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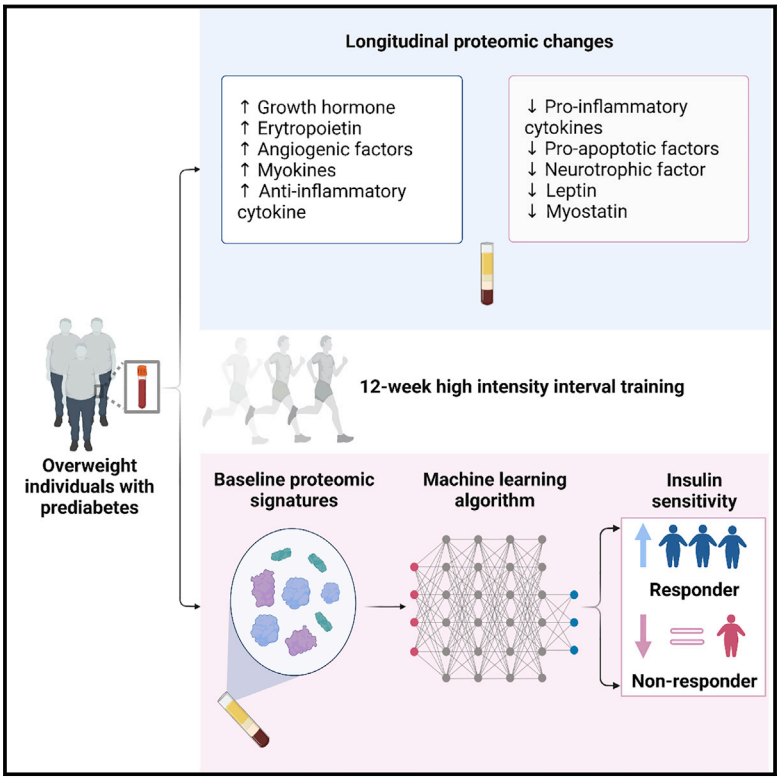
A machine-learning algorithm integrating baseline serum proteomic signatures predicts exercise responsiveness in overweight males with prediabetes

Graphical abstract



Highlights

- Exercise causes changes in hundreds of serum proteins in overweight individuals
- Gut immunity-related proteins are associated with metabolic adaptations to exercise
- Exercise responders and non-responders exhibit differential proteomic changes
- Baseline protein signatures accurately predict the metabolic outcomes of exercise

Authors

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In brief

Diaz-Canestro et al. identify circulating proteins that predict the metabolic responsiveness to exercise training in individuals with prediabetes. These findings may facilitate the clinical implementation of personalized exercise interventions for diabetes prevention.

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A machine-learning algorithm integrating baseline serum proteomic signatures predicts exercise responsiveness in overweight males with prediabetes

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SUMMARY

The molecular transducers conferring the benefits of chronic exercise in diabetes prevention remain to be comprehensively investigated. Herein, serum proteomic profiling of 688 inflammatory and metabolic biomarkers in 36 medication-naive overweight and obese men with prediabetes reveals hundreds of exercise-responsive proteins modulated by 12-week high-intensity interval exercise training, including regulators of metabolism, cardiovascular system, inflammation, and apoptosis. Strong associations are found between proteins involved in gastro-intestinal mucosal immunity and metabolic outcomes. Exercise-induced changes in trefoil factor 2 (TFF2) are associated with changes in insulin resistance and fasting insulin, whereas baseline levels of the pancreatic secretory granule membrane major glycoprotein GP2 are related to changes in fasting glucose and glucose tolerance. A hybrid set of 23 proteins including TFF2 are differentially altered in exercise responders and non-responders. Furthermore, a machine-learning algorithm integrating baseline proteomic signatures accurately predicts individualized metabolic responsiveness to exercise training.

INTRODUCTION

Exercise training (ET) is a cost-effective intervention for the prevention and management of obesity and type 2 diabetes mellitus (T2DM).[1,2] In patients with T2DM, regular exercise increases insulin sensitivity and secretion, leading to improved glucose tolerance. While insulin sensitivity can be enhanced by a single bout of exercise,[3] long-term exercise interventions are required to improve pancreatic beta cell function in patients with T2DM.[4,5] It has been proposed that metabolic adaptations to exercise involve complex organ crosstalk mediated by exercise-responsive factors, known as exerkines, secreted from skeletal muscle, adipose tissue, liver, gut, and other organs.[6] In recent years, research efforts have been focused on exerkines secreted by skeletal muscle, referred to as myokines. The established myokine par excellence, i.e., interleukin-6 (IL-6), is released during muscle contractions, leading to improved glucose homeostasis via autocrine, paracrine, and endocrine effects.[7] In addition, skeletal muscle releases other factors including, but not limited to, interleukin-15 (IL-15), interleukin-8 (IL-8), growth/differentiation factor 15, myostatin (MSTN), apelin, and irisin, which have been implicated in promoting lipolysis and browning of white adipose tissue, increasing muscle mass, and improving glucose tolerance. Molecules secreted by other organs, such as adipose tissue and liver, also play critical roles in mediating metabolic adaptations.[8] For example, the effects of ET on glucose and lipid homeostasis as well as insulin sensitivity were partly attributed to increased circulating levels of adipocyte-secreted adiponectin via enhancing fibroblast growth factor 21 (FGF21) sensitivity in adipose tissue.[9] Interestingly, FGF21 levels increase in response to acute exercise but decrease after chronic exercise in obese and prediabetic individuals, possibly due to improved FGF21 sensitivity.[10,11] Indeed, the response to acute and chronic exercise differs in a large number of circulating proteins,[10] and this inconsistency may partly reflect differences in metabolic adaptations. A recent multi-omics study has described the acute exerkine response in healthy and insulin-resistant individuals.[12] However, how exerkines coordinate spatially and temporally to confer the chronic metabolic benefits of exercise remains poorly understood. A targeted analyses of potential exerkines mediating the effects of chronic exercise have yet to be performed in humans, notably in individuals with impaired glucose tolerance.

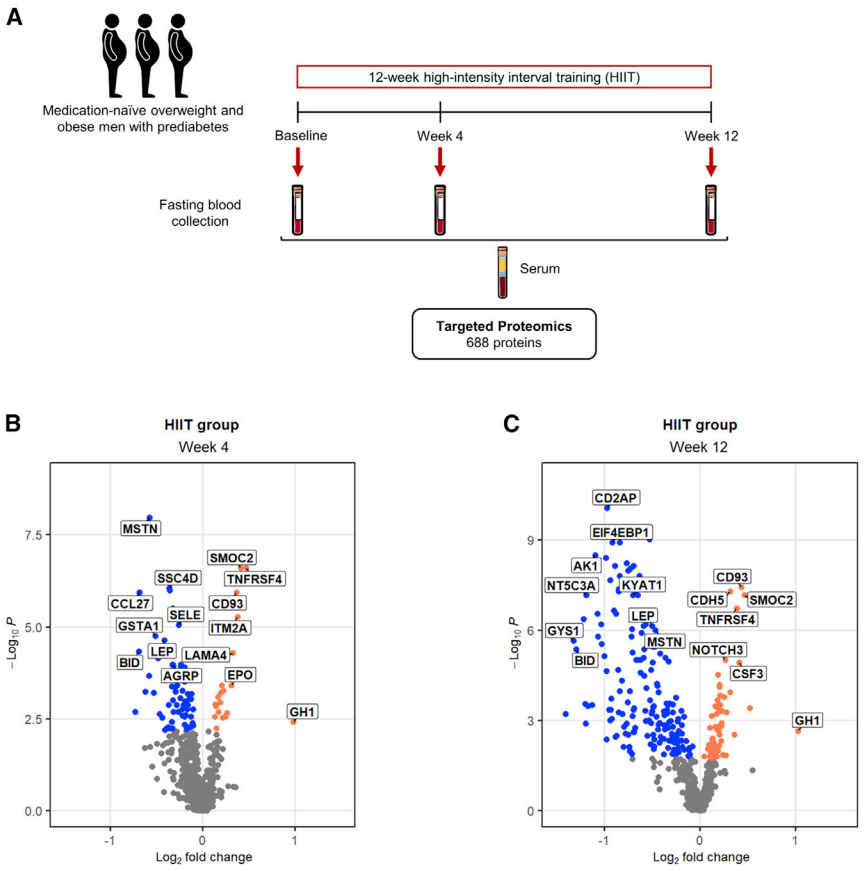

Figure 1. Proteomic changes in response to 12-week high-intensity interval training (HIIT)

(A) Schematic diagram of the study design.

