Interventions targeting hepatic and cardiovascular complications of metabolic syndrome

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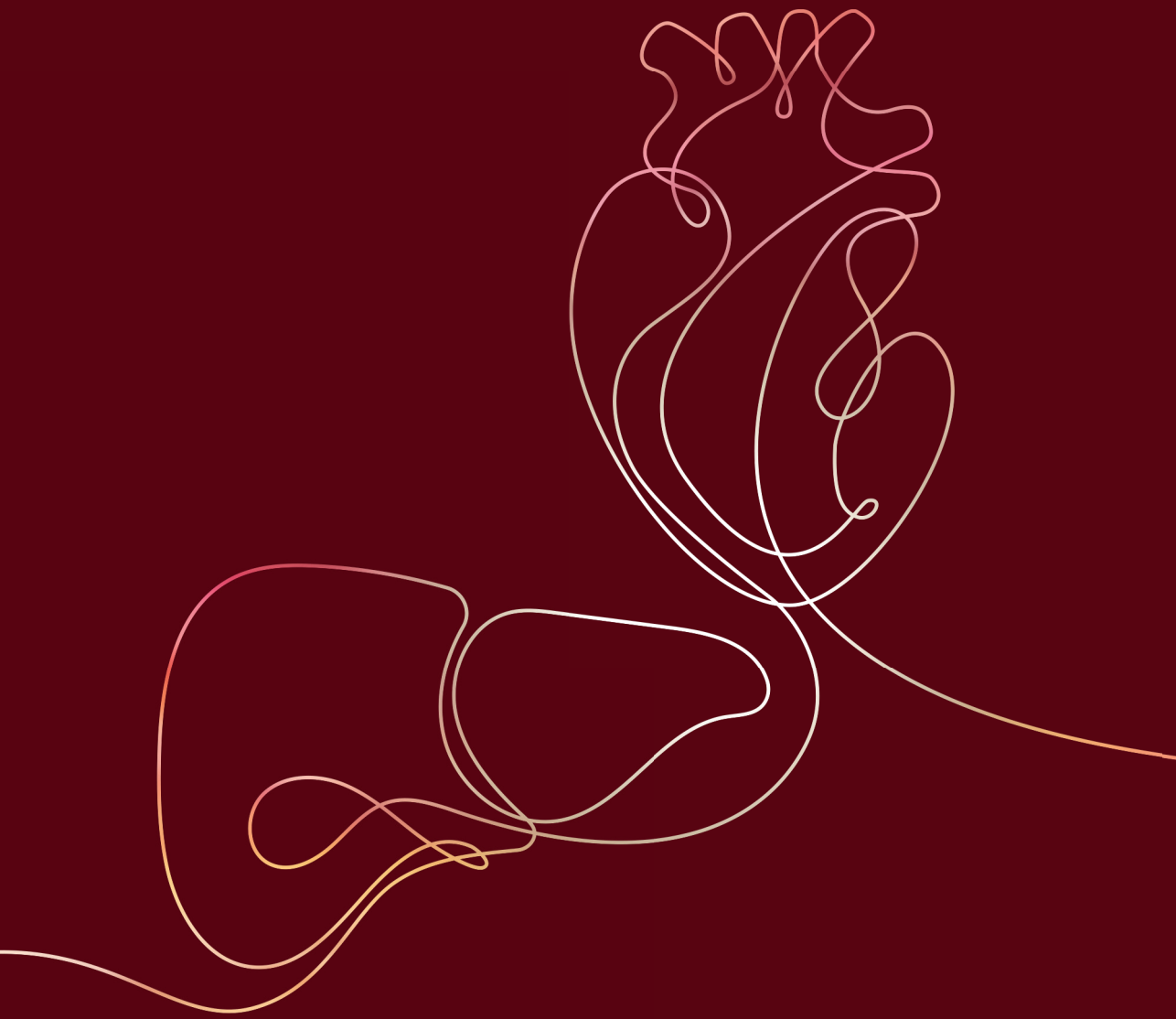

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Effects of repeated weight cycling on non-alcoholic steatohepatitis in diet-induced obese mice

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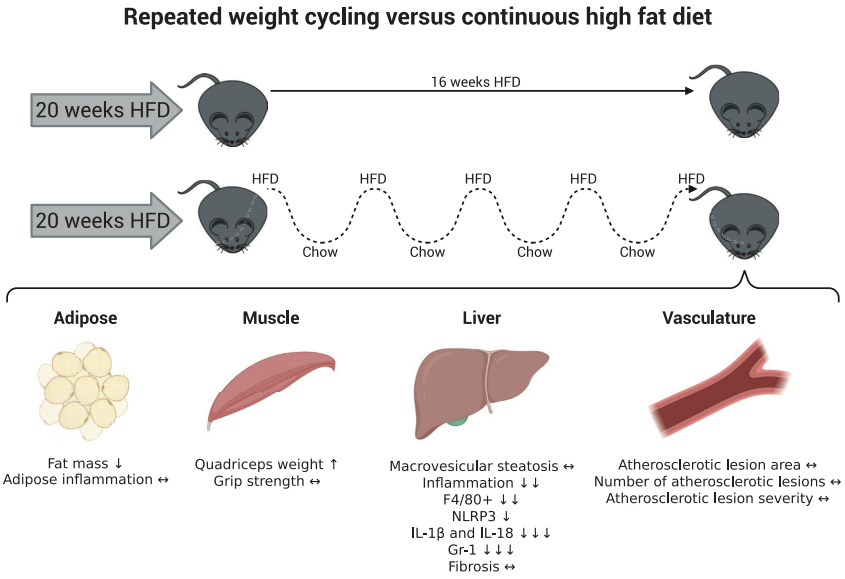
Abstract

Lifestyle interventions remain the treatment of choice for patients with obesity and metabolic complications, yet are difficult to maintain and often lead to cycles of weight loss and regain (weight cycling). Literature on weight cycling remains controversial and we therefore investigated the association between weight cycling and metabolic complications using preexistent obese mice. Ldlr-/-.Leiden mice received a high-fat diet (HFD) for 20 weeks to induce obesity. Subsequently, weight-cycled mice were switched between the healthy chow diet and HFD for four 2-week periods and compared to mice that received HFD for the total study period. Repeated weight cycling tended to decrease body weight and significantly reduced fat mass, whereas adipose tissue inflammation was similar relative to HFD controls. Weight cycling did not significantly affect blood glucose or plasma insulin levels yet significantly reduced plasma free fatty acid and alanine transaminase/aspartate transaminase levels. Hepatic macrovesicular steatosis was similar and microvesicular steatosis tended to be increased upon weight cycling. Weight cycling resulted in a robust decrease in hepatic inflammation compared to HFD controls while hepatic fibrosis and atherosclerosis development were not affected. These results argue against the postulate that repeated weight cycling leads to unfavorable metabolic effects, when compared to a continuous unhealthy lifestyle, and in fact revealed beneficial effects on hepatic inflammation, an important hallmark of non-alcoholic steatohepatitis.



Graphical abstract. Overview of the effects of repeated weight cycling versus continuous high-fat diet feeding on adipose tissue, muscle, liver, and vasculature.

1. Introduction

Obesity has reached epidemic proportions and with 2 billion overweight adults, worldwide prevalence has almost tripled since 1975[1]. Obesity is a major risk factor for diseases such as diabetes, cardiovascular diseases, dementia, and certain types of cancer[2]. Despite recent developments in FDA-approved drugs for weight management, the preferred therapy of choice remains implementation of lifestyle changes with the goal of losing weight to preclude development of metabolic complications. However, due to evolutionary pressure to store energy, lifestyle changes often prove difficult to maintain. Besides, weight that is lost during periods of dieting is often rapidly regained upon continuation of the unhealthy diet[3], resulting in weight cycling episodes, that is, yo-yo dieting.

