A Japanese cross-sectional multicentre study of biomarkers associated with cardiovascular disease in smokers and non-smokers

Frank Lüdicke, John Magnette, Gizelle Baker#, and Rolf Weitkunat

Philip Morris Products S.A., Research & Development, Neuchatel, Switzerland

Abstract

We performed a cross-sectional, multicentre study in Japan to detect the differences in biomarkers of exposure and cardiovascular biomarkers between smokers and non-smokers. Several clinically relevant cardiovascular biomarkers differed significantly between smokers and non-smokers, including lipid metabolism (high-density lipoprotein cholesterol concentrations – lower in smokers), inflammation (fibrinogen and white blood cell count – both higher in smokers), oxidative stress (8-epi-prostaglandin F2α – higher in smokers) and platelet activation (11-dehydro-thromboxane B2 – higher in smokers) (p < 0.0001). These results provide further evidence showing that cardiovascular biomarkers can discriminate smokers from non-smokers, and could be used to evaluate the risks associated with tobacco products.

Introduction

Japan has a high smoking rate, and more adult males smoke in Japan (32.4% males and 9.7% females) than in the USA (21.6% males and 16.5% females) or the UK (21% males and 19% females) (World Health Organisation, 2009, 2013). Although cigarette smoking is an established risk factor for cardiovascular diseases (CVDs) (Hozawa, 2011; Iso, 2011; Ueshima et al., 2008; US Department of Health and Human Services, 2010), the mortality rate attributable to coronary heart disease (CHD) in Japan is two-thirds of that in the USA.

Smoking accelerates atherosclerosis, which leads to CVD, by affecting endothelial functions, cholesterol metabolism and platelet functions and increasing inflammation and oxidative stress (Ambrose & Barua, 2004; Howard et al., 1998). By measuring the CVD-related biomarkers in smokers and non-smokers, it may be possible to determine the pathophysiological mechanisms that underlie the adverse health effects of smoking and identify intermediate smoking-related risk factors for CVD in Japanese individuals. In the first stage of this research programme, we performed a multicentre, cross-sectional study of smokers and non-smokers with the aim of examining the associations between smoking and cardiovascular biomarkers in Japanese individuals. This was done as a prelude to future longitudinal studies designed to prospectively examine the effects of smoking on the biomarkers identified in the present study.

In this study, we screened a number of cardiovascular biomarkers that are commonly used in the evaluation of lipid metabolism (high-density lipoprotein [HDL] cholesterol, low-density lipoprotein [LDL] cholesterol and triglycerides), inflammation (fibrinogen, high-sensitivity C-reactive protein [hs-CRP] and white blood cell [WBC] count), oxidative stress (8-epi-prostaglandin F2α [8-epi-PGF2α], malondialdehyde [MDA], oxidised LDL [ox-LDL] and glutathione peroxidase [GPx]), platelet activation (11-dehydrothromboxane B2 [11-DTXB2]) and endothelial function (von Willebrand factor [vWF], soluble intercellular adhesion molecule-1 [sICAM-1] and homocysteine) for their potential associations with smoking. We also examined several biomarkers of exposure to confirm that the subject-reported exposure was correlated with the actual exposure to cigarette smoke. These biomarkers of exposure included plasma cotinine (pCOT), nicotine and nicotine equivalents, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and carboxyhaemoglobin (COHb).

Methods

Subjects

Healthy smokers and non-smokers aged ≥30 years were recruited via flyers and posters advertising the study. Smokers were eligible if they had smoked only commercially available cigarettes for ≥5 years and smoked ≥10 cigarettes per day (cpd). Non-smokers were eligible if they had not used nicotine-containing products for ≥1 year before the study. Pregnant or nursing women were excluded. Subjects who were using drugs such as acetylsalicylic
Table 1. Biomarkers and assay methods.

| Biomarker | Matrix | Method |
|-----------|--------|--------|
| Biomarkers of smoking exposure | | |
| Carboxyhaemoglobin (COHb) | Blood | Spectrophotometry |
| Cotinine (pCOT) | Plasma | LC-MS/MS |
| Nicotine plus five nicotine-derived metabolites (nicotine equivalents, Neq)* | Urine | LC-MS/MS |
| Cardiovascular biomarkers | | |
| 11-dehydro-thromboxane-B2 (11-DTXB2) | Urine | LC-MS/MS |
| High-sensitivity C-reactive protein (hs-CRP) | Plasma | Konelab CRP test kit |
| High-density lipoprotein (HDL) cholesterol | Plasma | Konelab HDL test kit |
| Low-density lipoprotein (LDL) cholesterol | Plasma | Friedewald formulab |
| Fibrinogen | Plasma | Clauss method |
| 8-epi-prostaglandin F2α (8-epi-PF2α) | Plasma | Enzyme-linked immunosorbent assay |
| Glutathione peroxidase (GPx) | Plasma | Microparticle enzyme immunoassay |
| Homocysteine | Plasma | Flow cytometry |
| Malondialdehyde (MDA) | Plasma | Human MAP panelc |
| Soluble intercellular adhesion molecule-1 (sICAM-1) | Plasma | Human MAP panelc |
| von Willebrand factor (vWF) | Blood | Flow cytometry |
| White blood cell count (WBC) | Blood | Flow cytometry |

*aThe molar sum of nicotine, cotinine, trans-3'-hydroxycotinine and their respective glucuronide conjugates.

bLDL-cholesterol = “Total cholesterol” − “HDL-cholesterol” − “Triglycerides”/2.2.

cThe human MAP panel was developed by Rules-Based Medicine (Austin, TX).

