SCFA-HCA116 Treatment

- **Flow cytometry sample preparation and data analysis:**

HTC116 treated and untreated cells were prepared for flow cytometry as follows: Cells were washed 3x in PBS by spinning cells at 1000rpm in a bench-top Eppendorf centrifuge and discarding the supernatant carefully. Cells were fixed by adding 70% ice-cold ethanol dropwise while vortexing to minimise any clumping. Samples were stored at 4°C for at least 30mins prior to staining. Propidium iodide (PI) staining was carried out by first removing the ethanol by washing in PBS (as before), then adding 300µl of 50µg/ml PI per sample. 5 µl RNase was also added at this stage to prevent RNA contamination and ensure that only the DNA was stained. Samples were stored at 4°C overnight. Flow cytometry was carried out using a FACsCalibur flow cytometer. Data analysis was performed using CellQuest Pro (Becton, Dickenson and Company, UK office at Oxford Science Park, Oxford). Results are given in figures 1 and 2.

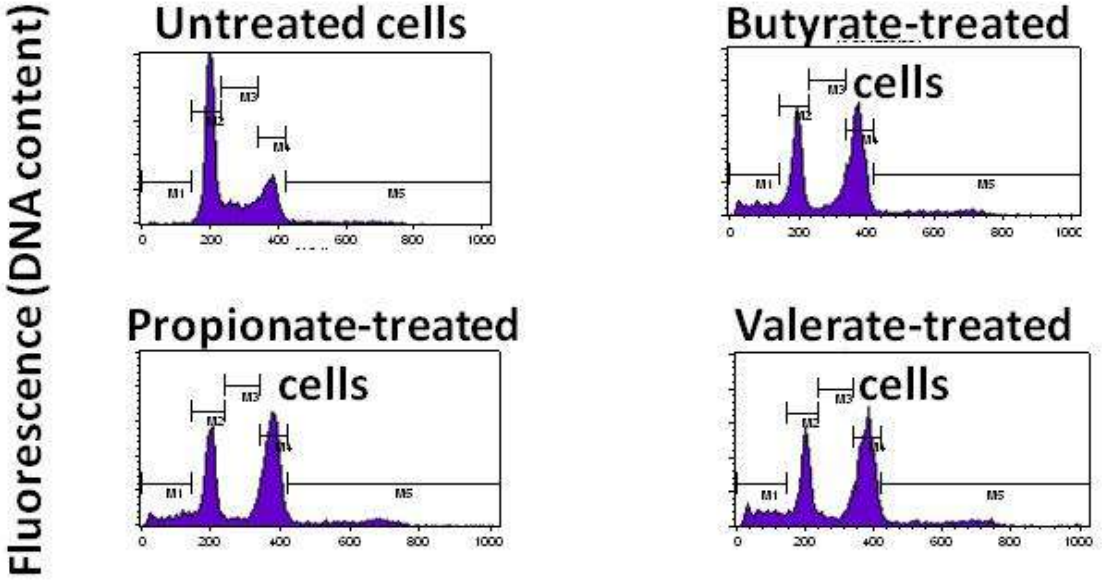


Fig. 1 Flow cytometric analysis (CellQuest Pro) of DNA content vs. phase (cell-cycle) of SCFA-treated cells compared to untreated HCT116 colon cancer cells: the untreated samples (top left) display a typical profile with the majority of cells lying in the G1 phase, as reflected by the height of the peak; on treatment with SCFAs there is a clear shift to G2 phase. [Cell-cycle phase: M1=subG1; M2=G1; M3=S; M4=G2; M5=>G2].

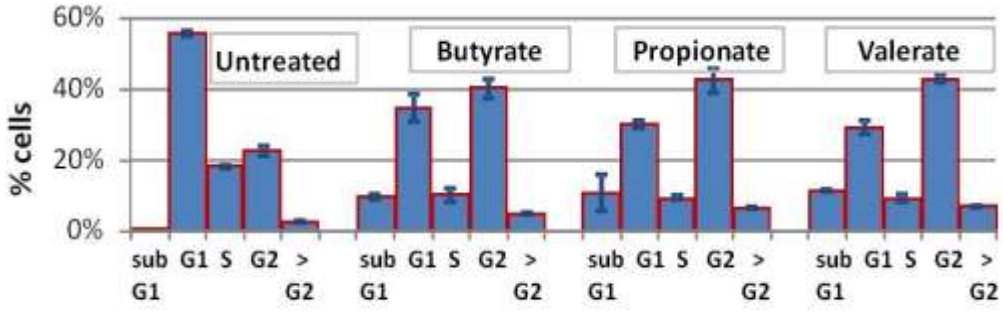


Fig. 2 Histogram showing the relative percentage of cells at each phase of the cell cycle, again clearly showing the shift from G1 phase to G2 and also an increase of cells >G2 (hyperploid cells) and <G1 (apoptosis).