(B and C) Volcano plots of the change in individual proteins after (B) 4 and (C) 12 weeks of HIIT in overweight and obese men with prediabetes (n = 36).

Orange and blue dots indicate proteins that are significantly upregulated or downregulated, respectively, as determined by paired Wilcoxon rank-sum test, FDR < 0.05. Gray dots indicate proteins without significant changes.

Large inter-individual variability in the metabolic response to exercise has been established by previous studies.[13–15] From 7% up to 44% of individuals with prediabetes do not respond favorably to exercise interventions in terms of metabolic outcomes.[16] Such heterogeneous results have been hypothetically attributed to genetics, epigenetics, and physiological factors; however, the underlying mechanisms remain elusive.[17] A recent study demonstrated that microbial species and associated metabolites were differently modulated by a 12-week high-intensity interval training (HIIT) program in metabolic "responders" and "non-responders," while baseline microbiome features accurately predicted metabolic responsiveness to the exercise intervention.[11] These findings disclosed a key role for gut microbiota in conferring the metabolic benefits of exercise. However, additional investigations are required to further elucidate the molecular mechanisms that drive the lack of metabolic response to ET in individuals with prediabetes, as well as to discover baseline circulating biomarkers that may discriminate between those most likely to benefit from those who do not.

To address the above questions, we performed a targeted proteomic analysis (688 inflammatory and metabolic proteins) in serum samples from well-characterized medication-naive overweight and obese Chinese men with prediabetes before and after chronic (12-week HIIT) exercise. We also determined the relationship between serum proteins and clinical parameters to identify potential mediators and predictors of metabolic outcomes. Furthermore, molecular responses were compared between responders and non-responders in terms of insulin resistance. Finally, a machine-learning (ML) algorithm that integrated baseline proteomics was developed to predict individual responsiveness to ET.

RESULTS

Cohort characteristics

Thirty-six medication-naive overweight and obese men with prediabetes underwent 12-week HIIT (Figure 1A). After the exercise intervention, body weight and adiposity were reduced, together with significant improvements in insulin sensitivity, glucose tolerance, and lipid profiles (Table S1). Considering the important role of circulating proteins as potential transducers of the metabolic benefits of exercise, we next explored the proteomic changes in response to 12-week HIIT in our cohort.

Serum proteomic changes in response to 12-week HIIT

To identify plasma proteins that were altered in response to HIIT, we compared the serum levels of 688 proteins at baseline versus 4 and 12 weeks after HIIT using Olink's antibody-based proteomics platform (Table S2). HIIT induced changes in 89 circulating proteins (22 upregulated and 67 downregulated) after 4 weeks (12.9% of total proteins, false discovery rate [FDR] < 0.05; Figure 1B) and 247 proteins (75 upregulated and 173 downregulated) after 12 weeks (35.9% of total proteins, FDR < 0.05; Figure 1C) compared with baseline. Growth hormone 1 (GH1) exhibited the highest upregulation (week 12, log2 fold change [FC] = 1.03, FDR < 0.05), whereas pro-apoptotic factors such as BH3-interacting domain death agonist (BID) were among the proteins with the highest downregulation (week 12, log2 FC = −1.30, FDR < 0.05). Next, to assess the specificity of this technology, we tested the reproducibility of 15 inflammatory proteins assessed by Olink technology in 19 individuals using a different platform (i.e., Mesoscale Discovery [MSD]). Eleven out of 15 assays were highly correlated, and 4 out of 15 were moderately correlated, suggesting a high reproducibility between the two technologies (Figure S1).

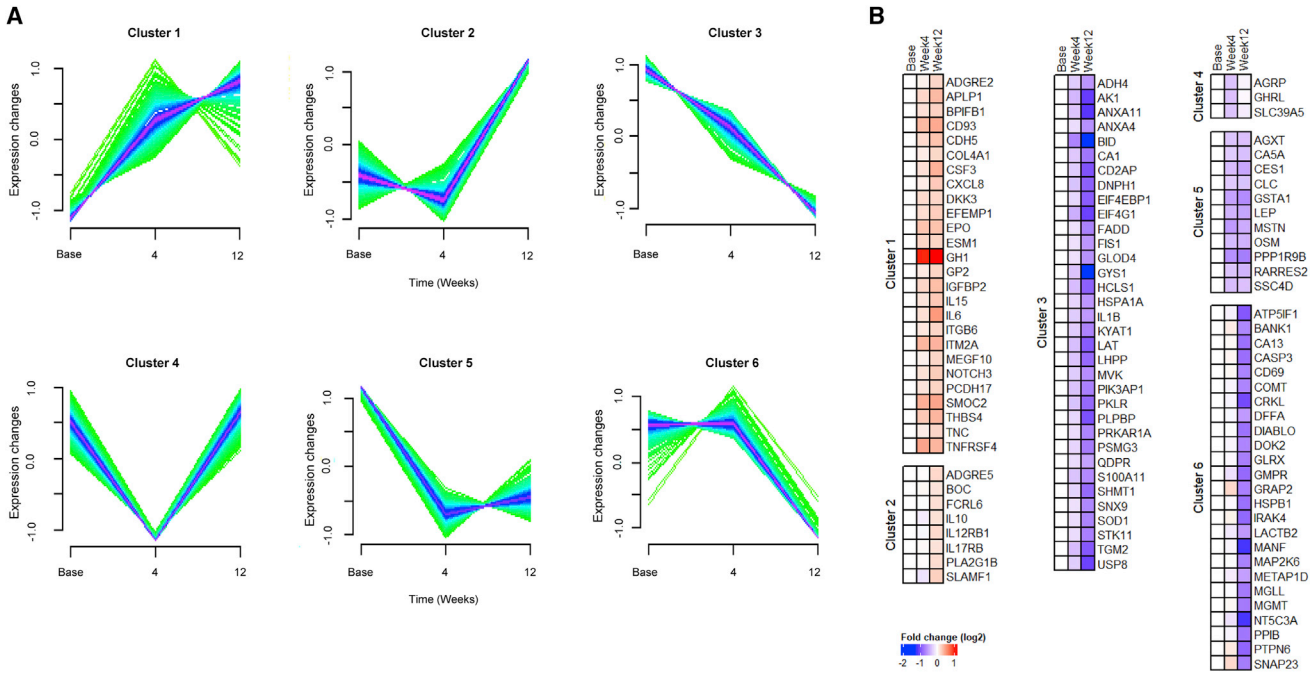

Figure 2. Longitudinal trajectories of proteomic changes in response to 12-week HIIT

(A) Clustering of longitudinal trajectories using circulating proteins.

(B) A subset of significant proteins (FDR < 0.05) that showed the highest change in response to 12-week HIIT (in terms of $\log_2$ fold change [FC]) in overweight and obese men with prediabetes (n = 36) are shown in the heatmap.

Data are represented as mean $\log_2$ FC relative to baseline. Proteins were grouped by clusters. Significant proteins were determined by paired Wilcoxon rank-sum test, FDR < 0.05.

Different trajectories of serum proteomic changes in response to 12-week HIIT

To better understand the changes induced by 12-week HIIT, proteins that significantly changed during 12-week HIIT (FDR < 0.05) were further examined using cluster analysis. Six main clusters of longitudinal trajectories were identified that delineated different patterns in response to the exercise intervention (Figure 2A) and a subset of significant proteins (FDR < 0.05) that showed the highest change (in terms of log2 FC) in each cluster are shown in Figure 2B.

