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F_2-isoprostanes as an indicator and risk factor for coronary heart disease

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Abstract

Coronary heart disease (CHD) is the leading single cause of death in the United States and most Western countries, killing more than 400,000 Americans per year. Although CHD often manifests suddenly as a fatal myocardial infarction, the atherosclerosis that gives rise to the infarction develops gradually and can be markedly slowed or even reversed through pharmacological and lifestyle interventions. These same atherosclerotic processes also drive related vascular diseases such as stroke and peripheral artery disease, and individuals surviving occlusive events often develop additional complications including ischemic cardiomyopathy and heart failure. Therefore, better detection of subclinical atherosclerosis, along with more effective treatments, could significantly reduce the rate of death from CHD and related vascular diseases in the United States. In recent years, oxidation of polyunsaturated fatty acids (PUFA) in plasma lipoproteins has been postulate to be a critical step in the development atherosclerosis. If so, then monitoring lipid peroxidation should be a useful indicator of disease risk and progression. This review will focus on the evidence that specific PUFA peroxidation products, the F_2-isoprostanes, are useful biomarkers that could potentially be utilized as indicators of CHD.

Keywords

isoprostanes; lipid peroxidation; coronary heart disease; cardiovascular disease; oxidative stress; biomarkers; antioxidants; polyunsaturated fatty acids; atherosclerosis

Introduction

For many years, screening and treatment of atherosclerosis focused on cholesterol levels in lipoproteins rather than reducing the peroxidation of the polyunsaturated fatty acid (PUFA) in these lipoproteins. The focus on cholesterol reduction was based on the two seminal discoveries by Brown and Goldstein: the first, in 1974, was that persons with familial hypercholesterolemia lacked the cell surface receptor for low density lipoprotein (LDL) [1] and therefore failed to regulate cholesterol synthesis and the second, in 1979, was that macrophages possessed scavenger receptors that bound and internalized acetylated LDL, producing massive cholesterol deposition similar to those found in the foam cells of

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atherosclerotic fatty streak lesions [2]. The notion that oxidation of PUFA in lipoproteins might be important to atherosclerosis arose in 1987, when Parthasarathy et al showed that LDL exposed to oxidants (oxidized LDL) was also taken up by macrophage scavenger receptors [3,4]. Recognition of oxidized LDL by scavenger receptors was postulated to result from modification of the apoB-100 protein in LDL in a similar manner as with acetylation, except that modification was due to lipid aldehydes such as malondialdehyde that were generated during PUFA oxidation. Oxidized LDL was suggested to form in vivo by penetration of LDL into the subintimal space of the vascular wall where it was oxidized by redox metals. Evidence that LDL oxidized in vitro could induce many proatherogenic effects in cultured cells led to the incorporation of oxidized LDL into some models of atherogenesis [5]. Some of these proatherogenic effects of oxidized LDL could also be induced by organic phase extracts of the oxidized LDL, suggesting that oxidized lipid themselves were proatherogenic, in addition to oxidatively modified ApoB. Therefore, even lipid peroxidation products in the vasculature that did not arise directly from LDL could contribute to atherogenesis. The current oxidative injury model of atherosclerosis posits that various risk factors for atherosclerosis promote the oxidation of LDL and other lipoproteins which creates proinflammatory lipid mediators that drive a chronic inflammatory state. In time, this chronic inflammatory state leads to complex plaque formation, rupture, and vessel occlusion. This model predicts: that risk factors for CHD should increase lipid peroxidation, that high concentrations of lipid peroxidation products are risk indicators for onset and severity of disease, and that interventions that lower lipid peroxidation should also modulate disease.

Measuring lipid peroxidation in vivo

In order to assess the extent to which clinical studies in humans support the hypothesis that lipid peroxidation mediates atherogenesis and that oxidized PUFA products can be used as indices of CHD, we must first identify appropriate in vivo biomarkers of lipid peroxidation. Peroxidation of the various PUFA esterified in the phospholipids, triglycerides, and cholesterols of lipoproteins generate literally hundreds of compounds including hydroxy-, hydroperoxy-, and epoxy- fatty acids, hydroxyalkenals, various dicarbonyl products, oxysterols, and fragmented phospholipids. Many of these oxidation products have biological activities that could contribute to atherogenesis. Ideally, clinic studies should measure the lipoxidation products deemed most likely to mediate inflammation and atherogenesis. However, no current consensus exists on which lipoxidation products are most important in terms of mediating disease. Therefore, most clinical trials simply measure one or two established indicators of lipid peroxidation in vivo, with the assumption that their levels reflect other lipid peroxidation products as well. Over the past few years, the measurement of F_2-isoprostanes (IsoPs) have emerged as one of the most sensitive and reliable biomarkers of lipid peroxidation in vivo [6,7]. For this reason, the measurement of IsoPs has been incorporated into a wide number of clinical trials. The results of these trials provide significant insights into the role of lipid peroxidation in disease.

