Wednesday, August 3, 2016

Poster Session: 9:30 AM - 5:00 PM

Lipids/Lipoproteins

B-115

Detection and Identification of Phosphatidylcholine Hydroperoxides in Plasma and Triglyceride-rich Lipoproteins using Orbitrap Mass Spectrometry

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Background: A growing body of evidence supports the association of triglyceride-rich lipoproteins (TRL) with atherosclerosis and coronary heart disease (CHD). Oxidized lipids in the TRL may be one of the promoting factors for its atherogenicity. We previously reported that TRL, including very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL), carries several molecular species of cholesteryl ester hydroperoxides and triglyceride hydroperoxides. Since the lipid peroxidation can induce chain-peroxidation reaction to the adjacent lipid molecules, it is likely that TRL may also carry phosphatidylcholine hydroperoxides (PCOOH). Furthermore, phospholipids exist on the surface of lipoproteins and thus are relatively more susceptible to the peroxidation. Therefore, analysis of PCOOH can be valuable in overall evaluation of future coronary diseases and oxidative injury. In light of this importance, this study was carried out to identify and characterize PCOOH in plasma and TRL.

Methods: We developed a novel approach for the identification and characterization of PCOOH from the lipid extract of plasma and lipoprotein fractions, using reversed-phase liquid chromatography coupled with a hybrid linear ion trap-Orbitrap mass spectrometer (LC-LTQ Orbitrap). A fasting EDTA blood sample was collected from 9 healthy human volunteers. VLDL (d <1.006) and IDL (d =1.006-1.019) were isolated from the plasma (n=6) by sequential ultracentrifugation. The purity of isolated lipoproteins was assessed by its molecular composition, mobility in polyacrylamide gel electrophoresis (LipoPhor) and apolipoproteins study using SDS-PAGE. Total lipids were extracted from plasma and lipoprotein samples, and subjected to the LC-LTQ Orbitrap analysis equipped with an electrospray ionization source. The mass spectrometry was operated in a positive-ion mode, extracted ion chromatogram was drawn with the mass tolerance set at 5.0 ppm, and PCOOH were detected as $[M+H]^+$ ion. Authentic synthetic standards of PCOOH were used for equivocal identification.

Results: On the basis of specific elemental composition, m/z mass spectra, and retention time in LC, we detected several molecular species of PCOOH that exist in human plasma and TRL. PC16:0/18:2-OOH and PC16:0/20:4-OOH were detected in all plasma samples. PC 16:0/18:2-OOH, PC 18:0/18:2-OOH, PC 16:0/20:4-OOH, PC 18:0/20:4-OOH, PC 16:0/22:6-OOH, and PC 18:0/22:6-OOH were the most predominant PCOOH detected in TRL. The distribution of PCOOH is higher in VLDL compared to IDL with consistent detection of PC16:0/20:4-OOH and PC16:0/22:6-OOH in all samples. PC16:0/20:5-OOH and PC18:0/22:6-OOH were not present in any of plasma samples.

Conclusion: We identified and characterized 12 molecular species of PCOOH in native lipoproteins and plasma. The existence of PCOOH in TRL is possibly associated with its atherogenicity; therefore, can serve as markers for early prediction and risk assessment of CHD. Further work is needed to reveal the association of these PCOOH in the atherosclerotic process.

B-116

Prevalence and Pattern of Dyslipidemia in Nepalese Individuals with Type 2 Diabetes

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Background: Atherogenic dyslipidemia is an important modifiable risk factor for cardiovascular disease among patients of type 2 diabetes mellitus. Its timely detection and characterization helps clinicians estimate future risk of CVD and take appropriate preventive measures. We thus aim to study the prevalence and pattern of dyslipidemia in a cohort of Nepalese patients with type 2 diabetes.

Patients and methods: We conducted a cross-sectional study on 497 diabetic patients aged 30-74 years at Manipal Teaching Hospital, Pokhara, Nepal. The relevant personal, medical, demographic and anthropometric data were collected. Blood samples were analyzed for plasma glucose, HbA1c and lipid profile. The National Cholesterol Education Program Adult Treatment Plan III guidelines were used to define prevalence of dyslipidemia. Statistical analyses of the data were performed using SPSS, with level of significance set at $p<0.050$.

