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Clinical Research

Long-term effect of sweeteners and sweetness enhancers on gene expression markers of adipose tissue function, adipocyte morphology, and metabolic health: a SWEET substudy

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BACKGROUND/OBJECTIVES: We investigated the effects of sweeteners and sweetness enhancers (S&SEs) in replacement of sugar on gene expression markers of adipose tissue (AT) function, adipocyte morphology, and metabolic health in adults during weight maintenance (WM) following weight loss (WL).

SUBJECTS/METHODS: As part of a randomized controlled trial (European SWEET-project), 83 adults were enrolled in the study, which consists of a dietary intervention, comprising a 2-month WL-phase and a 10-month WM-phase during which participants followed a healthy diet (<10E%-added sugar) with (S&SEs group) or without S&SEs (sugar group). At baseline, after WL, and at the end of WM, we determined body composition, whole-body/tissue-specific insulin sensitivity (oral glucose tolerance test), abdominal subcutaneous adipocyte size and AT gene expression.

RESULTS: WL decreased adipocyte size and improved insulin sensitivity (both $P < 0.001$), which was accompanied by a significant downregulation of genes involved in adipogenesis (*CEBPa*, $P = 0.004$), fatty acid uptake (*LPL* and *SREBF1*, both $P < 0.001$), fatty acid synthesis (*FASN* and *SCD*, both $P < 0.001$), intracellular lipolysis (*ATGL*, $P < 0.001$; *HSL*, $P = 0.001$), leptin (*LEP*, $P < 0.001$), and mitochondrial function (*CS*, $P = 0.048$) in AT. The S&SEs-group tended to regain less weight than the sugar group during the WM-phase (3.0 ± 1.2 vs. 5.9 ± 1.0 kg, respectively; $P = 0.050$). During the WM-phase, the S&SEs group showed a less pronounced increase in AT *LPL* gene expression ($P = 0.041$), while *ABHD5* expression decreased compared to the sugar group ($P = 0.036$). No group differences in adipocyte size, expression of genes involved adipogenesis/oxidative metabolism/inflammation, the sweet taste receptor TAS1R3 and insulin sensitivity were found.

CONCLUSIONS: S&SE-intake during a 10-month WM-phase following WL altered AT gene expression of lipolytic markers, without affecting adipocyte morphology and insulin sensitivity in adults with overweight/obesity.

TRIAL REGISTRATION: ClinicalTrials.gov NCT04226911, Sweeteners and Sweetness Enhancers: Prolonged Effects on Health, Obesity and Safety (SWEET).

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INTRODUCTION

Obesity is a chronic relapsing disease that acts as a gateway to many noncommunicable diseases due to an excessive accumulation of body fat [1–3]. Adipose tissue (AT) expansion results in adipocyte hypertrophy and AT dysfunction, marked by impaired lipolysis, inflammation, and mitochondrial dysfunction, thereby contributing to the onset of metabolic diseases such as type 2 diabetes and metabolic dysfunctional-associated fatty liver disease [2, 4, 5]. To prevent or improve this, a healthy lifestyle is recommended, encompassing strategies to reduce overall energy intake. Substituting sugars with sweeteners and sweetness enhancers (S&SEs) is one approach to lower diet energy density and potentially reduce body weight. However, it remains unclear whether S&SEs effectively reduce energy intake and whether this translates into improved AT function and metabolic health. Hence, the World Health Organization (WHO) issued a conditional recommendation discouraging the use of non-sugar sweeteners for weight control or reducing the risk of non-communicable

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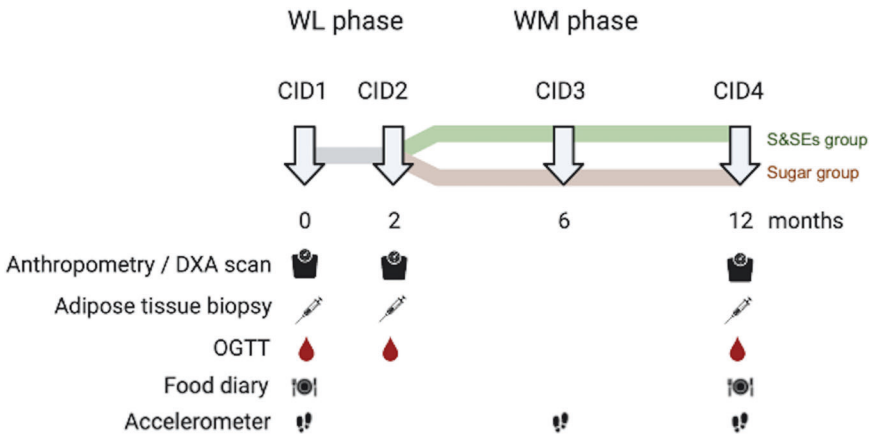

Fig. 1 Overview of study design and measurements. WL weight loss, WM weight maintenance, CID clinical investigation day, S&SEs sweeteners and sweetness enhancers, DXA dual energy x-ray absorptiometry, OGTT oral glucose tolerance test.

diseases [6]. This conservative approach is based on the lack of certainty and the overall balance of desirable and undesirable effects associated with long-term sweetener use, and was recently challenged [7].

The majority of short-term clinical studies report no or positive effects of S&SEs on body weight and glycemic control, whereas long-term cohort studies report associations between S&SE-intake and increased risk of non-communicable diseases [6, 8, 9]. Importantly, long-term randomized controlled trials (RCTs) investigating the role of S&SEs in body weight control and metabolic health are lacking. Limited clinical studies investigating the effect of S&SEs on AT function exist, with one study showing both up- and downregulated transcription of inflammatory transcriptomic pathways in abdominal subcutaneous AT in women with overweight or obesity after an 8-week diet soda consumption (including sucralose and acesulfame-K), although no differences were found in metabolic or inflammatory circulating biomarkers [10].

While evidence from human trials is limited, in vitro and rodent studies yield conflicting findings. Some in vitro studies found increased adipogenesis, reactive oxygen species, and reduced lipolysis upon saccharin and acesulfame-K (4.5 mM) stimulation in 3T3-L1 cells, or sucralose (0.45 or 4.5 mM) stimulation in human mesenchymal stem cells [11, 12]. Conversely, reduction in adipogenesis in 3T3-L1 cells was reported with saccharin (20 mM), sucralose (20 mM), or aspartame (10 µg/ml) stimulation [13, 14]. Furthermore, increased adiposity, lipogenesis, leptin concentration, protein abundance and gene expression of inflammatory markers was observed in white AT with 4 weeks and 4 months of S&SE-intake in mice, with or without diet-induced obesity [15, 16]. In contrast, another rodent study showed reduced adiposity and plasma leptin levels after 14 weeks of consuming an aspartame solution compared to control [17].

Thus, studies investigating the long-term effects of S&SEs on AT function and metabolic health are lacking, and existing preclinical studies provide inconsistent results [11–17]. Therefore, we investigated the effect of S&SEs in replacement of sugar on gene expression markers of adipose tissue function, adipocyte morphology, and metabolic health in adults with overweight or obesity in the European SWEET project (Sweeteners and sweetness enhancers: Impact on health, obesity, safety and sustainability) [18]. We hypothesized that replacing sugars with S&SEs has neutral or even beneficial effects on gene expression markers of adipose tissue function, adipocyte morphology, body composition, and insulin sensitivity.

METHODS

Study design

The current study was conducted at Maastricht University, The Netherlands, between August 2020 and October 2022, as a secondary analysis to the 1-year multicenter, parallel, randomized controlled trial within Work Package 3 of the European SWEET project (http://www.sweetproject.eu) [18]. The study was approved by the Medical Ethical Committee of Maastricht University Medical Centre (NL70977.068.19/METC19-056s) and performed according to the Declaration of Helsinki. The study was registered on ClinicalTrials.gov (NCT04226911).

