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Published in:
Nature Aging

DOI:
10.1038/s43587-021-00028-4

Publication date:
2021

Document version:
Accepted manuscript

Citation for pulished version (APA):
Kaeser, S. A., Lehallier, B., Thinggaard, M., Häsler, L. H., Apel, A., Bergmann, C., Berdnik, D., Jeune, B., Christensen, K., Grönke, S., Partridge, L., Wyss-Coray, T., Mengel-From, J., & Jucker, M. (2021). A neuronal blood marker is associated with mortality in old age. *Nature Aging*, *1*(2), 218–225. https://doi.org/10.1038/s43587-021-00028-4

A neuronal blood marker is associated with mortality in old age

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Neurofilament light chain (NfL) has emerged as a promising blood biomarker for the progression of various neurological diseases. NfL is a structural protein of nerve cells and elevated levels in blood are thought to mirror damage to the nervous system. We find that plasma NfL levels increase in humans with age (n=122; 21–107 years of age) and correlate with changes in other plasma proteins linked to neural pathways. In centenarians (n=135) plasma NfL levels are associated with mortality equally or better than previously described multi-item scales of cognitive or physical functioning, and this observation was replicated in an independent cohort of nonagenarians (n=180). Plasma NfL also increases in aging mice (n=114; 2–30 months of age) and dietary restriction, a paradigm that extends lifespan in mice, attenuates the age-related increase of plasma NfL. These observations suggest a contribution of nervous system functional deterioration to late life mortality.

An involvement of the nervous system in the regulation of aging and longevity has been suggested from invertebrates[1,2] and has gained momentum by more recent work in mammals including humans[3,4]. To date, the best indicators for death in elderly individuals are multi-item scales for physical and cognitive functioning[5-7]. A molecular biomarker in blood related to the underlying biology of aging and survival has been elusive, as aging and longevity are complex biological traits[8].

Neurofilament light chain (NfL) is a structural protein of nerve cells that can be detected in human bodily fluids and is a potential biomarker candidate of aging and longevity. NfL levels in cerebrospinal fluid (CSF) and blood are strongly correlated[9,10]. NfL increases with age in both CSF and blood and levels are predictive for cognitive decline[11,12]. NfL also increases in response to brain damage and in neurodegenerative diseases[13-15] and elevated NfL in CSF and blood have been linked to shorter survival among patients with Alzheimer´s disease, Parkinson´s disease, and other neurological diseases[16-18].

We set out to test whether nervous system deterioration exemplified by increased blood NfL contributes to all-cause mortality in old age. First, we measured NfL in blood of a human cohort and show that NfL is related to the neural blood proteome. Then we show an association of blood NfL to prospective mortality in centenarians and confirm the findings in nonagenarians. To further bolster the association of blood NfL with mortality, blood NfL was measured in a cohort of aging mice and in dietary restricted mice with a prolonged life-span.

RESULTS

Age-related increase of plasma NfL is linked to the plasma proteome of neural pathways

NfL levels were measured in blood plasma from a cohort of broad age range including nonagenarians and centenarians (n=122, 21–107 years of age; Table 1). We confirmed previous observations of a non-linear increase of NfL levels with age and an increased variability with aging[19] (Fig. 1a-c; Extended Data Fig. 1a-c). No effect of gender on NfL levels was found (Wilcoxon rank sum per decade, $p>0.05$).

To better understand the biological significance of blood NfL and its increase with age we then leveraged previously reported plasma proteome data of the same individuals using an aptamer technology[20]. Linear modeling controlled for age and gender revealed a significant correlation between log transformed NfL levels and 53 out of the 1,305 plasma proteins measured (Fig. 1d; Supplementary Table 1 and 2). The proteins most strongly associated with the age-related NfL change included Unc-5 Netrin Receptor D (*UNC5D*), which plays a role in neuronal cell survival and in axon guidance[21]; PILR Alpha Associated Neural Protein (*PIANP*), which acts as a ligand in neuronal tissues for *PILRA*, a gene associated with Alzheimer's Disease[22,23]; Cell Adhesion Molecule 3 (*CADM3*), a brain-specific protein related to the calcium-independent cell-cell adhesion molecules and involved in myelination[24]; TNF Receptor Superfamily Member 1A (*TNFRSF1A*), which plays a role in inflammation and cell death[25], and Beta-2-Microglobulin (*B2M*), a pro-aging protein impairing cognitive function and neurogenesis[26].

To determine the biological meaning of this association a Sliding Enrichment Pathways Analysis (SEPA) for the top 10 to top 200 proteins associated with blood NfL was calculated using three of the most comprehensive biological annotation and pathway databases (GO, KEGG, and Reactome). Within the top proteins, enrichment for those involved in death receptor activity (apoptosis-activating receptor activity), and more specifically in tumor necrosis factor-activated receptor activity were found (Fig. 1e). In addition, axon guidance and EPH-ephrin

signaling pathways emerged, which are altered with aging and most strongly in nonagenarians[20]. Although EPH-ephrin pathway and sub-pathways are associated with a variety of biological processes, Eph receptors and their ligands have important roles in synapse formation, function, and plasticity[27]. These results link plasma NfL to changes in other neuronal plasma proteins. Together with transcriptomics analyses in humans and mice showing a specific expression of NfL in the brain and in neurons (Extended Data Fig. 2), our findings suggest that plasma NfL levels are associated with pathways involved in neuronal function in the brain.

Plasma NfL is associated with mortality in centenarians

An independent cohort of centenarians was assessed for health status including the physical and cognitive composite parameters activity of daily living (ADL) and Mini-Mental State Examination (MMSE), respectively. From 135 centenarians (Table 1) blood was collected and blood donors were followed until death or up to 4 years after blood donation (Fig. 2a). MMSE and ADL measures indicated that 67% of the blood donors were not or only moderately impaired or disabled (Table 1), which is partly because the most disabled centenarians rarely donate blood (57% of participating centenarians donated blood). Nevertheless, this observation supports findings that at least some centenarians escape major age-related diseases[28,29]. NfL in plasma revealed a median concentration of 49 pg/ml and levels varied widely among the centenarians (Table 1). We also measured the phosphorylated form of neurofilament heavy chain (pNfH) and results showed again high variation among the centenarians (Table 1). For all measured parameters in Table 1 no significant gender differences were found except for creatinine as females had lower levels than males (median, females: 81.7, males: 107.3; quantile regression, p=0.011).

For survival analysis, we then stratified NfL and pNfH levels into quartiles (Fig. 2b,c). Using Kaplan-Meier survival estimates NfL exhibited a robust separation of the groups with subjects with low NfL levels surviving longer than subject with elevated levels. Using Cox regression analyses, NfL was strongly associated with 53% higher mortality per standard deviation (Table 2). No significant association was found for pNfH (Table 2) and thus pNfH was not further analyzed in the present study.

A similar survival analysis was done for ADL disability divided into no, moderate, or severe physical disability, and MMSE divided into no, mild, or severe cognitive impairment. For both measures Kaplan-Meier survival analysis found a separation of the groups (Extended Data Fig. 3) and Cox regression analyses revealed also for MMSE and ADL a significant association with mortality (Table 2). As expected, NfL was inversely correlated with MMSE categories but less so with ADL categories (Extended Data Fig. 3). In subsequent multivariate analysis NfL remained significant when adjusting for covariates including creatinine indicating that NfL blood levels reflect a biological process independent of muscle mass and putative kidney dysfunction (Table 2). Similarly, ADL disability also remained significant in the multivariate analysis, although the level of significance was slightly less than that of NfL (Table 2). Consistently, survival prediction assessed by area under the curve was better for NfL compared to MMSE and ADL (Extended Data Fig. 4).