Pertaining literature on weight cycling is nevertheless conflicting and consensus is lacking whether repeated weight cycling leads to unfavorable metabolic effects. For instance, some studies suggest that even partial weight regain may return metabolic risk factors to baseline values[4,5] and that body weight fluctuation increases the risk for cardiovascular disease and mortality[6]. Conversely, a study in patients with obesity and type 2 diabetes demonstrated that improvements in glycemic control persist after regaining initially lost weight[7]. The lack of a standardized definition of weight cycling allows for large discrepancies between studies, making it difficult to conclude the true effects of weight cycling and therefore necessitates a more systematic approach.

Animal studies can be most helpful since weight cycling can then be studied in a controlled manner by strictly regulating diet composition and food intake. Since patients with overweight and obesity are prone to yo-yo dieting[8], it is sensible to assess its effects in obese preclinical models. Nevertheless, most preclinical weight cycling studies used non-obese animal models from the start, which are per definition less suitable to draw conclusions from in the context of obesity. To the best of our knowledge, no studies have used an obese preclinical model that develops characteristics of non-alcoholic steatohepatitis (NASH), recently also rephrased as metabolic dysfunction-associated steatohepatitis (MASH), that is accompanied as well by cardiovascular disease[9]. Accordingly, we used the low-density lipoprotein receptor knockout Leiden (Ldlr-/-.Leiden) mouse model that develops obesity, hyperlipidemia, and insulin resistance in response to high-fat diet (HFD) feeding[9]. By subjecting these mice to cycles of healthy and unhealthy eating, we mimicked yo-yo dieting to assess the effects on several metabolic parameters, NASH, and atherosclerosis.

2. Materials and Methods

2.1 Experimental design

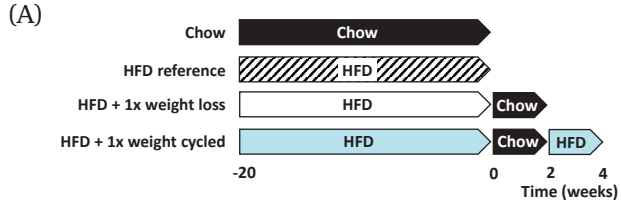
The study was approved by the governmental central committee on animal experiments (AVD5010020172064) and independent Animal Welfare Body of TNO (IVD TNO; TNO-465). Ldlr-/-.Leiden mice were bred and housed at the AAALAC-accredited SPF animal facility at TNO (TNO Metabolic Health Research, Leiden, the Netherlands). Ldlr-/-.Leiden mice were selected since they develop obesity and associated metabolic complications when fed an HFD without requiring extra dietary cholesterol supplementation. Male mice were chosen because of their increased susceptibility to developing obesity and inflammation compared with female mice[10].

Mice (16–20 weeks old) were group housed in a temperature-controlled room at 50%–60% humidity on a 12 h light–dark cycle. Mice had free access to heat-sterilized water and food. Body weight, food intake per cage, and clinical signs were monitored regularly. After a run-in period of 20 weeks on HFD containing 45 kCal% fat from lard, 35 kCal% from carbohydrates, and 20 kCal% casein (Research Diets, New Brunswick, NJ, USA) to induce obesity, mice were matched on body weight, lean body mass, 5-h-fasted blood glucose, plasma cholesterol, and triglycerides into groups of $n = 15$ mice each. A group that received the grain-based chow diet (Ssniff Spezialdiäten, Soest, Germany) was included as healthy reference. Appropriate group sizes were calculated a priori by power analysis (GPower), using a minimal effect size of 30% and two-sided test with 95% confidence interval, power of 90%, and α of 0.05.

The study was separated into two experiments, where we first evaluated the effects of one weight cycle and next that of repeated weight cycling. For the first experiment, two groups on either chow or HFD were sacrificed after the 20-week run-in period ($t = 0$) and served as healthy reference (chow) or HFD reference (Figure 1A). A third group was sacrificed after receiving chow for 2 weeks following the run-in period on HFD (HFD + 1x weight loss). The last group received HFD for the 20-week run-in period, followed by 2 weeks on chow and 2 additional weeks on HFD (HFD + 1x weight-cycled group).

For the second experiment, a healthy control group received chow for 16 additional weeks after the initial 20-week run-in period on chow (Figure 2A). The initial HFD group sacrificed after the 20-week run-in period in the first experiment served as HFD reference group for the second experiment too. Comparison with this HFD reference group gave an indication of whether repeated weight cycling modified parameters beyond levels at the start of the intervention period. Another group received HFD feeding for 16 additional weeks beyond the run-in period as age-matched HFD control group. The final group received HFD for the 20-week run-in period, followed by four alternating 2-week intervals on chow or HFD, aiming for a weight loss of approximately 10%, translational for human weight cycling[11]. At the study

(A)



(B)

	Chow	HFD reference	HFD + 1x weight loss	HFD + 1x weight cycled
Body weight (g)	35,4 ± 0.9***	49,6 ± 2,0	45,4 ± 1.1^{T}	49,8 ± 1,4
Blood glucose (mM)	7,5 ± 0,3	8,0 ± 0,3	6,0 ± 0.2***	6,8 ± 0.2**
Plasma insulin (ng/mL)	1,8 ± 0.2***	16,6 ± 2,3	4,9 ± 0.8***	9,1 ± 1.1**
Plasma cholesterol (mM)	8,0 ± 0.5***	40,1 ± 4,2	17,2 ± 1.2***	29,4 ± 3.7*
Fat mass (g)	7,6 ± 0.6***	21,4 ± 1,4	15,6 ± 1.2**	18,9 ± 1,3
Lean mass (g)	26,8 ± 0.4*	29,4 ± 0,8	30,4 ± 0,8	30,8 ± 0,7
WAT inflammation (#)	1,1 ± 0.4***	38,1 ± 5,8	37,8 ± 5,8	18,5 ± 2.8*
Hepatic steatosis (%)	3,1 ± 1.7***	54,5 ± 4,9	41,2 ± 3.0**	43,2 ± 4,8
Hepatic inflammation (%)	0,2 ± 0.1***	19,4 ± 4,1	3,3 ± 0.3**	9,1 ± 2.8*
Hepatic fibrosis (%)	0,8 ± 0.1*	2,0 ± 0,5	1,5 ± 0,2	3,0 ± 1,00

Figure 1. Weight cycling once improves several metabolic characteristics in obese Ldlr-/-. Leiden mice. Experimental timeline for weight cycling once (A). Two groups of n=15 Ldlr-/-. Leiden mice each received healthy chow (chow group; n=15) or high-fat diet (HFD reference; n=15) for 20 weeks. A third group was kept on HFD for the 20-week run-in period, followed by 2 weeks on chow (HFD+1x weight loss; n=15). The fourth group was fed HFD for 20 weeks, followed by 2 weeks on chow and another 2 weeks on HFD (HFD+1x weight-cycled; n=15). Metabolic parameters at the study endpoint (B). Hepatic steatosis and fibrosis are expressed as percentage of surface area, and hepatic inflammation is expressed as the number of inflammatory cell aggregates per mm^2. WAT, white adipose tissue. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, T: tendency (p<0.10) versus HFD reference. #p<0.05, ##p<0.01, ###p<0.001 HFD+1x weight loss versus HFD+1x weight cycled.

endpoint, animals were sacrificed by gradual-fill CO_2 asphyxiation and organs were collected and weighed.

