Title: Postprandial vascular-inflammatory and thrombotic responses to high-fat feeding are augmented by manipulating the lipid droplet size distribution

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ABSTRACT

Background and Aims: Postprandial responses are influenced not only by the type and amount of fat ingested, but also lipid droplet size distribution. However, little research has investigated the impact of differential lipid size distributions within a mixed-macronutrient meal context on postprandial vascular health. Therefore, we examined whether manipulating the lipid droplet size distribution within a mixed-macronutrient meal impacts vascular-inflammatory and thrombotic parameters. **Methods and Results:** In a randomised and counterbalanced fashion, sixteen adults (8 males; age 34±7 years; BMI of 25.3±4.5 kg/m^2) completed three separate fasted morning-time feeding challenges, each separated by a minimum washout of 7-days. On each occasion, test-meals matched for carbohydrate and protein content differing only in fat amount and the lipid droplet size distribution were administered, such that participants consumed (1) a low-fat meal (**LF**) with negligible fat content, (2) an emulsified-high-fat meal with a fine lipid droplet size (**FE**), or (3) an emulsified-high-fat meal with a coarse lipid droplet size (**CE**). Periodic blood samples were retrospectively analysed for plasma triglycerides, tumour necrosis factor alpha (TNFα), tissue factor (TF), fibrinogen, and plasminogen activator inhibitor-1 (PAI-1). Triglyceride concentrations increased rapidly overtime under **FE** (*P-time*<0.05); this rise was attenuated under **CE** (*P-time*>0.05) and was comparable to **LF** (*P-condition*>0.05). Similarly, **FE** induced a significant rise in TNFα, TF, fibrinogen, and PAI-1 (*P-time*<0.05); these parameters remained unchanged under **LF** and **CE** (*P-time*>0.05). **Conclusion**: A high-fat mixed-macronutrient meal with a larger lipid droplet size distribution ameliorates the associated rise in vascular-inflammatory and thrombotic parameters. **Trial registration:** ISRCTN88881254.

INTRODUCTION

The chronic consumption of processed foods – rich in calories and saturated fats – promotes a pro-inflammatory and hypercoagulable state [1-2] predisposing to vascular complications [3-5]. Indeed, in humans a single high-fat meal has been shown to increase inflammatory mediators and invoke a pro-thrombotic response in a dose-dependent manner [6-11]. Furthermore, in patients with established atherosclerosis, a single high-fat meal has been reported to trigger acute coronary events [12].

Factors affecting postprandial lipaemia are numerous, and include meal macronutrient and micronutrient composition, as well as fatty acid saturation, chain length, and their physiochemical structure [13]. The latter significantly influences lipid bioaccessibility and metabolism and has been shown to impact postprandial inflammation and circulating adhesion molecules [14], independent of meal fatty acid composition [15]. Fats of various molecular species are incorporated in food products under different physiochemical structures [16]. Previous work demonstrates that isoenergetic meals matched for nutrient composition yield divergent postprandial effects when foodstuffs differ by fat structuring [16], with in vitro studies showing that the size and interface of lipid droplets play an important role in determining the magnitude of postprandial lipaemia, by influencing solubilisation and digestion [17-18]. In humans, emulsification of fat and alteration of the lipid droplet size has been shown to have acute effects on metabolic responses, notably, lipaemia, glycaemia, and insulinemia [19]. However, it is not known how the size distribution of lipid droplets affects parameters of vascular-inflammation thrombosis. Moreover, previous work is limited in that studies have omitted the oral processing stage or delivered test-meals in the form of an isolated or co-ingested beverage-based emulsion [19-26]. This an important consideration which

limits real-world applicability given the potential influence of oral sensory stimulation [27] and that many processed high-fat foods comprise of emulsions *within* mixed-macronutrient meals [28]. We therefore hypothesised that manipulating the lipid droplet size distribution within a mixed-macronutrient meal could modulate postprandial lipaemia and thus vascular-inflammatory and thrombotic factors with a more pronounced effect in a small versus large lipid droplet size distribution.