To appropriately interpret the results of these clinical studies, several key features of the biochemistry of IsoPs and their measurements should be kept in mind. The vast majority of arachidonic acid is esterified in tissue phospholipids. Accordingly, IsoPs are predominantly formed initially esterified in phospholipids and are then subsequently hydrolyzed to their free acid form by platelet-activating factor acetylhydrolase [8]and possibly other phospholipases. The free IsoPs are released from tissue into the circulation, where they undergo partial metabolism, predominantly in the liver. Therefore, both unmetabolized IsoPs and IsoP metabolites are excreted into the urine [9-11]. Total body IsoP production can be assessed by quantifying unmetabolized free IsoPs in plasma or unmetabolized free IsoPs and IsoP metabolites in urine. Individual organ IsoP production is assessed by quantifying

esterified IsoPs. Caution must be applied when interpreting changes in unmetabolized IsoPs from urine as evidence for overall systemic increases in lipid peroxidation. This is because the IsoPs formed in the kidney are directly excreted into the urine without metabolism, so that if renal disease associated with oxidative damage is present, then the total levels of unmetabolized IsoPs in the urine could increase disproportionately to the total body change in lipid peroxidation. In this case, urinary levels of IsoP metabolites should better reflect actual changes in total body lipid peroxidation.

Valid clinical studies utilizing IsoPs require appropriate collection and storage procedures. Hemolysis of blood samples can release free iron and hemoglobin, both of which can catalyze artifactual oxidation of lipids during storage. To minimize this, a large bore needle should be used and blood manually collected using very gentle suction to ensure that no frothing and hemolysis occurs. Collected blood should be immediately transferred to tubes containing anticoagulants such as EDTA or citrate (but not heparin) and kept chilled until centrifugation which should be performed as soon as possible. Plasma should be aliquoted and stored at −80°C. Tissue samples should be flash frozen immediately after collection and also stored at −80°C. Long-term storage of plasma and tissue samples at −20°C or repeated freezing and thawing of samples will artifactually generate IsoPs via auto-oxidation of arachidonate. Because urine contains relatively little arachidonate, urine samples can be stored at −20°C. In general, even though IsoPs are chemically quite stable, long term storage of biological samples should still be avoided if possible.

The most reliable method for measurement of IsoPs is stable isotope dilution mass spectrometry. Several features of this assay make it more reliable than immunoassays. Directly adding the stable isotope internal standard to sample at the beginning of the assay greatly increases the reliability of these assays by preventing analytical error due to sample to sample variation in the efficiency o f extraction, derivitization, or ionization. Appropriate solid phase extraction of the IsoP at the beginning of the work-up removes potentially interfering substances from the sample, particularly arachidonate that could lead to artifactual formation of IsoP by autooxidation during the work-up procedure. Finally, coupling appropriate chromatography to selectively monitoring only the specific mass of the IsoP (and its internal standard) ensures that closely related arachidonate metabolites including prostaglandins do not interfere with quantitation. As plasma levels of free IsoPs in healthy humans are quite low (in the range of 30-40 pg/ml for healthy humans), mass spectrometry coupled to gas chromatography (GC/MS) rather than liquid chromatography (LC/MS) is generally employed because of its greater sensitivity. The urinary levels of unmetabolized IsoPs and metabolites of IsoPs are much higher (~ngs/ml), so that both GC/MS and LC/MS have been widely utilized for these measurements.

Because of the equipment costs associated with mass spectrometry assays, immunoassays have also been developed for measuring IsoPs. However, great care must be used in interpreting the results of IsoP immunoassays without adequate validation. Because IsoPs have limited antigenic structures, their immunoassays are carried out by competitive displacement of a labeled conjugate of IsoP from surface bound antibody, rather than by the more selective method of sandwich ELISA. Thus, compounds in the sample that non-specifically interfere with antigen-antibody binding will lead to aberrantly high values. Of particular concern are the fatty acids that are present at more than 10,000-fold greater concentration than IsoPs, and that can be released from albumin by standard partial purification strategies employed during immunoassay such as protein precipitation [12]. Other interfering contaminants may be released from solid phase cartridges or from plastics used in the assay [12]. Studies comparing the values obtained by immunoassay and GC/MS measurements have shown very different results depending on the antibodies used, the extent of sample purification, and the fluid measured, with some studies finding excellent

correlation [10,13,14] and other finding either modest [15] or very poor correlation [16-18]. Because of the poorer precision of immunoassays, larger numbers of subjects may be required to detect significant differences between populations when using immunoassays than when using mass spectrometric assays and validation by mass spectrometry of the results obtained by immunoassay, at least in a subset of samples, improves confidence in the overall conclusions.