2.2 Biochemical analyses in plasma and liver

Throughout the study, blood was drawn regularly from the tail vein after 5 h fasting into EDTA-coated tubes (Sarstedt, Nümbrecht, Germany). Fat and lean mass were determined using an NMR Echo MRI whole-body composition analyzer (EchoMRI 2-in-1, Echo Medical Systems LTD, Houston, TX, USA). Four paw grip strength was determined using a grip force meter (TSE Systems GmbH, Bad Homburg, Germany) by placing mice on a grid attached to a force gauge and steadily pulling the mouse by the tail. Grip strength was defined as the maximum strength produced before releasing the grid. Per mouse, five trials were performed with 1-min resting intervals. Lowest

and highest values were excluded and averages of the three remaining trials were taken and expressed per gram body weight. At sacrifice, livers, hearts, perigonadal white adipose tissue (WAT), visceral WAT, and quadriceps femoris were collected, weighed, formalin fixed, and paraffin embedded for histological analysis or snap-frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

Fasted blood glucose levels were determined at the time of blood sampling by using glucose strips and glucose hand analyzer (FreeStyle Lite, Abbott, Chicago, IL, USA). Plasma insulin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) (#90080; Crystal Chem, Elk Grove Village, IL, USA). Enzymatic colorimetric assays (Roche Diagnostics, Almere, the Netherlands) were used to determine plasma concentrations of cholesterol and triglycerides. Free fatty acids were measured in plasma treated with orlistat (1 mg/L; Sigma-Aldrich, St. Louis, MO, USA) and quantified with the NEFA-HR kit (Instruchemie, Delfzijl, the Netherlands). Plasma concentrations of alanine transaminase (ALT) and aspartate transaminase (AST) were established by reflectance photometry with a Reflotron® Plus analyzer (Hoffman-La Roche, Mannheim, Germany). ELISAs were used to determine hepatic concentrations of IL-1β (ab197742; Abcam, Cambridge, UK) and IL-18 (ab216165; Abcam). For this, tissue of the left liver lobe was homogenized in lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl (*v/v*), 5 mM $CaCl_2$ (*v/v*), 1% Triton X-100, and cOmplete™ mini protease inhibitor cocktail (Roche Diagnostics), centrifuged for 30 min at 4°C, 16 000*g*, and of which the supernatant was used for analysis.

2.3 Histological assessment of NASH

Formalin-fixed tissue of the left liver lobe that was paraffin embedded was cross-sectioned (3 µm) and stained with hematoxylin and eosin (H&E) or Sirius Red (SR). Sections were scored blindly for NASH by a board-certified pathologist by examining two liver slides per mouse and using an adapted grading system of human NASH[12,13]. Microvesicular and macrovesicular steatosis were determined at 40–100× magnification and expressed as percentage relative to the total liver area analyzed. Liver inflammation was determined at 100× magnification by counting the number of inflammatory foci per field (view size 4.2 mm^2). For scoring of hepatic steatosis and inflammation, average scores of five random, non-overlapping fields were taken and expressed per mm^2. For evaluation of hepatic fibrosis, two SR-stained liver cross-sections per mouse were evaluated by computerized image analysis of collagen content expressed as percentage of liver surface area. The board-certified pathologist identified fibrosis stage in two cross-sections per mouse using the protocol described by Tiniakos et al.[14]. In accordance with this protocol, F0 indicates absence of fibrosis, F1 indicates fibrosis in the perisinusoidal or periportal area, F2 indicates fibrosis within both perisinusoidal and periportal areas, F3 indicates bridging fibrosis, and F4 indicates cirrhosis.

For immunohistochemical assessment of inflammation, tissue of the left liver lobe was embedded in paraffin and cross-sectioned (4 µm). After deparaffinization and endogenous peroxidase blockage with 0.03% H_2O_2 in methanol for 20 min and subsequent rehydration, epitope retrieval was performed at 97°C for 20 min at pH 6.0 using the Dako PT Link (Agilent Technologies, Amstelveen, the Netherlands). Sections were blocked in 5% normal goat serum (NGS) in PBS and incubated overnight at 4°C with primary antibodies in 1% NGS in PBS. After washing with 0.05% (v/v) Tween-20 in PBS, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies in 1% NGS in PBS for 1 h at room temperature. For F4/80 staining, a rat monoclonal antibody (#14-4801; eBioscience (ThermoFisher), Landsmeer, the Netherlands) and goat anti-rat secondary antibody (ab7097, 1:200; Abcam) were used. HRP activity was visualized with 3,3′-diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA, USA), and slides were dehydrated and counterstained with hematoxylin (Sigma-Aldrich). The board-certified pathologist evaluated F4/80-positive crown-like structures (CLS) in five non-overlapping fields at 100× magnification. For NLRP3 staining, a rabbit polyclonal antibody (#PA5-79740, 1:400 v/v; Invitrogen, Waltham, MA, USA) and goat anti-rabbit secondary antibody (ab205718, 1:1000 v/v; Abcam, Cambridge, UK) were used. For Gr-1 staining, a rat Ly-6G FITC-coupled primary antibody (RB6-8C4, 1:1000 in PBS; Invitrogen) was used, after which slides were incubated with a rabbit anti-FITC antibody (#701078, 1:1000 in PBS; Invitrogen). Subsequently, slides were incubated with a goat anti-rabbit poly-HRP antibody (Brightvision poly-HRP, 1:1 in PBS; Immunologic, Duiven, the Netherlands) and HRP activity was visualized using NovaRED Substrate Kit (SK-4805; Vector Laboratories) and counterstained with hematoxylin. Slides were mounted with the Leica CV5030 Fully Automated Glass Coverslipper (Leica Biosystems), after which imaging was done using the Aperio AT2 imaging system (Leica Biosystems) at 20× magnification. ImageJ (version 1.48, NIH, Bethesda, MD, USA) with customized macros was used for quantification, where two cross-sections per mouse were evaluated and positively stained area was determined in five non-overlapping fields (view size 1.0 mm^2).

2.4 Histological assessment of atherosclerosis

Features of atherosclerosis were histologically assessed in line with previously described protocols[15–17]. In short, hearts were formalin fixed, paraffin embedded, and sectioned perpendicular to the aorta axis. Cross-sections of 5 µm cut at 50 µm intervals were stained with hematoxylin–phloxine–saffron (HPS) and scanned with an Aperio AT2 slide scanner (Leica Biosystems, Amsterdam, the Netherlands). Per mouse, four sections were assessed for atherosclerotic lesions, and total lesion area per cross-section was calculated. Lesion severity was determined in accordance with the American Heart Association classification, where I indicates early fatty streak, II indicates regular fatty streak, III indicates mild plaque, IV indicates moderate plaque, and V indicates severe plaque[15,17].

2.5 Histological assessment of adipose tissue pathology

Formalin-fixed and paraffin-embedded perigonadal WAT was cut into 5 µm sections and HPS stained. Adipose tissue morphometry was analyzed with Adiposoft[18], an open-source plugin for the image processing package Fiji[19] for ImageJ[20]. The number of CLS was determined as measure of WAT inflammation and expressed as number of CLS per 1000 adipocytes.

2.6 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Differences between groups were determined non-parametrically by Kruskal–Wallis testing followed by Mann–Whitney U testing for independent samples. Statistical analyses were performed using SPSS software (version 25, ICM Corp., Armonk, NY, USA). Two-tailed *p*-values are reported and a p-value<0.05 was considered statistically significant.