Cluster 1

Proteins in cluster 1 (n = 55, FDR < 0.05 at week 12) increased in the first 4 weeks of HIIT and continued to increase until the end of the intervention, although to a lesser extent. GH1 showed the highest upregulation, followed by cluster of differentiation 93 (CD93) (week 12, log2 FC = 0.44, FDR < 0.05), a receptor expressed in myeloid and endothelial cells that contributes to the removal of apoptotic cells,[18] and SPARC-related modular calcium-binding protein 2 (SMOC2) (week 12, log2 FC = 0.47, FDR < 0.05), a matricellular protein relevant to bone homeostasis.[19] Furthermore, proteins that stimulate hematopoiesis such as erythropoietin (EPO) and colony-stimulating factor 3 (CSF3), as well as myokines (i.e., IL-15, IL-6, IL-8/CXCL8) implicated in energy metabolism and angiogenesis, also presented this trajectory.

Cluster 2

Proteins in cluster 2 (n = 15, FDR < 0.05 at week 12) presented a delayed increase in response to 12-week HIIT compared with proteins in cluster 1. A large portion of the molecules in cluster 2 are involved in inflammation, including the anti-inflammatory cytokine IL-10.

Cluster 3

Cluster 3 contained proteins (n = 54, FDR < 0.05 at week 12) that decreased in the first 4 weeks of HIIT and continued to decrease to the same degree until the end of the intervention. The strongest decreases were observed in BID and the enzyme catalyzing muscle glycogen synthesis (muscle glycogen synthase [GYS1]) (week 12, log2 FC = −1.32, FDR < 0.05). Many other proteins had the same trajectory including kynurenine-oxoglutarate transaminase 1 (KYAT1), an enzyme involved in tryptophan metabolism,[20] and interleukin-1 beta (IL-1β).

Cluster 4

Cluster 4 was enriched in proteins (n = 12, FDR < 0.05 at week 4) that decreased after the first 4 weeks of HIIT and returned to baseline by the end of the intervention. Two appetite-stimulating hormones, agouti-related protein (AGRP; week 4, log2 FC = −0.33, FDR < 0.05) and ghrelin (GHRL; week 4, log2 FC = −0.34, FDR < 0.05), exhibited the strongest decreases in this cluster.

Cluster 5

Cluster 5 was abundant in proteins (n = 23, FDR < 0.05 at week 12) that decreased in the first 4 weeks of HIIT but did not return to baseline by the end of the intervention. Well-known hormones such as MSTN (week 12, log2 FC = −0.46, FDR < 0.05) and leptin (LEP; week 12, log2 FC = −0.50, FDR < 0.05) presented this pattern.

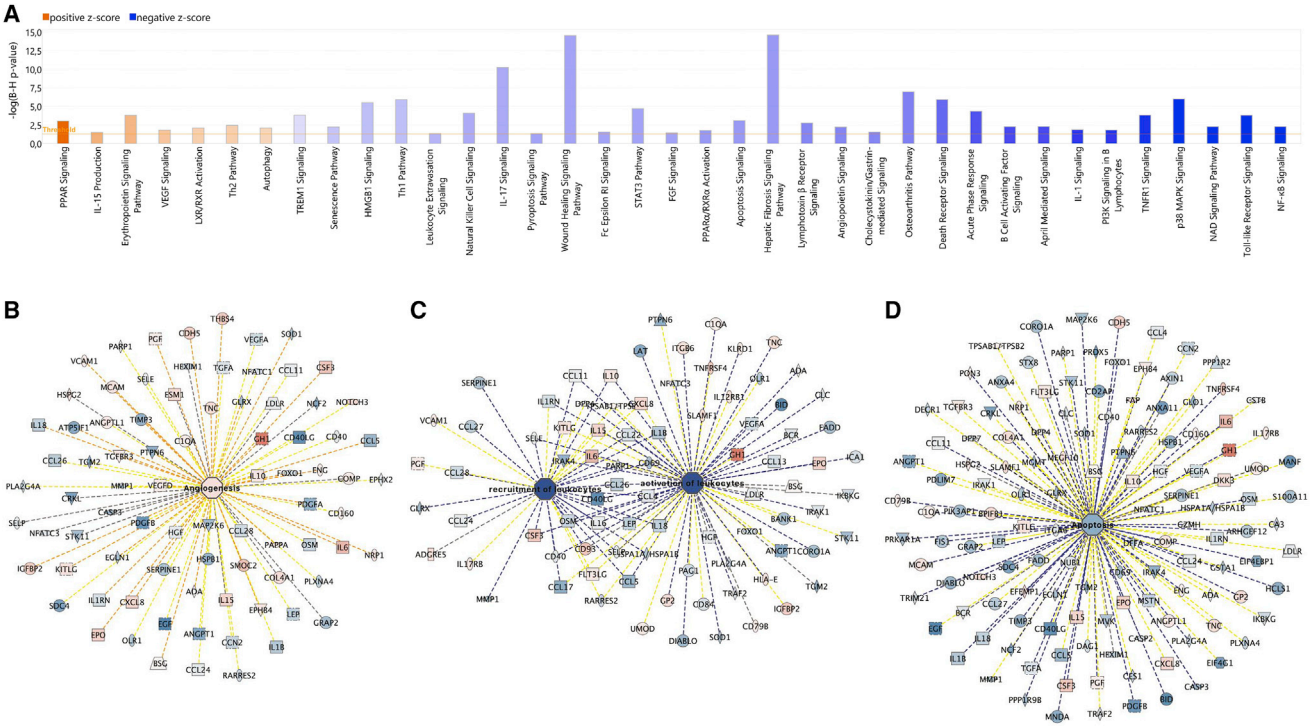

Figure 3. Ingenuity pathways analysis (IPA) for serum proteins altered during 12-week HIIT

(A) IPA analysis using proteins that significantly changed in response to 12-week HIIT. *Z* score is the activation score for a pathway, which reflects how much the pathway is activated (orange) or deactivated (blue) due to the changes in the expression of proteins involved in those pathways. -log (B-H p value) refers to Benjamini-Hochberg adjusted p value <0.05 .

(B–D) IPA of diseases and biological functions of significant proteins, which are displayed as nodes (proteins) and edges (biological relationship between nodes). The color intensity of each node represents the direction of change in response to 12-week HIIT, with red (upregulated), and blue (downregulated). The edges connecting the genes to the respective functions represent the predicted relationships, with blue representing inhibition, orange representing activation, gray representing effect not predicted, and yellow indicating finding inconsistent/contradictory with state of downstream molecule, respectively.

Cluster 6

Cluster 6 contained proteins (n = 43, FDR < 0.05 at week 12) that required more time to respond to exercise, exhibiting a significant reduction by the end of the intervention. Of these, mesencephalic astrocyte-derived neurotrophic factor (MANF) showed the highest decrease (week 12, log2 FC = −1.19, FDR < 0.05). In addition, some pro-apoptotic markers such as caspase-3 (CASP3) and diablo homolog (DIABLO) followed this pattern. Taken together, the above findings demonstrated that hundreds of proteins changed in response to 12-week HIIT, mainly delineating six different trajectories. To further understand the biological significance of these changes, we next performed pathway enrichment analysis.

Ingenuity pathway analysis (IPA) in response to 12-week HIIT

IPA was performed among the significantly altered serum proteins during 12-week HIIT. In the early phase of HIIT (the first 4 weeks), the "EPO signaling pathway" was the only pathway identified as activated (Figure S2A). Conversely, seven pathways were significantly downregulated including the "hepatic fibrosis signaling pathway." Likewise, analysis of diseases and biological functions revealed an increase in "quantity of myeloid cells" (Figure S2B), whereas several functions including "activation and migration of leukocytes" (Figure S2C) as well as "generation of reactive oxygen species" were decreased (Figure S2D).

After 12 weeks of HIIT, 7 pathways were identified as activated (Figure 3A), with most of them playing a key role in metabolism (i.e., "peroxisome proliferator-activated receptor (PPAR) signaling," "IL-15 production," and "liver X receptor (LXR)/retinoid X receptor (RXR) pathway"). Conversely, 30 pathways with a high number implicated in inflammatory (e.g., "NF-κB," "Toll-like receptor signaling," and "pyroptosis signaling") and apoptotic responses (e.g., "death receptor signaling," "apoptosis signaling," and "tumor necrosis factor receptor 1 (TNFR1) signaling") were inhibited. Analysis of diseases and biological functions demonstrated increased "angiogenesis" (Figure 3B), whereas "activation and recruitment of leukocytes" (Figure 3C) as well as "apoptosis" (Figure 3D) showed a decrease.

