Free Radical Research

ISSN: 1071-5762 (Print) 1029-2470 (Online) Journal homepage: http://www.tandfonline.com/loi/ifra20

F_2-Isoprostanes in HDL are Bound to Neutral Lipids and Phospholipids

Julie M Proudfoot, Anne E Barden, Kevin D Croft, Jean-M Galano, Thierry Durand, Valerie Bultel-Ponce, Martin Giera & Trevor A Mori

To cite this article: Julie M Proudfoot, Anne E Barden, Kevin D Croft, Jean-M Galano, Thierry Durand, Valerie Bultel-Ponce, Martin Giera & Trevor A Mori (2016): F_2-Isoprostanes in HDL are Bound to Neutral Lipids and Phospholipids, Free Radical Research, DOI: 10.1080/10715762.2016.1250262

To link to this article: http://dx.doi.org/10.1080/10715762.2016.1250262

Accepted author version posted online: 17 Oct 2016.

Submit your article to this journal

Article views: 3

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at http://www.tandfonline.com/action/journalInformation?journalCode=ifra20

Download by: [Ryerson University Library] **Date:** 21 October 2016, At: 11:41

F_2-Isoprostanes in HDL are Bound to Neutral Lipids and Phospholipids

Julie M Proudfoot[1], Anne E Barden[1], Kevin D Croft[1], Jean-M Galano[2], Thierry Durand[2], Valerie Bultel-Ponce[2], Martin Giera[3], Trevor A Mori[1].*

1. School of Medicine and Pharmacology, University of Western Australia, Royal Perth Hospital Unit, Perth, Australia
2. Institut des Biomolécules Max Mousseron (IBMM) UMR 5247, CNRS, Université de Montpellier, ENSCM, Montpellier, France
3. Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands

Julie M Proudfoot
School of Medicine and Pharmacology, University of Western Australia, MRF Building, GPO Box X2213, Perth, Australia 6847.
Tel: +61 8 92240391. Fax: +61 8 92240246.
Email: julie.proudfoot@uwa.edu.au

Anne E Barden
School of Medicine and Pharmacology, University of Western Australia, MRF Building, GPO Box X2213, Perth, Australia 6847.
Tel: +61 8 92240272. Fax: +61 8 92240246.
Email: anne.barden@uwa.edu.au

Kevin D Croft
School of Medicine and Pharmacology, University of Western Australia, MRF Building, GPO Box X2213, Perth, Australia 6847.
Tel: +61 8 92240275. Fax: +61 8 92240246.
Email: kevin.croft@uwa.edu.au

Jean-Marie Galano
Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 - CNRS - UM – ENSCM, Faculté de Pharmacie, 15 Av. Ch. Flahault, BP 14491, F-34093 Montpellier Cedex 05, France.
Tel: 33-4-11-75-95-33. Fax: 33-4-11-75-95-53.
Email: Jean-Marie.Galano@umontpellier.fr

Thierry Durand
Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 - CNRS - UM – ENSCM, Faculté de Pharmacie, 15 Av. Ch. Flahault, BP 14491, F-34093 Montpellier Cedex 05, France.
Tel: 33-4-11-75-95-58. Fax: 33-4-11-75-95-53.
Email: Thierry.Durand@umontpellier.fr

JUST ACCEPTED

Valérie Bultel-Poncé
Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 - CNRS - UM – ENSCM, Faculté de Pharmacie, 15 Av. Ch. Flahault, BP 14491, F-34093 Montpellier Cedex 05, France.
Tel: 33-4-11-75-95-32. Fax: 33-4-11-75-95-53.
Email: Valerie.Bultel@umontpellier.fr

Martin Giera
Center for Proteomics and Metabolomics, Leiden University Medical Center, Albinusdreef 2, 2300RC Leiden, The Netherlands.
Tel: 0031 71 5269526.
Email: m.a.giera@lumc.nl
ORCiD: 0000-0003-1684-1894.

Trevor A Mori
School of Medicine and Pharmacology, University of Western Australia, MRF Building, GPO Box X2213, Perth, Australia 6847.
Tel: +61 8 92240273. Fax: +61 8 92240246.
Email: trevor.mori@uwa.edu.au

*Address correspondence to:
Julie M Proudfoot, School of Medicine and Pharmacology, University of Western Australia, MRF Building, GPO Box X2213, Perth, Australia 6847.
Tel: +61 8 92240391. Fax: +61 8 92240246.
Email: julie.proudfoot@uwa.edu.au

WORD COUNT : 6,273

JUST ACCEPTED

Abbreviations:

CAD, coronary artery disease; Chol, cholesterol; F_2-IsoP, F_2-isoprostane; Chol-IsoP, Cholesterol-F_2-isoprostane; MG-IsoP, monoglyceride-F_2-isoprostane; CE, cholesterol ester; TG, triglyceride; MG, monoglyceride; DG, diglyceride; FA, fatty acid; PL, phospholipid; PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; oxPAPC, oxidized PAPC; AAPH, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; ADP, adenosine diphosphate; PFBBr, Pentafluorobenzylbromide; DIPEA, *N*,*N*-diisopropylethylamine; BSTFA, *N*,*O*-*bis*-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-TMCS, 99:1); BHT, butylated hydroxytoluene; LC/MS, liquid chromatography/mass spectrometry; GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; MeOH, methanol; THF, tetrahydrofuran; NH_4^+, ammonium ion; Li^+, lithium ion; SPE, solid phase extraction.

JUST ACCEPTED

F_2-Isoprostanes in HDL are Bound to Neutral Lipids and Phospholipids

Low HDL cholesterol (HDL-C) is a risk factor for coronary artery disease (CAD). However, interventions that raise HDL-C have failed to reduce cardiovascular events. We previously reported that HDL is the main carrier of plasma F_2-isoprostanes (F_2-IsoPs) that are markers of oxidative stress formed upon oxidation of arachidonic acid. F_2-IsoPs are predominantly associated with phospholipids. However, there is evidence that F_2-IsoPs in the liver of rats treated with carbon tetrachloride associate with the neutral lipids. To date it is not known whether F_2-IsoPs are found in the neutral lipids in HDL in humans. Possible candidate neutral lipids include cholesteryl esters, triglycerides, diglycerides and monoglycerides.This study aimed to identify the lipid classes within native and oxidized HDL that contain F_2-IsoPs. We showed that F_2-IsoPs in HDL are bound to neutral lipids as well as phospholipids. HDL-3 contained the highest concentration of F_2-IsoPs in all lipid classes before and after *in vitro* oxidation. Using targeted LC/MS and high resolution MS, we were unable to provide conclusive evidence for the presence of the synthesized standards 15(R)-15-F_{2t}-isoP cholesterol and 1-ent-15(RS)-15-F_{2t}-isoprostanoyl-sn-glycerol in the neutral lipids of HDL. Our findings show that oxidized lipids such as F_2-IsoPs are found in the core and surface of HDL. However, the exact molecular species remain to be definitively characterised. Future studies are required to determine whether the presence of F_2-IsoPs in neutral lipids alters HDL function.

JUST ACCEPTED

Keywords:

HDL, oxidized lipids, F_2-isoprostanes, lipoproteins, mass spectrometry.

Funding Details:

This work was supported by the National Heart Foundation of Australia under grant number G09P4425 and the Medical Research Foundation at Royal Perth Hospital.

JUST ACCEPTED

1. INTRODUCTION

Many human population studies have demonstrated that higher HDL cholesterol levels are associated with decreased risk of CAD [1,2] and that low levels are correlated with increased CAD risk [3]. However, randomised clinical trials designed to raise HDL cholesterol, have failed to reduce the incidence of CAD [4-6]. This has prompted a shift in focus to examine whether an increase in functional HDL particles that may be distinct from HDL cholesterol levels [7], is responsible for the apparent protective effect of HDL on CAD risk.

HDL has many antiatherogenic functions, including the ability to promote cholesterol efflux from cells [8], protecting LDL from oxidation [9] and exhibiting anti-inflammatory properties [10]. These attributes are related to HDL composition and structural characteristics as described recently [11,12]. Cholesterol efflux capacity, and antioxidative, antithrombotic, anti-inflammatory and antiapoptotic activities, are predominantly associated with small, dense, protein-rich HDL3 [11,12].

