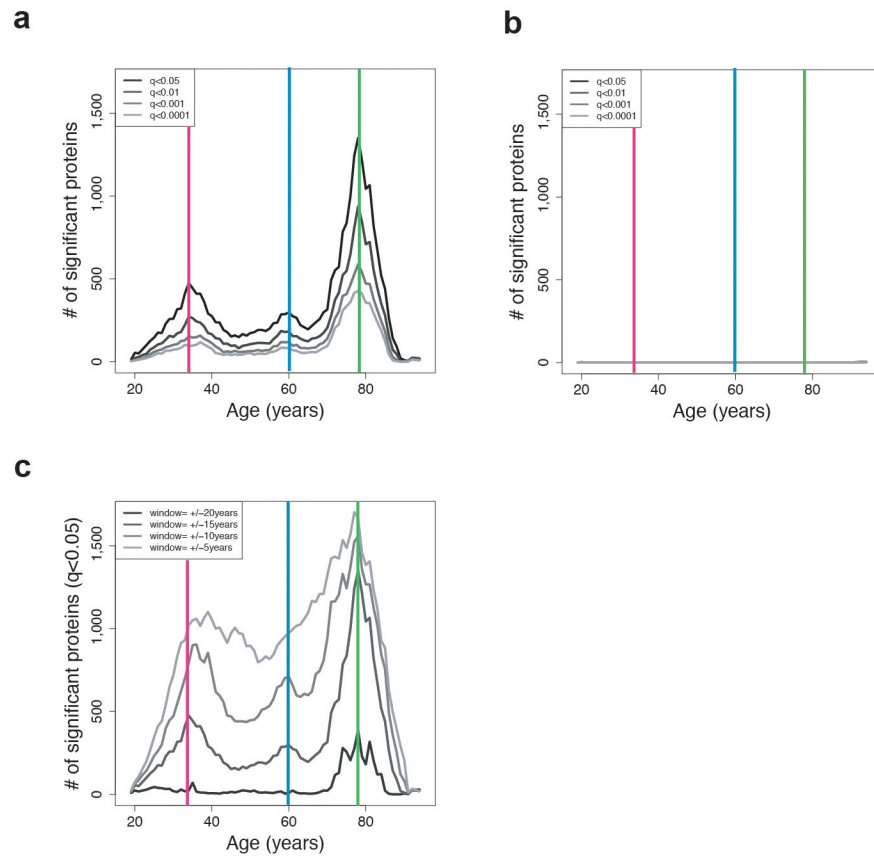
■ fdr>0.1
 ■ fdr<0.1
 ■ fdr<0.05
 ■ fdr<0.01

1 2 3 4
Top Pathways (Rank)

Extended Data Fig. 6. Pathways in clusters

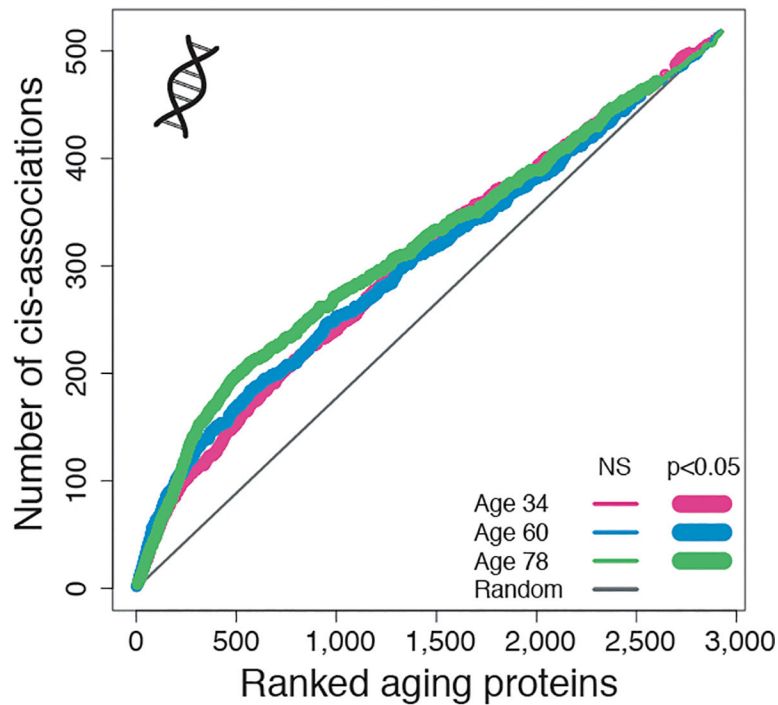
Pathway enrichment was tested using GO, Reactome and KEGG databases (n=4,263).

