**n-3 FATTY ACIDS REDUCE PLASMA 20-HYDROXYEICOSATETRAENOIC
ACID AND BLOOD PRESSURE IN PATIENTS WITH CHRONIC KIDNEY
DISEASE**

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ABSTRACT

Metabolism of arachidonic acid (AA) by cytochrome P450 ω -hydroxylase leads to the formation of 20-hydroxyeicosatetraenoic acid (20-HETE) that regulates vascular function, sodium homeostasis and blood pressure (BP). Supplementation with n-3 fatty acids is known to alter AA metabolism and reduce formation of the lipid peroxidation products F₂-isoprostanes, but the effect of n-3 fatty acids on 20-HETE has not been studied. We previously reported a significant effect of n-3 fatty acids but not coenzyme Q10 (CoQ) to reduce BP in a double-blind, placebo-controlled intervention, where patients with chronic kidney disease (CKD) were randomized to n-3 fatty acids (4 g), CoQ (200 mg), both supplements or control (4 g olive oil), daily for 8 weeks. This report examines the effect of n-3 fatty acids on plasma and urinary 20-HETE in the same study, as well as plasma and urinary F₂-isoprostanes, and relates these to changes in BP. Seventy four patients completed the 8 week intervention. n-3 Fatty acids but not CoQ significantly reduced plasma 20-HETE ($P=0.001$) and F₂-isoprostanes ($P<0.001$). In regression models adjusted for BP at baseline, post-intervention plasma 20-HETE was a significant predictor of the fall in systolic ($P<0.0001$) and diastolic BP ($P<0.0001$) after n-3 fatty acids. This is the first report that n-3 fatty acid supplementation reduces plasma 20-HETE in humans and that this associates with reduced BP. These results provide a plausible mechanism for the reduction in BP observed in patients with CKD following n-3 fatty acid supplementation.

Key words: 20-hydroxyeicosatetraenoic acid, n-3 fatty acid supplementation, F₂-isoprostanes, chronic kidney disease.

INTRODUCTION

Arachidonic acid (AA) metabolism by the cytochrome P450 enzymes leads to the formation of 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) that play an important role in the regulation of cardiac, renal and pulmonary function and vascular tone [1]. 20-HETE is a vasoconstrictor that is synthesised in smooth muscle cells. Its formation is stimulated by angiotensin II, noradrenalin and endothelin-1 [2-4]. In contrast, EETs are vasodilators that hyperpolarise vascular smooth muscle cells [1]. 20-HETE and EETs are also synthesised in the proximal tubule and thick ascending loop of Henle where they regulate sodium transport. In animal models, hypertension has been linked to deficient or to excessive synthesis of renal 20-HETE [5, 6]. In the Dahl salt sensitive rat, reduced renal 20-HETE associates with hypertension [5], whereas in the spontaneously hypertensive rat, increased renal 20-HETE contributes to raised blood pressure (BP) [6]. Therefore the effect of 20-HETE on BP depends on the balance of its vasoconstrictor action in the afferent arterioles and its natriuretic actions in the thick ascending loop of Henle.

Supplementation with n-3 fatty acids reduces the formation of arachidonic acid metabolites such as prostaglandins, thromboxane B₂ and leukotriene B₄ [7], and formation of F₂-isoprostanes, non-enzymatic free radical oxidation products of arachidonic acid [8, 9]. In addition, n-3 fatty acid supplementation has been shown to reduce BP in a number of randomised controlled trials in humans [10, 11]. However, to date the effect of n-3 fatty acid supplementation on 20-HETE synthesis has not been reported in animal or human studies. Coenzyme Q10 (CoQ) supplementation has also been shown to improve BP [12] and brachial artery endothelial function in type 2

diabetes [13] and to enhance the benefit of fenofibrate on the vascular wall [14]

We previously reported that n-3 fatty acids but not coenzyme Q10 (CoQ) significantly reduced BP in a double-blind, placebo-controlled intervention, where patients with chronic kidney disease (CKD) were randomized to n-3 fatty acids (n-3 FA) (4 g), CoQ (200 mg), both supplements (n-3 FA+CoQ) or control (4 g olive oil), daily for 8 weeks [15]. This report examines the effect of n-3 fatty acid supplementation on 20-HETE synthesis in patients from that study and relates the changes observed to changes in BP and oxidative stress, as well as urinary nitrate and nitrite as markers of nitric oxide synthesis.