3. Results

3.1 Improvements in metabolic parameters and hepatic inflammation are partially maintained after regaining initially lost weight

The effect of weight cycling once was first investigated. To this end, Ldlr-/-.Leiden mice were first fed a high-fat diet (HFD) for 20 weeks to induce obesity and were then switched to healthy chow for 2 weeks and subsequently switched back to HFD for 2 additional weeks (Figure 1A). Twenty weeks on HFD induced obesity (body weight + 40%, HFD reference vs. chow) and while 2 weeks on chow diet tended to decrease body weight (-8%, HFD + 1x weight loss vs. HFD reference), body weight returned to baseline levels when resuming the HFD (Figure 1B). After regaining initially lost weight, blood glucose concentrations remained significantly lower (-15%, HFD + 1x weight cycled vs. HFD reference), reaching concentrations even lower than those of chow-fed mice. The 20-week run-in period on HFD induced severe hyperinsulinemia (8.1-fold increase, HFD reference vs. chow), while losing weight once significantly reduced these levels by 70% (HFD + 1x weight loss vs. HFD reference). This HFD-induced hyperinsulinemia remained significantly lower in once weight-cycled mice (-45%, vs. HFD reference). Similarly, plasma cholesterol remained significantly lowered in once weight-cycled mice (-27%, vs. HFD reference). Plasma concentrations of triglycerides, ALT, and AST were unaltered in once weight-cycled mice compared to HFD reference mice (Figure S1). Likewise, improvements in fat and lean mass attributable to losing weight once were negated after resuming the HFD (Figure 1B). Similar patterns were observed for individual weights of different WAT depots (Figure S1). Interestingly, although changes in WAT inflammation were not

observed between HFD reference mice and mice that lost weight once, WAT inflammation was significantly reduced in once weight-cycled mice, both compared to HFD reference mice (-52%) and HFD+1x weight loss group (-51%) (Figure 1B), suggesting a delayed response on WAT inflammation on the initial weight loss. In the liver, losing weight once immediately reduced steatosis, yet this reduction was not maintained when mice returned to the HFD. In contrast, hepatic inflammation was significantly reduced in obese mice that lost weight once (-83%, vs. HFD reference), an improvement that partially persisted in once weight-cycled mice (-53%, vs. HFD reference). Microscopical analysis of hepatic fibrosis revealed no changes in mice that lost weight or were weight-cycled once (Figures 1B and S1). These data demonstrate that 20 weeks of HFD feeding induced severe metabolic complications in the Ldlr-/-.Leiden mouse model. Moreover, a single and modest weight loss episode immediately improved some of these complications, an improvement that was partially maintained after a single weight gain and return to baseline levels.

3.2 Repeated weight cycling improves metabolic parameters in obese Ldlr-/-.Leiden mice

In humans, the pattern of dieting followed by a period of weight regain often occurs repeatedly and therefore the effects of repeated yo-yo dieting were investigated in obese Ldlr-/-.Leiden mice by subjecting them to alternating 2-week periods of chow and HFD feeding (Figure 2A). As intended, body weight tended to decrease during periods of chow feeding and increased again during HFD feeding (Figure 2B). These patterns could primarily be attributed to food intake variability, with significantly lower caloric intake on chow and significantly higher caloric intake on HFD, also relative to continuously HFD-fed mice (Figure 2C). Although repeatedly weight-cycled mice showed significantly decreased blood glucose levels relative to mice sacrificed at the start of intervention (HFD reference: -13%), this reduction did not persist compared to mice that received HFD for the complete study period (36 weeks) (Figure 2D). Insulin levels were 6.6-fold higher in HFD controls compared to chow-fed mice and did not decrease significantly in repeatedly weight-cycled mice (Figure 2E). Conversely, HFD-induced increases in plasma cholesterol and triglyceride concentrations (3.3-fold and 4.5-fold increase, respectively, vs. chow) were significantly improved in weight-cycled mice (cholesterol: -29%; triglycerides: -25%, vs. HFD control) (Figure 2F,G). Compared to chow controls, plasma free fatty acid levels were increased significantly in HFD reference (+74%) and HFD control mice (2.2-fold increase) and repeated weight cycling significantly lowered these levels (-38%, vs. HFD control) (Figure 2H).

Liver weight and plasma ALT and AST levels were determined at the study endpoint. Sixteen additional weeks of HFD feeding resulted in a 60% increase in liver weight (vs. HFD reference), an increase not perceived in repeatedly weight-cycled

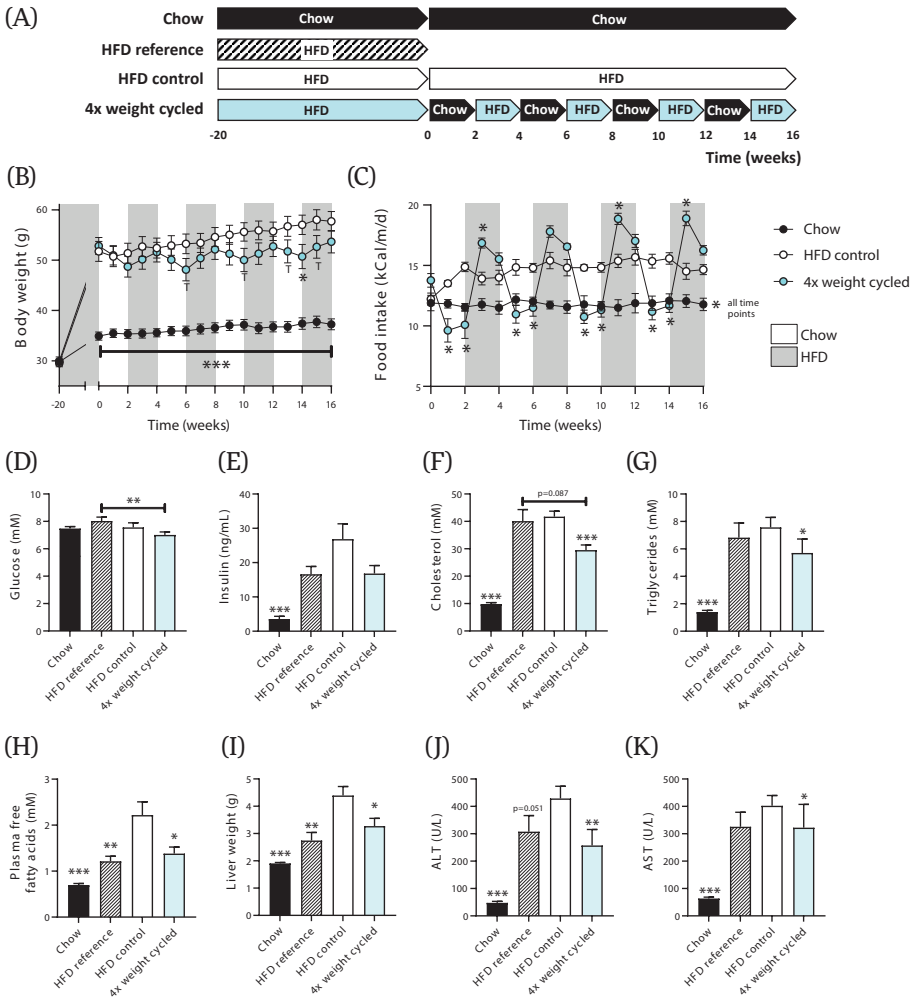


Figure 2. Repeated weight cycling improves metabolic parameters in the obese Ldlr-/-. Leiden mouse model. Experimental timeline for repeated weight cycling (A). One group of Ldlr-/-.Leiden mice received healthy chow diet for a total of 36 weeks (chow group; n=15). A second group received high-fat diet (HFD) and was sacrificed after a 20-week run-in period (HFD reference; n=15). A third group was fed the HFD for a total of 36 weeks (HFD control; n=15). The fourth group was fed HFD for 20 weeks, after which yo-yo dieting was initiated by alternating 2 weeks of healthy chow with 2 weeks of HFD, a pattern that was repeated four times until the study endpoint (4×weight-cycled group; n=15). Body weight (B), food intake (C), blood glucose (D), plasma insulin (E), plasma cholesterol (F), plasma triglycerides (G), plasma free fatty acids (H), liver weight (I), plasma alanine transaminase (ALT) (J), and plasma aspartate transaminase (AST) (K) were determined throughout the study or at study endpoint (t=16 weeks). Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, T: tendency (p<0.10) versus HFD control.

mice (-26%, vs. HFD control) (Figure 2I). Thirty-six weeks on HFD induced significant increases in plasma ALT (8.0-fold increase) and AST (5.3-fold increase) compared to chow controls (Figure 2J,K). Relative to the HFD reference group, an additional 16 weeks on HFD therefore resulted in a borderline significant increase in ALT (+39%, p=0.051) yet did not further aggravate AST concentrations. Repeated weight cycling improved both ALT (-40%) and AST (-20%) relative to HFD controls. Overall, these data indicate that repeated weight cycling has beneficial effects on metabolic parameters and is therefore preferred over continuous exposure to an unhealthy diet.

