

RESEARCH PROJECT ON ALS FINANCED BY THE VIALLI AND MAURO FOUNDATION

Final report on the research activity carried out at the Mario Negri Institute

From September 2007 to August 2010 Dr. Pozzi carried out a research activity at the Mario Negri Institute in Milan, with the supervision of Dr. Bendotti and Dr. Bonetto, thanks to a grant disbursed by the Vialli and Mauro Foundation for Research and Sport. Over the last three years, Dr. Pozzi focused on the research of the translational biomarkers of amyotrophic lateral sclerosis (ALS).

ALS is a neurodegenerative disease that selectively affects motor neurons and leads to death 2-4 years after being diagnosed. Up to now, ALS is diagnosed only through specialist clinical assessments that become definitive after one year from the beginning of the symptoms. In addition, the number of false-positive or false-negative diagnoses is still very high for this type of disease. For the patient, therefore, the ALS diagnosis available today makes it impossible to undergo prompt and specific treatments. For these reasons, for several years the international scientific community studying ALS has felt the need to precisely identify the markers of the disease with the aim of developing a rapid and accurate diagnostic test. A set of ALS-specific biomarkers can help not only specialists in their diagnosis, prognosis and monitoring of pharmacological treatments, but also researchers in more precisely identifying the mechanisms that distinguish this disease from other neurodegenerative diseases, in order to develop more targeted and efficient treatment measures. For many years, thanks to a profitable cooperation between Dr. Bendotti and Dr. Bonetto, the Mario Negri Institute of Milan allowed to start and complete a study focused on the identification of ALS-specific biomarkers. Patients and animal models were analysed in a comparative way, in order to find protein alterations that could be used to diagnose the disease and understand its causes.

90% of ALS cases are occasional, therefore the causes of the disease are unknown. In the remaining 10% of cases, however, there is a link with gene mutations. Among these, 5% of cases depend on the mutation of the superoxide dismutase 1 (SOD1) gene that, over the years, allowed to generate animal models to identify and study the pathogenetic mechanisms at the basis of motor neuron degeneration. Over the last 3 years, Dr. Pozzi focused on the identification and characterisation of protein biomarkers by comparing the alterations observed in patients with those observed in ALS animal models of transgenic mice and rats, bearing the human SOD1 gene with G93A mutation (replacement of glycine-alanine in position 93). They represent the models of the disease that can be more characterised and reproduced. The animal models are critical in the study of this disease because they allow to examine

the phenomena associated to it before the disease shows up, as well as to correlate the alterations in central nervous system districts vulnerable to the disease with possible changes in peripheral tissues that can be easily found in human beings, such as blood. The study was divided into two phases: the first was aimed at identifying protein alterations in the patient, whereas the second envisaged the analysis of the same alterations in animals. During the research period financed by the Foundation, Dr. Pozzi focused on identifying translational biomarkers, i.e. proteins that have the same behaviour or change in patient and in animal model, in order to use them as indices for the disease trend and facilitate the study of possible therapies against ALS.

In order for the diagnosis to be rapid and easily accessible for the patient, the biomarkers have to be found in a sample easily available. That is why blood samples were analysed, in particular a cellular sub-population available in this sample, such as lymphocytes and monocytes, generally classified as peripheral blood mononuclear cells (PBMC). The choice of the sample to analyse was based on the easy availability in patients and on the fact that, despite plasma or serum, the cellular assembly is more stable and its analysis requires less technical artefacts. Thanks to proteomic methods, it was possible to precisely identify proteins that vary their expression in patients compared to healthy individuals, and proteins altered by pathologic events such as oxidative stress.

During the first research period, the focus was on proteins altered by an excess of nitrate stress mediated by nitric oxide. It is known that because of an aberrant activity of the changed SOD1 or other toxic stimuli not identified in occasional ALS, a high quantity of RNSs develop in the motor neuron cells. These forms damage the cell modifying its proteins. Thanks to the analysis carried out in our laboratory, it was possible to identify those proteins specifically altered by this type of stress in peripheral cells both in patients and mice suffering from ALS. In addition, some of these proteins were identified also in the spinal cord of mice as well as in spinal cord autopsy findings of ALS patients. Proteins altered by nitrate stress identified in peripheral cells of patients testify alterations at cell structure (actine, alpha-actine, vinculin, filamin-A) and cell functions (ATPasi, CD41). In the SOD1G93A dependent animal model the same structural and functional proteins were identified (actine, vinculin, ATPasi). The analysis carried out in rats' PBMC showed PDI, GRP78 and HSC70 as other nitrate functional proteins. Furthermore, interestingly, actine, ATPasi and HSC70 in nitrate form were identified in the spinal cord of SOD1G93A mouse. Therefore, the study highlighted that a specific event characterising the disease can be identified not only in the pathologic tissue but also in peripheral cells of an easy accessible sample such as blood. The presence of certain proteins affected by this type of stress both in occasional patients and animal models at a pre-asymptomatic stage can help better clarify its possible causes and consequences. In addition, evidence of an overlapping between mechanisms that develop in central nervous system and peripheral cells gives more value to the emerging theory according to which ALS is considered a multi-cellular and multi-systemic disease. This research study was turned into a scientific publication in 2009 and was edited by *Antioxidant & Redox Signaling*, under the title "Nitroproteomics of Peripheral

