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Influence of a low carbohydrate diet on endothelial microvesicles in overweight women

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1 **Title page**

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3 **Title**

4 Influence of a low carbohydrate diet on endothelial microvesicles in overweight women

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8 Low carb diets and endothelial microvesicles

9

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Abstract

Low carbohydrate diets (LCD) are increasing in popularity, but their effect on vascular health has been questioned. Endothelial microvesicles (EMV) are membrane-derived vesicles with the potential to act as a sensitive prognostic biomarker of vascular health and endothelial function. The aim of this study was to examine the influence of a LCD on EMV and other endothelial biomarkers of protein origin. Twenty-four overweight women (aged 48.4±0.6 y, height 1.60±0.07 m, body mass 76.5±9.1 kg, BMI 28.1±2.7 $kg \cdot m^{-2}$, waist circumference 84.1±7.4 cm; mean±SD) were randomised to either 24 weeks on their normal diet (ND) or a LCD after which they crossed over to 24 weeks on the alternative diet. Participants were assisted in reducing carbohydrate intake but not below 40 $g \cdot day^{-1}$. Body composition and endothelial biomarkers were assessed at the cross-over point and at the end of the study. Daily carbohydrate intake (87±7 vs. 179±11 g) and the percentage of energy derived from carbohydrate (29 vs. 44%) were lower ($p<0.05$) on the LCD compared to the ND but absolute fat and saturated fat intake were unchanged. Body mass and waist circumference were 3.7±0.8 kg and 3.5±1.0 cm lower ($p<0.05$) respectively after the LCD compared with the ND phases. $CD31^{+}CD41^{-}$EMV, soluble (s) thrombomodulin, sE-selectin, sP-selectin, serum amyloid A and C-reactive protein were lower ($p<0.05$) after the LCD compared to the ND, but serum lipids and apolipoproteins were not different. EMV along with a range of endothelial and inflammatory biomarkers are reduced by a LCD that involves modest weight loss.

KEYWORDS: vascular health; endothelial biomarkers; weight loss; microparticles

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1 **Introduction**

2 Low carbohydrate diets (LCD) are increasing in popularity as a means of achieving
3 weight loss in overweight women. LCD typically have a carbohydrate content of less
4 than 100 $g \cdot day^{-1}$ or less than 30% of total daily energy intake (Bilsborough & Crowe,
5 2003). Diets such as the Atkins diet, sometimes termed very low carbohydrate diets or
6 very low carbohydrate ketogenic diets, target a carbohydrate content of 20 – 50 $g \cdot day^{-1}$,
7 representing less than 20% of total energy intake. LCD generally show greater weight
8 loss at 6 mo when compared to low fat diets (Brehm *et al.*, 2003; Foster *et al.*, 2003;
9 Gardner *et al.*, 2007; Samaha *et al.*, 2003; Yancy *et al.*, 2004). The results of a meta-
10 analysis of early trials demonstrate 3.3 kg greater weight loss at 6 mo when LCD are
11 compared to low fat calorie-restricted diets and at least as much weight loss at 12 mo
12 (Nordmann *et al.*, 2006). Despite these results with respect to body weight, the safety of
13 LCD have been questioned (Bilsborough & Crowe, 2003; Frigolet *et al.*, 2011). A
14 reduction in body mass of 5-10 % following dietary restriction has the potential to
15 improve vascular risk profile (National Institutes of Health, 1998). However, individuals
16 on LCD typically obtain a greater proportion of energy from fat, saturated fat and protein
17 which may have pro-atherogenic effects on some risk markers. In their meta-analysis,
18 Nordman and colleagues (Nordmann et al., 2006) demonstrated pro-atherogenic changes
19 in serum total cholesterol and low density lipoprotein cholesterol (LDL-C) following
20 LCD compared to low fat diets but anti-atherogenic changes in serum triglycerides, high
21 density lipoprotein cholesterol (HDL-C) and blood pressure. Given these conflicting
22 findings, there is a need to monitor more than traditional risk factors when assessing the
23 impact of dietary interventions on vascular risk.

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2 The endothelium expresses and releases a wide variety of factors relevant to vascular
3 inflammation, coagulation, vessel tone and barrier function. Endothelial-derived factors
4 associated with the atherothrombotic process have the ability to monitor changes in
5 vascular risk following lifestyle interventions. Soluble forms of selectins and cell
6 adhesion molecules have been employed previously in intervention studies (Davis *et al.*,
7 2011; Keogh *et al.*, 2008; Porreca *et al.*, 2004; Sharman & Volek, 2004; Wycherley *et al.*,
8 2009). Selectins and adhesion molecules are involved in the rolling and tethering of
9 circulating leukoctyes and their migration into the subendothelial layer, key processes in
10 atherogenesis (Brevetti *et al.*, 2006). Thrombomodulin is a key component of endothelial
11 thromboresistance with cell surface expression reduced, and circulating levels increased,
12 with endothelial injury and dysfunction (Martin *et al.*, 2013).