Serum proteomic changes associated with changes in metabolic outcomes

To identify potential mediators for the metabolic benefits of exercise, we analyzed the relationship between exercise-induced proteomic and metabolic changes using regression analyses

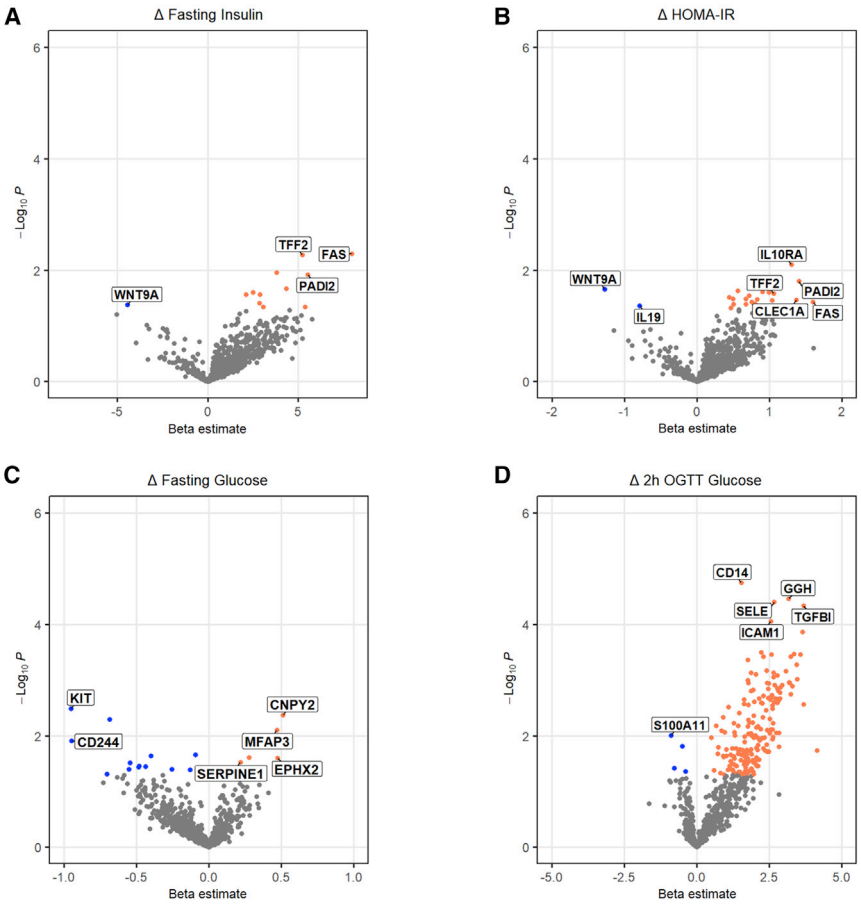

Figure 4. Proteomic changes associated with metabolic outcomes in response to 12-week HIIT

(A–D) Changes in serum proteins after 12-week HIIT associated with changes in (A) fasting insulin, (B) homeostatic model assessment for insulin resistance (HOMA-IR), (C) fasting glucose, and (D) 2h oral glucose tolerance test (OGTT) glucose in a linear regression model adjusted for age and Δ weight and Δ fat mass. Number of biological observations for each graph (n = 36). Orange and blue dots indicate proteins that are positively or negatively associated with metabolic outcomes ($p < 0.05$), respectively, whereas gray dots indicate not significant proteins ($p > 0.05$).

adjusted for age, Δ weight, and Δ percentage of fat mass. We identified changes in 11 and 21 proteins that were positively associated and 1 and 2 that were negatively associated with Δ fasting insulin and Δ homeostatic model assessment for insulin resistance (HOMA-IR), respectively ($p < 0.05$; Figures 4A and 4B). Interestingly, proteomic changes positively associated with changes in both outcomes included those of trefoil factor family member 2 (TFF2), a protein secreted by the gut and involved in metabolism;[21] Fas cell surface death receptor (FAS), a pro-apoptotic receptor activated in adipocytes from obese mice;[22] and protein-arginine deiminase type-2 (PADI2), an enzyme promoting the secretion of pro-inflammatory cytokines in macrophages.[23] In addition, we identified 9 and 194 proteomic changes that were positively and 13 and 4 that were negatively associated with Δ fasting glucose (FG) and Δ 2h OGTT glucose (2hG), respectively ($p < 0.05$; Figures 4C and 4D). The proteomic changes positively associated with Δ FG included those of canopy FGF signaling regulator 2 ($CNPY_2$), an angiogenic growth factor expressed in the liver and pancreas and modulated by FGF21,[24,25] and well-known proteins associated with cardiometabolic diseases including plasminogen activator inhibitor-1 (PAI-1/SERPINE1) and soluble epoxide hydrolase (sEH/EPHX2). Likewise, changes in circulating levels of endothelial adhesion molecules (e.g., E-selectin [SELE] and intercellular adhesion molecule 1 [ICAM-1]) were positively associated with changes in 2hG.

Serum baseline proteins associated with changes in metabolic outcomes

We also identified baseline serum protein levels that predicted metabolic responses to 12-week HIIT using regression analyses adjusted for age, weight, and percentage of fat mass at baseline. We identified 1 protein that positively associated with Δ fasting insulin and Δ HOMA-IR, while 55 and 54 showed negative association, respectively ($p < 0.05$; Figures 5A and 5B). Baseline proteins that associated negatively with changes in fasting insulin included molecules implicated in cardiovascular disease (CVD) such as the receptor for advanced glycation end products (AGER); tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE1), an angiopoietin receptor expressed in endothelial cells and associated with a pro-atherogenic phenotype;[26] and SELE. In addition, we identified 9 and 5 proteins that associated positively and 44 and 93 that associated negatively with Δ FG and Δ 2hG, respectively ($p < 0.05$; Figures 5C and 5D). Notably, the protein with the strongest positive association (in terms of p value) with changes in FG and glucose tolerance was pancreatic secretory granule membrane major glycoprotein GP2 (GP2), a secreted protein expressed in zymogen granules of pancreatic acinar cells as well as plasma membrane of M cells in the intestinal epithelium.[27]

Exercise induced differential changes of serum proteins in responders and non-responders

Participants presented a substantial inter-individual variation in terms of fasting insulin and HOMA-IR in response to the 12-week HIIT intervention.[11] Therefore, the participants were further classified into responders (Rs; n = 28) and non-responders (NR; n = 8) depending on whether they could or could not demonstrate a decrease in HOMA-IR greater than 2-fold