METHODS

This study is an investigator-initiated, double-blind randomised controlled trial with crossover design. Ethical approval was granted by the Research Ethics Committee at the University of Leeds, with study procedure conducted in accordance with the declaration of Helsinki and all participants providing written informed consent prior to enrolment. The study was conducted between February 2019 and December 2020 at the University of Leeds and is registered at ISRCTN.com with the study identifier: ISRCTN88881254.

Study Population

Sixteen adults (8 males) aged 34±7 years (range: 25-51 years), with a BMI of 25.3±4.5 kg/m^2 (range 22.9-28.5 kg/m^2), who were normoglycaemic (fasting glucose: 5.1±0.3), normolipidaemic (fasting triglycerides: 0.92±0.34), and normotensive (systolic blood pressure 119±2; diastolic blood pressure 81±2) participated in this study. Participants were eligible to participate if they were free of any aliments and/or not currently taking medication relating to gut mobility or digestion, an eating disorder or dietary allergies, a haematological, cardiovascular (including dyslipidaemia), metabolic, or psychiatric

disorder, and had maintained a stable weight for 3-months and were not currently taking non-steroidal anti-inflammatory drugs (NSAIDs).

In a randomised and counterbalanced fashion, participants underwent three laboratory-based experimental conditions, each separated by a minimum washout of 7-days. We used a validated online diet programme developed at the University of Leeds (MyFood24) [29] to capture self-report dietary intake and eating patterns in the 48-hours preceding each participant's first laboratory visit. This information was used to replicate dietary intake and mealtimes across conditions. During this time, participants were instructed to reframe from alcohol and caffeine consumption, abstain from strenuous physical activity, and were provided with a standardised meal to consume on the evening before each laboratory visit. The standardised meal comprised of a pre-prepared vegetarian lasagne (Energy = 2.4MJ; Fat = 11.8g; Carbohydrate = 93.4g; Protein = 25.4g) and participants were instructed to consume this meal no later than 20:00PM on the evening before each laboratory visit, replicating mealtime across study arms.

On each experimental condition, participants arrived at the laboratory on the morning (08:00-09:00AM) having adopted an overnight fast. Upon arrival, participants adopted a seated position, and a resting fasted blood sample was obtained via an indwelling catheter. Participants then consumed one of three mixed-macronutrient experimental test meals with further periodic blood sampling at 30-minute intervals. Blood samples at each timepoint were retrospectively analysed for plasma triglycerides, with tumour necrosis factor alpha (TNFα), tissue factor (TF), fibrinogen, and plasminogen activator inhibitor-1 (PAI-1) analysed at baseline, 180-minutes, and 360-minutes using methods

75 previously described [30]; the intra-assay coefficient for all analyses was <10%. During
76 laboratory stays, participants were instructed to remain seated and rested in an upright
77 position with ad libitum water intake recorded on the first visit and replicated on
78 subsequent visits.
79
80 Each test-meal was matched for carbohydrate and protein content differing only in the
81 amount of fat and the lipid droplet size of the fat, such that participants consumed (1)
82 a low-fat meal (**LF**) with negligible fat content, (2) an emulsified-high-fat meal with a
83 fine lipid droplet size (**FE**), or (3) an emulsified-high-fat meal with a coarse lipid droplet
84 size (**CE**). The macronutrient contribution to each meal is presented in Table 1; all
85 meals were based on the composition of a pasta dish consisting of penne pasta,
86 tomato sauce, and olive oil (Tesco, UK). The amount of each food item was identical
87 in each condition such that carbohydrate (69 g) and protein (11 g) content were
88 standardised. **FE** and **CE** included the addition of an absolute amount of 50 g of olive
89 oil (olive oil; Tesco, UK) in the form of an 80 g emulsion differing only in the size
90 distribution of lipid droplets. Our intention was that both meals would be visually
91 identical, but that deliberate manipulation of the lipid droplet size contained within the
92 meal would impact lipid bioaccessibility thus impacting the postprandial triglyceride,
93 inflammatory, and thrombotic response.
94
95 The emulsion consisted of a 30 g protein solution containing 26 g distilled water and
96 4 g whey protein isolate (Fonterra Limited; 96.3 wt% protein). The protein solution was
97 mixed for 120 minutes on a magnetic stirrer until complete protein absorption, as
98 indicated by a clear solution. Following which, an absolute amount of 50 g of olive oil
99 was added and then homogenised using a high-shear rate mixer at 7000 rpm for 2

