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Research Paper

When a Calorie Is Not a Calorie: Metabolic and Molecular Effects of Intermittent Fasting in Humans; Exploratory Outcomes of a Randomized Clinical Trial

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Intermittent fasting (IF) extends healthspan and lifespan in rodents and has been associated with metabolic benefits in humans, yet results so far have been inconsistent. In this study, we tested the effects of IF-induced weight loss on metabolic and molecular determinants of healthy aging. We performed a randomized clinical trial with a partial cross-over design in overweight men and women (30–65 y, average 49.3 ± 8.2 y) to test the effects of six-month IF (NCT01964118). Fifty participants were randomized to the IF (n = 28) or usual diet (n = 22) group. The primary outcome was the assessment of change in serum C-reactive protein levels from baseline to six months; secondary outcomes were changes in insulin sensitivity using oral glucose tolerance test (OGTT)-based indexes, and plasma metabolomics and gene expression changes in colon mucosa. No difference in serum levels of C-reactive protein or multiple cytokines and chemokines was observed over this period, despite a significant IF-induced 8% weight loss. IF caused a statistically significant but clinically irrelevant small improvement in OGTT-derived insulin sensitivity indexes. Preliminary multi-omic data analysis suggests that a nonlinear relationship exists between IF-induced weight loss and inhibition of multiple key nutrient-sensing aging pathways. More trials are needed to understand the impact of different degrees of energy restriction on metabolic and molecular health in humans, and how fasting should be complemented with diet quality changes during feast days to maximize clinical and longevity outcomes.

Introduction

The current pandemic of unhealthy lifestyles and obesity is a major public health challenge because of its numerous associated comorbid conditions and growing healthcare costs, estimated to exceed $150 billion per year in the United States alone[1]. Data from several randomized clinical trials have shown that daily calorie restriction (CR) with adequate nutrient intake, when associated with significant weight loss, results in robust improvements in cardiometabolic risk factors in obese and normal-weight people as well[2–6]. In particular, daily CR consistently and markedly improves two of the most important determinants of healthy aging: inflammation and insulin sensitivity, among many others[7,8].

Intermittent fasting (IF) has recently been proposed as an easier and practical alternative to chronic CR to promote weight loss, improve metabolic health, and modify key aging pathways (including mTOR and autophagy) in model organisms and humans[9,10]. However, unlike in rodents, results from several short-term randomized clinical trials of IF in overweight and obese adults have reported variable effects on health indicators, with little information on the molecular adaptations induced by IF in human tissues[2,11–13].

The present randomized clinical trial was designed to determine whether sustained weight loss induced by long-term IF confers beneficial metabolic and molecular effects in overweight and obese men and women free of major chronic diseases. For the first

six months, participants aged 30–65 y were randomly assigned to IF or the usual ad-libitum Western-like diet. In the second six months of the study, all participants underwent IF. The IF regiment allowed only non-starchy raw and/or cooked vegetable salads for lunch and dinner during the fasting days, dressed with two tablespoons of olive oil, lemon juice, or vinegar. This novel "vegetable fasting-mimicking" approach helped to markedly improve compliance while avoiding calorie counting and minimizing activation of the insulin/IGF/mTOR pathway because of the very low calorie, protein, and carbohydrate content of non-starchy vegetables[2]. Participants with a body mass index (BMI) between 24 and 27.9 kg/m^2 (n = 11 IF and 6 C for combined group A + D) were asked to fast for two nonconsecutive days per week, whereas those with a BMI between 28 and 35 kg/m^2 (n = 10 IF and 14 C for combined group A + D) fasted for three nonconsecutive days per week. We assessed the effects of IF on inflammatory markers (primary outcome), glucose tolerance and insulin sensitivity, body composition, and plasma adipokine and cortisol concentrations. By using a novel automated biological knowledge generation platform (CompBio)[14–17], we also analyzed the differential effects of IF-induced weight loss on plasma metabolomics and colon mucosa RNA-seq-based transcriptome molecular signatures.

Methods

Study design and participants

This was a randomized controlled trial done at Washington University in St. Louis in the United States, aimed at evaluating the effects of IF over a six-month period in healthy, overweight, or mildly obese (BMI 24.0–35.0 kg/m^2), weight-stable (<2 kg weight change in the previous six months) men and women (aged 30–65 y). Baseline characteristic of the participants are reported in **S Table 1**. None of the participants had serious chronic diseases or other health conditions that could interfere with the interpretation of the results. Use of glucoregulatory medication and smoking habits were exclusionary. The study protocol (NCT01964118) was approved by the institutional review board of Washington University Medical School, St. Louis, MO, United States (IRB #201303081). Study volunteers provided written informed consent. Study oversight was provided by a data and safety monitoring board. The CONSORT diagram for enrolled participants is shown in **S Figure 1**.

Study design

After a series of screening visits, participants were randomly assigned by a random list generator, to IF (group A) or the usual diet (group B) in a 1:1 allocation ratio. About 50 nonsmoker adults (30 women, 20 men) were randomized, and 49 began the intervention, and their data are included in these analyses (Fig. 1). After completing the six-month randomized study, participants who graduated from period 1 volunteered to continue the IF intervention for another six months (group C), while participants who had been randomized to the no-intervention control group crossed over to IF for six months (group D). All study visits were conducted at the Clinical Research Unit (4th floor, Barnard Hospital) at Washington University in St. Louis.

IF intervention

Participants in the IF group were prescribed by the study dietitians a fasting regimen that consisted of three nonconsecutive days per week, if their BMI was higher than 28 kg/m^2, whereas participants with a BMI between 24 and 27.9 kg/m^2 were asked to fast for two nonconsecutive days per week, for the entire duration of the study. All IF participants were asked to skip breakfast, lunch, dinner, snacks, and calorie-containing beverages on the fast days, but they were allowed to consume at lunch and dinner non-starchy raw and/or cooked vegetables "ad libitum," dressed with a maximum of two tablespoons of olive oil (~240 kcal) plus vinegar or lemon juice. Noncaloric drinks, such as black coffee, unsweetened tea, or zero-calorie soda, were allowed. Because non-starchy vegetables contain very small quantities of bioavailable calories, proteins, fats, and carbohydrates, this "vegetable fasting-mimicking" protocol (that does not require counting calories) was selected to mimic water-only fasting while minimizing participants' social life disruption. During the "feast" days, research volunteers were asked to consume their habitual diet without overcompensation of calories. Strategies to implement fast day planning and recipes for salads and cooked vegetables were covered at the weekly meetings with the dietitians. Participants' compliance to the intervention was assessed by monitoring weekly body weights, with weekly discussion with the study dietitians and the revision of four-day food diaries collection. Participants assigned to the control group continued on their regular diets; they received no specific dietary intervention or counseling.

Anthropometrics, body composition, and dietary assessment

Body weight was measured in duplicate after an overnight fast, with the participant wearing a hospital gown (Scale-Tronix, Welch Allyn, Inc.). IF group participants were also provided with scales (HD-357, Tanita Corporation of America, Inc., Illinois) for measuring body weight at home once weekly to be reported regularly to the study dietitians. Height was measured to the nearest 0.1 cm. BMI was calculated as weight/height^2. Whole-body fat mass (FM), lean mass (LM), and %FM were assessed by dual-energy x-ray absorptiometry (DXA; Lunar iDXA, software version 13:31, GE Healthcare, Madison, WI). Four-day food diaries were used to estimate self-reported intake. Participants received detailed instructions on how to weigh, measure, and record all food and beverages consumed during the collection. Research dietitians reviewed the diaries with participants and then analyzed them using the Nutrition Data System for Research (NDS-R program, version 2013–2015, Nutrition Data System for Research from the Nutrition Coordinating Center at the University of Minnesota).