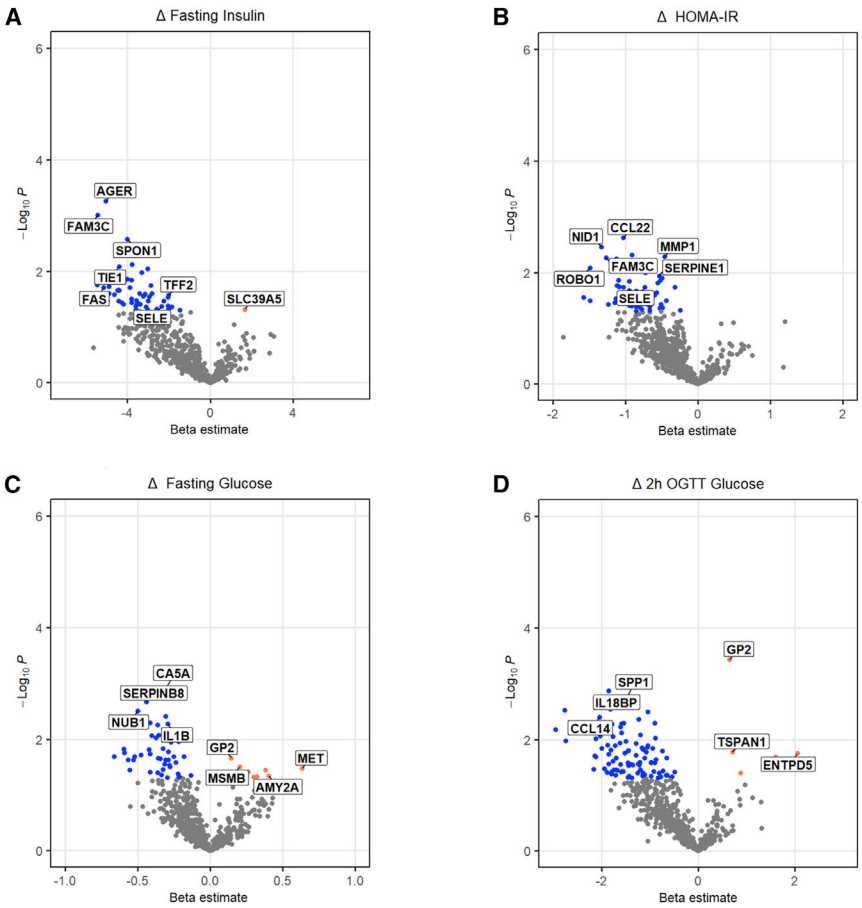

Figure 5. Baseline proteins associated with exercise-induced metabolic outcomes

(A–D) Baseline serum proteins associated with changes in (A) fasting insulin, (B) HOMA-IR, (C) fasting glucose, and (D) 2h OGTT glucose in a linear regression model adjusted for age, weight, and fat mass. Number of biological observations for each graph (n = 36). Orange and blue dots indicate proteins that are positively or negatively associated with metabolic outcomes ($p < 0.05$), respectively, whereas gray dots indicate not significant proteins ($p > 0.05$).

technical error. Responders and non-responders did not differ in clinical parameters at baseline and displayed a similar reduction in weight and adiposity as well as similar increase in cardiorespiratory fitness (Table S3). Notably, responders showed a striking 41.51% and 47.18% decrease in fasting insulin and HOMA-IR index, respectively, whereas non-responders showed a lack of improvement in both outcomes. In parallel, we detected differential changes in 23 proteins between responders and non-responders ($p < 0.05$; Figure 6A). Of these, TFF2 and FAS exhibited an opposite trend of changes between responder and non-responder groups. In addition, pancreatic α-amylase (AMY2A), a digestive enzyme involved in the metabolism of starch and glycogen,[28] and TIE1 were exclusively upregulated in non-responders, whereas their levels did not change in responders.

Finally, we also investigated whether proteomic differences at baseline could be integrated into a ML algorithm to predict individualized exercise responsiveness. To this end, a random forest algorithm integrating baseline serum proteomic data was built using the discovery cohort and achieved an area under the receiver operating characteristic curve (AUROC) of 0.87 (Figure 6B). The performance of this model was further evaluated in the validation cohort and achieved an AUROC value of 0.79 for the discrimination between responders and non-responders (Figure 6C). We compared the performance of the random forest with other ML models. In this regard, the random forest performs best, and the logistic regression model performs slightly better than the generalized linear model (Figure S3). One of the most informative proteins contributing to this classifier was major histocompatibility complex class I polypeptide-related sequence A_B (MICA/B) (Figure 6D), a stress-induced ligand for the natural killer (NK) group 2D receptor found on cell lineages with NK activity as well as gamma delta and alpha beta T cells.[29,30] Notably, lipocalin 2 (LCN2), a secretory glycoprotein associated with obesity and insulin resistance,[31] was elevated in responders compared with non-responders at baseline ($p < 0.05$). Conversely, MICA/B levels were increased in non-responders compared with responders, although it did not reach statistical significance (Figure 6E).

DISCUSSION

The present study provided characterization of inflammatory and cardiometabolic molecules responsive to chronic exercise in a cohort of 36 medication-naive overweight and obese men with prediabetes. Our Olink-based quantitative proteomic profiling revealed dynamic changes in hundreds of molecules and signaling pathways during the 12-week HIIT intervention, including regulators of metabolism, cardiovascular system, inflammation, and apoptosis. Proteins involved in gastro-intestinal (GI) mucosal immunity were strongly associated with metabolic outcomes of exercise intervention. Particularly, exercise-induced changes in TFF2 were positively associated with changes in insulin resistance and fasting insulin, whereas baseline levels of GP2 were positively associated with changes in FG and 2hG. We also observed a highly heterogeneous response to chronic exercise with respect to insulin sensitivity, which was accompanied by differential changes in 23 serum proteins, including TFF2. Finally, an ML algorithm integrating baseline proteins was developed and accurately predicted personalized metabolic responsiveness.

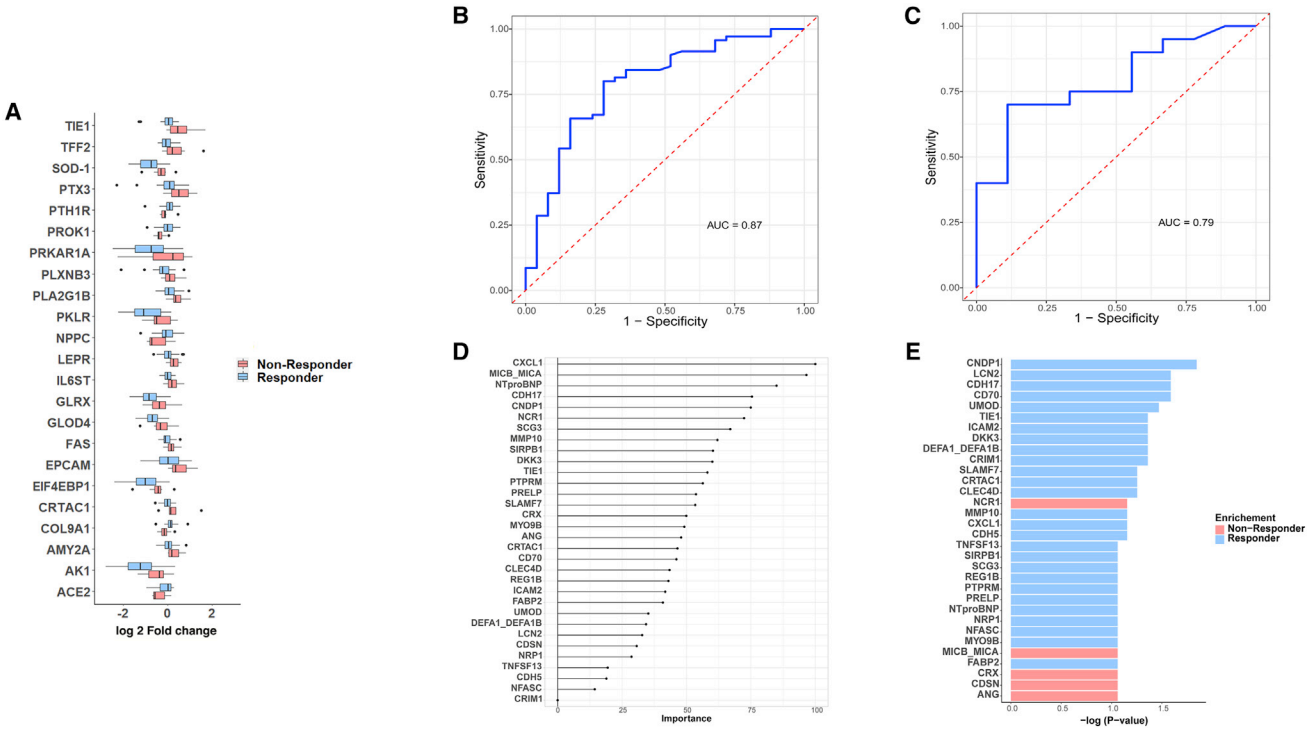

Figure 6. Differential changes of serum proteins in exercise responders and non-responders

(A) Significantly altered serum proteins ($p < 0.05$, unpaired Wilcoxon rank-sum test) between responders and non-responders. Log_2 FC was defined as the ratio between levels after exercise to those at baseline.

(B and C) The receiver operating characteristic (ROC) curves and area under curve (AUC) of the proteomics-based algorithm for the discrimination between responders and non-responders in (B) discovery cohort (n = 19) and (C) validation cohort (n = 29).

