Prandial Hypertriglyceridemia in Metabolic Syndrome is due to an Overproduction of both Chylomicron and VLDL Triacylglycerol

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Postprandial triacylglycerol kinetics

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Abstract

The aim was to determine whether fed very low density lipoprotein (VLDL) and chylomicron (CM) triacylglycerol (TAG) production rates are elevated in metabolic syndrome (MetS). Eight men with MetS (BMI 29.7± 1.1) and 8 lean age matched healthy men (BMI 23.1± 0.4) were studied using a frequent feeding protocol. After 4 hours of feeding an intravenous bolus of $^2$H$_5$-glycerol was administered to label VLDL1, VLDL2 and CM TAG. $^{13}$C glycerol tripalmitin was administered orally as an independent measure of CM TAG metabolism. Hepatic and intestinal lipoproteins were separated by an immunoaffinity method. In MetS, fed TAG and the increment in TAG from fasting to feeding were higher (p=0.03, p<0.05 respectively) than in lean men. Fed CM, VLDL1 and VLDL2 TAG pool sizes were higher (p=0.006, p=0.03, p<0.02) and CM, VLDL1 and VLDL2 TAG production rates were higher (p<0.002, p<0.05, p=0.06) than in lean men. VLDL1, VLDL2 and CM TAG clearance rates were not different between groups. In conclusion prandial hypertriglyceridemia in men with MetS was due to an increased production rate of both VLDL and CM TAG. Since both groups received identical meals this suggests that in MetS the intestine is synthesising more TAG de novo for export in CMs.
Introduction

Abnormally elevated blood triacylglycerol (TAG) levels in the postprandial period is a feature of MetS and is predictive of an increased risk of cardiovascular disease (CVD)(1,2,3). Hypertriglyceridemia is due to excess triglyceride-rich lipoproteins (TRL); very low-density lipoproteins, (VLDL) synthesised by the liver which contain the higher molecular weight form of apolipoprotein (apo)B, apoB100 and chylomicrons (CMs) which are synthesised in the intestine in response to an intake of dietary fat, which contain the lower molecular weight form of apoB, apoB48. Both VLDL and CMs share a common lipolytic pathway and are hydrolysed by lipoprotein lipase (LPL), an enzyme predominantly found on the endothelial surfaces of the capillaries of adipose tissue, heart and skeletal muscle (4). Postprandial hypertriglyceridemia in MetS may be due to the overproduction of intestinal or hepatic TRLs, impaired clearance or a combination of both. Insulin resistance is associated with a postprandial increase in apoB48 particles (5) and some studies suggested that this is due to impaired catabolism of intestinally derived TRL and remnant lipoprotein TAG (6,7). However the small intestine is capable of utilising endogenous substrates for TAG synthesis. Duez et al (8) have used a constant feeding protocol combined with an infusion of a labelled leucine to measure apoB48 kinetics, a surrogate measure of intestinal TRL kinetics, in men with a range of insulin sensitivity. The study showed that insulin resistance was associated with increased intestinal apoB48 production rate. In addition diet-induced insulin resistance in the Syrian golden hamster has been shown to be associated with a marked increase in intestinal lipoprotein production rate in both the fasting and the fed states (9) which could be reduced by treatment with rosiglitazone (10). Since insulin resistance is associated with
elevated non esterified fatty acid (NEFA) flux from adipose tissue the mechanism that leads to apoB48 overproduction may be increased delivery of NEFAs to the enterocyte since an acute elevation of NEFAs in hamsters has been shown to increase basal intestinal apoB48 production (11).

