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Mitochondrial bioenergetic pathways in blood leukocyte transcriptome decrease after intensive weight loss but are rescued following weight regain in female physique athletes

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Abstract

Prolonged periods of energy deficit leading to weight loss induce metabolic adaptations resulting in reduced energy expenditure, but the mechanisms for energy conservation are incompletely understood. We examined 42 healthy athletic females (age 27.5 ± 4.0 years, body mass index 23.4 ± 1.7 kg/m^2) who volunteered into either a group dieting for physique competition (n = 25) or a control group (n = 17). The diet group substantially reduced their energy intake and moderately increased exercise levels to induce loss of fat mass that was regained during a voluntary weight regain period. The control group maintained their typical lifestyle habits and body mass as instructed. From the diet group, fasting blood samples were drawn at baseline (PRE), after 4- to 5-month weight loss (PRE-MID), and after 4- to 5-month weight regain (MID-POST) as well as from the control group at similar intervals. Blood was analyzed to determine leukocyte transcriptome by RNA-Sequencing and serum metabolome by nuclear magnetic resonance (NMR) platform. The intensive weight loss period induced several metabolic adaptations, including a prominent suppression of transcriptomic signature for mitochondrial OXPHOS and ribosome biogenesis. The upstream regulator analysis suggested that this reprogramming of cellular energy metabolism may be mediated via AMPK/PGC1-α signaling and mTOR/eIF2

Abbreviations: CR, caloric restriction; DEG, differentially expressed genes; ETC, electronic transport chain; FDR, false discovery rate; GEE, generalized estimating equations; IPA, ingenuity pathway analysis; METh, metabolic equivalent hours; NMR, nuclear magnetic resonance; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TCA, tricarboxylic acid.

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signaling-dependent pathways. Our findings show for the first time that prolonged energy deprivation induced modulation of mitochondrial metabolism can be observed through minimally invasive measures of leukocyte transcriptome and serum metabolome at systemic level, suggesting that adaptation to energy deficit is broader in humans than previously thought.

KEYWORDS

diet, exercise, leukocytes, oxidative phosphorylation, ribosomes

1 | INTRODUCTION

Every year, many individuals engage in intense diet and exercise training programs in an attempt to pursue fat mass loss and improved aesthetic appearance. Results of these diets are often temporary, thus leading to a cycle of weight loss and subsequent regain. This so called yo-yo dieting/weight cycling has been regarded as a risk for sustained reduced energy expenditure at least in overweight individuals.[1-4] This decline in energy expenditure (ie, adaptive thermogenesis) is caused by a number of coordinated actions of metabolic, behavioral, neuroendocrine, and autonomic responses designed to maintain body energy stores at optimal levels.[1] Adaptive thermogenesis is considered a biologically meaningful survival mechanism that conserves energy in the face of starvation and dangerously low energy supplies.[5]

Dieting and weight cycling studies have thus far focused on overweight individuals. Weight cycling in normal weight individuals has been ignored perhaps due to the questionable ethics of subjecting normal weight individuals to a combination of very high dose of exercise with low-calorie diet, that is, very low energy availability.[6,7] However, even previously normal-weight athletes pursue lower levels of adiposity and a leaner phenotype as it has been considered beneficial in terms of superior performance and aesthetic appearance.[8-11] A period of very high-volume exercise and low energy availability leading to weight loss followed by a voluntary weight regain period is a very common practice in many sports.[6] In popular aesthetic sports such as fitness and physique sports, athletes after years of training, go through intensive dieting periods (>10 weeks) with reduced energy intake (<30 kcal/kg)[10] preceding competitions. Through intensive precompetition dieting, athletes aim to improve their muscular definition and aesthetic appearance by reducing body fat mass accomplished by high volume of both resistance and endurance training and a low-energy intake.[10,12,13] The weight loss period is usually followed by a voluntary weight regain period during which energy intake, exercise, and body fat mass levels are restored back to baseline levels aiming to recover from the intensive competition preparation diet.[10] To date, only a few studies on physique athlete weight regain period has been conducted, but it has been recommended that energy intake is increased up to 42-48 kcal/kg.[14] Thus, these individuals are ideal candidates for investigating the effects of metabolic adaptations during periods of voluntary weight loss and weight regain in previously normal weighed physically active individuals.

Although metabolic adaptation following caloric restriction (CR) is a well-established phenomenon, its molecular mechanisms are not completely understood. Given that mitochondria act as central bioenergetic organelles in the cells, they are thought to play an important role in CR-induced metabolic adaptations. Previous data from animal models indicate that the decreased mitochondrial respiration and oxygen consumption due to a decline in uncoupled respiration, that is, improved bioenergetic efficiency of mitochondria, might contribute to decreased energy expenditure during CR-induced weight loss in muscle, thus making weight maintenance and further weight reduction more challenging.[9,15,16] This metabolic adaptation may also make individuals more prone to regain the already lost fat mass after otherwise successful weight loss. We have previously shown that intense fat mass loss and regain have a distinct effect on hormonal system regulating energy metabolism.[10] In the present study,[10] we aimed to investigate the magnitude of mitochondrial and energy metabolism adaptations during a period of intense weight loss and voluntary weight regain at a systemic level in a population of normal-weight, healthy physique athletes.[10] More specifically, pathways of energy metabolism were examined by utilizing an integrative omics approach consisting of leukocyte-derived transcriptomics and serum nuclear magnetic resonance (NMR) metabolomics as minimally invasive markers of mitochondrial and metabolical adaptation.

2 | MATERIALS AND METHODS

2.1 | Study participants and design

Young normal-weight female physique athletes (n = 60) were recruited[10] to participate and volunteer in a self-administered weight loss and weight regain regimen (Figure 1). By the end of the study period (PRE-POST), a total of 10 athletes failed to complete the study regimen in a required manner. Specifically, the study drop-outs consisted of (a) one control not arriving to baseline testing, (b) three diet group participants failing to follow the diet program and thus did not compete, and (c) six controls were not able follow the control period or for other unknown reason to finish the study. In addition, participants with missing dietary data from at least one point of the study (n = 8) were excluded from the current omics study. After these exclusions, based on completion of the diet/control period and availability of data from all study points, 42 healthy athletic females (age 27.5 ± 4.0 years and body mass index [BMI] 23.4 ± 1.7 kg/m^2) were included in the current follow-up omics study. These individuals had volunteered into either a group dieting for physique competition (n = 25) or a control group (n = 17). Due to the intense nature of the study regimen, and ethical restrictions for randomization, the participants were allocated to diet and control groups based on their own choice.

