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Specific Plasma Oxylipins Increase the Odds of Cardiovascular and Cerebrovascular Events in Patients with Peripheral Artery Disease

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Specific Plasma Oxylipins Increase the Odds of Cardiovascular and Cerebrovascular Events in Patients with Peripheral Artery Disease

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Abstract

Oxylipins and fatty acids may be novel therapeutic targets for cardiovascular disease. The objective was to determine if plasma oxylipins or fatty acids can influence the odds of cardiovascular/cerebrovascular events. In 98 patients (25 female, 73 male) with peripheral artery disease, the prevalence of transient ischemic attacks, cerebrovascular accidents, stable angina and acute coronary syndrome was n= 16, 10, 16, and 24, respectively. Risk factors such as being male, diagnosed hypertension, diabetes mellitus, and hyperlipidemia were not associated with events. Plasma fatty acids and oxylipins were analyzed with gas chromatography and HPLC-MS/MS, respectively. None of 24 fatty acids quantified were associated with events. In contrast, 39 plasma oxylipins were quantified and 8 were significantly associated with events. These 8 oxylipins are known regulators of vascular tone. For example, every 1 unit increase in Thromboxane B_2/Prostaglandin $F_{1\alpha}$ and every 1 nM increase in plasma 16-hydroxyeicosatetraenoic acid, thromboxane B_2, or 11,12-dihydroxyeicosatrienoic acid (DiHETrE) increased the odds of having had $\geq$2 events versus no event ($p<0.05$). The greatest predictor was plasma 8,9-DiHETrE which increased the odds of acute coronary syndrome by 92-fold. In conclusion, specific oxylipins were highly associated with clinical events and may represent specific biomarkers and/or therapeutic targets of cardiovascular disease.

Registered clinical trial: #NCT00781950 at clinicaltrials.gov

Key Words: Lipidomics, Myocardial Infarction, Cerebrovascular Accident, Oxylipin, Fatty acid

Abbreviations: 6 keto=(6k), acute coronary syndrome=(ACS), cerebrovascular accident=(CVA), dihommo gamma linolenic acid=(DGLA), dihydroxydocosapentanoic acid=(DiHDPA), dihydroxyeicosatrienoic acid=(DiHETrE), dihydroxyoctadecenoic acid=(DiHOME), HETrE=(hydroxyeicosatrienoic acid), hydroxydocosahexaenoic acid,=(HDOHE), hydroxyeicosapentaenoic acid=(HEPE), hydroxyeicosatetraenoic acid=(HETE), hydroxyeicosatrienoic acid=(HETrE), hydroxyoctadecadienoic acid=(HODE), hydroxyoctadecatrienoic acid=(HOTrE), oxooctadecadienoic acid=(OXOODE), prostaglandin=(PG), standard error of the mean=(SEM), transient ischemic attack=(TIA), trihydroxyoctadecenoic acid=(TriHOME), thromboxane=(TX)

Introduction

Oxylipins are an intriguing group of molecules due to their ability to regulate inflammation and vascular tone (Gabbs et al. 2015; Nishimaki and Seki 1999; Norris and Dennis 2014; Dennis and Norris 2015; Eskildsen et al. 2014). There are three oxylipin subclasses: eicosanoids, octadecanoids and docosanoids. The eicosanoids are the most characterized in terms of their physiological and pathophysiological effects. Due to recent advancements in detection technology, many novel oxylipins within the octadecanoid and docosanoid subclasses have now also been identified (Gabbs et al. 2015). The substrate of origin and often the enzyme that produces these novel oxylipins are known, but the role for many of these molecules in physiology and disease remains unclear. It is generalized that most omega 6 fatty acid-derived oxylipins are more pro-inflammatory and vasoconstrictive than those produced from omega 3 fatty acids (Gabbs et al. 2015). Changes in vascular tone and inflammation have been implicated in endothelial dysfunction, hypertension, and atherosclerosis. These processes play a fundamental role in the etiology and progression of both cerebrovascular accidents and myocardial infarction (Puddu et al. 2000; Montezano and Touyz 2012; Shearer and Newman 2009). It is plausible, therefore, that plasma oxylipins may act as significant mediators in the progression of these events.

A patient population that is at a high risk for cardiovascular and cerebrovascular events are those living with peripheral artery disease (PAD). PAD is defined as chronic arterial occlusion typically from atherosclerosis to the arteries exclusive of intracranial and coronary circulation, ie: typically in the arms and legs (Ouriel 2001). However, patients with PAD often progress to coronary artery disease or cerebrovascular disease. PAD patients have a high incidence of hypertension and hypertension is a particularly dangerous risk factor for

cardiovascular outcomes (Caligiuri et al. 2016). Patients with PAD have a 35% lower survival rate and a higher incidence of myocardial infarctions and cerebrovascular accidents than matched patients with coronary artery disease alone (Welton et al. 2008). For this reason, a patient population living with PAD was chosen for the current investigation. The hypothesis of the current study was novel eicosanoids derived from omega-6 fatty acids will increase the odds of cardiovascular and cerebrovascular events in patients with PAD. In order to assess this hypothesis, the prevalence of stable angina, acute coronary syndrome, transient ischemic attacks and cerebrovacular accidents in patients living with PAD were assessed with regard to their relationship to coventional risk factors, plasma fatty acid and oxylipin concentrations.

Material and Methods

Baseline data from patients enrolled in the randomized, double-blinded, placebo controlled FlaxPAD trial (#NCT00781950 at clinicaltrials.gov) were included in this analysis (Rodriguez-Leyva et al. 2013). The trial was approved and in accordance with the University of Manitoba Research Ethics Boards, Health Canada Natural Health Product Directorate, and the St. Boniface Hospital Research Review Committee. All participants provided written consent. The total sample size for the current study was 98 participants (n=25 females and n=73 males). Further details of the patient population and CONSORT flow diagram have been published elsewhere (Rodriguez-Leyva et al. 2013; Caligiuri et al. 2014).

