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Endothelial inflammation correlates with subject triglycerides and waist size following a high fat meal

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TGRL modulates EC inflammation

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Abstract

A rise in postprandial serum triglycerides (PP-sTG) can potentiate inflammatory responses in vascular endothelial cells (ECs) and thus serve as an independent risk factor for predicting increased cardiovascular morbidity. We examined postprandial triglyceride-rich lipoproteins (PP-TGRLs) in subjects ranging from normal to hypertriglyceridemic for their capacity to alter EC acute inflammatory responses. Cultured human aortic ECs (HAECs) were conditioned with PP-TGRLs, isolated from human serum at the peak following a moderately high-fat meal. Very low density lipoprotein (VLDL) particle size increased postprandially and varied directly with subject's PP-sTG level and waist circumference (WC). PP-TGRL particles bound to HAEC and were internalized via LDL receptor-mediated endocytosis. PP-TGRL alone did not induce an inflammatory response over the range of individuals studied. However, combined with low dose TNFα stimulation (0.3 ng/mL), it elicited a net 10-15% increase above cytokine alone in membrane expression of VCAM-1, ICAM-1, and E-selectin, which was not observed with fasting TGRL. In contrast to upregulation of ICAM-1 and E-selectin, VCAM-1 transcription and expression varied in direct proportion with individual PP-sTG and WC. The extent of monocyte arrest on inflamed HAEC under shear stress also correlated closely with VCAM-1 expression induced by conditioning with PP-TGRL and TNFα stimulation. This *ex vivo* approach provides a quantitative means to assess an individual's inflammatory potential, revealing a greater propensity for endothelial inflammation in hypertriglyceridemic individuals with abdominal obesity.

Key Words: atherosclerosis, dyslipidemia, triglyceride rich lipoprotein, VCAM-1, TNFα

Introduction:

Atherosclerosis has been postulated to be a postprandial phenomenon (27, 36), promoted by repetitive exposure to transiently elevated serum triglycerides (sTG) (1). Epidemiological studies have established postprandial serum triglycerides (PP-sTG) as an independent risk factor for cardiovascular disease (CVD) (26), and demonstrated the strongest correlation with adverse cardiovascular events at the postprandial peak(2). The triglyceride spike following a high-fat meal is associated with transient endothelial dysfunction, notably the impairment in flow-mediated vasodilatation (1, 32), which precedes atherosclerotic lesion formation (22). However, our understanding of the cellular and molecular level events in endothelium that link epidemiological risk factors for CVD such as PP-sTG with the early mechanisms driving atherogenesis is limited.

Triglyceride-rich lipoproteins (TGRLs) constitute a heterogeneous group of particles differing in their lipid and protein components, which in turn determine particle size, density, receptor interactions and metabolism (17). Biochemical studies of TGRL have revealed compositional changes concomitant with the postprandial spike in sTG, more profoundly in hypertriglyceridemic (HTG) subjects. These include enrichment of cholesterol, ApoE, ApoCI and ApoCIII and depletion of ApoCII with decreased VLDL ApoB catabolism (3, 5). However, there is a lack of understanding as to how individual heterogeneity in TGRL can impact endothelial inflammation contributing to atherogenesis. Moreover, it is unclear the extent to which early inflammatory changes in endothelium are reflected by current clinical measurements used to assess cardiovascular risk.

We previously established an *ex vivo* model that assessed the inflammatory potential of an individual's TGRL by examining perturbations in endothelial cell (EC) response to TNFα, a cytokine expressed locally at sites of atherosclerotic plaque formation(28), and a biomarker of inflammation that correlates with risk of CVD(6, 19). We studied the effects of conditioning human aortic EC (HAEC) in a repetitive manner over several days with native PP-TGRLs isolated from normal triglyceridemic (NTG) subjects after a high-fat meal, reporting an increase in VCAM-1 upregulation by TNFα that resulted in elevated efficiency of monocyte recruitment under shear stress (31). Here we extend the previous study to interrogate the inflammatory potential of TGRL derived from a cohort of subjects ranging from NTG to HTG at the peak in sTG following a single high-fat meal. We hypothesized that the acute response of HAEC to simultaneous stimulation with an individual's PP-TGRL superposed with low dose TNFα would provide an *ex vivo* measure of the influence of anthropometric characteristics and metabolic profile on systemic inflammation. We report that PP-TGRLs are not acutely inflammatory to HAEC, but can augment cytokine-induced VCAM-1 expression and associated monocyte recruitment either positively or negatively, in proportion to the level of subject PP-sTG and abdominal obesity.

Materials and Methods:

An expanded Methods section is available online and as previously described (31).

Human subjects: The study included both normal and hypertriglyceridemic (fasting serum triglycerides (F-sTG) > 150 mg/dL) subjects, but excluded those on lipid-lowering or anti-inflammatory medications or with a fasting blood glucose > 110 mg/dL. Volunteers were enrolled according to IRB approved protocols under informed consent. They fasted for 12 hr and consumed a 1230-calorie test meal, moderately high in fat (47% of calories) and saturated fat (32% of total), representative of a western fast-food diet. Standard lipid panels were determined before and 3.5 after the meal, a time point previously demonstrated to correlate with peak levels of PP-sTG(2).

TGRL isolation and characterization: TGRLs (<1.0063g/mL) were isolated by ultracentrifugation (31), quantified for ApoB content (Alerchek), and tested for endotoxin using a chromogenic test kit (Associates of Cape Cod). The lipids were stored under nitrogen at 4ºC and used within 3 days after isolation. TGRL particle size distribution was measured using a Nanotrac Particle Size Analyzer with FLEX software (Microtrac Inc), which uses a controlled reference method to determine particle size based on dynamic light scattering, with a repeatability of 1% for 100 nm polystyrene. Each sample was quantified 5 times with a run time of 2-3 minutes, and the results of the 5 runs were averaged.

