Original Research

Short and long-term effects of high-intensity interval training applied alone or with whole-body cryostimulation on glucose homeostasis and myokine levels in overweight to obese subjects

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TABLE OF CONTENTS

1. Abstract

Background: COVID-19 pandemic has exacerbated the problem of physical inactivity and weight gain. Consequently, new strategies to counteract weight gain are being sought. Because of their accessibility, interval training and cold therapy are the most popular such strategies. We here aimed to examine the effect of 6 units of high-intensity interval training (HIIT), applied alone or in combination with 10 sessions of whole-body cryotherapy (WBC; 3 min at –110 °C per session) on incretins, myokines, and adipokines levels. **Materials and methods**: The study involved 65 subjects (body mass index of approximately 30 $kg \cdot m^{-2}$). The subjects were randomly divided into training group (TR; n = 27) and training supported by WBC group (TR-WBC; n = 38). Blood samples were collected before, immediately following, and 4 weeks after the intervention. **Results**: Fibroblast growth factor 21 (FGF21) levels sig-

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nificantly increased (p = 0.03) and adiponectin levels increased in the TR group (p = 0.05) compared with those recorded in TR-WBC group 24 h after the end of experimental protocol. Beneficial changes in the lipid profile (p = 0.07), a significant drop in visfatin levels ($p < 0.05$), and the improvement in β-cell function (HOMA-B; p = 0.02) were also observed in the TR group in the same time point of study. While TR-WBC did not induce similar changes, it ameliorated blood glucose levels (p = 0.03). Changes induced by both interventions were only sustained for 4 weeks after treatment. **Conclusion**: Collectively, HIIT, alone and in combination with WBC, positively affects metabolic indicators, albeit, most likely, different mechanisms drive the beneficial effects of different treatments.

2. Introduction

According to global estimates prior to the outbreak of COVID-19 pandemic, 27.5% of adults [1] and 81% of adolescents were physically inactive [2], and did not meet the recommended 150 min weekly dose of physical activity (PA). According to some authors, pandemic-related lockdown induced additional, major changes in lifestyle behavior among adults, with a 43% decrease in PA and 19% increase in unhealthy food consumption, ultimately resulting in weight gain [3]. The average weight gain among adults associated with the COVID-19 pandemic is 4.7 kg (unpublished, statistic data). Obese individuals with low cardiorespiratory fitness are typically a challenging population to be treated; in the presence of accompanying diseases, these individuals struggle to survive [4]. Further, obesity increases the risk of severe infection with SARS-CoV-2, the virus that causes COVID-19 [5]. Excessive fat accumulation, especially as visceral adipose tissue, impairs glucose homeostasis [6] and results in a low-grade inflammation that may, over time, lead to insulin resistance and type 2 diabetes (T2DM). Nonetheless, during the ongoing COVID-19 pandemic, home-based PA programs supported by digital solutions are commonly used to maintain an adequate level of PA and weight balance [7]. Further, limited access to fitness centers and infrastructure has focused the attention on intermittent forms of PA that could be performed at home, e.g., high-intensity interval training (HIIT). Among the different forms of physiotherapy, cold exposure is thought to enhance the beneficial effects of exercise. Consequently, cold water immersion in the sea or lake became popular in the winter of 2020/2021, when access to professional physiotherapy was limited. For these reasons, in this project we aimed to evaluate short- and long-term effects of interval training in combination with exposure to extreme cold, considering pro-health changes in the lipid profile, myokine profile and glucose homeostasis among overweight to obese, inactive participants.

Studies suggest that exercise [8] and cold exposure [9] elicit comparable muscle contractions, the latter in association with shivering, and induce similar endocrine responses, by stimulating the release of muscle-derived peptides. These act as endocrine-like factors, such as myokines [10] and exerkines [11], and are involved in the prevention or reversion of the negative effects of high food intake, being overweight, and obesity, as well as several other pathological conditions [12]. Fibroblast growth factor 21 (FGF21) [13] and irisin [14] are myokines modulated by cold exposure. They are important metabolic regulators that stimulate glucose uptake by adipocytes and myofibers, and improve glucose homeostasis. However, the physiology of the effects of physical exercise and cold exposure on myokine expression, in particular, that of FGF21, is still only marginally understood.

Costello *et al*. [15] studied the effect of whole-body cryotherapy (WBC; –110 °C, 3 min exposure) and cold-water immersion (8 °C, 4 min) on the temperature of different body parts. They observed comparable changes in the muscle and core temperature, but not in the skin temperature. Further, we have previously demonstrated that regular WBC causes a drop in FGF21 blood levels, but only among middle-aged participants [16]. The decrease in FGF21 levels was accompanied by an improvement of glucose homeostasis-related parameters, and a reduction of valine and asparagine levels [16]. The latter effect is particularly important since valine and asparagine are considered to be early markers of glucose homeostasis disturbance [17]. Other studies involving human subjects have focused on mild cold exposure: 12 h exposure to 19 °C [18] and 12 min exposure to 18 °C, lowered by 2 °C every 3 min to 12 °C [19]. The latter treatment enhanced circulating FGF21 levels and brown adipose tissue mass [19]. Further, treadmill exercise test performed over 2 weeks, following the Bruce's protocol, induces a significant increase in serum FGF21 levels in young, inactive women, and this is accompanied by an increase in free fatty acid levels in the blood, heart rate (HR), and energy expenditure during exercise, as well as changes in epinephrine levels [20].

Considering the above, one may hypothesize that a combination of HIIT and cryostimulation would positively impact metabolic homeostasis, consequently improving the inflammatory (i.e., myokine) status and glucose metabolism. As maintaining a good health status may reduce the risk of developing a severe disease associated with diverse infections, the search for accessible and effective pro-health, non-pharmacological strategies during the ongoing COVID-19 pandemic offers a valuable insight. To the best of our knowledge, this specific aspect and in particular, the HIIT- and cryostimulation-dependent changes in FGF21 levels (as an emerging pivotal mediator of metabolic homeostasis), as well as myokine, incretins, and appetite-controlling hormone levels, have not yet been investigated. Accordingly, the aim of the current study was to understand if, and how, the combination of HIIT and extreme cold exposure, vs. the HIIT alone, affects FGF21 serum levels,

and the adipo-myokine profile and metabolic status of overweight to obese subjects.

3. Materials and methods

3.1 Subjects

Sixty-five inactive, overweight to obese participants [body mass index (BMI) of approximately 30 kg·m^{-2}], who had not undergone WBC in the preceding 12 months, took part in the study. Eligible subjects underwent physical examination to evaluate their global health status, to exclude individuals with contraindications to cold exposure (e.g., acute cardiovascular and respiratory disease, unstable hypertension, blood pressure >160/100 mmHg, stroke or cold intolerance, circulatory and deep veins disorders, claustrophobia, cryoglobulinemia, hypothyroidism, neuropathies, Reynaud disease, and pregnancy) [21, 22]. Other than being overweight or obese, the inclusion criteria were: age >18 years, functional autonomy and physical inactivity (less than 60 minutes PA a week) assessed by the questionnaire. The exclusion criteria were: taking insulin or other chronic medications, immune-mediated pathologies, T2DM, and traumatic fractures in the preceding 2 years. Participants were randomly assigned to either the training group (TR, n = 27; BMI = 31.4 ± 3.5 kg·m^{-2}; age = 42 ± 13 years) or training combined with WBC group (TR-WBC, n = 38; BMI = 31.9 ± 5 kg·m^{-2}; age = 45 ± 9 years). Anthropometric data for the participants is shown in Fig. 2.

Body composition analyses were performed and the blood was collected 1 week prior to the study, and 24 h directly after and 4 weeks after completion of the intervention. The training workload for each subject was determined before the first HIIT session. The participants were asked to maintain and not to change their usual daily habits during their participation in the study.

The study protocol was approved by the Bioethical Committee of the Regional Medical Society in Gdansk (approval number KB-28/17) and the study was conducted in accordance with the Declaration of Helsinki. All subjects provided written informed consent for the publication of any associated data after being informed about the procedures.

