# The CALERIE™ Genomic Data Resource

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**Conflict of Interest.** DWB and DLC are listed as inventors of the Duke University and University of Otago invention DunedinPACE, which is licensed to TruDiagnostic. DWB is consulting CSO and SAB chair of BellSant and serves on the SAB of the Hooke Clinic.

## Abstract

Caloric restriction (CR) slows biological aging and prolongs healthy lifespan in model organisms. Findings from CALERIE-2™ – the first ever randomized, controlled trial of long-term CR in healthy, non-obese humans – broadly supports a similar pattern of effects in humans. To expand our understanding of the molecular pathways and biological processes underpinning CR effects in humans, we generated a series of genomic datasets from stored biospecimens collected from participants during the trial. These data constitute the first publicly-accessible genomic data resource for a randomized controlled trial of an intervention targeting the biology of aging. Datasets include whole-genome SNP genotypes, and three-timepoint-longitudinal DNA methylation, mRNA, and small RNA datasets generated from blood, skeletal muscle, and adipose tissue samples. The CALERIE Genomic Data Resource described in this article is available from the Aging Research Biobank. This multi-tissue, multi-omic, longitudinal data resource has great potential to advance translational geroscience.

# Introduction

Caloric restriction (CR) involves a reduction of caloric intake by 10-20% or more while maintaining adequate protein and micronutrient levels[1]. In laboratory experiments, CR slows biological aging and prolongs lifespan in multiple model organisms[2]. In humans, observational studies of individuals who self-impose CR[3,4], short-term trials involving participants with obesity[5], and involuntary CR among participants in the Biophere-II experiment[6] broadly support some of the health and longevity promoting effects shown in model organisms[7]. The CALERIE-2™ (Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy) study, is the first-ever randomized controlled trial of CR in healthy, non-obese humans. CALERIE-2™ is a phase II, multicenter, randomized controlled trial (RCT) that tested the effects of a 24-month CR intervention in healthy non-obese adult men and women. Although the trial did not achieve durable modification of its primary endpoints, resting metabolic rate and core temperature[8], analysis of participants' clinical laboratory values broadly support the hypothesis that CR promotes healthy aging; participants randomized to the CR condition experienced broad improvements in their cardiometabolic health relative to those in the control "ad libitum" (AL) condition[9] and exhibited a slowed pace of biological aging, as measured from algorithms based on clinical laboratory values[10,11]. These observations motivated efforts to elucidate underlying cellular-level mechanisms, including through projects funded by the National Institute on Aging at Columbia Univeristy, Duke University, and the University of California Los Angeles. The outcomes of these projects, including SNP genotype, DNA methylation, and messenger and small RNA sequencing data derived from blood, muscle, and adipose tissues collected from participants at pre-intervention baseline and at the 12- and 24-month follow-up assessments comprise the the CALERIE™ Genomic Data Resource.

**Description of the Trial**

Extending CALERIE™ phase I[12] in both scale and duration, CALERIE-2™ recruited a total of 220 subjects and assigned them in a 2:1 allocation to a CR treatment group or ad libitum (AL) control arm[13]. Subjects were randomly-assigned to CR or AL groups stratified on study site, sex, and body mass index. Participants in the CR group were assigned to a protocol designed to result in a 25% reduction in caloric intake relative to estimated energy requirements at enrollment. CR participants received an intensive behavioral intervention that included individual and group sessions, a meal provision phase, digital assistants to monitor caloric intake, and training in portion estimation and other nutrition and behavioral topics[14]. Adherence was assessed using measures of energy expenditure using the doubly-labelled water method as well as expected changes in body composition. The duration of the study for both CR and AL participants was 2-years.

Throughout the 2-year study duration, starting at baseline prior to randomization and recurring at months 1, 3, 6, 9, 12, 18, and 24, participants were evaluated for a range of pre-specified anthropometric, psychological, and physiological outcomes[8]. Blood samples were collected every six months. At baseline, 12-months, and 24-months, whole blood and samples were collected and banked. In addition, a subset of participants agreed to biopsies of adipose and

muscle tissue at baseline, 12-months, and 24-months. From these samples SNP-based genotypes, DNA methylation, mRNA, and small RNAs were assayed. Here, we describe these datasets and provide an overview of this data resource (**Fig. 1**).

# Results

## Overview

Of 238 eligible enrolled participants, n=220 were allocated to the trial and randomized to CR and AL treatment groups (**Fig. 1**). Two participants in the CR treatment group withdrew from the study, leaving n=143 participants in the CR group and n=75 in the AL group. In total, genomic data was produced for n=218 unique individuals. SNP data were available for 216 participants. DNAm and RNA sample coverage varied by treatment group, time-point, and tissue type (**Fig. 2**).

## Genotyping

Genotypic data is available for all but two participants who were randomized, provided consent, and completed the trial (n=216). **Figure 3** illustrates genetic ancestry of CALERIE participants as reflected in the first two principal components of the genome-wide single-nucleotide polymorphism (SNP) data.

## DNA methylation

DNA methylation (DNAm) was assayed from DNA extracted from whole blood, skeletal muscle, and adipose tissue using Illumina EPICv1 arrays. Assays of whole blood DNAm were completed by the Kobor Lab at the University of British Columbia as part of NIH grant R01AG061378. Assays of muscle and adipose DNAm were completed by the UCLA Neuroscience Genomics Core (UNGC) as part of NIH grant U01AG060908 using DNA extracted from banked tissue by the VB Kraus lab in the Duke Molecular Physiology Institute. Quality control and preprocessing for muscle and adipose tissue DNAm was carried out by the Geroscience Computational Core of the Robert N. Butler Columbia Aging Center.

Blood DNAm is available for baseline and at least one follow-up for n=197 (n=128 participants in the CR group and n=69 participants in the AL group)[15]. Sample sizes for blood DNAm for individual timepoints by treatment group are provided in **Fig. 2B.** Muscle DNAm is available for baseline and at least one follow-up for n=30 participants in the CR group and n=17 participants in the AL group. Sample sizes for muscle DNAm for individual timepoints by treatment group are provided in **Fig. 2B.** Adipose DNAm is available for baseline and at least one follow-up for n=31 participants in the CR group and n=17 participants in the AL group. Sample sizes for adipose DNAm for individual timepoints by treatment group are provided in **Fig. 2B.** Muscle and adipose DNAm data have not been published previously.