We have previously shown that HDL is the main carrier of the oxidized lipid species, F_2-IsoPs, in plasma (about 60% of total plasma F_2-IsoPs) and that HDL3 particles contain significantly elevated concentrations of F_2-IsoPs compared with HDL2 particles [13]. Thus the presence of F_2-IsoPs in HDL has the potential to influence many of the functions of HDL. However, the location of these oxidized lipid species within HDL particles is unknown, as is their possible effect on the antiatherogenic properties of HDL.

F_2-IsoPs result from free radical oxidation of arachidonic acid [14] so any lipids within lipoproteins containing arachidonic acid are a potential source of F_2-IsoPs following oxidation. In liver extracts from rats treated with carbon tetrachloride, F_2-IsoPs were predominantly associated with phospholipids [15]. F_2-IsoPs detected in less polar fractions were not further analysed and were thought to contain F_2-IsoPs esterified to

JUST ACCEPTED

other phospholipids and/or neutral lipids [15]. To date these neutral lipids have not been characterized. F_2-IsoP-containing PLs in human HDL have been described [15,16,17]. However, to our knowledge, no one has measured F_2-IsoPs in neutral lipids in HDL in humans. Possible candidate neutral lipids include cholesteryl esters (CE), triglycerides (TG), diglycerides (DG) and monoglycerides (MG).

This study aimed to examine F_2-IsoPs in the lipids of HDL and LDL in humans. In order to achieve this, lipids were extracted from lipoproteins, separated into lipid classes and each fraction was assayed for the presence of F_2-IsoPs. Cholesterol-F_2-isoprostane (Chol-IsoP) (Figure 1) and monoglyceride-F_2-isoprostane (MG-IsoP) (Figure 1) were synthesized as potential F_2-IsoP-containing molecules within HDL and LDL.

JUST ACCEPTED

2. MATERIALS AND METHODS

2.1. Reagents

Cholesteryl linoleate (standard CE) was purchased from Sigma-Aldrich (St. Louis, MO). 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (standard TG), 2-arachidonoyl glycerol (standard MG), 1-stearoyl-2-arachidonoyl-sn-glycerol (standard DG), 15-F_{2t}-isoprostane (8-*iso* $PGF_{2\alpha}$) and 15-F_{2t}-isoprostane-d_4 were purchased from Cayman Chemical Co. (Ann Arbor, MI); 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) (standard PL) from Avanti Polar Lipids; 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) from Aldrich Chemical Co. (Milwaukee, WI); Disodium EDTA from ICN Biomedicals (CA); PBS from Gibco™ Invitrogen (Carlsbad, CA); silylating agent *N,O-bis*-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-TMCS, 99:1) (BSTFA) from Pierce Chemicals (Rockford, IL); Cholesteryl linoleate, Pentafluorobenzylbromide (PFBBr), *N,N*-diisopropylethylamine (DIPEA), butylated hydroxytoluene (BHT) and all other chemicals were from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC grade or higher, methanol (MeOH) (Burdick and Jackson, Muskegon, MI, USA) and water (Optima, Thermo Fisher Scientific, Victoria, Australia) were LC/MS grade. Lithium hydroxide reagent grade, 2-propanol, ammonium formate and formic acid LC/MS grade were from Sigma Aldrich (St. Louis, MO, USA). *tert*-Butyldimethylsilyl chloride, dicyclohexylcarbodiimide, 4-dimethylaminopyridine, and tetra-*n*-butylammonium fluoride were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Measurement of F_2-isoprostanes

F_2-IsoPs were measured by GC-MS using electron capture negative ionization as previously described [13,18].

2.3. Preparation of Lipoproteins to Determine Distribution of F_2-isoprostanes

JUST ACCEPTED

Blood was collected from healthy, non-smoking men and women with written informed consent and ethics approval from the Human Ethics Committee of the University of Western Australia. Plasma was prepared as previously described, from blood collected into EDTA (final concentration 1 mg/ml), reduced glutathione (final concentration 1 mg/ml) and BHT (final concentration of 40 µg/ml) and stored at -80 ^{o}C [13]. Lipoproteins were isolated from plasma adjusted to density 1.26 g/ml with gradient solutions layered on top [13]. Samples were ultracentrifuged at 250 000 *g* for 24 h at 15 ^{o}C using a Beckman L-90K ultracentrifuge (Beckman Instruments, Australia) and fractions aspirated. Lipoproteins were stored at 4 ^{o}C and used within 14 days. Total Cholesterol was measured using the Cholesterol reagent enzymatic method (Roche Diagnostics GmbH, Germany). PLs were measured using a kit (Wako Pure Chemical Industries Ltd, Japan) and MG, DG and TG were quantitated using triglycerides reagent (Thermo Scientific, France).

In some experiments, lipoproteins were diluted to 0.35 mM Cholesterol in PBS and oxidized with 5 mM AAPH at 37 ^{o}C (n=3-4).

2.4. Preparation of Oxidized Lipid Standards (Figure 1)

2.4.1.Synthesis of Monoglyceride - F_2-isoprostane (MG-IsoP) 1-ent-15(RS)-15-F_{2t}-isoprostanoyl-sn-glycerol

Following our previously described synthesis of 15-F_{2t}-IsoP [19], fully *tert*-butyldimethylsilyl-protected *ent*-15(*RS*)-15-F_{2t}-IsoP was coupled with 1,2-bis-*tert*-butyldimethylsilyl-2-(*R*)-glycerol with dicyclohexylcarbodiimide and 4-dimethylaminopyridine. The second step consisted of *tert*-butyldimethylsilyl cleavage using tetra-*n*-butylammonium fluoride followed by Kaburagi and Kishi [20] work-up to furnish 1-*ent*-15(*RS*)-15-F_{2t}-isoprostanoyl-*sn*-glycerol in 35% overall yield.

JUST ACCEPTED

^{1}H-NMR (300MHz, CD3OD): 5.52 – 5.37 (m, 4H), 4.12-3.78 (m, 5H), 3.65-3.48 (m, 3H), 2.70-2.60 (m, 1H), 2.50-2.41 (m, 1H), 2.37-2.23 (m, 2H), 2.13-1.99 (m, 5H), 1.70-1.6 (m, 2H), 1.55-1.42 (m, 3H), 1.36-1.25 (m, 6H), 0.88 (br t, 1H).

2.4.2.Synthesis of Cholesterol - F_2-isoprostane (Chol-IsoP) 15(R)-15-F_{2t}-isoprostane cholesterol

Following our previously described synthesis of 15-F_{2t}-IsoP [19], fully *tert*-butyldimethylsilyl-protected 15(*R*)-15-F_{2t}-IsoP was coupled with cholesterol with dicyclohexylcarbodiimide and 4-dimethylaminopyridine. The second step consisted in *tert*-butyldimethylsilyl cleavage using tetra-*n*-butylammonium fluoride followed by Kaburagi and Kishi [20] work-up to furnish 15(*R*)-15-F_{2t}-isoprostane cholesterol in 26% overall yield.

^{1}H-NMR (300MHz, CD3OD): 5.50 (dd, J=5.0 Hz, 1H), 5.52-5.32 (m, 4H), 4.57-4.48 (m, 1H), 4.00-3.91 (m, 2H), 3.87-3.81 (m, 1H), 2.71-2.64 (m, 1H), 2.50-2.41 (m, 1H), 2.30-2.25 (m, 4H), 2.10-1.98 (m, 6H), 1.97-1.78 (m, 4H), 1.68-1.24 (m, 23H), 1.21-0.96 (m, 9H), 1.03 (s, 3H), 0.92 (d, J=6.6 Hz, 3H), 0.88 (br t, 3H), 0.87 (d, J=1.0 Hz, 3H), 0.85 (d, J=1.0 Hz, 3H), 0.70 (s, 3H).

2.4.3.Preparation of Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxidized PAPC)

PAPC was oxidized according to the method of Longmire et al. [21]. Briefly, PAPC (10 mg in 0.4 ml ethanol) was incubated at 37 ^{o}C with a final concentration of 5 µM ferric chloride, 2 mM ADP and 1 mM ascorbic acid in 50 mM Tris-HCl pH 7.4 containing 150 mM KCl in a total volume of 3.6 ml, with intermittent mixing. After 45 min, lipids were extracted with chloroform/methanol (2:1) [22], containing 0.005% BHT, followed by extraction with 4 ml equivalents of aqueous NaCl (0.9%) to 20 ml equivalents of chloroform/MeOH (2:1). The lower layer was dried under vacuum. Oxidized PAPC

JUST ACCEPTED

(oxPAPC) was resuspended in 90% methanol/10% water and purified on a Hypersil Gold C8 column (5 µm) (250 x 4.6 mm) (Thermo Fisher Scientific, Scoresby, Victoria, Australia) eluted isocratically with 90% methanol/10% water at 1 ml/min using a Hewlett Packard 1100 series HPLC (Hewlett Packard). Fractions were dried and resuspended in methanol and F_2-IsoPs were measured by GC-MS [13,18].