Blood analyses

Venous blood was sampled for metabolic and hormone concentrations after an overnight fast. Blood draws were performed on the day of the oral glucose tolerance test (OGTT). Samples were collected in serum, edetic acid, and heparinized plasma tubes, immediately centrifuged to separate the plasma, aliquoted, and stored in a −80 °C freezer until use. All serum and plasma samples were analyzed by the Core Laboratory for Clinical Studies at Washington University in St. Louis; technicians doing assessments were masked to treatment assignment. Cortisol was measured by ECLIA electrochemiluminescence (Elecsys Roche Diagnostic, Lewes England, on the Roche cobas e601), while high-sensitive C-reactive protein (hsCRP) was measured using a particle-enhanced immunoturbidimetric essay (Roche cobas c501).

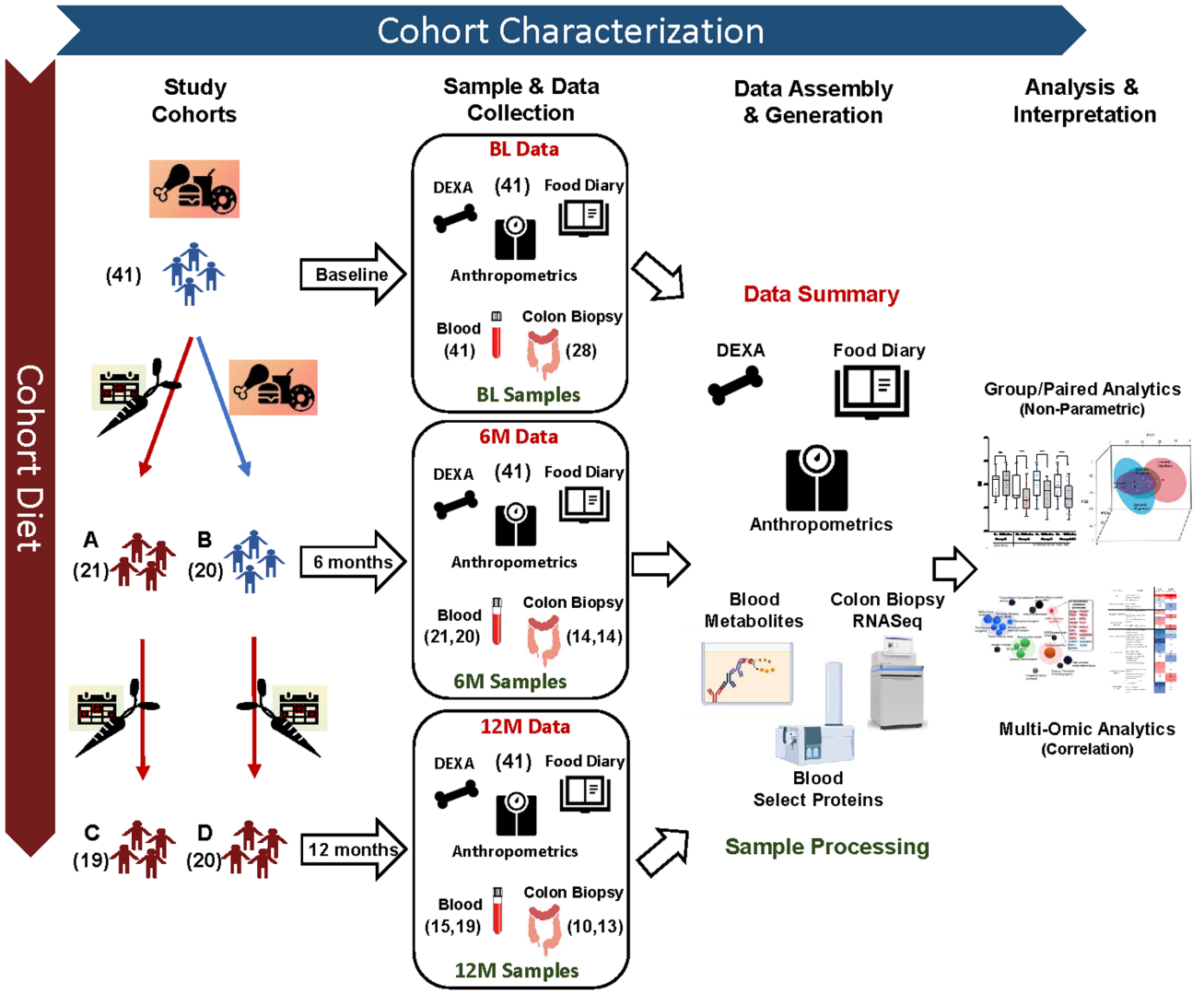

Figure 1. Intermittent fasting (IF) study associates IF-induced body composition and metabolic changes with multi-omic signatures.

Leptin was analyzed by RIA using Millipore kits. Commercial ELISA Quantikine kits (R&D System, Inc., Minneapolis, MN) were used to measure high-molecular-weight (HMW) adiponectin. Plasma cytokines and chemokines were measured using a multiplex immunoassay kit (K15048D) from Meso Scale Discovery (Rockville, MD). The assays were performed using a QuickPlex SQ120 analyzer with MSD Discovery Workbench 4.0 software (Meso Scale Discovery, Rockville, MD) according to the manufacturer's instructions with samples run in duplicate. Technicians who performed the analyses were blinded to the group/treatment assignment.

OGTT

Two-hour, 75-g OGTTs were performed at baseline and at 6 and 12 months with blood samples collected at baseline and 30, 60, 90, and 120 min after glucose consumption. Adequate carbohydrate intake over the previous three days (≥150 g per day) was ensured by prescription and a subsequent interview with a study dietitian. Plasma glucose was measured by the glucose oxidase method (glucose oxidase method, Stat Plus, Yellow Springs Instruments, Co., Yellow Springs, OH), and insulin was measured by radioimmunoassay (ECLIA electrochemiluminescence, Elecsys Roche Diagnostic, Lewes England, on the Roche cobas e601). Insulin resistance was calculated using homoeostasis model assessment (fasting glucose [mmol/L] × fasting insulin [mIU/L]/22.5)[18]. β-cell function was calculated using homoeostasis model assessment-β (% = [360 × fasting insulin (mIU/L)]/[fasting glucose (mg/dL) – 63])[18]. Area under the curve insulin and area under the curve glucose values from the OGTT were determined using the trapezoidal method[19]. Insulin response was calculated as the ratio of the change in plasma insulin from baseline to 30 min to the change in plasma glucose over the same period. The insulin sensitivity index was calculated as 1 divided by fasting insulin concentration (mIU/L)[20,21]. The Oral Disposition Index was calculated as the product of insulin response and insulin sensitivity[22,23].

Colon mucosa biopsy and gene expression analyses

Endoscopic cold biopsy specimens were obtained from macroscopically normal sigmoid mucosa (Flexible Video Sigmoidoscope, Olympus OSF V60) after an overnight fast and preparation with an enema containing water, in a subgroup of 14 IF participants (8 females and 6 males) and 14 controls (9 females and 5 males). Sigmoidoscopy was performed at baseline, 6- and 12-month visits.

