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Abnormal lipoprotein oxylipins in metabolic syndrome and partial correction by omega-3 fatty acids

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ABSTRACT

Metabolic syndrome (MetSyn) is characterized by chronic inflammation which mediates the associated high risk for cardiovascular and other diseases. Oxylipins are a superclass of lipid mediators with potent bioactivities in inflammation, vascular biology, and more. While their role as locally produced agents is appreciated, most oxylipins in plasma are found in lipoproteins suggesting defective regulation of inflammation could be mediated by the elevated VLDL and low HDL levels characteristic of MetSyn. Our objective was to compare the oxylipin composition of VLDL, LDL, and HDL in 14 optimally healthy individuals and 31 MetSyn patients, and then to determine the effects of treating MetSyn subjects with 4 g/day of prescription omega-3 fatty acids (P-OM3) on lipoprotein oxylipin profiles. We compared oxylipin compositions of healthy (14) and MetSyn (31) subjects followed by randomization and assignment to 4 g/d P-OM3 for 16 weeks using LC/MS/MS. Compared to healthy subjects, MetSyn is characterized by abnormalities of (1) pro-inflammatory, arachidonate-derived oxylipins from the lipoxygenase pathway in HDL; and (2) oxylipins mostly not derived from arachidonate in VLDL. P-OM3 treatment corrected many components of these abnormalities, reducing the burden of inflammatory mediators within peripherally circulating lipoproteins that could interfere with, or enhance, local effectors of inflammatory stress. We conclude that MetSyn is associated with a disruption of lipoprotein oxylipin patterns consistent with greater inflammatory stress, and the partial correction of these dysoxylipinemias by treatment with omega-3 fatty acids could explain some of their beneficial effects.

1. Introduction

MetSyn is a combination of abdominal obesity, elevated triglycerides, mildly elevated blood pressure and fasting glucose, and low HDL-cholesterol [1] which taken together represents an increased risk for cardiovascular disease and co-morbidities [2]. While it remains controversial whether these risk factors act synergistically or independently, the dyslipidemia is clinically important since high TG and low HDL define a CVD risk independent of the classic lipid risk marker, LDL-cholesterol. After lifestyle changes, the primary means to reduce lipid-based risk is statin therapy however given the nature of MetSyn, this approach seems less appropriate since it leaves TG and HDL relatively un-corrected but reduces LDL-C, a dyslipidemia that is not always present in MetSyn.

Omega-3 fatty acids reduce serum triglycerides [3] by 20–30% in pharmaceutical doses [4,5], ameliorating one component of MetSyn – dyslipidemia. Alone, this effect seems insufficient to account for the reduction in mortality reported in the JELIS trial, where subjects with MetSyn taking 1.8 g/day EPA had a 50% reduction in relative risk for major coronary events [6]. Given the estimated contribution of hypertriglyceridemia to overall risk [7,8] this is a greater risk-reduction than would be expected (especially considering that the net change in TG levels was only 5%), and it suggests omega-3 fatty acid therapy reduces risk by other mechanisms.

One systemic effect of P-OM3 is to increase the abundance of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in tissues. They do the same for most [9,10], but not all [11] lipid mediators produced from these fatty acids. Production of lipid mediators by

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oxygenating enzymes such as cyclooxygenase, lipoxygenase or cytochrome p450 are a major means whereby polyunsaturated fatty acids (PUFA) exert their effects; collectively, the superclass of oxygenated lipid mediators is termed *oxylipins*. Oxylipins are transported throughout the plasma in lipoproteins where they are available for delivery to target tissues by the lipolytic actions of lipases [12]. We hypothesized that the MetSyn could also be characterized by an abnormal profile of lipoprotein oxylipins. In a previous study, P-OM3 favorably altered the vasoactive oxylipin pattern in VLDL [13]. Here we sought confirmation of this in the controlled trial setting and further asked whether intervention with P-OM3 improved abnormalities in lipoprotein oxylipins. We employed a targeted lipidomic approach, measuring the metabolites of multiple oxylipin-producing pathways, multiple parent fatty acids (FA) and each major lipoprotein subclass (HDL, LDL, and VLDL) in a placebo controlled trial.

2. Methods

2.1. Metabolic syndrome participants and study location

The study was conducted by Sanford Research/USD in cooperation with Sanford Clinic – Clinical Research Services in Sioux Falls, South Dakota, as originally reported [14]. The protocol was approved by the IRB at the University of South Dakota and registered at clinicaltrials.gov (NCT00286234). Informed consent was required.

2.2. Study design

This is an ancillary study [14] with the addition of a group of optimally healthy controls for comparison since 'healthy' oxylipin levels are unknown. Fig. 1 represents the flow diagram of the substudy reported here. The original study employed P-OM3 treatment along with extended release niacin (ERN) in a randomized, double-blind, placebo-controlled clinical trial, with a 2 × 2 factorial treatment design; for this study the ERN arm was not included.

2.3. Selection of metabolic syndrome subjects

As previously reported [14], after initial screening informed consent and a full screening panel were obtained. Inclusion criteria were: age 40–69 years; BMI 25–40 kg/m^2; fasting TG, 150–750 mg/dL; HDL-C > 10 mg/dL; and the ratio of TG/HDL-C > 3.5. Qualifying subjects began a 6-week, diet-stabilization, dual-placebo, run-in phase (single-blind) in which non-compliant subjects (< 80% compliant) were identified and excluded. Those completing the run-in phase were randomly assigned to treatment using permuted blocks of four with stratification for gender (Fig. 1). All subjects were > 80% compliant by pill count.

2.4. Healthy controls

In addition to the MetSyn subjects, we recruited a set of healthy controls for comparison with MetSyn. Our intention was for the subjects to be 'optimally' healthy rather than 'apparently' healthy, and so our recruitment criteria were stringent to reflect this. Recruitment took place during the same time as the MetSyn subjects were entering their final visit. Subjects were age-matched (aged 40–69) with inclusion criteria as: TG < 130, a TG/HDL ratio < 3.0 and BMI between 21 and 25. They could not be taking any lipid lowering medications (*e.g.* statins, fibrates, niacin, n-3 FAs, *etc.*) or vasoactive medications (*e.g.* ACE inhibitors, alpha or beta blockers, Ca^{2+} channel blockers, or long acting nitrates, *etc.*). Subjects were screened by telephone and those qualifying were invited to a screening visit where informed consent was obtained, a medical history taken, height and weight measured and a fingerstick lipid and glucose panel obtained. Subjects still qualifying were invited to participate in the study.