Cell culture and treatment protocol: Cryopreserved HAECs derived from a 21-year old female (Genlantis, passage 3) were expanded in collagen coated (50ug/ml, Clontech) culture flasks. Cells were maintained in Endothelial Cell Growth Medium-2

(EGM-2, Lonza) with 10% FBS (Hyclone), and 1x antibiotic/ antimycotic solution (AA, Invitrogen). They were subcultured at ~80-90% confluence per the supplier's instructions, and used for experiments at passage 4-6 (within 15 population doublings). With exceptions noted, HAECs were conditioned with TGRLs (10 mg/dL ApoB) alone or simultaneously with the inflammatory cytokine TNF-α (0.3 ng/mL, R&D system) for 4hrs. All treatments were conducted in complete media (EGM-2 supplemented with 10% FBS and 1x AA). Treatments not receiving TGRL were supplemented with an equal amount of buffer in which the isolated TGRL was suspended (196mM NaCl, 0.3mM EDTA), to compensate for any changes in volume and media composition.

TGRL labeling and confocal imaging: PP-TGRLs were labeled with Alexa-Fluor488 reactive dye (Invitrogen). Total protein content was quantified by modified Lowry assay (Sigma). Excess dye was removed by column chromatography. Uptake of labeled PP-TGRLs was visualized by confocal microscopy. Images were analyzed in ImageJ and integrated fluorescence intensity for each cell in the field was quantified.

Flow cytometry: Cells were detached using an enzyme-free cell dissociation buffer (GIBCO), Fc-blocked, labeled with fluorescein-conjugated antibodies against human E-selectin, ICAM-1, VCAM-1, or isotype-matched IgG control, and analyzed by FACScan flow cytometer (Becton Dickinson) with CellQuest software. Data represents the median fluorescence intensity (MFI) from a single Gaussian population of 10,000 HAECs for each sample.

Cholesterol assay: Cellular cholesterol content was determined using Amplex Red Cholesterol Assay Kit (Invitrogen), a fluorometric method that measures H_2O_2 produced upon hydrolysis of cholesterol esters and the subsequent oxidation of cholesterol. Fluorescence signal was measured by a FLUOstar Optima multifunctional microplate reader (BMG Labtech, UK) and cholesterol concentration determined by reference to a standard curve.

RNA isolation and real time PCR: Total RNA was isolated using the High Pure Total RNA isolation kit (Roche) and converted to 1st strand cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche). qPCR was performed using Taqman Gene Expression Assays and Master Mix (Applied Biosystems) and a RealPlex Mastercycler (Eppendorf). Alternatively, Roche Fast Start Universal SYBR Green Master Mix reagents were used with exon-flanking primers designed using Primer3. Housekeeping genes RPS27a and RPLP0 were screened for their constant expression across the experimental conditions. Relative quantification was determined by the $\Delta\Delta C_t$ method (Taqman) or by reference to standard curves (SYBR).

RNA stability assay: In order to observe the impact of TGRL on message stability, HAECs were preincubated with control media or TNFα (1 ng/ml) for 1 hr to induce a strong, consistent inflammatory gene expression response. Actinomycin D (1 μg/ml) and/or TGRLs (10 mg/dL ApoB) was added at T=0hr. Transcript levels were monitored at 2, 4 and 6 hrs by qPCR.

Monocyte adhesion assay: Healthy normolipidemic (F-sTG < 100 mg/dL) subjects not on any medication were enrolled as monocyte donors according to IRB approved protocols under informed consent. Mononuclear cells were isolated from fasting blood by sedimentation over Lymphosep density separation media (MP). Monocytes were purified by repetitive centrifugation to deplete platelets and a bead-based negative isolation (Invitrogen) to remove other mononuclear cells. Each preparation was examined for purity by flow cytometry after incubating with an Alexa Fluor 488-labeled antibody to the monocytic marker CD14, and used if the number of $CD14^+$ cells exceeded 90%. They were resuspended in HEPES buffer + 0.1% HSA + 1.5 mM $CaCl_2$ at a concentration of 10^6 monocytes/mL, and perfused over preconditioned HAEC monolayers at a shear stress of 1 dyne/cm^2 for 5 min in a parallel plate flow channel(29). The number of arrested monocytes per field was quantified by identifying their phase-bright appearance in the same focal plane as the endothelial monolayer (31). Each data point represents an average of 8 fields per channel, 2-3 channels per condition.

Data analysis: Data were analyzed using GraphPad Prism v5.0 software. In general, multiple groups were analyzed by repeated measure ANOVA and differences assessed by the Student-Newman-Keuls (SNK) post test. Two experimental groups were compared using the Student t-test, pairing where appropriate. Two-tailed p values < 0.05 were considered statistically significant unless otherwise indicated. Correlations between groups were assessed using Pearson's correlation coefficient.

Results:

TGRL particle composition varies with the level of donor HTG and abdominal obesity

In order to investigate the effects of individual heterogeneity in TGRL on EC inflammatory responses, we recruited 61 subjects with normal fasting glucose, representing a broad range in body mass index (BMI, 19.8 – 57.9 kg/m^2), waist circumference (WC, 0.64 – 1.32 m) and F-sTG (41 – 538 mg/dL) (**Supplementary Tables I & II**). Consumption of the test meal led to a 99 ± 66% increase in sTG 3.5 hr postprandially that was significantly reduced by 7 hr (**Figure 1A**). Glucose, LDL and HDL cholesterol levels were modestly decreased at 3.5 hr, while total cholesterol and ApoB100 levels remained constant. The mean diameter of VLDL particles was increased by 26.6 ± 21.8% in response to the meal (**Figure 1B**), and increased in proportion to subject PP-sTG (Pearson r =0.71, P<0.0001, **Figure 1C**) and WC (Pearson r =0.58, P=0.0007, **Figure 1D**). VLDL diameter correlated more weakly with subject's F-sTG, and inversely with postprandial and fasting HDL, but no other anthropometric characteristics or lipid parameters. The results demonstrate that the test meal induced a transient increase in TGRL particle size concurrent with a postprandial spike in sTG, implicating altered TGRL composition that reflects the level of HTG and abdominal obesity.

PP-TGRL uptake by LDLRs increases under inflammation.

