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The isoprostanes—25 years later

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Abstract

Isoprostanes (IsoPs) are prostaglandin-like molecules generated independent of the cyclooxygenase (COX) by the free radical-induced peroxidation of arachidonic acid. The first isoprostane species discovered were isomeric to prostaglandin $F_{2\alpha}$ and were thus termed F_2-IsoPs. Since the initial discovery of the F_2-IsoPs, IsoPs with differing ring structures have been identified as well as IsoPs from different polyunsaturated fatty acids, including eicosapentaenoic acid and docosahexanenoic acid. The discovery of these molecules *in vivo* in humans has been a major contribution to the field of lipid oxidation and free radical research over the course of the past 25 years. These molecules have been determined to be both biomarkers and mediators of oxidative stress in numerous disease settings. This review focuses on recent developments in the field with an emphasis on clinical research. Special focus is given to the use of IsoPs as biomarkers in obesity, ischemia-reperfusion injury, the central nervous system, cancer, and genetic disorders. Additionally, attention is paid to diet and lifestyle factors that can affect endogenous levels of IsoPs. This article is part of a Special Issue entitled “Oxygenated metabolism of PUFA: analysis and biological relevance”.

Keywords

Isoprostane; Oxidative stress; Biomarker; Lipid peroxidation; Mass spectrometry

1. Introduction

January 2015 marks the 25th anniversary of the first report by Morrow and Roberts on the discovery of the isoprostanes (IsoPs), prostaglandin-like molecules generated *in vivo* in humans independent of the cyclooxygenase (COX) by the free radical-induced peroxidation of arachidonic acid [1]. Over the course of the past 25 years, more than 3800 articles have been published in the field of IsoP research by numerous investigators around the world. Numerous excellent reviews have been written describing the formation, chemical synthesis, and biological activities of the IsoPs as well as their potential use as biomarkers of disease.

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This review will provide a brief historical perspective on the discovery of the IsoPs with the primary focus on recent clinical research in the field.

2. Historical perspective

2.1. Discovery and formation of isoprostanes

Oxidative stress is characterized by the inability of the body's natural antioxidant defenses to detoxify and protect against pro-oxidant, and often pro-inflammatory, species. Production of reactive oxygen species (ROS), typically free radicals, is a hallmark of oxidative stress. Overproduction of ROS has been implicated in a variety of diseases yet much remains to be understood about mechanisms of oxidant injury in humans. Polyunsaturated fatty acids (PUFAs), such as arachidonic acid, are one target of free radical insult during oxidative stress. In the mid-1970s, it was shown that prostaglandin (PG)-like compounds could be formed *in vitro* by the non-enzymatic peroxidation of purified PUFA; however, this work had never been carried out beyond *in vitro* studies [2].

In the 1980s, a study showed that PGD_2 derived from COX is primarily metabolized *in vivo* in humans to 9α,11β-$PGF_{2\alpha}$ by the enzyme 11-ketoreductase [3]. In aqueous solutions, however, PGD_2 is an unstable compound that undergoes isomerization of the lower side chain and these isomers can also be reduced by 11-ketoreductase to yield isomers of 9α, 11β-$PGF_{2\alpha}$ [4]. In studies undertaken to further characterize these compounds utilizing mass spectrometry, it was found that when plasma samples from normal volunteers were processed and analyzed immediately, a series of peaks were detected possessing characteristics of F-ring PGs. Interestingly, however, when plasma samples that had been stored at −20 °C for several months were reanalyzed, identical chromatographic peaks were detected but levels of putative PGF_2-like compounds were up to 100-fold higher [1]. In addition, base-catalyzed hydrolysis of plasma lipids also yielded significant amounts of the PGF_2-like compounds. Antioxidants and reducing agents suppressed the formation of these compounds and CCl_4, a toxic agent known to generate free radicals in the liver through metabolism to the CCl_3•, dramatically increased the formation of these compounds in rats [1,6]. These experiments confirmed that the observed PGF_2-like compounds were generated *in vivo*, not by a COX-derived mechanism, but rather non-enzymatically by autoxidation of arachidonic acid. Morrow and Roberts termed this new class of compounds F_2-isoprostanes (IsoPs) because they were isomeric to $PGF_{2\alpha}$ [5,6].

A mechanism to explain the formation of the F_2-IsoPs from arachidonic acid is outlined in Fig. 1 [7]. Following abstraction of a bisallylic hydrogen atom and the addition of molecular oxygen to arachidonic acid to form a peroxyl radical, the peroxyl radical undergoes 5-*exo* cyclization and a second molecule of oxygen adds to the backbone of the compound to form PGG_2-like compounds. These unstable bicycloendoperoxide intermediates are then reduced to the F_2-IsoPs. Based on this mechanism of formation, four F_2-IsoP regioisomers, each of which is comprised of eight racemic diastereomers, a total of 64 compounds, are generated. The four regioisomer classes are named according to the carbon number on which the side chain hydroxyl group is attached (Fig. 1). This nomenclature system has been approved by the Eicosanoid Nomenclature Committee, which is sanctioned by JCBN of IUPAC [8]. An alternative nomenclature system for the IsoPs has been proposed by FitzGerald and

colleagues in which the abbreviation iP is used for isoprostane, and the regioisomers are denoted as III–VI (Fig. 1) [9]. It is important to note that 5- and 15-series regioisomers are formed in significantly greater abundance than the 8- and 12-series regioisomers as 8- and 12-series regioisomers readily undergo further oxidation [10].

In addition to F_2-IsoPs, various classes of IsoPs that differ in regards to the functional groups on the prostane ring have been discovered; the structures of these different compounds are summarized in Fig. 2. The compounds are named based upon the structure of the functional groups on the cyclopentane ring in a manner analogous to the prostaglandins. Like F_2-IsoPs, multiple isomers of these compounds are formed. E_2-/D_2-IsoPs that are isomeric to PGE_2 and PGD_2, are formed competitively with F_2-IsoPs from the isomerization of the arachidonyl endoperoxide intermediate ***1*** (Fig. 2) [11]. Depletion of cellular reducing agents, such as glutathione (GSH) or α-tocopherol, favors the formation of E_2/D_2-IsoPs over that of F_2-IsoPs, which is formed via reduction of the endoperoxide ***1*** (Fig. 2) [12]. E_2/D_2-IsoPs, however, are not terminal products of the IsoP pathway. These compounds readily dehydrate *in vivo* to yield A_2/J_2-IsoPs, which are also known as cyclopentenone IsoPs because they contain an α,β-unsaturated cyclopentenone ring structure [13,14]. A_2/J_2-IsoPs can be further metabolized, through rearrangement and dehydration, to yield deoxy-A_2 and deoxy-J_2-IsoPs [15]. Hardy and colleagues have shown that 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-d-PGJ_2), a highly studied molecule originally identified as a metabolite of PGD_2, can also be generated non-enzymatically during free radical-induced oxidation and is one of the deoxy-J_2-IsoP isomers [15]. Finally, in addition to the molecules described above, compounds isomeric to the COX-derived thromboxane B_2, termed isothromboxanes are products of the free radical-catalyzed peroxidation of arachidonic acid [16].