2.5. Extraction and Separation of Lipids from Plasma HDL and LDL

Lipids were extracted from plasma HDL and LDL using chloroform/MeOH (2:1) [22], dried under vacuum, and separated using two different solid phase extraction (SPE) methods.

a) The first SPE method used a Supelco 55261-U hybrid SPE phospholipid cartridge (Figure 2), washed with water and conditioned with 2-propanol containing 1% formic acid, to retain phospholipids on the cartridge. Samples free of phospholipids were then analysed for Chol-IsoP using high resolution LC-MS. HDL lipid extracts dissolved in 2-propanol containing 1% formic acid were applied to the SPE column and the eluate collected. The neutral lipids were dried under nitrogen and resuspended in 50% 2-propanol.

b) The second SPE method used Bond Elut NH2 columns (200 mg, Varian, Agilent Technologies) (Figure 3) to separate lipid extracts into lipid classes as described by Agren et al. [23], prior to LC/MS analysis. Lipid standards of CE, TG, MG, DG and PL, as well as MG-IsoP, Chol-IsoP, oxPAPC and 15-F_{2t}-IsoP, were applied to separate columns and fractions collected to identify their elution pattern. Standards and lipid extracts were eluted under the same conditions. SPE columns were prewashed with 0.6 ml acetone/water (7:1) and *n*-hexane (2×1ml). The standards or dried lipid extracts (approximately 3 mg lipid) were resuspended in 0.2 ml of *n*-hexane/methyl *tert*-butyl ether/acetic acid (100:3:0.3) and applied to the column under gravity. The column was

JUST ACCEPTED

eluted sequentially with 2.5 ml *n*-hexane (CE fraction), 3 ml *n*-hexane/chloroform/ethyl acetate (100:5:5) (TG fraction), 2.5 ml chloroform/2-propanol (2:1) (MG and DG fraction), 3 ml chloroform/MeOH/acetic acid (100:2:2) (FA fraction) and 3.5 ml MeOH/chloroform/water (10:5:4) (PL fraction). All column elution solutions contained 4µg/ml BHT to prevent artefactual production of IsoPs. The CE standard was identified in fractions using the Chol reagent (Roche Diagnostics GmbH, Germany), the TG, MG and DG standards were identified using the TGs reagent (Thermo Scientific, France) and the PL standard identified using a kit (Wako Pure Chemical Industries Ltd, Japan); MG-IsoP and Chol-IsoP standards, oxPAPC and 15-F_{2t}-IsoP standard were identified following concentration under vacuum, hydrolysis and GC-MS [13,18]; HDL lipid extracts were eluted, fractions concentrated under vacuum, then hydrolyzed and analyzed for F_2-IsoPs by GC-MS [13,18].

2.6. Purification of the neutral lipid (MG/DG) fraction

For more detailed analysis, the MG/DG fraction from chromatography on Bond Elut NH2 columns was further purified using HPLC (Figure 4). The MG/DG fraction was resuspended in acetonitrile and fractionated on a Hypersil Gold C8 column (5 µm) (250 × 4.6 mm) using a Hewlett Packard 1100 series HPLC to separate CE, MG and DG. The column was eluted at 1 ml/min with the following mobile phases A: acetonitrile, B: 2-propanol and C: MeOH. Chromatography used a gradient of 100% A at 0 min to 100% B at 40 min, followed by 100% B from 40 to 60 min and 100% C from 60 to 80 min. 1 ml fractions were collected, dried and F_2-IsoPs were measured by GC-MS [13,18].

2.7. LC/MS analysis of HPLC purified neutral lipid

HPLC fractions 4 and 11 (Figure 4) were analysed using LC/MS consisting of two Agilent 1290 UPLC binary pumps coupled to an Agilent 6460 triple quadrupole tandem

JUST ACCEPTED

mass spectrometer with a Jetstream source (Figure 5). The column used was an Eclipse XDB-C18 (3.5 µm) (2.1 × 150 mm). Data processing was performed using Agilent MassHunter software.

2.7.1. LC/MS of the Chol-IsoP standard using tandem mass spectrometry

(i) Conditions identifying the Chol fragments: (Figure 5A) Chromatography used the following mobile phases: A: 50% H_2O, 20% MeOH, 30% THF, 10 mM ammonium formate, 0.1% formic acid; B: 5% H_2O, 20% MeOH, 75% THF, 10 mM ammonium formate, 0.1% formic acid with a gradient of 100% A at 0 min to 100% B at 8 min, followed by 100% B from 8 to 10 min and finally 100% A from 10 to 12 min. The flow rate was 500 µl/min and the column temperature was 40 ^{o}C. The mass spectrometer was operated in positive electrospray ionization mode (ESI+) with the gas temperature at 350 ^{o}C, gas flow 5 l/ min, nebulizer 45 psi, sheath gas 250 ^{o}C, sheath gas flow 11 l/min, capillary 3,500 V and charging 500 V. Mass transitions for detection were $M+NH_4^+$ 740.5→369.2 (cholestane) (Collision-induced dissociation (CID) 30), $M+NH_4^+$ 740.5→161 (CID 30) (collision energy 15V).

(ii) Conditions identifying the IsoP fragments: (Figure 5B) Chromatography used the following mobile phases: A: 50% H_2O, 20% MeOH, 30% THF, 10 mM lithium hydroxide, 0.1% formic acid; B: 5% H_2O, 20% MeOH, 75% THF, 10 mM lithium hydroxide, 0.1% formic acid, with a gradient of 100% A at 0 min to 60% B at 1 min, to 100% B at 5 min then 100% B to 7 min and finally 100% A from 7 to 10 min. Flow rate was 350 µl/min and the column temperature was 20 ^{o}C. The mass spectrometer was operated in ESI+ mode with the gas temperature at 300 ^{o}C, gas flow 8 l/min, nebulizer 45 psi, sheath gas 250 ^{o}C, sheath gas flow 8 l/min, capillary 3500 V and charging 500 V. Mass transitions for detection were $M+Li^+$ 729.5→361 (CID 40), $M+Li^+$ 729.5→343 (CID 45) (collision energy 45V).

JUST ACCEPTED

2.7.2. LC/MS of the Chol-IsoP standard using high-resolution mass spectrometry

HDL lipid extracts were analysed using a Dionex Ultimate system (Ultimate 3000 RS nano LC system, Dionex, Amsterdam, The Netherlands) equipped with a Phenomenex Kinetex C18 column (1.7 µm) (100 Å) (2.1 mm × 50 mm) (Figure 2, Table 1). Temperature 50 ^{o}C. Mobile phases were: A: 80% H_2O, 20% acetonitrile, 0.05% formic acid, 5 mM ammonium formate; B: 90% 2-propanol, 9% acetonitrile, 1% water, 0.05% formic acid, 5 mM ammonium formate. Flow rate was 250 µl/min. Chromatography used 50% A from 0 min to 1 min, and a gradient from 50% A at 1 min to 5% A at 8 min and maintained 5% A to 10 min. The LC was coupled to a Bruker QToF Ultima mass spectrometer (Bruker, Bremen, Germany) operated under the following conditions: ESI+ mode, scanning from *m/z* 150 to 1250. Nebulizer 2.1 bar, dry gas 9 l/min (both nitrogen 99.9990%), dry heater 200 ^{o}C and capillary voltage 3500 V.

2.7.3. LC/MS of the MG-IsoP standard (Figure 5C)

Chromatography used the mobile phases as described under 2.7.1 (ii) with 100% A at 0 min to 4 min, then a gradient of 100% A at 4 min to 100% B at 5 min, then 100% B from 5 to 7.5 min and 100% A from 7.5 to 10 min. The flow rate was 350 µl/min and the column temperature was 20 ^{o}C. The mass spectrometer was operated in ESI+ mode with the gas temperature at 300 ^{o}C, gas flow 8 l/min, nebulizer 45 psi, sheath gas 250 ^{o}C, sheath gas flow 8 l/min, capillary 3500 V and charging 500 V. Mass transitions for detection were $M+Li^+$ 435.1→ 417 (CID 35), $M+Li^+$ 435.1→ 153 (CID 35) (collision energy 35V).