A number of studies have shown that VLDL apoB100 and VLDL TAG production rate is increased by insulin resistance (12,13) in the fasted state but no studies have measured VLDL TAG production rate quantitatively, in the fed state, in MetS. We have recently demonstrated that intravenously administered $^{2}H_{5}$ glycerol is incorporated into CM TAG and can be used to measure both CM and VLDL TAG kinetics in a frequent feeding study (14). In the present study we have used this methodology to investigate whether the greater increase in postprandial TAG in MetS compared to lean healthy subjects is due to an increase in CM and/or VLDL TAG production rate or a decrease in clearance rate. We hypothesised that an increased synthesis of both CM and VLDL TAG in MetS would be the major cause of the increased postprandial TAG.
Methods

Participants

This study was approved by the East Kent NHS Research Ethics Committee and University of Surrey Ethics Committee. Written informed consent was provided by all participants prior to inclusion in the study. Eight men with MetS and eight age matched lean healthy men were studied. MetS was defined as central obesity (increased waist circumference ≥ 94 cm) plus any two of the following four factors: triglycerides ≥ 1.7 mmol/L, HDL cholesterol <1.03 mmol/L, systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or fasting plasma glucose ≥ 5.6 mmol/L. With the exception of one subject, all subjects could be defined as having MetS without using the BP criteria. This subject had raised waist circumference, raised blood glucose and plasma TG at screening was 1.65 mmol/l. All men had an apoE3E3 genotype. The exclusion criteria were a diagnosis of diabetes, hepatic and renal disorders, fasting plasma triglyceride >5 mmol/L, cholesterol > 7 mmol/L, use of statins, fibrates or metformin.

Experimental Design

All subjects underwent an 11-hour feeding study after an overnight fast. Waist circumference was measured around the umbilicus. Body composition was measured using Tanita BC-418 Scales (Tokyo, Japan). An indwelling cannula was inserted into an antecubital vein for blood sampling. Baseline samples were taken to measure the fasting lipid profile, glucose and insulin. All subjects were fed 6 high fat (83.3%; 23% saturated, 33% monounsaturated, 24% polyunsaturated fatty acids), low carbohydrate (12.2%) and low protein (4.5%) liquid meals, 497kcal per meal at two hourly intervals (at -240, 120, 0, 120, 240 and 360 minutes). The aim of the feeding protocol was to increase fasting plasma TAG levels 2 fold and to maintain this for the duration of the study. This feeding protocol was successful at achieving steady state TAG levels. The
first 4 hours (-240-0 minutes) were to enable a TAG steady state to be achieved prior to administration of the tracers. The meal, composed of 125ml milk shake (Tesco, UK), 20ml double cream, 15ml olive oil and 20ml sunflower oil, was prepared as an emulsion. Each unit meal was prepared immediately prior to consumption and consumed within 1 minute by the subject. Tripalmitate ($^{13}$C$_3$ glyceryl) (10mg/kgBW) was mixed with the 3rd meal administered at 0 min. An intravenous bolus of 750mg $^2$H$_5$-glycerol was also administered at 0 min. Blood samples were then taken at intervals for 420 minutes for the measurement of plasma glycerol enrichment, Svedberg (Sf)>60 and Sf 20-60 lipoprotein TAG enrichment and concentration.

**Lipoprotein separation**

Lipoproteins were separated by sequential flotation ultracentrifugation to isolate S$_f$>60 (CMs and VLDL1), S$_f$ 20-60 (CM remnants and VLDL2), in a fixed angle rotor 50.4Ti (Beckman, USA) using a LE80-K ultracentrifuge (Beckman Coulter Optima, USA) as previously described (14).

**Isolation of endogenous and exogenous lipoproteins**

ApoB100 and apoB48 containing lipoproteins were separated using a sequential immunoaffinity binding method as previously described (14). Monoclonal antibodies to apoB100, 4G3, 5E11 and Bsol16 (Heart Institute, University of Ottawa, Canada) were coupled individually to protein G sepharose 4 Fast Flow (Amersham, UK) as previously described (14). After ultracentrifugation, lipoprotein fractions were loaded immediately onto the 4G3-matrix and incubated overnight. Then after centrifugation to separate the unbound and bound lipoproteins, the supernatant was loaded on to the 5E11-matrix, incubated at 4°C overnight, then centrifuged and the supernatant loaded
onto the Bsol16-matrix and incubated at 4°C overnight. The unbound fraction
(apoB48 containing lipoprotein) in the final Bsol16-matrix separation was separated
by centrifugation. The bound fractions from each separation on the 4G3-, 5E11- and
Bsol16- matrices (apoB100 containing lipoproteins) were combined.

