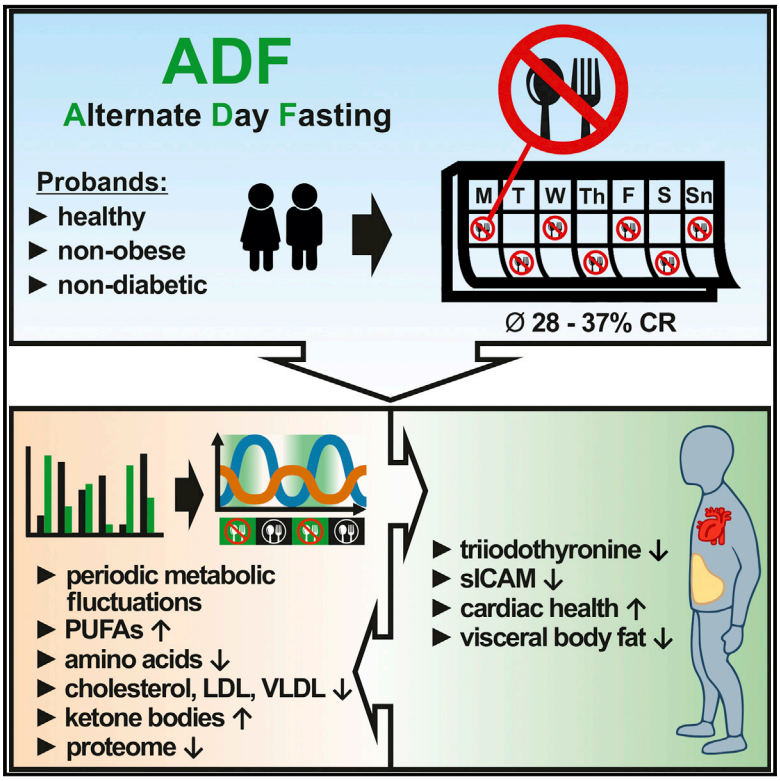
Clinical and Translational Report

Cell Metabolism

# Alternate Day Fasting Improves Physiological and Molecular Markers of Aging in Healthy, Non-obese Humans

## Graphical Abstract




## Highlights

- For healthy, non-obese adults, ADF is safe to practice for several months
- 4-week ADF decreases the body weight by 4.5% and improves the fat-to-lean ratio
- Cardiovascular parameters and the CVD risk are improved upon ADF
- ADF reduces T3 and periodically depletes amino acids, while increasing PUFAs

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## In Brief

Stekovic et al. show in the clinic that alternate day fasting (ADF) is a simple alternative to calorie restriction and provokes similar improvements on cardiovascular parameters and body composition. ADF was shown to be safe and beneficial in healthy, non-obese humans, not impairing immune function or bone health.

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# Alternate Day Fasting Improves Physiological and Molecular Markers of Aging in Healthy, Non-obese Humans

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## SUMMARY

Caloric restriction and intermittent fasting are known to prolong life- and healthspan in model organisms, while their effects on humans are less well studied. In a randomized controlled trial study (ClinicalTrials.gov identifier: NCT02673515), we show that 4 weeks of strict alternate day fasting (ADF) improved markers of general health in healthy, middle-aged humans while causing a 37% calorie reduction on average. No adverse effects occurred even after >6 months. ADF improved cardiovascular markers, reduced fat mass (particularly the trunk fat), improving the fat-to-lean ratio, and increased β-hydroxybutyrate, even on non-fasting days. On fasting days, the pro-aging amino-acid methionine, among others, was periodically depleted, while polyunsaturated fatty acids were elevated. We found reduced levels sICAM-1 (an age-associated inflammatory marker), low-density lipoprotein, and the metabolic regulator triiodothyronine after long-term ADF. These results shed light on the physiological impact of ADF and supports its safety. ADF could eventually become a clinically relevant intervention.

## INTRODUCTION

While chronically increased caloric intake (and in particular one that is carbohydrate-centered) has negative effects on human

### Context and Significance

Of all the anti-aging and longevity-promoting interventions tested so far, calorie restriction (CR) seems to be the most conserved among species and robust. However, humans struggle to constantly achieve a certain level of CR. Here, we show in a clinical trial that a related intervention, alternate day fasting (ADF), also leads to striking reduction in overall calorie intake over the course of the study but is more easily tolerated than continuous CR and provokes similar beneficial changes on the cardiovascular system and on body composition while being safe for a period of >6 months. We also found positive alterations in cardiovascular disease risk factors and in fat mass after only 4 weeks of ADF. In the future, this practice, which is already growing in use as a lifestyle intervention, could eventually accommodate modern healthcare in various settings.

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health (Kroemer et al., 2018), caloric restriction (CR) is known to extend healthspan and lifespan in model organisms, including rhesus monkeys (Anton and Leeuwenburgh, 2013; Mattison et al., 2017). The mechanisms of these longevity effects have not been fully elucidated yet. However, several clinical trials have been launched to translate the positive aspects of CR and fasting to humans. Thus, a recent study showed that CR reduced risk factors for cardiovascular diseases (CVD) and improved overall fitness and cardiac function parameters (i.e., blood pressure, heart rate, and energy expenditure) (Redman et al., 2018). Conversely, continuous CR has also been associated with depleted circulating leukocytes, immunosuppression, and reduced bone density (Devlin et al., 2010; Ritz et al., 2008; Tang et al., 2016; Villareal et al., 2016).

Among other CR alternatives, such as pharmacological mimicry of CR effects (Madeo et al., 2019) and periodic nutrient deprivation (Di Francesco et al., 2018), intermittent fasting (IF) could represent an easily manageable alternative to constant CR. Pre-clinical studies have shown that these dietary interventions reduce oxidative stress and metabolic rate, activate autophagy (which are biomarkers of CR-mediated lifespan extension), and expand healthspan and lifespan in various model organisms (Joslin et al., 2017; Madeo et al., 2015; Rubinsztein et al., 2011). However, there is still debate about the safety and efficiency of CR and IF, particularly on healthy humans. Among various forms of IF, alternate day fasting (ADF), defined as strict 36-h periods without caloric intake ("fast days") followed by 12-h intervals with ad libitum food consumption ("feast days"), seems to be one of the most extreme dietary interventions (Antoni et al., 2018; Tinsley and La Bounty, 2015). In principle, a less stringent protocol of ADF allowing 25% calorie intake on fast days has been reported to be safe in obese patients (i.e., BMI $\geq$ 30) for at least 8 weeks, with only minimal adverse incidences (Hoddy et al., 2015). Other studies have conducted various lengths and forms of ADF in different cohorts, with little to no obvious adverse effects, though substantial hunger was reported by (Heilbronn et al., 2005) on fast days, which did not decrease throughout the 3 week trial.

At the same time, some studies have indeed shown adverse effects of recurring fasting periods on different outcomes. For instance, extended overnight fasting periods might increase the risk of gallstone disease (Sichieri et al., 1991). Additionally, although discussed controversially, skipping breakfast is associated with an elevated risk of coronary heart disease, type 2 diabetes and other adverse factors in some cohorts (Cahill et al., 2013; Ballon et al., 2019; Sievert et al., 2019). Inline, accumulating data indicate that not only are fasting periods per se important for maintenance and improvement of metabolic health but also the timing of meals matters, favoring early intake of calories (i.e., breakfast) rather than late (i.e., dinner) (Di Francesco et al., 2018).

Though many studies have addressed the application of IF on overweight participants with or without type 2 diabetes (Tinsley and La Bounty, 2015), mechanistic trials on healthy humans have been elusive. To fill this gap, we designed the present study to examine the effects of strict ADF on cardiovascular parameters, such as heart rate, blood pressure, cholesterol levels, CVD risk, body composition, and the metabolome and proteome of healthy, non-overweight adults, thereby assessing both the effectiveness and safety of such intervention. This is the first study, to the best of our knowledge, that comprehensively reports the effects of short- and long-term strict ADF on the physiology, cardiovascular system, and body composition in a non-obese cohort. Moreover, the comparison of a long-term ADF cohort with matched controls from the general healthy population is unique. We also examined the potential influence of ADF on the immune system, bone mineral density (BMD), and bone mass in both study arms.

## RESULTS AND DISCUSSION

### Study Overview and Probands

We performed a cross-sectional analysis of a long-term ADF cohort of healthy adults (who had been performing ADF for more than 6 months on their own, prior to the enrollment in the study) and a healthy control group (none of them had any history performing ADF) (Figure 1A). The duration of ADF performance of the participants in the long-term cohort before the enrollment was in median 19 months (IQR 7 to 44 months) (Figure S6).

After the completion of the cross-sectional study arm, all subjects of the control group were then enrolled in a randomized controlled trial (RCT), where they were randomized either to ADF for 4 weeks or to continue their current *ad libitum* eating

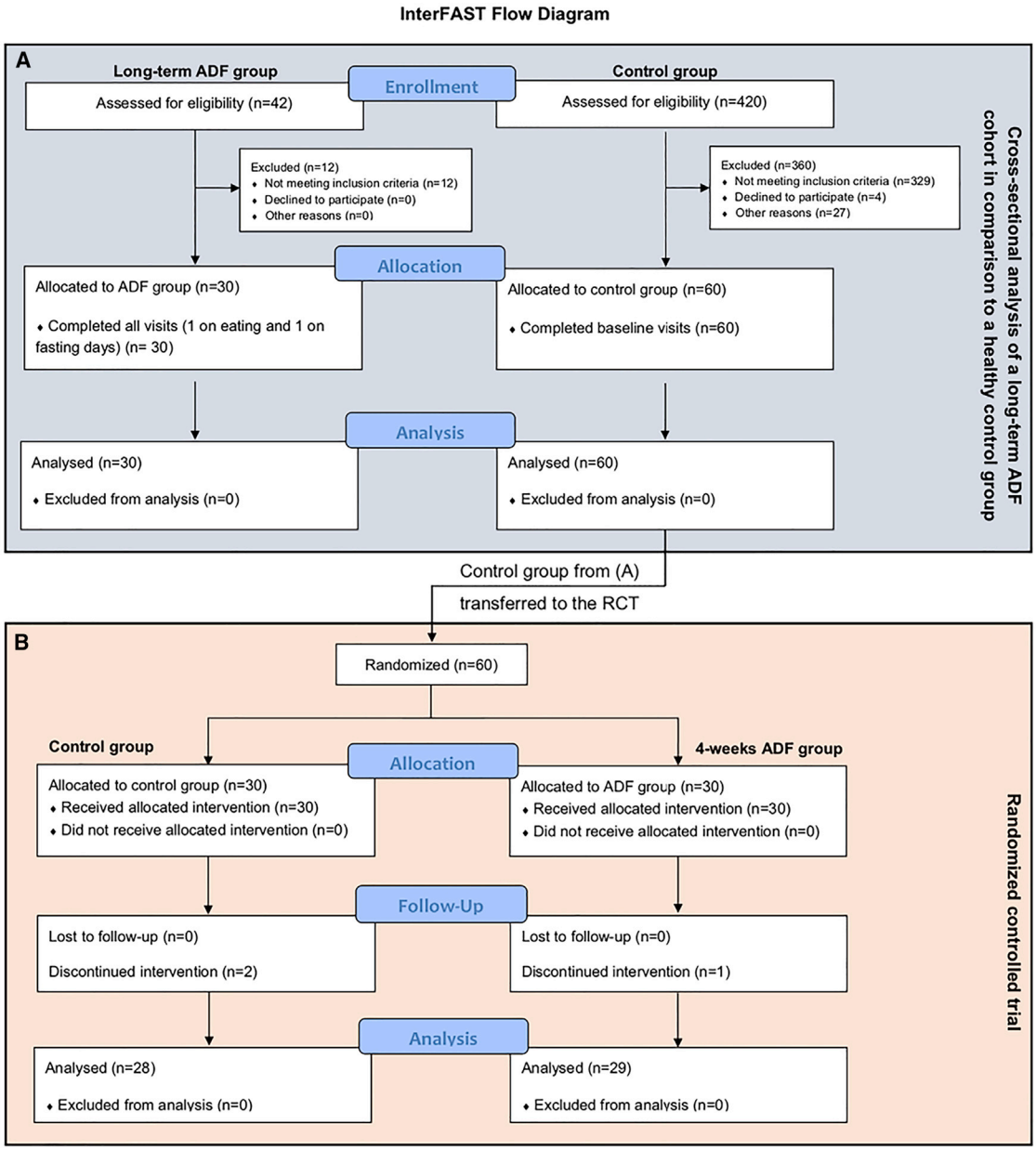


**Figure 1. CONSORT Diagram Indicating the Design of the Trial and Number of Participants in the Cohort Trial and RCT Arms of the InterFAST Study**

(A) Forty-two people who had been performing ADF on their own for more than 6 months prior to the study were screened for eligibility for the cross-sectional study. Twelve were excluded due to ineligibility. Thirty were analyzed and compared to 60 healthy, non-ADF performing controls who were enrolled after screening 420 adults for study eligibility based on previously set inclusion and exclusion criteria (see Supplemental Information). The main reasons for exclusion during the eligibility screening were BMI not within the defined frame (22.0 to 30.0 kg/m$^2$), people were smoking, or people were taking blood pressure-lowering medication or thyroid hormone substitution.