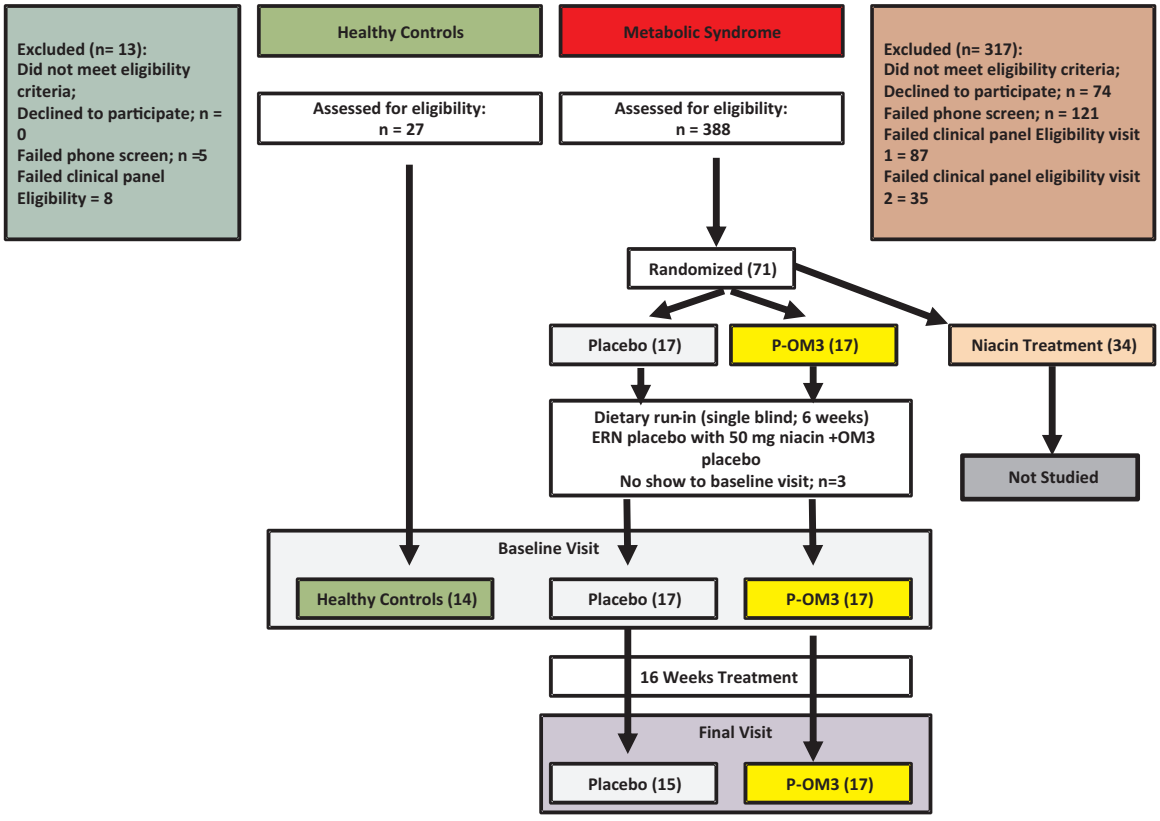


Fig. 1. Subject recruitment. Flow diagram showing the relationship between this ancillary trial and the parent trial. Based on the Consolidated Standards of Reporting Trial.

2.5. Plasma lipids

Plasma was collected into EDTA and stored under nitrogen. Plasma lipoproteins were obtained using the sequential flotation method; density gradients contained EDTA and BHT to prevent oxidation of lipids. Phospholipids were measured in each lipoprotein fraction using standard colorimetric assays. Lipoprotein apoprotein composition was measured by SDS-PAGE to ensure lipoprotein quality, and are reported elsewhere [15,16].

2.6. Fatty acids

The fatty acid composition of each lipoprotein class [17] and of erythrocytes [14] was determined as previously described. Briefly, fatty acid methyl esters were generated from lipoprotein samples by acid trans-esterification with boron trifluoride and analyzed by gas chromatography using a GC2010 Gas Chromatograph (Shimadzu, Columbia, MD) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA) using hydrogen as carrier gas. Fatty acids were identified by comparison with a standard mixture of fatty acids characteristic of erythrocytes. The omega-3 index (OMX) is defined as the RBC content of EPA plus DHA expressed as a percentage of total identified fatty acids after response factor correction.

2.7. Oxylipin analysis

Oxylipin isolation and determination were performed using modifications of previously reported procedures, which included expansion of the previous analyte list [18,19]. Briefly, plasma (200 µL) or lipoprotein fractions (50 µL) were spiked with butylated hydroxytoluene/EDTA, 10 deuterated prostanoid, eicosanoids, and octadecanoid surrogates, and subjected to hydrolysis in 0.1 M methanolic sodium hydroxide to release ester linked oxygenated lipids and free oxylipins were isolated using Oasis HLB solid phase extraction columns (Waters, Milford, MA), solvents removed under vacuum, and residues reconstituted with a 400 nM solution of 1-cyclohexyluriedo-3-dodecanoic acid (Sigma-Aldrich, St. Louis MO) in methanol, were filtered at 0.1 µm using Amicon Ultrafree-MC durapore PVDF filters (Millipore) [18]. Analytes were separated by reverse phase ultra-performance liquid chromatography on a 1.7 µm Acquity BEH column (Waters) and detected by negative mode electrospray ionization tandem quadrupole mass spectroscopy on an API 4000 QTRAP (Applied Biosystems Inc., Foster City, CA) using modifications of previously reported method [19]. A complete list of analytical targets and their acquisition parameters are presented in Supplemental Table 1. Representative chromatograms of HDL, LDL and VLDL particles together with analytical standard are presented in Fig. 2.

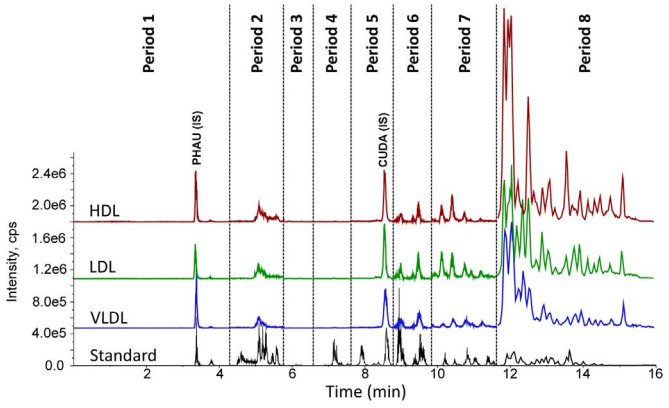


Fig. 2. Representative chromatograms of HDL, LDL and VLDL particles together with analytical standard. Internal standards (PHAU and CUDA) are indicated. List of oxylipins quantified during each period is included in the Supplemental Table 6.

2.8. Statistical methods

In this investigation, we considered the molar ratio of each oxylipin to phospholipids for each fraction. This was preferred for two reasons: first, the oxylipins reported here are often incorporated into phospholipids [20], and is reflected by the fact that this normalization reduced variance more so than other lipids such as cholesterol or triglycerides; second, P-OM3 treatment affects the composition and concentration of the lipoproteins themselves so as an outcome, the per-phospholipid concentration best measures compositional abundance independent of changes in plasma lipids.