13

14 Microvesicles are a novel class of vascular biomarker released from a range of vascular
15 cells including endothelial cells, platelets, vascular smooth muscle cells, erythrocytes and
16 leukocytes, into a range of body fluids including blood, urine, cerebrospinal fluid, lymph,
17 saliva and tears. These small vesicles, between 100 – 1000 nm in diameter, are released
18 by outward budding of the parent cell plasma membrane. In contrast, exosomes, a related
19 class of extracellular vesicle between 30 – 100 nm in diameter, are released by
20 exocytosis. Microvesicles are not simply biomarkers but also potentially mediators of
21 disease activity and cell-cell communication, transporting bioactive lipids, membrane
22 proteins, mRNA and miRNA from the parent to target cells (Akers *et al.*, 2013;
23 Andriantsitohaina *et al.*, 2012; Ayers *et al.*, 2015). Endothelial microvesicles (EMV)

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1 have prognostic potential as vascular biomarkers that may exceed the more established
2 soluble protein biomarkers. They are elevated in a range of vascular inflammatory
3 disorders and add to risk prediction in prospective studies when added to models
4 involving more established markers (Nozaki *et al.*, 2009) . Recently, we have shown an
5 association between EMV and unstable atherosclerosis (Wekesa *et al.*, 2014). EMV
6 impair endothelial dependent dilation, increase arterial stiffness, promote inflammation
7 and initiate coagulation (Chironi *et al.*, 2009).
8
9 In contrast to studies that have assessed blood lipids, blood glucose and blood pressure,
10 only a few studies have employed endothelial biomarkers to assess the effects of LCD on
11 vascular risk, and to our knowledge no studies have employed EMV. Reductions in
12 soluble intercellular adhesion molecule (sICAM-1), soluble E-selectin and soluble P-
13 selectin, (Davis *et al*., 2011; Keogh *et al*., 2008; Sharman & Volek, 2004; Wycherley *et*
14 *al*., 2009) been demonstrated following LCD interventions, but not soluble vascular cell
15 adhesion molecule (sVCAM-1) (Wycherley *et al*., 2009). Although elevated in the obese
16 state (Stepanian *et al.*, 2013), EMV have not been widely employed in dietary
17 intervention research. Following bariatric surgery-induced weight loss however, one
18 study reported a reduction in EMV (Cheng *et al.*, 2013) and another no change
19 (Stepanian *et al*., 2013).
20
21 The purpose of this study was to examine the influence of a 24-week LCD on
22 cardiovascular risk profile determined from changes in EMV and other endothelial-

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1 inflammatory biomarkers. Middle-aged overweight women were chosen as the subject
2 group of interest, given the popularity of LCD and the paucity of data in this population.
3
4
5 **Methods**
6 *Study overview*
7 In this randomised crossover study, participants were randomised to either 24 weeks on
8 their normal diet (ND) or a LCD after which they crossed over to the alternative diet for a
9 further 24 weeks. A fasting blood sample was obtained at the cross-over point (Week 24)
10 and at the end of the study (Week 48) from which circulating microvesicles and protein
11 biomarkers were determined. Body composition and blood pressure were also evaluated
12 at these time points.
13 *Study population and ethics*
14 Twenty-eight women were recruited into this intervention study though 4 dropped out at
15 various stages. The final analysis was therefore based on 24 participants (aged 48.4 ± 0.6
16 y, height 1.60 ± 0.07 m, body mass 76.5 ± 9.1 kg, body mass index (BMI) 28.1 ± 2.7
17 $kg \cdot m^{-2}$, waist circumference 84.1 ± 7.4 cm, % body fat 43.5 ± 5.0; mean ± SD) who
18 completed both phases of the study. The inclusion criteria were women in their late
19 reproductive or post-menopausal years, a BMI between 25 and 30 $kg \cdot m^{-2}$ and not having
20 engaged in dieting practices in the previous 6 months. Exclusion criteria included
21 participation in intense physical activity (> 3 times per week of vigorous physical
22 activity), high alcohol consumption (> 14 units weekly), currently pregnant, lactating or
23 taking hormone replacement therapy, previous hysterectomy or a history of chronic

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menstrual irregularities, diabetes mellitus, kidney disease, chronic illness, inflammatory conditions, renal, gastrointestinal or hormonal disorders. Each participant completed a health screening form to determine eligibility for the study. From an ethical standpoint, the study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from Waterford Institute of Technology Research Ethics Committee and written informed consent was obtained from each participant. Details of the trial protocol have been published (www.isrctn.com, ISRCTN 48034106).

Diet

During the LCD period participants were assisted in to reducing their carbohydrate intake considerably, but not below 40 g·day^{-1}. Participants were also provided with information on what constituted carbohydrate foods, a list of the macronutrient content in the most commonly consumed foods and a booklet with LCD recipes, meal plans and advice. In addition, they attended cooking classes that demonstrated how to make LCD meals that were not readily available in stores. Participants completed weekly food frequency questionnaires for self-monitoring purposes and to assist with compliance. These were discussed with participants at the mid-point of each phase but were not analysed for research purposes. Four individuals exited the study because of an inability to adhere to a LCD. In contrast, the ND group were monitored via the weekly food frequency questionnaires.