To facilitate the rapid appraisal of the underlying data structure and missingness, we generated a series of summary datasets. For each tissue, we provide a set of tables listing the probes that

passed quality control (**Supplemental Data Files 1-4, 9-12, 17-20**) and their beta-value distributions and missingness at each timepoint (**Supplemental Data Files 5-8, 13-16, 21-24**).

In addition to CpG methylation levels, we also computed a series of composite variables from the blood DNAm data. To help account for technical variability across samples, we provide the top principal componets of EPIC-array control-probe beta values for blood, adipose, and muscle[16]. We also include in this database the series of epigenetic clocks and estimated white blood cell proportions reported previously[17] as well as a newly-derived set of estimated white blood cell proportions[18]. Intercorrelations of chronological age and five epigenetic clocks at the baseline assessment, prior to treatment, are provided in **Figure 4**. Relative proportions of each of the 12 white blood cell types estimated at baseline measurement, prior to treatment, using previously described methods[18] are graphed in **Figure 5.**

## RNAseq

RNA sequencing was performed for three tissues: plasma, skeletal muscle, and adipose tissue. All RNA was extracted from plasma at the Duke Molecular Physiology Institute from stored plasma, skeletal muscle, and adipose-tissue biospecimens. Plasma and adipose RNA were sequenced at the Duke University Center for Genomic and Computational Biology. Muscle RNA was sequenced at the Center for Cancer Research Sequencing facility (National Cancer Institute). Messenger RNA (mRNA) and small RNA (smRNA) datasets were generated for skeletal muscle and adipose tissues. Only smRNAs were generated from plasma due to mRNA sample quality issues.

Small RNAs. Plasma smRNAs were available for baseline and at least one follow-up for n=129 participants in the CR group and n=71 participants in the AL group. Sample sizes for plasma smRNAs for individual timepoints by treatment group are provided in **Fig. 2B**. Muscle smRNAs are available for baseline and at least one follow-up for n=28 participants in the CR group and n=17 participants in the AL group. Sample sizes for muscle smRNA for individual timepoints by treatment group are provided in **Fig. 2B**. Adipose smRNAs are available for baseline and at least one follow-up for n=21 participants in the CR group and n=13 participants in the AL group. Sample sizes for adipose smRNAs for individual timepoints by treatment group are provided in **Fig. 2B.**

Messenger RNA. Muscle mRNA is available for baseline and at least one follow-up for n=28 participants in the CR group and n=17 participants in the AL group[19]. Sample sizes for muscle mRNA for individual timepoints by treatment group are provided in **Fig. 2B**. Adipose mRNA is available for baseline and at least one follow-up for n=23 participants in the CR group and n=14 participants in the AL group. This dataset was generated from an independent and larger set of samples than what has been published previously[20]. Sample sizes for this new adipose mRNA for individual timepoints by treatment group are provided in **Fig. 2B.** Adipose mRNA analysis has not been published previously. An overview of adipose mRNA response to CALERIE intervention in this dataset is reported in **Fig. 6**.

To facilitate the rapid appraisal of the underlying data structure of smRNA and mRNA for plasma, adipose, and muscle samples, we generated a series of summary datasets for transcripts passing quality control and their distributions across each timepoint (**Supplemental Data Files 25-44**).

## Discussion

CALERIE-2™ is the first ever Randomized Control Trial (RCT) of long-term CR in healthy, non-obese humans. The CALERIE™ Genomic Data Resource reported here represents the first such resource for a trail of an intervention hypothesized to modulate biological processes of aging. Below, we briefly outline applications of this resource to advance the frontiers of translational geroscience.

First, the CALERIE™ Genomic Data Resource provides opportunities to compare impacts of novel drug and behavioral interventions designed to modulate aging biology with a gold-standard intervention well-established in the animal literature. Admittedly, CALERIE™ is an imperfect implementation of CR compared to the controlled laboratory settings of model organisms. Nevertheless, CALERIE™ provides a baseline against which alternative, potentially more scalable geroprotective interventions can be compared. The molecular functions, biological processes, and cellular components generated from genomic datasets provide a 'common language' for comparisons with other intervention and observational studies in humans[21]. For example, the CALERIE™ Genomic Data Resource will facilitate comparison with a growing list of behavioral and pharmacological intervention trials now underway, many of which have specified genomic variables as endpoints[22]. For example, blood DNA methylation (DNAm) are being generated in a range of intervention trials, including trials of diet and exercise[23–26], supplements, including Vitamin D[27] and nicotinamide mononucleotide[28], and pharmacological interventions, including Dasatinib, Quercetin, and Fisetin[29]. RNA from blood, as well as RNA and DNAm from adipose and muscle are also available for a range of diet and exercise-related interventions[30–35]. As genomic data will become available for an increasing range of trials, the value of the CALERIE™ Genomic Data Resource will continue to grow.

Second, the CALERIE™ Genomic Data Resource will allow researchers to compare the molecular effects of CR on human subjects with the molecular responses of in vitro and in vivo model systems[36–38]. This complementary approach allows researchers to validate findings from rigorous human trails like CALERIE™ in laboratory settings and vice versa, and can provide insights into phylogenetically-conserved molecular responses to CR that can be used as benchmarks for interventions on biological aging. In one example, researchers examined the effect of CR in mice on muscle satellite cell expansion through the binding of circulating plasminogen to the Plg-RKT receptor. The role of plasminogen on muscle satellite cell expansion in mice was validated in humans using measures of blood plasminogen, a DNAm surrogate marker of plasminogen inhibitor PAI-1[39], and muscle biopsies from the CALERIE™ trail[40]. Through the recapitulation of findings in mice and humans, the researchers were able to demonstrate the mediating role of the plasminogen system on muscle stem cell regeneration in response to CR. The parallel analysis of human and in vivo or in vitro models has been successfully employed in the study of cancer and other diseases[41,42] and is likely to play a key role in the future of translational geroscience.