Several low density lipoprotein receptors (LDLRs) are constitutively expressed on HAEC, mediating EC uptake of native TGRLs and their remnant particles (RP) by endocytosis(30). We examined PP-TGRL uptake by LDLR-related mechanisms in resting

and cytokine stimulated HAEC using fluorophore-conjugated particles. Fluorescence from bound and internalized particles significantly increased as early as 15 min after adding TGRL, and was distributed in a punctate pattern, consistent with receptor-mediated endocytosis, which was inhibited by 78% in the presence of the LDLR antagonist RAP and not control IgG (**Figure 1E-G**). This binding was associated with a rise in cellular cholesterol content at 4 hr following exposure to PP-TGRL that was increased by 15% in the presence of TNFα (**Figure 1H**). These results demonstrate that PP-TGRL particles are rapidly bound by EC via a LDLR-specific mechanism, and that their internalization is increased under inflammation.

PP-TGRL enhances cytokine-induced surface expression of CAMs.

TNFα is found at pg/ml level in human serum and is elevated after a high-fat meal(6). It is expressed several hundred fold higher in atherosclerotic lesions(28), and plays an important role in atherogenesis by upregulating EC vascular cell adhesion molecules (CAMs), which support monocyte recruitment from the circulation(19, 21). To test the hypothesis that PP-TGRL derived from our subjects would differentially impact endothelial inflammatory responses, PP-TGRLs from each subject were examined for the ability to modulate the TNFα-induced surface expression of CAMs. PP-TGRL alone did not elicit a significant increase in CAM expression in resting HAEC after 4 hr incubation (**Supplementary Figure I**). In fact, on average we observed a small but statistically significant reduction in VCAM-1 (-7.2%) and E-selectin (-9.8%) expression from baseline. However, acute exposure to PP-TGRLs enhanced TNFα-induced surface expression of VCAM-1 (9.6%), ICAM-1 (13.4%) and E-selectin (13.2%). These data demonstrate that

acute exposure of HAECs to PP-TGRLs isolated at their peak after a single high-fat meal can modulate inflammatory response to cytokine.

TGRL-enhanced, cytokine-induced VCAM-1 expression correlates with subject WC and PP-sTG.

While PP-TGRL on average enhanced CAM expression in response to TNFα in our subject pool, we observed significant inter-individual variability in its priming capacity. This reflects the compositional nature of a subject's TGRLs rather than a dosage effect, since each aliquot was normalized by ApoB content to deliver the same number of particles (1 ApoB molecule/ TGRL particle). We investigated whether such PP-TGRL priming capacity varied predictably with subject anthropometric characteristics or lipid profile. The increase in VCAM-1 expression (**Table 1**) correlated most strongly with subject WC (**Figure 2A**, Pearson r = 0.66, P < 0.001), followed by PP-sTG (**Figure 2D**, Pearson r = 0.59, P = 0.003), F-sTG (Pearson r = 0.46, P = 0.026), and inversely with postprandial HDL cholesterol (PP-sHDL) (Pearson r = -0.42, P = 0.046). No significant correlation was observed with other serum lipids, glucose or BMI. Notably, these correlations revealed that PP-TGRL could either positively or negatively modulate VCAM-1 expression over a considerable range about the mean (from a 20% drop to a 28% increase from TNFα alone). On average, enhancement in VCAM-1 expression was observed for PP-TGRL from subjects with PP-sTG ≥ 225 mg/dL (17.8%) or WC ≥ 0.8 m (16.7%) (**Figure 2A, D inset**). Clinical characteristics of groups defined by this cutoff in PP-sTG are given in **Supplementary Table III**. Overall, this cutoff was more predictive of enhanced VCAM-1 expression than one based on a clinically used criterion for

hypertriglyceridemia (F-sTG > 150 mg/dL). Although ICAM-1 and E-selectin upregulation by TNFα were enhanced in the presence of PP-TGRL over all subjects, these did not correlate significantly with subject WC (**Figure 2B, C**), PP-sTG (**Figure 2E, F**) or any other anthropometric or metabolic parameters (data not shown). The same priming effect on VCAM-1 expression was not observed with F-TGRL derived from the same donors (**Supplementary Figure II**), which overall reduced CAM expression relative to TNFα, indicating that this is a transient response attributable to the postprandial nature of the TGRL. Taken together, these data reveal that acute exposure to PP-TGRL exacerbates an inflammatory response in EC specific to VCAM-1 that correlates directly with individual subject's metabolic characteristics reflected in sTG and WC.

PP-TGRL modulates cytokine-induced VCAM-1 expression at the transcriptional level without affecting mRNA stability.

The dynamics of VCAM-1 gene expression and post-transcriptional stability in response to PP-TGRL were assessed to determine if these could predict the upregulated protein expression. qPCR results revealed that 4 hr treatment with TGRL alone did not induce VCAM-1 gene expression, but together with TNFα stimulation significantly enhanced upregulation of VCAM-1 mRNA overall by 15%(**Supplementary Figure III**). In HAEC pre-exposed to TNFα for 1 hr to initiate an inflammatory response, subsequent addition of PP-TGRL overall enhanced TNFα-stimulated VCAM-1 upregulation that peaked at 27%, 4 hrs after its addition (**Figure 3A**). In order to assess whether the increase in VCAM-1 was due to increased production or a net decrease in mRNA degradation, transcript stability was examined using actinomycin D (ActD), an inhibitor of *de novo*

transcription. Addition of ActD revealed rapid turnover of VCAM-1 mRNA evident by a ~90% reduction in the amount of mRNA produced by TNFα at any given time point from 2-6 hours after its addition (**Figure 3B**). There was no difference in transcript levels in the presence or absence of PP-TGRLs, indicating that PP-TGRL priming did not change the degradation rate of VCAM-1 transcript. The priming effect of PP-TGRL on VCAM-1 mRNA expression at 4 hr was examined for variability in its response as a function of subject PP-sTG levels. This correlation (**Figure 3C,** Pearson r=0.3787, P = 0.0822) was not as strong as that observed for the surface expression of VCAM-1. However, TGRL from individuals with the highest PP-sTG (> 300 mg/dL) induced a significantly greater increase (average 32%) in VCAM-1 mRNA expression (**Figure 3C, inset**). In contrast, ICAM-1 and E-selectin mRNA in response to TNFα were modestly decreased by the presence of PP-TGRL at 4 hr and did not correlate with PP-sTG levels (**Supplementary Figure III**). Together these results suggest that PP-TGRL has a priming effect specific to VCAM-1 gene expression that is a function of amplified *de novo* transcription rather than modified RNA stability.