JUST ACCEPTED

3. RESULTS

3.1. LC/MS to determine presence of Chol-IsoP in HDL lipids

To ascertain if the synthesized oxidized lipid standards were present in HDL lipid extracts, LC/MS was first used to determine conditions for elution (Figure 2A) and detectability after SPE treatment (Figure 2B) of the Chol-IsoP standard. SPE was applied to remove PLs from the HDL total lipids due to co-elution, causing possible ion-suppression (Figure 2C). As can be seen from Figure 2D, neither the SPE treated nor untreated HDL fractions gave rise to a conclusive signal related to Chol-IsoP. Additionally, the high resolution MS data was examined for the presence of potential F_2-IsoP-containing lipids, including Chol-IsoP and MG-IsoP, however, no molecules corresponding to the exact masses of relevant compounds were found (Table 1).

3.2. Distribution of F_2-isoprostanes in plasma HDL and LDL lipid classes

A second approach to identify Chol-IsoP in HDL lipid extracts used separation of HDL and LDL lipid extracts on Bond Elut-NH2 SPE columns. Partitioning of lipid classes on these columns was confirmed using lipid standards: cholesteryl linoleate (CE), 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (TG), 2-arachidonoyl glycerol (MG), 1-stearoyl-2-arachidonoyl-sn-glycerol (DG) and PAPC (PL). HDL2 and HDL3 were extracted and separated into fractions corresponding to CEs, TGs, MGs/DGs, FAs and PLs [23], and F_2-IsoPs were measured following hydrolysis of each fraction. LDL was extracted and separated in a similar manner for comparison. Figure 3A shows the distribution of F_2-IsoPs within these fractions for HDL2, HDL3 and LDL. HDL2 and HDL3 had higher levels of F_2-IsoPs than LDL before and after oxidation with AAPH (Figure 3). F_2-IsoPs eluted mainly in the fractions corresponding with the MG/DG and PL standards. A significant amount (about 30%) of HDL F_2-IsoPs were found in the neutral lipids, while about 60% appeared in the PL fraction (Figure 3). These results were confirmed in 6 other HDL preparations (results not shown). Given that F_2-IsoPs

JUST ACCEPTED

were in the neutral lipid fraction, we postulated that they may be in the form of Chol-IsoP or MG-IsoP and confirmed that these synthesized standards also eluted in this fraction.

3.3. LC/MS to determine presence of Chol-IsoP and MG-IsoP in HPLC-purified HDL neutral lipids

HPLC was employed to separate the neutral lipids in the HDL MG/DG fraction from the Bond Elut-NH2 SPE, and provide purer fractions for LC/MS analysis. F_2-IsoPs were measured in each HPLC fraction after concentration and hydrolysis. Figure 4 shows that MG-IsoP and Chol-IsoP eluted in different fractions after HPLC, in contrast to the Bond Elut-NH2 SPE column where they co-eluted. F_2-IsoPs in the HDL MG/DG fraction from Bond Elut-NH2 SPE eluted predominantly in HPLC fractions 4 and 11 that corresponded to the retention times for the MG-IsoP and Chol-IsoP standards, respectively (Figure 4).

The structure of Chol-IsoP in Figure 1 indicates that fragmentation of the molecule can occur via cleavage of the Chol moiety or the IsoP component. Mass transitions for the Chol moiety of the Chol-IsoP synthetic standard are shown in Figure 5A and for the IsoP component in Figure 5B.

HPLC fractions 4 and 11 were analysed using ESI+, CID with multiple reaction monitoring (MRM) employing a triple quadrupole LC/MS. Figure 5A shows the fragmentation of the Chol component of HPLC fraction 11 with two $M+NH_4^+$ transitions. The composite of the fragmentation of the Chol-IsoP standard and HPLC fraction 11 shows a small but inconclusive overlap (Figure 5A). However, the close elution of another isobaric peak indicates strongly that this may be an isomer of the CE species. Fragmentation of the IsoP component of HPLC fraction 11 with two $M+Li^+$ transitions, and overlay of this chromatogram with that for the Chol-IsoP standard is

JUST ACCEPTED

shown in Figure 5B. Again the data are inconclusive, however, there is an isobaric peak with a retention time close to the standard, suggesting a CE isomer.

Figure 5C shows fragmentation of the MG-IsoP standard compared to that of HPLC fraction 4 with two $M+Li^+$ transitions. The resulting chromatograms and composite provide inconclusive evidence for the presence of MG-IsoP in HDL neutral lipids, although the broad peaks for HPLC fraction 4 transitions suggest multiple isobaric species.

4. DISCUSSION

This is the first study describing the presence of F_2-IsoPs in the neutral lipid fraction in human HDL and LDL. We have shown that approximately 30% of the total F_2-IsoPs in HDL are in neutral lipids. F_2-IsoPs were identified in fractions that corresponded with the elution profile of Chol-IsoP and MG-IsoP standards, however, using a targeted approach with these specific synthesized standards we could not find conclusive evidence for the presence of these lipids in HDL. However, in the Chol-IsoP fragmentations there was an isobaric peak that eluted closely to the retention time of our synthesized Chol-IsoP standard in both the cholesterol moiety transitions and the isoprostane moiety transitions, suggesting the presence of an IsoP isomer of the CE species. Our studies were conducted using samples collected under reducing conditions with care taken to prevent possible *ex-vivo* oxidation during isolation of lipoproteins and their lipid class fractions. *In vitro* oxidation of HDL and LDL showed the profile of F_2-IsoPs was similar to that of native lipoproteins, indicating that the lipid fractions were equally susceptible to oxidation. Our data supports the only other report identifying F_2-IsoPs in neutral lipids. Morrow et al. showed F_2-IsoPs were primarily bound to PLs and neutral lipids in liver extracts from carbon tetrachloride treated rats [15].

JUST ACCEPTED

The major lipid components of HDL have recently been reviewed [24]. Most plasma HDL particles are spherical with a neutral lipid core containing CE and TG surrounded by protein, PL, sphingolipids and cholesterol on the surface. A recent study [25] reported that Type 2 diabetics had smaller HDL particles, forcing CE and TG from the HDL core to the surface, termed "herniated" HDL. This extrusion of core lipids increased hydrophobicity of the HDL surface and may increase susceptibility of neutral lipids to oxidation.

The neutral lipids comprise about 50 mol% of total lipids in HDL of which CE account for around 36 mol% [24]. The CE cholesteryl arachidonate represented 7.3% of total cholesteryl ester species in HDL2 and HDL3 [26]. Other studies reported 7 mol% [27] and 9.4 mol% [28] cholesteryl arachidonate in HDL. Arachidonate-containing DG comprised 3.8% of native HDL DGs [26], however, DG accounted for only about 0.23 mol% of HDL total lipids [24]. These arachidonate-containing lipids are possible sources of the F_2-IsoPs measured in HDL neutral lipids, particularly the relatively abundant cholesteryl arachidonate being oxidized to Chol-IsoP.

We synthesized two candidate oxidized neutral lipids; cholesterol-15-F_{2t}-isoprostane (Chol-IsoP) and monoglyceride-15-F_{2t}-isoprostane (MG-IsoP) that were used as standards to determine their presence in lipid fractions isolated from native HDL. During SPE chromatography and Bond Elut NH2 column elution, the Chol-IsoP standard did not elute in the CE fraction but was present in the MG/DG fraction where the MG-IsoP standard eluted. This is most likely due to increased polarity of Chol-IsoP compared with cholesteryl arachidonate, similar to the reported increased polarity of F_2-IsoP-containing phosphatidylcholine compared to nonoxidized phosphatidylcholine [15]. We reasoned that F_2-IsoP-containing lipids in the MG/DG fraction could derive from oxidized arachidonate-containing CE, TG, MG and/or DG. When the HDL

JUST ACCEPTED

MG/DG fraction from the Bond Elut NH2 column was purified using HPLC, two F_2-IsoP-containing peaks were identified, that corresponded to the elution times of the MG-IsoP and Chol-IsoP standards. However, we were unable to provide conclusive evidence for the presence of either MG-IsoP in the first peak (fraction 4, Figure 4) or Chol-IsoP in the second peak (fraction 11, Figure 4). Elution of peaks close to the retention time of the standards suggested that the species in HDL neutral lipids may have been isomers of the synthesized standards. The sensitivity of current LC/MS technology for measuring oxidized neutral lipids may be a limiting factor and exacerbated by the low levels present compared with other lipids. There is potential for multiple F_2-IsoP-containing neutral lipids in HDL and also the possibility that other residues in the lipids are oxidized, along with the arachidonic acid, for example oxidized cholesterol in CE. This expands the number of potential oxidized neutral lipids in lipoproteins. There are many IsoP isomers and some of these co-elute during measurement with GC-MS [18]. Our Chol-IsoP and MG-IsoP standards were prepared using the 15-F_{2t}-IsoP isomer.

