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Prolonged fasting promotes systemic inflammation and platelet activation in humans: a medically supervised, water-only fasting and refeeding study

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SUMMARY

Prolonged fasting (PF), defined as abstaining from energy intake for ≥4 consecutive days, has gained interest as a potential health intervention. However, the biological effects of PF on the plasma proteome are not well understood. In this study, we investigated the effects of a medically supervised water-only fast (mean duration: 9.8 ± 3.1 days), followed by 5.3 ± 2.4 days of guided refeeding, in 20 middle-aged volunteers (mean age: 52.2 ± 11.8 years; BMI: 28.8 ± 6.4 kg/m²). Fasting resulted in a 7.7% mean weight loss and significant increases in serum beta-hydroxybutyrate (BHB), confirming adherence. Untargeted high-dimensional plasma proteomics (SOMAScan, 1,317 proteins) revealed multiple adaptations to PF, including preservation of skeletal muscle and bone, enhanced lysosomal biogenesis, increased lipid metabolism via PPARα signaling, and reduced amyloid fiber formation. Notably, PF significantly reduced circulating amyloid beta proteins Aβ40 and Aβ42, key components of brain amyloid plaques. In addition, PF induced an acute inflammatory response, characterized by elevated plasma C-reactive protein (CRP), hepcidin, midkine, and interleukin 8 (IL-8), among others. A retrospective cohort analysis of 1,422 individuals undergoing modified fasting confirmed increased CRP levels (from 2.8 ± 0.1 to 4.3 ± 0.2 mg/L). The acute phase response, associated with transforming growth factor (TGF)-β signaling, was accompanied by increased platelet degranulation and upregulation of the complement and coagulation cascade, validated by ELISAs in blood and urine. While the acute inflammatory response during PF may serve as a transient adaptive mechanism, it raises concerns regarding potential cardiometabolic effects that could persist after refeeding. Further investigation is warranted to elucidate the long-term molecular and clinical implications of PF across diverse populations.

INTRODUCTION

Prolonged fasting (PF), defined as abstaining from energy intake for ≥4 consecutive days(1), has been practiced throughout history for cultural, spiritual, and health-related reasons. Recently, it has gained renewed attention as a potential intervention to promote health and longevity by mitigating cellular aging, reducing inflammation, and lowering the risk of cardiovascular disease and cancer.(2, 3) However, the systemic biological adaptations to PF and its effects on inflammation remain unclear. Advances in high-throughput proteomics now enable the simultaneous measurement of thousands of plasma proteins with high specificity, providing a unique opportunity to investigate molecular adaptations to fasting and refeeding. Such an approach addresses the limitations of earlier studies, which were constrained to examining only a small number of specific preselected proteins.

In this study, we examined 20 volunteers attending a fasting clinic before, during, and after an average 10-day water-only fast, followed by an average of 5 days of supervised refeeding with a plant-based diet.

Using untargeted plasma proteomics with SOMAScan, we identified both potential benefits and drawbacks of PF and refeeding at the molecular level. PF triggered a significant shift in 6.6% of the plasma proteome; however, less than 1% of these proteins remained significantly altered after refeeding, supporting a transient effect. Contrary to our hypothesis, the primary outcome was a significant increase in inflammation and cytokine signaling via TGF-β, confirming that PF elevates inflammation. Additionally, we observed alterations in neutrophil and platelet degranulation, along with well-documented changes in IGF and PPARα signaling, key regulators of growth and lipid metabolism, respectively. Limited nutrient availability activated the PTEN, STAT3, and MAPK pathways, critical signal transducers that regulate cell proliferation in response to external stimuli. Notably, this study is the first to report that PF significantly reduced amyloid fiber formation and lowered circulating amyloid beta proteins Aβ40 and Aβ42, even though it did not affect their ratio. The findings were validated through targeted mass spectrometry and ELISAs in blood and urine samples, as well as in two external cohorts undergoing similar PF regimens. While PF is commonly associated with health benefits such as weight loss, our findings suggest its effects are more complex and multifaceted, with potential physiological benefits and drawbacks that require an individualized approach to fasting interventions.

RESULTS

We recruited 20 middle-aged volunteers (mean age: 52.2 ± 11.8 years; mean BMI: 28.8 ± 6.4 kg/m²), including 11 women and 9 men. Participants were approached by the study team at TrueNorth Health Center, a facility offering medically supervised fasting. The study team operated independently from the Center. Volunteers followed a fasting and refeeding regimen, consisting of an average 9.8 ± 3.1-day water-only fast, followed by an average 5.3 ± 2.4 days of guided refeeding (Methods). Both fasting and refeeding procedures were based on previously established protocols.(4, 5) Blood and urine samples were collected between 6 and 8 am to minimize diurnal variability, processed immediately, and stored at -80°C for analysis. We conducted an untargeted high-dimensional proteomic analysis using SOMAScan, measuring plasma levels of 1,317 proteins. (6) In addition, targeted mass spectrometry and ELISAs were employed to quantify specific biomarkers in blood and urine. The results were compared with two independent datasets of PF and modified fasting in humans, from Pietzner et al. (2024) and Wilhelmi de Toledo et al. (2019).

At baseline, the average body weight of the volunteers was 85.6 ± 25.6 kg in women and 87.9 ± 15.4 kg in men. By the end of the fasting period, participants experienced significant weight loss, with women losing 6.3 ± 1.7 kg and men losing 6.9 ± 2.2 kg ($p < 0.0001$), corresponding to reductions of 7.6% and 7.8% of

baseline body weight, respectively. BMI decreased by an average of 2.2 ± 0.5 kg/m² ($p < 0.0001$), a fractional decrease of 7.6%, while waist circumference was reduced by 6% ($p < 0.0001$). These reductions in body weight, BMI, and waist circumference persisted through the refeeding period (**Table 1**). Mild adverse events were common, including headaches, weakness, fatigue, insomnia, dry mouth, and orthostatic hypotension (**Fig S1**), prompting the transition to a broth and/or juice fast in six participants. Other adverse events included severe abdominal pain and diarrhea (n=1), hypokalemia (n=1), arrhythmias (n=1), and dizziness and palpitations (n=1). When feasible, blood and urine samples collected before this transition were used for analysis (**Fig S2**). Adherence was high, with all participants exhibiting a physiological fasting response, as evidenced by significantly elevated serum beta-hydroxybutyrate (BHB) concentrations ($p < 0.0001$), which normalized during refeeding (**Table 1**). The fasting-induced increase in BHB and its normalization with refeeding were significantly correlated with changes in inflammatory markers (midkine and IL-8), metabolic regulators (FGF19, leptin receptor, chemerin, growth hormone receptor), and MAPK signaling, a crucial mediator of cell proliferation (**Fig S3**).