Lididomics

Plasma fatty acids were detected and quantified using gas chromatography and indicated as ng/mL. Details of methodology for fatty acid extraction and quantification are as previously described (Caligiuri et al. 2013). An expansive lipidomics analysis searching for over 100

mediators were included in this study. Plasma oxylipins were extracted, detected, and quantified as previously described (Caligiuri et al. 2014). Oxylipins are represented as nM.

Cardiovascular Events

Accurate coronary syndrome (ACS) was defined by S-T elevation myocardial infarction, non S-T Elevation myocardial infarction, and unstable angina. These were defined according to the Third Universal definition (Thygesen et al. 2012). ACS was defined by detecting a change of cardiac biomarkers with one biomarker greater than the 99th percentile upper reference limit in addition to a minimum of one of the following: ischemic symptoms, new ST changes, new left bundle branch block, pathological Q waves, new loss of healthy myocardium, left ventricular wall abnomality, and/or intracoronary thrombus (Thygesen et al. 2012). Stable angina pectoris was defined as symptoms of chest discomfort without elevated cardiac biomarkers or evidence of other extraneous plausible causes (eg: gastroesophageal reflux) upon exertion (Thygesen et al. 2012). A cerebrovascular accident (CVA) was defined in accordance with the American Heart Association and American Stroke Association guidelines and diagnosed with a computed tomography scan or magnetic resonance imaging (Sacco et al. 2013). A transient ischemic attack (TIA) was defined as an event without permanent cerebral damage but rather a brief period of focal cerebral impedance of blood flow that resulted in temporary neurological dysfunction (Easton et al. 2009).

Statistical Analysis

Statistical analyses were performed using SAS version 9.3 (Cary, NC, USA). Logistic regression with score selection was utilized to determine the three most significantly associated oxylipins or fatty acids with patient events. Univariate and multivariate forward selection logistic

regression were utilized to determine the association of oxylipins and fatty acids to events while controlling for hypertension, diabetes mellitus, hyperlipidemia, age, sex, smoking status and medications. Bootstrap analysis was utilized for distribution estimation. Probability was assessed with the logit (p) equation produced from the logistic regression analyses. The Wilcoxin-Rank Sum test was utilized for 2 group comparisons due to the non-Gaussian distribution with a post-hoc Bonferroni correction. All tests were set at a significance level of 0.05.

Results

Prevalence of Cardiovascular and Cerebrovascular Events

Figure 1 illustrates the prevalence of cardiovascular and cerebrovascular events in the study population. ACS affected the population studied here more than any other clinical event (n=24 patients). No patients presented with all 4 events. Four patients presented with 3 of the 4 possible events, 17 patients had 2 events, 20 patients presented with 1 event, and 57 patients had no cardiovascular or cerebrovascular event.

Plasma Fatty Acid Concentrations by Presence of Event

Because polyunsaturated fatty acids are the substrates to the bioactive oxylipins, plasma fatty acid concentrations were compared by presence of event. Twenty-four plasma fatty acids were detected. Twleve of these were polyunsaturated fatty acids. Plasma fatty acid concentrations (ng/mL) did not differ significantly among patients for presence of angina, ACS, TIA or CVA (Online Supplementary Table 1), nor were plasma fatty acids significant predictors of events in logistic regression models.

Plasma Oxylipin Concentrations by Presence of Event

Thirty-nine plasma oxylipins were quantified in the current study population living with peripheral artery disease. Concentrations are presented in Supplemental Table 2. Using the Wilcoxin-Rank sum test followed by a Bonferroni correction, four plasma oxylipins were significantly different between the presence of an event or absence (Figure 2). For example, plasma 16-hydroxyeicosatetraenoic acid (HETE) was more than 4 times higher in patients that suffered from a CVA versus patients that did not (Figure 2). Using the score selection method of logistic regression, the oxylipins most significantly associated with an event were identified (Tables 1-4). For example, only 6 keto (6k)-$PGF_{1\alpha}$ resulted in an odds ratio less than 1 for the prevalence of TIAs, indicating a protective effect (Table 3). This contributed to the ratio of TXB_2/6k$PGF_{1\alpha}$ significantly increasing the odds of a TIA by 3.8-fold for every 1 unit increase (Table 3).

It was important to take into account the physiological concentrations of the plasma oxylipins, as logistic regression is per 1 nM increase. Therefore, Figure 3 illustrates the probability of an event by plasma oxylipin concentration observed in the current population ($p<0.05$). 16-HETE resulted in the greatest increase in the probability of angina and cerebrovascular accidents based on the observed physiological concentration range (Figure 3). However, 8,9-DiHETrE had the largest slope at 4.53 (as indicated by b in the $y = a + bx$ equation) for prediction of ACS prevalence.

Plasma oxylipin profiles were also compared between those with multiple events and those with no event. 16-HETE (OR: 16.1 (14.9, 17.3)), thromboxane B_2 (OR: 10.5 (9.77, 11.3)), and 11,12-DiHETrE (OR: 10.1 (9.52, 10.7)), all increased the odds of having had multiple events (ie: 2 or 3 events) versus no events. β-blockers were significantly associated with the prevalence of angina, ACS and TIAs but not CVAs. Age, sex, smoking status, hyperlipidemia, hypertension,

diabetes mellitus, and other medications were not significantly associated with any event. In the presence of these factors, 16-HETE and 8,9-DiHETrE, still significantly influenced the odds of events (Tables 1-4).

The ratio of TXB_2 to $6kPGF_{1\alpha}$ has previously held predictive value for cardiovascular disease and therefore was assessed as a predictor of events in this population (Nishimaki and Seki 1999; de Leval et al. 2004). $TXB_2/6kPGF_{1\alpha}$ increased the odds of a TIA with an odds ratio of 3.79 (3.66, 3.93; p-value = 0.019) as well as having multiple events (ie: 2 or 3 events) versus no event OR: 5.13 (4.90, 5.36; p-value = 0.024). Adding all four DiHETrEs together provided predictive value for having any event OR: 2.0 (2.0, 2.1) p-value = 0.034 as well as an ACS OR: 1.95 (1.05, 3.64) p-value = 0.035. Adding the concentration of HETEs held no predictive value for events. Likewise, summation of the oxylipins by their respective enzyme (cyclooxygenase, lipoxygenase, and cytochrome P450) held no predictive value.