A natural logarithmic transformation was used to stabilize the variance and reduce skewness across all data. Geometric means and 95% confidence intervals for baseline and follow-up for each treatment group were calculated. Some variables had observations below the limit of detection (LOD), these values were imputed using a single imputation process based on a normal distribution with the mean equal to ½ the LOD and a standard deviation equal to 1/3 of the mean. Once data were imputed they were adjusted for the level of phospholipids in each sample. Finally, an adjustment was made for batch effects. Analyses were performed using the COMBAT package in R available in the SVA package [21,22]. The COMBAT package uses an empirical Bayes framework that borrows strength across input variables to improve batch adjustment in studies with small sample sizes per batch.

After batch adjustment, the data were analyzed using cluster analysis for dimension reduction. Two types of analyses were performed; one focusing on the difference between healthy controls and patients with MetSyn, and the other assessing the change over time in MetSyn patients randomized to P-OM3 or placebo. Clustering was performed using the WGCNA package in R [23]. This package performs weighted correlation network analysis and groups variables into modules based on their connectedness to other variables. The resulting modules were then analyzed using principal component analysis and component scores were used to compare groups. Clustering was performed separately for baseline data (used to compare normal controls to MetSyn patients) and for trial data (used to compare changes over time between the P-OM3 and placebo groups). Comparisons between groups were performed using a *t*-test on the first principal component score of each module. For interpretation, clusters are composed of oxylipins that vary together. Significant differences in clusters can be interpreted to mean the constituent oxylipins change together. Data analysis was performed using the R software and the Bioconductor platform within R [24,25] and JMP 12.1.0.

3. Results

3.1. Subject characteristics

The baseline demographic values for healthy and MetSyn subjects were previously described [14]. Healthy controls were metabolically ideal compared to the MetSyn group. While our recruitment criteria focused on triglyceride and HDL levels and BMI, the subjects with MetSyn were additionally insulin resistant and had high blood pressure (Table 1), and so in the aggregate reflected the full spectrum of MetSyn symptoms (see Supplemental Table 2). Importantly subjects with MetSyn did not have elevated LDL-C, consistent with this syndrome. As previously shown, treatment with P-OM3 reduced serum triglycerides and VLDL-C, but did not affect other parameters (Table 1) except heart rate. Lipoprotein fatty acids on a per-phospholipid basis are available in Supplemental Tables 3 and 4.

3.2. Lipoprotein oxylipins in healthy controls versus MetSyn patients

We first sought to identify whether and how the lipoprotein oxylipins from MetSyn patients are abnormal. We previously reported the distribution of lipoprotein oxylipins in hypertriglyceridemic

Table 1
Baseline characteristics of participants.[a] See Shearer et al. [14], Borja et al. [52] and Savinova et al. [15] for population compairson.

		Baseline group differences (median, IQR)			*P-OM3 treatment effect (mean, 95% CI)*	
Parameter[b]	**N**	**Con (n = 14)**	**MetSyn (n = 31)**	**P-value**[a]	**Change (FO n = 17; Pb n = 14)**[c]	**P-value**
Glycemia						
Glucose (mmol/L)	**43**	**4.8 (4.6, 5.2)**	**5.5 (5.1, 5.9)**	**0.001**	[d]	0.10
Insulin (uU/mL)	**43**	**3 (2, 3.5)**	**14 (6, 18.5)**	**< 0.0001**		0.08
HOMA1-IR (none)	**43**	**0.63 (0.49, 0.77)**	**3.15 (1.47, 4.57)**	**< 0.0001**	[d]	0.19
Lipids						
TG (VAP)	**45**	**71 (59, 89)**	**206 (134, 274)**	**< 0.0001**	**−18% (−33, −1)**[d]	**0.04**
Total Chol	45	193 (175, 231)	200 (176, 232)	> 0.80		> 0.80
PL	45	279 (260, 353)	322 (275, 374)	0.18	[d]	0.73
VLDL-C	**45**	**16 (14, 20)**	**26 (22, 38)**	**< 0.0001**	**−14% (−24, −2)**[d]	**0.02**
LDL-C	45	112 (83, 132)	119 (91, 142)	0.28		0.76
Lpa-C	45	7 (5, 9)	5 (4, 7)	0.23	[d]	0.75
IDL-C	**43**	**7 (4, 18)**	**18 (12, 23)**	**0.02**		0.33
HDL-C	**45**	**55 (47, 64)**	**41 (37, 46)**	**< 0.0001**	[d]	0.59
Non HDL-C	45	129 (98, 153)	148 (125, 175)	0.04		0.37
Non HDL:HDL Ratio	**45**	**2.2 (1.8, 2.8)**	**3.7 (3.4, 4)**	**< 0.0001**		0.45
Time to Peak LDL-C	**45**	**119 (118, 120)**	**113 (110, 115)**	**< 0.0001**	[d]	0.44
Vascular						
BP-Systolic (mmHg)	**45**	**112 (107, 118)**	**130 (124, 139)**	**< 0.0001**		> 0.80
Heart Rate (bpm)	**45**	**55 (47, 58)**	**69 (62, 76)**	**< 0.0001**	**−4.2 (−8.1, −0.3)**	**0.04**
MAP (mmHg)	**45**	**85 (82, 94)**	**99 (95, 106)**	**0.0002**	[d]	> 0.80
PP (mmHg)	45	44 (40, 47)	47 (42, 54)	0.08	[d]	0.35

[a] Wilcoxon rank sums test.
[b] mg/dL unless specified.
[c] age & sex adjusted parameter estimate for effect.
[d] log transformed for normality.

(250–500 mg/dL) subjects on statin therapy [13], however it was not known whether their baseline levels were abnormal. No prior studies have compared the content of optimally healthy and MetSyn subjects, so population distributions and/or ranges are unknown. Since the cluster analysis is an unbiased approach, when properties such as a common parent FA or common lipoprotein sources are shared within clusters the common factors are more likely to have a biological basis.

A complex matrix of oxylipins was detected with cluster memberships determined by lipoprotein, by the parent PUFA, and by metabolic production pathway; membership and differences are summarized in Supplemental Table 5. Summaries by pathway for esterified oxylipins are presented by lipoprotein in Fig. 3. MetSyn was characterized by differences in two color-coded clusters: Cluster 3 (Mustard; p = 0.01) and Cluster 7 (Brown; p = 0.0001). Differences represented in the Mustard cluster were comprised entirely of HDL oxylipins (P = 0.01) (Supplemental Table 5 and Fig. 3A), and include greater levels of arachidonic acid (AA) products of lipoxygenase (LOX) metabolism, including alcohols, ketones and leukotrienes. The 5-LOX pathway, which is nearly universally pro-inflammatory, was represented in the cluster by 5-HETE, 5-KETE and leukotrienes. Products of 12-LOX (12-HETE) and 15-LOX (12-HETE), as well as autoxidation (9-HETE and isoprostanes) were also present in this cluster. These oxylipins were not affected in LDL or VLDL.