Enrichment was tested using Fisher's exact test (GO) and hypergeometric test (Reactome and KEGG). The top 4 pathways for each cluster are shown. Pathway IDs and number of plasma proteins associated are represented in the table.



Extended Data Fig. 7. DE-SWAN age effect for multiple q-values cutoffs, windows size and after phenotypes permutations
 Different Q-value cutoffs are represented in (a). Similar analysis with different after phenotype permutations (b) and different windows size in (c). The 3 local peaks identified at age 34, 60 and 78 are indicated by colored vertical lines.

Cis-associations and aging waves



Extended Data Fig. 8. Cis-associations and aging waves

Enrichment for cis-association in the waves of aging proteins identified by DE-SWAN. Aging proteins were ranked based on p-values at age 34, 60 and 78 and the cumulative number of cis-associations was counted. One-sided permutation tests ($1e+5$ permutations) were used to assess significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The top 10 pathways per condition are represented. Enrichment was tested using Fisher's exact test (GO) and hypergeometric test (Reactome and KEGG).

(f) Schematic representation of the of biological age modeling using the plasma proteome.

(g) Prediction of age in the validation cohort (n=1,446) using 373 plasma proteins. Pearson correlation coefficient between chronological and predicted age is indicated.

(h) Association between delta age (difference between chronological age and chronological age) and functional readouts in old. Top associations in both Discovery and Validation datasets are represented.

(i) Schematic representation of the comparison between the human and mouse aging proteomes.

(j) Conserved markers of aging. Both human and mouse aging effects are signed by the beta age of their corresponding linear analysis. Forty-six plasma proteins are changing in the same direction in mouse and humans (red dots) and define a conserved aging signature.

(k) Alteration of the conserved aging signature by parabiosis. Normed principal component analysis was used to characterize changes of the conserved aging signature when mice are exposed to young or old blood.

(l) Age-related changes of the conserved aging signature. Plasma protein levels were z-scored and aging trajectories were estimated by locally estimated scatterplot smoothing.