Monocyte recruitment to HAEC under shear flow increases with VCAM-1 expression under elevated PP-sTG.

Uptake of modified lipids and recruitment of monocytes to inflamed endothelium is the hallmark of the early atherosclerotic lesion and underscores the importance of inflammation in atherogenesis(19). In order to provide further insight into the functional significance of VCAM-1 modulation, we assessed monocyte recruitment to HAEC monolayers primed with PP-TGRL in a microfluidic flow channel that allows direct

imaging of TNFα-induced monocyte arrest under fluid shear stress(29) (**Figure 4**). Monocyte arrest positively correlated with both PP-sTG (Pearson r = 0.67, P = 0.0091, **Figure 4A**) and to a greater degree with the membrane expression of VCAM-1(Pearson r = 0.75, P = 0.0034, **Figure 4B**). A 1% change in VCAM-1 expression resulted in a corresponding 2% change in monocyte arrest. Again, we observed a critical threshold in PP-sTG of ~225 mg/dL above which PP-TGRL potentiated the efficiency of monocyte arrest. Remarkable was the observation that PP-TGRLs from low PP-sTG (<225mg/dL) subjects exerted an anti-inflammatory effect, decreasing the influence of TNFα on VCAM-1 upregulation and monocyte arrest. Stable adhesion of monocytes was dependent on integrin binding to VCAM-1 as revealed by pretreatment with a blocking antibody which reduced adhesion by ~80% to baseline levels (**Figure 4C**). These data demonstrate that PP-TGRL can exert both pro- and anti-inflammatory effects on TNFα-induced VCAM-1 expression and the efficiency of monocyte recruitment.

Discussion:

This study examines the effects of hypertriglyceridemia on the endothelial inflammatory response in a cohort of 61 subjects representing a diverse population from normal to hypertriglyceridemic, but otherwise healthy. Cultured HAECs were treated *ex vivo* with PP-TGRLs isolated at their peak following a single high-fat meal. Changes in VLDL composition reflected elevated PP-sTG levels in response to the meal. PP-TGRL bound to EC via LDLR-mediated endocytosis and specifically modulated cytokine-induced VCAM-1 expression and monocyte arrest in a manner sensitive to individual donor metabolic characteristics. This was reflected in an exacerbated inflammatory response for subjects with PP-sTG ≥ 225mg/dL or WC ≥ 0.8 m. Our findings link common epidemiological measurements of cardio-metabolic risk directly with acute markers of endothelial inflammation in a vascular mimetic model.

TGRL was isolated at a time point representative of the postprandial peak in sTG, which has been demonstrated to correlate most closely with incidence of cardiovascular morbidity(2), and to have a greater effect on endothelial dysfunction compared to the fasting state(1). The absence of a priming effect on VCAM-1 expression in response to fasting TGRL from HTG individuals in our model provided a rationale to focus on the postprandial state. Moreover, our study meal was chosen to be representative of a common fast-food meal consisting of a large percentage of the calories from fat, and a high ratio of saturated fat. The composition of TGRL postprandially reflects the nature of the meal. Previous studies comparing the effects of an isocaloric low fat meal to a high fat meal on EC function demonstrated no change in sTG, oxidative stress, or flow-

mediated dilatation in response to the low fat meal (1, 32). The fatty acid content of a meal was previously shown to affect the composition and distribution of VLDL postprandially(7), to enrich apoE and apoC-III levels in TGRL(16), most profoundly for saturated fatty acids, and to enhance VCAM-1 and E-selectin expression by EC (35). These observations served as motivation to focus our investigation on a high fat test meal.

Elevated TGRL has been associated with endothelial inflammation and dysfunction, particularly in the postprandial state(32). Previous studies have reported that TGRL increased endothelial permeability (12), inflammatory cytokine production, vascular adhesion molecule expression (20), and oxidative stress (33), decreased NO activity, and impaired flow-mediated brachial artery vasoactivity (32). However, our studies indicate that PP-TGRL alone is not inherently inflammatory and, based on VCAM-1 expression and monocyte recruitment under shear flow, actually exerts anti-inflammatory effects. We observed a small but statistically significant reduction in VCAM-1 and E-selectin gene and protein expression in resting HAEC in response to PP-TGRL in 60-85% of subjects. As monocyte arrest is negligible in unstimulated EC, these observations may highlight a homeostatic feedback mechanism that maintains a low basal CAM expression and function under enhanced metabolic activity, thereby countering inflammation (9).

The lack of an overt inflammatory response to PP-TGRL implies the absence of oxidation, as oxidized lipoproteins themselves enhance inflammatory CAM expression to levels comparable to TNFα (31). Our study used native TGRL stored such that no oxidation

occurred *ex vivo*. In contrast to oxidized lipoproteins which are cleared by scavenger receptors on EC and have been directly implicated in atherogenesis(19), native TGRLs and their RPs may be bound and internalized by the LDLR family. These not only function as cargo receptors that deliver macromolecules to the cell via endocytosis, but affect cell signaling and the maintenance of cholesterol homeostasis(30). We demonstrate that LDLR-mediated endocytosis accounted for ~80% of the cellular uptake of native TGRL in our model, which was increased under inflammation. We reason that uptake via LDLRs results in acute receptor-mediated signaling events (i.e. within 1 hr of TNFα stimulation) that can regulate a subsequent cytokine mediated inflammatory response.

The absence of an inflammatory response to PP-TGRL treatment is consistent with our previous report in which a chronic vascular injury model was applied (31). We previously reported that repetitive conditioning with PP-TGRL over 3 days alone did not elicit inflammation, but primed HAEC to respond to TNFα stimulation at concentrations comparable to those observed in atherosclerotic plaques(28). Our results contrast with other recent studies reporting that TGRL alone was sufficient to increase the expression of a large number of inflammatory genes and to enhance VCAM-1 surface expression in HAEC(24, 25). In the latter studies, NTG subjects were compared with type IV HTG subjects administered a much higher oral fat load (82% calories from fat). A higher fat load is one factor that might account for a greater inflammatory response in NTG subjects or in response to lipid alone. Our subject pool represents a continuous distribution from normal to hyperlipidemic, and our meal is typical of that associated with

a western high fat diet. Although both studies demonstrate an increased inflammatory potential of PP-TGRL from HTG subjects, ours demonstrates the emergence of inflammatory outcomes only upon co-stimulation with a low dose of TNFα. Since TNFα is considered a biomarker of inflammation in human serum, our results may indicate that subjects with pre-existing low grade inflammation are more susceptible to postprandial-induced endothelial dysfunction.