*LC-MS/MS, liquid chromatography-tandem mass spectrometry; HPLC, high-performance liquid chromatography; MAP, multi-analyte profiling.

acid, non-steroidal anti-inflammatory drugs, steroids, statins and nutritional supplements (e.g. >800 units/day of vitamin C or E) were excluded from the study. Subjects provided written informed consent and were compensated for their participation.

Study design

This cross-sectional study was conducted at nine sites in six Japanese cities between July and December 2007. The study was approved by institutional review boards at each participating site and was conducted in accordance with Good Clinical Practise and the principles of the Declaration of Helsinki (1996).

All the subjects visited their study site for a screening visit (Visit 1) and two subsequent visits. Visit 2 was scheduled 3–14 d after Visit 1 and Visit 3 was scheduled 2–7 d after Visit 2. Each subject’s medical history and use of concomitant medications were recorded at Visit 1. Smokers also completed the Fagerström test for nicotine-dependence (FTND) at Visit 1. Smoking status, sex and age, and the smokers were further stratified by daily cigarette consumption (at 10.19, 20–30, >30 cpd).

Bioanalytical methods

All bioanalytical methods were validated according to the US Food and Drug Administration guidance (Food and Drug Administration (FDA), 2001). Urinary biomarkers of exposure were nicotine, five nicotine-derived metabolites (nicotine equivalents [Neq]), NNAL and glucuronide conjugates (total NNAL). The urinary concentrations of biomarkers of exposure and pCOT concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). These biomarkers of exposure were analysed by Philip Morris Research Laboratories (Cologne, Germany) except for COHb, which was analysed at MDS Pharma Services (Fehraltorf, Switzerland) and was measured in whole blood by spectrophotometry.

The cardiovascular biomarkers measured in this study and the methods used are summarised in Table 1. Triglycerides, total serum cholesterol and HDL-cholesterol concentrations were determined using enzymatic kits (Thermo Clinical Labsystems, Vantaa, Finland). LDL-cholesterol was calculated using the equation: “LDL-cholesterol” = “total cholesterol” − “HDL-cholesterol” − “Triglycerides”/2.2. Plasma fibrinogen concentrations were measured using the Clauss clot detection method on an ACL-Futura analyser (Instrumentation Laboratories, Bedford, MA). Plasma hs-CRP concentrations were measured using a Konelab hs-CRP test kit (Thermo Scientific, Waltham, MA). Plasma Ox-LDL and plasma GPx were measured using commercially available ELISA kits (Biomedica, Vienna, Austria and Cayman, Tallinn, Estonia, respectively). Urinary 11-DTXB2 concentrations were determined by LC-MS/MS. The WBC count, homocysteine, 8-epi-PGF2α, MDA, sICAM-1 and vWF were determined at Philip Morris Research Laboratories. Fibrinogen concentrations were measured at Covance Laboratories (Harrogate, UK). In this study, we report the urinary exposure biomarkers measured in the samples collected at Visit 2, while blood and plasma biomarkers are reported for samples collected at Visits 2 and 3.

Statistical analysis

All subjects who completed the study and for whom reliable measurements of the cardiovascular biomarkers were available were included into the analysis. Subjects were stratified by smoking status, sex and age, and the smokers were further stratified by daily cigarette consumption (<10, 10–19, 20–30, >30 cpd).

For each subject, the mean values at both Visits 2 and 3 or the only value if a valid value was available for only one visit
were entered into the statistical analyses. CVD-related biomarker concentrations below the lower limit of quantification (BLLOQ) were replaced with one-half of the lower limit of quantification (LLOQ) or 0.125 mg/L for hs-CRP.

Analysis of covariance was used to analyse the cardiovascular biomarkers. Smoking status, age and gender were included as covariates. Statistical significance was defined as \( p < 0.05 \). Values are presented as the means (standard deviations). All statistical analyses were conducted using SAS software version 9.1 (SAS Institute Inc., Cary, NC).

**Results**

**Subjects**

A total of 731 adult smokers were enrolled of which 670 were considered evaluable, and 367 adult non-smokers were enrolled of which 356 were evaluable. The characteristics of the subjects are summarised in Table 2. The mean (standard deviation) cigarette consumption was 20.7 (8.9) cpd in all smokers, 21.8 (9.7) cpd in males and 18.6 (6.8) cpd in females. Overall, 341 (50.9%) smokers smoked 10–19 cpd, and the others smoked 20–30 cpd (245 [36.6%]) or \( \geq 30 \) cpd (84 [12.5%]). The mean FTND score for all smokers was 4.6 (Table 2).