3.2 Baseline assessment

Body mass and composition (lean body mass, BMI, body fat, and visceral adipose tissue) were determined using dual energy X-ray absorptiometry (DXA) with a Lunar Prodigy whole-body scanner (GE HealthCare, Madison, WI, USA) and enCORE v16 SP1 software (version 3.1.9.4, Heinrich Heine University, Düsseldorf, Germany). Subjects were assessed using DXA in the morning, after an overnight fast, prior to blood collection, usually within 1 h of arrival for clinical assessment and after medical check-up. The day before each assessment, DXA was calibrated using phantoms, according to the manufacturer's guidelines. Scanning mode was automatically chosen by the DXA apparatus. The subjects were exposed to a radiation dose of approximately 2 μSv per scan; the scan took approximately 6–11 min. During DXA assessment, the subjects were lying on the scanning table in supine position, wearing light indoor clothing, and with no metal objects on their body [23]. The DXA measurements were performed three times: at baseline, and immediately after and 4 weeks after completion of the intervention.

Prior to the experiment, a pilot HIIT test was performed to establish individual HR and the training workload. Each individual pedaled at 80–100 rpm with a load of 1.5 W·kg^{-1} (women) or 2.0 W·kg^{-1} (men), so as to achieve intensity of 90% of HR$_{max}$.

Supervised HIIT sessions were performed according to a protocol of Little *et al.* [24], three times a week for 2 weeks (6 sessions in total). In the TR-WBC group, exercise training was performed at the Pomeranian Rheumatologic Centre (Sopot, Poland) directly before WBC sessions 1, 3, 5, 6, 8, 9, and 11. Each training comprised: (A) 3 min warm up at 50 W; (B) 10 × 60 s cycling intervals interspersed with 60 s of recovery; and (C) 2 min cooling down at 50 W. The entire session lasted 25 min. During recovery, the subjects were allowed to rest by slowly pedaling against a resistance of 50 W. The TR group performed the 6 HIIT units without WBC treatment.

WBC exposure took place at the Pomeranian Rheumatologic Centre. The center is equipped with an electric cryochamber (Zimmer Medicine System, Cryochamber ELECPOL, Poznan, Poland), located in a temperature- and humidity-controlled room. The study schedule involved 10 treatments over 2 weeks, with a 2-day rest during the weekend. Sessions took place at the same time of day (in the morning, between 8:30 AM and 9:00 AM, after a light breakfast). In the TR-WBC group, the WBC session was conducted directly after the HIIT session, after careful sweat removal from the body by wiping. During WBC, the participants were minimally dressed (e.g., bathing suit, socks, clogs, headband, and a surgical mask), spent 30 s in a vestibule at –60 °C to allow the body to adapt to low temperature, and then moved to the cryochamber maintained at –110 °C, where they stayed for 3 min. Blood pressure was measured before each WBC session to exclude participants with an elevated blood pressure caused by the activation of sympathetic nervous system (blood pressure ≥130/90 mmHg). Access to the cryochamber was allowed only under the supervision of skilled personnel in control of the procedures.

3.3 Blood collection and analysis

Blood samples were collected by standard venipuncture by a trained nurse, before the study protocol was initiated, at the completion of the intervention, and 4 weeks after the completion of the intervention. However,

most participants from the TR group did not attend the sampling at the third time point; therefore, only participants from the TR-WBC group (n = 35) were considered in the ensuing analysis (Fig. 1). At each sampling time, 14 mL of blood was drawn into two plain serum and two K_2EDTA tubes (Becton, Dickinson & Co., Franklin Lakes, NJ, USA). After mixing by inverting 10 times, the serum in the plain tubes was allowed to clot, in vertical position, for 45 min at approximately 20 °C, while the contents of the K_2EDTA tubes were homogenized for 15 min. The tubes were then centrifuged at 2000 × *g* at 4 °C for 10 min to separate the serum and the plasma, and stored at –80 °C until analysis.

The serum lipid profile [total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG)] was determined by enzyme immunoassays using commercial kits (Alpha Diagnostics, Warsaw, Poland). Glucose levels were determined using Cobas 6000 analyzer (Roche Diagnostics, Warsaw, Poland) according to the manufacturer's instructions. Insulin levels were assessed using an immunoassay kit (Diametra, catalog no. DKO076, Perugia, Italy). The intra-assay coefficient of variation (CV) for insulin was ≤5.0% and the detection limit was 0.25 μIU·mL^{-1}. Insulin sensitivity (HOMA-S), β-cell function (HOMA-B), and insulin resistance (HOMA-IR) were calculated from paired fasting glucose and C-peptide level readings using HOMA2 calculator v2.2.3 (University of Oxford; www.dtu.ox.ac.uk/homacalculator). The reference values are 100% for HOMA-S and HOMA-B, and 1.0 for HOMA-IR [25].

Serum FGF21 levels were determined by enzyme immunoassay using a commercial kit (R&D Systems, Minneapolis, USA, catalog no. DF2100), following the manufacturer's recommendations. The detection limit was 8.69 pg·mL^{-1} and the average intra-assay CV was 3.9%. Serum irisin levels were assessed using an immunoassay kit from Phoenix Pharmaceuticals Inc., Burlingame, USA (catalog no. EK 067-29). The intra-assay CV and the detection limits were <10.0% and 1.29 ng·mL^{-1}, respectively.

The levels of other mediators [adiponectin, C-peptide, ghrelin, gastric inhibitory peptide (GIP), glucagon-like peptide 1 (GLP-1), glucagon, leptin, resistin, and visfatin] were assayed using multiplex immunofluorescence technology and Bio-Plex Pro Diabetes Assay Panels (Bio-Rad, USA, catalog no. 171A7002M for adiponectin and 171A7001M for others). The detection limits were 31.0 pg·mL^{-1} for adiponectin; 4.0 pg·mL^{-1} for C-peptide; 3.0 pg·mL^{-1} for ghrelin, GIP, and leptin; 12.0 pg·mL^{-1} for GLP-1; 47.0 pg·mL^{-1} for glucagon; 1.0 pg·mL^{-1} for resistin; and 8.0 pg·mL^{-1} for visfatin. The average intra-assay CV was 3.0% for adiponectin, C-peptide, GIP, GLP-1, glucagon, resistin, and visfatin; and 4.0% for ghrelin and leptin.

3.4 Statistical analysis

The sample size of the study group was predetermined using power calculations with the software G*power (version 3.1.9.4, Heinrich Heine University, Düsseldorf) [26] (*a priori* repeated-measures, within–between interaction; α = 0.05, 1-β = 0.95, r = 0.8, η_p^2 = 0.06, ε = 1; with a further 20% surplus for the possibility that a participant would not complete the intervention course).

Statistical analyses were performed using the statistics software package Statistica v13.1 (TIBCO Software, Palo Alto, CA, USA). Shapiro-Wilk test was used to assess the homogeneity of dispersion from normal distribution. Brown-Forsythe test was used to evaluate the homogeneity of variance. Repeated measures analysis of variance (rANOVA) was calculated. In case of a significant time × group interaction, *post hoc* tests for unequal sample sizes were performed to identify significantly different results. The effect size (partial eta squared) was also calculated, with $\eta_p^2 \geq 0.01$ indicating a small effect; ≥0.059 indicating a medium effect; and ≥0.138 indicating a large effect [27]. Paired tests were used to analyze the prolonged effect of cryotherapy in the TR-WBC group. For a homogenous sample, paired *t*-test analysis was performed to identify significant changes; for a heterogeneous sample, Wilcoxon signed-rank test was used. In addition, 95% confidence interval was calculated for changes within each study group. The level of significance was set at $p < 0.05$.

4. Results

Anthropometric data are presented in Fig. 2. No significant differences were noted among the participants at baseline. The interventions did not affect the participants' body composition.