Proteomics adaptations to prolonged fasting in humans

We found that 6.6% of protein targets (n = 86/1317) exhibited significant changes by the end of fasting (adjusted $p < 0.05$), with 74 proteins decreasing and 12 increasing (**Figure 1A**). However, after five days of gradual refeeding, only 12 proteins (<1%) remained significantly altered, indicating that most fasting-induced proteomic changes are transient. The most significantly reduced proteins included key regulators of muscle homeostasis, such as inhibin beta A (INHBA, -3.3 fold, adjusted p = 9.07E-05), myostatin (-2 fold, adjusted p = 0.000466), and GDF11/8 (-1.6 fold, adjusted p = 0.000757), all members of the TGF-β superfamily (**Figure 1B**). These proteins are vital for muscle regulation, likely reflecting the body's adaptive response to fasting, balancing muscle preservation with tissue repair during nutrient deprivation.(7) Interestingly, inhibiting GDF11 and myostatin has been linked to increased bone density and strength through enhanced osteoblast activity and suppressed osteoclastogenesis.(8, 9) Consistent with this, plasma parathyroid hormone (PTH) levels decreased by 2.1-fold (adjusted p = 0.0045), suggesting a compensatory hormonal adjustment during fasting to slow bone loss. Under conditions of energy deprivation, the insulin-sensitizing adipokine adiponectin also decreased from 5643 ± 3282 ng/mL to 4275 ± 2519 ng/mL ($p < 0.0001$) (**Figure 1C**).

In contrast, proteins involved in energy, glucose, and bile acid metabolism significantly increased, with PPARα emerging as a key activated pathway (**Figure 1D, Fig S4**). Regulated by free fatty acids, PPARα

drives hepatic lipid metabolism and ketogenesis, a vital fasting adaptation. Another major fasting-activated pathway was the CLEAR (Coordinated Lysosomal Expression and Regulation) network, which governs lysosomal biogenesis and function. This pathway is crucial for autophagy and exocytosis, both essential for cellular maintenance and adaptation to nutrient scarcity. The gut hormone FGF19 also increased 1.8-fold (adjusted p = 0.048) (**Figure 1B**), playing a crucial role in energy metabolism by regulating bile acid synthesis, enhancing glucose utilization, and promoting hepatocyte proliferation.(10) In addition, the soluble leptin receptor increased significantly (2.2-fold, adjusted p = 0.002), facilitating appetite regulation by regulating leptin bioavailability in the bloodstream.(11, 12) The significant increases in FGF19 and the soluble leptin receptor were previously reported in an independent cohort undergoing a 7-day water-only fast by Pietzner et al. (2024). (13) This comparative cohort of 12 participants experienced an average weight loss of 5.7±0.8 kg, representing a 7.4% reduction in baseline body weight. Comparative proteomics analysis of both cohorts at the 7-day endpoint identified an overlap of 44 significantly decreased and 5 significantly increased proteins, with no discrepancies between studies (**Fig S5**). Despite methodological differences (Olink vs. SOMAScan), Reactome pathway enrichment analysis revealed that most altered pathways were common to both datasets, particularly those related to neutrophil and platelet degranulation, as well as interleukin, MAPK, and PI3K/AKT signaling (**Fig S6**). The findings highlight the highly conserved and universal nature of the physiological response to water-only PF.

Additionally, PF was associated with a reduction in synaptogenesis pathways and amyloid fibril formation (**Figure 1D, Fig S4**). An increasing trend in brain-derived neurotrophic factor (BDNF) levels was observed (1.32-fold, adjusted p = 0.18), which may support previous evidence of fasting's neuroprotective effects.(14) While the predicted decrease in fibrillar formation was not specific to amyloid beta proteins, we hypothesize that it could reflect lower circulating amyloid beta levels. To investigate this, plasma levels of amyloid beta (Aβ) 42, 40, and the Aβ42/Aβ40 ratio - a diagnostic biomarker for brain amyloid plaques (15)- were measured using mass spectrometry. (16, 17) Interestingly, PF significantly reduced plasma concentrations of both Aβ42 and Aβ40 (**Figure 1E-G**), suggesting either a decreased production rate or accelerated degradation of these amyloid peptides during fasting, with levels returning to baseline after refeeding. Even though the reduction in individual Aβ components may have potential beneficial implications for amyloidosis, the Aβ42/Aβ40 ratio, which is the validated biomarker used clinically to identify individuals with brain amyloid plaques (18, 19), remained unchanged.

Prolonged fasting increases inflammation

The primary outcome of our study was inflammation. SOMAScan plasma proteomics analysis revealed significant increases in well-established inflammatory markers, including hepcidin, ferritin, midkine, matrix metalloproteinase 9, IL-8, and platelet-activating factor acetylhydrolase (PAFAH or PLA2G7) (**Figure 2A-B**). Contrary to our initial hypothesis that fasting would exert an anti-inflammatory effect, PF led to a pronounced 129% increase in circulating high-sensitivity C-reactive protein (hsCRP) levels measured by ELISA (Wilcoxon's p = 0.0004, ANOVA p = 0.0070), with levels returning to baseline after refeeding in all but one participant (**Table 1, Figure 2C**). The significant rise in hsCRP was positively correlated with C5 (involved in inflammation) and LILRB2 (regulating inflammation and axonal regeneration), and negatively associated with PCI (SERPINA5), hemojuvelin, and MED1 (adipogenesis) (**Figure 2D**). At the pathway level, CRP was significantly associated with the TGF-β signaling pathway and the complement and coagulation cascade (**Figure 2E**), suggesting that PF may activate the innate immune response through inflammation. To validate these findings in a broader population, we retrospectively analyzed data from 1,422 individuals who underwent medically supervised modified fasting at the Buchinger-Wilhelmi Clinic in Germany.(5) In this comparative cohort, the average fasting duration was 8.2 ± 0.1 days, with an average weight loss of 4.3 ± 2.0 kg. Notably, 66.6% of participants experienced a significant increase in plasma CRP levels (**Figure 2F**), confirming the acute inflammatory effect of PF across a larger cohort. Importantly, this increase in CRP was observed regardless of fasting duration (5, 10, 15, or 20 days).

We also observed a significant increase in liver transaminases AST and ALT during fasting, with levels rising by 65% and 64%, respectively, and remaining elevated during refeeding (**Table 1**). This elevation in liver enzymes, indicative of hepatic stress, was also observed in the Buchinger-Wilhelmi Clinic validation cohort (**Fig S7**). The concurrent rise in liver enzymes and inflammatory markers highlights the need for medical monitoring of individuals undergoing PF interventions.

Fasting and refeeding elevate biomarkers of platelet activation and degranulation

An unexpected finding was the observed increase in biomarkers and pathways associated with platelet activity during PF (**Figure 3**). Reactome pathway enrichment analysis of the SOMAScan data revealed that PF influenced platelet degranulation, a process that facilitates thrombin generation by releasing fibrinogen and von Willebrand factor (vWF) from alpha granules at injury sites.(20) Even though prothrombin levels remained unchanged (1-fold, adjusted p = 0.85), vWF and its receptor, soluble glycoprotein Ib alpha (GP1Bα), were mildly increased (**Figure 3A**), correlating with elevated chemokines

(e.g., IL-8, CCL7, CCL11) (**Figure 3B**). To confirm platelet degranulation, we measured urinary 11-dehydro-TXB2 levels via ELISA, an enzymatic product in the TXA2/TXB2 pathway primarily derived from activated platelets via cyclooxygenase-1 activity. Surprisingly, 11-dehydro-TXB2 levels rose by 21% during fasting and 36% post-refeeding (**Figure 3C**), with no change in platelet counts (**Figure 3D**), indicating that increased degranulation, rather than heightened platelet production, drove the effect.(21) Urinary 11-dehydro-TXB2 is a gold standard biomarker of platelet activation and cardiovascular risk.(22) The Framingham study highlights its predictive value for all-cause mortality, cardiovascular death, and major arterial events.(23) In the ASCEND trial, it was significantly associated with future vascular events in nearly 8,000 diabetic participants.(24, 25) Therefore, our findings reveal a PF-induced phenotype characterized by interconnected inflammation and platelet activation, potentially affecting thrombotic risk in individuals with pre-existing conditions.