To illustrate how robustly a combination of NfL and ADL disability jointly predict mortality in centenarians, we estimated how the chance of reaching 103 years of age increased with this combined measure. Centenarians with both elevated NfL (above median) and severe disability had 0% chance of reaching 103 years, whereas those with both lower NfL (below median) and disability scores had a 46% chance of reaching 103 years (Table 3).

Plasma NfL is also associated with mortality in nonagenarians

To replicate the association of blood NfL with mortality in another cohort of old age, plasma NfL was assessed in 93-year-old human subjects (n=180; Table 1). Nonagenarian blood donors were followed until death (Fig. 2d). NfL in plasma revealed a median concentration of 35 pg/ml. Cox regression analyses indicated a 22% higher mortality per standard deviation and NfL remained significant when adjusting for MMSE and ADL as covariates (Table 2). Similar to the centenarians, survival prediction was better for NfL compared to ADL and MMSE (Extended Data Fig. 4). Excluding in total three high NfL measures (above 300 pg/ml; outliers according to Grubbs test) from nonagenarians and centenarians revealed similar results in the multivariate comparison between NfL, MMSE and ADL and to some degree an even stronger association between NfL and mortality for the nonagenarians (Supplementary Table 3).

Age-related increase of plasma NfL in mice is attenuated through dietary restriction

Mice do not show signs of human-like age-related neurodegenerative diseases, but exhibit features of normal human brain aging[30-32]. To study whether plasma NfL also increases with age in mice, NfL was measured in 2-30 months old C57BL/6J mice. Similar to humans, an age-related increase was found with an increased variability at old ages (Fig. 3a-c; Extended Data Fig. 1d-f).

Dietary restriction, i.e. reduced food intake while avoiding malnutrition, has been shown to extend life span in many species including C57BL6/J (B6) mice or B6 F1 hybrids[33,34]. To study whether a reduction in age-specific mortality through dietary restriction is also reflected in reduced NfL levels, C3B6 mice were dietary restricted starting from 3 months of age and analyzed at 16, 20, and 28 months of age (Fig. 3d-f). Roughly 50% reduced plasma NfL levels were found compared to *ad libitum* fed mice at both 16 and 20 months of age (Fig. 3e). No such reduction in NfL levels were found at 28 months of age, albeit an age where 70% of *ad libitum* fed mice have died, potentially selecting the cohort in favor of individuals with lower NfL levels (Fig. 3f). These observation in mice suggest that the increase of blood NfL with aging also occurs in the absence of human-like age-related neurodegenerative diseases and that reduction of age-specific mortality is reflected in lower levels of blood NfL.

DISCUSSION

Multi-item scores of cognitive and physical fitness have been found to be major predictors of lifespan at old age[5-7]. Thus, the equal or even stronger association of the single neuronal protein NfL in plasma with survival in nonagenarians and centenarians suggests that deterioration of neuronal function contributes to late-life mortality. Indeed, recent human brain transcriptomics reported that extended life span (>85 years of age) is linked to inhibition of neural circuit activity with conserved pathways from human to non-mammalian species[3,4].

Increased blood NfL levels have largely been associated with neurological diseases[9,13-15]. Most relevant, in age-related neurodegenerative diseases NfL increases in blood already at presymptomatic stages[35,36]. Thus, the association of increased NfL levels with mortality in the oldest old may indicate clinically silent neuropathological changes that are common in postmortem centenarian brains[37,38].

However, even young adults have NfL blood levels of 7–10 pg/ml, and thus NfL levels in blood are difficult to explain solely with disease-associated neurodegeneration. NfL has been found in synapses, and it modulates glutamatergic transmission[39,40] and its release into the CSF and blood may mirror remodeling and activity of neural circuits. Thus, besides apparently silent

CNS damage, increased blood NfL with aging may be associated with altered neural network activity[4]. The finding that reduction of mortality through diet-restriction is reflected in lower plasma NfL levels supports the involvement of nervous system dysfunction in old age mortality. The contribution of peripheral nerve NfL to the total blood NfL is not known[10].

Predicting mortality at higher ages is important for medical decision making. A blood-based biomarker may complement current geriatric assessments that are time-consuming and often difficult in the oldest old. In a previous cohort of centenarians, TNF-alpha in blood was also associated with mortality per standard deviation (SD), albeit with a lower hazard ratio compared to 1 SD of NfL in the current work[41]. For NfL measurements, minimal blood volume is needed and NfL immunosassays are becoming routine in clinical settings. Although longitudinal NfL measurements (rate of change) have been found to be superior over single measurement for predicting disease progression[35], repeated blood donations, albeit desirable, are difficult to obtain at extreme ages.

Our findings in nonagenarian and centenarians may or may not be generalized to other age groups, in which the association of fundamental mechanism of longevity to mortality prediction is influenced by earlier onset diseases and to a minor degree also by accidental death[42]. Moreover, although centenarians and nonagenarians were recruited among participants alive at enrollment, not all participated and completed the assessment or donated blood, which is likely a selection bias towards the healthiest individuals. Nevertheless, since we defined cognitive impairment as MMSE <24, it is possible that up to 15% more centenarians and nonagenarians could be classified as having dementia using more extensive classifications (e.g. https://www.cochrane.org/CD011145/DEMENTIA_mini-mental-state-examination-mmse-detection-dementia-people-aged-over-65). Finally, the present study focused on one specific blood biomarker and future studies will need to elucidate the relationship between blood NfL and other previously described biomarkers signatures of aging and mortality risk such as the multi-item scales of metabolic biomarkers[43-48].

In conclusion, our observations suggest that nervous system functional deterioration contributes to late life mortality and that blood NfL maybe a suitable single fluid biomarker for prospective all-cause mortality.

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METHODS

Age Cohorts

One hundred twenty-two human plasma samples (60 males and 62 females) from 4 different sub-cohorts (VASeattle n=52; age 21–88, PRIN06 n=23; age 56–102, PRIN09 n=21; age 56–107, GEHA n=26; age 89–104) were used for this study. The characteristics of these four sub-cohorts have been described previously[20]. Note that out of the 171 blood samples described previously and measured with the SOMAscan assay[20], only 122 had enough sample volume left and were used for the present study. The age ranged from 21 to 107 years with a median of 72 (1st quartile=60, 3rd quartile=90). Written informed consent was obtained from each subject. The IRB has determined that this research does not meet the definition of human subject research per STANFORD's HRPP policy and no IRB approval was required for measuring these samples with the SOMAscan assay.

Centenarian Cohort

The Danish 1915 West birth cohort study included all who were born in 1915 and alive on their 100th birthday and living in the Western part of Denmark. Centenarians were assessed in their own home or in care homes. This comprised of 303 centenarians and of these 238 participated (185 in-person and 53 proxy interviews) in a health survey conducted by a geriatrician together with a geriatric nurse. The study was approved by the Danish ethical committees (Ethics approval number S-20140099). All participants gave written informed consent. The health measures included measures of activity of daily living (ADL) and cognitive performance (MMSE)[49,50]. ADL was assessed by self-report of eleven physical tasks and of these the five-item assessing bathing, dressing, toileting, transfer and feeding was used to derive the ADL disability score which is based on the Katz ADL index[51]. MMSE was divided into three categories: 0–17 (severe cognitive impairment), 18–23 (mild cognitive impairment) and 24–30 (no cognitive impairment). From the 185 in-person-interviewed centenarians 131 blood samples could be collected. In addition, 4 centenarians from a pilot study performed in 2014 who were born in 1914 were included in the study giving a total of 135 centenarians with blood sample.

Date of death of the centenarians was followed up until January 2019 by linkage with the Danish central registries via the unique identification number assigned at birth to all Danish citizens[52]. No one immigrated in the follow-up period so there was no loss to follow-up. The study was approved from the Danish Data Protection Agency (Copenhagen, Denmark; Trial number 2016-41-4552) as well as from the Ethics Committee at the medical faculty of the University of Tübingen, Germany (Project-Number 837/2017BO2).

