Raman Spectroscopy on human tear films: Healthy subjects VS Amyothrophic Lateral Sclerosis patiens

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“Tear-Based Vibrational Spectroscopy Applied to Amyotrophic Lateral Sclerosis”

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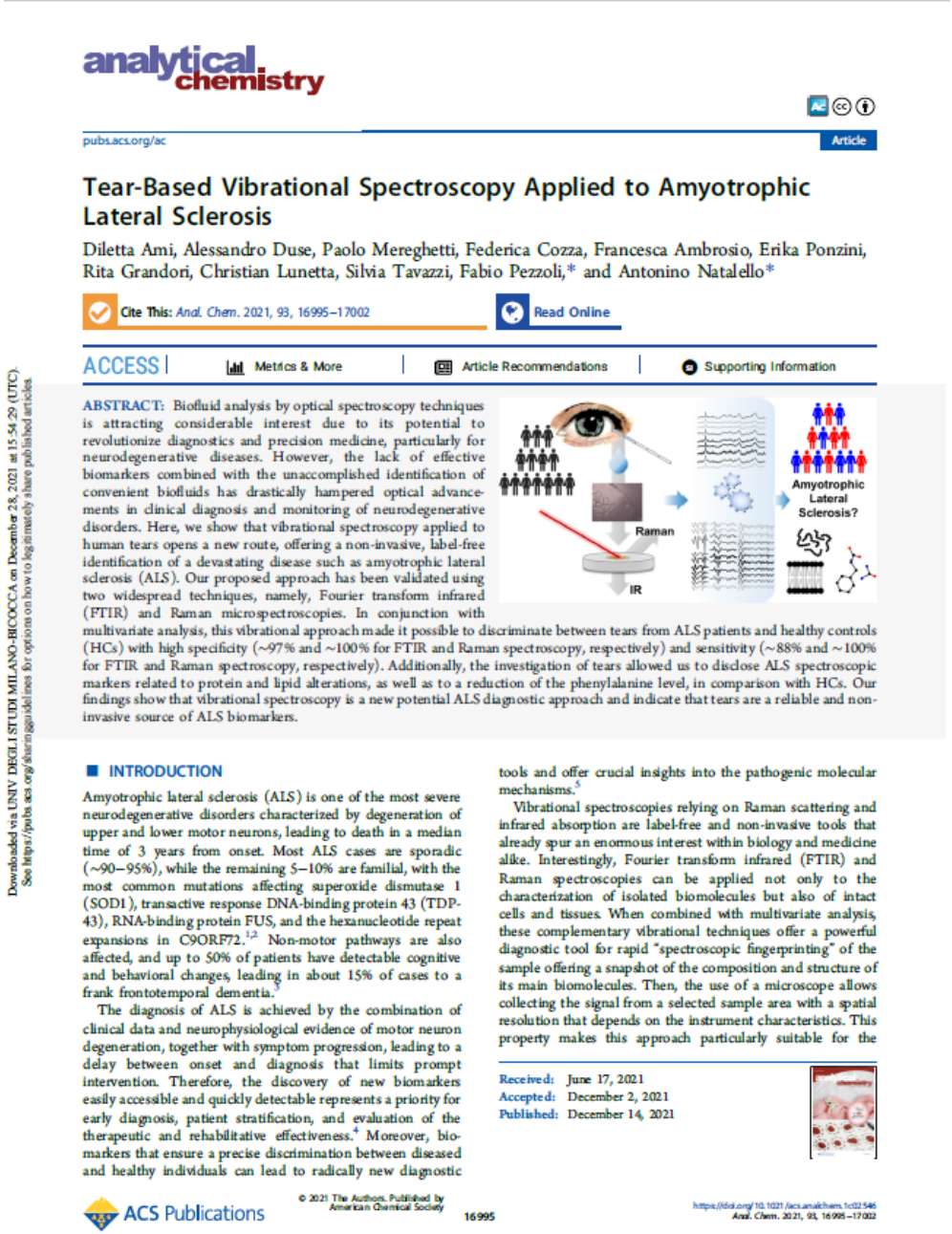

