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Impact of physical fitness and exercise training on subcutaneous adipose tissue beiging markers in humans with and without diabetes and a high-fat diet-fed mouse model

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Abstract

Aims: Exercise training induces white adipose tissue (WAT) beiging and improves glucose homeostasis and mitochondrial function in rodents. This could be relevant for type 2 diabetes in humans, but the effect of physical fitness on beiging of subcutaneous WAT (scWAT) remains unclear. This translational study investigates if beiging of scWAT associates with physical fitness in healthy humans and recent-onset type 2 diabetes and if a voluntary running wheel intervention is sufficient to induce beiging in mice.

Materials and Methods: Gene expression levels of established beiging markers were measured in scWAT biopsies of humans with (n = 28) or without type 2 diabetes (n = 28), stratified by spiroergometry into low (L-FIT; n = 14 each) and high physical fitness (H-FIT; n = 14 each). High-fat diet-fed FVB/N mice underwent voluntary wheel running, treadmill training or no training (n = 8 each group). Following the training intervention, mitochondrial respiration and content of scWAT were assessed by high-resolution respirometry and citrate synthase activity, respectively.

The GDS Group consists of M. Roden (speaker), H. Al-Hasani, B. Belgardt, G. Bönhof, V. Burkart, G. Geerling, C. Herder, A. Icks, K. Jandeleit-Dahm, J. Kotzka, O. Kuß, E. Lammert, W. Rathmann, S. Schlesinger, V. Schrauwen-Hinderling, J. Szendroedi, S. Trenkamp, R. Wagner and their co-workers who contributed to the design and conduct of the GDS.

Results: *Secreted CD137 antigen* (*Tnfrsf9/Cd137*) expression was three-fold higher in glucose-tolerant H-FIT than in L-FIT, but not different between H-FIT and L-FIT with type 2 diabetes. In mice, both training modalities increased *Cd137* expression and enhanced mitochondrial content without changing respiration in scWAT. Treadmill but not voluntary wheel running led to improved whole-body insulin sensitivity.

Conclusions: Higher physical fitness and different exercise interventions associated with higher gene expression levels of the beiging marker *CD137* in healthy humans and mice on a high-fat diet. Humans with recent-onset type 2 diabetes show an impaired adipose tissue-specific response to physical activity.

KEYWORDS
beiging, exercise, insulin sensitivity, physical fitness, subcutaneous adipose tissue, type 2 diabetes mellitus

1 | INTRODUCTION

Obesity is a major risk factor for type 2 diabetes, but also independently predicts insulin resistance, the hallmark of type 2 diabetes.[1] While increasing body fat is associated with insulin resistance, physical activity improves insulin sensitivity independently of weight loss, mainly via increased mitochondrial respiration and content in skeletal muscle.[2,3] A number of rodent studies report enhanced mitochondrial function in response to exercise training in skeletal muscle but also in white adipose tissue (WAT), where it is termed 'browning/beiging'.[4–6] The subcutaneous WAT (scWAT) is more sensitive to different external stimuli for the induction of mitochondrial biogenesis than visceral WAT.[7] Likewise, beiging of scWAT increases the whole-body energy expenditure in rats,[8] which may have beneficial effects on glucose homeostasis. Transplantation of scWAT from trained to untrained mice improved whole-body glucose uptake, which did not occur upon transplantation of scWAT from sedentary mice.[9]

Stimulation of brown adipose tissue (BAT) may be less effective than beiging of WAT in adults for regulating energy homeostasis,[10] but the efficiency of beiging might depend on the respective fat depot. Compared with BAT, murine beige adipocytes showed an even higher stimulatory potential by physical activity.[10] Exercise studies vary not only concerning the health status of participants or animal model, but also with regard to the particular type of exercise as well as frequency, intensity and duration of the intervention.[11–13]

In humans, systemic insulin resistance can be the result of accumulating intracellular lipotoxic lipid species (e.g. diacylglycerols, ceramides), inflammatory processes and impaired mitochondrial function in muscle, adipose and liver.[1] WAT has a central role in interorgan crosstalk by regulating systemic lipid and energy metabolism.[14,15] Compromised mitochondrial function in adipose tissue impairs secretion of endocrine mediators regulating insulin sensitivity in other tissues[15] and may be an early perturbation in type 2 diabetes. Several human studies indicate that exercise does not induce beiging, at least in response to rather short-term training interventions.[16,17] Although longer endurance training has been shown to increase expression of beiging genes in abdominal scWAT in healthy adults,[18] these changes did not translate to improved insulin sensitivity as derived from the homeostasis model assessment of insulin resistance (HOMA-IR). This indicates that shorter interventions may induce only transient changes in expression of beiging genes in adipocytes, ceasing upon stimulus withdrawal.[19] The impact of physical fitness as a more chronic outcome on induction of scWAT beiging and its relevance in early type 2 diabetes is less clear. As experimental induction of beiging via cold exposure is unphysiological in adults, and pharmacological treatment is often accompanied by adverse side effects, limiting its use in humans, this study focused on the potential of physical activity to induce beiging of WAT.[20,21]

The present study used a translational approach linking findings of scWAT beiging in mice to those in humans. In humans, this study aimed to unravel the relationship between (a) physical fitness and scWAT beiging (primary outcome) and assess (b) its relevance in recent-onset type 2 diabetes; and (c) in mice, we aimed to identify the potential of exercise training to induce beiging by directly comparing two different long-term exercise training interventions in a mouse model challenged by a high-fat diet.

2 | MATERIALS AND METHODS

2.1 | Study participants

From the ongoing German Diabetes Study (GDS), 28 persons with recent-onset type 2 diabetes and 28 glucose-tolerant humans matched for age, body mass index (BMI) and sex were included in this study. The GDS is a prospective observational study investigating the natural course of recently diagnosed diabetes and the development of diabetes-associated complications in humans with type 1 or 2 diabetes and a control population. The study design and cohort profile of the GDS are described elsewhere in detail.[22] For the present study, a subcohort of participants was selected based on physical fitness status with an age range of 30-69 years and available data of spiroergometry

and adipose tissue biopsy samples and then subdivided according to disease and fitness status derived from spiroergometry.

