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Tear-based vibrational spectroscopy for non-invasive biomarker discovery in Amyotrophic Lateral Sclerosis

A. Duse([1])([2]), F. Ambrosio([3]), D. Ami([3]), A. Borghesi([1])([2]), F. Cozza([2])([4])([5]), R. Rolandi([6]), R. Grandori([3]), C. Lunetta([4])([5])([7]), P. Mereghetti([8]), A. Natalello([3]), F. Pezzoli([1]), F. Zeri([1])([2])([9]), E. Ponzini([1])([2]) and S. Tavazzi([1])([2])

([1]) *Dipartimento di Scienza dei Materiali, Università degli Studi di Milano-Bicocca Milano, Italy*
([2]) *Optics and Optometry research center, COMiB, Università degli Studi di Milano-Bicocca Milano, Italy*
([3]) *Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca Milano, Italy*
([4]) *NEuroMuscular Omnicentre, Fondazione Serena Onlus - Milano, Italy*
([5]) *NeMO LAB S.r.l. - Milano, Italy*
([6]) *CdL in Ottica e Optometria, Dipartimento di Scienza dei Materiali, Università degli Studi di Milano-Bicocca Milano, Italy*
([7]) *Istituto Maugeri IRCCS - Milano, Italy*
([8]) *Bioinformatics Consultant - Arquata Scrivia, Italy*
([9]) *College of Health and Life Sciences, Aston University - Birmingham, UK*

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Summary. — In recent literature, tear's biomarkers analysis through spectroscopic techniques is emerging as a reliable tool to identify neurodegenerative diseases such as Alzheimer's and Parkinson's syndromes. The focus of this work is to discuss the results of the application of Raman Spectroscopy on tears collected from patients affected by Amyotrophic Lateral Sclerosis (ALS), comparing to healthy controls. Showing high specificity and sensitivity, both differential and multivariate analysis have reported differences in spectral components, principally identified in two peaks related to Phenylalanine and Amide I. Furthermore, as distinctive for the neurodegenerative diseases, protein and lipids alterations have been found. The results, being confirmed by a parallel study conducted by infrared (IR) spectroscopy on the same samples, lead to the prospect of defining the technique supportive in the diagnosis of ALS, exploiting tears as a source of biomarkers.

1. – Introduction

Human tear film is a complex structure that provides ocular surface's homeostasis through oxygenation, hydration, and protection against pathogens. The analysis of tears

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provides an insight on traditional conditions that can cause alterations of anterior ocular surface [1-12]. In recent studies, biomarkers have been recognized as discriminantitems even for neurodegenerative diseases [1, 2, 13]. Differently, this study is focused on Amyotrophic Lateral Sclerosis (ALS): it consists in a degeneration of motoneurons in which biomarkers are recognized in hematic levels of proteins, *i.e.*, albumin and urea, and other disease-related metabolites [14]. Adding to this, protein abnormal aggregation occurs, such as the neuronal presence of TDP-43 agglomerates [15]. This work aims to assess if such possible variations are found even in tears, when considered as indicators of ALS [16, 17]. The analysis methods on tears differ according to the object of the investigation; in the present work, the aim is the application of Raman Spectroscopy on dry samples to assess proteins, lipids, or other components, and possibly evaluate differences between ALS patients and a Healthy Control (HC) group. Raman Spectroscopy allows rapidly obtaining qualitative and roughly quantitative information on the molecular composition of a sample, exploiting the vibrational properties of the examined system.

2. – Materials and methods

Raman measurements were carried out using a Horiba T64000 instrument in a single spectrometer configuration with a 1800 lines/mm grating and a Si charge-coupled device cooled by liquid nitrogen. The laser excitation, operated at 532 nm wavelength and 6 mW power to avoid heat-induced degradation of the sample, was focused onto the specimen using a 100 × /0.9 NA objective, which was also used to collect the Raman signal in the so-called backscattering geometry. In this study, the number of samples per group was 20 for ALS (average = 64.0 years old) and 18 for HC (average = 54.0 years old). The age range of the subjects was 40–70 years. Tear samples were collected using a glass capillary (5–7 microliters) and dried on a BaF_2 substrate, in a temperature and humidity-controlled extractor hood to produce a ferning pattern according to the literature [18]. The Raman measurements were carried out with acquisition time of 60×5 seconds, over the 900–1800 cm^{-1} range.

3. – Results

Figure 1 shows a tear sample as an example. In particular, fig. 1, panel B shows the green laser spot impinging on it in four different measurements. In the Raman spectra, well-defined peaks in the 900–1800 cm^{-1} range were observed: in particular, the average Raman spectra of both ALS and HC groups were found to be dominated by a rather narrow feature at 1000 cm^{-1} and by additional broader peaks located above 1200 cm^{-1}. The stronger Raman band observed at ∼1000 cm^{-1} is likely given by urea [16, 18] with contributions from phenylalanine [16, 19]. The protein Raman response of tears was detected in the amide I band due to the stretching vibrations of the backbone C=O groups, with peaks at 1657 cm^{-1} (alpha-helices) and 1670 cm^{-1} (beta-sheets) [20]. A band at ∼ 1250 cm^{-1} was assigned to amide III [16, 18]. Finally, a weak feature at ∼ 1770 cm^{-1} was ascribed to the C=O stretching of lipids [10, 16]. All spectra were included in a MatLab algorithm for background subtraction, to remove noise produced by random stray light and fluorescence, and then normalized to the integral relative to Amide I peak; the result was a single average spectrum containing all structures' contributes, belonging to ALS and HC's, which were subsequently submitted to differential analysis. The entire pool of corrected spectra was finally analysed by multivariate technique.