(B) After the baseline assessment, the same 60 control participants were further randomized to either control (continue current eating behaviors) or 4-week ADF intervention group in a 1:1 ratio. The randomization was conducted using an online tool ''Randomizer for Clinical Trials'' (https://www.randomizer.at/) and stratifying participants by sex. During these 4 weeks of intervention, 3 participants (ADF group, n = 1; control group, n = 2) discontinued study participation due to lack of motivation or dissatisfaction with the randomization result. Remaining 57 participants completed the intervention and were included in the present analysis.

behavior (Figure 1B). This means that the 60 participants that served as the control group for the long-term fasters subsequently entered a 4-week RCT. In both the >6 months and 4 weeks of ADF groups, participants were instructed to eat every second-day ad libitum, but to completely exclude solid and liquid foods and caloric beverages on the fast days.

The study has been registered in the clinicaltrials.gov database (NCT02673515) and conducted according to the principles

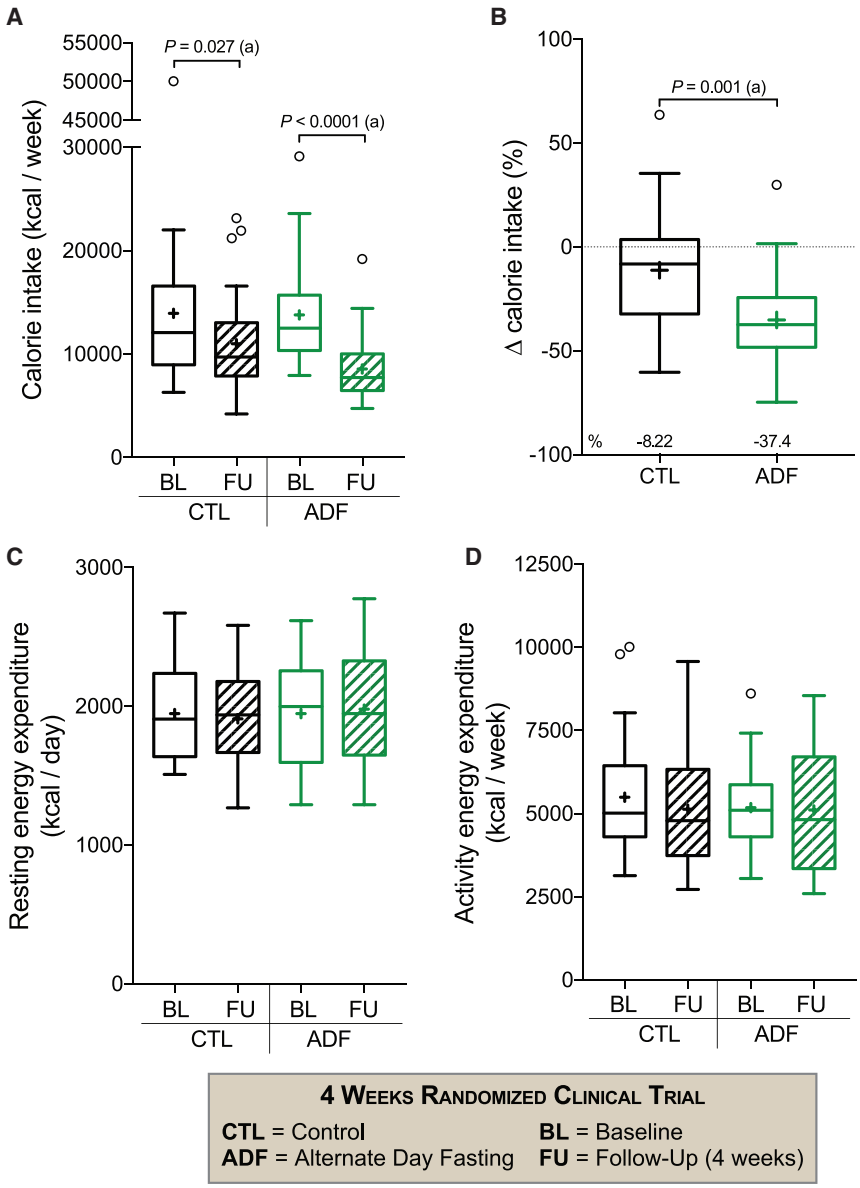


**Figure 2. Levels of Caloric Restriction, Activity, and Resting Energy Expenditure after 4 Weeks of ADF**

Calorie intake, measured by food frequency questionnaires, is reduced during the RCT (A and B). Resting energy expenditure on non-fasting days, as well as activity energy expenditure (measured during the last week of the trial) stay indifferent during the RCT (C and D). Data are visualized as Tukey box plots (line at median, x at mean). For parameters with non-gaussian distribution, a nonparametric Mann-Whitney test was used. For normally distributed data, a two-sided Student's t test was applied. When applicable, a paired Student's t test or a non-parametric Wilcoxon matched-pairs signed rank test was applied. a = non-gaussian data distribution. Percentage inlets depict median in-group differences to baseline after 4 weeks of intervention. Results were obtained from a randomized clinical trial (see STAR Methods).

of the International Declaration of Helsinki and good clinical practice (GCP). It was approved by the ethics committee of the Medical University of Graz (27–166 ex 14/15). Informed consents were collected from all participants of the study prior to enrollment. A full study protocol was published previously (Tripolt et al., 2018). Briefly, we measured various anthropometric, physiological, hormonal, metabolic, and biochemical parameters to estimate health status of included participants and the effects of the intervention. Both groups within the cohort study (control versus >6 months of ADF) as well as within the RCT (control versus 4 weeks of ADF) were comparable for gender distribution, age (cohort medians between 48 and 52 years), body mass index (cohort averages between 25.37 and 25.66), and waist-to-hip ratio at the baseline visit (Table S1). The compliance rate during the RCT was very high (2 dropouts in the control group and 1 in the ADF group; Figure 1).

## Short-Term Effects of ADF Include Improved Fat Distribution and Cardiovascular Health as well as Lowered Levels of Triiodothyronine

Here, we report the first results of an RCT, designed to study the effects of short-term (4 weeks) strict ADF on the physiology, cardiovascular parameters and body composition of healthy, non-obese adults. The RCT data showed no statistically significant differences (neither at baseline nor at study end) in the different parameters used to assess insulin sensitivity (HOMA-IR, QUICKI, ISI, and Matsuda) when comparing the 4-week ADF with the control group (which received no intervention apart from the study visits and kept their normal *ad libitum* lifestyle) (Table S3). This accounted for all analyses, irrespective of whether non-completers were excluded from the analysis or not, or whether non-parametric or parametric statistical tests were performed. Moreover, the in-group changes from baseline to study end at 4 weeks within the ADF group were also not significantly different (Table S3). To assess the levels of CR achieved by ADF, we estimated the calorie intake via rigorous food frequency questionnaires over the whole course of the RCT. We observed a significant drop of calorie intake, as calculated per week: on average, the ADF group had a reduced calorie intake of −37.40% (−48.32 to −24.36) during the intervention compared to the respective baseline values. Interestingly, the control group, staying on ad libitum diet, also reported reduced calorie intake of −8.22% (−32.23 to 3.57) (Figures 2A and 2B; Table 1). However, unlike a recent CR-study by Redman et al., we did not find reduced resting energy expenditure values after 4 weeks of ADF on the "feast day" (Figure 2C). No adverse effects occurred during the 4 weeks trial.

**Table 1. Results after 4 Weeks of Intervention Show Significant Changes in Body Composition, Physiological, and Cardiovascular Parameters in the ADF Group**

