**“In-gel” protein spot Trypsin digestion protocol**

 Protein spots excised manually from the gels were subjected to a tryptic digestion “in-gel”. Each sample was first de-stained by a destain solution (1:1, v/v; 30 mM potassium hexacyano-ferrate(III)/100 mM sodium thiosulphate) and then washed with ultra-pure deionized water and acetonitrile (ACN), before the reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide. After drying in a vacuum dryer (Eppendorf Concentrator Plus, Eppendorf, Milan, Italy), samples were rehydrated in 20 L of 25 mM ammonium bicarbonate solution containing 120 ng of Trypsin gold mass spectrometry grade and incubated over-night at +37°C. After digestion, the peptides were extracted using a solution composed of 1% trifluoroacetic acid/50% ACN. Finally, the samples were concentrated in a vacuum dryer and then stored at ‒80°C until mass spectrometry analysis.

*Chemicals and reagents*

 Potassium Hexacyano-Ferrate(III), Sodium Thiosulphate, DTT, Iodoacetamide, and Ammonium Bicarbonate were from Merck KGaA (Darmstadt, Germany). Trypsin Gold Mass Spectrometry Grade was from Promega (Madison, WI, USA). Trifluoroacetic Acid and Acetonitrile were from Carlo Erba Reagents (Milan, Italy).

**MS protein identification by LC-ESI-QO-MS/MS**

Analyses were perfomed by an UHPLC-MS QExactive™ (ThermoScientific) system, composed of UHPLC 3000 Ultimate System coupled to an ESI-QExactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (LC-ESI-QO-MS/MS System), which combines high-resolution quadrupole precursor ion selection and accurate-mass (HRAM) orbitrap detection. Dried samples were resuspended in water/acetonitrile/formic acid (95:3:2), sonicated for 10 minutes at room temperature and centrifuged at 12,100 x g for 10 minutes prior to analyses. Separations were carried out on a ZORBAX RRHD Eclipse Plus C18 column (50 × 2.1 mm ID.; 1.8 μm particle size; Agilent) with a mobile phase of 0.1 % aqueous formic acid solution (A) and acetonitrile (B) using the following gradient elution at a flow-rate of 0.3 mL/min: 0 to 3 min, isocratic at 2% (B); 3 to 21 min, linear gradient from 2 to 27% (B); 21 to 25 min, linear gradient from 27 to 90% (B); 25 to 28 min, isocratic at 90% (B); 28 to 28.1 min, linear gradient from 90 to 2% (B). An equilibration period of 6.9 min was used between each run; total run time: 35 min. The injection volume was 15 µL and the injector needle was washed with methanol/water 1:1. Nitrogen was used for spray stabilization, for collision-induced dissociation experiments in the higher energy collision dissociation (HCD) cell, and as damping gas in the C-trap. ESI source operated in positive ionization mode and the operative parameters were set as follows: capillary voltage, 3.5 kV; capillary temperature, 320 °C; sheath gas flow, 40 arbitrary units (arb); auxiliary gas flow, 30 (arb); auxiliary gas temperature, 290°C; sweep gas flow, 3 (arb); S-Lens RF level, 55 (arb). The MS settings were: 70,000 resolution, 3 × 106 automatic gain control (AGC) target and 200 ms maximum injection time (IT) for MS1 scans (range: *m/z* 250−1500) and 17,500 resolution, 5 × 105 AGC target, 50 ms IT for dd-MS² scans (range set according to *m/z* and charge value of the precursor). The five most intense multi-charged ions were selected for MS2 nitrogen-promoted collision-induced dissociation (normalized collision energy: 28%). A precursor active exclusion of 20 seconds was set and peptide-like isotope pattern ions were preferred. Analyses were controlled by Xcalibur™ software (v. 29 build 2926). Raw data were converted into mascot generic format using MsConvert (v. 3.0.10730, ProteoWizard tools) and then were searched by MASCOT search engine (Version 2.4, Matrix Science, London, UK) against the databases Swiss-Prot for peptide sequences and C-RAP for contaminants, respectively. The search parameters were set as follows: trypsin as proteolytic enzyme, carbamidomethyl-cysteine as fixed modification, deamidated (NQ) and oxidated (M) methionine as variable modifications, one missed trypsin cleavage allowed, mass tolerance set at 10 ppm for the precursor ions and 0.05 Da for the product ions. An automatic decoy database search was used to estimate the false discovery rate (FDR), which was adjusted to < 1% FDR.