Except for AA epoxides, MetSyn patients had lower levels of epoxides in VLDL Cluster 7 (Brown; P < 0.0001) (Fig. 3C). LA, aLA, EPA, and DHA epoxides were all lower in MetSyn and the corresponding vicinal-diols of each epoxide were also lower, despite more than 3-fold greater levels of their parent PUFA (Fig. 3C). The levels of epoxy derivatives in the VLDL clustered together with the levels of AA and LA derived ketones in LDL and VLDL Cluster 7(Brown; P < 0.0001). Those AA and LA derived ketones were higher in subjects with MetSyn than in controls (Fig. 3B and C).

Although not different in MetSyn patients, the levels of DHA epoxides and their corresponding vicinal diols vary together in HDL and LDL, suggestive of a link between LDL and HDL oxylipin trafficking in Cluster 2 (Sky Blue; Fig. 3A and B).

3.3. P-OM3 treatment effects in metabolic syndrome

P-OM3 therapy induced profound changes in some, but not all lipoprotein oxylipins; in some cases treatment resulted in oxylipin patterns that were similar to those in healthy subjects. P-OM3 induced changes (Supplemental Table 6) were found in three of eleven clusters: Cluster 3 (Mustard, p = 0.02), Cluster 10 (Light Blue, p < 0.0001), and Cluster 11 (Salmon, p = 0.01). Changes in esterified oxylipins by lipoprotein and by pathway are summarized in Fig. 4.

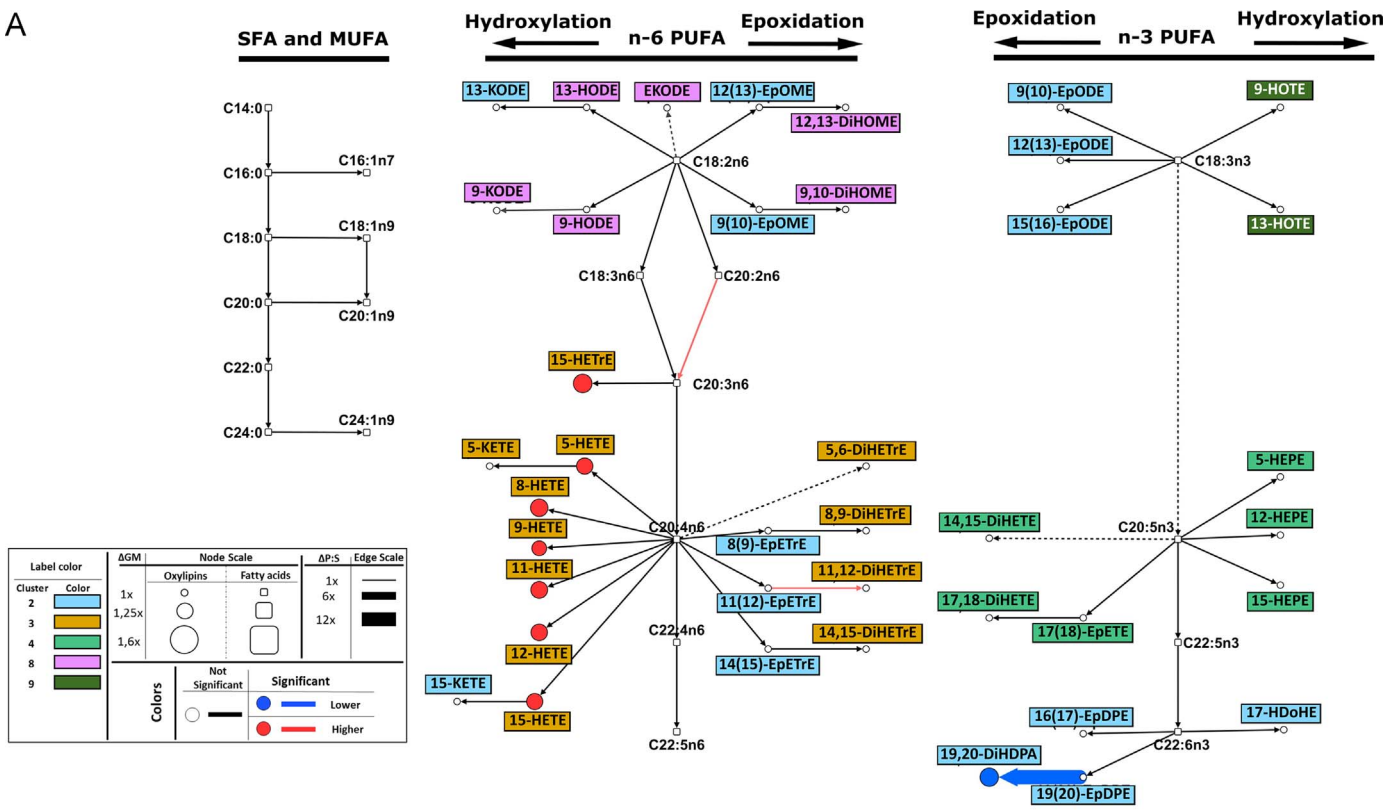
P-OM3 induced a treatment dependent change in the Cluster 3 (Mustard; p = 0.02) and contained similar oxylipins as before, including AA products of LOX metabolism: alcohols, ketones, and leukotrienes. These oxylipins involve almost uniquely pro-inflammatory 5-LOX derivatives (5-HETE, 5-KETE and 6-trans-LTB4), product of autoxidation (9-HETE) as well as 12-HETE and 15-HETE. These same oxylipins were increased in MetSyn HDL, but were decreased by P-OM3 treatment. Thus, Cluster 3 reflects a P-OM3-induced improvement in oxylipin profiles in MetSyn patients.

P-OM3 also changed the Cluster 10 (Light Blue; P < 0.001) which, except for Resolvin E1, consisted predominantly of EPA- and DHA-oxylipins, across all lipoproteins (Fig. 4A, B and C; Cluster 10 – Light Blue, Supplemental Table 6). Treatment increased the EPA and DHA metabolites from both LOX and CYP pathways. Two non-EPA/DHA members of the Light Blue cluster were HDL-12(13)-EpODE (aLA epoxy-derivative) which was positively correlated with the omega-3 oxylipins, and VLDL-15-KETE (AA 15-LOX derivative) which was negatively correlated. Among MetSyn subjects, DHA epoxides and alcohols and EPA epoxides in VLDL were depressed compared to controls, and P-OM3 hyper-corrected this deficit.