| | RCT Control Baseline | RCT Control Follow-Up (4 Weeks) | Control Δ 4 Weeks | Time p Value (Control) | RCT ADF Baseline | RCT ADF Follow-Up (4 Weeks) | ADF Δ 4 Weeks | Time p Value (ADF) | Time versus Intervention p Value (Δ) |
|---|---|---|---|---|---|---|---|---|---|
| Calorie Intake (kcal/Week) | 12,087 (8,967 to 16,590)[a] | 9,729 (7,895 to 13,038)[a] | −839 (−3,924 to 485.3)[a] | 0.0269[a] | 12,502 (10,353 to 15,701)[a] | 7,726 (6,469 to 10,013)[a] | −4,271 (−7,038 to −3,018)[a] | <0.0001[a] | 0.0008[a] |
| Δ Calorie Intake (%) | | | −8.222 (−32.23 to 3.568) | | | | −37.4 (−48.32 to −24.36)[a] | | 0.0012[a] |
| Activity Energy Expenditure (kcal/Week) | 5,013 (4,296 to 6,431)[a] | 4,792 (3,738 to 6,333) | −365 (−942.3 to 329.8) | 0.4568 | 5,102 (4,307 to 5,867) | 4,820 (3,347 to 6,709) | −211 (−1,057 to 805) | 0.3554[a] | 0.9024[a] |
| Body Weight (kg) | 75.93 ± 12.50 | 76.23 ± 12.57 | −0.196 ± 1.101 | 0.3636 | 77.21 ± 10.25 | 73.71 ± 9.97 | −3.5 ± 1.475 | <0.0001 | <0.0001 |
| Total Bone Mass (kg) | 2.79 ± 0.59 | 2.81 ± 0.59 | −0.00033 ± 0.0225 | 0.9393 | 2.76 ± 0.52 | 2.75 ± 0.53 | −0.005 ± 0.0259 | 0.3073 | 0.4764 |
| Bone Mineral Density at the Lumbar Spine ($g/cm^2$) | 1.25 (1.09 to 1.34) | 1.26 (1.03 to 1.32) | −0.0056 (−0.0154 to 0.0017)[a] | 0.2744[a] | 1.23 (1.16 to 1.42) | 1.22 (1.09 to 1.39) | −0.0114 (−0.0348 to 0.0074) | 0.0341 | 0.2584[a] |
| Total Fat Mass (kg) | 22.69 (18.86 to 26.71)[a] | 22.64 (18.65 to 26.54)[a] | −0.1506 (−0.8358 to 0.409) | 0.3762 | 26.49 (21.7 to 29.46) | 22.63 (19.38 to 26.91) | −2.112 (−3.119 to −1.352) | <0.0001 | <0.0001 |
| Total Lean Mass (kg) | 47.6 (37.96 to 58.48) | 48.36 (39.02 to 58.23) | −0.0691 (−0.799 to 0.7908) | 0.7924 | 47.39 (41.95 to 59.18)[a] | 45.82 (40.66 to 58.19) | −1.572 (−1.988 to −0.691) | <0.0001 | <0.0001 |
| Trunk Fat Mass (kg) | 12.1 ± 3.91 | 12.08 ± 3.88 | −0.03425 ± 0.5377 | 0.7433 | 12.82 ± 3.21 | 11.38 ± 3.05 | −1.435 ± 0.668 | <0.0001 | <0.0001 |
| Trunk Lean Mass (kg) | 23.11 ± 5.07 | 23.32 ± 5.15 | 0.0082 ± 0.7114 | 0.9527 | 23.17 ± 3.88 | 22.72 ± 4.21 | −0.4555 ± 0.8458 | 0.0072 | 0.0312 |
| Android Fat Mass (kg) | 1.91 (1.29 to 2.38) | 1.87 (1.39 to 2.3) | −0.0135 (−0.0984 to 0.0221)[a] | 0.1414[a] | 2.04 (1.51 to 2.81)[a] | 1.7 (1.3 to 2.43) | −0.3991 (−0.5502 to −0.1915) | <0.0001 | <0.0001[a] |
| Android Lean Mass (kg) | 3.41 ± 0.77 | 3.42 ± 0.80 | −0.0216 ± 0.1802 | 0.5394 | 3.4 ± 0.58 | 3.31 ± 0.64 | −0.0918 ± 0.1708 | 0.0073 | 0.1402 |
| Gynoid Fat Mass (kg) | 3.74 (3.3 to 4.73)[a] | 3.78 (3.14 to 4.51)[a] | 0.0251 (−0.2302 to 0.1129)[a] | 0.1551[a] | 4.23 (3.9 to 5.29) | 3.94 (3.46 to 4.98) | −0.348 (−0.529 to 0.221) | <0.0001 | <0.0001[a] |
| Gynoid Lean Mass (kg) | 7.61 (5.89 to 9.27) | 7.72 (6.01 to 9.21) | 0.0358 (−0.0909 to 0.169)[a] | 0.441[a] | 7.5 (6.35 to 8.99) | 7.33 (6.29 to 8.71) | −0.2418 (−0.3837 to −0.1276)[a] | 0.0001[a] | <0.0001[a] |
| BMI ($kg/m^2$) | 25.21 (23.57 to 27.34) | 25.2 (23.73 to 27.68) | −0.02 (−0.33 to 0.26)[a] | 0.4958[a] | 25.48 (24.21 to 27.06) | 24.25 (23.11 to 25.66) | −1.23 (−1.515 to −0.875) | <0.0001 | <0.0001[a] |
| Systole (MmHg) | 117.5 (113.3 to 127) | 118 (112 to 127.5) | −1 (−6 to 7)[a] | 0.9594[a] | 121 (112.5 to 126)[a] | 115 (111 to 120) | −4.5 (−7.75 to −0.5)[a] | 0.006[a] | 0.0465[a] |
| Δ Systole (%) | | | −0.73 (−4.826 to 6.2)[a] | | | | −3.62 (−6.916 to −0.397) [a] | | 0.0444[a] |
| Diastole (MmHg) | 74.5 (70 to 84.75) | 78 (70.5 to 84) | 0 (−3.5 to 2.5)[a] | 0.7914[a] | 74 (68 to 81.5) | 72 (69 to 77.75) [a] | −2.5 (−4 to 1) | 0.0302 | 0.1951[a] |

(*Continued on next page*)

**Table 1.** ***Continued***

| | RCT Control Baseline | RCT Control Follow-Up (4 Weeks) | Control Δ 4 Weeks | Time p Value (Control) | RCT ADF Baseline | RCT ADF Follow-Up (4 Weeks) | ADF Δ 4 Weeks | Time p Value (ADF) | Time versus Intervention p Value (Δ) |
|---|---|---|---|---|---|---|---|---|---|
| Δ Diastole (%) | | | 0 (−4.786 to 3.49)[a] | | | | −3.37 (−5.081 to 1.494) | | 0.2189[a] |
| Heartrate (1/min) | 60 (55.25 to 65) | 57 (54.25 to 65.75) | −3.5 (−5.75 to 0) | 0.0843 | 63 (60 to 71) | 60 (53.25 to 67) | −4.5 (−6 to −1)[a] | 0.0019[a] | 0.2638[a] |
| Δ Heartrate (%) | | | −3.195 ± 8.983 | | | | −7.132 ± 9.917 | | 0.1562 |
| Mean Arterial Pressure (MmHg) | 95 (89.25 to 103.8) | 96 (90.5 to 103.5) | −1 (−3 to 5)[a] | 0.9944[a] | 94 (89.5 to 101) | 92.5 (87.25 to 96.75)[a] | −3 (−5 to 1.5)[a] | 0.0087[a] | 0.0585[a] |
| Δ Mean Arterial Pressure (%) | | | −0.893 (−3.489 to 5.019)[a] | | | | −3.315 (−4.876 to 1.63)[a] | | 0.0616[a] |
| Pulse Pressure (MmHg) | 41.5 (37.25 to 44)[a] | 42 (39 to 46) | 0 (−2.5 to 4.5)[a] | 0.4967[a] | 45 (39.5 to 48) | 42 (39 to 45) | −2.5 (−4.75to 0.75)[a] | 0.0088[a] | 0.0241[a] |
| Δ Pulse Pressure (%) | | | 0 (−5.714 to 11.65)[a] | | | | −5.586 (−10.05 to 1.562) | | 0.0266[a] |
| Pulse Wave Velocity (m/s) | 7.15 (6.35 to 7.8) | 7.3 (6.8 to 7.95) | 0 (−0.15 to 0.2)[a] | 0.4681[a] | 6.9 (6.4 to 7.5) | 6.7 (6.2 to 7.6) | −0.1 (−0.225 to 0)[a] | 0.0157[a] | 0.0406[a] |
| Δ Pulse Wave Velocity (%) | | | 0 (−2.07 to 2.819)[a] | | | | −1.538 (−3.333 to 0)[a] | | 0.0362[a] |

Changes of anthropometric and cardiovascular parameters within the RCT were different among the randomized groups, favoring a greater mass and BMI loss, lower systole, diastole, heart rate, mean arterial pressure, pulse pressure, and pulse wave velocity in the ADF group [data are represented as means ± standard deviation or median (IQR)]. Results were obtained from a randomized clinical trial (see STAR Methods).

[a]Non-Gaussian data distribution (Mann-Whitney or Wilcoxon matched-pairs signed test)

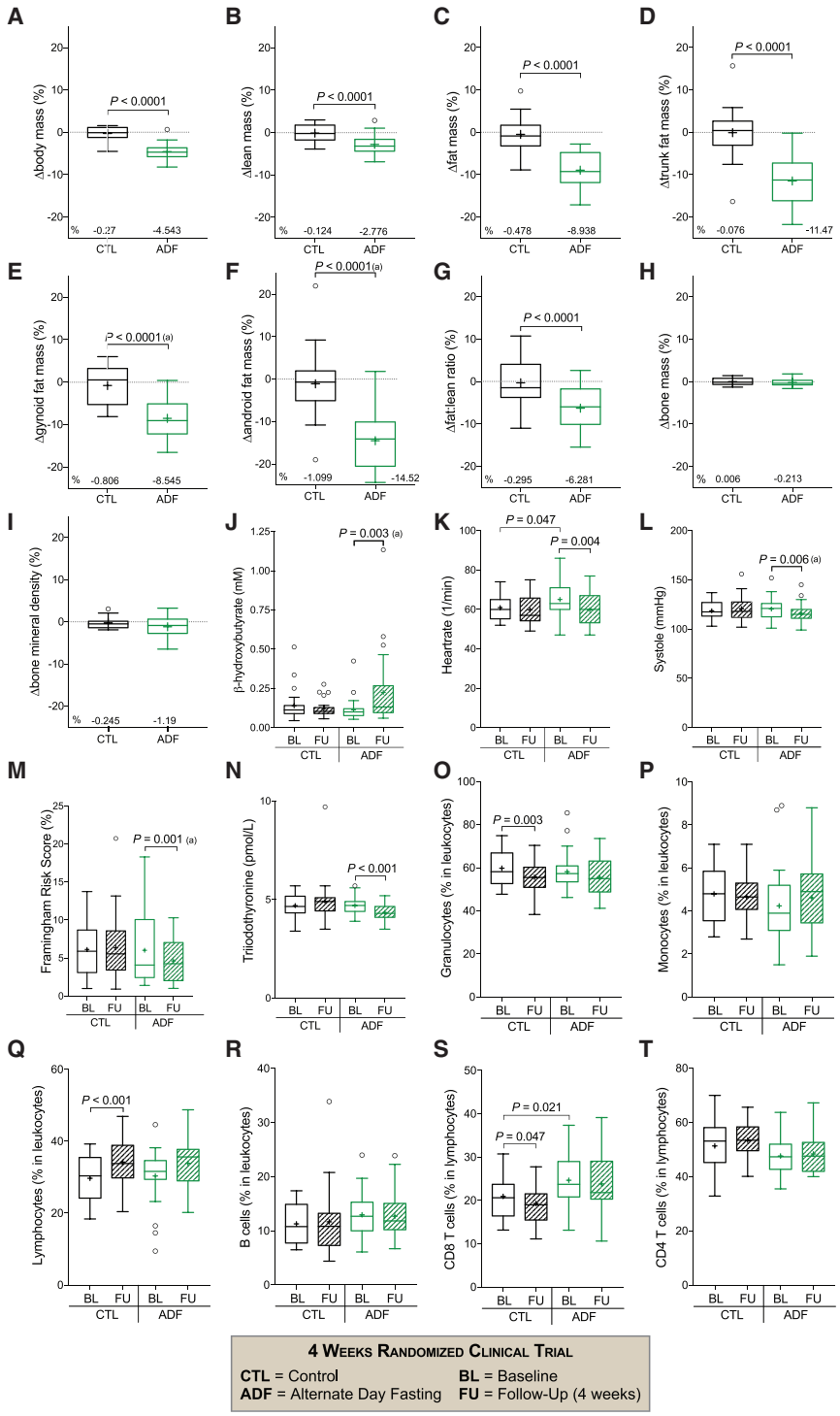


**Figure 3. Four Weeks of ADF Impact Body Composition and Reduce the Risk for Cardiovascular Diseases**

ADF reduces body weight (A), lean (B), and fat (C) mass, leading to a decrease in fat:lean ratios (G). Major fat loss was observed in the android area (F) while gynoidal fat showed lower relative mass reduction (E). No influence on bone mass (H) or bone mineral density at the lumbar spine (I) was observed. Serum levels of β-hydroxybutyrate were found to be elevated in the ADF group after 4 weeks (J) and a reduced risk for cardiovascular diseases was measured by means of the reduced heartrate (K), systolic blood pressure (L), and Framingham Risk Score (M). Circulating levels of triiodothyronine (N) were reduced in the ADF group. No change in the abundance of granulocytes (O), monocytes (P), lymphocytes (Q), B cells (R), $CD8^+$ cytotoxic T cells (S), $CD4^+$ helper T cells (T) was observed in the ADF group. BL = baseline; FU = follow-up; p values depict differences between group-deltas (A–H) or within groups. Data are visualized as Tukey box plots (line at median, x at mean). For parameters with non-gaussian distribution, a nonparametric Mann-Whitney test was used. For normally distributed data, a two-sided Student's t test was applied. When applicable, a paired Student's t test or a nonparametric Wilcoxon matched-pairs signed rank test was applied. a = non-gaussian data distribution. Percentage inlets depict mean in-group differences to baseline after 4 weeks of intervention. Results were obtained from a randomized clinical trial (see STAR Methods).