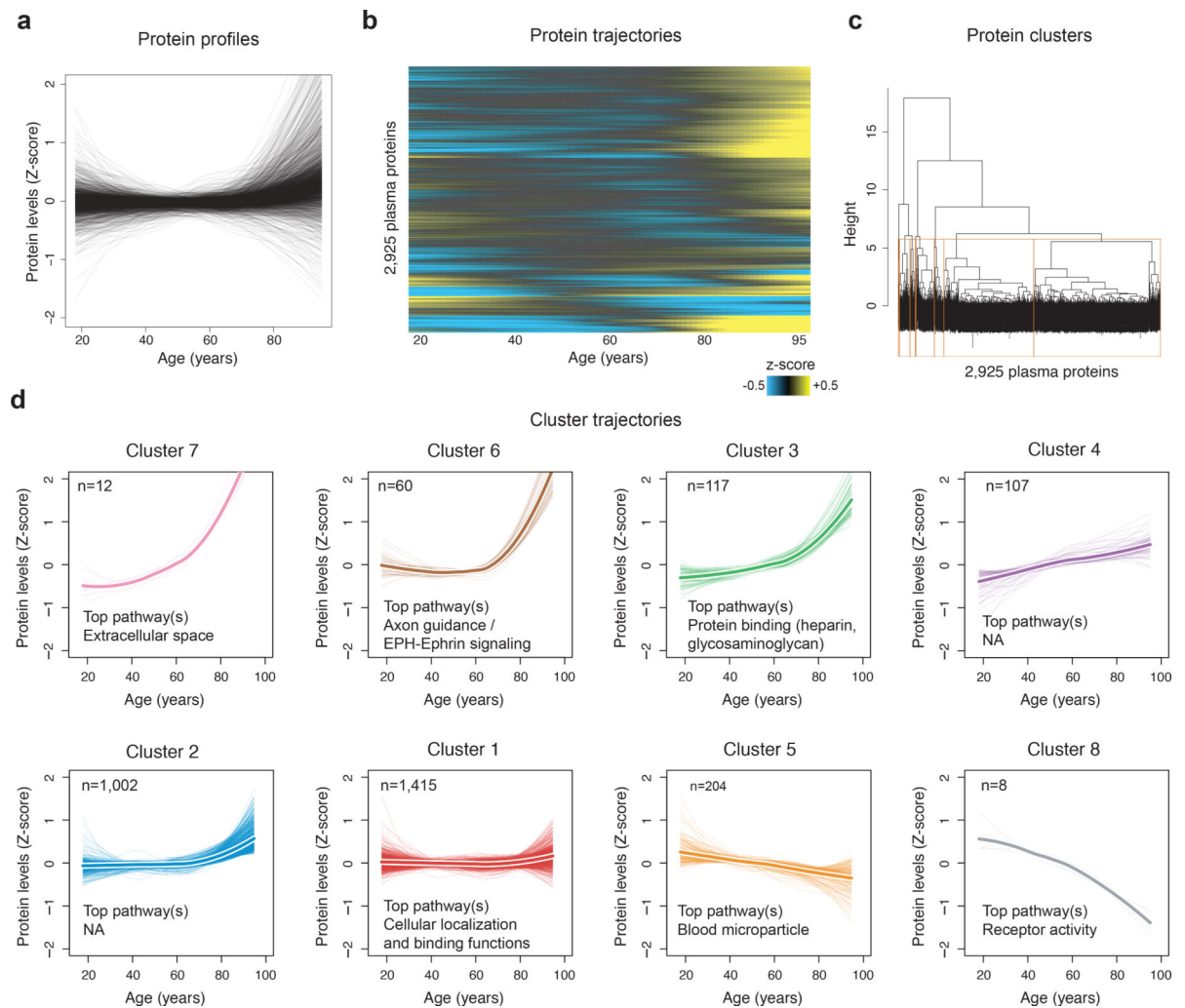


Figure 2: Clustering of protein trajectories identifies linear and non-linear changes during aging.

(a) Protein trajectories during aging. Plasma protein levels were z-scored and trajectories of the 2,925 plasma proteins were estimated by LOESS.

(b) Trajectories are represented in two dimensions by a heatmap and unsupervised hierarchical clustering was used to group plasma proteins with similar trajectories.

(c) Hierarchical clustering dendrogram. The 8 clusters identified are represented by orange boxes.

(d) Protein trajectories of the 8 identified clusters. Clusters are grouped by the similarity of global trajectories, the thicker lines representing the average trajectory for each cluster. The number of proteins and the most significant enriched pathways are represented for each cluster. Pathway enrichment was tested using GO, Reactome and KEGG databases. The top 20 pathways for each cluster are listed in Supplementary Table 12.