VO_2ATRef., which is VO_2 at the aerobic threshold (VO_2AT) as the percentage of the predicted VO_2peak, was used as an estimate of physical fitness. The higher this threshold relative to the maximal oxygen uptake, the higher the intensity that can be sustained without metabolic perturbations, which would result in termination of exercise. As such, a less conditioned individual will reach VO_2AT at a lower exercise intensity relative to the maximum than a more conditioned individual, despite similar maximal oxygen uptake values, which makes this parameter an optimal performance indicator.[23] Thus, humans with VO_2ATRef. <60% were considered as unfit humans (n = 14 L-FIT with and n = 14 without type 2 diabetes), while values of VO_2ATRef. ≥60% marked physically fit (n = 14 H-FIT with and n = 14 without type 2 diabetes) persons.[24] All participants gave written informed consent before inclusion in the study (ClinicalTrials.gov registration no. NCT01055093), which was performed according to principles of the Declaration of Helsinki and approved by the local ethics board of the Heinrich Heine University, Düsseldorf, Germany. All glucose-tolerant humans underwent a 75-g oral glucose-tolerance test (Accu-Chek Dextro O.G-T.; Roche) with blood sampling at time points of 5, 30, 60 and 120 min to assess glucose tolerance and to exclude persons with impaired glucose tolerance.[22]

2.2 | Study protocol

For all visits, the participants refrained from any exercise 3 days before all examinations and arrived at the Clinical Research Center in the morning after 10 h of overnight fasting. At the first visit, each participant underwent recording of medical history, bioimpedance analysis and physical laboratory examinations and cycling spiroergometry.[22] At the second visit, a hyperinsulinaemic-euglycaemic clamp test was conducted to assess whole-body insulin sensitivity (M-value) and open-circuit indirect calorimetry (Vmax Encore 29n; CareFusion) was employed to measure resting energy expenditure (REE) as described.[22] At the third visit, ^{1}H-magnetic resonance spectroscopy/magnetic resonance imaging measurements (Achieva X-series; Philips Healthcare) were performed to assess hepatocellular lipid content and a biopsy was obtained from paraumbilical abdominal scWAT by the needle suction technique and after immediate freezing in liquid nitrogen stored at −80 °C for later analyses.[22]

2.3 | Spiroergometry

An incremental exhaustive exercise test was performed on a cycle ergometer (Viasprint 200; Ergoline) to assess VO_2peak and VO_2AT via breath-by-breath spirometry (MasterScreen CPX; Jäger/Viasys) as described before.[22] The VO_2AT, also known as the gas exchange threshold, is characterized by an increased ventilation in response to an increasing exercise workload.[25] It marks the point of first onset of blood lactate. This is in contrast to the second ventilatory threshold, also referred to as the respiratory compensation point, which marks a disproportionate increase in ventilation as lactate is more rapidly increasing with intensity after this point.[26] As an indicator of physical fitness, we used the VO_2AT as the percentage of the predicted age-specific VO_2peak (VO_2ATRef.). The closer VO_2AT is to the maximal oxygen uptake (VO_2max), the higher the intensity the individual can sustain without accumulation of blood lactate, hence this parameter provides a valid estimate of the aerobic performance of an individual.[27] According to recommendations from the American College of Sports Medicine, measures of VO_2max are expressed relative to total body mass.[28] Participants were separated into physically fit and unfit humans as previously described.[24]

2.4 | Laboratory analyses

Fasting plasma glucose (FPG), insulin, free fatty acid (FFA) levels, glycated haemoglobin (HbA1c), high-density and low-density lipoprotein cholesterol (HDL-C and LDL-C), triglycerides and high-sensitivity C-reactive protein were measured as previously described.[22] Fasting adipose tissue insulin resistance (Adipo-IR) was calculated from fasting FFA and insulin levels.[15]

2.5 | Experimental animals

FVB/N mice, known for their high exercise performance on both voluntary wheel running and treadmill training[29] were purchased from a commercial breeder (Charles River). All experiments were approved by the Animal Welfare Committee of the State Ministry of Agriculture, Nutrition and Forestry (State of North Rhine-Westphalia, Germany, Reference number 84-02.04.2017.A102). Three to six male mice per cage (Makrolon type III) were housed at 22 °C and a reversed 12-h light/dark cycle with lights off from 10:30 am to 10:30 pm. Animals had ad libitum access to food and water. After weaning at the age of 3 weeks, animals received a high-fat diet (D12492 from Research Diets) as described in Table S1.

2.6 | Physical training and materials for the mouse study

Concise protocols, chemicals, reaction kits and buffer ingredients are described and listed in Table S2. In brief, mice were either subjected to a 6-week endurance interval training with progressively increasing speed and duration of exercise bouts on treadmills or no training. For the running wheel intervention, one group had access to running wheels with recorded running distance, while the other group was not provided with wheels.

2.7 | Body weight and body composition

Body weight was measured with an electronic scale (Sartorius), body composition was analysed using a nuclear magnetic resonance spectrometer (Echo MRI).

2.8 | Intraperitoneal glucose tolerance tests

To assess the metabolic impact of regular exercise training on whole-body glycaemia in mice, animals were subjected to intraperitoneal glucose tolerance tests and measurements of 6 h-fasted FPG and insulin levels, respectively. For intraperitoneal glucose tolerance tests, glucose (2 g/kg body weight, sterile 20% solution) was injected intraperitoneally into 6-h fasted animals. Blood samples were taken at time points 0, 15, 30, 60 and 120 min from the tail tip.

2.9 | High-resolution respirometry and citrate synthase activity

High-resolution respirometry was used to measure tissue-specific mitochondrial function (Oxygraph 2k; Oroboros Instruments) at 37 °C after standardized air calibration and considering instrumental oxygen background flux as described.[30,31] Oxygen concentration and oxygen flux in adipose tissue samples from mice were computed by DatLab software (DatLab). Citrate synthase activity (CSA), an established surrogate marker of mitochondrial content,[32] was assessed using a commercial CSA kit (cat. no. CS0720; Sigma-Aldrich). On average, the chamber oxygen concentrations ranged from 193 μM initially to 130 μM at the end of the protocol.

