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Markers of adipose tissue inflammation are transiently elevated during intermittent fasting in women who are overweight or obese

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ABSTRACT

Objective: This study compared the effects of daily calorie restriction (DR) versus intermittent fasting (IF) on markers of inflammation and extracellular matrix deposition in adipose tissue and skeletal muscle in a controlled feeding trial in women with overweight or obesity.
Methods: Women (N = 76) were randomised to one of three diets and provided with all foods at 100% (IF100) or 70% (IF70 and DR70) of calculated energy requirements for 8 weeks. IF groups ate breakfast prior to fasting for 24-h on 3 non-consecutive days/week. Weight, body composition, serum non-esterified fatty acids (NEFA), tumour necrosis factor-alpha (TNFα), interleukin-6 (IL-6), interleukin-10 (IL-10), M1- and M2-macrophage markers by qPCR and immunohistochemistry in adipose tissue and skeletal muscle were measured following a 12-h overnight fast (fed day, all groups) and a 24-h fast (IF groups only).
Results: IF70 resulted in greater weight and fat losses and reductions in serum NEFA versus DR70 and IF100 ($P < 0.05$) after fed days. Markers of inflammation in serum (TNFα, IL6 and IL10), subcutaneous adipose tissue and skeletal muscle (CD68, CD40 and CD163) were unchanged by DR or IF after fed days. After fasting, NEFA, M1-macrophages ($CD40^+$) in adipose tissue, and M2-macrophages ($CD163^+$) in muscle were increased in IF70 and IF100 (all $P < 0.05$) and the changes in NEFA and mRNA of pan-macrophage marker CD68 in adipose tissue were positively correlated ($r = 0.56$, $P = 0.002$).
Conclusions: Unlike caloric restriction, IF transiently elevated markers of macrophage infiltration in adipose tissue and skeletal muscle, possibly in response to marked increases in adipose tissue lipolysis.

Introduction

Monocytes are recruited into adipose tissue and skeletal muscle in humans and in animal models with obesity [1,2]. These monocytes are polarised towards inflammatory M1-macrophages that release pro-inflammatory cytokines, which impair the insulin signalling cascade [1–4]. Obesity is also associated with increased deposition of the extracellular matrix (ECM) and fibrosis. This may impair adipocyte expansion and promote ectopic lipid accumulation and insulin resistance [5,6].

Daily calorie restriction (DR) results in weight and fat losses, reductions in adipocyte size and reduces the risk of type 2 diabetes and cardiovascular diseases in individuals with obesity [7–9]. DR also reduces macrophage infiltration in adipose tissue and muscle in mice [10–12], although this result is more controversial in humans [13–16]. There is some evidence that DR may result in phenotype switching of macrophages from the inflammatory M1- to anti-inflammatory M2-macrophage profile in adipose tissue in mice [10]. DR also decreases mRNA levels of collagen genes and increases the expression of genes involved in ECM degradation in adipose tissue [13,17], but the effects in muscle are less clear.

Intermittent fasting (IF) involves intermittent periods of zero or minimal energy intake, typically for 24 h, followed by ad libitum food access. IF reduces body weight, fat mass and improves cardiovascular and diabetes risk markers in mice and in humans [18–22]. The impacts of IF on markers of adipose tissue or skeletal muscle

Abbreviations: COL6A1, collagen type VI alpha 1; DR, daily calorie restriction; ECM, extracellular matrix; hs-CRP, high sensitive C-reactive protein; HOMA-IR, homeostasis model of assessment-insulin resistance; IF, intermittent fasting; IL-6/10, interleukin-6/10; MCP-1, monocyte chemoattractant protein-1; MMPs, matrix metalloproteinases; NEFA, non-esterified fatty acids; TIMPs, tissue inhibitors of metalloproteinases; TNFα, tumour necrosis factor-alpha.

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remodelling have not been examined in humans. This is of interest since three studies have reported that acute 24 h fasting increases macrophage infiltration into adipose tissue in mice [11,23,24].

This study compared the effects of (1) eight-week DR versus IF on markers of inflammation and extracellular matrix in adipose tissue and skeletal muscle in women with overweight or obesity, and (2) the acute effects of the 24-h fast during IF on these outcomes. We hypothesized that DR and IF would reduce markers of ECM deposition, but that IF will increase markers of inflammation in adipose tissue and muscle.

Research design and methods

Participants and study design

This study was approved by the Royal Adelaide Hospital Research Ethics Committee, and all participants provided written, informed consent prior to their inclusion. The study was registered as a clinical trial with Clinicaltrials.gov (NCT01769976). The design of this study has been described previously [25]. Briefly, 88 women aged 35–70 years, with a BMI of 25–42 kg/m^2, were randomised to one of four groups in a 2:2:2:1 ratio (DR70, IF70, IF100 and control) for 8 weeks. This report excludes the control group (n = 12), as biopsies were not obtained. A flowchart describing the exact number of participants that were screened, enrolled, completed the intervention and underwent biopsies has been provided in Fig. 1.