One participant in the control group did not complete the follow-up sigmoidoscopy, and therefore only 13 controls data were used for analyses. Single-use biopsy forceps (Radial Jaw4, Boston Scientific, Natik, MA, United States) were used to collect ~10 pieces of tissue, immediately washed in PBS (Life Technologies, Carlsbad, CA, United States) and flash frozen in liquid nitrogen and stored at −80 until further analyses. RNA was extracted from colon samples using Trizol reagent (Invitrogen, Carlsbad, CA, United States) by technicians of the Tissue Procurement Core of Washington University in St. Louis, following the manufacturer's instructions. RNA sequencing experiments and bioinformatic analysis were performed at the Genomic Technology Access Center, Washington University in St. Louis.

Samples were prepared according to the library kit manufacturer's protocol, indexed, pooled, and sequenced on an Illumina HiSeq. Basecalls and demultiplexing were performed with Illumina's bcl2fastq software and a custom Python demultiplexing program with a maximum of one mismatch in the indexing read. RNA-seq reads were then aligned to the Ensembl release 76 top-level assembly with STAR version 2.0.4b[24]. Gene counts were derived from the number of uniquely aligned, unambiguous reads by Subread:featureCount version 1.4.5[25]. Isoform expression of known Ensembl transcripts was estimated with Sailfish version 0.6.13[26]. Sequencing performance was assessed for the total number of aligned reads, the total number of uniquely aligned reads, and the features detected. The ribosomal fraction, known junction saturation, and read distribution over known gene models were quantified with RSeQC version 2.3[27].

All gene counts were then imported into the R/Bioconductor package EdgeR[28], and TMM normalization size factors were calculated to adjust for samples for differences in library size. Genes or transcripts not expressed in any sample were excluded from further analysis. The TMM size factors and the matrix of counts were then imported into the R/Bioconductor package Limma[29]. Weighted likelihoods based on the observed mean-variance relationship were then derived for every gene with Voom[30]. The performance of all genes was assessed with plots of the residual standard deviation of every gene to their average log-count with a robustly fitted trend line of the residuals.

Gene CPM ratios (six months to baseline) were calculated for each subject and used for correlation analysis with each of the phenotypes.

Plasma metabolomics

About 30 uL of EDTA-Plasma were analyzed at the University of California, Davis, for primary metabolism profiling by gas chromatography/time-of-flight (GCTOF) mass spectrometry using Gerstel CIS4—with dual MPS Injector/ Agilent 6890 GC-Pegasus III TOF MS, as previously described[31,32].

COMPBIO Analysis

While the core algorithms are proprietary, the basic workflow of the software is as follows. CompBio analyzes all available PubMed abstracts and full-text articles that reference any of the input entities and performs automated knowledge extraction using a feature known as contextual language processing. Based on a biological dictionary, not restricted to fixed pathway lists or ontologies, and conditional probability analysis, CompBio computes the enrichment of biological concepts (biologically relevant words or names collected from a multitude of sources) associated with the input entities. Related concepts are further aggregated into higher-level themes (e.g., biological pathways and processes, cell types and structures, etc.) based on their contextual relationships within the literature specifically associated with the input entities.

The scoring of entity, concept, and overall theme enrichment is based on a multicomponent function referred to as the enrichment score. This score reflects both the rarity of the concept event associated with the entity list, as well as its degree of overall enrichment as compared to appropriate randomized datasets. Enrichment scores are first computed for concept-entity relationships and then further aggregated for themes. These theme-level scores are also compared to appropriate randomized data to compute a Normalized Enrichment Score (NES) as well as the associated p-value. Themes with NES >= 1.2 and a p-value < 0.1 were considered for further analysis.

CompBio maps are generated in a fully automated fashion, and the annotation of the themes is semi-automated. Themes that clearly map, via the most central concepts and entities, to know pathways and processes in common knowledge databases such as GO, Reactome, and others have those annotations provided. However, as CompBio often identifies known biology not represented in any of these knowledge bases, the remaining themes are annotated through human assessment.

Statistical Analysis

All participants who provided both baseline and six-month data were included in the analyses. Pairwise within-group comparisons within each group and comparisons across groups with classical controls were performed using the Wilcoxon test and Mann–Whitney U test, respectively. Spearman's correlation was used to examine relationships in the IF and control groups among changes in phenotypic variables and changes in molecular markers.

All statistical tests were two-tailed, and significance was accepted at $p < 0.05$. Data are expressed as mean ± SD unless indicated otherwise. All analyses were performed using R version 4.1.1 (2021-08010) and GraphPad Prism 9 (version 9.2.0 (283)).

Results

Study participants

Totally, 66 participants were consented and assessed for eligibility; 50 were randomized, and 49 started the intervention (CONSORT flow diagram, **S Figure 1**), with 75% of IF participants (group A) and 91% of controls (group B) completing the six-month protocol (**Fig. 1**). After completing the six-month randomized study, 19 of the 21 participants in the intervention group who graduated from period 1 volunteered to continue the IF intervention for another six months (group C), while 20 of the 22 participants who had been randomized to the control group crossed over to IF for the second six months of the study (group D). Retention of study participants in the second period of the study was good, with 12-month dropout rates of 28.6% and 5% in groups C and D, respectively. As shown in **Figure 2A**, principal component analysis (PCA) shows a near complete overlap of all the measurements presented in **Table 1** between group A and group D that were therefore combined (IF-combined) in **Table 1** and **S Tables 2 and 3**.