minutes. For **FE**, the emulsion was further homogenised through a high-pressure homogeniser at 250 bar (first stage) and 50 bar (second stage) for 2 passes at room temperature. The lipid droplet size distributions were measured using a Mastersizer 2000 based on a laser light scattering technique; 10 ml of distilled water was prepared in a flask and 1ml of the emulsion was added and mixed, creating a diluted solution for viewing under an optic microscope at 20 μm which confirmed differing lipid droplet sizes between **CE** and **FE** (Figure 1). For **LF**, the 30 g protein solution was added and mixed manually via light stirring, in addition to 50 ml of water to ensure meals were isovolumetric and matched protein content. For both test-meals, care was taken when integrating the emulsions into the sauce component of the meal as to not impact the lipid droplet size or distribution of the emulsion. Given the impact of time on lipid droplet size and distribution of the emulsions (Figure 1), each emulsion was added to the test-meals and consumed within 30-minutes of emulsion preparation to ensure consistency of emulsion stability.

In absence of data regarding the impact of lipid droplet size on vascular-inflammatory and thrombotic biomarkers, the primary outcome measure was the triglyceride incremental area under the curve (AUC) from 0 to 360-minutes postprandially. A total number of 16 participants was sufficient to detect 5% differences in triglyceride AUCs between conditions, with a power of 95% at $P<0.05$, given a standard deviation of the studied variable of at 29%. We calculated the achieved power across vascular-inflammatory and thrombotic variables; a sample size of 16 was sufficient to achieve 5% differences in AUCs between conditions, with a minimum power of 82%.

Data was analysed using SPSS software version 24 (IBM SPSS Statistics, IBM Corp) and is presented as mean±SD unless otherwise stated. The trapezoid rule [31] was used to calculate the total AUC for all metabolites. Following checks for normality, a repeated measures ANOVA was employed to investigate time, condition, and time-by-condition interactions with significant interactions explored using Bonferroni-corrected post-hoc comparisons for time-course data. A one-way ANOVA was used to assess conditional differences in postprandial AUC for each metabolite. A *P*-value of <0.05 was considered statistically significant.

RESULTS

The droplet size distribution of **FE** and **CE** are shown in Figure 1A-D. **FE** had an average *D*[4,3] of 18.88±0.98 μm and ζ-potential of -55.8±6.14 mV, and **CE** *D*[4,3] of 12.79±2.87 μm and ζ-potential of -54.1±65.3 mV. The characteristics of both emulsions showed good stability over 4-hours.

Biochemical responses are presented in Figures 2, 3A-E, and 4A-E. There was a significant time (P<0.001) condition (P<0.001), and time-by-condition interaction (P<0.001) effect for plasma triglycerides (Figure 4A). **FE** induced a significant increase in plasma triglycerides with peak concentrations recorded at 180-minutes post-meal (P-time<0.05), whereas concentrations remained largely unchanged under **LF** in which a peak was not observed (P-time>0.05; Figure 4A). Resultantly, total AUC was significantly greater under **FE** compared to **LF** (**FE:** 460±181 vs. **LF:** 219±61 mmol/Lhr^{-1}, P<0.001; Figure 3, 4A). Plasma triglycerides concentrations increased temporally under **CE** (P-time<0.05; Figure 4A), however this rise was largely attenuated as demonstrated by a lower AUC (**FE:** 460±180 vs. **CE** 338±69

mmol/L.hr^{-1}, P<0.001; Figure 2, 3A), a lower peak concentration (**FE:** 1.86±0.65vs. **CE:** 1.20±0.31 mmol/L, P=0.001; Figure 4A), and a prolonged time to peak (210-minutes; Figure 4A).

There was a significant time, condition, and time-by-condition interaction effect for TNFα, TF, fibrinogen, and PAI-1 (P<0.05, for all analyses; Figure 4B-E). **FE** induced a significant rise in TNFα, TF, fibrinogen, and PAI-1 at 180-minutes and 360-minutes post-meal (P-time<0.05), whereas responses under **CE** remained unchanged throughout the observation period (P-time>0.05) and were comparable to responses elicited under **LF** (P-condition>0.05; Figure 4). Resultantly, total vascular-inflammatory and thrombotic exposure was greatest under **FE**, with **CE** lower and comparable to **LF** (Figures 2 and 3). There was a significant inter-individual variability observed between **FE** and **CE**, as expressed as the magnitude of change (CV% triglycerides AUC: 124%; CV% TNFα AUC: 65%; CV%TF AUC: 25%; CV% fibrinogen AUC: 180%; and CV% PAI-1 AUC: 135%).