Dietary assessment

A 3 day food diary was completed at baseline and at weeks 12 (mid-point phase 1), 24 (cross over), 36 (mid-point phase 2) and 48 (end of study). Participants were instructed to maintain current phase-dependent dietary practices and to estimate food quantities.

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1 Visual and instructional cues were given to assist with portion size estimation. The food
2 diaries were analysed using CompEat™ analysis software. Intake of selected macro-
3 nutrients (energy, carbohydrate, fat, saturated fat and protein) and micro-nutrients during
4 the ND and LCD phases were calculated by averaging daily values at the mid-point and
5 end of each phase from these 3 day food diary analyses.

6 *Physiological Measurements*

7 Height, body mass, waist circumference and blood pressure were measured at baseline,
8 week 24 and week 48. Blood pressure was determined using an automated blood
9 pressure monitor (Omron Healthcare, USA) and appropriate cuff size, with participants
10 seated and resting for 10 min before assessments were made. Body fat was determined
11 by DEXA (Norland XR-46, USA).

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12 *Blood collection and processing*

13 All blood samples were taken in the morning following an overnight fast. The first 3.0
14 mL was discarded. Samples were kept at room temperature prior to centrifugation and
15 processed within 2 hours of collection. The serum tubes were centrifuged for 15 minutes
16 at 1500g at 20°C. The upper part of the serum and plasma was collected down to 1 cm
17 above the buffy coat layer. The sodium citrate tubes (3.2%), for MV analysis, underwent
18 a double centrifugation protocol. They were first centrifuged for 15 minutes at 1500g at
19 20°C, followed by 2 minutes at 13,000g at 20°C to produce platelet poor plasma, with the
20 pellet discarded. Aliquots of ~350 mL of serum and plasma were stored at -80°C for later
21 analysis.

1 *Microvesicle and soluble protein biomarker analysis*

2 MV were defined based on size and fluorescence using flow cytometry (FC500, Beckman
3 Coulter, USA). Microvesicles were defined as particles < 1.0 μm in diameter with
4 fluorescence greater than that of negative control samples. A microvesicle size gate was
5 established on forward vs. side scatter using 0.5 and 0.9 μm sizing beads (Megamix,
6 Biocytex, France). This bead combination can be used to establish the upper limit of the
7 size gate and to standardize MV enumeration by calibrating the lower limits of the gate
8 just above the noise of the instrument. Two different EMV subsets were enumerated,
9 MV expressing CD31 but not expressing the platelet marker CD41 ($CD31^{+}CD41^{-}$ EMV)
10 and MV expressing the endothelial specific markers CD144, CD146 or CD105 in a
11 monochrome multimarker assay (mmEMV). This monochrome multimarker approach
12 was previously employed (Duval *et al.*, 2010) to improve the signal to noise ratio, given
13 the low number of these endothelial specific markers on particles of small surface area.
14 Representative plots (figure 2) are included as supplementary data.

15

16 Plasma samples were thawed on ice prior to analysis. Exactly 330 μl of thawed plasma
17 was washed twice in PBS-citrate (19,000g for 30 min). After the first wash the pellet (~
18 30 μL) was resuspended in PBS-citrate. Following the second wash the pellet was
19 resuspended in 80 μL PBS-citrate and vortexed thoroughly, resulting in a sufficient
20 sample volume for all assays ($CD31^{+}41^{-}$, mmEMV and negative control samples). The
21 following reagents were used to stain the samples, CD31-PE, CD41-PECy5, CD144-PE,
22 CD146-PE, CD105-PE, IgG1-PECy5 and IgG1-PE (all BD Biosciences, UK). All
23 antibodies were diluted in PBS and centrifuged at 13,000g for 3 min to remove

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aggregates. For the CD31^{+}41^{-} EMV assay, 20 μL of resupended pellet was incubated in the dark for 30 min with CD31-PE (final conc. 0.68 μg·mL^{-1}) and CD41-PECy5 (final conc. 0.12 μg·mL^{-1}). The negative control sample employed IgG1-PECy5. For the mmEMV assay, 20 μL of resupended pellet was incubated in the dark for 30 min with CD144-PE (final conc. 0.19 μg·mL^{-1}, CD146-PE (final conc. 0.12 μg·mL^{-1}) and CD105-PE (final conc. 0.38 μg·mL^{-1}). The negative control employed IgG1-PE (final conc. 0.69 μg·mL^{-1}). Then, 500 μL of phosphate buffered saline was added to each sample, followed immediately by flow cytometric analysis for 3 min at medium speed. Instrument flow rate was calculated using flow count beads (SPHERO Accucount, Spherotech, USA). EMV counts were converted to event·μl^{-1} taking into account the flow rate of the instrument, the 500 μl of PBS added prior to analysis and the concentrating effects of the wash protocol which reduced a starting plasma volume from 330 μl to 110 μl.