Nonagenarian Cohort

The Danish 1905 birth cohort included all who were born in 1905 and alive 1. August 1998. This comprised of 3,725 nonagenarians and of these 2,262 participated in a health survey conducted by trained lay persons (1,884 in-person and 448 proxy interviews). The study was approved by the Danish ethical committees (Ethics approval number VF20040240). All participants gave written informed consent. The health measures included the same measures as for the centenarian cohort, which were the ADL disability score and the MMSE (see above). Whole blood samples were collected by a physician shortly after the in-person interview of those living in Funen. This comprised of 231 nonagenarians and 180 donated whole blood samples.

Date of death of the nonagenarians was followed as described for the centenarians, i.e. until January 2019 by linkage with the Danish central registries via the unique identification number assigned at birth to all Danish citizens[52]. The study was approved from the Danish Data Protection Agency (Copenhagen, Denmark; Trial number 2020-522-0256) as well as from the Ethics Committee at the medical faculty of the University of Tübingen, Germany (Project-Number 838/2017BO2).

Genotyping of APOE variants

Genotyping of the *APOE* variants rs429358 and rs7412 in the blood from the centenarian and nonagenarian cohorts was carried out using predesigned TaqMan® SNP Genotyping assays for 98 individual who participated a survey five years earlier (Applied Biosystems, Foster City, CA, USA)[53]. Only 4 subjects were genotyped homozygous for *APOE e4*.

Mouse cohorts and dietary restriction protocol

Male and female C57BL/6J mice were group-housed and aged at the Hertie Institute for Clinical Brain Research (Tübingen, Germany). All mice were kept under specific pathogen-free conditions with 22±2°C temperature, 55±10% humidity, a 12 h light/dark cycle, and with food and water *ad libitum*. Commercially available rodent chow (Altromin #1324) was used. For environmental enrichment, mice had constant access to nesting material and chew sticks. Mice were euthanized at defined ages or when they reached the humane endpoint (https://www.humane-endpoints.info/en). All experiments were done in accordance with the veterinary office regulations of Baden-Württemberg (Germany), and were approved by the local Animal Care and Use Committees.

Dietary restriction (DR) was performed in accordance with the recommendations and guidelines of the Federation of the European Laboratory Animal Science Association (FELASA), with all protocols approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany (reference no. 84-02.04.2015.A437). Female F_1 hybrid mice (C3B6F1) were generated in the mouse facility of the Max-Planck-Institute for Biology of Ageing by crossing C3H/HeOuJ females with C57BL/6NCrl males (strain codes 626 and 027, respectively, Charles River Laboratories). Litter size was adjusted to a maximum of 8 pups by removing male pups within 3 d of birth. Pups were weaned at 3–4 weeks of age and were randomly assigned to cages upon weaning. Animals were housed in groups of 5 females in individually ventilated cages under specific-pathogen-free conditions with constant temperature (21 °C), 50–60% humidity and a 12-hour light–dark cycle. For environmental enrichment, mice had constant access to nesting material and chew sticks. All mice received commercially available rodent chow (ssniff R/M-H autoclavable, ssniff Spezialdiäten) and were provided with water ad libitum. Mice were randomly assigned to cages and cages were randomly assigned to dietary restriction (DR) or *ad libitum* (AL) feeding. Food consumption of the AL group was measured weekly, and DR animals received 60% of the food amount consumed by AL animals. DR treatment was started at 12 weeks of age. DR animals were fed once per day, and all animals were checked daily for their well-being and any deaths. Mice were euthanized at the ages of 16, 20 and 28 months by cervical dislocation.

Blood collection in humans and mice

Blood from the centenarians and nonagenarians was collected in 10 ml EDTA tubes (Venoject) and centrifuged at 1,000 x g for 15 min on the same day the blood was donated. Plasma was then aliquoted and stored at –80 °C. Similar processing (centrifugation at 1,500 x g for 10 minutes) was performed for the samples of the age cohort.

Blood from the mice was obtained for the aging study by anesthetizing the mice with a mixture of ketamine (250 mg/kg) and xylazine (20 mg/kg) with blood collection by heart puncture with EDTA-treated syringes and transfer into EDTA-coated vials (MiniCollect® K3EDTA, Greiner Bio-one, Kremsmünster, Austria). To obtain plasma vials were centrifuged at 2000 *x* g for 10 min at 4 °C, assessed for hemolysis, aliquoted and stored at -80 °C in our in-house mouse biobank. All available biobank plasma samples were used (n=114). For the dietary restriction study, mice were killed via cervical dislocation and decapitated to collect blood in an Eppendorf tube using a funnel. 1µl 0.5M EDTA pH 7.45 was added per 100 µl of blood, mixed and centrifuged at 1500 *x* g for 10 min at 4 °C. Plasma was transferred into fresh tubes and subsequently snap-frozen in liquid nitrogen and stored for further analysis. The sample sizes (n=5 for the 16 month-old groups; n=10 for the 20 month-old groups; n=10 for the 28 month-old groups) were the total blood samples available.

NfL and pNfH measurements

Plasma NfL concentrations were determined by Single Molecule Array (Simoa) technology using the Simoa™ NF-Light Advantage Kit according to manufacturer's instructions (Quanterix, Billerica, MA). In addition, for the Centenarian cohort plasma pNfH concentrations were measured using the Simoa™ pNF-heavy Discovery Kit (Quanterix). Before the measurements all human and mouse plasma samples were centrifuged for 5 min at 10,000 x g and 4 °C and the supernatant was used for the measurement. NfL samples of the centenarian and nonagenarian cohorts were diluted 1:4 with Simoa NF-Light sample diluent. Samples of the Age cohort were diluted up to 1:16 (for samples with less volume; no significant effect of the dilution factor was found, multivariate linear regression analysis of log-transformed NfL values with *age* and *sex* as covariates, F(1,118)=0.0006, P=0,98). Mouse plasma samples were diluted 1:10 (mouse aging study) or 1:20 (dietary restriction study, less sample volume) with Simoa NF-Light sample diluent. For pNfH, samples of the Centenarian cohort were diluted 1:4 with Simoa pNF-heavy sample diluent. All samples were measured on a Simoa HD-1 or HD-X Analyzer in duplicates except for one NfL and one pNfH sample, for which only one technical replicate could be measured. For three other samples Quanterix was able to recover the second technical replicate on the basis of the original images that were taken within the Simoa instrument. Within the mouse cohorts for five samples only one technical replicate could be measured and one sample only had enough volume for a single measurement. Internal reference samples were used as controls on every plate. All human samples had a random code and the measurements of human and mouse samples were done blinded.

SOMAscan® assay

The SOMAscan platform was used to quantify relative levels of 1,305 human plasma proteins in the samples of the human Age cohort. This platform is based on modified single-stranded DNA (SOMAmers) binding to specific protein targets with high sensitivity and specificity as described previously[54].

Aliquots (150 µl) of plasma were shipped to Somalogic Inc (Boulder, CO, US) on dry ice. Plasma samples were analyzed in three different batches. HybNorm.plateScale.medNorm data files were bridged to data from the 1st batch of samples using calibrators. In addition to the Age cohort samples, 12 additional aliquots from 4 of these samples were measured in the different batches to estimate intra- and inter-assay variability. Reproducibility analysis based on these technical replicates showed a median CV of ~5% within runs and 10% between runs. Relative Fluorescent Units (RFU) of each plasma protein were log10-transformed. The SOMAscan proteomics data have been described in detail previously and are available in Supplementary table 16 of a previous study[20].