DISCUSSION

This is the first in-human study to test and observe that manipulating the lipid droplet size within a high-fat mixed-macronutrient meal impacts postprandial vascular-inflammatory thrombotic markers. Specifically, we demonstrate that a high-fat meal with a smaller lipid droplet size induces a sustained pro-vascular-inflammatory and prothrombotic milieu. In contrast, manipulating the structure of the fat component within the meal to feature a coarse emulsion characteristic of a large lipid droplet size completely abates this rise in vascular-inflammatory and thrombotic parameters such that the response is comparable to a meal with negligible fat content. These findings

have important implications for the food industry and public health as we demonstrate that manipulating fat structure within a mixed-macronutrient meal representative of foods widely and frequently consumed, can impact the detrimental postprandial vascular response associated with high-fat foods.

It is generally well accepted that a single high-fat meal induces a pro-vascular-inflammatory and prothrombotic response in a dose-dependent manner[6-11], and that repeated exposure to such insults promotes a hypercoagulable state[1-2] and predisposes to vascular damage[3-5]. As such, nutritional recommendations typically focus on the amount and type of dietary fat, but little consideration is given to the manner in which fat is structured [32-35]. Our data show that the postprandial vascular-inflammatory and thrombotic response to a high-fat mixed-macronutrient meal is significantly influenced by the physiochemical structure of the meal independent of fatty acid composition. As such, our findings carry important considerations for both the food industry and the consumer in terms of food processing and daily dietary decisions. We show that manipulating lipid droplet size and distribution within mixed-macronutrient meals, which are representative of those widely and frequently consumed in real-life, can significantly impact postprandial vascular health. Historically, food processing has been considered important in terms of enhancing the sensory properties of food and for nutrient release and bioavailability, and to a lesser extent, health. However, the present study demonstrates that altering food processing techniques may also play an important role in reducing the vascular health impacts of mass-produced processed food products which are generally considered unhealthy.

198 Given that both high-fat meals in the present study were isovolumetric and matched
199 for macronutrient composition, the attenuated triglyceride response elicited under **CE**
200 as compared to **FE**, demonstrates a clear divergence in lipid bioaccessibility kinetics
201 and metabolism between the two test meals, which, we postulate drove the
202 exacerbated vascular-inflammatory response. Elevated and sustained postprandial
203 lipaemia promotes increased high-density lipoprotein clearance and the formation of
204 atherogenic low-density lipoprotein particles [36]. This process increases
205 subendothelial retention of lipoproteins inducing activation of the vascular endothelium
206 as manifested by the increased expression of pro-vascular-inflammatory and
207 prothrombotic mediators [1-2]. This enhanced thrombotic environment contributes to
208 poor clinical outcomes in people at increased risk of vascular complications [37].
209 Namely, increases in TF activity upregulates production of thrombin accelerating the
210 risk of clot formation [38], raised fibrinogen concentrations, reflective of low-grade
211 vascular inflammation, contributes to formation of denser clots, and increased PAI-1
212 levels impair the fibrinolytic process [38]. Although these haemostatic changes have
213 been reported previously in response to a single high-fat load in at-risk populations
214 [39], to the best of our knowledge, this is the first study to report these haemostatic
215 aberrations in response to fat *structure* and independent of the amount of fat ingested.
216 Further, the fact that we detected notable differences between meal types in our
217 relatively young and healthy participants, free of known cardiovascular and
218 haemostatic abnormalities, raises the intriguing possibility that manipulating the
219 structure of fat within commonly consumed processed foods may translate to
220 improvements in vascular risk in more compromised populations such as diabetes,
221 and atherosclerosis [40-41].
222