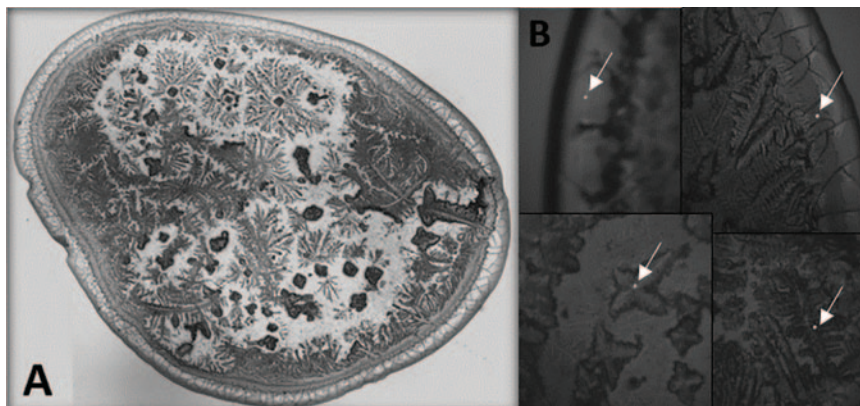

Fig. 1. – (A) Example of a microscopic image of a tear sample; (B) examples of 10× microscope images taken by T64000, in which green laser spots are indicated by the arrows.

In fig. 2, the differential average spectrum of the two sample classes allowed identifying ALS-specific molecular markers: mainly, ALS patients demonstrate a marked reduction of the Raman band at $\sim 1000\,\text{cm}^{-1}$ on average. This is possibly linked to a remarkable global reduction of the phenylalanine level in the tear fluid caused by the disease, suggesting a striking malfunctioning of the metabolism of amino acids. Such phenomena can cause alterations in the neurotransmitter regulation, which are known to result in severe neurological dysfunctions [10] and support the observation that ALS can be fully regarded as a metabolic neurodegenerative disease [21].

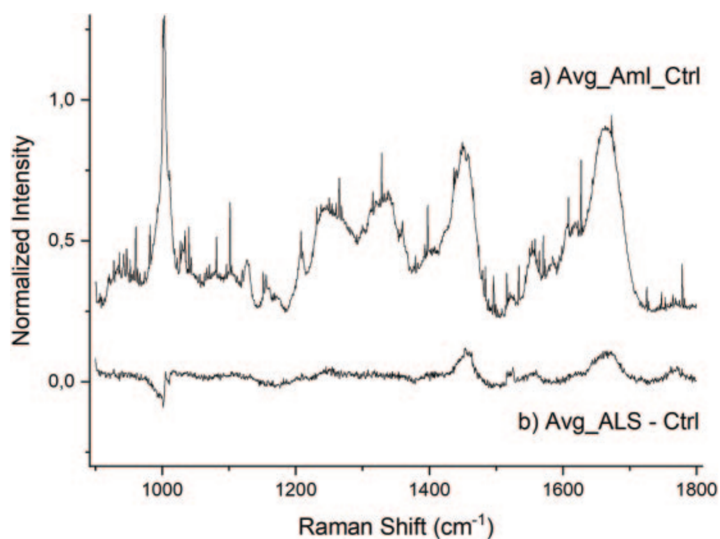

Fig. 2. – (a) Mean Raman spectrum of the healthy control group normalized to the integral of Amide I peak and shifted upwards to higher values on the ordinate axis for a better visualization of the graph; (b) difference between the mean Raman normalized spectra of the two investigated groups.

Multivariate analysis was also carried out by PLS-DA and results were validated by the xgbTree method. The PLS-DA analysis of the Raman spectra showed a high classification accuracy with a specificity and sensitivity of ∼100%. According to the PLS-DA method, phenylalanine and amide I are the most important signatures for classification: the metabolic dysfunctions and alterations of whole proteins' structures and content seem to play a crucial role in this neurodegenerative disease, and the reflection of this into the tear film allows for a prospective identification of ALS development and progression. Furthermore, the xgbTree method showed a high classification performance (0.95) with 0.87 sensitivity and 0.86 specificity.

4. – Conclusions

Raman microspectroscopy, coupled with multivariate analysis, for the characterization of tears from ALS patients and HCs, proved to be an easily accessible diagnostic method with the notable potential of providing unmatched insights into pathology. The technique allows discriminating between pathological and healthy samples with a high overall performance without the necessity of molecular labeling nor expensive and unpractical identification of molecular biomarkers. Furthermore, multivariate analysis discloses the spectral components responsible for discrimination, which can then be assigned to specific biomolecule classes, a result that is highly desirable for a multifactorial pathology such as ALS.

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