Prolonged fasting affects cardiometabolic biomarkers

In our study, PF induced significant changes in lipid profiles, including increases in plasma total cholesterol, non-HDL cholesterol, LDL cholesterol, and the total cholesterol/HDL ratio, all of which reversed after refeeding (**Table 1**). Plasma triglycerides steadily increased, peaking with a 32% rise post-refeeding. Proteomics analysis revealed a significant 1.49-fold reduction in proprotein convertase subtilisin/kexin type 9 (PCSK9) (adjusted p = 0.008) at the end of fasting (**Figure 1B**). This reduction likely decreased PCSK9 binding to LDL receptors, thereby preventing their degradation in liver cells.(26)

In addition to lipid changes, serum glucose levels decreased from 85.7 mg/dL to 70.3 mg/dL (18%) during fasting (**Table 1**), reflecting the rewiring of whole-body metabolism upon depletion of glucose stores. Similarly, the adipokines chemerin (-1.7-fold, adjusted p = 0.0002) and fetuin B (FETUB) (-1.8-fold, adjusted p = 6.05E-05) were also decreased (**Figure 1A-B**).(25, 26) During refeeding, glucose levels and HOMA-IR, an indicator of insulin resistance, increased significantly, reflecting enhanced glucose availability with the reintroduction of food.

Fasting and refeeding do not induce systemic changes in oxidation status

While fasting has been suggested to reduce oxidative stress in animal models (27), our proteomic analysis revealed a decrease in superoxide dismutase 3 (SOD3) levels (-1.3-fold, adjusted p = 0.003), an extracellular antioxidant enzyme crucial for redox balance. Additionally, the expected improvements in *in vivo* oxidation status were not observed. Using the validated urinary biomarker of lipid oxidation 8-iso-

prostaglandin F2α (25, 28), we found a heterogeneous oxidative response to fasting and refeeding. The result suggests that PF does not universally reduce oxidative stress in humans (**Fig S8**).

DISCUSSION

Throughout human evolution, extended periods of food scarcity were common, shaping metabolic flexibility as a survival mechanism. In the context of the global obesity epidemic, fasting has resurged as a popular, sometimes extreme, weight-loss strategy. (2, 29) However, the body's adaptations to PF and its potential health effects remain poorly understood. Our study offers a comprehensive proteomic analysis of the responses to PF and refeeding, uncovering both beneficial and potentially detrimental effects. Consistent with previous research (13), we uncovered changes in multiple proteins involved in skeletal muscle and bone homeostasis (INHBA, myostatin, GDF11/8, PTH). Interestingly, exogenous GDF11 has been shown to function as a calorie restriction mimetic in mice, stimulating adiponectin secretion and improving insulin sensitivity(30). The acute inflammatory response triggered by PF warrants further investigation to clarify its clinical significance, and it is consistent with a clinical study demonstrating that a 10-day water-only fast triggers an inflammatory transcriptional signature in adipose tissue.(31) In our study, inflammation was accompanied by evidence of platelet degranulation, raising concerns as elevated urinary TXB2 has been linked to accelerated atherogenesis and increased cardiovascular risk.(24, 32) The inflammatory profile characterized by elevated hsCRP, IL-8, and activation of TGF-β and complement pathways resembles the hallmark features of trained immunity (33), suggesting that PF may serve as an endogenous trigger for this adaptive immune mechanism. However, the immune effects of PF are complex and context-dependent, with evidence supporting fasting-induced modulation of immunosenescence and immune response during immunotherapy.(34-37)

Prior studies of the adipose tissue transcriptome have linked inflammatory pathways to insulin resistance.(38-40) Consistently, our data show that PF is associated with elevated HOMA-IR. Additionally, increased triglycerides and liver transaminases suggest that prolonged nutrient deprivation, unlike moderate calorie restriction, may disrupt lipoprotein metabolism and liver function (41-44).

Furthermore, this study is the first to demonstrate that PF lowers plasma Aβ42 and Aβ40, key components of amyloid plaques implicated in Alzheimer's disease pathology.(45) These findings suggest that nutrient deprivation alters amyloid precursor protein (APP) expression or processing, influencing either the production or clearance of plasma Aβ42 and Aβ40. Importantly, PF did not affect the Aβ42/40 ratio, a

validated biomarker for brain amyloid plaques (18). This supports the notion that the Aβ42/40 ratio, rather than individual concentrations, is a robust biomarker, as it accounts for inter-individual variability in pre-analytical conditions (46) and presence of comorbidities.(47)

Our study has several strengths and limitations. Strengths include the use of two methodologies (mass spectrometry and ELISA) across biological samples (plasma and urine), which yielded consistent findings. Multiple biomarkers were assessed in an untargeted approach, reducing reliance on a single marker, and results were validated in two independent cohorts. Limitations include the single-arm design with a lack of control group, the small sample size, and the variability in fasting and refeeding durations decided by the volunteers.

In summary, our study reveals a multifaceted proteomic response to PF, extending beyond the traditional adipose-centric or energy homeostasis framework. We identified elevated biomarkers related to muscle and bone preservation, reduced amyloid formation, increased inflammation and platelet activity, and lipid metabolism. By conducting a comparative analysis in an independent cohort, we identify a universal signature of the physiological response to water-only PF, observing no differences between studies despite variations in cohort characteristics and methodology. However, we also observed substantial inter-individual variability at the molecular level, emphasizing the need for personalized fasting regimens. In contrast, oxidation status remained unchanged, with no evidence of antioxidant effects. The acute inflammatory response, also observed in an independent cohort, may reflect a positive adaptive mechanism. However, it also raises concerns about a potentially adverse cardiometabolic phenotype, particularly for individuals with thrombotic conditions or unstable atherosclerotic plaques. This mirrors data from exercise interventions, where acute vigorous physical activity can transiently increase cardiovascular risk, particularly in untrained individuals or those with underlying conditions. However, with appropriate progressive training, long-term beneficial adaptations occur, leading to reduced cardiovascular mortality.(48) Unlike exercise, where dose-response relationships and adaptations are well-established, our understanding of how repeated PF bouts impact long-term molecular, metabolic, and clinical outcomes remains limited, highlighting the need for further research.

Resource availability: All data are presented in the manuscript and supplementary materials, and are available upon reasonable request to the corresponding author.

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Declaration of interests: A.G. is the founder of TrueNorth Health Center, a private facility that offers medically supervised fasting interventions. K.M.K. and M.R.M. are employed by C_2N Diagnostics. The other authors report no conflicts of interest. The funding sources were not involved in any form with the findings presented in the study. The article was not commissioned. No author was precluded access to data.

FIGURE LEGENDS

Table 1. Anthropometric measures and cardiometabolic effects of prolonged fasting in humans. Data expressed as mean ± SD. Statistical significance was calculated using paired, 2-tailed Student's t or Wilcoxon signed rank test for non-normally distributed data. N = 20 participants, except for cholesterol measurements and triglycerides (N = 19). Significance levels are indicated as p-values.