Besides IsoPs, another group of related compounds are also generated from the peroxidation of arachidonic acid via a similar mechanism. IsoPs are formed when the indicated carbon-centered radical undergoes 5-*exo*-cyclization to form the cyclopentane ring, as shown in Fig. 3. Alternatively, this carbon-centered radical could react with a molecule of oxygen to generate oxidized products of a different structure (Fig. 3) [17]. These products have a substituted tetrahydrofuran ring and thus have been named isofurans (IsoFs). A total of eight regioisomers are formed that are comprised of 16 racemic diastereomers for a total of 256 compounds [18].

2.2. Isoprostane metabolism

In contrast to COX-derived PGs that are generated from free arachidonic acid, IsoPs are initially formed from arachidonic acid *in situ* on lipids [5,19]. Molecular modeling of IsoP-containing phospholipids reveals them to be remarkably distorted molecules. IsoPs can be released from the phospholipid backbone as free acids by phospholipase action. Platelet activating factor-acetylhydrolase (PAF-AH) has also been shown to release F_2-IsoPs from phospholipids [20].

Free F_2-IsoPs are found circulating in plasma, are further filtered in the kidney, and appear in the urine [21,22]. F_2-IsoPs can also undergo metabolism in the liver to yield a variety of metabolites (Fig. 4) [23–29]. In humans, two major urinary metabolites of 15-F_{2t}-IsoP (also

referred to as 8-iso-$PGF_{2\alpha}$ or $iPF_{2\alpha}$-III), one of the most abundant endogenous F_2-IsoPs, have been identified to be 2,3-dinor-15-F_{2t}-IsoP and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP [24,26,27]. Metabolism studies of eight different 15-series F_2-IsoP stereoisomers have also been conducted in isolated rat hepatocytes, an accepted model of metabolism [28]. In addition to 2,3-dinor- and 2,3-dinor-5,6-dihydro metabolites, 13,14-dihydro-15-keto- and 2,3,4,5-tetranor- derivatives of 15-F_{2t}-IsoP were identified (Fig. 4) [28]. Taurine conjugates of 15-F_{2t}-IsoP and its various metabolites were also observed (Fig. 4) [28]. In human clinical trials, different F_2-IsoP stereoisomers, including 5- and 15-F_{2t}-IsoP, as well as 2,3-dinor-15-F_{2t}-IsoP and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP have been used as biomarkers of IsoP formation *in vivo*.

Importantly, Yan and colleagues have proposed that F_2-IsoPs are excreted in the urine conjugated with glucuronide [29]. The structure of the glucuronide metabolite(s) has not been directly confirmed (proposed structure in Fig. 4), but the authors noted that levels of urinary F_2-IsoPs were significantly increased after treatment of the urine with β-glucuronidase (0.43 ± 0.02 vs. 0.61 ± 0.03 nmol/mmol Cr) [29]. Typically, glucuronide conjugates are formed by reaction with free hydroxyl groups or carboxylic acid groups. Due to the similarity in structure between 15-F_{2t}-IsoP and the 2,3-dinor- and 2,3-dinor-5,6-dihydro- metabolites, it is possible that glucuronide conjugates of 2,3-dinor-15-F_{2t}-IsoP and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP could exist but these compounds have not been studied to date. Glucuronidation thus represents another route of F_2-IsoP metabolism that needs to be considered when measuring these compounds in urine.

The metabolism of D_2/E_2-IsoPs is less well studied. As discussed above, it is known that these compounds dehydrate *in vivo* to yield A_2/J_2-IsoPs [13,14] and that A_2/J_2-IsoPs can be further metabolized, through rearrangement and dehydration, to yield deoxy-A_2 and deoxy-J_2-IsoPs [15]. The metabolism of cyclopentenone IsoPs has been studied in HepG2 cells, a cell line derived from human hepatocytes, as well as in the rat [30,31]. These molecules are rapidly metabolized by glutathione transferase enzymes yielding water-soluble modified glutathione conjugates. The metabolism of isothromboxanes and isofurans has not been studied.

3. Isoprostanes as biomarkers of oxidative stress in human disease

A major impediment in the field of free radical research has historically been the lack of specific and sensitive methods to assess endogenous oxidative damage. Over the course of the past 25 years, F_2-IsoPs have been shown to be a reliable biomarker of endogenous lipid peroxidation because they are ubiquitous in the body and are chemically stable in biological fluids when stored correctly. Both animal and clinical studies have tested the reliability of this biomarker. An NIH-sponsored study, termed the Biomarkers of Oxidative Stress Study (BOSS), found that plasma F_2-IsoPs are increased in a time- and dose-dependent manner in rats administered carbon tetrachloride, a toxic agent known to systematically generate free radicals in the liver [32]. Il'yasova and coworkers then conducted a follow-up study to the BOSS trial in humans using doxorubicin-based chemotherapy as the model because this drug is known to generate hydroxyl radicals at pharmacological doses [33]. Several commonly measured indices of oxidative stress were examined in 23 women newly

diagnosed with breast cancer undergoing standard chemotherapy. F_2-IsoPs, as well as the F_2-IsoP metabolite 2,3-dinor-15-F_{2t}-IsoP, were significantly increased in the urine 1 h following treatment and returned to baseline levels 24 h following treatment. Together these findings confirm that F_2-IsoPs are reflective of endogenous lipid peroxidation following free radical production.

A multitude of papers have been published describing different analytical methods for the quantification of F_2-IsoPs in biological fluids [34–58]. Three primary techniques are used to measure these compounds: (1) gas chromatography–mass spectrometry, (2) liquid chromatography–mass spectrometry, and (3) immunoassays (ELISAs). Mass spectrometric-based assays are widely accepted as the most accurate methodologies as the results of immunoassays could be confounded due to the structural similarities between F_2-IsoPs and COX-derived prostaglandins as well as other related molecules [59–62]. F_2-IsoPs can be measured in plasma, urine, any tissue, cerebral spinal fluid, exhaled breath condensate, amniotic fluid, and saliva. Measurement in plasma and urine is most common in humans as these fluids are the least invasive. Caution must be taken, however, when collecting and storing plasma for F_2-IsoP analysis as these molecules can be generated from *ex vivo* oxidation of arachidonic acid in the plasma. In a recent commentary by Barden and colleagues, it is recommended to collect blood into tubes containing the anticoagulant EDTA and the antioxidants butylated hydroxytoluene (BHT) and GSH to min-imize artifactual elevation of F_2-IsoPs [63]. All samples should be stored at −80 °C, not −20 °C, upon collection because autoxidation can occur at −20 °C leading to artifactual generation of F_2-IsoPs *ex vivo*.

In addition to the collection and storage of the samples, there are several factors that are important to consider when measuring F_2-IsoPs as a biomarker of oxidative stress in disease. These factors include choosing the appropriate sample matrix, the timing of sample collection, and considerations regarding the hydrolysis, metabolism, and excretion of these compounds [64]. In plasma, most clinical studies have quantified F_2-IsoPs as free fatty acids; however, as mentioned previously, F_2-IsoPs are found esterified in phospholipids in the plasma. Base hydrolysis of plasma lipids prior to analysis provides a measure of total F_2-IsoPs in the plasma. In urine, the use of F_2-IsoPs as a biomarker of systemic oxidative stress is even more complex as these molecules can be directly excreted or, as discussed above, metabolized to yield a variety of different compounds, the formation of which is regulated by numerous enzymes. In a recent commentary, Halliwell and Lee propose that in order to obtain a clear picture of F_2-IsoP formation *in vivo* it is best to measure total F_2-IsoPs (both molecules esterified in phospho-lipids and those which are unesterified) in plasma along with free and metabolized F_2-IsoPs in urine [64].