As will be discussed in detail below, clinical studies that have measured IsoP levels by a variety of methods provide a significant body of evidence that many risk factors for CHD increase overall lipid peroxidation, that higher IsoP levels correlate with greater extent of CHD, that IsoP levels predict disease outcomes, and that IsoP levels can be used to assess the effectiveness of various therapies aimed at reducing the level of lipid peroxidation.

Risk factors for CHD increase lipid peroxidation

Well-established risk factors for CHD include older age, male gender, high LDL cholesterol levels, low HDL cholesterol levels, obesity, diabetes, smoking, and hypertension. Other risk factors include elevated levels of high sensitivity C-reactive protein (hs-CRP) and homocysteine. Although each of these risk factors may contribute to cardiovascular disease by mechanisms independent of their effects on lipid peroxidation, there is significant evidence that many of these risk factors also increase lipid peroxidation (Table 1). For instance, smoking is associated with 2 to 3-fold higher IsoP levels in numerous studies [19-23]. Many studies have also shown that adult subjects with high levels of the proatherogenic LDL cholesterol have about two-fold higher levels of IsoPs compared to aged matched controls [24-28]. Similar two-fold elevations in IsoP levels were seen in persons with low levels of the protective lipoprotein HDL [29]. Both type 1 and type 2 diabetes are associated with a 2 to 3-fold increases in IsoP levels [30-34], and obesity correlates with increased IsoP levels even when adjusting for blood glucose levels [35-38].

Other risk factors have more subtle effects on IsoP levels and may therefore make lesser contributions to the overall extent of lipid peroxidation in disease. Several studies looking at IsoP levels in normotensive versus hypertensive persons have found somewhat higher IsoP levels with hypertension [39-42]. IsoP levels also positively correlate with CRP levels [43,44] and with homocysteine levels [45]. Although IsoP levels are generally not increased in healthy older individuals under resting conditions [35,46], even apparently healthy older adults have significantly increased IsoP levels compared to young adults when subjected to short bouts of ischemia/reperfusion [47]. IsoP levels are generally similar or slightly higher in healthy women than men [35,48], so that the gender differences in risk for CHD are most likely to be due to factors other than lipid peroxidation.

Overall, the finding that many risk factors for the CHD associate with increased IsoP levels in various clinical studies supports the notion that lipid peroxidation is an important contributor to the process of atherogenesis. The additive effect of various risk factors for CHD can also be explained by this mechanism, because of their additive effect on the extent of lipid peroxidation.

Lipid peroxidation as an independent risk factor for CHD

If lipid peroxidation contributes to atherogenesis, then identifying patients with high lipid peroxidation levels should tell us who is at risk for CHD and who would therefore benefit from therapeutic intervention (Table 2). To determine if IsoP values were an independent predictor of CHD, Schwedhelm et al performed a case-control studies with 93 subjects with verified CHD and 93 age- and sex-matched healthy controls [49]. They measured IsoPs along with more traditional markers such as hypercholesterolemia, low HDL, body mass

index, diabetes, systolic blood pressure, hs-CRP, and smoking status. Patients with a greater number of risk factors had higher IsoP values. Each biomarker was associated with a higher odd ratio for CHD in univariate analysis, but only two biomarkers (IsoPs and hs-CRP) were associated with higher odd ratios for CHD when multivariate analysis was applied in a stepwise regression model. This finding established IsoP values as a potential independent risk factor for CHD.

A subsequent study further established IsoPs values as an independent risk factor for CHD [50]. In this study, nine different lipid peroxidation products (IsoPs and eight different hydroxy fatty acids) were each measured by mass spectrometry in the plasma of consecutive patients who underwent diagnostic coronary angiography. Two of the nine lipoxidation products (IsoPs and 9-HETE) were significantly higher in those diagnosed with CHD compared to those without. The other lipoxidation products did not reach statistical significance. When all patients were stratified by IsoP quartile, the odds ratio for patients in the highest IsoP quartile to have angiographic evidence of CHD was 9.7 compared to subjects in the lowest IsoP quartile. Adding IsoP (or 9-HETE) values to the standard Framingham global risk score significantly improved the ability to predict angiographic CHD compared to using the Framingham risk score alone, demonstrating the potential clinical utility of these measurements.