Figure 1. Proteomics adaptations to prolonged fasting in humans. (A) Volcano plot of differentially expressed SOMAScan plasma proteins during fasting from N = 15 participants. Significance cut-off adjusted $p < 0.05$. FC = End of Fasting / Baseline. (B) Individual changes in highlighted proteins from (A) normalized to baseline during fasting and refeeding. Each dot represents protein levels in each participant (N = 15). Adjusted p-value calculated with one-way ANOVA. (C) Absolute levels of plasma adiponectin

measured by ELISA across three time points. Each dot represents levels in each participant (N = 20). (D) Volcano plot of differentially enriched canonical pathways in IPA with predicted activation (orange) or inhibition (blue). Input = 1,255 mapped SOMAScan proteins from (A). (E-F-G) Absolute levels of plasma Aβ42, Aβ40, and their ratio measured by IP-LC-MS/MS across three timepoints. N = 20 participants. Statistical analysis is described in the Methods for each analysis. Significance levels are indicated as adjusted p-values, *p < 0.05, **p < 0.01, and ***p < 0.001. For all graphs, BL = Baseline, EF = End of Fasting, ER = End of Refeeding.

Figure 2. Prolonged fasting increases inflammation. (A) All significantly upregulated SOMAScan proteins (n = 12) during fasting normalized to baseline (targets from volcano plot in Figure 2A). Pro-inflammatory proteins are shown with light red background. N = 15 participants. FC = End of Fasting / Baseline (B) Individual changes in 6 inflammatory proteins from panel A during fasting and refeeding. Each dot represents protein levels in each participant. (C) Absolute hsCRP levels in the blood of each participant (N = 20) measured by ELISA across three time points. (D) Significantly correlated proteomic targets to CRP changes during fasting and refeeding (positive effect size = same changes; negative effect size = inverse changes). (E) Significantly enriched KEGG pathways relative to CRP changes. (F) Validation of CRP changes in an independent fasting cohort of 1,422 participants. Measurements of weight and CRP at baseline (BL, blue) and end of fasting (EF, orange) timepoints. The same variables are plotted by fasting length category. 5d = 5 day fast, 10d = 10 day fast, 15d = 15 day fast, 20d = 20 day fast. Median, interquartile range, and outliers are shown, with notches representing the 95% confidence intervals. Statistical analysis is described in the Methods for each analysis. Significance levels are indicated as adjusted p-values, *p < 0.05, **p < 0.01, and ***p < 0.001. For all graphs, BL = Baseline, EF = End of Fasting, ER = End of Refeeding.

Figure 3. Fasting and refeeding elevate biomarkers of platelet activation and degranulation. (A) Individual changes in 4 platelet-associated proteins from SOMAScan normalized to baseline during fasting and refeeding. Each dot represents protein levels in each participant (N = 15). Adjusted p-value calculated with one-way ANOVA. (B) Volcano plot of vWF (effect size) on all 1,317 SOMAScan proteins during combined fasting and refeeding. Significance cut-off adjusted p < 0.01. N = 15 participants. (C) KEGG pathway enrichment analysis for proteins associated with vWF. Fold enrichments in KEGG pathway analysis are shown relative to fold changes for vWF. (D) Absolute TXB2 levels in the urine of each participant (N = 20) across three time points. (E) Absolute platelet counts in the blood of each participant (N = 20) across three time points. Statistical analysis is described in the Methods for each analysis.

Significance levels are indicated as adjusted p-values, *p < 0.05, **p < 0.01, and ***p < 0.001. For all graphs, BL = Baseline, EF = End of Fasting, ER = End of Refeeding.

Supplementary Table 1. Inclusion and exclusion criteria.

Supplementary Table 2. Medications.

Supplementary Figure 1. Adverse Events (AEs) during fasting and refeeding. AEs were assessed by a qualified medical practitioner.

Supplementary Figure 2. Sample collection time points. Blood and urinary samples were collected at baseline (BL), End of Fasting (EF), and End of Refeeding (ER) timepoints. Six participants (IDs 1, 6, 7, 12, 13, 21) switched from water-only fasting to juice and/or broth fasting following medical advice. For these participants, except for ID13, samples for End of Fasting were collected prior to the switch. Three participants (IDs 8, 14, 20) consumed 1-2 juice and/or vegetable broth during the fast. One participant (ID5) consumed juice daily during the fast.

Supplementary Figure 3. BHB association with inflammatory markers and cytokine and MAPK signalling. Volcano plots of BHB (effect size) on all 1,317 SOMAScan proteomics variables during (A) fasting and (B) combined fasting and refeeding. Significance cut-off adjusted p < 0.01. N = 15 participants. (C-D) KEGG pathway enrichment analysis for proteins associated with BHB. Fold enrichments in KEGG pathway analysis are shown relative to fold changes for BHB.

Supplementary Figure 4. Full list of IPA canonical pathways. Volcano plot of differentially enriched canonical pathways with predicted activation (orange) or inhibition (blue) using proteomics input from Figure 2A.

Supplementary Figure 5. Comparative proteomics between the SOMAScan dataset and the Olink dataset by Pietzner et al. (2024). (A) Venn diagram of shared proteins present in both datasets. (B) Significantly altered proteins in each dataset, according to each study's statistical methods. (C) List of significantly altered shared proteins (44 decreased in blue and 5 increased in red).

Supplementary Figure 6. Comparative Reactome pathway analysis between the SOMAScan dataset and the Olink dataset by Pietzner et al. (2024). Reactome pathway analysis comparison between (A) SOMAScan and (B) Olink proteomics datasets at fasting day 7 using Uniprot as the reference dataset (accessed October 2024). Pathways are colour matched. BH corrected p-values are shown (grey line).

Supplementary Figure 7. **Hepatic markers during fasting by Wilhelmi de Toledo et al. (2019).** Validation of ALT and AST changes in an independent fasting cohort of 1,422 participants. Measurements of ALT and AST at baseline (BL, blue) and end of fasting (EF, orange) timepoints. Median, interquartile range, and outliers are shown, with notches representing the 95% confidence intervals. Statistical analysis is described in the Methods for each analysis. Significance levels are indicated as adjusted p-values, *p < 0.05, **p < 0.01, and ***p < 0.001.

Supplementary Figure 8. Oxidative stress and lipid peroxidation status upon prolonged fasting. (A) SOD3 abundance from SOMAScan proteomics dataset across time points from N = 15 participants. (B) Absolute 8-iso-prostaglandin F2α levels in the urine of each participant (N = 20) across three timepoints. Statistical significance was calculated using Wilcoxon signed rank test for non-normally distributed data for all parameters. Dots represent study participants for all graphs. Significance levels are indicated as p-values.

STAR METHODS

STUDY PARTICIPANT DETAILS

The study protocol, approved by the institutional review board of the Marin General Hospital, Greenbrae, CA, USA, received written informed consent from volunteers. The study was performed per the principles in the Declaration of Helsinki. Volunteers were approached by the study team at TrueNorth Health Center, a private facility offering medically supervised fasting. The study team operated independently from the Center. Individuals at the Center were given the opportunity to volunteer for the study, and those interested were screened for eligibility. Out of 168 individuals screened for eligibility, 33 met the inclusion criteria, encompassing individuals of both genders, aged 18 or older, with a body mass index (BMI) of ≥ 20 kg/m^2. Exclusion criteria included any history of chronic disease, physical or psychiatric conditions, use of medications incompatible with fasting, or other factors such as alcoholism or life situations that could interfere with the intervention or compliance (**Supplementary Table 1**). Twenty participants (N = 20), with a baseline BMI of 28.8±6.4 kg/m^2 (range 21.1-50.3 kg/m^2) consented to and commenced the medically