The observation of a threshold in subject PP-sTG above which PP-TGRL modulated an inflammatory response implies that metabolic stress can sensitize endothelium to pathological changes (9). Changes in endothelial VCAM-1 expression proved to be the most sensitive indicator of individual variation in the inflammatory potential of PP-TGRL. Monocyte arrest revealed a functional response that corroborated our findings at the level of VCAM-1 gene and protein expression. Notably, we observed that only a 1% change in VCAM-1 expression elicited a significant 2% increment in monocyte arrest over a dynamic range within which PP-TGRL modulated TNFα-induced VCAM-1 expression from a -20% drop to 28% increase from cytokine alone. We chose to use isolated monocytes from healthy, fasting, normolipidemic subjects for this study in order to decrease variability, and in recognition that PP-sTG can impact inflammatory responses in monocytes as well. For example, it was recently reported that monocyte avidity for VCAM-1 is increased postprandially via a mechanism involving upregulation of CD11c (13), where subject PP-sTG was also a reliable indicator of monocyte activation. Thus, complementary mechanisms exist in EC and monocytes that are consistent with the enhancement of transient inflammatory responses to postprandially elevated sTG.

This supports the notion that atherosclerosis may develop in metabolically disposed subjects gradually in response to repetitive postprandial inflammatory insult.

Although it is widely recognized that dyslipidemia induced by an atherogenic diet upregulates endothelial inflammatory responses, a novelty of our study is the observation of the variability in the inflammatory response and the direct correlation with donor PP-sTG and abdominal obesity. The differential capacity of PP-TGRL to alter HAEC inflammatory responses may be attributed to individual heterogeneity in particle lipid and apolipoprotein composition postprandially, reflecting both the meal and the subject's metabolic status(17). We observed that HTG subjects with more visceral fat storage produced larger triglyceride-enriched VLDL particles that were more inflammatory. Previous studies provide evidence that ApoCIII, ApoE, and cholesterol are enriched in postprandial TGRL, particularly in HTG subjects (3, 6) or after a meal high in saturated fat (16). Variation in apolipoprotein composition could lead to differences in receptor-mediated binding and signaling events. For example, enrichment of ApoE, which serves as a potent ligand for LDLRs(4), facilitates internalization of TGRLs and their remnant particles, while ApoCIII has been reported to upregulate EC VCAM-1 and ICAM-1 expression via activation of PKCβ and the NFκB pathway (18). Heterogeneity in inflammatory responses to TGRL can also reflect the fatty acid content of the particles released upon metabolism. An increase in circulating free fatty acids is associated with HTG and obesity (23), and fatty acids have been demonstrated to act as pro- and anti-inflammatory modulators of the EC response to cytokine (10, 34) . It was recently demonstrated that fatty acids released upon the *ex vivo* lipolysis of PP-TGRL contained

neutral and oxygenated lipids that activated ROS production in EC (33). In contrast, certain fatty acids released from TGRL upon lipolysis may serve as agonists for peroxisome proliferator-activated receptors (PPARs), which downregulate TNFα- and VLDL-induced VCAM-1 expression (37). Thus, the EC response to lipolysis should reflect a balance between inflammatory and anti-inflammatory lipids contained in the particles (33). Notably, our study used native intact TGRL, though it does not rule out the involvement of fatty acids released by endogenous EC metabolic activity at the membrane or following endocytosis. Additional studies are needed to demonstrate the relative role of different signaling mechanisms, activated as a consequence of changes in TGRL apolipoprotein or fatty acid composition, in modulating TGRL-induced pro- or anti-inflammatory signaling in our model.

The cytokine-induced surface expression of ICAM-1 and E-selectin were equally elevated by PP-TGRLs at 4 hr. However, only VCAM-1 significantly correlated with the variation in a subject's lipids or anthropometric characteristics, and was elevated at the transcript level. The relative membrane expression of VCAM-1 in response to TNFα stimulation may be regulated either transcriptionally(8), or post-transcriptionally, through mechanisms that affect mRNA stability or translation(15). We propose that the relative capacity of PP-TGRL to modulate the inflammatory response to cytokine is through altering expression and activation of transcription factors that act cooperatively with NFκB in the CAM promoter. In this regard, TGRL has been reported to activate the p38 pathway and the binding of NFκB, AP-1 and CREB to the promoters of inflammatory genes in hyperlipidemic subjects (25). We also previously demonstrated that PP-TGRL

enhanced p38 MAPK activation that, together with cytokine stimulation, resulted in greater transcriptional activity of NFκB (31). Post-transcriptional regulatory mechanisms may also contribute to differential expression of CAMs during inflammation. For example, miRNA126 was recently shown to endogenously suppress VCAM-1 expression in EC and inhibit translation without affecting TNFα-induced transcription or transcript stability (15).

We demonstrated that PP-TGRL isolated at the peak after a high fat meal modulated a transient inflammatory response in EC in proportion to the level of subject HTG or visceral obesity. Abdominal obesity is associated with metabolic abnormalities and an increased risk of type II diabetes and atherosclerotic CVD (11, 14). Waist circumference (>40 in for males, >35 in for females) is a major criterion for diagnosis of metabolic syndrome and, together with elevated F-sTG (>150 mg/dL), may reflect a subject's propensity for more metabolically active visceral adipose tissue and elevated risk (11, 14). Our findings link simple clinical metrics with biologically relevant markers of endothelial inflammation that may provide a means for assessing an individual's response to a repeated metabolic challenge, and an early measure of associated cardiovascular risk. Specifically, we demonstrated that TGRL particles from subjects with elevated PP-sTG (>225 mg/dL) and WC above a subclinical threshold of 0.8m (~31.5 in) for abdominal obesity correlated with enhanced VCAM-1 expression and the recruitment of monocytes, a harbinger of atherosclerosis (19).