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Article

Tear-Based Vibrational Spectroscopy Applied to Amyotrophic Lateral Sclerosis

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ABSTRACT: Biofluid analysis by optical spectroscopy techniques is attracting considerable interest due to its potential to revolutionize diagnostics and precision medicine, particularly for neurodegenerative diseases. However, the lack of effective biomarkers combined with the unaccomplished identification of convenient biofluids has drastically hampered optical advancements in clinical diagnosis and monitoring of neurodegenerative disorders. Here, we show that vibrational spectroscopy applied to human tears opens a new route, offering a non-invasive, label-free identification of a devastating disease such as amyotrophic lateral sclerosis (ALS). Our proposed approach has been validated using two widespread techniques, namely, Fourier transform infrared (FTIR) and Raman microspectroscopies. In conjunction with multivariate analysis, this vibrational approach made it possible to discriminate between tears from ALS patients and healthy controls (HCs) with high specificity (~97% and ~100% for FTIR and Raman spectroscopy, respectively) and sensitivity (~88% and ~100% for FTIR and Raman spectroscopy, respectively). Additionally, the investigation of tears allowed us to disclose ALS spectroscopic markers related to protein and lipid alterations, as well as to a reduction of the phenylalanine level, in comparison with HCs. Our findings show that vibrational spectroscopy is a new potential ALS diagnostic approach and indicate that tears are a reliable and non-invasive source of ALS biomarkers.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is one of the most severe neurodegenerative disorders characterized by degeneration of upper and lower motor neurons, leading to death in a median time of 3 years from onset. Most ALS cases are sporadic (~90−95%), while the remaining 5−10% are familial, with the most common mutations affecting superoxide dismutase 1 (SOD1), transactive response DNA-binding protein 43 (TDP-43), RNA-binding protein FUS, and the hexanucleotide repeat expansions in C9ORF72.[1,2] Non-motor pathways are also affected, and up to 50% of patients have detectable cognitive and behavioral changes, leading in about 15% of cases to a frank frontotemporal dementia.[3]

The diagnosis of ALS is achieved by the combination of clinical data and neurophysiological evidence of motor neuron degeneration, together with symptom progression, leading to a delay between onset and diagnosis that limits prompt intervention. Therefore, the discovery of new biomarkers easily accessible and quickly detectable represents a priority for early diagnosis, patient stratification, and evaluation of the therapeutic and rehabilitative effectiveness.[4] Moreover, biomarkers that ensure a precise discrimination between diseased and healthy individuals can lead to radically new diagnostic tools and offer crucial insights into the pathogenic molecular mechanisms.[5]

Vibrational spectroscopies relying on Raman scattering and infrared absorption are label-free and non-invasive tools that already spur an enormous interest within biology and medicine alike. Interestingly, Fourier transform infrared (FTIR) and Raman spectroscopies can be applied not only to the characterization of isolated biomolecules but also of intact cells and tissues. When combined with multivariate analysis, these complementary vibrational techniques offer a powerful diagnostic tool for rapid “spectroscopic fingerprinting” of the sample offering a snapshot of the composition and structure of its main biomolecules. Then, the use of a microscope allows collecting the signal from a selected sample area with a spatial resolution that depends on the instrument characteristics. This property makes this approach particularly suitable for the

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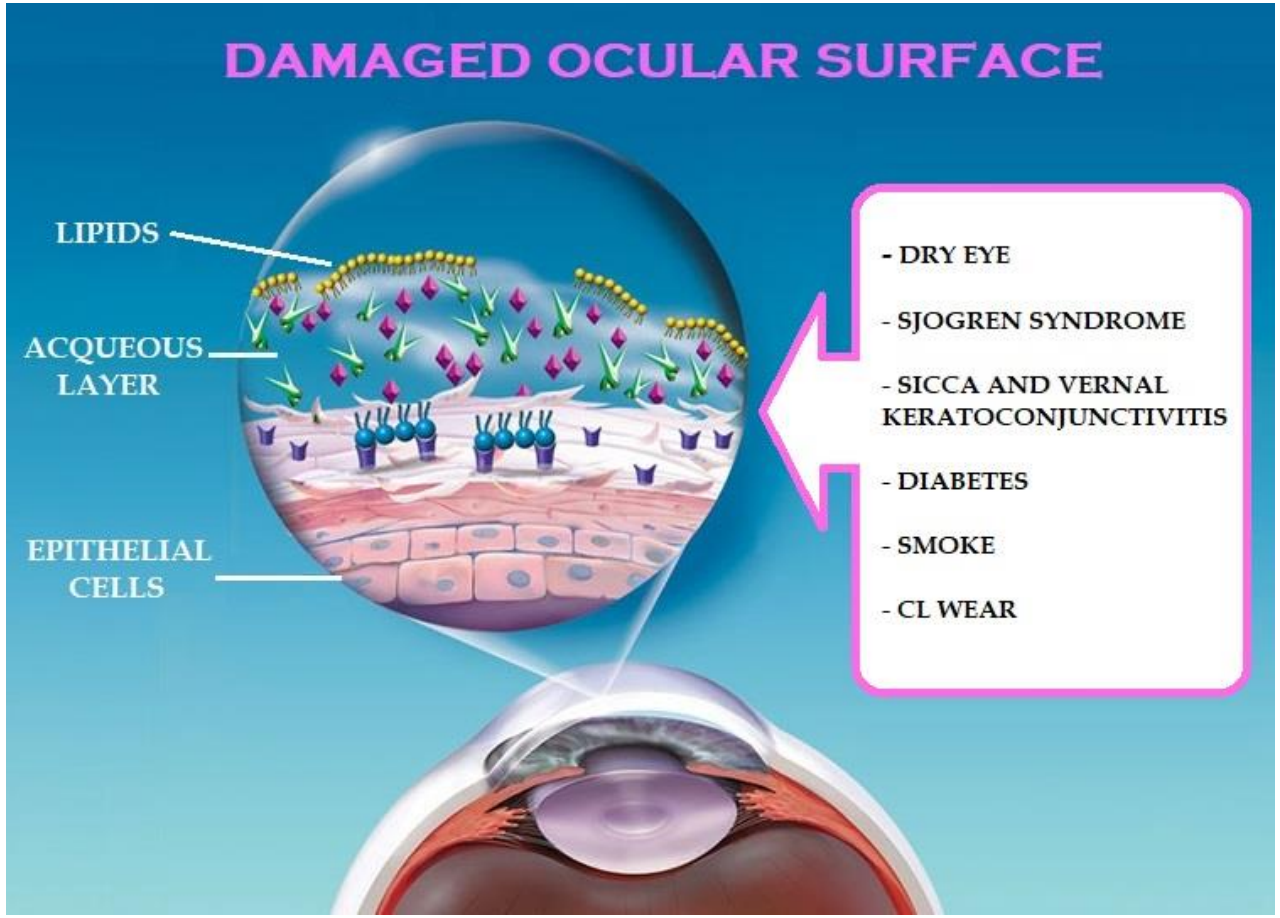
Introduction

