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Original Research Communication

Different Patterns of Oxidized Lipid Products in Plasma and Urine of Dengue Fever, Stroke, and Parkinson's Disease Patients: Cautions in the Use of Biomarkers of Oxidative Stress

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Abstract

Many products of lipid oxidation have been associated with human diseases. These include F_2-isoprostanes (F_2-IsoPs), hydroxyeicosatetraenoic acid products (HETEs), and cholesterol oxidation products (COPs). Here we present measurements of F_2-IsoPs, HETEs, COPs, and arachidonate in single plasma samples of patients with acute (dengue fever and ischemic stroke) and chronic (Parkinson's) diseases, and in age-matched study controls. Urine samples were collected for F_2-IsoPs analysis. Our analysis demonstrated elevated F_2-IsoPs levels in ischemic stroke, HETEs in Parkinson's disease, dengue fever, and ischemic stroke, and COPs in Parkinson's disease and dengue fever patients, as compared with those in age-matched study controls. Strong but complex correlations were observed between levels of certain oxidized lipid products and age. The relations between various oxidized lipids and dengue fever, stroke, and Parkinson's disease are discussed in relation to the selection and application of biomarkers of oxidative lipid damage, in particular the need for corrections for age and lipid levels. *Antioxid. Redox Signal.* 11, 407–420.

Introduction

MEASUREMENT of oxidized lipids has become increasingly important to help in understanding the dynamics of oxidative stress in human diseases. For example, F_2-isoprostanes (F_2-IsoPs) are a group of metabolites (64 regioisomers) produced by nonenzymatic free radical oxidation of arachidonic acid. Some F_2-IsoPs are potent vasoconstrictors that may be involved in the pathology of stroke, diabetes mellitus, and atherosclerosis (2, 26, 28). Arachidonic acid also can be oxidized by free radicals, lipoxygenases, and cytochrome P450 enzymes to produce epoxyeicosatrienoic acid products (EETs) or hydroxyeicosatetraenoic acid products (HETEs) (8, 13, 48, 54). Although different types of HETE isomers have been described (such as 5-, 8-, 9-, 11-, 12-, 15-, 20-HETE), the precise roles of these isomers *in vivo* are poorly understood. Recently some of these isomers have been linked with vascular function and cancer [*e.g.*, 20-HETE is reported to be a vasoconstrictor in the cerebral circulation (39); in-

creased 9-HETE was observed in coronary artery disease (41); and 5-, 8-, 12-, and 15-HETE are involved in tumor development (35)].

Another group of oxidized lipids that has drawn interest is the cholesterol oxidation products (COPs). Cholesterol can be oxidized *via* enzymatic P450 reactions to give 7α-, 24-, 25-, and 27-hydroxycholesterol or by nonenzymatic free radical reactions to give 7β-hydroxycholesterol and 7-ketocholesterol (7). Some COPs can be formed by both pathways (*e.g.*, 7α-hydroxycholesterol is formed as a precursor to bile synthesis and also by free radical attack). COPs are found in different forms (esterified, sulfated, conjugated, and free *in vivo*) (7), and some of the COPs appear to be specific to certain disease models. For example, 24- and 27-hydroxycholesterol are proposed to be involved in brain vascular function (4), and others have suggested a role of COPs in coronary artery diseases and in the development of dementia and stroke (37).

Simultaneous measurement of different lipid oxidation products may allow a better understanding of the signifi-

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cance of these molecules with respect to human diseases (2, 14, 15, 22, 30). Our group has previously reported techniques for the measurement of multiple oxidation products of arachidonic acid and cholesterol in a single plasma sample (22). Such a method is valuable, first because clinical samples are often limited. Second, the stated levels, and ranges of levels, of various biomarkers vary between laboratories, many depending on the exact analytic method applied, and so it is often difficult to compare clinical disease sample results between different published reports. In this study, by using a standardized analytical protocol, we measured a range of oxidized lipid biomarkers (F_2-IsoPs, HETEs, COPs) in body fluids from patients with acute (dengue fever or ischemic stroke) and chronic (Parkinson's disease) diseases, and study controls. Our findings provide further insight into the relation between lipid oxidation products and human diseases, and emphasize the careful controls that are needed when measuring such products and presenting the data.

Materials and Methods

Materials

High purity grade ($\geq$95%) heavy labeled standards of F_2-isoprostanes (F_2-IsoPs), 8-iso-PGF$_{2\alpha}$-d$_4$, IPF$_{2\alpha}$-VI-d$_4$, and IPF$_{2\alpha}$-IV-d$_4$, hydroxyeicosatetraenoic acid (HETEs) standards, 5(S)-HETE-d$_8$, 12(S)-HETE-d$_8$, 15(S)-HETE-d$_8$, 20-HETE-d$_6$, and arachidonic acid-d$_8$ were obtained from Cayman Chemicals, (Ann Arbor, MI). Oxysterol standards (purity $\geq$95%), 7β-OH cholesterol-d$_7$, 7α-OH cholesterol-d$_7$, 26 (27)-OH cholesterol-d$_5$, and 7-keto-cholesterol-d$_7$, were purchased from CDN Isotopes, Canada, and 24-OH cholesterol-d$_7$, from Medical Isotopes Inc. (Pelham, NH).

Analytic grade formic acid was purchased from Lancaster (England), ammonium hydroxide, potassium hydroxide, butylated hydroxytoluene (BHT), hydrochloric acid, ethanol, acetic acid from Merck (Darmstadt, Germany), and hexane from Tedia (Fairfield, OH). HPLC-grade methanol was purchased from EM Science (Darmstadt, Germany), and iso-octane and ethyl acetate, from Fisher Scientific (UK). *N,O*-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + TMCS) silylating agent was obtained from Pierce Chemicals (Rockford, IL). Pentafluorobenzylbromide (PFBBr) and *N,N*-diisopropylethylamine (DIPEA) were purchased from Sigma Chemicals (St. Louis, MO). Oasis Mixed Anion Exchange (MAX) cartridges for solid-phase extraction (SPE) were from Waters Corp. (Milford, MA).

Study design and patients

The study was carried out in a single center (Clinical Trial Unit, National University Hospital, Singapore), where selection of healthy controls was randomized. For Parkinson's disease, dengue fever, and stroke it was designed as an age-matched case–control study. The gender ratio (male/female) for healthy controls and Parkinson disease patients was 1:1, whereas for dengue fever and stroke patients, it was 1:2. No restriction was placed on the diet of the healthy control and the recruited patients (except to exclude the use of dietary supplements), but where possible, all were asked to fast overnight before blood and urine sampling to minimize any absorption of COPs or other oxidized lipid products (50). We included patients with Parkinson's disease, ischemic stroke,

and dengue infection from the National University Hospital, Singapore. All patients provided informed consent before recruitment to the study. Acute ischemic stroke was diagnosed clinically and supported by neuroimaging modalities such as computed tomography and magnetic resonance imaging. We included patients with first-ever stroke with a National Institute of Health Stroke Severity (NIHSS) score exceeding 6 who were seen within 24 h from the onset of their symptoms. Acute dengue infection was diagnosed in patients who manifested a fourfold increase in IgG antibodies against dengue, measured in acute and convalescent sera. Parkinson's disease was diagnosed in patients who fulfilled the United Kingdom Parkinson's Disease Society Brain Bank criteria in the presence of bradykinesia and at least one of the following: muscular rigidity, rest tremor, postural instability unrelated to primary visual, cerebellar, vestibular, or proprioceptive dysfunction. We recruited healthy controls who did not smoke and who were not taking any medications or dietary supplements. The study protocol was approved by the Institutional Review Board of the National University Hospital.