In conclusion, we applied a reductionist approach to evaluate the inflammatory potential

of an individual's PP-TGRL and demonstrated a direct correlation between the spike in circulating triglycerides after a meal and its capacity to specifically alter expression of VCAM-1 and monocyte recruitment on aortic endothelium using a rapid and reliable lab-on-a-chip assay.

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Disclosures:

None.

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Figure Legends:

Figure 1. A-D, TGRL particle size increases postprandially and varies with subject characteristics. A, Serum triglyceride levels (mean ± SEM) in 16 subjects at 0 (fasting), 3.5 and 7 hr after the meal. Significance by repeated measure ANOVA with SNK post test, * P<0.001 from fasting, # P<0.005 from 3.5 hr. **B,** Average diameter of VLDL isolated from those subjects at the designated time postprandially. Significance by repeated measure ANOVA with SNK post test, $ P<0.05 from fasting. Pearson correlations between VLDL diameter and subject (**C**) postprandial serum triglycerides or (**D**) waist circumference. **E-H, TGRL uptake by HAEC via LDLRs. E, F,** Representative confocal images and **G**, statistical analysis of TGRL binding (mean ± SEM, n=4) of HAEC incubated with Alexafluor488-labeled TGRL (10 mg/dL ApoB) for 1hr (**E**) alone or (**F**) with RAP (50μg/ml). Significance by paired Student t-test. **H**, Cellular cholesterol content (mean ± SEM, n=4) of HAEC incubated with TGRLs, TNFα or both for 4hrs. Significance by repeated measure ANOVA with SNK post test.

Figure 2. TGRL-enhanced VCAM-1 expression correlates with subject WC and PPsTG.

Pearson correlations between TGRL-modulated surface expression of cell adhesion molecules (VCAM-1, ICAM-1, E-selectin) and donor waist circumference (**A, B, C**) or postprandial serum triglycerides (**D, E, F**). HAECs were treated for 4 hr with TNFα alone or simultaneously with TGRLs. CAM expression is presented as a percent change with TGRL relative to TNFα alone. Insets represent the same data for samples binned into two categories based on cutoffs in PP-sTG (225 mg/dl) or WC (0.8m). Significance by

Student t-test, mean ± SEM (n=13-22); NS, not significant.

Figure 3. PP-TGRL enhances TNFα induced VCAM-1 transcription without affecting mRNA stability.

A, B, Kinetics of VCAM-1 mRNA expression assessed by qPCR at 2, 4 and 6 hrs after 1hr pretreatment with TNFα (1 ng/ml), followed by addition of (**A**) TGRL alone, (**B**) actinomycin D (1 μg/ml, solid diamond), or both (open circle). VCAM-1 expression is presented as a percent change with TGRL (and/ or ActD) relative to TNFα alone at each time point (mean ± SEM, n = 10). Significance by repeated measure ANOVA with SNK post test, * $P<0.05$ from time = 0hr; **C,** Pearson correlation between TGRL-modulated VCAM-1 gene expression and donor postprandial serum triglycerides. HAECs were treated for 4 hr with TNFα alone or simultaneously with TGRLs. VCAM-1 expression is presented as a percent change with TGRL relative to TNFα alone. Inset represents the same data for samples binned into two categories based on cutoff in PP-sTG (300 mg/dl). Significance by Student t-test, mean ± SEM (n=22).

Figure 4. Monocyte recruitment to HAEC under shear flow increases with VCAM-1 expression under elevated PP-sTG.

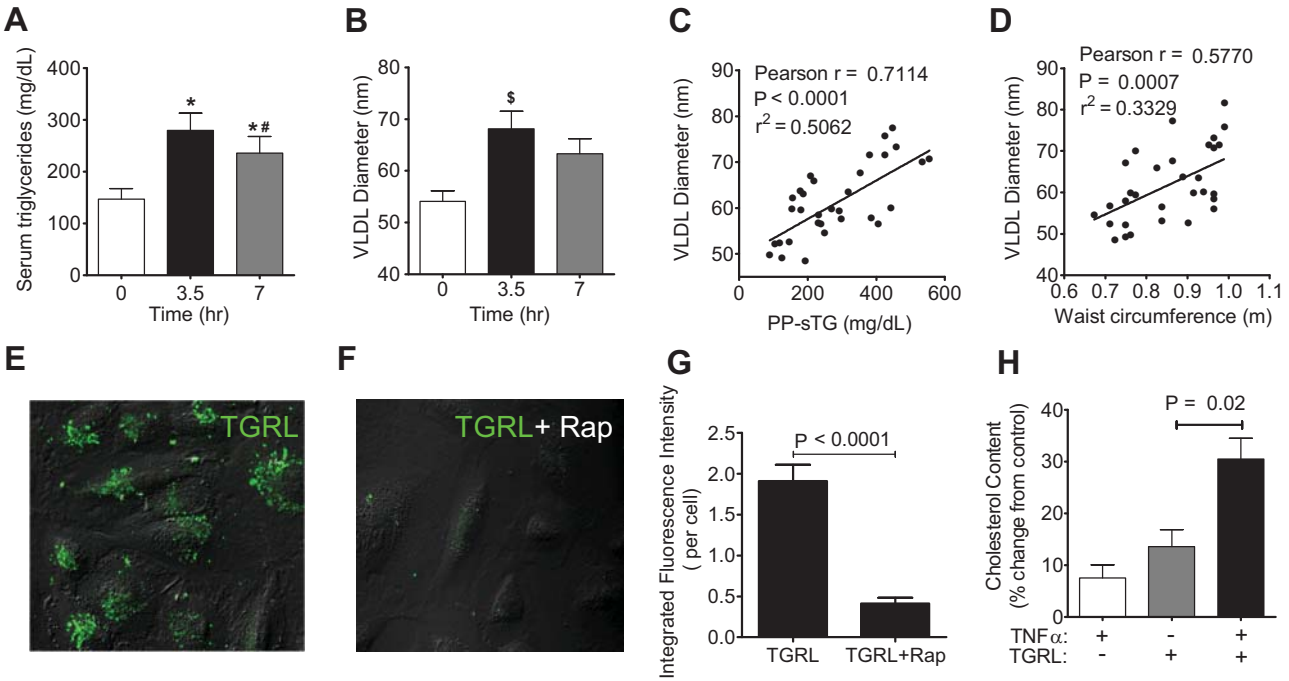
A, B, Pearson correlations between TGRL-modulated monocyte arrest and **A)** TGRL donor postprandial serum triglycerides or **B)** HAEC VCAM-1 surface expression. HAECs were treated for 4 hr with TNFα alone or simultaneously with TGRLs and exposed to monocytes isolated from healthy subjects under flow (n=14). Monocyte arrest and VCAM-1 expression are presented as a percent change with TGRL relative to TNFα