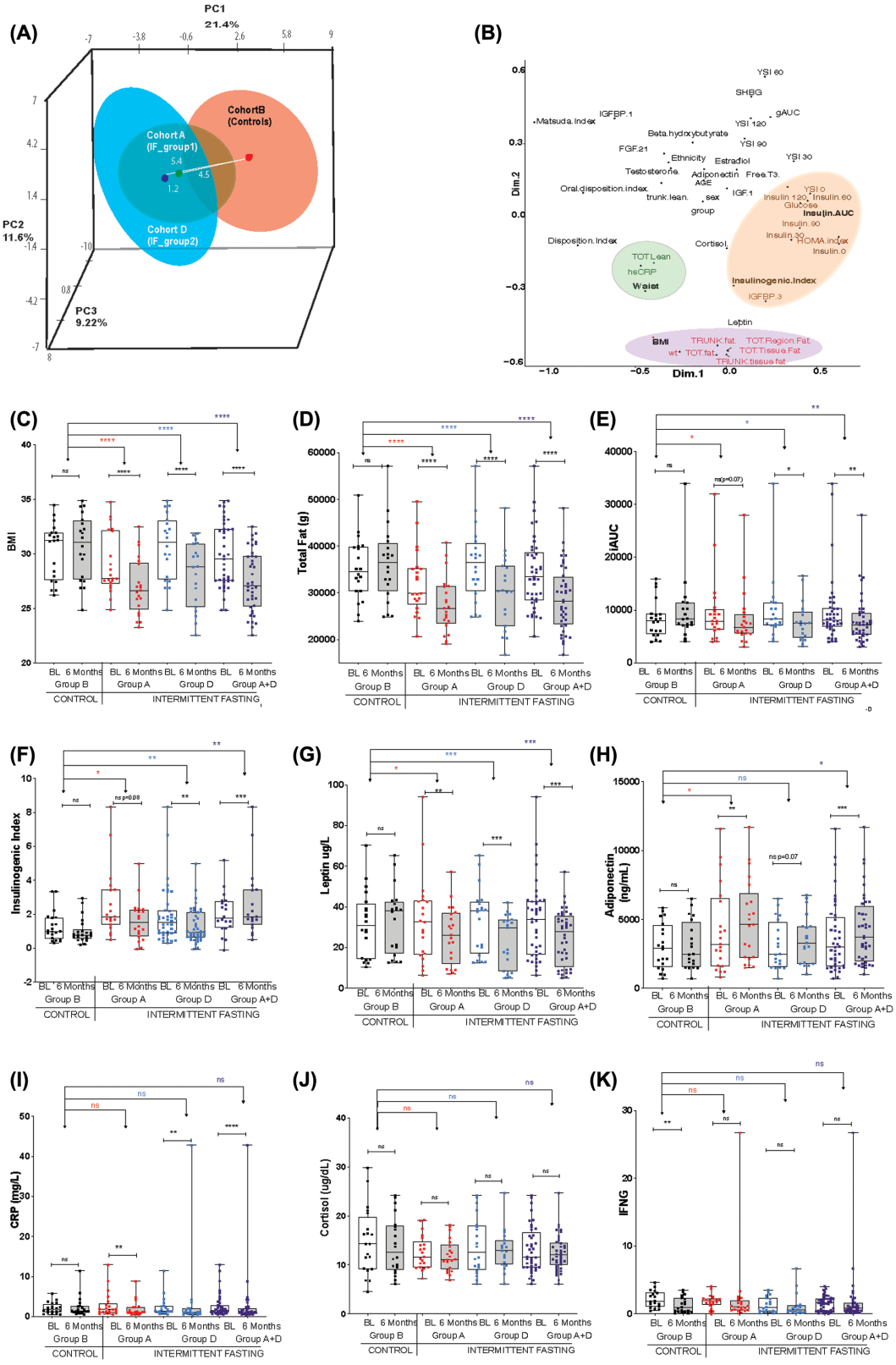

Figure 2. Effects of IF on body composition and metabolic parameters. (A) PCA plot based on six-month differences in body composition and metabolic parameters for the IF and control groups. The size of the colored spheres represents two standard deviations from the centroid of all subjects in the cohort (Group A, green; Group D, blue; Group B [control], red). **(B)** multidimensional scaling plot based on six-month differences in body composition and metabolic values for the combined IF cohorts (A + D). Green (waist-related), beige (insulin-related), and purple (BMI-related) ellipses encompass apparent clusters of parameters with similar patterns of variance. **(C–K)** Box plots of key study parameters. Intra-group comparisons computed between baseline and six months of IF or control diet (Wilcoxon signed-rank test, paired). Intergroup comparisons computed between IF and control diet cohort six-month differences (Mann–Whitney U test; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Table 1. Body composition and metabolic changes before and after intervention.

	IF (Group A) n = 21	p Value Between Groups	Controls (Group B) n = 20	p Value Between Groups	IF_combined (Group A + D) n = 41
Body composition and adipokines					
Weight (kg)					
Baseline	85.3±11.2		91.8±13.11		89.0±13.1
Final	79.4±11.5		92.9±14.0		82.4±12.2
Change	−5.9±3.6	**<0.0001**	1.1±3.2	**<0.0001**	−7.0±3.5
p value	**0.000**		0.171		**0.000**
BMI (kg/m^2)					
Baseline	29.0±2.8		30.3±2.5		29.8±2.9
Final	27.0±2.6		30.6±2.9		27.5±2.8
Change	−2.0±1.3	**<0.0001**	0.3±1.1	**<0.0001**	−2.3±1.2
p value	**0.000**		0.167		**0.000**
Waist (cm)					
Baseline	101.7±9.3		103.4±9.6		103.1±9.4
Final	93.3±9.7		104.5±9.4		95.2±9.9
Change	−8.4±7.8	**<0.0001**	1.1±6.9	**<0.0001**	−7.7±6.6
p value	**0.000**		0.178		**0.000**
Total fat mass (kg)					
Baseline	32.2±7.0		35.1±6.7		34.2±7.7
Final	27.5±5.9		36.2±8.1		28.8±7.0
Change	−4.7±3.2	**<0.0001**	1.1±3.2	**<0.0001**	−5.4±2.9
p value	**0.000**		0.295		**0.000**
Trunk fat mass (kg)					
Baseline	17.2±3.9		19.2±4.4		18.4±4.7
Final	14.4±3.7		19.8±5.3		15.2±4.2
Change	−2.8±1.8	**<0.0001**	0.6±2.0	**<0.0001**	−3.3±1.9
p value	**0.000**		0.294		**0.000**
Total lean mass (kg)					
Baseline	50.0±10.1		52.8±10.3		51.5±10.2
Final	49.0±10.5		53.0±10.3		50.7±10.1
Change	−1.0±1.1	**0.001**	0.2±1.1	**0.000**	−1.2±1.3
p value	**0.000**		0.498		**0.000**
Adiponectin HMW (ng/mL)					
Baseline	4228±3096		3037±1668		3668±2564
Final	4868±2968		3080±1744		4243±2528
Change	641±895	**0.018**	43±797	**0.045**	528±928
p value	**0.003**		0.433		**0.001**
Leptin (μg/L)					
Baseline	33.3±21.1		31.6±16.2		33.3±18.6±
Final	26.2±13.9		33.2±16.2		24.9±13.3
Change	−7.1±11.7	**0.011**	1.6±9.3	**0.000**	−8.4±9.9
p value	**0.003**		0.648		**0.000**
Inflammatory markers					
hsCRP (mg/L)					
Baseline	2.9±3.1		2.1±1.4		2.6±2.8
Final	1.9±2.0		2.4±2.5		2.7±6.7
Change	−1.0±2.3	0.297	0.3±2.2	0.221	0.1±6.7
p value	**0.003**		0.622		0.981

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Table 1. Continued.