Numerous excellent reviews have been written on F_2-IsoPs as biomarkers of oxidative stress in human health and disease [65–94]. In the coming sections, we will highlight several recent human studies that have examined the formation of endogenous F_2-IsoPs and explored their applications as biomarkers of oxidative stress.

3.1. Isoprostanes and obesity

The marked increase in the incidence of overweight and obese persons is recognized as perhaps the most serious public health issue in the United States. It is estimated that 69% of American adults over age 20 are either overweight or obese. Additionally, the incidence of overweight and obesity in children and adolescents is rising; in 2013, it was estimated that 18% of children 6–19 are either overweight or obese [95]. Both morbidity and mortality increase with excessive body weight. Multiple studies have clearly shown that levels of F_2-IsoPs, as measured in the plasma or urine, increase in obese adults [74,96–101]. F_2-IsoPs have been positively correlated with body mass index (BMI), waist circumference (WC), visceral fat area, and percent body fat [102–118]. Interestingly, recent epidemiological studies have implicated possible racial differences in the association of F_2-IsoPs with obesity. In the Insulin Resistance Atherosclerosis Study (IRAS) three different F_2-IsoP isomers along with the metabolite 2,3-dinor-15-F_{2t}-IsoP were measured in the urine of 237 African Americans, 342 non-Hispanic whites, and 275 Hispanic whites [115]. Levels of the urinary F_2-IsoPs and the F_2-IsoP metabolite increased with BMI in non-Hispanic whites and His-panic whites but there was no increase with BMI observed in African Americans. A study by Warolin et al. in African American (n = 82) and white (n = 76) youth, ages 8–17 years, found plasma F_2-IsoPs correlated with percent body fat and truncal fat but no correlation was found with BMI nor were racial differences observed [119]. In a population of 845 Chinese women living in Shanghai, urinary 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP but not urinary F_2-IsoPs correlated with increased in BMI [120]. Finally, in a study of 294 Canadian Artic Inuits with metabolic syndrome, plasma F_2-IsoPs and IsoFs correlated positively with BMI and abdominal obesity while IsoFs alone correlated with WC [116]. Together these studies indicate that lipid peroxidation associated with oxidative stress is increased in obese populations. These studies also highlight the importance of assessing multiple IsoP entities including plasma and urine F_2-IsoPs as well as their urinary metabolites and other molecular species such as IsoFs to detect pathophysiological associations with oxidative stress.

3.2. Isoprostanes and ischemia-reperfusion

It is well understood that ischemia/reperfusion (I/R) elicits formation of reactive oxygen species (ROS). F_2-IsoPs have been shown to increase post-I/R in humans in disease settings [121]. Levels of F_2-IsoPs have been shown to increase in patients with chronic lower limb ischemia [122], following ischemic stroke [123,124], and after aneurysm rupture [125]. Model studies have also been conducted to better understand the human response to I/R in different populations. For example, in a human model using suprasystolic inflation of an arm blood pressure cuff to safely induce localized forearm I/R, Davies and colleagues showed that levels of F_2-IsoPs in the plasma increase 15 min post-I/R and remain elevated for at least 3 h [126]. Interestingly, the observed increase in F_2-IsoPs is more dramatic in healthy older adults (ages 62–81) compared to healthy young adults (ages 20–33) and levels remain elevated in the older population for a longer period of time. In a follow-up study comparing physically fit older adults with unfit age-matched controls, classified based upon VO_{2max} and maximal leg power, the F_2-IsoP response to forearm I/R was lower in the physically fit group [127]. This observation is postulated to result from differences in the activity of antioxidant enzymes.

I/R injury is a major concern in certain surgical settings, such as coronary artery bypass grafting (CABG) surgery and organ transplantation, when blood flow is restored following a period of blood vessel occlusion. Multiple studies have shown increases in F_2-IsoPs during CABG or cardiopulmonary bypass (CPB) surgery [121,128–131]. Importantly, this observed increase in oxidative stress is thought to contribute to postoperative complications. One such complication is acute kidney injury (AKI). AKI occurs in up to 30% of all patients undergoing CPB surgery and independently predicts associated morbidity and mortality. In a case–control study of 10 cardiac surgery AKI patients and 10 cardiac surgery risk-matched controls, Billings and colleagues determined that the increase in plasma F_2-IsoPs, and IsoFs, during surgery were greater in the AKI patients in comparison to controls [132]. In a follow-up study of 445 patients undergoing cardiac surgery, it was determined that baseline F_2-IsoPs as well as intraoperative levels of F_2-IsoPs independently predicted AKI [114]. Pulmonary dysfunction is another common complication associated with cardiac surgery. It is particularly problematic in infants and children having surgical procedures performed to repair congenital heart defects. In a study of 20 infants (ages 3–12 months) undergoing elective stage 2 palliation on CPB to repair univentricular physiology, plasma F_2-IsoPs were quantified at baseline (prior to surgical incision), 30 min after initiation of CPB, immediately after separation from CPB, and 24 h post-operatively [133]. Like adults on CPB, levels of F_2-IsoPs increased significantly during surgery and returned to baseline levels 24 h following the procedure. Interestingly, higher levels of plasma F_2-IsoP levels immediately after separation from CPB were correlated with decreased dynamic lung compliance. Due to the small size of this study, it was not possible to determine if F_2-IsoPs can predict long-term outcomes in this population but the findings, together with the adult studies presented herein, suggest that further study of F_2-IsoPs, and potentially IsoFs, as predictive biomarkers of poor outcome following CPB is warranted.

In follow-up to the finding that lipid peroxidation is increased during CABG and CPB surgeries, multiple studies have monitored treatments that could reduce levels of surgery-induced oxidative stress. For example, in a study of 20 patients scheduled for CABG surgery, Berg and colleagues showed that subjects treated with aspirin (160 mg daily) for 1 week prior to surgery experienced a less robust elevation of F_2-IsoPs during surgery compared to subjects not taking aspirin [134]. Interestingly, the aspirin treated group also showed lower baseline levels of F_2-IsoPs at initiation of surgery. Others have studied the effect of acetaminophen treatment on the day of CPB surgery [135]. No effect of acetaminophen on levels of urinary F_2-IsoPs was noted but the drug significantly lowered the magnitude of increase in urinary IsoFs during surgery. Finally, it has been suggested that certain anesthesia agents can exert antioxidant activity. Ballester et al. recently reported a study comparing the effect of the anaesthetics sevoflurane and propofol on F_2-IsoP levels in blood samples taken from the coronary sinus of 38 patients undergoing elective off-pump CABG surgery [136]. Interestingly, F_2-IsoPs did not increase in the patients on sevoflurane while signifi-cant increases were noted in the group who received propofol. No other studies that we are aware of have compared the effect of the chosen an-esthesia in cardiac surgery patients. In a study of patients undergoing total knee arthroplasty (TKA), a surgery that also represents a setting of I/R, Mas and co-workers compared levels of F_2-IsoPs in patients receiving either general anesthesia or spinal anesthesia [137]. All patients showed a

significant increase in F_2-IsoPs following TKA; however, patients receiving general anesthesia had more significant increases in F_2-IsoPs than those receiving spinal anesthesia. Together, these studies indicate that procedures can be taken to help lower the resulting oxidative stress from surgery-associated I/R, but further work needs to be done in clinical populations to determine the most effective treatments to help prevent adverse clinical outcomes following surgery.

