

1 SUPPLEMENTARY MATERIALS AND METHODS

2 Normalisation and Filtering Method Selection

3 Three normalisation methods were systematically compared across filtering thresholds
4 (proteins detected in all replicates of 1–13 experimental groups):

5 **Median normalisation:** Column medians calculated excluding zero values; scaling factors
6 computed as ratio of each column median to global median; data scaled and \log_2 -
7 transformed.

8 **TMM normalisation:** Implemented via `edgeR::calcNormFactors` followed by counts per
9 million with \log_2 scaling.

10 **VSN normalisation:** Variance stabilising normalisation applied using `vsnMatrix` with offset
11 of 1.

12 **Performance metrics:** Three metrics were calculated from both UMAP and PCA
13 dimensionality reductions to assess biological separation of conditions:

14 • **Within-to-between group distance ratio (W/B):** Mean within-group distances
15 divided by mean between-group distances; lower values indicate tighter replicate
16 clustering.

17 • **Silhouette score:** Ranges -1 to 1; measures similarity of samples to own cluster
18 versus other clusters; higher values indicate better-defined clusters.

19 • **Davies-Bouldin index:** Average similarity between each cluster and its most similar
20 cluster; lower values indicate better separation.

21 **Composite score calculation:** Each metric was min-max scaled to 0–1 range. Composite
22 = scaled(W/B ratio) – scaled(Silhouette) + scaled(Davies-Bouldin), where lower scores
23 indicate better performance.

24 **UMAP parameters:** Principal components explaining $\geq 75\%$ cumulative variance;
25 n_neighbors=15; min_dist=0.1; metric="euclidean"; random seed=42.

26 **Method selection results:** VSN performed poorly, particularly with lenient thresholds
27 (Figure S1A). Both median and TMM showed excellent scores across most thresholds.
28 Cross-validation using PCA (PC1 and PC2) provided consistent patterns (Figure S1B).
29 Permutation testing (n=1,000 random label shuffles) confirmed observed clustering was
30 significantly better than random (Figure S1C). Coefficient of variation analysis showed
31 excellent data quality (median CVs: 0.01–0.04 across conditions; Figure S1D).

32 **Final selection:** Median normalisation with a threshold of 3 conditions (proteins detected
33 in all replicates of ≥ 3 groups), retaining 2,204 proteins (38.6% of 5,714 detected) for
34 subsequent analysis (Figure S1E). This provided optimal group-to-group segregation and
35 within-group clustering (Figure S1F-G), excellent composite score, and robust quality
36 control metrics (Figure S2A-D).

37

38 **Differential Expression Analysis**

39 DEqMS accounts for peptide spectral match (PSM) count-dependent variance, improving
40 statistical power in MS-based proteomics. Design matrix specified 13 experimental groups
41 (undifferentiated control; LD, HD, LA, HA at weeks 1, 3, 4). Linear models were fitted using
42 limma::lmFit, followed by DEqMS::spectraCounteBayes for variance modelling based on
43 PSM counts.

44 **Complete contrast list (42 comparisons):**

- 45 • **Temporal vs Week 1 (8):** W3 and W4 vs W1 for each condition (LD, HD, LA, HA)
 - 46 • **Temporal vs Control (12):** Each condition at W1, W3, W4 vs undifferentiated control
 - 47 • **Cross-condition at each timepoint (12):** HD vs LD, LA vs LD, HA vs HD, HA vs LA at W1,
48 W3, W4
 - 49 • **Glucose effect comparisons (6):** HD vs LD at each week, with and without AZD8055
 - 50 • **AZD8055 effect comparisons (4):** LA vs LD and HA vs HD at W3 and W4
- 51 **Significance thresholds:** $|\log_2FC| > 1$ and Benjamini-Hochberg adjusted p-value < 0.05 .

52 **Gene Ontology Enrichment Analysis**

53 **enrichGO parameters:** ont="BP" (Biological Process); OrgDb=org.Mm.eg.db;
54 pAdjustMethod="BH"; pvalueCutoff=0.05; minGSSize=5; maxGSSize=500 (excluding overly
55 broad terms); readable=TRUE. Gene symbols were converted to Entrez IDs using bitr().

56 **Background universe:** All detected proteins with valid Entrez ID mappings were used as
57 background, following proteomics best practice to prevent inflation of enrichment statistics
58 from proteins not detectable in the experimental system. For the Wnt signalling analysis
59 (Supplementary Figure 4A), enrichment was also calculated using the full genome as
60 background for comparison (detected: $p_{adj}=0.057$; genome: $p_{adj}=0.037$).

61 **Term simplification:** Redundant GO terms were removed using simplify() with semantic
62 similarity cutoff=0.7 (terms with $\geq 70\%$ similarity clustered; term with lowest adjusted p-
63 value retained from each cluster) and select_fun=min applied to adjusted p-values.

64 **Bidirectional analysis:** Where ≥ 5 genes were available in each direction, upregulated and
65 downregulated proteins were analysed separately to capture direction-specific pathway
66 alterations.