Gene expression data

NfL gene (Nefl) expression in mouse tissues was assessed using the shiny app created by Schaum and collaborators[55]. In this study, bulk RNA-sequencing from 17 organ types isolated from C57BL/6JN mice was performed. To determine which specific cell types were expressing NfL in the mouse brain, the interface developed by the Tabula Muris Senis consortium was used[56]. NEFL in human tissue was obtained from the human gene expression data from the GTEx portal website (http://www.gtexportal.org/).

Statistical analysis

No statistical methods were used to pre-determine sample sizes. Sample sizes were given by the available samples from the human and mouse biobanks (see above for numbers).

Association between NfL and the plasma proteome

To determine the association between NfL and other proteins measured in plasma using the SOMAscan assay, we used the following linear model:

$$\text{log10Protein level} \sim \alpha + \beta 1\ \text{logNfL} + \beta 2\ \text{Age} + \beta 3\ \text{Sex} + \beta 4\ \text{Subcohort} + \varepsilon$$

Type II sum of squares (SS) were calculated using the ANOVA function of the R car package (R version 3.6.1 with packages: car (3.0-3), topGO (2.36), clusterProfiler(3.12.0) , org.Hs.eg.db (3.8.2)[57]. This SS type tests for each main effect after the other main effects. Q-values were estimated using Benjamini–Hochberg approach[58].

Pathway analysis

To determine the biological meaning of the plasma proteins associated with NfL, the top 200 proteins were ranked based on their significance and queried Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome[59-61]. Using these databases, enrichment for pathways using a Sliding Pathway analysis (SEPA) approach for the top 10 to top 200 proteins in increments of 1 protein was used. The 1,305 proteins measured by SomaScan served as the background set of proteins against which to test for over-representation. To identify the most biologically meaningful terms and pathways, only those consistently highly significant proteins are reported (q<0.05 for at least 5 different incremental list of proteins). SEPA has been described in detail previously[20]. Note that the 1,305 proteins measured in this study cover ~80% of the human GO terms and ~90% of the Reactome and KEGG pathways with more than ten genes."

NfL and pNfH in centenarians and nonagenarian

Quantile regression of the median was performed to estimate the association between sex, MMSE, ADL disability, and NfL and pNfH. The analyses were also performed with linear regression with log-transformed NfL and pNfH showing similar results as the quantile regression.

Kaplan-Meier survival estimates were performed to illustrate the association between survival and NfL, pNfH, creatinine, MMSE and ADL disability in the centenarian cohort. Cox regression models were used to determine the size of the associations between survival and the biomarkers and the health indicators for the centenarian cohort. The proportional hazards assumption, underlying the Cox regression models, was not compromised in the applied models (tested using the Schoenfeld residual test). Hazard ratios were estimated per 1 standard deviation except for ADL and APOE where categories were used. Analyses were both performed as univariate and multivariate models. The Kaplan-Meier survival estimates and Cox regression analysis was repeated in the nonagenarian cohort for NfL, MMSE, ADL and APOE. For both the centenarian and nonagenarian a time-dependent area under the curve (AUC) from the ROC curves was estimated to evaluate the prediction of survival over time. According to

Grubbs test, 3 outliers of NfL were observed and the survival analysis of NfL, MMSE and ADL was repeated excluding these outliers. Subsequently, for the centenarian the chance of surviving to 103 years of age was estimated and how it changes with a joint measure of NfL and ADL disability. STATA 16.0 (Stockholm, Sweden) was used for all analyses.

NfL in dietary restricted mice

Data distribution was assessed with the Shapiro-Wilk test. Nonnormally distributed variables were logarithmic-transformed and analyzed with parametric statistical tests (ANOVA with Bonferroni's post hoc test for pairwise comparison). JMP software (Version 14.3; SAS Institute Inc., Cary, North Carolina, USA) was used for statistical analysis. Graphical displays were generated with GraphPad Prism version 6 (GraphPad Software, La Jolla, California USA).

DATA AVAILABILITY

The SOMAscan proteomics data are available in Supplementary Table 16 of a previous study[20]. According to Danish legislation, transfer and sharing of individual-level data requires prior approval from the Danish Data Protection Agency and requires that data sharing requests are dealt with on a case-by-case basis. For this reason, the data cannot be deposited in a public database and data presentation at an individual level is avoided. However, we welcome any enquiries regarding collaboration and individual requests for data sharing.

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ACKNOWLEDEMENT

We would like to thank Gerhard Eschweiler, Bettina Wegenast-Braun, Oliver Preische, Thomas Gasser (Tübingen, Germany), Lary Walker (Atlanta, GA), Oliver Hahn (Stanford) and all other members of our laboratories for helpful comments. This work was made possible by a generous grant from Cure Alzheimer's Fund (S.A.K. and M.J.), the National Institute on Aging (DP1-AG053015 to T.W.-C.), and the NOMIS Foundation (T.W.-C.). The Danish Aging Research Center is supported by a grant from the VELUX Foundation and funded the centenarian study. Most important, the altruism of the participants is greatly appreciated.

AUTHOR CONTRIBUTION

T.W-C., J. M-F. and M.J. designed the overall study. S.G. and L.P. designed and supervised the study of the effects of dietary restriction. S.A.K., B.L., M.T., A.A., L.M.H., C.B. performed the experimental work and the statistical analysis. D.B., B.J., K.C., were involved in sample and data collection. T.W-C., J. M-F. and M.J. wrote the manuscript with the help of all co-authors. This work was partly done while M.J. was a Guest Professor in Stanford.

COMPETING INTERESTS

The authors declare no competing interests

	Age cohort	Centenarian cohort	Nonagenarian Cohort
N of participants	122	135	180
Gender			
Female (%):Male (%)	62 (51):60 (49)	106 (79):29 (21)	136 (76):44 (24)
Age [years]			
median (range)	72 (21–107)	100	92.9 (92.2-93.7)
Survival			
Chance of reaching 100, (%)	—	—	16.1
Chance of reaching 103, (%)		23.0	—
Mortality			
Rate per 100 person-year	—	43.2	25.1
MMSE			
median (IQR)	29 (3)	20 (9.5)	23 (7.5)
Not impaired [24–30], n (%)	100 (82)	46 (34.1)	77 (42.8)
Moderately impaired [18–23], n (%)	11 (9)	45 (33.3)	62 (34.4)
Severely impaired [0–17], n (%)	9 (7.4)	37 (27.4)	37 (20.6)
Missing value	2 (1.6)	7 (5.2)	4 (2.2)
ADL			
Not disabled, n (%)	—	38 (28.1)	90 (50.0)
Moderately disabled, n (%)		53 (39.3)	68 (37.8)
Severely disabled, n (%)		44 (32.6)	22 (12.2)
Plasma NfL [pg/ml]			
mean (SD), median (IQR)	23.9 (27.2), 15.8 (17.6)	62.5 (50.2), 49.4 (34.3)	44.9 (38.5), 34.8 (26.6)
Plasma pNfH [pg/ml]			
mean (SD), median (IQR)	—	384.7 (310.3), 342.8 (345.7)	—
Plasma Creatinine [pg/ml]			
mean (SD), median (IQR)	—	98.3 (39.2), 89.7 (46.3)	—
ApoE status[2]			
ε4 carriers (%):none ε4 (%)	—	24 (24.5):74 (75.5)	43 (23.9):134 (74.4)

[1]Survival status of centenarians and nonagenarians in January 2019

[2]ApoE status was evaluated in 98 out of the 135 centenarians using blood from a health survey at the age of 95 years. ApoE status was obtained from 177 of the 180 nonagenarians.

Table 1. Characteristics of the Age, Centenarian, and Nonagenarian cohorts.