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Endothelial-inflammatory biomarkers of protein origin were analysed in duplicate using commercially available immunoassays (Meso Scale Discovery, Rockville, MD, USA) employing multiplexing technology and electrochemiluminescence detection. Serum cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides, apolipoprotein A1 (ApoA1), and apolipoprotein B (ApoB) were determined via spectrophotometric assays performed on an automated clinical chemistry system (ACE® Wassermann B.V., Netherlands) using appropriate reagents, calibrators and controls (Randox Laboratories, UK).

1 *Statistics*

2 All data was checked for normality using the Kolmogorov-Smirnov test. The effect of
3 the LCD on all variables was determined by comparing values at the end of the ND and
4 LCD phases using paired t-tests. The potential for carryover between phase 1 and phase
5 2 of the study was checked by comparing the delta scores (ND – LCD) for each variable
6 between the group that was first randomised to the ND phase (n=12) and the group first
7 randomised to the LCD phase (n=12) using independent t-tests (Wellek & Blettner,
8 2012). Associations between the between-phase differences in body mass and the
9 differences in circulating biomarkers were determined using Pearson correlations.
10 Significance was set at $p<0.05$. Data are presented as mean ± SEM.

11

12 **Results**

13 *Carryover*

14 With respect to body mass, blood pressure, EMV and all soluble endothelial and
15 inflammatory biomarkers, delta scores between the ND and LCD phases were not
16 different between the group first randomised to the ND phase and the group first
17 randomised to the LCD phase.

18 *Mean daily intake of various nutrients*

19 Daily intake of energy and macronutrients on the ND and LCD (table 1) were calculated
20 by averaging the 3 day food diary analysis at the mid-point and end of each phase. Mean
21 daily energy and carbohydrate intake were 398 kcal and 92 g lower ($p<0.05$) respectively
22 on the LCD compared to the ND. Mean daily intake of fat, saturated fat and protein was

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1 not different on the LCD and ND (table 1) but the percentage of energy obtained from fat
2 and protein was higher ($p<0.05$) on the LCD compared to the ND (figure 1).
3 *Body composition*
4 Body mass, body mass index and waist circumference were 3.7 ± 0.8 kg, 2.4 ± 0.3 $kg{\cdot}m^{-2}$
5 and 3.5 ± 1.0 cm lower respectively ($p<0.05$) at the end of the LCD compared to the ND
6 phases (table 2). Systolic blood pressure was not different but diastolic blood pressure
7 was 5 ± 2 mmHg lower at the end of the LCD compared to the ND phases.
8 *EMV and endothelial-inflammatory biomarkers*
9 $CD31^{+}CD41^{-}$ EMV ($p<0.05$) but not mmEMV ($p=0.08$) were lower at the end of the
10 LCD compared to the end of the ND (table 3). Soluble E-selectin, sP-selectin, soluble
11 thrombomodulin (sTM), C-reactive protein (CRP) and serum amyloid A (SAA) were
12 lower ($p<0.05$) following 24 weeks on the LCD compared to 24 weeks on the ND (table
13 3). Soluble ICAM-1, sICAM-3 ($p=0.08$) and sVCAM-1 were not different following 24
14 weeks on the LCD compared to the ND (table 3). Serum lipids, apolipoproteins and
15 glucose were not different at the end of the LCD compared to the ND phases (table 4).
16 *Associations*
17 The difference in $CD31^{+}CD41^{-}$EMV between dietary phases was correlated with the
18 difference in mmEMV ($r=0.51$) but with no other biomarker or dietary variable. The
19 difference in waist circumference was correlated ($p<0.05$) with the difference in
20 mmEMV ($r=0.47$). The difference in body mass between phases was correlated ($p<0.05$)
21 with the difference in diastolic blood pressure ($r=0.44$), sE-selectin ($r=0.68$) and CRP
22 ($r=0.56$).
23

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1 **Discussion**

2 The purpose of this study was to determine the influence of a 24 week LCD on EMV and
3 soluble endothelial biomarkers of protein origin in overweight women. $CD31^{+}41^{-}$EMV,
4 sE-selectin, sP-selectin, sTM, SAA and CRP were all reduced as a result of the LCD.
5 These changes were accompanied by a modest reduction in body mass, waist
6 circumference and diastolic blood pressure.

7

8 This dietary intervention targeted a reduction in carbohydrate intake but not the near
9 elimination of carbohydrate from the diet. Participants were not permitted to reduce
10 carbohydrate intake below 40 $g \cdot day^{-1}$ in order to avoid ketogenesis. Otherwise the
11 participants were free to devise their own strategies to help them achieve the reduction in
12 carbohydrate intake. Carbohydrate intake was ~90 $g \cdot day^{-1}$ lower on the LCD compared to
13 the ND. With no meaningful increase in absolute fat or protein intake when consuming
14 the LCD, the resultant daily energy deficit was ~400 kcal. However, the percentage of
15 energy obtained from carbohydrate decreased from 44 % to 28 % with the percentage of
16 energy from fat increasing from 38 % to 47 % and protein from 17 % to 23 %. There was
17 no difference in saturated fat intake between the LCD and ND phases, whether expressed
18 in $g \cdot day^{-1}$ or as a % of total fat intake. The energy deficit over the course of the 24 weeks
19 resulted in a moderate reduction in body mass and waist circumference, respectively.
20 The percentage reduction in body mass was ~5 %, which according to some sources
21 (National Institutes of Health, 1998) is the threshold at which health benefits are
22 observed.