Logistic regression was also performed comparing patients with only one event, as indicated in Figure 1, versus the rest of the population without that particular event. Because this decreased the number of cases to less than 10, this reflected an exploratory analysis only. In these assessments, every 1 nM increase in plasma 8,9-DiHETrE increased the odds of an ACS by 454-fold (377, 547) with a p-value of 0.041. Every 1 nM increase of plasma 16-HETE increased the odds of a CVA by 25-fold (22, 27) with a p-value of 0.043. There were no significant predictors for angina or TIA in these exploratory models.

Discussion

Oxylipins may have a role in cardiovascular and cerebrovascular events because of their actions on inflammation and vascular tone (Norris and Dennis 2014; Dennis and Norris 2015).

Patients with PAD are at a higher risk for cardiovascular events versus a healthy population or even patients with coronary artery disease (Welton et al. 2008). For this reason, patients with PAD presented an important population to investigate. In this population, typical risk factors such as hypertension, diabetes, and hyperlipidemia did not influence the risk of events. This could be due to the fact that hypertension, hyperlipidemia, and diabetes mellitus were well controlled in the population. Despite this, the oxylipins which are markers for vascular tone and inflammation were significantly associated to events. Oxylipins may present a new era of risk markers/therapeutic targets particulary in the presence of well controlled risk factors.

This is the first study to observe the relationship of cardiovascular/cerebrovascular events to a comprehensive plasma lipidomics profile in patients with PAD. There was no significant relationship between fatty acid profiles and cardiovascular events; however their products, plasma oxylipins, did significantly influence the presence of events. A similar observation was observed in an obese animal model provided diets of varying fatty acid levels. Particular renal phospholipid and triglyceride fatty acid concentrations did not differ among groups but renal oxylipins significantly differed (Caligiuri et al. 2013; Caligiuri et al. 2014b). Similarly in the current study, patients with past clinical events did not have significantly different concentrations of omega-6 fatty acids versus patients with no events. However, patients with events did have significantly higher concentrations of many pro-inflammatory and vasoconstrictive oxylipins produced from omega 6 fatty acids. In contrast, one omega-6 derived oxylipin, $6kPGF_{1\alpha,}$ had a protective odds ratio of 0.066 against transient ischemic attacks. $6kPGF_{1\alpha}$ is the stable product of prostacyclin (PGI_2), which is an anti-aggregatory, endothelial-derived vasodilator (Chen et al. 2002). PGI_2 counteracts the aggregatory and vasoconstrictive effects of thromboxane A_2 (Chen

et al. 2002). This was supported by our finding that the ratio of TXB_2 to $6kPGF_{1\alpha}$ held predictive value for patients having multiple events or a TIA alone.

16-HETE, thromboxane B_2 and 11,12-DiHETrE are all produced from arachidonic acid and all significantly increased the odds of having had multiple cardiovascular events versus no events. 16-HETE is produced by polymorphonuclear leukocytes *in vitro* (Bednar et al. 2000) and is released upon angiotensin II stimulation which may explain its positive relationship to CVAs and angina. 16-HETE is released following angiotensin II stimulation. However, there was no statistically significant correlation between ACE inhibitors and cardiovascular events. This dilemma may be explained in several ways. First, ACE inhibitors were trending to be statistically significant ($p = 0.12$) (Table 4) but insufficient sample size may have limited the capacity to power these results to statistical significance. In addition, we have no data on patient adherence to ACE inhibitors. Furthermore, although 16-HETE is released in response to Angiotensin II, how an ACE inhibitor may impact 16-HETE concentrations in patients is unknown. An ACE inhibitor may not necessarily prevent 16-HETE release. The production of 16-HETE is regulated by many means including the concentration of other HETEs, CYP450, and arachidonic acid availability. It is also unclear if other medications concomitantly administered to these patients may have had additional and perhaps contrasting effects on plasma 16-HETE concentrations. The exact mechanism for the relationship with cardiovascular events has yet to be determined and represents an intriguing area for future research. Less surprising was the positive relationship of TXB_2 with events because TXB_2 is the stable product of the potent vasoconstrictor thromboxane A_2. Similarly, thromboxane B_2 is present in significantly higher concentrations in the coronary circulation in patients with angina versus healthy subjects and during times of chest pain versus no pain (Tada et al. 1981). 11,12-DiHETrE is a product of

11,12-epoxyeicosatrienoic acid (EpETrE). When 11,12-EpETrE is converted by soluble epoxide hydrolase to 11,12-DiHETrE, it causes a significant loss of vasodilation (Sudhahar et al. 2010; Falk et al. 2003). Therefore, higher concentrations of 11,12-DiHETrE have been implicated in vasoconstriction and even hypertension (Caligiuri et al. 2014). It may be precisely the vascular constricting properties of 11,12-DiHETrE that explain the increased odds of events in the present study. The positive relationship of plasma 18-HEPE to ACS was unexpected. 18-HEPE is a pre-cursor to the E-series resolvins which regulate resolution of inflammation and, therefore, are generalized as protective (Oh et al. 2011; Serhan and Petasis 2011). It is possible that following ACS, plasma 18-HEPE levels increase as a protective adaptive mechanism to reduce inflammation. Future research is required to determine if 18-HEPE can improve cardiac remodelling post-MI. Other oxylipins may not have been associated with cardiovascular outcomes due to a lack of affect in vascular tone regulation, or they may have a very short half-life, or they may have concentrations that are too variable in the population of 98 patients.

It is also important to note that the largest slopes in the probability analysis shown in Figure 3 belonged to 8,9-DiHETrE and 16-HETE. This indicates that the odds of events changed more significantly with smaller changes in the plasma concentration of these two oxylipins versus other oxylipins. 8,9-DiHETrE and 16-HETE were also significant predictors of ACS and CVAs, respectively, in the multivariate logistic regression as well as the exploratory analysis of patients with only one event. As a result, these two molecules may prove to be particularly powerful targets for therapeutic investigation or risk marker assessment of ACS and CVAs in the future.