	Centenarian cohort		Nonagenarian cohort	
	HR (95% CI)	*p*-value*	HR (95% CI)	*p*-value*
Univariate regression[1]				
NfL (1 SD increase)	1.53 (1.29, 1.82)	1.10×10^{-6}	1.22 (1.10, 1.35)	2.01×10^{-4}
pNfH (1 SD increase)	1.18 (0.98, 1.41)	0.0744	—	
Creatinine (1 SD increase)	1.23 (1.01, 1.49)	0.0386	—	
MMSE (1 SD decrease)	1.43 (1.18, 1.75)	3.55×10^{-4}	1.27 (1.10, 1.48)	0.0016
ADL (1 category increase)[2]	1.59 (1.23, 2.04)	3.54×10^{-4}	1.57 (1.25, 1.97)	1.11×10^{-4}
ApoE (ε4 carriers)	1.07 (0.66, 1.75)	0.7795	1.09 (0.77, 1.54)	0.6261
Multivariate analyses[1]				
NfL (1 SD increase)	1.34 (1.08, 1,68)	0.0092	1.20 (1.07, 1.35)	0.0019
pNfH (1 SD increase)	1.04 (0.85, 1.28)	0.6830	—	
Creatinine (1 SD increase)	1.03 (0.81, 1.31)	0.7848	—	
MMSE (1 SD decrease)	1.22 (0.98, 1.53)	0.0768	1.18 (0.998, 1.39)	0.0522
ADL (1 category increase)[2]	1.40 (1.04, 1.88)	0.0274	1.30 (1.01, 1.68)	0.0406

Hazard ratios (HR) are per standard deviation (SD) for the continuous phenotypes, per increase in categories for ADL, and per ε4 carriers vs. non-carriers for ApoE.

* two-sided *p*-values (no adjustment for multiple comparison)

[1]adjusted for gender

[2]categories: not disabled, moderately disabled, and severely disabled

Table 2. Associations between plasma NfL, functional indicators, and mortality among centenarians and nonagenarians estimated from Cox regression models.

Plasma NfL*	ADL	n	Likelihood of reaching 103 years (95% CI)
> 72.6 pg/ml	-	33	6.1 (0.7, 20.2)
49.4-72.6 pg/ml	-	34	14.7 (5.0; 31.1)
38.3-49.4 pg/ml	-	34	35.3 (19.7, 53.5)
< 38.3 pg/ml	-	34	35.3 (19.7, 53.5)
-	Severely disabled	44	11.4 (3.8, 24.6)
-	Moderately disabled	53	22.6 (12.3, 36.2)
-	Not disabled	38	36.8 (21.8, 54.0)
> 49.4 pg/ml	Severely disabled	24	0.0 (0.0, 14.2)
> 49.4 pg/ml	Moderately disabled	29	13.8 (3.9, 31.7)
> 49.4 pg/ml	Not disabled	14	21.4 (4.7, 50.8)
≤ 49.4 pg/ml	Severely disabled	20	25.0 (8.7, 49.1)
≤ 49.4 pg/ml	Moderately disabled	24	33.3 (15.6, 55.3)
≤ 49.4 pg/ml	Not disabled	24	45.8 (25.6, 67.2)

* NfL was divided into 4 equal size groups (quartiles). For the combined prediction, NfL was divided into two equal size groups (below or above the median).

Table 3. Prediction model for centenarians (100 years of age) of reaching 103 years of age

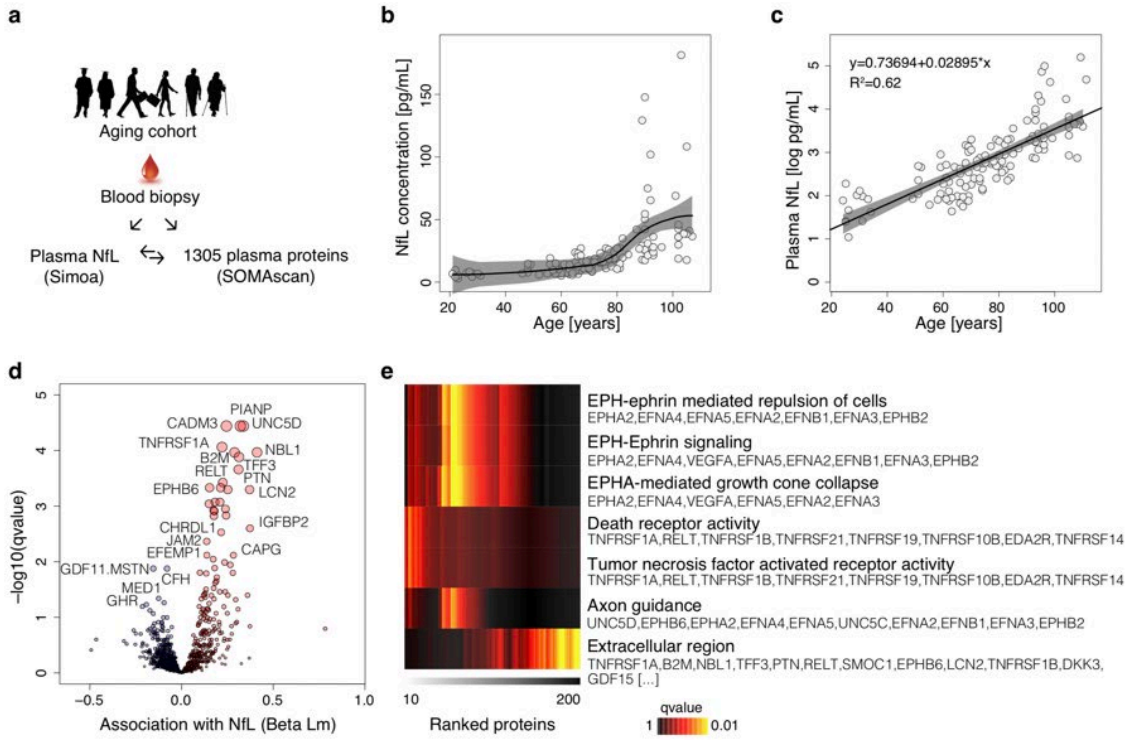


Figure 1: *Linkage of the age-related changes of plasma NfL to the plasma proteome of neural pathways*. **(a)** Schematic representation of the association analysis between Neurofilament Light chain (NfL) and the plasma proteome. EDTA plasma samples (n=122) were measured using the SOMAscan and Simoa platforms (see Online Methods). **(b)** Absolute NfL concentration with local polynomial regression fitting using LOESS. The black line represents LOESS fitted values and the shaded areas represent the standard errors around the model estimates. **(c)** Plasma NfL values (log-transformed) as a function of age. The black line represents fitted values using a linear model and the shaded areas represent fitted values within the 95% confidence interval of the slope estimate. Age explains 62% (R^2) of plasma NfL values. Adjusting the linear model for gender and sub-cohorts increases the adjusted R^2 to 66%. For a more detailed analysis see Extended Data Fig. 1. **(d)** Association between plasma NfL and the 1305 plasma proteins. Plasma proteins levels measured by SOMAscan were log10-transformed and levels of each plasma protein were modeled as function of the effect of log NfL, age, sex, and sub-cohort. Beta NfL and corresponding adjusted *p*-values are represented by a volcano plot. Red and black colors distinguish proteins increased and decreased with NfL, respectively. **(e)** Pathways associated with NfL identified by Sliding Enrichment Pathway Analysis (SEPA). Enrichment for pathways in the top 10 to top 200 proteins was tested in increments of 1 protein. Only pathways with a relatively large number of proteins annotated in the top 200 hits (at least 5 proteins) and consistently highly significant (q<0.05 for at least 5 different incremental list of proteins) are represented.