3.3. Isoprostanes and the central nervous system

Due to the high concentration of PUFA in the brain compared to other organs, lipid peroxidation is a primary outcome of free radical-induced brain injury. To that end, F_2-IsoPs have been shown to increase in a number of chronic neurodegenerative diseases including Alzheimer's disease (AD) [138–141], Huntington's disease [142], Parkinson's disease [143,144], and amyotrophic lateral sclerosis (ALS) [145]. Importantly, measurement of F_2-IsoPs in the plasma or urine does not consistently show increases in these diseases while their measurement in cerebral spinal fluid (CSF) is more indicative of the changes in oxidative stress in the brain [146,147]. A very recent study by Montine and colleagues measured F_2-IsoPs, along with biomarkers of AD, in the CSF of more than 400 healthy adults (ages 21–100) to examine the association between these biomarkers and cognitive function in relation to aging. Higher levels of F_2-IsoPs in the CSF were found to be associated with poorer executive function [148]. Additionally, Guest et al. also recently determined that F_2-IsoPs in the CSF also increase with age [149].

F_2-IsoPs in the CSF are increased in acute brain injury as well. Corcoran et al. quantified F_2-IsoPs, IsoFs, and F_4-neuroprostanes (NPs), F_2-IsoP-like compounds generated from the free radical-catalyzed oxidation of docosahexaenoic acid (DHA), in the CSF of patients within 24 h following aneurysmal subarachnoid hemorrhage (aSAH) or traumatic brain injury (TBI) and compared them to age-matched controls [150]. All three classes of lipid peroxidation products were increased in the CSF of aSAH patients while only IsoFs and F_4-NPs were increased following TBI. Interestingly, in a separate study monitoring the production of lipid mediators in the CSF following TBI, Farius and colleagues tentatively identified levels of D_2/E_2-IsoPs to be elevated compared to controls [151]. This same group previously reported a dramatic increase in the levels of D_2/E_2-IsoPs compared to F_2-IsoPs in a rat model of TBI [38]. Further, Reich et al. determined that D_2/E_2-IsoPs, and not F_2-IsoPs, were the favored products of the IsoP pathway post-mortem brain samples from humans with AD [152,153]. As discussed at the beginning of this review, D_2/E_2-IsoPs are formed competitively with F_2-IsoPs; lower levels of cellular reducing agents, such as GSH or α-tocopherol, may favor the formation of E_2/D_2-IsoPs over F_2-IsoPs in the brain [154].

3.4. Isoprostanes and cancer risk

Few studies have investigated the associations between levels of F_2-IsoPs and risk of cancer. One case–control study reported that the level of F_2-IsoPs was increased among subjects with breast cancer compared to controls [155]. Compared to the lowest quartile, the increasing quartiles of F_2-IsoPs were associated with odds ratios (OR) (95% confidence interval (95% CI)) of 1.25 (0.81–1.94), 1.53 (0.99–2.35) and 1.88 (1.23–2.88) for the 2nd, 3rd, and 4th quartile (p for trend, 0.002). In a subsequent cohort study conducted in Taiwan,

high urinary excretion rates of F_2-IsoPs was significantly linked to an increased risk of hepatocellular carcinoma with an OR of 2.53 (95% CI: 1.30–4.93) [156]. Two nested case–control studies were conducted within the multiethnic cohort [157,158]. One study found, when compared to the lowest tertile of urinary F_2-IsoPs, the second and third tertiles of F_2-IsoPs were associated with two-fold elevated risk of lung cancer among men, but not women [157]. The other study found that high urinary F_2-IsoPs was not related to risk of prostate cancer [158]. In contrast, two subsequent case–control studies found that urinary levels of F_2-IsoPs significantly increased in prostate cancer patients compared to controls [159,160]. A recent case–control study determined that urinary levels of F_2-IsoPs were significantly higher in gastric cancer patients compared to controls [161]. In a prospective study, occurrence of adenoma was not significantly associated with urinary levels of F_2-IsoPs and other three metabolites in the pathway [162]. In a very recent case–control study, the investigators found that an oxidative stress balance score which took into account of pro-oxidant and antioxidant exposure was significantly lower in colorectal adenoma patients compared to controls and this score was inversely correlated with plasma concentrations of F_2-IsoPs [163].

Taken together, these case–control studies conducted so far consistently found higher levels of F_2-IsoPs were associated with risk of cancer, including cancers of the breast, prostate, and stomach as well as colorectal adenoma. On the other hand, the findings from prospective studies have been few and inconsistent. The possible explanation for the inconsistent findings between case–control studies and prospective nested case–control studies is that it is not clear in case–control studies if oxidative stress leads to cancer or results from cancer.

In recent years, Dai and colleagues conducted the first study to prospectively examine both urinary F_2-IsoP and the metabolite 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP as biomarkers of breast cancer risk in a large-scale nested case–control study within the Shanghai Women's Health Study (SWHS), a population-based cohort study of 74,942 Chinese women between 40 and 70 years of age [164]. In this study, F_2-IsoPs nor 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP [165] significantly differed by breast cancer status. Levels of F_2-IsoPs and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP were actually related to a reduced risk of breast cancer among women with a BMI of <25. Among women with a BMI of <23, high F_2-IsoPs was associated with a reduced risk of breast cancer in a dose–response manner (p for trend, 0.006) with an OR of 0.46 (95% CI: 0.26–0.80) for the highest tertile vs. the lowest (p for interaction, 0.006). This reduction in risk appeared in both pre- and postmenopausal women. In contrast, however, F_2-IsoPs and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP were associated with an increased risk of breast cancer among women with a BMI of ≥25. The associations became stronger with increasing levels of BMI and were more significant for 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP compared to F_2-IsoPs. These findings indicate that the role of ROS in the development of breast cancer is different by BMI status. As discussed previously, numerous studies have consistently observed that overweight or obese men and women have a significantly elevated level of F_2-IsoPs, indicating women with a high level of BMI have an excessive production of ROS and, thus, are at high risk of oxidative stress [97–99]. Therefore, among overweight/obese women, high levels of F_2-IsoP-M and/or F_2-IsoPs may be related to an increased risk of breast cancer [166]. Conversely, among women with normal BMI, basal levels of ROS are necessary to trigger p53 activation, directly mediate apoptosis and induce senescence [167].

Additionally, F_2-IsoPs was found to increase the glucose-induced synthesis of TGF-β1, a critical tumor suppressor at initial stage [168]. Several protective factors for breast cancer risk, such as physical activity, parity (normal pregnancy) and preeclampsia, are also linked to significantly elevated levels of lipid peroxidation [169]. Thus, the role of F_2-IsoPs, lipid peroxidation, and oxidative stress are complex in the pathophysiology of cancer.