The effects of intense fat mass loss and regain were examined from the diet group (n = 25, age 27.2 ± 4.2 years, BMI 23.5 ± 1.8 kg/m^2) at three test time points: (a) baseline tests were conducted before the weight loss regimen began (PRE), (b) immediately after the weight loss period (PRE-MID, 20.2 ± 3.3 weeks), and (c) after the weight regain period (MID-POST, 17.8 ± 2.4 weeks) (Figure 1). The weight loss period (PRE-MID) is usually characterized with low energy availability together with increasing the amount of aerobic exercise training while frequency and intensity of resistance training is kept at a relatively constant level to minimize the amount of muscle mass lost. The vigorous progressive weight loss routine is usually followed by a voluntary weight-regain period (MID-POST), during which exercise training and energy intake levels are reverted back to normal levels. This subsequent weight regain has been considered mandatory and beneficial to restore potentially disrupted metabolic homeostasis caused by prolonged low energy availability, intense exercise training, and extremely low levels of fat mass.

The control group of physique athletes (n = 17, age 27.8 ± 3.8 years, BMI 23.1 ± 1.4 kg/m^2) was instructed to maintain their typical weight and fitness lifestyle throughout the whole study period (PRE-MID, 22.4 ± 2.5 weeks; MID-POST, 19.2 ± 3.5 weeks). At each experimental time point (PRE, MID, and POST), blood samples were obtained, and all the participants went through a series of the same anthropometric and clinical tests that were described

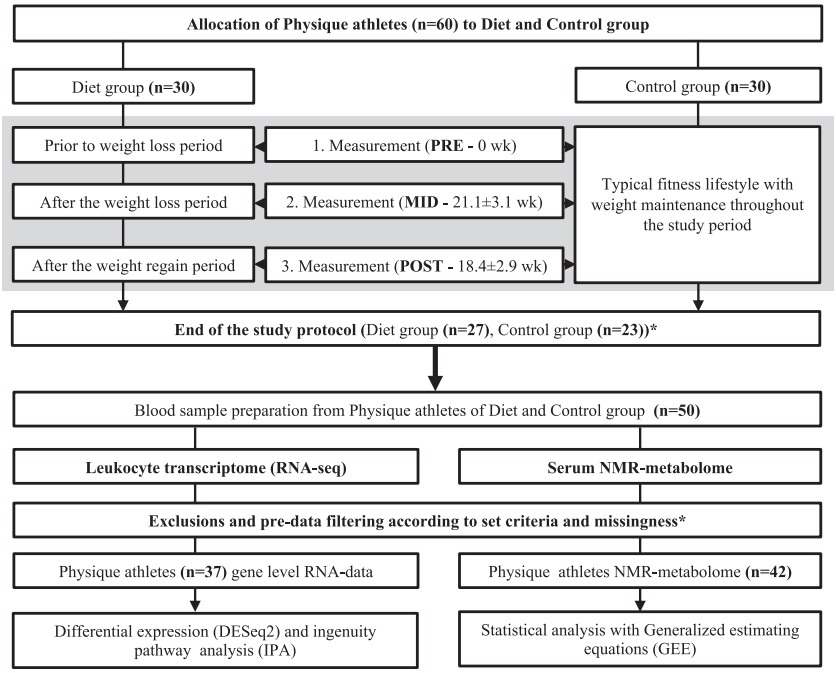

FIGURE 1 Study design and workflow. The study design and workflow are illustrated in a flowchart to demonstrate the utilized study protocol. *Overall, 10 participants failed to complete the study regimen (PRE-POST) in a required manner of the 60 individuals who volunteered for the study. A number of participants lacking complete dietary records (n = 8) were also excluded from the current omics study. Only individuals with minimal missing information were included in the final study sample of 42 participants (diet group n = 25, control group n = 17), for which bioinformatic analyses were conducted. Study period lengths (PRE-MID, MID-POST) are reported as mean (standard deviation) of both the diet and control group individuals. GEE, generalized estimating equations; IPA, ingenuity pathway analysis; NMR, nuclear magnetic resonance

earlier (Figure 1).[10] The following methods on reported nutrient intakes, exercise levels, and muscular fitness have been described in full detail earlier by Hulmi et al.[10] The Ethical Committee at the University of Jyväskylä approved the study protocol, and all participants gave written informed consent in accordance with the Declaration of Helsinki.

2.2 | Anthropometric measurements

In the current study, body composition and anthropometrics (ie, fat mass, visceral fat mass, and lean mass) were assessed with dual-energy X-ray absorptiometry (DEXA, Lunar Prodigy Advance, GE Medical Systems, Lunar, Madison, WI, USA).[10]

2.3 | Dietary intake

The physique study participants recorded nutrient intake repeatedly using self-reported food diaries from representative days throughout the study (a) at baseline (PRE), (b) after the weight loss period (MID), and (c) after the weight regain period (POST).[10] The diet group participants followed strict dietary routines during the weight loss period (PRE-MID), where majority of the food ingested was weighed and reported. The control group reported food diaries over three weekdays and one weekend day (a) at the baseline (PRE), (b) in the middle of the study (MID), and (c) during the last part of the study (POST). Nutritional supplements (ie, sport supplements) were included in the dietary analysis. The food diaries provided by the study participants were analyzed by dietary analysis software (Aivodiet, Flow-team Oy, Oulu, Finland).

2.4 | Physical activity and muscular fitness

The duration and intensity of daily physical activity and exercise training were also reported by the physique study participants throughout the study period (PRE, MID, and POST) from which overall physical activity (METh/week) level was calculated.[10] As reported earlier, the training regimen of the study participants (n = 42) included (a) resistance training 4.8 ± 0.7 (diet group) and 3.9 ± 1.9 (controls) times per week at the baseline (PRE), after which the resistance training frequency was maintained throughout the study period (MID and POST) in both groups. In contrast, the aerobic training frequency was increased from 3.8 ± 2.8 to 4.8 ± 3.0 times per week for the duration of the weight loss period (PRE-MID), which was mostly due to the increased amount of steady state aerobic training, and partly due to increased amount of HIT (high intensity training)-exercise.[10] The level of aerobic training decreased during the recovery period (MID-POST) in these participants down to 2.3 ± 2.2 times per week, whereas the controls maintained their levels of aerobic training relatively constant.[10] Changes in lower and upper body strength (ie, maximal voluntary contraction) was determined using isometric tests consisting of (a) a horizontal leg press extension dynamometer and (b) maximal isometric bilateral smith bench press together with (c) maximal explosive strength using a vertical counter movement jump. See further information on the training regimens and measures of muscular fitness from Hulmi et al.[10]

2.5 | Venous blood sampling and analysis

Fasting blood samples were taken from both the diet and control group after at least 8 hours of fasting at each time point. All the participants were asked to sleep for at least 8 hours during the preceding night and were required to refrain from strenuous physical activity for at least 24 hours before venous blood sampling at baseline (PRE), after the weight loss period (MID), and after the weight regain period (POST). Venous blood samples were taken from the antecubital vein into serum tubes (Venosafe; Terumo Medical Co., Leuven, Hanau, Belgium) using standardized laboratory procedures.[10]

2.6 | Transcriptome

2.6.1 | Library preparation, sequencing, read alignment, and batch effect

Transcriptome was quantified in peripheral leukocytes extracted from the samples collected in PAXgene blood RNA tube. The sequencing RNA library of each sample was processed using Illumina TruSeq according to the protocol provided by the manufacturer (https://www.illumina.com). The utilized Illumina protocol was paired end and strand specific, and the applied read depth for library preparation was set to 2 × 100 bp. Sequencing of the RNA libraries was carried out with the Illumina HiSeq2000 sequencing platform.