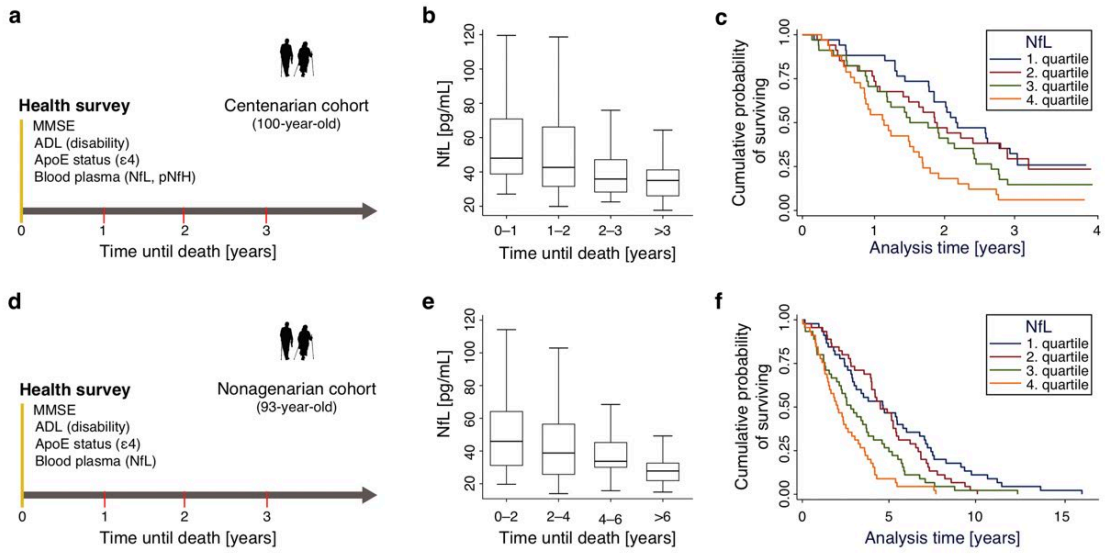


Figure 2. *Plasma NfL is associated with survival in centenarians and nonagenarians.* **(a)** Health survey in centenarians (n=135) including Mini-Mental State Examination (MMSE), assessment of activity of daily living (ADL), ApoE status, and blood collection for measurement of plasma NfL and pNfH preceding up to 4-year follow-up of survival status. **(b)** Plasma NfL concentrations (Box plots showing center line as median, the box as the first and third quartile and the whiskers as the adjacent values which are the largest observation that is less or equal than the third quartile + 1.5 x interquartile range and the lowest observation that is greater or equal than the first quartile - 1.5 x interquartile range) in centenarians that died within 0-1 (n=37), 1-2 (n=41), or 2-3 (n=30) years after blood donation and the ones that were still alive (>3 years) (n=27). **(c)** Kaplan-Meier survival curves for centenarians with plasma NfL concentrations <38.3 pg/mL (1st quartile, q1, n=34, blue), 38.3–49.4 pg/mL (q2, n=34, purple), 49.4–72.6 pg/mL (q3, n=34, green), and >72.6 pg/mL (q4, n=33, yellow). For Cox regression analysis see Table 2. **(d)** Health survey of 93-year-old nonagenarians (n=180) including MMSE, ADL, ApoE status, and blood collection for measurement of plasma NfL preceding up to 15-year follow-up of survival status. **(e)** Plasma NfL concentrations (Box plots as in panel b) in nonagenarians that died within 0-2 (n=54), 2-4 (n=50), or 4-6 (n=38) years after blood donation and the ones that were still alive after 7 years (n=38). **(f)** Kaplan-Meier survival curves for nonagenarians with plasma NfL concentrations <25.5 pg/mL (1st quartile, q1, n=45, blue), 25.5–34.8 pg/mL (q2, n=45, purple), 34.8–51.9 pg/mL (q3, n=45, green), and >51.9 pg/mL (q4, n=45, yellow). For Cox regression analysis see Table 2.