Finally, P-OM3 treatment affected Cluster 11 (Salmon; P = 0.01), which contained oxylipins in LDL derived predominantly from AA, including mid chain alcohols, ketones, leukotrienes, and isoprostanes (Fig. 4B; Cluster 11, Supplemental Table 6).

4. Discussion

In this study, we sought to understand the abnormalities in lipid



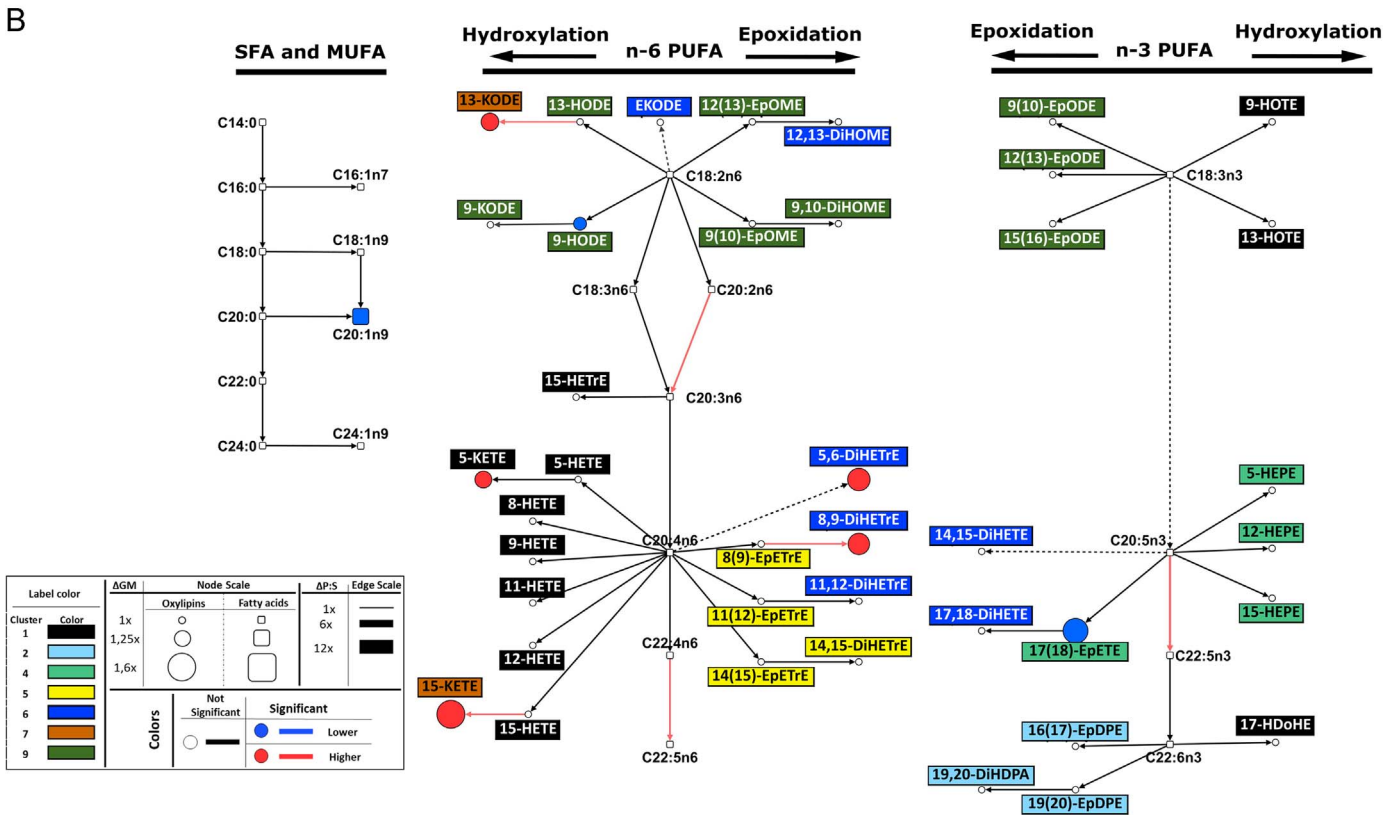


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Fig. 3. Pathway analysis of differences between healthy controls (n = 14) and MetSyn (n = 31) subjects in lipoproteins (A – HDL, B – LDL, C – VLDL), normalized to lipoprotein phospholipid content (as ng oxylipin in lipoprotein/mg phospholipid in lipoprotein). Pathways of fatty-acid metabolism (rounded rectangle shapes) are represented along with oxylipin metabolites (circles). Direct metabolites are connected with solid lines, indirect metabolites (with one or more intermediate products) are connected with dashed lines. The line thickness represents the fold-change in product to substrate ratio between the MetSyn and the healthy controls with the blues lines being significantly lower in MetSyn and the red lines being significantly greater (see Δ product to substrate ratio, ΔP:S, in the network legend). The node size represents the fold of change in the level of fatty acids and their metabolites between the MetSyn and the healthy controls (see Δ geometric mean, ΔGM, in the network legend) with the blues nodes being significantly lower in MetSyn and the red nodes being significantly greater. Metabolite labels are color coded according to their hierarchical clusters affiliations (see Supplemental Table 5). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