Measurement of lipid and hormone concentrations
Enzymatic assays were used to measure fasting and fed plasma NEFA, total
cholesterol, plasma TAG, the total fraction TAG (Sf>60 and Sf 20-60) and the CM
fraction TAG (Sf>60 and Sf 20-60) (ABX, Chicksands, Shefford, Bedfordshire, UK)
using a Cobas MIRA (Roche, Welwyn Garden City, UK). The CM fraction TAG was
concentrated 7 fold prior to measurement. VLDL1 TAG concentration was calculated
as the difference between the total Sf>60 TAG concentration (measured prior to
antibody separation) and the Sf>60 CM TAG concentration. VLDL2 TAG was
calculated as the difference between the total Sf 20-60 TAG concentration and the Sf
20-60 CM TAG concentration. HDL-cholesterol was measured on a Bayer Advia
1650 analyzer (Bayer, Newbury, United Kingdom). Insulin, adiponectin, and leptin,
were measured by immunoassay using commercially available kits (Millipore
corporation, Billerica, MA, USA).

Isotopic enrichment determination
TAG in the lipoprotein fractions isolated by immunoaffinity chromatography was
extracted in chloroform: methanol (2:1, vol%) and separated by thin-layer
chromatography, then hydrolysed with 3%HCl in methanol (vol%) at 50°C overnight.
Glycerol was then purified by ion-exchange chromatography and concentrated by
freeze-drying overnight (15). For analysis of plasma free glycerol, samples (0.5ml)
were deproteinised and purified by ion-exchange chromatography. Freeze-dried glycerol was derivatised to glycerol triacetate (15), and enrichment measured by gas chromatography mass spectrometry (GC-MS) in PCI mode with methane as the reagent gas. Ions were monitored at $m/z$ 159 and at $m/z$ 164 (m+5). Enrichment of the CM remnants ($S_t$ 12-20) with $^2$H$_5$-glycerol was below the detection limit of the assay in most participants.

The $^{13}$C/$^{12}$C isotope ratio determination of oral glycerol tracer was performed with a Trace GC Ultra with auto sampler AS3000, coupled to an isotope ratio mass spectrometer Delta Plus XP (Thermo Electron, Corp, USA) via an oxidation reactor, reactor temperature 960°C and a combustion interface III (Thermo Electron Corporation, Bremen, Germany). Enrichment of the CM remnants ($S_t$ 12-20) with $^{13}$C-glycerol was below the detection limit of the assay in most participants.

**Data analysis**

VLDL1, VLDL2 and CM TAG kinetics were calculated using a mathematical model as previously described (14). A single pool model was used to describe CM TAG kinetics and a two pool model to describe VLDL1 and VLDL2 TAG kinetics using the SAAM II program (SAAM Institute, Seattle, WA). The models represent the kinetics of the tracer-to-tracee ratio (TTR) profiles which change as labelled glycerol is removed from plasma and incorporated into the TAG fractions. The models included a delay pathway for the incorporation of glycerol into VLDL TAG by the liver and CM TAG in the intestine. The VLDL TAG model included a compartment for VLDL1-TAG and a compartment for VLDL2-TAG with an input into both compartments from the glycerol precursor pool, a loss from each compartment and a
transfer from the VLDL1-TAG compartment to the VLDL2-TAG compartment. In a steady state, the fractional secretion rate (FSR) is equal to the fractional catabolic rate (FCR), a measure of clearance. The production rate was calculated from the product of the FSR and the TAG pool size. TAG pool size was calculated from the TAG concentration of the bound and unbound fraction multiplied by plasma volume. Plasma volume was calculated as described by Pearson et al (16). Fasting insulin sensitivity was assessed by homeostatic model assessment (HOMA-IR) (17).