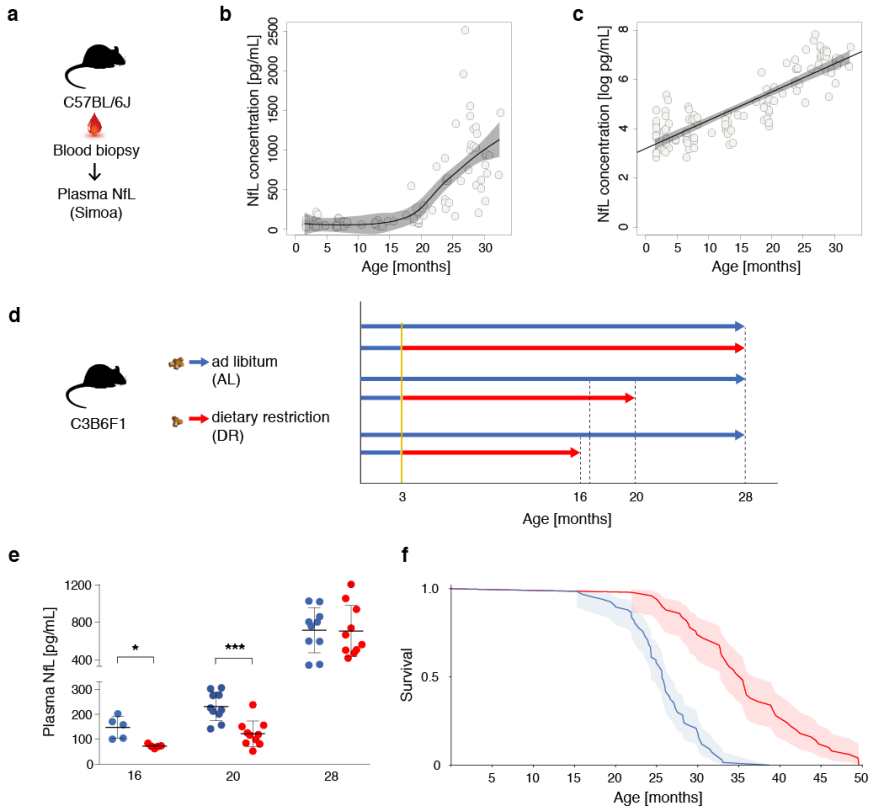
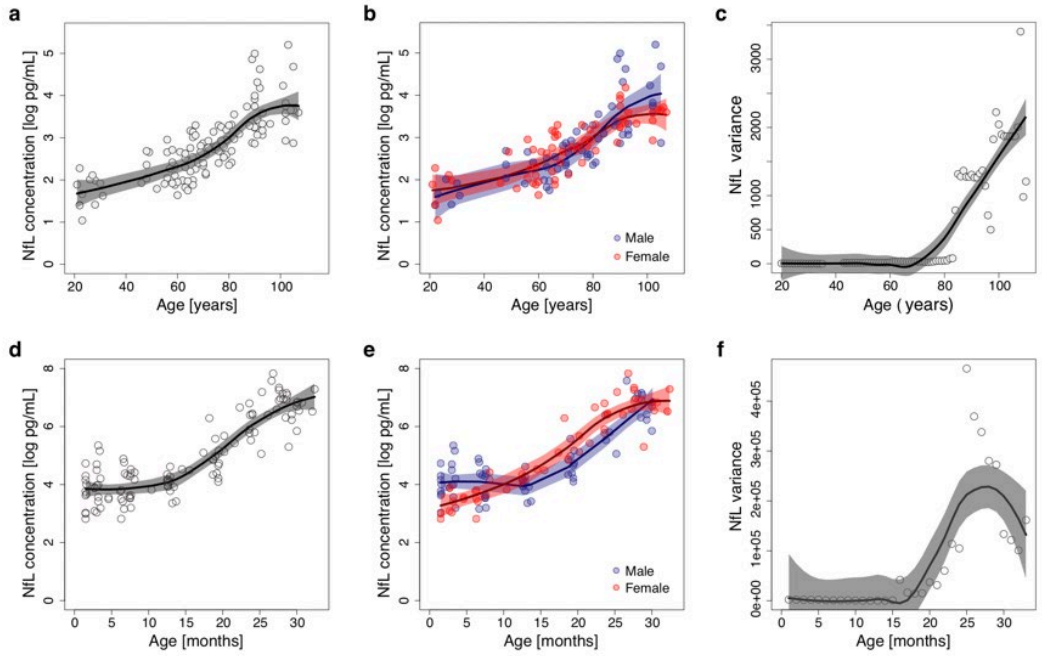
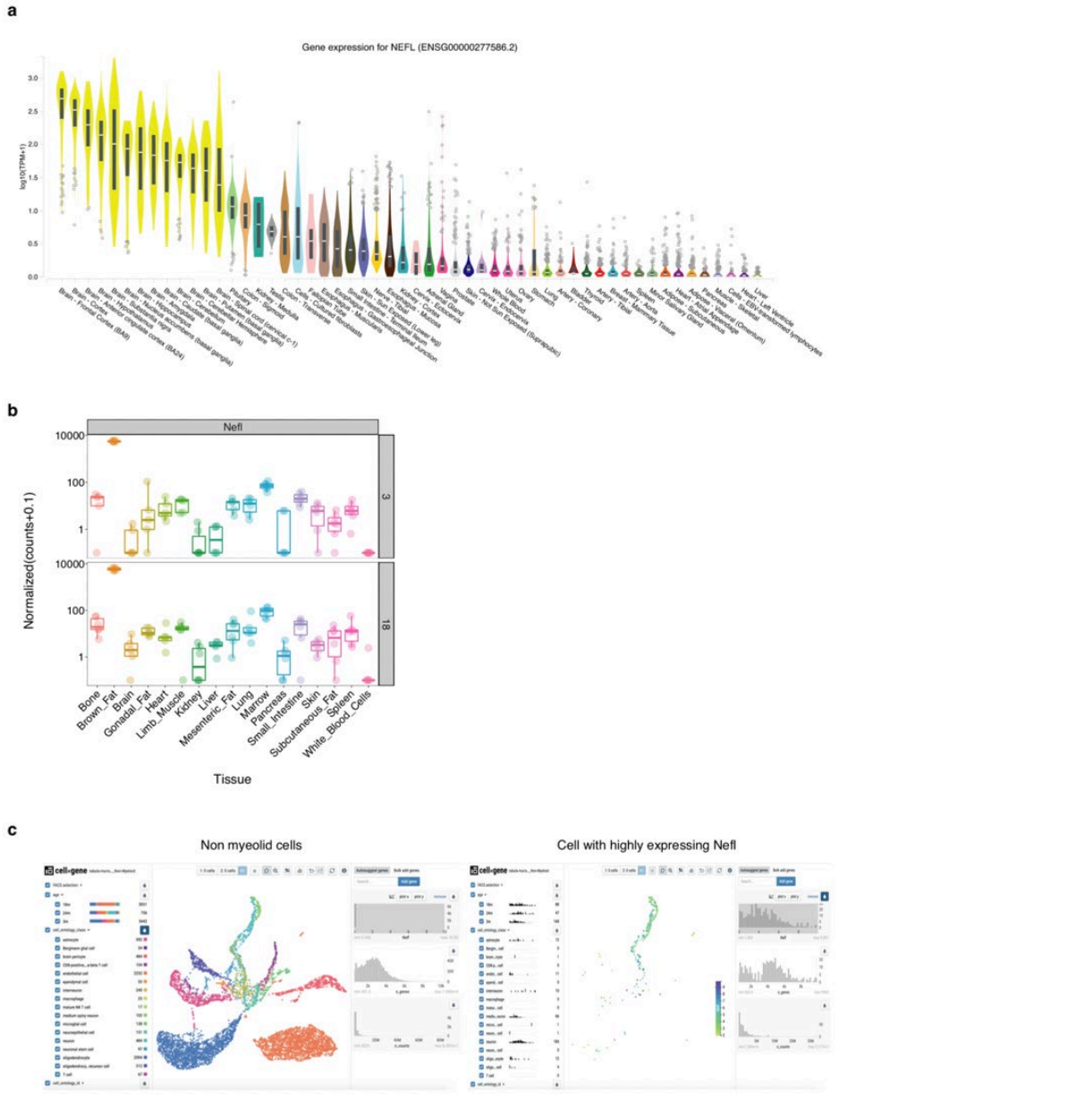


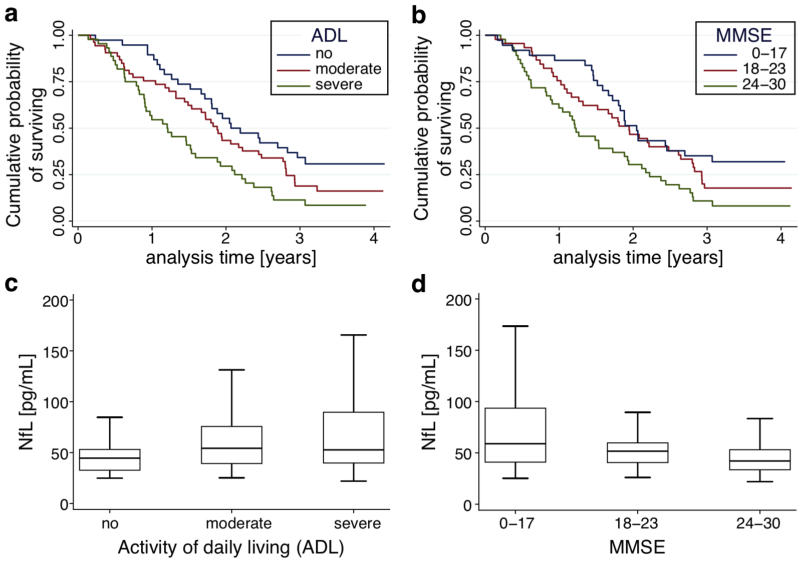
Figure 3. *Age-related increase of plasma NfL in mice is attenuated through dietary restriction*. **(a)** Plasma NfL was measured in aging C57BL/6J (B6) mice (n=114). **(b)** Absolute NfL concentration with local polynomial regression fitting using LOESS. The black line represents LOESS fitted values and the shaded areas represent the standard errors around the model estimates. **(c)** Log-transformed NfL concentration. The black line represents fitted values using a linear model and the shaded areas represent fitted values within the 95% confidence interval of the slope estimate. Age explains 73.7% (R^2) of plasma log NfL values. Adjusting the model for gender (males, n=65; females, n=49) does not increase the adjusted R^2 (73.5%). For a more detailed analysis see Extended Data Fig. 1. **(d)** Schematic representation of dietary restriction paradigm using female C3B6F1 mice. Starting at 3 months of age mice were randomly assigned to a dietary restriction (DR) or *ad libitum* (AL) feeding. Analysis was done at 16, 20, and 28 months of age. **(e)** Plasma NfL was significantly reduced in the DR compared to AL mice at 16 months of age (N=5/group) and 20 months of age (N=10/group), but not 28 months of age (N=10/group). Two-way ANOVA of log-transformed values, interaction *age group x diet,* F(2,48)=5.65; P=0.0065; Bonferroni´s *post hoc* test for DR vs. AL at 16 and 20 months, **p*=0.0101 and *** *p*=0.0002, respectively. Means ± SD are shown. **(f)** In a parallel cohort survival of female C3B6F1 mice was measured. Median lifespan of DR mice (n=50; 1058 days ≙ 34.8 months) was increased by 37.5% compared to AL (n=68; 770 days ≙ 25.3 months) animals (p=3.65 x 10^{-16}, Log-rank test). Shaded areas indicate 95% confidence intervals.