(D) Feature importance contributing to this classifier.

(E) The differentially abundant proteomic biomarkers ($p < 0.1$, by unpaired Wilcoxon rank-sum test) between responders and non-responders at baseline with significance calculated as −log10 (p value). Red: enriched in non-responders; blue: enriched in responders.

This study examines the molecular effects of chronic exercise in individuals with prediabetes using a large-scale quantitative analysis of serum proteins. Previous studies performed metabolite profiling in response to chronic exercise in healthy young and obese individuals.[32,33] Likewise, a recent study shed light on baseline proteins that may predict exercise responsiveness in terms of cardiorespiratory fitness.[34] However, in the latter study, there was no assessment of proteins after the exercise intervention, and metabolic outcomes were not investigated, highlighting the need for a human study that examines proteomic changes in relation to metabolic adaptations.

Numerous proteins changed in response to 12-week HIIT, with the number of upregulated proteins being lower than the downregulated ones. This may be explained by the functional properties of the included proteins, as inflammatory markers are often increased in obese/overweight individuals, and ET can counteract these changes.[35,36] Notably, the strongest changes in the upregulated proteins were observed in molecules involved in metabolism. GH1, a hormone secreted by the anterior lobe of the pituitary gland that stimulates lipolysis in adipose tissue and free fatty acid oxidation in the liver,[37] presented the strongest upregulation, followed by other lipolytic factors such as IL-6 and IL-15.[38,39] IL-6 is upregulated in obese individuals and patients with T2DM.[40] While serum IL-6 is elevated by acute exercise, both decreased and increased levels of circulating IL-6 have been observed in participants with different types of chronic ET.[41–44] Our findings are in agreement with a previous report that indicates that vigorous chronic exercise, contrary to light and moderate chronic exercise, increases circulating levels of IL-6.[44] A potential explanation for these findings may be the increased muscle-derived IL-6 with high-intensity ET. It should be noted that although IL-6 is considered a pro-inflammatory cytokine, muscle-derived IL-6 is associated with anti-inflammatory properties.[45,46] Furthermore, loss of visceral adipose tissue following exercise is dependent on IL-6.[38] On the other hand, our results on IL-15 confirm previous studies in women indicating that this cytokine increases in response to chronic exercise, in particular with resistance training.[47,48] Notably, lipolytic factors are key molecules bridging the anti-inflammatory effects of exercise and metabolism since a reduction in visceral adipose tissue decreases the secretion of pro-inflammatory cytokines and deleterious adipokines.[49] Previous studies have demonstrated a pro-inflammatory state in individuals with prediabetes and T2DM.[40,50,51] In this regard, plasma profiling of patients with newly diagnosed T2DM using the Olink technology indicated increased inflammatory markers (i.e., oncostatin M, IL-16, IL-18, CCL4, E-selectin)

compared with healthy controls.[40] Conversely, our findings demonstrated the decrease of these pro-inflammatory markers as well as other cytokines such as IL-1β following 12-week HIIT. Decreased levels of IL-1β, which has been associated with the pathogenesis of CVD and T2DM,[52–54] were also observed after a 12-month HIIT program in patients with T2DM.[55] In parallel, we observed a marked reduction of pro-apoptotic pathways and molecules including BID, CASP3, and DIABLO. Whereas BID is a pro-apoptotic gene belonging to the Bcl-2 protein family, DIABLO leads to the activation of caspases, which are the effector of apoptosis.[56] Of note, apoptosis of adipocytes is a process that links obesity and metabolism.[57] In mice fed on a high-fat diet (HFD), the inhibition of BID prevented the infiltration of macrophages in adipose tissue.[57] Moreover, ET reduced apoptotic and inflammatory markers in adipose tissue of obese rats.[58] However, as we cannot determine the tissue source of these circulating proteins, further research should investigate whether changes in adipose tissue remodeling may explain the exercise-induced reduction in circulating apoptotic and inflammatory markers.

Our study also revealed that several appetite stimulating hormones, such as GHRL and AGRP, were decreased in the early phase of HIIT but returned to baseline by end of the intervention. In contrast, appetite-suppressing hormones such as LEP did not return to baseline. In plasma samples of patients with T2DM, levels of AGRP and LEP were increased, while GHRL was decreased, compared with healthy controls.[40] Conflicting results have been reported regarding the effect of ET on GHRL, which may depend on the isoform of GHRL analyzed, i.e., total, acylated, or non-acylated.[59,60] Otherwise, accumulating evidence has demonstrated that chronic exercise reduced circulating levels of LEP in prediabetic and diabetic individuals,[61] which is usually accompanied by improvements in LEP sensitivity in the brain.[62] Another well-known hormone that exhibits a similar change with LEP in response to 12-week HIIT is MSTN, a myokine that inhibits muscle growth and is associated with insulin resistance.[63] In agreement with our findings, circulating levels of MSTN decreased after resistance training in sedentary men and women.[48,64] Additionally, a marked reduction of MANF was also observed after 12-week exercise intervention. MANF is a secreted protein expressed in tissues involved in the neuro-endocrine axis and has recently been associated with insulin resistance in patients with prediabetes.[65] Our results describe the remarkable changes of MANF in response to ET, which warrants further studies to address the potential role of this exercise-responsive factor on metabolic adaptations.

To a lesser extent, molecules regulating the cardiovascular system were also affected by 12-week HIIT. Red blood cell volume (RBCV) and total blood volume (BV) expansion is a fundamental adaptation of chronic exercise leading to an increased capacity for oxygen (O_2) delivery and aerobic metabolism.[66] In this regard, hypovolemia is a prevalent characteristic of patients with diabetes that may contribute to the reduced exercise tolerance observed in this population.[67] The observation of increased circulating levels of EPO in our study suggests that 12-week HIIT stimulated erythropoiesis, plausibly resulting in RBCV and BV expansion in individuals with prediabetes. In addition, we found increased angiogenesis and angiogenic factors, including IL-8, vascular endothelial growth factor D (VEFGD), and SMOC2, which may contribute to a higher capillary density in skeletal muscle,[68] possibly increasing the peripheral capacity to extract O_2 from the circulation, another step in the O_2 cascade that can be impaired in our study population.

Both clinical and animal data indicate that ET may beneficially modulate the gut microbiome. For instance, elite endurance athletes are characterized by a more diverse microbiome, with high abundance of health-associated microbial species such as *Akkermansia*, and increased levels of short chain fatty acids (SCFAs) compared with sedentary controls.[69,70] Likewise, ET modulates gut microbiota composition in mice by increasing the numbers of beneficial microbial species.[71] In addition, recent publications indicate that the gut microbiota play a key role in mediating the beneficial effects of exercise on cardiovascular as well as metabolic adaptations. For instance, gut microbiome mediated the beneficial effects of exercise on cardiac function following myocardial infarction in mice.[72] On the other hand, *Veillonella*, a lactate-metabolizing microbiome, was increased after exercise in two independent cohorts of marathoners and Olympic trial rowers. In parallel, mice treated with *Veillonella* increased treadmill run time.[73] *Veillonella* species metabolize lactate into SCFAs, which in turn enhance mitochondria oxidative capacity and improve aerobic metabolism.[74] Similarly, we recently found that the gut microbiota may play a key role in conferring the metabolic benefits of exercise. Exercise-induced changes in gut microbiota composition were associated with the metabolic improvements after the exercise intervention. In addition, transplantation of fecal human microbiota from individuals that had improved insulin sensitivity after 12-week HIIT ameliorated glucose intolerance and insulin resistance in obese recipient mice.[11] In this line, our regression analyses showed that exercise-induced changes in TFF2, a protein primarily expressed in neck cells of the stomach and duodenum Brunner' gland and involved in innate and adaptive immunity,[75] positively correlated with changes in insulin resistance and fasting insulin. The main function of TFF2 is to protect the GI mucosa from internal and exogenous aggressions of the epithelium.[76] Considering that the mucosal immune response plays a key role in modulating the composition and function of gut microbiota,[77] TFF2 may partly mediate the metabolic effects of exercise by inducing changes in the microbiota. In addition, basal levels of GP2, a protein expressed in gut immune cells,[78] were positively associated with changes in FG and 2hG, independently of body weight and adiposity. Notably, GP2 has been shown to be upregulated at the specific inflamed region in the gut.[79] It is reasonable to speculate that individuals with higher levels of GP2 at baseline may also exhibit pro-inflammatory composition of the gut microbiome. Given that subtle differences in microbial signatures may lead to different metabolic responses to ET,[11] further studies should investigate the role of TFF2 in the exercise-gut microbiota-metabolic response axis as well as validate the predictive value of GP2 for glycemic responsiveness to exercise.