Third, the CALERIE™ Genomic Data Resource provides opportunities to evaluate the sensitivity of proposed genomics-based indices of biological aging to CR intervention. There are now abundant data to establish that intervention in the CALERIE™ trial improved a range of health parameters for the treatment group[9–11,43,44]. These data provide a background against which to evaluate proposed novel indices of biological aging, as we did in our prior analysis of epigenetic clocks[17]. The CALERIE™ Genomic Data Resource will provide a platform for analysis of the many new blood-based clocks recently published and others still forthcoming[45–48]. Furthermore, DNAm and RNA from muscle and adipose data from the CALERIE™ Genomic Data Resource will provide researchers with the rare opportunity to study the relationship between new and existing blood-based measures and molecular responses to CR in other tissues.

Fourth, the CALERIE™ Genomic Data Resource will provide developers with a powerful setting in which to develop, implement, and validate new and existing multi-omics tools. Multi-omics approaches leverage complementary information provided by different molecular data types to gain a more comprehensive understanding of complex biological systems and processes. Multi-omics tools use similarities, network structures, correlations, and probabilistic relationships across molecular datasets to generate novel insights not evident from analysis of individual omics data[49]. Most multi-omics tools have been developed and implemented in cancer research, where they are used for studying disease biology, disease subtyping, and in the development of diagnostic and prognostic biomarkers[49]. The application of such multi-omics tools in the CALERIE™ Genomic Data Resource could be used to better understand the complex biology of CR in living humans, the heterogeneity of responses to CR, or pharmacological targets for CR mimicking drugs.

Finally, the CALERIE™ Genomic Data Resource can provide researchers with preliminary data for planning interventions on biological aging. For example, CALERIE™ results for current epigenetic clocks[17] highlight the need for large sample sizes in trials planning to use these measures as outcomes. CALERIE™ data can similarly illuminate sample size planning around other genomic measures of aging.

In conclusion the CALERIE™ Genomic Data Resource is a new platform for discovery and validation in aging biomarker research and a reference resource for new geroscience clinical trials. It is our hope that publication of omics datasets for geroscience trials will become standard practice, advancing open science within geroscience research and enabling rapid comparative and integrative analyses to accelerate translation of therapies to extend healthspan.

# Methods

## Sample Collection

### Blood Collection

Approximate 70ml of blood was collected from participants after an overnight fast (12 hours) and immediately cryopreserved in liquid nitrogen and stored at −80°C from the time of acquisition until further processing.

### Muscle biopsies

Skeletal muscle biopsies of the vastus lateralis (VL) muscle were obtained using a 25-gauge, 2 inch needle as described in Bergstrom[50]. Participants were fasting overnight (12 hours) at the time of biosample collection. Briefly, the biopsy site was treated with local anesthesia. A small incision was made using a scalpel and the biopsy needle was inserted. Approximately 50-100 mg of muscle tissue was extracted and placed in a cryovial and immediately flash frozen in liquid nitrogen. Samples were stored at -80°C until processing.

### Adipose biopsies

Abdominal subcutaneous adipose tissue biopsies were obtained using 3 or 4mm x 15cm liposuction needles. Participants were fasting overnight (12 hours) at the time of biosample collection. Briefly, an area approximately one hand-width lateral to the umbilicus was sterilized and local anesthesia obtained using 2% lidocaine. After making a small insicion with a scapel, the liposuction needle was inserted and suction was applied to the attached syringe to withdraw approximately 900mg of tissue. Tissue was rinsed with phosphate buffered saline, weighed, placed in a cryovial, and immediately flash frozen in liquid nitrogen. Samples were stored at -80°C until processing.

## Genotyping

The CALERIE™ genotype dataset was produced by the Kobor Lab at the University of British-Columbia and the Genomics Analysis Shared Resource at Duke University. DNA was extracted from n=217 baseline blood samples obtained from the CALERIE™ Biorepository at the University of Vermont. Participants were fasting overnight (12 hours) at the time of biosample collection. Genotyping was conducted using the Illumina Global Screening Array-24 v3.0 (GSA) BeadChips containing 654,027 markers, with ~30,000 add-on markers from Infinium PsychArray-24 focused content panel (Illumina, San Diego, CA). Briefly, 200ng DNA was processed and hybridized to the GSA chips according to the manufacturer's instructions, and scanned using the Illumina iScan platform. Genotypes were called for SNPs matched to dbSNP v151 [51] and for which valid calls were made in >98% of participants using GenomeStudio v2.0 (Illumina, San Diego, CA). Additional SNPs were imputed using the IMPUTE2 software suite G3[52] and the 1000 Genomes Phase 3 reference panel[53]. Pre-phasing and imputation were conducted by breaking the genome into 5 megabase fragments. The final SNP database consisted of directly genotyped SNPs and SNPs imputed with a >90% probability of a specific genotype for which call rates were >95% and minor allele frequencies were >1%.

### *SNP-principal components*

The top 20 principal components were calculated on the autosomal SNPs using PLINK v1.90b3.36 with default parameters[54].

## DNA methylation

### Preprocessing, Normalization, and Quality Control

#### *Blood*

DNA methylation profiling for blood was conducted in the Kobor Lab (https://cmmt.ubc.ca/kobor-lab/) from whole-blood DNA stored at -80 degrees Fahrenheit. Briefly, 750 ng of DNA was extracted from whole blood and bisulfite converted using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). Methylation was measured from 160ng of bisulfite-converted DNA using the Illumina EPIC Beadchip (Illumina Inc, San Diego, CA, USA).

QC and normalization were performed using methylumi[55](v. 2.32.0) and the Bioconductor (v 2.46.0)[56] package from the R statistical programming environment (v 3.6.3). Probes with detection p-values >0.05 were coded as missing; probes missing in >5% of samples were removed from the dataset (final probe n=828,613 CpGs). Normalization to eliminate systematic dye bias in the 2-channel probes was carried out using the methylumi default method.