Sample preparation

Samples of blood and urine of healthy volunteers and Parkinson disease patients were collected in the morning. As for dengue fever and ischemic stroke patients, blood and urine samples were taken on the day of clinical diagnosis before any clinical intervention and also (where possible) after recovery. Venous blood was collected into Na-EDTA blood tubes that were primed with 15 μl of 5 mM indomethacin dissolved in ethanol. Plasma was separated immediately by centrifugation and then placed into tubes with 20 μl/ml plasma of 2 mM BHT (in ethanol). The samples were stored at $-80°$C and were analyzed within 6 months of sample collection.

Before analysis, the plasma samples were thawed at room temperature. Mixed heavy isotopes, 8-iso-PGF$_{2\alpha}$-d$_4$, IPF$_{2\alpha}$-VI-d$_4$, 5(S)-HETE-d$_8$, 12(S)-HETE-d$_8$, 15(S)-HETE-d$_8$, 20-HETE-d$_6$ and arachidonic acid-d$_8$, and 7β-OH cholesterol-d$_7$, 7α-OH cholesterol-d$_7$, 24-OH cholesterol-d$_7$, and 26 (27)-OH cholesterol-d$_5$, and 7-keto-cholesterol-d$_7$, all prepared in ethanol, were added to plasma and mixed. To measure (22) the total (free+esterified) form of oxidized lipids (F_2-IsoPs, HETEs, and COPs) and total arachidonate, 1 ml plasma was hydrolyzed at 37°C for 30 min with 1 ml of 1 M potassium hydroxide prepared in methanol for the release of esterified lipids. Afterwards, 0.5 ml methanol, 0.2 ml of 5 M HCl, and 2.5 ml of 40 mM formic acid (pH 4.6) were further added and mixed. For measurement of free forms in plasma and urine (22, 23) for F_2-IsoPs and in plasma for HETEs, 1 ml of formic acid (40 mM, pH 4.5) was added to 1 ml of sample, mixed and then immediately processed by SPE. For standardizing the dilution of urine, creatinine levels were measured by using Sigma Diagnostic kit (St. Louis, MO), and total cholesterol levels, by the National Referral Laboratory (NRL), Singapore.

Extraction and derivatization of oxidised lipid products

The prepared samples were extracted and derivatized by using a previously described method (22). In brief, MAX SPE cartridges were used to purify the prepared samples, by

washing with 2 ml of 2% ammonium hydroxide and then by 2 ml of methanol: 20 mM formic acid (pH 4.6) mix (40:60 vol/vol). Afterward, different solvents were sequentially added for the elution of COPs, and F$_2$-IsoPs, HETEs, and arachidonate. COPs were eluted with 2 ml of hexane followed by 2 ml of hexane:ethyl acetate (70:30 vol/vol). The two fractions were combined for derivatization for GC-MS analysis. Thereafter, total F$_2$-IsoPs, total HETEs, and total arachidonate were eluted with 2 ml of hexane/ethanol/acetic acid (20:29.4:0.6 vol/vol). The procedure described was repeated for extraction of free F$_2$-IsoPs and HETEs in plasma and urine.

The collected samples were completely dried under ultra–high-purity nitrogen gas. Samples for COPs measurement were derivatized with 50 μl pyridine and 50 μl N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) and incubated for 2 h at room temperature. The derivatized mix was then dried and reconstituted in 30 μl undecane with 5 μl BSTFA + 1%TMCS before GC-MS analysis. Samples for total F$_2$-IsoPs, total HETEs and total arachidonate measurement were derivatized with 15 μl DIPEA (10% vol/vol acetonitrile) and 30 μl PFBBr (10% vol/vol acetonitrile) at room temperature for 30 min and dried under nitrogen gas. Acetonitrile (20 μl) and BSTFA with 1% TMCS (40 μl) were then added and incubated at room temperature for 2 h. The derivatized samples were then dried and reconstituted in 70 μl iso-octane and incubated at room temperature for 20 min. Before GC-MS analysis, an aliquot of 5 μl of the sample was taken into another vial and diluted with 195 μl of iso-octane for total arachidonate measurement, and the rest was used to measure total F$_2$-IsoPs and total HETEs.

Analysis with gas chromatography–mass selective detection

F$_2$-IsoPs, HETEs, and arachidonate. The derivatized samples for F$_2$-IsoPs, HETEs, and arachidonate were analyzed with a mass selective detector (Hewlett-Packard 5973N; Agilent Technologies,) connected to a gas chromatograph (Hewlett-Packard 6890; Agilent Technologies, Santa Clara, CA), fitted with an automatic sampler and a computer workstation. The temperature settings were programmed (22). The mass spectrometer was used in the negative chemical ionization (NCI) mode set at selective ion monitoring (SIM), and chromatographic separations were carried out on a fused silica capillary column coated with cross-linked 5% phenylmethylsiloxane (HP-5; Agilent Technologies). Quantitation was achieved by relating the peak area of the total and free forms of F$_2$-IsoPs or HETEs, and total arachidonate with their respective deuterated internal standard peaks (22).

Cholesterol oxidation products. For measurement of COPs, a mass selective detector (Hewlett-Packard 5975; Agilent Technologies) interfaced with a gas chromatograph (Hewlett-Packard 5890 II) and equipped with an automatic sampler and a computer workstation was used. Separations were carried out on a fused silica capillary column coated with cross-linked 5% phenylmethylsiloxane (Ultra 2, Agilent Technologies), and the temperature settings were programmed (22). The detector was set at electron ionization (EI) mode, and measurement was performed by SIM. Quantification of COPs

was calculated by comparing peak area of each compound with the deuterated internal heavy standard.

Statistical analysis

All analysis was performed by using GraphPad Prism version 5.0 for Macintosh (GraphPad Software, San Diego, CA). Student's t test was performed between healthy subjects and for all illnesses. The significance of onset to recovery stage for dengue and stroke patients was tested with analysis of variance at confidence level of 95%. Spearman's ranked correlation was performed between age and F$_2$-IsoPs, HETEs, arachidonate, and COPs, and Pearson's correlation, between urinary F$_2$-IsoPs, plasma F$_2$-IsoPs, and arachidonate at 95% confidence interval.

Results

The purpose of this article is to examine the levels of various oxidized lipid biomarkers in healthy controls and patients with examples of acute diseases (dengue fever and recent-onset ischemic stroke) and a chronic disease (Parkinson's disease). All these diseases are complex and multifactorial, which must be borne in mind when evaluating the results.

Before beginning studies on clinical samples, we determined whether age or gender affects levels of F$_2$-IsoPs, arachidonate, and COPs (Fig. 1, Tables 1 and 2) in healthy controls. No significant gender effects were found (data not shown). The data for plasma arachidonate and cholesterol were initially ranked according to age, and Spearman's correlation study was performed for every 5-year increment starting from 25 years. Graphic linear regression and correlation significance was found to change at 50 ± 5 years. Hence the data are presented as three groups, all (25–86 years), younger than 50 years (25–49 years), and 50 years and older (50–86 years). A significant positive correlation was found between urinary F$_2$-IsoPs and age (Fig. 1). This correlation was observed in the age range 25–49 years but not in the range from 50 to 86 years. By contrast, little correlation was shown between plasma total, esterified, and free F$_2$-IsoPs and age (Table 1). After arachidonate adjustment of the data, plasma total and esterified F$_2$-IsoPs still showed only a weak correlation with age (Table 1). Significant negative correlations between age (25–86 years) and plasma arachidonate, total cholesterol, 7β-hydroxycholesterol, and 27-hydroxycholesterol were demonstrated (Fig. 1 and Table 2), but in this case, the decrease for all four biomarkers was much more obvious in the subjects aged 50 or older (Fig. 1 and Table 2). Significant negative correlations for 7β-hydroxycholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol with age (25–86 years) were seen even after adjustment for total cholesterol (Table 2). The decrease was noticeable in 50+-aged subjects for 24-hydroxycholesterol and subjects younger than 50 years for 27-hydroxycholesterol.