Statistical analysis
Results are presented as mean±SEM. Data were analysed using SPSS 16 (SPSS inc; Chicago USA). Non-parametric data were logarithmically transformed before statistical analysis. Analysis of concentrations during frequent feeding was by repeated measures ANOVA. The increment in plasma TAG from fasting to feeding was calculated as the difference between the mean TAG during frequent feeding and fasting plasma TAG. Comparison of measurements in the two groups was by unpaired two-tailed t-test. A P value lower than 0.05 was considered to be significant.
Results

Body composition, insulin sensitivity and fasting lipid profile (Table 1)

Body weight, BMI, waist circumference and fat mass were higher in the men with MetS than the healthy lean men (all p<0.001). Fat free mass was not different between groups. Glucose and insulin concentration and HOMA-IR were higher in MetS (p=0.01, p=0.05, p=0.03). Fasting plasma TAG was higher and HDL cholesterol was lower (p=0.04, p<0.04) but plasma cholesterol, and NEFAs were not significantly different in MetS.

Prandial concentrations

Plasma TAG, NEFA, glucose and insulin during frequent feeding are shown in Figure 1. Plasma TAG, Sf>60 TAG and Sf 20-60 TAG were not significantly different between 0 and 420 minutes in either group. During feeding mean plasma TAG, plasma glucose and serum insulin were significantly higher in the men with MetS compared with the lean men (p=0.03, p=0.01, p=0.05 respectively). The increment in plasma TAG from fasting to frequent feeding was also higher in the men with MetS (2.94±0.87 mmol/l) than in the lean men (0.88±0.19 mmol/l) (p=0.04). Plasma NEFA concentration did not differ between the 2 groups during feeding.

CM enrichment profile with the oral $^{13}$C-glycerol tracer

The enrichment of the $^{13}$C glycerol tracer in the CM and VLDL1 TAG is shown in Figure 2. The AUC for CM TAG enrichment was not different in the two groups. $^{13}$C glycerol enrichment in VLDL1 TAG was <8% of CM enrichment 150 minutes after tracer administration in the lean men demonstrating that the immnoaffinity method was efficiently separating the CM and VLDL fractions. There was a small rise in
enrichment of VLDL1 TAG with $^{13}$C glycerol during the remainder of the study. In the men with MetS, $^{13}$C glycerol tracer enrichment in VLDL1 TAG, measured as AUC, was higher than in the lean men (p=0.05).

**VLDL and CM kinetics (measured with an intravenous $^2$H$_5$-glycerol tracer)**

Plasma glycerol enrichment was not different in the 2 groups (data not shown). The enrichment of CM TAG, VLDL1 and VLDL2 TAG, with the iv tracer, $^2$H$_5$-glycerol, in the lean men and men with MetS is shown in figure 3, figure 4a and 4b respectively. In the men with MetS CM, VLDL1 and VLDL2 TAG production rates were higher (p<0.002, p<0.05, p=0.057) (Figure 5) and CM, VLDL1 and VLDL2 TAG pool sizes were higher (p=0.006, p=0.03, p<0.02) than in the lean men. There was no significant difference between the 2 groups in VLDL1, VLDL2 and CM TAG FCR, a measure of clearance.

**Discussion**

In men with MetS the increment in plasma TAG in response to frequent feeding was considerably higher than in age matched healthy subjects despite both groups receiving identical meals. The higher fed TAG level in MetS was due to a higher production rate of VLDL1, VLDL2 and CM TAG rather than reduced clearance. Previous studies have shown that prandial VLDL and CM particle production rate, measured by tracer labelling of apolipoproteinB100 and B48 respectively, is increased in insulin resistant subjects (18, 8). However these studies did not measure the kinetics of the TAG substrate within the particle. In the present study we show for the first time that prandial VLDL and CM TAG production rate is increased using in vivo tracer labelling of TAG in MetS.
The oral tripalmitin tracer was labelled on the glyceryl moiety and previous studies suggest that approximately 75% of this would be absorbed as monoacylglycerol (MAG) and resynthesized into TAG with the remaining 25% being completely hydrolysed to glycerol and NEFA (19). The amount of oral tracer administered was determined by body weight, so was 27% higher in the men with MetS. However the enrichment (tracer:tracee ratio) of CM TAG with this tracer was similar in the two groups demonstrating that there was greater dilution of this tracer with unlabelled TAG in the CM of men with MetS. This suggests that more intestinal TAG were being exported in CM which was derived from a non-oral route in these subjects. A greater production rate of CM TAG in MetS was confirmed from the kinetic measurements made using the $^2$H$_5$ intravenous tracer. There are several possible sources of this non-oral TAG a) TAG synthesised from NEFA and glycerol imported from the circulation b) TAG synthesised from NEFA generated within the enterocyte from glucose (de novo lipogenesis; DNL) or c) the release of TAG or exchange of labelled MAG from the meal with unlabelled MAG, from pre-existing storage pools as shown in Figure 6.