2.10 | RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction in murine and human subcutaneous white adipose tissue

RNA was isolated by TRIzol extraction using miRNeasy-Mini Kit (Qiagen) and cDNA was synthesized using GoScript™ Reverse Transcriptase Kit (Promega) with 1 μg RNA using random hexanucleotide primers (Roche). Gene expression of established markers for beige adipocytes in scWAT such as *Carbonic anhydrase 4* (*CAR4*), *tumour necrosis factor receptor superfamily member 9*, also known as *Cluster of Differentiation 137* (*TNFRSF9* or *CD137*), *Cell Death-Inducing DFFA-Like Effector A* (*CIDEA*), *Cytochrome C Oxidase Subunit VIIIb* (*COX8B*), *purinergic receptor P2X* (*P2RX5*), *Precursors expressing platelet-derived growth factor receptor alpha* (*Pdgfra*), *transmembrane protein 26* (*TMEM26*) and *Uncoupling Protein-1* (*UCP1*)[19] were quantified via quantitative real-time polymerase chain reaction, using the GoTaq® qPCR Master Mix (Promega) and specific SYBR Green primers (Table S3). Quantification was conducted by the $2^{-\Delta\Delta Ct}$ method[33] using *EIF4A2* as the housekeeping gene.

2.11 | Laboratory analyses

For the determination of FPG and plasma insulin concentrations in mice, blood was collected from the tail tip after 6 h of fasting with ad libitum access to water. FPG was determined with a glucometer (Contour; Bayer) and plasma insulin levels were measured using the Insulin Ultrasensitive ELISA Kit (DRG Instruments). HOMA-IR was calculated from FPG and insulin levels according to the formula: HOMA-IR = fasting insulin (ng/ml) × fasting blood glucose (mg/dl)/405[34] from the 6-h fasted animals.

2.12 | Statistical analyses

Results are given as median (first and third quartiles) or mean ± standard error of the mean. Variables were compared using non-parametric ANOVA model (Kruskal-Wallis) with Dunn's correction, two-tailed Mann-Whitney U and Student's t-test with Bonferroni adjustment as appropriate to determine differences between groups. Nominal variables were compared by Fisher's exact test. Relations between variables were investigated using Pearson or Spearman rank correlation analyses as appropriate. The total area under the curve for a specific variable was calculated as the integral of the time course of the respective variable during the test. The standardized mean difference (Cohen's d) was used for power analyses using estimates for mean and standard error of *Cd137* expression levels from trained and untrained mice in our pilot study. Based on the two-sample-two-sided t-test, the power calculation showed that a standardized mean difference of 1.5 (a large effect size) can be detected in a sample size of n = 14 per group with a power of 95%. As our experiments later showed, the effect size for *CD137* expression in scWAT of healthy L-FIT versus H-FIT humans (primary outcome; Cohen's d: 2.0) was even larger. All statistical tests were two-sided and $p \leq .05$ was accepted to indicate significant differences. All statistical analyses and graphs were generated using GraphPad Prism, Version 7.01 (GraphPad Software, Inc.).

3 | RESULTS

3.1 | Participants' characteristics

By design, all groups showed similar distribution of age, sex, BMI and diabetes, but also similar waist and hip circumference, waist-to-hip ratio, lean body mass and body fat percentage, independent of fitness status (Table 1). In addition, all groups had comparable fasting FFA, triglycerides, HDL-C, LDL-C and high-sensitivity C-reactive protein as well as similar hepatocellular lipid content, respiratory quotient and REE under fasting and hyperinsulinaemic clamp conditions (Table 1).

Within the respective groups, without and with diabetes, H-FIT versus L-FIT had similar HbA1c, FPG, whole-body insulin sensitivity and Adipo-IR (Table 1). Compared with humans with type 2 diabetes, HbA1c and FPG were lower in glucose-tolerant participants in both H-FIT and L-FIT (Table 1).

Whole-body and adipose tissue insulin sensitivity was lower in persons with type 2 diabetes compared with glucose-tolerant humans in L-FIT only (Table 1).

TABLE 1 Characteristics of study population

	L-FIT			H-FIT			L-FIT vs. H-FIT	
Variable	CON	T2D	*p*	CON	T2D	*p*	p (CON)	p (T2D)
Male/female, n	10/4	10/4	-	10/4	10/4	-	-	-
Age, years	52 (44; 57)	54 (47; 62)	.99	52 (47; 59)	53 (46; 57)	.99	.99	.99
BMI, kg/m^2	30 (27; 34)	32 (26; 36)	.99	30 (27; 31)	30 (30; 34)	.99	.99	.99
Lean body weight, kg	62 (56; 72)	66 (55; 74)	.99	62 (54; 71)	65 (55; 72)	.99	.99	.99
Body fat, %	33 (29; 36)	34 (30; 37)	.99	30 (25; 38)	31 (29; 37)	.99	.99	.99
Diabetes duration, days	-	202 (89; 256)	-	-	119 (57; 174)	-	-	.19
Waist circumference, cm	107 (103; 113)	109 (97; 118)	.99	100 (92; 103)	103 (102; 108)	.88	.22	.99
Hip circumference, cm	108 (102; 113)	112 (101; 117)	.99	106 (102; 113)	109 (104; 117)	.99	.99	.99
Waist to hip ratio	1.0 (0.96; 1.0)	0.98 (0.91; 1.0)	.99	0.9 (0.88; 0.96)	0.96 (0.91; 1.0)	.99	.12	.99
HbA1c, %	5.4 (5.2; 5.6)	6.2 (5.6; 6.5)	**<.05**	5.2 (5.2; 5.4)	6.1 (5.8; 6.5)	**<.001**	.99	.99
HbA1c, mmol/mol	35 (33; 38)	44 (38; 47)	**<.05**	34 (33; 35)	43 (40; 48)	**<.001**	.99	.99
FPG, mg/dl	88 (86; 95)	122 (108; 146)	**<.001**	91 (86; 96)	109 (100; 125)	**<.01**	.99	.99
FFA, µmol/L	480 (367; 616)	567 (458; 643)	.99	532 (302; 779)	632 (529; 710)	.99	.99	.99
TG, mg/dl	131 (91; 171)	161 (110; 180)	.99	115 (93; 158)	111 (74; 158)	.99	.99	.70
Total cholesterol, mg/dl	197 (185; 219)	210 (179;)	.98	213 (185; 236)	185 (162; 209)	.40	.99	.92
HDL cholesterol, mg/dl	47 (43; 70)	42 (37; 51)	.53	51 (42; 62)	50 (40; 53)	.99	.99	.99
LDL cholesterol, mg/dl	135 (120; 143)	132 (106; 151)	.99	136 (119; 159)	110 (98; 139)	.43	.99	.99
hsCRP, mg/dl	0.1 (0.1; 0.1)	0.2 (0.1; 0.4)	.07	0.1 (0.1; 0.2)	0.2 (0.1; 0.3)	.73	.99	.99
HCL, % of water signal	2.51 (1.1; 7.3)	9.7 (5.1; 21.2)	.46	4.3 (1.7; 5.9)	6.5 (4.4; 16.8)	.48	.99	.99
M-value, mg/kg/min	8.8 (7.4; 10.0)	5.5 (4.7; 7.4)	**<.05**	10.7 (8.5; 11.7)	8.6 (5.5; 10.1)	.34	.22	.55
Adipo-IR, [µmol/L] × [mU/L]	1001 (614; 1444)	1525 (1328; 3009)	**.05**	937 (554; 951)	1311 (813; 2297)	.13	.76	.99
REE, kcal/day	1453 (1180; 1558)	1417 (1111; 1585)	.99	1279 (1114; 1484)	1484 (1175; 1546)	.99	.79	.99
REE insulin-stimulated, kcal/day	1544 (1385; 1622)	1478 (1291; 1569)	.99	1544 (1265; 1730)	1531 (1237; 1649)	.99	.99	.99