Results: We found mixed dyslipidemia as the most prevalent (88.2%) and isolated dyslipidemia (10.1%) as the least prevalent forms of dyslipidemia in our patients. The most prevalent form of single dyslipidemia was high LDL-C (73.8%) and combined dyslipidemia was high TG, high LDL-C and low HDL-C (44.7%). Prevalence of all single and mixed dyslipidemia was higher in patients with poor glycemic control and hypertension. The glycemic status of patients correlated with their fasting serum lipid profile. Dyslipidemia was associated mainly with male gender, poor glycemic control and hypertension.

Conclusion: The prevalence of dyslipidemia is very high in Nepalese diabetic patients. Hence, urgent lifestyle intervention and aggressive lipid lowering treatment plans are needed to minimize their future risk of cardiovascular disease.

B-119

Serum amyloid A increases the antioxidant ability of high density lipoprotein

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Background: Atherosclerosis is associated with chronic inflammation. Sustained inflammation is the essential feature of atherosclerotic lesions as well as the accumulation of cholesterol. Although many study reported that the activation of inflammatory cells and the release of various cytokines contributed to the progress of atherosclerosis, it remains to be completely elucidated how the inflammation impacts on lipid metabolism. Serum amyloid A (SAA) is one of the acute phase proteins and its concentration in plasma is also elevated modestly in chronic inflammatory disorders. Furthermore SAA is notably increased at inflamed regions with a higher level than that in plasma under chronic inflammatory conditions. Elevated SAA becomes the major apolipoprotein component of high density lipoprotein (HDL) due to a displacement of apolipoprotein A-I (apoA-I). Consequently, this remodeling could affect HDL metabolism, however there is almost no report about the direct influences of SAA on the function of HDL. In this study, we evaluated the effects of SAA on antioxidant ability, which is one of various anti-atherogenic properties of HDL using both SAA protein and SAA-containing HDL.

Methods: LDL (d = 1.006-1.063 g/mL) and HDL (d = 1.063-1.210 g/mL) were isolated by ultracentrifugation. Reconstituted SAA-HDL was prepared by incubating recombinant human SAA (rhSAA) with HDL isolated from serum of normal subjects. HDLs obtained from various patients serum were divided into three groups by SAA levels (Low: ≤ 8 μg/mL, n=11, Middle: 8-100 μg/mL, n=10, and High: > 100 μg/mL, n=20). The antioxidant ability was estimated by the effect of each protein (SAA or apoA-I) or HDL on LDL oxidation, which was monitored as the formation of conjugated dienes at 234 nm. Antioxidant ability was defined as relative prolongation of the lag time and as relative decrease of the maximum velocity (V_{max}) compared to LDL alone.

Results: The relative lag time (mean ± SD) of rhSAA was 1.33 ± 0.05 whereas that of apoA-I was 1.22 ± 0.02 ($p < 0.05$). The relative V_{max} was 0.79 ± 0.03 and 0.86 ± 0.02, respectively ($p < 0.05$). As for HDL particles, the relative lag times of rhSAA-HDL and normal HDL were 1.83 ± 0.20 and 1.46 ± 0.27 ($p < 0.05$), and the relative V_{max} were 0.54 ± 0.15 and 0.66 ± 0.09, respectively. With regard to results in patients, the relative lag times of HDL in each group (Low, Middle, and High) were 1.24 ± 0.06, 1.25 ± 0.06, 1.36 ± 0.14 ($p < 0.05$), the relative V_{max} were 1.18 ± 0.08, 1.11 ± 0.15, and 1.06 ± 0.08, respectively ($p < 0.01$).

Conclusion: rhSAA had higher ability for inhibition of LDL oxidation than apoA-I. Similarly, rhSAA-HDL and HDL obtained from the patients with high SAA levels prolonged the relative lag time of oxidation profile. These indicate that SAA increased antioxidant ability of HDL. It might mean that SAA, whether the conformation such as lipid-free or HDL bound, plays an atheroprotective role as an antioxidant agent both at tissues and in the circulation.

B-120

Effect of Differentiation and Foam Cell Formation on Cholesterol Efflux Capacity of Apolipoprotein A-I.

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Backgroud: Cholesterol efflux capacity is one of the atheroprotective functions of apolipoprotein A-I (apoA-I). In recent years, a number of studies have suggested a relationship between cholesterol efflux capacity and cardiovascular disease. However, the assay method of cholesterol efflux capacity has not been optimized. It might be one of the reasons why the different results have been given by the similar experiments. The purpose of this study is to investigate the effect of different states of cells on the cholesterol efflux capacity of apoA-I and *N*-homocysteinylated apoA-I (*N*-Hcy apoA-I), known as one of the risk factors for cardiovascular disease.