4.1 Short-term changes induced by HIIT

The HIIT protocol, performed alone, lowered TG levels from 162.6 ± 131.2 to 129.0 ± 72.9 pg·mL^{-1} (TR: –26.0%, Δ = –33.5, Cl = –67.9; 0.9 vs. TR-WBC: 0.9%, Δ = 1.2, Cl = –22.0; 22.5, p = 0.07, ES = 0.05; Fig. 3C) and HOMA-B values from 93.8 ± 36.9 to 83.7 ± 26.8% (TR: –12.1%, Δ = –10.2, Cl = –23.4; 3.0 vs. TR-WBC: 6.0%, Δ = 5.8, Cl = –1.2; 12.9, p = 0.02, ES = 0.07; Fig. 3B) in comparison to HIIT protocol with 10 WBC sessions (TG: from 125.5 ± 80.7 to 126.7 ± 66.5 pg·mL^{-1}; HOMA-B: from 91.1 ± 19.0 to 96.9 ± 22.9%). Other indicators of the lipid profile and glucose homeostasis (i.e., glucose, insulin, HOMA-S, HOMA-R, and glucagon) remained unaltered after the completion of the HIIT protocol (Fig. 4C, Tables 1,2). The levels of C-peptide, released into the blood as a by-product of insulin secretion, showed a downward trend in the TR group (–15.7%; Table 2). Pre-to-post changes (Δ) in HOMA-B and C-peptide levels were strongly and positively correlated (r = 0.74; Table 3).

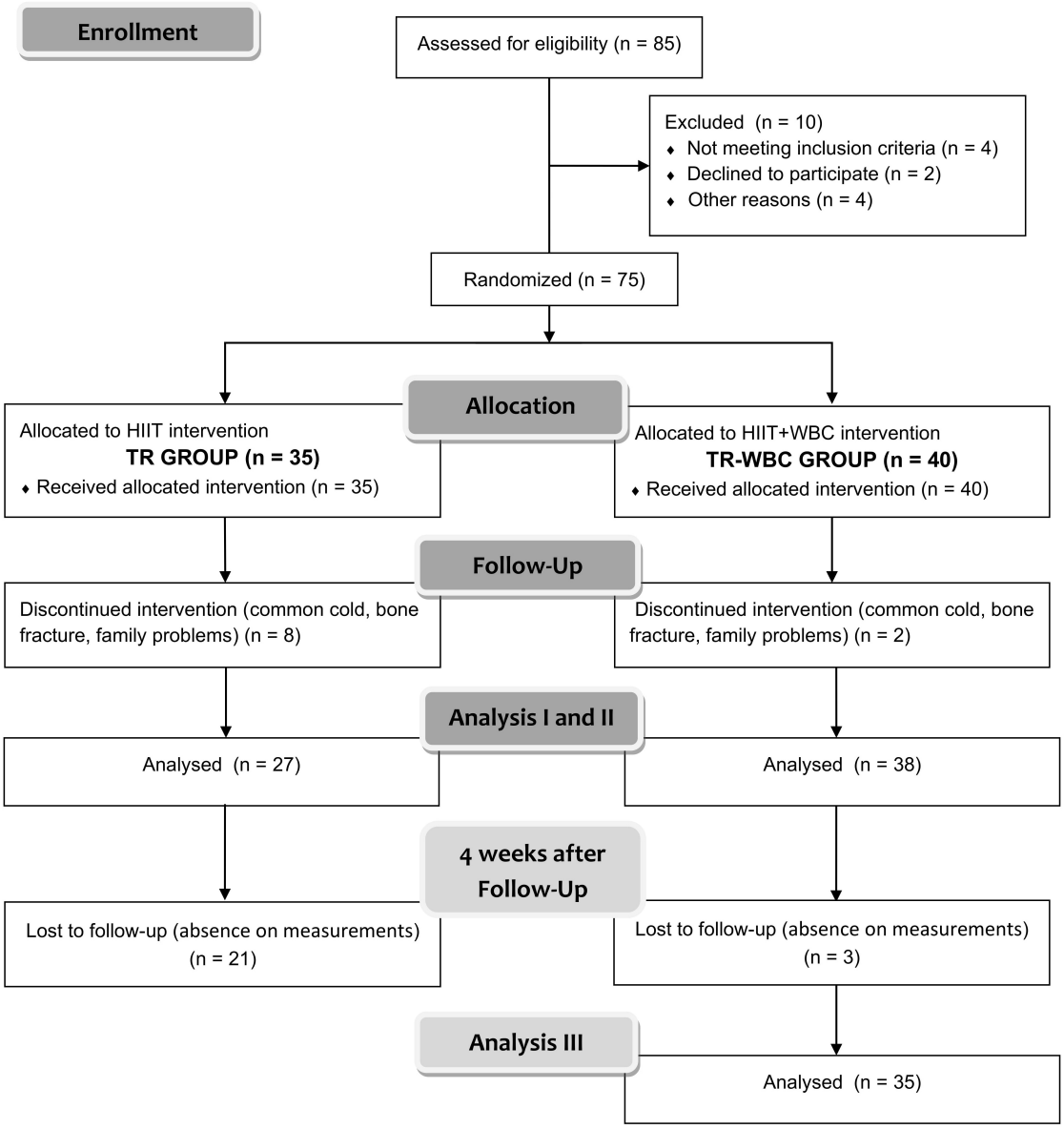

Fig. 1. The schedule of examinations. Analysis: (I) before protocol, (II) directly after protocol and (III) 4 weeks post finishing the whole experimental protocol only in training with whole-body cryotherapy group (TR-WBC).

The HIIT protocol resulted in a significant increase in FGF21 blood levels, from 191.0 ± 91.8 to 275.0 ± 178.8 pg·mL^{-1} (Δ = 83.9, Cl = 13.4; 154.4, $p < 0.05$). Shifts in FGF21 and adiponectin levels in the TR group differed significantly from the values recorded for the TR-WBC group ($p = 0.03$, ES = 0.08 for FGF21; Fig. 4A; $p = 0.05$, ES = 0.06 for adiponectin; Fig. 4B). In the TR-WBC group, FGF21 levels remained unchanged, while adiponectin levels decreased.

The HIIT protocol induced changes in the levels of proinflammatory cytokines, namely, a drop in the visfatin from 5734.5 ± 2921.7 to 5107.4 ± 2713.6 pg·mL^{-1} (Δ = –627.2, Cl = –1103.1; –151.2, $p < 0.05$; Fig. 3D), leptin, and resistin levels (Table 2), although statistical significance in comparison to the TR-WBC group was only reached for visfatin ($p = 0.04$, ES = 0.07; Fig. 3D). The remaining factors were not affected by the training intervention (Table 2).

4.2 Short-term changes induced by the HIIT–WBC combination

The combination of HIIT and WBC did not significantly alter the lipid profile (Table 1) but it improved glucose homeostasis indicators.

Nonetheless, the observed upward trend in HDL levels in the TR-WBC group (from 55.6 ± 15.9 to 57.8 ± 17.3 mg·dL^{-1}, Δ = 2.2, Cl = –0.2; 4.2) was significantly different from the response in the TR group (decrease from 52.7 ± 13.7 to 51.3 ± 14.6 mg·dL^{-1}, Δ = –1.4, Cl = –

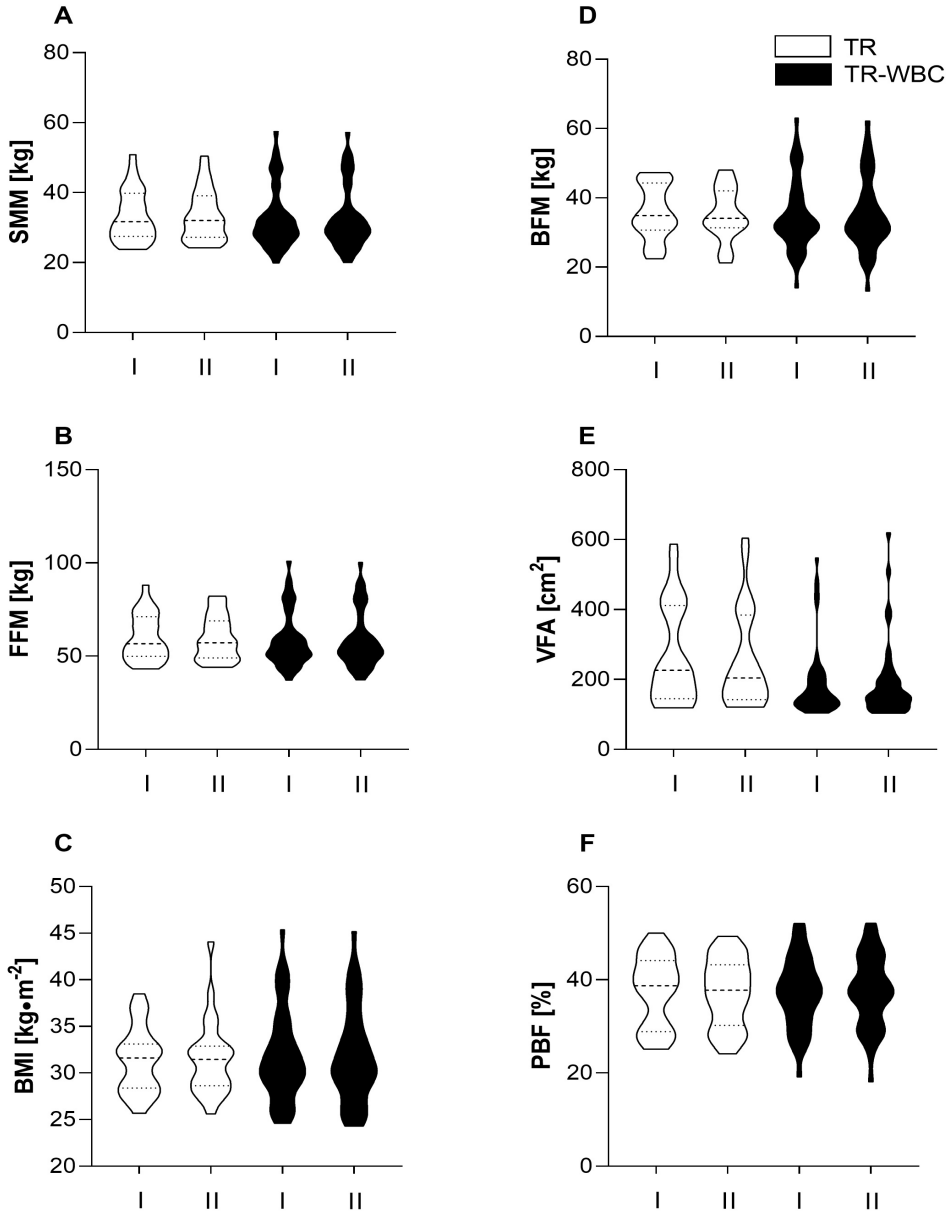

Fig. 2. Anthropometric characteristics of participants. (A) Skeletal muscle mass (SMM). (B) Free fat mass (FFM). (C) Body mass index (BMI). (D) Body fat mass (BFM). (E) Visceral fat area (VFA) and (F) percent of body fat (PBF) recorded (I) before and (II) after experimental protocol in training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38). Data are presented as median and range.

3.8; 1.0, p = 0.02, ES = 0.08; Fig. 3A). Resting glucose levels were significantly reduced from 99.2 ± 10.9 to 95.9 ± 9.9 mg·dL^{-1} (Δ = –3.3, CI = –5.5; –1.1) in the TR-WBC group ($p < 0.05$) and this shift was significantly different from values recorded for the TR group (p = 0.03, ES = 0.07; Fig. 4C).

Of note, changes in TG (Fig. 3C) and FGF21 levels (from 204.0 ± 4.1 to 212.6 ± 113.7 pg·mL^{-1}, Δ = 8.6; Fig. 4A) recorded in the TR-WBC group were relatively blunted. Similar trends were noted for C-peptide (TR: –15.7% vs. TR-WBC: –2.3%), leptin (TR: –16.2% vs. TR-WBC: –6.8%), resistin (TR: –7% vs. TR-WBC: –0.4%, Table 2) and visfatin levels (TR: –12.3% vs. TR-WBC: –1.6%, from 6220.9 ± 2516.9 to 6125.2 ± 2683.6 pg·mL^{-1}, Δ = –95.8, CI = –389.0; 197.5; Fig. 3D).

Considering the diabetic panel markers, an upward trend in the levels of ghrelin (TR-WBC: 8.5% vs. TR: –0.6%) and GIP (TR-WBC: 7.9% vs. TR: –9.6%) was noted in the TR-WBC group, and the opposite was observed in the TR group. The differences were not statistically significant (Table 2); however, in the TR-WBC group, ΔGIP positively correlated with Δglucose (r = 0.34; Table 3) and negatively correlated with ΔFGF21 (r = –0.33; Table 3). The remaining factors were not affected by the combination of HIIT and WBC (Tables 1,2).

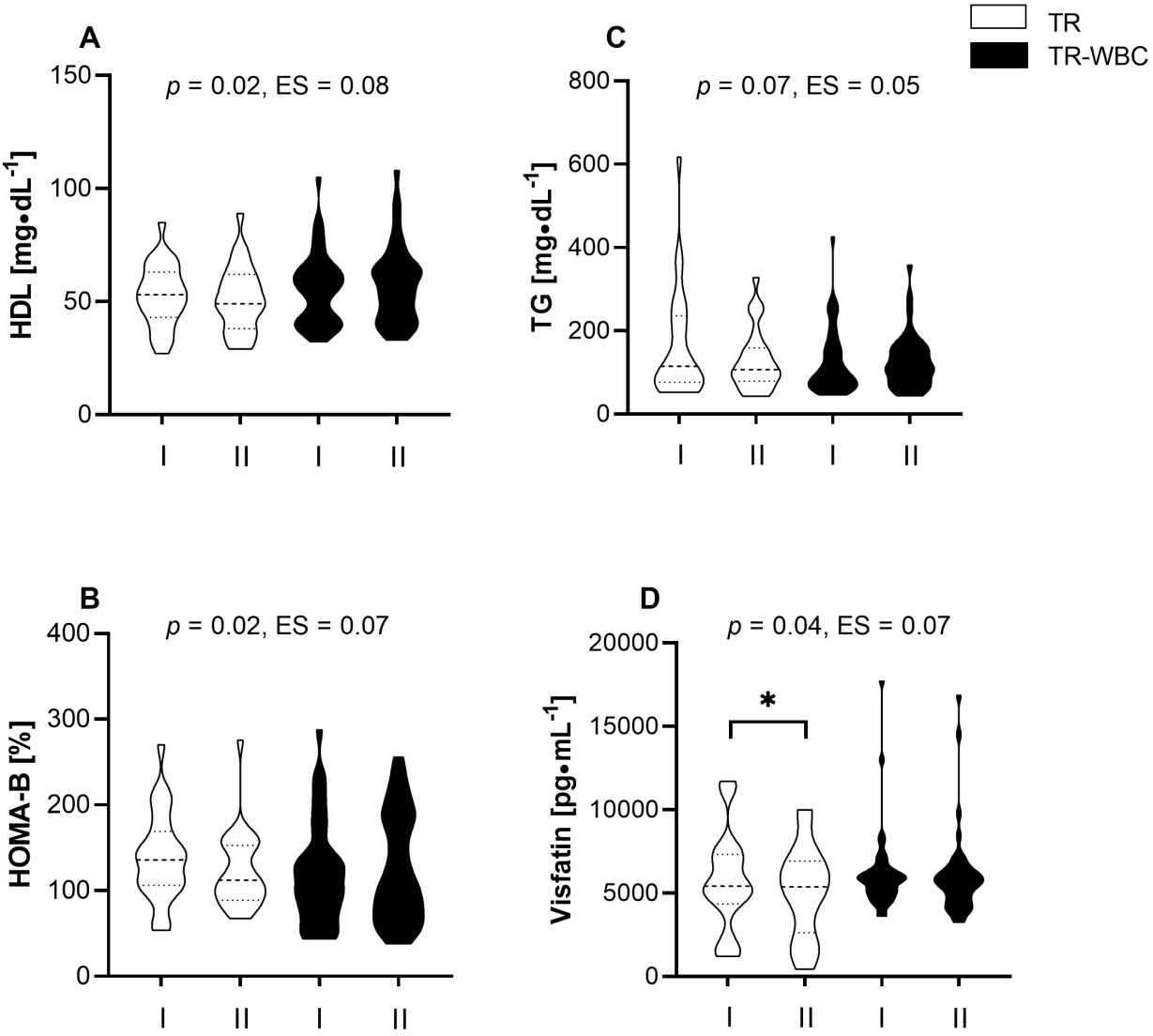

Fig. 3. Changes in the concentration of selected metabolic indicators. (A) High density lipoprotein cholesterol (HDL). (B) The level of Homeostasis Model Assessment estimates β-cell function as percentages of a normal reference population (HOMA-B). (C) Triglycerides (TG) and (D) Visfatin recorded (I) before and (II) after experimental protocol in training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38). Data are presented as median and range; *statistical significance in the group, $p < 0.05$; ES, effect size (partial eta squared).

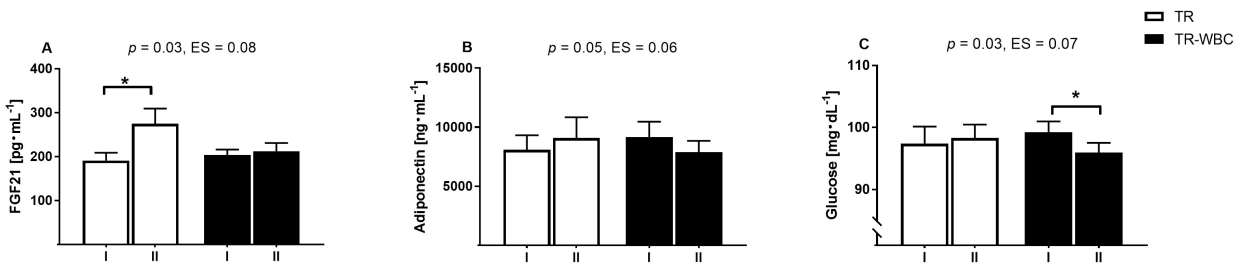

Fig. 4. Changes in adipokines concentration and glucose level before and after intervention. (A) Fibroblast growth factor 21 (FGF21). (B) Adiponectin and (C) glucose recorded (I) before and (II) after experimental protocol in training group (TR; n = 27) and training with whole- body cryotherapy (TR-WBC; n = 38). Data are presented as mean $\pm$ SD; *statistical significance in the group, $p < 0.05$; ES, effect size (partial eta squared).

Table 1. The effect of interventions on lipid profile and glucose homeostasis indicators among training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38).

	TR				TR-WBC				ANOVA	
	Before	After	Δ	95% CI	Before	After	Δ	95% CI	Group × time	ES
TC [mg·dL^{-1}]	196.2 ± 44.6	192.7 ± 39.0	–3.56	–11.96; 4.85	194.5 ± 42.6	197.9 ± 38.3	3.39	–4.12; 10.91	0.22	0.02
LDL [mg·dL^{-1}]	112.6 ± 43.1	112.5 ± 34.7	1.23	–8.38; 10.84	113.8 ± 35.4	114.7 ± 31.7	0.93	–5.39; 7.24	0.95	0.00
Insulin [µIU·mL^{-1}]	17.5 ± 11.0	16.1 ± 9.8	–2.2	–5.67; 1.27	18.3 ± 12.2	17.6 ± 10.0	–0.7	–3.63; 2.24	0.50	0.01
HOMA-S [%]	106.5 ± 56.4	114.2 ± 58.0	7.66	–11.56; 26.89	93.1 ± 48.6	91.2 ± 36.9	–1.87	–13.1; 9.4	0.36	0.01
HOMA-IR	1.3 ± 0.9	1.1 ± 0.5	–0.17	–0.46; 0.13	1.3 ± 0.5	1.3 ± 0.5	–0.05	–0.2; 0.08	0.42	0.01

Data are presented as mean ± SD; Δ, difference between after and before measurements; 95% CI, 95% confidence interval; ANOVA, analysis of variance with repeated measure; ES, effect size (partial eta squared); TC, total cholesterol; LDL, low density lipoprotein cholesterol; HOMA, The Homeostasis Model Assessment estimates; HOMA-S, insulin sensitivity as percentages of a normal reference population; HOMA-IR, insulin resistance.

Table 2. The effect of training and whole-body cryotherapy on metabolic indicators among training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38).

	TR				TR-WBC				ANOVA	
	Before	After	Δ	95% CI	Before	After	Δ	95% CI	Group × time	ES
C-Peptide [pg·mL^{-1}]	1687.1 ± 1104.2	1457.8 ± 680.3	–229.3	–619.9; 161.4	1716.5 ± 659.2	1678.4 ± 602.4	–38.1	–206.4; 1302	0.31	0.02
Ghrelin [pg·mL^{-1}]	918.4 ± 527.6	923.8 ± 573.0	5.3	–74.8; 85.5	601.9 ± 512.1	657.7 ± 656.5	55.8	–15.3; 126.9	0.34	0.01
GIP [pg·mL^{-1}]	430.1 ± 524.0	392.3 ± 297.9	–50.6	–362.3; 261.1	224.0 ± 185.9	243.1 ± 292.7	19.1	–54.8; 93.0	0.52	0.01
GLP-1 [pg·mL^{-1}]	289.8 ± 143.2	255.1 ± 92.9	0.9	–30.9; 32.7	297.4 ± 110.1	294.9 ± 144.7	–2.5	–22.0; 16.9	0.85	0.00
Glucagon [pg·mL^{-1}]	1087.0 ± 430.9	1047.7 ± 427.2	–39.3	–108.8; 30.1	1413.8 ± 336.2	1428.4 ± 403.1	14.6	–25.1; 54.4	0.15	0.03
Leptin [pg·mL^{-1}]	13637.6 ± 12742.4	11736.5 ± 10775.9	–1901.1	–4278.0; 475.8	11494.9 ± 8614.3	10766.7 ± 8407.2	–728.2	–2051.7; 595.2	0.35	0.01
Resistin [pg·mL^{-1}]	8311.8 ± 2648.4	7769.5 ± 2175.8	–542.3	–1703.0; 618.4	8809.4 ± 4635.1	8777.9 ± 4953.8	–31.5	–939.3; 876.3	0.48	0.01
Irisin [ng·mL^{-1}]	26.1 ± 14.5	24.1 ± 13.7	–2.1	–4.4; 0.2	23.4 ± 13.9	24.6 ± 13.3	1.1	–2.4; 4.6	0.16	0.03

Data are presented as mean ± SD; Δ, difference between after and before measurements; 95% CI, 95% confidence interval; ANOVA, analysis of variance with repeated measure; ES, effect size (partial eta squared); GIP, gastric inhibitory peptide; GLP-1, glucagon-like peptide 1.

Table 3. Correlation coefficients of ΔHOMA-B, ΔGlucose, ΔFGF21 and ΔC-peptide, ΔGIP among training group (TR; n = 27) and training with whole-body cryotherapy group (TR-WBC; n = 38).

	ΔHOMA-B [%]		ΔGlucose [mg·dL^{-1}]		ΔFGF21 [pg·mL^{-1}]	
	TR	TR-WBC	TR	TR-WBC	TR	TR-WBC
ΔC-peptide [pg·mL^{-1}]	0.74*	0.69*	0.10	0.26	0.10	–0.13
ΔGIP [pg·mL^{-1}]	0.46	0.33*	0.14	0.34*	–0.13	–0.33*

Values are Spearman correlation; *statistically significant correlations, $p < 0.05$; GIP, gastric inhibitory peptide; HOMA-B, β-cell function; FGF-21, fibroblast growth factor 21.

4.3 Prolonged effects of the HIIT–WBC combination

Four weeks after the end of the training intervention, elevated skeletal muscle mass (SMM) and free fat mass (FFM) were registered in the TR-WBC group (p = 0.01 for both components, accordingly; Fig. 5A,B). At that time point, the TC and HDL levels were significantly higher than the baseline levels (194.5 $\pm$ 42.6 vs. 197.9 $\pm$ 38.0 mg·dL^{-1} at baseline, p = 0.03, and 55.6 $\pm$ 15.9 vs. 58.2 $\pm$ 17.4 mg·dL^{-1} at baseline, p = 0.02, accordingly), but were not statistically different from the values immediately after the end of the training intervention (Fig. 5C,D). The decrease in glucose levels induced by the HIIT-WBC combination was not maintained. In fact, glucose levels 1 month after the intervention increased from 95.9 $\pm$ 9.9 to 98.7 $\pm$ 11.0 mg·dL^{-1} (p = 0.01), i.e., returned to baseline values (Fig. 5E). Four weeks after the training intervention, irisin levels tended to decrease from the level recorded 24 h after last HIIT-WBC procedure (from 24.6 $\pm$ 13.3 to 21.7 $\pm$ 10.4 ng·mL^{-1}, p = 0.08; Fig. 5F).

5. Discussion

Physical exercise is an important and effective strategy for counteracting metabolic imbalance in overweight and obese individuals; this is of particular relevance in periods, such as the ongoing COVID-19 pandemic that sees overweight, obese and metabolically dysfunctional patients as one of the more vulnerable groups. In recent years, WBC has been described as a valuable form of physiotherapy because of its exercise-mimicking effects. However, as reported by different studies, WBC manifests its real potential only when combined with a physical exercise program [28, 29]. Accordingly, in the current study, we set out to determine the effect of HIIT in combination with WBC vs. HIIT alone, on FGF21 serum levels, adipo-myokine profile, and metabolic status of overweight to obese individuals as a preventative strategy against the most severe outcomes of SARS-CoV-2 infection.

Data presented in the current study only partially support the claim that the beneficial effects of WBC are fully realized only in combination with physical exercise. The main finding of the study is that 6 units of HIIT training (the TR intervention) suffice to cause a significant increase in FGF21 levels in obese inactive individuals. These changes were associated with an increase in the circulating levels of the anti-inflammatory adipokine adiponectin. At the same time, the metabolic profile improved, i.e., TG levels dropped and HOMA-B values improved. Of note, HIIT in conjunction with WBC did not induce such changes. As recently reported by Sun *et al.* [30], FGF21 acts as a hepatokine, adipokine, and myokine; however, the main tissue source of circulating FGF21 that mediates the effect of exercise is not known. Further, FGF21 responses to exercise are inconsistent, and different studies have reported a decrease [31], no change [32], and increase [33] in its levels upon exercise. Micielska *et al.* [34] demonstrated that 15 units of high-intensity circuit resistance training induce a drop and an increase in FGF21 levels. Only the drop was associated with an amelioration and impairment of cognitive function. The mechanisms underlying the diverse effects of exercise remain unclear and warrant further research.

Physical inactivity exerts a catabolic effect on muscle tissue [35]. Data on the effect of physical inactivity alone on the blood levels of FGF21 are limited. However, Asle *et al.* [36] reported that FGF21 levels increase after 12 weeks of HIIT (3 sets of 10 $\times$ 60 s, 3 times/week) in obese non-active participants particularly in conjunction with low carbohydrate diet but also in normal diet and low fat diet groups except for the high fat diet group where decrease of FGF21 level was noted. This partially agrees with the changes in FGF21 levels observed herein. According to a recent study in a mouse model, the expression and systemic release of muscle-derived FGF21 are very low in normal healthy muscle, and mainly increase under stress conditions (e.g., starvation, aging, and obesity) [37]. Exercise sensitizes adipose tissue to FGF21, which is the basis for a multi-organ crosstalk coordination responsible for maintaining metabolic homeostasis [38]. Therefore, the source of FGF21 release into the bloodstream depends on both, internal and external stimuli [39].

The high-intensity workload featured in the HIIT protocol applied in the current study to overweight to obese inactive individuals, could be a stress-generating factor contributing to the increase in blood FGF21 levels, with the growth factor most likely released from the muscle. On the other hand, FGF21 expression in the muscle is reportedly elevated during mitochondrial dysfunction, and protects against obesity and insulin resistance [40, 41]. We here observed a strong, positive correlation between the reduction of HOMA-B values and the downward trend of C-peptide levels in the TR group participants. The changes in HOMA-B values were significantly different from those in the TR-WBC group, where the FGF21 levels remained unchanged. C-peptide is commonly used as a marker of insulin resistance and metabolic syndrome [42]. The rate of C-peptide secretion is more constant than that of insulin and, hence, it is a reliable marker of pancreatic β-cell function [43]. The observed drop in HOMA-B values to those close to the reference (100%) [25] in the TR group may indicate a reduced metabolic load on β-cells, to maintain normoglycemia.

The increase in FGF21 levels may be also caused by its enhanced expression in the liver [44] and white adipose tissue [45]. Indeed, liver-derived FGF21 improves glucose tolerance [46] and enhances oxidation of free fatty acids [47]. In the TR group, we observed a downward trend in the TG levels, while glucose levels remained unchanged. Therefore, we cannot rule out the possibility that the liver was the main source of FGF21 under these conditions. This