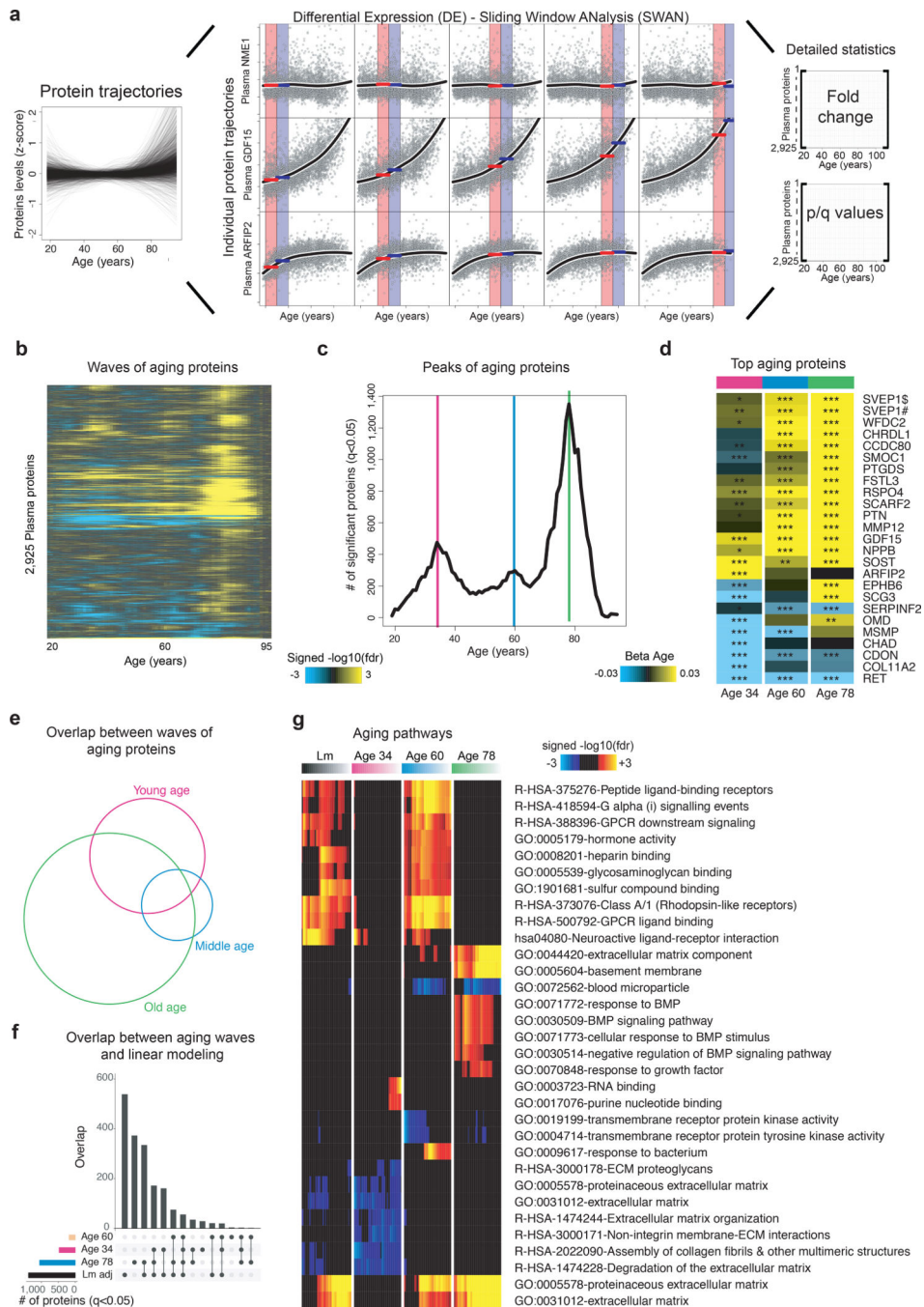


Figure 3: Sliding window analysis distinguishes waves of aging plasma factors.

(a) Differential Expression - Sliding Window ANalysis (DE-SWAN). DE-SWAN compares proteins levels between groups of individuals in parcels of 10 years, e.g. 30–40 compared with 40–50. DE-SWAN identifies linear and non-linear changes during aging. Examples of the DE-SWAN for 3 proteins and 5 age windows. Red and blue rectangles show the two parcels and the red and blue lines symbolize the mean within each parcel. DE-SWAN provides statistics for each age window and each plasma protein, allowing detailed analysis of plasma proteomic changes during aging.

- (b) Waves of aging plasma proteins characterized by DE-SWAN (n=4,263). Within each window, $-\log_{10}(\text{p-values})$ and $-\log_{10}(\text{q-values})$ were estimated by linear modeling adjusted for age and sex and significance was tested using F-test. Local changes attributable to age were signed based on corresponding beta age.
- (c) Number of plasma proteins differentially expressed during aging. Three local peaks at the ages of 34, 60 and 78 were identified by DE-SWAN.
- (d) Top 10 plasma proteins identified by DE-SWAN at age 34, 60 and 78 (n=4,263). Linear models adjusted for age, sex and subcohort were used and significance was tested using F-test. Blue and yellow colors represent local decrease and increase, respectively. # and \$ indicate different SOMAmers targeting the same protein. * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$.
- (e) Intersections between waves of aging proteins (n=4,263, $q < 0.05$). Linear models adjusted for age, sex and subcohort were used and significance was tested using F-test.
- (f) Intersections between linear modeling and the aging waves (n=4,263, $q < 0.05$). Linear models adjusted for age, sex and subcohort were used and significance was tested using F-test.
- (g) Visualization of pathways significantly enriched for aging proteins identified by linear modeling and DE-SWAN at age 34, 60 and 78 (n=4,263). Proteins upregulated and downregulated were analyzed separately. The top 10 pathways per condition are represented. Enrichment was tested using Fisher's exact test (GO) and hypergeometric test (Reactome and KEGG).