DR70 were provided 70% of calculated energy requirements, daily. The IF70 group were provided foods at ∼32% of requirements at breakfast on fasting days and at ∼100% of energy requirements on fed days, such that their overall energy deficit was equivalent to the DR70. The IF100 group was provided foods at ∼37% of requirements at breakfast on fasting days and at ∼145% of energy requirements on fed days. On fasting days, IF groups consumed breakfast before 8 am prior to initiating a fast for 24-h, until 8 am the following day, every other week-day (3 fasting days per week). During the 24-h fasting period, participants were allowed to consume water and energy-free foods, black coffee and/or tea, plus one very low-calorie broth (250 ml, ∼20 kcals). To monitor compliance, participants were required to complete daily checklists and to return them to the researchers on the weekly individual counselling. Self-reported adherence to DR and IF70 diets was excellent, whereas the IF100 group ate 9% less than prescribed on fed days [25].

Metabolic testing in participants

Participants were required to attend the research clinic at 7:30 a.m., at baseline and after 8-weeks following an overnight 12-h fast. IF groups undertook an additional visit following a 24-h fast. Body weight, height, waist and hip circumference were measured with the participant dressed in a hospital gown after voiding. Intravenous cannulae were placed, baseline samples collecsted and a primed 120-min hyperinsulinaemic-euglycaemic (60 mU/m^2/min) clamp commenced as previously described [25]. Total body composition was assessed by dual-energy X-ray absorptiometry (Lunar Prodigy; GE Healthcare, NSW, Australia) at baseline and after 8-weeks following an overnight fast for all groups.

Adipose and muscle tissue biopsies

Subcutaneous abdominal adipose and vastus lateralis muscle samples were obtained in a subset of participants (N = 16 DR70, N = 16 IF70, N = 14 IF100) at baseline and at week 8 after 12-h fast and 24-h fast (IF groups only) as previously described [26,27]. One sample (∼30 mg) was fixed for histology, and the remainder frozen in liquid nitrogen and stored at −80 °C.

Table 1
Taqman primers used for mRNA expression analysis.

Gene symbol	Gene name	Assay ID
Housekeeper		
ACTB	Actin beta	Hs01060665_g1
PPIB	Peptidylprolyl isomerase B	Hs00168719_m1
LRP10	LDL receptor related protein 10	Hs01047362_m1
Pan macrophage		
CD68	CD68 molecule	Hs02836816_g1
M1-macrophage		
CD40	CD40 molecule	Hs01002913_g1
TNF	Tumour necrosis factor	Hs01113624_g1
IL6	Interleukin 6	Hs00985639_m1
M2-macrophage		
CD163	CD163 molecule	Hs00174705_m1
IL10	Interleukin 10	Hs00961622_m1
Macrophage recruitment		
CCL2	C-C motif chemokine ligand 2	Hs00234140_m1
CCL3	C-C motif chemokine ligand 3	Hs00234142_m1
Extracellular matrix		
COL6A1	Collagen type VI alpha 1	Hs01095585_m1
MMP2	Matrix metallopeptidase 2	Hs01548727_m1
TIMP1	TIMP metallopeptidase inhibitor 1	Hs00171558_m1

Biochemical analysis

Blood glucose was examined by photometric assay in the laboratory of SA Pathology (Adelaide, South Australia, Australia). Serum insulin was measured by radioimmunoassay (HI-14K, Millipore, MA, USA). Plasma high-sensitivity C-reactive protein (Hs-CRP, Beckman Coulter Inc., CA, USA) was measured using commercially available enzymatic kits on a Beckman AU480 clinical analyser (Beckman Coulter Inc). Serum TNFα, interleukin-6 (IL-6) and interleukin-10 (IL-10) were analysed by multiplex bead array assays (R & D Systems, Minneapolis, USA) with MAGPIX Multiplex Reader (Luminex, Austin, TX, USA). Serum non-esterified fatty acids (NEFA) were measured by enzymatic colorimetric assay (NEFA-HR (2), Wako Diagnostics, CA, USA), and serum monocyte chemoattractant protein-1 (MCP-1) by ELISA kit (BD, San Diego, CA, USA) on a VersaMax ELISA Microplate Reader (Sunnyvale, CA, USA). Samples were run in duplicate and samples from each subject were tested within the same run to reduce instrument variation. Homeostasis model of assessment-insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5.

Quantitative real-time PCR

As described previously [28], total RNA was extracted from adipose (100–150 mg) and muscle (30–50 mg) samples using TRI Reagent (Sigma, St. Louis, USA) following manufacturer's instructions. The concentration and purity of RNA were assessed by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, CA, USA). cDNA synthesis was conducted using T100 Thermal Cycler (Bio-Rad, CA, USA) with 1000 ng of each RNA sample using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA) according to kit instructions. Quantitative real-time PCR was performed using the Taqman primers for pan-macrophage (*CD68*), inflammatory M-1 macrophage (*CD40*, *TNF* and *IL6*), anti-inflammatory M2-macrophage (*CD163* and *IL10*), macrophage recruitment (*CCL2* and *CCL3*), extracellular matrix (*COL6A1*, *MMP2* and *TIMP1*, Table 1) and Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The samples were run in duplicate on an ABI 7500 sequence detection system (Applied Biosystems,