Different factors may determine the effectiveness of a lifestyle interventions in diabetes prevention including genetics, epigenetics, and physiological conditions.[17] Specific genetic patterns including variations in metabolic genes such as adiponectin receptor 1 and

muscle expression of genes involved in mitochondrial biogenesis may lead to differential responses to exercise.[14,80] On the other hand, physiological factors such as high basal liver fat and visceral fat have been associated with metabolic resistance to lifestyle interventions.[81] Notably, we previously demonstrated that 12-week HIIT induced a heterogeneous metabolic response associated with a maladaptation of the gut microbiota in non-responders.[11] In addition, our current findings showed that 23 proteins, including TFF2, differentially changed between responders and non-responders. The increase in TFF2 observed in non-responders might modulate the microbiome via regulating genes involved in innate host antimicrobial defense,[75] while changes in gut microbiota composition may also possibly contribute to increased levels of TFF2. Additional research is needed to understand whether TFF2 is associated with the deleterious changes observed in the microbiota of non-responders. Furthermore, 12-week HIIT induced increased levels of AMY2A in non-responders. Regarding this finding, acarbose, an oral α-amylase inhibitor that acts by slowing down the intestinal absorption of glucose, is used in the management of T2DM.[82,83] In addition, acarbose improves insulin sensitivity in subjects with impaired glucose tolerance.[84] Therefore, exercise-induced upregulation of AMY2A in non-responders may additionally contribute to the lack of improvement in insulin sensitivity. Finally, it is worth to mention that the lack of response in a metabolic outcome is not necessarily reflected in other variables such as cardiorespiratory fitness (i.e., VO_{2max}) since responders and non-responders displayed similar improvements in VO_{2max} in our study. Consistently, in the HART-D study, individuals with diabetes exhibited improved metabolic parameters following an exercise intervention independently of the changes in VO_{2max}.[85] Of relevance, VO_{2max} response to ET is, to a large extent, determined by hematological and cardiac adaptations,[86,87] whereas skeletal and adipose tissue adaptations are the important contributors to metabolic improvements.[9,88] Circulating proteins are particularly attractive as biomarkers to predict the metabolic response to ET. In our protein biomarker analyses, baseline levels of circulating proteins accurately discriminated between those individuals that benefited from the exercise intervention in terms of metabolic adaptations from those who did not. MICA/B was one of the most informative proteins to this classifier. Interestingly, MICA/B contributes to the detection of damaged, infected, or transformed intestinal epithelial cells in a process independent of traditional major histocompatibility complex (MHC) class I antigen processing.[89] Therefore, these results may further support the assumption of a pro-inflammatory composition of the gut microbiome in non-responders. Future clinical interventions may apply the present ML algorithm to identify exercise non-responders, facilitating that alternative strategies can be implemented in those individuals. For instance, dietary modifications specifically targeting the gut microbiota, as recently described,[90] may maximize the metabolic benefits of exercise in these individuals.

In conclusion, the present study provides a detailed proteomic map of serum proteins in response to chronic exercise including potential transducers of the metabolic benefits of exercise. These findings, together with the recognition of a baseline proteomic signature that predicts individualized metabolic responsiveness to ET, may facilitate the clinical implementation of personalized exercise interventions for diabetes prevention.

Limitations of the study

Our investigation is limited by a small sample size and restrictive inclusion criteria for study participants (only men were included), which constrains the generalizability of these results to women and other populations. Female participants were not included mainly due to the large fluctuations in several sex hormones at this age range (20–60 years old) and the menstrual period of females, which may influence the attendance and compliance to our 12-week HIIT and the interpretation of data. In the future, we will certainly expand our pilot study to include men and women and other types of exercise to test whether our findings can be extrapolated to both sexes. Another limitation refers to the confined protein panel (688 cardiometabolic and inflammatory proteins), which does not provide a complete coverage of the human proteome. Moreover, our proteomic analysis does not include a lean or obese control group without exercise, which would have provided information on proteins that change naturally within a 12-week period. Therefore, future studies in different ethnic groups with large sample sizes and inclusion of sedentary subjects are needed to confirm the present findings and to explore the potential relevance in clinical applications.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Ethics statement
  - Study participants
- METHOD DETAILS
  - Participants selection
  - HIIT protocol
  - Collection of clinical data
  - Definition of responders and non-responders
  - Olink Proteomics
  - Mesoscale Discovery (MSD)
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - ML algorithm
- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

C.D.-C. performed bioinformatics analysis, interpreted results, and wrote the manuscript. J.C. performed bioinformatics analysis. Y.L., Y.W., and H.H. contributed to the acquisition of data. M.A.T. designed and carried out the exercise intervention and edited the manuscript. A.X. conceived and supervised the study and wrote and edited the manuscript. C.D.-C., C.-H.L., K.S.L.L., E.H., Y.L., M.A.T., and A.X. revised the manuscript critically for important intellectual contents.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human Blood	This paper	N/A
Critical commercial assays		
Human Insulin ELISA Kit	Immunodiagnostics	Cat# 31380
Olink Explore 384 Cardiometabolic &cardiometabolic II	Olink	N/A
Olink Explore 384 Inflammation & Inflammation II	Olink	N/A
Deposited data		
Proteomics data Table S2	This paper	N/A
Software and algorithms		
R Software	R Core Team	https://www.r-project.org/
Mfuzz	N/A	https://www.bioconductor.org/packages/release/bioc/html/Mfuzz.html (Continued on next PAGE) Ce
Ingenuity pathway analysis	QIAGEN	N/A
igraph	N/A	https://cran.r-project.org/web/packages/igraph/index.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for data should be directed to and will be fulfilled by the lead contact, Aimin Xu (amxu@hku.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Olink raw proteomics data are provided in Table S2.
- This study reports original code deposited at (https://github.com/candeladiazcanestro/Proteomics).
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement

This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW 15-370) and endorsed the principles of the Declaration of Helsinki. Written informed consents were obtained from all participants.

Study participants

Overweight and obese Chinese men aged 20 to 60 years were recruited from the local community via flyers and online advertisements, as previously described.[11] A total of 36 subjects (28 responders and 8 non-responders) who completed the exercise intervention and had samples at different time points were included in the longitudinal proteomic profiling. In addition, a total of 48 subjects (34 responders and 14 non-responders) who had baseline samples were included in the random forest model for predicting exercise responsiveness.

METHOD DETAILS

Participants selection

The inclusion criteria comprised: (i) non-smoking and Chinese men aged 20 to 60 years; (ii) overweight or obese as defined by Asian criteria (body mass index (BMI) > 23 kg/m^2) and with stable body weight (<5% weight change over the last 3 months); (iii) prediabetes,

defined by impaired glucose tolerance (2-h blood glucose $\geq$7.8 mmol/L and $\leq$11.0 mmol/L after a 75-gram (g) oral glucose challenge) and/or impaired fasting glucose (fasting blood glucose $\geq$5.6 mmol/L and $\leq$6.9 mmol/L) following the American Diabetes Association guidelines;[91] (iv) absence of any systemic, metabolic and cardiovascular diseases (CVD), as well as any infection within one month prior to recruitment; and (v) not on any medication or specific diet. Exclusion criteria comprised: (i) acute illness or current evidence of acute or chronic inflammatory or infectious diseases; (ii) any neurological, musculoskeletal or cardio-respiratory condition that would entail risk during exercise or preclude the participant from adapting to an exercise program; (iii) specific diet program and/or participation in regular exercise for more than 2 times per week in the latest 3 months prior to recruitment; and (iv) mental illness rendering them unable to carry out exercise activities or understand the nature, scope, and possible consequences of the study.