The relationship of β-blockers to events was significant for angina, ACS, and TIAs. β-blockers are a first-line therapy for hypertension and angina, and are often prescribed to prevent

a secondary myocardial infarction (Bradley et al. 2007). In this patient population, β-blockers were prescribed for anginal symptoms more often than calcium channel blockers, thus explaining the lack of a significant relationship of calcium channel blockers to angina. For these reasons, it likely explains the relationship between this class of medications and events.

Limitations

It is important to note that limitations exist within this study. First, because oxylipins were elevated in patients following an event, it is diffcult to ascertain if they are a cause or a consequence of the event. Because of their physiological role (ie: vasoconstricting or inflammatory), it is reasonable to propose that either or both roles are valid. Secondly, the length of time from the clinical event to the measurement of the oxylipins in this study was variable and may have influenced the oxylipin concentrations. Third, information regarding the type of CVA or ACS and the diagnostic method to detect the events was not standardized in the medical records used. Lastly, the distribution of sex was skewed toward males. Future trials in a different population of hypertensive patients should aim to recruit a more even sex distribution to obtain data with high power for both sexes. Future prospective studies following a large cohort of at risk patients may resolve these questions by obtaining plasma oxylipin profiles prior to and immediately after an event.

Conclusion

In conclusion, this research supports a significant relationship for specific plasma oxylipins such as 16-HETE and 8,9-DiHETrE to cardiovascular and cerebrovascular events in the presence of well controlled risk factors. Oxylipins may have the potential to serve as new risk markers/therapeutic targets beyond conventional standard of care practice. This was not a

phenomenon generalized to all lipids as plasma fatty acid concentrations were not associated with events in the current study. It is important to note that the oxylipin concentrations observed here may increase the risk of a clinical event or be important mediators in the healing process following an event. This study has identified specific oxylipins that are attractive targets to investigate for their involvement in cardiovascular and cerebrovascular events. Therefore, these oxylipins may be used as targets in future clinical or experimental investigations utilizing a lipidomics approach.

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Disclosures

None.

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Figure Legends

Figure 1: Venn Diagram of Cardiovascular and Cerebrovascular Event Prevalence in 98 Patients With Peripheral Artery Disease

Figure 2: Concentration of Plasma Oxylipins by Presence of Cardiovascular/Cerebrovascular Outcomes * denotes p-value<0.05. Abbreviations: 6-keto=(6k), acute coronary syndrome=(ACS), cerebrovacular accident=(CVA), dihydroxyeicosatrienoic acid=(DiHETrE), hydroxyeicosatetranoic acid=(HETE), prostaglandin=(PG), transient ischemic attack=(TIA), thromboxane=(TX).

Figure 3: Probability of Cardiovascular and Cerebrovascular Events by Plasma Oxylipin Concentrations (nM). Oxylipins were selected based on the score selection method of logistic regression to determine the strongest predicting oxylipins. Probabilities were obtained by taking the EXP of logit (p) to first obtain the odds. Probability was calculated by odds/(1+odds). Abbreviations: 6-keto=(6k), dihydroxyeicosatrienoic acid=(DiHETrE), hydroxyeicosapentanoic acid=(HEPE), hydroxyeicosatetranoic acid=(HETE), prostaglandin=(PG), thromboxane=(TX).

383 Table 1: Univariate and Multivariate Logistic Regression Models for the Risk Assessment of

384 Stable Angina

Variable	Cases	Controls	Univariate OR (95%CI)	p-value	Multivariate OR (95%CI)	p-value
Plasma Oxylipin	39	N/A				
PGE_2			80 (71, 90)	0.022	80 (71, 90)	0.022
16-HETE			9.1 (8.5, 9.7)	0.025		
Other Characteristics						
Age ≥ 65 years	65	33	1.8 (0.54, 6.2)	0.33		
Female Sex	25	73	0.63 (0.16, 2.4)	0.50		
Smoking Status(current)	25	73	0.40 (0.083, 1.9)	0.24		
Hypertension	74	24	2.2 (0.47, 11)	0.31		
Diabetes Mellitus	33	65	1.9 (0.62, 5.8)	0.27		
Hyperlipidemia	78	20	3.9 (0.49, 32)	0.20		
Medications						
Beta Blocker	33	65	3.1 (1.0, 9.3)	0.043		
CCB	27	71	0.33 (0.07, 1.5)	0.16		
ACE inhibitor	47	51	1.6 (0.56, 4.8)	0.37		
ARB	15	83	1.4 (0.33, 5.4)	0.68		
Diuretic	39	39	0.89 (0.30, 2.7)	0.84		
Aspirin	71	27	0.65 (0.21, 2.0)	0.45		
Statin	70	28	2.5 (0.52, 12)	0.26		

385 Angiotensin Converting Enzyme=(ACE), Angiotensin Receptor Blocker=(ARB), Calcium

386 Channel Blocker=(CCB), Hydroxyeicosatetraenoic acid=(HETE), Prostaglandin=(PG)

Table 2 : Univariate and Multivariate Logistic Regression Models for the Risk Assessment of Acute Coronary Syndrome

Variable	Cases	Controls	Univariate OR (95%CI)	p-value	Multivariate OR (95%CI)	p-value
Plasma Oxylipin	39	N/A				
8,9-DiHETrE			92.4 (82.0, 104)	0.0192	92.4 (82.0, 104)	0.0192
18-HEPE			4.1 (4.0, 4.3)	0.045		
Other Characteristics						
Age ≥ 65 years	65	33	1.1 (0.44, 3.0)	0.78		
Female Sex	25	73	0.71 (0.23, 2.2)	0.55		
Smoking Status(current)	25	73	1.0 (0.35, 3.0)	0.97		
Hypertension	74	24	4.4 (0.96, 21)	0.057		
Diabetes	33	65	1.6 (0.60, 4.0)	0.36		
Hyperlipidemia	78	20	2.0 (0.52, 7.4)	0.32		
Medications						
Beta Blocker	33	65	8.8 (3.1, 25)	<0.00010	12 (3.6, 40)	<0.0001
CCB	27	71	0.44 (0.14, 1.4)	0.18		
ACE inhibitor	47	51	1.6 (0.62, 3.9)	0.35		
ARB	15	83	0.43 (0.090, 2.0)	0.29		
Diuretic	39	39	1.7 (0.69, 4.4)	0.24		
Aspirin	71	27	0.62 (0.23, 1.6)	0.33		
Statin	70	28	3.1 (0.82, 11)	0.094		