2.6.2 | Differential expression analysis

We further processed sequence alignments with the DESeq2 software to assemble transcripts, quantify the expression levels, and analyze differentially expressed genes (DEGs). DESeq2 applies its own normalization methods and independent filtering to raw read RNA-Sequencing data. Before statistical analysis, some of the low expressed genes were excluded from the analysis if they matched the criteria: (a)

gene had zero read counts across samples, and (b) gene had lower than five read counts in at least five samples. We further excluded additional samples from the analysis according to three criteria: (a) prior information on the incompetence to follow the study protocol; (b) sample outliers based on Cook's distance, pairwise MA plots, and sample distance heat maps; and (c) subjects without all three time-point measurements. After applying these exclusions, the final differential expression analysis set of samples included 111 samples from 37 participants (diet group n = 24, control group n = 13) in the differential expression analysis.

2.6.3 | Statistical analysis of transcriptome

Primary genome-wide expression analysis of the leukocyte transcriptome suggested suppressed mitochondrial bioenergetic profile following the intense weight loss period.[13] Thus, for the purposes of our study, we aimed to investigate mitochondrial bioenergetic pathways further and performed a targeted differential expression analysis for gene sets associated with mitochondrial function (eg, electronic transport chain [ETC], tricarboxylic acid [TCA] cycle, and β-oxidation,), mitochondrial biogenesis, and associated energy metabolism pathways and ribosomal metabolism. Human MitoCarta 2.0, Reactome, KEGG, and WikiPathway databases were used as the main gene lists for targeting genes of interest in the aforementioned pathways. In total, 1,700 genes were selected for the differential expression analysis (Table S1).

To identify DEGs among these gene sets, we used the Likelihood ratio test to conduct a nested time-course study with DESeq2 (H_0 = Group + Time + Group * Subject, H_1 = Group + Time + Group * Subject + Group * Time). We investigated whether genes were differentially expressed between the diet and control group across any of the time points when accounting for the between-subject variability. Post-hoc analysis of the Likelihood ratio test was also conducted for the diet and control group only (H_0 = Subject, H_1 = Subject + Time) to further explore within-group changes. In addition, Wald tests were applied within the DESeq2 interface for testing contrasts for the between/within group comparison across any two individual time points. A *Q* value of .05 for false discovery rate (FDR) was used to adjust for multiple testing.

2.6.4 | Pathway analysis of gene level data

Downstream pathway analysis of the DEGs was conducted to identify the affected biological pathways. The Ingenuity Pathway Analysis (IPA, QIAGEN Bioinformatics, Aarhus, Denmark) tool was used for analysis as it combines a wide set of integrated databases.

2.6.5 | Analysis of transcriptome centroids and their association with phenotype

We also determined how phenotype characteristics and their changes (eg, anthropometric and exercise level) associated with the observed changes in energy metabolism gene pathway centroids using correlation analysis. For each mitochondrial and energy metabolism gene pathway, centroids were calculated and normalized as standard deviation (SD) change from the reference Z-score (PRE) within each sample. In a similar manner, phenotype variables were calculated and normalized as SD change from the reference Z-score (PRE) within each sample. For gene pathway and phenotype variable centroids, statistically significance was calculated using Pearson correlation, where significance threshold was set as P value < .05.

2.7 | Metabolomics: NMR data preparation, quality control, and management

A high-throughput serum NMR metabolomics platform was used for the absolute quantification of serum metabolites. The full process and methods of sample preparation and quantification have been described previously.[17] The NMR metabolome assay yields a total of 228 different metabolites, including an array of lipoprotein subclasses, apolipoproteins, serum-free fatty acids, and a wide variety of small molecules such as glycolysis precursors, amino acids, and inflammation biomarkers. Of these metabolites, we selected for the present article a total of 22 metabolites known to be associated with mitochondrial function (eg, oxidative phosphorylation, and β-oxidation) and energy metabolism (Table S2). For this aim, we analyzed the metabolome of 42 physique athletes (diet group n = 25, control group n = 17) measured at three time points (PRE, MID, and POST). Prior to the analysis, we assessed data skewness, normality and outliers with dot plots and histograms. To dispose of excess variance caused by outliers, metabolite values were excluded from the analysis if SD was greater or less than four (±4) from the mean. Statistical analysis of the metabolomic data was performed using generalized estimating equations (GEE).

3 | RESULTS

3.1 | Phenotype characteristics of study participants throughout the study

As reported previously,[10,12,13] after the 20-week weight loss period (PRE-MID), the diet group achieved a 52% decrease in total body fat mass with only minor changes in lean mass, muscle size and maximal and explosive

strength.[10] Aforementioned decrease in fat mass measures was accomplished by a ~19% decrease in energy intake (mainly from carbohydrates) and a ~15% increase in total volume of exercise, as measured by metabolic equivalent hours per week (METh/week).[10] Following the weight regain period (MID-POST), all measures on fat mass were reverted back to baseline levels as reported previously.[10] In the controls, no significant changes were observed in the anthropometric traits measured throughout the study (PRE-MID-POST).[10]

3.2 | Overview of the targeted transcriptome analysis of mitochondrial and energy substrate metabolism

First, we examined the transcriptomic profile of leukocytes in the diet group using a targeted approach with ~1,700 preselected genes with importance in mitochondrial function and energy metabolism. Of those, a total of 303 genes were differentially expressed (161 downregulated and 142 upregulated) (FDR < 0.05) following the weight loss period (Table S3). All of the intense weight loss induced changes in gene expression levels were reverted back to baseline levels during the subsequent weight regain period in the diet group (Table S4). Following the entire weight cycling period, the expression levels of four genes were differentially expressed (FDR < 0.05) as (a) *ODC1* and *ATP5F1E* were reverted above baseline levels after the weight loss period, whereas (b) the expression levels of *MCUR1* and *ELOVL7* were increased only by the weight regain period when compared with baseline levels (Figures 2E and S1). Thus, this shows that none of the downregulated genes after the diet routine remained downregulated following the weight regain and the same was true for the upregulated genes. In the controls, no significant (FDR > 0.05) changes were observed in the investigated gene expression profiles measured throughout the study (Tables S5 and S6).