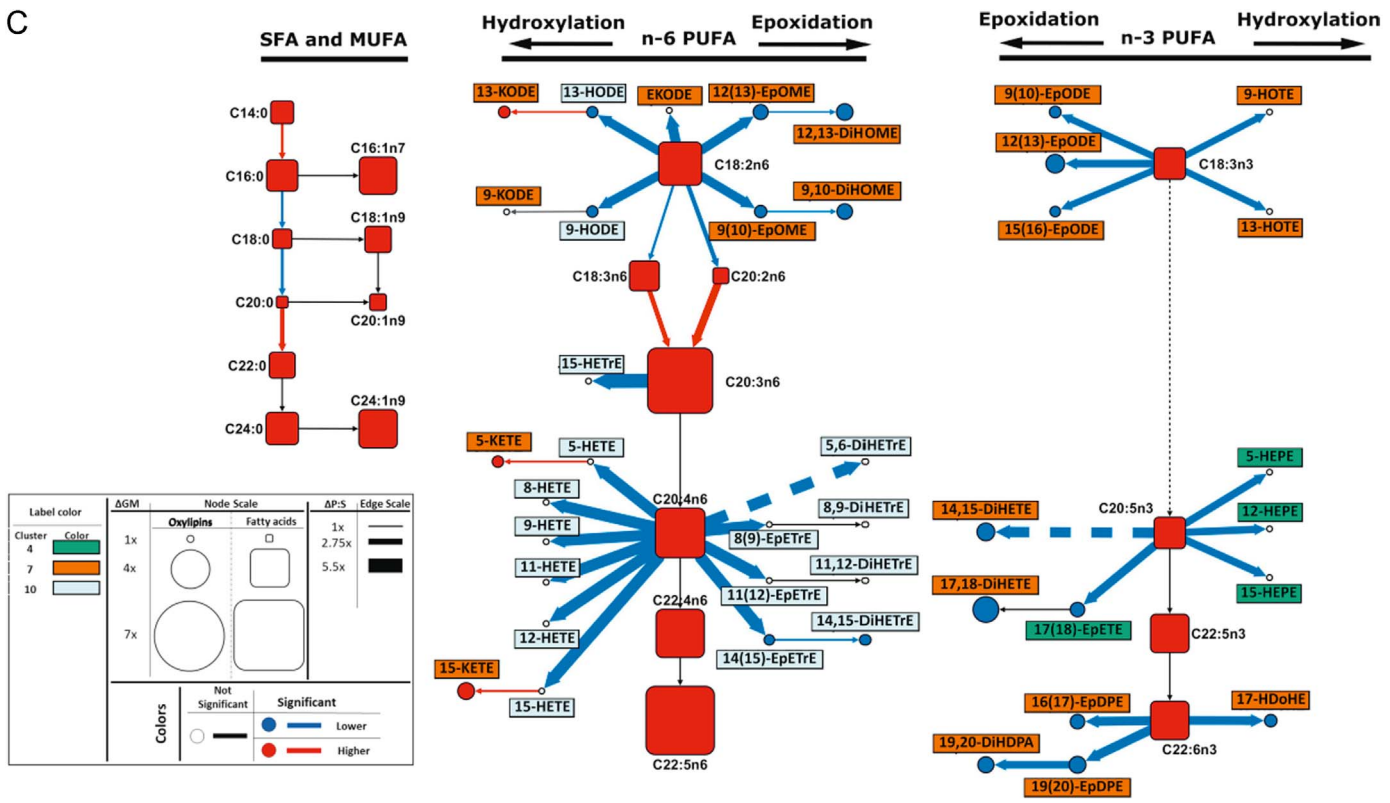


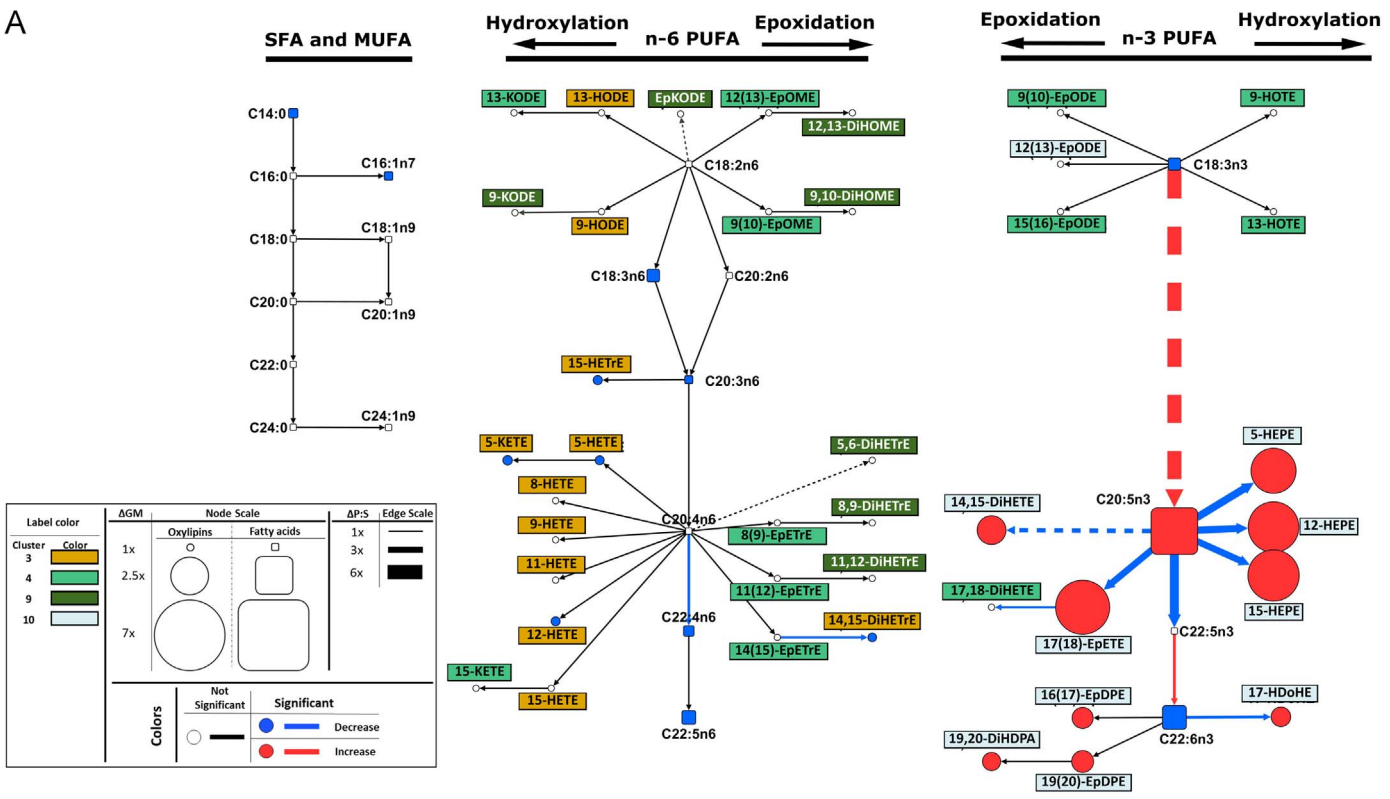
Fig. 3. (*continued*)

signaling metabolites that are present in MetSyn and whether those can be normalized by P-OM3. Clinically, the dyslipidemia of MetSyn is derived from the VLDL-TG:HDL axis [26]. Although P-OM3 partially resolves the VLDL elevation [14] it profoundly affects the fatty acid composition of all lipoproteins [27]. Based on our findings, MetSyn patients are characterized by elevated HDL and VLDL LOX derived metabolites and reduced fatty acid epoxides in VLDL. P-OM3 treatment resulted in the correction of some, but not all, of these MetSyn deficits.

Acylation of alcohols, as well as other oxylipins such as epoxides, into membrane phospholipids is relatively well established; their presence in lipoprotein glycerolipids has been previously demonstrated [28]. Oxylipins present in lipoproteins are subject to lipolysis by lipoprotein lipase which traffic glycerolipids [12], and the lipolysis products of lipoproteins induce responses *in vitro* similar to those associated with oxylipins and lipoproteins *in vivo* [29]. Given the delivery and action of oxylipins by the same systems that target FAs to tissues, such findings collectively have profound implications for endocrine-like signaling by the lipoprotein-oxylipin axis.