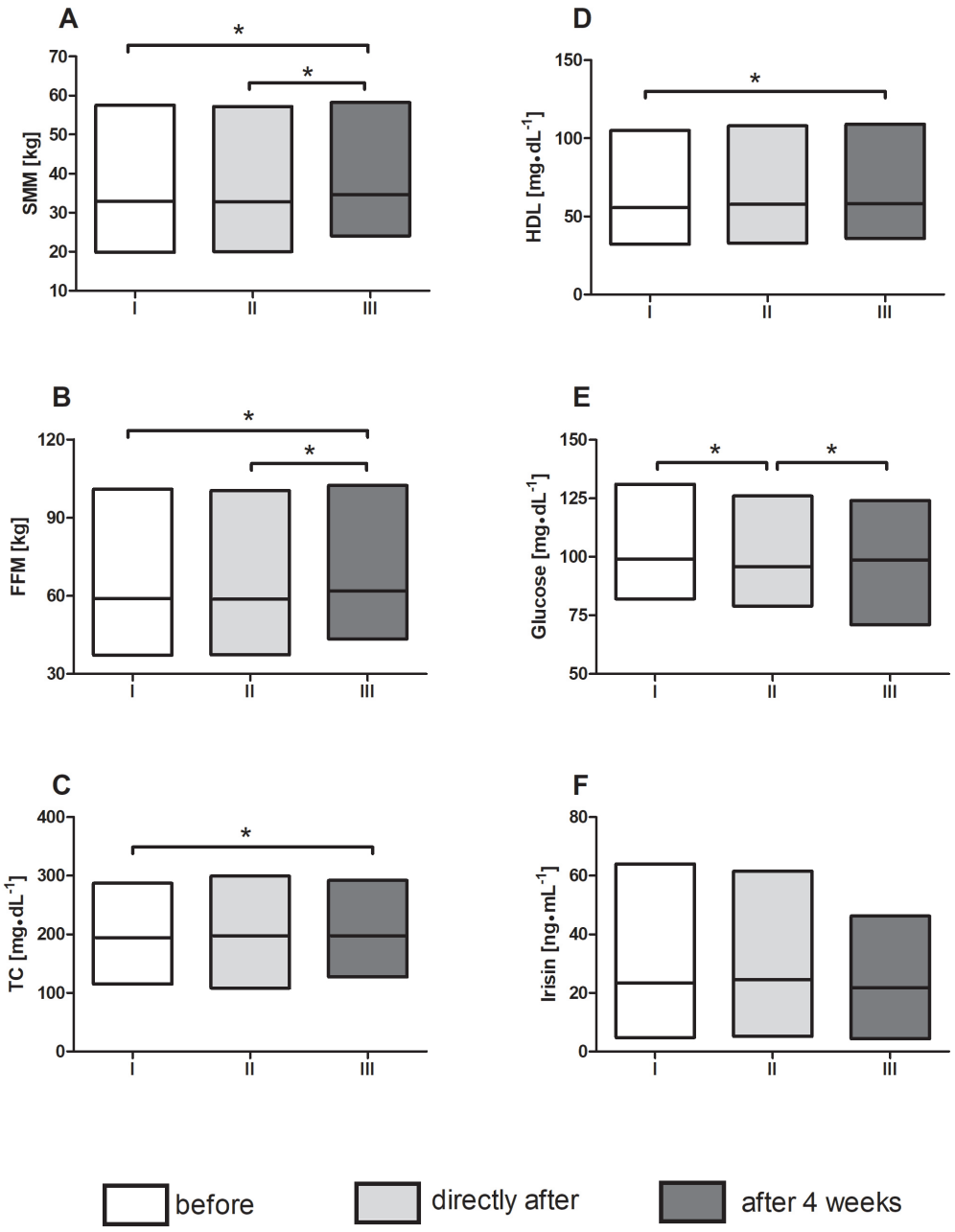

Fig. 5. Changes of body composition and selected metabolic indicators. Changes in the (A) skeletal muscle mass (SMM). (B) Free fat mass (FFM). Concentration of (C) total cholesterol (TC). (D) High- density lipoprotein cholesterol (HDL). (E) Glucose and (F) irisin at each point of blood collection: (I) before protocol, (II) directly after protocol and (III) 4 weeks post finishing the whole experimental protocol in training with whole-body cryotherapy group (TR-WBC). Data are presented as mean, min and max value; *significant differences between time point measurements, $p < 0.05$.

response differed from the effect in the TR-WBC group. Savikj *et al.* [48] reported that the effect of HIIT training on blood glucose levels depends on the time of day the training is performed. Specifically, in their study, afternoon HIIT was more effective in improving blood glucose levels in men with T2DM than morning HIIT; by contrast, morning HIIT had an opposite effect, increasing blood glucose levels [48]. In the current study, the training sessions took place in the morning but we did not observe any changes in glucose levels. However, it is worth noting that blood glucose levels significantly decreased in the TR-WBC group. Hence, the beneficial changes in glucose homeostasis noted in this group may be associated with the activation of the sympathetic nervous system either by cold exposure [49, 50] or by physical activity. We have previously reported that changes in glucose levels may be related to fluctuating FGF21 levels [16]. Fisher *et al.* [51] reported that cold exposure induces expression of endogenous FGF21 in different adipose de-

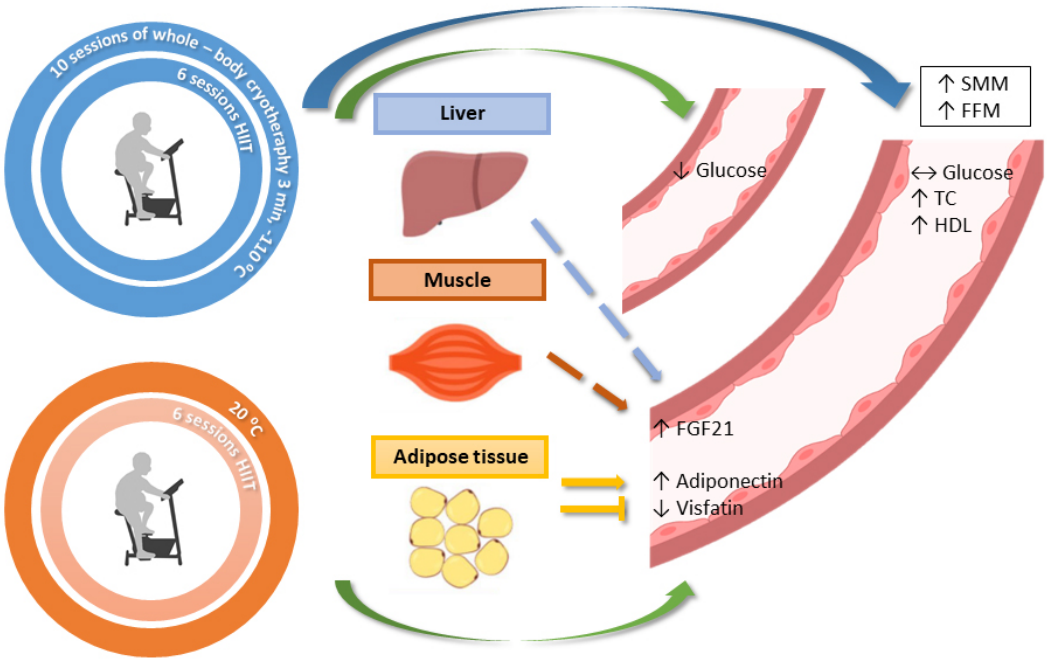

Fig. 6. Graphical conclusion of induced changes recorded in blood and body mass composition. Green arrows indicate short-term effects, purple long-term effects observed 4 weeks after the end of the intervention. The dashed arrows indicate the likely source of FGF21 (liver or muscle as a possible) released to bloodstream. The drop-in glucose level in the training with whole-body cryotherapy group turned out to be short-term effect and did not last until 4 weeks after the end of the protocol. HIIT, high intensity interval training; FGF21, fibroblast growth factor 21; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; SMM, skeletal muscle mass; FFM, free fat mass.

pots or browning of white adipose tissue (WAT). FGF21 was originally described as a factor that enhances insulin-independent glucose uptake in cultured adipocytes, acting via glucose transporter 1 (Glut1) [46]. Therefore, FGF21 can increase the uptake of glucose by adipose tissue in an autocrine manner, independently of insulin, causing its concentration to drop.

In the current study, cold exposure in conjunction with HIIT led to an increase in HDL cholesterol levels but did not affect TG levels. This partially agrees with previous observations of Lubkowska *et al.* [52], who noted a significant decrease of the LDL/HDL ratio after at least 10 WBC sessions. In the current study, changes in HDL levels were significantly different in the two experimental groups. Since HDL cholesterol levels did not change in the TR group, it is likely that WBC contributed to the changes in the TR-WBC group lipid profile. Similar, Rymaszewska *et al.* [53] showed that WBC positively affects the lipid profile, especially in individuals with high BMI. TG, TC, and LDL levels were reduced after WBC [53]. Consequently, it has been suggested that cryotherapy could be an effective treatment for lipid disorders.

Together with increased FGF21 levels, we here observed an increase in adiponectin levels in the TR group. While adiponectin is an adipokine [54], it also acts as a myokine as it is expressed by skeletal muscle during contraction [55], similarly to the previously described mediator, FGF21. Based on the data from a mouse model, FGF21 regulates adiponectin expression in adipocytes in endocrinal manner, de facto coupling FGF21 activity in WAT with metabolic effects in the liver and muscle [56]. Further, circulating FGF21 upregulates adiponectin expression in different fat depots (subcutaneous and visceral adipose tissue) and serum level in obese mouse, as a protective mechanism against systemic insulin resistance [57]. In the TR group in the present study, the increase in adiponectin and FGF21 levels was accompanied by a decrease in HOMA-B values, and a downward trend of C-peptide levels. Furthermore, we noted a decrease in proinflammatory adipokine levels (visfatin, leptin, and resistin) in the TR group. However, only changes in visfatin levels were statistically significant in comparison with those in the TR-WBC group.