The potential utility of IsoP values as an independent risk indicator for CHD found in these small pilot studies have been subsequently confirmed in larger populations. For instance, Gross et al compared IsoP values and the extent of coronary artery calcification (CAC) in a biracial cohort of 2850 young healthy men and women [48]. Approximately 23% of the men in the highest IsoP quartile manifested calcification compared to only about 12% of the men in the lowest IsoP quartile. Although prevalence of calcification was much lower in women overall, they still found that a greater percentage of women in the highest IsoP quartile manifested calcification than those in the lowest IsoP quartile. Another case control study with 799 patients with angiographically confirmed CHD and 925 healthy controls found similar two-fold increases in odds ratios for the highest IsoP quartile compared to the lowest quartile [51].

Although almost all studies to date indicate a correlation between IsoP values and CHD, we are aware of two studies that have failed to find such a relationship. Ruef et al reported (but did not show data) that IsoP levels were not significantly higher in 162 patients with stable angina or acute coronary syndromes compared to 46 control patient [52] . These measurements were performed by immunoassay, which may have meant the study was simply underpowered. No differences were also found in a nested case-control study that included 647 patients even though a GC/MS assay method was used measure IsoP levels [53]; however, IsoP values in the matched control patients ranged from 0.3 nM to 65 nM. From our extensive experience measuring IsoP levels in humans, this 200-fold variation is highly unusual for normal healthy patients and therefore suggests that a significant portion of the matched control subjects may in fact have had subclinical disease at the time of the study. Thus, in our opinion, the results of these two studies do not significantly alter the conclusion that high IsoP values are an independent risk factor for CHD.

Isoprostanes as an indicator of disease severity and outcome

In addition to signifying an increased risk for disease, high IsoP values may also provide information about the severity of disease. Vassalle et al compared plasma IsoP levels in 38 patients with angiographically measured CHD and 30 healthy control subjects[54]. They not only found that plasma levels of IsoP increased with the number of risk factors, but also that subjects with greater number of diseased vessels had higher plasma IsoP levels. This finding

was confirmed by a larger study comparing IsoP levels and severity of coronary artery stenosis in 241 consecutive patients undergoing coronary angiography for suspected coronary artery disease [55]. Once again, IsoP levels correlated with the number of risk factors and the number of affected vessels.

If there is a relationship between IsoP values and severity of coronary disease, then do IsoP values predict clinical outcome of patients diagnosed with CHD? One of the first studies to address this question measured IsoP and other biomarkers in 108 patients admitted to the emergency room with chest pain and subsequently diagnosed with acute coronary syndrome (ACS) based on changes in their echocardiogram or elevated troponin levels [56]. IsoP levels at admission were more than 3-fold higher in patients diagnosed with ACS than the 101 age- and gendered-matched patients who did not have ACS. The ACS patients were then tracked over the next 30 days for four primary endpoints (nonfatal myocardial infarction, heart failure, revascularization or death). 42% of patients in the highest IsoP tertile at admission had reached one or more of these endpoints by 30 days, compare to only 14% and 17% of patients in the lowest and middle IsoP tertiles, respectively. Using this data to create receiver operating characteristic curves, the optimal cutoff point for IsoP levels was 124.5 pg/ml (74% sensitive, 81% specific in predicting cardiac events ; 57% positive predictive value, 90% negative predictive value.) Interestingly, IsoP values had greater predictive power than hs-CRP values. Thus, high IsoP values appear to be a useful indicator that a patient is at high risk and that greater vigilance and intervention are needed.

The relationship between CHD and IsoP levels suggests that atherosclerotic tissue is a significant source of the increased IsoPs found in circulation and in urine. Evidence that formation of IsoPs occurs in atherosclerotic vascular tissue to a greater extent than in normal vascular tissues comes from a study that used directional coronary atherectomy [57]. Mean IsoP levels in lesion specimens from patients with unstable angina pectoris or recent myocardial infarction were approximately 16-fold higher than in specimens from apparently normal peripheral artery. Lesions from patients with stable angina were 7-fold higher than the control tissue. Therefore, the increased circulating or urinary IsoP levels for in CHD seems to directly reflect increased lipid peroxidation that occurs in blood vessels undergoing atherogenesis.

Isoprostanes as an endpoint in interventional trials

If increased IsoP values directly reflect lipid peroxidation in vessel walls that is driving atherogenesis, then a reduction in IsoP values should indicate successful intervention and modulation of disease progression (Table 3). Can IsoP values be used as a surrogate endpoint in interventional studies? Several clinical studies for therapeutic agents that are effective at ameliorating disease and that may target lipid peroxidation indirectly have shown reduction in IsoP values after intervention. For instance, statins, which lower LDL levels by inhibition cholesterol synthesis, have been consistently shown to reduce IsoP levels in hypercholesterolemic patients [27,28,58-60]. Smoking cessation also rapidly reduces IsoP levels [21,61,62]. AT1 receptor blockers, used to treat hypertension, also significantly reduce IsoP levels in hyperholesterolemic patients [63]. Weight loss in obese subjects also profoundly lowers IsoP levels [64-69]. These results strongly suggest that effective interventions for CHD will either directly or indirectly lower IsoP values, so that measurement of IsoP values should be a highly useful surrogate endpoint in clinical trials.