Methods: Cholesterol efflux capacity was determined by measuring a rate of ^{3}H-cholesterol removed by apoA-I from THP-1 (a human monocytic leukemia cell line) macrophages. We estimated the cholesterol efflux capacity under various cell conditions as follows: 1) THP-1 cells were differentiated with phorbol myristate acetate (PMA) for different terms (1 to 5 days) and the degree of cell differentiation was evaluated by detecting the expression of ATP-binding cassette transporter A1 (ABCA1) and CD11b, an adhesion molecule, with flow cytometry and western blotting analysis. 2) After differentiation with PMA treatment for a fixed term, THP-1 macrophages were incubated with acetylated low density lipoprotein (acLDL) for different terms (1 to 5 days) and the lipid droplet accumulation in foam cells was determined by oil red O staining. 3) Under these conditions, cholesterol efflux capacity was compared between *N*-Hcy apoA-I and normal apoA-I.

Results: 1) When foam cell formation was fixed for 1 day, cholesterol efflux capacities were the highest and the second highest at day 2 and day 1 differentiation respectively, and decreased after day 3. The amount of ^{3}H-cholesterol took inside the cells was also the highest at day 2, but the lowest at day 1, and decreased after day 3. ABCA1 expression was decreased on a parallel with PMA treatment period ($p < 0.05$). 2) The amount of lipid droplet accumulation was lineally increased during acLDL loading ($p < 0.05$). The amount of ^{3}H-cholesterol took inside the cells was also lineally increased for 3 days but then almost steadied. Cholesterol efflux capacity was the highest at day 1 loading with acLDL and decreased in a loading time-dependent manner. ABCA1 expression did not change significantly. 3) Cholesterol efflux capacity of *N*-Hcy apoA-I was significantly lower than that of apoA-I by differentiation and foam cell formation for 1 or 2 days. However, no significant difference was observed under the states of excessive differentiation or excessive foam cell formation.

Conclusion: We found that cholesterol efflux capacity was greatly affected by the state of differentiation and foam cell formation. Actually, the different cell conditions did not always produce the fixed result that *N*-homocysteinylation influenced on cholesterol efflux capacity of apoA-I. These results suggest that cholesterol efflux capacity varies depending on the

state of cells and the assays should be performed using the defined protocols including stimulation period.

B-121

LDL particle size associates with apoE-containing HDL in patients who undergo coronary computed tomographic angiography

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Background: Patients with coronary artery disease have several lipoprotein abnormalities, such as increased triglyceride (TG) and LDL-cholesterol, and reduced HDL-cholesterol (HDL-C). Small-dense LDL, with increased TG and reduced HDL-C levels, constitute the atherogenic lipoprotein phenotype. However, associations of LDL particle size (LDL-size) with HDL-associated apolipoproteins and their subclasses have not well been understood. We compared LDL-size with several HDL variables, including apoE-containing HDL-C.

Methods: We studied 52 consecutive patients (age: 66.4 ± 11.9 years, male 50.0%) who underwent coronary computed tomographic angiography. Plasma samples were analyzed by a gel-permeation liquid chromatography system, equipped with a Superose 6 column (GE healthcare), and major lipoprotein cholesterol levels and LDL-size were calculated from each of peak area and elution times on the chromatographic cholesterol patterns, respectively. HDL-associated apolipoproteins (apoA-I, A-II, C-I, C-III and E) were determined by sandwich ELISA on the HDL fractions separated from whole plasma with the polyethylene glycol (PEG) precipitation method. For measurement of apoE-containing HDL-C, the isolated HDL fraction was applied into a heparin affinity column (HiTrap Heparin, GE healthcare) equilibrated with 10 mM MOPS (pH 7.0) containing 50 mM sodium chloride, and bound HDL (apoE-containing HDL) was eluted by 10 mM MOPS (pH 7.0) containing 1.0 M sodium acetate, followed by an on-line enzymatic detection for cholesterol. Pearson's correlation coefficients were used to evaluate the correlation between two variables. A stepwise multiple regression analysis was used to identify HDL variables influencing for LDL-size.

