Plasma proteome profiling of healthy individuals across the lifespan in a Sicilian cohort with long-lived individuals

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**Abstract**

**Main points:**

The study of healthy human aging is extremely important to shed light on the molecular mechanisms behind aging in order to predict and/or avoid the development of aging-related disorders such as atherosclerosis and diabetes. Herein, we have employed an untargeted mass spectrometry-based approach to study a Sicilian plasma cohort containing long-lived individuals (LLIs). This approach has identified proteins correlated to age such as fibulin-1, dystroglycan and gamma glutamyl hydrolase, confirming what has been previously reported. Furthermore, our findings revealed a unique signature of proteins correlating with location and uric acid, such as metalloprotease 9 and actin respectively.

**1 INTRODUCTION**

Aging, defined as a time-dependent functional decline of living organisms (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013), is characterized by a progressive deterioration of physiological functions, often leading to development of age-related diseases. Major age-related disorders include atherosclerosis, neurodegenerative disorders and diabetes (Vasto et al., 2010).

Several factors have been characterized as risks for the development of age-related diseases such as: genomic predispositions, telomeric and epigenetic alterations, mitochondrial dysfunction and cellular senescence (López-Otín et al., 2013). Also, chronic systemic inflammation might lead to higher risk of developing cardiovascular disease, both representing a major cause of death in people over 65 years old (Ferrucci & Fabbri, 2018).

Long-lived individuals (LLIs) represent a good model of healthy aging since, during the years, they have escaped the development of age-related diseases and show good health. Investigating aging mechanisms and, even more, how LLIs have developed a healthy aging process, is of great interest to unravel potential biomarkers that could prolong human lifespan and/or promote healthy aging. Even though plasma proteomics is very challenging, due to the large dynamic range with some highly abundant proteins that can mask lower abundance proteins (Anderson & Anderson, 2002), and due to the lack of high-throughput approaches, the study of circulating proteins from plasma could contribute to the identification of unique protein signatures in the elderly population. The potential of plasma proteomics for the study of aging can also be seen from a growing number of recent studies on plasma proteome changes as function of aging (Lehallier et al., 2019; Moaddel et al., 2021) .

In the present study, we analyzed the plasma proteome of a Sicilian cohort of healthy individuals in the age range 20-100+ with the aim of identifying protein patterns across the lifespan that are specifically related to healthy aging.

In detail we have used a high-throughput automated protein digestion approach, to reduce the variability between samples, combined with a data-independent acquisition (DIA) method to deepen protein coverage. Using this plasma proteomics approach in combination with statistical analysis we could confirm some of the findings from other aging studies and we have been able to further identify new proteins that could play an important role in healthy aging.

**2 RESULTS**

In the present study a primary aim was to use an untargeted approach for analysis of plasma to reveal proteins that could be associated to aging and other health factors. Specifically, we used an automated high-throughput plasma protein preparation method and combined it with DIA LC-MS/MS to identify differentially abundant proteins in plasma between different age groups from a healthy Sicilian cohort. We further aimed to use the dataset to investigate if plasma proteome differences correlating to other anthropometric factors could be revealed.

**Plasma aging proteome overview**

The current study included a total of 86 plasma samples from healthy men and women aged 20 to 95+ years. A schematic overview of the samples and the workflow employed is displayed in **Figure 1**. Further individuals’ information regarding clinical and biochemical parameters as well as BMI, age and location (origin of the samples) are reported in **Supplementary Table 1**.

By using an automated sample processing and a label-free approach on undepleted plasma combined with data independent acquisition (DIA) a total amount of 435 proteins were identified and quantified. After inspection of the data, some proteins were removed due to more than 50% missing values and the final protein list contained 410 proteins.

**Figure 2A** displays the overall sample distribution in the principal component analysis (PCA) plot. Percent variance explained in the principal components (PCs), shows a good distribution of the data in the first 10 PCs (**Figure 2B**). Thus, the association between the 10 PCs and the clinical variables collected from each individual was further investigated. Results show that parameters like age, category (young, middle age, old, very old and centenaries), location (city or village), uric acid (UA2) and gender play a role in proteins distribution, while biochemical factors, such as LDL, HDL and BMI, do not significantly contribute to the distribution of proteins (**Figure 2C**).

**Protein correlation with age**

The correlation between proteins and age was further investigated.