These data illustrate the importance of careful age matching of groups when performing clinical studies. Thus we eliminated data for healthy subjects younger than 40 years for comparison with the acute and chronic disease patients, in whom the median age was 56 years, and range, 40–69 years. No gender difference was found in the level of the oxidized lipids measured in the age-matched control groups and disease patients. Our correlation analysis also showed
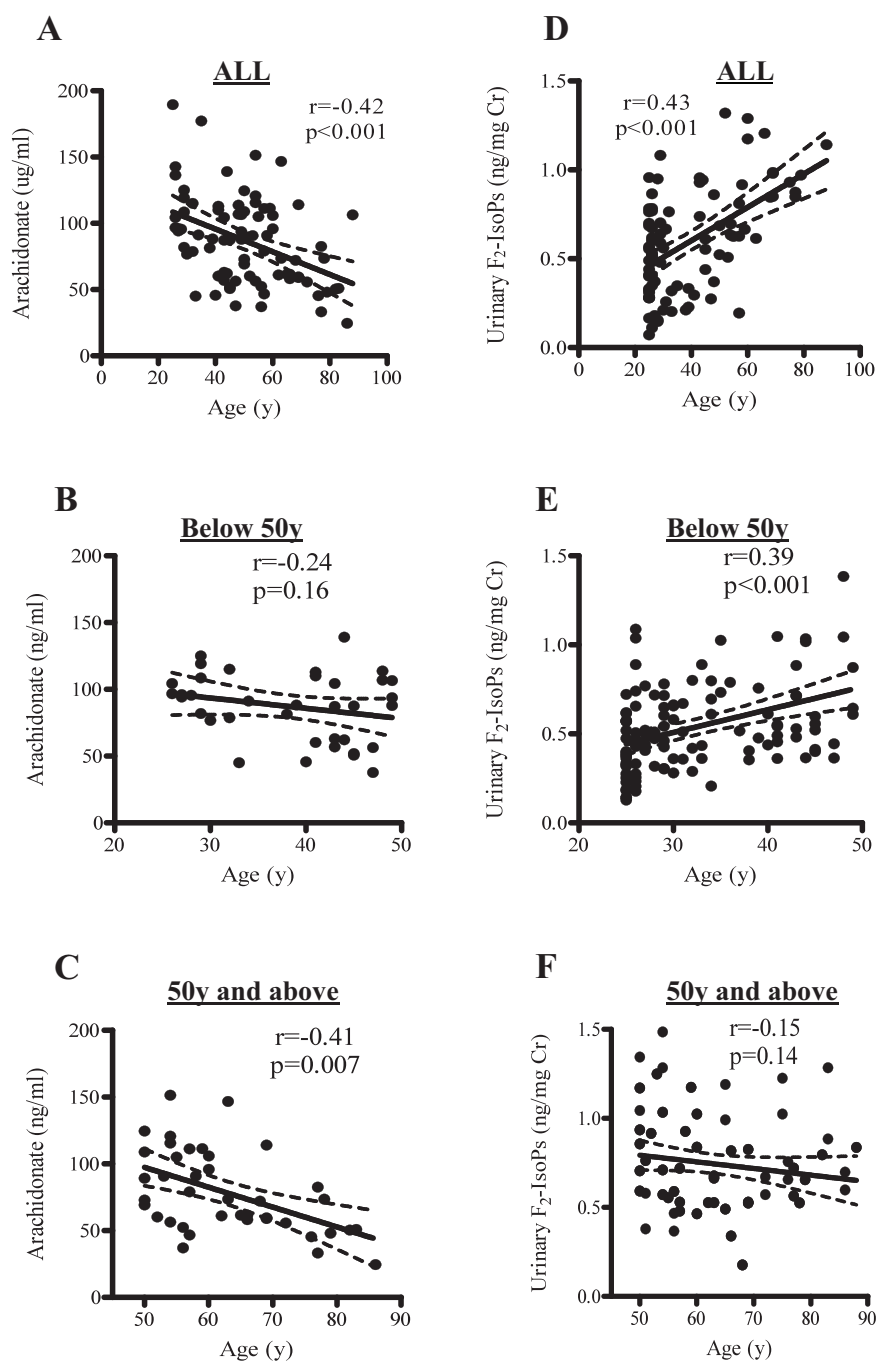
FIG. 1. Spearman's correlation of oxidized lipid products in plasma of healthy controls. (**A**) Arachidonate *vs.* age [25–86 years (y); $n = 81$]. (**B**) Arachidonate *vs.* age younger than 50 y (25–49 y; $n = 40$). (**C**) Arachidonate *vs.* age 50 y and older (50–86 y; $n = 41$). (**D**) Urinary F_2-IsoPs *vs.* age (25–86 y; $n = 92$). (**E**) Urinary F_2-IsoPs *vs.* age younger than 50 y (25–49 y; $n = 52$). (**F**) Urinary F_2-IsoPs *vs.* age 50 y and older (50–86 y; $n = 40$). Mean ± SEM at 95% confidence level for each correlation is indicated by *dotted line.* ALL indicates all subjects.

plasma total F_2-IsoPs to be positively correlated with plasma arachidonate and urinary F_2-IsoPs levels (Fig. 2) over the whole age range.

F_2-isoprostanes and arachidonate in disease

Plasma F_2-IsoPs levels (total, esterified, or free) of Parkinson's disease subjects showed no significant difference from healthy age-matched controls (Fig. 3). It should be noted that these patients were being treated with various drugs (*e.g.*, L-DOPA), which could perhaps have pro- and antioxidant properties against lipid peroxidation (1, 14, 33, 42, 43). Unlike for normal controls, a significant positive correlation was found for Parkinson's disease between age and plasma total F_2-IsoPs ($r = 0.52; p < 0.01$). Correlations also were noted between urinary F_2-IsoPs and age ($r = 0.41; p < 0.05$), between plasma total F_2-IsoPs and arachidonate ($r = 0.67; p < 0.001$), and between urinary F_2-IsoPs and plasma free F_2-IsoPs ($r = 0.41; p < 0.05$), but this did not cause F_2-IsoPs levels to become significantly greater than those in normal controls. Parkinson's disease did not change arachidonate levels compared with those in healthy controls (Fig. 3). Analysis of the age-matched controls (40–69 years) did not show any significant correlation between age, F_2-IsoPs, HETEs, arachidonate, and COPs, and between urinary F_2-IsoPs and plasma F_2-IsoPs.

TABLE 1. SPEARMAN'S CORRELATION OF BETWEEN PLASMA F_2-ISOPROSTANES, ARACHIDONATE, AND AGE

	All subjects			<50 years			≥50 years		
	n	r	p	n	r	p	n	r	p
Total F_2-IsoPs	140	0.21	ns	80	0.23	ns	60	−0.24	ns
Esterified F_2-IsoPs	140	0.02	ns	80	0.03	ns	60	−0.28	ns
Free F_2-IsoPs	140	−0.06	ns	80	0.11	ns	60	−0.01	ns
Arachidonate	81	−0.42	<0.001	40	−0.24	ns	41	−0.41	<0.01
Total F_2-IsoPs/arachidonate	81	0.29	ns	40	0.25	ns	41	0.09	ns
Esterified F_2-IsoPs/arachidonate	81	0.16	ns	40	0.19	ns	41	−0.02	ns

Gender ratio (male : female) for all subjects is 1 : 1, <50 years is 1 : 2 and ≥50 years is 1 : 1.
ns, not significant at 95% confidence interval.