#### *Muscle*

DNA methylation profiling for muscle was conducted in the UCLA UCLA Neuroscience Genomics Core (UNGC) from skeletal muscle DNA extracted in the VB Kraus lab at Duke University and stored at -80 degrees Celcius. Briefly, 250 ng of DNA was extracted from muscle tissue and bisulfite converted using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). Methylation was measured from 250 ng of bisulfite-converted DNA using the Illumina EPIC Beadchip (Illumina Inc, San Diego, CA, USA). Quality control and preprocessing for muscle tissue DNAm was carried out from IDATs in the Geroscience Core in the Butler Columbia Aging Center, and performed in the R computing environment using minfi[57], wateRmelon[58], and EWAStools[59] packages. Prior to normalization, sample quality control was carried out. Sample quality control involved checking samples for bisulfite conversion efficiencies <80%, signal intensities <10.5, average detection p-values <0.005, <5 beads for >5% of probes, or sex mismatches based on methylation levels at 3 CpGs in the X-chromosome inactivation center (chrXIC:72987573-72988170)[60]. No samples failed sample quality control. Samples were then normalized using the *PreprocessNoob* function in the minfi package[57]. Probe quality control was then run. Probe quality control consisted of dropping cross-hybridizing probes, sex-chromosome linked probes, probes with a beadcount of <3, and probes with SNP-associated CpGs or probes with SNPs in single-base extension regions. Finally, intra-sample normalization using beta-mixture quantile (BMIQ) was carried out[61].

#### *Adipose*

DNA methylation profiling for adipose was conducted in the UCLA Neuroscience Genomics Core (UNGC) from adipose tissue DNA extracted in the VB Kraus lab at Duke University and stored at -80 degrees Celcius. Briefly, 250 ng of DNA was extracted from adipose tissue and bisulfite

converted using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). Methylation was measured from 250 ng of bisulfite-converted DNA using the Illumina EPIC Beadchip (Illumina Inc, San Diego, CA, USA). Quality control and preprocessing for adipose tissue DNAm was carried out from IDATs in the Geroscience Core in the Butler Columbia Aging Center, and followed identical protocol to that for muscle DNAm. No adipose samples failed quality control.

## Derived Variables

### *DNAm control-probe principal components (PCs)*

For blood, muscle, and adipose, we conducted principal component analysis of EPIC-array control-probe beta values to compute controls for technical variability across the samples. To do so, we loaded raw idats, normalized using *normalizeMethyLumiSet*, and extracted normalization quality control probes. Principal components (PCs) were extracted from normalization probes, and standard deviations of these PCs were standardized by squaring and dividing by the sum of their squares. PCs that explained 90% of the variance in normalization probes were retained.

### *Immune cell proportion estimates*

For blood, proportions of CD8T, CD4T, natural killer cells, b cells, monoctyes, and neutrophils were estimated using the Houseman equation via the *FlowSorted.Blood.EPIC* package and standard "Blood" reference panel[62,63](**Supplementary Data File 39**). An 'extended' version of *estimateCellCounts2* using *FlowSorted.BloodExtended.EPIC* using the "BloodExtended" reference panel was also used to calculate basophils, B naïve, B memory, CD4T naïve, CD4T memory, CD8T naïve, CD8T memory, eosinophils, monocytes, neutrophils, T regulatory cells and natural killer cells (**Supplementary Data File 40**). Cell estimates were estimated on a red green channel set (RGset) and noob normalized with the reference dataset as specified by the package developers[18].

### *Epigenetic clock estimates*

Blood epigenetic clocks including Horvath, Hannum, PhenoAge, GrimAge were calculated using the matrix of beta values provided in the data repository and the online calculator (https://dnamage.genetics.ucla.edu/, since been superseded by https://projects.clockfoundation.org). DunedinPACE was calculated using the matrix of beta values provided in the data repository and the DunedinPACE R package (https://github.com/danbelsky/DunedinPACE). Principal components-based clocks (PC clocks) developed by Higgins-Chen and colleagues were calculated in R on noob normalized samples that passed sample quality control using previously described code and methods[64].

## RNAseq

### Blood

#### *RNA extraction*

Circulating RNA was extracted from 200 µL plasma using the Qiagen miRNeasy Serum/Plasma Advanced Kit (Catalog no. 217204). RNA samples were stored at -80°C until miRNA sequencing was performed.

#### *sRNA sequencing*

sRNA sequencing was performed on samples isolated from plasma (n=584; AL=36%:CR=64%). Sequencing libraries were prepared from 5µLs of miRNeasy RNA using the QiaSeq miRNA Library Auto Kit (384; cat # 331509) according to the manufacturer's instructions. Library amplification (22 cycles) was performed on a Beckman i7 liquid handler, using QiaSeq miRNA 96 Index IL Auto A (384; cat # 331569). Library quality was evaluated using a fragment analyzer (Agilent); the library concentrations were quantified by Qubit ds DNA HS assay (ThermoFisher). Libraries were sequenced on a NovaSeq 6000 (Illumina) using S4 lanes and 75 base pair single reads.

#### *sRNA processing and normalization*

QIAseq smRNA sequencing FASTQ files were preprocessed using the GeneGlobe® Data Analysis platform, Legacy version 2.0. SmRNA reads were first processed by trimming at the 3' adapter using cutadapt. Next, the trimmed reads were used to identify insert and unique molecular index (UMI) sequences. Reads with <16 base pair insert sequences or less than 10 base pair UMI sequences were discarded. Annotations of insert and UMI sequences were mapped to the human reference genome (GRCh38/hg38) using the alignment tool, bowtie. All miRNA/piRNA mapped reads and associated UMIs were then aggregated to count unique molecules using miRbase V21 and piRNABank, respectively. Following the data processing steps, UMI counts were filtered to remove low expression smRNAs (row sum counts <50 were removed). Filtered UMI counts were then used to calculate normalization factors using the trimmed mean of M-values (TMM) method within the BioConductor edgeR package.

### Muscle

#### *RNA extraction*

Flash frozen skeletal muscle samples (~50 mg) were homogenized using a bead-based Qiagen TissueLyser II in l mL TRIzol™ Reagent for a total of 4 minutes, rotating the TissueLyser Adapter after 2 minutes (Invitrogen™ ThermoFisher #15596026). Following homogenization, RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen #74104) and stored at -80°C until mRNA sequencing.

#### *mRNA sequencing*

Illumina libraries were generated with the TruSeq Stranded Total RNA Library Preparation Kit. RNA was sequenced using the *Illumina NovaSeq 6000* sequencing system with paired-end reads. The samples yielded 387 to 618 million pass filter reads with more than 86% of bases above the quality score of Q30.

