CABG tissue collection

LV tissue from patients undergoing CABG was sampled from two or more pre-determined areas (as guided by CMR and coronary angiography data) on the beating heart during surgery: 1) a region of viable myocardium with inducible ischemia; 2) a region of remote myocardium with normal contractility, no qualitative evidence of inducible hypoperfusion on perfusion imaging, and without infarct pattern LGE. Each biopsy location was determined from analysis of the CMR and coronary angiography images, with co-localisation performed in theatre with the consultant cardiac surgeon. The *a priori* plan was to acquire paired biopsies, permitting high energy phosphate (HEP) quantification and LC-MS for each individual patient. Surgical discretion, however, resulted in some patients not undergoing paired tissue collection. Conversely, where deemed safe, extra tissue was acquired for single-nuclei RNA sequencing. The CABG biopsies were performed on the beating heart (prior to aortic cross-clamp, cardioplegia and hypothermia in the cases using cardiopulmonary bypass) with a Tru-Cut® needle or scalpel by the consultant cardiac surgeon. The majority of operations were performed off-pump without cardiopulmonary bypass. Samples were immediately clamp-frozen in theatre using a Wollenberg clamp (manufactured by Josh Firman, LMB Workshop, UK) which had been pre-cooled in liquid nitrogen until tissue acquisition. The clamps were then reopened, and the tissue rapidly transferred into Eppendorf tubes (pre-cooled in dry ice) before storage at $^{-}80$ºC awaiting further analysis.

Tissue extraction and metabolite analysis by LC–MS

The protocol used in this experiment has previously been published by our group and portions of the following text have been recycled to ensure clarity.[38] Frozen LV tissue samples (~1-5mg) were weighed into Precellys tubes (Stretton Scientific Ltd., Derbyshire, UK), and an exact volume of extraction solution (50% methanol, 30% acetonitrile and 20% water) was added to obtain 40 mg specimen per ml of extraction solution, permitting

comparisons between experimental conditions for the same metabolite. The samples were subsequently lysed after the addition of 3 ceramic beads using a Precellys 24 tissue homogenizer (Bertin Corp, Rockville, MD 20850, USA. 5500 r.p.m for 15 seconds × 2) and finally centrifuged (16,162 × g for 10 min at 4 °C). The supernatant was transferred into glass vials (Microsolv Technology Corp., Leland, NC 28451, USA) and stored at −80 °C until LC–MS analysis.

HILIC chromatographic separation of metabolites was achieved using a Millipore Sequant ZIC-pHILIC analytical column (5 µm, 2.1 × 150 mm) equipped with a 2.1 × 20 mm guard column (both 5 mm particle size) with a binary solvent system. Solvent A was 20 mM ammonium carbonate, 0.05% ammonium hydroxide; Solvent B was acetonitrile. The column oven and autosampler tray were kept at 40 °C and 4 °C, respectively. The chromatographic gradient was run at a flow rate of 0.200 mL/min as follows: 0–2 min: 80% B; 2-17 min: linear gradient from 80% B to 20% B; 17-17.1 min: linear gradient from 20% B to 80% B; 17.1-22.5 min: hold at 80% B. Samples were randomized and analysed with LC–MS in a blinded manner with an injection volume was 5 µl. Pooled samples were generated from an equal mixture of all individual samples and analysed interspersed, at regular intervals, within the sample sequence as a quality control. Each sample was analysed with three analytical replicates.

Metabolites were measured using a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap Mass spectrometer (HRMS) coupled to a Dionex Ultimate 3000 UHPLC. The mass spectrometer was operated in full-scan, polarity-switching mode, with the spray voltage set to +4.5 kV/-3.5 kV, the heated capillary held at 320 °C, and the auxiliary gas heater kept at 280 °C. The sheath gas flow was programmed to 55 units, the auxiliary gas flow was programmed to 15 units, and the sweep gas flow was programmed to 0 unit. HRMS data acquisition was performed in a range of m/z = 70–900, with the resolution set at 70,000, the AGC target at 1×10^6, and the maximum injection time (Max IT) at 120 ms. Metabolite identities were confirmed

using two parameters: (1) precursor ion m/z was matched within 5 ppm of theoretical mass predicted by the chemical formula; (2) the retention time of metabolites was within 5% of the retention time of a purified standard run with the same chromatographic method. Each sample underwent 3 analytical repeats with subsequent peak annotation and chromatogram review and peak area integration were performed using the Thermo Fisher software Tracefinder 5.0. The peak area for each detected metabolite was subjected to the "Filtering 80% Rule", half minimum missing value imputation, and normalized against the total ion count (TIC) to correct any variations introduced from sample handling through instrument analysis. Samples were excluded after performing testing for outliers based on geometric distances of each point in the PCA score analysis as part of the muma package (v.1.4)[39].

The normalized LC-MS results were first explored by principal component analysis (PCA) to assess for clusters within the CAD patients, with subsequent labelling of the 'ischemic' and 'remote' samples. PCA analysis was performed using the R base package stats (v.4.0.5) (https://www.r-project.org/) with the function prcomp and visualized using the autoplot function of ggplot2 (v.3.3.5)[37] after loading the ggfortify package (v.0.4.14)[40]. Next, differential metabolite analysis was performed to investigate patterns of metabolite changes within patients (e.g. between 'ischemic' and 'remote' samples) that were consistent across the cohort. Differential metabolomics analysis was performed using the R package „gtools" (v.3.9.2)[41] to calculate the Log2FC using the functions „foldchange" and „foldchange2logratio" (parameter base=2). The corresponding p-value was calculated using the R base package stats (v.4.1.3) with the function "wilcox.test" or "t.test" and adjusted using Benjamini Hochberg "BH". Volcano plots were generated using the EnhancedVolcano package (v. 1.12.0)[42] and correlation plots using corrplot (v.0.92). Detailed code can be found under https://github.com/ChristinaSchmidt1/AMBITION_study. Univariate analysis of specific metabolites of interest was conducted, comparing 'ischemic' and 'remote' segments.

In this experiment, statistical analysis was conducted on the mean values of the analytical repeats and no adjustment for multiple testing was plotted. Analysis was performed using the paired t-test for normally distributed data and the Wilcoxon-signed-rank test for non-normally distributed data. Finally, we performed exploratory analysis assessing the LV metabolic landscape of i) CAD patients with impaired LVEF, comparing to the CAD patients with preserved LVEF, ii) CAD patients with 3 or more LV hypocontractile viable segments compared to patients with <3 hypocontractile viable regions and iii) CAD patients with a global MPR >1.5, comparing to patients with a global MPR ≤ 1.5. In these experiments, 'ischemic' and 'remote' biopsies were individually included as biological replicates. Analysis was performed using the Welch's t-test for normally distributed data and the Wilcoxon-Mann Whitney U test for non-normally distributed data.