Results: Total cholesterol, HDL-C, and TG levels of the studied subjects were 223 ± 53 (mg/dL), 68.6 ± 20.5 (mg/dL), and 145 ± 61 (mg/dL), respectively. LDL-size was significantly correlated with plasma TG ($r = -0.683$) and HDL-C levels ($r = 0.542$), consistent with previous reports. Moreover, we found a significant positive correlation between LDL-size and apoE levels in the HDL fractions (HDL-apoE) ($r = 0.510$, $P<0.001$). ApoC-I and apoC-III levels in the HDL fractions were also associated with LDL-size, but their correlation coefficient values were smaller than HDL-apoE levels. In contrast, apoA-I and apoA-II, which are major protein components of HDL particles, were not significantly associated with LDL-size. ApoE-containing HDL-C levels and the ratio of apoE-containing HDL-C to total HDL-C were significantly correlated with LDL-size ($r = 0.606$, $P<0.001$, $r = 0.355$, $P<0.05$, respectively). There was a positive correlation between apoE-containing HDL-C and HDL-apoE levels ($r = 0.853$, $P<0.001$). Stepwise multiple regression analyses selected only apoE-containing HDL-C (beta coefficient = 0.606, $P<0.001$) that significantly correlated with LDL-size (adjusted $R^2 = 0.355$), suggesting apoE-containing HDL-C as independent predictor of LDLs.

Conclusion: We found a significant linkage of LDL-size with apoE-containing HDL-C levels in patients with or without various degree of coronary stenosis. These findings may contribute to understanding of lipoprotein metabolisms involved in apoE-containing HDL.

B-123

Cholesterol uptake capacity, a new concept of HDL functionality, for risk stratification in coronary artery disease

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Background: Recent studies have shown that the capacity of high density lipoprotein (HDL) to stimulate cholesterol efflux from lipid-laden macrophages is a better predictor of cardiovascular disease status than overall HDL cholesterol concentration. However, the standard procedures for measuring efflux capacity involve radioisotope-labeled cholesterol and cultured macrophages, thus more simplified method to measure HDL functional levels is strongly demanded for its clinical application.

Methods: We hypothesized that the efficiency of HDL-induced cholesterol efflux from macrophages is mainly dependent on the capacity of HDL to accept cholesterol, which we named "cholesterol uptake capacity". In order to test the hypothesis, we constructed a cell-free plate assay system to evaluate cholesterol uptake capacity of HDL using a fluorescence-labeled cholesterol and an apolipoprotein A1 (apoA1) specific antibody. Briefly, apoB depleted serum was incubated in reaction buffer containing the labeled cholesterol, followed by their capture to the immobilized anti-apoA1 antibody, and then fluorescence signals were detected from the labeled cholesterol incorporated into HDL or apoA1. To investigate the feasibility of cholesterol uptake capacity for coronary risk assessment, we quantified cholesterol uptake capacity of serum samples from 210 patients with coronary artery disease who had previously undergone revascularization.

Results: The assay system had high reproducibility (CV<10%) and a short processing time (<6 hours). Steroid structure of the labeled cholesterol, not fluorescence moiety itself, was essential for its incorporation into HDL and apoA1. Cholesterol uptake capacity declined with the myeloperoxidase-mediated oxidation of HDL or in the presence of lecithin-cholesterol acyltransferase. Cholesterol uptake capacity correlated significantly with cholesterol efflux capacity ($r=0.82$, $P<0.0001$, $n=29$). There was a significant inverse association between requirement of revascularization and cholesterol uptake capacity ($p=0.023$). In patients with the optimal control of low-density lipoprotein cholesterol ($n=125$), only cholesterol uptake capacity remained significant in multivariable analysis adjusted for age, sex, smoking history, HbA1c level, blood pressure, and concentrations of LDL-C and HDL-C (odds ratio 0.53, 95% CI 0.29-0.96, $p=0.0037$).

Conclusion: Cholesterol uptake capacity assay can evaluate functionality of HDL and lipid-free apoA1 in a sensitive and high-throughput manner without using radioisotope-label and cells. The assay system could be applicable for assessing CVD risk in the clinical setting.

B-124

Development of a New Biochip Array for ApoE4 Classification from Plasma Samples Using Immunoassay Based Methods

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Background: Apolipoprotein E (APOE) plays a key role in lipid metabolism and is recognised as one of the most powerful genetic risk factors for dementia and other neurodegenerative diseases. It has become one of the most widely studied gene variants in Alzheimer's disease and constitutes a major consideration for preventive medicine. ApoE exists in three common isoforms (ApoE2, ApoE3 and ApoE4) which are coded by three co-dominant alleles (e2, e3, e4). As such six common ApoE phenotypes exist within the general population E2/E2, E3/E3, E4/E4 (homozygous) and E2/E3, E2/E4, E3/E4 (heterozygous). The presence of the ApoE4 isoform is recognised as a major genetic risk factor for development of Alzheimer's disease. The availability of analytical methods for rapid and reliable ApoE4 classification is therefore advantageous.