Pathway analysis of the aforementioned 303 DEGs in the diet group following the intense weight loss period (PRE-MID) revealed significant (Q value < .05) changes in a number of canonical pathways connected to the regulation of mitochondrial oxidative function and energy metabolism (Table S7). More specifically of the DEGs enriched in canonical pathways, IPA predicted (a) eIF2 signaling (Z-score = −5.20, Q value = 1.00×10^{-38}), (b) oxidative phosphorylation (OXPHOS) (Z-score = −2.84, Q value = 2.69×10^{-9}) pathways to be most strongly (Z-score > |2|) suppressed, whereas NRF2-mediated oxidative stress response (Z-score = 2.24, Q value = 5.25×10^{-2}) pathway was suggested to be activated after the weight loss period (Table S7). In addition, other DEG enriched pathways with tendency (Z-score < |2|) to be suppressed included, (c) mTOR signaling (Z-score = −1.34, Q value = 7.94×10^{-15}) pathway whereas activation was suggested for (a) Sirtuin signaling (Z-score = 0.83, Q value = 5.89×10^{-10}) and (b) AMPK Signaling (Z-score = 1.39, Q value = 5.62×10^{-6}) (Table S7).

3.3 | Intensive weight loss is characterized by transcriptomic and metabolomic signatures of decreased mitochondrial bioenergetics

Given the high enrichment of DEGs in pathways associated with mitochondrial oxidative function and energy metabolism upon weight loss, we aimed to characterize changes taking place in the major cellular energy-yielding pathways, glycolytic metabolism and mitochondrial OXPHOS, in more detail.

First, enhanced glycolytic metabolism was indicated through moderate upregulation of genes participating in reactions and an activation of glycolysis (FDR < 0.05: *PGAM2*, *LDHAL6A*, $P < .05$: *HK3*, *PFKM*, *PGK1*, *PKLR*, and *LDHD*). In addition, diminished utilization of glycolysis end product, pyruvate, within mitochondria was suggested through increased expression (FDR < 0.05) levels of *HIF1A*[13] that is a central metabolic switch driving glycolytic metabolism and mitochondrial oxidative quiescence (Figure S2B). This observation was corroborated by altered expression profile of several genes (FDR < 0.05: *PDK4*, $P < .05$: *MCP2*, *PDK1*) responsible for (a) pyruvate transport to mitochondria and (b) pyruvate conversion to acetyl-CoA that fuels the TCA cycle (Figure S2B; Table S3). However, only nominal downregulation was observed in two rate limiting key enzymes of TCA cycle (eg, *IDH2* and *SDHA*) ($P < .05$) without further changes in other enzymes (Table S3).

Second, we aimed to understand how the predicted suppression of OXPHOS pathway affected mitochondrial bioenergetics by mapping expression of all genes encoding the five mitochondrial respiratory complex subunits in the ETC pathway (Figure 2A-E; Table S3). Downregulatory (FDR < 0.05) effect was observed for several nuclear genes coding the mitochondrial respiratory chain subunits, where the most distinct suppression of transcriptomic profile was detected in the Complex V (Figure 2E; Table S3). Suppression of mitochondrial oxidative metabolism was further indicated through downregulation of genes responsible for regulating mitochondrial fatty acid β-oxidation (FDR < 0.05: *CPT1A*, *SLC25A20*, *ETFA*, and *ACAA2*; $P < .05$: *ACADVL* and *ACADS*) (Figure S3B; Table S3). Consequently, together with the leukocyte transcriptomic signatures for suppressed OXPHOS and fatty acid oxidation—also systemic level mitochondrial bioenergetic quiescence was suggested through elevated levels