- **iTRAQ Workflow**

HCT116 Cell Lysate Preparation

HCT116 cells were plated at 1.6 x103 cells/cm in T175 flasks in DMEM media and incubated at 37 °C for 24 h to allow them to adhere. Treatments with SCFAs (B, P, V) at concentrations established by HCA for IC50 at 24 h for G2/M cell-cycle arrest were then applied. An untreated sample was included as a control. After 24 h treatment, the cells were harvested by being scraped into PBS and centrifuged to remove any residual supernatant. The cell pellets were flash-frozen in liquid nitrogen and stored at -80 °C. For iTRAQ, the treated cells were lysed by resuspending them in 1 M triethylammonium bicarbonate (TEAB) pH 8.5, 0.05% SDS lysis buffer and then sonicated in a Bioruptor (Diagenode) for 10-15 x 30-second cycles.

Protein Assays

Protein content was determined using Bradford protein assay reagent (Bio-Rad) according to manufacturer's instructions. Absorbance at 595 nm was measured against standard BSA curves using a Multi-detection Reader (BioTek). Whole cell lysates at a protein concentration of 20 mg protein/ml were stored in 105 µg aliquots at -80 °C.

Peptide labelling with iTRAQ Reagents

Two biological replicates of total cell lysates were prepared as described above. These were proteolytically digested with trypsin and labelled according to procedures outlined by Applied Biosystems with minor modifications. This entailed making the treated samples up to a final volume of 21 µl at 5mg protein/ml in 1 M TEAB/0.05% SDS. The samples were reduced by adding 2 µl 50 mM TCEP (tris-2-carboxyethyl phosphine, Sigma) and incubating for 1 h at 60 °C. Cysteine residues were blocked by adding 1 µl MMTS (methyl methane thiosulfate, Sigma) at room temperature for 10 minutes. Trypsin (10 µg) was added to each sample and incubated overnight at 37 °C. rypsin digestion was verified by SDS-PAGE on 5 µg of sample. 50 µl of isopropanol was added to each of the 8 iTRAQ reagents (Applied Biosystems). These were added to the three SCFA-treated and untreated samples from each biological replicate (untreated: 113, 114; butyrate: 115, 116; propionate: 117, 118; valerate: 119, 121). Samples were incubated at room temperature for 2 h. All the samples were pooled in a 1:1 ratio and dried in a vacuum centrifuge (Eppendorf Concentrator 5301). The samples were reconstituted in 90 µl of 20% acetonitrile, 0.1% formic acid and the pH was adjusted to pH 2. Finally this 8-plex peptide mixture was sonicated and any precipitate present pelleted by centrifugation.

Strong Cation Exchange (SCX) Liquid Chromatography (HPLC)