3.3 Four times weight cycling decreases total fat mass and adipose tissue inflammation

Subsequently, effects of repeated weight cycling on body composition were evaluated. Compared to HFD reference mice, 16 additional weeks on HFD tended to increase fat mass even further (+16%, p=0.08) and significantly increased lean mass as well (+11%) (Figure 3B). In weight-cycled mice, lean mass was not affected, whereas fat mass was significantly reduced compared to mice that continuously received HFD (-15%). The total weight of perigonadal WAT depots tended to decrease with 16 additional weeks on HFD compared to HFD reference mice (-11%, p=0.09) and was not affected in repeatedly weight-cycled mice (Figure 3C). Visceral WAT weights were similar in HFD reference, HFD control, and four times weight-cycled mice (Figure 3C). More detailed histological analysis of perigonadal WAT (Figure 3A) revealed that adipocyte size and WAT inflammation remained stable between mice that received HFD feeding for 20 and 36 weeks (Figure 3D,E). Weight-cycled mice had a borderline significant increase in adipocyte size compared to HFD controls (+10%, p=0.0502). Relative to HFD reference mice, WAT inflammation was significantly reduced in repeatedly weight-cycled mice (-57%), yet this difference was not observed relative to HFD controls.

Weight cycling has been postulated to have detrimental effects on muscle mass and function, which were investigated here as well. After 36 weeks on HFD, relative mass of the quadriceps femoris muscle had declined by -37% (HFD control vs. chow) and an additional -14% compared to the HFD reference group that was sacrificed 16 weeks prior (Figure 3F). This decline in relative muscle mass was ameliorated in repeatedly weight-cycled mice compared to HFD controls (+11%). Muscle functionality as measured by grip strength was worsened in HFD-fed mice (-18% for HFD reference and -38% for HFD control, vs. chow) (Figure 3G). The significantly higher muscle mass for repeatedly weight-cycled mice versus HFD controls did not translate into increased muscle functionality, since this group showed reduced normalized grip strength compared to HFD reference mice (-18%), rendering repeated weight-cycled mice at comparable normalized grip strength levels as HFD control mice.

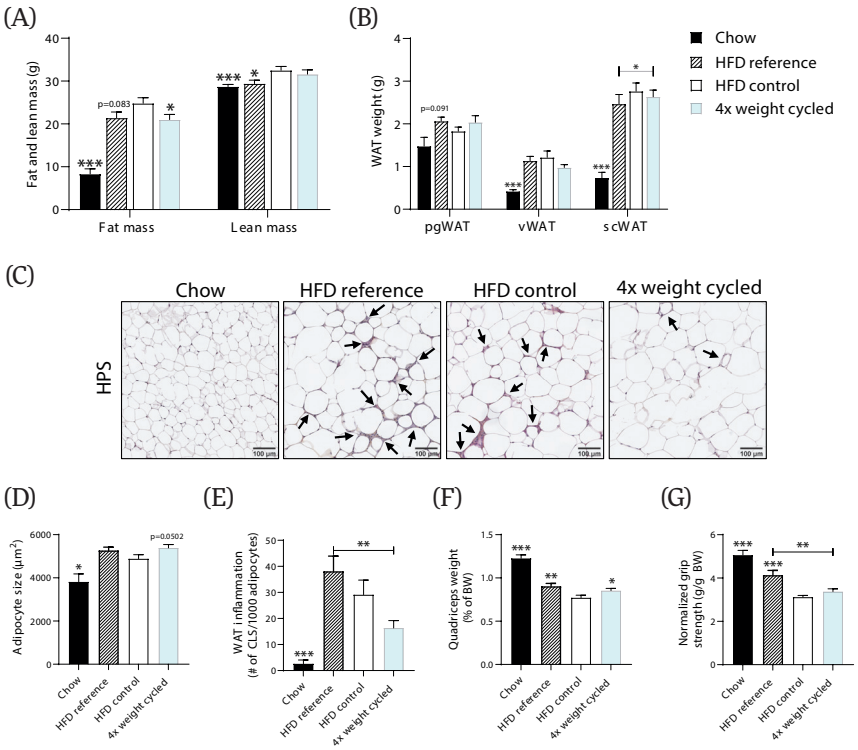


Figure 3. Four times weight cycling decreases total fat mass and improves muscle weight. Fat and lean mass expressed as percentage of body weight (BW) (A), weights of perigonadal (pg), visceral (v), and subcutaneous (sc) white adipose tissue (WAT) (B), representative hematoxylin–phloxine–saffron (HPS)-stained images of pgWAT with arrows indicating crown-like structures (CLS) (C), adipocyte size (D), WAT inflammation (E), quadriceps weight expressed as percentage of body weight (F), and normalized grip strength (G) were all determined at study endpoint (t=16 weeks). Values are presented as mean ± SEM for n=15 mice on chow diet, n=15 mice on high-fat diet (HFD) sacrificed at t=0 weeks (HFD reference), n=15 on HFD sacrificed at t=16 weeks (HFD control), and n=15 on HFD for a 20-week run-in period followed by alternated 2-week periods of chow or HFD feeding (4×weight-cycled group). *p<0.05, **p<0.01, ***p<0.001 versus HFD control.

3.4 Four times weight cycling does not affect hepatic steatosis and fibrosis yet strongly improves hepatic inflammation

Hepatic complications are common in patients with metabolic syndrome and therefore effects of repeated weight cycling on parameters of NASH were evaluated. Histological examination of liver tissue revealed that HFD feeding induced severe macrovesicular and microvesicular steatosis (Figure 4A-C). Repeated weight cycling did not affect macrovesicular steatosis, and microvesicular steatosis tended to be

increased in comparison to HFD controls (+42%, p=0.08). In chow-fed mice, hepatic inflammation quantified by counting aggregated inflammatory cells in liver cross-sections was not detected while these aggregates were abundantly present in mice that received HFD for either 20 or 36 weeks (Figure 4D). Subsequent evaluation of the presence of macrophages in the liver was performed by histological identification of F4/80-positive crown-like structures. There was no positive F4/80 staining in livers of chow-fed mice and abundant staining in HFD reference and HFD control mice, indicating significant macrophage infiltration in livers of these mice (Figure 4A,E). Remarkably, four times weight cycling significantly ameliorated hepatic inflammation, with extensive reductions in the number of inflammatory cell aggregates (-67% vs. HFD control) and F4/80-positive crown-like structures (-75% vs. HFD control). Furthermore, SR-stained liver tissue was analyzed for fibrosis, and 20 weeks on HFD was observed to induce some fibrosis that was aggravated in the 16 weeks thereafter (2.6-fold increase, HFD control vs. chow) (Figure 4A,E). Correspondingly, fibrosis stage deteriorated significantly in mice that received HFD for the total study duration (2.4-fold increase, HFD control vs. chow) (Figure 4G). Four times weight cycling did not affect hepatic fibrotic surface area nor stage, rendering these mice at a similar fibrosis stage as HFD control mice, with fibrosis predominantly in perisinusoidal and periportal areas.