From this initial exploratory study, we cannot at this stage claim that manipulating the lipid droplet size within high-fat mixed-macronutrient meals definitively improves vascular health and future prospective studies with larger cohorts in at-risk populations with different meal compositions should focus attempts on addressing this aim. For example, the composition of olive oil consists primarily of oleic acid (~80%) with smaller amounts of other fatty acids including linoleic acid and palmitic acid, and varying with the cultivar, extraction process, time of harvest, and heating [42]. Therefore, foods with different fatty acid profiles may elicit different postprandial lipaemic and inflammatory response [43]. Further, the relative contribution of carbohydrate, fat, and protein also impact postprandial metabolic inflammatory responses and this is likely mediated by the population studied, the disease and nutritional status of individuals [44-50]. A limitation of this study is the low sampling frequency; more frequent samples would have enabled a more comprehensive temporal profile of our chosen parameters, and a longer observation window would have enabled us to determine at what point our chosen response variables reached resolution to baseline. Further, owing to our sampling methods, it was not possible to directly measure platelet aggregation and clot lysis, and we were unable to verify whether changes in lipid droplet size or distribution occurred following integration of the emulsion into the test-meal due to the composition and viscosity of the meal. However, given that little agitation occurred during meal preparation, and that we observed profound differences in our outcomes between experimental conditions, it is likely that any changes in lipid size and distribution following incorporation of the emulsions to test-meals were negligible and that they had little impact on study findings. To facilitate serial blood sampling, we utilised an indwelling catheter, which may have caused local inflammation. However, owing to the randomised cross-over

248 design of this study, participants were exposed to this factor across all three study
249 arms. Moreover, the fact that we did not observe any changes in our chosen vascular-
250 inflammatory and thrombotic biomarkers under the low-fat condition would suggest
251 that this had a negligible effect on our findings. This study has a number of strengths,
252 including its rigorous crossover design, an extended observation window as compared
253 to previous studies, the isocaloric and isovolumetric matching of high-fat test-meals,
254 and tight inclusion criteria to limit potentially confounding factors.
255
256 In conclusion, we present the first in-human study to show that manipulating the lipid
257 droplet size within a high-fat mixed-macronutrient meal impacts postprandial vascular-
258 inflammatory and thrombotic markers. We show that a high-fat meal with a smaller
259 lipid droplet size induces a sustained pro-vascular-inflammatory and pro-thrombotic
260 milieu, whereas a large lipid droplet size completely attenuates the rise in vascular-
261 inflammatory and thrombotic parameters similarly to a meal with negligible fat content.