	IF (Group A) n = 21	p Value Between Groups	Controls (Group B) n = 20	p Value Between Groups	IF_combined (Group A + D) n = 41
IL-6 (pg/mL)					
Baseline	0.66±0.65		0.50±0.37		0.51±0.51
Final	0.43±0.33		0.36±0.27		0.35±0.28
Change	−0.24±0.51	0.518	−0.14±0.29	0.556	−0.17±0.38
p value	**0.030**		0.107		**0.001**
TNF-α (pg/mL)					
Baseline	2.31±1.31		1.86±1.07		2.28±1.56
Final	2.99±3.03		2.25±1.85		3.98±2.86
Change	0.68±3.47	0.918	0.27±1.98	0.087	1.85±3.34
p value	0.539		0.890		**0.002**
IL-1β (pg/mL)					
Baseline	0.05±0.12		0.01±0.01		0.04±0.10
Final	0.02±0.02		0.02±0.01		0.02±0.02
Change	−0.03±0.13	0.838	0.01±0.01	0.576	−0.02±0.10
p value	0.812		0.666		0.529
IL-17 (pg/mL)					
Baseline	0.46±0.30		0.41±0.30		0.41±0.31
Final	0.35±0.23		0.35±0.32		0.47±0.74
Change	−0.11±0.26	0.314	−0.04±0.26	0.588	0.07±0.69
p value	0.080		0.554		0.658
MCP-1 (pg/mL)					
Baseline	303.9±83.8		322.8±93.6		296.5±87.1
Final	281.4±77.0		288.7±92.0		290.7±85.5
Change	−22.5±53.7	0.449	−34.1±72.4	0.085	−7.49±49.01
p value	0.070		**0.013**		0.519
IFN-γ (pg/mL)					
Baseline	1.8±1.0		2.1±1.2		1.6±1.1
Final	2.4±5.6		1.3±1.1		1.9±4.3
Change	0.7±5.7	0.682	−0.7±0.8	0.380	0.3±4.3
p value	0.133		**0.001**		0.057
IL-10 (pg/mL)					
Baseline	0.18±0.10		0.15±0.07		0.16±0.09
Final	0.17±0.07		0.14±0.08		0.19±0.17
Change	−0.01±0.11	0.818	−0.01±0.08	0.091	0.03±0.16
p value	0.948		0.409		0.063
Glucose metabolism and Insulin sensitivity markers					
Fasting glucose (mg/dL)					
Baseline	95.5±10.4		91.1±11.1		93.4±9.5
Final	93.8±11.0		91.2±8.0		91.8±10.4
Change	−1.8±9.5	0.321	0.1±10.0	0.293	−1.7±9.0
p value	0.258		0.970		0.172
Glucose AUC ($\times 10^3$ mg · min/dL)					
Baseline	17.8±4.3		14.5±2.7		16.5±3.9
Final	17.1±2.8		15.1±2.6		15.9±2.8
Change	−0.74±0.3	0.109	0.54±1.9	0.080	−0.50±2.8
p value	0.288		0.225		0.227

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Table 1. Continued.

	IF (Group A) n = 21	p Value Between Groups	Controls (Group B) n = 20	p Value Between Groups	IF_combined (Group A + D) n = 41
Fasting insulin (μU/mL)					
Baseline	12.6±6.4		11.3±6.1		12.8±8.8
Final	11.7±9.9		13.1±10.9		11.2±8.6
Change	−0.9±9.8	0.092	1.8±8.0	0.054	−1.9±8.2
p value	0.060		0.837		**0.011**
Insulin AUC ($\times 10^3$ μU · min/mL)					
Baseline	9.7±6.5		8.3±3.6		9.8±6.4
Final	8.3±5.4		9.9±6.4		8.1±4.7
Change	−1.4±3.3	**0.015**	1.5±4.7	**0.005**	−1.8±4.0
p value	0.070		0.067		**0.007**
HOMA index					
Baseline	3.1±1.7		2.7±1.5		3.1±2.3
Final	3.0±3.2		3.2±2.8		2.7±2.6
Change	−0.1±3.2	0.100	0.5±2.1	**0.045**	−0.5±2.6
p value	**0.046**		0.841		**0.007**
Matsuda index					
Baseline	3.5±2.2		4.1±1.7		3.8±2.3
Final	4.0±1.8		4.1±2.5		4.8±4.2
Change	0.5±1.6	0.081	0.0±1.6	0.077	1.1±4.1
p value	0.137		0.465		0.058
Insulinogenic index					
Baseline	1.3±0.9		2.0±1.2		1.9±1.6
Final	1.0±0.7		2.6±2.0		1.3±1.0
Change	−0.2±0.8	**0.021**	0.6±1.7	**0.002**	−0.6±1.4
p value	0.089		0.134		**0.001**
Disposition index (ISI × IGI)					
Baseline	4.5±5.8		7.8±5.6		6.7±6.7
Final	3.9±3.1		9.1±6.8		6.3±8.2
Change	−0.6±6.1	0.440	1.2±4.5	0.193	−0.6±6.2
p value	0.838		0.312		0.735
Oral disposition index					
Baseline	0.13±0.15		0.22±0.18		0.20±0.20
Final	0.12±0.09		0.28±0.21		0.36±1.35
Change	−0.02±0.16	0.323	0.06±0.18	0.096	0.17±1.3
p value	0.958		0.182		0.264

Body composition and metabolic changes before and after six months of intermittent fasting (IF) for groups A (IF intervention group), B (control group), and A + D (intervention group and the controls that started IF as a delayed intervention). Values are expressed as mean ± SD. Bold values represent significantly different p values. Pairwise comparison within groups using nonparametric Wilcoxon test. Pairwise comparisons across groups were performed using the Wilcoxon test and Mann–Whitney U test.

IF markedly reduces body weight and adiposity

At baseline, most participants were overweight, with a mean BMI of 29.6 ± 2.7 kg/m^2 and body fat of 42.9 ± 3.3% in women and 31.6 ± 4.1% in men. After six months, weight losses were −5.9 ± 3.6 kg in group A, −7.0 ± 3.5 kg in the IF-combined group A + D, and +1.1 ± 3.2 kg in group B (Table 1), which corresponded to reductions of −7%, −8%, and +1% of baseline body weights, respectively. As shown in Table 1 and Figure 2C, BMI reduction after six months were 2.1 ± 1.3, 2.6 ± 1.0, and 2.3 ± 1.2 kg/m^2 in groups A, D, and A + D, respectively ($p < 0.0001$), with no change in the control group B. As with the decreases in weight, BMI, and waist circumference, reductions in whole-body, trunk fat (Fig. 2D), and LM, as assessed by DXA, were significantly greater in the groups A, D, and A + D than in the control group B ($p < 0.0001$). FM comprised 79.6% and 77.1% of total weight loss in groups A and A + D, respectively. Our data demonstrate a marked improvement in weight and body composition parameters with IF. As shown in **S Tables 2 and 3**, self-reported energy

intake estimated from the food diaries decreased significantly ($p < 0.0001$) in all the IF groups. Averaged over the six months, the IF groups achieved 22.8% CR on a weekly basis.

IF reduces serum leptin and increases adiponectin

To investigate the hormonal effects caused by IF-induced reductions of total and trunk fat, serum levels of leptin and HMW adiponectin, two key adipocyte-secreted hormones[33], were measured. As expected, serum levels of leptin, a metabolic marker of the amount of energy stored in the adipose tissue, markedly decreased in all the IF groups ($p < 0.01$), with no changes in the control group B (**Table 1**, **Fig. 2G**). IF-induced changes in DXA total body and trunk fat strongly correlated with changes in serum leptin concentrations ($R = 0.60$; 0.48 $p = 3.98E{-}27$; $1.15E{-}21$, as shown in **S Table 5**). In contrast, serum levels of HMW adiponectin significantly increased with IF in groups A and A + D ($0.045 \geq p \geq 0.018$), with no changes in the control group B (**Table 1**, **Fig. 2H**). Several studies have reported an association between HMW adiponectin, the biologically active form of adiponectin, and improved insulin sensitivity[34]; our findings showed a modest correlation with insulin 60 ($r = -0.32$; $p < 0.0001$) but not with any other OGTT-derived glucose or insulin parameter or index.

