# Top-Down proteomic analysis of CSF proteins from ALS patients

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Amyotrophic lateral sclerosis (ALS) is a form of motor neuron disease. ALS is a progressive, fatal, neurodegenerative disease caused by the degeneration of motor neurons, the nerve cells in the central nervous system that control voluntary muscle movement. Diagnosis is achieved with clinical evaluation, as well as electromyography and neuroimaging. However, a significant number of patients are misdiagnosed or diagnosed after a long delay. Therefore, it is crucial to find potential biomarkers to improve early and reliable diagnosis. Using a Bottom-Up analysis and isobaric quantification of cerebrospinal fluid (CSF) from ALS patients, we recently identified a panel of differentially expressed proteins (Borg et. al, submitted). In the present study we applied Top-Down proteomics to examine modifications of these proteins in CSF samples. Post translational modifications (PTM) and various isoforms of targeted proteins from control and sporadic ALS (SALS) were characterized.

ALS has largely unknown pathogenesis that typically result in death within a few years from diagnosis [1]. There are currently no effective therapies for ALS. Clinical diagnosis usually takes several months to complete and the long delay between symptom onset and diagnosis, limits the possibilities for effective intervention and clinical trials [2]. The establishment of protein biomarkers for ALS may aid an earlier diagnosis, facilitating the search for effective therapeutic interventions and monitoring drug efficacy during clinical trials.

Our goal was to identify by Top-Down proteomics PTMs and isoform variants of targeted CSF proteins in ALS patients and controls, with the aim of using them as a diagnosis tool. We focused on proteins which were found differentially expressed in our previous studies of CSF from ALS patients (Borg et. al, submitted).

In order to achieve this goal, the following workflow was applied (Figure 1): CSF protein samples were precipitated with acetonitrile [3] and submitted to liquid chromatography- mass spectrometry (LC-MS) analysis. In detail, Top-Down analysis was performed by online LC-nanoESI-MS coupling on a LTQ-FT Ultra (ThermoScientific) with simultaneous fraction collection using the Triversa Nanomate (Advion BioSciences). A BioSuite pPhenyl 1000 (Waters) 10 µm RPC 2.0 x 75 mm column with a 5-80% ACN gradient over 60 min (100 µl/min) was used for intact protein separations. A 1/250 part from the column eluent was injected directly to the mass spectrometer through the Triversa Nanomate using nanoESI chip technology (on line LC-nanoESI-MS, dynamic Top-Down) and the rest of the eluent was fraction collected in a multi-well plate through the same Nanomate. According to data obtained by dynamic Top-Down, the specific retention times of m/z ions of interest were visualized; afterwards, these specific fractions containing ions of interest were infused into the MS from the multi-well plate and analyzed by static Top-Down. Protein fragments were obtained by applying CID or ECD and/or IRMPD on selected intact proteins ions according to our interest of identification/ PTM studies. From 500 to 1000 microscan averaging was necessary to increase signal to noise ratio (S/N) and obtain informative fragment spectra from infused fractions. Obtained data was processed by ProsightPC2 (ThermoScientific).

In this work, we focused mainly in 4 proteins named apolipoprotein A1, cystatin C, β-microglobulin and β-microglobulin short isoform.

A truncated isoform of apolipoprotein A1 (Apo A1) was observed in both control and SALS samples. An isoform of -128 a.m.u of this truncated Apo A1 form was also detected in both samples and fragment analysis was consistent with K-10 deletion (natural variant; K missing in Marburg/ Munster 2 isoform). Short and major isoforms of β-microglobulin were encountered. Several fragments indicated a modification of residue at po-