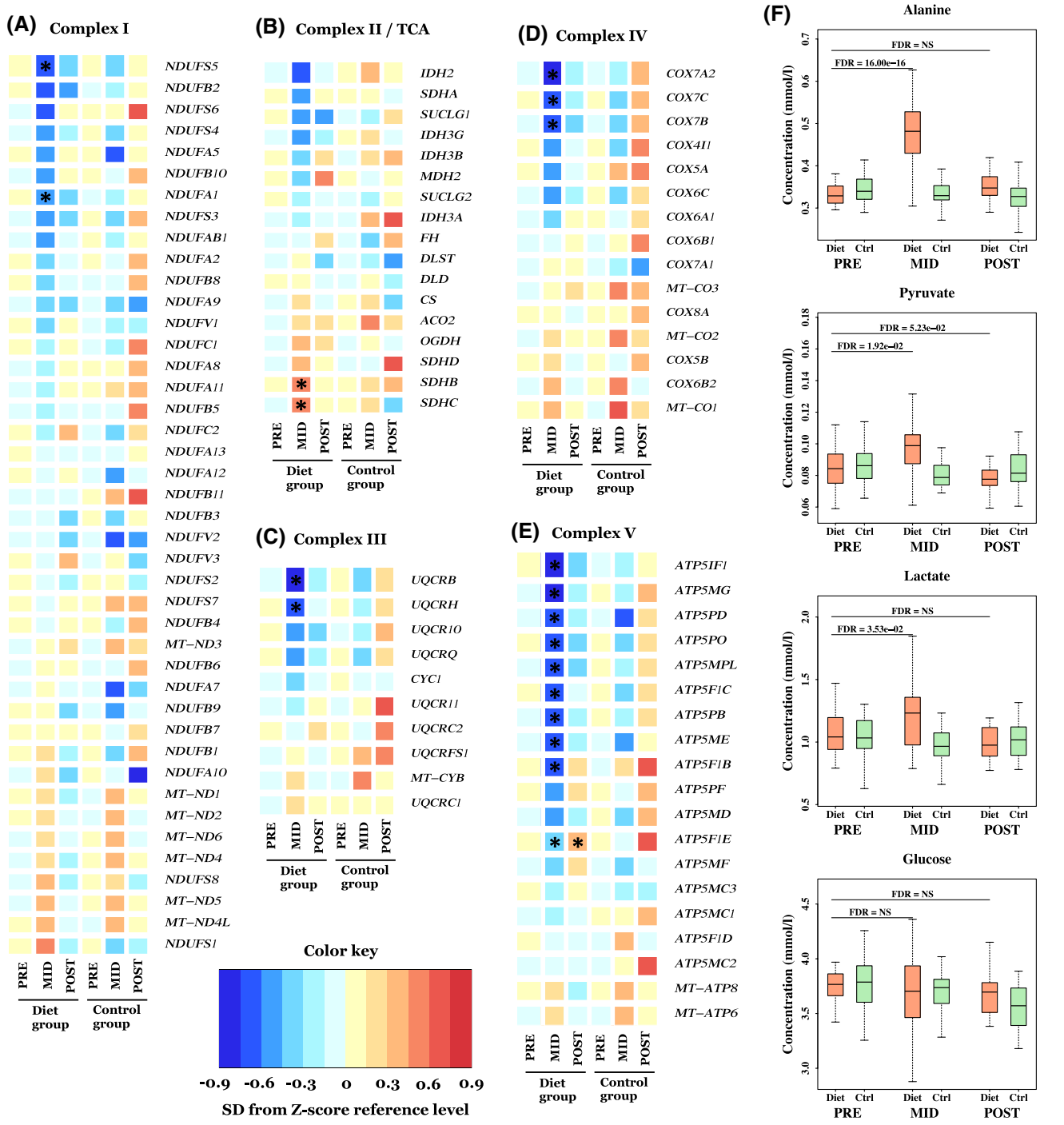

FIGURE 2 Transcriptomic activity of oxidative phosphorylation (OXPHOS) gene pathway following the intense weight loss period. Here, we demonstrate how the prolonged period of low-energy availability and intense exercise leading to substantial weight loss modulated gene expression profile of genes associated with OXPHOS pathways. Specifically, transcriptomic changes of genes in Complex I (panel A), Complex II and TCA cycle (panels B), Complex III (panel C), Complex IV (panel D), and Complex V (panel E) have been demonstrated with heatmaps. The intense weight loss period (PRE-MID) had the most distinct downregulatory effect on the expression levels of Complex V (panel E) genes. Gene expression levels are represented as standard deviation (SD) change from the reference Z-score. Baseline calculated Z-score values (PRE) from both the diet and control groups were separately set as the reference level to which each individual group/time-point level was compared. On the heat map, blue colors indicate decrease and red colors increase in gene expression level compared with the calculated reference value as depicted with the color key. Statistically significant change is annotated with asterisks (*) within heatmap cells using FDR adjusted *P* values (false discovery rate [FDR] < 0.05) for within-group time-point comparisons. Levels of substrates (serum alanine, pyruvate, lactate, and glucose) reflecting the balance between oxidative and glycolytic metabolism during the study period are depicted in panel F

of serum alanine ($\beta = 0.143 \pm 0.011$, FDR $= 1.0 \times 10^{-16}$), pyruvate ($\beta = 0.015 \pm 0.006$, FDR $= 0.02$), and lactate ($\beta = 0.149 \pm 0.062$, FDR $= 0.04$) (Figure 2F; Table S8). These clinical markers for mitochondrial dysfunction are subsequently increased in circulation when glycolysis end product, pyruvate, is not utilized in sufficient manner for energy production in mitochondria.

Third, it was also investigated in more depth how changes in the clinical phenotype[10] correlated with the aforementioned changes in the leukocyte transcriptomic bioenergetic profile following the intense weight loss and weight regain period (Table 1). Subsequently, significant correlations ($r = 0.3$-0.5, $P < .05$) between changes in fat mass parameters (eg, weight, fat mass, android fat mass, and BMI) and mitochondrial oxidative metabolism signature were observed when data from all time points were analyzed (Table 1). Physique athletes with the most drastic drop in fat mass experienced also the most distinct suppression of mitochondrial oxidative metabolism signature, together with a similar positive correlation between weight regain and increase in mitochondrial oxidative bioenergetic signature (Table 1). Thus, collectively, our results suggest that a major systemic hallmark of intensive weight loss is the decline in mitochondrial bioenergetics, whereas the weight regain has the potential to rescue these changes.

3.4 | Intensive weight loss is characterized with increased regulatory signaling for mitochondrial biogenesis

Consistent with previous CR studies,[18] intensive weight loss lead to the aforementioned upregulation of pathways (Q value $< .05$: Sirtuin signaling and AMPK signaling) regulating mitochondrial biogenesis (Table S7). Closer examination of individual genes involved in "Sirtuin and AMPK signaling" pathways revealed a significant upregulation (FDR < 0.05) of three AMPK subunits, whereas no significant changes in the expression of genes coding Sirtuins 1-7 themselves were detected (Figure 3A,B, Table S3). The key downstream mediator of positive effects of AMPK on mitochondrial biogenesis and cellular bioenergetic metabolism—PGC1-α (*PPARCG1A*) was observed to be upregulated (FDR < 0.05) upon weight loss. Similarly, nominal changes were observed in its transcriptional targets (a) *NRF1* ($P < .05$), the transcription factor involved in the activation of mitochondrial transcription factor A (TFAM) and transcription of mtDNA encoded genes, and (b) in *NFE2L2* (aka. *NRF2*) ($P < .05$), the key driver of transcriptional activity of oxidative stress response genes (Figure 3A,B; Table S3). These results suggest that intensive weight loss triggers systemic AMPK/PGC-α-coordinated induction in mitochondrial biogenesis and oxidative stress defence signaling.

3.5 | Intensive weight loss is characterized with increased signature for cellular exposure to ROS

As we observed positive upstream signals by AMPK/PGC1-α/NRF2 and predicted the activation of NRF2-mediated oxidative stress response pathway (Table S3), we further analyzed transcription signatures of reactive oxygen species (ROS) and antioxidant defence system. We observed suppressed transcriptomic profile for genes coding major hydrogen peroxide (ROS)-scavenging enzymes (FDR < 0.05: *GPX1*, *TXN2*, *PRDX2*, *PRDX5*, and *PRDX6*) (Figure S4; Table S3). In addition, enhanced formation of hydrogen peroxide (ROS) was suggested by upregulation (FDR < 0.05) of (a) gene coding mitochondrial superoxide dismutase 2 (*SOD2*) (Figure S4C) and (b) genes regulating initial stages of peroxisomal β-oxidation (*ACOX1* and *ACOXL*) (Figure S4B). Taken together, these results imply that long-term energy deficit combined with high-volume exercise training leading to weight loss results in increased cellular transcriptomic signature potentially suggesting increased exposure to ROS and exhaustion of ROS defense system.

3.6 | Intensive weight loss is characterized with transcriptomic profiles of suppressed capacity for cytosolic protein translation

Lastly, given that CR has been previously shown to regulate protein synthesis pathways, we assessed more in detail pathways involved in protein metabolism. Suppressed cytosolic and mitochondrial ribosome biogenesis and translation capacity was suggested through (a) the predicted inactivation of the major nutrient sensing pathways regulating protein translation and ribosome biogenesis (Q value $< .05$: eIF2 signaling and mTOR signaling) that were specifically highly enriched with genes encoding cytosolic ribosomal proteins, (b) repression of genes coding key regulatory factors of protein translation (FDR < 0.05: *EIF2AK1*, *EIF2A*, *EIF3*, *eEF2*),[13] (c) decreased gene expression of cytosolic and mitochondrial ribosomal proteins (Figure 4A,B; Table S7). Overall, our results depicted that intensive weight loss triggers reduction in the gene expression signatures important for global protein synthesis and translation, which is known to be an energetically expensive process.[19]

4 | DISCUSSION

The current integrated high-throughput omics study, utilizing a unique group of female physique athletes who voluntarily performed high volumes of exercise together with a prolonged period of low energy availability,[10,12,13] represents an

TABLE 1 Mitochondrial oxidative metabolism transcriptome centroid expression profile change correlation with phenotype changes across the entire study period (PRE-MID-POST)