Conditions that produce alteration in tear film's components may lead to ocular surface damage



Alzheimer
+
Parkinson

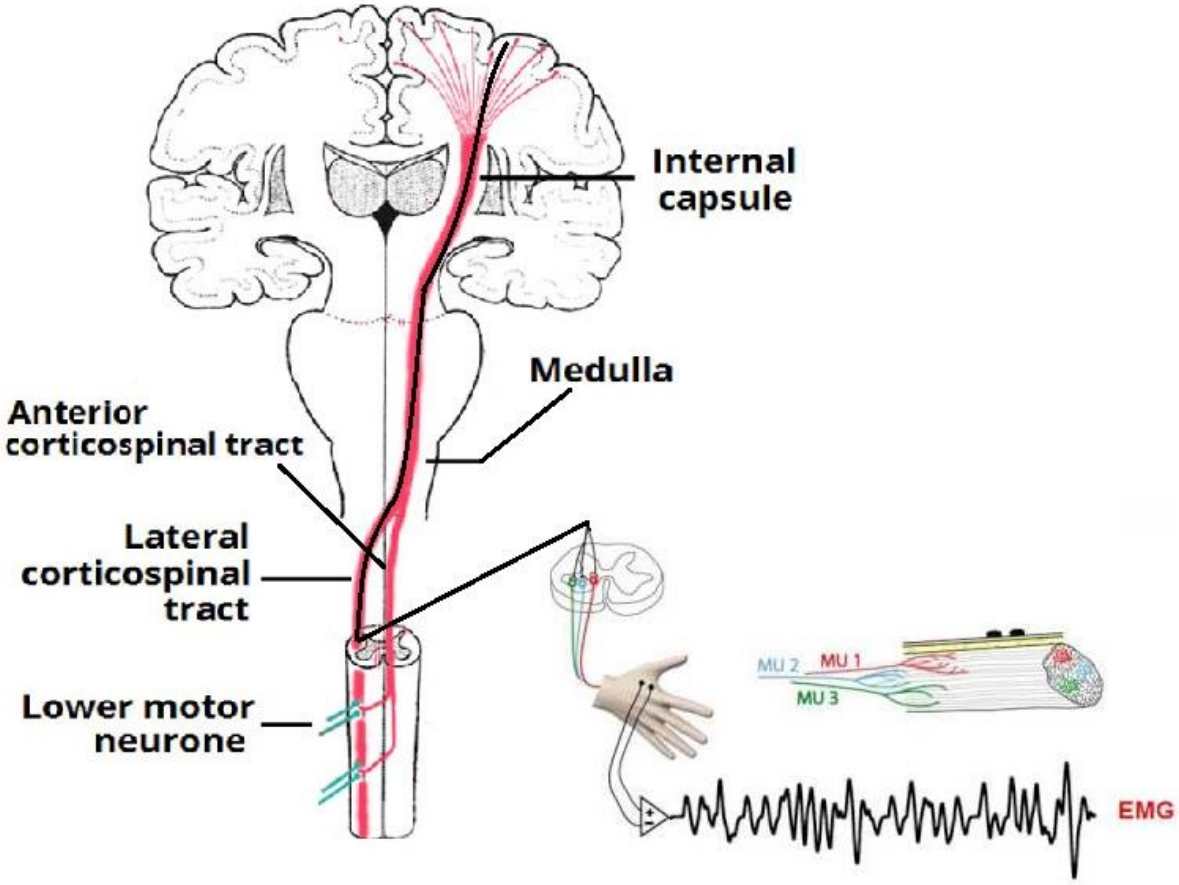
«Roda M, Ciavarella C, Giannaccare G, Versura P. Biomarkers in Tears and Ocular Surface: A Window for Neurodegenerative Diseases. Eye Contact Lens. 2020 Mar;46 Suppl 2:S129-S134. doi: 10.1097/ICL.0000000000000663. PMID: 31658175.»