METHODS

Patient Recruitment

Men and women aged 25–75 years, with chronic renal impairment, were recruited from the renal units of Royal Perth, Sir Charles Gairdner and Fremantle Hospitals, in Perth, Western Australia. The patients had an estimated (e)GFR > 15 and < 60 ml/ min/1.73m² and serum creatinine < 350mmol/l. They were non-smokers and were excluded if they had diabetes; angina pectoris; major surgery, a cardiovascular event or symptoms of cardiovascular disease within the last 3 months; BP > 170/100mmHg; liver disease; nephrotic syndrome (proteinuria >3 g/day or protein/creatinine ratio >300 mg/mmol); or haemoglobin < 110 g/l. Patients were also excluded if they regularly took non-steroidal anti-inflammatory or immunosuppressive drug therapy, nitrates; the phosphodiesterase inhibitor sildenafil; ate more than one fish meal per week or regularly took fish oil supplements; or if they consumed an average of more than four standard alcoholic drinks per day. Antihypertensive or lipid-lowering medication were not exclusion criteria. The study was approved by the ethics committee

of Royal Perth Hospital. All patients gave informed written consent. The study was registered with the Australian Clinical Trials Register (ACTRN012605000088640).

Study design

The study was double-blind, placebo-controlled and of 8 weeks duration. During a 3-week familiarization period, patients continued their usual dietary habits. After baseline measurements were obtained, they were stratified by age and BMI, and randomized to one of four study groups: n-3 fatty acids (4 g daily, Omacor, Solvay Pharmaceuticals, Pymble, NSW, Australia), CoQ (200 mg daily, Blackmores Australia (Balgowlah, NSW, Australia), the two treatments combined, or control (4 g daily olive oil, Cardinal Health Australia, Braeside, Victoria, Australia). The n-3 fatty acid capsules were 1g and contained 460 mg eicosapentaenoic acid (EPA), 38 mg docosapentaenoic acid and 380 mg docosahexaenoic acid (DHA). Capsules were taken as 2 x 1 g n-3 fatty acid or control, and 2 x 50 mg CoQ or placebo, twice daily with meals. Patients were asked to maintain their usual diets and not to alter their lifestyle, during the intervention. Compliance with the supplements was monitored by measurement of plasma fatty acids and plasma CoQ.

Fasted blood samples and a 24 hr urine were collected at baseline and at the end of the intervention for measurement of plasma and urinary 20-HETE and F₂-isoprostanes.

Measurement of plasma and urinary 20-HETE and F₂-isoprostanes, and urinary nitrite and nitrate

Blood samples were collected into cold tubes containing EDTA with butylated hydroxytoluene (BHT) and reduced glutathione (GSH), centrifuged at 4°C and the plasma stored at -80°C. An aliquot of the 24 h urine was stored at -80°C until assay.

Urinary 20-HETE was measured by gas chromatography–mass spectrometry (GCMS) using electron capture negative chemical ionization. The assay was a modification of our previously described method [16] and extracted a smaller volume of urine (0.5 ml) and used 2ng of d₆-20-HETE (Cayman Chemical Co.) as an internal standard. Plasma free 20-HETE was measured in 125 µl of plasma. After the addition of 1 ng of d₆-20-HETE internal standard the plasma was acidified with 0.1 M sodium acetate buffer, pH 4.6, and applied under vacuum to Bond-Elut Certify II columns (200 mg) (Varian, Australia), that were prewashed with methanol and 0.1 M sodium acetate, pH 7.0, with 5% methanol. The column was then washed with methanol/water (50/50) and dried under vacuum (6 mm Hg for 2 min) before elution of 20-HETE with hexane/ethyl acetate/acetic acid (75/25/1). The sample was dried and derivatized as previously described for urine [16] .

Plasma and urinary F₂-isoprostanes were measured using GCMS as previously described [17].

Urinary nitrite and nitrate were measured from a 24hr urine collection at baseline and the end of the intervention by GCMS as previously described [18].