The lack of such surrogate endpoint clouds the interpretation of an entire group of therapeutic trials involving vitamin E supplementation that have been used as evidence that lipid peroxidation does not mediate CHD. These trials were initiated based on epidemiological studies that suggested an inverse relationship between intake of the dietary

antioxidant vitamin E and CHD [70,71] and on animal studies showing a reduction of atherosclerosis with vitamin E supplementation[72-77]. However, in a large number of placebo controlled clinical trials, vitamin E supplementation failed to reduce CHD [78-85]. One key, but untested, assumption of these trials was that the doses of vitamin E used were sufficient to reduce lipid peroxidation in hypercholesterolemic subjects. A recent pharmacokinetic /pharmacodynamic studies for vitamin E using hypercholesterolemic subjects with elevated plasma IsoP values suggest this key assumption was not correct [86]. Sixteen weeks of vitamin E supplementation at 1600 IU/day or greater were required to significantly reduced plasma IsoP levels. The typical dose of vitamin E used in clinical trials has been 400 IU/day or less, so that the doses of vitamin E used in almost every clinical trial for CHD to date appear to have been too low to significantly alter lipid peroxidation. Therefore, while these trials provide clear evidence that low dose vitamin E is not an effective treatment for CHD, they do not provide evidence about the effectiveness of lowering lipid peroxidation as a treatment for CHD.

Limitations of current knowledge and future studies needed

Clinical studies in the past decade have greatly strengthened the evidence that lipid peroxidation plays a key role in atherogenesis. While the consistent findings that high IsoP values are independent risk factors for CHD and correlate with severity of disease suggest the potential clinical utility of measuring IsoP levels, this remains to be demonstrated with appropriate clinical studies. For instance, there clearly need to be more studies to determine whether IsoP levels at admittance to the emergency room for chest pain or acute coronary syndrome are a useful predictor of short and long-term outcomes and if applying more aggressive treatments based on high IsoP levels actually improves outcomes. In a similar manner, there need to be large scale clinical studies to determine if initiating or escalating preventative treatments (e.g. statins or antihypertensives) based on IsoP levels in patients with otherwise borderline indicators significantly reduces the incidence of CHD. A key aspect of these trials should be the establishment of what specific IsoP values should trigger intervention, along with identifying what level of reduction is required for efficacy. We believe that the use of mass spectrometry based methods rather than immunoassay to quantify IsoP levels in these studies is critical to establishing appropriate treatment guidelines. Although this may slightly add to the cost of conducting large scale trials, this seems to be the only reasonable way to ensure that measurements conducted at multiple testing centers can be reliably compared to one another, be free of artifacts, and be subsequently standardized for widespread use.

The results of the past decade also demonstrate that many pharmacological interventions that reduce risk factors of CHD (e.g. elevated cholesterol levels, diabetes, etc…) concomitantly reduce lipid peroxidation. It remains to be established whether lowering lipid peroxidation is by itself clinically relevant and how significant of reductions in lipid peroxidation levels are required to provide maximum clinical benefit. An important aspect of such studies would be to determine if mechanistically unrelated interventional strategies that achieve similar reductions in IsoP levels provide similar benefits in terms of outcome. It must also be determined whether reduction of lipid peroxidation must be achieved very early in atherogenesis to be beneficial, or whether reduction in later stages of disease is also beneficial. In addition to high dose vitamin E, there are a number of novel dietary antioxidants (e.g. pomegranate juice [87-89]) and synthetic antioxidant interventions that have been proposed to reduce lipid peroxidation and that might merit use in clinical trials. However, defining the clinical pharmacology and the doses needed to suppress lipid peroxidation by these agents is needed prior to initiating trials. To appropriately test the efficacy of such antioxidant strategies in the prevention of CHD, we suggest that a minimum IsoP value of more than 2 SD above the normal mean (i.e. plasma IsoP >47 pg/ml as

measured by GC/MS assay) be used as the inclusion criteria for subjects [86] and that treatment doses be titrated to reduce IsoP values to within 1SD of the normal range (i.e. plasma IsoPs <41 pg/ml). Such studies should provide a clear test of the hypothesis that reducing lipid peroxidation is an effective strategy in the treatment of CHD and if successful, would establish appropriate target levels for physicians to use in treating their patients, in the same manner that target cholesterol and blood pressure levels are currently used.