3.5. Isoprostanes, diet, and lifestyle

In general, increased levels of F_2-IsoPs are considered to be detrimental to human health. For that reason, numerous studies have focused on identifying dietary or lifestyle modifications that could alter levels of endogenous F_2-IsoPs. Dietary antioxidants have naturally been an area of intense research in the field. Unfortunately, the results of published studies often vary due to inherent differences in study design including factors such as antioxidant dose, duration of dietary supplementation, matrix analyzed (ie. urine, plasma, other), and IsoP analyzed (i.e. F_2-IsoPs or its urinary metabolite) [64,170]. This is particularly true for studies involving supplementation with Vitamin E (α-tocopherol), a commonly used antioxidant [170]. A dose-finding study of vitamin E actually helped to clarify the optimal parameters for studying vitamin E as it relates to the lowering of lipid peroxidation associated with oxidative stress [171]. This study determined that daily doses of 1600 IU or greater for at least 8 weeks were required to statistically reduce plasma levels of F_2-IsoPs. Lower doses have also been effective at lowering F_2-IsoPs in settings where levels of lipid peroxidation are expected to be elevated. For example, Block and colleagues have shown that 1000 mg/day vitamin C or 800 IU/day vitamin E for 2 months can lower levels of plasma F_2-IsoPs by 22% ($p = 0.01$) or 9.8% ($p = 0.46$), respectively, when baseline levels of F_2-IsoPs are high (>50 mg/ml), such as in obese populations [172]. Importantly, neither treatment decreased levels of F_2-IsoPs in individuals with normal baseline levels. In other studies, pre-treatment of skin with topical vitamin E reduced levels of F_2-IsoPs generated post-UV irradiation [173]. Also, supplementation with vitamin E acetate decreased both baseline and allergen-induced F_2-IsoPs in the brochoalveolar lavage fluid of atopic asthmatics [174]. Dorjgochoo et al. reported that urinary excretion rates of 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP, but not F_2-IsoPs, were lower in Chinese women who used a vitamin E supplement, as determined from diet and food questionnaire responses, compared to those who did not [120]. In that same population, plasma concentrations of several antioxidants (i.e., β-carotenes, both trans and cis β-carotenes, lycopene other than trans, 5-cis and 7-cis isomers, cis anhydrolutein, and cis β-cryptoxanthin) were inversely associated with 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP but not with F_2-IsoPs, whereas β-, γ-, and δ-tocopherols were positively associated with 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP but not with F_2-IsoPs.

A variety of antioxidants other than Vitamin E have been studied in relationship to F_2-IsoPs. Similarly to Vitamin E, other antioxidants tend to have the greatest effects in populations with an increased level of basal oxidative stress (ie. obese, asthmatics, etc.) than in normal healthy populations. For example, vitamin C has been shown to decrease pancreatic cancer tumor progression in pre-clinical models [175]. Results of a phase I clinical study of twice-weekly intravenous pharmacological doses of vitamin C, given in combination with gemcitabine, showed that levels of F_2-IsoPs decreased post-infusion in all patients tested (n

= 5) [176]. Anthocyanins, polyphenolic antioxidants typically found in dark fruits such as blackberries, strawberries, blueberries, blackcurrants, and cherries, also lower levels of F_2-IsoPs [177–180]. Consumption of tart cherry juice, and not placebo, lowers the observed increase in plasma F_2-IsoP levels following forearm I/R induced by blood pressure cuff inflation [177]. In another study of well-trained distance runners, subjects who consumed 250 g of blueberries per day for 6 weeks had reduced increases in plasma F_2-IsoPs following a 2.5 h run compared to controls [178]. Consumption of a blackcurrant juice drink (20% juice) also reduced basal levels of F_2-IsoPs in plasma in a population of healthy adults who typically consume <2 servings of fruit and vegetables per day [179].

Acetaminophen, similar to vitamin E and anthocyanins, is an electron donor capable of reducing radicals. Commonly, acetaminophen is known to modulate the activity of the COX enzyme by reducing the radical cation in the peroxidase site of the enzyme that is necessary for the synthesis of PGs. In recent studies, acetaminophen has been shown to modulate non-enzymatic lipid peroxidation [181,182]. The antioxidant properties of acetaminophen were recently studied in a human population with sepsis [183]. Levels of F_2-IsoPs were significantly lower in subjects who received acetaminophen during treatment to patients who did not.

Recently, the association between diet, typically reported through the use of food questionnaires, and F_2-IsoPs has been examined. In a large study of more than 5000 young and middle-aged Caucasian and African Americans followed for over the course of 20 years, diets high in fruits and vegetables and low in red meat were associated with lower levels of plasma F_2-IsoPs [184]. Diets with a higher intake of red meat showed increased levels of F_2-IsoPs. In a smaller study of premenopausal women (n = 258) participating in the 5 A Day for Better Health Program, women who met the daily requirement of at least five servings of fruits and vegetables had lower plasma levels of F_2-IsoPs [185]. In a study of 1005 pre- and post-menopausal Chinese women, however, consumption of cruciferous vegetables was not associated with lower urinary F_2-IsoPs or F_2-IsoP-M [186]. Other dietary factors that reduce levels of endogenous F_2-IsoPs include marine fish oil [101,187–197]. Marine fish oil is rich in the omega-3 PUFAs eicosapentaenoic acid (EPA) and DHA. Importantly, IsoP-like oxidation products can be generated from the oxidation of EPA and DHA. F_3-, E_3/D_3-, and A_3/J_3-IsoPs are generated from the peroxidation of EPA; the related products produced from DHA have been identified and are termed neuroprostanes, as DHA is enriched in the brain [198–202]. Interestingly, while arachidonic acid-derived F_2-IsoPs induce vasoconstriction and platelet aggregation and increase mean systemic arterial pressure, EPA-derived F_3-IsoPs are less potent vasoconstrictors and do not cause platelet aggregation or an increase in arterial pressure [193,203]. The biological activities of the neuroprostanes are less well studied.

The studies highlighted herein represent the many complex factors in the diet that can affect levels of lipid peroxidation in vivo in humans. It is important to note that even caloric restriction alone has been shown to decrease systemic F_2-IsoPs [113]. Thus, while quantifying levels of F_2-IsoPs and/or related compounds has given great insight into the physiological and pathophysiological roles of oxidative stress in human disease, care must be taken when interpreting results from any given study.

3.6. Isoprostanes and genetic disorders

Human genetic disorders are characterized by an abnormality in an individual's DNA. Genetic disorders can involve a mutation in a single gene or involve an entire chromosome. Oxidative stress has been implicated in the pathophysiology of multiple human genetic disorders. F_2-IsoPs levels are increased in patients with autism-spectrum disorders [204,205], Smith-Lemli-Opitz Syndrome (SLOS) [206], sickle cell anemia [207], cystic fibrosis [208–210], and in various inborn errors of metabolism [211]. Interestingly, levels of F_2-IsoPs are increased in the amniotic fluid of pregnancies carrying fetuses with Down syndrome [212]. Oxida-tive stress and IsoPs are well-studied in patients with Rett syndrome (RTT) by De Felice and colleagues [213–216]. RTT is a severe autism spectrum disorder caused by a mutation in the X-linked MECP2 gene that encodes the methyl-CpG-binding protein-2 in most cases. The disease primarily affects girls and is characterized by loss of acquired cognitive, social, and motor skills together with development of autistic behavior; symptoms typically appear between 6 and 18 months of age. Levels of F_2-IsoPs as well as levels of F_4-NPs and F_2-dihomo-IsoPs, derived from adrenic acid, are increased in plasma of RTT patients [213–216]. F_4-NPs correlate with disease severity and F_2-dihomo-IsoPs are elevated in early disease stages. Notably, in a cohort of 21 typical RTT patients supplemented with 20–40 mg/kg marine fish oil twice daily, levels of plasma F_4-NPs decreased by ~80% over the course of 12 months, with more than one-third of those patients reaching levels in the normal range compared to age-matched controls [214]. This finding that marine fish oil can reduced levels of oxidative stress biomakers in RTT patients has led to additional clinical trials in which supplementation with EPA and DHA at early stages in the disease has led to a partial clinical rescue of RTT [193].