The 1-year randomized controlled trial included a 2-month weight loss (WL) phase followed by a 10-month weight maintenance (WM) phase (Fig. 1). During the WL phase, the participants followed a low-energy diet (LED) (3347 – 4184 kJ/day), consisting of powdered shakes, soups, and smoothies (Cambridge Weight Plan), intending to reduce body weight by a minimum of 5%. After successful WL (≥5%), participants were randomized into two intervention diets with or without S&SEs for 10-months (S&SEs or sugar group, respectively). Clinical investigation days (CIDs) were scheduled at baseline (month 0 (M0)), after M2, M6 and M12. Anthropometry, body composition, gene expression markers of AT function, adipocyte morphology, and insulin sensitivity were measured at M0 and M2 (before and after WL), and at M12 (end of the WM phase) (Fig. 1). Prior to the CIDs, participants fasted for a minimum of 10 h with restrictions on high-intensity physical activity, coffee, and smoking 12 h before. The detailed protocol is described elsewhere [18].

Study participants

All 83 participants in the SWEET intervention study at Maastricht University, aged 18–65 years with a BMI ≥ 25 kg/m^2 and fasting glucose ≤7.0 mmol/L, and provided informed consent according to local legislation after verbal and written instructions. Exclusion criteria included diabetes, heart or coronary diseases, kidney or liver diseases, psychiatric illnesses, systemic infections, endocrine diseases, weight change >5% the past 2 months prior to the study, surgical treatment of obesity, change in smoking habits one month prior to the study, regularly drinking (>14 or 21 units/week for women and men respectively), intensive physical activity (>10 hours per week), night work or late shift work, and the use of prescription medication that interfere with the outcome of the study [18].

Randomization

Participants were randomly assigned to intervention groups after inclusion in a 1:1 ratio using a site-specific randomization list, created by an independent person in Copenhagen. The list is stratified by sex, age (<40 years or ≥40 years), and BMI (<30 or ≥30 kg/m^2). Stratification was implemented by sequentially assigning the participants from each stratum to the two interventions in blocks of 4 (Software R). In case participants shared a household, they were allocated to the same dietary intervention group based on the oldest member of the household. Staff and study participants were not blinded because of the nature of the intervention.

Diet groups and dietary instruction

The two diet intervention groups were 1) a healthy diet with <10E% added sugar allowing foods and drinks with S&SEs (S&SEs group) and 2) a healthy diet with <10E% added sugar not allowing foods and drinks with S&SEs (sugar group). The healthy diet was based on the recommendations of macronutrients, e.g. energy from fat (30–35E%), protein (15–20E%), and carbohydrate (50–55E%), as well as dietary fiber intake (at least 25 or 35 g/day for women and men respectively), saturated fats (<10E%), and added sugar intake (<10E%, as defined by the WHO [19]). The diet was *ad libitum* and included all types of S&SEs (artificial, natural, low-calorie, sugar alcohols, non-caloric) available on the market. A unit system was developed to ensure both intervention groups maintained a sugar intake below 10E% of daily intake. Each participant's maximum sugar allowance was calculated based on individual energy requirements and converted to a specific number of units per day (one unit equals 10 g sugar). The sugar group was instructed to limit their unit-consumption of sugar-containing products, while the S&SEs group should replace these units with equivalent S&SE-containing products using a food exchange list. Dietary WM counseling sessions occurred at M2, M4, M6, and M9 to offer dietary guidance and support in adhering to the assigned intervention. Further details on calculations and the unit system are available elsewhere [18].

Anthropometric characteristics and body composition

Body weight, height, waist and hip circumference, and body composition were measured as described previously [18]. Briefly, body composition, including body fat mass and percentage, fat free mass, android fat, gynoid fat, and visceral adipose tissue (VAT) were assessed by Dual energy X-ray Absorptiometry (Hologic Discovery A system (Bedford, MA, USA), Software: Hologic-Apex, version 4.5.3, with viewer software Hologic Physician's viewer, version 7.1; Hologic Horizon A, Software: application SW version 5.6.1.2, Physicians Viewer version 7.3, Apex version 5.5). Android and gynoid fat are described as the fat mass around the mid-section and hips, respectively.

Abdominal subcutaneous AT biopsy

Abdominal subcutaneous AT biopsies were collected (~1 g) using needle aspiration, 6–8 cm lateral from the umbilicus, under local anesthesia (2% lidocaine). The specimens were washed with sterile saline and visible blood vessels were removed using sterile tweezers. A small sample of AT was fixed overnight in 4% buffered paraformaldehyde and embedded in paraffin for adipocyte size analysis. The remaining samples were snap-frozen in liquid nitrogen and stored at −80 °C.

Adipocyte size. Histological sections (8μm) were cut from paraffin-embedded tissue on microscope glass slides and dried overnight in an incubator at 37 C°. Staining with hematoxylin and eosin was performed and digital images were captured at 20× magnification using a Leica DFC320 digital camera (Leica DM3000 microscope, Leica, Rijswijk, The Netherlands). Individual adipocyte area and diameter (~200 adipocytes/sample) were assessed using computerized morphometric analysis (Leica QWin V3, Cambridge, England).

Abdominal subcutaneous AT gene expression. Total RNA was extracted from frozen AT biopsies using TRIzol reagent (Invitrogen, Breda, The Netherlands) and reversed transcribed to 500 ng cDNA (iScript cDNA Synthesis Kit, BIO-RAD). Expression of genes encoding proteins involved in body weight regulation and/or obesity-related complications was determined using SYBR Green-based real-time PCR (iCycler/MyIQ BIO-RAD). Specifically, we determined gene expression of markers of adipogenesis (peroxisome proliferator-activated receptor (*PPARγ*), fatty acid binding protein 4 (*FABP4*), and CCAAT/enhancer-binding protein alpha (*CEBPα*)), intracellular lipolysis (adipose triglyceride lipase (*ATGL*), hormone-sensitive lipase (*HSL*), 1-acylglycerol-3-phosphate O-acyltransferase (*ABHD5/CGI-58*), perilipin-1 (*PLIN1*), and G0/G1 switch 2 (*G0S2*)), fatty acid uptake (lipoprotein lipase (*LPL*), angiopoietin-like 4 (*ANGPTL4*), and sterol regulatory element binding transcription factor 1 (*SREBF1*)), fatty acid synthesis (fatty acid synthase (*FASN*), acetyl-CoA carboxylase (*ACC*), and stearoyl-CoA desaturase (*SCD*)), adipokines (adiponectin (*ADIPOQ*) and leptin (*LEP*)), inflammation (interleukin-6 (*IL-6*), monocyte chemoattractant protein 1 (*MCP-1*), cluster of differentiation 68 (*CD68*), interleukin 1β (*IL-1β*), and plasminogen activator inhibitor-1 (*PAI-1*)), mitochondrial oxidative phosphorylation (OXPHOS) (nicotinamide adenine dinucleotide[NADH] dehydrogenase [ubiquinone] 1 (NDUF) beta subcomplex subunit B5 (*NDUFB5*), V-type proton ATPase catalytic subunit A (*ATP6V1A*), carnitine palmitoyltransferase 2 (*CPT2*), citrate synthase (*CS*), uncoupling protein-2 (*UCP2*), and NDUF alpha subcomplex subunit 1 (*NDUFA1*)), and the sweet taste receptor (*TAS1R3)*. The sequences of the forward and reverse primers are listed in Supplementary Table 1. Verified primer for the *TAS1R3* (qHsaCED0002321) was obtained from Bio-Rad Laboratories. The $2^{-\Delta CT}$ method was used for the calculation of the results, and data was normalized to 18S ribosomal RNA.