From this analysis we could identify 106 proteins that significantly (q-value<0.05) correlate with age (**Supplementary Figure 1**). To further discriminate between up- and down- regulated proteins, a cut-off on effect size >/< 0.01 was applied. 20 age-associated proteins were found to be negatively regulated, while 27 were positively regulated as shown in the volcano plot (**Figure 3A**). Among these last group of proteins, fibulin-1 (FBLN1, P23142), lysozyme C (LYZ, P61626), dystroglycan (DAG1, Q14118) and gamma glutamyl hydrolase (GGH, Q92820) were already reported in another study as related to aging (Lehallier et al., 2019). Indeed, FBLN1 and DAG1 have been shown to play a crucial role in aging since both have a positive effect on brain homeostasis and neurotrophic activities (Moore et al., 2002; Ohsawa, Takamura, & Kohsaka, 2001). Interestingly, also extracellular superoxide dismutase (SOD3, P08240) is increasing with age, suggesting a higher protection from oxygen reactive species in older individuals.

Most of the age-associated proteins that are negatively correlated with age are immunoglobulins. It has been reported that accumulation of immunoglobulins activates the immune system and results in chronic inflammation during ageing (Aprahamian, Takemura, Goukassian, & Walsh, 2008). The reduced expression of immunoglobulins in our older cohort suggests a better response to inflammation and cellular stress.

Furthermore, IGFBP-3 and IGFBP-5 are less expressed in our elderly cohort pointing towards reduced cell senescence in these individuals as well as reduced cellular inflammation. Indeed, both proteins have been associated with cellular senescence and they have been reported to induce fibroblast activation and inflammatory response (Elzi et al., 2012; Kojima, Kunimoto, Inoue, & Nakajima, 2012; Sanada et al., 2018).

To further characterize proteome changes during individuals’ lifespan we used the differential expression-sliding window analysis (DEswan) algorithm, which has been demonstrated to provide useful information about protein changes at particular stages of life (Lehallier et al., 2019). The algorithm employed was set to analyze protein levels within a window of 10 years. Using this approach we were able to identify significant proteins changing mainly in two age waves with a peak at age 40 and then a consistent peak at age 80 (**Figure 3B**). The upset plot (**Figure 3C**) is also showing the number of significant proteins that are unique or shared between age waves. Most of the significant proteins in the age-waves are unique to LLIs the age range X80 comprises of 16 proteins, while X80 and X90 share 22 proteins (**Supplementary Table 2**). These results suggest a unique protein signature for LLIs. Among these proteins, many of them are related to extracellular matrix (ECM) structures and functions. The ECM comprises of proteins providing structural and biochemical support to cells. Since the cellular behavior is highly affected by the surrounding environment, age-related changes of the ECM impact the ability to give support to the cells and might influence most processes in the body (Birch, 2018).

On the other hand, 7 proteins that are unique to age range 40 are related to the immune response and they result to be (up or down regulated compared to old? Ashfaq could you look into that?).

**Protein correlation with other parameters**

In the present dataset, most of the plasma samples were collected from the city (Palermo), while some of the LLIs and their relatives plasma were coming from a village in the Sicilian mountains. 9 proteins correlate to the location at a set q-value<0.05 (**Supplementary Figure 2**). **Figure 4A** shows the intensity of 3 representative proteins (B2MG, LYSC and CYTC) and their distribution in regard to age (x-axis) and to the location (blue village and red city). These proteins are found in higher levels in the elderly population in the village than in the city, their expression is increasing with age and they are related to the cellular protein metabolic process.

Furthermore, protein correlation with age and location was investigated in parallel, and 9 proteins were significantly correlating to both variables. The heatmap (**Figure 4B**)shows the differential expression of these proteins. Matrix metalloprotease 9 (MMP9) was negatively regulated across samples, confirming a previously found correlation for LLIs in a larger cohort which also includes the here studied individuals (Cancemi et al., 2020) but using another analysis method.

Furthermore, 11 proteins were significantly correlating to gender. As expected, 2 proteins are hormonal proteins (SHBG and PZP). Can we have a plot for the proteins related to gender?

UA is the end product of purine metabolism. The role of UA is controversial since it has been reported to enhance oxidative stress (Yu, Sánchez-Lozada, Johnson, & Kang, 2010) while other studies suggest that UA is a reactive oxygen species (ROS) scavenger, thus playing an antioxidant effect (El Ridi & Tallima, 2017; Glantzounis, Tsimoyiannis, Kappas, & Galaris, 2005; Sautin & Johnson, 2008). Even UA correlation with age is controversial since it has been shown to physiologically increase during adulthood and it has been associated to endothelial dysfunctions and hypertension (Kawamoto et al., 2013; Kuzuya, Ando, Iguchi, & Shimokata, 2002; Zhou et al., 2017). On the contrary, increased levels of UA in elderly populations have proved a greater muscle strength (Lee, Hong, Park, & Kang, 2019), while in *Caenorhabditis elegans* high levels of UA seem to increase life span (Wan et al., 2020).