We previously showed that blood collection into EDTA alone resulted in significant increases in F_2-IsoPs compared to collection into EDTA/BHT/reduced glutathione [29]. In the present study, plasma was collected into EDTA/BHT/reduced glutathione to minimize artefactual production of F_2-IsoPs and BHT was included in column elution solutions for the same reason.

A recent investigation demonstrated that surface lipids, such as oxidized phosphatidylcholines and oxidized Chol, were readily transferred from oxidized LDL to HDL, while internal oxidized CEs were less readily transferred [30]. An earlier report found that HDL can accept oxidized CEs from oxidized LDL, but only in the presence of cholesteryl ester transfer protein (CETP) [31]. CETP did not distinguish between

JUST ACCEPTED

oxidized or unoxidized CEs as it facilitated their transfer between HDL and LDL [31]. Transfer of PL hydroperoxides (PLOOH) from oxidized LDL to HDL3 was shown to be dependent on the rigidity of HDL surface lipids, and was followed by inactivation of PLOOH by apoAI [32]. Transfer of oxidized lipids between lipoproteins is a possible source of F_2-IsoPs in HDL. Du et al. [12] showed that small, dense HDL3 subfractions were the most efficient mediators of Chol efflux. Our results indicated that HDL3 particles contained the greatest concentration of F_2-IsoPs in neutral lipids and PLs, compared to HDL2 particles. Whether this reflects their greater involvement in efflux of not only Chol, but also PLs and other lipids, oxidized or un-oxidized, is unknown. The magnitude of F_2-IsoPs may reflect a balance between lipid transfer between lipoproteins, lipid efflux from cells and *in situ* HDL oxidative damage.

This is the first report, to our knowledge, describing F_2-IsoPs in human lipoprotein neutral lipids. Measurement of these markers in core and surface lipids may provide valuable information on the degree of oxidative damage in different lipid classes and contribute to our understanding of the role of oxidative stress in CAD. Future studies will also need to determine whether the presence of F_2-IsoPs in neutral lipids alters HDL function.

JUST ACCEPTED

ACKNOWLEDGEMENTS

We thank Marieke Heijink and Evelyne Steenvoorden for assistance with the high resolution MS analysis.

We would also like to acknowledge Associate Professor Peter Meikle, Baker IDI Heart and Diabetes Institute, Melbourne, Australia for helpful advice in planning some experiments.

Disclosure statement: The authors report no conflicts of interest.

REFERENCES

1. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med 1977;62:707–714.
2. Miller M, Seidler A, Kwiterovich PO, Pearson TA. Long-term predictors of subsequent cardiovascular events with coronary artery disease and 'desirable' levels of plasma total cholesterol. Circulation. 1992;86:1165–1170.
3. Barter P, Gotto AM, LaRosa JC, Maroni J, Szarek M, Grundy SM et al. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. N Engl J Med 2007;357:1301–1310.
4. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M et al. Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med 2007;357:2109–2122.
5. Boden WE, Probstfield JL, Anderson T, Chaitman BR, Desvignes-Nickens P, Koprowicz K et al. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. N Engl J Med 2011;365:2255–2267.
6. Schwartz GG, Olsson AG, Abt M, Ballantyne CM, Barter PJ, Brumm J et al. Effects of dalcetrapib in patients with a recent acute coronary syndrome. N Engl J Med 2012;367:2089–2099.
7. Rached FH, Chapman MJ, Kontush A. HDL particle subpopulations: Focus on biological function. Biofactors. 2015;41:67-77.

JUST ACCEPTED

8. Duffy D, Rader DJ. Emerging therapies targeting high-density lipoprotein metabolism and reverse cholesterol transport. Circulation. 2006;113:1140–1150.
9. Navab M, Hama SY, Anantharamaiah GM, Hassan K, Hough GP, Watson AD et al. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. J Lipid Res 2000;41:1495–1508.
10. Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, Fogelman AM. Antiinflammatory properties of HDL. Circ Res 2004;95:764–772.
11. Camont L, Lhomme M, Rached F, Le Goff W, Nègre-Salvayre A, Salvayre R et al. Small, dense high-density lipoprotein-3 particles are enriched in negatively charged phospholipids: relevance to cellular cholesterol efflux, antioxidative, antithrombotic, anti-inflammatory, and antiapoptotic functionalities. Arterioscler Thromb Vasc Biol 2013;33:2715-2723.
12. Du X, Kim MJ, Hou L, Le Goff W, Chapman MJ, van Eck M et al. HDL particle size is a critical determinant of ABCA1-mediated macrophage cellular cholesterol export. Circ Res 2015;116:1133-1142.
13. Proudfoot JM, Barden AE, Loke WM, Croft KD, Puddey IB, Mori TA. HDL is the major lipoprotein carrier of plasma F2-isoprostanes. J Lipid Res 2009;50:716–722.
14. Morrow JD, Hill KD, Burk RF, Nammour TM, Badr KF, Roberts LJ. 1990. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc Natl Acad Sci USA 1990;87:9383–9387.
15. Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ. Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. Proc Natl Acad Sci USA 1992;89:10721–10725.

JUST ACCEPTED

16. Ahmed Z, Ravandi A, Maguire GF, Emili A, Draganov D, La Du BN et al. Apolipoprotein A-I promotes the formation of phosphatidylcholine core aldehydes that are hydrolyzed by paraoxonase (PON-1) during high density lipoprotein oxidation with a peroxynitrite donor. J Biol Chem 2001;276:24473-24481.
17. Ahmed Z, Ravandi A, Maguire GF, Emili A, Draganov D, La Du BN et al. Multiple substrates for paraoxonase-1 during oxidation of phosphatidylcholine by peroxynitrite. Biochem Biophys Res Commun 2002;290:391-396.
18. Mori TA, Croft KD, Puddey IB, Beilin LJ. An improved method for the measurement of urinary and plasma F2-isoprostanes using gas chromatography-mass spectrometry. Anal Biochem 1999;268:117-125.
19. Oger C, Brinkmann Y, Bouazzaoui S, Durand T, Galano JM. Stereocontrolled access to isoprostanes via a bicyclo[3.3.0]octene framework. Org Lett 2008;10:5087–5090.
20. Kaburagi Y, Kishi Y. Operationally simple and efficient workup procedure for TBAF-mediated desilylation: application to halichondrin synthesis. Org Lett 2007;9:723-726.
21. Longmire AW, Swift LL, Roberts LJ, Awad JA, Burk RF, Morrow JD. Effect of oxygen tension on the generation of F2-isoprostanes and malondialdehyde in peroxidizing rat liver microsomes. Biochem Pharmacol 1994;47:1173-1177.
22. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.
23. Agren JJ, Julkunen A, Penttila I. Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column. J Lipid Res 1992;33:1871-1876.