Previous studies repeatedly reported that continuous CR (~15% calorie reduction) leads to significant body weight reduction after 3–12 months (Redman et al., 2009, 2018). Inline, we found that ADF caused similar effects on fat and lean mass as well as total body weight after 4 weeks (Figures 3A–3C). Overall, these effects were sufficient to reduce the body mass index (BMI) by more than 1 kg/m$^2$ (ΔBMI after 4 weeks ADF −1.23 (−1.515 to −0.875), p value < 0.0001) (Table 1). Analyses of the body composition by dual-energy X-ray absorptiometry (DEXA) revealed that fat reduction preferably affected the trunk fat; in particular the android area with an average reduction of 14.5% ± 6.4 percentage points (p value < 0.0001) (Figures 3D–3F). Of note, excessive adiposity in the android zone is believed to be the most lipotoxic one (Kang et al., 2011; Kelli et al., 2017). The fat-to-lean ratio was significantly improved upon ADF (−6.3% ± 5.0 percentage points, p value < 0.0001) (Figure 3G). Further, bone mass was not affected (Δbone mass −0.005 ± 0.026 kg, p value 0.307) (Figure 3H; Table 1) and the BMD at the lumbar spine region only marginally decreased due to ADF by −0.867% (−2.756 to 0.6429) (p value 0.034) as compared to −0.474% (−1.391 to 0.1325) in the CTL group (Figure 3I; Table 1). Of note, the decrease in BMD is only significant when analyzed within the ADF group, while comparing ΔBMDs of both groups does not yield significant differences (p value 0.258). BMD may be significantly reduced by more than 1% during relatively mild CR (approximately

20% CR for the first 6 months, 8%–11% for the remaining 18 months) in long-term studies after 12 months (Villareal et al., 2016).

Elevated circulating ketone bodies have been reported after prolonged fasting periods in humans (London et al., 1986; Puchalska and Crawford, 2017; Steinhauser et al., 2018). Among manifold biological roles, they act as energy substrates under nutrient deprivation conditions (Puchalska and Crawford, 2017). Interestingly, we found elevated serum levels of β-hydroxybutyrate, previously reported to harbor anti-aging and cardioprotective properties (Edwards et al., 2014; Newman et al., 2017), after 4 weeks of ADF in the intervention group (Figure 3J). Of note, the sampling was performed on non-fasting days, arguing for persistently changed ketone metabolism by the rhythmic fasting periods in this group.

In rodents, different forms of periodic fasting were shown to provoke positive effects on metabolic and cardiovascular health by reducing circulating glucose, cholesterol and triglyceride concentrations, heart rate, and blood pressure (Di Francesco et al., 2018; Longo and Panda, 2016). Even in combination with a high-fat diet, such dietary interventions may prevent or improve obesity (Li et al., 2017), insulin sensitivity (Li et al., 2017), hepatic steatosis (Hatori et al., 2012), and inflammation (Hatori et al., 2012) as compared to a continuous isocaloric diet. Similarly, ADF reduced the total body weight, waist-to-hip ratio, fat mass, and fat-free mass in obese individuals and in patients at medium- to high-risk of CVD (Antoni et al., 2018; Rozing et al., 2010).

In the RCT, ADF improved cardiovascular markers, reducing systolic and diastolic blood pressure, heart rate, arterial and pulse pressure as well as pulse wave velocity (Figures 3K and 3L; Table 1). Though, it cannot be excluded that the dampening effect of ADF on the heart rate was due to elevated values in the ADF group at baseline (Figure 3K). Consequently, according to the Framingham Risk Score, which measures the risk in percent to develop a cardiovascular disease in the next 10 years (Wilson et al., 1998), the observed changes are sufficient to significantly (p value 0.009) reduce the risk for future adverse cardiovascular events even in healthy adults by 1.416% ± 2.484% (Figure 3M). Given the general good health conditions and low baseline risk of the groups, the observed improvement suggests short-term ADF as a valid intervention to improve cardiovascular health in the general population.

We wondered whether these improvements might also be due to increased physical activity. Thus, we recorded the activity energy expenditure during the last week of the RCT and compared it to baseline values, but we found no differences between the groups (Figure 2D; Table 1). The concentration of most blood lipids, health, and aging parameters (Figures S1–S3) as well as the abundance of various immune cell types remained unchanged (Figures 3O–3T and S2A). Interestingly though, short-term ADF was sufficient to reduce circulating triiodothyronine (fT3) levels (Figure 3N). Of note, low levels of fT3, without an impaired thyroid gland function, have been intensively linked to longevity in humans (Rozing et al., 2010).

Although these results are promising, a cautious note is still needed here, since we do not know the consequences of strict ADF exceeding the period of 6 months.

## Long-Term Effects of ADF Include Lower Levels of Cholesterol and Triiodothyronine without Adverse Side Effects

Four weeks of ADF, accompanied by relatively high, but oscillating, levels of CR, lead to generally benign effects, including the reduction of body weight, Framingham Risk Score, and fT3 levels without significant changes in other widely used health and aging parameters. Similar to the RCT cohort, we did not detect clinically and statistically relevant differences in the insulin sensitivity parameters between the long-term ADF group and the control group (Table S4). The long-term ADF group had significantly (p value < 0.001) lower caloric intake of 8,792 (6,689 to 11,967) kcal/week, as compared to control levels of 12,306 (9,258 to 15,925) (Figure 4A; Table S1). This corresponds to −28.56% (−45.56 to 2.76) calorie intake, relative to the control median, in comparison to −37.4% in the RCT. In line with the RCT results, we again did not observe a significant difference in resting energy expenditure in the long-term ADF cohort (Figure 4B).

However, different to the RCT cohorts, in the >6 months of ADF group, we observed lower levels of circulating total cholesterol, low density (LDL), very low-density lipoprotein (VLDL), and triglycerides compared to the control group, while high-density lipoprotein (HDL) was similar in both groups (Figures 5A–5E). In line with the effects of 4 weeks of ADF, we observed lower heart rate in the >6 months of ADF group (Figure 5F) supporting the hypothesis that ADF may lead to improved cardiovascular health. Again, these differences could not be explained by differences in overall activity levels, as the activity energy expenditure was not significantly different in the long-term ADF group [CTL 5,034 (4,307 to 6,065) versus ADF 4,545 (3,759 to 5,923) kcal/week, p value 0.206] (Figure 4C; Table S1).

CR is known to compromise immune function and cause negative effects on BMD (after 12 months (Villareal et al., 2016)), which is accompanied by reduced numbers of white blood cells (WBC) (Meydani et al., 2016). However, comparable to the results of the 4-week-long intervention, bone mass, BMD of the lumbar spine region, white blood cell counts (Figures 5I–5K), and abundance of various other immune cell types (Figures 5L–5Q) in the long-term ADF group showed no significant differences to healthy, non-fasting controls. RBC counts, as well as iron metabolism markers in the blood plasma (hematocrit, haemoglobin, iron, and transferrin saturation), were lower in the >6 months of ADF group but stayed within the reference range (Figures S4A–S4D and S4F). Yet, ferritin and transferrin were not different in the >6 months of ADF as compared to the control group (Figures S4E and S4G), and no iron deficiency was diagnosed. The mean platelet volume was slightly greater in participants undergoing ADF, while the number of thrombocytes remained unchanged (Figures S4H and S4I).

The secretion of parathyroid hormone (PTH) was greater (Figure S4J) and circulating fT3 levels were lower in those practicing ADF for >6 months (Figure 5H). Unlike fT3, there was no difference in the circulating levels of thyroid-stimulating hormone (TSH) and free thyroxine, providing evidence for normal function of the thyroidal gland after >6 months of ADF (Figures S4K–S4L). No changes of other aging biomarkers like plasma protein oxidation (Figure S4M), IGFBP1 and IGFBP3 (data not shown), and CRP levels (Figure S4N) were detected. Interestingly, different to the RCT, we did not find significantly higher levels of

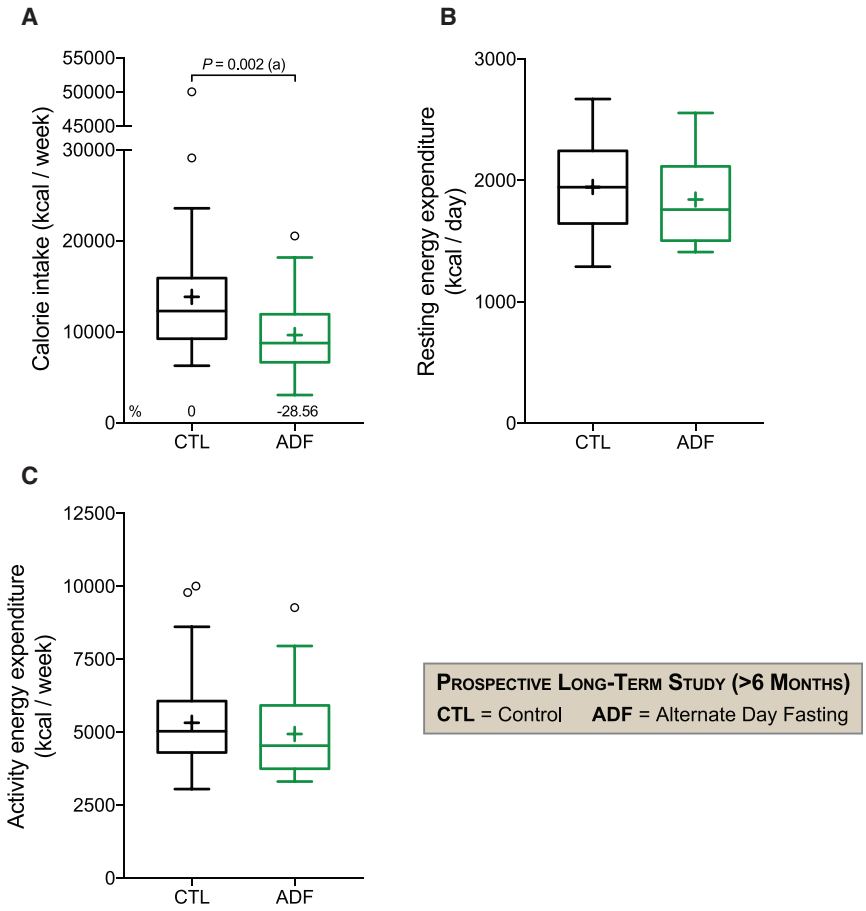


**Figure 4. Levels of Caloric Restriction, Activity, and Resting Energy Expenditure in a Cohort of Healthy, Non-obese Humans on ADF for >6 Months**

Calorie intake, measured by food frequency questionnaires, is reduced in probands performing long-time ADF (A). Resting energy expenditure on non-fasting days, as well as activity energy expenditure are indifferent from the control group (B, C). Data are visualized as Tukey box plots (line at median, x at mean). For parameters with non-gaussian distribution, a nonparametric Mann-Whitney test was used. For normally distributed data, a two-sided Student's t test was applied. a = non-gaussian data distribution. Percentage inlets depict median differences to control median. Results were obtained from a cohort study (see STAR Methods).