Ambulatory Blood pressure monitoring

Ambulatory BP and heart rate (HR) were monitored over 24 hours at baseline and at the end of intervention using the SpaceLabs Monitor (Model 90217, SpaceLabs Medical Inc, Issaquah, Washington, USA) fitted by a trained nurse who instructed the patient in its use. The recorder was pre-set to record BP and HR every 20 min during waking hours and every 30 min during sleep.

Statistical Analysis

The study had more than 80% power to detect main effect changes of 30% in

plasma 20-HETE and 20% in plasma F₂-isoprostane. Analyses included only participants who completed the trial. Post-intervention data were analysed using SPSS15.0 (SPSS Inc., Chicago, Illinois, USA) or SAS 9.0 (SAS Inc, USA) with general linear models adjusting for baseline values and assessing main and interactive effects of n-3 fatty acids and CoQ. Baseline measures were compared by one-way analysis of variance. Significance levels were adjusted for multiple comparisons by the Tukey test. Values are means (SEM) or geometric mean [95% confidence interval (CI)].

Regression models adjusted for baseline values of the dependent variable were used to examine predictors of post-intervention plasma 20-HETE as described by Pocock et al. [19] Variables examined as predictors included, age, gender, and post-intervention levels of plasma F₂-isoprostanes, platelet AA and EPA, BMI, eGFR, and weight. The relationship between post-intervention BP and plasma 20-HETE was examined using regression models that adjusted for baseline BP, BMI, eGFR, weight and gender.

RESULTS

Eighty-five patients were randomized to the four groups. Mean values for baseline characteristics were: age 56.5±1.4 years; BMI 27.3±0.5 kg/m²; supine BP was 125.0±1.7/72.3±0.9; eGFR 35.8±1.2 ml/min/1.73m². Seventy-four patients completed the study. Bodyweight and eGFR were similar at baseline in the 4 groups, and did not change significantly during the intervention [15]. Antihypertensive medication was taken by 72 of the 74 patients and did not change throughout the study. At baseline, 74% of patients allocated to n-3 fatty acids were taking angiotensin converting enzyme inhibitors compared with 75% of those allocated to the control oil.

Post-intervention levels of platelet total n-3 fatty acids and plasma CoQ confirmed compliance with each of the different treatment regimes. Baseline adjusted, post-intervention platelet total long-chain n-3 fatty acids were significantly elevated in the groups taking n-3 fatty acids ($p<0.001$): $8.3\pm0.5\%$ (n-3 FA) and $7.6\pm0.5\%$ (n-3 FA+CoQ) compared with control and CoQ ($4.8\pm0.5\%$ and $4.9\pm0.5\%$, respectively). Baseline adjusted, post-intervention plasma CoQ was significantly elevated in the groups taking CoQ ($p<0.001$): 4355 ± 211 nmol/L (CoQ) and 3393 ± 224 nmol/L (n-3 FA+CoQ) compared with 1474 ± 251 nmol/L (control) and 1015 ± 213 nmol/L (n-3 FA).

The Effect of n-3 fatty acids on plasma and urinary 20-HETE and F₂-isoprostanes, and urinary nitrate and nitrite

There were no significant group differences at baseline. There were no group or interactive effects of CoQ on plasma or urinary 20-HETE, F₂-isoprostanes (Table 1). Therefore, the analysis was confined to the effects of n-3 fatty acid supplementation on plasma and urinary 20-HETE and F₂-isoprostanes.

n-3 Fatty acid supplementation for 8 weeks reduced plasma 20-HETE by 341 (95% CI -543, -138) pmol/l and plasma F₂-isoprostanes by 361 (95% CI -458, -265) pmol/L, equivalent to 29% and 22% of baseline values, respectively (Table 1, Figure 1). Urinary 20-HETE and urinary F₂-isoprostanes were not significantly altered by n-3 fatty acid supplementation (Table 1).

Urinary nitrite and nitrate excretion was not significantly different between the groups at baseline (Table 2). There were no significant group or interactive effects of CoQ on urinary nitrite or nitrate. In a main effects analysis, n-3 fatty acid supplementation for 8 weeks did not significantly alter urinary nitrite or nitrate excretion. Urinary nitrite and nitrate were not significantly correlated with baseline or

post-intervention SBP or DBP.