By contrast, F_2-IsoPs (total, esterified, adjusted for arachidonate, and urinary) levels were significantly higher than those in age-matched controls during the first 24 h of acute stroke (onset) and tended to be higher even after recovery from stroke (Fig. 3). Onset in this context means when the patient was first admitted to hospital with symptoms, and blood and urine samples could be drawn, and is not necessarily when the stroke began. Even for the patients who recovered (those who no longer showed symptoms of neurologic deterioration and no further vascular events), levels of these F_2-IsoPs (total, esterified, and adjusted for arachidonate) did not decrease completely to the healthy control range, although some decline was seen (Fig. 3).

Onset of dengue fever refers to the development of severe symptoms leading to hospital admission. Dengue fever (onset) did not cause any change of esterified F_2-IsoPs or total F_2-IsoPs, but interestingly, an increase in plasma free F_2-IsoPs and urinary F_2-IsoPs was noted, both of which decreased in the recovered patients (Fig. 3). Such change might be due to possible modification of renal function during the dengue fever or to changes in lipolysis that could have accelerated the hydrolysis of intact F_2-IsoPs esterified to phospholipids (21, 22, 44).

Hydroxyeicosatetraenoic acid products in disease

Total and esterified HETEs levels were found to be about double the age-matched healthy control level for stroke (onset) subjects, even after arachidonate adjustment. At recovery stage, the levels significantly decreased to values similar to these of the healthy controls (Fig. 4). Free HETEs level did not change in stroke (Fig. 4).

Onset of dengue fever also was associated with high total and esterified HETEs, and the total and esterified arachidonate-adjusted values compared with the age-matched healthy control. At the recovery stage, these levels again decreased close to the healthy control level. On the contrary, free HETEs were significantly lower in dengue fever (onset) group compared with the healthy age-matched controls, and the level decreased even further on recovery. Elevated levels of total and esterified HETEs and total and esterified arachidonate-adjusted HETEs in Parkinson's disease patients were observed in comparison to the healthy control, but the free HETEs level did not differ (Fig. 4).

Cholesterol oxidation products in disease

Acute stroke patients had lower 7β-hydroxycholesterol levels compared with the age-matched controls, and these

TABLE 2. SPEARMAN'S CORRELATION COEFFICIENT OF BETWEEN PLASMA CHOLESTEROL OXIDATION PRODUCTS, TOTAL-CHOLESTEROL, AND AGE

	All subjects			<50 years			≥50 years		
	n	r	p	n	r	p	n	r	p
7β-Hydroxycholesterol	95	−0.42	<0.001	56	−0.15	ns	39	−0.50	<0.01
7-Ketocholesterol	95	0.15	ns	56	0.03	ns	39	−0.03	ns
7α-Hydroxycholesterol	95	−0.17	ns	56	−0.19	ns	39	−0.18	ns
24-Hydroxycholesterol	95	0.07	ns	56	0.01	ns	39	−0.04	ns
27-Hydroxycholesterol	95	−0.39	<0.001	56	−0.22	ns	39	−0.58	<0.01
Total-cholesterol	75	−0.26	<0.05	43	0.19	ns	32	−0.67	<0.001
7β-Hydroxycholesterol/cholesterol	75	−0.29	<0.05	43	−0.29	ns	32	−0.06	ns
7-Ketocholesterol/cholesterol	75	0.07	ns	43	0.004	ns	32	0.10	ns
7α-Hydroxycholesterol/cholesterol	75	−0.19	ns	43	−0.26	ns	32	−0.04	ns
24-Hydroxycholesterol/cholesterol	75	−0.26	<0.05	43	−0.16	ns	32	−0.42	<0.05
27-Hydroxycholesterol/cholesterol	75	−0.43	<0.001	43	−0.54	<0.001	32	0.05	ns

Gender ratio (male : female) for all subjects is 1 : 1, <50 years is 1 : 1 and ≥50 years is 1 : 1.
ns, not significant at 95% confidence interval.

A

r=0.54
p<0.001

B

r=0.38
p<0.001
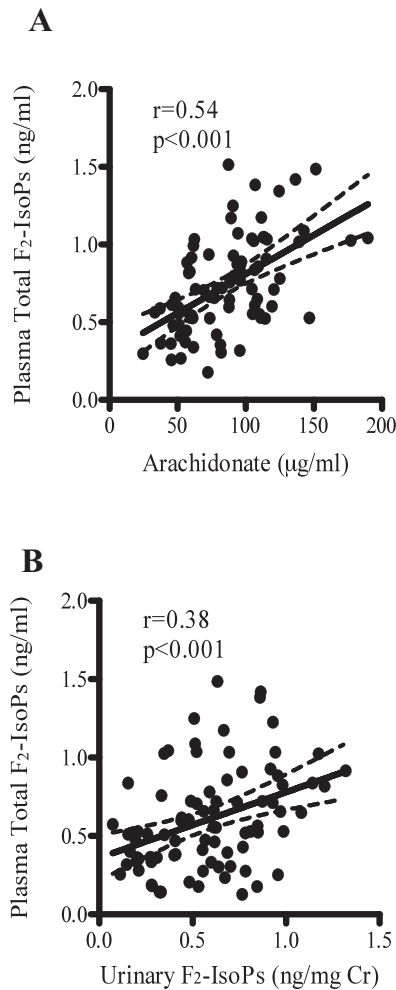
FIG. 2. Pearson's correlation of oxidized lipid products in plasma of healthy controls (25–86 years). (**A**) Plasma total F_2-IsoPs *vs.* arachidonate ($n = 81$). (**B**) Plasma total F_2-IsoPs *vs.* urinary F_2-IsoPs ($n = 92$). Mean ± SEM at 95% confidence level for each correlation is indicated by *dotted line.*

remained low even at the recovery stage (Fig. 5). However, it is important to relate these effects to disease-induced changes in cholesterol levels. After an acute stroke, the total plasma cholesterol levels tended to be elevated compared with those of age-matched controls, but at recovery stage, the levels significantly decreased (Fig. 6). Levels of 7β-hydroxycholesterol adjusted with cholesterol levels still tended to be low compared with those of the healthy controls, but the levels tended to increase after recovery. Other products of COPs and COPs standardized with total cholesterol were not affected by stroke (Figs. 5 and 6).

Compared with those in the age-matched healthy controls, acute dengue fever infection significantly lowered 7β-hydroxycholesterol, 7-ketocholesterol, 7α-hydroxycholesterol, and 24-hydroxycholesterol levels, whereas at recovery, the levels increased closer to the levels of age-matched controls (Fig. 5). However, onset of dengue fever caused markedly lower cholesterol levels, which increased after recovery. As a result, 7β- and 7α-hydroxycholesterol levels adjusted with total cholesterol were still lower but to a smaller extent, and 7-ketocholesterol and 27-hydroxycholesterol adjusted with total cholesterol were actually higher than those in the healthy control (Fig. 6). At the recovery stage of dengue fever, 7α-hydroxycholesterol and 27-hydroxycholesterol adjusted with total cholesterol decreased, the latter near to the levels of the healthy controls (Fig. 6).

Parkinson's disease subjects showed significantly higher 7β-hydroxycholesterol, 7-ketocholesterol, and 27-hydroxycholesterol, but low 24-hydroxycholesterol levels compared with the healthy controls (Fig. 5). No difference in cholesterol level was found in Parkinson's disease compared with the healthy control, and so adjustment for total cholesterol also showed significantly high 7-ketocholesterol and 27-hydroxycholesterol and low 24-hydroxycholesterol levels compared with those of the healthy controls (Fig. 6).