«Cennamo G, Montorio D, Morra VB, Criscuolo C, Lanzillo R, Salvatore E, Camerlingo C, Lisitskiy M, Delfino I, Portaccio M, Lepore M. Surface-enhanced Raman spectroscopy of tears: toward a diagnostic tool for neurodegenerative disease identification. J Biomed Opt. 2020 Aug;25(8):1-12. doi: 10.1117/1.JBO.25.8.087002. PMID: 32767890; PMCID: PMC7406892.»

Amyothrophic Lateral Sclerosis

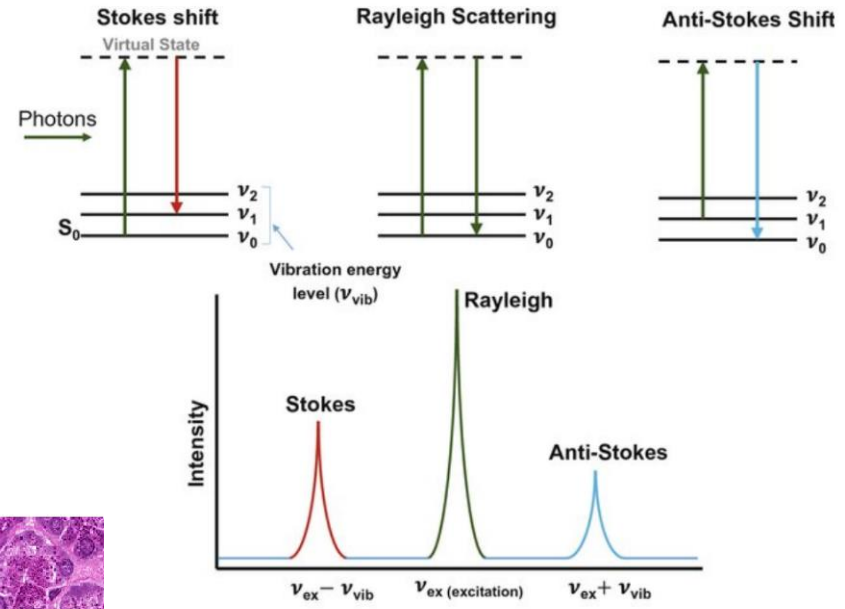
- ALS is a progressive **degeneration of motoneurons**
- **Diagnosis** is difficult and possible by body signs and EMG
- Blood biomarkers are assessed as **prognosis factors** (e.g. emoglobine, creatinine and urea)

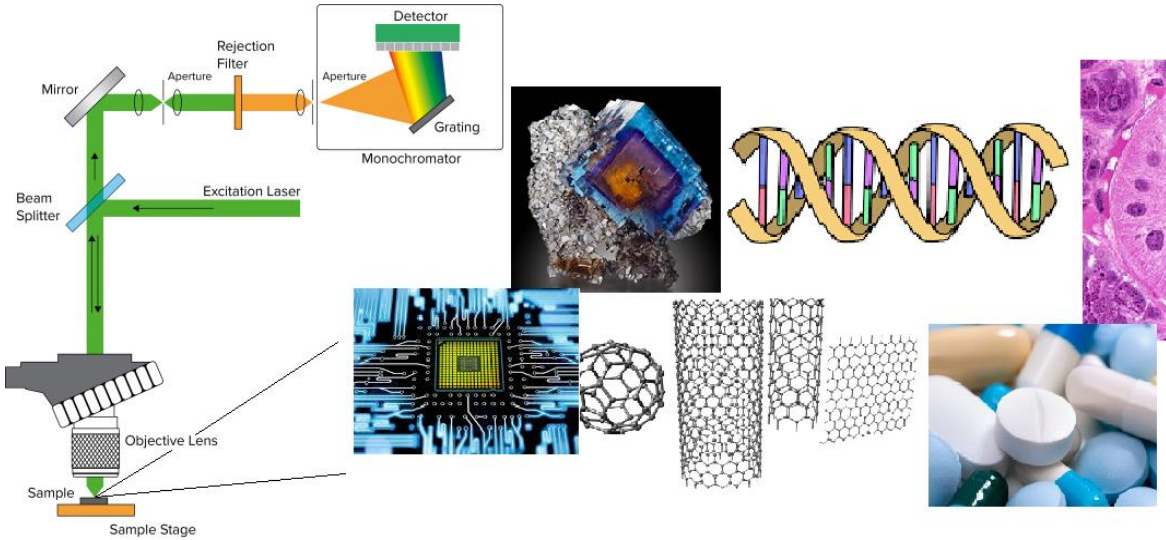
Abnormal proteins aggregation
(e.g.TDP-43) in neurons