In addition to more clinical trials, the results of the past decades also provide the rationale for continuing animal and cell culture studies to fully elucidate how lipoxidation products contribute to atherosclerosis. Although IsoPs clearly serve as excellent markers of lipid peroxidation, this should not distract from efforts to identify the lipoxidation products that are the most important contributors to atherosclerosis and to perform clinical studies to test their usefulness as biomarkers. With such studies in hand, the questions of whether lipid peroxidation is a critical component of CHD and if lipid peroxidation is a suitable target for intervention in CHD should finally be satisfactorily answered.

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List of Abbreviations

ACS	Acute coronary syndrome
CHD	coronary heart disease
IsoPs	F_2-isoprostanes
GC/MS	gas chromatography/mass spectrometry
HDL	high density lipoprotein
hs-CRP	high sensitivity C-reactive protein
LC/MS	liquid chromatography/mass spectrometry
LDL	low density lipoprotein
PUFA	polyunsaturated fatty acids

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Table 1

Studies Assessing the Effect of Risk Factors on IsoP Levels

Study Design and Population	Major Finding	IsoP Assay	Ref
Smoking			
•Smokers (n=10) vs age/sex-matched non-smokers (n=10)	2.4-fold ↑ plasma IsoP levels in smokers	GC/MS	[19]
•Smokers (n=6) vs age-matched non-smokers (n=6)	2.3-fold ↑ urine IsoP levels in smokers	GC/MS	[20]
•Heavy (n=5) vs moderate (n=5) vs non-smokers (n=8)	3.2-fold (heavy) and 1.7-fold (moderate) ↑urine IsoP	GC/MS	[21]
•COPD current smoker (n=15) vs COPD ex-smoker (n =25) vs healthy smokers (n=12) vs healthy non-smokers (n=10).	4.1-fold (COPD smoker), 3.6-fold (COPD ex), 2.2-fold ↑ breath condensate IsoP	ELISA	[23]
Elevated LDL			
•HC patients (n=40) vs age/sex matched controls	2.2-fold ↑ urine IsoP levels in HC patients	ELISA	[24]
•Familial HC patients (n=38) vs controls (n=38) and moderate HC patients (n=24) vs controls (n=24)	1.5-fold (familial HC) and 1.4 (moderate HC) ↑ urine IsoP	GC/MS	[25]
•HC patients (n=25) vs controls (n=12)	3.2-fold ↑ plasma IsoP levels in HC patients	ELISA	[26]
•HC patients (n=67) vs controls (n=32)	1.4-fold ↑plasma IsoP levels in HC patients	ELISA	[28]
Reduced HDL			
•Low HDL (n=8) vs normolipidemic (n=15)	2.1-fold ↑plasma IsoP levels in low HDL subjects	ELISA	[29]
Diabetes			
•Type 2 diabetics (n=39) vs healthy controls (n=15)	3.3-fold ↑total plasma IsoP levels in T2DM patients	GC/MS	[30]
•Type 2 diabetics (n=62) vs age/sex-matched controls (n=62) and Type 1 diabetics (n=23) vs matched controls (n=23)	2.0-fold ↑urine IsoP levels in both T2DM and T1DM patients	RIA	[31]
•Type 2 diabetics (n=40) vs age/sex-matched controls (n=25)	5.9-fold ↑plasma IsoP levels in T2DM patients	ELISA	[32]
•Type 1 diabetics (n=14) vs age/BMI-matched controls (n=14)	2.3-fold ↑ urine IsoP levels in T1DM patients	LC/MS/MS	[33]
•Type 2 diabetics (n=26) vs age-matched controls (n=52)	2.7-fold ↑urine IsoP levels in T2DM patients	ELISA	[34]
Obesity			
•Framingham Study participants (n=2828)	Log urine IsoP correlates with BMI (0.087 per 5kg/m^2)	ELISA	[35]
•Obese men (n=14) vs non-obese men (n=17)	4.2-fold ↑ plasma IsoP levels	ELISA	[36]
•Met. Syndrome patients (n=10) vs matched controls (n=11)	3.7-fold ↑ plasma IsoP levels	ELISA	[37]
•Obese children (n=44) vs age-matched non-obese (n=28)	3.2-fold ↑ plasma IsoP levels	ELISA	[38]
Hypertension			
•Framingham Study participants (n=2828)	Log urine IsoP correlates with systolic BP (0.029 per 20 mm Hg)	ELISA	[35]
•Renovascular disease patients vs essential hypertension patients vs healthy subjects.	2.5-fold (renovascular) and 1.5-fold (essential hypertension) ↑urine IsoP levels	RIA	[39]
•Hypertensive patients vs normotensive controls	2.0-fold ↑ plasma IsoP levels	ELISA	[40]
•Pulmonary Hypertensives (n=25) vs age/sex-matched controls (n= 25)	2.3-fold ↑ urine IsoP levels	GC/MS	[42]
CRP			
•Japanese workers undergoing yearly exam (n=551)	Urine IsoP/creatinine vs CRP (r = 0.139)	ELISA	[44]
•TRAIN study participants (n=60)	Log urine IsoP vs log hsCRP (r = 0.31)	GC/MS	[43]
Homocysteine			