JUST ACCEPTED

24. Kontush A, Lindahl M, Lhomme M, Calabresi L, Chapman MJ, Davidson WS. Structure of HDL: particle subclasses and molecular components. Handb Exp Pharmacol 2015;224:3-51.
25. Amigó N, Mallol R, Heras M, Martínez-Hervás S, Blanco-Vaca F, Escolà-Gil JC et al. Lipoprotein hydrophobic core lipids are partially extruded to surface in smaller HDL: "Herniated" HDL, a common feature in diabetes. Sci Rep 2016;6:19249.
26. Vieu C, Jaspard B, Barbaras R, Manent J, Chap H, Perret B, Collet X. Identification and quantitation of diacylglycerols in HDL and accessibility to lipase. J Lipid Res 1996;37:1153-1161.
27. Wiesner P, Leidl K, Boettcher A, Schmitz G, Liebisch G. Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry. J Lipid Res 2009;50:574-585.
28. Ståhlman M, Fagerberg B, Adiels M, Ekroos K, Chapman JM, Kontush A, Borén J. Dyslipidemia, but not hyperglycemia and insulin resistance, is associated with marked alterations in the HDL lipidome in type 2 diabetic subjects in the DIWA cohort: impact on small HDL particles. Biochim Biophys Acta. 2013;1831:1609-1617.
29. Barden AE, Mas E, Croft KD, Phillips M, Mori TA. Minimizing artifactual elevation of lipid peroxidation products (F2-isoprostanes) in plasma during collection and storage. Anal Biochem 2014;449:129-131.
30. Rasmiena AA, Barlow CK, Ng TW, Tull D, Meikle PJ. High density lipoprotein efficiently accepts surface but not internal oxidised lipids from oxidised low density lipoprotein. Biochim Biophys Acta 2016;1861:69-77.

JUST ACCEPTED

31. Christison JK, Rye KA, Stocker R. Exchange of oxidized cholesteryl linoleate between LDL and HDL mediated by cholesteryl ester transfer protein. J Lipid Res 1995;36:2017-2026.
32. Zerrad-Saadi A, Therond P, Chantepie S, Couturier M, Rye KA, Chapman MJ, Kontush A. HDL3-mediated inactivation of LDL-associated phospholipid hydroperoxides is determined by the redox status of apolipoprotein A-I and HDL particle surface lipid rigidity: relevance to inflammation and atherogenesis. Arterioscler Thromb Vasc Biol 2009;29:2169-2175.

JUST ACCEPTED

Table 1. High Resolution Mass Spectrometry of HDL Lipid Extract using QToF.

High resolution MS data was examined for F_2-IsoP-containing lipids.

The masses indicated were checked to give a chromatographic signal in the correct region with an accuracy of <0.01 Da deviation.

Name	Formula	Exact mass $[M+NH_4^+]$, $^1[M+H^+]$	Detected?
Chol-IsoP	C47 H78 O5	740.619925	no
MG-IsoP	C23 H40 O7	446.312405	no
DG 18:0-IsoP	C41 H74 O8	712.57337	no
DG 18:1-IsoP	C41 H72 O8	710.557719	no
DG 16:0-IsoP	C39 H70 O8	684.54207	no
16:0-IsoP PC	C44 H82 O11 N P	^{1}832.570552	no
16:0-IsoP PE	C41 H76 O11 N P	^{1}790.523602	no
18:0-IsoP PC	C46 H86 O11 N P	^{1}860.601852	no
18:0-IsoP PE	C43 H80 O11 N P	^{1}818.554902	no
18:0-IsoP PI	C47 H85 O16 P	^{1}937.565528	no
18:1-IsoP PC	C46 H84 O11 N P	^{1}858.586202	no
18:1-IsoP PE	C43 H78 O11 N P	^{1}816.539252	no
18:1-IsoP PI	C47 H83 O16 P	^{1}935.549878	no

JUST ACCEPTED

FIGURE LEGENDS

Figure 1

Structures of Synthesized Lipid Standards

Figure 2

Analysis with high resolution mass spectrometry using QToF. **A** Chol-IsoP standard (100 ng/ml) extracted ion chromatogram (EIC) *m/z* 740.619. **B** Plasma samples (200 µl) spiked with 100 ng Chol-IsoP with (blue) and without (red) Supelco 5526-U hybrid SPE sample pretreatment to remove PL, EIC m/z 740.619. **C** Comparison of the base peak intensity chromatograms of an HDL sample with (red) and without (blue) SPE sample pretreatment. **D** EIC *m/z* 740.619 of an HDL sample with (red) and without (blue) SPE sample pretreatment.

Figure 3

LDL, HDL2 and HDL3 were purified from plasma by ultracentrifugation and each were diluted to 0.35 mM cholesterol in PBS and incubated with 5 mM AAPH at 37 °C. Aliquots were collected at **A** Time 0 min (unoxidized) and **B** Time 150 min after addition of AAPH, and F_2-IsoPs were measured after lipids were extracted and separated using Bond Elut NH2 SPE, as described in the methods. Oxidized PAPC (oxPAPC) was prepared and purified using HPLC according to the methods. MG-IsoP, Chol-IsoP, oxPAPC and F_2-IsoP standard were eluted separately on Bond Elut NH2 SPE columns and F_2-IsoPs were measured in each fraction. Their elution peaks are indicated. Results are shown as mean ± SEM, n=3-4.

JUST ACCEPTED

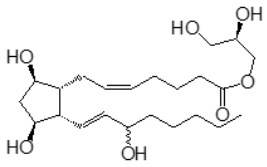
Figure 4

Native plasma HDL lipids were extracted and separated using Bond Elut NH2 SPE, as described in the methods. The MG/DG fraction was purified using HPLC and F_2-IsoPs were measured in each fraction. MG-IsoP and Chol-IsoP were separately fractionated using HPLC and their F_2-IsoPs elution peaks are indicated.

Figure 5

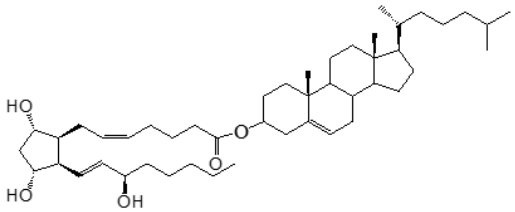
A Analysis of fractions from HPLC purified HDL MG/DG using ESI+, CID with MRM employing a triple quadrupole LC/MS. Fragmentation of the Chol moiety of the Chol-IsoP standard examined $M+NH_4^+$ transitions of the standard and the same mass transitions in HPLC fraction 11 from HDL MG/DG. Composites of the standard and HPLC fraction 11 chromatograms are shown. **B** Fragmentation of the IsoP component of the Chol-IsoP standard examined M+Li+ transitions of the standard and the same mass transitions in HPLC fraction 11 from HDL MG/DG. Composites of the standard and HPLC fraction 11 chromatograms are shown. **C** Fragmentation of the IsoP moiety of the MG-IsoP standard examined M+Li+ transitions of the standard and the same mass transitions in HPLC fraction 4 from HDL MG/DG. Composites of the standard and HPLC fraction 4 chromatograms are shown.

JUST ACCEPTED



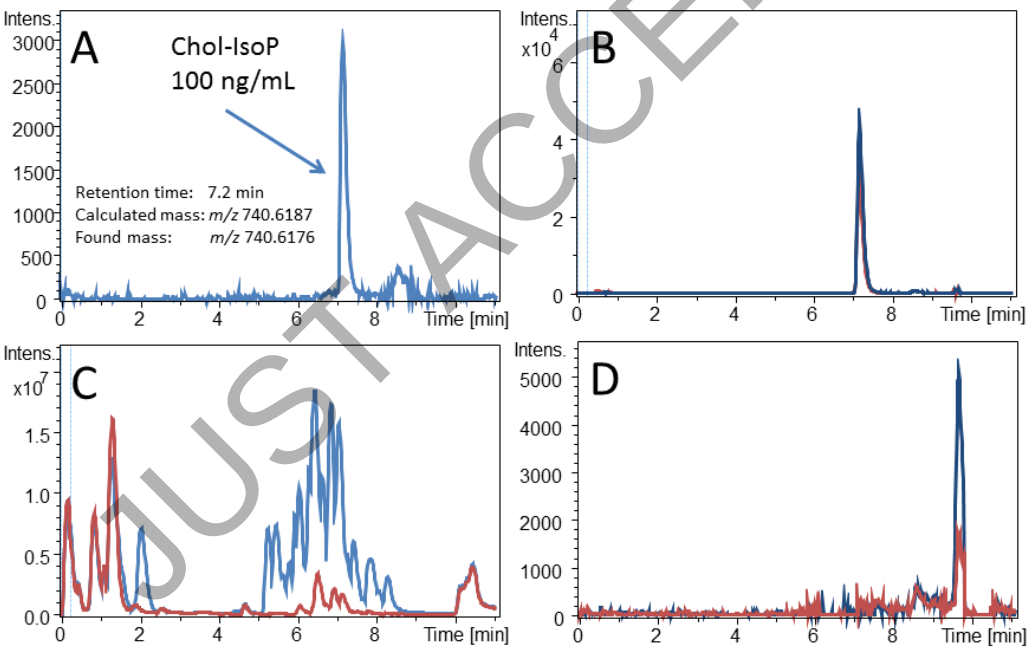
1-*ent*-15(RS)-15-F_{2t}-isoprostanoyl-*sn*-glycerol