COMPETING INTERESTS

Nothing to disclose

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FIGURES

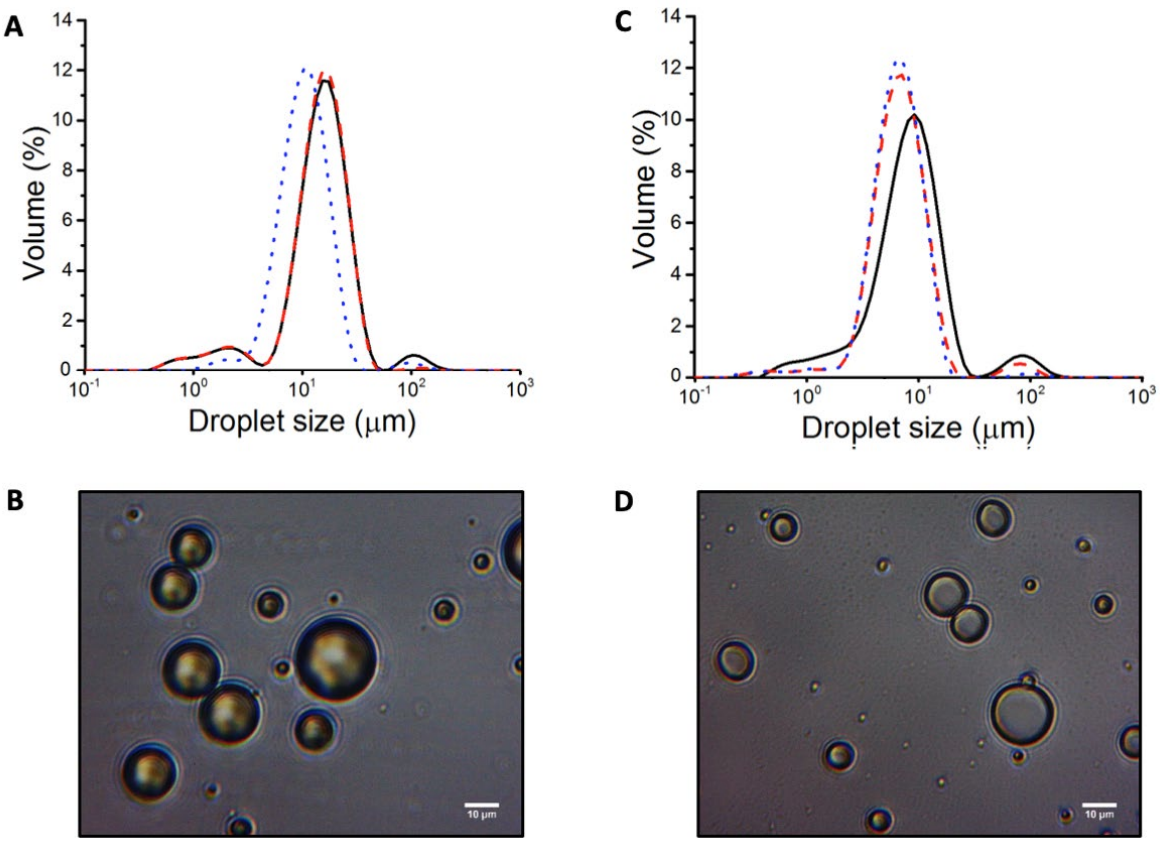

Figure 1. Droplet size and distribution of **CE** and **FE** emulsions using laser light scattering microscopy. Panels A-B: Droplet size and distribution of **CE**; Panels C-D: Droplet size and distribution of FE. Solid black trace = droplet size distribution of fresh emulsion; broken red trace = droplet size distribution of emulsion at 4-hours; broken blue trace = droplet size distribution of emulsion at 12-hours.