### *mRNA filtering, alignment, and genome annotation*

The quality of reads in fastq RNA-Seq files were initially assessed using *FastQC* tool (v. 0.11.5; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), Preseq (v. 2.0.3)[65], Picard tools (v. 2.17.11; https://broadinstitute.github.io/picard/) and RSeQC (v. 2.6.4)[66]. Reads were trimmed using *bbduk* (from bbtools package (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/)[67]. Following trimming, cleaned reads were examined one more time using *FastQC*. Next, cleaned fastq files, along with reference human genome 38 and Ensembl annotation v104, were used as input for STAR (version 2.7.10a), a splice-aware aligner implemented with a novel algorithm for aligning high-throughput long and short RNA-Seq data to a reference genome[68]. The STAR aligner was run with the *–quantmode TranscriptomeSam* parameter to also generate transcriptome BAM files, and was used for RSEM analysis. Genome BAM files were sorted and indexed using samtools. Finally, genome BAM files were used as input for *featureCounts* from the Rsubread package (version 2.0.1)[69], a suitable program for counting reads for various genomic features such as genes.

### *sRNA sequencing*

sRNA sequencing was performed on samples isolated from muscle (n=116; AL=38%:CR=62%) using the same protocols as for blood, described above.

### *sRNA Processing and Normalization*

sRNA data were processed and normalized using the same protocols as for blood, described above.

## Adipose

### *RNA extraction*

Segments of flash frozen abdominal adipose tissue (~50 mg each) were homogenized using a bead-based Qiagen TissueLyser II in l mL TRIzol™ Reagent for a total of 4 minutes, rotating the TissueLyser Adapter after 2 minutes (Invitrogen™ ThermoFisher #15596026). Following homogenization, RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen #74104) and stored at -80°C until smRNA sequencing.

### *mRNA sequencing*

mRNA sequencing was performed on the Illumina NovaSeq 6000 Sequencer at the Duke Sequencing Core. Barcoded libraries for mRNA sequencing were produced from the Universal Plus mRNA-seq NuQuant kit (0408-A01, Tecan Genomics) according to manufacturers recommended protocol with 17 amplication cycles and 200ng of total starting RNA. The protocol was automated on a Perkin Elmer Sciclone G3 liquid handler.

### *mRNA filtering, alignment, and genome annotation*

Alignment of fastq files against the GRCh38 assembly of the human genome was performed using Subread[70] on the Columbia Department of Systems Biology high performance cluster. Most samples aligned with >90% mapped reads. One sample aligned with 69.6% mapped reads,

but demonstrated satisfactory base sequence quality, tile sequence quality, and per sequence quality scores. Quantification was performed using featureCounts[69] method as implemented in Subread. A matrix of counts was constructed, with genes labeled using Entrez Gene ID numbers. Initial quality control steps and outlier detection was carried out in 5 groups of 30 samples each. Samples that passed the first screen were then combined for a final quality and outlier detection round. Quality was assessed and outliers detected by multidimensional scaling[71] and hierarchical clustering[72]. Multidimensional scaling was performed using limma[73]. Hierarchical clustering was performed with Euclidean distance and complete linkage clustering[72] as implemented in the Heatmap.2 command in gplots[74]. Optimal clusters determined by the gapstat[75] method as implemented in NBClust[76]. During quality control, 2 samples – both baseline samples – were removed. One sample had too few reads, while the other was a clear outlier on multidimensional scaling plots and linkage clustering maps.

### *mRNA Differential Expression*

Samples were normalized using the trimmed mean method[77] and analyzed for differential expression with limma-voom with sample weighting to downweight the effect of sample outliers[73,78,79]. Participant was modeled as a random effect using the duplicate correlation method[80,81]. CR at follow-up (12- or 24-months) was compared to AL at follow-up (12- or 24-months), relative to CR at baseline compared to AL at baseline. Raw p-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate[82].

### *sRNA sequencing*

sRNA sequencing was performed on samples isolated from muscle adipose (n=65 AL=38%:CR=62%) using the same protocols as for blood, described above.

### *sRNA processing and normalization*

sRNA data were processed and normalized using the same protocols as for blood, described above.