MG-IsoP

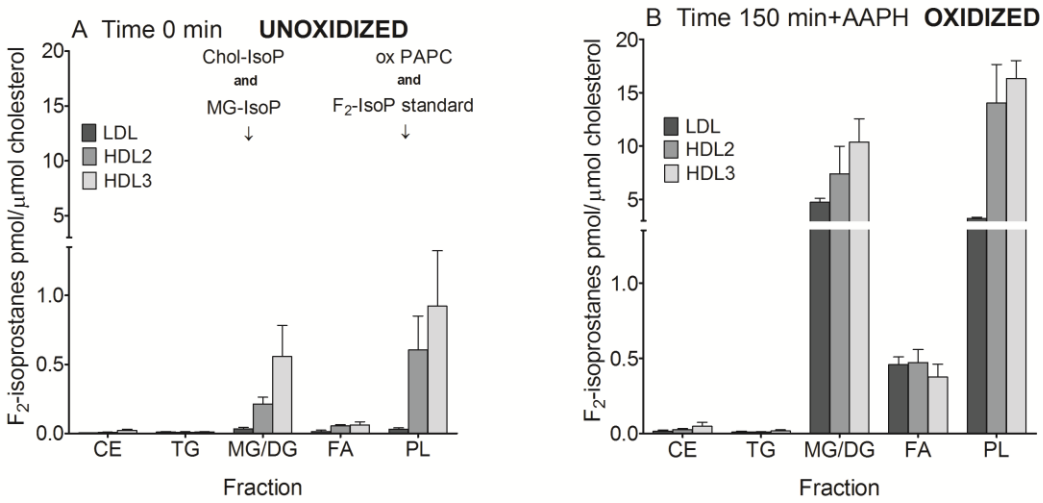


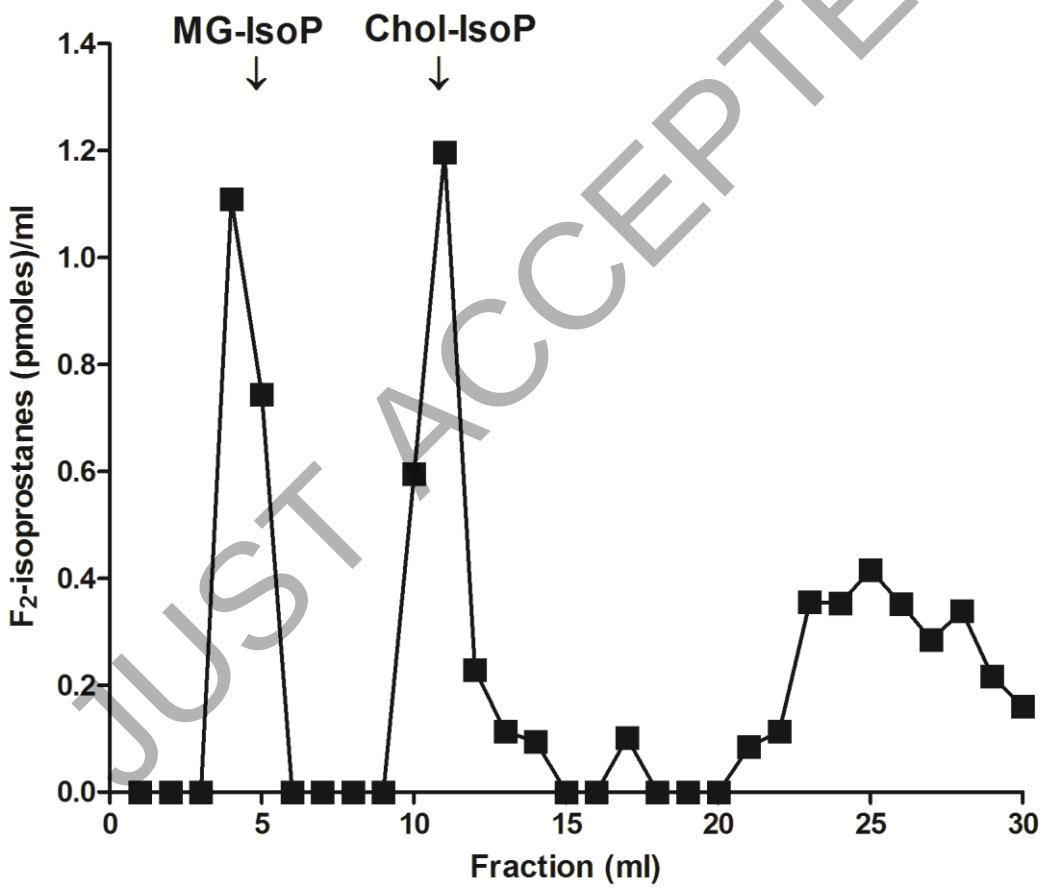
15(R)-15-F_{2t}-isoprostane cholesterol

Chol-IsoP

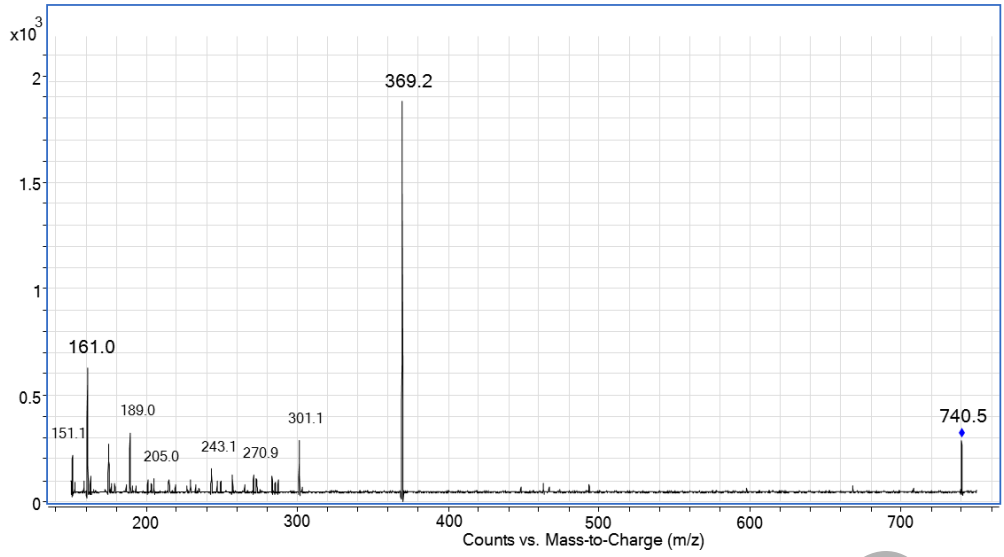


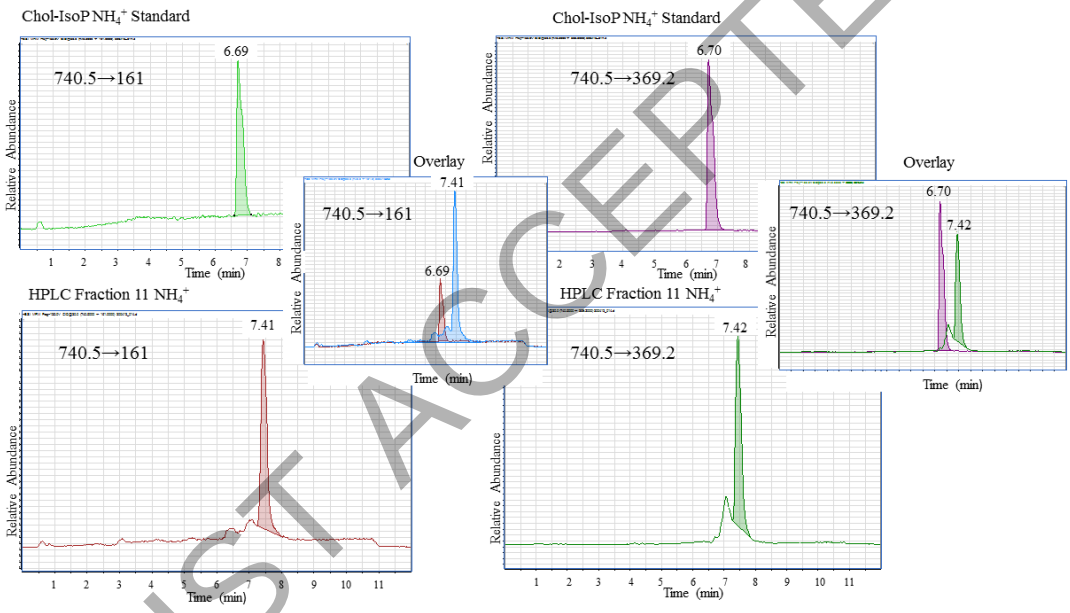
JUST ACCEPTED