Insulin sensitivity

A 7-point oral glucose tolerance test (OGTT) was performed to determine whole-body and tissue-specific insulin sensitivity. Participants ingested 200 ml ready-to-use 75 g glucose solution (Novolab). Blood was drawn from the antecubital vein at $t = 0$, 15, 30, 45, 60, 90, and 120 min to determine plasma glucose and insulin levels. Whole-body insulin sensitivity was estimated using the homeostatic model assessment of insulin resistance (HOMA-IR) and Matsuda-index, as described previously [20]. HOMA-IR was calculated by: (fasting glucose [mmol/L]*fasting insulin [mU/L])/22.5 [21]. Matsuda-index was calculated by: 10.000/√((fasting glucose [mmol/L]*fasting insulin [pmol/L])(mean glucose [mmol/L]*mean insulin [pmol/L])) [22]. Tissue-specific insulin sensitivity was estimated according to the method of Abdul-Ghani et al. [23], which has previously been validated against the hyperinsulinemic-euglycemic clamp [22, 24], as described previously [20]. Hepatic insulin resistance index (HIRI) was calculated by: √(glucose 0–30 [AUC, mg/d × h]*insulin 0–30 [AUC, μU/mL × h]). Muscle insulin sensitivity index (MISI) used the rate of decay of plasma glucose concentrations (dG/dt) during the OGTT, calculated as the slope of the least square fit to the decline in plasma glucose concentration from peak to nadir, divided by the mean plasma insulin, expressed in pmol/L. The calculation of MISI is optimized by means of cubic splining by O'Donovan et al. [19].

Dietary records

Participants were instructed to record their dietary intake at M0 (baseline) and M12 for 4 days (3 weekdays and one weekend day) (Fig. 1). Daily average intake of energy and macronutrients, including dietary fiber and saturated fat content were assessed using the Eetmeter food diary and analysis tool (Voedingscentrum, Den Haag, the Netherlands). From the dietary records, sugar intake and S&SE-intake in units, listed in the food exchange list, were also assessed at M0 and M12. Compliance with S&SE-intake was further corroborated by urinary biomarkers of S&SEs in the total SWEET study population, as reported previously [25].

Physical activity

Physical activity was determined at M0, M6, and M12 using a triaxial accelerometer (activPAL3™ micro, PAL Technologies Ltd., Glasgow, Scotland, UK) (Fig. 1). The activPAL, attached to the anterior thigh for 7 continuous days, measured posture allocation, step count, and 24 h/day physical activity and distinguished between sleeping time, low-to-moderate physical activity (LMPA) (time spent standing or walking <100 steps/min), moderate-to-vigorous physical activity (MVPA) (time spent walking ≥100 steps/min or cycling), and sedentary time (time spent sitting or lying).

Statistical analysis

The 1-year multicenter RCT, powered for the primary outcome of body weight change, requires 330 overweight participants (accounting for a 30% dropout) [18], with 83 participants for each intervention center. For this substudy, 42 participants are needed to detect a 20% difference in adipocyte size, with an α of 0.05 and a power of 0.80. Numerical variables were reported as means ± standard error of the mean (SEM). For the WL phase, paired t-test was used to assess pre- vs. post-WL changes in anthropometry, body composition, adipocyte size and size distribution, AT gene expression, and insulin sensitivity indices in the total study population pre-intervention. Physical activity was analyzed using repeated-measures linear mixed model. For the WM phase, linear mixed-model analysis was conducted to compare dietary intake, added sugar or S&SE-intake in units, anthropometric measures, body composition, adipocyte size, size distribution, AT gene expression, and insulin sensitivity indices among groups. The models used the change in anthropometry or body composition during the WM phase (M2–M12) as the dependent variable, group as the fixed variable, and baseline values and changes during the WL phase (M0–M2) as covariates. Additionally, metabolic parameter models were adjusted for baseline body weight and changes

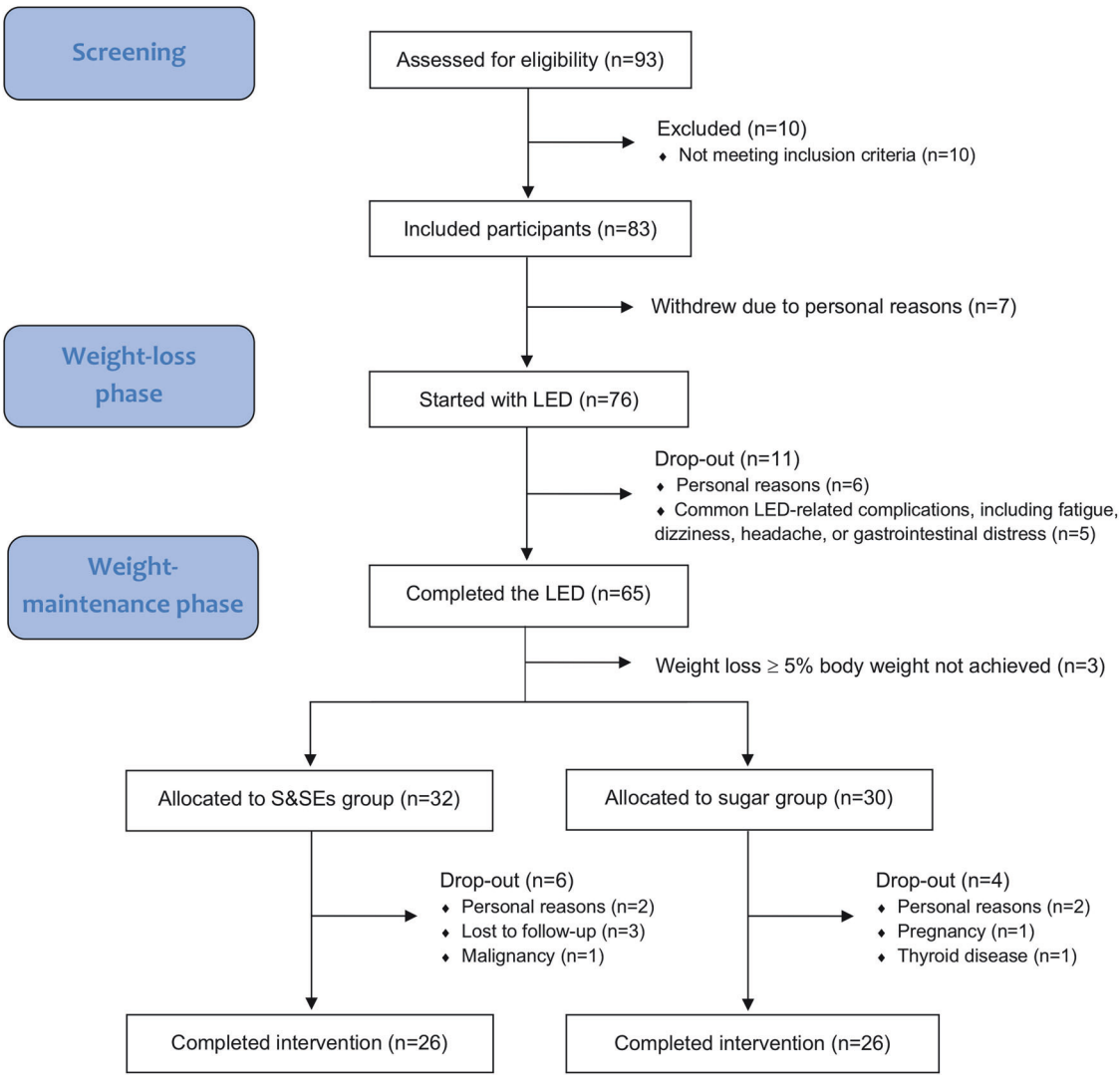

Fig. 2 Flowchart of participant enrollment and eligibility. LED low-energy diet, S&SEs sweeteners and sweetness enhancers.

during the WL phase. Multiple linear regression, adjusted for baseline variables and changes during the WL phase, was performed to determine the association between the change in adipocyte size, AT gene expression, and insulin sensitivity indices. Non-normally distributed data were ln-transformed to approximate a normal distribution and to stabilize variance across groups. Analyses were performed in IBM SPSS Statistics 25, and significance was defined as $P < 0.05$ (two-sided).