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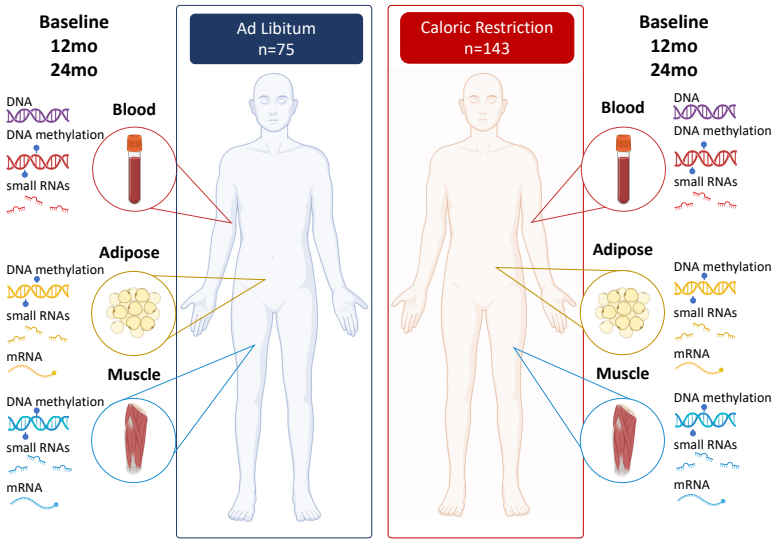
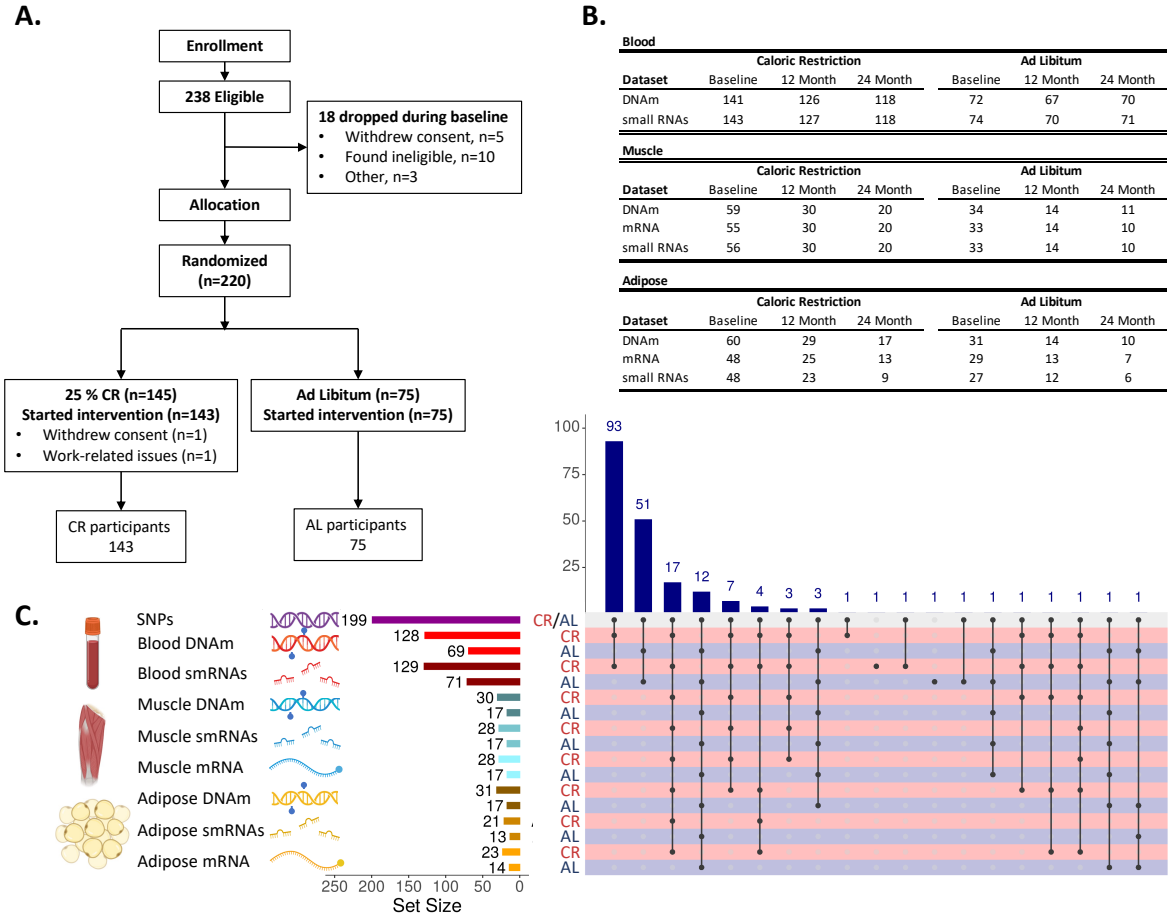



**Figure 1.** Overview of molecular datasets generated for the CALERIE™ caloric restriction trial. Genetic material (DNA) was derived from blood samples for both ad libitum (AL) and caloric restriction (CR) groups at baseline. DNA methylation was derived from blood, muscle, and adipose samples for both AL and CR at baseline, 12-months, and 24-months. Small RNAs were derived from blood, muscle, and adipose samples for both AL and CR at baseline, 12-months, and 24-months. mRNA was derived from muscle and adipose samples for both AL and CR at baseline, 12-months, and 24-months. Figure created using images from Biorender.com.




**Blood**

| | Caloric Restriction | | | Ad Libitum | | |
|---|---|---|---|---|---|---|
| **Dataset** | Baseline | 12 Month | 24 Month | Baseline | 12 Month | 24 Month |
| DNAm | 141 | 126 | 118 | 72 | 67 | 70 |
| small RNAs | 143 | 127 | 118 | 74 | 70 | 71 |

**Muscle**

| | Caloric Restriction | | | Ad Libitum | | |
|---|---|---|---|---|---|---|
| **Dataset** | Baseline | 12 Month | 24 Month | Baseline | 12 Month | 24 Month |
| DNAm | 59 | 30 | 20 | 34 | 14 | 11 |
| mRNA | 55 | 30 | 20 | 33 | 14 | 10 |
| small RNAs | 56 | 30 | 20 | 33 | 14 | 10 |

**Adipose**

| | Caloric Restriction | | | Ad Libitum | | |
|---|---|---|---|---|---|---|
| **Dataset** | Baseline | 12 Month | 24 Month | Baseline | 12 Month | 24 Month |
| DNAm | 60 | 29 | 17 | 31 | 14 | 10 |
| mRNA | 48 | 25 | 13 | 29 | 13 | 7 |
| small RNAs | 48 | 23 | 9 | 27 | 12 | 6 |

**Figure 2. Study design and molecular data resources for the CALERIE™ trail. (A)** Consort diagram showing enrollment, eligibility, and final sample sizes by treatment group. **(B)** Individual sample numbers by molecular data type, treatment group (Caloric Restriction or Ad Libitum) and follow-up visit (Baseline, 12 Month, or 24 Month) across different tissue types. For some individuals, samples were available at follow-up but not baseline (or vice versa), so baseline numbers for a tissue and treatment group combination will not always match sample sizes in Fig. 2C. **(C)** Upset plot showing overlap of available datasets across treatment group, tissues, molecular data type. In this figure, only samples with baseline and at least one follow-up (12- or 24-months) are counted. The left-hand side shows tissue type (blood, muscle, or adipose), type of molecular data type (SNPs, DNAm, smRNAs, or mRNA), set size for each tissue and data type combination in number of unique individuals, and group (CR or AL). The bottom right-hand side shows points and connecting lines indicating overlapping intersections across tissues and data types color coded by treatment group (CR = red, AL = navy). The top right hand side shows a barchart indicating sample sizes for the overlapping intersections of tissue and datatypes. Each tissue and molecular data type combination is linked to a corresponding color scheme as follows: genomic variation (purple DNA), blood DNA methylation (red DNA with lollipops), blood small RNAs (smRNAs; dark red RNA fragments), muscle DNA methylation (blue DNA with lollipops), muscle smRNAs (blue RNA fragments), muscle mRNA (blue single RNA strand), adipose DNA methylation (yellow DNA with lollipops), adipose smRNAs (yellow RNA fragments), adipose mRNA (yellow single RNA strand).