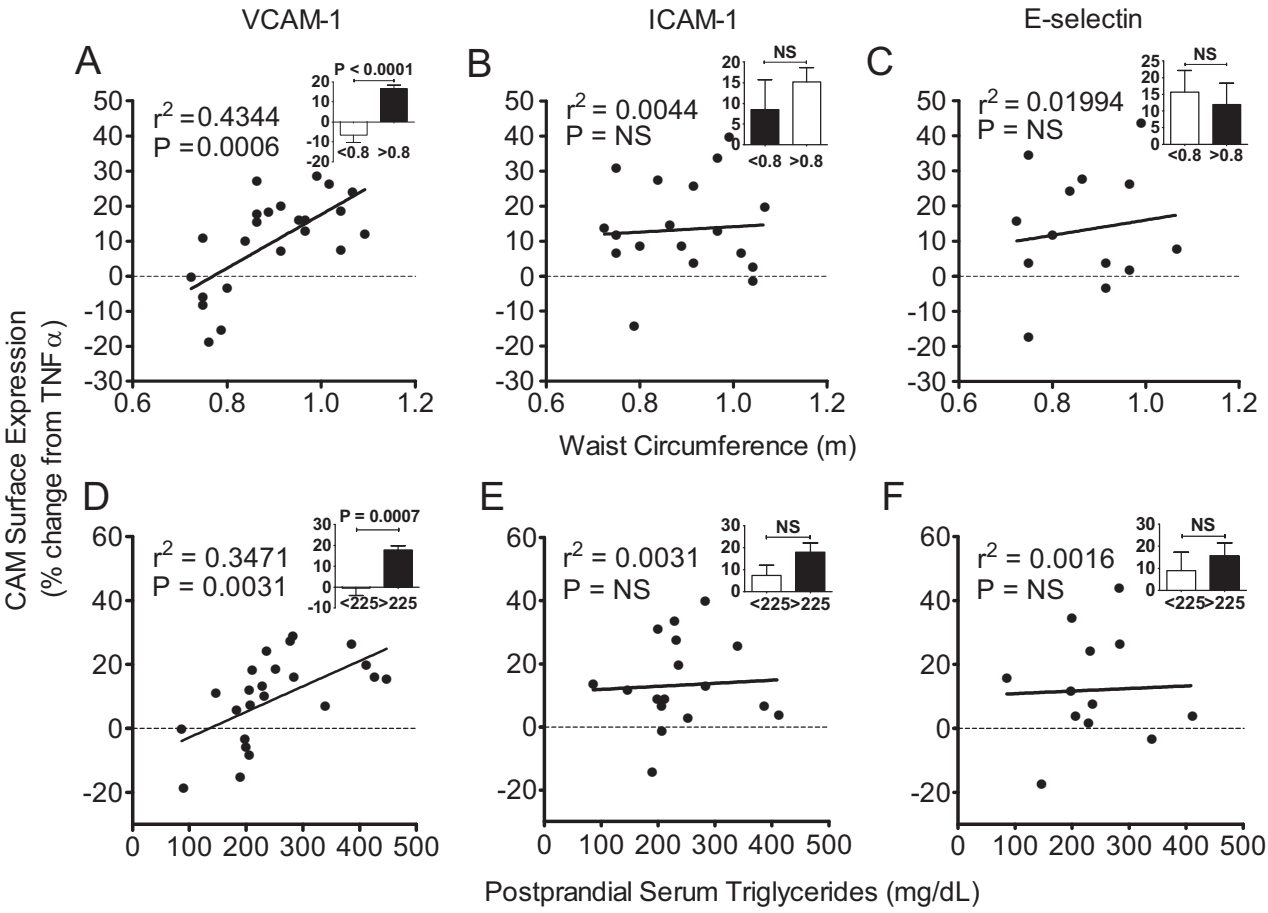
alone. C, Monocyte arrest quantified in the presence or absence of anti-VCAM-1 antibody (20μg/ml), (n=3-5). Significance by paired Student t-test; NS, not significant.

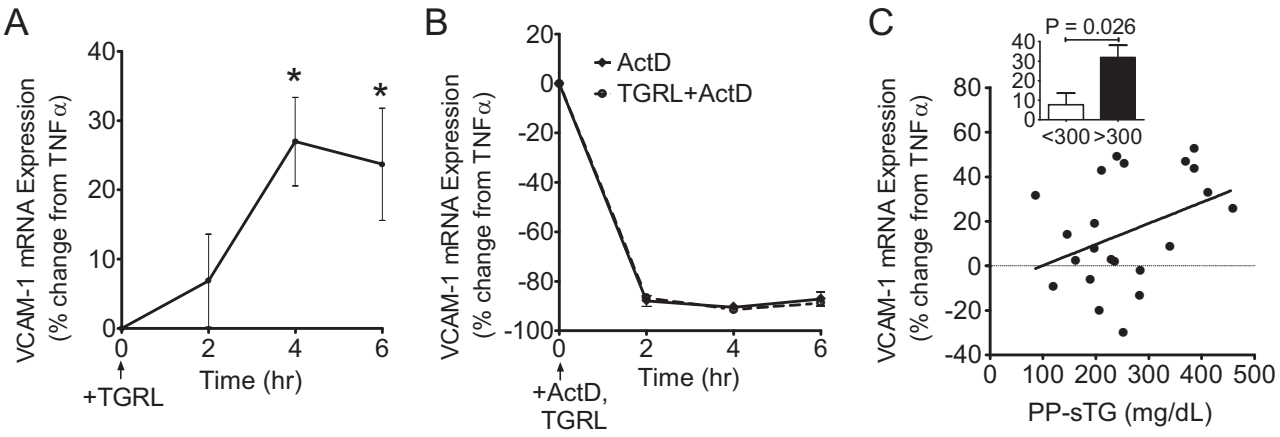
TABLE 1. Correlations between subject clinical characteristics and TGRL-modulated VCAM-1 expression