Note: Data are median (1st; 3rd quartile), non-parametric ANOVA (Kruskal-Wallis test) and two-tailed Mann-Whitney U test with Dunn's and Bonferroni adjustment as appropriate. HbA1c, FPG, TG, HDL, LDL, FFA, hsCRP, HCL and whole-body insulin sensitivity (m-value) were analysed in the fasted state. Bold indicates a significant or trend for difference between the respective comparisons.

Abbreviations: BMI, body mass index; CON, glucose-tolerant humans; FFA, free fatty acids; FPG, fasting plasma glucose levels; HbA1c, glycosylated haemoglobin; HCL, hepatocellular lipid content; HDL, high-density lipoprotein; H-FIT, physically high-fit humans; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; L-FIT, physically low-fit humans; REE basal, resting energy expenditure in fasting conditions; REE insulin stimulated, resting energy expenditure during clamp; T2D, type 2 diabetes mellitus; TG, triglycerides.

Of note, more L-FIT than H-FIT received glucose-lowering medication (Table S4).

3.2 | Physical fitness in humans

As an indicator of physical fitness and as per definition and group allocation, VO_2ATRef. was higher in persons without (Cohen's d: 2.7) and with (Cohen's d: 2.3) diabetes in H-FIT versus L-FIT (Figure 1A).

In glucose-tolerant humans, VO_2max relative to body weight was higher in H-FIT compared with L-FIT (Cohen's d: 1.2), while VO_2max tended to be higher in H-FIT compared with L-FIT with type 2 diabetes (Cohen's d: 1.0) (Figure 1B). In glucose-tolerant humans, VO_2AT tended to be higher in H-FIT compared with L-FIT (Cohen's d: 0.9) ($p = 0.050$), while VO_2AT was higher in H-FIT compared with L-FIT with type 2 diabetes (Cohen's d: 1.2) (Figure 1C).

3.3 | Expression levels of beiging markers in human subcutaneous white adipose tissue

In persons with and without diabetes, H-FIT and L-FIT had comparable expression levels of *CAR4*, *CIDEA*, *COX8B*, *P2RX5*, *TMEM26* and *UCP1* in scWAT. In glucose-tolerant humans, *CD137* expression was three-fold higher in H-FIT than L-FIT (Cohen's d: 1.9) (Figure 1D). Of note, H-FIT and L-FIT people with type 2 diabetes had similar *CD137* expression levels (Cohen's d: 0.3). H-FIT glucose-tolerant participants showed two-fold higher *CD137* (Cohen's d: 1.3) and four-fold higher