RESULTS

Between June 2020 and September 2021, 83 participants were enrolled in the study. Seven participants did not start the intervention due to loss of interest, resulting in 76 participants who started with the low-energy diet, of which 65 participants (78%) successfully completed the WL phase. Out of these 65 participants, 52 individuals (26 individuals in each group) completed the WM phase (Fig. 2). During the WL phase, 11 participants dropped out due to personal reasons ($n = 6$) or common LED-related complications such as fatigue, dizziness, headache, or gastrointestinal distress ($n = 5$). During the WM phase, 6 participants in the S&SEs group dropped out due to personal reasons ($n = 2$), lost to follow-up ($n = 2$), or malignancy ($n = 1$), whereas in the sugar group 4 participants dropped out due to personal reasons ($n = 2$), pregnancy ($n = 1$), or incident thyroid disease ($n = 1$). The average age of the study population that completed the WL phase was 48.4 ± 1.5 years and the average BMI was 32.3 ± 0.6 kg/m^2 at baseline (Table 1).

Weight loss phase

During the WL phase, total WL for the study population was 10.0 ± 0.6 kg and BMI was reduced by 3.5 ± 0.2 kg/m^2 (both $P < 0.001$) (Table 1), with no group differences. Furthermore, there were no group differences at baseline or at M2 (start of the WM phase). During WL, Matsuda-index increased ($P < 0.001$), whereas HOMA-IR, HIRI, and adipocyte diameter decreased (all $P < 0.001$) (Table 1) (Fig. 3A), with a shift towards a higher proportion of small adipocytes (Fig. 3B). Furthermore, the expression of AT genes involved in adipogenesis (Fig. 3C, *CEBPa*, $P = 0.004$), fatty acid uptake (Fig. 3D, *LPL* and *SREBF1*, both $P < 0.001$), fatty acid synthesis (Fig. 3E, *FASN* and *SCD*, both $P < 0.001$), intracellular lipolysis (Fig. 3F, *ATGL*, $P < 0.001$; *HSL*, $P = 0.001$), leptin (Fig. 3G, *LEP*, $P < 0.001$), and OXPHOS (Fig. 3H, CS, $P = 0.048$) decreased.

Weight maintenance phase

Dietary intake and physical activity. During the WM phase, both groups showed similar energy intake, macronutrient composition, and fiber and saturated fat intake (Supplementary Table 2). As

Table 1. Characteristics of participants that have completed the WL phase.

	Baseline (M0)	After WL (M2)	Change M0 to M2	
	Mean ± SEM	***Mean ± SEM***	***Mean ± SEM***	***P*-value**
Demographic variables				
Age (years)	48.4 ± 1.5			
Sex, females (n (%))	50 (76.9)			
Anthropometric variables				
Weight (kg)	92.2 ± 2.0	82.2 ± 1.7	10.0 ± 0.6	**<0.001**
BMI (kg/cm^2)	32.3 ± 0.6	28.8 ± 0.5	3.5 ± 0.2	**<0.001**
Waist circumference (cm)	103.8 ± 1.8	94.4 ± 1.7	9.4 ± 0.7	**<0.001**
Hip circumference (cm)	114.2 ± 1.2	107.0 ± 1.2	7.2 ± 0.5	**<0.001**
Body composition variables				
Body fat (kg)	37.6 ± 1.1	31.5 ± 1.0	6.1 ± 0.4	**<0.001**
(%)	40.2 ± 0.7	37.7 ± 0.8	2.7 ± 0.3	**<0.001**
Fat free mass (kg)	55.4 ± 1.4	51.9 ± 1.3	3.6 ± 0.3	**<0.001**
Android fat (kg)	3.4 ± 0.1	2.6 ± 0.1	0.8 ± 0.5	**<0.001**
Gynoid fat (kg)	6.3 ± 0.2	5.3 ± 0.2	1.0 ± 0.3	**<0.001**
Android-gynoid fat ratio	1.09 ± 0.02	1.02 ± 0.02	0.07 ± 0.01	**<0.001**
Visceral adipose tissue (g)	637 ± 52	466 ± 40	171 ± 35	**<0.001**
Metabolic variables				
Fasting glucose (mmol/L)	5.2 ± 0.1	5.0 ± 0.1	0.3 ± 0.1	**<0.001**
Fasting insulin (mU/L)	11.4 ± 1.2	6.5 ± 0.4	4.9 ± 1.0	**<0.001**
HOMA-IR	2.6 ± 0.3	1.4 ± 0.1	1.2 ± 0.2	**<0.001**
Matsuda-index	4.4 ± 0.3	6.1 ± 0.4	1.8 ± 0.3	**<0.001**
HIRI	588 ± 58	372 ± 35	216 ± 39	**<0.001**
MISI	0.12 ± 0.01	0.12 ± 0.01	0.00 ± 0.01	0.947
Blood pressure variables				
Systolic blood pressure (mmHg)	125 ± 2	116 ± 2	9 ± 1	**<0.001**
Diastolic blood pressure (mmHg)	83 ± 1	78 ± 1	6 ± 1	**<0.001**

Values are presented in mean ± SEM. Bold values denote significant change. *P*-value for difference before and after WL (paired *T*-test). $n = 65$, except for Matsuda-index ($n = 64$), HIRI ($n = 61$), and MISI ($n = 63$).
M month, *WL* weight loss, *BMI* body mass index, *HOMA-IR* homeostatic model assessment insulin resistance, *HIRI* hepatic insulin resistance index, *MISI* muscle insulin sensitivity index.

expected, the intake of S&SEs (units) increased in the S&SEs group compared to the sugar group (3.1 ± 0.5 vs. −0.6 ± 0.5 units, respectively, $P < 0.001$). No difference was observed in the intake of added sugar (units) between both groups. Furthermore, physical activity (including sedentary time, LMPA, MVPA, and step count) and sleeping time remained constant over time with no group disparities (Supplementary Table 3).

Body composition and metabolic health. During the WM period, the S&SEs group showed a tendency towards a lower total weight regain compared to the sugar group (3.0 ± 1.2 vs. 5.9 ± 1.0 kg, $P = 0.050$, Table 2). Similarly, a tendency towards a smaller increase in BMI and hip circumference was found for the S&SEs group compared to the sugar group (BMI, $P = 0.069$; hip circumference, $P = 0.079$). No differences between groups were found in the change in whole-body (HOMA-IR, $P = 0.335$; Matsuda-index, $P = 0.875$), hepatic (HIRI, $P = 0.537$) or skeletal muscle insulin sensitivity (MISI, $P = 0.717$) during the WM-period.

Adipocyte morphology and AT gene expression. During the WM period, no differences were found in changes in adipocyte diameter and adipocyte diameter distribution between groups (Fig. 4A, B). Furthermore, no difference was found in the expression of genes involved in adipogenesis (Fig. 4C), whereas the increase in *LPL* expression was less pronounced in the S&SEs compared to the sugar group (1.41 ± 0.14 vs. 1.79 ± 0.25-fold change (FC), $P = 0.041$) (Fig. 4D). Moreover, *ABHD5* expression showed a decrease in the S&SEs compared to the sugar group (1.25 ± 0.14 vs. 1.14 ± 0.20 FC, $P = 0.036$, respectively) (Fig. 4F). Additionally, no differences in changes of expression were in genes involved in fatty acid synthesis (Fig. 4E), OXPHOS (Fig. 4H), inflammation (Fig. 4I), adipokines (Fig. 4G), and the sweet taste receptor *TAS1R3* (Fig. 4J).