4. Biological functions of the isoprostanes

The chemical synthesis of specific IsoPs by many different investigators around the globe has allowed the exploration of the biological activity of these molecules. Most studies have focused on the analysis of IsoPs in the form of the free fatty acid. Among the most studied of the IsoPs is 15-F_{2t}-IsoP. Much of the work surrounding the biological activity of this molecule has focused on the cardiovascular system. Recently, Bauer and colleagues published an elegant review regarding IsoPs in the cardiovascular system [217]. Briefly, 15-F_{2t}-IsoP is a potent vasoconstrictor in most vascular beds. Additionally, this molecule can modulate platelet activity, inhibit angiogenesis, and promote atherosclerosis by stimulating adhesion of monocytes and neutrophils to endothelial cells. These biological activities of 15-F_{2t}-IsoP are primarily mediated through interaction with the thromboxane (TP) receptor. Similarly, 15-E_{2t}-IsoP (also referred to as 8-iso-PGE_2 or iPE_2-III) mediates its functions through the TP receptor.

15-F_{2t}-IsoP and 15-E_{2t}-IsoP have been shown to exert biological activity through other PG receptors as well, including the PGE_2 (EP2-4) receptors and $PGF_{2\alpha}$ (FP) receptors [218–227]. Interestingly, the activity of the molecules can vary depending upon the receptor through which it acts. For example, while 15-F_{2t}-IsoP and 15-E_{2t}-IsoP are vaso-constrictors through the TP receptors, both molecules can induce vaso-dilation through the EP receptor. These opposing effects have been particularly well studied in the lung [219]. Additionally,

Chen and coworkers have observed both the vasoconstrictive and vasodilatory effects of 15-F_{2t}-IsoP and 15-E_{2t}-IsoP in murine ductus arteriosus (DA), a central vascular shunt that constricts soon after birth, allowing redirection of blood flow from the placenta to the lungs [228]. These authors showed that isolated term murine DA constricts in response to both 15-F_{2t}-IsoP and 15-E_{2t}-IsoP in a concentration-dependent manner and that this effect could be reversed by administration of a TP receptor antagonist. Interestingly, when TP receptors were blocked prior to IsoP exposure, both 15-F_{2t}-IsoP and 15-E_{2t}-IsoP produced potent vasorelaxation of the term DA. This action was determined to be due to activation of the EP4 receptor by the IsoPs. Further, the authors noted a difference between the actions of 15-F_{2t}-IsoP and 15-E_{2t}-IsoP in term and preterm isolated DA. Whereas 15-F_{2t}-IsoP induced vasoconstriction in both the term and preterm DA via the TP receptor, 15-E_{2t}-IsoP induced a dose-dependent vasodilation in the preterm DA and an EP4 receptor antagonist could reverse this effect. The authors explained that the response of the term and preterm vessels could be explained by a gestational shift in expression of the target receptors as EP4 receptors expression is upregulated in the preterm DA and maintained through birth while expression of the TP receptor is low in the preterm DA. In addition to these mouse studies, 15-F_{2t}-IsoP and 15-E_{2t}-IsoP were shown to be potent dose-dependent constrictors of the DA in chicken embryos, again acting through the TP receptor, and, interestingly, the presence of H_2O_2 can modify this response [229,230]. Historically, COX-derived PGs have been thought to play a major role in postnatal DA homeostasis, however, these findings suggest that IsoPs could play a role in the mediation of DA constriction. In related work, Comporti and colleagues found that F_2-IsoPs levels are significantly higher in neonates than in older infants and further are inversely correlated with gestational age [231]. More recently, this group has shown that levels of F_2-IsoPs were significantly decreased in a study of 43 preterm infants (gestational age <33 weeks) being treated with ibuprofen to induce DA closure. The authors suggest that ibuprofen is dual-acting as both an antioxidant and COX inhibitor and could be beneficial in the treatment of this at-risk population. Further studies are required to confirm these results and explore the pathophysiology of IsoPs in newborns where the DA fails to close.

In addition to the studies described above, 15-F_{2t}-IsoP and 15-E_{2t}-IsoP have been shown to produce hyperalgesia, an increased sensitivity to pain. 15-E_{2t}-IsoP, when injected into the hindpaw of rats, was shown to significantly reduce the withdrawal threshold in response to both mechanical and thermal stimuli, while 15-F_{2t}-IsoP reduced the withdrawal threshold to mechanical stimuli alone [232]. Further, 15-E_{2t}-IsoP enhanced the release of transmitters from isolated rodent sensory neurons; this effect could not be reversed by the treatment of COX inhibitors. Additionally, 15-F_{2t}-IsoP and 15-E_{2t}-IsoP enhanced the firing of C-nociceptors in pentobarbital-anesthetized rats [233]. Together these findings broaden our understanding of IsoP biology and pave the way for new areas of research regarding potential consequences of oxidative stress and lipid peroxidation.

The biological activities of other IsoPs have been explored as well. A_2/J_2-IsoPs, dehydration products of D_2/E_2-IsoPs, are reactive electro-philes that readily form Michael adducts with cellular thiols due to the presence of an α,β-unsaturated cyclopentenone ring structure. Current knowledge regarding the biological actions of these molecules has been reviewed

elsewhere [70,87,234–238]. The biological activity of isothromboxanes and isofurans has not been studied.

5. A look towards the future

The discovery of the IsoPs as products of non-enzymatic lipid peroxidation has been a major contribution to the field of lipid oxidation and free radical research. Twenty-five years after their initial discovery in human plasma, our knowledge regarding the formation of these potent molecules continues to expand, providing new insights into the nature of lipid peroxidation *in vivo* and revealing new biological activities. Basic research into the biochemistry, pharmacology, and metabolism of the IsoPs, coupled with clinical studies employing these molecules as biomarkers, will continue to provide important insights into the role of oxidant stress in human physiology and pathophysiology.