Relevance: Biochip Array Technology (BAT) enables the determination of multiple analytes from a single sample. This technology has been successfully applied to a new biochip array to directly identify from a plasma sample whether patients are ApoE4 heterozygous, homozygous or null through simultaneous detection of both total ApoE levels and specific ApoE4 levels.

Methods: Simultaneous chemiluminescent biochip-based sandwich immunoassays for measurement of ApoE4 and total ApoE directly from plasma samples were employed and applied to the Evidence Investigator analyser. An initial cohort of 272 plasma samples of known genotype were used to establish initial assay parameters. A ratio was calculated using total ApoE ApoE4 protein levels to classify samples as ApoE4 heterozygous, homozygous or null. A further cohort of 112 plasma samples of unknown genotype were utilised to verify performance characteristics established employing the initial cohort. Genotype concordance was further investigated by genotyping these same 112 plasma samples from circulating cell free DNA (cfDNA) through the use of another biochip array platform, based on a combination of multiplex PCR and biochip array hybridisation, which allows simultaneous detection of APOE specific single nucleotide polymorphisms (SNPs). Receiver Operating Characteristics (ROC) curve was used to establish the sensitivity and specificity of the assay using the combined cohort of 384 plasma samples.

Results: From the initial cohort of 272 samples with known genotypes, 100% were correctly identified as null, heterozygous or homozygous for ApoE4 by the biochip array. From the additional 112 plasma samples, analysed using BAT for protein and SNPs detection, 100% concordance was found between both approaches. ROC analysis showed that patient samples could be identified as APOE4 positive or negative with 100% sensitivity and 100% specificity, all in approximately 3 hours.

Conclusions: An individual's APOE status has been shown to affect pre-symptomatic risk, diagnosis, prognosis, and treatment response for a variety of diseases, in particular Alzheimer's disease. The results show that BAT can be successfully applied to provide a platform to rapidly and accurately detect an individual's APOE4 status directly from a plasma sample. In combination with medical and family history, medication and lifestyle, this can deliver valuable information for personalised medicine approaches.

B-125

Performance of Calculated Low Density Lipoprotein Cholesterol in a Pediatric Population

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Background: The Friedewald equation is the most widely-used method for determining low-density lipoprotein cholesterol (LDL-C) concentrations. Recent evidence suggests that this formula is constrained by a significant negative bias when LDL-C falls below 70 mg/dL; an effect which is amplified with increasing triglyceride levels.

Objective: The goal of this study was to examine the bias of Friedewald and Hopkins-calculated LDL-C in pediatric samples with high triglycerides (150-399 mg/dL) and low LDL-C levels (≤ 100 mg/dL) relative to the reference method (ultracentrifugation (UC)).

Subjects and Methods: LDL-C concentrations in 69 pediatric plasma samples were measured by UC (LDL-UC) and calculated using Friedewald (LDL-C_F) and Hopkins (LDL-C_H) equations. Bias was assessed by difference plots and regression analysis produced using EP Evaluator software (Data Innovations).

Results: As seen in the table below, LDL-C_F performed well when LDL-UC levels >100 mg/dL (-0.4%), however a significant mean negative bias was observed with LDL-UC ≤100 mg/dL and LDL-UC ≤70 mg/dL (-15 and -16%). The Hopkins formula produced a bias of approximately 5% when LDL-UC>70 mg/dL and this increased to about 11% for LDL-UC ≤70mg/dL. To address this bias, we performed a modification to the Friedewald formula using a mean TG: VLDL ratio of 6.3 (derived from calculation), which negated most bias in low LDL-UC concentration samples (≤100 mg/dL = -1.3% and ≤70 mg/dL = 0.9%).

Conclusions: A modified Friedewald equation, using a TG: VLDL of 6.3, may improve the concordance of calculated results to the reference method in pediatric samples with low LDL-C and increased TG levels. Larger studies are underway to confirm these novel findings in the pediatric populations.