supervised water-only fasting. Their average age was 52.2±11.8 years (range 31–72 years), with eleven being women. Detailed baseline characteristics are provided in **Table 1**. Before the study, ten individuals were not taking any medications, nine were using medications listed in Supplementary Table 2, and one used a CPAP at night. All individuals on medications (except volunteer 17, who continued using an estrogen cream) stopped their medications 1-4 days prior to the fast to allow baseline samples to be collected after the medication was discontinued. During fasting, two volunteers took medications prescribed by their doctor (fexofenadine, dichloralphenazone, acetaminophen, isometheptene) as needed, while 18 volunteers did not. During refeeding, one volunteer took medication (tamsulosin) for urinary pain. Therefore, the majority of samples were obtained without medication. However, we acknowledge that the pause in medication intake prior to baseline sample collection could have influenced the data. Fourteen individuals were non-smokers, five were former smokers, and one was a current smoker (volunteer 20, who smoked 4-5 times per week for 20 years). The former smokers include volunteer 04 (smoked for 4 years, 10 cigarettes/day, quit 10 years ago), volunteer 05 (smoked for 6 months, 1-2 cigarettes/day, quit 21 years ago), volunteer 06 (smoked for 10 years, 15 cigarettes/day, quit 19 years ago), volunteer 12 (smoked for 30 years, 10 cigarettes/day, quit 12 years ago), and volunteer 13 (smoked for 20 years, 15 cigarettes/day, quit 40 years ago). The primary objective was to determine whether PF significantly reduces inflammation, a process deeply involved in the pathogenesis of multiple age-associated chronic diseases and in the biology of aging itself. The primary outcome measure was circulating C-reactive protein (CRP) levels and other inflammatory markers at baseline and during fasting and refeeding phases.

Medically supervised, water-only fasting and refeeding protocol. The water-only fasting and refeeding protocol was conducted at TrueNorth Health Center in Santa Rosa, California, a specialized medical facility for prolonged water-only fasting. The protocol was administered by physicians who thoroughly examined participants' physical, neurological, and psychological health. However, all the data and measurements were independently collected by Dr. Serena Commissati from May to December 2017. Participants underwent a medical history review, urinalysis, complete blood count with differentials, and a comprehensive metabolic panel. Before fasting, participants consumed a diet of fresh raw fruits and steamed vegetables for at least two days. During fasting, participants remained at the facility, consuming a minimum of 1182 mL of water per day. Participants were instructed to avoid strenuous exercise, with only minimal physical activity (such as light walks and stretching) allowed. Medical staff closely monitored vital signs and symptoms twice daily, repeating urinalysis and blood tests weekly or as directed. Sample

collection was optimized to minimize heterogeneity in fasting duration as volunteers decided the number of fasting days, ranging from 7 to 16 days. Samples were collected at baseline, fasting day 7, last day of fasting, and last day of refeeding for each participant (**Supplementary Figure 2**). Fasting was discontinued based on symptom stabilization, patient request, or medical necessity. Gradual refeeding began post-fasting, starting with juice consumption on the first day, gradually introducing solid, whole-plant foods without added sugar, oil, and salt. Moderate exercise was reintroduced gradually, with clinicians providing twice-daily monitoring during the refeeding phase.

METHOD DETAILS

Anthropometrics. Anthropometric measurements included height, measured without shoes to the nearest 0.1 cm, and body weight, obtained on a balance scale in the morning after a 12-hour fast. Body Mass Index (BMI) was calculated by dividing body weight (in kilograms) by the square of height (in meters). Waist circumference was measured to the nearest 0.1 cm at the iliac crest level during minimal respiration.

Blood analyses. Venous blood was sampled after an overnight fast, and processed for storage at –80°C. The Core Laboratory for Clinical Studies at Washington University in St. Louis analyzed all serum samples. Technicians, unaware of the timepoint assignment, conducted assessments. High-sensitivity C-reactive protein (hsCRP) was measured using a particle-enhanced immunoturbidimetric assay (Roche cobas c501). Commercial Enzyme-Linked Immunosorbent Assay (ELISA) Quantikine kits (R&D System Inc, Minneapolis, MN) were used for measuring other hormones.

Proteomics. Blood proteome was conducted on a subset of 15 individuals arbitrarily selected using the SOMAScan protein array platform at baseline (BL), during fasting (day 7 and end of fasting (EF)), and after refeeding (ER). Plasma samples were processed by SomaLogics technicians following recommended standard protocols for SOMAScan Assay Human Plasma, as described elsewhere.(49, 50) A SOMAScan Quality Statement was provided for normalization and calibration. The assay profiled 1,317 protein analytes.

Plasma Aβ42 and Aβ40 quantitation. This immunoprecipitation liquid chromatography-tandem mass spectrometry (IP-LC-MS/MS) assay has been clinically and analytically validated (15-17, 19) and is performed in a CLIA-certified, CAP-accredited, ISO 13485-compliant laboratory at C_2N Diagnostics (St.

Louis, MO, USA). Briefly, plasma samples are spiked with known quantities of stable isotope labeled internal standard Aβ42 and Aβ40 proteins (r-Peptide, Watkinsville, GA), plasma Aβ isoforms are immunoprecipitated, enzymatically digested into Aβ42- and Aβ40-specific peptides that are separated using micro-flow liquid chromatography (Waters Corp., Milford, MA, USA), and sprayed into the source of a Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Fragment ions formed from endogenous and exogenous Aβ42 and Aβ40 peptides are monitored, their peak areas are quantified and compared to the same peak areas monitored in a series of four calibrators formulated in human serum albumin, processed and analysed (as above) in parallel with the human plasma samples. In plasma, the peak area ratios for endogenous and internal standard peptides are compared to the same for the calibration standard curve, and plasma Aβ42 and Aβ40 concentrations are obtained from the respective standard curve. Plasma Aβ42/40 ratio is calculated by dividing the plasma Aβ42 concentration by the Aβ40 concentration (both in pg/mL).

Urine measurements. The 11-dehydro-thromboxane(TX)B_2 is one of the major urinary enzymatic metabolite of TXA_2/TXB_2 in humans and an index of *in vivo* platelet activation(22). The urinary 8-iso-prostaglandin (PG)$F_{2\alpha}$ is a non-enzymatic, oxidation product of arachidonic acid and an *in vivo* biomarker of lipid peroxidation(51). Briefly, urine samples were thawed, centrifuged, 2000 cpm of ^{3}H-PGE_2 (3.70-6.86 TBq/mmol, Perkin Elmer, Boston, USA) were added to 1 mL-urine samples that were loaded onto a 1 mL/50 mg C18 column (BakerbondTM-spe, J.T.Baker, Gliwice, Poland) and eluted with 2.5 mL of isooctane/ethyl acetate (1:1, vol/vol). The eluate was then transferred to a 1 mL/100 mg SiOH column (BakerbondTM-spe, J.T.Baker) and eluted with 2 mL of ethyl acetate/methanol (60:40, vol/vol), dried and resuspended in 1 mL of PBS/0.1% BSA buffer for subsequent immunoassay and recovery count. Biomarkers were measured with a standard ELISA as previously described(52) using specific antibodies(53). Urinary creatinine was measured with a commercial kit (Creatinine Colorimetric Detection Kit; Enzo Life Sciences, Farmingdale, NY). The final value of each biomarker was corrected for the percentage of recovery based on the ^{3}H-PGE_2 cpm and expressed as pg/mg of creatinine.