23

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1 This is the first study to demonstrate a reduction in EMV and adds to the body of
2 evidence demonstrating an improvement in cardiovascular risk profile following a
3 reduction in dietary carbohydrate intake leading to moderate weight loss. EMV are
4 emerging as a novel marker of vascular risk in cross-sectional but also in prospective
5 studies (Lee *et al.*, 2012; Nozaki et al., 2009) where they can add to risk prediction. This
6 reduction in $CD31^{+}CD41^{-}$ EMV was accompanied by a reduction in soluble endothelial
7 markers and acute phase inflammatory proteins. The difference in the mmEMV subset
8 between diet phases did not reach statistical significance. There is evidence that specific
9 EMV subsets exist and respond differently to cell activation and apoptosis (Jimenez *et*
10 *al.*, 2003). Two other studies have examined changes in EMV following bariatric surgery
11 induced weight loss. Paradoxically, Cheng and colleagues (Cheng et al., 2013)
12 demonstrated a reduction in $CD144^{+}$ EMV (CD144 was part of our EMV multimarker
13 panel that was unchanged by our LCD) while Stephanian and colleagues (2013) reported
14 no change in $CD31^{+}41^{-}$ EMV.
15
16 Of the various circulating MV populations, those of endothelial origin are typically the
17 most difficult to enumerate. This may relate to the low abundance in the circulation of
18 EMV above the noise of the instrument and/or to the low expression of endothelial-
19 specific markers on the MV membrane. $CD31^{+}$ EMV are the most widely reported in the
20 literature, but the $CD31^{+}$ EMV assay must include a platelet-specific marker to exclude
21 PMV, as CD31 is also abundantly expressed on platelets. Unlike CD42b, sometimes
22 employed in EMV research for this purpose, the expression of CD41 on platelets is
23 relatively stable and not affected by platelet activation triggers, hence our definition of

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EMV as $CD31^{+}41^{-}$ events. A novel strategy that avoids the need to gate out PMV in a two colour assay is the monochrome multimarker assay proposed by Duval and colleagues (2010). This employs more than one endothelial-specific antibody, all tagged with the same fluorochrome to increase the signal of EMV bearing weakly expressed markers. Using this monochrome multimarker approach, Duval *et al*. reported a 20 fold difference between EMV of lupus patients and healthy controls. Although the use of single endothelial-specific markers is the ideal, this has not to date yielded clear EMV populations in our laboratory with the technology available. The lack of consensus on how to quantify EMV is a limitation in the field. There is scope for methodological research, possibly corroborating flow cytometric methods with ELISA-based MV assays or investigating other endothelial-specific markers such as thrombomodulin (Martin *et al.*, 2014).

The effects of the LCD on protein markers of endothelial activation and acute phase inflammatory proteins are broadly in line with the results of a small number of other studies in this field (Davis et al., 2011; Keogh et al., 2008; Sharman & Volek, 2004; Wycherley et al., 2009) with sE-selectin, sP-selectin, sTM, SAA and CRP lower at the end of the LCD phase. Previous studies have reported reductions in sTM (Porreca et al., 2004) and SAA (Yang *et al.*, 2006) following a traditional hypocaloric diet leading to weight loss, though to our knowledge, this is the first study to demonstrate their reduction following a LCD intervention. Soluble P-selectin is present on both endothelial cells and platelets and the source of the LCD difference is unclear. Regardless, these changes are consistent with the cross talk that occurs between endothelial cells, platelets and

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1 leukocytes that occur at all stages of atherosclerotic disease (Siegel-Axel & Gawaz,
2 2007). Contrary to other studies (Davis et al., 2011; Keogh et al., 2008; Sharman &
3 Volek, 2004; Wycherley et al., 2009), there was no difference in sICAM-1 during the
4 LCD and ND phases. Crucially however, no marker measured was higher during the
5 LCD phase. Differential responses of soluble selectins and cell adhesions molecules to
6 diet and pharmacological intervention are common in the literature (Abe *et al.*, 1998;
7 Eschen *et al.*, 2004; Hackman *et al.*, 1996; Wycherley *et al*., 2009) with sVCAM-1 the
8 least likely to be reduced. Hackman and colleagues (1996) speculated that the much of
9 sICAM-1 and sVCAM-1 may arise not from the endothelium but from leukocytes and
10 vascular smooth muscle cells, which do not always respond in the same way to treatment.
11
12 The mechanisms underpinning the reduction in EMV during the LCD phase are unclear.
13 The reduction in EMV and other markers were accompanied by a modest reduction in
14 body mass and waist circumference. Adipose tissue, particularly visceral adipose tissue
15 is known to release pro-inflammatory cytokines such as tissue necrosis factor alpha
16 (TNF-α) that stimulate the release of EMV (Brown *et al.*, 2011). Although not measured
17 in this study, TNF-α is known to be reduced following weight loss (Dandona *et al.*,
18 1998). These cytokines can also stimulate the production of the acute phase proteins
19 CRP and SAA, with both proteins lower in the LCD phase of this study. A correlation
20 was observed between the difference in body mass and the differences in CRP and sE-
21 selectin between diet phases, with a correlation also observed between the differences in
22 waist circumference and mmEMV. Cheng and colleagues (Cheng et al., 2013) reported
23 a considerable reduction in EMV one month following bariatric surgery coinciding with a