Discussion

This study showed that different types of diseases (using examples of two acute diseases and one chronic one) can lead to altered levels of oxidized lipids. It highlights the advantages of a single methodologic approach for the analysis, which minimizes the discrepancy of values due to application of different analytic methods. Our studies were intended to observe potential differences in biomarkers of lipid oxidation in different diseases and are not intended at this stage to investigate the relation of biomarkers to the disease process or to propose diagnostic and prognostic assays, especially given the complex nature of Parkinson's disease (effects of various treatments, especially), dengue infection, and stroke and the wide variations in severity and outcome between patients. Further detailed studies are being conducted in larger clinical settings for each disease.

Nevertheless, our data draw attention to several important issues when performing any studies of this type. First, levels of many of the oxidized lipid products are affected by age, in particular, F_2-IsoPs, 7α-hydroxycholesterol and 27-hydroxycholesterol, and the effects vary in different age bands. The literature contains conflicting reports on the relation of age and F_2-IsoPs, where an increase in plasma and urine was reported (3, 47, 49), a decrease in urine (19), or no association with age in plasma (10, 25) and in exhaled breath condensate (29). In our study, healthy controls also showed an increase in urinary F_2-IsoPs with age (25–86 years) but
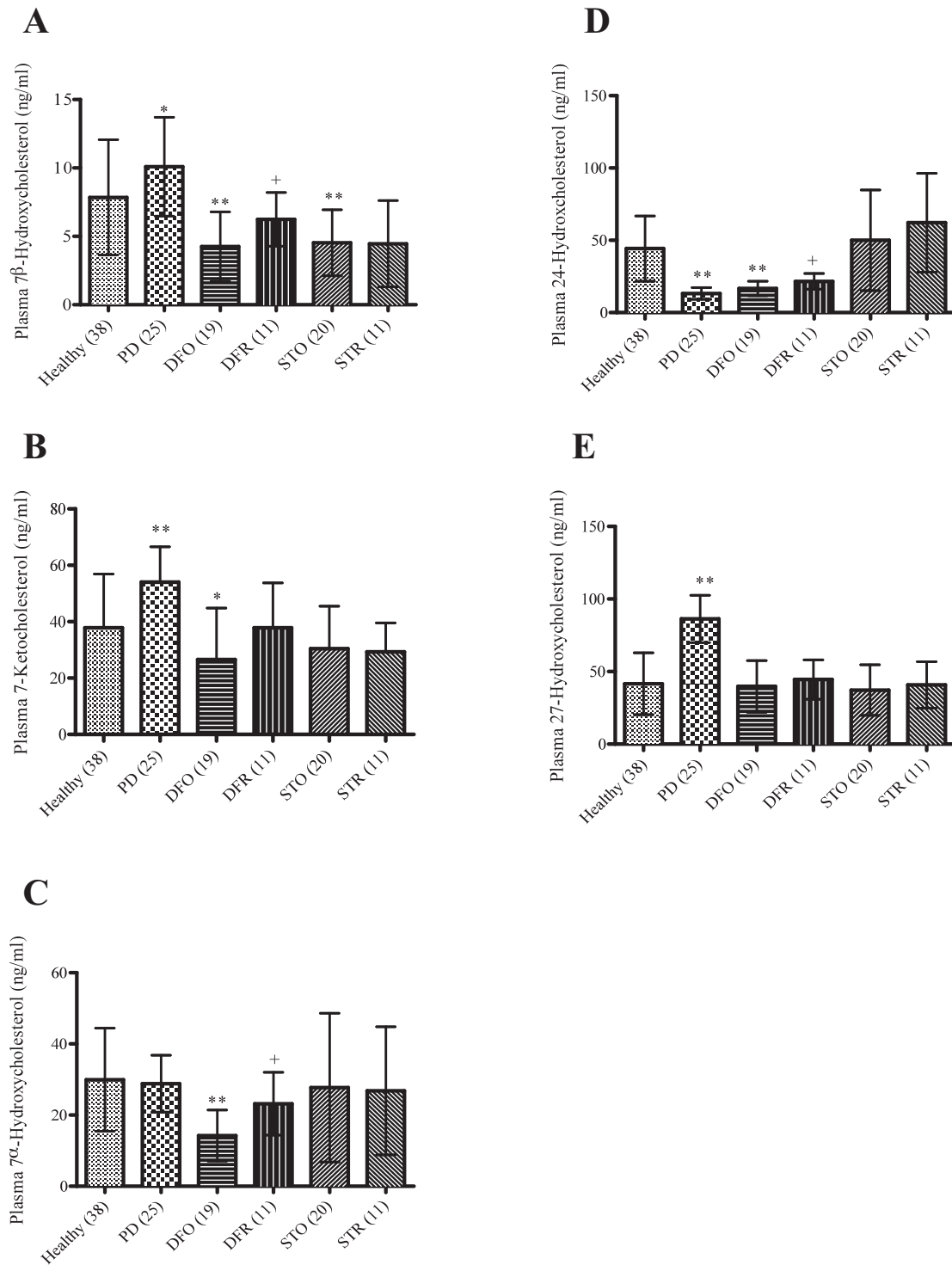
FIG. 3. Comparison of F_2-isoprostane levels between age-matched healthy controls and patients with acute and chronic diseases. (**A**) Plasma total F_2-IsoPs, (**B**) plasma esterified F_2-IsoPs, (**C**) plasma free F_2-IsoPs, (**D**) arachidonate, (**E**) plasma total F_2-IsoPs