Factors predicting plasma 20-HETE after n-3 fatty acids

Using regression analysis, we examined the factors that best predicted post-intervention plasma 20-HETE. Post-intervention plasma 20-HETE was inversely related to post-intervention platelet EPA ($\beta=-0.261$, $P=0.013$) and positively related to post-intervention platelet AA ($\beta=0.273$, $P=0.008$) after adjusting for baseline plasma 20-HETE (Table 3). This model accounted for 28.4% of the variation in plasma 20-HETE after the intervention. As post-intervention plasma F₂-isoprostanes were correlated with platelet EPA ($r=-0.297$, $P=0.011$) and AA ($r=-0.302$, $P=0.009$), the relationship between plasma F₂-isoprostanes and post-intervention plasma 20-HETE was examined in a separate regression model. Post-intervention plasma F₂-isoprostanes were positively associated with post-intervention plasma 20-HETE ($\beta=0.313$, $P=0.003$) after adjusting for baseline plasma 20-HETE (Table 3) and accounted for 26.8% of the variance in plasma 20-HETE. Age, gender, BMI and eGFR were not significant predictors of plasma 20-HETE in either model.

The effect of n-3 fatty acids on the relationship between plasma 20-HETE and BP

Supplementation with n-3 fatty acids significantly reduced BP in this study [15]. In main effects analysis, n-3 fatty acids reduced 24 h SBP (-3.3 ± 0.7 mmHg, $P<0.0001$) and 24 h DBP (-2.9 ± 0.5 mmHg, $P<0.0001$). The fall in BP was apparent during awake hours: SBP (-2.6 ± 0.9 mmHg, $P=0.003$) and DBP (-2.4 ± 0.6 mmHg, $P<0.0001$); and while asleep: SBP (-4.3 ± 1.2 mmHg, $P=0.0003$) and DBP (-4.3 ± 0.8 mmHg, $P<0.0001$).

The relationship between plasma 20-HETE and BP measured over 24 hr, and while awake or asleep, was examined at baseline and after n-3 fatty acid supplementation using regression analysis. At baseline, after adjusting for age and body

weight plasma 20-HETE was significantly negatively associated with awake SBP ($\beta=-0.256$, $P=0.018$) but not 24h or night time SBP or DBP.

In the groups combined the correlation between the post intervention change in 20-HETE and the change in SBP was $r=0.073$, $P=0.54$ for 24h SBP, $r=0.133$, $P=0.26$ for daytime SBP and $r=0.07$, $P=0.57$ for night-time SBP. The relationship between plasma 20-HETE and post-intervention SBP and DBP used regression models that adjusted for BP measured at baseline [19]. The model that best explained post-intervention 24h SBP and accounted for 61.1% of the variance, showed a positive association between 24h SBP and post-intervention plasma 20-HETE ($\beta=0.179$, $P=0.018$) (Table 4). There was also a significant positive relationship with DBP and post-intervention plasma 20-HETE ($\beta=0.150$, $P=0.028$) after adjusting for baseline 24h DBP. This model accounted for 68.2% of the variation in post-intervention 24h DBP. The significant relationship between post-intervention plasma 20-HETE and post-intervention 24h SBP and 24h DBP was strongly influenced by daytime SBP and DBP, respectively (Table 4); there was no significant association between plasma 20-HETE and night time SBP ($P=0.895$) or DBP ($P=0.747$). The addition of BMI, eGFR, weight and gender did not significantly improve the regression models. The inclusion of a variable that indicated use of ACE inhibitors did not significantly alter the relationship between post-intervention plasma 20-HETE and SBP or DBP. We found no significant relationship between post-intervention plasma F₂-isoprostanes and post-intervention 24h SBP ($\beta= 0.013$, $P= 0.871$) or DBP ($\beta= -0.013$, $P=0.861$).

DISCUSSION

We have shown for the first time that supplementation with n-3 fatty acids for 8 weeks

significantly reduces plasma 20-HETE in patients with CKD. A strength of our study is the randomised placebo controlled intervention that had adequate power to detect relevant main effect differences in 20-HETE and F₂-isoprostanes. The post-intervention concentration of plasma 20-HETE was a significant predictor of post intervention 24hr and daytime SBP and DBP. n-3 Fatty acid supplementation did not alter urinary 20-HETE, suggesting that the effects of n-3 fatty acids to reduce plasma 20-HETE may be unrelated to renal synthesis in patients with CKD. In spite of a previous report that CoQ supplementation improved BP [12] and brachial artery endothelial function [13], we did not find any effect of CoQ on 20-HETE, BP or endothelial function [15]

20-HETE is a potent vasoconstrictor that acts by blocking the large conductance calcium activated potassium channel (K_{Ca}) in vascular smooth muscle cells leading to a fall in membrane potential and enhancing influx of Ca²⁺ via voltage sensitive Ca²⁺ channels. 20-HETE can affect endothelial function by uncoupling endothelial nitric oxide synthase leading to increased oxidative stress and reducing nitric oxide bioavailability¹⁴. However, we have no evidence of altered NO synthesis in our study as there were no changes in urinary nitrite or nitrate excretion after n-3 fatty acid supplementation. 20-HETE can induce angiotensin converting enzyme and activates the renin angiotensin system thereby further contributing to endothelial dysfunction [20, 21]. A limitation of our study is that we did not assess the effects of n-3 fatty acids on the renin angiotensin system. However, the high proportion of patients in the two groups taking ACE inhibitors (75%) during the study suggests that the renin-angiotensin system was not a major determinant of the relationship between post-intervention 20-HETE and blood pressure. This is supported by the regression analysis which was unaffected when ACE inhibitor status was included in the model. In humans, elevated

urinary 20-HETE has been associated with impaired endothelial function [22]. However, we previously reported that in this study there were no changes in endothelial function assessed by flow mediated dilatation after n-3 fatty acids.[15] In hypertensive patients [23] and patients with the metabolic syndrome [17], 20-HETE positively associates with F₂-isoprostanes, that are reliable measures of oxidative stress. Plasma 20-HETE was not significantly associated with SBP or DBP at baseline. However, the strong positive relationship between post-intervention plasma 20-HETE and systolic and diastolic BP after adjusting for baseline values suggest that n-3 fatty acid supplementation alters the relationship between 20-HETE and BP in a manner that may be causally linked. Interestingly, plasma F₂-isoprostanes were significantly reduced by n-3 fatty acids and post-intervention plasma F₂-isoprostane was a significant predictor of post-intervention plasma 20-HETE. However, plasma F₂-isoprostanes were not significantly related to post-intervention BP. This suggests that the relationship between the fall in plasma 20-HETE and BP may be independent of any effects on oxidative stress.

The mechanisms by which n-3 fatty acids reduce plasma 20-HETE without altering urinary 20-HETE most likely relate their effects on cytochrome P450 enzymes metabolising arachidonic acid to 20-HETE, and their effects on enzymes involved in 20-HETE clearance from the circulation. In humans, cytochrome P450 enzymes metabolising arachidonic acid to 20-HETE belong mainly to the CYP4F and CYP4A families of ω -hydroxylases. These enzymes are mainly found in the liver (CYP4A11, 4F2 and 4F3) [24] and kidney (CYP4A11 and 4F2) [25, 26], but they are also present in human platelets (CYP4A11 and 4F2) [27], neutrophils (CYP4F3) [28, 29], smooth muscle cells (CYP4A11) [30] and microvascular endothelial cells (CYP4F2 and 4F3)

[21]. A study using human recombinant CYP450 enzymes and human liver microsomes showed that both EPA and DHA strongly inhibited ω -hydroxylation of arachidonic acid to 20-HETE by CYP4F2 and CYP4F3B [31], the two main CYP4 enzymes involved in ω -hydroxylation in the liver. We previously reported significant increases in both EPA and DHA in platelets in this study after n-3 fatty acid supplementation [15], that are likely to reflect changes in fatty acid composition in the liver and other tissues. In this study n-3 fatty acid supplementation may have led to inhibition of the CYP4F2 and CYP4F3 enzymes and ω -hydroxylation of arachidonic acid to 20-HETE, impacting mainly on hepatic synthesis of 20-HETE and resulting in lower circulating levels of plasma 20-HETE, although inhibition of 20-HETE synthesis in other cells may also have contributed to reduced circulating 20-HETE levels.

We found no effect on urinary 20-HETE levels after supplementation with n-3 fatty acids. In humans, the majority of 20-HETE is excreted as the glucuronide conjugate [32]. Glucuronidation is a metabolic process involved in phase II drug metabolism and is important for clearance of 20-HETE from the circulation in humans. It occurs mainly in the liver but can take place in all major organs including the kidney. The assay used to measure urinary 20-HETE in this study treats urine with glucuronidase to enable measurement of total 20-HETE excretion [33]. In contrast measurement of 20-HETE in plasma represents free 20-HETE. It is possible that renal impairment affects renal synthesis and/or clearance of 20-HETE from the circulation masking any effect of n-3 fatty acids on urinary 20-HETE in patients with CKD.

Only one other study has examined CYP450 eicosanoids in relation to renal disease in humans [34]. In that study, plasma and urinary 20-HETE were measured in patients with renovascular disease (diagnosed by angiographic evidence of severe renal

artery stenosis), essential hypertension and healthy controls. The study showed that plasma 20-HETE was elevated and urinary 20-HETE was reduced in renovascular disease compared with controls. This suggests that plasma and urinary 20-HETE are also differentially affected in human renovascular disease.

In this study n-3 fatty acids reduced plasma but not urinary F₂-isoprostanes. We have previously shown that n-3 fatty acids reduce both plasma and urinary F₂-isoprostanes [35-38] in patients with normal renal function. The absence of changes in urinary F₂-isoprostanes after n-3 fatty acids may be related to the renal impairment in CKD.

We have previously reported that n-3 fatty acid supplementation in these CKD patients did not affect renal function [15]. We found no significant relationship between plasma or urinary 20-HETE or F₂-isoprostanes and renal function (eGFR) at baseline or after the intervention. Limitations in our trial include the small sample size studied and the relatively short duration of n-3 fatty acid supplementation that may have impacted on our ability to determine any effect of n-3 fatty acids and reduced plasma 20-HETE on renal function.

In conclusion, we have shown for the first time that n-3 fatty acid supplementation in patients with CKD significantly reduces plasma 20-HETE and that this reduction is significantly associated with reduced BP. These results suggest that a reduction in 20-HETE may be a plausible mechanism for the reduction in BP observed in patients with CKD following n-3 fatty acid supplementation.

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TABLE 1. Baseline and post-intervention plasma and urinary 20-HETE and F₂-Isoprostanes by group showing the main effects of n-3 FA and CoQ

	Control	n-3 FA	CoQ	n-3FA + CoQ	Main Effect	
	(n=15)	(n=20)	(n=21)	(n=18)	n-3FA	CoQ
Plasma 20-HETE (pmol/L)						
Baseline	1343	1096	1185	1134		NS
	(1118,1567)	(959,1232)	(940, 1430)	(1017,1252)		
Post-Intervention	1274	791	1238	898	-341 (-543, -138)	64 (-134, 263)
	(1006, 1543)	(595, 985)	(982, 1495)	(729, 1066)	P=0.001	P=0.522
Urinary 20-HETE (pmol/mmol creatinine)						
Baseline	151	146	142	185		NS
	(104, 197)	(100, 192)	(109, 175)	(122, 249)		
Post-Intervention	124	130	136	150	-3 (-39, 33)	-10 (-46, 25)
	(81, 167)	(91, 168)	(88, 184)	(122, 249)	P=0.871	P=0.57
Plasma F₂-Isoprostane (pmol/L)						
Baseline	1602	1714	1448	1479		NS
	(1423,1780)	(1539,1888)	(1346,1550)	(1325,1632)		
Post-Intervention	1658	1215	1533	1253	-361 (-458, -265)	-44 (-145, 57)
	(1553,1764)	(1119,1312)	(1443,1624)	(1156,1350)	P<0.001	P=0.389
Urine F₂-Isoprostane (pmol/mmol creatinine)						
Baseline	125	114	133	104		NS
	(89,161)	(81,148)	(93,173)	(83,126)		
Post-Intervention	133	100	121	143	-5.0 (-44, 34)	15 (-23, 54)
	(91,176)	(64,137)	(85,156)	(105,182)	P=0.798	P=0.424

Values expressed as mean (95% confidence interval). n-3 FA, n-3 fatty acid; ANOVA, analysis of variance; CoQ, coenzyme Q10 . Baseline measures were compared by one-way ANOVA and were not significantly different between groups. . General linear model analysis tested for main effects and interactions on post-intervention values adjusted for baseline value. There were no significant interactions between the treatment groups that affected 20-HETE or F₂-isoprostane responses

TABLE 2. Baseline and post-intervention urinary nitrite and nitrate by group showing the main effects of n-3 FA and CoQ

	Control	n-3 FA	CoQ	n-3FA + CoQ	Main Effect	
	(n=15)	(n=20)	(n=21)	(n=18)	n-3FA	CoQ
Urinary Nitrite (μmol/mmol creatinine)						
Baseline	1.77	2.97	1.33	0.93	NS	
	(0.61,2.94)	(-0.66,6.60)	(0.80,1.87)	(0.35,1.51)		
Post-Intervention	1.24	2.30	1.16	1.39	-0.63 (-2.1, 0.84)	-0.45 (-1.9,1.04)
	(0.38,2.09)	(-0.18,4.77)	(0.56,1.76)	(0.28,2.50)	P=0.40	P=0.55
Urinary Nitrate (μmol/mmol creatinine)						
Baseline	31.5	20.6	23.8	23.9	NS	
	(21.4,41.6)	(15.4,25.8)	(17.5,30.2)	(16.4,31.6)		
Post-Intervention	33.8	21.2	32.9	21.0	-7.1(-19, 0.092)	1.9 (-10, 9.1)
	(20.0,47.6)	(15.2,27.2)	(19.3,46.6)	(15.6,26.5)	P=0.107	P=0.66

TABLE 3. Regression models examining predictors of post-intervention plasma 20-HETE adjusted for baseline plasma 20-HETE.

Dependent Variable	Predictor Variables	β	t	P Value
Model 1				
Post-intervention plasma 20-HETE				
	Post-intervention platelet AA	0.273	2.737	0.008
	Post-intervention platelet EPA	-0.261	-2.557	0.013

Adjusted $r^2 = 0.284$, ANOVA $F_{3,70} = 10.662$, $P < 0.0001$.

Model 2				
Post-intervention plasma 20-HETE				
	Post-intervention plasma F ₂ -Isoprostanes	0.313	3.102	0.003

Adjusted $r^2 = 0.268$, ANOVA $F_{2,70} = 14.165$, $P < 0.0001$.

Age, gender, BMI and GFR were not significant predictors of post-intervention plasma 20-HETE in either Model.

TABLE 4. Regression models examining the relationship between post-intervention plasma 20-HETE and 24 hour, and daytime systolic and diastolic blood pressure adjusted for respective baseline values of blood pressure

Dependent Variable	Predictor Variables	β	t	P Value
Post-intervention				
24h SBP				
	Post-intervention plasma 20-HETE	0.179	2.430	0.018
Adjusted $r^2 = 0.611$, ANOVA $F_{2,70}=57.66$, $P<0.0001$.				
Post-intervention				
24h DBP				
	Post-intervention plasma 20-HETE	0.150	2.249	0.028
Adjusted $r^2 = 0.682$, ANOVA $F_{2,70}=78.34$, $P<0.0001$.				
Post-intervention				
Awake SBP				
	Post-intervention plasma 20-HETE	0.278	3.223	0.002
Adjusted $r^2 = 0.659$, ANOVA $F_{2,70}=70.66$, $P<0.0001$.				
Post-intervention				
Awake DBP				
	Post-intervention plasma 20-HETE	0.210	3.056	0.003
Adjusted $r^2 = 0.659$, ANOVA $F_{2,70}=70.66$, $P<0.0001$.				

Figure 1. Baseline adjusted, post-intervention plasma 20-HETE (top panel) and plasma F₂-isoprostanes (lower panel) in patients taking n-3 fatty acids (n-3 FA) (grey bars) compared with patients not taking n-3 fatty acids (NO n-3 FA) (white bars). Values are means and 95% CI. General linear model analysis tested for the main effects of n-3 fatty acids. Significance levels refer to post intervention means adjusted for baseline values, † $P \leq 0.001$ for the effect of n-3 fatty acid supplementation.

Figure 1