Association between S&SE-induced changes in AT gene expression of LPL and ABHD5 and changes in anthropometry, metabolic health, and adipocyte morphology. No correlations were found between changes in *LPL* or *ABHD5* expression and changes in body weight, BMI, and waist and hip circumferences upon S&SE-intake during the WM phase (Table 3). However, a positive association was found between the change in *LPL* expression and fasting glucose upon S&SE-intake (adj. std$\beta = 0.625$, $P = 0.012$), indicating that less pronounced increase in *LPL* expression was related to smaller increase in fasting glucose during the WM phase. Additionally, no correlations were found between changes in *ABHD5* expression and metabolic variables. Finally, the change in *ABHD5* expression upon S&SE-intake was positively correlated with the change in adipocyte diameter (adj. std$\beta = 0.472$, $P = 0.038$), while no

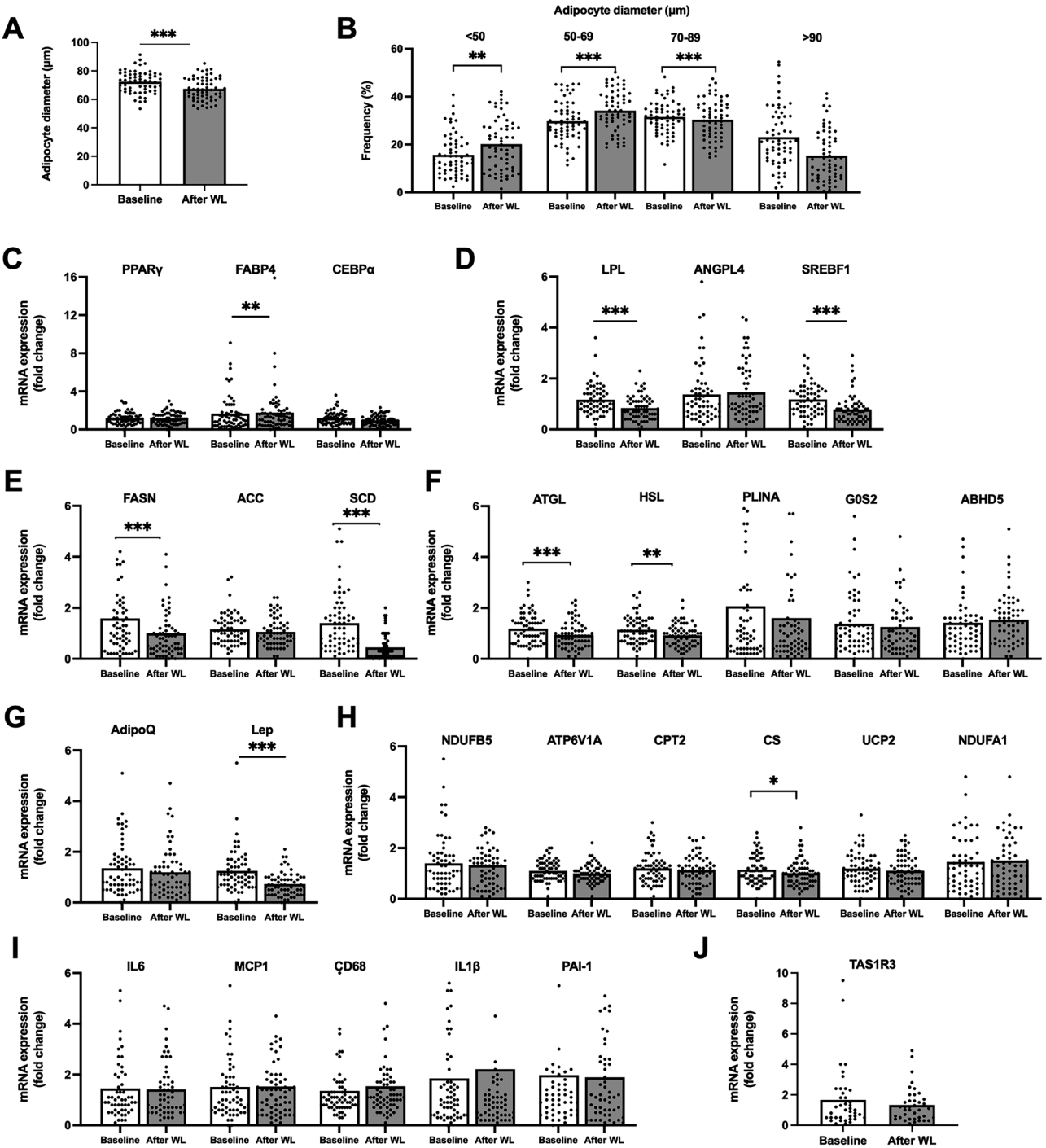

Fig. 3 Abdominal subcutaneous adipose tissue morphology and gene expression of the total study population before and after WL. **A** Mean adipocyte diameter and **B** adipocyte size distribution are presented as mean with individual data points displayed within the plunger plots (<50%, $P = 0.001$; all $n = 62$). Expression of **C** genes involved in adipogenesis (*PPARγ*, $P = 0.775$, $n = 62$; *FABP4*, $P = 0.621$; *CEBPα*, $P = 0.004$), **D** genes involved in fatty acid uptake (*LPL*, $P < 0.001$, $n = 62$; *ANGPL4*, $P = 0.380$, $n = 61$; *SREBF1*, $P < 0.001$), **E** genes involved in FA synthesis (*FASN*, $P < 0.001$; *ACC*, $P = 0.154$, $n = 61$; *SCD*, $P < 0.001$), **F** genes involved in intracellular lipolysis (*ATGL*, $P < 0.001$; *HSL*, $P = 0.001$; *PLIN1*, $P = 0.361$, $n = 62$; *G0S2*, $P = 0.195$, $n = 61$; *ABHD5*, $P = 0.111$, $n = 60$), **G** adipokines (*AdipoQ*, $P = 0.111$, $n = 62$; *LEP*, $P < 0.001$), **H** genes involved in OXHPOS (NDUFB5, $P = 0.725$, $n = 61$; *ATP6V1A*, $P = 0.076$; *CPT2*, $P = 0.647$, $n = 61$; *CS*, $P = 0.048$; *UCP2*, $P = 0.289$; *NDUFA1*, $P = 0.940$, $n = 58$), **I** genes involved in inflammation (*IL6*, $P = 0.785$, $n = 53$; *MCP1*, $P = 0.941$, $n = 61$; *CD68*, $P = 0.107$; *IL1β*, $P = 0.711$, $n = 60$; *PAI-1*, $P = 0.928$, $n = 54$), and **J** sweet taste receptor (*TAS1R3*, $P = 0.540$, $n = 39$) are presented as mean fold-change relative to the baseline values with individual data points displayed within the plunger plots. Results were normalized to 18S ribosomal RNA. White bars represent baseline values and gray bars represents the values after WL. $n = 63$, unless stated otherwise. *P*-value for difference before and after WL (*$P < 0.05$, **$P < 0.010$, and ***$P < 0.001$). WL weight loss, OXPHOS oxidative phosphorylation.

Table 2. Characteristics of the S&SEs group and sugar group that have completed the WM phase, at baseline (M0) and before (M2) and after WM phase (M12).