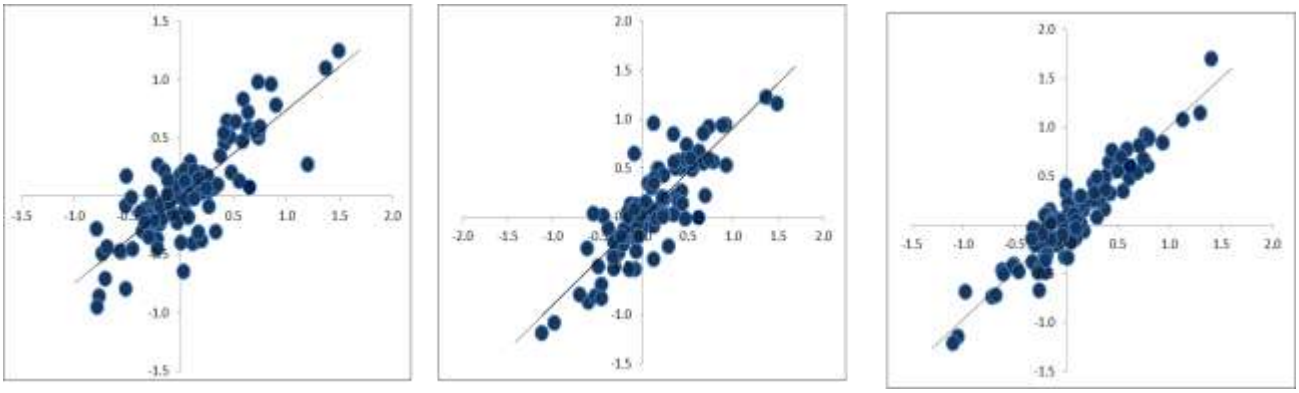
Peptide pre-fractionation was achieved using a PolySULFOETHYL-A Pre-Packed Column (PolyLC, Columbia, MD) with a 5 µm particle size and a column dimension of 100 mm×4.6 mm i.d., 200 Å pore size, on a BioLC HPLC (Dionex, Surrey, U.K.). SCX was achieved using a low ionic buffer A (20% acetonitrile, 0.1% formic acid), a high ionic buffer B (20% acetonitrile, 0.1% formic acid, 500 mM KCl). The sample was loaded onto the column and washed for at least 60 minutes at a flow rate of 0.4 mL/min with 100 % SCX Buffer A (20 % acetonitrile, 0.1 % formic Acid) to remove salts, TCEP and unincorporated iTRAQ reagent. Peptides were then separated using a gradient of SCX Buffer B (20% acetonitrile, 0.1% formic acid, 0.5 M KCl) at the same flow rate of 400 µL/min. Buffer B levels increased from 0% to 25% from 5 minutes to 30 minutes then from 25% to 100% over 5 minutes, followed by an increase from 26% to 100% over the next 15 min. Buffer B was held for another 5 min for isocratic washing prior to column re-equilibration with buffer A. The sample injection volume was 100 µL, and the liquid flow rate was 0.4 mL/min. The SCX chromatogram was monitored using UVD170U ultraviolet detector and Chromeleon software v. 6.50 (Dionex, LC Packings, The Netherlands). Fractions were collected using a Foxy Jr. (Dionex) fraction collector in 30 sec (16-32 mins) or 1 min (0-16 mins and 32-49 mins) intervals on low-binding 1.5 mL microcentrifuge (Eppendorf) tubes to minimize unspecific binding loss. Fractions were vacuum-concentration prior to LC-MS/MS analysis.

iTRAQ Tandem-Mass Spectrometry

Fractions collected from offline separation techniques were eluted through the Ultimate 3000 nano-LC system (Dionex, LC Packings, The Netherlands) interfaced with a QSTAR XL (ABSciex, Foster City, US) tandem ESI-QUAD-TOF MS. Vacuum dried fractions were resuspended in loading buffer (3% acetonitrile, 0.1% trifluoroacetic acid), injected and captured into a 0.3×5 mm trap column (3 µm C18 Dionex-LC Packings). Samples were then eluted onto a 0.075×150 mm analytical column (3 µm C18 Dionex-LC Packings) using an automated binary gradient with a flow of 300 nL/min from 95% buffer A (3% acetonitrile, 0.1% formic acid), to 35% buffer B (97% acetonitrile, 0.1% formic acid) over 90 min, followed by a 5 min ramp to 95% buffer II (with isocratic washing for 10 min). Predefined 1 s 350−1600 m/z MS survey scans were acquired with up to two dynamically excluded precursors selected for a 3 s MS/MS (m/z 65−2000) scan. The collision energy range was increased by 20% as compared to the unlabelled peptides in order to overcome the stabilizing effect of the basic N-terminal derivatives, and to achieve equivalent fragmentation as recommended by ABSciex.

C) Comparative analysis - Correlation plots

The mean iTRAQ ratio for each SCFA replicate was calculated relative to both untreated control replicates and plotted for each set of replicates (Butyrate, Propionate and Valerate).