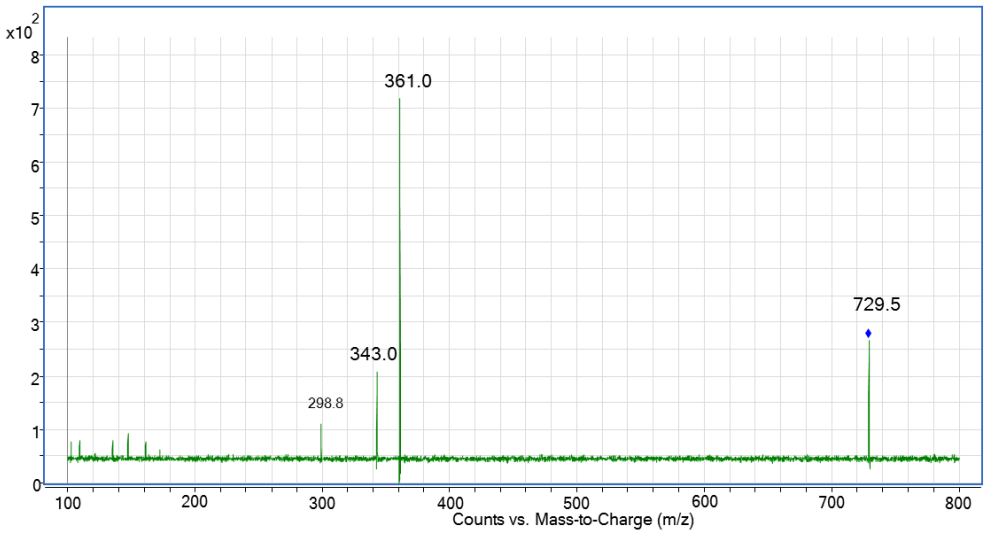


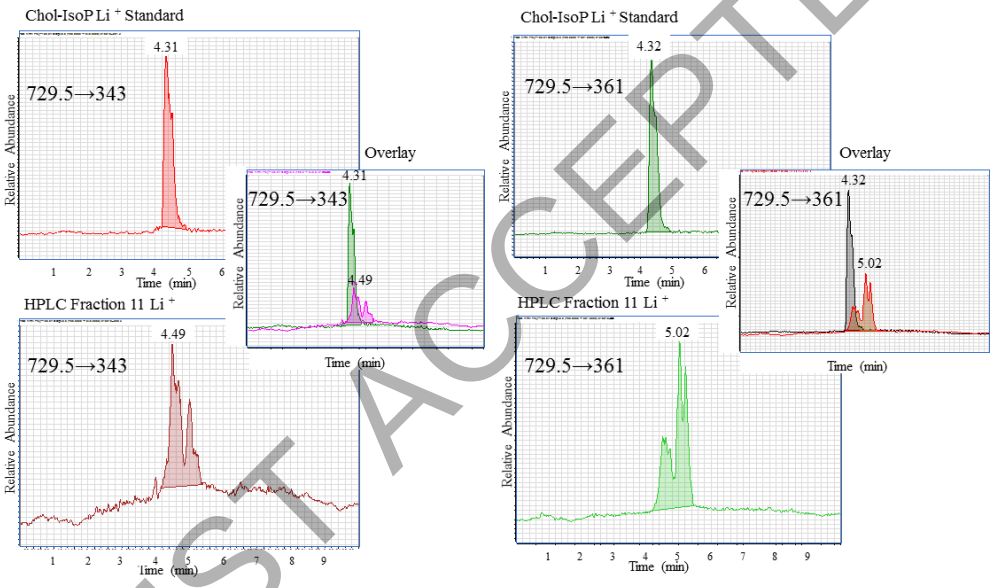
JUST ACCEPTED



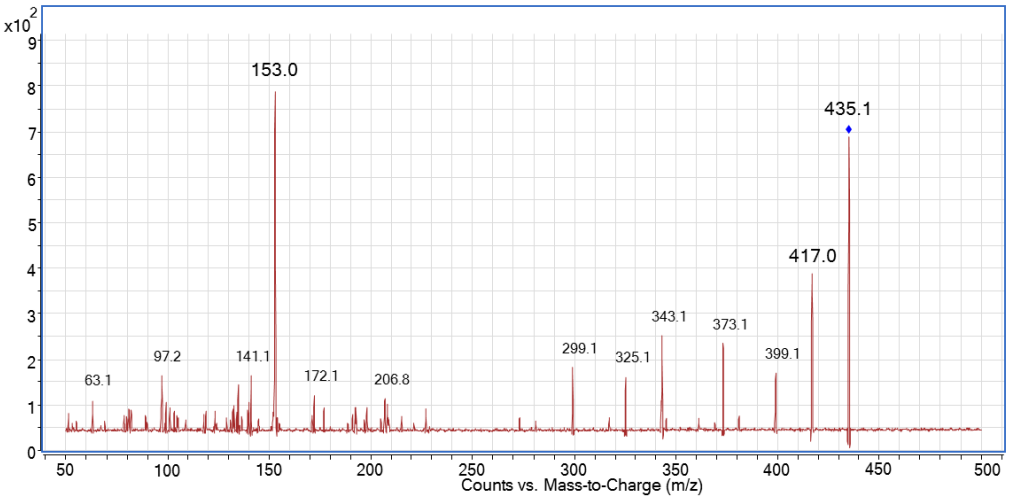


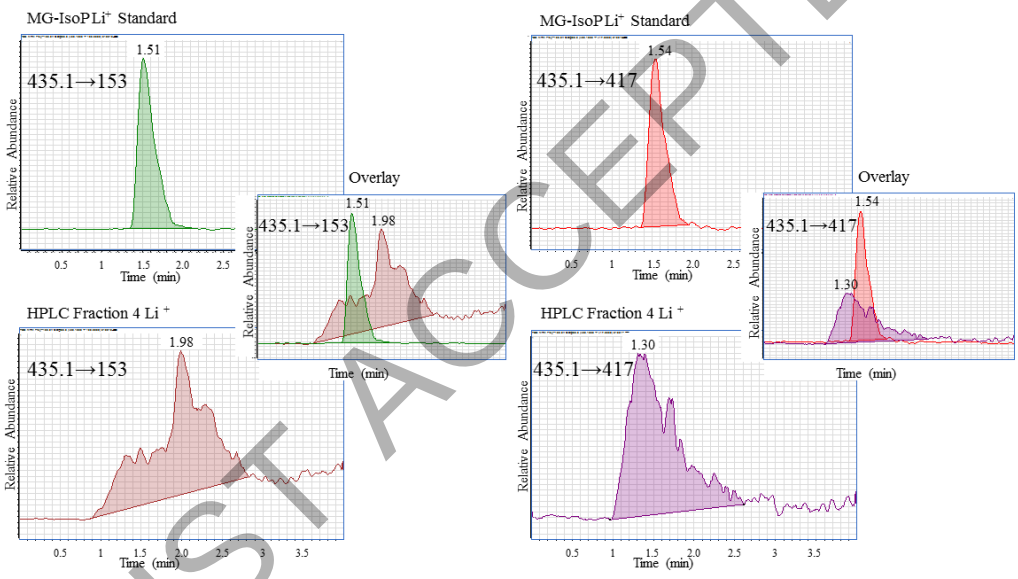
JUST ACCEPTED





JUST ACCEPTED





JUST ACCEPTED