**Protein identification by LC-ESI-QO-MS/MS**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Entry name**a | **Acc.****number**b | **Protein name** | **Score**c | **Pep./****pep. sign.**d | **Seq./****seq. sign.**e | **Cov.**f**(%)** | **emPAI**g |
| **THRB** | P00734 | Prothrombin | 448 | 41/21 | 18/12 | 26 | 0.89 |
| **SAMP** | P02743 | Serum amyloid P-component | 322 | 15/12 | 8/8 | 30 | 1.66 |
| **IGKC** | P01834 | Ig kappa chain C region  | 514 | 45/23 | 7/6 | 85 | 13.75 |
| **APOA1** | P02647 | Apolipoprotein A-I | 1309 | 105/72 | 31/25 | 76 | 44.71 |
| **SAA4** | P01834 | Serum amyloid A-4 protein  | 183 | 24/7 | 9/4 | 56 | 1.98 |
| **ITIH4** | Q14624 | Inter-alpha-trypsin inhibitor heavy chain H4 | 149 | 10/7 | 6/5 | 7 | 0.18 |
| **CO4A** | P0C0L4 | Complement C4-A (fragment) | 186 | 21/14 | 8/7 | 4 | 0.14 |
| **TTHY** | P02766 | Transthyretin | 82 | 10/6 | 5/5 | 26 | 1.34 |
| **TETN1** | P05452 | Tetranectin | 90 | 11/4 | 8/3 | 35 | 0.62 |
| **TETN2** | P05452 | Tetranectin | 106 | 7/4 | 6/4 | 35 | 0.62 |
| **A1AT** | P01009 | Alpha-1-antitrypsin | 517 | 41/28 | 17/12 | 36 | 2.15 |
| **HPT** | P00738 | Haptoglobin | 145 | 13/8 | 6/4 | 42 | 1.34 |
| **APOA4** | P06727 | Apolipoprotein A-IV  | 1448 | 126/80 | 42/36 | 74 | 29.73 |
|  |  |  |  |  |  |  |  |

aEntry name from UniProt knowledge database

bPrimary accession number from UniProt database

cThe highest scores with MASCOT search engine

dNumber of total peptides/significant peptides matching the identified protein

eNumber of total sequences/significant sequences

fSequence coverage: percentage of sequenced amino acids for each identified protein