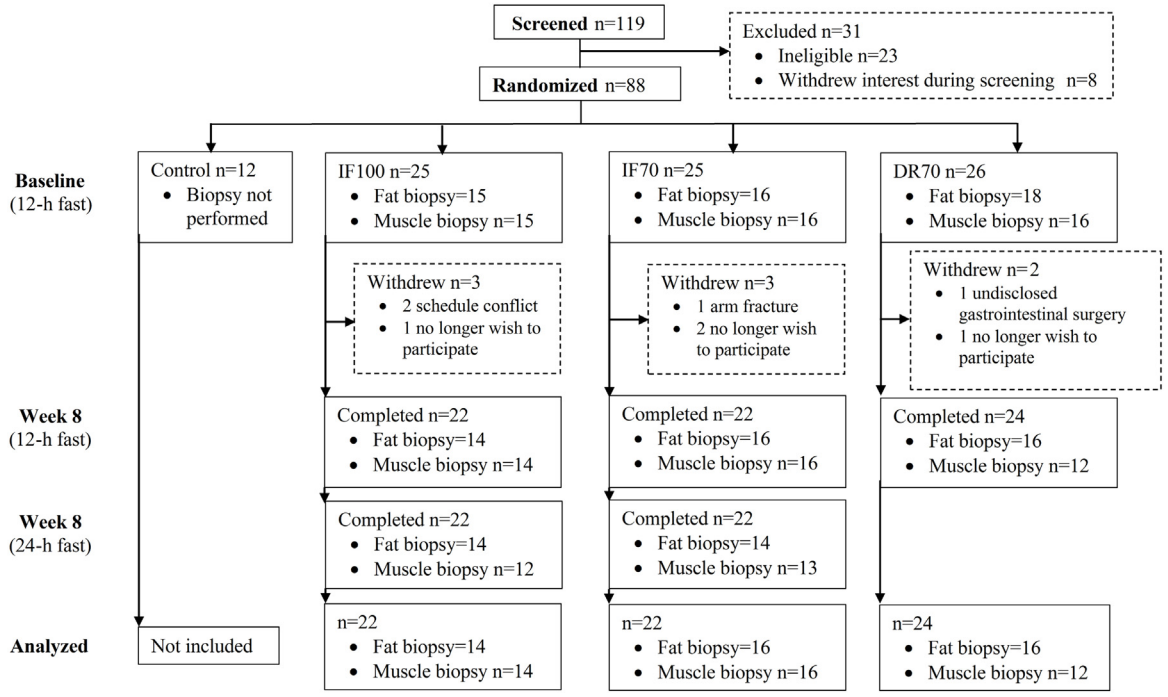

Fig. 1. Flowchart of the study.
Control: continuous energy intake at 100% of baseline energy requirements; DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements.

Foster City, CA, USA) with internal negative controls and a standard curve (pooled from ten participants at baseline). Relative gene expression was analysed using the $2^{-\Delta CT}$ method and normalised for the mean of *ACTB* and *PPIB* for adipose tissue, and the mean of *ACTB* and *LPR10* for skeletal muscle, which were not different at baseline, or following the intervention. *CCL3*, *TNF* and *IL6* were below detectable limits in adipose tissue and muscle.

Histological analysis and immunofluorescent staining

Adipose tissue biopsy samples were fixed in Bouin's solution (HT10132, Sigma-Aldrich), dehydrated, paraffin embedded, sectioned at 5 μm and mounted on positively-charged glass slides. All slides were randomly assigned numeric codes by a research officer to blind the investigator (BL) quantifying outcomes. Haematoxylin and eosin (H&E) staining was performed using a standard protocol. Digital images were acquired using a camera (U-TV1X-2, Olympus, Tokyo, Japan) and diameters measured using cellSens Software (Olympus, Tokyo, Japan). Adipocyte diameter was measured in at least three fields of view at 20×. The mean diameter was calculated from an average of 300 cells per sample as described previously [26]. Masson's trichrome staining was performed using a commercial kit (HT15-1KT, Sigma-Aldrich) following manufacturer's instructions. Slides were scanned using the Pannoramic 250 Flash II scanner (3DHISTECH, Budapest, Hungary) and whole sections were analysed using Masson's trichrome macro in Image J (National Institutes of Health, USA). Particular care was taken to exclude areas which contained blood vessels, as collagen is associated with vasculature [29].

For CD40 (M1-macrophage) and CD206 (M2-macrophage) co-staining, deparaffinised and rehydrated slides were incubated with ELOXALL solution (SP-600, Vector) for 10 min at room temperature to eliminate endogenous peroxidase and alkaline phosphatase. Antigen retrieval was achieved using modified citrate-based buffer (S1700, Dako) and incubation in a 95 °C water bath for 20 min. Slides were blocked with 5% bovine serum albumin (A7030-10 G, Sigma) in phosphate-buffered saline (PBS, 79382-50TAB, Sigma) for 60 min at room temperature before being incubated with a mouse anti-human CD40 (1:200, MAB6321, R& D) for 90 min at room temperature. Slides were then washed with PBS for 5 min for 3 times prior to being incubated with a chicken anti-mouse secondary antibody (1:500, A-21200, ThermoFisher) for 60 min at room temperature. Following 5 min × 3 washing, goat anti-human CD206 (1:200, AF2534, R&D) and the corresponding donkey anti-goat secondary antibody (1:500, A-11057, ThermoFisher) were applied. CD40 and CD206 co-labelled slides were counterstained with ProLong Gold Antifade Mountant with DAPI (P36941, ThermoFisher). Eight to ten fields at 40× were analysed using the camera and software mentioned above. Positive cells were expressed per 100 adipocytes as described previously [30].