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1 10% reduction in body mass, though there were no further reduction in EMV at 12
2 months, despite another 10% reduction in body mass. The timecourse for reductions in
3 EMV may therefore not always mirror reductions in body mass. Hypertension is
4 associated with elevated EMV (Chen *et al.*, 2011), consistent with the mechanosensitive
5 properties of endothelial cells. However, the small difference in diastolic blood pressure
6 between dietary phases in this study was not correlated with the difference in EMV.
7 Changes in macronutrient intake are other possibilities that could potentially influence
8 EMV release, independent of weight loss. High glucose can induce oxidative stress in
9 endothelial cells triggering EMV release (Jansen *et al.*, 2013), with EMV elevated in
10 diabetes (Leroyer *et al.*, 2008). Although carbohydrate intake was reduced considerably
11 during the LCD phase, fasting serum glucose was not different between phases however.
12 It is not clear if a reduced carbohydrate intake can reduce endothelial stress in the absence
13 of changes in fasting glucose. Further research is clearly needed to elucidate the
14 mechanisms by which a LCD, leading to moderate weight loss, can lower EMV. In
15 particular, it would be intriguing to examine the influence of a LCD on EMV and other
16 endothelial biomarkers where body mass is maintained via increases in fat and protein
17 intake.
18
19 The difference in EMV and other markers cannot be attributed to differences in blood
20 lipids as no blood lipid or apolipoprotein variable was significantly different between the
21 LCD and ND conditions. The absence of any absolute increase in fat, saturated fat and
22 protein intake during the LCD phase may be relevant here. The effects on EMV of
23 VLCD that result in pro-atherogenic changes in blood lipids have yet to be determined.

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1 As considerable biomarker reductions can occur in the absence of any change in blood
2 lipids, these results also highlight the need to go beyond blood lipids when evaluating
3 dietary interventions.
4
5 This study has a number of limitations. The study design did not involve a washout
6 between trial phases with the mid-trial blood sample representing the end of the initial
7 phase (ND or LCD phase) and the start of the next phase. This was in part due to the
8 absence of data indicating the duration of a wash out necessary following the LCD phase
9 to return biomarkers to pre-study concentrations but also a desire not to prolong the study
10 beyond 48 weeks. Twenty-four weeks (duration of second phase) back on normal diets
11 was likely to be sufficient however for a return to normal metabolic and endothelial
12 profiles in those who were randomised to undertake the LCD phase first. Crucially, there
13 was no evidence of carryover between phase 1 and phase 2, with no significant
14 differences in ND – LCD delta scores in those first randomised to the ND and those first
15 randomised to the LCD. As a result, the effects of the LCD were determined simply by
16 comparing participant values at the end of their LCD and ND phases. The results of this
17 study do not generalise to all LCD. With a mean carbohydrate intake of 87 $g \cdot day^{-1}$,
18 representing 29% of daily energy needs, this LCD was not particularly extreme, though
19 still reducing carbohydrate intake by ~90 $g \cdot day^{-1}$. More extreme ketogenic diets may
20 have adverse effects on endothelial health, regardless of the magnitude of weight loss.
21
22 In conclusion, a diet that reduces carbohydrate intake by ~90 $g \cdot day^{-1}$ so that carbohydrate
23 accounts for ~30% of total daily energy intake, results in modest reductions in body

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1 mass, waist circumference and blood pressure accompanied by reductions in
2 $CD31^{+}CD41^{-}$ EMV and multiple other endothelial inflammatory biomarkers. It does not
3 increase any endothelial biomarker. Though the precise mechanisms involved are
4 unclear from this study, the changes in endothelial markers can occur in the absence of
5 any change in blood lipids.
6
7

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Conflict of Interest

None

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Authorship

ALW, OOD, KSC and MH formulated the research question. MH and LD were responsible for the study design. ALW, DF, JPP and MDR were responsible for data collection and sample analysis. ALW and MH analysed the data. AW, MH, OOD and KSC prepared the manuscript. All authors approved the final manuscript version.