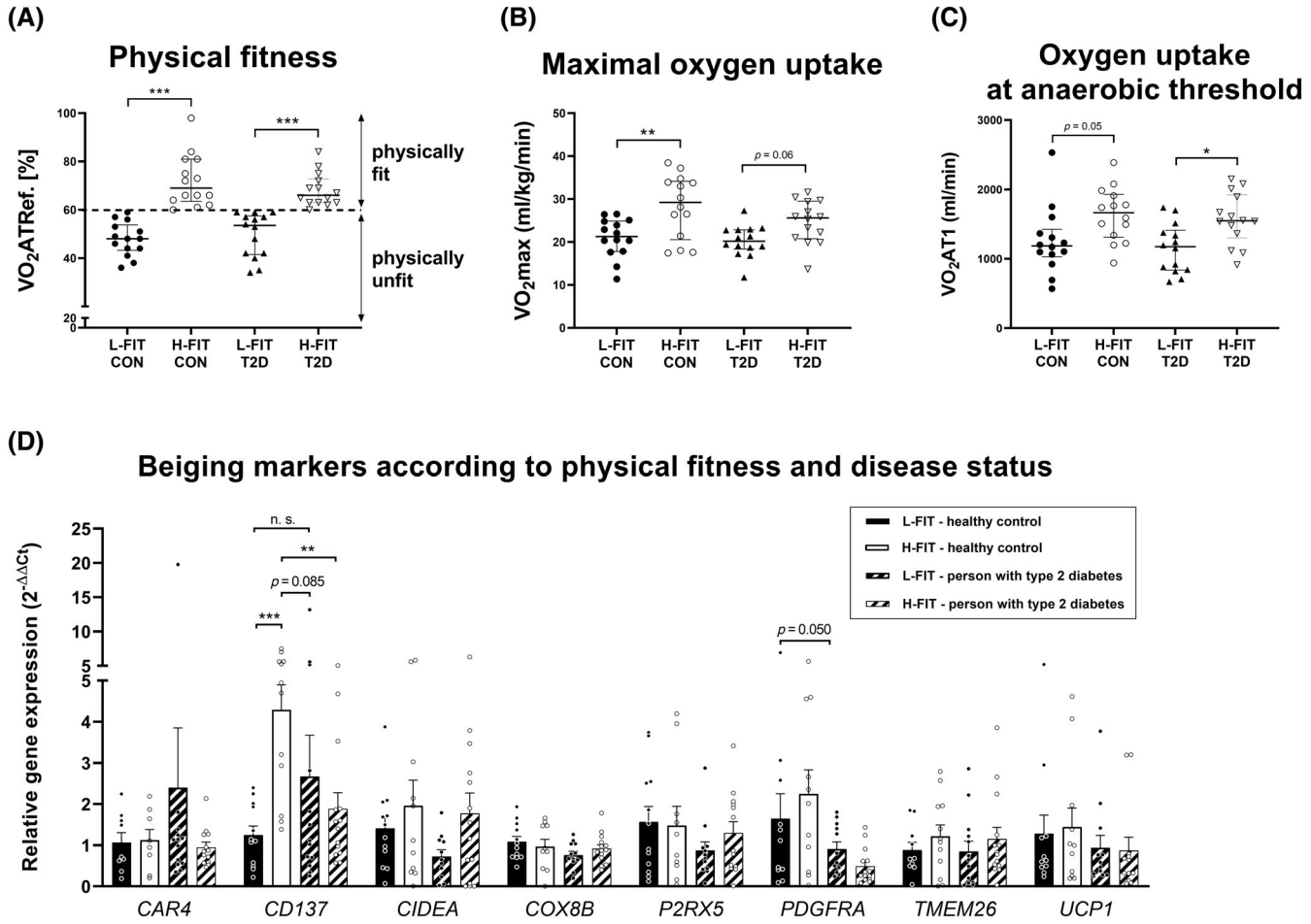

FIGURE 1 Physical fitness and beiging in humans. Spiroergometry derived (A) maximal oxygen uptake (VO_2max) relative to body mass and (B) oxygen uptake at anaerobic threshold. (C) Humans were preassigned based on their VO_2ATRef. into physically unfit (L-FIT) and fit persons (H-FIT) and by disease status into glucose-tolerant healthy humans (CON) and persons with type 2 diabetes (T2D). (D) Gene expression levels of established markers for beige adipocytes such as *Carbonic anhydrase 4* (*CAR4*), *Cluster of Differentiation 137* (*CD137*), *Cell Death-Inducing DFFA-Like Effector A* (*CIDEA*), *Cytochrome C Oxidase Subunit VIIIb* (*COX8B*), *purinergic receptor P2X* (*P2RX5*), *Precursors expressing platelet-derived growth factor receptor alpha* (*PDGFRA*), *transmembrane protein 26* (*TMEM26*), and *Uncoupling Protein-1* (*UCP1*) were measured in human abdominal subcutaneous adipose tissue biopsies. Data are shown as individual values and as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$, data were compared by non-parametric ANOVA (Kruskal-Wallis test) with Dunn's correction.

PDGFRA expression levels than H-FIT with type 2 diabetes (Cohen's d: 0.7) (Figure 1D). Noteworthy, L-FIT persons with and without diabetes had similar expressions levels of beiging genes (Figure 1D).

Expression levels of *CD137* correlated positively with VO_2ATRef. in controls ($r = 0.597$, $p < .01$), but not in humans with type 2 diabetes ($r = -0.078$, $p = .700$). In addition, *CD137* expression neither associated with HbA1c ($r = -0.111$, $p = .973$) nor with M-value ($r = 0.162$, $p = .450$) or Adipo-IR ($r = -0.046$, $p = .840$) in glucose-tolerant persons. Similarly, *CD137* expression neither associated with glycaemic control ($r = 0.146$, $p = .469$) nor with whole-body ($r = 0.049$, $p = .809$) or adipose tissue insulin sensitivity ($r = -0.047$, $p = .816$) in persons with diabetes. None of the assessed beiging markers associated with glycaemic control, whole-body or adipose tissue insulin sensitivity in persons with and without diabetes (data not shown). To further investigate the potential metabolic impact of beiging, we assessed the effects of different chronic training interventions in mice fed a regular chow diet and in animals challenged by a high-fat diet.

3.4 | Physical performance and whole-body glycemia

Mice with access to running wheels ran on average 3.4 km/day throughout the 6 weeks of access to running wheels (Figure 2A). However, voluntary wheel running did not result in improved glucose tolerance (Figure 2B,C) or fasting insulin sensitivity (HOMA-IR) (Figure 2D). In contrast, 6 weeks of running on a motorized treadmill significantly increased the degree of physical fitness, as shown by elevated time to exhaustion (Figure 2E). This also led to substantial improvements in glucose tolerance (Figure 2F,G) and fasting insulin sensitivity (Figure 2H).

3.5 | Body weight and body composition

After the high-fat diet with running wheel intervention, mice showed increased body weight, unchanged lean body mass and increased