standardized with arachidonate level, (**F**) plasma esterified F_2-IsoPs standardized with arachidonate level, and (**G**) urinary F_2-IsoPs. Each graphic column expresses mean ± SD. Number of subjects for each disease group is shown in parentheses. AA, Arachidonate; Cr, creatinine; PD, Parkinson's disease; DFO, onset of dengue fever; DFR, recovery stage of dengue fever; STO, onset of ischemic stroke; and STR, recovery stage of ischemic stroke. Unpaired Student's *t* tests showed $^*p < 0.05$ and $^{**}p < 0.01$ *vs.* healthy controls. Repeated analysis of variance showed $^+p < 0.05$ and $^{++}p < 0.01$ between onset and recovery stages of dengue or ischemic stroke.
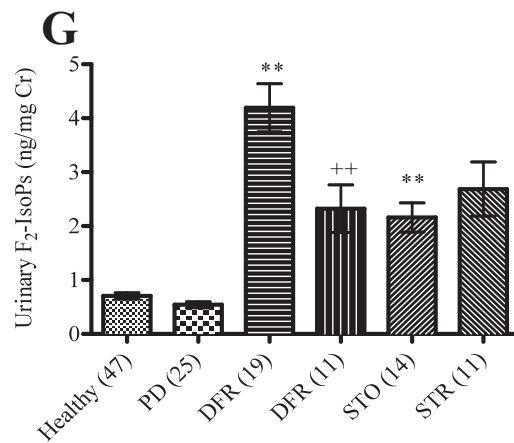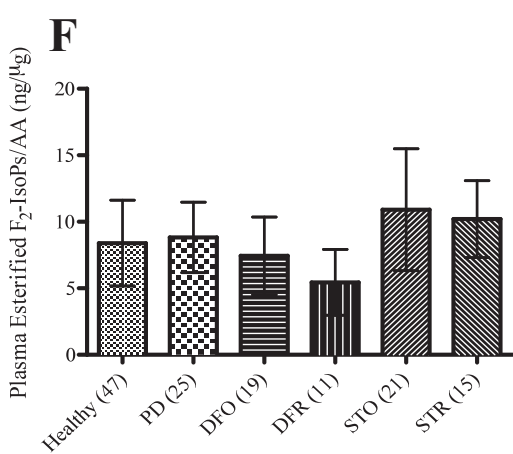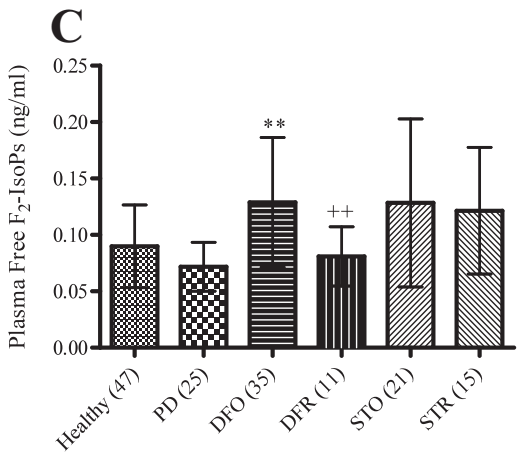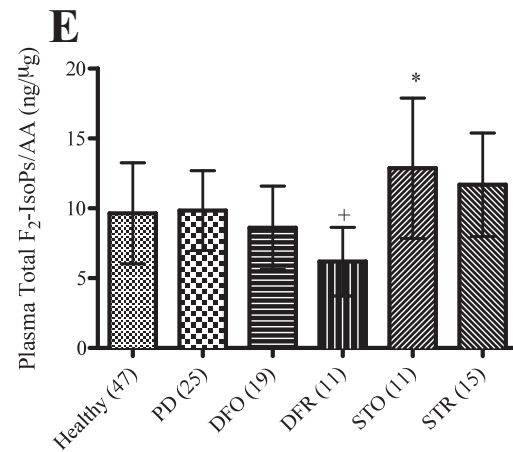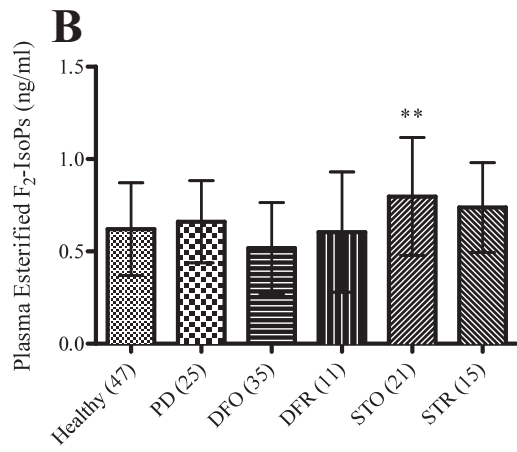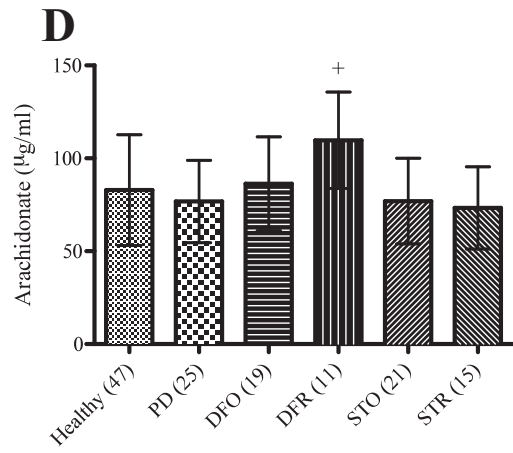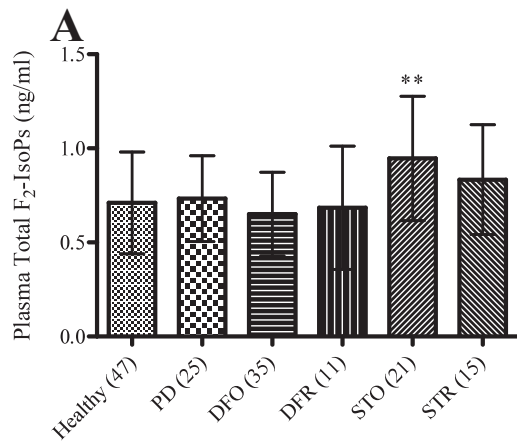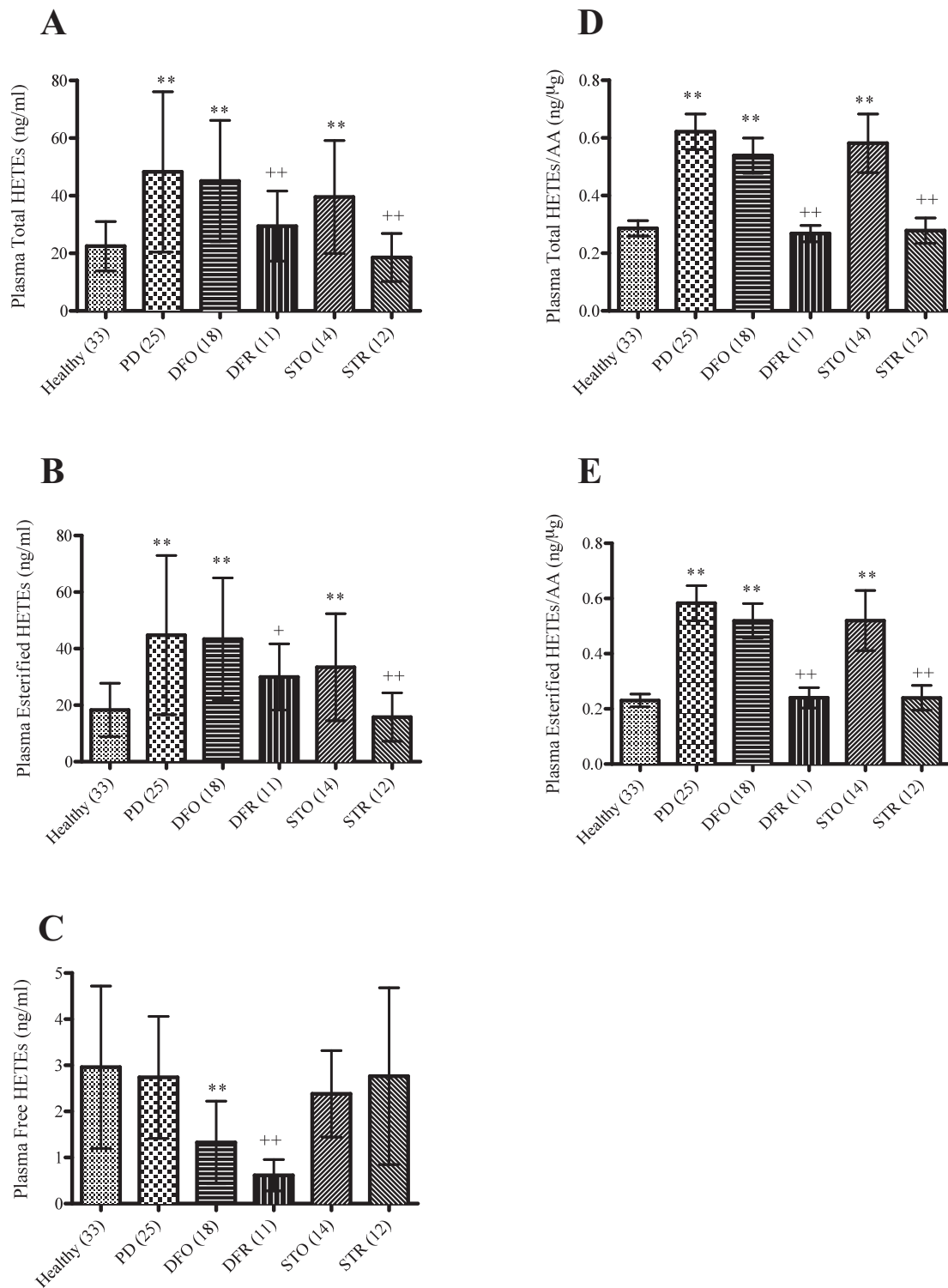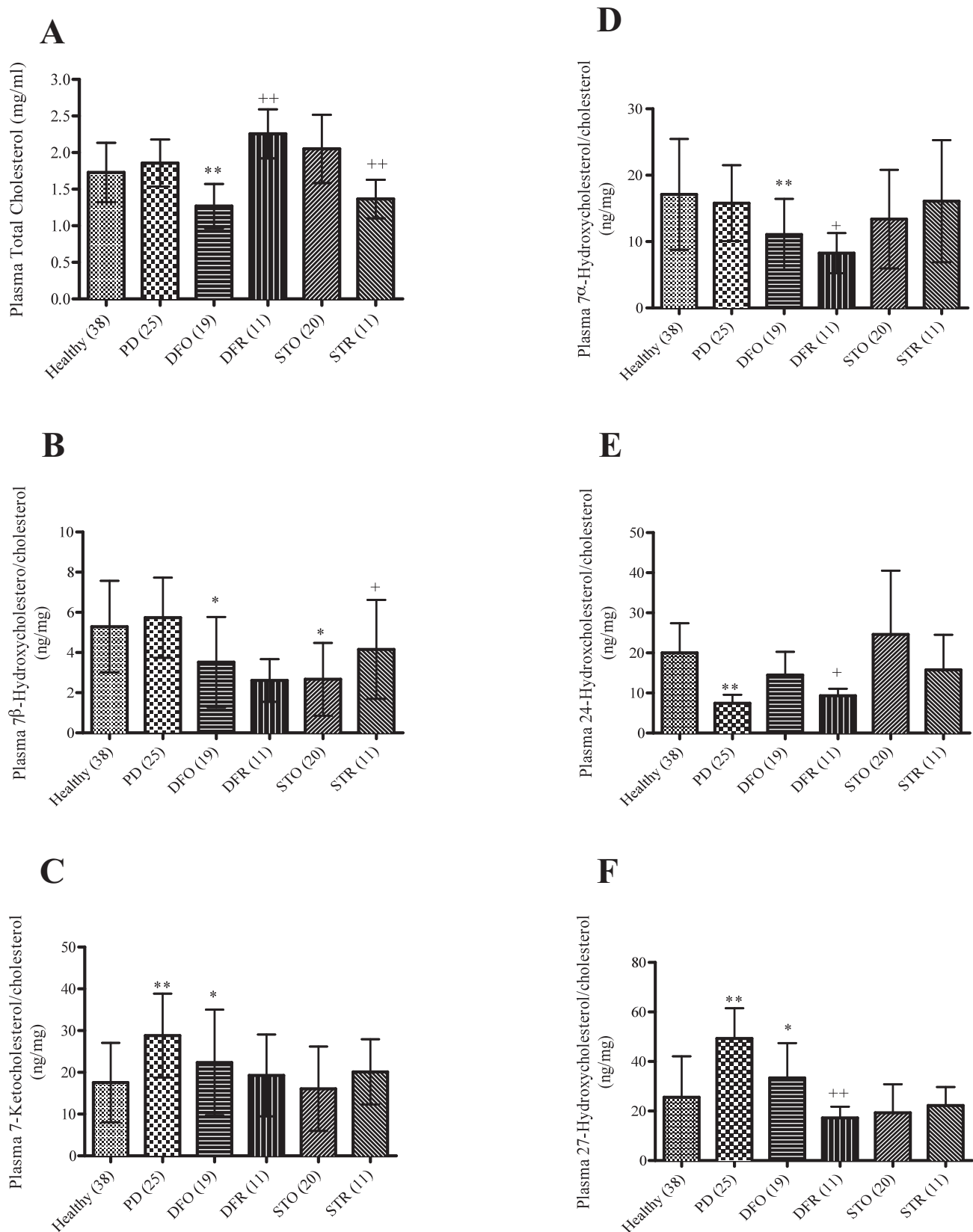
FIG. 4.   Comparison of HETEs levels between age-matched healthy controls and patients with acute and chronic diseases on (A) plasma total HETEs, (B) plasma esterified HETEs, (C) plasma free HETEs, (D) plasma total HETEs standardized with arachidonate level, and (E) plasma esterified HETEs standardized with arachidonate level. Each graphic column expresses mean ± SD. Number of subjects for each disease group is shown in parentheses. AA, Arachidonate; PD, Parkinson's disease; DFO, onset of dengue fever; DFR, recovery stage of dengue fever; STO, onset of ischemic stroke; and STR, recovery stage of ischemic stroke. Unpaired Student's t test showed $^*p < 0.05$ and $^{**}p < 0.01$ *vs.* healthy controls. Repeated analysis of variance showed $^+p < 0.05$ and $^{++}p < 0.01$ between onset and recovery stages of dengue or ischemic stroke.