The purpose of this study

Exploiting Raman Spectroscopy as an **effective technique** to compare healthy and ALS tears in order to evaluate a possible difference





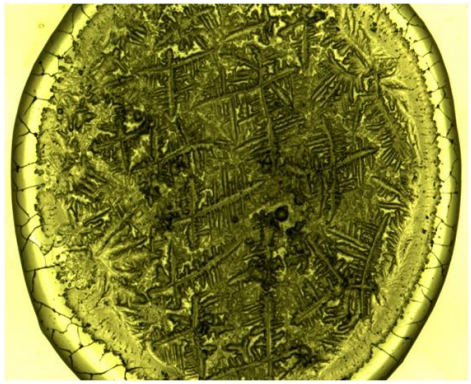
Raman investigations conducted in lab of Prof. Pezzoli

Department of Materials Science, University of Milano-Bicocca

Collection of tear samples by sterile glass capillary: 19 ALS and 20 HCs

NEuroMuscular Omnicentre (NEMO), ASST Grande Ospedale Metropolitano Niguarda, Milano

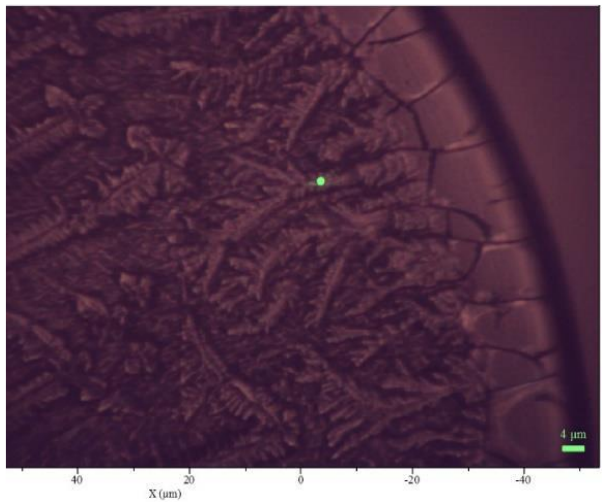
STORING AT 193 K

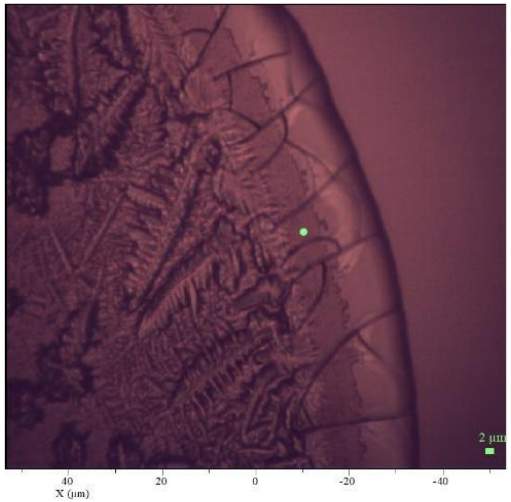


Controlled envirorment-ferning pattern formation on BaF_2

Department of Biotechnology and Biosciences, University of Milano-Bicocca

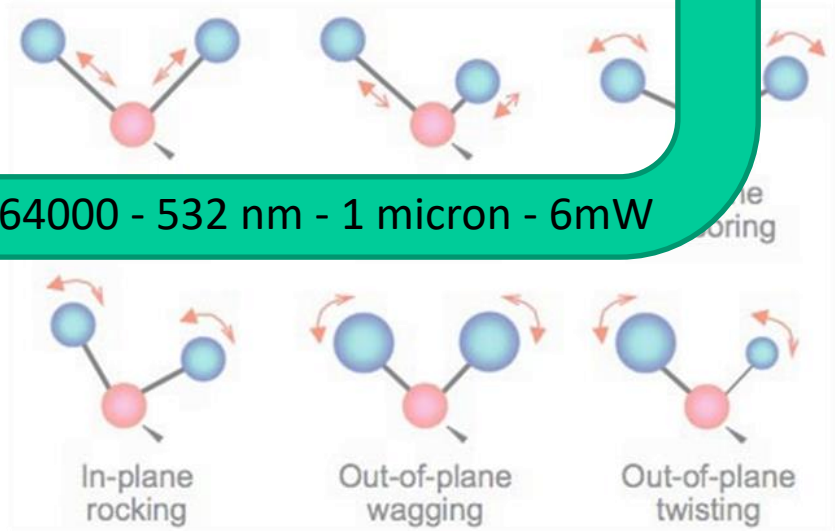
Method



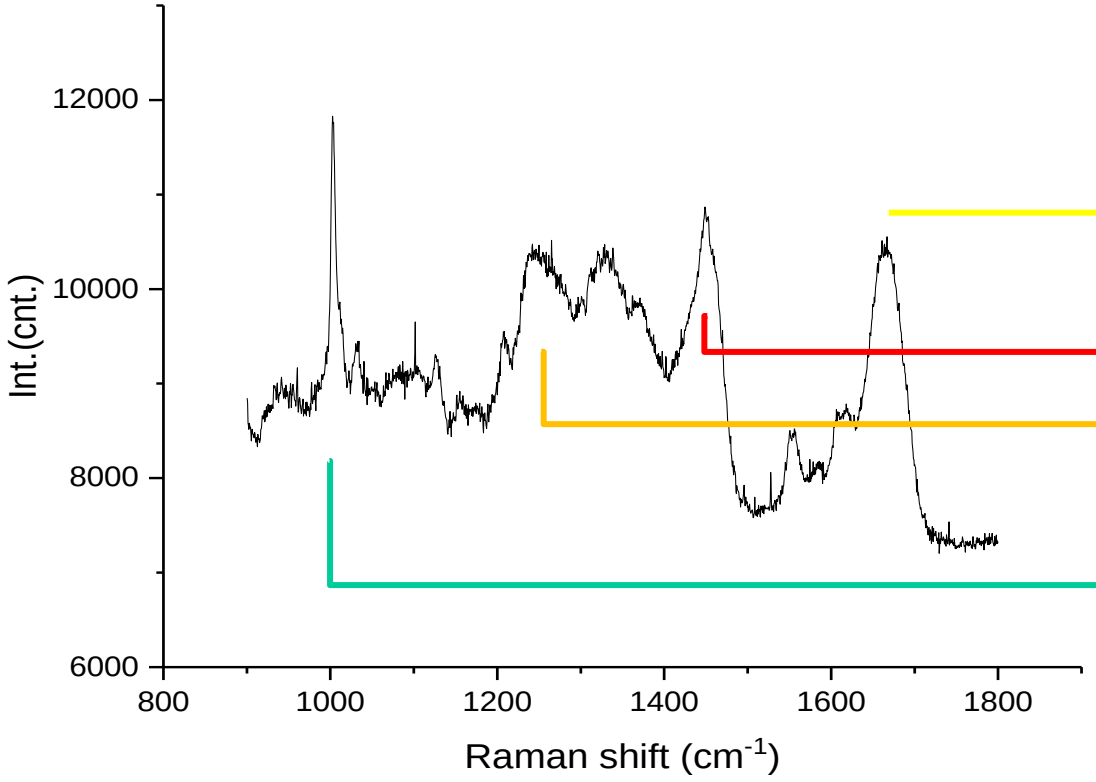


Acquisition time 60x5 sec. in 900-1800 cm^{-1} range on **recurrent objects**

Horiba T64000 - 532 nm - 1 micron - 6mW



Spectral peaks assignment



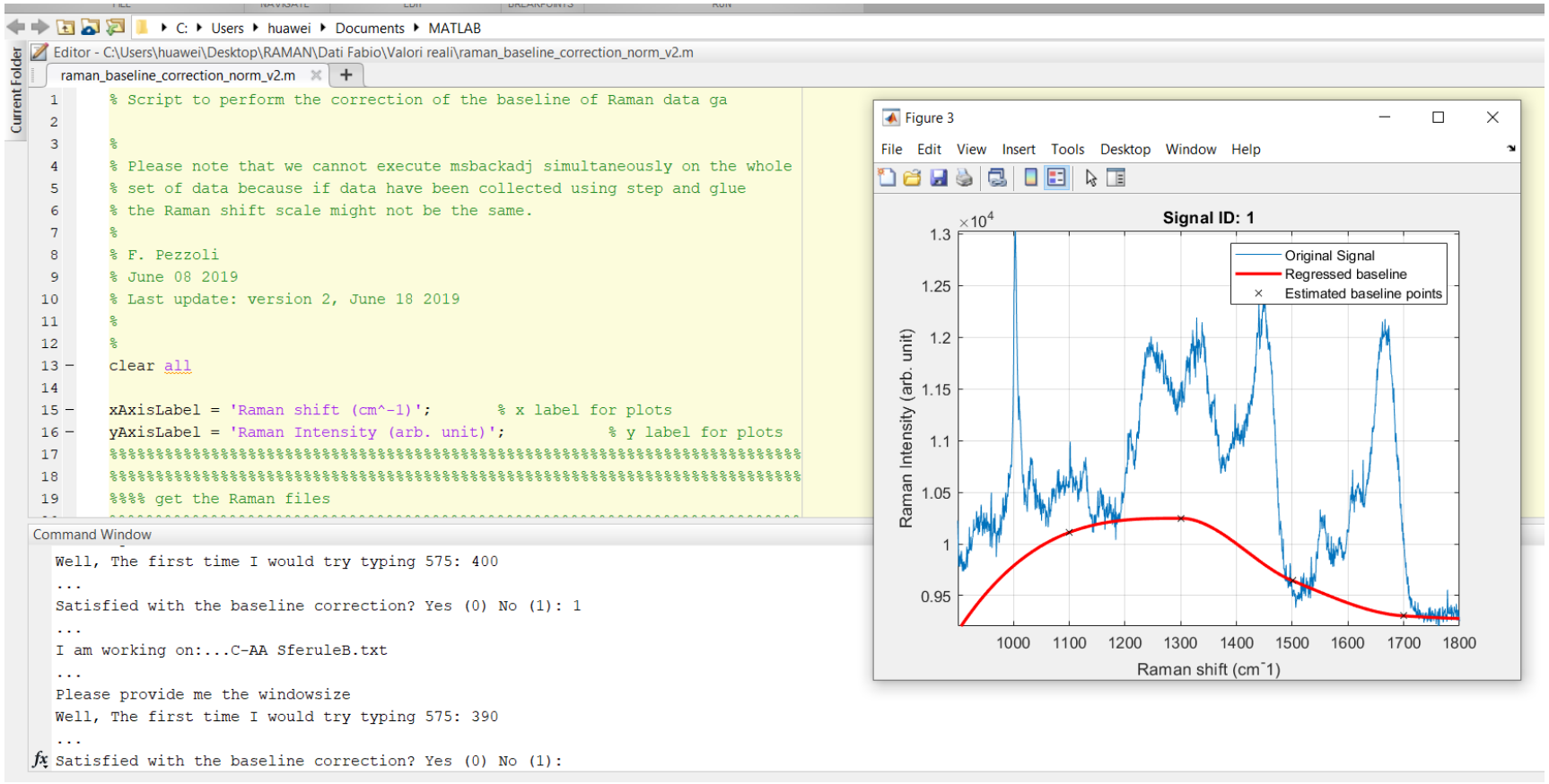
4 main peaks of interest aligned with literature and assigned according to chart

Table 3. Tentative Assignment of Some Bands Found in FT-Raman Spectra of Biological Specimen

Band Numbering (cf. Fig. 1B)	Frequency (cm^{-1})	Assignment[a]
1	~3059	$(C{=}C{-}H)_{(arom.)}$ str
2	~2975	CH_3 str
3	~2935	CH_3 and CH_2 str
4	~2870–2890	CH_2 str
5	~1735	>C=O ester str
6	~1650–1680	Amide I
7	~1614	Tyrosine
8	~1606	Phenylalanine
9	~1575	Guanine, adenine (ring stretching)
10	~1440–1460	C—H def
11	~1295	CH_2 def
12	~1230–1295	Amide III
13	~1129	C—N and C—C str
14	~1102	$>PO_2^-$ str (sym)
15	~1085	C—O str
16	~1061	C—N and C—C str
17	~1004	Phenylalanine
18	~852	"Buried" tyrosine
19	~829	"Exposed" tyrosine
20	~785	Cytosine, uracil (ring, str)
21	~720	Adenine
22	~665	Guanine
23	~640	Tyrosine (skeletal)
24	~620	Phenylalanine (skeletal)
25	~520–540	S—S str

"Naumann, D. FT-infrared and FT-Raman spectroscopy in biomedical research. *Applied spectroscopy reviews* 2001, *36*(2-3), 239-298. https://doi.org/10.1081/ASR-100106157"

Application of Biotech MatLab routine for non linear background subtraction + Integral normalization to 1000 cm^{-1} of all spectra



Courtesy of Prof. Fabio Pezzoli

Differential Analysis

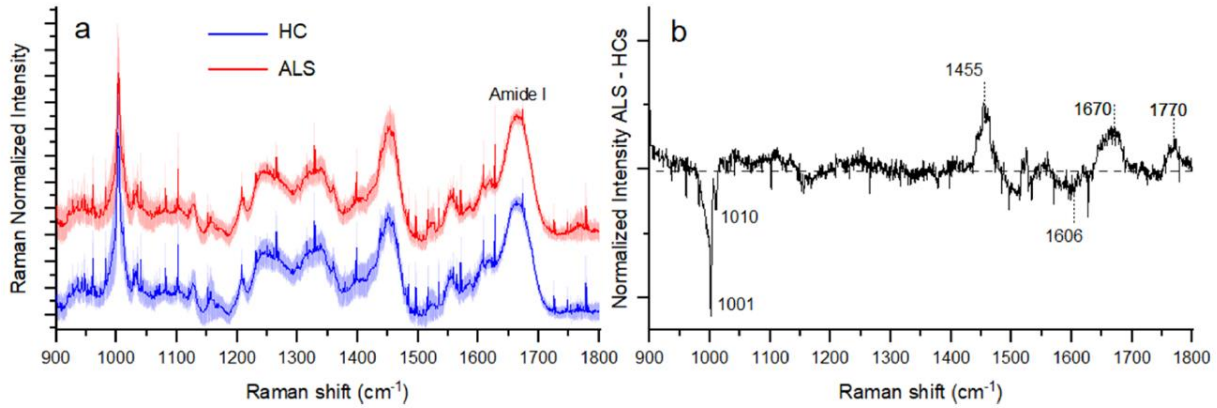


Figure 4. Comparison of the mean Raman spectra obtained by considering all the measured tears from ALS patients and HCs (a). The shadowed area refers to the standard deviation of the data. (b) Spectrally resolved differential average Raman spectra of the two investigated groups.