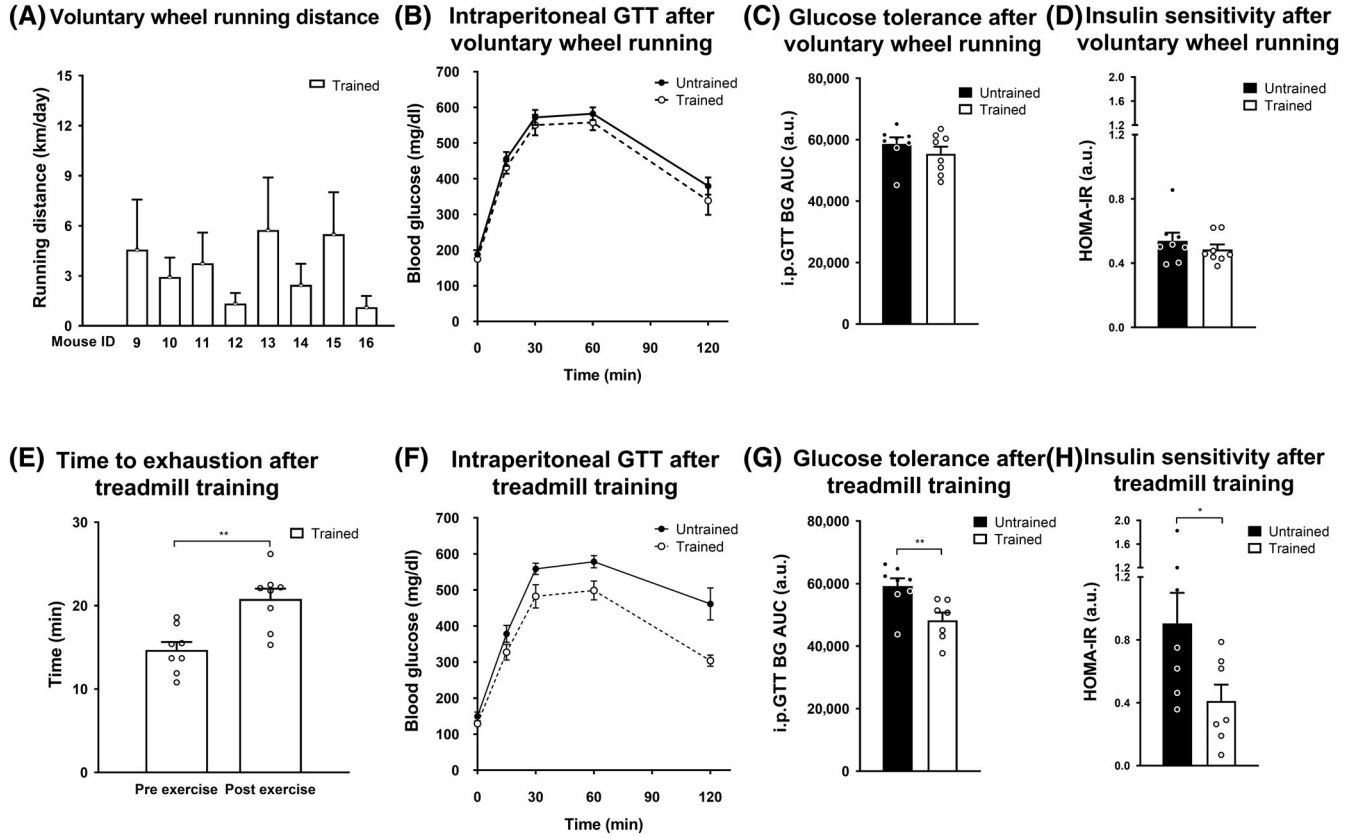

FIGURE 2 Glucose tolerance and insulin sensitivity in untrained and trained mice. Intraperitoneal glucose tolerance test was applied to assess glucose tolerance from individual glucose values over time, as well as from the area under the curve and homeostasis model assessment of insulin resistance (HOMA-IR) was used to assess fasting insulin sensitivity from fasting glucose and insulin concentrations in mice. (A) Running performance (km per mouse per day), (B) glucose tolerance test with (C) area under the curve from individual glucose values and (D) fasting HOMA-IR of FVB/N mice of the voluntary running wheel and (E-H) treadmill running group. Data are shown as individual values and mean ± SEM as indicated. $^{*}p < .05$, $^{**}p < .01$, data were compared by non-parametric ANOVA (Kruskal-Wallis test) with Dunn's correction and by two-tailed Student's t-test with Bonferroni correction.

body fat mass (Figure S1A-C) irrespective of the training status compared with baseline measurements. Moreover, the increase of body fat mass after high-fat diet in mice subjected to the running wheel intervention was not as pronounced in trained animals when compared with their untrained littermates (Figure S1C). Following high-fat diet with treadmill running, body weight, lean body mass and body fat mass were increased in both untrained and trained mice compared with the baseline measurements (Figure S1D-F). After high-fat diet with treadmill running, body weight and body fat mass were lower in trained compared with untrained mice (Figure S1A-F).

3.6 | Mitochondrial content and function

CSA as a marker of mitochondrial content was about two-fold higher in scWAT from trained mice compared with untrained animals, independent of the respective training modality (Cohen's d >1.3) (Figure 3A,B). Neither voluntary wheel running nor treadmill training resulted in alterations of mitochondrial respiration in scWAT from high-fat diet-fed mice (Cohen's d >0.5) (Figure 3C,D).

3.7 | Gene expression in murine subcutaneous white adipose tissue

Analogous to humans, we assessed scWAT beiging markers in animals. These markers were differently affected by voluntary wheel running and treadmill training, respectively. Mice subjected to voluntary wheel running had increased gene expression levels of *Cd137* (Cohen's d: 0.4) and *Ucp1* (Cohen's d: 1.0) (Figure 3E) while *Car4*, *Cidea*, *Cox8b*, *P2rx5*, *Pdgfrα* and *Tmem26* expression was similar between trained and untrained mice (Figure 3E). Treadmill training, however, resulted in a substantial increase in *Cd137* expression in trained mice only (Cohen's d: 1.4), whereas expression levels of *Car4*, *Cidea*, *Cox8b*, *P2rx5*, *Pdgfrα*, *Tmem26* and *Ucp1* were similar between trained and untrained animals (Figure 3F).

4 | DISCUSSION

This study reveals that the previously suggested adipocyte beiging marker *CD137* in scWAT associates with physical fitness in humans