	Average % Bias		
Method	**LDL-UC ≤ 70mg/dL (n = 22)**	**LDL-UC ≤ 100mg/dL (n = 54)**	**LDL-UC >100 mg/dL (n = 15)**
LDL-C_F	-16.2	-15.4	-0.4
LDL-C_H	10.6	4.7	5.2
TG: VLDL = 6.3	0.9	-1.3	6.3

B-126

Detailed Faecal Fat analysis Using FT-IR Spectroscopy: Exploring The Possibilities

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Background: Fourier transform infrared (FT-IR) spectroscopic determination of faecal fat has been reported to be a simple and elegant alternative for the classical Van De Kamer approach. Next to quantification of the fat content, it allows detailed analysis of the chemical nature of fats by molecular fingerprinting. Analysis of lipase hydrolysis efficiency (fatty acid/triglyceride ratio; FA/TG ratio), fatty acid chain length and trans-unsaturated fatty acids could provide better monitoring of dietary treatment in patients with (non-)pancreatic malabsorption. We performed an in-depth exploration of these possibilities.

Methods: Stool samples were prepared for analysis according to the method described by Jakobs et al. (Ann Clin Biochem, 2000). Analyses were carried out on a Perkin Elmer Spectrum Two® spectrometer in the range 3500-450 cm^{-1}, using chloroform for background substraction. Human stool samples (24hrs collection, stored at -20°C, n=96) from patients on which routine fat analysis was requested (n=41) or from patients with known pancreatic and non-pancreatic steatorrhea (n=55) were used. Samples with a fat content < 1g/100g faeces were excluded (n=24), due to limited clinical value and loss of interpretability of the calculations. **A)** FA/TG ratio could be determined using the ratio of absorbance at resp. 2855 and 1746 cm^{-1}. To estimate hydrolysis efficiency of lipase (%), sample ratios were compared with the ratio of butter (containing 82% total fat) and pure free FA (i.e. stearic acid, palmitic acid and mixture of stearic:palmitic acid 65:35). **B)** Mean FA chain length could be calculated using the ratio of absorbance at resp. 2855 and 1709 cm^{-1}. **C)** To determine the specific absorbance peak of trans double bounds, trielaidine (a monoacid trans-unsaturated TG) was analyzed. The area of the peak at 966 cm^{-1} was used to trace the presence of trans-type unsaturated FA.

Results: A) Butter showed a low FA/TG ratio (1,21) and free FA a high FA/TG ratio (6.76). The mean sample ratio was 4,61 (i.e. 61% efficiency), with values ranging from 1,05 (7%) to 7,19 (108% efficiency, similar with ratio of pure FA).

B) The amount of absorbance was correlated with the mean acyl chain length of stearic acid (C18) or palmitic acid (C16)). The relative absorbance contribution per C-atom was calculated (ratio 1,06 for C18-standard and 0,91 for C16-standard), indicating an absorbance-contribution of 0,06 per C-atom. The mean ratio of the samples was 1,12 (i.e. mean acyl chain length of C19), with values ranging from 0,73 (C12) to 1,68 (C28).

C) Two samples from pancreas-insufficient patients contained traceable amounts of trans-type unsaturated FA.

Conclusion: For the analysis of faecal material, FT-IR provides unique information difficult to obtain using other techniques. It allows to elucidate the efficiency of fat digestion by pancreatic lipase, the determination of fatty acid chain length and the detection of trans-unsaturated fatty acids in stool samples. These findings offer new perspectives for diet monitoring in patients with (non-)pancreatic malabsorption.

B-127

Coupling of PAF receptor and modified LDL receptor LOX-1 to transduce oxidized LDL-induced cell signaling.

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Aim: To investigate the potential synergism between LOX-1 and PAF receptor in intracellular signal transduction in response to oxLDL.

Background: Lectin-like oxidized low density lipoprotein (LDL) receptor 1 (LOX-1) is the major receptor for oxidized LDL (oxLDL) in endothelial cells and LOX-1-mediated oxLDL actions induce endothelial dysfunction. Recently, we have reported that the angiotensin II type 1 receptor (AT_1) promotes oxLDL-induced cell responses through interaction with LOX-1 (Yamamoto K., FASEB J 2015). On the other hand, some reports suggested that PAF receptor (PAFR), also a member of G-protein coupled receptor, might be involved in mediating the signal transduction of oxLDL.

Methods: ***Detection of phosphorylation of ERK1/2:*** The cells seeded on 12-well dishes were starved for serum for 24 h, and then incubated with oxLDL (10 μg/ml) for 10 min at 37°C. ERK1/2 phosphorylation of the cell lysates was analyzed by immunoblotting with the antibodies: anti-phosphoERK1/2 (Thr202/Tyr204) antibody, anti-total-ERK1/2 antibody, anti-V5 antibody, and anti-HA antibody.