Previous studies have shown that an acute increase in NEFA levels in healthy subjects increases apoB48 production rate (20). Interestingly in the present study neither fasting nor prandial NEFA concentrations were different in the two groups suggesting this was not the driver for a higher synthesis rate of CM TAG in MetS. While the contribution of DNL to hepatic TAG synthesis is well known (21), it is only recently that this pathway has been demonstrated to be important in enterocytes, and in an insulin resistant hamster model enterocyte DNL was shown to be increased (9, 22). In
the liver DNL is an insulin- and glucose-dependent process under the control of specific transcription factors, sterol regulatory element binding protein 1c (SREBP-1c), which is activated by insulin and carbohydrate response element binding protein (ChREBP) which is activated by glucose (23). ChREBP is also expressed in the small intestine and the elevated glucose in MetS may be responsible for an increase in DNL and CM TAG production rate.

Both indirect and direct evidence exists supporting the presence of cytoplasmic TAG storage pools in enterocytes, so an elevated number or size of these pools in MetS may also be a contributing factor to the increased CM TAG export. Storage pools of TAG have been demonstrated in mice enterocytes and the TAG content was found to directly correlate with the amount of TAG consumed (24). In humans, sequential meal tests have shown that CMs secreted after a second meal carry TAG ingested in the first meal (25). Since mammalian enterocytes turn over every 3 to 4 days these can only be very short term stores (26). Higher food intake in the subjects with MetS could lead to larger TAG stores. To determine whether this source of TAG makes a major contribution to the elevated CM production rate, dietary intake would need to be fixed at the same level in the 2 groups of subjects for 3 days prior to the kinetic study.

It has been clearly demonstrated that fasting VLDL TAG production rate is increased in insulin resistance (13). To the authors knowledge only one previous study has measured VLDL TAG production rate after feeding in insulin resistant subjects. In that study a constant infusion of ex vivo labelled VLDL TAG was used and VLDL-TAG secretion rate was shown to be higher in men with type 2 diabetes than healthy
men (27). Although HOMA-IR was not reported in the latter study patients with type 2 diabetes would be expected to be more insulin resistant than the subjects with MetS in the current study. The higher VLDL TAG production rate after feeding in MetS in the current study may be due to an increase in imported NEFA, DNL or TAG storage pools as discussed for the enterocyte. In addition the liver will take up recycled TAG from TRL including CM. The enrichment of VLDL1 TAG with \(^{13}\)C glycerol was very low initially but gradually increased with time indicating recycling of CM remnants to the liver as demonstrated previously (28). That this was higher in MetS is also supported by the study of Hodson et al (28) which showed 3.5 fold higher recycling of CM remnants in insulin resistant subjects compared to insulin sensitive subjects. In non-alcoholic fatty liver disease, the hepatic manifestation of MetS, 15% of hepatic TAG was from the diet ie recycled CM TAG, while DNL was shown to account for 26% (29). However insulin resistance measured by HOMA-IR was considerably higher than in the subjects with MetS in the current study. Hepatic DNL has been shown to be increased in subjects with insulin resistance (30, 31) and has been directly linked to glucose and insulin levels (23). Fasting VLDL TAG production rate has also been shown to be correlated with hyperglycaemia (32).