Angiotensin Converting Enzyme=(ACE), Angiotensin Receptor Blocker=(ARB), Calcium Channel Blocker=(CCB), Dihydroxyeicosatrienoic acid=(DiHETrE), Hydroxyeicosapentanoic acid=(HEPE)

392 Table 3: Univariate and Multivariate Logistic Regression Models for the Risk Assessment of

393 Transient Ischemic Attacks

Variable	Cases	Controls	Univariate OR (95%CI)	p-value	Multivariate OR (95%CI)	p-value
Plasma Oxylipin	39	N/A				
TXB_2/6k$PGF_{1\alpha}$			3.8 (3.6, 3.9)	0.0048	3.8 (3.6, 3.9)	0.019
6k$PGF_{1\alpha}$			0.066 (0.061, 0.072)	0.040		
Other Characteristics						
Age ≥ 65 years	65	33	1.8 (0.54, 6.2)	0.33		
Female Sex	25	73	0.37 (0.077, 1.7)	0.21		
Smoking Status(current)	25	73	0.36 (0.076, 1.7)	0.20		
Hypertension	74	24	1.3 (0.33, 5.0)	0.71		
Diabetes	33	65	0.96 (0.30, 3.1)	0.95		
Hyperlipidemia	78	20	0.97 (0.24, 3.8)	0.96		
Medications						
Beta Blocker	33	65	3.1 (1.0, 9.3)	0.043		
CCB	27	71	1.7 (0.57, 5.4)	0.33		
ACE inhibitor	47	51	0.90 (0.31, 2.6)	0.85		
ARB	15	83	1.3 (0.33, 5.4)	0.68		
Diuretic	39	39	0.64 (0.20, 2.0)	0.45		
Aspirin	71	27	0.47 (0.16, 1.4)	0.18		
Statin	70	28	0.95 (0.62, 1.5)	0.83		

394 6-keto=(6k), Angiotensin Converting Enzyme=(ACE), Angiotensin Receptor Blocker=(ARB),

395 Calcium Channel Blocker=(CCB), Prostagladin=(PG), Thromboxane=(TX)

25

Table 4: Univariate and Multivariate Logistic Regression Models for the Risk Assessment of Cerebrovascular Accidents

Variable	Cases	Controls	Univariate OR (95%CI)	p-value	Multivariate OR (95%CI)	p-value
Plasma Oxylipin	39	N/A				
16-HETE			55 (51, 60)	0.0016	55 (51, 60)	0.0016
$PGF_{2\alpha}$			3.3 (3.2, 3.5)	0.047		
Other Characteristics						
Age ≥ 65 years	65	33	0.33 (0.86, 1.3)	0.10		
Female Sex	25	73	2.1 (0.55, 8.3)	0.28		
Smoking Status(current)	25	73	0.81 (0.16, 4.2)	0.80		
Hypertension	74	24	1.1 (0.21, 5.7)	0.91		
Diabetes	33	65	0.97 (0.23, 4.1)	0.96		
Hyperlipidemia	78	20	0.84 (0.16, 4.4)	0.83		
Medications						
Beta Blocker	33	65	1.4 (0.36, 5.2)	0.66		
CCB	27	71	0.63 (0.13, 3.2)	0.58		
ACE inhibitor	47	51	3.1 (0.75, 13)	0.12		
ARB	15	83	<0.001 (<0.001, >999)	0.96		
Diuretic	39	39	4.1 (0.99, 17)	0.052		
Aspirin	71	27	0.38 (0.10, 1.4)	0.15		
Statin	70	28	1.1 (0.29, 4.4)	0.86		

Angiotensin Converting Enzyme=(ACE), Angiotensin Receptor Blocker=(ARB), Calcium Channel Blocker=(CCB), Hydroxyeicosatetraenoic acid=(HETE), Prostaglandin=(PG)

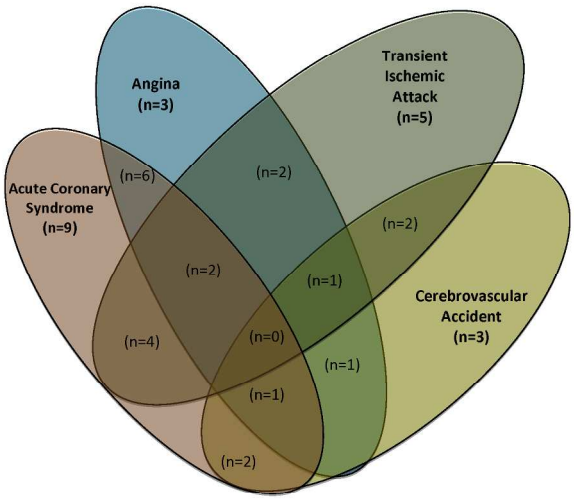

Figure 1: Venn Diagram of Cardiovascular and Cerebrovascular Event Prevalence in 98 Patients With Peripheral Arterial Disease

Figure 1

215x279mm (300 x 300 DPI)

2

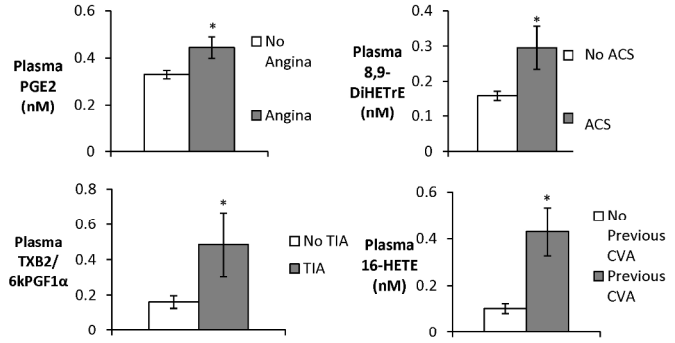

Figure 2: Concentration of Plasma Oxylipins by Presence of Cardiovascular/Cerebrovascular Outcomes
* denotes p-value <0.05. Abbreviations: 6-keto (6k), acute coronary syndrome (ACS), cerebrovacular accident (CVA), dihydroxyeicosatrienoic acid (DiHETrE), hydroxyeicosatetranoic acid (HETE), prostaglandin (PG), transient ischemic attack (TIA), thromboxane (TX).