HIIT protocol

The 12-week HIIT consisted of three sessions per week on non-consecutive days at the Active Health Clinic, Center for Sports and Exercise, The University of Hong Kong, supervised by certified exercise specialists in a one-to-one manner, as previously described.[11] Compliance in the exercise session was highly encouraged and participants were required to take part in at least 85% of all the exercise sessions for inclusion into the study. The 70-min (min) high-intensity aerobic and resistance interval training sessions consisted of a 10 min warm-up, followed by the participants being rotated through three high-intensity interval stations involving treadmill, resistance/calisthenics, and stationary bike exercises, each lasting 10 min with 3–4 min recovery between stations. Each training session concluded with 10–15 min of cool-down and stretching exercises. Participants wore a wireless heart rate telemetry sensor (Polar H7 heart rate sensor, Polar Electro Oy, Kempele, Finland) throughout the exercise sessions to monitor their heart rate (HR) and to ensure that they were working at the appropriate intensity level. The intensity was adjusted according to individual participant's real-time HR telemetry and they were encouraged to work at 80-95% of maximal heart rate (HR_{max}). The treadmill interval station consisted of 3–4 bouts of 2 min running at 85-95% maximal aerobic capacity (VO_{2max}) separated by 30–45 s (s) periods of active recovery at 50% VO_{2max}, during which speed and incline were adjusted according to the participant's fitness level. The stationary bike station comprised 4-5 bouts of 45–60 s cycling efforts at 90-95% peak power output (W_{peak}) interspersed with 60–75 s active recovery at 30% W_{peak}, during which resistance and cadence were adjusted according to the participant's fitness level. The station of resistance and calisthenics exercises comprised 2-3 sets of several types of high-intensity exercises such as squats, kettlebell swings, planks and burpees, and resistance with 30 s rest between each set. The intensity and work/rest ratio of the resistance and calisthenics station progressed weekly over the 12-week training period in order to keep the exercises challenging and maintain appropriate high intensity interval training stimuli. Aside from ET, all recruited participants were instructed to continue their normal routine with regard to their physical activity and diet.

Collection of clinical data

Fasting serum samples were collected at baseline, 4 and 12 weeks after the exercise program and were conducted at 48-72 h after the final exercise session to prevent the influence of the acute effects of exercise. 75-g oral glucose tolerance test (OGTT) was conducted after 10-12 h overnight fasting. Blood samples were collected for the determination of plasma glucose and insulin levels at 0, 60 and 120 min after taking the 75 g glucose solution (TRELAN-G75, Ajinomoto Pharmaceutical Co. Ltd., Tokyo, Japan). Glucose, serum lipid profiles, including triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol, were determined with standard laboratory techniques on a Hitachi 717 Analyzer (Roche Diagnostics, Germany). Serum insulin levels were measured with ELISA kits (Immunodiagnostics, Hong Kong, China). Insulin resistance index (HOMA-IR) (Wallace et al., 2004) was calculated using homeostasis model assessment methods, defined as fasting insulin (μU/mL) x fasting glucose (mmol/L)/22.5. Body composition was assessed via whole-body dual-energy X-ray absorptiometry (DXA) scans (Explorer S/N 91,075, Hologic Inc., Waltham, MA, USA). Strength and cardiovascular physical assessments were conducted at baseline and within one week after completion of the 12-week intervention. Maximal voluntary muscle strength (chest press and leg press) was assessed using the 1-RM method (Keiser A-300, Keiser Corp., Fresno, CA, USA) following the manufacturer's instructions. An integrative cardio-respiratory fitness test using the Balke treadmill protocol was performed on a motor driven, electronically controlled treadmill (TrackMaster, Full Vision Inc., Newton, KS, USA) to assess VO_{2max} before and after the exercise intervention.

Definition of responders and non-responders

Non-response to the exercise intervention was defined as a failure to demonstrate a decrease in HOMA- IR greater than the 2-fold technical error.[11]

Olink Proteomics

Antibody-based technology (Olink Proteomics AB, Uppsala, Sweden) was used for proteomics profiling in serum samples. Specifically, protein biomarkers were detected via the Olink Explore 384 cardiometabolic and inflammation panels. Briefly, the proximity extension assay (PEA) technology uses DNA oligonucleotide-labelled antibody pairs to bind target proteins. After two matched antibodies bind the target protein, the oligonucleotide pairs hybridize and are extended by DNA polymerase to create a unique DNA barcode that is subsequently readout using next-generation sequencing. Since only correctly matched DNA string pairs could generate detectable and quantifiable signals, the PEA technology exhibited high specificity and excellent sensitivity.[92] After excluding 46 proteins that were not detected in >50% of the samples and 3 proteins that were duplicated, the final analysis included

688 proteins (352 from the cardiometabolic panel, 333 from the inflammatory panel and 3 included in both panels). The median intra-assay coefficient of variability (CV) was 12.5%, as assessed by multiple replicates of a pooled sample included in the experiment. Values were presented as normalized protein expression (NPX) units on a log2 scale.

Mesoscale Discovery (MSD)

We subsequently performed additional proteomics profiling using an MSD technology (MesoScale Discovery, Rockville, MD, USA) in 19 individuals from the study cohort to determine the reproducibility of our olink-based results. MSD procedure follows that of a sandwich ELISA, with any analytes of interest captured on the electrode being detected with an analyte specific ruthenium-conjugated secondary antibody. Values were presented as pg/ml on a log2 scale.

QUANTIFICATION AND STATISTICAL ANALYSIS

Main statistical analyses were performed with R software. Participants' baseline characteristics and outcomes were expressed as mean ± SEM. Data were tested for normal distribution with the Kolmogorov-Smirnov test and for homogeneity of variances with the Levene's test. Change from baseline was evaluated by paired Student's *t* test. Difference between groups at baseline and after intervention was evaluated by independent Student's *t* test and ANCOVA model controlling for the baseline measurements, respectively. The proteomics data were not normally distributed. The paired Wilcoxon rank-sum test was used to compare protein levels at 4- and 12-week vs baseline. The Benjamini-Hochberg false discovery rate (FDR) method, with a 5% FDR significance threshold, was applied to control for multiple testing. Fuzzy c-mean clustering was performed using the R package 'Mfuzz' (v2.20.0) after log2-transformation and *Z* score scaling of the data. Ingenuity pathway analysis (IPA, QIAGEN, Hilden, Germany) platform was performed to search for enriched pathways using significant proteins. p values were corrected for multiple hypothesis using the Benjamini-Hochberg method and pathways with FDR below 0.05 were considered significant. Linear regression analyses were performed to determine the relationship between changes in metabolic parameters and (i) baseline levels of proteins and (ii) intervention-induced changes in proteins ($p < 0.05$). Covariates in regression models included age, baseline or changes in body weight and body fat percentage. Differences in exercise-induced proteins changes between metabolic responder and non-responders were evaluated with unpaired Wilcoxon rank-sum test ($p < 0.05$).

ML algorithm

The proteomic profiles at baseline of 48 subjects were used to build an ML algorithm for predicting exercise responsiveness, with R caret package. Three ML models including (1) generalized linear regression, (2) logistic regression and (3) random forest were constructed using the baseline proteomics data. Samples were divided in two cohorts as performed in our previous manuscript.[11] The discovery cohort included 14 responders and 5 non-responders recruited between September 2016 and November 2016, whereas the validation cohort included 20 responders and 9 non-responders that shared similar characteristics with the discovery cohort, but were recruited between October 2018 and December 2018. A total of 32 differentially abundant proteomics biomarkers between responders and non-responders at baseline within the discovery cohort ($n = 19$, $p < 0.1$, Wilcoxon rank-sum test) were used for model construction, with 5-repeated 10-fold cross validation and ROSE sampling strategy to account for the imbalance in the two classes. The generalization of the random forest model was further tested in the validation cohort ($n = 29$). The area under the receiver operating characteristic curve (AUROC) was used as the main indicator of model performance.

ADDITIONAL RESOURCES

Complete clinical trial registration was deposited at ClinicalTrials.gov (NCT03240978), as previously described.[11]