RESEARCH ARTICLE

# FTIR spectroscopy confirms age-related changes in protein conformation in a new independent dataset of human plasma samples

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**Abstract**
The loss of proteostasis is a key hallmark of aging and has been well documented using several model systems. However, in biofluids such as blood plasma, little is known about how the loss of proteostasis affects protein conformation during aging.
Our previous work has demonstrated age-related changes in protein conformation in human plasma samples using FTIR spectroscopy and conformation-specific antibodies. To prove the potential of spectroscopy to detect slight changes in protein secondary structure during aging, we have applied the same methodology to a new and independent set of 24 human plasma samples from donors with ages ranging from 40 to 75 years.
The results clearly show that from middle to old age there is a decrease in antiparallel β-sheet structures and an increase in intermolecular β-sheets, indicative of an increase in aggregation-prone proteins in human plasma over time. This confirms the potential of FTIR spectroscopy as a technique to evaluate protein conformational changes related to health and disease and as a suitable method to be used in a clinical setting to produce quick and reliable results.

**Keywords:** Protein conformation, Aging, FTIR spectroscopy

## 1. Introduction:

### Proteostasis and aging

The loss of proteostasis is one of the hallmarks of aging as described by López-Otín and colleagues(1). As organisms age, cells lose their ability to maintain protein homeostasis and, as a consequence, there is accumulation of toxic and aberrant proteins, which are closely related to aging diseases, as Alzheimer's or Parkinson's disease(2).

An objective of preventive medicine is to control the consequences of age-related diseases, so that individuals can live with good health during as much time as possible. One of the challenges in this field is to establish healthy aging biomarkers in order to predict which individuals are supposed to experienciate longer healthy lifespan. As consequence of the so known loss of proteostasis protein can be traced with that purpose(3,4).

In fact, several studies characterizing protein aggregation changes during aging in tissues have been carried out(5–7), and until now little is known about what happens in biofluids, such as blood serum, plasma or urine.

Since peripheral fluids are an optimal choice for biomarker identification and metabolic fingerprinting, it would be of great interest to identify age-related changes in protein conformation in these samples. Fourier Transform Infrared (FTIR) spectroscopy appears as a promising approach for the study of biofluids and has already been successfully used in a clinical setting for diagnosis(8). In our previous work we used FTIR spectroscopy to evaluate age-related changes in protein conformation in human plasma from a dataset of 127 samples(9) and our results suggested that FTIR spectroscopy was able to detect changes in protein secondary structure from middle to old age. When age-associated co-morbidities were excluded, a correlation between the age of the donors and secondary structure of plasma proteins was found: samples from younger donors were characterized by antiparallel β-sheet-containing structures while intermolecular β-sheets characterized older samples(9).

To validate the obtained results and to validate and support the applicability of FTIR as a reliable approach to study proteostasis during aging we have applied the same methodology to a new, independent dataset of human plasma samples to find out if these results corroborate our previous conclusions.

## 2. Methods:

### FTIR analysis of human plasma samples

#### *2.1. Collection and characterization of human plasma samples*

A total of 24 healthy adults with ages ranging from 40 to 75 years (mean age: 61.6 ± 9.1 years, 62.5% female, 37.5% male) were recruited from a Primary Health Care Centre in the Aveiro region of Portugal. To be included in the study, participants had to be free of any health complications such as cancer, chronic inflammatory diseases, pulmonary diseases, neurologic diseases, and cardiovascular diseases. Ethical approval was given by the local Ethics Committees: ARS Centro Ref: 174211, CES of ARS Centro Ref: 012804-04.04.2012, and by the National Committee for Data Protection Ref: 369/2012. All participants signed a consent from, and all procedures were conducted in accordance with the Declaration of Helsinki.

Medical history, clinical data, medication, and demographic data were collected from clinical files and confirmed with the patients. Blood samples were collected by venipuncture of the antecubital vein into EDTA coated tubes, centrifuged at 2000g for 15 minutes at 4°C, and plasma aliquoted and stored at -80°C until analysis.