3.5 Repeated weight cycling reduces NLRP3 inflammasome pathway activity and neutrophil infiltration

Repeated weight cycling vastly reduced hepatic inflammation and therefore further investigation of these anti-inflammatory effects was performed. Free fatty acids can serve as triggers for NLRP3 inflammasome pathway activation[21], causing the downstream production of pro-inflammatory cytokines IL-1β and IL-18[22]. Here, HFD controls displayed significantly increased levels of free fatty acids (Figure 2H) and consequently showed significantly increased NLRP3 protein expression (Figure 5A,B). Accordingly, levels of the downstream effectors IL-1β and IL-18 were significantly increased as well (IL-1β: 6.0-fold increase; IL-18: 2.3-fold increase, HFD control vs. chow) (Figure 5C,D). Four times weight cycling had favorable effects on plasma free fatty acid levels relative to HFD controls (-38%) and NLRP3 protein expression was reduced as well, both relative to HFD reference mice (-32%) and HFD controls (-33%). As a result, downstream in the NLRP3 inflammasome pathway, repeated weight cycling almost completely abolished the rise in IL-1β protein levels (-97%) and significantly reduced IL-18 protein levels (-37%) compared to HFD controls.

Since neutrophils drive non-alcoholic fatty liver disease (NAFLD) progression into NASH[23], expression of the neutrophil marker Gr-1 was evaluated. HFD feeding induced a significant increase in neutrophil infiltration after 20 weeks which was maintained with 16 additional weeks on this diet (3.6-fold increase, vs. chow) (Figure

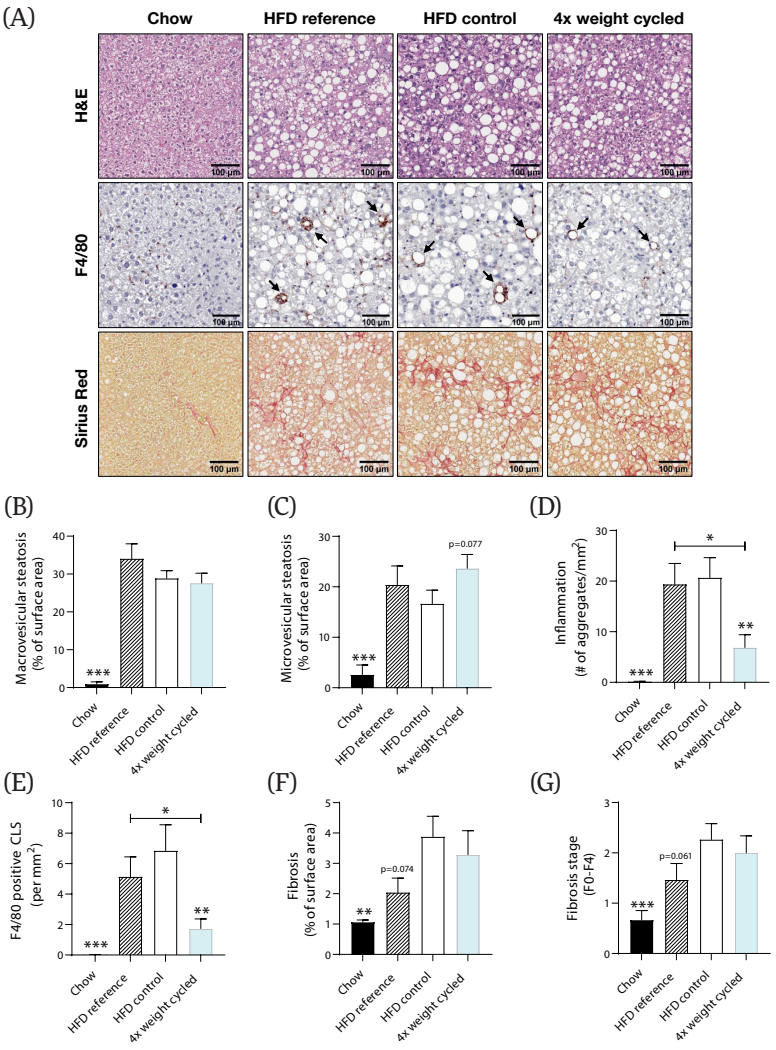


Figure 4. Repeated weight cycling has beneficial effects on hepatic inflammation but does not affect hepatic steatosis or fibrosis. Representative images of liver cross-sections stained for hematoxylin and eosin (H&E), F4/80 with arrows indicating positive staining of crown-like structures (CLS) and Sirius Red (A). Macrovesicular steatosis (B) and microvesicular steatosis (C) as percentage of surface area, number of inflammatory cell aggregates per mm^2 microscopic field (D), F4/80-positive CLS (E), fibrosis as percentage of surface area (F), and fibrosis stage (F0-F4) (G) were determined at the study endpoint. Values are presented as mean ± SEM for n=15 mice on chow diet, n=15 mice on high-fat diet (HFD) sacrificed at t=0 weeks (HFD reference), n=15 on HFD sacrificed at t=16 weeks (HFD control), and n=15 on HFD for a 20-week run-in period followed by alternated 2-week periods of chow or HFD feeding (4×weight-cycled group). *p<0.05, **p<0.01, ***p<0.001 versus HFD control.

5A,E). Gr-1 immunoreactivity was significantly reduced in repeatedly weight-cycled mice (-61%, vs. HFD control) to levels more comparable to those of chow-fed mice.

3.6 Repeated weight cycling does not affect severe atherosclerosis

Given their genetic background, Ldlr-/-.Leiden mice are known to already develop atherosclerosis during aging[9]. Here, chow-fed mice indeed developed atherosclerotic lesions that were severely aggravated by HFD feeding (Figure 6A). Elevated cholesterol levels are highly associated with development of cardiovascular disease and although cholesterol concentrations at the study endpoint were significantly lower in repeatedly weight-cycled mice compared to HFD controls (Figure 2F), the total cholesterol exposure (concentration*weeks) during the whole study was similar in these two groups (Figure 6B). Accordingly, atherosclerotic lesion area was significantly increased

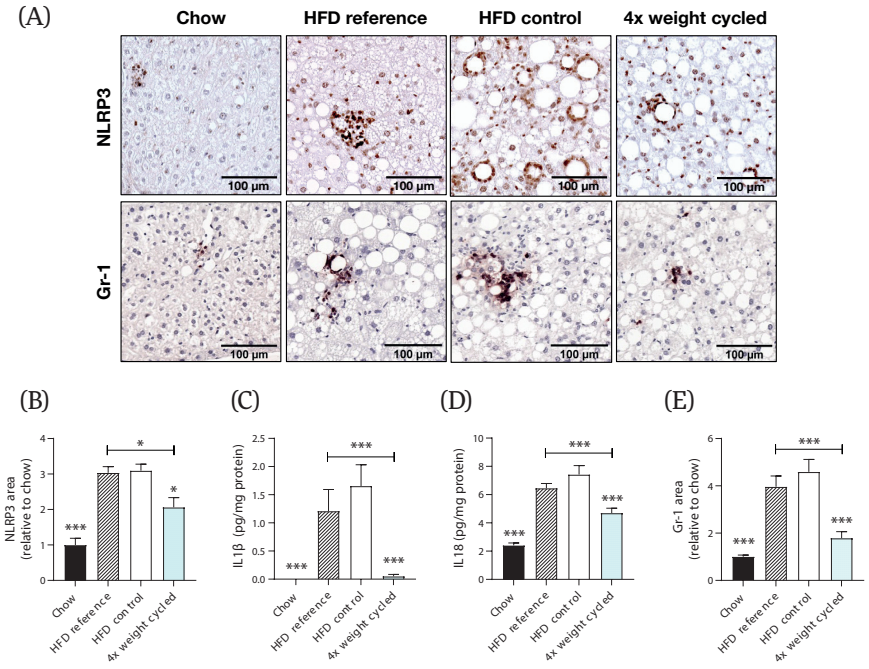


Figure 5. Four times weight cycling reduces NLRP3 inflammasome pathway activity and neutrophil infiltration. Representative images of liver cross-sections stained for NLRP3 and GR-1 (A). NLRP3-positive area relative to chow (B), hepatic IL-1β (C), and hepatic IL-18 (D), and Gr-1-positive area relative to chow (E) were determined at the study endpoint. Values are presented as mean ± SEM for n=15 mice on chow diet, n=15 mice on high-fat diet (HFD) sacrificed at t=0 weeks (HFD reference), n=15 on HFD sacrificed at t=16 weeks (HFD control), and n=15 on HFD for a 20-week run-in period followed by alternated 2-week periods of chow or HFD feeding (4×weight-cycled group). *p<0.05, **p<0.01, ***p<0.001 versus HFD control.

in the HFD reference group and aggravated even further in HFD controls (+95%, vs. chow) and four times weight cycling did not affect this (Figure 6C). The total number of atherosclerotic lesions was similar for all groups (Figure 6D) and no significant differences were detected for the percentage of undiseased segments either (Figure 6E).