Boold Mononuclear Cells from Patients and Rat Model of ALS" (Nardo G., Pozzi S., et al. vol.11, num.7, pp.1559-1567).

A further effort to identify ALS biomarkers was made at the second stage of the study. Thanks to proteomic methods it was possible to highlight some proteins that show a different expression in PBMCs of patients at different stages of the disease compared to healthy sample individuals and patients suffering from other neurodegenerative diseases often confused with ALS (diabetes-driven neuropathy, peripheral axon neuropathy, Charcot-Marie-Tooth disease, spinocerebellar ataxia, multiple sclerosis, polymyositis, etc...). The analysis showed 129 proteins with an expression behaviour different between patients and healthy individuals. The analysis with mass spectrometry identified 71 proteins with different cell functions. In patients' blood cells, proteins linked to energy metabolic rate, oxidative stress regulation and maintenance and degradation of other proteins, but also to cell structure, inflammatory response and interaction with DNA/RNA are altered. Among these, 14 proteins were selected as candidate biomarkers. This choice depended on the fact that, according to existing literature, some of these proteins are involved in the central nervous system, in mechanisms typical of neurodegenerative diseases and altered in ALS. The 14 candidates were validated on a single patient and with easy-to-use techniques in order to have a rapid and efficient diagnostic test in the future. The study highlighted that within the selected biomarker set, it was possible to isolate, with 98% of accuracy and sensitivity, patients from healthy individuals, with 91% ALS patients from patients suffering from other neurodegenerative diseases and with 90% the two different stages of the disease. Therefore Dr. Pozzi verified the behaviour of these markers also in the animal model. The analysis involved not only PBMCs, in order to establish a correspondence with the patient, but also the central nervous system, and particularly the dorsal and ventral roots of the lumbar spinal cord. It is known that in lumbar ventral roots there is the highest number of motor neurons that degenerate during the disease. The analysis of this area, together with peripheral blood cells allows to establish a correlation between periphery and cell population of the central nervous system that undergo pathologic events. 5 out of 14 biomarkers were identified which show the same correspondence between the patient and the animal model even at the pre-symptomatic stage. These are proteins linked to oxidative stress, maintaining equilibrium during oxidative stress and structural stress of other proteins, as well as interacting with DNA and RNA. Therefore these markers have a translational nature because they have the same behaviour in patients and animal models. They can testify the presence of mechanisms they regulate that are responsible for the disease but highlight also the elements to target with specific therapies. Currently this study has been submitted for approval with the title "Mechanism-related multiprotein biomarkers of amyotrophic lateral sclerosis in peripheral blood mononuclear cells" (Nardo G., Pozzi S., et al. 2010).

In summary, the research study carried out by Dr. Pozzi in the period financed by the Vialli and Mauro Foundation contributed to identify the translational biomarkers between ALS patient and animal model. These markers are

proteins altered by disease-typical events, specifically the oxidative stress, and proteins that show an altered expression compared with healthy individuals. Thanks to this study, several scientific considerations can be made: first of all, a new cell assembly shows its importance, different from the motor neuron population, that is similar to it but is more useful for research and clinical diagnosis. Secondly, the presence of correspondence between occasional disease onsets, represented by the examined patients, and cases of familiarity, particularly the SOD1G93A-dependent. Therefore, the identification of key targets in humans and animal models at a pre-symptomatic stage, allow a deeper analysis of mechanisms that cause the disease. On this matter, Dr. Bonetto laboratory work is focused on the involvement of a translational marker that shows an altered expression in ALS cases and that could prove critical in the etiopathogenesis and could also become a potential target of an effective pharmacological treatment.