differential average spectra of the two classes identifying ALS-specific molecular markers:
ALS patients demonstrate on average a marked reduction of the Raman band at ~1000 cm^{-1}

- The positive maximum at ~1003 cm^{-1} in fern-like patterns due to overtake of urea in ALS patients
- Sign reversal in the external ring due to deficiency of phenylalanine vibrations

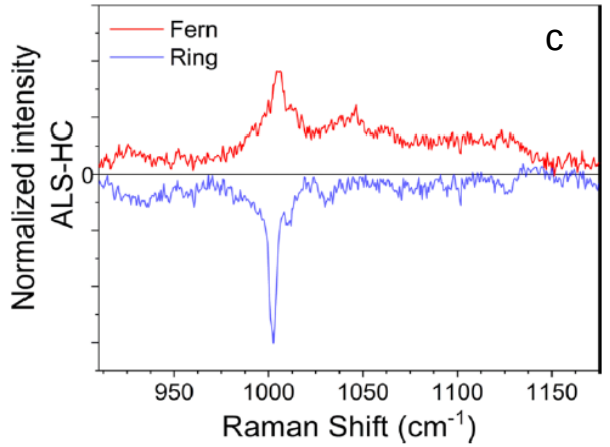


Figure 5. Differential average Raman spectra of ALS patients and HCs measured in the fern-like patterns at the center of the dried drop (red line) and in the coffee ring (blue line).

Multivariate Analysis

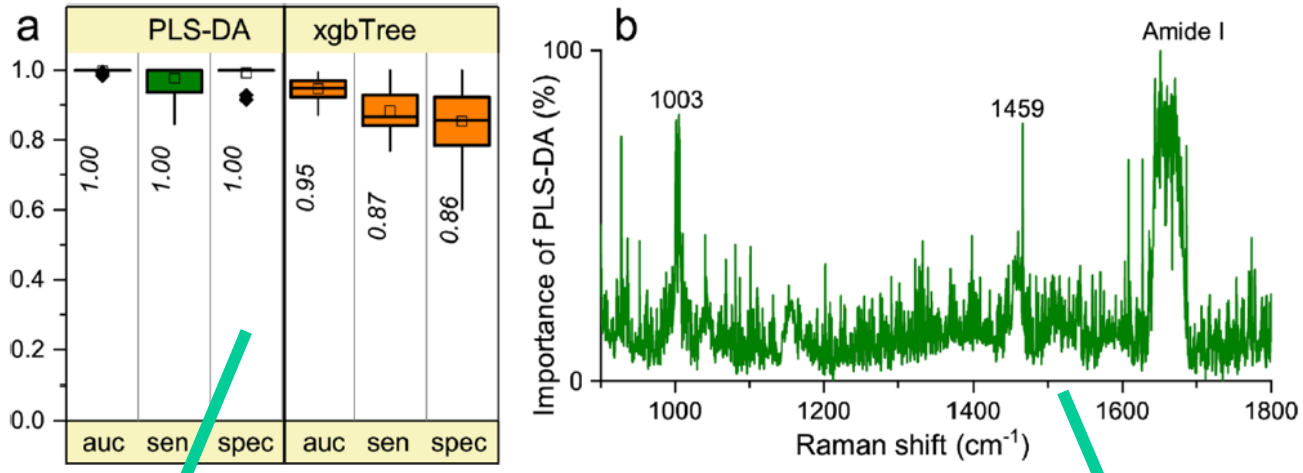


Figure 6. Multivariate analysis of Raman spectra. (A) Overall performances of PLS-DA and xgbTree methods in the 900−1800 cm^{-1} spectral range. In particular, the resampled area under the curve (auc), sensitivity (sens.), and specificity (spec.) are reported as in Figure 2. (B) Wavenumber importance (domain 0−100) for PLS-DA method in the 900−1800 cm^{-1} spectral range.

The PLS-DA analysis shows a high classification accuracy with a specificity and sensitivity of ~100%

Spectral components discriminating ALS from HCs: phenylalanine and amide I are the most important signatures for the classification

Analysis of PhD.Paolo Mereghetti

Conclusions

- Confirmation of the hypothesis: metabolic dysfunctions and alterations of the whole protein structures and/or content might play a crucial role in this neurodegenerative disease
- Detection of differences in biomarkers proved to be high in sensitivity and specificity

- Interpretation on the origin of this possible difference is still to be pursued

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Thank you for your attention!