Metabolomics Workbench Upload Metadata

Project / Study title

Individualized exercise intervention for people with multiple myeloma improves quality of life in a randomised controlled trial

Project / Study summary

Although new treatments have improved survival for multiple myeloma (MM), quality of life remains poor for people with this incurable cancer. We conducted a multi-site randomized, waitlist-controlled trial of an individualized exercise program for people at all stages of MM (n=60). Compared to the waitlist control group, participants of the 12-week intervention had significant improvement in health-related quality of life, mediated through improved MM symptoms, cardiorespiratory fitness and bone pain, with were mostly maintained at follow-up (up to 12 months). Exploratory plasma metabolomics and lipidomics was conducted to delineate molecular mechanisms and biomarkers.

Collection summary

Fasted plasma samples were collected from participants at multiple time points as depicted in Figure 1, taken from the protocol paper Nicol, J.L.;Woodrow, C.;Cunningham, B.J.; Mollee, P.;Weber, N.; Smith, M.D.; Nicol, A.J.; Gordon, L.G.; Hill, M.M.; Skinner, T.L. An Individualized Exercise Intervention for People with Multiple Myeloma—Study Protocol of a Randomized Waitlist-Controlled Trial. Curr. Oncol. 2022, 29, 901–923. https://doi.org/10.3390/curroncol29020077.

After overnight fasting, plasma was collected by an experienced phlebotomist from an antecubital vein using a 23-gauge needle into EDTA-coated blood collection tubes and immediately stored on ice. Aliquots were prepared in Eppendorf tubes within 4 hours, and frozen at -80°C until use.

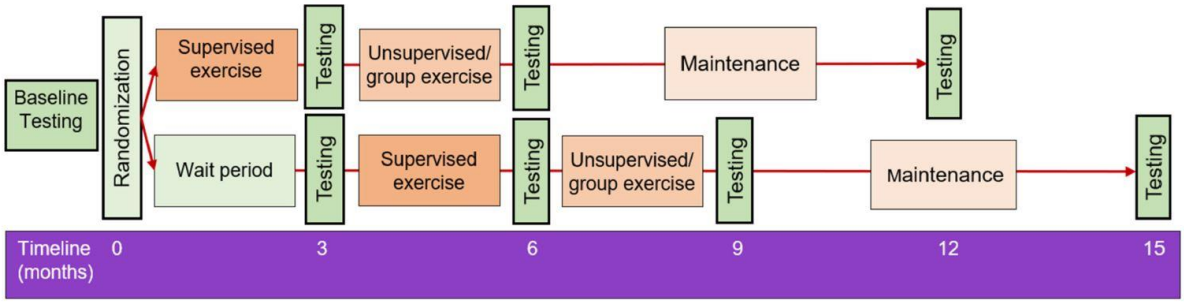

Figure 1. Schematic representation of the study protocol.

Treatment summary

Multiple myeloma patients were randomized to exercise (EX) or waitlist (WT) groups as detailed in the protocol paper: Nicol, J.L.;Woodrow, C.;Cunningham, B.J.; Mollee, P.;Weber, N.; Smith, M.D.; Nicol, A.J.; Gordon, L.G.; Hill, M.M.; Skinner, T.L. An Individualized Exercise Intervention for People with Multiple Myeloma—Study Protocol of a Randomized Waitlist-Controlled Trial. Curr. Oncol. 2022, 29, 901–923. https://doi.org/10.3390/curroncol29020077.

The individualized exercise program was for 3 months, and follow-up plasma was collected at 12 months. WT group had usual care for 3 months prior to the same exercise and follow-up regime.