In the present cohort, 29 proteins are significantly correlating with UA2, 18 of them are positively correlated to UA2 while 11 proteins are correlating negatively to UA2, as show in **Figure 4C**. Of the 18 proteins positively correlated to UA2, 9 of them are part of the cytoskeleton machinery such as ACT, CFL1, FLN-A, PFN1, TLN1, TPM4 and VCL. The fact that most of these cytoskeleton-associated proteins are mostly expressed in older individuals, could imply a higher need to recruit proteins to the cytoskeleton to support and maintain cellular homeostasis (Amberg, Leadsham, Kotiadis, & Gourlay, 2012).

**Cluster of significant proteins and their trajectories**

Normalized significant proteins were divided into clusters with an age step size of 10 years. As depicted in **Figure 5**, the first 7 clusters show high intensity and were further investigated. Cluster 1 and 3 are the most represented ones (41 and 18 proteins respectively) and show a linear increase with age; while cluster 2, 4, 5, 6 and 7 (19, 15, 5, 13 and 15 proteins respectively) show a step-wise decrease trajectory with age. Most of the proteins in cluster 1 and 3 are related to coagulation and clotting such as fibrinogen, von Willebrand factor as well as coagulation factor V and IX. These proteins are well known to increase with the physiological process of aging and are higher in centenarians, where a state of high coagulation enzyme activity has been reported (Mannucci et al., 1997; D. Mari et al., 1995; Daniela Mari, Coppola, & Provenzano, 2008). Among the proteins that are representing the decreasing trajectories, there are several immunoglobulins and serpins. Serpins are known to be highly expressed in senescent cells, providing a fine balance between thrombosis and thrombolysis cascades, and have been identified as plasma biomarkers of ageing (Basisty et al., 2020; Tanaka et al., 2018). We could speculate that the lower expression of many serpins in our elderly population could indicate an escape mechanism from cell senescence.

**Overlap with SOMASCAN data**

As protein changes recently has been studied using the SOMASCAN technology (Ref), we investigated the overlap in terms of quantified proteins in the recent Lehallier study which is based on the SOMASCAN affinity technology and our LC-MS/MS based study. Mapping was done through gene symbols, and interestingely, despite the lower number of proteins quantified in our study, only 121 genes were quantified in both studies, while our study contributed 325 unique genes, and the other study 2686 unique genes. We are indicating for the proteins in Supplementary Table if they were uniquely quantified in our study, or in both studies, repectively, despite the fact that mapping of proteins to genes may be ambiguous sometimes. It can be noted that about 70 of the unique genes in our study are immunoglobulin genes. The overlap of proteins with significant correlation to age was also examined for the two studies. A total of fifteen proteins were found to correlate significantly with age in both studies (q<0.05), see Supplementary Table 2.

**3 DISCUSSION**

**3**

**4 EXPERIMENTAL PROCEDURES**

**Sample preparation for mass spectrometry analysis**

Plasma diluted 10-fold with 5% SDS in 100 mM Tris (pH=7.55) and sonicated using a probe sonicator (Branson Digital Sonifier® 250-D, Branson Ultrasonics Corporation, Danbury, USA), at amplitude 10%, with 10s pulse on and 20s off, for a total of 36 cycles. The solution was centrifuged at 13,000 rpm for 8 minutes to remove debris and the supernatant, containing proteins, was recovered.

A fixed volume (50 μl) of the supernatant, containing approximately 50 μg of plasma proteins as determined by protein concentration measurements of randomly selected samples, was digested into peptides using on-bead digestion on HILIC Microspheres (ReSyn Biosciences, Gauteng, South Africa). The process was fully automated using 96-well plates in King-Fisher Flex (Thermo Fisher Scientific, Bremen). The automated procedure consisted of the following steps: magnetic microspheres (1:10 protein:beads ratio) were incubated and equilibrated in equilibration buffer (15% ACN, 100 mM NH4Ac, pH=4.5); protein sample was incubated in binding buffer (30% ACN, 200 mM NH4Ac, pH=4.5) where proteins would bind to HILIC beads. Beads were then washed twice in 95% ACN to remove unspecific and low binding proteins. Bead-binding proteins were then incubated for 1h at 47°C with trypsin (20:1 protein:trypsin w/w ratio) dissolved in 50 mM AMBIC. Peptides were recovered from the plate and dried in a Speedvac (Thermo Fisher Scientific, Germany) prior to C18 desalting.

Desalting was performed using BioPureSPN Mini, PROTO 300 C18 (The Nest Group, Inc., MA, USA). Briefly, columns were equilibrated with 100 μl 70% ACN, 5% FA and conditioned using 100 μl 5% FA. Samples were resuspended in 100 ul 5% FA and loaded on the column. Columns were washed in 5% FA and cleaned peptides were eluted using 100 μl 50% ACN, 5% FA. All the steps were performed using an Eppendorf bench top centrifuge at 50 x g for 2 minutes.

Cleaned peptides were dried and stored at -20°C prior to quantification and injection into the mass spectrometer.