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Abbreviations

AD	Alzheimer’s disease
AKI	Acute kidney injury
aSAH	Aneurysmal subarachnoid hemorrhage
BMI	Body mass index
CAPG	Coronary artery bypass grafting
COX	Cyclooxygenase
CPB	Cardiopulmonary bypass
CSF	Cerebral spinal fluid
DA	Ductus arteriosus
DHA	Docosahexaenoic acid
EP	Prostaglandin E_2 receptor
EPA	Eicosapentaenoic acid
GSH	Glutathione
I/R	Ischemic-reperfusion
IsoF	Isofuran
IsoP	Isoprostane
NP	Neuroprostane

PAF	Platelet activating factor
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RTT	Rett syndrome
TBI	Traumatic brain injury
TP	Thromboxane receptor
WC	Waist circumference

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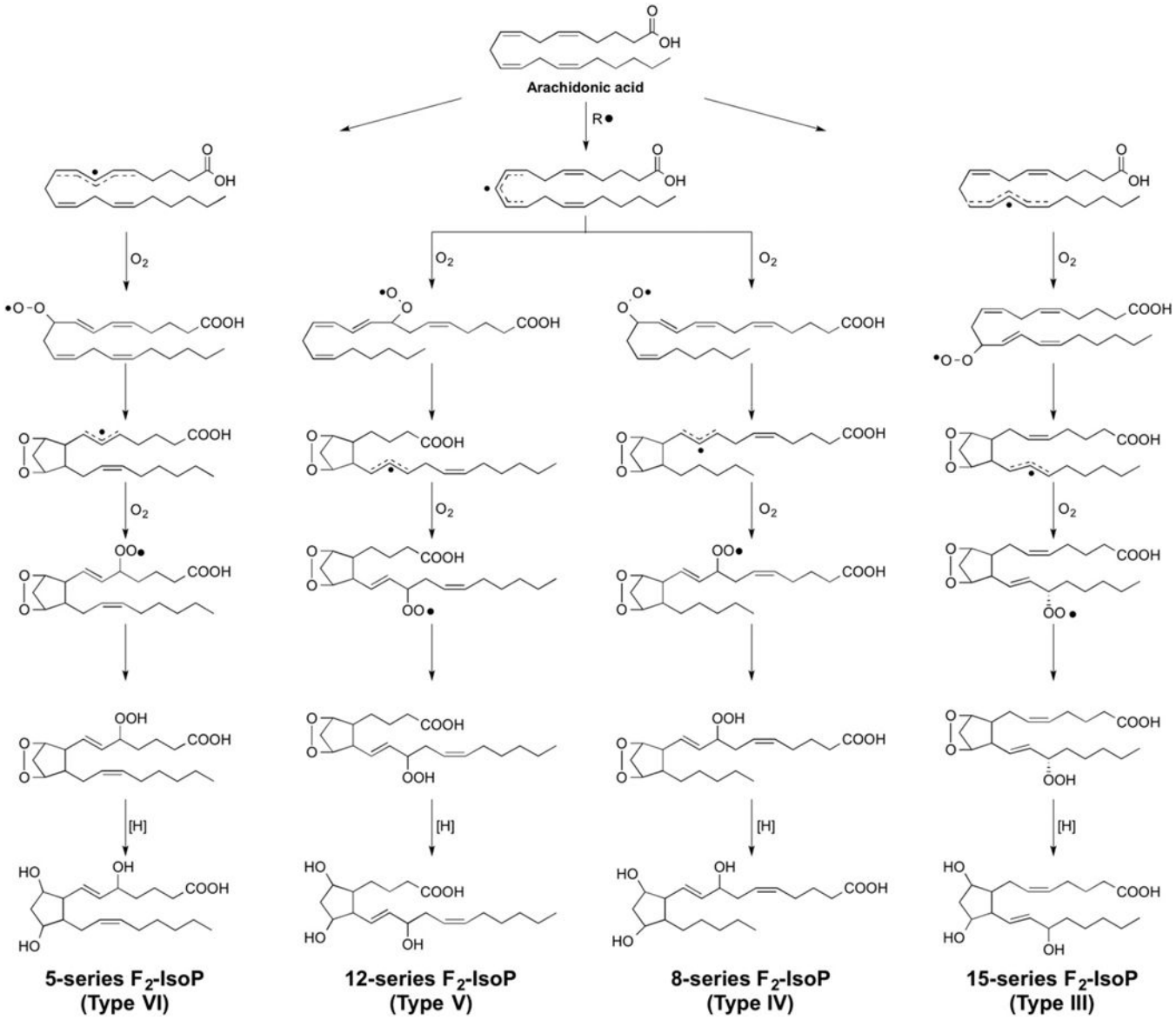

Fig. 1.

Mechanism of formation of F_2-isoprostanes from the free radical-catalyzed peroxidation of arachidonic acid. Two primary nomenclature systems have been developed to classify isoprostanes [8,9]. In the nomenclature system used throughout this manuscript, IsoP is used as the abbreviation for isoprostane and the four regioisomer classes are named according to the carbon number on which the side chain hydroxyl group is attached, with the carboxyl carbon being ***1*** [8]. This nomenclature system has been approved by the Eicosanoid Nomenclature Committee, which is sanctioned by JCBN of IUPAC. The alternative nomenclature system uses the abbreviation iP for isoprostane and the regioisomers are denoted as III–VI based upon the number of carbons between the omega carbon and the first double bond [9].