Butyrate -115 vs Butyrate-116
R =0.83

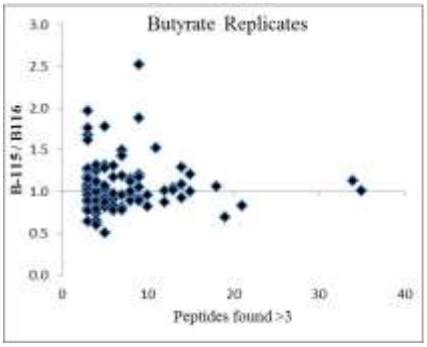
Propionate-117 vs Propionate-118
R =0.84

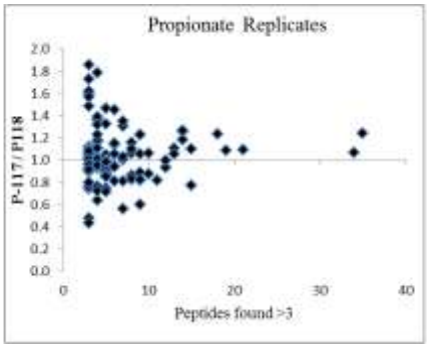
Valerate-119 vs Valerate-121
R = 0.94

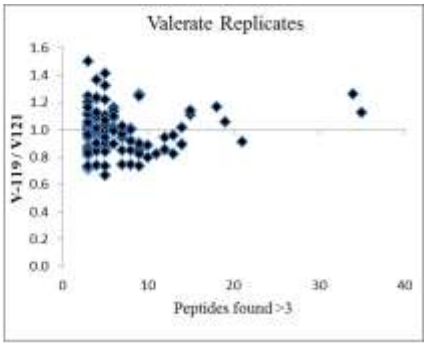
Fig. 1 Experimental quality was assessed by plotting log value ratios from two biological replicates against each other, with minimal scatter indicating higher confidence in the results.

- **Replicate Scatter Plots**

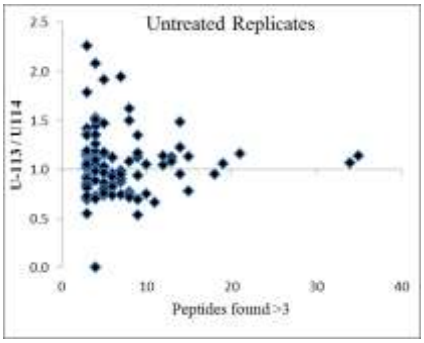
a) Ratio of SCFA replicates vs. peptides found.



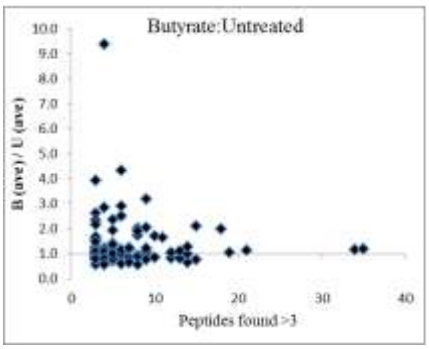


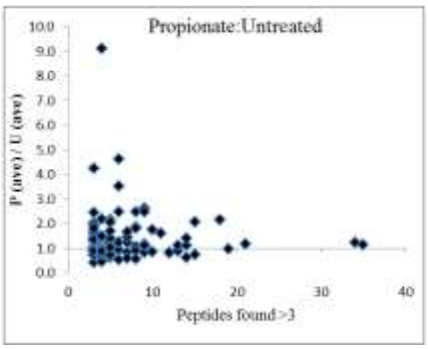


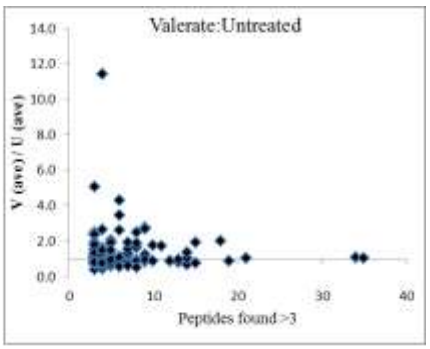
b) Ratio of Untreated control replicates vs. peptides found.



c) Ratio of SCFA-treated cells to untreated cells vs. peptides found.







Scatter plots show how closely replicates match, providing a measure of the sample preparation procedures and reproducibility in an iTRAQ experiment. The range of fold changes show how closely the SCFA replicates compare and their scatter profiles gives a measure of disparity. Three variations of scatter plots are shown: Panel (a) shows plotted ratios of the SCFA replicates Butyrate; Propionate; Valerate; and untreated. Panel (b) sratios of the untreated replicates.Panel (c) shows plotted ratios of SCFA-treated cells relative to untreated cells