IF-induced weight loss does not reduce circulating inflammatory markers

Previous trials have demonstrated that moderate weight loss (similar to the degree achieved in this study) induced by daily chronic CR with adequate nutrition can markedly reduce circulating markers of inflammation in both obese and nonobese men and women[4,35,36]. To investigate the effects of IF on these markers, we measured changes in serum concentration not only of the acute phase response reactant high-sensitivity C-reactive protein (hsCRP) but also of a panel of circulating cytokines and chemokines assessed by multiplex immunoassay technology. As shown in **Table 1** and **Figure 2I**, hsCRP (primary outcome) did not change with IF. Interferon-gamma levels were also unaffected by IF (**Fig. 2K**). None of the other cytokines or chemokines circulating levels demonstrated a significant difference among groups, although, compared to the control group, both serum TNFα ($p = 0.087$) and IL-10 ($p = 0.091$) concentrations trended toward an increase in the IF-combined A + D group (**Table 1**). Previous trials have shown that chronic daily CR significantly increases circulating cortisol levels[37,38]. However, in this trial of chronic IF, serum cortisol levels were not significantly different within and among study groups (**Fig. 2J**).

IF-induced weight loss improves some indicators of insulin sensitivity

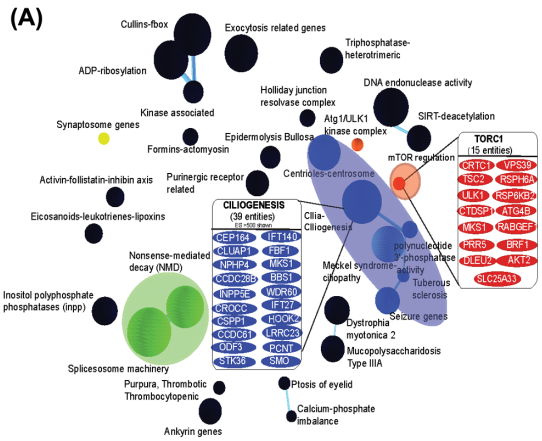
Insulin resistance and compensatory hyperinsulinemia, mainly driven by excessive central adiposity, precede by many years the pathological increase in blood glucose and are of particular concern because they are major drivers of the accumulation of cellular damage leading to cardiovascular disease, cancer, and accelerated ageing[2,39,40]. To investigate the effects of IF on glucose and insulin metabolism, we performed a 2-hour OGTT at baseline, 6 and 12 months. Glucose metabolism (assessed as fasting glucose, glucose area under the curve, oral disposition index, and Matsuda index) was not different between groups (**Table 1**). In contrast, insulin action and sensitivity, assessed as HOMA-IR, insulin area under the curve (AUCi), and insulinogenic index during the OGTT procedure, were significantly improved by IF with no change in the control group (**Table 1**, **Fig. 2E,F**). However, it is important to note that although the overall change difference in AUCi was statistically significant, the actual treatment effects were small and possibly clinically irrelevant, with only plasma insulin concentration at 30 and 90 min being statistically different from the control group (**S Figure 2**).

Multi-omic changes in response to IF

IF in rodents increases healthspan and lifespan by activating multiple transcriptional factors that activate autophagy, mitophagy, and tissue repair capacity and reduce oxidative stress and inflammatory pathways[2,41]. To investigate the molecular impact of IF in humans, we carried out comprehensive gene expression profiling (mRNA sequencing) of colorectal mucosa biopsies of a subset of 27 participants, 14 participants who underwent six-month IF and 13 controls eating a usual Western diet. Rapidly renewing tissues such as colon mucosa show greater molecular changes in response to diet, affecting genomic stability, DNA repair, and senescence mechanisms[42], which in turn can modulate colon cancer risk, the third most commonly diagnosed cancer and the second most deadly cancer in the Western world[43,44]. In parallel, in-depth plasma metabolomics (untargeted) profiling was also performed on blood samples (**S Table 13**). Then, IF-induced changes in multi-omic measures (six-month to baseline ratios) were independently correlated, utilizing Spearman's rank correlation method, with changes in six-month to baseline ratios, in key body composition and metabolic measures (BMI, Waist, iAUC, and insulinogenic index) selected based on multidimensional scaling analysis (**Fig. 2B**). Entities (genes, proteins, and metabolites) whose six-month changes were strongly correlated ($|R| >= 0.45$, $p < 1.66E{-}11$) with four parameters (BMI, Waist, Insulin AUC, and Insulin index) were subjected to a comprehensive assessment of biological pathways and processes utilizing the software platform CompBio V2.0 (PercayAI, Inc., St. Louis, MO, PubMed version June 2021). Entities positively and negatively correlated with the four parameters were analyzed separately, and CompBio project maps were generated for each. A complete list of the identified biological themes and associated genes is provided in the supplementary material (**S Tables 9–12**).

IF modulates multiple mTOR-related pathways

Given that IF-induced negative energy balance resulted in significant weight loss and body composition changes, we investigated whether IF-induced BMI reduction correlates with specific molecular adaptations. The resulting annotated biological maps for positively and negatively correlated molecular entities with BMI are given in **Figure 3A** and **3B**, respectively. The observed biological themes cover a range of diverse but interrelated pathways and processes. Key themes positively correlated with BMI included *mTOR* regulation, *ATG1/ULK* kinase complex, and cilia-ciliogenesis, with an additional small cluster of nonsense-mediated decay and spliceosome machinery themes. The *mTOR* theme includes well-known regulators of this key pathway associated with aging and disease[45] including effectors and responders such as *TSC2*, *ULK1*, *BPS39*, and *ATG4B*. The cilia-ciliogenesis supercluster was one of the strongest components of the complete map, with at least five related themes: Cilia-ciliogenesis, Centrioles-centrosome, Seizure genes, and Ciliopathies, such as Meckel syndrome and tuberous sclerosis. Several key members (ES > 300) involved in ciliogenesis are highlighted in



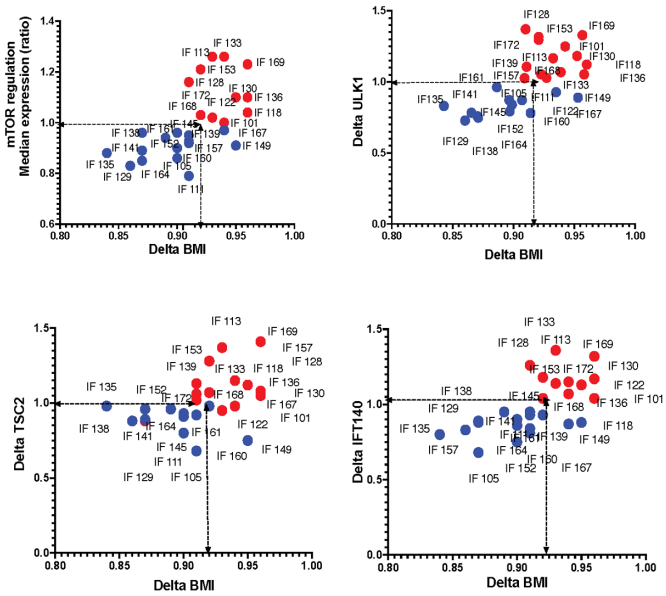
(C)

	THEME	BMI (NESCORE)
CILIA	Cilia-Ciliogenesis	3.30
	Centrioles-cilia	2.84
	Seizure genes	1.17
	Meckel syndrome-ciliopathies	1.78
SIGNALING	mTOR pathway regulation	1.25
	ATG1-ULK1 kinase complex	1.24
	GRB2-Shc-Sos	2.52
INFLAMMATION-IMMUNE	MyD88-TLR4 cascase	2.81
	Eicosanoids-leukotrienes	1.21
VESICLE-TRANSPORT	Clathrin coat -trans-Golgi network vesicle	3.36
	Autophagosome assembly	2.26
	COPI dependent Golgi-ER transport	2.68
GLUCOSE_LIPID	Defective SLC2A1 causes GLUT1DS1	2.67
	Fructose-bisphosphate aldolase activity	2.68
	Defective PGM1 causes PGM1-CDG	2.64
DNA_RNA	Spliceosome machinery	2.17
	Holliday junction resolvase complex	1.19
	NHEJ	2.88
UPR	CCT complex	2.6
	PERK mediated UPR response	2.49