67 **Bone-Related Gene Signature**

68 A data-derived signature of 164 bone-related genes was compiled by extracting all genes
69 contributing to GO terms containing bone-related keywords across the entire dataset.
70 Keywords: bone, biomineral, mineralisation, ossification, skeletal, tissue remodelling,
71 cartilage, osteoblast, osteocyte, osteoclast, extracellular matrix, ECM organisation,
72 collagen, basement membrane, cell adhesion, integrin, Wnt, beta-catenin. This signature
73 was used to quantify bone programme engagement by counting differentially expressed
74 proteins matching the signature.

75 **GO Term Categorisation System**

76 GO terms were categorised into biological themes based on keyword matching:

77 **Bone-related:** bone, biomineral, mineralisation, ossification, skeletal, tissue remodelling,
78 cartilage, osteoblast, osteocyte, osteoclast

79 **ECM organisation:** extracellular matrix, collagen, basement membrane, integrin, cell
80 adhesion, external encapsulating structure, supramolecular fibre

81 **PI3K/Akt/mTOR:** mTOR, mechanistic target of rapamycin, phosphatidylinositol 3-kinase,
82 insulin receptor signalling, insulin-like growth factor receptor signalling, S6 kinase

83 **Lysosomal/autophagic:** lysosome, autophagy, macroautophagy, cathepsin, vacuolar
84 acidification, phagosome, mitophagy

85 **Mitochondrial metabolism:** mitochondrial, oxidative phosphorylation, electron transport,
86 respiratory chain, tricarboxylic acid cycle, ATP synthesis

87 **Glycolysis:** glycolytic process, glucose catabolic, hexose catabolic, canonical glycolysis

88 **Proteasome:** proteasome, proteasomal, ubiquitin-dependent protein catabolic

89 **Oxidative stress:** reactive oxygen species, superoxide, hydrogen peroxide, glutathione
90 metabolic, antioxidant, response to oxidative stress

91 **DNA replication:** DNA replication, replication fork, DNA synthesis, MCM complex, cell cycle

92 **Telomere maintenance:** telomere, telomerase, chromosome end, replicative senescence,
93 cellular senescence

94 **TNF regulation:** tumor necrosis factor

95 **Category heatmap score:** intensity = (number of enriched pathways / 5) × (median
96 enrichment / 10). Direction: UP if $n_{up} > n_{down} \times 2$; DOWN if $n_{down} > n_{up} \times 2$; otherwise no
97 enrichment shown.

98 **Visualisation Methods**

99 **Dimensionality reduction:** PCA and UMAP plots with convex hulls drawn around each
100 experimental group. PCA: PC1 vs PC2 with variance explained indicated. UMAP: using PCs
101 explaining $\geq 75\%$ cumulative variance.

102 **Expression heatmaps:** Top 100 most variable proteins; row scaling (z-scores); hierarchical
103 clustering (pheatmap).

104 **Circle plots (Figure 2B-C):** Circle size = gene ratio (proportion of pathway genes among
105 DEPs); colour intensity = significance ($-\log_{10}$ adjusted p-value); colour = direction
106 (red=upregulated, blue=downregulated); empty circles = no significant enrichment.

107 **Pathway enrichment heatmaps:** Signed $-\log_{10}$ (adjusted p-value) with colour indicating
108 direction.

109 **Gene-concept networks (Figure S5):** Generated using cnetplot (enrichplot package); top
110 15 enriched GO terms connected to contributing genes; gene nodes coloured by \log_2 FC
111 (red=up, blue=down); edges coloured by GO term category.

112 **Volcano plots (Figure S6):** Generated for each pairwise comparison showing \log_2 fold
113 change (x-axis) versus $-\log_{10}$ adjusted p-value (y-axis). Proteins meeting significance
114 thresholds ($|\log_2FC| > 1$ and adjusted p < 0.05) are coloured red (upregulated) or blue

115 (downregulated); non-significant proteins appear in grey. The top 20 most significantly
116 differentially expressed proteins are labelled.

117 **Bar plots:** $-\log_{10}(\text{adjusted p-value})$ with colour indicating enrichment direction.

118 **Complete Software Environment**

119 R version 4.5.1. Core analysis: DEqMS 1.26.0, limma 3.64.3, edgeR 4.6.3, vsn 3.76.0,
120 matrixStats 1.5.0. Pathway analysis: clusterProfiler 4.16.0, enrichplot 1.28.4, DOSE 4.2.0,
121 GO.db 3.21.0, org.Mm.eg.db 3.21.0, AnnotationDbi 1.70.0. Visualisation: ggplot2 4.0.1,
122 pheatmap 1.0.13, ggrepel 0.9.6, cowplot 1.2.0, plotly 4.11.0, factoextra 1.0.7, RColorBrewer
123 1.1.3. Dimensionality reduction: umap 0.2.10.0. Data handling: dplyr 1.1.4, tidyr 1.3.1,
124 readxl 1.4.5, stringr 1.6.0.