SUPPORTING INFORMATION
Proteomic Analysis of Human Plasma During Intermittent Fasting
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Supplementary Figure 1. 3D-printed 96-well StageTip spinner design and characterization. (a) Model of the assembled device in the configuration used for StageTip equilibration, sample loading and wash steps. The 96-well plate for collection of the solutions is not shown. (b) Section view of the assembled device in the configuration used for StageTip peptide elution. The red arrow highlights the end of the StageTip that is below the top of the 96-well PCR plate. (c) Cubes of 2 cm were printed in either polyethylene terephthalate glycol-modified (PETG), high impact polystyrene (HIPS), or acrylonitrile butadiene styrene (ABS). Each was submerged in either water, 100% acetonitrile (ACN), 1% trifluoroacetic acid (TFA) in water, 50% ACN in water, 99% isopropanol (IPA) & 1% TFA, 80% ACN & 5% NH3, or 100% acetone for 1 minute. Each cube was dried for the same period and imaged. (d) C18 StageTips were centrifuged at various speeds to determine optimal timings. Each tip was spun consecutively with acetonitrile, water and 50% acetonitrile in water. The error bars at each data point (mean volume remaining) represents standard deviation. n = 6 for each solvent at each speed. (e) StageTips (SDB-RPS based) were processed with either the 96-well device, or individual 2 mL tube adaptors using a standard protocol (see Supplementary method). The total processing time for 3 separate individuals is shown as separate datapoints for 8, 16 and 24 StageTips using each method. The trendline for each method of processing is shown.
Supplementary Figure 2. Reproducibility and efficacy testing of the 96-well StageTip processing device, Spin96. (a) Plasma samples were processed through SDB-RPS StageTips with either the 96-well device, or individual 2 mL tube adaptors. Eluted peptides were subjected to nanoLC-MS-MS analysis and label-free quantitation (LFQ) using MaxQuant which was used to calculate; (b) proteins identified, (c) average number of peptide identifications per protein and (d) peptide spectral matches (PSMs). (e) Each row and column represent a technical replicate for each StageTip processing method.
**FIGURE S3.** Clinical measures of metabolic health before and after the 8-week intermittent fasting (IF) intervention. The lines in each clinical parameter plot represent the value (units indicated on each y-axis) for each measure in each participant at each condition. Grey bands indicate the 95% confidence interval for the median.
FIGURE S4. Intermittent fasting (IF) induces changes in abundance of key metabolic proteins. The lines in each protein plot represent the adjusted label free quantitation (Adj. LFQ) intensity for each participant in each condition. Grey bands indicate the 95% confidence interval for the median.
FIGURE S5. Changes in protein abundance correlate with clinical measures of metabolic health. Each dot represents an individual sample (either before, or after intermittent fasting), where the protein measures are displayed as the label free quantitation (LFQ) intensity. Clinical measures are shown in the indicated units on each axis. The trend line is shown with surrounding 95% confidence interval.
Supplementary Figure 6...continued

PON1 M55

PON1 L55
Supplementary Figure 6...continued

SERPINA1
V237

SERPINA1
A237
Supplementary Figure 6...continued

APOE C130

APOE R130
SUPPLEMENTARY METHODS

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Design of the Spin96 StageTip Processing Device

Our aim was to design a 96-well device (Spin96) for rapid and consistent processing of StageTips, whilst minimizing the possibility of cross-contamination. To this end, we designed the system to enable rapid transition between two configurations (Supplementary Figure 1a & 1b). The first configuration is used for StageTip equilibration, sample loading, and washing steps (Supplementary Figure 1a). It consists of a top component and associated holder that supports the StageTips, which is placed over a wash-bottom component containing a standard 96-well plate for collecting waste liquid. Important design considerations for this assembly were that the tips remain >5 mm away from the waste liquid being collected, and that any spray from one tip/well is contained and cannot reach other tips (Supplementary Figure 1a red arrow). This will prevent contamination of the tips with waste liquids and peptides from other samples. The second configuration is used for peptide elution from the StageTips into either an unskirted 96 well PCR plate, or PCR strip tubes (Supplementary Figure 1b). In this mode the top component and holder with StageTips is placed over a bottom component and its associated holder that supports either a 96-well PCR plate, or PCR strip-tubes for collecting peptide eluents. The most important design consideration for this configuration was the height of the tip inside the collection well/tube. We aimed to have the tip several millimeters below the top of the well/tube to ensure all eluted peptide were collected in the desired well/tube with no loses of liquid and/or cross contamination (Supplementary Figure 1b red arrow).

When assembled in either configuration, the device was designed to be approximately the height of a standard 2 mL deep-well plate, so that the Spin96 can be used in most swinging-bucket centrifuges with a standard deep-well plate rotor. The plates/tubes containing the eluates can subsequently be placed directly into vacuum concentrator instruments (such as an Eppendorf Vacuum Concentrator Plus, or Genevac EZ-2). Once dried and resuspended in aqueous buffer these plates can be used directly in an LC autosampler, which minimizes sample handling and sample loss that would otherwise occur upon transferring to separate tubes/vials for LC-MS/MS analysis.

Optimization of Spin96 Parameters

Each of the components for Spin96 were designed to be 3D-printed using fused-deposition modelling (FDM). Given that StageTip processing involves the use of solvents that may induce plastic defects, we needed to determine an optimal polymer for construction of the tip spinner. To this end, we evaluated three widely used and commercially-available 3D-printing plastics: polyethylene terephthalate glycol-modified (PETG), high-impact polystyrene (HIPS) and acrylonitrile butadiene styrene (ABS), for their ability to resist deformation following exposure to solvents required for various StageTip clean-up methods (1-4). The layer structure of
each treated plastic was compared to the untreated control. An inability to visualize individual layer-lines is consistent with the plastic’s surface dissolving in the solvent (Supplementary Figure 1c). Acetone was used as a positive control as all of the plastics tested are sensitive to acetone treatment (5). Both PETG and HIPS were not visibly affected by any solvent except the acetone control. However, printing of PETG generated very small loose strands of plastic (stringing), visible in our images (Supplementary Figure 1c), which could lead to sample contamination. Therefore, we used HIPS for the 3D-printing of our tip spinner apparatus. We developed a suitable centrifugation protocol for our tip spinner device by determining the time required to flow solvents through StageTips in the Spin96 using different centrifugal g-forces. A standard amount (100 μL) of either 100% acetonitrile, water, or 50% acetonitrile in water, was used for each of these measurements (n=6). The solvents were tested in order of their normal use for a C18 clean-up protocol (4). To determine the optimal centrifugation speed, the protocol was tested at 500, 1000 and 1500 g. For each solvent tested the StageTips were emptied of all liquid at 1000 x g over a minute faster than at 500 x g and showed less variation in solvent flow (Supplementary Figure 1d). Using the optimized g-force settings, water was cleared from each tip within two minutes of spinning at 1000 g (Supplementary Figure 1d), which was chosen as the optimal time with minimal drying of the resin. A 50% (v/v) acetonitrile/water mixture also cleared the StageTips within two minutes (Supplementary Figure 1d), but since it is used at the final peptide elution step we set the centrifugation time to 5 minutes to ensure complete recovery of peptides.

To determine if the Spin96 is more time efficient for StageTip processing than StageTips processed using individual tubes, mixed-mode (SDB-RPS based) SPE was performed using both techniques (Supplementary Figure 1e). We chose this method due to the many steps needed in the protocol, which provided for a more extensive comparison. Both techniques were performed by 3 individuals with each round of SPE being completed with either 8, 16, or 24 tips. We used a maximum of 24 StageTips per round as this is the limit that can be processed in parallel using 2 mL tubes and a standard benchtop centrifuge. Overall, the total SPE processing time was similar between the individual tubes and the 96-well tip spinner, with the 96-well device becoming more efficient as more tips are processed in parallel. The time required for processing 24 tips was between 40-50 min using either the individual tubes, or the 96 well tip spinner. Obviously, once >24 StageTips need to be processed using individual StageTips in tubes, the total time required will be at least double (~100 min) that required for the 96-well tip spinner. Processing of >48 samples will require further increases in time required, with processing of 96 samples estimated to require >200 min. In contrast, processing 96 samples with the tip-spinner device would take ~60 min, as all 96 are processed in parallel.

**StageTip processing comparison: individual tubes versus 96-well device**

We next evaluated the use of Spin96 for peptide sample clean-up, compared to previously established centrifugation-based procedures using individual StageTips in 2 mL tubes. This was performed using digest of human plasma from healthy volunteers. In this comparison we used mixed-mode SDB-RPS StageTip SPE across five biological replicates (separate individuals), with 3 technical replicates of each performed (Supplementary Figure 2a). Each sample was subsequently subjected to nanoLC-MS/MS analysis using a 1 h gradient separation. Analysis of these data showed that our 96-well device performs equally well compared with tube-based StageTip processing regarding identified protein groups (Supplementary Figure 2b and Supplementary Table 1), razor and unique peptides (Supplementary Figure 2c and Supplementary Table 2) and PSMs (Supplementary Figure 2d). None of these measures had statistically significant differences between
the Spin96 and tube processing methods. To demonstrate the high level of correlation in the performance of the Spin96 versus tube processing we plotted the protein-level LFQ intensities for all data derived from one individual (subject 7), which was representative of all subjects who demonstrated a Pearson correlation of >0.99 (Supplementary Figure 2e). These data demonstrate that the Spin96 generated the same level of sensitivity and clean-up performance compared to lower-throughput methods of StageTip processing.

The original CAD design files for our Spin96 device are made freely available with this manuscript and provide a basis for further changes to the device structure to accommodate different SPE workflows. For example, the workflow may require the use of larger tips, different collection plates, or higher centrifugation speeds. The polymer we have chosen to manufacture the 96-well tip spinner, HIPS, is a low cost and widely available commercial filament available for common FDM 3D printers, and displays high strength making it compatible with repeated centrifugation. Users may want to use different plastics, such as polypropylene, which is even further resistant to a more diverse range of solvents, including acetone. Such adaptations may require further structural reinforcement especially if the alternative material is more flexible. The ability to modify the 3D CAD designs also presents the opportunity to adapt it for other purposes. Here we have focused on peptide-level SPE, however other applications may also be suitable by using other matrices, such as sample preparation workflows for either nucleic acids, or metabolites. To this end, the apparatus itself could be modified to hold different tips and collection plates to suit different workflows, such as methods requiring larger volumes of solvent for sample binding and wash steps. The use of 3D printing for laboratory manufacturing also offers great reductions in cost. For example, a comparable 96-well SPE plate for sample clean-up using a vacuum manifold, typically costs around US$5 per sample, whereas using home-made StageTips with our device costs approximately US$0.50 per sample, including manufacturing costs.

**Human plasma collection and ethics for Spin96 optimisation and testing**

Human plasma was obtained from 5 healthy volunteers using standard phlebotomy techniques. Blood samples were immediately centrifuged and frozen at -80°C. The Royal Prince Alfred Hospital Research Ethics Review Committee approved the study protocol (X17-0129 & HREC/17/RPAH/183), and all participants provided written, informed consent prior to their inclusion.

**Plastic chemical testing**

A 2 cm cube was generated in SolidWorks (Dassault Systèmes, Velizy-Villacoublay, France) (version 2017) and exported as an STL file for slicing in Z-suite (Zortrax, Olsztyn, Poland). The sliced models were printed on a M200 (Zortrax) in polyethylene terephthalate glycol-modified (Z-PETG, Zortrax), high-impact polystyrene (Z-HIPS, Zortrax) and acrylonitrile butadiene styrene (Z-ABS, Zortrax). All plastics were printed at a layer height of 0.14 mm. For testing of each solvent, a separate cube made of either PETG, HIPS or ABS was placed in 10 mL of each solvent for 1 min, removed and allowed to air dry for 2 min before being imaged using the DinoLite USB microscope (AnMo Electronics Corporation, Taiwan).

**Tip spinner solvent and centrifugation testing**

StageTips were produced using two layers of either Empore 3M C18 disks (Cat No. 66883-U, Sigma), or Empore 3M styrene divinylbenzene reversed-phase sulfonate (SDB-RPS) disks (Cat No. 66886-U, Sigma). These discs were punched out with a 14G blunt-end needle and packed into 200 μL pipette tips (Cat No. Z640417,
Eppendorf, Hamburg, Germany) as described previously (3). For testing, StageTips were placed into the holder associated with the top component (Supplementary Figure 1a). A 96-well unskirted PCR plate (Cat No. AB-0700, ThermoFisher) whose edges were trimmed to fit was placed into a separate holder and inserted into the bottom component (Supplementary Figure 1a & 1b). The top and bottom halves were then stacked together. For each solvent tested, StageTips were centrifuged at various g-force settings in a Heraeus Megafuge 16R (ThermoFisher Scientific), with a deep-well plate centrifuge adaptor for 15 s, and the eluted solvent weighed using an analytical balance (A&D, Tokyo, Japan). This was repeated until the tip was emptied of solvent. Each tip used was tested with 100 μL of either 100% acetonitrile, 100% water, or a 50% (v/v) acetonitrile/water mixture. Evaporation of the 100% acetonitrile and rapid transit time (~ 1 min) precluded accurate measurement, and so data for this solvent is not shown.