Study Design and Population	Major Finding	IsoP Assay	Ref
•Subset of ASAP study participants (n=100)	1.5-fold ↑ plasma IsoP in high vs low homocysteine quintile (r=0.33)	GC/MS	[45]
Age			
•Framingham Study participants (n=2828)	Log urine IsoP inversely correlates with age (−0.037 per 10 years age)	ELISA	[35]
•Subset of Louisiana Healthy Aging Study >90 years old (n= 74) vs 60-74 years old (n =49) vs 20-34 years old (n= 47)	Urine IsoP metabolite 26% lower in the >90 year old group	LC/MS/MS	[46]
•Older (>60 years old, n=20) vs young (20-33 years old, n=20)	Plasma IsoP in age groups not different at rest, but area under response the curve 7.8–fold ↑ in older group after ischemia/ reperfusion	GC/MS	[47]
Gender			
•Framingham Study participants (n=2828)	Log urine IsoP inversely correlates with being male (0.157 for female)	ELISA	[35]
•CARDIA study participants Male (n=1302) vs Female (n= 1548)	Plasma IsoP 25% lower in males	GC/MS	[48]

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; CRP, high sensitivity C-reactive protein; ELISA, enzyme linked immunoassay; GC/MS, gas chromatography mass spectrometry; HC, hypercholesterolemia; LC/MS/MS, HPLC tandem mass spectrometry; RIA; radioimmunoassay; BP, blood pressure

Table 2

Studies Assessing Relationship Between IsoP Levels and Presence or Extent of CHD

Study Design and Population	Major Findings	Ref
Studies That Found IsoP Levels to be Independent Risk Factors for CHD		
Verified CHD cases (n=93) vs controls (n=93) Measured IsoP, BMI, BP, TC, HDL, LDL, TG, hsCRP, oxLDL and presence of DM, smoking, and medications.	• Urine IsoP 1.8-fold↑ in CHD patients (GC/MS). • IsoP correlate with #CHD risk factors. • 27.3 Odds Ratio (OR) for CHD in highest IsoP tertile.	[49]
Consecutive patients undergoing diagnostic coronary angiography. CHD (≥50% stenoses, n=54) vs control (<30% stenoses, n=50).	• Plasma IsoP 1.5-fold↑ in CHD patients (LC/MS/MS). • 9.7 OR (adjusted) for CHD in highest IsoP quartile. • 9-HETE also predicted CHD	[50]
CARDIA study participants (age ≤ 30 at entrance) were analyzed for coronary artery calcification (CAC) at 15 year follow-up (n=2850).	• 1.24 OR (adjusted) for CAC per 33 pg/mL plasma IsoP (GC/MS).	[48]
Verified CHD patients (n=799) vs controls (n=925).	• Urine IsoP 1.2-fold↑ in CHD subjects (ELISA) • 2.55 OR (adjusted) for CHD in highest IsoP quartile • PAF-AH activity also predicted CHD	[51]
Studies that Found IsoP Levels to Correlate with Extent of Disease		
CHD cases (n=38) vs healthy controls (n=30). CHD patients underwent angiography to determine # vessels involved.	• Plasma IsoP ↑ with #CHD risk factors (ELISA). • IsoP 1.5-fold↑with 1-vessel disease and 2.0-fold↑with multi-vessel disease.	[54]
Consecutive patients undergoing diagnostic coronary angiography. #vessel with ≥50% stenoses determined. Controls (0 vessel n=72, 1-vessel CHD n=65, 2-vessel CHD n=61, 3-vessel CHD n=43.	• Plasma IsoP ↑ with #CHD risk factors (ELISA). • OR 2.47 (adjusted) per 100 pg/ml increase in IsoP. • IsoP 1.2-fold↑ 1-vessel CHD, 1.3-fold↑ 2-vessel CHD, 1.4-fold↑3-vessel CHD.	[55]
Studies that Found IsoP Levels May Predict Outcome of CHD		
Patients admitted to ER with chest pain and diagnosed with ACS (n=108.) ACS patients followed for 30 days for composite end-point of MI, CHF, revascularization, or death.	• Serum IsoP 4.8-fold↑ in ACS at admission vs controls (ELISA). • 42% pts in highest IsoP tertile reach endpoint vs 14% for lowest tertile.	[56]
Studies That Failed to Find Significant Correlation Between IsoP Levels and CHD		
CHD patients with ACS (n=54), CHD with stable angina (n=108) vs. control (n=46). Measured 17 plasma biomarkers including IsoP.	• Stated did not find an increase in plasma IsoP levels (ELISA) for patients with CHD, (no data shown.)	[52]
Nested case control from Fletcher Challenge Cohort study. CHD cases (n=227) vs controls (n=417).	• Found similar plasma IsoP levels (GC/MS) for case vs. control. • There was an extremely unusual range of IsoP levels in control subjects ..	[53]