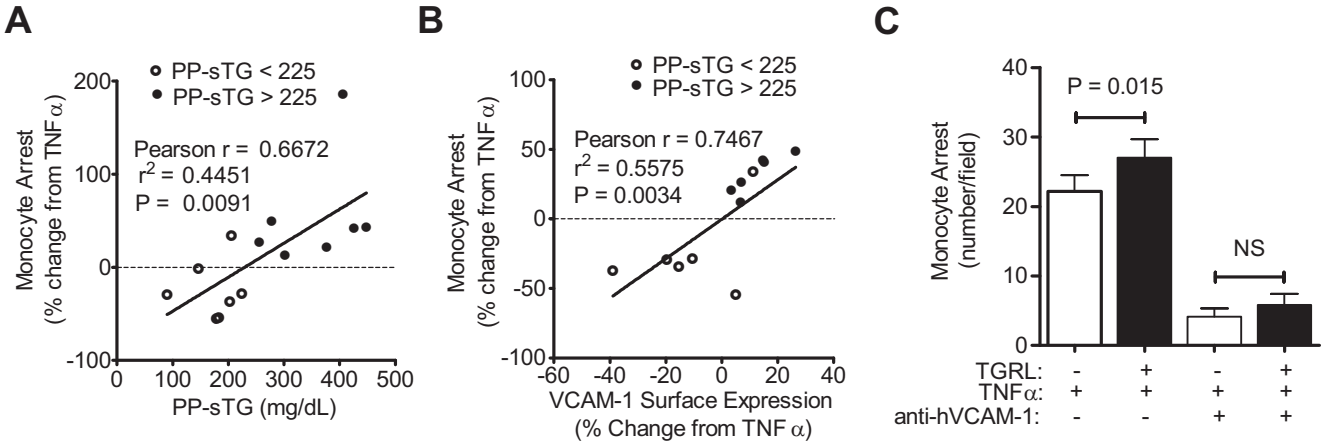
	Pearson r	*P* value (two-tailed)	R^2	*P*-value Summary
Waist circumference	0.66	0.0006	0.43	†
Body mass index	0.39	0.067	0.15	
Fasting				
Triglycerides	0.46	0.026	0.22	*
Total cholesterol	-0.41	0.055	0.17	
LDL cholesterol	-0.41	0.056	0.17	
HDL cholesterol	-0.39	0.067	0.15	
Apolipoprotein B 100	-0.24	0.264	0.06	
CHOL:HDL	0.24	0.262	0.06	
Glucose	-0.01	0.949	0.00	
Postprandial				
Triglycerides	0.59	0.003	0.35	†
HDL cholesterol	-0.42	0.046	0.18	*
LDL cholesterol	-0.40	0.083	0.16	
Total cholesterol	-0.37	0.079	0.14	
Glucose	-0.30	0.167	0.09	
CHOL:HDL	0.29	0.181	0.08	
Apolipoprotein B 100	-0.02	0.940	0.00	

* $P < 0.05$; †$P < 0.01$;


A
Serum triglycerides (mg/dL)
400
300
200
100
0
*
*#
0
3.5
7
Time (hr)
B
VLDL Diameter (nm)
80
70
60
50
40
$
0
3.5
7
Time (hr)
C
VLDL Diameter (nm)
90
80
70
60
50
Pearson r = 0.7114
P < 0.0001
r2 = 0.5062
0
200
400
600
PP-sTG (mg/dL)
D
VLDL Diameter (nm)
90
80
70
60
50
40
Pearson r = 0.5770
P = 0.0007
r2 = 0.3329
0.6
0.7
0.8
0.9
1.0
1.1
Waist circumference (m)
E
TGRL
F
TGRL+ Rap
G
Integrated Fluorescence Intensity
(per cell)
2.5
2.0
1.5
1.0
0.5
0.0
P < 0.0001
TGRL
TGRL+Rap
H
Cholesterol Content
(% change from control)
40
30
20
10
0
P = 0.02
TNFα:
TGRL:
+
-
-
+
+
+


VCAM-1
ICAM-1
E-selectin
A
B
C
D
E
F
r2 =0.4344
P =0.0006
r2 = 0.0044
P = NS
r2 = 0.01994
P = NS
r2 = 0.3471
P = 0.0031
r2 = 0.0031
P = NS
r2 = 0.0016
P = NS
P < 0.0001
P = 0.0007
NS
<0.8 >0.8
<225>225
CAM Surface Expression
(% change from TNF α)
Waist Circumference (m)
Postprandial Serum Triglycerides (mg/dL)


A
VCAM-1 mRNA Expression
(% change from TNFα)
40
30
20
10
0
0
2
4
6
Time (hr)
+TGRL
*
*
B
VCAM-1 mRNA Expression
(% change from TNFα)
20
0
-20
-40
-60
-80
-100
ActD
TGRL+ActD
0
2
4
6
Time (hr)
+ActD,
TGRL
C
VCAM-1 mRNA Expression
(% change from TNFα)
P = 0.026
40
30
20
10
0
<300
>300
80
60
40
20
0
-20
-40
0
100
200
300
400
500
PP-sTG (mg/dL)


A
PP-sTG < 225
PP-sTG > 225
Pearson r = 0.6672
r2 = 0.4451
P = 0.0091
Monocyte Arrest
(% change from TNF α)
PP-sTG (mg/dL)
B
PP-sTG < 225
PP-sTG > 225
Pearson r = 0.7467
r2 = 0.5575
P = 0.0034
Monocyte Arrest
(% change from TNF α)
VCAM-1 Surface Expression
(% Change from TNF α)
C
P = 0.015
NS
Monocyte Arrest
(number/field)
TGRL:
TNFα:
anti-hVCAM-1: