ONLINE SUPPLEMENTAL MATERIAL

EVALUATION OF CYTOCHROME P450-DERIVED EICOSANOIDS IN HUMANS WITH STABLE ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

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Supplemental Methods

Study population and protocol

A cohort of individuals ≤65 years of age with established coronary artery disease (CAD), defined as ≥50% stenosis in at least one major epicardial coronary artery by coronary angiography, were identified in the UNC Cardiac Catheterization Laboratory. Exclusion criteria included pregnancy, atrial fibrillation, left-ventricular systolic dysfunction (ejection fraction ≤35%), current use of long-acting nitrates or insulin, active autoimmune disease, history of severe aortic stenosis, history of solid organ transplant or dialysis, or history of cancer within the previous 5 years.

A cohort of healthy volunteers (HV) ≤65 years of age were identified from the local Chapel Hill, NC community by advertisement. Following a detailed medical and medication history and a fasting serum chemistry and cholesterol panel, individuals with a history of cardiovascular disease, risk factors for CAD (including physician-diagnosed hypertension or diabetes, cigarette smoking within the previous 6 months, high cholesterol [defined as total cholesterol >6.2 mmol/L (240 mg/dL), triglycerides >2.3 mmol/L (200 mg/dL), or LDL cholesterol >4.1 mmol/L (160 mg/dL)], or body mass index ≥30 kg/m^2), or currently taking any medication for a chronic medical condition were excluded.

The study protocol was approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board. Eligible participants provided written informed consent and returned to the UNC Clinical and Translational Research Center (CTRC) for a single morning study visit after fasting overnight and withholding their morning medications. Participants in the CAD cohort returned to the CTRC 62±34 days after their index catheterization and were clinically stable at the time of their study visit. All participants were instructed to refrain from tobacco products, caffeine, and vigorous exercise the morning of the study visit, and from use of vitamin C, vitamin E, fish oil, niacin or arginine supplements, oral decongestants, non-steroidal anti-inflammatory drugs (other than low-dose aspirin), or erectile dysfunction medications for at least 7 days prior to the study visit. Individuals experiencing a respiratory tract infection within 4 weeks of the study visit were not eligible to participate. At the study visit, blood was collected by venipuncture. Plasma was separated by centrifugation and stored at -80^{o}C pending analysis.

Quantification of plasma CYP-derived eicosanoids

Plasma eicosanoid levels were quantified as previously described [1, 2]. Briefly, plasma (0.25 mL) was diluted in 0.1% acetic acid/5% methanol solution containing 0.009 mM butylated hydroxytoluene. Samples were loaded onto HyperSep Retain PEP SPE cartridges (Thermo Fisher Scientific, Waltham, MA) that had been conditioned with 0.1% acetic acid/5% methanol and spiked with 30 ng each of 10,11-epoxyheptadecanoic acid and 10,11-dihydroxynonadecanoic acid as internal standards. Columns were washed with two column volumes of 0.1% acetic acid/5% methanol, and analytes were eluted with 1 mL acetonitrile. Extracts were dried under nitrogen gas at 37°C and reconstituted in 40% ethanol. CYP-derived metabolites of arachidonic acid (14,15-EET, 11,12-EET, 8,9-EET, 14,15-DHET, 11,12-DHET, 8,9-DHET, 5,6-DHET, and 20-HETE) and linoleic acid (9,10- and 12,13-epoxyoctadecenoic acid (9,10-EpOME and 12,13-EpOME) and 9,10- and 12,13-dihydroxyoctadecenoic acid (9,10-DHOME and 12,13-DHOME)) were separated by reverse phase HPLC on a 1x150 mm, 5µm Luna C18(2) column (Phenomenex, Torrance, CA) and quantified using a MDS Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with negative mode electrospray ionization and multiple reaction monitoring [1].

Data were acquired and analyzed with Analyst software, version 1.5 (Applied Biosystems), and the relative response ratios of each analyte to the appropriate internal standard were used to calculate concentrations. Extraction efficiency for each sample was calculated based on the recovery of the internal standards. Samples for which the extraction efficiency was greater than two standard deviations below the mean were considered to be analytical failures and excluded

from the statistical analysis. A concentration equal to half that of the lowest standard was imputed for samples in which the calculated concentration fell below that value. Analytes for which more than 20% of the values were imputed were dropped from the statistical analysis [3]. Consistent with a prior analysis in a healthy population [4], plasma concentrations of 11,12-EET were below the lower limit of detection in 60 of 82 CAD patients (73.2%) and 28 of 36 healthy volunteers (77.8%), and thus it was excluded from the analysis.

Statistical analysis

Data are presented as mean ± standard deviation or median (interquartile range) unless otherwise indicated. In addition to analyzing each separately, EET regioisomers (14,15-EET and 8,9-EET) and DHET regioisomers (14,15-DHET, 11,12-DHET, 8,9-DHET, and 5,6-DHET) were summed to evaluate total plasma concentrations of EETs and DHETs, respectively. The sum of all EET and DHET regioisomers (EETs+DHETs) was calculated as an index of total epoxygenase activity. Epoxide:diol ratios (14,15-EET:14,15-DHET, 9,10-EpOME:9,10-DHOME, and 12,13-EpOME:12,13-DHOME) were calculated as indices of sEH activity. Previous studies in *Ephx2*$^{-/-}$ mice and in humans demonstrate that these ratios in plasma are sensitive and specific biomarkers of sEH activity *in vivo*, with lower ratios indicative of higher sEH activity [2, 5]. Parameters that were not normally distributed, including plasma CYP-derived eicosanoid metabolite levels and epoxide:diol ratios, were log-transformed prior to statistical analysis.

Inter-metabolite correlations were determined by Spearman rank correlation to assess redundancy in each biomarker of CYP-mediated eicosanoid metabolism. To identify clinical factors that influence CYP epoxygenase (sum EETs, sum DHETs), sEH (14,15-EET:14,15-DHET ratio), and CYP ω-hydroxylase (20-HETE) metabolic function in CAD patients, stepwise multiple regression analysis was performed. Potential covariates included demographic factors (age, gender, race), indices of CAD severity (presence of multivessel disease, presence of acute coronary syndrome at index catheterization), comorbidities (hypertension, diabetes, cigarette smoking), body mass index, and angiotensin converting enzyme (ACE) inhibitor/angiotensin receptor blocker (ARB) use. These comorbidities were considered because alterations in CYP epoxygenase and ω-hydroxylase pathway expression and metabolic activity have been observed in rodent models of hypertension [6], obesity [7], and chronic tobacco smoke exposure [8]. ACE inhibitor/ARB use was included because angiotensin II regulates CYP-mediated eicosanoid metabolism in preclinical models [9] and in humans [10]. Due to the high prevalence of beta-blocker, aspirin, and statin use in CAD patients, use of these medications was not considered as a covariate. Covariates with $p<0.15$ were included in the final model. Comparisons of eicosanoid biomarker levels stratified by categorical covariates within the CAD cohort were performed by one-way ANOVA.

Study population characteristics were compared across the CAD and HV cohorts using a one-way ANOVA or chi-squared test, as appropriate. Sum EETs, sum DHETs, 20-HETE, and epoxide:diol ratios in the CAD and HV cohorts were compared by regression in an unadjusted model and a model adjusted for age, gender, and race. Analyses stratified by the categorical covariates identified in the multiple regression analysis were also performed. Stratified analyses were also completed to assess the potential contribution of confounding factors to the observed differences in each biomarker across the CAD and HV cohorts. The primary analysis was the case-control comparison of the sum EETs, sum DHETs, 20-HETE, and 14,15-EET:14,15-DHET ratio. Therefore, the significance level was set at $P=0.0125$ (0.05/4) to account for the impact of multiple statistical tests. At an $\alpha=0.0125$ level, we had approximately 80% power to detect a 40% difference in each biomarker between the non-obese and obese CAD groups and between each CAD subgroup and the HV cohort, assuming a coefficient of variation of 50% [2]. In a secondary analysis, plasma levels of each EET and DHET regioisomer were compared across the CAD and HV cohorts by one-way ANOVA. All analyses were performed using SAS 9.1.3 (SAS Institute, Cary, NC).

Supplemental Table 1. Univariate relationships between clinical factors and biomarkers of CYP epoxygenase, sEH and CYP ω-hydroxylase function in CAD patients

	Parameter Estimate	SE	R^2	p
Sum EETs				
Age	-0.013	0.007	0.039	0.075
Race	-0.138	0.151	0.010	0.363
Gender	-0.073	0.117	0.005	0.534
BMI	-0.030	0.009	0.122	0.001
Multivessel disease	0.052	0.118	0.002	0.660
ACS	0.013	0.124	<0.001	0.919
Hypertension	-0.226	0.133	0.035	0.092
Diabetes	-0.066	0.128	0.003	0.608
Smoking	-0.052	0.128	0.002	0.685
ACEI/ARB use	-0.185	0.115	0.032	0.110
Sum DHETs				
Age	-0.002	0.007	0.001	0.737
Race	-0.076	0.149	0.003	0.609
Gender	0.080	0.115	0.006	0.485
BMI	-0.009	0.009	0.011	0.360
Multivessel disease	-0.107	0.116	0.011	0.359
ACS	-0.142	0.121	0.017	0.244
Hypertension	0.037	0.133	0.001	0.779
Diabetes	-0.237	0.124	0.044	0.059
Smoking	0.255	0.123	0.051	0.041
ACEI/ARB use	-0.156	0.113	0.023	0.174
14,15-EET:14,15-DHET				
Age	-0.014	0.013	0.015	0.281
Race	-0.106	0.256	0.002	0.679
Gender	-0.191	0.198	0.012	0.337
BMI	-0.028	0.016	0.038	0.081
Multivessel disease	0.222	0.199	0.015	0.267
ACS	0.209	0.208	0.013	0.318
Hypertension	-0.308	0.226	0.023	0.177
Diabetes	0.147	0.217	0.006	0.500
Smoking	-0.404	0.213	0.043	0.061
ACEI/ARB use	-0.036	0.197	<0.001	0.856

ACEI = angiotensin converting enzyme inhibitor; ACS = acute coronary syndrome at index catheterization; ARB = angiotensin receptor blocker; BMI = body mass index

Sum EETs, sum DHETs, and 14,15-EET:14,15-DHET ratios were log-transformed prior to analysis.

Supplemental Table 1 (cont.). Univariate relationships between clinical factors and biomarkers of CYP epoxygenase, sEH and CYP ω-hydroxylase function in CAD patients

	Parameter Estimate	SE	R^2	p
20-HETE				
Age	0.001	0.007	<0.001	0.851
Race	-0.118	0.148	0.008	0.427
Gender	-0.003	0.115	<0.001	0.978
BMI	-0.016	0.009	0.034	0.097
Multivessel disease	-0.142	0.115	0.019	0.220
ACS	-0.132	0.121	0.015	0.277
Hypertension	-0.127	0.132	0.012	0.336
Diabetes	-0.224	0.124	0.040	0.073
Smoking	-0.125	0.125	0.012	0.323
ACEI/ARB use	-0.320	0.109	0.098	0.004

ACEI = angiotensin converting enzyme inhibitor; ACS = acute coronary syndrome at index catheterization; ARB = angiotensin receptor blocker; BMI = body mass index

20-HETE levels were log-transformed prior to analysis.

Supplemental Table 2. Comparison of plasma CYP-derived eicosanoid levels between healthy volunteers and both non-obese (BMI <30 kg/m^2) and obese (BMI ≥30 kg/m^2) CAD patients.

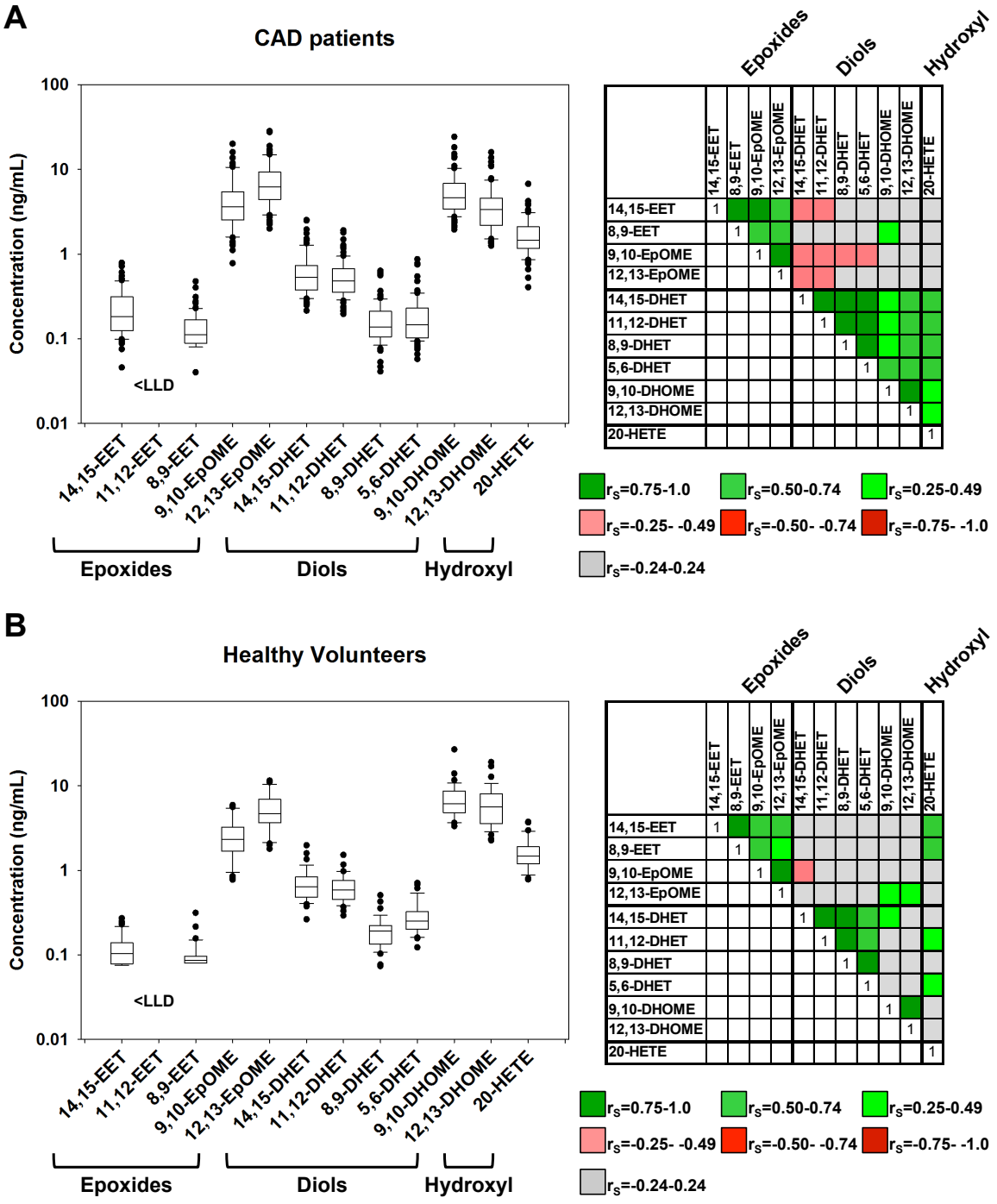
Analyte (ng/mL)	Healthy Volunteer (n=36)	Non-obese CAD (n=36)	Obese CAD (n=46)
CYP Epoxygenase			
Epoxides			
14,15-EET	0.10 (0.06)	0.24 (0.19) * †	0.14 (0.11) * †
8,9-EET	0.09 (0.02)	0.12 (0.10) * †	0.10 (0.04)
Sum EETs	0.19 (0.08)	0.34 (0.32) * †	0.25 (0.17) * †
9,10-EpOME	2.33 (1.52)	4.48 (4.17) * †	3.15 (2.37) * †
12,13-EpOME	4.68 (3.15)	6.83 (6.72) * †	6.17 (4.11)
Sum EpOMEs	7.02 (4.59)	11.3 (10.8) * †	9.12 (5.75)
Diols			
14,15-DHET	0.64 (0.34)	0.52 (0.33)	0.54 (0.36)
11,12-DHET	0.59 (0.30)	0.47 (0.30)	0.49 (0.31)
8,9-DHET	0.19 (0.09)	0.15 (0.12)	0.13 (0.11)
5,6-DHET	0.25 (0.12)	0.15 (0.15) * †	0.15 (0.12) * †
Sum DHETs	1.65 (0.75)	1.26 (0.80)	1.32 (0.81)
9,10-DHOME	6.14 (3.32)	4.93 (3.46)	4.42 (2.63)
12,13-DHOME	5.61 (4.26)	3.46 (2.44) *	3.36 (2.41) * †
Sum DHOMEs	12.2 (7.27)	8.66 (5.50) *	7.42 (5.65) * †
Total			
Sum EETs + DHETs	1.86 (0.74)	1.83 (0.84)	1.56 (0.59)
Soluble Epoxide Hydrolase			
Epoxide:Diol ratios			
14,15-EET:14,15-DHET	0.18 (0.14)	0.52 (0.59) * †	0.23 (0.49) * †
9,10-EpOME:9,10-DHOME	0.42 (0.27)	0.93 (0.95) * †	0.61 (0.92) * †
12,13-EpOME:12,13-DHOME	0.94 (0.67)	2.19 (1.93) * †	1.57 (2.33) * †
CYP ω-Hydroxylase			
Hydroxyl			
20-HETE	1.48 (0.70)	1.60 (0.97)	1.41 (0.66)

Data presented as median (interquartile range). Following log-transformation, plasma levels of each analyte were compared by one-way ANOVA.

*P<0.0125 versus healthy volunteers (unadjusted)

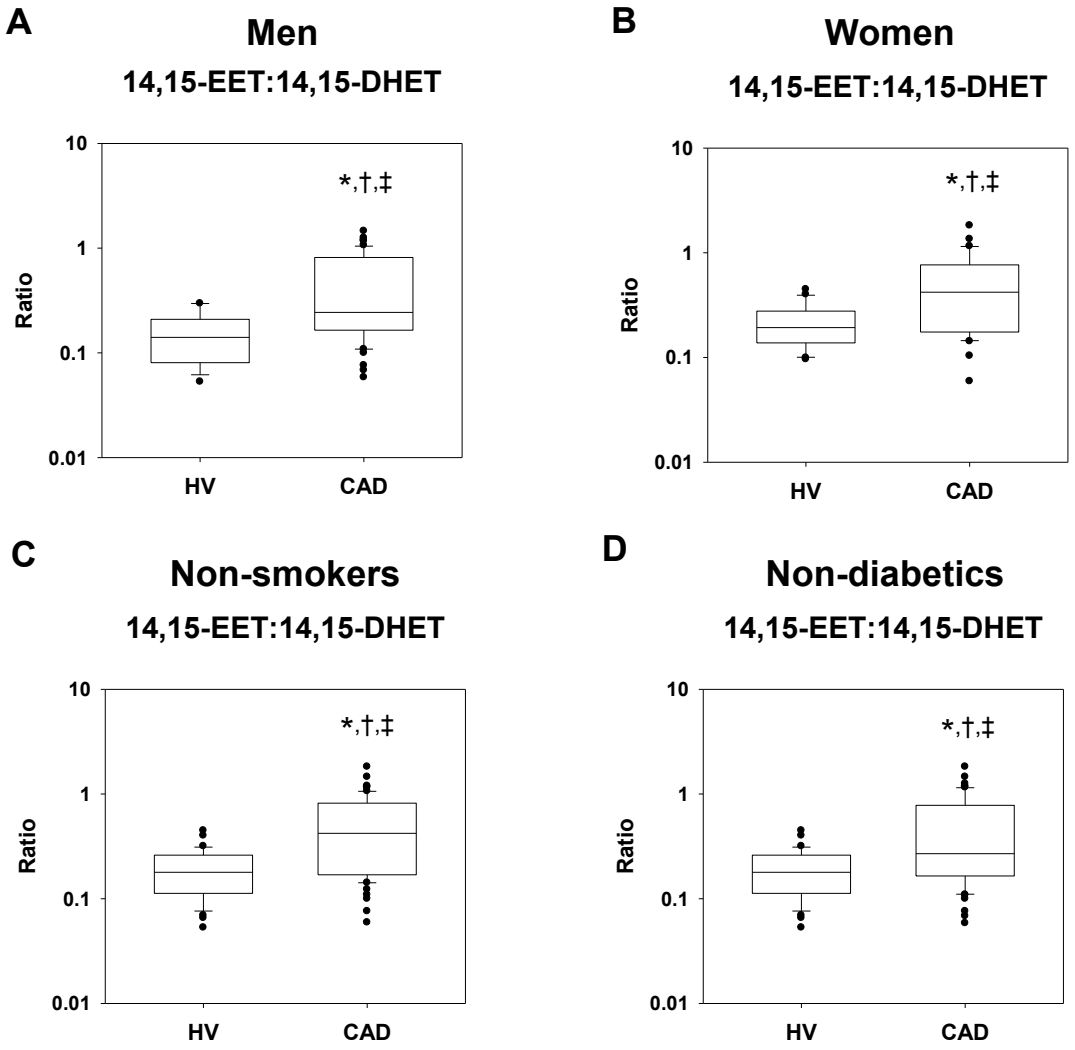
†P<0.0125 versus healthy volunteers (adjusted for age, gender, and race)

Supplemental Figure 1



Supplemental Figure 1. Distribution of plasma concentrations of CYP-derived epoxide, diol, and hydroxyl metabolites of arachidonic and linoleic acid plotted on a $\log_{10}$-scale and heat maps depicting strength of Spearman rank correlations between metabolites in the cohort of (A) CAD patients (n=82) and (B) healthy volunteers (n=36). Gray boxes indicate no correlation. Colored boxes indicate a significant correlation, with the strength of the correlation indicated by the intensity of the color. 11,12-EET levels were below the lower limit of detection (<LLD) in 60 of 82 CAD patients (73.2%) and 28 of 36 healthy volunteers (77.8%).

Supplemental Figure 2



Supplemental Figure 2. Distribution of plasma 14,15-EET:14,15-DHET ratio in (A) men (HV: n=16; CAD: n=51), (B) women (HV: n=20; CAD: n=31), (C) non-smokers (HV: n=36; CAD: n=60), and (D) non-diabetics (HV: n=36; CAD: n=60) plotted on a $\log_{10}$-scale. *Unadjusted P<0.0125 versus HV. †P<0.0125 versus HV after adjusting for age and race (A, B) or age, gender and race (C, D). ‡P<0.0125 versus HV after adjusting for body mass index. Significant differences were also observed for the 9,10-EpOME:9,10-DHOME and 12,13-EpOME:12,13-DHOME ratios (P<0.001 for all comparisons, data not shown).

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