Luciferase reporter assay for NF-κB and SRF: LOX-1 and PAFR expressing cells seeded in a 24-well plate were transiently co-transfected with 500 ng of NF-κB or SRF promoter Firefly luciferase reporter vectors, pGF1-NF-κB or pGF1-SRF, together with pRL-CMV Renilla luciferase control reporter vector, and cultured for 6 h. Then, the cells were starved for 24 h, and stimulated with oxLDL for 20 h. The luciferase activity was measured with the Dual-Luciferase Reporter Assay Kit.

Detection of DiI-labeled oxLDL: LOX-1 and PAFR expressing cells were treated for 60 min with 3 μg/ml of DiI-labeled oxLDL on ice. Nuclei of the cells were stained with DAPI (1 μg/ml). Quantitative fluorescence cell image analysis was performed using the INCell analyzer 2000 system.

Co-immunoprecipitation: Cell lysate were prepared with a lysis buffer containing 1%Triron- X100. Proteins were precipitated with anti-FLAG-M2 affinity gel and eluted with FLAG peptide. The purified proteins were then analyzed by immunoblotting with antibodies against HA and V5.

Results: OxLDL-induced cell responses, ERK, NF-κB and SRF activation, were promoted by additional expression of PAFR compared with cells expressing solely LOX-1 or PAFR. In this condition, the binding of DiI-labeled oxLDL to the cells expressing both LOX-1 and PAFR was not significantly different from the cells expressing solely LOX-1. In addition, PAFR antagonists, ABT-491, suppressed oxLDL-induced ERK phosphorylation in endothelial cells. Furthermore, immunoprecipitation analysis showed that LOX-1 was co-immunoprecipitated with PAFR from the cells transfected with both PAFR and LOX-1, while Dectin-1, which has highest homology to LOX-1 in C-type lectin-like protein family, was not. These results suggested that LOX-1 and PAFR expressed physically proximal to each other in cell surface might cooperatively strengthen the signal of oxLDL action.

Conclusion: Coupling of LOX-1 and PAFR, as the case of AT_1, might be of importance in oxLDL-induced biological reactions.

B-128

Influence of triglycerides on LDL-C measured by direct homogeneous method and estimated by the Friedewald equation and a novel calculation

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Background: Low-density lipoprotein cholesterol (LDL-C) is the primary biomarker for assessing cardiovascular risk. The gold-standard LDL-C method (beta-quantification; LDL-C_{BQ}) involves ultracentrifugation and lipoprotein specific precipitation and is not influenced by triglycerides (TG). Due to the expense and expertise requirements of LDL-C_{BQ}, estimation of LDL-C by the Friedewald equation (LDL-C_F) is routinely used. Alternative LDL-C methods with purportedly superior performance across TG concentrations include direct homogeneous measurement (LDL-C_D), and the novel estimation formula (LDL-C_N). **Objective**: To assess the influence of TG on performance of LDL-C_F, LDL-C_D, and LDL-C_N compared to LDL-C_{BQ}. **Methods**: Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), TG, and LDL-C_D were measured using Roche Cobas c501 analyzers. LDL-C_{BQ} was performed using Beckman LE-80K ultracentrifuge and dextran sulfate-Mg/Cl precipitation. LDL-C_F was calculated as TC - HDL-C - (TG/5); LDL-C_N was calculated as TC - HDL-C - (TG/X) where X is an empirical value determined based on TG, TC and HDL-C as described in by Martin, et al (JAMA, 2013). Bias was assessed by comparison to LDL-C_{BQ}. Clinical categories were <100, 100-129, 130-159, 160-189, and ≥190 mg/dL LDL-C. **Results:** The median LDL-C_{BQ} for all samples was 97 mg/dL and did not significantly differ with TG. As TG concentrations increased, LDL-C_F significantly underestimated LDL-C_{BQ}, LDL-C_N overestimated LDL-C_{BQ}, and LDL-C_D remained unchanged (**Table 1**). Variability (indicated by SD of bias) increased significantly with TG for LDL-C_F and LDL-C_N and a lesser extent for LDL-C_D. LDL-C_F was most concordant, followed by LDL-C_N and finally LDL-C_D. LDL-C_N was significantly less concordant among samples with TG >300 mg/dL, while LDL-C_F and LDL-C_D were not significantly impacted by TG. **Conclusions**: LDL-C_F was most concordant with LDL-C_{BQ} overall and when TG >400 mg/dL. TG strongly influenced bias and variability for both estimated LDL-C methods. LDL-C_D was least influenced by TG, however, there appeared to be a systematic bias.