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Table 1 Daily energy, carbohydrate, protein, fat and saturated fat intake during the normal diet and low carbohydrate diet phases (n=24)

	Normal diet	**Low carbohydrate diet**
Energy (kcal)	1626 ± 86	1228 ± 58 *
Carbohydrate (g)	179 ± 11	87 ± 7 *
Protein (g)	71 ± 3	71 ± 3
Fat (g)	70 ± 5	66 ± 4
Saturated fat (g)	27 ± 2	23 ± 1

**p*<0.05 compared to Normal diet*

Table 2: Body composition and blood pressure following 24 weeks on a normal diet and 24 weeks on a low carbohydrate diet (n=24)

	Normal diet	**Low carbohydrate diet**
Body mass (kg)	73.8 ± 1.8	70.1 ± 1.8*
Body mass index ($kg \cdot m^{-2}$)	27.1 ± 0.6	25.7 ± 0.6 *
Waist circumference (cm)	85.8 ± 0.5	82.3 ± 0.6 *
Systolic blood pressure (mmHg)	130 ± 2	127 ± 3
Diastolic blood pressure (mmHg)	84 ± 2	79 ± 2*

**p*<0.05 compared to Normal diet*

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Table 3: Endothelial and inflammatory biomarkers following 24 weeks on a normal diet and 24 weeks on a low carbohydrate diet (n=24)

	Normal diet	Low carbohydrate diet
$CD31^{+}41^{-}$EMV (events·µL^{-1})	29.9 ± 4.0	20.9 ± 3.0*
mmEMV (events·µL^{-1})	57.0 ± 7.8	43.9 ± 6.8†
sE-selectin (ng·mL^{-1})	13.1 ± 1.4	11.9 ± 1.4*
sP-selectin (ng·mL^{-1})	59.9 ± 6.4	52.3 ± 5.7*
sICAM-1 (ng·mL^{-1})	307 ± 26	294 ± 37
sVCAM-1 (ng·mL^{-1})	320 ± 18	320 ± 14
sThrombomodulin (ng·mL^{-1})	3.62 ± 0.23	3.39 ± 0.22*
sICAM-3 (ng·mL^{-1})	1.25 ± 0.18	1.21 ± 0.17†
CRP (mg·L^{-1})	1.49 ± 0.31	1.14 ± 0.24*
SAA (ng·mL^{-1})	2556 ± 570	1546 ± 415*

**p<0.05 compared to Normal diet. †p=0.08 compared to Normal diet. Values are mean ± SEM. EMV = endothelial microvesicles, mmEMV = monochrome multimarker EMV and represents microvesicles positive for CD105, CD144 or CD146. Samples were stained with these three endothelial specific monochrome antibodies in order to increase EMV counts, sICAM-1 = soluble intercellular adhesion molecule 1, sVCAM-1 = soluble vascular cell adhesion molecule, CRP = C-reactive protein, SAA = serum amyloid A*

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Table 4 Serum lipids and apolipoproteins following 24 weeks on a normal diet and 24 weeks on a low carbohydrate diet (n=24)

	Normal diet	Low carbohydrate diet
Apolipoprotein A1 (mg·dL^{-1})	138 ± 3	141 ± 4
Apolipoprotein B (mg·dL^{-1})	112 ± 3	110 ± 4
Cholesterol (mmol·L^{-1})	5.5 ± 0.1	5.4 ± 0.1
HDL-cholesterol (mmol·L^{-1})	1.67 ± 0.07	1.66 ± 0.07
LDL-cholesterol (mmol·L^{-1})	3.3 ± 0.1	3.2 ± 0.2
NEFA (mmol·L^{-1})	0.69 ± 0.06	0.61 ± 0.03
Triglycerides (mmol·L^{-1})	0.92 ± 0.06	0.90 ± 0.10
Glucose (mmol·L^{-1})	5.25 ± 0.07	5.26 ± 0.12

Values are mean ± SEM. HDL = high density lipoprotein; LDL = low density lipoprotein; NEFA = Non-Esterified Fatty Acid

Figure legends

Figure 1: Percentage of total daily energy intake obtained from carbohydrate, fat and protein when following a normal diet (ND) and a low carbohydrate diet (LCD)

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Supplementary data legend

Figure 2: Representative flow cytometric plots of monochrome multimarker EMV and $CD31^{+}41^{-}$ EMV

Plot A, FS vs. SS plot of EMV with size gate (E) < 1.0 μm and above the noise of the instrument established using sizing beads. Subsequent plots B, C, D and E are gated on size gate (E). Plots B and C, monochrome multimarker EMV (in J2) expressing CD144, CD146 and/or CD105 and negative control sample containing IgG1-PE. Plots D and E, $CD31^{+}41^{-}$ EMV (quadrant X4) and negative control sample containing IgG1-PECy5.