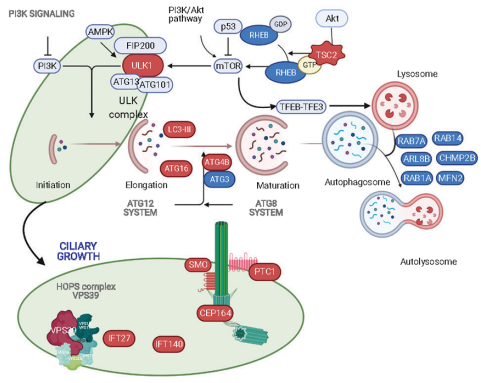
(D)

	TSC2	RPS6KB2	VPS39	ULK1	ATG4B	RHEB	CHMP2B	RALB	RAB7A	ARL8B
Weight	0.52	0.46	0.56	0.68	0.45	-0.56	-0.65	-0.47	-0.57	-0.52
BMI	0.52	0.46	0.56	0.68	0.45	-0.56	-0.65	-0.47	-0.57	-0.52
Waist	0.19	0.19	0.35	0.29	0.2	-0.07	-0.36	-0.37	-0.35	-0.14
Total Fat	0.28	0.19	0.48	0.49	0.17	-0.32	-0.21	-0.14	-0.22	-0.25
Glucose	-0.29	-0.23	-0.12	-0.15	-0.2	0.29	0.14	0.05	0.19	0.02
gAUC	-0.1	0.06	-0.14	-0.08	-0.08	0.31	0.25	0.12	0.12	0.16
Insulin AUC	0.06	-0.08	0.08	0.15	0.02	0.19	0.12	-0.08	0.13	0.21
Insulinogenic Index 30	0.1	0.05	-0.01	0.13	0.04	-0.1	-0.1	-0.26	-0.09	-0.07

(E)



(F)



(G)

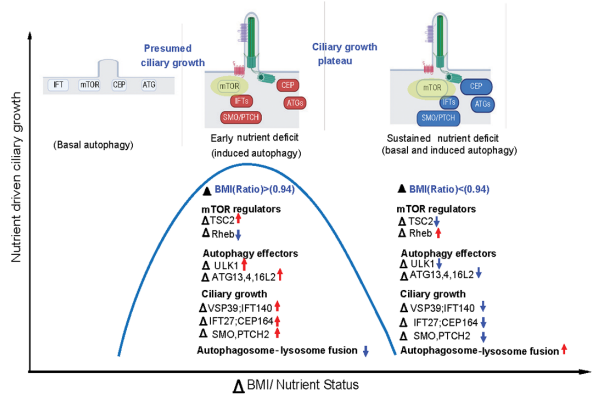


Figure 3. Multi-omics analysis of IF-induced metabolic and molecular adaptations associated with BMI changes. (A,B) CompBio biological process maps generated from entities positively and negatively correlated, respectively, with delta BMI (Spearman $|r| > 0.45$; $p < 1.67E{-}11$). Biological themes generated from correlated entities are shown as circles. The size reflects the raw enrichment score for the theme, while the thickness of the blue connecting lines indicates shared entities between them. In panel (A), themes associated with mTOR and autophagy regulation (red), cilia-ciliogenesis (blue), and RNA processing (green) are highlighted. In panel (B), themes enriched for autophagosome formation (red), vesicular transport (blue), and glucose metabolism (yellow) are highlighted. **(C)** Table of NESs for key biological themes and clusters from CompBio analyses (red and blue indicate positive and negative correlations, respectively). **(D)** Individual correlations of representative mTOR/autophagy/cilia genes with key body composition and metabolic parameters (Spearman r value). **(E)** mTOR/autophagy/cilia gene expression ratios (six-month IF/baseline) relative to BMI ratios

(legend continued on next page)

(six-month IF/baseline). Thirteen-gene mTOR theme median ratio profile (upper left), ULK1—autophagy regulator (upper right), TSC2—mTOR regulator (lower left), and IFT140—ciliogenesis (lower right) are provided. Red and blue dots indicate patients separated by a median mTOR ratio of 1. Subgroup 1 (red dots) also indicates a lower BMI reduction (ratio > 0.92, mean = 0.94), while subgroup 2 demonstrated a larger BMI reduction (ratio < 0.92, mean = 0.89). Note that for gene expression, values >1.0 indicated up-regulation over baseline, and values <1.0 indicate down-regulation from baseline, indicating regulation in opposite directions based on BMI reduction. **(F)** Pathway map illustrating the relationship between mTOR, autophagy, and ciliogenesis. Genes positively correlated with BMI in this study are shown in red, while genes negatively correlated with BMI are shown in blue. Genes not observed to be correlated with BMI are in light gray. **(G)** Hypothetical visual representation of the nutrient-driven effects of IF on mTOR/autophagy/ciliogenesis regulation based on the magnitude of BMI reduction. The level of BMI reduction in subgroup 1 is associated with a gene expression signature that indicates inhibition of mTOR and ciliary growth. Within subgroup 2, however, it appears that ciliary growth has plateaued, leading to a reversal of gene expression changes.

blue (Fig. 3A) including *CEP164*, *IFT140*, *MKS1*, *CLUAP1*, and *SMO*. The theme map for negative correlation with BMI identified a large cluster associated with Golgi-ER trafficking and vesicular transport, and a smaller cluster with GLUT1-sugar metabolism-related themes and autophagosome assembly themes, including the *RAB7A*, *RAB8A*, *RHEB*, *CHMP2B*, *ARL8B*, and *ATG3* genes. Figure 3C provides a summary of related themes with their higher-order biological associations for the BMI in conjunction with their CompBio NESs. A complete table is provided in **S Figure 3**. As shown in Figure 3D similar correlations were observed between weight and BMI and *mTOR*/autophagy-lysosome and cilia-related gene changes (ranging from 0.45 to 0.68 and −0.47 to −0.65, respectively); weaker but significant correlations were also noted with waist circumference and DXA total fat changes but not with insulin sensitivity markers.