	Weight (kg)		Fat mass (kg)		Lean mass (kg)		Android fat mass (kg)		Body mass index (kg/m^2)		Resistance training (METh/week)		Aerobic training (METh/week)	
	r	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Beta oxidation	0.33	4.90e-03*	0.41	2.91e-04*	−0.00	0.97	0.47	3.85e-05*	0.43	1.39e-04*	0.11	.38	−0.06	.63
ETC—Complex 1	0.27	1.95e-02*	0.36	1.82e-03*	−0.01	0.92	0.38	1.09e-03*	0.37	1.18e-03*	0.01	.93	−0.08	.63
ETC—Complex 2 and TCA	0.36	1.95e-03*	0.46	5.25e-05*	0.00	0.98	0.45	8.08e-05*	0.48	1.82e-05*	0.17	.20	−0.10	.42
ETC—Complex 3	0.27	1.95e-04*	0.32	5.38e-03*	0.03	0.82	0.34	3.47e-03*	0.36	1.74e-03*	0.04	.78	−0.09	.50
ETC—Complex 4	0.26	1.95e-05*	0.32	5.72e-03*	0.02	0.87	0.36	1.79e-03*	0.36	1.66e-03*	0.01	.95	−0.08	.56
ETC—Complex 5	0.28	1.95e-06*	0.39	7.26e-04*	−0.04	0.74	0.43	1.57e-04*	0.39	7.32e-04*	−0.00	.98	−0.14	.30

Note: For each mitochondrial energy metabolism gene pathway, centroids were calculated and normalized as standard deviation (SD) change from the reference Z-score (PRE) for each time point measurement. In a similar manner, phenotype variables were calculated and normalized as standard deviation (SD) change from the reference Z-score (PRE) for each time point measurement.

Abbreviations: ETC, electron transport chain; METh, metabolic equivalent hours; TCA, tricarboxylic acid cycle.

*For gene pathway centroids versus phenotype variables, statistically significance was calculated using Pearson correlation, where significance threshold was set as P value < .05.

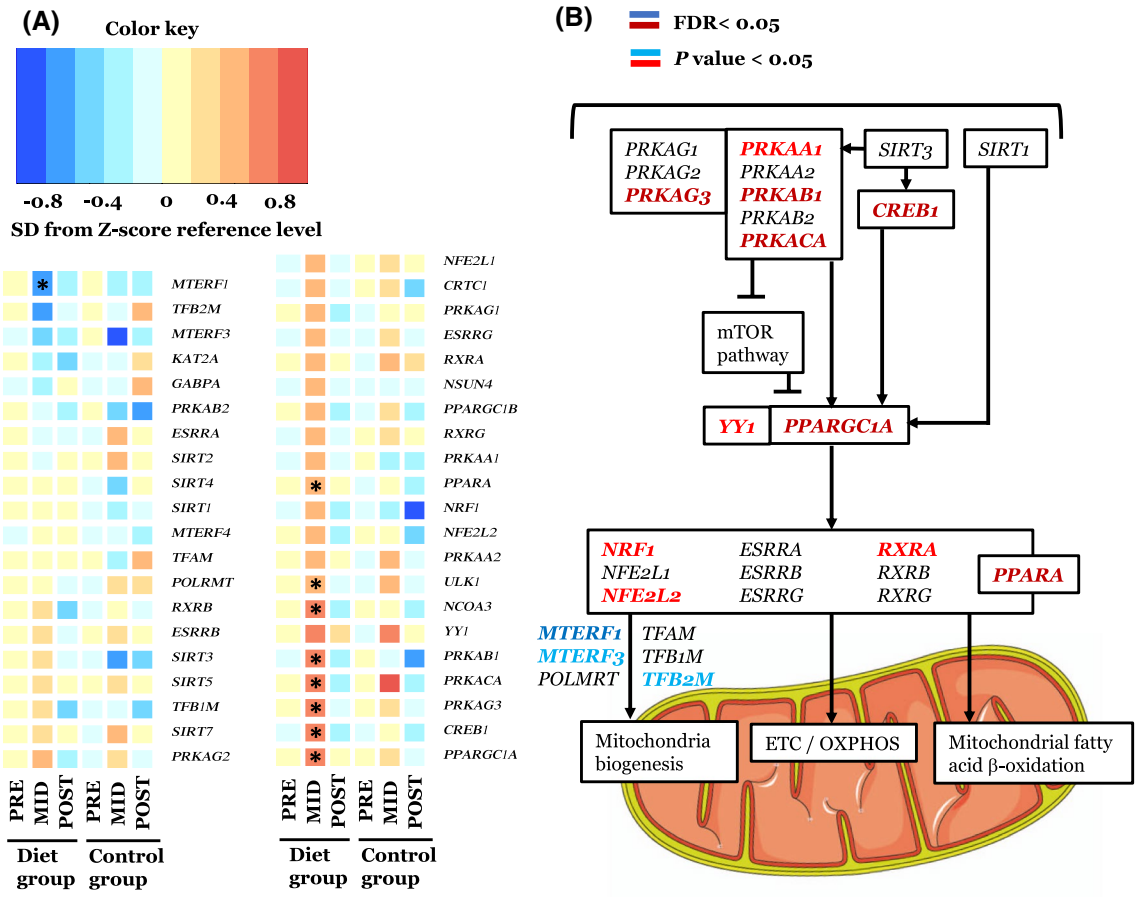

FIGURE 3 Transcriptomic activity of genes participating in mitochondrial biogenesis regulation following the intense weight loss period. In Figure 3, it is demonstrated how the prolonged period of low-energy availability and intense exercise leading to substantial weight loss modulated gene expression profile of genes associated with mitochondrial biogenesis (panels A–B). Gene expression levels are represented as standard deviation (SD) change from the reference Z-score (panel A). Baseline calculated Z-score values (PRE) from both the diet and control groups were separately set as the reference level to which each individual group/time-point level was compared. On the heat map, blue colors indicate decrease and red colors increase in gene expression level compared with the calculated reference value as depicted with the color key. Statistically significant change is annotated with asterisks (*) within heatmap cells using adjusted *P* values (false discovery rate [FDR] < 0.05) for within-group time-point comparisons. Mitochondrial biogenesis pathways and associated reactions are depicted in panel B. Within mitochondrial biogenesis pathways (panel B), upregulated (FDR < 0.05, dark red; *P* value < .05, light red) genes are indicated with red color, downregulated (FDR < 0.05, dark blue, *P* value < .05, light blue) genes with blue color, and genes with no clear change in expression pattern with color black

ideal model to explore the effects of intensive weight loss on systemic mitochondrial metabolism in normal weight physically active individuals—an area previously unexplored. Adaptive thermogenesis is considered a biologically meaningful survival mechanism conserving energy when energy supply is very low.[5] In the present study, adaptive changes to energy restriction were shown through various downregulated energy metabolism pathways at the systemic level. More specifically, by constructing networks of leukocyte transcriptomics and serum metabolomics profiles, our study characterized numerous adaptation mechanisms in the cellular bioenergetic profile during the intense weight loss showing that peripheral leukocytes can reflect whole body metabolism. These adaptations include strongly repressed transcriptomic signature for mitochondrial OXPHOS, especially in complex V, and ribosomal proteins as well as activated ROS-scavenging system. This reprogramming of cellular energy metabolism is likely mediated via (a) AMPK/PGC1-α and (b) mTOR/eIF2 signaling pathways, respectively, aiming at conserving energy consumption and minimizing oxidative stress during the prolonged period of exercise training and energy deprivation. Given that the alterations in the cellular and mitochondrial bioenergetic profile were reversed following the subsequent weight regain period, our results demonstrate that normal weight healthy individuals possess a high degree of metabolic flexibility and can subsequently adapt their cellular energy production mechanisms in according to alterations in energy availability.