gExponentially modified protein abundance index

**Raw data of spot volume for each pool of serum samples**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Protein** | Pool C1 | Pool C2 | Pool C3 | Pool C4 | Pool C5 | Mean | Pool MM1 | Pool MM2 | Pool MM3 | Pool MM4 | Pool MM5 | Mean | Pool PM1 | Pool PM2 | Pool PM3 | Pool PM4 | Pool PM5 | Mean |
| **A1AT** | 2,215 | 2,731 | 2,226 | 2,644 | 2,548 | 2,473 | 1,795 | 1,827 | 1,527 | 1,728 | 1,702 | 1,716 | 4,863 | 5,872 | 5,313 | 5,472 | 5,226 | 5,349 |
| **APOA4** | 10,792 | 9,514 | 10,232 | 9,873 | 10,352 | 10,153 | 10,887 | 8,598 | 10,268 | 9,102 | 9,065 | 9,584 | 3,915 | 4,429 | 3,342 | 4,115 | 3,673 | 3,895 |
| **ITIH4** | 0,396 | 0,489 | 0,399 | 0,544 | 0,385 | 0,443 | 0,665 | 0,876 | 0,977 | 0,697 | 0,978 | 0,839 | 0,935 | 0,829 | 0,754 | 0,646 | 0,702 | 0,773 |
| **CO4A** | 0,463 | 0,334 | 0,478 | 0,336 | 0,385 | 0,399 | 0,754 | 0,775 | 0,889 | 0,811 | 0,848 | 0,815 | 0,430 | 0,412 | 0,496 | 0,512 | 0,544 | 0,479 |
| **SAMP** | 0,111 | 0,384 | 0,269 | 0,354 | 0,120 | 0,248 | 0,902 | 1,094 | 1,012 | 0,923 | 0,881 | 0,962 | 0,534 | 0,621 | 0,465 | 0,558 | 0,536 | 0,543 |
| **APOA1** | 16,023 | 16,446 | 16,618 | 16,101 | 15,989 | 16,235 | 1,162 | 0,958 | 0,780 | 1,135 | 0,798 | 0,967 | 8,050 | 8,656 | 8,066 | 7,476 | 8,124 | 8,074 |
| **IGKC** | 0,394 | 0,708 | 0,374 | 0,721 | 0,557 | 0,551 | 1,143 | 1,054 | 1,078 | 0,917 | 0,935 | 1,025 | 1,071 | 0,839 | 0,865 | 0,966 | 1,101 | 0,968 |
| **TETN1** | 0,368 | 0,647 | 0,425 | 0,485 | 0,617 | 0,508 | 0,519 | 0,374 | 0,328 | 0,410 | 0,406 | 0,407 | 1,404 | 0,848 | 0,701 | 0,969 | 0,998 | 0,984 |
| **TETN2** | 0,235 | 0,372 | 0,369 | 0,287 | 0,258 | 0,304 | 0,338 | 0,331 | 0,392 | 0,399 | 0,390 | 0,370 | 0,772 | 0,705 | 0,720 | 0,806 | 0,828 | 0,766 |
| **HPT** | 0,298 | 0,284 | 0,312 | 0,301 | 0,262 | 0,291 | 0,388 | 0,348 | 0,431 | 0,402 | 0,377 | 0,389 | 0,600 | 0,705 | 0,655 | 0,732 | 0,696 | 0,678 |
| **SSA4** | 1,108 | 1,073 | 1,131 | 1,020 | 1,124 | 1,091 | 0,541 | 0,482 | 0,580 | 0,486 | 0,566 | 0,531 | 0,241 | 0,345 | 0,155 | 0,305 | 0,202 | 0,250 |
| **TTHY** | 0,446 | 0,565 | 0,487 | 0,555 | 0,477 | 0,506 | 0,361 | 0,587 | 0,653 | 0,579 | 0,488 | 0,534 | 6,123 | 6,734 | 6,648 | 5,578 | 5,874 | 6,191 |
| **THRB** | 1,197 | 1,181 | 1,179 | 1,189 | 1,201 | 1,189 | 2,229 | 2,447 | 3,153 | 2,870 | 2,351 | 2,610 | 4,121 | 4,503 | 5,870 | 5,141 | 4,522 | 4,831 |

The raw data of spot volume for each pool were obtained by the PDQuest 2D analysis software, for each gel image.

The mean value of spot volume of each protein was used to evaluate the fold-change of spot abundance, that is the different protein expression (up- or down-regulation) among the different groups.

The fold-change data for each protein (calculated as ratio between the mean values), are graphically reported in the manuscript in Figure 3.

**Inflammation in menstrual cycle and menopause**

 Inflammatory profiles in women appear to fluctuate based on reproductive events across the lifespan, with estradiol exposure playing a significant role. In particular, estradiol appears to be linked to suppression of proinflammatory cytokine production, while greater inflammation is associated with the menopausal transition [G.F. Mattina, R.J. Van Lieshout, M. Steiner. Inflammation, depression and cardiovascular disease in women: the role of the immune system across critical reproductive events. Ther. Adv. Cardiovasc. Dis. 13 (2019) 1–26. doi:10.1177/1753944719851950.].