β-hydroxybutyrate after an overnight (12 h) fast in the long-term ADF cohort when compared to the control group (Figure S4O). However, comparing the β-hydroxybutyrate levels under 12-h-fasted and 36-h-fasted conditions in the long-term ADF group, we observed a fasting-induced surge of β-hydroxybutyrate levels (Figure S4P). Finally, the concentration of sICAM-1, a biomarker for various age-associated diseases and inflammation (Jenny et al., 2006), was significantly (p value 0.048) lower in the long-term ADF cohort (Figure 5G).

## Periodic Changes in the Metabolome during ADF Include Depletion of the Pro-aging Amino Acid Methionine

Contrary to constant CR, IF represents a periodic stimulus to the organism, and preclinical studies suggest that the beneficial effects of IF cannot be solely attributed to a reduction of total caloric intake (Di Francesco et al., 2018; Longo and Panda, 2016). To explore the possible mechanisms of the ADF-induced physiological and biochemical alterations, we analyzed the metabolic and proteomic changes that occur between fast and feast days. Untargeted analyses of the plasma metabolome and the PBMC (peripheral blood mononuclear cell) proteome revealed clear-cut differences between the fasting and feeding days even after >6 months of ADF. In the metabolome, 54 out of 113 detected significantly modified metabolites (p value < 0.05) were at least 20% higher after 36 h of fasting, of which the majority (>95%) were lipids or free fatty acids, including polyunsaturated free fatty acids (PUFAs), α-tocopherol, and a type of vitamin E (Table S2A). On the contrary, 49 metabolites were at least 20% lower, consisting mainly (44.9%) of amino acids or related metabolites (e.g., ornithine, citrulline, and taurine; Table S2A). Interestingly, low systemic concentrations of amino acids, especially methionine have been shown to be sufficient for lifespan extension in model organisms (Levine et al., 2014; Ruckenstuhl et al., 2014). Several short-chain FFAs and lipids were also found to be downregulated. These include propionic (C3:0), nonanoic (C9:0), and nonadecylic acid (C19:0), which are all associated with intestinal bacterial metabolism. Additionally, carnitine levels decreased while palmitoylcarnitine was elevated nearly 2-fold (Table S2A).

Further analyses by metabolite set enrichment analysis (MSEA) confirmed the overall pattern of changes in lipid and amino acid metabolism (Figures 6A and S5). Specifically, pathways of essential PUFAs omega-3/6 linolenic and linoleic, respectively, and arachidonic acid concomitantly with long-chain FAs β-oxidation were enriched (Figures 6A, 6C, and S5), whereas metabolites from urea cycle, ammonia recycling, and several pathways associated with amino acid metabolism were substantially depleted (Table S2A). Of note, pathways of saturation and elongation of PUFAs were enriched (Figure 6C). The overall shift in the plasma metabolome with FA enrichment and amino acid depletion, resembles data collected from mice and non-human primates (monkeys) subjected to fasting or CR (Mitchell et al., 2016, 2019). Most likely, these changes can be attributed to enhanced lipolysis in adipose tissues coupled to enhanced hepatic capture of amino acids for gluconeogenesis.

## Periodic Changes in the Proteome during ADF Point to General Proteomic Depletion and Modulated Lipid Metabolism

Strikingly, only 13 out of 2,089 significantly (p value < 0.05) modulated protein hits within the PBMC proteome showed an

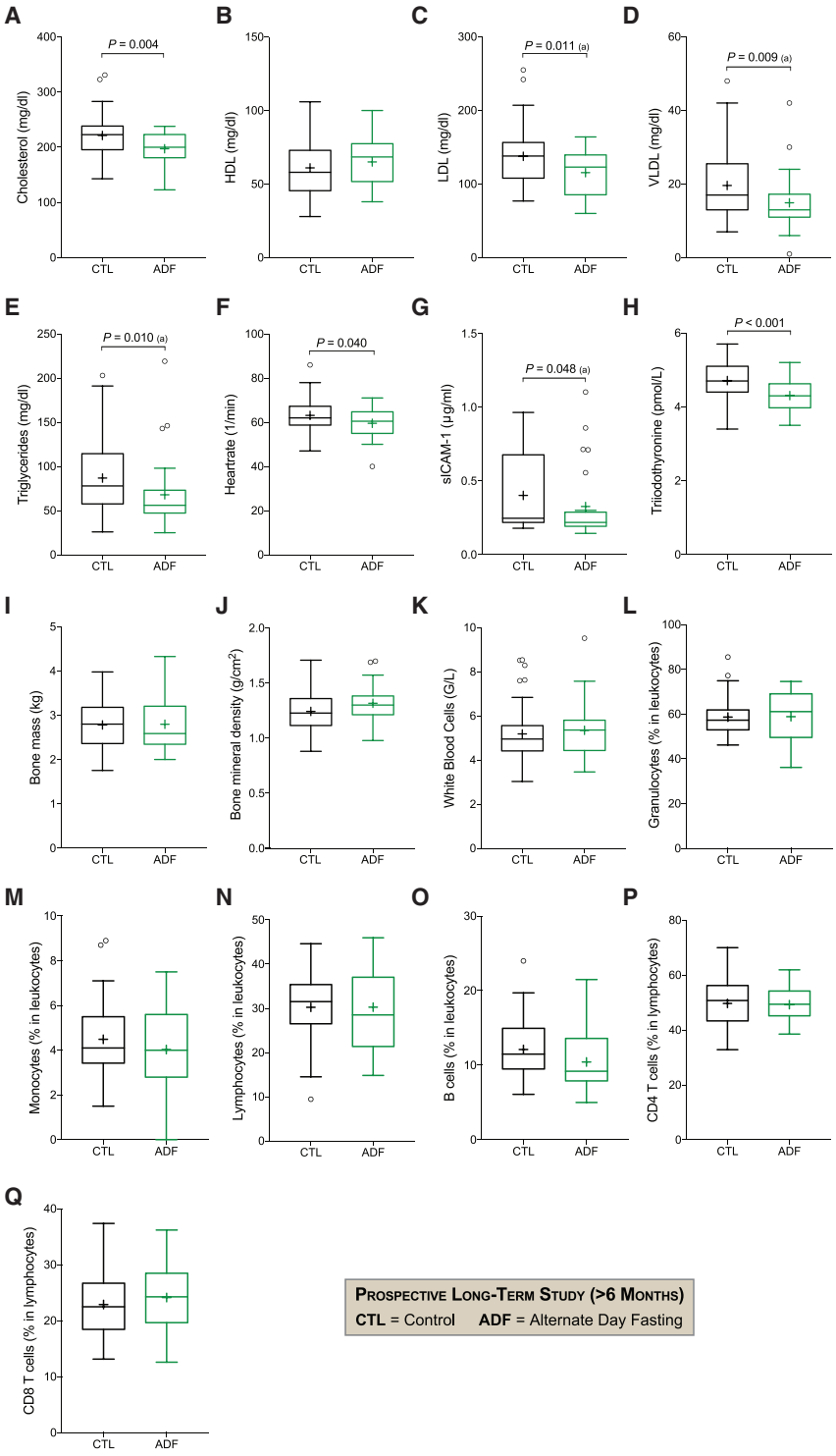


**Figure 5. Differences in the Lipid Profile of Healthy, Non-obese Humans on ADF for >6 Months Compared to Healthy Controls**

ADF is associated with lower levels of total cholesterol (A), LDL (C), VLDL (D) and triglycerides (E) in the blood plasma, while HDL levels (B) remain comparable to the control group even after >6 months of ADF. The heart rate (F), the inflammation marker sICAM-1 (G) and the circulating levels of triiodothyronine (H) are lower in the >6 months of ADF group. The total bone mass (I), bone mineral density at the lumbar spine (J) and white blood cell count (K) are comparable to controls. No differences were observed in the abundance of granulocytes (L), monocytes (M), lymphocytes (N), B cells (O), $CD8^+$ cytotoxic T cells (P), $CD4^+$ helper T cells (Q). Data are visualized as Tukey box plots (line at median, x at mean). For parameters with non-gaussian distribution, a nonparametric Mann-Whitney test was used. For normally distributed data, a two-sided Student's t test was applied. a = non-gaussian data distribution. Results were obtained from a cohort study (see STAR Methods).

increase of ≥15%, while the remaining proteins were downregulated (Table S2B) after 36 h of fasting. Gene set enrichment analysis (GSEA) performed on the PBMC proteome unveiled changes in pathways related to lipid metabolism (i.e., lipid transport, lipoprotein, and steroid-binding processes), pathways related to energy metabolism (i.e., mitochondrial β-oxidation, glycogen metabolism, and TCA cycle), and stress response (i.e., heat shock protein binding, caspase activation) among others (Figure 6B; Table S2C). GSEA-detectable immunological pathways were associated with natural killer (NK) cell inhibitory receptor signaling and NK cell activation through integrins, among others (Table S2D). Hypothetically, the status of PBMCs during fasting days might be a consequence of or reflect the impact of overabundant PUFAs in the plasma (such as omega 3/6 linoleic-linolenic acids), which are known modulators of immune function (Baker et al., 2016; Burdge, 2006).

## PERSPECTIVE

The importance and impact of periodic changes on physiology, health maintenance, and aging has recently been discussed (Di Francesco et al., 2018). Experiments in model organisms show that periodic food intake interacts with the circadian oscillator at the transcriptional, metabolic, and microbial levels (Longo and Panda, 2016). The relevance of meal timing, in particular with respect to CVD, has also been shown for humans, confirming the beneficial health effects of intermittent as compared to constant food intake (Melkani and Panda, 2017). The optimal length of recurring fasting periods in humans may depend on the desired effect and parameters measured and be subject to individual differences. Still, accumulating data hint that a reduced meal number throughout