For the first time in previously normal-weight athletes, we demonstrated that a prolonged period of high-volume exercise training combined with energy deprivation has the potential to induce a disruption of the cellular bioenergetic profile.[20] Our

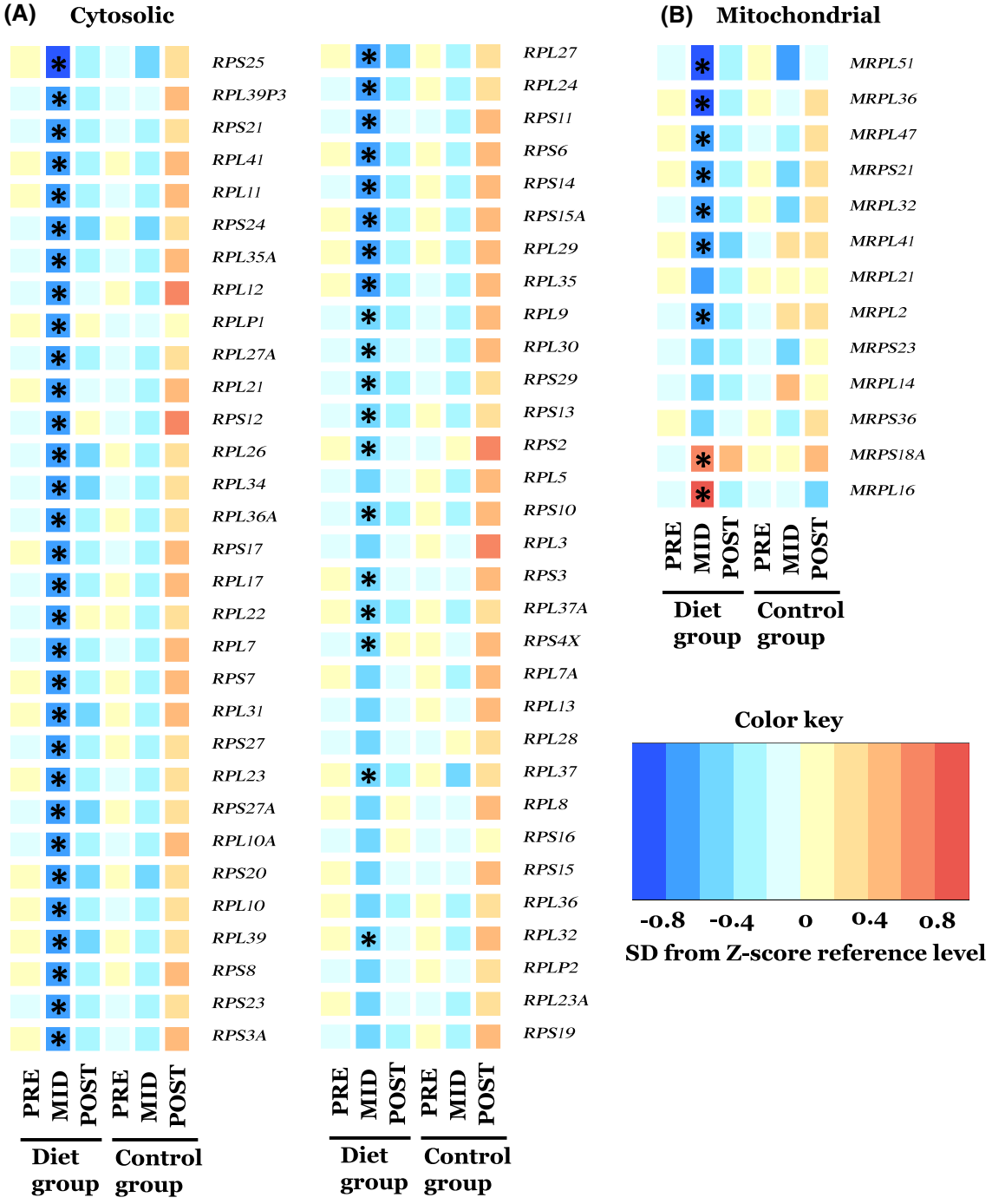

FIGURE 4 Transcriptomic activity of genes coding mitochondrial and cytosolic ribosomal proteins following the intense weight loss period. Here, we demonstrate how the prolonged period of low-energy availability and intense exercise leading to substantial weight loss modulated gene expression profile of genes associated with cytosolic and mitochondrial ribosome protein metabolism (panels A–B). Gene expression levels are represented as standard deviation (SD) change from the reference Z-score (panel A). Baseline calculated Z-score values (PRE) from both the diet and control groups were separately set as the reference level to which each individual group/time-point level was compared. On the heat map, blue colors indicate decrease and red colors increase in gene expression level compared with the calculated reference value as depicted with the color key. Statistically significant change is annotated with asterisks (*) within heatmap cells using adjusted *P* values (false discovery rate [FDR] < 0.05) for within-group time-point comparisons

findings invoke the question whether the observed transcriptomic profile for diminished mitochondrial oxidative pathways was translated into actual changes in mitochondrial bioenergetic function. A similar downregulation of mitochondrial oxidative pathways has been reported upon long-term conventional weight loss in subcutaneous white adipose tissue[21]; although, in skeletal muscle, the results have been more conflicting, reporting both decreased[16] and unchanged[22]

mitochondrial respiratory function. Given that CR has been shown to reduce muscle mitochondrial respiration by increasing the efficiency of ETC function in animals,[23] we speculate that our observation of suppressed of mitochondrial energy production pathways leads to similar alterations in the mitochondrial bioenergetic profile and function. The purpose of this underlying primary mechanism can be speculated to minimize mitochondrial respiration derived ROS production and conserve energy upon increased cellular stress due to the long-term energy deprivation and extensive exercise training as suggested previously.[24,25] This mechanism may be mediated by the ROS stabilized HIF1-α which drives a switch,[26-29] between glycolytic metabolism and mitochondrial oxidative quiescence with concomitant improvement in mitochondrial respiration efficiency. In accordance with this mechanistic hypothesis, the transcriptomic profile following the intense weight loss in our study was characterized by simultaneous (a) activation of ROS producing pathways together with (b) the compromised antioxidant defence system, thus predisposing cells to increased exposure to ROS and (c) upregulation of HIF1-α.