Buchinger-Wilhelmi Clinic study participants. The Buchinger-Wilhelmi Clinic (BWC) study, as outlined by Wilhelmi de Toledo et al. in 2019,(5) involved 1422 participants aged 18-99 years. Ethics approval for the original study (German Clinical Trials Register ID: DRKS00010111) was obtained by Wilhelmi de Toledo et al., with approval granted by the medical council of Baden-Württemberg and the Ethics Committee of the Charité-University Medical Center, Berlin. The original study was performed following the Declaration of

Helsinki and written informed consent was obtained from participants. The individuals, without predefined contraindications to Buchinger fasting, voluntarily joined the clinic for preventive or therapeutic fasting. Fasting durations ranged from 4 to 21 days, with participants categorized into fasting lengths of 5, 10, 15, and 20 days for analysis. Fasting guidelines included a daily intake of 200–250 kcal and 25–35 g of carbohydrates, obtained from fruit juice and vegetable soup, along with 3 liters of water or non-caloric herbal teas. This study independently analyzed published, publicly available data from the original study.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis. The SOMAScan proteomics dataset included N = 15 participants at baseline, fasting day 7, and last day of fasting and refeeding. All other assays reported in this manuscript included N = 20 participants. Statistical analyses for all variables were performed using pairwise differences between baseline, fasting (either day 7 or last day of fasting), and last day of refeeding assessed using Student's t-test (2 timepoints, normal data), Wilcoxon signed-rank test (non-normally distributed data), and one-way ANOVA (3 timepoints). Changes were expressed as absolute quantities, relative quantities normalized to baseline, or fold-change (FC) relative to baseline. All statistical tests were two-tailed, and significance was considered at adjusted p-value < 0.05, unless specified otherwise. The same statistical approach was applied to analyze data from the previously published Buchinger-Wilhelmi cohort. Data were analyzed and visualizations were produced with GraphPad Prism (version 10), QIAGEN IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA, accessed October 2024), FunRich: Functional Enrichment analysis tool (version 3.1.4, FunRich :: Functional Enrichment Analysis Tool :: Home), and Excel.

The correlation between BHB, CRP, and vWF with the circulating SOMAScan proteome was examined using mixed-effect regression models for longitudinal data, with individuals as the random effect. A False Discovery Rate (FDR) correction for multiple testing was applied to control for the type I error rate. Pathway enrichment analyses utilized the pathfindR package,(54) mapping significant ($p < 0.01$) proteins into active sub-networks based on the reference protein-protein interaction database. The active subnetworks were filtered based on the number of significant genes and their interaction likelihood scores. Finally, the list of subnetworks was used as the input for the enrichment analyses. We used the BioGrid (https://thebiogrid.org/) database as the reference for protein-protein interactions, and the KEGG database (https://www.genome.jp/kegg/) for biological pathways. We defined pathways with FDR-adjusted $p < 0.01$ as statistically significant. All analyses and graphs were performed using the open-source R software version 4.2.1 and RStudio.

Ingenuity Pathway Analysis. Alterations in canonical pathways were generated with IPA software (QIAGEN Inc., https://www.qiagenbio informatics.com/products/ingenuity-pathway-analysis, October 2024) using the SOMAScan proteomics dataset as an input (n = 1,317 proteins), including protein identifiers (Uniprot), fold-changes (fasting/baseline), and adjusted p-values. IPA mapped 1,255 entities (1,255/1,317 = 95%) that were analyzed using Core Analysis Expression Analysis based on log2(FC) values. The adjusted p-value cutoff was 0.05, producing 82 analysis-ready molecules (71 downregulated and 11 upregulated). The full list of significantly altered canonical pathways and details of synaptogenesis and amyloid fiber formation are presented in Supplementary Material.

Reactome pathway analysis. Alterations in Reactome pathways were generated with FunRich software (FunRich :: Functional Enrichment Analysis Tool :: Home, October 2024) using the SOMAScan proteomics dataset (n = 1,255 proteins) as an input, including protein identifiers (Uniprot), fold-changes (fasting/baseline), and adjusted p-values. The Reactome pathways database mapped 1,081 proteins and calculated the percentage of proteins per pathway (No. of genes in the dataset divided by No. of genes in the Uniprot background dataset) and the -log10(adjusted p-value). Significance threshold < 0.05.

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	Baseline Mean	SD	Fasting Mean	SD	P-value (Fasting vs Baseline)	Refeeding Mean	SD	P-value (Refeeding vs Baseline)
Sex, Female (%)	11 (55%)	-	-	-	-	-	-	-
Age (years)	52.2	11.8	-	-	-	-	-	-
Height (cm)	173.4	10.8	-	-	-	-	-	-
Weight (kg)	86.6	20.6	80.0	19.5	<0.0001	80.5	18.9	<0.0001
BMI (kg/m2)	28.8	6.4	26.6	6.2	<0.0001	26.7	6.0	<0.0001
Waist (cm)	96.4	12.7	90.6	12.9	<0.0001	90.7	11.7	<0.0001
DPB (mmHg)	72.1	8.7	64.5	6.7	0.0030	67.7	7.3	0.1857
SBP (mmHg)	123.9	10.2	118.1	12.4	0.0301	116.8	12.1	0.0296
ß-hydroxybutyrate (mmol/L)	0.6	0.9	5.0	1.0	<0.0001	0.4	0.3	0.6215
Glucose (mg/dL)	85.7	10.4	70.3	10.5	0.0002	92.8	9.7	0.0155
HOMA-IR	1.7	1.5	0.8	1.1	0.0006	2.2	1.4	0.0266
C-reactive protein (mg/dL)	1.7	1.5	3.9	3.8	0.0004	3.4	9.9	0.6542
Total cholesterol (mg/dL)	192.0	33.5	216.6	47.8	0.0327	168.2	34.5	0.0006
HDL cholesterol (mg/dL)	56.8	19.2	47.8	12.3	0.0015	44.6	10.9	0.0007
Triglycerides (mg/dL)	102.8	46.2	125.4	37.4	0.0745	135.2	47.2	0.0008
LDL cholesterol (mg/dL)	108.4	37.4	131.6	55.5	0.0898	96.7	30.4	0.041
Non-HDL cholesterol (mg/dL)	135.3	34.0	168.8	47.9	0.0061	123.6	33.5	0.0523
Total cholesterol:HDL ratio	3.8	1.5	4.8	1.5	0.0024	4.0	1.1	0.6152
ALT (IU/L)	21.9	11.4	35.9	20.3	0.0003	34.4	19.4	0.0006
AST (IU/L)	22.8	8.5	37.6	17.2	<0.0001	31.5	17.9	0.0350

Table 1.