FIG. 5. Comparison of COPs levels between age-matched healthy controls and patients with acute and chronic diseases. (**A**) 7β-Hydroxycholesterol, (**B**) 7-ketocholesterol, (**C**) 7α-hydroxycholesterol, (**D**) 24-hydroxycholesterol, and (**E**) 27-hydroxycholesterol. Each graphic column expresses mean $\pm$ SD. Number of subjects for each disease group is shown in parentheses. PD, Parkinson's disease; DFO, onset of dengue fever; DFR, recovery stage of dengue fever; STO, onset of ischemic stroke; STR, recovery stage of ischemic stroke. Unpaired Student's t test showed $^*p < 0.05$ and $^{**}p < 0.01$ vs. healthy controls. Repeated analysis of variance showed $^+p < 0.05$ between onset and recovery stages of dengue or ischemic stroke.

only in the younger group (25–49 years) and not in the older group (50–86 years). In contrast, arachidonate, total-cholesterol, 7β-hydroxycholesterol, and 27-hydroxycholesterol decreased with age, but more so in subjects older than 50 years

than in those younger than 50 years. One factor might be the different changes of HDL levels in these groups (8). Hence, the discrepancies (3, 10, 15, 19, 47, 49) might relate to the use of different age ranges. It also should be noted that the great

FIG. 6. Comparison of total cholesterol and COPs-cholesterol adjusted levels between age-matched healthy controls and patients with acute and chronic diseases. (**A**) Total cholesterol, (**B**) 7β-hydroxycholesterol, (**C**) 7-ketocholesterol, (**D**) 7α-hydroxycholesterol, (**E**) 24-hydroxycholesterol, and (**F**) 27-hydroxycholesterol. Each graphic column expresses as mean ± SD. Number of subjects for each disease group is shown in parentheses. PD, Parkinson disease; DFO, onset of dengue fever; DF,: recovery stage of dengue fever; STO, onset of ischemic stroke; and STR, recovery stage of ischemic stroke. Unpaired Student's *t* test showed *p < 0.05 and **p < 0.01 *vs.* healthy controls. Repeated analysis of variance showed +p < 0.05 and ++p < 0.01 between onset and recovery stages of dengue.

majority of our patients are of Chinese origin (in Singapore), whereas previous studies examined United States and European populations. Whether this affects the results remains uncertain.