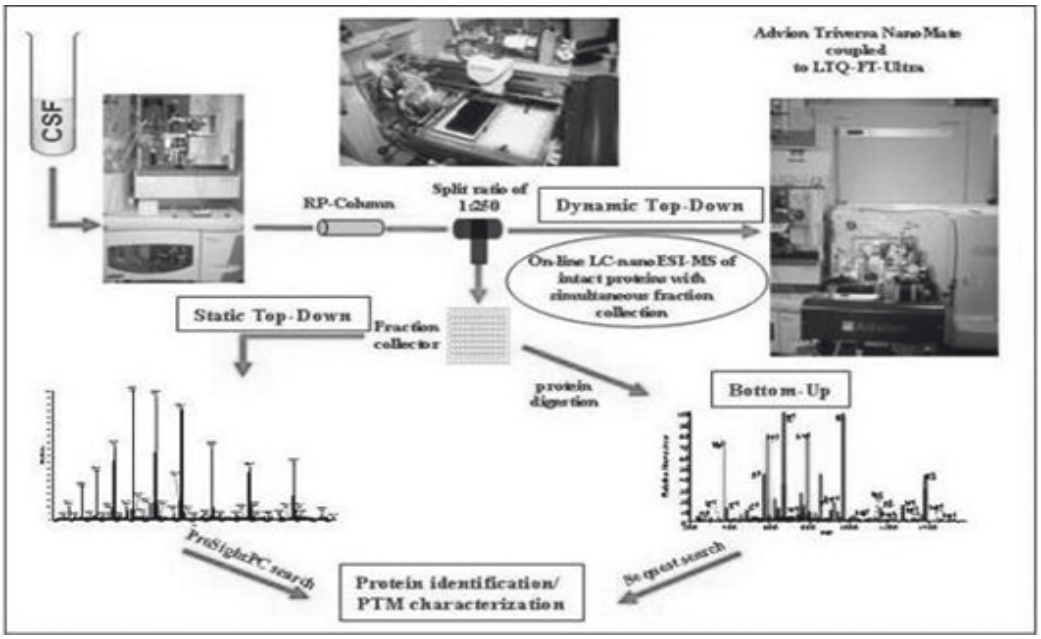


**Figure 1.** *Workflow applied for identification and characterization of potential biomarkes for ALS*

sition 22 (pyroglutamic acid formed from Gln), a glycosylation at $Ser_4$ or $Ser_2$ and a phosphorylation in the short isoform. Moreover, further analysis of cystatin C protein showed a truncated isoform, an oxidation of the $Met_{14}$ and a phosphorylation. Table 1 summarized PTMs characterized by Top-Down. Protein samples collected in multi-well plate at retention times of interest were pooled and digested with specific enzyme. These peptides were analyzed applying nanoLC-MS/MS and the identified proteins confirmed the results obtained by Top-Down.

The isoform variants and PTMs detected in this work, showed the potential of Top-Down applied in biomarker field. Nevertheless, these results must be validated in order to use them as a diagnosis tool for ALS using and independent methodology.

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**Table 1.** *Summary of the PTMs characterized by Top-Down methodology.*

| Protein | ret. times | Intact mass** | Major isoform detected control | ALS | PTMs |
|---|---|---|---|---|---|
| Apo A1 | 31.2-31.7 | 30758.93<br>28061.47 | 27933.43<br>28061.40<br>28224.44<br>28384.48 | 27933.43<br>28061.40<br><br>28386.63 | t, -128.09, missing $K_{107}$ or term Q<br>truncated Apo A1(t)<br>t, +162.9, glycation |
| ß-microglobulin | 24.8-25.2 | 13705.91 | <br>13871.84<br>13928.86 | 13752.83<br>13871.82<br>13928.84 | |
| ß-microglobulin (short isoform) | 21.2-21.9 | 11741.02 | 11721.78<br><br>11801.76<br>11883.84 | 11721.78<br><br>11801.76 | -17.02, pyro-Glu from Q<br><br>-17, +79.96 phosphorylation<br>-17, +162.05, Glc at $S_{2/4}$ |
| cystatin C | 23.5-24.5 | 15789.08<br>13334.56 | <br>13334.48<br>13350.56<br>13413.51 | <br>13334.48<br>13350.56<br>13413.51 | truncated cystatin C (t)<br>t, +15.99, M(O)<br>t, +79.96, phosphorylation |

## References

[1] Rowland L.P., Shneider N.A. Amyotrophic lateral sclerosis. N. Engl. J. Med, 2001; 344:1688–1700.

[2] Ryberg H and Bowser R. Protein biomarkers for amyotrophic lateral sclerosis. Expert Review of Proteomics, 2008: 5: 249-262.

[3] Abdi F, Quinn JF, Jankovic J et al. Detection of biomarkers with a multiplex quantitative proteomic platform in cerebrospinal fluid of patients with neurodegenerative disorders. J Alzheimers Dis. 2006; 9:293-348.


# Optimum method designed for 2D-DIGE of arterial intima and media isolated by laser microdissection


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## Introduction

Tissue proteomic studies on atherosclerosis have traditionally focused on whole artery extracts from biopsy or necropsy origin. Arterial intima and media layers are both involved in atherosclerotic development. In the present work, we describe an optimum method which employs the combination of Laser Microdissection and Pressure Catapulting (LMPC) and 2D-DIGE saturation labelling to investigate the human intima and media subproteomes isolated from atherosclerotic (coronary and aorta) or non-atherosclerotic (preatherosclerotic coronary) arteries.

## Methods

Coronary biopsies from patients undergoing bypass surgery and coronary and aorta necropsies were immediately washed in saline and freezed embedded with OCT. Intima and media were isolated by LMPC with a Microbeam System (PALM Microlaser). Several methods for staining (hematoxilin, cresyl violet), protein extraction and DIGE labelling were tested in order to achieve the better protocol for 2-DE analysis of human arterial layers. First of all, a spin column chromatographic step with Protein Desalting Spin Columns (Pierce) was assayed in order to minimize negative effects from remaining stain. In addition, different lysis buffers were tested to assure efficient extraction of arterial layers proteome, which is problematic due to highly acidic myofilament proteins and presence of calcium.

## Results

Concerning staining methods, both hematoxilin and cresyl violet were found to negatively affect protein extraction and IEF. A chromatographic spin column step prior to saturation DIGE labelling improved substantially 2-DE gels, permitting the use of both stains for human arterial layers proteomic analysis. Ethanol solved cresyl violet minimizes proteases action and facilitates visualization of arterial tissue. For this reasons this stain was chosen for further analysis. Optimal DIGE labelling conditions were set at 1nmol TCEP and 2nmol Dye, so that free dye deleterious effects were minimized. The addition of SDS, and in a higher extend DTT, on lysis buffer significantly increased protein solubilization,