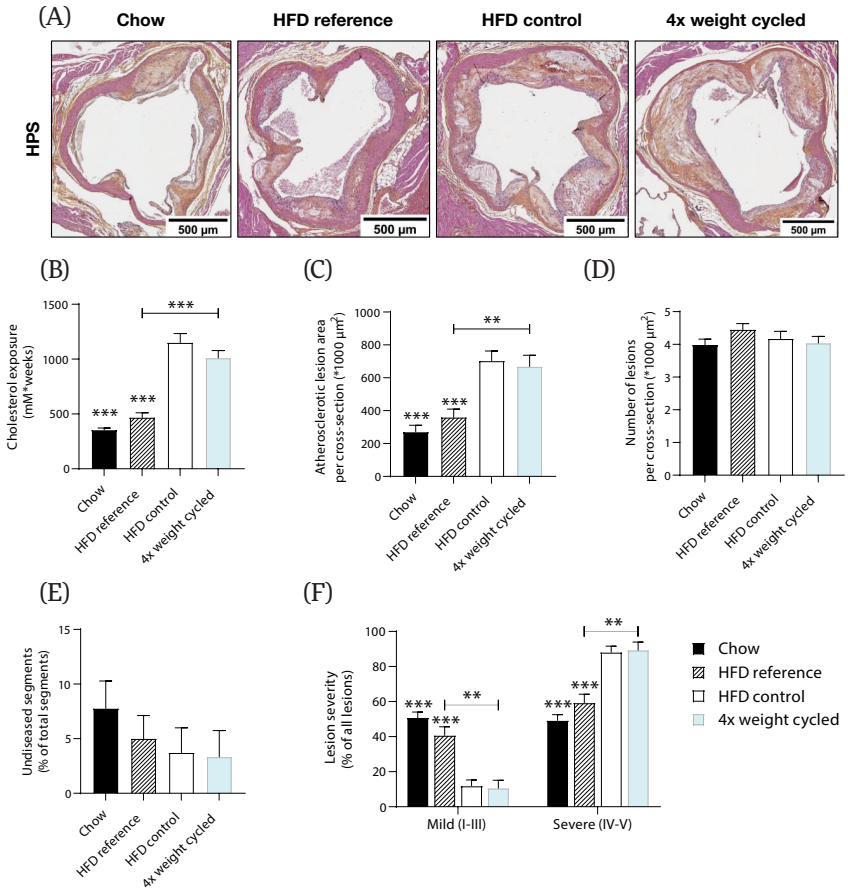


Figure 6. Atherosclerosis parameters are not affected in repeatedly weight-cycled mice. Representative images of hematoxylin–phloxine–saffron (HPS)-stained cross-sections of the aortic root area (A). Cholesterol exposure (concentration*weeks) (B), atherosclerotic lesion area per cross-section (C), number of atherosclerotic lesions per cross-section (D), percentage of undiseased segments (E), and atherosclerotic lesion severity as percentage of all lesions (F) were determined at the study endpoint. Values are presented as mean ± SEM for n=15 mice on chow diet, n=15 mice on high-fat diet (HFD) sacrificed at t=0 weeks (HFD reference), n=15 on HFD sacrificed at t=16 weeks (HFD control), and n=15 on HFD for a 20-week run-in period followed by alternated 2-week periods of chow or HFD feeding (4×weight-cycled group). **p<0.01, ***p<0.001 versus HFD control.

Regarding atherosclerotic lesion severity, prolonged exposure to the HFD had detrimental effects, with mice displaying significantly less mild (type I-III) lesions (-71%) and significantly more severe (type IV-V) lesions (+49%) in HFD controls versus HFD reference mice (Figure 6F). Four times weight cycling did not affect severity of atherosclerotic plaques.

4. Discussion

The aim of this study was to investigate the metabolic consequences of weight cycling using an initially obese mouse model prone to developing metabolic complications, NASH, and atherosclerosis. Here, we demonstrate that weight cycling was not harmful and in fact improved several metabolic parameters and hepatic inflammation compared with continuous exposure to an unhealthy diet.

The lack of a standardized definition for weight cycling makes it challenging to compare clinical studies. For instance, weight cycling may be established by determining body weight variability between various intervals, yet lengths of these intervals as well as the number of dietary cycles vary widely. Besides, weight cycling may encompass smaller or larger magnitudes of weight loss and regain. Likewise, yo-yo dieting does not limit itself to patients with overweight and obesity, yet is seen in a wider range of the general population. This heterogeneity is translated to the experimental design of preclinical studies. In some preclinical studies, caloric intake is restricted while others switch animals between diets with different macronutrient contents or use a combination of both approaches. Surprisingly, however, many preclinical weight cycling studies used a study design where weight cycling was initiated in lean animals from baseline onward, without including a run-in period on an energy-dense diet to allow for development of obesity. This complicates extrapolation to a patient population with obesity where yo-yo dieting is commonly observed, which makes the current study using obese Ldlr-/-.Leiden mice unique and more translational. These mice are genetically predisposed to develop hyperlipidemia with their cholesterol primarily confined to LDL particles, contrary to wild-type mice that contain their cholesterol primarily in HDL particles. To mimic yo-yo dieting, obese Ldlr-/-.Leiden mice were switched between healthy chow and HFD, an approach that is representative of yo-yo dieting in human patients with obesity, where periods of dieting with the goal of losing weight are alternated with relapses into unhealthy habits. We first evaluated if a single weight cycling episode affected metabolic, adipose, and hepatic parameters. However, since yo-yo dieting often occurs in repeated patterns, we evaluated the effects of repeated weight cycling as well.

Twenty weeks on HFD induced pronounced obesity and elevated glucose, insulin, and cholesterol concentrations in the Ldlr-/-.Leiden mouse model. Switching these mice to healthy chow tended to induce weight loss, attributed to a significant reduction in fat mass. This weight was regained again after returning to HFD, a phenomenon that has been described in other studies on weight cycling once as well[24–26]. Several rodent studies that have reported on the effects of weight cycling once demonstrate no effects on glucose levels[24–28] while effects on insulin concentrations are more conflicting, with some studies reporting an increase[25] whereas others report a decrease[24] or no effects at all[26–28]. In their preclinical study, Winn et al. report weight-cycling-induced glucose intolerance that could be attributed to inadequate insulin secretion resulting from loss of pancreatic plasticity[29]. It is, however, noteworthy that these studies have all been carried out in wild-type mouse models that lack the hyperinsulinemic and hyperlipidemic characteristics seen in patients with metabolic syndrome and that are recapitulated in the HFD-fed Ldlr-/-.Leiden model. Here, weight loss reduced both glucose and insulin levels and although weight regain increased levels of both parameters, they remained significantly lower compared to baseline levels. Consistent with other preclinical studies, weight cycling once did not affect plasma concentrations of hepatic function markers ALT and AST[26,27]. Subsequent microscopical investigation of the liver revealed no effects of weight cycling once on hepatic steatosis and fibrosis, which is in line with other preclinical studies[26,27]. Interestingly, livers of once weight-cycled mice were significantly less inflamed, and similar improvements were observed in the white adipose tissue. In short, a single weight loss already strongly improved parameters of metabolic health. While returning to the HFD partially reversed some of these improvements, others remained enhanced, suggesting that weight cycling once already has certain beneficial effects on some metabolic parameters and inflammation.