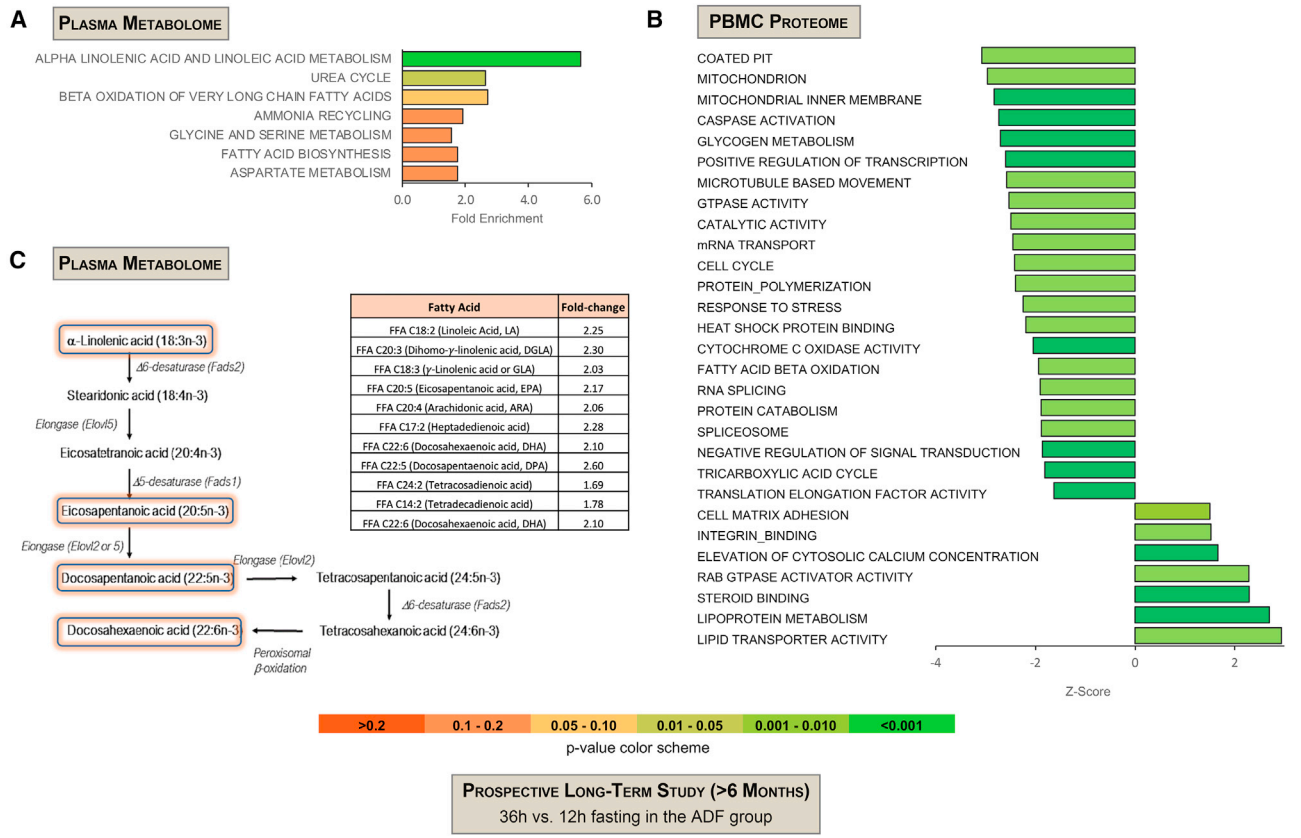


**Figure 6. Periodic Changes in Plasma Metabolome and Pbmc during ADF**

Variations between 36 h (after fast day) and 12-h fast (after feast day) during ADF show a pattern of periodic lipid enrichment and amino acids depletion and subsequent overall PBMC proteome downregulation in healthy humans. Metabolite set enrichment analysis results (A) are presented graphically displaying the p values according to the designated color scheme whereas the bar length represents the fold enrichment (extended list shown in Figure S5). Collected data shows drastic enrichment of polyunsaturated fatty acids and metabolites of lipid and fatty acid metabolism after a fast day. Gene set enrichment analysis (GSEA) of the PBMC's proteome (B) is represented by Z scores (relative changes of a particular gene set compared to the change of whole dataset) of significant gene and/or protein sets (GO terms) displayed by the length of the bar and p values represented according to the designated color scheme. GSEA identified periodic reduction in energy metabolism and stress response proteins after a fast day, while proteins involved in lipid metabolism were highly increased. Metabolites involved in metabolic conversion of alpha-linolenic acid to longer chain PUFAs were significantly enriched in the plasma after 36 h fast (C). Highlighted fatty acids in the pathway are listed in this figure with corresponding fold-changes. Genes encoding the enzymes are shown in parentheses (adapted from Baker et al., 2016). Results were obtained from a cohort study (see STAR Methods).

the day may provoke robust physiological benefits (Paoli et al., 2019).

Individuals performing ADF in our RCT did not fully compensate for the lack of caloric intake on the fasting days with elevated calorie intake on the feast days, thus reaching significant levels of caloric restriction (37.4%) throughout the intervention. Given the simple protocol and few guidelines the probands had to follow, ADF seems to be an easy way to achieve high levels of CR in non-obese humans. Despite a statistically significant body weight reduction in the ADF versus control group in the 4-week intervention trial, no changes of insulin sensitivity were observed. It seems plausible to assume here that in healthy people who are already highly insulin sensitive at baseline, ADF does not further improve parameters of insulin sensitivity. Notably, the measurements were taken after a 12-h fasting period both at baseline and study end to assess effects of the 4-week intervention, rather than differences in insulin sensitivity between a 12-h and a 36-h fasting period.

ADF proves to be effective in reducing the risk for CVD not only in obese (Trepanowski et al., 2017), but also in metabolically healthy, non-obese humans, as shown in this study. ADF managed to significantly reduce the Framingham risk score (risk in percent to develop a CVD in the next 10 years) already after 4 weeks, while having significant impact on blood lipids after >6 months of intervention. While the effect on heart rate in the RCT could be due to significantly higher baseline values in the RCT, improvements of other cardiovascular parameters are at no risk of potential baseline bias. The beneficial effects on cardiovascular health were also reflected by the greater weight-loss effect on android fat, which is associated with elevated CVD risks (Wiklund et al., 2008). Whether strict ADF is superior to CR in promoting cardioprotection will be an interesting course of upcoming research, although a recent study suggests that the effects on weight loss and cardiovascular improvements are very similar between a mild form of ADF (25% calories on fast days, 125% on feast days) and 25% continuous CR (Trepanowski

et al., 2017). On the other hand, >6 months of ADF did not cause a decline in BMD or white blood cell count, as it was reported for longer periods of constant CR (Meydani et al., 2016; Schafer, 2016; Villareal et al., 2016), which makes this nutritional intervention a suitable alternative to continuous CR. While there was a small decline of BMD in the 4-week RCT, in the long term, the ADF group even had a trend toward higher BMD values. Continuously mild CR was shown to negatively affect BMD only after 12 months, though the report also showed not-significant reductions after already 6 months (Villareal et al., 2016). Of note, there is a relevant difference in the cohorts' ages: the cohort from Villareal et al. is on average 38 years old, as compared to 48–50 years (medians) in this study. This age difference might have further marked influence on the interventions' effects on BMD. Still, given the more drastic reduction of calories achieved by strict ADF and the lack of effect on bone mass or BMD after 6 months, it is tempting to speculate that ADF is safe concerning bone health in the short term. However, the lack of baseline values for the long-term cohort does not allow us to make a final statement about the effect of strict ADF on bone health and extended RCTs on a longer term will be needed to comprehensively address this issue.

Previous studies suggested metabolic slowing through reduced thyroid hormones and improvement of cardiac health as some of the main hallmarks of CR- and fasting-induced longevity (Redman et al., 2018). The present study suggests that effects on thyroid hormones and cardiac health might also play a role in ADF, which could eventually slow the pace of aging (Fontana et al., 2006). However, in contrast to a CR-study by Redman et al. (2018), we did not detect changes in resting energy expenditure after ADF, neither in the RCT, nor in the long-term fasters (although measured by different methods). Still, as we could not assess baseline values of the long-term cohort, it is still possible that effects may surface after more than 4 weeks ADF, when baseline characterization is thoroughly performed.

Unlike continuous CR, ADF leads to periodic changes in the plasma metabolome and the leukocyte proteome that might explain its distinctive features. Periodic shifts toward increased PUFA blood levels could represent an additional mode of action of this intervention, given their immunomodulatory (Baker et al., 2016; Burdge, 2006) and cardioprotective (Ander et al., 2003; Elagizi et al., 2018; Kris-Etherton et al., 2004) properties. Importantly, periodically elevated levels of ketone bodies, such as β-hydroxybutyrate, might contribute to long-term healthspan improvements and cardioprotective effects (Newman et al., 2017). Indeed, we could show that even after >6 months of ADF, the human body still experiences a great surge in β-hydroxybutyrate levels on fasting days during ADF. Furthermore, in the RCT described herein, we found elevated β-hydroxybutyrate even during non-fasted conditions after 4 weeks of ADF. Increased β-hydroxybutyrate levels positively influence various parameters of health (Edwards et al., 2015) indirectly reduce blood pressure caused by high salt consumption (Chakraborty et al., 2018) and improve cerebral blood flow in aging brain upon CR (Lin et al., 2015).

Overall, this study puts forward further evidence regarding the modulation of the thyroid axis and the cardiovascular system by the periodic depletion of energy intake. ADF and CR may share large overlaps in their beneficial effects on the human body, with obvious differences regarding the oscillating nature of ADF. Whether these additional features of ADF are increasing the beneficial impact on the health status, needs to be studied in future trials. Thus, it will be important to compare ADF and CR interventions in humans with matched levels of caloric reduction and to further validate pre-clinical findings, such as the essential role of the gut-microbiome in the beneficial effects of periodic fasting (Li et al., 2017).

In the future a coordinated approach is needed to robustly compare the effects of different fasting regimes with each other, and across different cohort types. Strict ADF (0% calories on fast days) is still scarcely represented in the literature (Patterson and Sears, 2017). In contrast to our data, Varady et al. (2013) did not find reduced fat-free mass, but lowered TG levels after 12 weeks of ADF with 25% calorie intake on "fast" days. Another study (Varady et al., 2009) again showed lowered LDL, HDL, and TG levels after 8 weeks of ADF in obese probands. While we did not observe changes in the levels of blood lipids after the 4 weeks of ADF on the feast day, a 28-h fasting period was shown to increase LDL and HDL levels, while lowering triglycerides in healthy adults on the fast day (Horne et al., 2013). As some of the acutely changed parameters were immediately reversed on the next feeding day, this raises interesting questions about the acute, persistent, or adaptive nature of the effects of ADF. This also should emphasize that sampling time points and exact adherence to fasting periods before examinations are crucial in capturing (subtle) fasting effects and could explain some of the differences across the literature.

Although many studies, including this one, have provided promising clinical data for ADF and similar interventions, motivating a broader part of the population to conduct periods of IF to improve body composition and cardiovascular health might be difficult. Periods of elevated hunger feelings on the fasting days (Heilbronn et al., 2005), societies with constant, convenient access to nutrients, and eating patterns strongly intertwined with social structures might represent significant obstacles. As for many dietary interventions, regaining weight after the end of the intervention might be a problematic factor for certain groups and needs to be further addressed in future ADF studies. A recent study reported that there was no significant difference in the post-interventional weight gain between groups performing 6 months of ADF or CR. Both groups regained roughly 30% of their accumulated weight loss during a 6 months post-interventional observation period (Trepanowski et al., 2017). Nonetheless, shorter courses of periodic fasting might still represent a well-tolerated way of improving weight, body composition, and cardiometabolic health for a longer period following the intervention.

Finally, even healthy adults should not perform ADF without consultation by clinicians to rule out adverse effects due to critical medical conditions. Importantly, although not directly assessed in this study, a wholesome and balanced diet is likely crucial to foster the beneficial effects caused by ADF. Thus, appreciable clinical support and a generally healthy lifestyle should be considered before starting ADF.

This is especially important with regard to the results obtained from the long-term cohort, as we cannot exclude that many more people may have originally started ADF and stopped due to unknown reasons. Obviously, these people could not be assessed

in our cross-sectional study part. Moreover, we have not assessed the personal reasons for starting ADF of the cross-sectional trial participants, which could have introduced an additional bias. Hence, overall safety and efficacy of ADF should be assessed by performing future large-scale RCTs with long-term follow-up.

### Limitations of Study

Aging is considered a continuous and evolutionary robust biological process. Hence, the selection criteria used in our study (i.e., age, health status, and BMI) introduce a selection bias in our study design. On the other hand, showing effectiveness of IF in healthy, 35- to 65-year-old humans represents a major contribution to understanding how ADF can be utilized as a preventive rather than curative intervention. Furthermore, the relatively low number of participants could be viewed as a limitation of this study. Yet, given the homogeneous results despite a rather heterogeneous population (i.e., age and BMI), this may be viewed as supporting the applicability of the studied protocol. The approach to the recruitment of subjects could have introduced a selection bias toward participants who were already knowledgeable and/or interested in ADF. While we were not able to blind participants regarding their intervention, allocation, clinical, and scientific staff were blinded during the process of collection, archiving, and analyses of collected samples and measurements. Further, in the prospective trial, we do not have access to baseline values of the long-term ADF cohort. Thus, it cannot be excluded that effects on physiology and cardiovascular health could even be greater for long-term ADF application. In addition, the potential bias introduced by the per-protocol analysis may be viewed as a limitation. Further, our study focuses on metabolic and cardiovascular health parameters and contains no long-term mechanisms to monitor the quality and quantity of food intake during the study participation. However, as cardiovascular and metabolic diseases are among the main contributors of age-related health decline, this study generates a marked impact on the current state of knowledge in the field.