There is a strong association between the physical conditions associated with lipoproteins and the bioactivities of oxylipins; PPAR activation being one area of substantial overlap. For instance, lipoproteins activate PPARs in a lipoprotein class- and lipolysis-dependent manner. VLDL undergoing lipolysis by LpL activate PPARα [30,31], hepatic lipase-mediated VLDL lipolysis activates PPARδ [32] and endothelial lipase-mediated HDL lipolysis activates PPARα [33], suggesting discrete localization of PPAR activators in lipoprotein-glycerolipid classes. Concurrently, oxylipins are endogenous PPAR agonists: LA mid-chain alcohols (HODEs) are one example of potent PPARγ agonists [34] and were shown in the current experiment to be decreased in VLDL of MetSyn subjects (Fig. 3C).

Other actions overlapping with lipoproteins include vasoactivity and inflammation. HDL prevent endothelial cell dysfunction [35] while VLDL contribute to endothelial dysfunction by activating their inflammatory response [36–38]. Similarly, numerous oxylipins have vasoactive effects, particularly fatty acid epoxides which are hyperpolarizing factors [39] and activate Ca^{2+} channels [40,41]. Our study shows the levels of every oxylipin being decreased in the VLDL of MetSyn subjects (Fig. 3C), a condition where endothelial dysfunction is a major component of the pathology [42]. Further, the levels of select oxylipins are repleted by P-OM3 therapy, which is known to improve vascular dysfunction. Oxylipins regulate inflammation as well as it resolution [43]. Among LOX isoforms, 5-LOX is nearly universally associated with increased inflammatory activity [44] reflected in the blockade of its activity as treatment for asthma [45]. 12- and 15-LOX are more mixed in their effects on inflammation. They initiate inflammation in the form of endoplasmic reticulum-stress [46], and their follow-up metabolites, resolvins (also LOX – derivatives) initiate the resolution of inflammation [47]. The global impact of other LOX isoforms is less clear, having atherogenic and thrombogenic activities [48] along with atheroprotective activities [49]. In addition, LOX-derived keto FAs activate neutrophils, eosinophils, and monocytes and act as chemotractants [50]. Their delivery to lipoprotein targets could



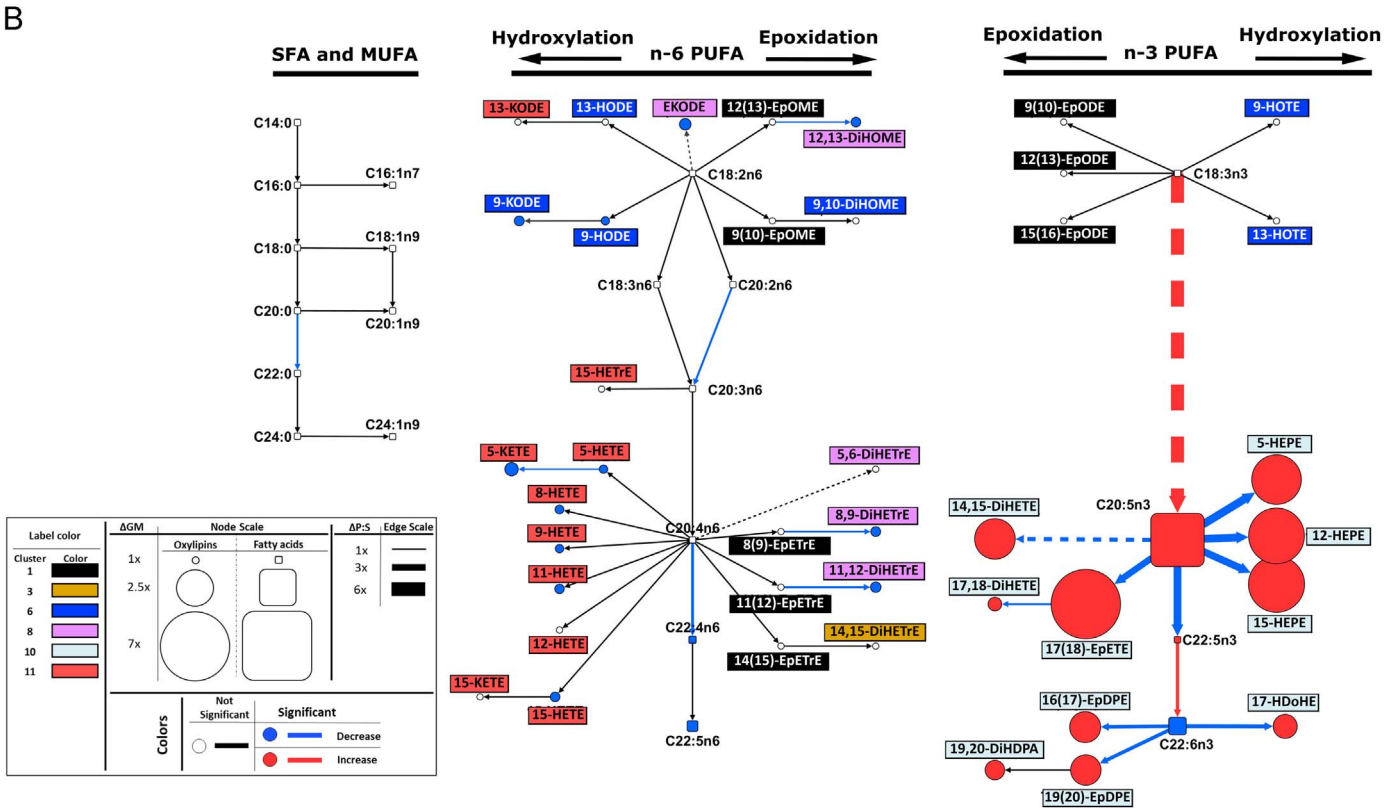


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Fig. 4. Pathway analysis of differences between placebo (n = 14) and P-OM3 (n = 17) treated MetSyn subjects in lipoproteins (A – HDL, B – LDL, C – VLDL), normalized to lipoprotein phospholipid content (as ng oxylipin in lipoprotein/mg phospholipid in lipoprotein). Pathways of fatty-acid metabolism (rounded rectangle shapes) are represented along with oxylipin metabolites (circles). Direct metabolites are connected with solid lines, indirect metabolites (with one or more intermediate products in between) are connected with dashed lines. The line thickness represents the fold of change in product to substrate ratio between the MetSyn subjects treated with placebo and those treated with P-OM3, adjusting for baseline (see Δ product to substrate ratio, ΔP:S, in the network legend) with the blues lines being significantly decreased by P-OM3 treatment and the red lines being significantly increased. The node size represents the fold of change in the level of fatty acids and their metabolites between the MetSyn subjects treated with placebo and those treated with P-OM3, adjusting for baseline (see Δ geometric mean, ΔGM, in the network legend) with the blues nodes being significantly decreased by P-OM3 treatment and the red nodes being significantly increased. Metabolite labels are color coded according to their hierarchical clusters affiliations (see Supplemental Table 6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