2

Figure 2

215x279mm (300 x 300 DPI)

3

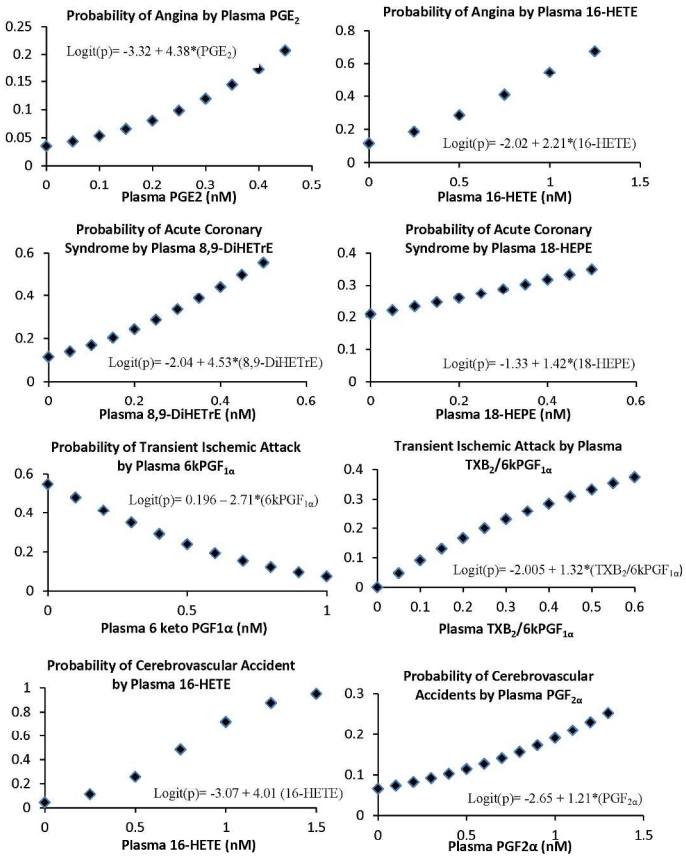

Figure 3: Probability of Cardiovascular and Cerebrovascular Events by Plasma Oxylipin Concentrations (nM). Oxylipins were selected based on the score selection method of logistic regression to determine the strongest predicting oxylipins. Probabilities were obtained by taking the EXP of logit (p) to first

3

Figure 3

215x279mm (300 x 300 DPI)

4

obtain the odds. Probability was calculated by odds/(1+odds). Abbreviations: 6-keto (6k), dihydroxyeicosatrienoic acid (DiHETrE), hydroxyeicosapentanoic acid (HEPE), hydroxyeicosatetranoic acid (HETE), prostaglandin (PG), thromboxane (TX).

4

Figure 3 continued

215x279mm (300 x 300 DPI)

Supplemental Material

Table 1: Plasma Fatty Acid Concentration (μg/mL) by Presence of Event

Fatty Acid	No Angina (n=82)	SEM	Yes Angina (n=16)	SEM	No ACS (n=74)	SEM	Yes ACS (n=24)	SEM
C12:0	0.7	0.3	0.3	0.3	0.5	0.3	1.0	0.6
C14:0	24.7	1.7	23.2	4.6	25.3	2.0	22.1	2.2
C14:1	1.7	0.3	1.5	0.8	1.5	0.3	2.0	0.7
C16:0	640.6	25.9	578.8	65.0	642.6	29.1	593.2	40.6
C16:1	59.9	4.2	56.8	17.2	62.1	5.5	50.9	5.9
C18:0	189.0	5.4	170.7	12.7	188.0	5.9	179.7	9.4
C18:1 Oleic	703.4	26.6	705.3	72.2	707.2	28.9	692.9	51.4
C18:1 Vaccenic	45.9	1.7	44.5	5.6	46.7	2.0	42.6	3.2
C18:2	702.3	21.3	637.2	52.0	703.3	22.4	655.7	41.7
C20:0	5.5	0.3	4.4	0.8	5.6	0.3	4.7	0.6
C18:3 DGLA	13.2	0.8	12.2	1.4	12.9	0.8	13.5	1.1
C20:1	3.4	0.3	4.0	0.7	3.6	0.3	3.2	0.5
C18:3 ALA	18.2	1.2	22.9	4.1	18.4	1.2	20.7	3.0
C20:2	3.6	0.3	3.9	0.7	3.6	0.4	3.8	0.6
C22:0	15.9	0.7	12.8	1.5	15.9	0.8	13.8	1.2
C20:3	36.5	1.4	33.4	3.7	36.8	1.5	33.3	2.4
C20:4	212.0	6.6	194.3	14.7	210.3	7.1	205.4	11.8
C22:2	0.1	0.0	0.3	0.3	0.1	0.1	0.2	0.2
C24:0	11.9	0.5	9.2	1.1	11.9	0.5	10.3	0.8
C20:5	20.8	1.2	20.4	2.1	20.7	1.3	20.8	2.0
C24:1	24.2	0.8	22.1	2.9	24.4	0.9	22.3	1.9
C22:4	3.9	0.3	3.1	0.8	3.9	0.4	3.5	0.6
C22:5	13.3	0.6	13.5	1.3	13.2	0.7	13.7	1.1
C22:6	41.6	2.2	40.3	3.6	42.7	2.4	37.5	2.5