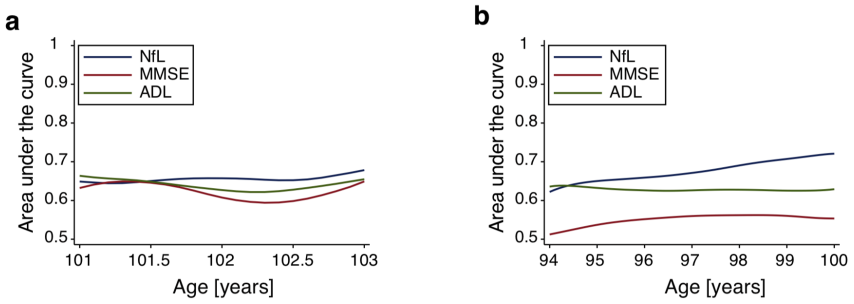
Extended Data Figure 1. *Plasma NfL trajectory with age in humans and mice.* **(a-c)** Plasma NfL concentration of the human age cohort presented in Figure 1b. Scatter plots for **(a)** log NfL concentration, **(b)** log NfL concentration for females versus males, and **(c)** NfL variance in plasma with aging (n=122). NfL variance was calculated using a 10-year sliding window. Local polynomial regression fitting using LOESS was done. Thick lines represent LOESS fitted values and shaded areas representing the standard errors around the model estimates. Although the cohort appears too small for a reliable description of the age-related changes, the data are consistent with previous work of a slow and more linear increase until approximately 70 years of age, followed by a much more rapid increase (Khalil, M.*, et al.* Serum neurofilament light levels in normal aging and their association with morphologic brain changes. *Nat Commun* **11**, 812, 2020). Interestingly, the present data may suggest that this rapid increase reaches a plateau in nonagenarians and centenarians. It is possible that at these ages individuals with very high NfL levels die out and thus reduce the further increase on a population level (Christensen, K., McGue, M., Petersen, I., Jeune, B. & Vaupel, J.W. Exceptional longevity does not result in excessive levels of disability. *Proc Natl Acad Sci U S A* **105**, 13274, 2008). **(d-f)** Plasma NfL concentration of the aging C57BL/6J mouse cohort presented in Figure 5a-c. Scatter plots for **(d)** log NfL concentration; **(e)** log NfL concentration for females versus males; and **(f)** NfL variance in plasma with aging (n=114). NfL variance was calculated using a 5-month sliding window. Local polynomial regression fitting using LOESS was done. Thick lines represent LOESS fitted values and shaded areas represent the standard errors around the model estimates. Similar to the human data, a slow and more linear increase until approximately 15–18 months of age is followed by a much more rapid increase with an increased variance towards the end of the lifespan.



Extended Data Figure 2. *NfL gene expression in tissues and cells.* **(a)** NfL gene expression in humans from the GTEx consortium (see Online Methods). NEFL is highly expressed in all brain regions. In all other tissues the expression is lower. **(b)** Tissue-specific NfL gene expression in C57BL/6JN mice (see Methods). Again, Nefl is highly expressed in brain and lowly expressed in other tissues. Data represent mice at 3 months of age (top) and 18 months of age (bottom). (Box plots showing center line as the median, the box as the first and third quartile and the whiskers as the adjacent values which are the largest observation that is less or equal than the third quartile + 1.5 x interquartile range and the lowest observation that is greater or equal than the first quartile - 1.5 x interquartile range). **(c)** NfL gene expression in non-myeloid brain cells from the Tabula Muris Senis consortium (see Online Methods). The FACS data is visualized using a UMAP. Murine brain cells are color-coded according cell ontology class (left). Cells expressing Nefl (log cpm threshold of 1.954) are visualized on the right where 22 % (66/303) of the cells correspond to medium spiny neurons and 61% (185/303) to neurons.



Extended Data Figure 3. *Physical activity and cognitive ability in centenarians as predictors of survival and their association with plasma NfL.* **(a, b)** Kaplan-Meier survival curve for Activity of Daily Living (ADL: no (n=38), moderate (n=53), severe disability(n=44)) and Mini-Mental State Estimation (MMSE: 0–17 (n=37), 18–23 (n=45), 24–30 (n=46)). For Cox regression analysis see Table 2. **(c, d)** Plasma NfL concentrations (Box plots showing center line as the median, the box as the first and third quartile and the whiskers as the adjacent values which are the largest observation that is less or equal than the third quartile + 1.5 x interquartile range and the lowest observation that is greater or equal than the first quartile - 1.5 x interquartile range) for the different ADL and MMSE categories. The association between NfL and ADL across all three groups was not significant with an estimated median increase of NfL of 3.7 pg/ml with increasing disability (95% CI: (-4.2; 11.7), p=0.354). In contrast, the association between NfL and MMSE was significant across all three groups. NfL (median) estimated decreased by 8.5 pg/ml (95% CI: (2.3; 14.7), p=0.008).



Extended Data Figure 4. *Survival prediction for NfL, MMSE, and ADL.* The size of the prediction was estimated as area under the curve (AUC) from the prediction of survival from blood sample until a certain age (the x-axis of time-dependent AUC). **(a)** Survival prediction for the centenarians up to 103 years of age. The average AUC over the follow-up period were for the centenarians 0.65, 95% CI: (0.56: 0.75), 0.62, 95% CI: (0.52: 0.72) and 0.64, 95% CI: (0.54: 0.73) for NfL, MMSE and ADL, respectively. **(b)** Survival prediction for the 93-year-old nonagenarians up to 100 years of age. The average AUC over the follow-up period were for the nonagenarians 0.68, 95% CI: (0.59: 0.76), 0.55, 95% CI: (0.46: 0.64) and 0.63, 95% CI: (0.54: 0.71) for NfL, MMSE and ADL, respectively. These observations indicate that blood NfL predicts survival better compared to MMSE and ADL.

Supplementary Table 1 and 2 are attached as Excel Files

	Centenarian cohort		Nonagenarian cohort	
	HR (95% CI)	*p*-value*	HR (95% CI)	*p*-value*
Univariate regression[1]				
NfL (1 SD increase)	1.34 (1.13, 1.60)	0.0010	1.44 (1.27, 1.64)	1.38×10^{-8}
MMSE (1 SD decrease)	1.40 (1.14, 1.71)	0.0011	1.28 (1.10, 1.48)	0.0016
ADL (1 category increase)[2]	1.56 (1.21, 2.01)	0.0007	1.57 (1.25, 1.97)	1.04×10^{-4}
Multivariate analyses[1]				
NfL (1 SD increase)	1.29 (1.07, 1,54)	0.0069	1.36 (1.17, 1.57)	5.19×10^{-5}
MMSE (1 SD decrease)	1.22 (0.98, 1.52)	0.0813	1.15 (0.97, 1.36)	0.1046
ADL (1 category increase)[2]	1.40 (1.05, 1.88)	0.0225	1.19 (0.91, 1.55)	0.2028

Hazard ratios (HR) are per standard deviation (SD) for the continuous phenotypes, per increase in categories for ADL, and per ε4 carriers vs. non-carriers for ApoE.

* two-sided *p*-values (no adjustment for multiple comparison)

[1]adjusted for gender

[2]categories: not disabled, moderately disabled, and severely disabled

Supplementary Table 3. Associations between plasma NfL, MMSE, ADL, and mortality among centenarians and nonagenarians estimated from Cox regression models. Same analysis as described in Table 2 after exclusion of 3 subjects that revealed NfL plasma levels above 300 pg/ml (outliers according to Grubbs test performed on log-transformed NfL).