Table 1. LDL-C performance across TG

Triglycerides, mg/dL	<150	150-199	200-299	300-399	≥400	Any
N	51	53	51	47	52	254
LDL-C, mg/dL; median (IQR)						
Beta-quantification	91(47)	106(39)	106(46)	88(41)	95(55)	97(46)
Friedewald	93(39)	107(41)	106(54)	85(42)	80(62)	93(52)
Direct	101(43)	118(36)	118(62)	103(44)	103(58)	108(52)
Novel	92(37)	112(38)	116(52)	108(36)	119(52)	108(47)
Bias, mg/dL ; mean±SD						
Friedewald	-1±14	-2±13	-2±13	-6±16	-17±41	-6±23
Direct	8±13	12±11	14±13	15±12	10±26	12±16
Novel	-1±14	4±12	10±13	16±14	23±33	10±21
Concordance						
Friedewald	80%	87%	84%	81%	75%	81%
Direct	69%	51%	51%	57%	58%	57%
Novel	76%	83%	71%	55%	44%	66%

B-129

Prevalence of Lipoprotein X in Samples with Extremely Low High Density Lipoprotein Cholesterol

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Background: A hallmark abnormal lipoprotein produced during cholestatic liver disease is lipoprotein X (LpX). LpX is rich in unesterified cholesterol and phospholipids and the protein component is high in albumin. Despite a lack of apolipoprotein B, LpX has similar density to low-density lipoprotein (LDL) making LpX cholesterol indistinguishable from LDL-C by most LDL-C methods. The presence of LpX in patient sera is detected as a reverse migrating band on lipoprotein electrophoresis methods. In late stages of primary biliary cholestasis, hypercholesterolemia is common; however, concentrations of high density lipoprotein (HDL-C) are severely decreased. Other causes of extremely low HDL-C values include various inherited disorders, malignancy, or androgen use. The prevalence of Lp-X in serum from patients with extremely low HDL-C is unknown. **Objective:** To establish the prevalence of LpX among patients with extremely low HDL-C (<10 mg/dL), and determine if other laboratory values such as apolipoprotein A1 (apoA1), total cholesterol, LDL-C, or serum icterus index correlate with the presence of LpX. **Design and methods:** Sera from 554 consecutive samples with HDL-C <10 mg/dL measured by automated direct method (Roche Cobas c501) were collected between 12/2011 and 12/2015. The presence of LpX was determined by lipoprotein electrophoresis (Helena SPIFE 3000

system using SPIFE Vis Cholesterol Kit). Additional measures included apoA1 and total cholesterol (Roche Cobas systems), serum icterus indices (I-index; Roche Cobas systems). The presence of LpX precludes the ability to measure or estimate LDL-C, however, for purposes of this study LDL-C was estimated by the Friedewald formula in all samples. **Results:** LpX was identified in 125 (22.6%) of 554 specimens. The average (interquartile range (IQR)) HDL-C was 6 (4-7) mg/dL and apoA1 was 40 (24-50) mg/dL in the entire cohort; values were not significantly different in samples with or without LpX ($p > 0.10$). The mean (IQR) I-index for LpX positive samples was 14 (8-18.5) and was significantly higher than LpX negative samples at 7 (1-8) $p<0.0001$). ROC curve analysis for LpX as a function of I-index found an optimal cutoff of 8 (AUC 0.776, sensitivity/specificity = 76%/73%). Samples with LpX had significantly higher serum concentrations for total cholesterol (248 mg/dL vs. 103 mg/dL, cutpoint 128 mg/dL, AUC 0.868, sensitivity/specificity = 87%/74%) and LDL-C (190 mg/dL vs. 55 mg/dL, cutpoint 84 mg/dL, AUC 0.871, sensitivity/specificity = 78%/82%). When using laboratory values obtained from a basic lipid panel to predict the presence or absence of LpX, 40 of 41 samples with an I-index of zero were negative for LpX. Conversely, 55 of 60 samples with an I-index >2 and LDL-C ≥160 mg/dL were positive for LpX. **Conclusion:** LpX is present in 1 of 5 samples (20%) with extremely low HDL-C (<10mg/dL) submitted for standard lipid panel analysis. The presence of LpX in samples with HDL-C <10 mg/dL can be nearly always ruled-out by undetectable I-index, while the presence of LpX is strongly suggested by elevations of both I-index (>2) and LDL-C (≥160 mg/dL).