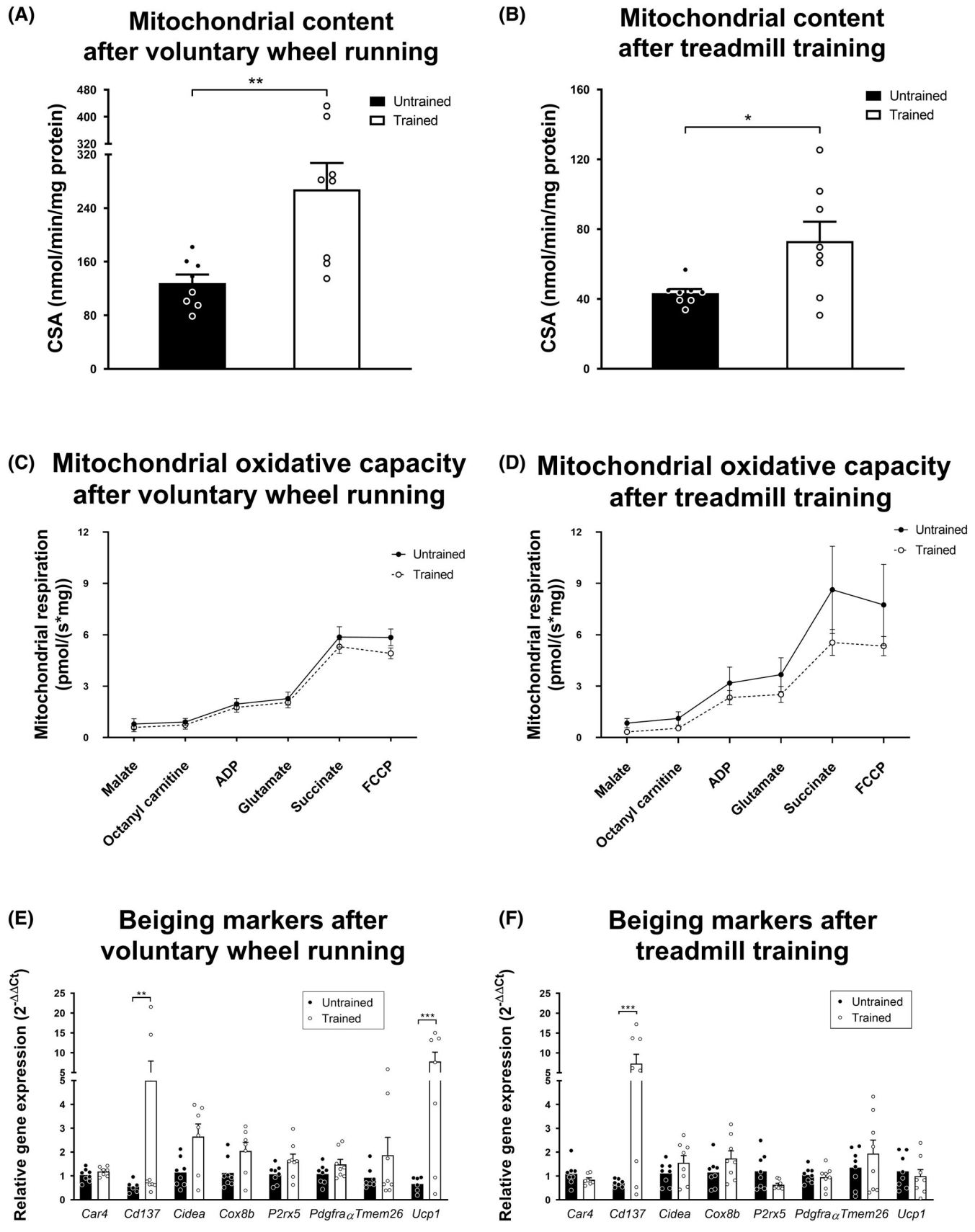

FIGURE 3 Legend on next page.

with normal glucose tolerance but not in persons with recent-onset type 2 diabetes. In accordance, gene expression levels of *Cd137* and mitochondrial content increased in scWAT from mice after two different modes of chronic exercise training.

Higher expression levels of *CD137* of glucose-tolerant physically fit versus unfit persons and higher expression levels in scWAT of trained versus untrained mice suggests that *Cd137* increases in parallel with physical fitness and chronic training in scWAT. Initially, *Cd137* was identified in scWAT as a marker of beige adipocyte precursors and mature beige adipocytes in murine models. As a member of the tumour necrosis factor receptor superfamily (TNFRSF) this beiging marker is also referred to as TNFRSF9 or 4-1BB.[19] Although studies in mice have consistently uncovered that exercise induces WAT beiging, several studies have been performed in healthy humans but not in persons with recent-onset type 2 diabetes. Most of the human studies found small or no changes in beiging markers, oxidative capacity or glucose metabolism in WAT.[11,12,16,17,35,36] However previous results remain inconclusive. While moderate endurance training increased gene expression levels of several beiging markers such as *Ucp1* in human WAT,[18] cold-induced [^{18}F]-FDG-derived BAT activity was not improved in endurance-trained athletes and gene expression levels of *TMEM26*, *CIDEA* and *CD137* were unchanged compared with sedentary humans.[36] However, the latter study included young trained athletes and untrained humans with different body fat mass and did not adjust for this confounder.

Results from our study suggest that *CD137* expression levels can be used as a marker for chronic training-induced beiging in mice and physical fitness-induced beiging in humans, which is supported by the finding that expression levels correlated strongly with physical fitness in glucose-tolerant humans. In contrast to our results, several previous studies found no changes in *CD137* expression after different acute exercise interventions in humans.[11,12,17,18] These discrepancies could be explained by different exercise interventions (exercise type, duration, frequency or intensity of training) and inclusion of different human cohorts (differences in sex, age, BMI, body fat content and health status). Effects on WAT beiging may be more prominent in humans with higher physical fitness levels, which rather reflect chronic physical exercise training. Thus, results from previous studies should not be interpreted as lack of effective beiging on metabolic parameters, but rather that chronic physical exercise training would be necessary for more effective induction of beiging in scWAT.[18] The current study stratified humans according to their physical fitness by VO_2ATRef., a robust and accepted marker of physical fitness[24] and uncovered effects of rather long-term physical stimulation on adipocyte beiging in humans. To the best of our knowledge, none of the previous studies assessed scWAT beiging in humans based on their fitness status as assessed by gold standard method of spiroergometry.

In this study, both physically fit and unfit persons with type 2 diabetes showed similar beiging markers as unfit glucose-tolerant humans. Beige adipocytes were speculated to ameliorate metabolic complications of obesity and type 2 diabetes such as insulin resistance in skeletal muscle and adipose tissue.[37] However, our data suggest that beiging of scWAT may be mainly relevant in humans without diabetes, possibly related to known perturbations of adipocyte plasticity in early-onset type 2 diabetes, ultimately causing derogated responses to physiological cues with subsequent pathological consequences.[37] Of note, the design of our study enables us to uncover the relevance of type 2 diabetes for physical fitness-induced beiging of scWAT without confounding factors such as effects of longstanding glucotoxicity and concomitant diseases.

The current study found no associations between browning/beiging markers and improvements in glycaemic control, whole-body or adipose tissue insulin sensitivity in persons with or without diabetes. This agrees with a previous study revealing that increased browning/beiging did not result in metabolic improvements.[18] Although regular physical activity of 150 min/week can reduce the susceptibility to develop type 2 diabetes,[38] our data suggest that physical fitness-induced beiging of scWAT is at least not directly related to metabolic improvements. This is also supported by our findings that physically fit and unfit persons with and without diabetes showed no differences in REE. Given the disparities between humans and rodents, the task of translating novel mechanisms for activating BAT to individuals with metabolic disorders is a challenging endeavour and the significance of beiging of WAT in humans remains a matter of debate. In persons with burn trauma, which experience severe adrenergic stress, beiging of WAT could account for about 10% of the hypermetabolism induced by burns.[39] Although the effects of BAT activation in humans are evident and include increased REE, whole-body glucose disposal[40] as well as insulin sensitivity,[41] the metabolic benefits of beiging of WAT are probably more subtle. Glucose tolerance was enhanced in high-fat diet-induced glucose intolerant mice implanted with human beige adipocytes.[42] The beneficial features of BAT or beiging, however, probably do not solely stem solely from their potential to impact on energy expenditure or glucose uptake, but could also be attributed to its metabolic communication and responsive interactions with other organ systems, including the central nervous system. As such, brown adipocytes possess the capacity to