 

*2.2. FTIR measurements and data analysis*

For details of FTIR spectral acquisition see section 2.4 of ref (9). Briefly, samples were thawed on ice and homogenized before spectral acquisition in ATR mode in the mid infrared range. 5µL of plasma were placed on the ATR crystal and air-dried prior to acquisition.

Similar to our previous work, spectra were visually analyzed individually to detect suspicious profiles or high background noise. An exploratory PCA analysis was also performed on raw spectra to detect outlier samples based on Q-residuals and Hotelling's $T^2$ values(10).

After outlier removal, the region of interest (1700-1600$cm^{-1}$) was selected, baseline corrected and area normalized, to correct for differences in que amount of sample placed on the crystal. Finally, a Savitzky-Golay 2$^{nd}$ derivative was applied to increase spectral resolution and resolve peaks associated with protein secondary structure.

*2.3. PLS-R multivariate analysis*

A PLS-R multivariate analysis was performed on processed spectra, using a Kernel algorithm and random intern cross-validation, to analyze changes in protein conformation during the aging process. All statistical analyses were performed using The Unscrambler X software (v.10.5 CAMO Analytics).

## 3. RESULTS:

**Age-related changes in protein conformation in human plasma**

The average spectra of human plasma samples are presented in Figure 1 (A-C). Similar to our previous work, samples were divided in 3 age groups for visualization: ≤59 years; 60-69 years and ≥70 years. Spectra of the different age groups were visually identical, highlighting the need for multivariate statistical analysis to detect any changes in protein conformation. 2$^{nd}$ derivative spectra show 6 bands associated with protein secondary structure: near 1628$cm^{-1}$ (intermolecular β-sheet); near 1634$cm^{-1}$ (random coil); around 1648$cm^{-1}$ (α-helix); 1662$cm^{-1}$ (β-turns) and the band around 1696$cm^{-1}$ (antiparallel β-sheet)(11).

To determine a possible correlation between FTIR spectra and the age of donors, a PLS-R analysis on baseline corrected, normalized 2$^{nd}$ derivative spectra of samples in the amide I region was performed, using 1 latent variable.

 