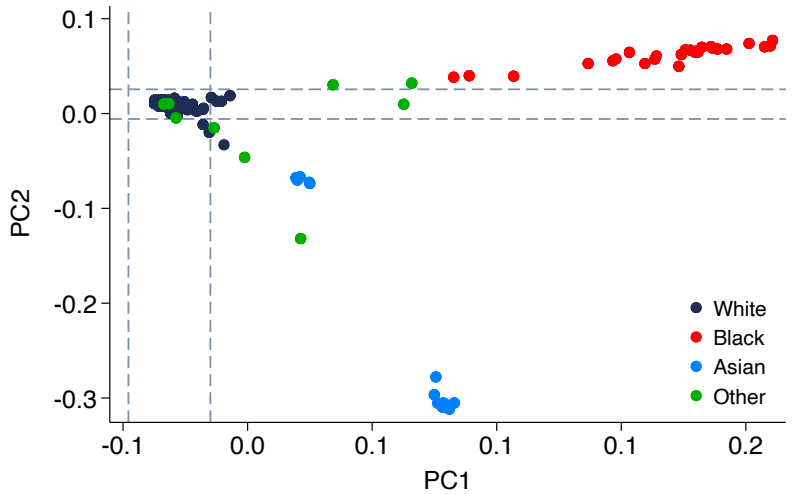



**Figure 3. SNP-based principal components scores for CALERIE™ participants.** The figure shows the top two SNP-based principal components (PCs) for CALERIE™ participants (n=217), colored by self-reported race/ethnicity. Dashed lines indicate three standard deviations from the mean for participants who self-reported race/ethnicity as White.

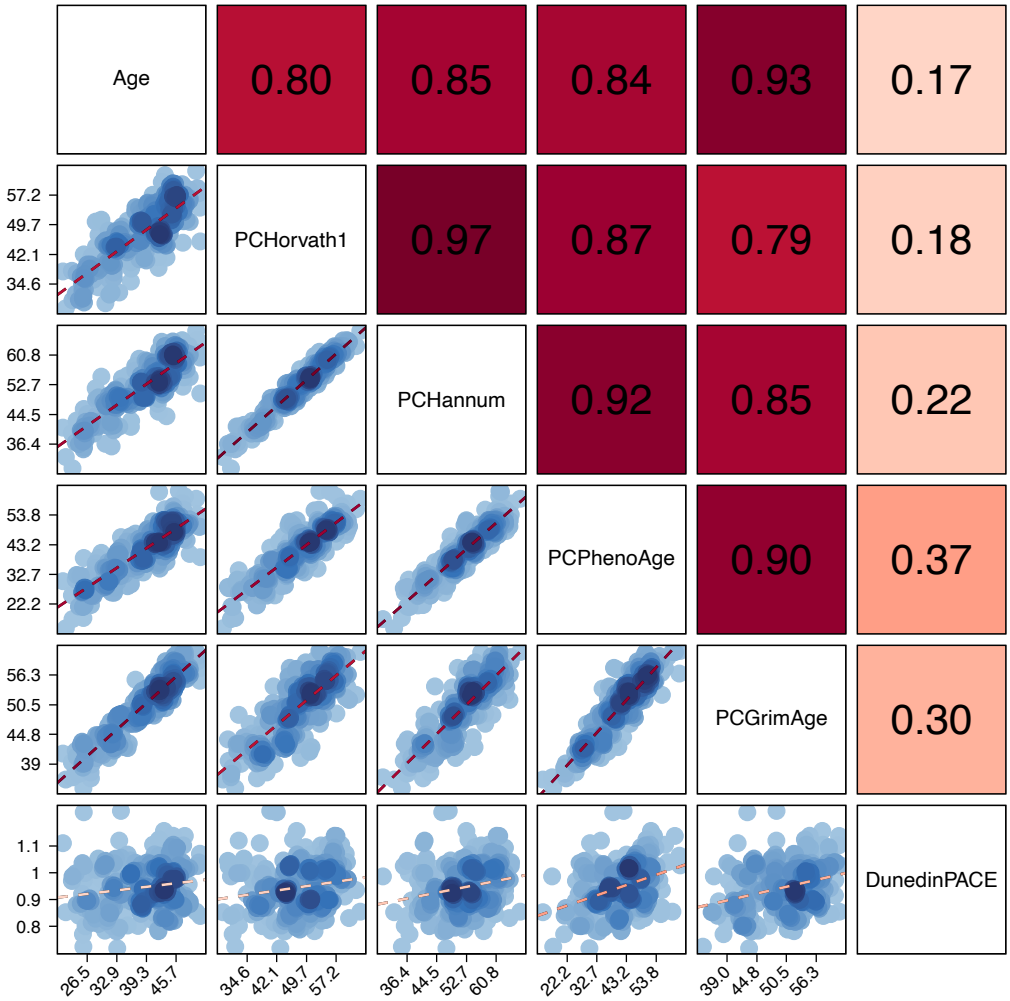



**Figure 4**. **Scatterplot and correlation matrix for chronological age and five epigenetic clocks at baseline measurement.** The figure shows a matrix of correlations and association plots among chronological age and Horvath, Hannum, PhenoAge, GrimAge, and DunedinPACE epigenetic clocks. Values are for participants at baseline, prior to treatment. Horvath, Hannum, PhenoAge and GrimAge were calculated from principal components based on the methods of Higgins-Chen et al. 2022. The diagonal cells of the matrix list chronological age and DNA methylation measures. The half of the matrix below the diagonal shows scatter plots of associations. For each scatter-plot cell, the y-axis corresponds to the variable named along the matrix diagonal to the right of the plot and the x-axis corresponds to the variable named along the matrix diagonal above the plot. The half of the matrix above the diagonal lists Pearson correlations between the DNA methylation measures. For each correlation cell, the value reflects the correlation of the variables named along the matrix diagonal to the left of the cell and below the cell.

