**Unique metabolomic profile of skeletal muscle in chronic limb threatening ischemia**

NMR sample preparation

Both polar and non-polar metabolites were extracted from the gastrocnemius muscle specimens using FOLCH extraction. In brief, wet weigh of the frozen tissues were determined and immediately homogenized in 1 mL of ice-cold methanol using a PowerLyzer 24 Homogenizer (QIAGEN Group, Hilden, Germany). All enzymatic activities are halted once the sample was homogenized in the methanol. Homogenization was followed by centrifugation (13.2K r.p.m., 4 oC, 30 minutes) and supernatant was transferred into a new glass vial consisting a mixture of 3 mL of ice-cold chloroform and methanol (2:1 v/v) ratio. The cold mixture was vortexed for several minutes and left in an ice bath 15 minutes to allow for phase separation. Next, 1 mL of ice-cold 0.9% saline was added to the mixture followed by vigorous mixing. The mixture was again left in the ice bath for 45 minutes for phase separation. The upper methanol/water layer was transferred to a new falcon tube. To the lower chloroform layer, 1 mL of ice-cold 0.9% saline was added and all steps were followed as mentioned above. Following a 45-minute incubation, upper methanol/water layer was again transferred to the previous falcon tube and dried using a Labconco freeze drier (Labconco Corporation, MO, USA). The chloroform layer was dried under a stream of nitrogen gas. The dried samples (both aqueous and organic phases) were stored at -80 oC until resuspension for NMR experiments.

Lyophilized aqueous phase samples were re-suspended in 50 µL of 50 mM phosphate buffer (pH 7.2) consisting 2 mM of EDTA along with 0.2% NaN3 and 0.5 mM D6-DSS in 100% deuterated environment. Lyophilized organic phase sample were re-suspended in 80 µL of CDCl3 along with 10 mM of pyrazine. All samples were loaded in 1.7 mm NMR tube to acquire spectra.

All solution state NMR experiments were acquired with a Bruker (Bruker BioSpin Corporation, Billerica, MA) Avance Neo 600 MHz/54mm console with a 1.7 mm TCl CryoProbe. First slice of 1D nuclear Overhauser effect spectroscopy (noesypr1D) pulse sequence with water pre-saturation during relaxation delay (d1) was used to collect 1D spectra for both aqueous and organic phase samples. Acquisition parameters were applied: 128 scans (nt), 1 s recycle delay (d1), 4 s acquisition time (aq), 100 ms mixing time, and 7142.9 Hz spectral width (sw) using 1H 90o pulse width (pw) at room temperature (25 oC).

Semi-solid HR-MAS spectra were collected on intact human gastrocnemius muscle specimens. For this, a Bruker 800 MHz system equipped with 4 mm HR-MAS probe was used. Preparation of HR-MAS was performed using 3.2 mm inside diameter plastic insert. The following acquisition parameters were used to collect 1D NOESY spectra (noesypr1D) with pre-saturation of water signal: 256 nt, 2 s d1, 2.04 s aq, 100 ms mixing time, and 8012.8 Hz sw using 90o pw at 4 oC. The sample was spun at 5 kHz speed maintaining 54.7o magic angle.