Episodes of yo-yo dieting often occur in repeated patterns and therefore we next explored the effects of repeated weight cycling in obese Ldlr-/-.Leiden mice. With every 2-week period on healthy chow, mice lost 4%–8% body weight, levels translational to dietary weight loss in patients with obesity[11]. During periods of weight loss, weight-cycled mice had significantly lower food intake compared to mice that continuously received HFD and body weight decreased accordingly. Conversely, returning from chow to HFD increased food intake, significantly exceeding levels observed in mice that received HFD continuously, while body weight remained comparable. These data are in line with what is often seen in humans, where patients may relapse into unhealthy habits and tend to overindulge following periods of weight loss[30]. Regarding body composition, conflicting data are reported in the literature, with some studies showing that repeated weight cycling reduces fat mass[31], while others report no changes[32]. In weight-cycled Ldlr-/-.Leiden mice, reductions in body weight could be attributed to a 15% reduction in fat mass while lean mass

remained stable. Little is known about the effects of weight cycling on muscle mass of patients with obesity. One study in patients with obesity revealed an association between more frequent weight cycling and lower muscle mass and strength[33]. Here, weight cycling improved quadriceps weight compared to the continuous HFD group, although this did not translate into increased muscle functionality. These data demonstrate improved body composition in weight-cycled mice relative to continuous HFD feeding.

In Ldlr-/-.Leiden mice, HFD-induced hyperglycemia and hyperinsulinemia were not affected by repeated weight cycling, which is consistent with other preclinical studies on repeated weight cycling in different models[34,35]. However, plasma cholesterol and triglyceride concentrations were significantly improved relative to HFD controls. Subsequent microscopical analysis of how repeated weight cycling influenced organ pathophysiology revealed a borderline significant increase in adipocyte size compared to HFD controls. This is consistent with other preclinical weight cycling studies that report increased adipocyte hypertrophy[24,36]. Although it is unclear what mechanisms underlie this, it is suggested that weight cycling may increase fat storage efficiency[37]. Moreover, clinical studies in non-obese adults describe that various adipose depots respond differently to weight fluctuations to accommodate long-term increases in energy storage[38,39]. In once weight-cycled mice, we found significant improvements in adipose inflammation, improvements that persisted after four weight cycles. These findings demonstrate that repeated weight cycling has favorable effects over continuous HFD feeding regarding body composition and adipose inflammation.

The effects of repeated weight cycling on NAFLD-NASH in patients with obesity have been sparsely described in literature. Weight cycling once was demonstrated to have no effects on hepatic steatosis[26,27] while a different study where mice were exposed to 10 weight cycles reported reduced hepatic steatosis[35]. In this study, repeated weight cycling had minimal effects on hepatic steatosis. To the best of our knowledge, no studies reported on the effects of repeated weight cycling on hepatic fibrosis, which we have shown here to be unaffected. Interestingly, the significant improvement in hepatic inflammation observed in once weight-cycled mice persisted after repeated weight cycling. This improvement was also observed compared to mice in the HFD reference group that were sacrificed prior to weight cycling and that had similar body weights and liver weights, indicating that the improved liver inflammation cannot solely be attributed to weight loss. NAFLD-NASH are diseases that are classically thought to develop following the two-hit hypothesis, where lipid accumulation is followed by activation of inflammatory cascades and subsequently fibrogenesis[40]. However, more recent studies challenge the assumption that liver fibrosis is always preceded by liver inflammation since many other factors contribute to NAFLD-NASH development, and fibrosis can develop in steatotic livers with

minimal inflammation as well[41,42]. This is in line with what we demonstrate in the current study, where we found that repeated weight cycling improved hepatic inflammation although there were no significant effects on hepatic fibrosis. In patients with overweight or obesity, cardiovascular disease is the most common cause of death[43]. Although many human observational studies suggest weight cycling worsens risk factors for cardiovascular disease[6,44,45], we found atherosclerosis development in our study to be unaffected by yo-yo dieting. In summary, repeated weight cycling did not affect hepatic steatosis and fibrosis nor atherosclerosis, yet significantly improved liver inflammation compared to continuous HFD feeding.

More detailed investigation on how repeated weight cycling affected liver inflammation revealed significant reductions in F4/80-positive crown-like structures compared to the continuous HFD group, signifying reduced numbers of infiltrated cells of the monocyte/macrophage lineage. Free fatty acids are known inducers of mitochondrial stress that may stimulate the production of reactive oxygen species, which in turn can lead to NLRP3 inflammasome activation[21,22]. The reduction in free fatty acids in four times weight-cycled mice resulted in lowered hepatic NLRP3 protein levels and consequent reduced expression of the downstream effectors IL-1β and IL-18. These data demonstrate that repeated weight cycling significantly diminishes the induction of an inflammatory response through NLRP3 activation. Since inflammation is a driving factor in NAFLD regression into NASH, dieting attempts that result in weight cycling may thus in fact be beneficial relative to continuous exposure to an unhealthy diet.

It should be considered that many other factors could influence the effects of weight cycling. For instance, physical exercise may play a role[46] as well as alterations in composition and function of fecal microbiota, which may even be used as interventional target to ameliorate excessive secondary weight gain[47,48]. Besides, many patients with obesity are prescribed medications, including the recently approved glucagon-like peptide-1 receptor agonist semaglutide[49,50] that may mediate the effects of weight cycling. Moreover, the psychological impact of weight cycling should not be underestimated. Associations have been found that weight cycling decreased perceptions of health and well-being and increased binge eating severity and depressive symptoms[51–53]. Here, we focused on the effects of weight cycling in the context of metabolic syndrome since this is representative of the general patient population prone to yo-yo dieting[8]. Nevertheless, weight cycling is not limited to patients with obesity and while studies in wild-type models may give more insight into the effects of weight cycling in lean populations, the results from this study are more translational to patients with metabolic syndrome. By limiting our study design to dietary intervention only, we provided a comprehensive overview of the effects of yo-yo dieting on parameters relevant to metabolic syndrome (Figure 7). With this approach, we obtained results that argue against the postulate that weight cycling

leads to unfavorable metabolic effects and in fact demonstrate significant beneficial effects of yo-yo dieting on hepatic inflammation compared to a continuous unhealthy lifestyle.

5. Article information

5.1. Disclosures

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.2. Acknowledgements

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7. Supplementary material

	Chow	HFD reference	HFD + 1x weight loss	HFD + 1x weight cycled
Plasma triglycerides (mM)	1,1 ± 0.1***	6,8 ± 1,1	1,5 ± 0.1***	5,5 ± 1
Plasma ALT (U/L)	42,9 ± 3.4***	307,6 ± 59	193,2 ± 33.7^{T}	254,7 ± 39,4
Plasma AST (U/L)	83,3 ± 5.8***	325,8 ± 52,7	207,6 ± 28.6^{T}	335,5 ± 74,9
pgWAT weight (g)	1,1 ± 0.1***	2,1 ± 0,1	1,6 ± 0.1**	1,9 ± 0,2
vWAT weight (g)	0,3 ± 0.0***	1,1 ± 0,1	0,7 ± 0.1**	1 ± 0,1
Fibrosis stage (F0-F4)	0,3 ± 0.1*	1,5 ± 0,3	1,6 ± 0,2	2,2 ± 0,2

Supplementary Figure 1. Effects of weight cycling once on metabolic parameters. Metabolic parameters at the study endpoint. White adipose tissue (WAT) inflammation is expressed as the number of crown-like structures. HFD, high fat diet; ALT, alanine transaminase; AST, aspartate transaminase; pgWAT, perigonadal white adipose tissue; vWAT, visceral white adipose tissue. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, T: tendency (p<0.10) vs. HFD reference. ##p<0.01, ###p<0.001 HFD + 1x weight loss vs. HFD + 1x weight cycled.