Sample preparation summary

In total, 126 human plasma samples from 46 patients were prepared. All samples (including QCs and blanks) were randomized in one batch and then evenly split between two 2 mL 96-deepwell plates (Eppendorf #0030 501.306). Human plasma samples were thawed on ice and briefly vortexed before aliquoting 100 µL plasma of each sample to a well. In addition, a global sample pool containing equal volumes (12 µL) of each sample was prepared into a 2 mL Eppendorf tube as quality control (QC) and 12 x 100 µL aliquots were transferred into wells across the 2 plates. Finally, blank negative control extraction samples were prepared by transferring 100 µL of 1X PBS to 6 wells equally across 2 plates. Ten-times the sample volume of ice-cold butanol/methanol (1:1) containing 50 µg/mL antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT) and 0.5 µg/mL ISTD 4-chloro-L-phenylalanine (PCPA) was added to each well using a multi-channel pipette. Plates were covered with organic solvent resistant sealing mats (Eppendorf #0030 127.960) and vortexed for 3 min at 1000 rpm. Samples were then sonicated for 15 min in an ice-cold water bath sonicator, stored overnight at -30^{o}C and centrifuged for 30 min at 4,000 x g (4^{o}C). Samples were aliquoted into 96-well V-bottom plates (Greiner #651201) using a liquid handler platform (AssayMap Bravo, Agilent). From each deepwell plate, 4 x 100 µL and 2 x 200 µL aliquots were prepared, totalling 8 and 4 plates, respectively. Samples were dried down (2.5 hrs) using a Genevac EZ-2 vacuum concentrator and fast-stack swings facilitating drying of 8 plates per batch. Dried sample plates were covered with AlumaSeal CS sealing film for cold storage (Finneran-Porviar #FC-100) and stored at -80^{o}C until LC/MS analysis.

Untargeted LC/MS analysis

For LC/MS analysis, most sample resuspension steps were performed on a liquid handler platform (AssayMap Bravo, Agilent). Plates with dried 100 µL and 200 µL sample aliquots were used for metabolite analysis on reversed-phase and HILIC, respectively. For each analysis mode, all plates were resuspended concurrently and all QC samples across plates were recovered into a single tube using the same resuspension procedure as stated for samples below. Following centrifugation for 20 min at 16,000 x g (4^{o}C), the QC supernatant was transferred into a new tube and HILIC QC samples were diluted to 80% ACN as described below.

Metabolites analysed on reversed-phase were resuspended in 50 µL of chilled 0.1% formic acid containing 1 µg/mL of tripeptide Val-Tyr-Val (VTV) internal standard to each well, followed by thoroughly vortexing plate for 3 min at 1200 rpm and 10 min equilibration on ice. Following plate centrifugation for 30 min at 4000 x g (4ºC), 40 µL of the supernatant volume was transferred to a new plate and sealed with zone-free film (Sigma #Z721646).

Metabolites analysed on HILIC were dissolved by adding 40 µL of chilled milliQ water to each well, followed by thoroughly vortexing plate for 3 min at 1200 rpm and 10 min equilibration on ice. After plate centrifugation for 30 min at 4000 x g (4ºC), 20 µL of the supernatant volume was transferred to a new plate and diluted with ice-cold acetonitrile containing 1 µg/mL VTV internal standard to a final concentration of 80% acetonitrile and sealed with zone-free film.

QC dilutions (0.8, 0.4, 0.2, 0.1, 0.01) were prepared for each analysis mode by diluting respective QC samples with chilled solutions of 0.1% formic acid and 80% acetonitrile (both containing VTV) for metabolite separation on reversed-phase and HILIC, respectively.

Internal standard VTV was used to flag autosampler inaccuracies. In general, samples and blanks were injected from plate, while QCs and QC dilutions were injected from vial. The autosampler temperature was set to 4ºC and injection volumes were 3 µL for positive and 5 µL for negative ionization mode.

Untargeted metabolomics was performed as published previously (Koenig et al. 2018) with modifications, using a 1290 Infinity II UHPLC coupled to a 6545 QTOF mass spectrometer via Dual AJS ESI source (Agilent, Santa Clara, USA) and MassHunter data acquisition software (v.10.1). Assessment of MS instrument performance and usage of reference ions were also performed as described previously. Chromatographic conditions are described in the following sections. Each analytical column was connected to a 2.1 x 5 mm guard column with respective resin.

Reversed-phase separation of metabolites was adapted from Evans et al. (2014) with modifications. Separation was achieved on a Zorbax Eclipse Plus C18 RRHD (1.8 µm, 95 Å, 2.1x50mm, #959757-902, Agilent) column held at 40°C and the MS was operated in positive ionization mode. Eluent A was milliQ water and eluent B was methanol both containing 0.1% formic acid. Total method runtime was 8.5 min with the following gradient: 0 min (0.5% eluent B) – 4 min (70% B) – 4.5 min (98% B) – 5.4 min (98% B) – 5.5 min (0.5% B) – 8.5 min (0.5% B). The flow was diverted to waste after 5 minutes and a flow rate of 0.4 mL/min was applied.