	Baseline		Weight maintenance phase						
	M0		M2		M12		Change M2 to M12		*P*-value
	S&SEs group *Mean ± SEM*	Sugar group *Mean ± SEM*	S&SEs group *Mean ± SEM*	Sugar group *Mean ± SEM*	S&SEs group *Mean ± SEM*	Sugar group *Mean ± SEM*	S&SEs group *Mean ± SEM*	Sugar group *Mean ± SEM*	
Demographic variables									
Age (years)	51.5 ± 2.3	46.0 ± 2.5							
Sex, Females/Males (*n*/*n*)	19/87	20/6							
Anthropometric variables									
Weight (kg)	92.7 ± 3.3	93.3 ± 3.3	82.4 ± 2.8	81.8 ± 2.7	85.4 ± 3.1	87.7 ± 3.2	3.0 ± 1.2	5.9 ± 1.0	0.050
BMI (kg/cm^2)	32.0 ± 1.0	32.6 ± 1.0	28.5 ± 0.8	28.6 ± 0.9	29.6 ± 0.9	30.7 ± 1.0	1.0 ± 0.4	2.0 ± 0.3	0.069
Waist circumference (cm)	105.3 ± 2.9	102.6 ± 3.0	94.1 ± 2.4	93.0 ± 3.0	96.4 ± 2.5	97.8 ± 3.0	2.3 ± 1.4	4.8 ± 0.8	0.283
Hip circumference (cm)	114.1 ± 2.1	115.1 ± 1.9	106.5 ± 1.7	107.2 ± 1.9	107.6 ± 1.7	110.9 ± 2.1	1.1 ± 1.1	3.7 ± 1.0	0.079
Body composition variables									
Body fat (kg)	38.1 ± 1.4	38.4 ± 1.7	31.7 ± 1.6	31.7 ± 1.6	33.2 ± 1.9	33.7 ± 1.8	1.5 ± 1.4	2.0 ± 0.6	0.866
(%)	40.8 ± 1.3	40.8 ± 1.1	38.1 ± 1.4	38.1 ± 1.2	36.9 ± 1.7	37.7 ± 1.3	1.1 ± 0.7	0.4 ± 0.4	0.346
Fat free mass (kg)	55.5 ± 2.4	55.5 ± 2.2	51.7 ± 2.2	51.3 ± 1.9	54.7 ± 2.4	55.2 ± 2.1	1.8 ± 1.3	3.9 ± 0.5	0.146
Android fat (kg)	3.4 ± 0.2	3.4 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	2.7 ± 0.2	2.9 ± 0.2	0.1 ± 0.1	0.3 ± 0.1	0.148
Gynoid fat (kg)	6.5 ± 0.4	6.4 ± 0.3	5.5 ± 0.3	5.4 ± 0.3	5.6 ± 0.4	5.7 ± 0.3	0.1 ± 0.2	0.3 ± 0.1	0.504
Android-gynoid fat ratio	1.07 ± 0.03	1.10 ± 0.04	1.01 ± 0.03	1.01 ± 0.04	0.98 ± 0.05	1.05 ± 0.04	0.03 ± 0.04	0.04 ± 0.02	0.214
Visceral adipose tissue (g)	603 ± 67	701 ± 85	431 ± 64	520 ± 67	562 ± 49	602 ± 62	132 ± 53	82 ± 50	0.407
Metabolic variables									
Fasting glucose (mmol/L)	5.2 ± 0.1	5.2 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	0.0 ± 0.1	0.0 ± 1.0	0.561
Fasting insulin (mU/L)	9.6 ± 0.9	10.6 ± 1.1	6.7 ± 0.7	5.7 ± 0.5	6.4 ± 0.5	6.4 ± 0.5	0.3 ± 0.7	0.7 ± 0.5	0.323
HOMA-IR	2.2 ± 0.2	2.4 ± 0.3	1.5 ± 0.2	1.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.1 ± 0.2	0.2 ± 0.1	0.335
Matsuda-index	3.8 ± 0.4	4.9 ± 0.6	5.7 ± 0.5	6.7 ± 0.6	6.0 ± 0.5	6.4 ± 0.6	0.3 ± 0.5	0.2 ± 0.5	0.875
HIRI	542 ± 51	569 ± 88	350 ± 40	336 ± 46	372 ± 48	375 ± 54	22 ± 38	39 ± 53	0.537
MISI	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.18 ± 0.03	0.15 ± 0.02	0.07 ± 0.03	0.02 ± 0.03	0.717
Blood pressure variables									
Systolic blood pressure (mmHg)	127 ± 3	124 ± 3	118 ± 3	114 ± 3	126 ± 3	119 ± 3	9 ± 2	5 ± 3	0.175
Diastolic blood pressure (mmHg)	85 ± 2	82 ± 2	80 ± 2	75 ± 2	82 ± 2	79 ± 2	2 ± 1	4 ± 1	0.960

Values are presented in mean ± SEM, unless specified otherwise. $n = 26$ in each group, except for fasting glucose and insulin ($n = 25$ for sugar group), HOMA-R ($n = 25$ for both groups), Matsuda-index ($n = 24$ for S&SEs group and $n = 25$ for sugar group), HIRI ($n = 24$ both groups), and MISI ($n = 23$ in S&SEs group and $n = 25$ in sugar group). Group differences were assessed using linear mixed model with adjustment for body weight (weight loss changes) and baseline differences.

S&SEs sweeteners and sweetness enhancers, *M* month, *BMI* body mass index, *HOMA-IR* homeostatic model assessment insulin resistance, *HIRI* hepatic insulin resistance index, *MISI* muscle insulin sensitivity index.