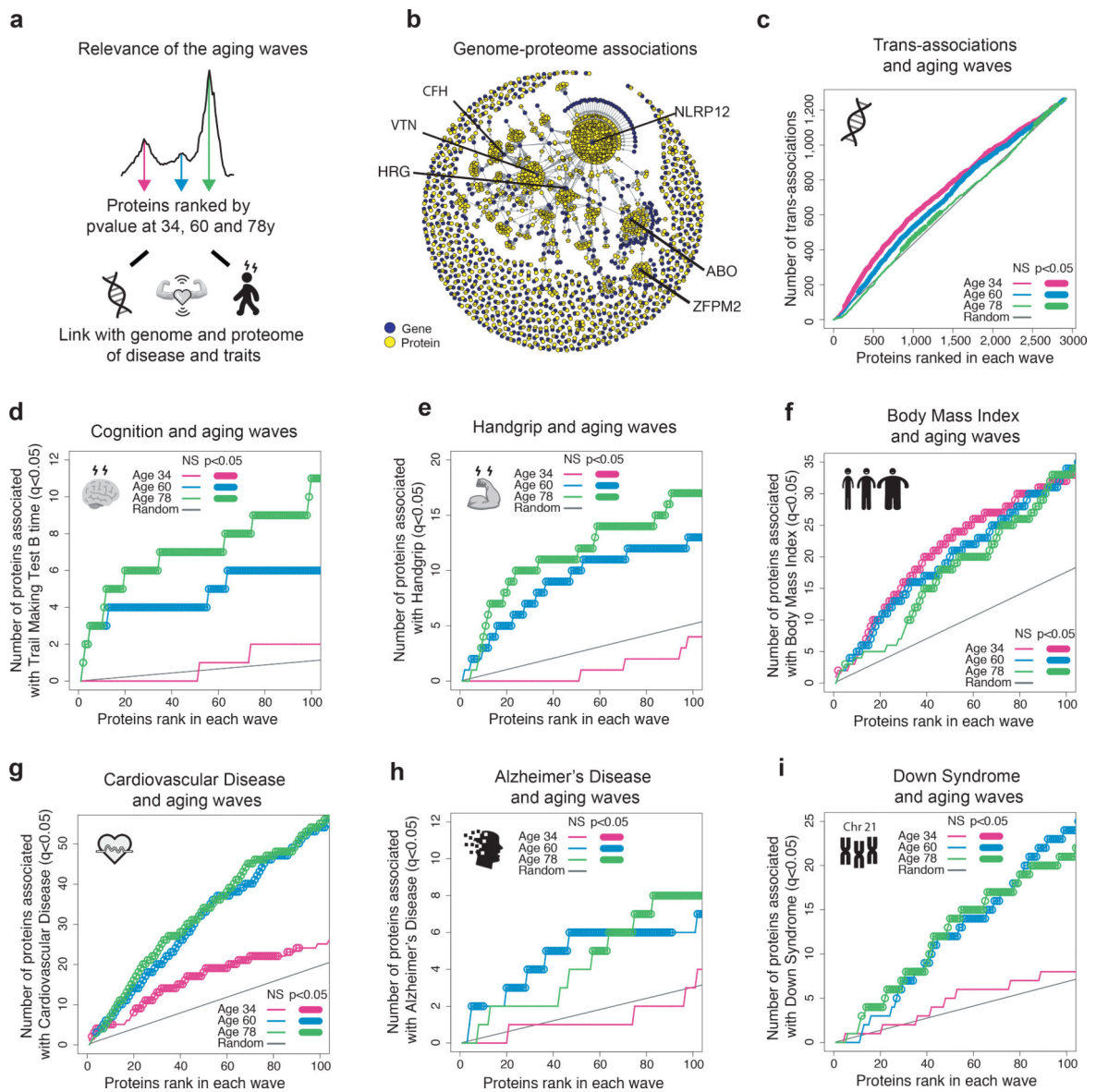


Figure 4: Waves of aging proteins are differentially linked to the genome and proteome of disease and traits

(a) Relevance of the aging waves. Schematic representation of analysis. The proteins changing at 34, 60 and 78y were ranked by p-value and were associated with the genome and the proteome of disease and traits.

(b) Association between the genome and the proteome. Network created using the pQTL associations identified by Sun et al. (2018).

(c) Enrichment for trans-association in the waves of aging proteins identified by DE-SWAN. Aging proteins at age 34, 60 and 78y were ranked based on p-value and the cumulative number of trans-associations was enumerated. One-sided permutation tests ($1e+5$ permutations) were used to assess significance.

Enrichment for proteins involved in cognitive and physical performance in the waves of aging proteins (d-e).

Enrichment for disease-associated proteins in the waves of aging proteins (f-i).

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