Insulin has been shown to suppress both apoB48 and apoB100 production rate during feeding in healthy subjects (33, 34) and this inhibitory effect is blunted in type 2 diabetes (35). Fasting VLDL1 TAG production rate has also been shown to be inhibited by insulin with a reduced effect in insulin resistance (36). The increased CM and VLDL TAG production rate in the presence of elevated prandial insulin levels in the men with MetS also suggests an impaired effect of insulin. Impaired insulin
signalling has been demonstrated in enterocytes and hepatocytes from an insulin resistant animal model (22).

While some previous studies have suggested that CM clearance is impaired in insulin resistance (7, 37) and thus responsible for the elevated postprandial TAG, CM TAG clearance rate was not different in the two groups in the current study. This may be due to the different study design or less insulin resistance. In these previous studies (7, 37) either a large oral fat load or an intravenous CM like emulsion was administered. TRL apoB48 FCR has been shown to be similar in healthy subjects and subjects with insulin resistance studied with a continuous feeding protocol (8). The mean half-life for CM TAG (Sf>60) in the current study can be calculated from the FCR to be 72 minutes in the healthy subjects and 86 minutes in MetS. This is considerably longer than the half-life for CM TAG (Sf>400) previously reported to be 5-6 minutes in healthy subjects (38). In the density Sf>400, CM are approximately 340nm in diameter. In the Sf>60 density CM will include particles of this size but also particles as small as 40nm and this can explain the longer half-life. It has been reported that larger particles are cleared faster than smaller ones (39). The FCR for CM apoB48 in the density range Sf>20 has been reported to be 5.0 pools/day (a half-life of 200 minutes) which is lower than in the current study, but a similar order of magnitude (40).

The FCR for VLDL1 TAG in the two groups was in the range of previously reported measurements in fasting studies (32, 41). VLDL TAG clearance was not impaired in the fed state in MetS in the current study. This supports the findings of a single meal study in healthy men and men with type 2 diabetes which showed that although the
basal fasting FCR was lower in men with type 2 diabetes the postprandial FCR was not different in the 2 groups (27).

A limitation of this study was that the percentage of fat in meal was considerably higher than would be expected in a physiological meal. Further studies need to be undertaken to confirm the findings of the study using a more physiological meal feeding protocol.

In conclusion prandial hypertriglyceridemia in men with MetS was due to an increased production rate of both VLDL and CM TAG. Since both groups received identical meals this suggests that in MetS the intestine is synthesising more TAG de novo.
Acknowledgements

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References


Table 1. Subject characteristics (mean±SEM)

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* 2 subjects were on anti-hypertensive medication
Figure legends

Figure 1. a) Plasma TAG  b) plasma NEFA c) plasma glucose and d) serum insulin in healthy subjects (○) and subjects with MetS (●) during frequent feeding (mean ± SEM).

Figure 2. $^{13}$C$_3$-glycerol enrichment in CM TAG (♦) and VLDL1 TAG (■) in a) healthy subjects and b) subjects with MetS (mean ± SEM).

Figure 3. $^2$H$_5$ glycerol enrichment in CM in healthy subjects (○) and subjects with MetS (●) (mean ± SEM).

Figure 4. $^2$H$_5$ glycerol enrichment in VLDL1 (♦) and VLDL2 TAG (■) in a) healthy subjects and b) subjects with MetS (mean ± SEM).

Figure 5. a) TAG PR b) TAG FCR and TAG pool size for VLDL1, VLDL2 and CM in healthy subjects (grey columns) and subjects with MetS (black columns) (mean ± SEM).

Figure 6. Schematic of CM TAG synthesis in enterocytes showing labeling of TAG with oral $^{13}$C glycerol and intravenous $^2$H$_5$ glycerol. A. TAG synthesis from dietary NEFA and MAG, B. TAG synthesis from endogenous (circulating) NEFA and glycerol, C. TAG synthesis from glycerol generated from glucose, D. TAG incorporation into CM from TAG stores. MAG (monoacylglycerol); CM (chylomicron); B48 (apolipoprotein B48); TAG (triacylglycerol)