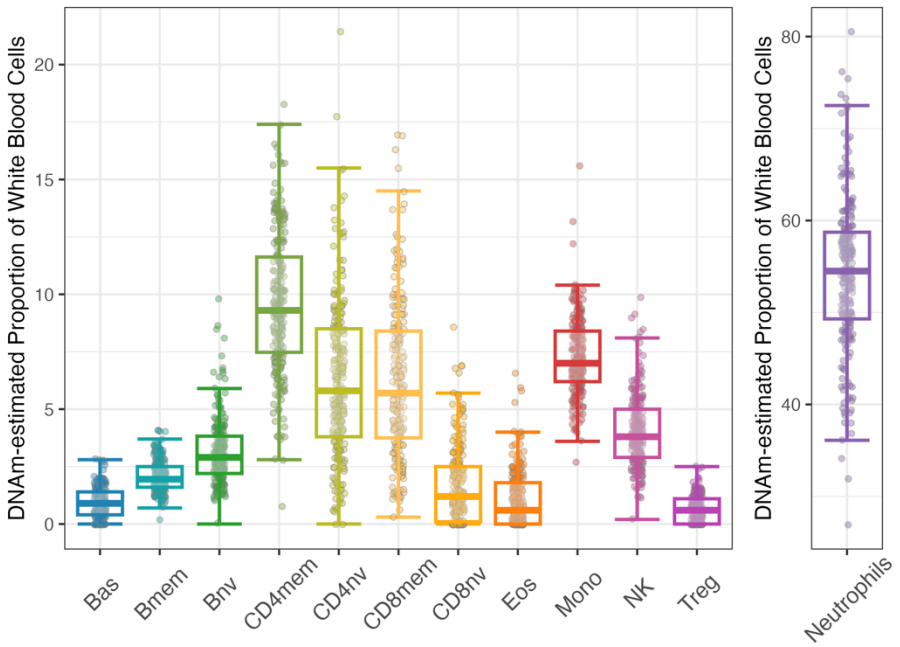



**Figure 5**. **Relative proportions of 12 white blood cell types estimated for all participants at baseline.** The figure shows the estimated relative proportions of 12 white blood cell types for all participants at baseline, prior to treatment (Bas=basophils, Bmem=memory B cells, Bnv=naïve B cells, CD4mem=memory CD4T cells, CD4nv=naïve CD4T cells, CD8mem=memory CD8T cells, CD8nv=naïve CD8T cells, Eos=Eosinophils, Mono=Monocytes, NK=Natural Killer cells, Treg=T regulatory cells, Neutrophils=Neutrophil cells). Individual points represent individual observations. Box and whisker plots showing median (horizontal line), 25$^{th}$ quartile (bottom of the box), 75$^{th}$ quartile (top of the box), and minimum and maximum values within 1.5 × IQR (interquartile range; top and bottom segments, respectively). Neutrophils are plotted on a separate scale.

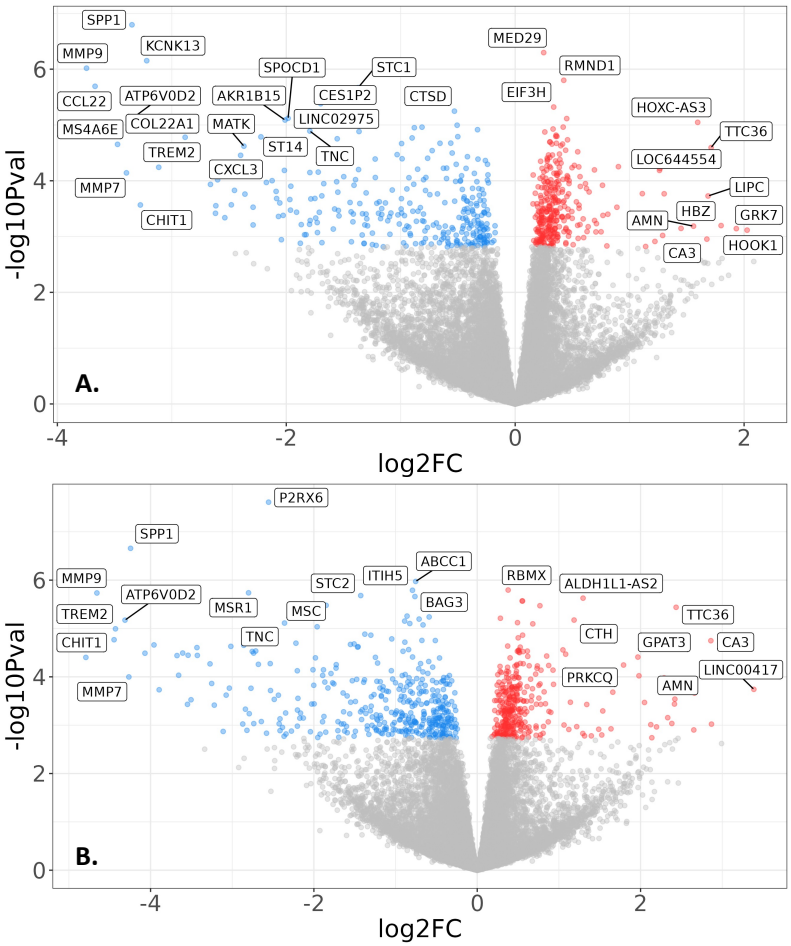



**Figure 6. Plot of RNA expression differences between CR and AL treatment groups at the 12-month (A) and 24-month (B) timepoint across 19471 genes.** The x-axis shows log2 fold-change differences between groups. The y-axis shows -log10 p-value. Blue points show genes with false-discovery rate q-values less than 0.05 were downregulated in CR participants. Red points show genes with false-discovery rate q-values less than 0.05 were upregulated in CR participants. Gray points show genes with q-values equal or greater than 0.05. Using a false-discovery cutoff of *q*=0.05, a total of 605 genes were differentially-expressed between CR and AL treatment groups at the 12-month (**Panel A**) and 24-month (**Panel B**) timepoints. A dispersed but otherwise arbitrary selection of 31 differentially-expressed genes labeled by gene symbol are shown for the 12-month treatment effect. A dispersed but otherwise arbitrary selection of 23 differentially-expressed genes labeled by gene symbol are shown for the 24-month treatment effect.