HILIC separation of metabolites was carried out on a Poroshell 120 HILIC-Z (100Å, 2.7µm, 2.1x100mm, #675775-924, Agilent) column at 30ºC and the MS was operated in negative ionization mode. Eluent A was 10 mM ammonium acetate and 5 µM medronic acid in ACN/milliQ 90:10 (pH 9) and eluent B was 10 mM ammonium acetate and 5 µM medronic acid in milliQ (pH 9). Gradient applied was: 0 min (10%

eluent B) – 3.5 min (25% B) – 5.5 min (50% B) – 7.5 min (50% B) – 7.6 min (10% B) – 14 min (10% B). Flow rate was set to 0.25 mL/min with a flow ramp to 0.5 mL/min during equilibration.

Full scan MS data acquisition (m/z 50-1700) was carried out at a scan rate of 2.5 spectra/sec with the following source conditions applied for metabolites analysed on reversed-phase: Gas temperature 250°C, gas flow 13 L/min, sheath gas temperature and flow at 400°C and 12 L/min, respectively, nebulizer 30 psi, fragmentor 135, capillary voltage at +4500 V, nozzle voltage and CE were zero. For metabolite separation on HILIC conditions were adjusted as follows: Gas temperature 200°C, gas flow 10 L/min, nebulizer 40 psi, capillary voltage at -2500 V.

QC samples were used to assess instrument variation (retention time and signal stability) and to acquire MS/MS fragmentation data. Before a sample sequence, 10 replicate QC injections were performed to condition the column. Then, QCs were injected in duplicate at the beginning and end of each sequence and after every set of 10 samples. All samples were run in a randomized order and no blank injections were performed between samples. Following the sample sequence, acquisition of iterative MS/MS data, QC dilutions and blank negative control extractions was performed. The latter was being used in downstream data cleaning to identify and remove background noise and potential contaminants.

For compound identification, the 'Iterative MS/MS' data acquisition mode was employed, i.e. a sample (here: QC pools) was injected multiple times and precursors previously selected for MS/MS fragmentation were excluded in subsequent runs. Eight iterative MS/MS acquisition runs per fixed collision energy (CE) value were performed with CE values set to 0, 10, 20, and 40 V. Spectral parameters were as follows: MS and MS/MS mass range was 50-1700; MS and MS/MS acquisition rate was 3 spectra/sec; quadrupole isolation width was narrow (~1.3 m/z). A maximum of 8 precursors per cycle were targeted which resulted in a cycle time of 3.1 s. Precursor threshold was set to 500 counts or 0.001% with an active exclusion of 0.2 min after 1 spectra. Iterative MS/MS settings were enabled with a mass error tolerance of +/- 5 ppm and retention time exclusion tolerance of +/- 0.1 min. Precursor charge state was set to 1, 2 and unknown. Precursor abundance-based scan speed with a target of 25,000 counts/spectrum and the use MS/MS accumulation time limit were enabled. Precursor purity stringency was kept at 70% and cutoff 0%. Reference ions were excluded from fragmentation with a delta mass tolerance of 10 ppm.

Data processing

Feature extraction was performed separately for each of the 2 LC/MS analysis modes. A total of 148 data files (126 patient samples, 16 QCs, and 6 blank negative control extractions) were loaded into MassHunter Profinder (v 10 SP1, Agilent) and assigned to sample groups. Patient samples were divided into 2 sample groups (waitlist and exercise group), totalling 4 samples groups altogether. First, retention time alignment was conducted using a QC run as reference file. Spectral feature extraction was then performed using the recursive feature extraction method employing default settings with minor adjustments: Peak extraction retention time (Rt) range was restricted to 0.1-5 min and 0.1-7 min for

reversed-phase and HILIC, respectively, compound binning and alignment tolerances were set to 1% + 0.3 min for Rt and 20 ppm + 2 mDa for mass, integrator Agile 2 was used for peak integration, peak filters were set to at least 2500 counts and features must have satisfied filter conditions in at least 75 % of files in at least one sample group.

Feature peak area was exported and data cleaning was performed in accordance with Southam et al. (2021) using an in-house R script compiled of the following steps. QC samples were removed from the data matrix area if the total peak area (of all features) exceeded +/-25% of the median QC total peak area. Features were deleted from the data matrix if: detected in less than 70% of QC samples; absent across all sample groups; the mean QC/extract blank area ratio was less than 5; and the peak area RSD across QC samples was larger than 30%. In addition, duplicate feature entries were removed.

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