Excessive weight loss is associated with a reversal of mTOR-related autophagy and ciliary growth pathway regulation

Autophagy is a crucial homeostatic mechanism, conserved across diverse eukaryotic species, that plays an important role against the age-dependent accumulation of misfolded proteins and dysfunctional organelles and mitochondria. It is commonly believed that fasting induces a dose-dependent autophagic response mainly driven by *FOXO* activation and *mTOR* inhibition. However, our preliminary findings show that excessive weight loss (BMI reduction in excess of 2.5 units) is accompanied by a reversal of the *mTOR* inhibition and autophagy activation observed with moderate CR (Fig. 3E). As shown in Figure 3E, not only the median expression of the *mTOR*-related gene group (top 14 genes) but also each of the individual *mTOR* and autophagy-related genes underwent a similar dose-dependent inverse regulation pattern. When splitting the IF group into two subgroups based on a median *mTOR* gene group differential expression of greater or less than 1.0, the difference in BMI reduction was highly significant (Group 1, *mTOR* group differential expression > 1.0, mean BMI ratio = 0.94; Group 2 BMI *mTOR* group differential expression < 1.0, mean BMI ratio = 0.89, p = 3.68E−04 U test; **S Table 4**). A similar BMI-driven inverse relationship pattern was observed for ciliary growth-related genes (Fig. 3G, **S Table 6**), suggesting the presence of a common regulatory mechanism of these three biological processes (Fig. 3F). Our preliminary molecular findings support the hypothesis of a nutrient-sensing-driven autophagy-cilia axis[46–48] that regulates cellular integrity and organ function by transducing extracellular stimuli inside the cell (Fig. 3G). Mild to modest dietary restriction triggers intestinal primary cilia growth via modulation of *mTOR*/Autophagy/Cilia gene expression pathways. However, excessive and prolonged nutrient deprivation inhibits ciliogenesis by downregulating the autophagy/ciliary molecular machinery once further growth of the primary cilia is no longer warranted by environmental inputs. To further determine if any other dietary parameter could explain the IF-induced BMI-related subgrouping, correlation data were carefully examined for other factors, including number of fasting days (three vs. two days of fasting per week), grams of food consumed, and calorie intake (**S Table 5**). While none of the parameters tested demonstrated a significant difference across the subgroups, calorie intake did trend lower in the IF subgroup with greater BMI reduction (Group 1 six-month IF mean kCal = 1800.98; Group 2 mean Kcal = 1488.7; p = 0.09).

Discussion

Our study was designed to measure the effects of IF-induced weight loss on inflammatory, metabolic, and molecular pathways of healthy aging. This study showed that, unlike with chronic daily CR, a similar 8% weight loss induced by IF did not reduce C-reactive protein or any other circulating inflammatory cytokine. We also found that IF caused a statistically significant but very small improvement in some insulin sensitivity indexes. This is consistent with the results of recent randomized trials of alternate-day fasting showing no improvements in either markers of inflammation or insulin sensitivity (as assessed with the OGTT or the hyperinsulinemic euglycemic clamp)[12,49], reinforcing the emerging concept that from a metabolic point of view, a calorie is not a calorie[50,51]. Finally, our preliminary molecular findings suggest that strong correlations exist between the degree of IF-induced weight loss and multiple key molecular aging pathways, with a seemingly paradoxical impact of excessive weight loss on mTOR-related autophagic and ciliary growth pathways.

Elevated markers of chronic inflammation are a hallmark of aging (inflammaging) and are widely recognized to influence the development of multiple age-associated diseases[7]. Several clinical studies have shown that weight loss induced by daily CR is associated with marked and sustained reductions in inflammatory biomarkers and cytokines, even in young and middle-aged men and women free of major chronic diseases[4,35,36,52]. However, in this trial, the substantial reduction in body weight (on average 8% with an ~16% FM loss) and circulating leptin induced by IF did not result in a lowering of serum C-reactive protein, TNF-α, IL-6, or other pro-inflammatory cytokine and chemokines levels. This confirms the results of other short-term human trials of IF and suggests that, unlike in rodents[53,54], human beings undergoing intermittent cycles of 24-hour fasting (that result in significant weight and fat loss) do not experience the same beneficial metabolic adaptations. After all, a 24-hour fast-feed cycle in mice most likely equates to recurrent ~5-day fast-feed cycles in humans[2]. Indeed, because of their extremely high-energy metabolism, most strains of mice starve to death after only

48–60 hours of fasting. By contrast, even lean humans can undertake a fast of 57–73 days before death occurs[2].

Another major determinant of health and longevity is insulin sensitivity. Deletions of the insulin receptor specifically in adipose tissue (*FIRKO*), or insulin receptor substrate 1 *(IRS1*) in the whole body and insulin receptor substrate 2 (*IRS2*) selectively in the mouse brain have been shown to slow aging and prolong lifespan in mice[2,55–57]. Improved insulin sensitivity is a widely conserved response to chronic daily CR in model organisms, rodents, monkeys, and humans and has been proposed as a key health and longevity mechanism of CR[2,58]. IF also improves insulin sensitivity in mice[59], but the results of our and other randomized trials show negligible improvements in insulin sensitivity biomarkers when more sophisticated measures that fasting glucose and insulin are employed[12,49]. A possible explanation for this discrepancy is that laboratory mice during the feast days eat nutritionally balanced chow diets. On the contrary, most people (including our study participants) practicing IF, during feast days usually consume their typical high-protein, ultra-processed Western obesogenic diets that have been shown to induce detrimental effects on metabolic and gut microbiome health[51]. Results from a weight loss trial of obese women showed that high-protein diets (1.3 g/kg/day) entirely prevented the usual improvement in insulin sensitivity observed in those consuming a normal protein diet (0.8 g/kg/day) who lost the same 10% body weight[50]. Consuming a high protein diet seems to offset the favorable effects of weight loss on insulin resistance and may drive cancer and accelerated aging by overstimulating the PI3K/AKT/mTOR pathway even in the face of significant reductions in visceral and hepatic fat[11,60].

Data from dietary, genetic, and pharmacological (rapamycin) animal studies have consistently shown that mTOR inhibition and autophagy activation are key molecular adaptation for lifespan extension[58]. It was previously believed that a dose-dependent response was linking CR to longevity, but recent studies have demonstrated that a nonlinear relationship exists between energy restriction and lifespan extension in rodents that is strain-specific[61]. The results of this trial support these findings and suggest that, even in humans, excessive weight loss induced by IF is associated with a reversal of mTOR inhibition and the observed components of autophagy activation, potentially counteracting the anti-aging effects of IF.

Major strengths of this study include the randomized controlled trial design minimizing the potential for selection bias, and the extensive metabolic and molecular phenotyping with multi-omic analysis. Our study had a high retention rate of enrolled participants and good adherence to the study interventions, as shown by the successful weight reduction over six months. The relatively small number of participants limits our ability to generalize some of the conclusions. However, this was an exploratory, technically challenging, and highly labor-intensive study. Despite this restriction, both IF groups underwent a number of body weight and composition changes that were large enough to be statistically and clinically significant despite the relatively small number of subjects.

In conclusion, the findings of this trial demonstrate that IF in overweight men and women results in significant body weight and fat loss but without clinically meaningful improvements in systemic inflammatory markers or glucose-insulin metabolism, reinforcing the concept that weight reduction does not always translate into improved metabolic health. Multi-omic data analysis suggests that a nonlinear relationship exists between IF-induced weight loss and inhibition of multiple key nutrient-sensing aging pathways. More work is needed to understand the impact of different degrees of energy restriction on metabolic and molecular health in humans and how fasting should be complemented with diet quality changes to maximize clinical outcomes.

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Declaration of Interests

R.D.H. and R.A.B. may receive royalty income based on the CompBIo method developed by them. and licensed by Washington University to PercayAI. All the other authors have no financial conflicts of interest to disclose.

Data Availability

RNA-seq data from the colon mucosa biopsies are deposited at NCBI-GEO. GSE196335. Metabolomics data are made available in **S Table 13**.

Code Availability

CompBio pathway analysis tool is a commercial software available from PercayAI (https://www.percayai.com).

Supplementary Materials

Supplemental information can be found online at https://doi.org/10.59368/agingbio.20230013.

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