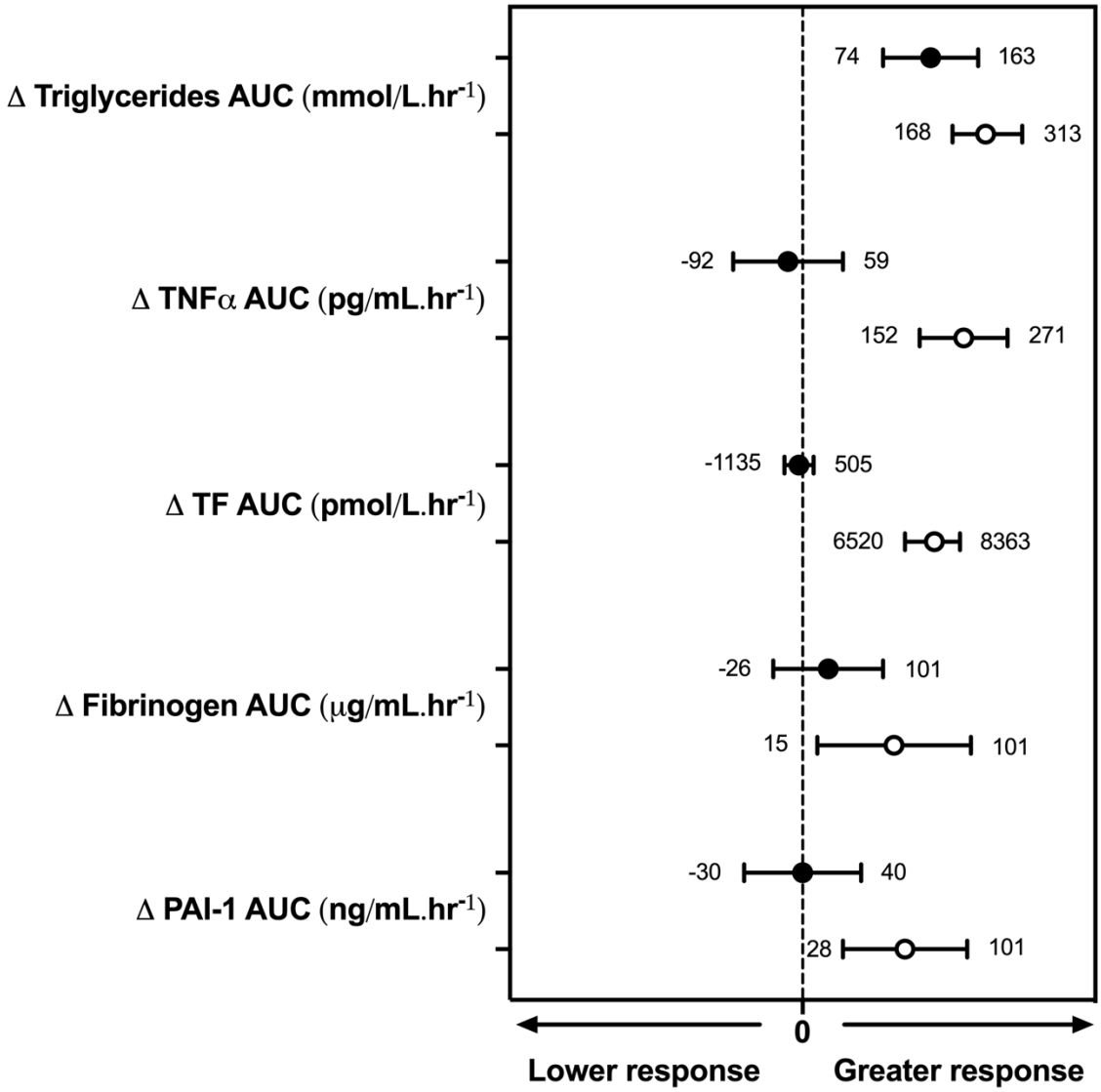

Figure 2. Postprandial responses in biochemical parameters to high-fat mixed macronutrient meals differing only in lipid droplet size distribution. Shown is the estimate difference in treatment effect as a change from **LF** (circle) accompanied with the 95% confidence interval (CI; bars). Numbers indicate absolute range of CI. Solid circles = **CE**; Open circles = **FE**.

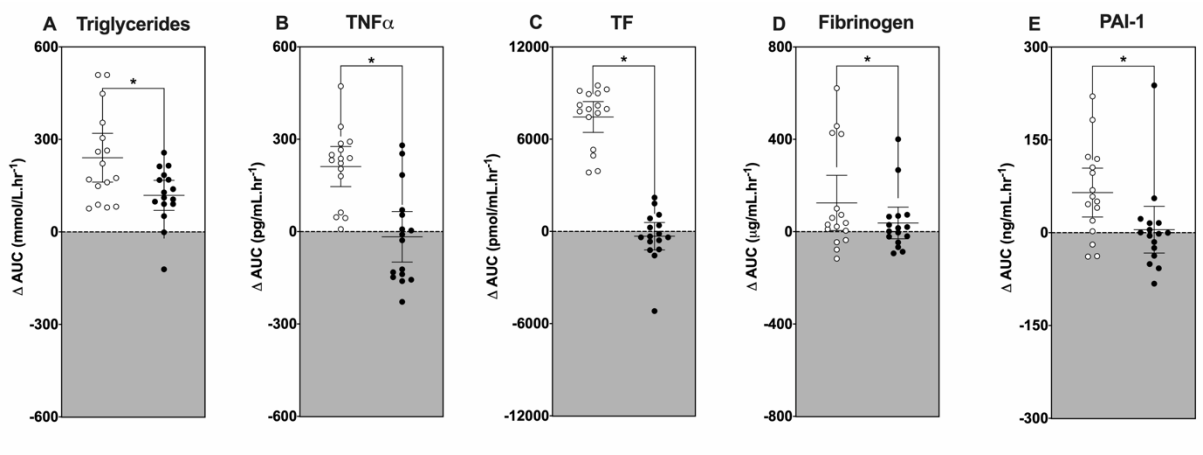

Figure 3. Proportionate change in postprandial biochemical parameters to high-fat mixed macronutrient meals differing only in lipid droplet size distribution relative to a low-fat meal control. Data represents individual participant-level data presented as mean 95% ± confidence interval (CI; bars). Solid circles = **CE**; Open circles = **FE**. * indicates a statistically significant difference between **CE** and **FE** ($P<0.05$).