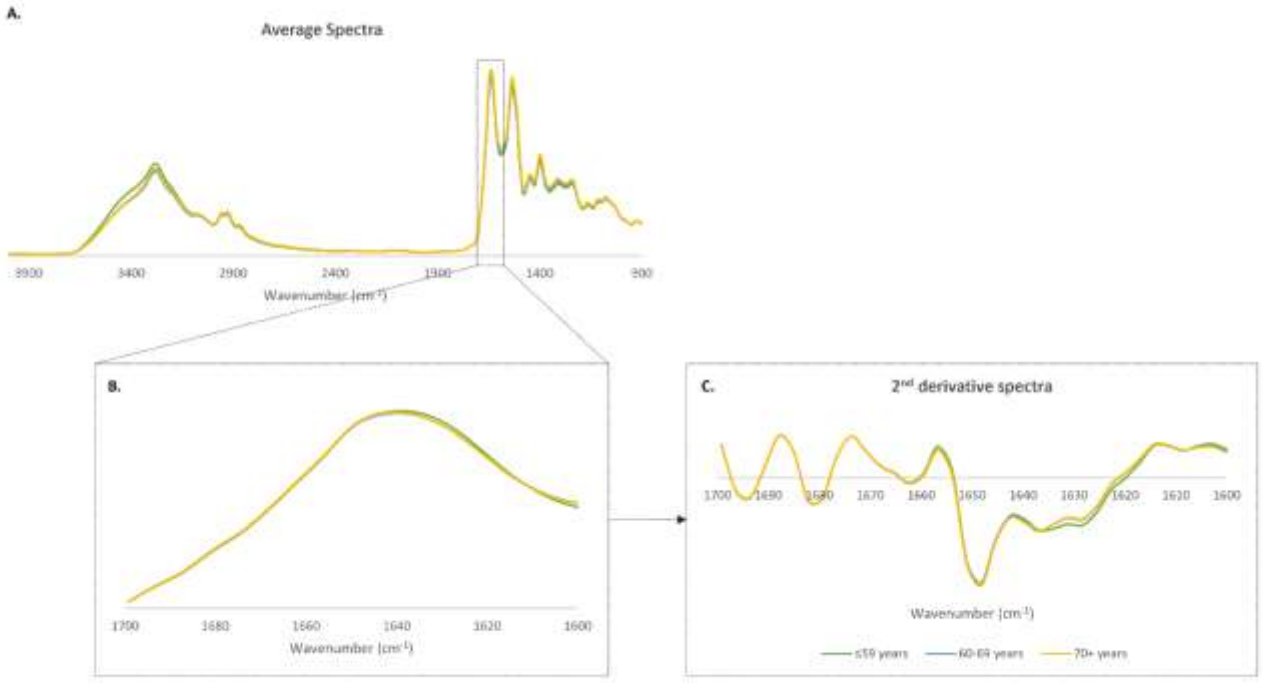


**Figure 1: FTIR spectra of human plasma with aging.** (A) Overview of FTIR spectra of samples in the 4000–900 $cm^{-1}$ region. (B) Amplification of the amide I band (1700–1600 $cm^{-1}$). (C) 2$^{nd}$ derivative of spectra of amide I region, using Savitzky-Golay algorithm with 3 smoothing points.

 