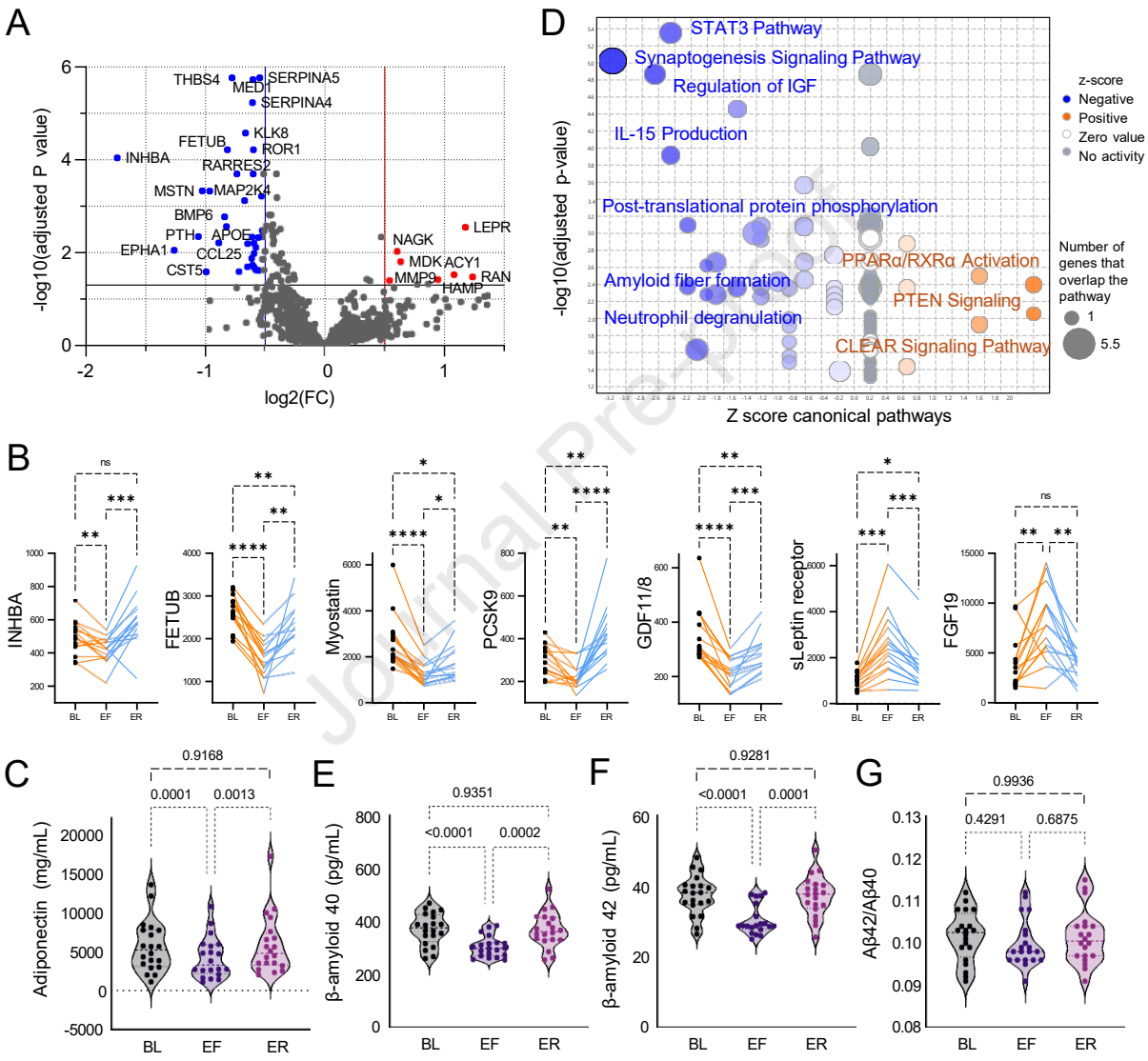

A
-log10(adjusted P value)
log2(FC)
THBS4
SERPINA5
MEDI
SERPINA4
KLK8
FETUB
ROR1
INHBA
RARRES2
MSTN
MAP2K4
BMP6
PTH
APOE
EPHA1
CCL25
CST5
NAGK
LEPR
MDK
ACY1
MMP9
RAN
HAMP
D
-log10(adjusted p-value)
Z score canonical pathways
STAT3 Pathway
Synaptogenesis Signaling Pathway
Regulation of IGF
IL-15 Production
Post-translational protein phosphorylation
Amyloid fiber formation
Neutrophil degranulation
PPARα/RXRα Activation
PTEN Signaling
CLEAR Signaling Pathway
z-score
Negative
Positive
Zero value
No activity
Number of genes that overlap the pathway
1
5.5
B
INHBA
FETUB
Myostatin
PCSK9
GDF11/8
sLeptin receptor
FGF19
BL
EF
ER
C
Adiponectin (mg/mL)
0.9168
0.0001
0.0013
E
β-amyloid 40 (pg/mL)
0.9351
<0.0001
0.0002
F
β-amyloid 42 (pg/mL)
0.9281
<0.0001
0.0001
G
Aβ42/Aβ40
0.9936
0.4291
0.6875

Figure 1.

A

UniProt	Entrez Gene Symbol	Protein Name	Fold Change (week 1 vs baseline)	Adjusted p-value
P62826	RAN	GTP-binding nuclear protein Ran	2.35	0.0331
P48357	LEPR	Leptin receptor	2.26	0.0028
Q03154	ACY1	Aminoacylase-1	2.11	0.0307
P81172	HAMP	Hepcidin	1.93	0.0391
O95750	FGF19	Fibroblast growth factor 19	1.82	0.0487
P02794 P02792	FTH1 FTL	Ferritin heavy chain / Ferritin light chain	1.71	0.0496
P21741	MDK	Midkine	1.55	0.0162
Q9UJ70	NAGK	N-acetylglucosamine kinase	1.52	0.0098
P14780	MMP9	Matrix metalloproteinase 9	1.46	0.0411
P20810	CAST	Calpastatin	1.39	0.0048
P10145	CXCL8	Interleukin-8	1.30	0.0418
Q13093	PLA2G7	Platelet-activating factor acetylhydrolase (PAFAH)	1.28	0.0386