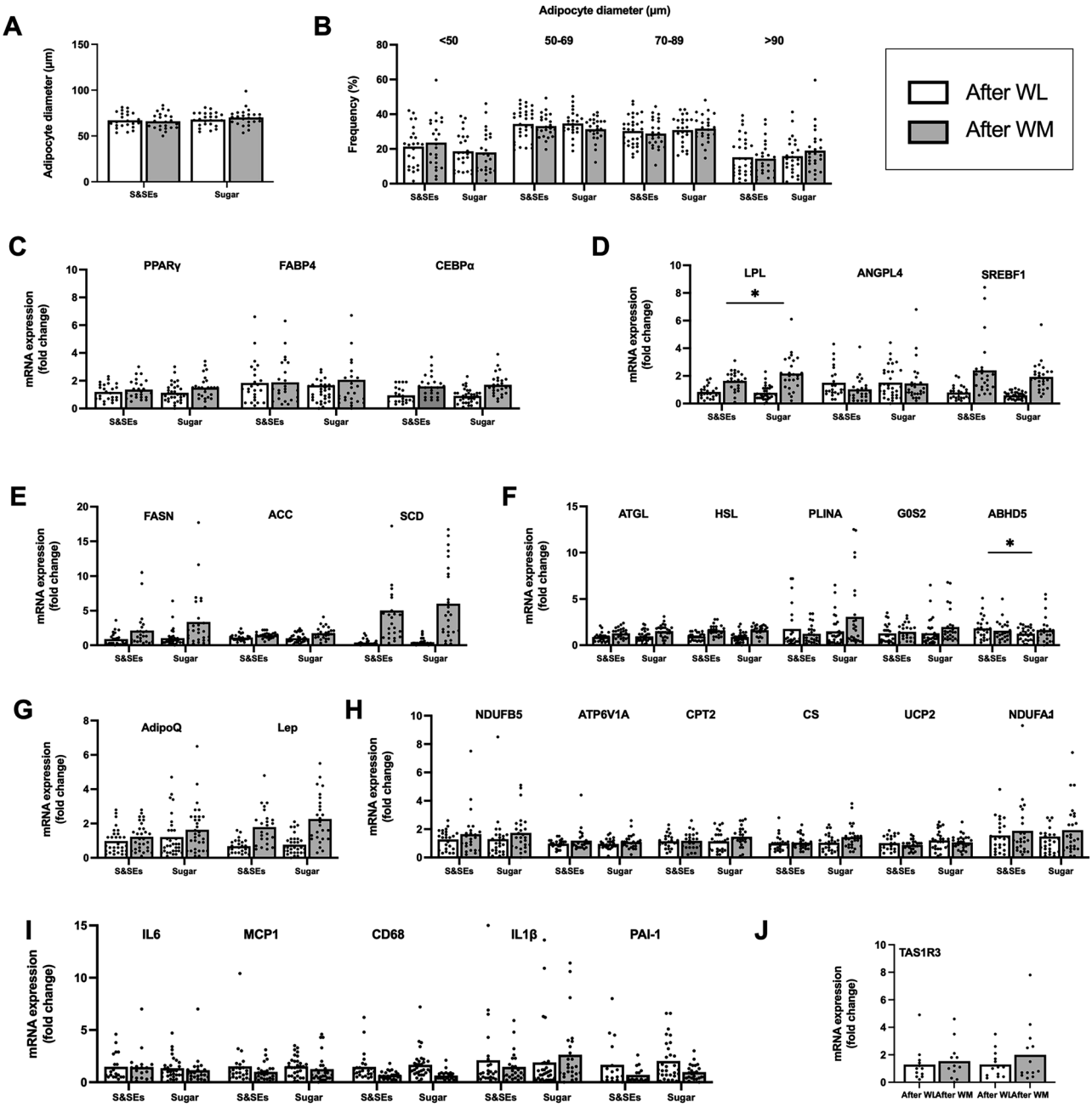

Fig. 4 Abdominal subcutaneous AT morphology and gene expression of the S&SEs group and sugar group before and after the WM phase. A Mean adipocyte diameter ($P = 0.116$) and **B** adipocyte size distribution (<50%, $P = 0.114$; 50–69%, $P = 0.307$; 70–89%, $P = 0.300$; >90%, $P = 0.290$) are presented as mean with individual data points displayed within the plunger plots. (all S&SEs group $n = 23$, sugar group $n = 24$). Expression of (**C**) genes involved in adipogenesis (*PPARγ*, $P = 0.381$; *FABP4*, $P = 0.913$; *CEBPα*, $P = 0.674$), **D** genes involved in fatty acid uptake (*LPL*, $P = 0.041$, S&SEs group $n = 24$ and sugar group $n = 24$; *ANGPL4*, $P = 0.349$, S&SEs group $n = 23$ and sugar group $n = 26$; *SREBF1*, $P = 0.502$), (**E**) genes involved in fatty acid synthesis (*FASN*, $P = 0.844$, S&SEs group $n = 24$ and sugar group $n = 25$; *ACC*, $P = 0.428$, S&SEs group $n = 24$ and sugar group $n = 25$; *SCD*, $P = 0.258$), (**F**) genes involved in intracellular lipolysis (*ATGL*, $P = 0.410$; *HSL*, $P = 0.728$; *PLIN1*, $P = 0.052$, S&SEs group $n = 24$ and sugar group $n = 24$; *G0S2*, $P = 0.228$, S&SEs group $n = 23$ and sugar group $n = 26$; *ABHD5*, $P = 0.036$, S&SEs group $n = 24$ and sugar group $n = 25$), (**G**) adipokines (*AdipoQ*, $P = 0.334$; *LEP*, $P = 0.218$, S&SEs group $n = 24$, sugar group $n = 25$), (**H**) genes involved in OXHPOS (NDUFB5, $P = 0.774$; *ATP6V1A*, $P = 0.815$; *CPT2*, $P = 0.447$, S&SEs group $n = 23$, sugar group $n = 25$; *CS*, P = 0.120; *UCP2*, $P = 0.826$; *NDUFA1*, $P = 0.949$), (**I**) genes involved in inflammation (*IL6*, $P = 0.387$, S&SEs group $n = 19$, sugar group $n = 19$; *MCP1*, $P = 0.237$, S&SEs group $n = 24$, sugar group $n = 25$; *CD68*, $P = 0.623$; *IL1β*, $P = 0.832$, S&SEs group $n = 24$, sugar group $n = 23$; *PAI-1*, $P = 0.706$, S&SEs group $n = 16$, sugar group $n = 22$), **J** sweet taste receptor (*TAS1R3*, $P = 0.589$, S&SEs group $n = 11$, sugar group $n = 10$) are presented as mean fold-change relative to values of the sugar group after WL with individual data points displayed within the plunger plots (S&SEs group $n = 24$ and sugar group $n = 26$, unless stated otherwise). Results were normalized to 18S ribosomal RNA. White bars represent data after the WL phase and gray bars represent data after the WM phase. *P*-value for group difference (*$P < 0.05$). WL weight loss, WM weight maintenance, S&SEs sweeteners and sweetness enhancers, OXPHOS oxidative phosphorylation.

Table 3. Multiple linear regression coefficients between S&SE-induced changes in anthropometry, metabolic parameters, and adipocyte morphology and S&SE-induced changes in AT gene expression of *LPL* and *ABHD5* during the WM phase.

	LPL				ABHD5			
	n	**std β**	**95% CI**	**P-value**	***n***	**std β**	**95% CI**	**P-value**
Anthropometric variables								
Body weight (kg)	24	0.399	–0.055;0.287	0.171	24	0.363	−0.062;0.692	0.096
BMI (kg/cm^2)	24	0.303	−0.031;0.255	0.118	24	0.343	−0.010;0.074	0.130
Waist circumference (cm)	20	0.041	−0.046;0.054	0.863	20	−0.153	−0.013;0.007	0.536
Hip circumference (cm)	20	−0.048	−1.834;1.548	0.858	20	0.156	−0.283;0.469	0.605
Metabolic variables								
Fasting glucose (mmol/L)	20	0.625	0.123;0.805	**0.012**	20	−0.201	−0.114;0.055	0.464
Fasting insulin (mU/L)	18	0.011	−0.106;0.110	0.968	18	−0.551	−0.043;0.002	0.070
HOMA-IR	18	0.157	−6.775;10.396	0.649	18	−0.561	−3.020;0.356	0.109
Matsuda-index	16	−0.233	−1.548;0.447	0.239	16	0.596	−0.010;0.539	0.057
HIRI	16	0.188	−2.332;4.195	0.529	16	−0.004	−0.631;0.625	0.991
MISI	15	−0.296	−6.133;2.482	0.350	15	0.447	−0.194;1.227	0.129
Adipocyte morphology								
Diameter (μm)	19	0.239	−0.017;0.060	0.252	19	0.472	0.001;0.021	**0.038**
<50% (%)	19	−0.042	−0.029;0.024	0.859	19	−0.413	−0.012;0.001	0.076
50–69% (%)	19	−0.345	−0.093;0.013	0.131	19	−0.206	−0.021;0.009	0.421
70–89% (%)	19	0.274	−0.354;1.247	0.253	19	0.440	–0.030;0.390	0.087
90% (%)	19	0.208	−0.355;0.946	0.348	19	0.324	−0.059;0.290	0.179

Values are presented in standardized β for effect size comparison and 95% confidence interval (CI) (based on unstandardized coefficients). Bold values denote significance ($P < 0.05$). Multiple linear regression was performed with adjustment for body weight (changes M0–M2) and baseline differences.
S&SEs sweeteners and sweetness enhancers, *AT* adipose tissue, *LPL* lipoprotein lipase, *ABHD5* α/β-Hydrolase domain-containing protein 5, *WM* weight maintenance, *BMI* body mass index, *HOMA-IR* homeostatic model assessment insulin resistance, *HIRI* hepatic insulin resistance index, *MISI* muscle insulin sensitivity index.

correlation was found between changes in *LPL* expression and adipocyte diameter.