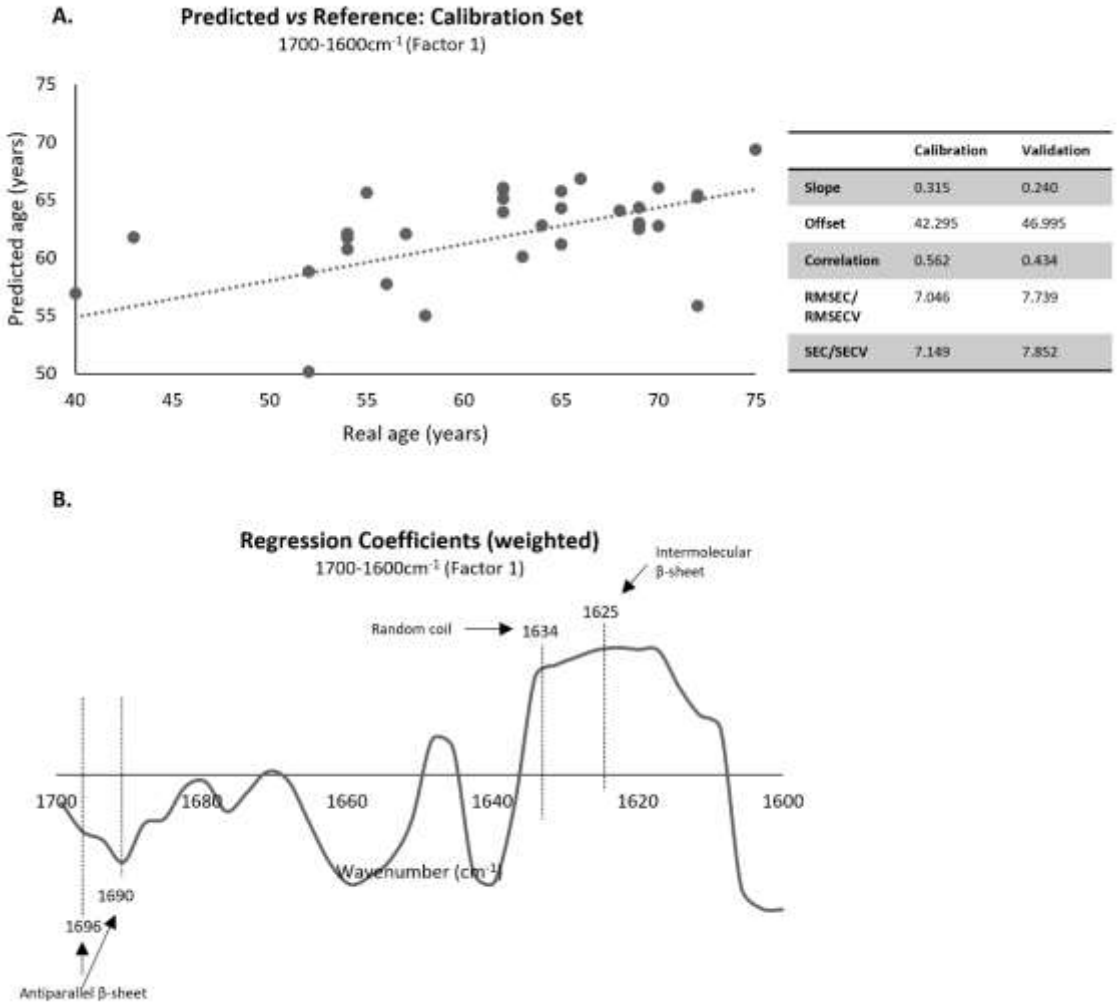


**Figure 2: PLS-R multivariate analysis of human plasma samples.** (A) PLS-R Predicted vs Reference plot of factor 1 of the second-derivative FTIR spectra of human plasma samples in the amide I region (1700–1600cm$^{-1}$); (B) Respective β-coefficients plot. RMSEC: Root Mean Squares Error of Calibration; RMSECV: Root Mean Squares Error of Cross Validation; SEC: Standard Error of Calibration; SECV: Standard Error of Cross Validation.

The results from PLS-R analysis of the spectra are presented in Figure 2. Plots of Predicted vs Reference age and respective β-coefficients (A-B, respectively) are shown. The correlation coefficients (0.562 for calibration set and 0.434 for validation set) indicate a moderate positive correlation between protein secondary structure and the age of the donor (12) (Figure 2A). Weighted coefficients show that peaks associated with antiparallel β-sheets are associated with younger donors and peaks associated with random coils and intermolecular β-sheets are associated with older donors (Figure 2B).

 

## 4. Discussion

Protein aggregation is associated with several age-related diseases(13) and, because of this, it has been widely studied. The proteostasis network is greatly affected by age, as cells progressively lose their ability to repair or degrade misfolded proteins(14). In relation to protein secondary structure, it is known that intermolecular β-sheets are associated with aggregation-prone proteins, however this relationship is not valid for all proteins(15). FTIR spectroscopy is widely used to assess secondary protein structure in aqueous samples(16–18) and thus plasma samples were analyzed using this technique to evaluate protein conformational changes across aging. In fact, in our previous work on protein aggregation in human plasma, we verified that the levels of protein aggregates in this biofluid are relatively low(9). Nevertheless, we successfully used FTIR spectroscopy to detect slight changes in protein conformation in samples from donors with different ages. Now, we intended to verify if spectroscopic approaches can be used for protein aggregation studies in aging biofluids, using a new independent dataset of plasma samples to validate our previous results.

Despite the experimental procedures being the same in our previous work(9) and here, we adjusted our spectral processing method to more accurately analyze the spectra, according to what is reported in the literature(10): specifically, we normalized spectra to the total area of the amide I band and not to the intensity of the amide I peak. Nevertheless, our results coincide with those from our first cohort of samples and show that from middle to old age, there seems to be a transition from prefibrillar to fibrillar structures in proteins in blood plasma. As we suggested before, this may represent a consequence of the aging process, indicating a failure of the proteostasis network in the elderly, or a protective mechanism, since oligomeric aggregates are more reactive and seem to be more toxic than large fibrillar structures(19,20). This confirms the age-dependent evolution in the conformation profile of human plasma proteins that may indicate a transition from prefibrillar to fibrillar structures. The identified presence of prefibrillar oligomers might represent an intermediate structure that can later give rise to more stable proteins, richer in β-sheet structures, which can then associate and elongate to produce organized amyloid fibrils with hydrogen-bonded intermolecular β-sheets(14).

In conclusion, using a different and independent dataset but with the same inclusion and exclusion criteria, this work has corroborated previous results and confirms that FTIR spectroscopy is a simple, inexpensive and quick approach to study protein conformational changes in biofluids and monitor protein aggregation during aging.

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