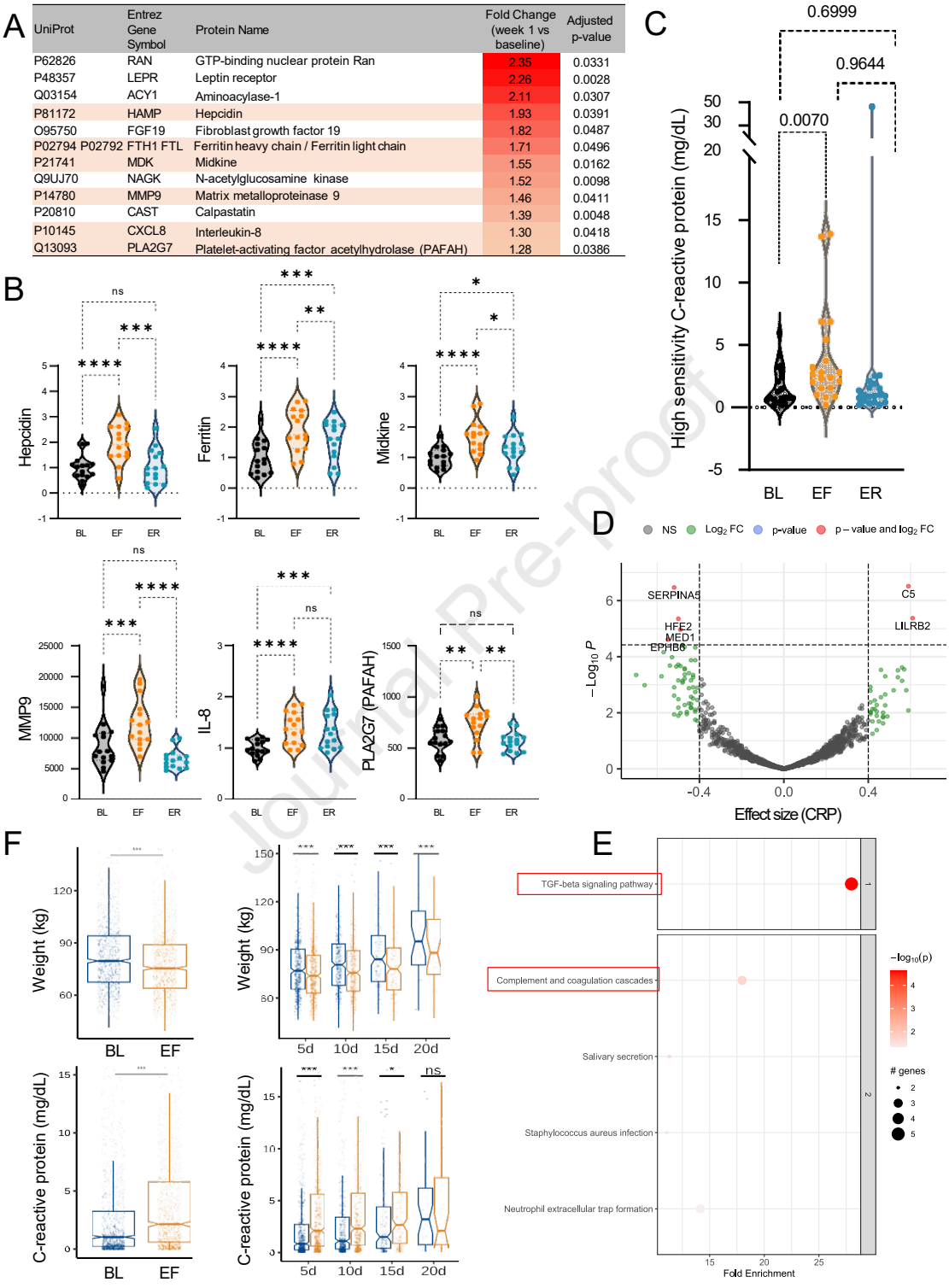


Figure 2

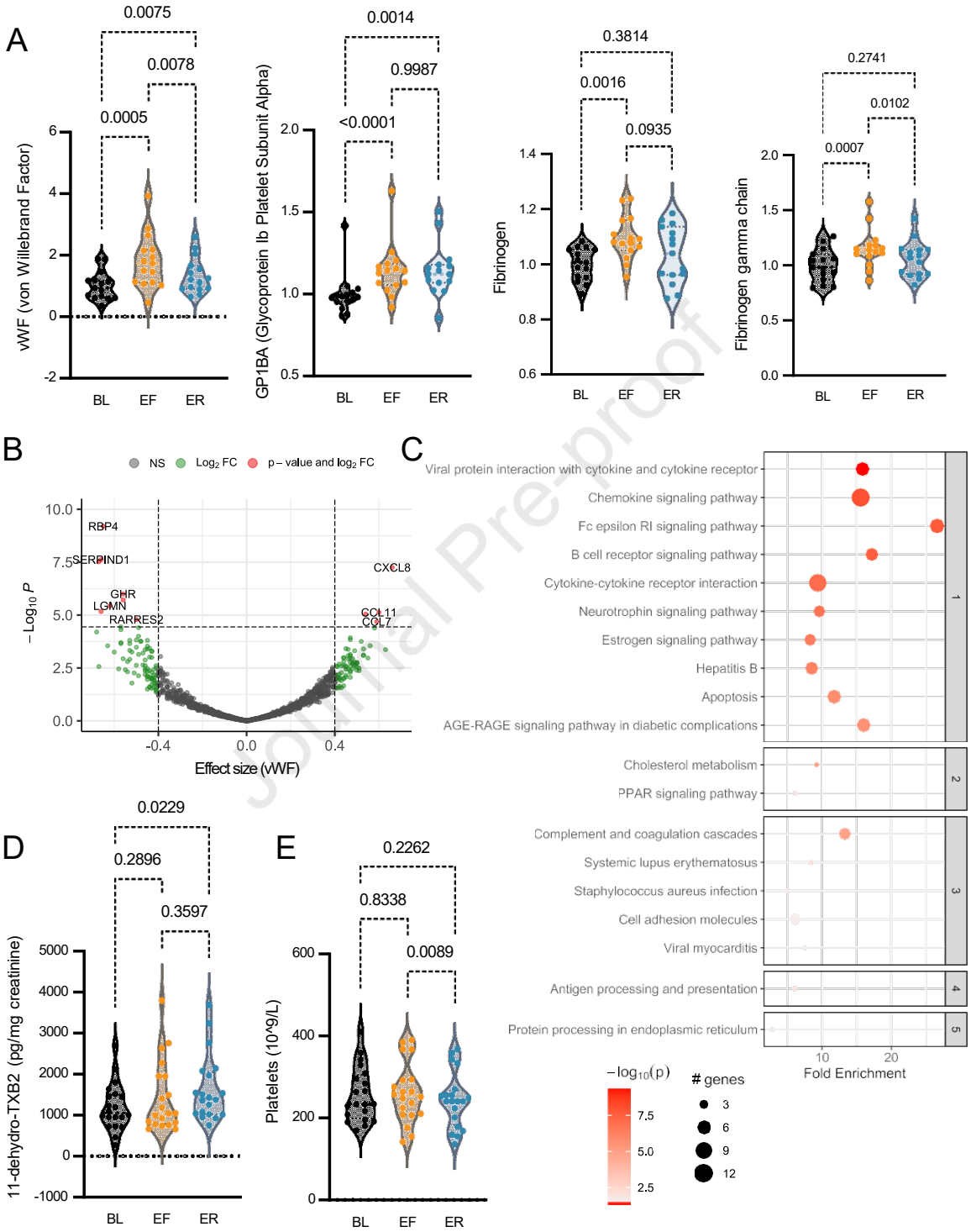

A
vWF (von Willebrand Factor)
GP1BA (Glycoprotein Ib Platelet Subunit Alpha)
Fibrinogen
Fibrinogen gamma chain
0.0075
0.0078
0.0005
0.0014
0.9987
<0.0001
0.3814
0.0016
0.0935
0.2741
0.0102
0.0007
BL
EF
ER
B
NS
Log2 FC
p – value and log2 FC
– Log10 P
Effect size (vWF)
RBP4
SERPIND1
GHR
LGMN
RARRES2
CXCL8
CCL11
CCL7
C
Viral protein interaction with cytokine and cytokine receptor
Chemokine signaling pathway
Fc epsilon RI signaling pathway
B cell receptor signaling pathway
Cytokine-cytokine receptor interaction
Neurotrophin signaling pathway
Estrogen signaling pathway
Hepatitis B
Apoptosis
AGE-RAGE signaling pathway in diabetic complications
Cholesterol metabolism
PPAR signaling pathway
Complement and coagulation cascades
Systemic lupus erythematosus
Staphylococcus aureus infection
Cell adhesion molecules
Viral myocarditis
Antigen processing and presentation
Protein processing in endoplasmic reticulum
Fold Enrichment
– log10(p)
genes
D
11-dehydro-TXB2 (pg/mg creatinine)
0.0229
0.2896
0.3597
E
Platelets (10^9/L)
0.2262
0.8338
0.0089

Figure 3

Journal Pre-proof

- Prolonged water-only fasting induces weight loss, metabolic ketosis, and enhances lipid metabolism in volunteers.
- Prolonged fasting reduces circulating amyloid beta proteins, a key component of brain amyloid plaques.
- Prolonged fasting triggers inflammation and platelet activation, potentially impacting cardiometabolic health.

Journal Pre-proof

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