Abbreviations: ACS, acute coronary syndrome; **CHF,** congestive heart failure; **CRP,** high sensitivity C-reactive protein; **ELISA,** enzyme linked immunoassay; **GC/MS,** gas chromatography mass spectrometry; **HC,** hypercholesterolemia; **LC/MS/MS,** HPLC tandem mass spectrometry.

Table 3

Interventions That Significantly Reduce IsoP Levels in Human Clinical Studies

Population	Duration of Treatment	Major Findings	Ref
Statins			
•HC patients (n=34)	1 month simvastatin q.s. ↓TC >20%	Urine IsoP (RIA) ↓34% and giving 600 mg/d vitamin E had no added effect.	[58]
•HC patients (n=25)	6 months pravastatin 10 or 20 mg/d	Plasma IsoP (ELISA) ↓47% and also ↓LV mass .	[27]
•HC patients (n=34)	6 months simvastatin 40 mg ±Vit. E	Plasma IsoP (ELISA) ↓~90 pg/ml (no Vit E) and ↓~140 pg/ml (with 400 IU/dVit E).	[28]
•CHD patients (n=64)	2 months atorvastatin 40 mg/d	Plasma IsoP (ELISA) ↓52% overall. Also ↓LDL C and hsCRP.	[59]
•Dialysis pts (n=28)	4 months simvastatin 5mg/d or10mg/d	Plasma IsoP (ELISA) ↓52% (LDL <200) and ↓40% (LDL>200). Also ↓oxLDL and ↑%FMD.	[60]
Smoking Cessation			
•Smokers (n=8)	2 weeks cessation	Plasma IsoP (GC/MS) ↓38%	[19]
•Smokers (n=6)	3 weeks cessation	Urine IsoP (GC/MS) ↓33%	[21]
•Smokers (n=36)	3 weeks cessation	Urine IsoP (ELISA) ↓~32% overall*. Also ↓plasma and serum IsoP.	[61,62]
AT 1 Receptor Antagonists			
•HC patients (n=17)	6 weeks candesartan 16 mg/d	Serum IsoP (ELISA) ↓13%. Also ↓MCP-1, sICAM-1, hsCRP.	[63]
Caloric Restriction/Weight Loss			
•Obese women (n=11)	12 weeks calorie intake 1200 kcal/d	Urine IsoP (RIA) ↓32% . Included only if weight ↓≥5 kg. Also ↓hsCRP and 11-d-TxB_2.	[64]
•Obese men (n=11)	3 weeks high fiber diet and exercise	Serum IsoP (ELISA) ↓~30% * Body weight ↓3.7%. Also ↓BP and TC	[65]
•Obese women (n=71)	6 months hypocaloric diet± orlistat	Plasma IsoP (ELISA) ↓72% (no orlistat) and ↓79% (with orlistat). Also ↓hsCRP, LDL, TG	[66]
•Overweight asthmatic patients (n=10)	8 weeks alternating day 80% CR	Serum IsoP↓79%. Body weight ↓8%. Also ↓protein carbonyl and nitrotyrosine.	[67]
•Obese pts (n=30)	8 weeks 30% CR	Urine IsoP (ELISA)↓ 35%. Body weight ↓7%. Also ↓MDA, TC, Urate.	[68]
•NAFLD pts (n=7)	subtotal gastrectomy	Serum IsoP (GC/MS) ↓~38% *. Also BMI ↓21% and changes in PUFAs.	[69]
Vitamin E			
•HC pts (n=5/dose)	16 weeks vitamin E (0- 3200 IU/d)	Plasma IsoP (GC/MS) ↓35% (1600 IU/d) and ↓49% (3200 IU/d). Trend for ↓IsoP at 800 IU/d. No change at 100-400 IU/d Vitamin E.	[86]

Abbreviations: BMI, body mass index; **BP,** blood pressure; **CR,** caloric restriction; **FMD,** Flow Mediated Dilation; **HC,** hypercholesterolemia; **TC**, total cholesterol; **NAFLD,** non-alcoholic fatty liver disease.

* estimated from graph in figure.