FIGURE 3 Mitochondrial content, function and beiging in untrained and trained mice. (A,B) Mitochondrial content and (C,D) function in subcutaneous adipose tissue biopsy samples of FVB/N mice were assessed after voluntary wheel running and treadmill training. Gene expression of established markers for beige adipocytes such as *Carbonic anhydrase 4* (*Car4*), *Cluster of Differentiation 137* (*Cd137*), *Cell Death-Inducing DFFA-Like Effector A* (*Cidea*), *Cytochrome C Oxidase Subunit VIIIb* (*Cox8b*), *purinergic receptor P2X* (*P2rx5*), *Precursors expressing platelet-derived growth factor receptor alpha* (*Pdgfrα*), *transmembrane protein 26* (*Tmem26*) and *Uncoupling Protein-1* (*Ucp1*) were measured in subcutaneous adipose tissue biopsy samples of untrained and trained mice after (E) voluntary wheel running and (F) treadmill training. Data are shown as individual values and mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$, data were compared by non-parametric ANOVA (Kruskal-Wallis test) with Dunn's correction and by two-tailed Student's t-test and remained significant after Bonferroni correction.

release endocrine factors referred to as 'batokines', which may improve systemic metabolic regulation by engaging other metabolic tissues such as skeletal muscle. These 'batokines' exhibit autocrine, paracrine and endocrine effects and include, among other, fibroblast growth factor 21, bone morphogenetic protein 8b, interleukin-6, vascular endothelial growth factor A, insulin-like growth factor 1, neuregulin 4, prostaglandins, endocannabinoids and different microRNAs.[43] Accordingly, elucidating formation and dynamics of human brown or beige adipocytes could be essential groundwork for future BAT-centred therapeutics.

In agreement with results in humans, this study uncovered *Cd137* as a marker for exercise-induced beiging in scWAT in mice subjected to two different exercise interventions. Previous studies showed training-induced increases of *Ucp1*, *Cidea* and *Cox8b* in WAT of rodents after both acute exercise and chronic endurance training.[44–47] In agreement, numerous studies showed the impact of exercise interventions on BAT activation and/or WAT beiging in rodents.[14] While our study indeed showed an increase in *CD137* expression in both healthy humans and mice on a high-fat diet following exercise interventions, we acknowledge that the overall changes in other established markers of beiging, such as *Ucp1*, were not consistent across all conditions. The observed variations in *Ucp1* expression may suggest that the beiging process in scWAT is context-dependent. It is possible that our experimental protocols in mice, albeit aiming at mimicking chronic exercise training, captured an early phase of beiging, when not yet all features of browning are fully established as seen in previous studies issuing acute interventions (e.g. cold exposure or pharmacological induction of BAT). In addition, differences in species and the modality of the interventions (voluntary wheel running vs. treadmill training) could contribute to the variation in *Ucp1* expression. Therefore, while our study highlights *CD137* as a possible marker associated with physical fitness and exercise training, further research is needed to elucidate the full spectrum of beiging processes in scWAT. These results underline the impact of different training interventions in mice to induce WAT beiging. In this study, we used FVB/N mice, which are known for their good exercise performance during both voluntary wheel running and treadmill training.[29] Although not directly comparable, non-exercising FVB/N mice may reflect physically unfit and trained mice physically fit humans. This suggests that beiging in scWAT is context-dependent, possibly reflecting an early adaptation. Differences in species and exercise interventions may contribute to variable UCP1 expression. Further research is needed to elucidate fully the scWAT beiging and its metabolic implications.

This study showed that only treadmill exercise training increased glucose tolerance and whole-body insulin sensitivity in mice. Previous studies showed divergent results regarding effects of exercise training on BAT activation and effects on insulin sensitivity. While previous studies in rodents revealed that endurance training induces proteins of the insulin signalling pathway in BAT, these studies lacked functional evidence as they did not assess glucose uptake.[48,49] Other studies found no changes in glucose transporter type 1 or 4 after moderate endurance training in BAT[46,50] or even decreased insulin-stimulated glucose uptake into BAT.[36] While our study found divergent effects of exercise training on insulin sensitivity, functional analyses of scWAT-specific glucose uptake were not performed, which is a limitation of this study.

In this study, both exercise interventions in mice revealed increased mitochondrial content, but not mitochondrial respiration after exercise. Our results are in line with a previous study reporting increased content but not respiration of mitochondria in WAT after exercise interventions in humans.[51] Previous reports speculated that increases of mitochondrial content before respiration could rest on beiging induction in WAT by enhanced mitochondrial biogenesis.[51] We confirm these findings in mice after different training interventions and even when animals are challenged by a high-fat diet. Conversely, humans with type 2 diabetes showed reduced mitochondrial content in scWAT.[52]

The strength of this study lies in the translational design, the deep phenotyping in a rare cohort of humans with recent-onset type 2 diabetes and a well-matched group of glucose-tolerant humans. Furthermore, gold-standard methodology allowed to assess insulin sensitivity and physical fitness levels. Limitations include the use of a surrogate parameter for adipose tissue insulin sensitivity and lack of measurements of insulin sensitivity from scWAT biopsies. Nevertheless, this study shows that *CD137* is indeed a relevant marker for beiging, depending on physical activity in two species. Because of the nature of a cross-sectional design in the human part, this study does not allow conclusions to be drawn as to causality or temporal relationships.

In conclusion, this study emphasizes that *CD137* can be a relevant beiging marker in scWAT in healthy humans impacted by physical fitness, but beiging per se may be defective already in recent-onset type 2 diabetes. This indicates that disease-development could associate with an impaired organ-specific response to physical activity, highlighting the need for tailored recommendations for physical exercise in type 2 diabetes.

AUTHOR CONTRIBUTIONS

AC, DP and KB designed the study. KB wrote the article and researched the data. AC and DP researched the data, contributed to the discussion and writing process, reviewed and edited the article. SB, AC-P, DA, CS, O-PZ, MS, NS, MB, CM, KP and AS researched the data and reviewed the article. JS, RW, HA-H and MR edited the article. All authors critically reviewed and revised the manuscript and gave final approval of this version to be published.

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as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Open Access funding enabled and organized by Projekt DEAL.

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CONFLICT OF INTEREST STATEMENT

MR received personal fees from Allergan, Eli Lilly, Fishawack Group, Gilead Sciences, Novo Nordisk, Pfizer, Prosciento and Target RWE and investigator-initiated research support from Boehringer-Ingelheim, Nutricia/Danone and Sanofi-Aventis. RW reports lecture fees from Novo Nordisk, Sanofi and Eli Lilly and served on an advisory board for Akcea Therapeutics, Daiichi Sankyo, Sanofi, Eli Lilly and NovoNordisk. The authors have no conflicts of interest to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets used for the current study are available from the German Diabetes Study (GDS; PI: Michael Roden) upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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