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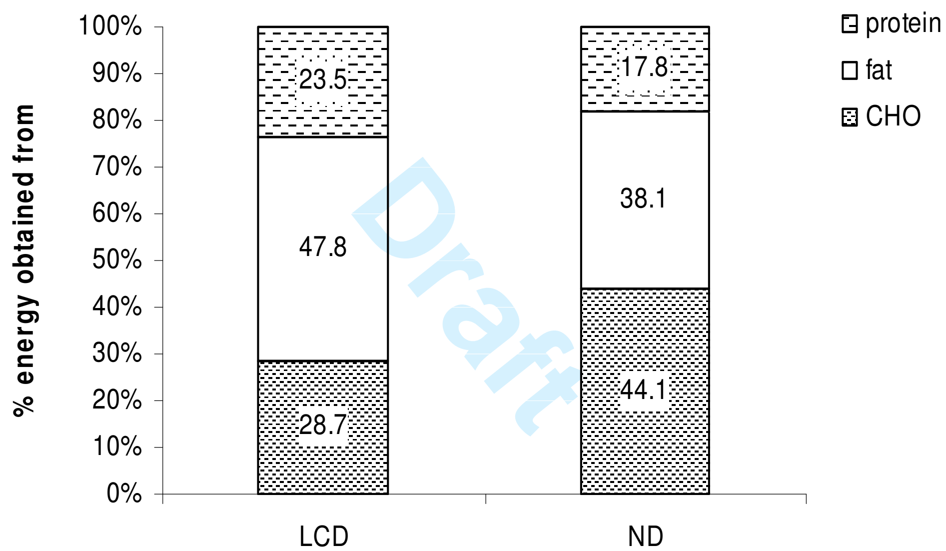

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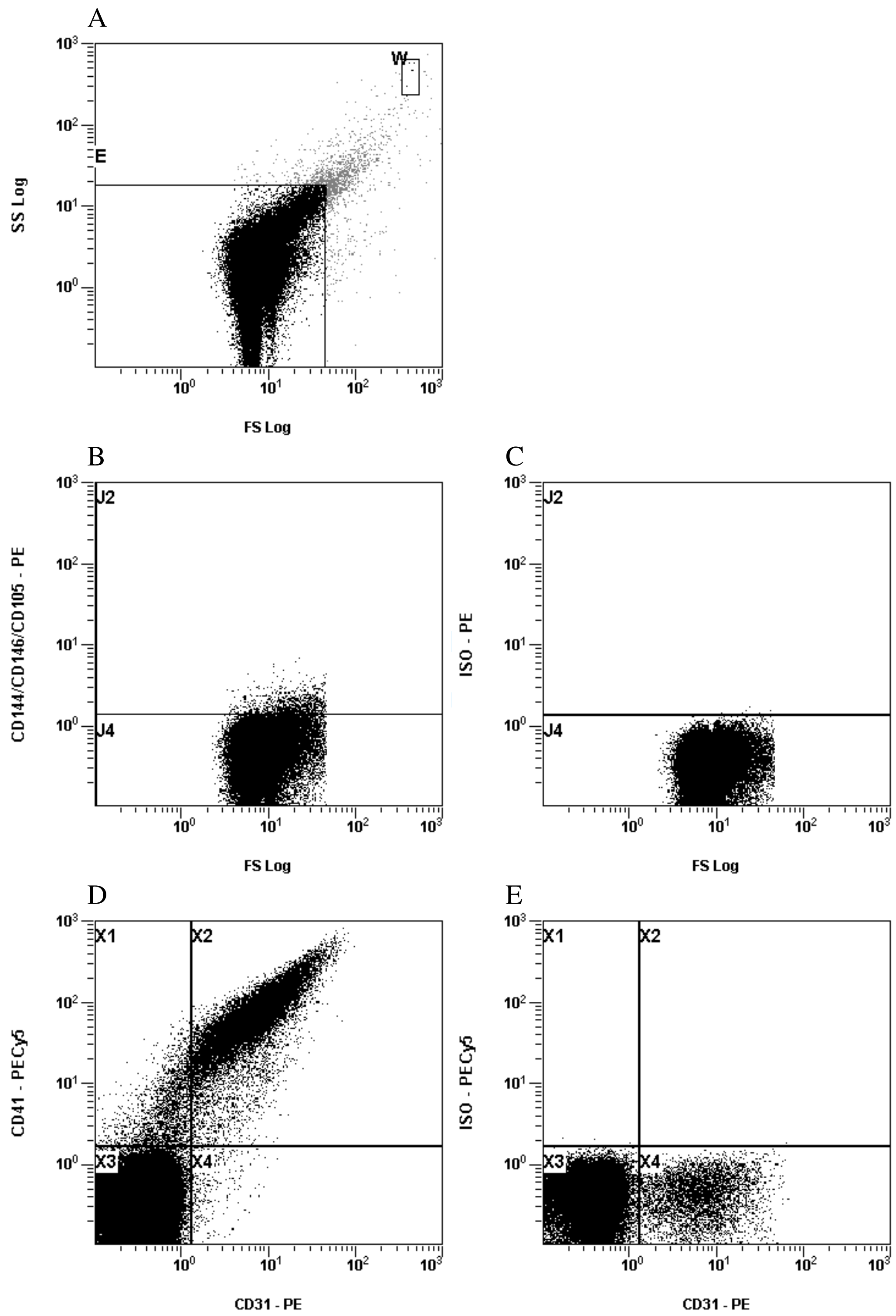
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CD31 - PE