Mitochondrial biogenesis is activated after aerobic exercise training[30] and CR.[18] Induced mitochondrial biogenesis and content is thought to defend against increased oxidative stress by reducing ROS production due to lowered ETC activity per each mitochondrion and the increased number of entry points for electrons.[31] This will reduce the probability of electron leakage at ETC complexes known to promote partial reduction of oxygen to form superoxide in mitochondria. Previous studies investigating long-term energy deficit leading to sustained weight loss have documented increased markers of mitochondrial biogenesis[21] in subcutaneous white adipose tissue as well as both increased[32] and unchanged[22] mitochondrial biogenesis in skeletal muscle after weight loss. Our findings also suggested invoked systemic mitochondrial biogenesis upon the intense weight loss period possibly through the activation of AMPK/PGC1-α signaling—the master regulatory pathway of mitochondrial biogenesis.[33-35] However, the idea that intensive weight loss increased mitochondrial biogenesis even in part due to the increased amount of aerobic exercise and regular resistance training in combination with CR in the present study is confusing because the generation of new mitochondria is an energetically expensive process requiring an incorporation of newly synthesized proteins into existing mitochondria. This phenomenon needs further mechanistical studies to be conducted in the future.

Another strong finding in the present study to support adaptive mechanisms to survive the energy deficit in addition to downregulated OXPHOS was a consistent decrease in the gene expression signature of ribosomal proteins. It has been recently characterized that under a state of nutrient starvation the suppression of mTOR pathway mediates the recycling and degradation of key cell storage units,[36,37] ribosomes, to provide nucleotides, amino acids, and energy for the cells. In the present study, energy deprivation induced attenuation in the global signatures of genes coding ribosomal proteins and (ii) regulatory genes of protein translation thus in theory, reducing translation capacity. In accordance with our findings, CR has been shown to result in the selective synthesis of both mitochondrial and cytosolic proteins.[38] This global response seems to favor only the synthesis of proteins that enforce the recovery from stress and are only essential for cellular functions,[39] due to the fact that protein synthesis and especially protein translation are one of the most energy-consuming cellular processes.[19] Subsequently, these processes were predicted to be regulated through the suppression of mTOR/eIF2 signaling pathway. Future studies should investigate whether the decreased transcription of ribosomal proteins is accompanied by increased ribophagy during energy restriction in humans and also the physiological significance of these processes to conserve energy[1] and to maintain important functions of the body when energy supply is low.[5]

Mitochondria are known to be crucial also for the function of the immune system.[40] Arising evidence has demonstrated that suppression of the mitochondrial bioenergetic profile is linked with immunodefiency and frequent infections.[41] Although regular moderate intensity and duration of exercise is beneficial for the host immune defense,[42] infection burden has been sometimes been reported to be high among high performance athletes going through intense exercise regimens or during competitions or a competition preparation.[42] Correspondingly, impaired immune response on the same study population was implied by our previous publication suggesting immunosuppression upon the prolonged period of intense training with low-energy availability with the very high volume and intensity of exercise.[12] Based on the occurring evidence[41] and our findings, we hypothesize that the observed alterations in the mitochondrial bioenergetic profile and AMPK and mTOR signaling might mediate to some extent the documented suppression of the immune responses and increased susceptibility to infections in highly trained athletes in which prevalence of low energy availability is high.[7] In support of our hypothesis, nutrient sensors AMPK and mTOR are known to modulate immune responses for example via control of T cell metabolic plasticity.[43,44] In the future, it should be determined whether repeated consecutive intense weight loss bouts could have profound long-lasting negative effects on leukocytes' mitochondrial bioenergetic functions and their immune responses.

The strengths of our study were the comprehensive integrative system biological data sets of leukocyte transcriptome and serum metabolomics, and the longitudinal study design including both the weight loss and weight regain periods and the control group. Although leukocyte-derived mRNA expression levels have been reported to dynamically reflect system wide biology,[45] we acknowledge that the major limitation of our study was that mRNA expression levels derived from

leukocytes do not always reflect actual levels of biologically active proteins on the global level in different tissues or function of mitochondria. Therefore, future studies should investigate whether these results are translated into leukocyte and other cell's proteome or functional parameters of the mitochondria.[46] In future, the direct measures of mitochondrial respiration and amount are required to show whether the observed changes in transcriptomic profiles translate into alterations in mitochondrial function, efficiency, and the number in leukocytes and peripheral tissues. No dietary control or standardizing could be applied due to ethical reasons, but the cohort of physique athletes exceptionally well reported their dietary intakes during the weight loss regimen of the study. In addition, dietary content was very similar across the participants throughout the weight loss regimen (ie, high protein content together with consistently reduced amount of carbohydrates and fats) with basically no exceptions.[10] However, we acknowledge that the results may not be generalizable to other types of diets. The present study was conducted in athletic females, why the future studies should investigate whether the present findings apply also to other populations such as males and overweight individuals. Although in the present study we did not directly measure metabolic rate from the participants, we have now conducted a new diet trial in both females and males in which it was demonstrated that resting metabolic rate measured by indirect calorimetry was decreased during the weight loss period similarly as has been shown earlier in obese women[16] without changes in absolute level of fat oxidation (Isola et al, in preparation) supporting our present systemic level findings.

In conclusion, our study revealed that a prolonged period of low-energy availability and high volume of combined aerobic and resistance training is characterized by numerous adaptation mechanisms aimed at conserving energy consumption of which the most central ones seem to be the repression of signatures associated with mitochondrial oxidative functions and protein translation. This reprogramming of cellular energy metabolism is suggested by us to be mediated via (a) activation of AMPK/PGC1-α signaling and (b) suppression of mTOR/eIF2 signaling pathways, respectively. Although, the weight regain restored the mitochondrial and energy metabolism pathways, future studies are required to evaluate the effect of repeated consecutive intense weight loss and regain cycles on leukocytes' mitochondrial and immune system functions, if pursued without sufficient time allowed for recovery. In the end, our study also reveals for the first time that diet-induced modulation of mitochondrial metabolism can be observed through minimally-invasive measures of leukocyte transcriptome and serum metabolome at the systemic level, suggesting that adaptation to energy deficit is broader in humans than previously thought.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

H.V. Sarin was responsible for the analysis and the majority of writing. J.J. Hulmi, M. Perola, K.H. Pietiläinen and E. Pirinen supervised the project and contributed significantly to the writing and editing of the manuscript. J.J. Hulmi, V. Isola, K. Häkkinen and M. Perola designed the original study, and V. Isola was responsible for conducting the trial. Other authors contributed to analysis and interpretation of the results based on their expertise (K.H. Pietiläinen—weight loss, endocrinology, and mitochondria; E. Pirinen —mitochondria). All the authors accepted the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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