**nanoLC mass spectrometry**

300 ng peptides (as determined by nanodrop) of each sample was resuspended in 0.1% FA loaded onto an EASY-nano LC system (Thermo Fisher Scientific, Germany). The analytical column was 15 cm long fused silica capillary (75 μm\* 16 cm Pico Tip Emitter, New Objective, USA) packed in house with C18 material ReproSil-Pur 1.9 μm (Dr. Maisch GmbH, Germany). Peptides were separated using an 80 minutes method, including a 60 minutes linear gradient from 10% to 250% solvent B (80% ACN, 0.1% FA) from 3 to 63 minutes at a constant flow rate of 250 nl/min. The gradient was preceeded by a 3 min gradient from 5-10% B and followed by a 5 min gradient to 40% B and finally a five minute gradient to 90% B followed by 7 minutes isocartic washing at 90% B. The nanoLC system was coupled to a Q-Exactive HF-X Mass Spectrometer (Thermo Fisher Scientific, Germany). Data were acquired using data-independent acquisition (DIA).

**DIA acquisition**

To generate a chromatogram library for DIA processing, the MS was set to acquire six DIA acquisitions with staggered 4 m/z MS/MS spectra (4 m/z precursor isolation windows at 30,000 resolution, AGC target 1e6, maximum inject time 60 ms) using an overlapping window pattern from narrow mass ranges (approximately 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000 m/z, as described in (Pino, Just, MacCoss, & Searle, 2020). Full window MS1 spectra (395-1005 m/z at 30,000 resolution using an AGC target value of 3x106 ions and a maximum injection time of 55 ms)

For quantitative samples, the MS was set to acquire full scan MS1 spectra as above, and DIA spectra at 15,000 resolution with AGC target of 1x106, maximum injection time of 20 ms, loop count 75, and using staggered isolation window of approximately 8 m/z.

DIA spectra were set with normalized collision energy (NCE) of 27. The Xcalibur software v3.0 (Thermo Fisher Scientific, Germany) controlled the nanoLC system, the mass spectrometer and was used to acquire and visualize the RAW data.

**Mass spectrometry data processing**

Raw DIA MS files were converted to mzML using Proteowizard (Chambers et al., 2012) version 3.0.20079, with vendor peak picking and PRISM demultiplexing. The mzML files were further processed in EncyclopeDIA version 0.9.0 (Searle et al., 2018). A chromatogram library was generated using the pool windows DIA files by matching to a predicted human proteome spectral library (Searle et al., 2020). The spectral library ”uniprot\_human\_25apr2019.fasta.z2\_nce33.dlib”, version as of 28 January 2020, was downloaded along with the corresponding UniProt human fasta file from <https://www.proteomicsdb.org/prosit>. A chromatogram library was generated using default settings in EncyclopeDIA and used for processing of the full DIA acquisitions of the different samples. A quantitative protein group file (1% protein group false discovery rate) for the samples was exported for further analysis. Intensities in the protein file were log2 transformed and normalized using Cyclic LOESS normalization (Gentleman, Carey, Huber, Irizarry, & Dudoit, 2005; Smyth, 2005) in NormalyzerDE (Willforss, Chawade, & Levander, 2019) before further analysis.

**Data analysis**:

Data missingness was analyzed using the "mice" package in R. We considered proteins present in at least 50% of the samples for further analyses. Principal component analysis (PCA) was used to perform exploratory analyses of the proteomics data. Explained variance by each principal component was calculated using the output from “prcomp” function in R.

Correlation between principal components and phenotype (clinical) variables was calculated using “lm” function in R and "adj.r.squared" values were extracted from the summary statistics produced by the ´lm´ function.

**Differential expression analysis**

For differential expression analyses the "limma" R package was used. The following models were used to analyze deferentially abundant proteins:

- \*Basic model\* protein ~ Age

- \*Gender and BMI adjusted model\* protein ~ gender + BMI + Age

- \*Interaction with environmental variables\* protein ~ gender + bmi + Age + ENV + Age\*ENV

**Age related protein clustering**

To identify patterns associated with age we performed cluster analyses of proteins associated with age. To visualize trends, age related intervals as following were used:

1. Plot for groups between 0-40, 61-60, 61-80 and 81-112.

2. Plots for groups for age span of 10 years each

Mean protein intensity was calculated for proteins in each cluster followed by average over each age intervals.

For clustering, protein data was scaled followed by calculating distance between protein observations using Euclidean distance. Clustering was then performed by using complete linkage between observations.

**SWANDE analyses**

Differential expression Sliding window analysis distinguishes (DEswan, (Lehallier et al., 2019)) was used to identify waves of aging plasma proteins.

Age span between 20 years to 120 years with interval size of 10 years was selected for sliding window analyses in the DEswan function implemented in DEswan package. Sex was used as covariate in the DEswan analyses.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHORS CONTRIBUTIONS**

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**Figure Legends**