Fatty Acid	No TIA (n=82)	SEM	Yes TIA (n=16)	SEM	No CVA (n=88)	SEM	Yes CVA (n=10)	SEM
C12:0	0.7	0.3	0.3	0.3	0.6	0.3	0.7	0.5
C14:0	25.2	1.9	20.8	2.1	24.2	1.7	27.0	4.4
C14:1	1.8	0.3	0.9	0.5	1.6	0.3	1.7	0.9
C16:0	636.0	27.4	602.5	45.4	627.1	26.0	660.9	61.2
C16:1	60.9	5.1	51.6	7.9	58.8	4.8	64.7	11.8
C18:0	186.5	5.6	183.1	11.5	185.2	5.4	192.9	12.8
C18:1 Oleic	705.6	27.8	693.7	58.4	699.4	27.0	741.3	63.5
C18:1 Vaccenic	45.6	1.9	46.2	3.9	45.1	1.7	51.1	6.4
C18:2	684.2	21.2	730.2	54.0	688.1	21.1	722.7	58.7
C20:0	5.3	0.3	5.6	0.8	5.3	0.3	5.5	0.7
C18:3 DGLA	12.9	0.7	13.9	1.7	12.7	0.7	16.8	2.0
C20:1	3.5	0.3	3.8	0.7	3.4	0.3	4.9	0.7
C18:3 ALA	18.3	1.3	22.4	2.8	18.9	1.3	19.2	2.5
C20:2	3.6	0.3	4.1	0.8	3.5	0.3	4.8	0.9
C22:0	15.4	0.7	15.0	2.0	15.4	0.7	14.8	2.2
C20:3	36.2	1.4	34.7	3.1	35.5	1.4	40.0	3.0
C20:4	204.9	6.6	230.7	14.2	205.5	6.1	240.6	24.1
C22:2	0.1	0.1	0.0	0.0	0.1	0.1	0.0	0.0
C24:0	11.4	0.5	11.7	1.1	11.4	0.5	11.9	1.3
C20:5	20.0	1.2	24.0	2.5	20.4	1.2	22.8	2.9
C24:1	23.7	0.8	24.9	2.7	23.9	0.8	24.3	3.5
C22:4	3.7	0.4	4.3	0.6	3.6	0.3	5.6	1.0
C22:5	13.2	0.6	14.2	1.2	13.1	0.6	15.7	2.0
C22:6	39.7	1.6	50.1	8.8	41.0	2.1	45.4	4.5

Abbreviations: acute coronary syndrome (ACS), alpha-linolenic acid (ALA), cerebrovascular accident (CVA), dihommo gamma linolenic acid (DGLA), transient ischemic attack (TIA), standard error of the mean (SEM)

Table 2: Plasma Oxylipin Concentration (nM) by Presence of Event

Oxylipin	No Angina (n=82)	SEM	Yes Angina (n=16)	SEM	No ACS (n=74)	SEM	Yes ACS (n=24)	SEM
10-HDOHE	0.4	0.1	0.9	0.5	0.4	0.1	0.9	0.4
11,12-DiHETrE	0.6	0.0	0.8	0.1	0.6	0.0	0.8	0.1
11-HDOHE	0.8	0.1	1.2	0.4	0.8	0.1	1.3	0.4
11-HETE	0.8	0.1	1.2	0.5	0.7	0.0	1.3	0.4
12-HETE	3.8	0.6	4.1	0.9	4.0	0.6	3.4	0.7
12,13-DiHOME	3.4	0.4	2.5	0.3	3.3	0.4	3.0	0.3
12-HEPE	0.8	0.1	1.3	0.4	0.8	0.2	1.1	0.3
13-HODE	10.1	1.0	9.2	1.0	10.2	1.0	9.3	0.9
13-HDOHE	0.3	0.1	0.5	0.3	0.2	0.0	0.6	0.3
13-OXOODE	9.0	2.2	6.7	0.8	9.3	2.5	6.4	0.7
14,15-DiHETrE	0.8	0.0	0.8	0.1	0.8	0.0	0.9	0.1
14-HDOHE	1.4	0.2	1.9	0.5	1.5	0.2	1.5	0.3
15-HETrE	0.5	0.1	0.9	0.4	0.5	0.1	1.0	0.3
15-HETE	1.1	0.1	1.5	0.4	1.1	0.1	1.5	0.4
16-HDOHE	0.3	0.1	0.6	0.4	0.2	0.0	0.7	0.3
16-HETE	0.1	0.0	0.3	0.1	0.1	0.0	0.2	0.1
17-HDOHE	0.9	0.1	1.3	0.8	0.7	0.1	1.5	0.6
18-HEPE	0.1	0.0	0.3	0.2	0.1	0.0	0.3	0.2
18-HETE	0.3	0.0	0.5	0.3	0.3	0.0	0.5	0.2
19,20-DiHDPA	1.5	0.1	1.6	0.2	1.5	0.1	1.5	0.1
20-HDOHE	0.4	0.1	0.7	0.4	0.3	0.0	0.6	0.3
4-HDOHE	0.6	0.1	0.8	0.3	0.5	0.1	1.0	0.4
5-HETE	1.3	0.1	1.9	0.7	1.2	0.1	2.1	0.6
5,6-DiHETrE	0.3	0.0	0.3	0.1	0.3	0.0	0.3	0.1
5-HEPE	0.6	0.1	1.1	0.5	0.5	0.1	1.1	0.4
6keto-$PGF_{1\alpha}$	0.7	0.0	0.7	0.0	0.7	0.0	0.7	0.0
7-HDOHE	0.2	0.1	0.5	0.4	0.1	0.0	0.6	0.3
8,9-DiHETrE	0.2	0.0	0.3	0.1	0.2	0.0	0.3	0.1
8-HETE	0.7	0.1	1.0	0.4	0.6	0.1	1.1	0.3
9-HODE	8.8	0.9	8.4	1.2	8.9	1.0	8.2	0.9
9,10,13-TriHOME	5.5	2.1	2.1	0.3	5.9	2.3	2.0	0.2
9,10-DIHOME	3.7	0.4	3.0	0.5	3.7	0.5	3.1	0.4
9,12,13-TriHOME	3.2	1.1	1.6	0.3	3.4	1.2	1.6	0.2
9-HOTrE	0.8	0.1	0.9	0.2	0.7	0.1	1.0	0.2
Maresin	27.6	4.4	20.6	6.1	26.5	4.6	26.3	6.6
PGD_2	10.4	3.7	4.6	1.1	27.5	17.9	8.6	3.1
PGE_2	0.3	0.0	0.4	0.0	0.3	0.0	0.4	0.0
$PGF_{2\alpha}$	0.3	0.1	0.1	0.1	0.3	0.0	0.3	0.1
TXB_2	0.1	0.0	0.2	0.1	0.1	0.0	0.2	0.1