Visfatin levels increase with obesity and elevated BMI [58]. In a recently published review, Kumari and Yadav [58] concluded that visfatin modulates obesity-related pathophysiological activities, contributing to the development of disease, such as diabetes (by regulating pancreatic β-cell function), cardiovascular disorders, or even some forms of cancer. Studies involving obese subjects confirmed that while PA reduces blood visfatin levels, this effect is mainly induced by aerobic [59] and resistance training [60]. No data regarding the effect of HIIT training on visfatin levels in obese adults are available. Studies with

young participants revealed some changes in visfatin levels in response to interval training [61, 62]. In the current study, a decrease in visfatin levels was recorded only in the TR group. Previously, Ziemann *et al.* [9] reported no changes in adiponectin levels and a reduction of visfatin levels (7.4%) in individuals with low cardiorespiratory fitness in response to 10 sessions of WBC. Data presented herein suggest that HIIT alone is more effective in reducing visfatin levels than HIIT applied together with WBC.

Irisin is another factor that regulates glucose homeostasis [63]. It inhibits the development of obesity-related inflammatory phenotype in adipocytes and macrophages *in vitro* [64]. Accordingly, we evaluated irisin and inflammatory marker levels in the current study. Levels of circulating irisin are modulated by diet, obesity, exercise, and pharmacological agents [65]. Of note, Dulian *et al.* [66] showed that resting irisin levels increase in response to WBC in obese subjects. Nevertheless, in the current study, 10 sessions of WBC combined with HIIT, and HIIT alone, did not impact circulating levels of this myokine.

Incretins, including GIP and GLP-1, were also assayed herein, since their secretion and activity are dysregulated in obesity and diabetes [67]. Incretins are hormones that regulate insulin and glucagon secretion by pancreatic cells in a glucose-dependent manner. Despite the significant decrease in blood glucose levels in the TR-WBC group, no significant changes in GIP, GLP-1, or glucagon levels were detected. These observations are in line with a report of Hindsø *et al.* [68], who showed that fasting and oral glucose-stimulated incretins levels are not affected in inactive and overweight to obese individuals after 6 weeks of low-volume 3-time per week HIIT. However, in the current study, an upward tendency in GIP levels was apparent in the TR-WBC group. This was opposite to the changes observed in the TR group. A similar tendency was observed for glucagon levels in the TR (a decrease) and TR-WBC (an increase) groups. Although the differences between the groups were not statistically significant, it is important to note that changes in GIP levels were positively correlated with changes in glucose levels in the TR-WBC group. Hence, an increase in GIP levels either causes a significant reduction in blood glucose levels or the relationship is opposite. In rat models, hyperglycemia reduces GIP receptor expression in β-cells [69]. Accordingly, in the current study, the increase in circulating GIP levels could have been stimulated by the decrease in blood glucose levels. On the other hand, in animal models, cold acclimation (4 ± 1 °C for 42 days) increases brown adipose tissue mass, improves glycemic response to oral glucose, and significantly reduces insulin responses [70]. These changes are associated with increased intestinal levels of GIP and GLP-1. These observations indicate that in rat, changes in GIP secretion and activity may be involved in the metabolic adaptation to cold acclimation [71]. Accordingly, we conclude that cold exposure may contribute to the upward trend of GIP levels in the TR-WBC group, consequently leading to a reduction in glucose levels.

Ghrelin is a peptide-hormone that, similarly to GIP and GLP-1, is mainly secreted by enteroendocrine cells [72], and plays an important role in the development of obesity and metabolic-related disorders. It also promotes feeding in cold environments [73, 74], a response associated with an increase in ghrelin levels and a reduction in leptin levels [74, 75]. In the current study, ghrelin levels were not affected in a statistically significant manner by either intervention; however, we observed a tendency of ghrelin blood levels to increase following WBC. This observation was partially in agreement with that of Kojima and co-workers [76], who demonstrated that post-exercise WBC (–140 °C for 3 min) does not affect plasma ghrelin and serum leptin levels, but significantly increases energy intake in human. This might suggest that the tendency of ghrelin levels to increase after WBC leads to an increased energy uptake [77]. Nevertheless, we were unable to evaluate the effect on the study participants' appetite because this aspect was not tested in the current study.

The current study has some limitations that should be addressed in the future. First, we did not assess the effects of WBC alone in the current study. This can be addressed by including a third, WBC-only, group in the study design, and comparing the effects of WBC on the various metabolic parameters with those of other interventions. Another potential limitation is the choice of the evaluated analytes. It is possible that the investigated panel of mediators, although broad, did not provide a complete overview of the body's response to the tested interventions. Although we detected some metabolic and endocrine changes, it is possible that the effect would have been more pronounced upon a longer training program and, above all, additional WBC sessions. Hence, we are unable to recommend the minimum therapeutic number of cold exposures at this time. Secondly, we did not conduct any monitoring of changes in participants' fitness level during and at the end of HIIT protocol which could have revealed more granular variation in the individual cardiorespiratory response to exercise protocol. On the other hand, in preliminary phase of our experiment, based on previously published studies [9, 78], we tried to select a group uniform in terms of body composition and the level of physical activity. Finally, we did not explore the mechanisms underlying the observed effects of cryostimulation and HIIT on the metabolic homeostasis.

Still, to the best of our knowledge, this study is the first to assess long-term effects of cold exposure applied in conjunction with physical training on adipo-myokine profile, and metabolic status of overweight to obese individuals. The findings presented herein indicate that the observed decrease in glucose levels induced by HIIT–WBC was reversed 1 month after the treatment, as a return to daily habits eroded the beneficial effects of the intervention.

6. Conclusions

To summarize, the HIIT protocol, both alone and in combination with WBC, affected the metabolic indicators and myokines' concentrations. These impacts manifested differently, likely due to the different underlying mechanisms. Training alone caused significant changes in FGF21 concentration, whereas in combination with WBC, it abolished this effect. Similarly, different responses in adiponectin were observed; it increased in response to the HIIT alone but it decreased in response to the combination of HIIT and WBC. Moreover, the combined approach of training and WBC induced beneficial, yet temporary, effects on glucose concentration and glucose homeostasis among obese participants (Fig. 6). In practice, although short-term, the presented effects support the use of pro-health procedures, such as physical activity and cold exposure, as preventative strategies to limit the severe effects of other incident diseases.

7. Author contributions

Conceptualization—MKF and EZ; methodology—MKF, ERF and VS; validation—MKF, KM, SP and GL; formal analysis—JK; investigation—MKF, ERF, JJ and KM; resources—JJ and AB; data curation—MKF; writing-original draft preparation—MKF, EZ; writing-review and editing—MKF, AB, SP, GL and EZ; visualization—MKF and JK; supervision—JK and VS; project administration—EZ; funding acquisition—EZ All authors have read and agreed to the published version of the manuscript and agree with the order of presentation of the authors.

8. Ethics approval and consent to participate

The study protocol was approved by the Bioethical Committee of the Regional Medical Society in Gdansk (approval number KB-28/17) and was conducted in accordance with the Declaration of Helsinki. All subjects provided written informed consent for the publication of any associated data after being informed about the procedures.

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11. Conflict of interest

The authors declare no conflict of interest.

12. References

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Abbreviations: HIIT, high intensive interval training; WBC, whole-body cryotherapy; TR, training group; TR-WBC, training supported by WBC group; FGF21, fibroblast growth factor 21; HOMA-IR, The Homeostasis Model Assessment estimates insulin resistance; HOMA-β, The Homeostasis Model Assessment estimates β-cell function; HOMA-S, The Homeostasis Model Assessment estimates insulin sensitivity as percentages of a normal reference population; T2DM, type 2 diabetes; PA, physical activity; HR, heart rate; BMI, body mass index; CV, coefficient of variation; DXA, dual energy X-ray absorptiometry; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; GIP, gastric inhibitory peptide; GLP-1, glucagon- like peptide 1; rANOVA, analysis of variance; ES, effect size (partial eta squared); SMM, skeletal muscle mass; FFM, free fat mass; WAT, white adipose tissue; Glut1, glucose transporter 1.

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