Our second conclusion is that diseases do not show elevations in all lipid oxidation products (*i.e.*, disease itself does not simply elevate all biomarkers of oxidative lipid damage). Thus, for F_2-IsoPs, we saw an increase in stroke but not in dengue or Parkinson's disease, and for COPs in Parkinson's disease but not in stroke. By contrast, elevated HETEs were found in all three diseases (discussed later). It is well known that free radicals play a role in lipid peroxidation involved in human ischemia/reperfusion injury (14, 15, 23). It was reported that F_2-IsoPs levels were elevated within the first 8 h of stroke (20), but in our study, high levels were still recorded within 24 h of stroke and tended to decrease on recovery (day 7) in patients who recovered. This was clearly shown in ischemic-stroke patients in whom the levels of F_2-IsoPs were elevated (20, 46). Oxidized lipid compounds, in particular F_2-IsoPs, have been linked to vascular function, whereas an increase is often related to vasoconstriction (25).

Our investigation showed plasma F_2-IsoPs were not elevated in Parkinson's disease compared with the age-matched study controls. F_2-IsoPs have been previously measure in the substantia nigra (27) in Parkinson's disease patients, but the levels were not elevated, although those of isofurans were. A relation was found between urinary F_2-IsoPs and plasma free F_2-IsoPs in Parkinson's disease in this study but not in age-matched healthy controls. However, a strong correlation (Fig. 4) between total F_2-IsoPs and urinary F_2-IsoPs in our total population of healthy controls (25–86 years) was found, and it appears to be attributed to the younger population, those younger than 50 years (25–49 years; $r = 0.34$; $p = 0.01$) and not older than 50 years (50–86 years; $r = 0.18$; $p = 0.15$). Our results in healthy subjects are consistent with those of Morrow *et al.* (31). The difference for Parkinson's disease is unknown, although it could be related to altered renal function or excessive production of local free F_2-IsoPs in the kidney (21, 38, 44). Our results also indicate the need to consider whether in disease, we should measure plasma F_2-IsoPs, urinary F_2-IsoPs, or both, as done here. Another interesting point is that both plasma total ($r = 0.52$) and urinary ($r = 0.41$) F_2-IsoPs levels tended to increase with age in the Parkinson's disease group but not in normal controls, perhaps suggesting that the longer one has Parkinson's disease, the more oxidative stress may tend to increase.

It was initially surprising to find that COPs levels in stroke patients were not elevated compared with those in the healthy group, in particular, 24-hydroxycholesterol, which is known to arise from brain (5). A similar report has been made (6), and it was concluded that a limit exists in measuring 24-hydroxycholesterol in plasma of stroke patients because of damaged regulation of the enzyme 24-hydroxylase (17). Our data also reveal the potential confounding effects of changes in cholesterol levels during stroke (perhaps affected by changes in nutrient intake, disordered lipid metabolism, or changes in the use of cholesterol-lowering drugs). In intracerebral metabolism of the blood–brain barrier in humans, where 27-hydroxycholesterol passes into the brain and 24-hydroxycholesterol out of the brain, the con-

centration gradient created modifies the metabolism in the liver and the brain (4, 16). Thus a ratio of 27-hydoxycholesterol/24-hydroxycholesterol is often expressed in disease models. It is suggested the ratio of 27-hydroxycholesterol/24-hydroxycholesterol is about 0.2 in brain and about 2 in circulation; this demonstrates the abnormality of cholesterol homeostasis in stroke in this study, where a ratio of 0.7 was recorded in plasma. Moreover, the ratio of 27-hydoxycholesterol/24-hydroxycholesterol is quoted to be high in the brain of neurologic disease patients (4, 16). We found it to be sixfold higher in the plasma of Parkinson's disease patients compared with the healthy group in our study.

High 7-ketocholesterol and 27-hydroxycholesterol, and low 24-hydroxycholesterol, even when normalized by cholesterol levels in Parkinson's disease, indicate the complexity of the changes in this disease. The high level of 7β-hydroxycholesterol in Parkinson's disease may indicate not only involvement of free radical reactions on cholesterol but also the 27-hydroxylase enzymes that produce 27-hydroxycholesterol (4), which could further break down to produce 7-ketocholesterol and 7β-hydroxycholesterol (18). Further, in the pathogenesis of neurologic disorders, it appears that plasma 24-hydroxycholesterol decreases with age, also corresponding to the size of the brain and loss of hepatic function (6).

It is suggested that lipid oxidation products may have inflammatory potency in certain diseases, although sometimes antiinflammatory effects have been described (2, 32, 40). The pathology of dengue fever patients involves intense inflammation and depression of platelet levels. These changes are linked to altered cytokines, immune function, lipid metabolism, and nutrition and may lead to low total cholesterol and COPs level in dengue fever (36, 45).

Interestingly, HETEs were elevated in Parkinson's disease, dengue fever, and stroke. HETEs are oxidized products of arachidonic acid *via* enzymatic reaction of cytochrome P450, hydroxylases, and lipoxygenases, or by free radical reactions. Like F_2-IsoPs, numerous isomers of HETEs exist, and individual isomers were not determined in our study (22). Moreover, production of 20-HETE is suggested to be induced in ischemia/reperfusion injury (11, 12, 13, 34) and to lead to vasoconstriction, especially in small arteries (39). 20-HETE is also known to mediate vasoconstriction in cerebral vascular function, and regulation of renal vascular tone and maintenance of renal blood flow (39, 52, 53). Thus, the elevated levels in stroke revealed in this study are potentially deleterious. Conversely, 5-, 8-, 12-, and 15-HETEs (which are constituents of the total HETEs measured in this study) are produced by nonenzymatic free-radical–mediated peroxidation of arachidonic acid (51). High levels were recorded in human atherosclerotic plaques (24), and HETEs may be associated with tumor development (35). Further, increases in plasma 9-HETE have been associated with increased risk of coronary artery disease (41), and 15-HETE increase is related to cerebral vasoconstriction (11).

These data illustrate our third major conclusion in measuring biomarkers of oxidized lipids in clinical samples; it is essential to present data for oxidized lipids not only per milliliter of plasma but also per unit substrate. This is reflected in dengue fever patients, in whom plasma total F_2-IsoPs were the same between onset and recovery stage when expressed

per milliliter plasma, but the levels at recovery stage were lower than those at the onset stage when expressed per microgram of arachidonate. Such observations were also made in COPs of dengue fever patients, in whom levels expressed per milliliter plasma showed higher 7β- and 7α-hydroxycholesterol levels in the recovery stage of dengue fever, but the opposite effect was found when expressed per milligram total cholesterol.

Finally, we conclude that to assess oxidized lipid damage products accurately in human diseases (or at least for Parkinson's disease, dengue fever, and stroke), we must examine several biomarkers, and the methods used in this study may have value in this respect.

Acknowledgments

Abbreviations

BHT, butylated hydroxytoluene; BSTFA + TMCS, *N,O*-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane; COPs, cholesterol oxidation products; DF, dengue fever; DFO, onset of dengue fever; DFR, recovery from dengue fever; DIPEA, *N,N*-diisopropylethylamine; EETS, epoxyeicosatrienoic acid; EI, electron ionization; F_2-IsoPs, F_2-isoprostanes; GC-MS, gas chromatography–mass spectrometry; HCl, hydrochloric acid; HDL, high-density lipoprotein; HETE, hydroxyeicosatetraenoic acid; HETEs, hydroxyeicosatetraenoic acid products; IgG, immunoglobulin G; LDL, low-density lipoprotein; MAX, mixed anion exchange; Na-EDTA: disodium ethylenediamine tetraacetate; NCI, negative chemical ionization; NIHSS, National Institutes of Health Stroke Severity; PD, Parkinson's disease; PFBBr, pentafluorobenzylbromide; SPE, solid-phase extraction; ST, ischemic stroke; STO, onset of ischemic stroke; STR, recovery from ischemic stroke.

Disclosure Statement

No competing financial interests exist

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