Oxylipin	No TIA (n=82)	SEM	Yes TIA (n=16)	SEM	No CVA (n=88)	SEM	Yes CVA (n=10)	SEM
10-HDOHE	0.5	0.1	0.4	0.1	0.5	0.1	0.9	0.4
11,12-DiHETrE	0.6	0.0	0.8	0.1	0.7	0.0	0.7	0.1
11-HDOHE	0.9	0.1	0.8	0.3	0.8	0.1	1.5	0.6
11-HETE	0.8	0.1	0.8	0.1	0.8	0.1	1.3	0.6
12-HETE	3.9	0.6	3.5	0.8	3.9	0.5	4.0	1.1
12,13-DiHOME	3.3	0.4	2.7	0.2	3.2	0.3	3.1	0.5
12-HEPE	0.9	0.2	0.8	0.3	0.9	0.1	1.3	0.5
13-HODE	9.8	0.9	10.8	1.3	10.1	0.9	9.4	1.4
13-HDOHE	0.3	0.1	0.2	0.1	0.3	0.1	0.5	0.3
13-OXOODE	9.0	2.2	6.9	1.1	8.9	2.1	5.9	1.0
14,15-DiHETrE	0.8	0.0	0.8	0.1	0.8	0.0	0.9	0.1
14-HDOHE	1.5	0.2	1.5	0.5	1.5	0.2	1.8	0.5
15-HETrE	0.6	0.1	0.5	0.1	0.6	0.1	0.9	0.4
15-HETE	1.2	0.1	1.2	0.1	1.1	0.1	1.8	0.5
16-HDOHE	0.4	0.1	0.3	0.1	0.3	0.1	0.6	0.3
16-HETE	0.1	0.0	0.2	0.0	0.1	0.0	0.4	0.1
17-HDOHE	0.9	0.2	1.0	0.4	0.9	0.2	1.4	0.8
18-HEPE	0.1	0.0	0.1	0.1	0.1	0.0	0.4	0.2
18-HETE	0.3	0.1	0.3	0.1	0.3	0.1	0.5	0.2
19,20-DiHDPA	1.5	0.1	1.7	0.2	1.5	0.1	1.8	0.2
20-HDOHE	0.4	0.1	0.5	0.2	0.4	0.1	0.6	0.2
4-HDOHE	0.6	0.1	0.5	0.1	0.5	0.1	1.3	0.7
5-HETE	1.4	0.2	1.4	0.3	1.3	0.1	2.3	0.9
5,6-DiHETrE	0.3	0.0	0.2	0.0	0.3	0.0	0.3	0.1
5-HEPE	0.7	0.1	0.7	0.2	0.6	0.1	1.4	0.6
6keto-$PGF_{1\alpha}$	0.7	0.0	0.6	0.1	0.7	0.0	0.7	0.1
7-HDOHE	0.3	0.1	0.2	0.1	0.2	0.1	0.6	0.4
8,9-DiHETrE	0.2	0.0	0.2	0.0	0.2	0.0	0.3	0.1
8-HETE	0.8	0.1	0.7	0.2	0.7	0.1	1.3	0.5
9-HODE	8.6	0.9	9.2	1.2	8.8	0.8	8.1	1.4
9,10,13-TriHOME	5.4	2.1	2.6	0.4	5.3	1.9	2.2	0.4
9,10-DIHOME	3.6	0.4	3.5	0.6	3.6	0.4	3.5	0.7
9,12,13-TriHOME	3.1	1.1	2.0	0.3	3.1	1.0	1.7	0.3
9-HOTrE	0.8	0.1	0.9	0.2	0.8	0.1	0.8	0.3
Maresin	26.2	4.4	27.8	6.9	26.2	4.0	28.5	12.0
PGD_2	26.2	16.1	5.7	1.0	23.9	15.0	13.9	7.3
PGE_2	0.3	0.0	0.4	0.1	0.3	0.0	0.4	0.0
$PGF_{2\alpha}$	0.3	0.1	0.3	0.1	0.2	0.0	0.6	0.3
TXB_2	0.1	0.0	0.3	0.1	0.1	0.0	0.2	0.1

Note: Plasma oxylipin concentrations in the FlaxPAD trial divided based on placebo or flaxseed group at baseline and 6 months have been previously published (Caligiuri et al. *Hypertension*. 2014; 64: 53-59). Abbreviations: 6 keto (6k), acute coronary syndrome (ACS), cerebrovascular accident (CVA), dihommo gamma linolenic acid

(DGLA), dihydroxydocosapentanoic acid (DiHDPA), dihydroxyeicosatrienoic acid (DiHETrE), dihydroxyoctadecenoic acid (DiHOME), HETrE (hydroxyeicosatrienoic acid), hydroxydocosahexaenoic acid, (HDOHE), hydroxyeicosapentaenoic acid (HEPE), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosatrienoic acid (HETrE), hydroxyoctadecadienoic acid (HODE), hydroxyoctadecatrienoic acid (HOTrE), oxooctadecadienoic acid (OXOODE), prostaglandin (PG), standard error of the mean (SEM), transient ischemic attack (TIA), trihydroxyoctadecenoic acid (TriHOME), thromboxane (TX).