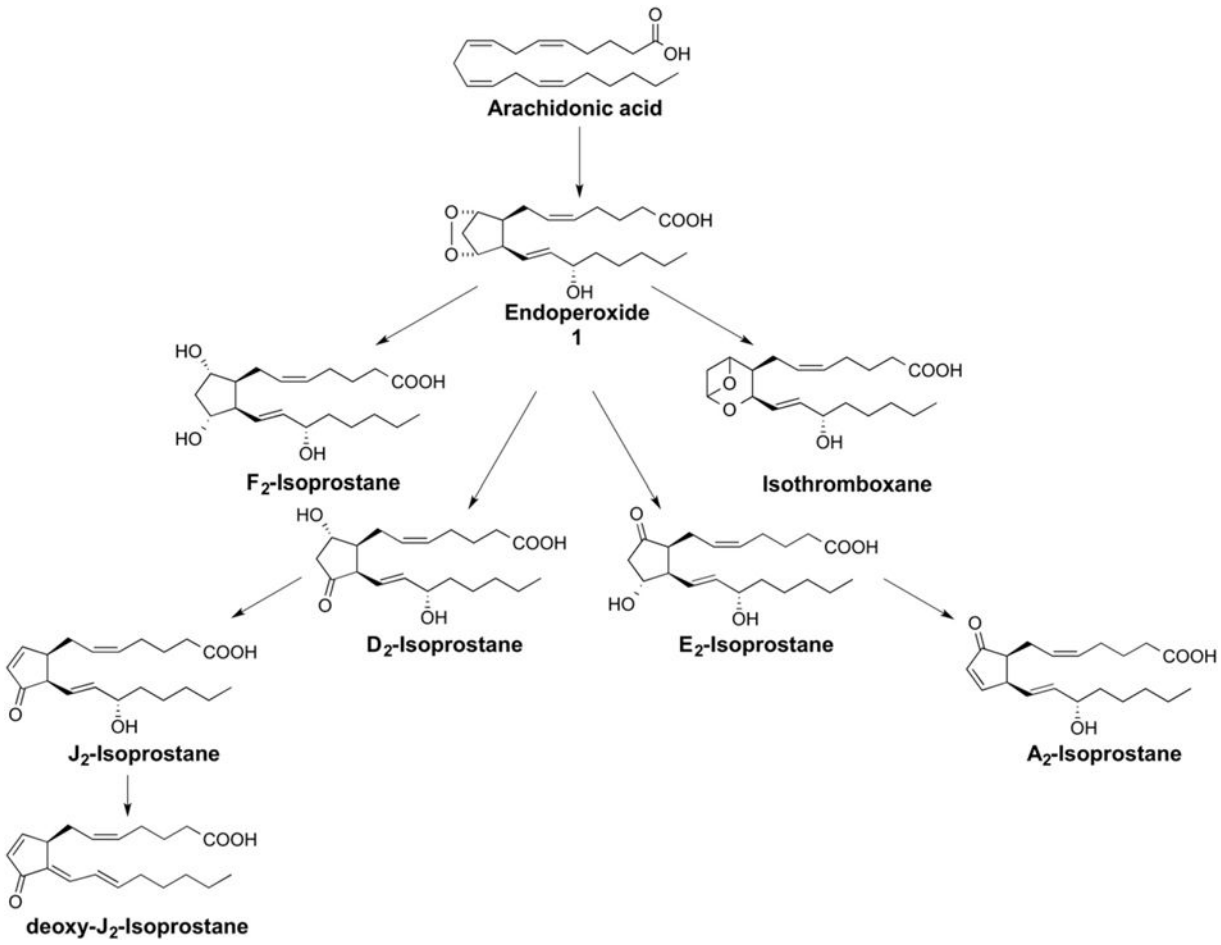

Fig. 2.

Arachidonic acid can be oxidized to many different classes of isoprostanes. F_2-isoprostanes are most commonly used as biomarkers of oxidative stress in human disease but E_2/D_2-isoprostanes have also been used as biomarkers of oxidative stress under certain conditions. Isothromboxanes as well as A_2/J_2-isoprostanes and deoxy-J_2-isoprostanes are also formed.

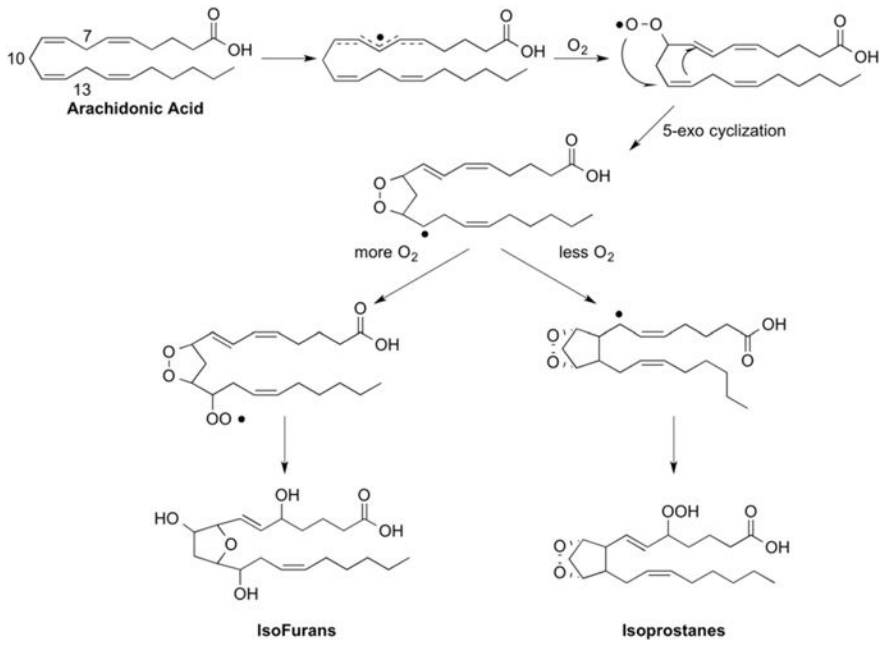

Fig. 3.

Under settings of increased oxygen tension isofurans are formed preferentially to isoprostanes from a common radical intermediate.

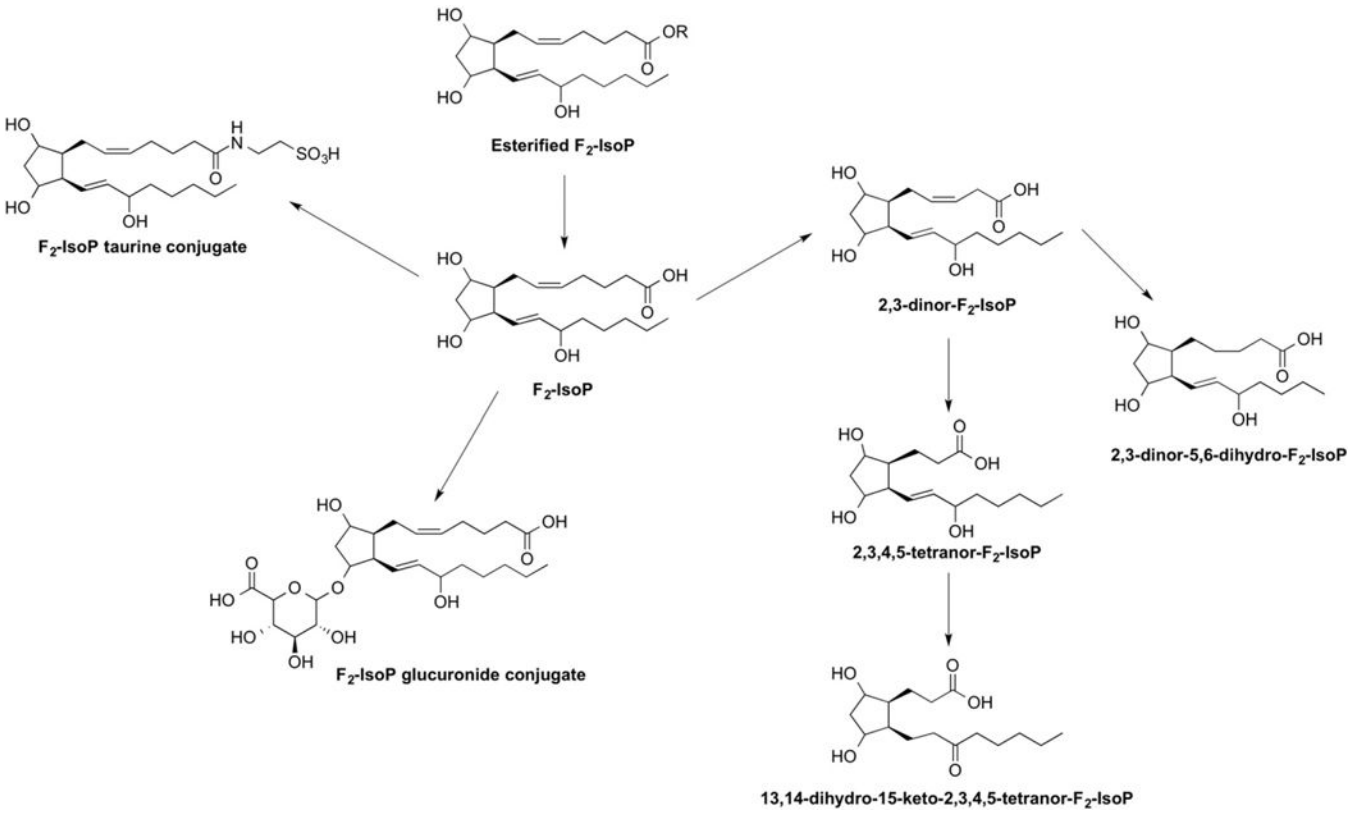

Fig. 4.
Urinary metabolites of F_2-isoprostanes.