DISCUSSION

In the present study, we investigated the effect of long-term S&SE-intake as a replacement of sugar on gene expression of markers of AT function, adipocyte morphology, and metabolic health in adults with overweight or obesity during weight maintenance (WM) following weight loss (WL). As expected, we found decreased abdominal subcutaneous adipocyte size and improved insulin sensitivity at the end of the WL phase, which was accompanied by a significant downregulation of AT genes involved in adipogenesis, fatty acid uptake, fatty acid synthesis, intracellular lipolysis, leptin, and mitochondrial function. During the WM period following WL, the S&SEs group tended to regain less body weight compared to the sugar group. Interestingly, the present findings demonstrate for the first time that S&SE-intake during a long-term WM period results in a less pronounced increase in AT gene expression of *LPL* and a decrease in expression of *ABHD5* (both involved in lipolysis) compared to no S&SE-intake, but did not alter adipocyte morphology, expression of genes involved adipogenesis/oxidative metabolism/inflammation, the sweet taste receptor TAS1R3 and insulin sensitivity.

During the 10-month WM phase, S&SE-intake resulted in altered expression of genes involved in extra- and intracellular lipolysis (*LPL* and *ABHD5*, respectively) compared to no S&SE-intake. *LPL* hydrolyzes circulating triacylglycerol, thereby releasing free fatty acids (FFA) that are taken up and stored in AT, while *ABHD5* acts as a co-activator of *ATGL*, thus contributing to intracellular triacylglycerol hydrolysis (that is, lipid breakdown) in AT [26]. If these changes in *LPL* and *ABHD5* gene expression translate to functional changes, the lower *LPL* gene expression after 10-months of S&SE-intake may reflect reduced extracellular lipolysis and hence lower AT lipid uptake, whereas the lower *ABHD5* gene expression may indicate a lower rate of intracellular lipolysis, despite unchanged ATGL gene expression. Thus, these data suggest that S&SE-intake may lower AT lipid turnover. Interestingly, we found that the smaller increase in AT *LPL* gene expression upon S&SE-intake was related to a more modest increase in fasting glucose during the WM phase. Taken together, our findings imply that reduced extracellular lipolysis following S&SE-intake may have contributed to lower adiposity and improved body weight control and glucose homeostasis [26]. Indeed, a trend towards less body weight regain upon S&SE-intake was found compared to no S&SE-intake in the present study population, which is in line with the overall findings in the total SWEET population of the RCT ($n = 341$) [25]. Nevertheless, no differences were found in adipocyte morphology, VAT, and insulin sensitivity between the S&SE and sugar groups during the WM phase. In agreement with this, previous findings showed that, intrahepatic lipid content (measured by ^{1}H-magnetic resonance spectroscopy) during the WM phase did not differ between the S&SEs and sugar groups in the current study population [25]. Furthermore, it has previously been shown that 6-month aspartame-sweetened diet cola consumption (1L/d) did not affect insulin sensitivity, VAT, and ectopic fat, including intrahepatic- and intramyocellular lipid content in adults with overweight of obesity, compared to water [27, 28]. The latter findings align with the majority of clinical studies reporting neutral or beneficial effects of S&SEs on body weight and glycemic control [8].

Limited evidence exists regarding the impact of S&SEs on adipocyte morphology and AT gene expression related to lipid metabolism, with conflicting findings in rodents. For instance,

saccharin administration in neonatal mice reduced adipocyte size [29], while maternal acesulfame-K consumption showed increased size in female offspring and no difference in males [30]. Steviol glycoside was found to have no effect on AT gene expression of *LPL* in diabetic rats [31], while saccharin stimulation reduced intracellular lipolysis in *3T3-L1* cells and human adipocytes [11], which is in line with our findings of lower AT *ABHD5* expression during the WM phase in the present study. Notably, the active concentration of saccharin (4.5 mM) in the latter study [11] exceeded the expected maximum human daily intake. Likewise, saccharin and acesulfame- K increased adipogenesis in mouse and human precursor adipocytes (*3T3-L1* cells, rodent mesenchymal stem cells, and human stromal vascular cells) [11]. This stimulation of adipogenesis and suppression of lipolysis seemed independent of the expression of sweet taste receptors (*T1R2* and *T1R3*) [11]. The present study is, to our knowledge, the first to investigate the expression of sweet taste receptors (*TAS1R3*) in human adipose tissue in response to S&SE-intake, and one of the few to determine *TAS1R3* expression in human adipocytes.

The strength of the present study includes the novelty of investigating the effects of long-term (10 months) S&SE-intake on AT gene expression of markers of AT function, adipocyte morphology, and metabolic health in a real-life controlled setting, reflecting realistic consumption patterns and dosages of various S&SEs in products available at the local market. However, our study was not designed to assess potential health effects of individual S&SEs. For some S&SEs, it also remains uncertain whether they exert direct physiological effects on peripheral tissues, as compounds such as aspartame or sucralose generally pass through the gut with little to no absorption and therefore are unlikely to reach peripheral tissues [8]. In addition, dietary changes were tracked using 4-day food records, which may be subject to reporting bias. Nevertheless, results of the urinary biomarkers of S&SEs measured in the total population of the SWEET study in four intervention sites revealed increased urinary excretion of S&SEs, including cyclamate, sucralose, acesulfame, saccharine, and steviol acyl glucuronide in the S&SEs group compared to the sugar group, as recently reported [25].

To better understand the potential health benefits and implications of substituting sugar with S&SEs, the conduct of long-term RCTs should be prioritized. Substantial long-term evidence empowers decision-making for healthcare professionals and policymakers in formulating guidelines, as underscored by the WHO guideline on sweeteners [6, 7]. Since this is the first study investigating the effects of long-term S&SE-intake on AT gene expression, more studies are warranted to build upon these findings and its metabolic implications. Although challenging, future studies should consider direct assessment of adipose tissue metabolism, in addition to gene expression and adipocyte morphology, to better understand how changes at the molecular level translate into physiological changes. In addition, metabolic pathways of different S&SEs, their impact on microbiota alterations, and their underlying physiological mechanism may aid in a better understanding of the potential effects on body weight control and glucose homeostasis.

In conclusion, the present findings demonstrate that 1) WL decreased adipocyte size, improved insulin sensitivity, and altered AT expression of genes encoding proteins involved in lipid and glucose metabolism, and 2) S&SE-intake during a 10-month WM period following WL induced by a LED altered AT gene expression of markers related to extra- and intracellular lipolysis (that is, lipid uptake and release), which was accompanied by less weight regain, compared to sugar intake in adults with overweight or obesity. To further our understanding, future research should prioritize long-term RCTs, and explore the metabolic impact of individual S&SEs.

DATA AVAILABILITY

Deidentified individual participant data that underlie the results reported in this paper and study protocol information should be requested through the corresponding author.

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AUTHOR CONTRIBUTIONS

The authors' contributions were as follows: MDP drafted and edited the manuscript. MDP analyzed the data and generated the results. MDP and JJAJB performed the measurements and collected the data. JWEJ supervised the adipose tissue gene expression analysis. AR, LK, JAH, JCGH, EEB and GHG designed this (sub)study. AR, LK, JAH and JCGH coordinate the SWEET project. JJAJB, JWEJ, LK, JAH, JCGH, AR, TCMA, EEB and GHG reviewed the manuscript. GHG had the primary responsibility for the final content. All authors approved the final version of the manuscript.

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