Statistical analysis

Data are expressed as mean ± SEM. Individuals who withdrew from the study were not included in the analyses. Participants completing baseline and week-8 (12-h fast) biopsies were included for gene expression analyses. All statistical analyses were performed using IBM SPSS Statistics 24 (Armonk, New York, USA). Baseline differences between groups were analysed using one-way ANOVA with Bonferroni–Holm post-hoc test. A maximum likelihood mixed effects model was employed to examine the group differences of 8-week intervention following an overnight 12-h fast, as well as the time effects within each group. The model included fixed effects for intervention, visit and the intervention by visit interaction, and a random effect for subject with an unstructured covariance matrix to account for the repeated visits. The effect of intervention was assessed with planned contrasts between groups in the change from baseline to week 8 (12-h fast). Bonferroni-adjusted pairwise comparisons were also conducted within each group to assess differences over time from baseline to following a 12-h fast (all groups) and a 24-h fast (IF groups only). All analysis was performed with two-tailed tests. Data were log-transformed for analysis if skew-

Table 2
Anthropometric and metabolic measures of participants at baseline.

Variable N	DR70 26	IF70 25	IF100 25	P
Age (years)	51 ± 2	50 ± 2	51 ± 2	0.827
Pre/post menopause	12/14	13/12	10/15	–
Weight (kg)	88.4 ± 2.8	89.4 ± 2.8	84.1 ± 2.8	0.289
Height (cm)	164.7 ± 1.0	166.0 ± 1.7	162.2 ± 1.5	0.158
Waist (cm)	99.0 ± 1.8	100.5 ± 2.2	98.8 ± 2.6	0.859
Hip (cm)	115.7 ± 2.4	115.1 ± 2.1	112.1 ± 1.9	0.433
Waist/hip ratio	0.86 ± 0.01	0.87 ± 0.02	0.88 ± 0.02	0.640
BMI (kg/m^2)	32.6 ± 1.0	32.4 ± 0.8	31.2 ± 0.9	0.746
Triglyceride (mmol/l)	1.3 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	0.093
Total-cholesterol (mmol/l)	4.9 ± 0.1	4.8 ± 0.1	5.0 ± 0.2	0.256
HDL-cholesterol (mmol/l)	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	0.728
LDL-cholesterol (mmol/l)	3.0 ± 0.1	2.9 ± 0.1	3.0 ± 0.2	0.726
Glucose (mmol/l)	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	0.956
hs-CRP (mg/l)	2.7 ± 0.5	2.9 ± 0.5	2.8 ± 0.5	0.907

Data are presented as mean ± SEM. One-way ANOVA with Bonferroni–Holm post-hoc test. DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements. BMI: body mass index; HDL-C: high density lipoprotein; LDL-C: low density lipoprotein.

ness in the residuals was observed. Correlations were calculated using Pearson correlation coefficients. Significance was accepted as $P < 0.05$.

Results

Anthropometrics and metabolites at baseline

Anthropometric and metabolic parameters of the participants at baseline are summarised in Table 2. There were no significant differences between groups with respect to any variables assessed at baseline.

Adiposity, glucose metabolism and non-esterified fatty acids

As previously reported [25], greater reductions in body weight and fat mass was observed in IF70 vs. DR70 and IF100 (both $P < 0.05$, Fig. 2A and B). Adipocyte size was reduced in all groups (all $P \leq 0.01$), with no difference between groups (Fig. 2C). Following the fed day, NEFA levels were decreased in all groups (all $P < 0.05$), with greater reductions in IF70 vs. DR70 ($P = 0.02$, Fig. 2D). Fasting HOMA-IR was reduced from baseline in IF70 ($P = 0.01$) and was elevated in IF100 vs. IF70 and DR70 (both $P < 0.05$, Fig. 2E). Insulin sensitivity by clamp was unchanged (Fig. 2F). Following the fasting day, NEFA levels were increased ($P < 0.05$, Fig. 2D) and HOMA-IR were reduced in both IF groups ($P \leq 0.01$, Fig. 2E). Insulin sensitivity by clamp tended to be impaired following the fast in the IF100 group ($P = 0.06$, Fig. 2F).

Systemic and tissue inflammation

Following the fed day, there were no within or between group changes in any inflammatory markers assessed in serum, adipose tissue or muscle (Fig. 3A–T), apart from an increase in serum MCP-1 in IF100 ($P = 0.01$, Fig. 3D). When measured following the 24-h fast, serum MCP-1, *CD40* mRNA levels, the total number of M1- and pan-macrophages in adipose tissue by histology, *CD163* mRNA and the *CD163*:*CD40* ratio in muscle were significantly increased in the IF70 group (Fig. 3E–P). The number of M1-, M2- and pan-macrophages in adipose tissue, *CD163* and *CD68* mRNA and the *CD163*:*CD40* ratio in muscle were also significantly increased after the 24-h fast in the IF100 group (Fig. 3E–P). The change in *CD68* mRNA levels in adipose tissue was positively correlated with the change in NEFA in both IF groups ($r = 0.56$, $P = 0.002$, Fig. 3I).