## STAR★METHODS

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### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cmet.2019.07.016.

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### AUTHOR CONTRIBUTIONS

S. Stekovic, F.S., H.S., T.R.P., and F.M. contributed to acquiring funding for the InterFAST trial. S. Stekovic and N.T. contributed to efficient project management and day-to-day operation. N.T. and H.S. contributed to acquiring ethical approval for the InterFAST trial. S. Stekovic, N.T., S. Schroeder, J.T., H.S., T.R.P., and F.M. significantly contributed to the creation of study protocol. S. Stekovic, S.J.H., N.T., M.A.A., P.R., L.P., J.T.S., T.P., B.P., J.U., J.T., T.E., C.M., M.S., E.Z., N.B., R.R., A.S., E.K., N.V., A.S., T.M., F.S., R.d.C., G.K., B.-O.P., J.D., H.S., T.R.P., and F.M. contributed to collection, analysis, and interpretation of data. All authors reviewed and contributed to the final manuscript.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, Peptides, and Recombinant Proteins | | |
| DTNB (Ellman's Reagent) | Thermo Fisher | Cat.# 22582 |
| Critical Commercial Assays | | |
| V-PLEX Human Biomarker 40-Plex Kit | Mesoscale Diagnostics | Cat.# K15209G-2 |
| Free Fatty Acids Kit | Wako Chemical | Cat.# 434-91795 and 436–91995 |
| Thyroid Stimulating Hormone (TSH) Immunoassay | ADVIA Centaur | Cat.# 0870387 |
| Triiodthyronine (T3) Immunoassay | ADVIA Centaur | Cat.# 03154228 |
| Thyroxine (T4) Immunoassay | ADVIA Centaur | Cat.# 06490106 |
| Software and Algorithms | | |
| msConvert | mzXML ProteoWizard Toolkit; http://proteowizard.sourceforge.net/tools.shtml | Version 3.0.5 |
| PeakScout | Fröhlich et al. 2016 | In-house software (Version 3.0.7.jar) |
| Bruker Topspin | Bruker GmbH | Version 4.0.2 |
| MestreNova | Mestrelab Research | Version 12.0.2 |
| MetaboAnalyst | Chong et al. 2018 | Version 4.0 |
| Spectronaut Pulsar X | Biognosys | Version 12.0 (11.0.15038.14_Enterprise) |
| Graphpad Prism 7.0 | GraphPad, https://www.graphpad.com/scientific-software/prism/ | Version 7.0 |
| R | The R Foundation; https://www.r-project.org/ | Version 3.4.1 |
| TibcoSpotfire | Tibco | Version 7.5.0 (23.28.9701.4854) |
| MetaSoft Studio | Cortex Biophysik GmbH | https://cortex-medical.com/DE/MetaSoft-Studio.htm |
| DataAnalyzer | Movisens GmbH | https://www.movisens.com/de/produkte/dataanalyzer-2/ |
| Hypertension Management Software CS Version 4.7 | I.E.M. Healthcare GmbH | https://www.iem.de/de/produkte/hms-software.html |
| Other | | |
| Accelerometer (Movisens) | Movisens GmbH | https://www.movisens.com/de |
| Indirect calorimetry (MetaMax 3b) | Cortex Biophysik GmbH | https://cortex-medical.com/DE/METAMAX-3B.htm |
| Body composition (Lunar iDXA) | GE Healthcare | http://www3.gehealthcare.de/de-de/produkte/kategorien/densitometrie/dxa/lunar_idxa_for_bone_health |
| MobiloGraph (24h PWA Monitor) | I.E.M. Healthcare GmbH | https://www.iem.de/de/produkte/mobil-o-graph.html |
| Blood pressure (Boso Medicus Uno) | Bosch & Sohn GmbH | https://www.boso.de/produkte/blutdruckmessgeraete-fuer-die-selbstmessung/oberarmgeraete.html |

### LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources, tissue and reagents should be directed to and will be fulfilled by the Lead Contact, Frank Madeo (frank.madeo@uni-graz.at).

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Study Design

In the ADF group, participants were asked to eat every second day *ad libitum*, so called ''feast days'' but to refrain from calorie intake on the fast days by completely excluding solid and liquid foods and caloric beverages (including diet sodas and ''calorie-free'' meals or beverages). Hence, study participants were only allowed to consume water, flavored carbonated water, unsweetened black or green tea or coffee on the fast day.

### Study Subjects and Recruitment

90 healthy study participants were selected from the general population for the participation in this study. Thirty participants thereof practiced Alternate Day Fasting for at least 6 months previous to this study and have been used to collect the data on the long-term effects of ADF in healthy individuals. Remaining sixty participants were included in the RCT as healthy participants without recent ADF experience. For this study, separate informed consent was created. There were no significant differences in anthropometric characteristics between respective study groups in the cohort trial and the RCT (Table S1). We did not compute a power analysis prior to the study because our study was designed as an explorative, hypothesis-generating trial.

Participants without previous ADF history assigned for the 4 weeks short-term fasting trial were identified via Primary Care and advertisements in local and social media. Long-term ADF study participants were recruited via advertisements (in public media and through ''10in2'' social media channels) and leaflets at events promoting ADF and online via different blogs. Study participants were recruited by the investigators and the study nurse in charge. Participants were informed about the study background and protocol in detail, and any questions or concerns brought forward by the study participants were answered previous to signing the informed consent. All participants have signed a written informed consent before any particular study procedure.

## METHOD DETAILS

All methods are additionally described in detail in previously published study protocol (Tripolt et al., 2018). Here we briefly summarize the study, measurement and data evaluation procedures.

### Study Registration

The study was conducted according to the principles of the International Declaration of Helsinki and to the principles of good clinical practice (GCP) under the approval of the ethics committee of the Medical University of Graz (27-166 ex 14/15). The clinical examinations have been conducted at the Division of Endocrinology and Diabetology at the Medical University of Graz (academic hospital), Austria. The study is registered in the Database for Clinical Trials of the U.S. National Institutes of Health (ClinicalTrials.gov identifier: NCT02673515).

### Data Collection

After obtaining signed informed consent from participants, recruiting clinicians completed case report forms (CRFs) for each participant to record socio-demographic information, anthropometric measurements, vital signs, information about medical history, concomitant medication, physical examination and dietary habits. Source documents comprise the CRF and hospital records as well as laboratory records. All documents were stored safely in a confidential manner and in accordance to national data privacy laws and regulation. Study participants were pseudonymized by using a unique study participant number/code. In addition, their initials and date of birth were reported on all study-specific documents. Source data was collected and archived securely for internal monitoring and possible external audits or inspections by regulatory authorities.

### Randomization Procedures

For the pilot RCT, the study participants without previous ADF history were randomly assigned to either ''ADF'' or ''control group'', in a 1:1 ratio, using the web-based randomization tool ''Randomizer for Clinical Trials'' (www.randomizer.at), provided by the Institute of Medical Informatics, Statistics and Documentation of the Medical University Graz, Austria. The randomization was performed stratified by sex.

### Inclusion Criteria

- Age between 35 and 65 years, both inclusive
- BMI between 22.0 and 30.0 kg/m$^2$, both inclusive
- Stable weight (change <±10% current bodyweight) for 3 months prior to the study
- Fasting blood glucose < 110mg/dl without glucose lowering medication
- LDL-cholesterol < 180mg/dl without lipid lowering medication
- Blood pressure < 140/90 mmHg without blood pressure lowering medication
- Stable weight (change < ± 10%) for 3 months immediately prior to the study

### Exclusion Criteria

- History of metabolic disorder
- History of cardiovascular disease
- Acute or chronic inflammatory disorder
- Known malignancy
- Use of tobacco products within 5 years (smokers not eligible for the study)
- Abuse of recreational drugs within 5 years
- Alcohol abuse (more than 15 drinks/week)
- Dietary restrictions (e.g. vegetarianism and veganism)
- Women and men on hormonal supplementation
- Women or men on hormone-based contraceptive agents within the past 2 months
- Therapy with antidepressants within the past 6 months
- Regular therapy with acetylsalicylic acid or current medication to regulate blood sugar, blood pressure or lipids
- Women who are pregnant, breast-feeding or aiming to become pregnant during the course of the trial

### Anthropometric Measurements

Body weight was measured in standing position rounding to the nearest 0,5 kg. Height was measured using a metric tape with the patient standing straight against the wall. BMI was calculated using the weight in kilograms divided by the second power of the height expressed in meters.

### Biochemical Measurements

Insulin and c-peptide were measured by chemiluminescence on an Advia Centaur system (Siemens Healthcare Diagnostics, Eschborn, Germany). Hormones such as anti-Müllerian hormone (AMH), testosterone, cortisol, thyreotropin, triiodothyronine and thyroxine, estrogen, sexual hormone-binding globulin (SHBG), luteotropic hormone (LH), follicle-stimulating hormone (FSH) and 25(OH)vitamin D were measured using automated analyzers: AMH by Beckmann-Coulter, Krefeld, Germany; testosterone, cortisol, thyreotropin, triiodothyronine and thyroxine by Siemens Advia Centaur, Eschborn, Germany; SHBG by Roche Diagnostics, Mannheim, Germany; estrogen, LH and FSH by Triturus, Biomedical Diagnostics, Antwerp, Belgium, 25(OH)vitamin D by iSYS, IDS, Boldon, UK, respectively. Samples for appetite hormones (leptin, ghrelin, PYY and others) were stored at −80°C until analysis after centrifugation. Free fatty acids were measured enzymatically (Wako Chemical, Neuss, Germany) on an Olympus AU640 (Olympus Diagnostica, Hamburg, Germany). Routine parameters were determined using a cobas® analyzer (Roche Diagnostics). Inflammation parameters were measured using V-PLEX Human Biomarker 40-Plex Kit (Meso Scale Diagnostics, USA) according to provided standard protocol. Oxidative stress as abundance of free thiol groups (RSH) was measured using Ellman's reagent as previously described (Simpson, 2008). Briefly, blood plasma samples were diluted in 300x volume of 0.1 M sodium phosphate buffer, pH 8.0 after which the absorption of each sample was measured at 412 nm wavelength. In the next step, 10x sample volume of Ellman's reagent was added to each sample and the absorption at 412 nm wavelength was measured until it plateaued.

### Blood Pressure and Heart Rate

Blood pressure and pulse were measured using an automated sphygmomanometer Boso Medicus Uno (Bosch & Sohn GmbH, Juningen, Germany) and palpating the radial artery respectively after a 5-min rest in a seated position, with legs uncrossed, the back and arms in an upright position.

### Blood Sampling and Storage

Blood sampling was performed during the morning (7:00 a.m.–8:30 a.m.) after an overnight fast. Before blood sampling, participants remained in the seated position for at least 10 min. Samples were collected as plasma, serum, whole blood and PBMCs fractions and either analyzed immediately after the blood drawing (for clinical chemistry, endocrinology and immunotyping) or stored at -80°C until the measurements (metabolomics, proteomics, inflammation, aging biomarkers).

### Bone Densitometry and Body Composition

Bone density was measured using dual-energy X-ray absorptiometry (DXA) using GE Lunar iDEXA (GE Healthcare) according to the departmental standard operating procedure. Body regions are defined using standard anatomical partitions. Scan areas are analyzed to determine lean mass, fat mass, bone mass, bone mineral density and total body composition.

### Energy Expenditure and Calorie Intake

Physical activity (total energy expenditure) was measured using a three-axial acceleration sensor (adxl345, Analog Devices; range: ±8 g; sampling rate: 64 Hz; resolution: 12 bit). The raw data from this device were analyzed using the associated software DataAnalyzer (Movisens GmbH, Karlsruhe, Germany).