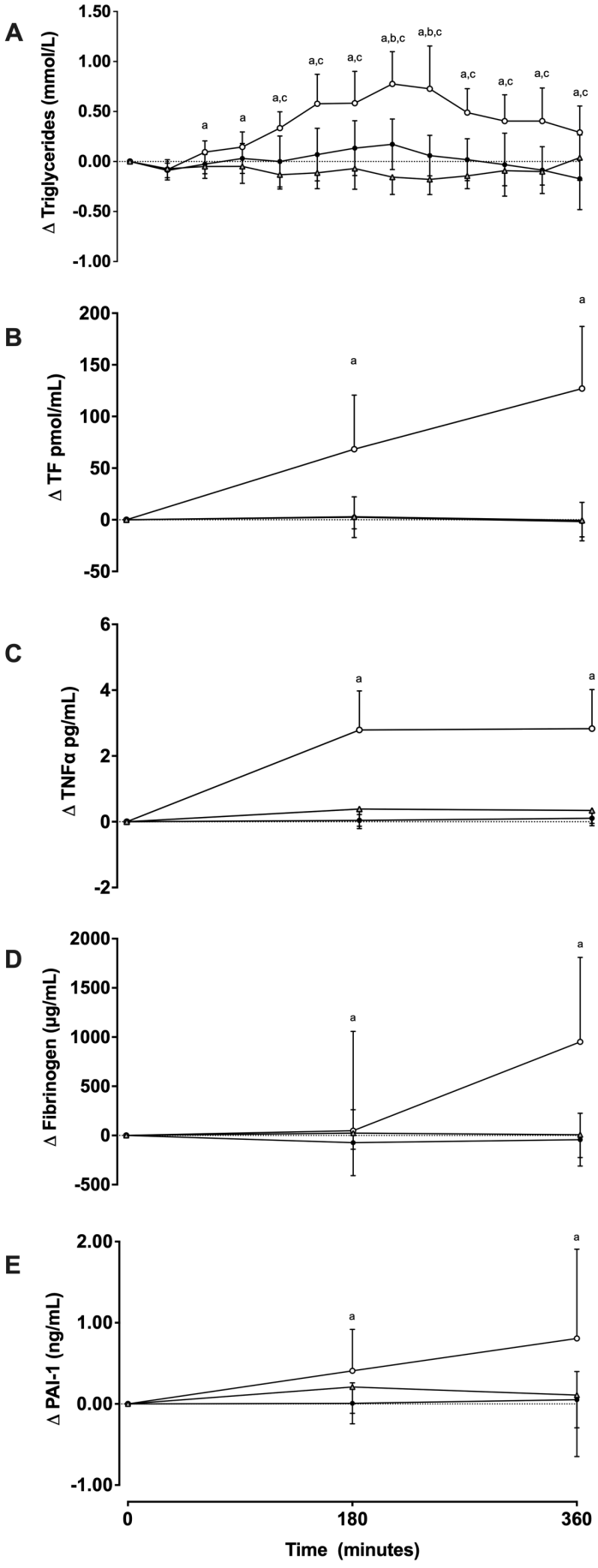
A
Δ Triglycerides (mmol/L)
1.50
1.00
0.50
0.00
-0.50
-1.00
a
a
a,c
a,c
a,c
a,b,c
a,b,c
a,c
a,c
a,c
a,c
B
Δ TF pmol/mL)
200
150
100
50
0
-50
a
a
C
Δ TNFα pg/mL)
6
4
2
0
-2
a
a
D
Δ Fibrinogen (µg/mL)
2000
1500
1000
500
0
-500
a
a
E
Δ PAI-1 (ng/mL)
2.00
1.00
0.00
-1.00
a
a
0
180
360
Time (minutes)

Figure 4. Time-course changes in postprandial biochemical parameters to high-fat mixed macronutrient meals differing only in lipid droplet size distribution relative to a low-fat meal control. Data shown as mean ± SD. Solid circles = **CE**; Open circles = **FE**; grey triangles = LF. a = indicates **FE** is significantly different to **LF**; b indicates **CE** is significantly different to **LF**; c indicates a significant difference between **CE** and **FE** ($P<0.05$).