Extracellular matrix remodelling in adipose tissue and muscle

There was a significant increase in the expression of MMP2 in adipose tissue in DR70 and IF70 following the fed day, which was maintained following the fasting day in the IF70 group (Fig. 4B). However, there were no changes in any of the other ECM markers examined, or in the integrated density or area of collagen in adipose tissue by trichrome staining (Fig. 4A, C, G, H and I). In skeletal muscle, *COL6A1* and *MMP2* mRNA levels were increased following the fed day only in IF100 group (both $P \leq 0.05$, Fig. 4D and F).

Discussion

The effects of IF, as an alternative weight loss strategy to DR, on macrophage infiltration and extracellular matrix deposition in humans are unclear. This is of interest since macrophage infiltration is stimulated in response to an acute 24-h fast in mice [11,23,24]. In this study, we observed an increase in M1 markers of macrophage infiltration in adipose tissue in response to the 24-h fast imposed by the IF schedule. This was associated with increases in NEFA, a marker of adipose tissue lipolysis.

The phenotype of macrophages cover a continuum of functional states from M1, or classically activated macrophages, which secrete pro-inflammatory molecules such as IFN-γ and TNFα, to M2, or alternatively activated anti-inflammatory macrophages or both [1,31], although studies have identified a group pro-inflammatory macrophages that were metabolically activated by insulin, glucose or fatty acids, but do not present classic M1 or M2 markers [32–34]. Obesity is associated with an increase in M1 macrophages in adipose tissue, which can be partially reversed following DR [6,9,13,17,31,35,36]. M1 macrophage infiltration also occurs in skeletal muscle in mice that are fed a high fat diet [4], and linked with development of sarcopenia [37]. Increases in M1 macrophages in skeletal muscle in humans are associated with poorer insulin sensitivity [4]. In this study, DR did not impact any of the markers of inflammation that were examined in adipose tissue, skeletal muscle, or systemically. This finding is in line with previous reports [13,38]. Tam et al reported that 24-weeks of DR led to 10% weight loss in individuals with obesity, but did not alter systemic inflammation or markers of macrophages in adipose tissue [38]. Magkos et al. reported that 10–15% weight loss by DR partially improved systemic inflammation in individuals with obesity, but did not alter mRNA levels of macrophage markers in subcutaneous adipose tissue [13]. There was also no change in M1 or M2 markers at the mRNA level in skeletal muscle following 3-months of DR [15]. In response to IF, we also observed that markers of inflammation in adipose tissue or skeletal muscle were unchanged when measured following a fed day. This finding supports a recently published article in adipose tissue [39].

Uniquely, we have assessed markers of the inflammatory response after the fasting day imposed by an IF schedule. We observed transient increases in M1 markers of inflammation in adipose tissue, which was associated with the change in NEFA levels. This is similar to results in mice that reported increases in circulating NEFA levels in concert with macrophage infiltration in adipose tissue in response to a 24-h fast [11]. Although, we could not recently recapitulate these results in mice [40]. In that study, the peak of adipose tissue macrophage number coincided with the peak in the circulating concentration of NEFA and glycerol released from adipose tissue, suggesting that lipolysis drives macrophage accumulation in adipose tissue [11]. Moreover, *in vivo* and *in vitro* studies have shown that stimulation of adipocyte lipolysis increases the uptake and storage of lipids by macrophages [11,41]. Thus, we speculate that macrophages could play a protective role to buffer elevated NEFA levels induced by fasting in

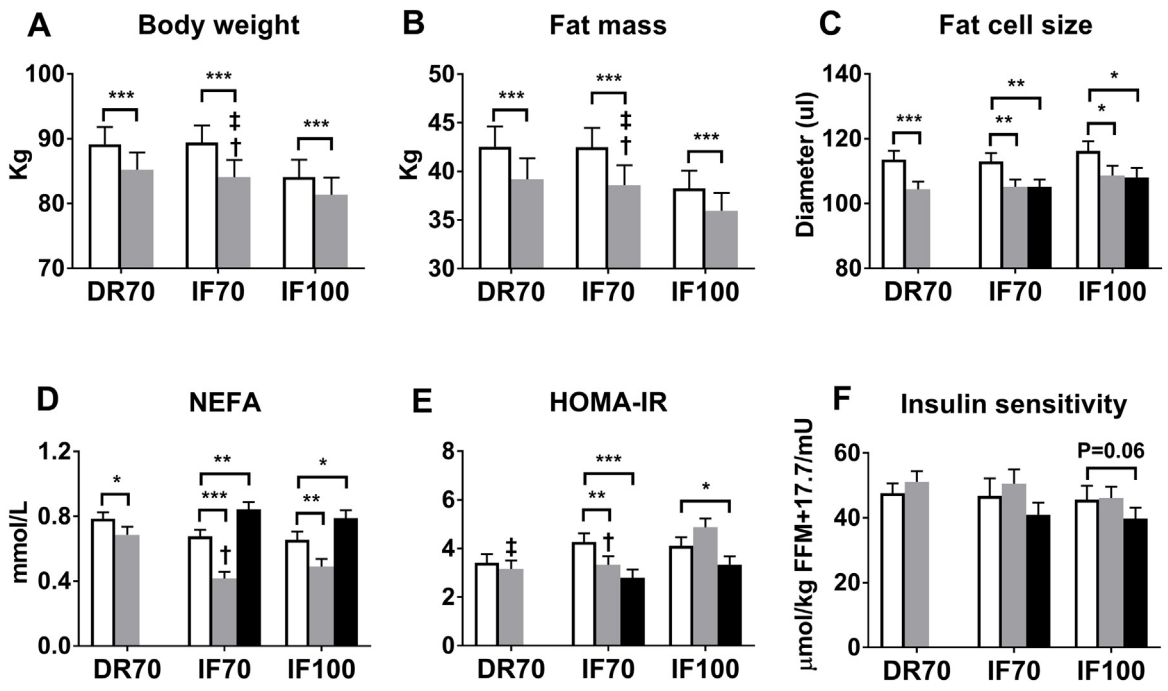

Fig. 2. Adiposity, non-esterified fatty acids and insulin sensitivity after 8-weeks of intervention. (A): Body weight, (B): fat mass, (C): fat cell size, (D): NEFA, (E): HOMA-IR and (F): insulin sensitivity assessed by hyperinsulinaemic-euglycaemic clamp.
DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements. NEFA: non-esterified fatty acids; HOMA-IR: homeostasis model of assessment-insulin resistance.
Open bar: baseline; grey bar: week 8 (12-h fast) and Black bar: week 8 (24-h fast). †$P < 0.05$ vs. DR70 in the change from baseline to week 8 following a 12-h fast; ‡ $P < 0.05$ vs. IF100 in the change from baseline to week 8 following a 12-h fast. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. baseline.

humans, as has been observed in mice [11], and may protect against the development of fasting-induced insulin resistance [42]. However, we did not observe a relationship between the change in insulin sensitivity by clamp and the change in macrophage numbers in adipose tissue.

The extracellular matrix is a non-cellular component that provides structural support to the neighbouring cells and influences cell morphology and function through cell-matrix connections [5]. Collagens are degraded by matrix metalloproteinases (MMPs), which are negatively regulated by tissue inhibitors of metalloproteinases (TIMPs) [9]. This balance between synthesis and degradation maintains ECM homeostasis, and is perturbed in the obese state [5]. In this study, *MMP2* mRNA expression was increased in adipose tissue in DR and IF70 groups. This finding suggests that weight loss, rather than the mode of dietary restriction, promotes ECM degradation in adipose tissue. Previous studies have also shown that 5% weight loss increases genes involved in the degradation of ECM in subcutaneous adipose tissue [13]. We did not detect changes in collagen deposition by histology. Adipose tissue fibrosis was also unchanged following substantial weight loss by bariatric surgery in patients who were morbidly obese with or without type 2 diabetes [43]. Interestingly, Clément's group observed increased collagen deposition in subcutaneous adipose tissue 3–12 months after bariatric surgery [44]. However, this was characterised by a decrease in cross-link of matrix fibres and increased degradation of the extracellular matrix and was associated with an increase in M2-macrophages. This study highlights that the structure or quality of collagen should also be taken into consideration when assessing changes in tissue fibrosis during weight loss in future studies.

Previous studies in mice show that IF promotes M2-polarisation of macrophages in adipose tissue, and that this was associated with adipose tissue browning [23,45]. In our hands, IF did not alter M2 markers in human adipose tissue. However, mRNA levels of CD163 were elevated in skeletal muscle in response to IF, which was not observed in the DR group. Macrophages have well-characterised roles in skeletal muscle repair and regeneration in response to injury [46,47]. M1-macrophages are responsible for phagocytosing cell debris and promoting satellite-cell proliferation, while M2-macrophages release pro-fibrotic molecules that activate fibroblasts to synthesize ECM components and promote skeletal muscle differentiation [48,49]. Exercise training also increases M2 macrophages in mouse skeletal muscle, in concert with muscle growth [42]. In this study, *COL6A1* and *MMP2* mRNA levels were increased in response to IF, although this reached statistical significance only in the IF100 group after a feeding day. The possibility that IF may have positive impacts on muscle repair and regeneration requires further investigation. Although it should be noted that IF did not differentially effect muscle mass vs DR [25], however functional assessments were not conducted.

This was a highly controlled, short-term intervention to examine mechanisms associated with improvements in IF versus continuous dietary restriction that was limited to women who were overweight and obese. Thus, the responses in free-living populations, men, or in individuals with normal body weight and the long-term effects may be different. In addition, macrophages activated by metabolic stimuli and presenting both M1 and M2 markers were not evaluated in this report.

This study highlights that the mode of dietary restriction differentially alters macrophage infiltration in adipose tissue and skeletal muscle in humans. These findings supports studies in mice to show that macrophages rapidly respond to fasting and NEFA release in adipose tissue. Future studies should also investigate the effects of IF on skeletal muscle growth and regeneration in obesity.

Funding

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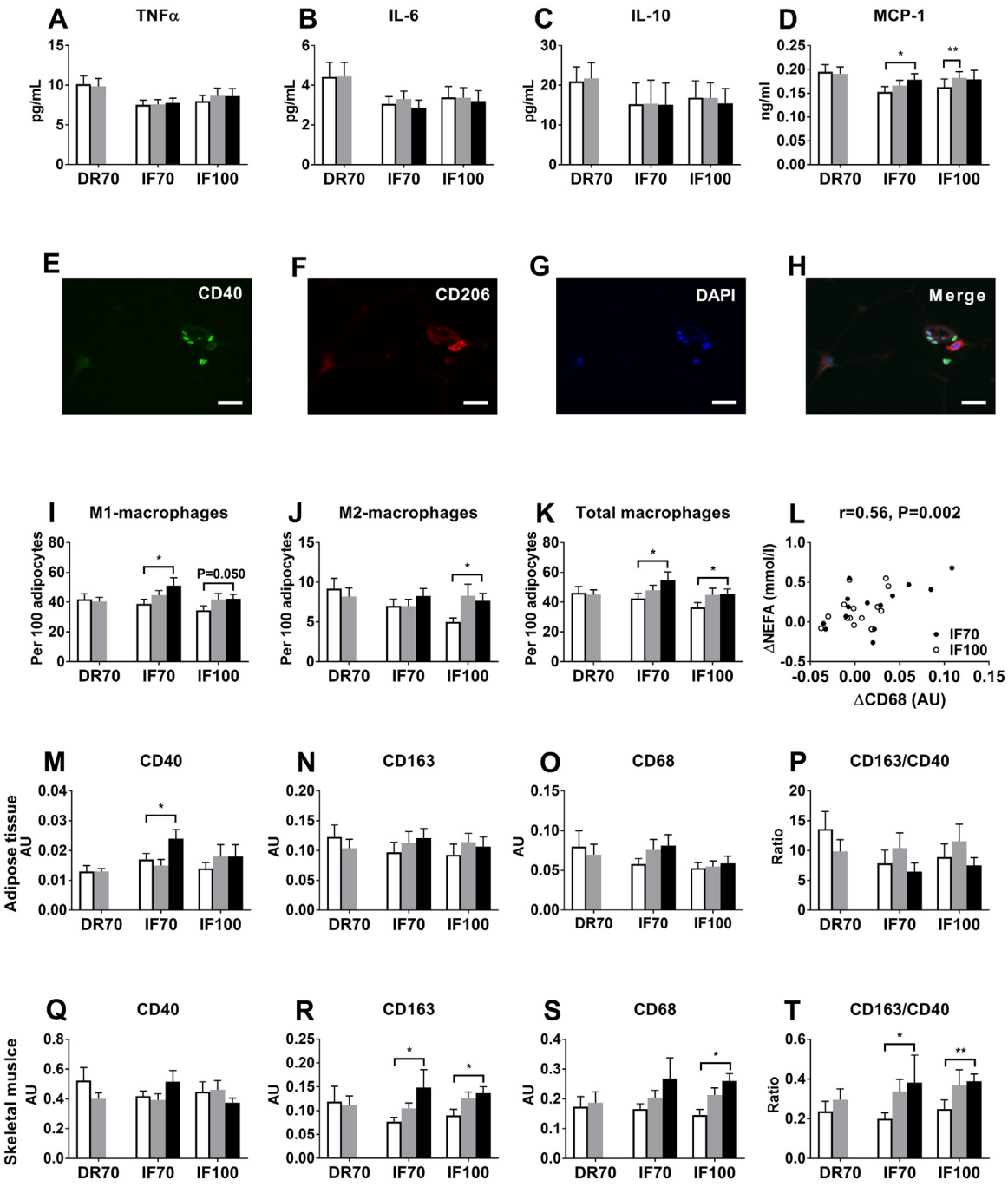

Fig. 3. Systemic and tissue inflammation after 8-weeks of intervention. (A–D): serum TNFα, IL-6, IL-10 and MCP-1. (E–H): representative images of fluorescent staining for M1-macrophage (CD40, Fig. E), M2-macrophage (CD206, Fig. F), nuclei (DAPI, Fig. G) and layout (Fig. H). (I–K): quantification of M1-, M2- and total macrophages in adipose tissue. (L): correlation between changes in NEFA levels and *CD68* mRNA levels in adipose tissue after a 24-h fast. (M–O): mRNA levels of *CD40* (M1-), *CD163* (M2-) and *CD68* (pan-macrophage) in adipose tissue. (P): the ratio of *CD163*:*CD40* in adipose tissue. (Q–S): mRNA levels of *CD40*, *CD163* and *CD68* in skeletal muscle. (T): the ratio of *CD163*:*CD40* in muscle. AU: arbitrary unit.
DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements. TNFα: tumour necrosis factor-alpha; IL-6/10: interleukin-6/10; MCP-1: monocyte chemoattractant protein-1; NEFA: non-esterified fatty acids.
Open bar: baseline; grey bar: week 8 (12-h fast) and Black bar: week 8 (24-h fast). Scale bar: 20 μm. *P < 0.05 and **P < 0.01 vs. baseline.

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Prior presentation

Parts of this study were presented as an oral presentation at the Australian & New Zealand Obesity Society 2016 Annual Scientific

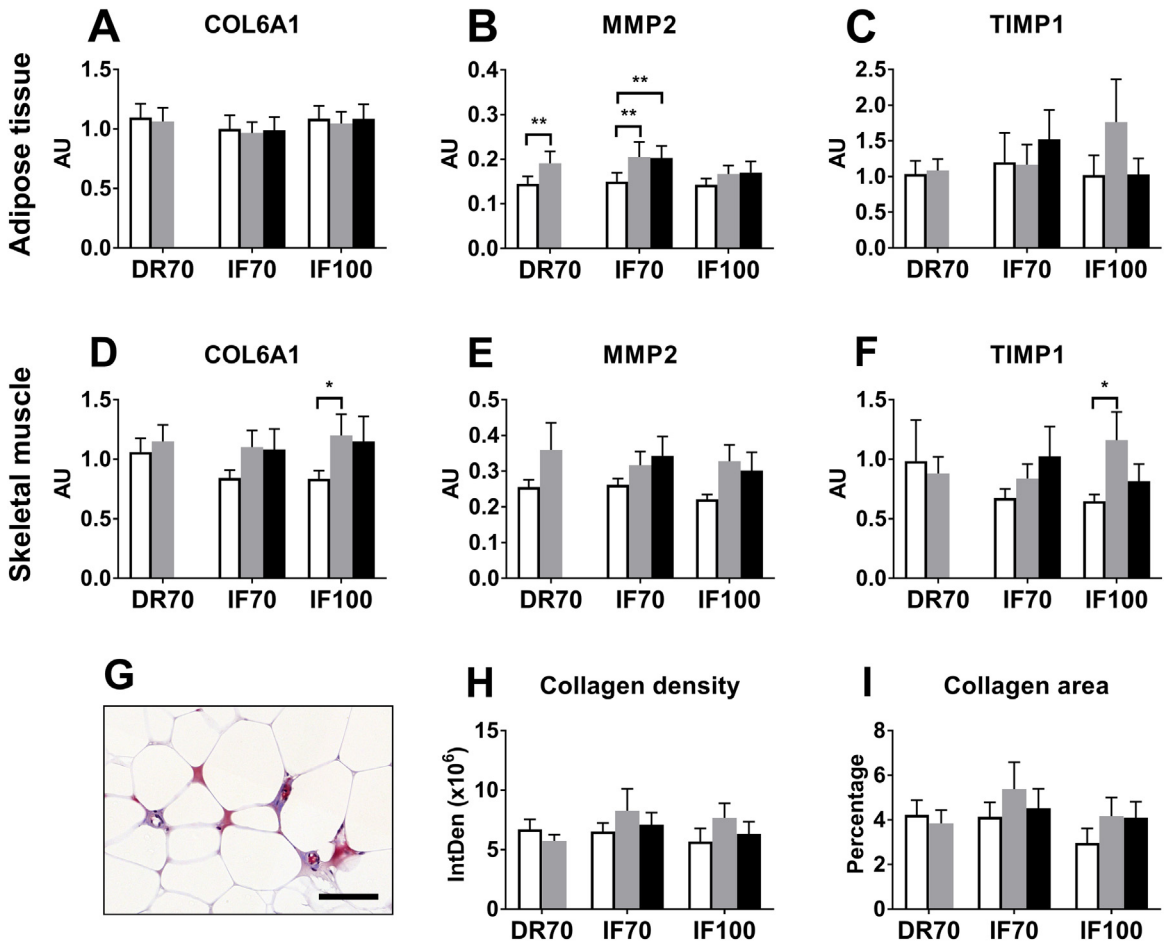

Fig. 4. Extracellular matrix remodelling in adipose tissue and muscle after 8-weeks of intervention. (A–C): mRNA levels of *COL6A1*, *MMP2* and *TIMP1* in adipose tissue; (D–F): mRNA levels of *COL6A1*, *MMP2* and *TIMP1* in muscle; (G): representative image for Masson's trichrome staining in adipose tissue; (H–I): the integrated density (H) and area (I) of collagen in adipose tissue.
DR70: continuous energy restriction at 70% baseline energy requirements: IF70: intermittent fasting diet at 70% baseline energy requirements; IF100: intermittent fasting diet at 100% baseline energy requirements. COL6A1: collagen type VI alpha 1; MMPs: matrix metalloproteinases; TIMPs: tissue inhibitors of metalloproteinases.
Open bar: baseline; grey bar: week 8 (12-h fast) and black bar: week 8 (24-h fast). Scale bar: 100 μm. *P < 0.05 and **P < 0.01 vs. baseline.

Meeting, Brisbane, 19–21 October, 2016, and as a poster at the 77th Scientific Sessions of the American Diabetes Association, San Diego, 9–13 June, 2017.

Ethical statement

This study was approved by the Royal Adelaide Hospital Research Ethics Committee, and all participants provided written, informed consent prior to their inclusion.

Conflict of interest

The authors have no conflict of interest to declare.

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