Resting energy expenditure was measured for 30 minutes using a mobile oxygen analyzer (MetaMax 3b; Cortex Biophysik, Leipzig, Germany). The analyzer was warmed up and calibrated according to the manufacturer instructions before each measurement.

On the basis of the FFQ (Food Frequency Questionnaire) the size of the portion (mg for solid food and ml for liquid food) and the consumption frequency were evaluated. Subsequently, caloric intake (kcal) was calculated on the basis of a German nutritive value database (www.naehrwertrechner.de).

### Insulin sensitivity indices

The calculation of insulin sensitivity indices has been described in the designated study protocol paper in detail (Tripolt et al., 2018). Briefly, the following indices were calculated:

The Matsuda Index (ISOGTT),

$$ISoGTT = \frac{10000}{\sqrt{(glucose0 * insulin0) * (mean\ glucose * mean\ insulin)}},$$

the HOMA-IR (homeostasis model assessment for insulin resistance),

$$HOMA - IR = \frac{FPG(mmol/l) * FSI(U/l)}{22.5},$$

the QUICKI,

$$QUICKI = \frac{1}{\log(insulin0) + \log(glucose0)},$$

and the insulin sensitivity index (ISI),

$$(ISI = 0.222 - 0.00333 * BMI - 0.0000779 * Ins120 - 0.000422 * age).$$

### Mass Spectrometric Proteome Analysis

PBMCs ($3x10^6$ per aliquot) were treated with 50 μl lysis buffer (100 mM ammonium bicarbonate, pH 7.5, containing 1% sodium deoxycholate and 1 mM DTT) and incubated on ice for 30 min. After centrifugation iodoacetamide (final concentration 5.5 mM) was applied to the supernatant for alkylation and incubated for 10 min at room temperature. Protein digestion was performed overnight at 37°C using 1.3 μg trypsin. Afterwards, trifuoroacetic acid was added to precipitate access of sodium deoxycholate. Supernatant containing peptide mixtures were processed on STAGE tips (1) and analyzed by LC-MS/MS. The LC-MS measurements were performed on a QExactive HF-X mass spectrometer coupled to an EasyLC 1200 nanoflow-HPLC. Peptides were separated on fused silica HPLC-column tip (I.D. 75 μm, New Objective, self-packed with reprosil-Pur 120 C18-AQ, 1.9 μm (Dr. Maisch) to a length of 20 cm) using a gradient of A (0.1% formic acid in water) and B (0.1% formic acid in 80% acetonitrile in water).The mass spectrometer was operated in the data-independent mode; after each survey scan (mass range m/z = 350 – 1,200; resolution: 120,000) 28 DIA scans with an isolation width of 31.4 m/z were performed covering a total range of precursors from 350-1,200 m/z. AGC target value was set to 3 x 106, resolution to 30,000 and normalized collision energy to 27%. The MS raw files were analyzed using Spectronaut Pusar X software version 12.0 with standard settings (2) using a home-made spectral library generated from two DDA experiments (in total 25 runs) made from PMDCs containing about 100,000 precursors from about 7,000 proteins.

Relative intensities of proteins isolated from PBMCs of donors fasting for 36 h were divided by protein intensities from PBMCs of the same donors fasting for 12 h to study cell responses to prolonged fasting. Respective protein ratios were normalized to sample intensity medians and $\log_2$ transformed. All proteins that were quantified in less than 3 out of 18 donors were removed. Missing values were imputed using a normal distribution (width 0.3, down shift 1.8), and resulting ratios were z-score normalized based on sample IDs. Hierarchical clustering was performed using Euclidean distance. Enrichment analyses of GO terms of proteins in single clusters compared to the entire dataset were performed using Fisher's exact test (FDR<0.04, BH corrected).

### Metabolite Analysis by HPLC/MS

Samples were randomly divided into three batches for extraction and work-up was done on three consecutive days. On each day, samples designated for extraction were thawed at room temperature, vortexed shortly and 100μl aliquots were prepared in protein LoBind Eppendorf tubes. 400μl of cold methanol (stored at -80°C for 4 hours prior to extraction) were pipetted onto the samples and the mix was vortexed for ~20 seconds. After incubation overnight at 80°C samples were centrifuged for 10 minutes at 14.000g at 4°C and the supernatants transferred to a new protein LoBind Eppendorf tube. Supernatants were dried under nitrogen and stored at -80°C until all three batches were finished. Extraction and drying times were kept to a maximum difference of 10 minutes. All samples were reconstituted in 100μl 30% methanol-70% H2O, vortexed for ~45 seconds and centrifuged for 5 minutes at 14.000g at 4°C. The supernatant was divided into two aliquots (40μl each) and 20μl of each sample were pooled for quality control (QC). All samples were frozen at -80°C prior to measurement.

All samples were measured in one analytical run. Every 24h samples were freshly thawed at room temperature and added to the autosampler at 4°C. Samples were measured randomized with blanks (30%-methanol-70% $H_2O$), QC and UltimateMix (UM) at set intervals.

Sample analysis was done with a Dionex Ultimate 3000 HPLC setup (Thermo Fisher Scientific, USA) equipped with a $NH_2$-Luna HILIC analytical column and crudcatcher. Injection volume was 10 μl and metabolite separation was achieved with a 37-min gradient: aqueous acetonitrile solution [(5% acetonitrile v/v), 20mM ammonium acetate, 20-mM ammonium hydroxide, pH 9.45] was used as eluent A (LMA) and acetonitrile as eluent B (LMB). Mass spectrometric detection was carried out with a Q-Exactive™ system (Thermo Fisher Scientific). Electrospray ionization (ESI) was used for negative and positive ionization and masses between 70 and 1100 m/z were detected.

After sample analysis, raw data were converted into mzXML by msConvert (ProteoWizard Toolkit v3.0.5) (Chambers et al., 2012), and known metabolites were searched for with the tool PeakScout (developed in-house; as previously published in (Fröhlich et al., 2016) or (Mueller et al., 2017)) using a reference list containing accurate mass and retention times acquired via reference substances. Statistics were performed with R [R Core Team] (v3.4.1, packages stats, FactoMineR, missMDA, nlme, lsmeans, readxl, openxlsx) and TibcoSpotfire (v7.5.0).

### NMR-Measurement of Serum β-Hydroxybutyrate

Methanol and hydrochloric acid (37 % m/v) were obtained from Carl Roth (Karlsruhe, Germany). Deuterium oxide ($D_2O$) was obtained from Sigma Aldrich (Saint Louis, MO). Sodium phosphate, dibasic ($Na_2HPO4$), sodium hydroxide, and sodium azide ($NaN_3$) were obtained from VWR International (Darmstadt, Germany). 3(trimethylsilyl)propionic acid-2,2,3,3-d4 sodium salt (TMSP) was obtained from eurisotop (Saint-Aubin, France). Deionized water was purified using an in-house Milli-Q® Advantage Water Purification System from Millipore (Schwalbach, Germany). All chemicals were used with no further purification. The phosphate buffer solution was prepared by dissolving 5.56 g of anhydrous $Na_2HPO_4$, 0.4 g of TMSP, and 0.2 g NaN3, in 400 mL of deionized $D_2O$ and adjusted to pH 7.4 with 1M NaOH and HCl. Upon addition of $D_2O$ to a final volume of 500 ml the pH was readjusted to pH 7.4 with 1M NaOH and HCl.

To remove serum proteins and to quench enzymatic reactions in the samples 200 μL serum were mixed with 400 μL methanol and stored at -20°C until further processing. Afterwards the samples were spun at 17949 rcf at 4°C for 30 minutes. Supernatants were lyophilized and 500 μL of NMR buffer in $D_2O$ were added to the lyophilized samples, which were then re-dissolved and transferred to 5 mm NMR tubes. Metabolites were measured as described previously (Lorenz et al., 2018) and detailed below.

All NMR experiments were performed at 310 K on a Bruker Ultrashield Plus 600 MHz Avance Neo spectrometer equipped with a TXI probe head. The 1D CPMG (Carr-Purcell–Meiboom–Gill) pulse sequence (cpmgpr1d, 512 scans, 73728 points in F1, 12019.230 Hz spectral width, 1024 transients, recycle delay 4 s), with water suppression using pre-saturation, was used for 1H 1D NMR experiments.

Bruker Top-spin version 4.0.2 was used for NMR data acquisition. The spectra for all samples were automatically processed (exponential line broadening of 0.3 Hz), phased, and referenced to TSP at 0.0 ppm using Bruker Topspin 4.0.2 software (Bruker GmbH, Rheinstetten, Germany). Spectra were imported into MestreNova 12.0.2 in order to quantify metabolites of interest.

### Omics Analysis

The PBMC proteome was analyzed for Gene Set Enrichment Analysis (GSEA) by, first, mapping the protein dataset to their formal gene symbol, and then normalizing by log-z transform to fit a normal distribution. The normalized fold-change is the input for parametric analysis of GSEA. Statistical comparison of the enrichment of each gene or protein expression change value vs. the total gene or protein expression change value is performed to obtain the corresponding p-value and to calculate each GO Term's aggregation Z score, as follows:

$$Z\ score\ (selected\ gene\ set) = \frac{expression\ change\ selected\ gene\ set - \ expression\ change\ of\ whole\ dataset}{\sigma_{expression\ change\ selected\ gene\ set - expression\ change\ whole\ dataset}}$$

Using the above calculated p-value we estimate the false discovery rate (FDR) to correct for multiple comparison error accumulation. The following statistical cut-off for significance of gene/protein enrichment were adopted: t-test p value $\leq 0.05$; at least 3 enriched gene/protein members; FDR $\leq 0.05$.

Metabolite profiles were analyzed using MetaboAnalyst versions 3.0 and 4.0 (Chong et al., 2018; Xia and Wishart, 2016), utilizing the Metabolite Set Enrichment Analysis (MSEA) module. Reactome analysis of the full dataset proteome-metabolome was performed with the OmicsNet module of MetaboAnalyst.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Data is visualized as Tukey box plots (line at median, x at mean), if not stated otherwise. Statistical calculations were performed using Graphpad Prism 7.0 (Graphpad software, La Jolla, Calif., USA). The D'Agostino & Pearson normality and Shapiro-Wilk tests were used to check for data distribution. For parameters with non-gaussian distribution, a nonparametric Mann-Whitney test was used. For normally distributed data, a two-sided student's t-test was applied. When applicable, a paired student's t-test or a nonparametric Wilcoxon matched-pairs signed rank test was applied. A *P*-value of <0.05 was considered statistically significant. Metabolomic and proteomic analyses were statistically corrected for multiple comparisons.

RCT dropouts (2 in the control group, 1 in the ADF intervention group) were excluded from the analyses of the RCT. However, their baseline data are included in the analysis when comparing the long-term ADF group to a control group (the participants of this control group were then randomized into control and intervention groups of the RCT).

Other missing values that occurred, e.g., due to technical problems during one of the visits were not imputed. In baseline and follow-up calculations, all available data (except for the three dropouts mentioned) were included. Due to some additional missing values during baseline or follow-up, the *n*(delta) may be smaller in comparison to *n*(baseline) or *n*(follow-up), which explains the slight discrepancy of some absolute values compared to the deltas in Table 1.

The deltas were calculated as the average of (value[follow-up] - value[baseline]) for every study participant with paired measurements available (i.e., baseline and follow-up).

## DATA AND CODE AVAILABILITY

All data needed to evaluate the conclusions of the paper are included in the article or the Supplemental Information.

## ADDITIONAL RESOURCES

ClinicalTrials.gov Identifier: NCT02673515.