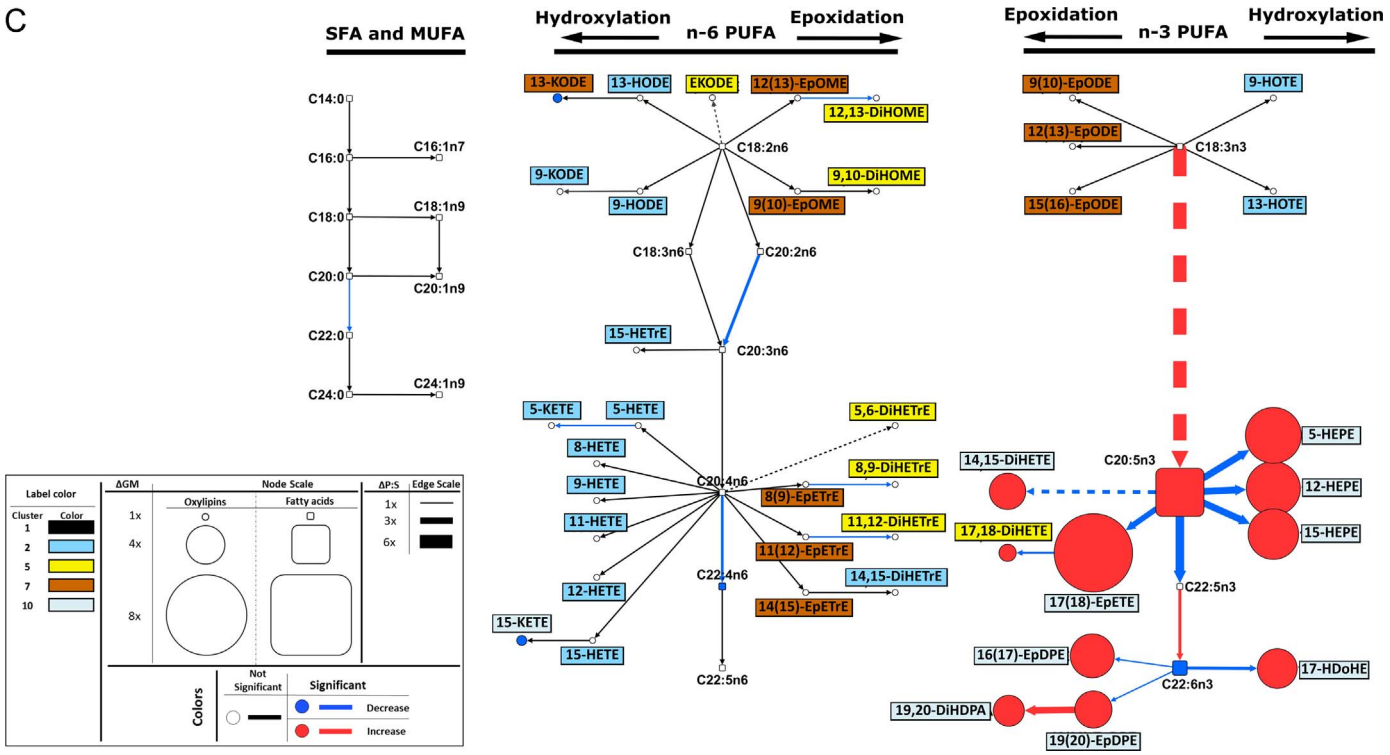

Fig. 4. (*continued*)

enhance atherogenesis, provided their action when delivered by lipoproteins is the same as their paracrine action. Our study shows that MetSyn subjects have elevated concentrations of pro-inflammatory LOX metabolites in HDL (5-HETE, 5-KETE, Fig. 3A) and VLDL (5-KETE, Fig. 3C), with parallel decreases in anti-inflammatory oxylipins in VLDL (Resolvin E1 and epoxy-FA, Supplemental Table 5 and Fig. 3C). Interestingly, in MetSyn subjects, the higher levels of 5-KETE were not accompanied by a correspondingly greater level of AA, AA-alcohols or leukotriene LTB4 (in the alternate 5-LOX pathway). The mechanism of this is unknown.

P-OM3 therapy significantly reduces pro-inflammatory oxylipins in HDL, in LDL, and partially in VLDL (Fig. 4). This suggests EPA + DHA reduce the pro-inflammatory activity of LDL and apoB containing lipoproteins, and could thus slow the development of atherosclerosis in these subjects.

Finally, despite two-fold elevations of DHA and EPA in VLDL of MetSyn subjects compared with controls, DHA and EPA-oxylipins were lower (Fig. 3C). This indicates that production of DHA-oxylipins is not simply a function of DHA availability, and identifies their depletion in VLDL as a key component of the MetSyn *dys*oxylipinemia. P-OM3 greatly increased their levels, more than correcting the deficit in this oxylipin pool.

Overall our findings support the hypothesis that the risk-reducing impact of P-OM3 includes more than simple reductions in serum TG. Here, despite having no impact on the cholesterol composition of HDL and LDL, both lipoprotein classes were altered by omega-3 FAs, including changes in oxylipins not derived from EPA or DHA. Increased LDL-cholesterol has been observed in some trials on P-OM3, however the changes in oxylipin composition reported here are likely to provide counter acting, anti-inflammatory effects which could improve outcomes. This could be a plausible alternative mechanistic to TG-reduction as the sole benefit of P-OM3 therapy. Some trials have observed P-OM3 induced increases in HDL-C; we have documented compositional changes independent of HDL-C which are also likely to improve HDL function. Interestingly, the F2-isoprostanes were not a component of any of the P-OM3 corrections observed here. This is somewhat unexpected since it is nearly universally considered to be a marker of oxidative stress, which P-OM3 is thought to reduce [51].

4.1. Limitations

This study is a secondary analysis of a trial intended to demonstrate combined effects of extended release niacin and P-OM3 on insulin resistance in MetSyn patients. While it has the benefit of random assignment and placebo control, it was not prospectively powered on these endpoints, and so non-significant outcomes should be interpreted cautiously. Other limitations include short treatment duration (4 months) when atherosclerosis develops over years.

4.2. Conclusion

The abnormalities in discrete lipoprotein oxylipin clusters in MetSyn suggest a novel molecular explanation for the risks and outcomes associated with it. Since at least one abnormality of MetSyn

lipoproteins is an enrichment with inflammatory oxylipins, it suggests these molecules may be delivered *via* lipoprotein receptors or lipases to tissues involved in the atherosclerotic process. Partial correction of the abnormality by P-OM3 provides a feasible explanation for the reduction in inflammatory stress by omega-3 fatty acids.

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Disclosure summary

GCS received research support from GlaxoSmithKline. GCS and WSH received speakership honoraria from GlaxoSmithKline within three years of project execution. No other potential conflicts.

Clinical trial registration

clinicaltrials.gov (NCT00286234).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.plefa.2017.10.006.

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