Spin96 Optimization. For the determination of correct timing in our Spin96 protocol, 6 technical replicates were used for each condition to account for measurement variability. For Spin96 optimizations, 3 replicates were determined to be suitable given the effect size and variance observed. For the comparison of the proteomics output of the differing StageTip SPE processing methods applied to human plasma, 5 technical replicates were used for each condition. This was determined as a suitable number of replicates given the variance observed.

Protocol timing test with SDB-RPS StageTips
SDB-RPS StageTips were generated as described above. Various numbers of StageTips were then subjected to either tube, or 96-well tip-spinner cleanup. Times were recorded at the end of each step, and 0.2% TFA was used instead of peptide samples. For the tube-based StageTip clean-up, StageTips were processed as described above. 3D printed StageTip tube adaptors were placed in 2 mL tubes and centrifuged using an Eppendorf 5430 R centrifuge at 1000 g at RT for the same times as above. The CAD design file for the tube adapters is included in Supplementary File 1 and the tube adaptors were printed with the same method as used for the Spin96 components with maximum infill.

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Larance Lab SDB-RPS Stage Tip Protocol

*Solutions to be prepared:
- 100% AcN (LC Grade) – Make up a separate bottle
  for this (KEEP IT CLEAN!!!)
- 30% methanol, 1% TFA in H2O (LC-Grade). (KEEP IT CLEAN!!!)
- 0.2% TFA in H2O (LC-Grade). (KEEP IT CLEAN!!!)
- 1% TFA in H2O (LC-Grade). (KEEP IT CLEAN!!!)
- 99% Isopropanol, 1% TFA. (KEEP IT CLEAN!!!)
- 5% Ammonium hydroxide (assume stock is 100%), 80% ACN. (KEEP IT CLEAN!!!)
- 5% formic acid in a 100ml bottle (KEEP IT CLEAN!!!)

Method:
1. Pack Eppendorf 200ul tips with punched SDB-RPS discs (stacked 2 discs thick) Sigma Cat No. 66886-U
2. Mount tips into 3D-Printed holder over a clean 96-well deepwell plate
3. WET - Add 100ul of 100% acetonitrile
4. Centrifuge in BECKMAN CENTRIFUGE at 1,000 x g for 1 min.
5. EQUIL1 - Add 100ul 30% methanol, 1% TFA
6. Centrifuge in BECKMAN CENTRIFUGE at 1,000 x g for 3 min.
7. EQUIL2 - Add 100ul 0.2% TFA
8. Centrifuge in BECKMAN CENTRIFUGE at 1,000 x g for 3 min.
9. SAMPLE - Add <=100ul peptides in 1% TFA
10. Centrifuge in BECKMAN CENTRIFUGE at 1,000 x g for 3 min.
11. WASH1 - Add 100ul 0.2% TFA
12. Centrifuge in BECKMAN CENTRIFUGE at 1,000 x g for 3 min.
13. WASH2 - Add 100ul 99% Isopropanol, 1% TFA
14. Centrifuge in BECKMAN CENTRIFUGE at 1,000 x g for 3 min.
15. Mount tips into 3D-Printed holder over a clean 96-well PCR plate
16. ELUTE - Add 100ul 5% Ammonium hydroxide, 80% ACN.
17. Centrifuge in BECKMAN CENTRIFUGE at 1,000 x g for 5 min.
18. Dry peptides in PCR plate (inside a 500ul deepwell plate) using Genevac on LVL5west.
Use a PCR plate in 500ul deepwell plate as a balance. Using settings of 35°C max temp, NH3-H2O mode, 1 hours until final (1h 15min total drying time).
19. Resuspend peptides in 10ul of 5% formic acid at RT.
20. Seal wells with a Thermo silicon PCR mat from the MSCF core facility. Store at 4°C.