**Biomarkers of exposure**

Table 3 summarises the urinary/plasma concentrations of the biomarkers of exposure in smokers and non-smokers. As would be expected, the mean plasma COHb (3.68 [1.19] versus 1.99 [0.40]%), pCOT (218.2 [126.6] versus 4.4 [17.8] ng/mL), 24-h urinary Neq (7.81 [6.3] versus 0.39 [0.56] mg/24 h) and 24-h urinary total NNAL (226.2 [194.3] versus 9.3 [32.8] ng/24 h) concentrations were consistently greater in the smokers than in non-smokers. All of these biomarkers of exposure tend to increase with increasing daily cigarette consumption. In smokers, the mean pCOT, 24-h urinary Neq and 24-h urinary total NNAL concentrations tend to be greater in males than in females. There were no appreciable differences in biomarkers of exposure between male and female non-smokers.

**Cardiovascular biomarkers**

Table 4 compares the cardiovascular biomarkers between smokers and non-smokers. The mean HDL-cholesterol concentration, which was measured as an anti-atherogenic lipid and as a biomarker for lipid metabolism, was significantly lower in smokers than in non-smokers by about 0.1 mmol/L (1.21 [0.39] versus 1.32 [0.40] mmol/L; \( p < 0.0001 \)). The mean LDL-cholesterol concentrations were similar in smokers and non-smokers (2.97 [0.9] versus 2.98 [0.8] mmol/L; \( p = 0.9 \)). The mean triglyceride concentrations were also similar in smokers and non-smokers (1.45 [1.0] versus 1.32 [1.0] mmol/L; \( p = 0.5 \)).

Regarding biomarkers of inflammation, the mean plasma fibrinogen concentration was about 0.15 g/L greater in smokers than in non-smokers (2.95 [0.95] versus 2.80 [0.58] g/L; \( p < 0.0001 \)). By contrast, the mean hs-CRP concentration was not significantly different between the two groups of subjects (0.96 [2.40] versus 0.77 [2.95] mg/L; \( p = 0.29 \)). The mean WBC count in smokers and non-smokers was 6.52 (1.65) and 5.26 (1.13) \( \times 10^{10} /L \), respectively (\( p < 0.0001 \)).

Four biomarkers of oxidative stress were examined in this study. The mean 8-epi-PGF2\( \alpha \) concentration was significantly greater in smokers than in non-smokers (1.65 [0.91] versus...
1.33 [0.80] nmol/L; p < 0.0001). The mean GPx concentration was significantly lower in smokers than in non-smokers (102.1 [41.7] versus 109.2 [44.7] nmol/min/mL; p = 0.0082). By contrast, the mean plasma MDA (0.51 [0.21] versus 0.51 [0.18] μmol/L; p = 0.85) and ox-LDL (0.65 [0.99] versus 0.70 [0.91] ng/L; p = 0.47) concentrations were almost identical in both group of subjects.

As biomarkers of endothelial function, although the mean plasma vWF (29.3 [21.0] versus 28.3 [17.0] μg/mL; p = 0.41) and homocysteine (10.35 [5.19] versus 9.67 [4.47] μmol/L; p = 0.043) concentrations were generally similar between smokers and non-smokers, the mean sICAM-1 concentration was about 14 ng/mL greater in smokers than in non-smokers (72.4 [26.0] versus 58.0 [18.4] ng/mL; p < 0.0001).

Finally, as a biomarker of platelet activation, the mean plasma 11-DTXB2 concentration was significantly greater in smokers than in non-smokers (2.60 [1.58] versus 1.94 [1.12] nmol/L; p = 0.0001).

Notably, the differences observed in several cardiovascular biomarkers between smokers and non-smokers (e.g. HDL-cholesterol, fibrinogen, 11-DTXB2 and 8-epi-PGF2α) were consistent for males and females (Table 4).

### Discussion

Exposure to cigarette smoke affects a number of biological processes, which in turn trigger response pathways causally linked to smoking-related diseases (Ambrose & Barua, 2004; Howard et al., 1998). Several clinically relevant factors within these pathways contribute to the pathophysiological mechanisms underlying smoking-related diseases. Many of these risk factors improve in the short to mid-term (e.g. within 1 week to 1 year) following smoking cessation. For a biomarker to be acknowledged as a risk factor, there should be a strong biological rational for its relationship with the clinical outcome and it must be supported by compelling data from clinical and epidemiological studies (Micheel & Ball, 2010).

Lipid metabolism, inflammation, oxidative stress, endothelial function and platelet function are biological processes involved in atherosclerosis. Cigarette smokers have a higher risk of coronary artery disease (CAD) than non-smokers. Blood coagulation disorders, impaired integrity of the arterial wall and changes in blood lipid and lipoprotein concentrations may contribute to smoking-related CVD (Ambrose & Barua, 2004; Howard et al., 1998).

In the content that follows, we review the associations between smoking and potential CVD biomarkers in Japanese subjects, review the differences between males and females to understand potential gender differences and place our findings in the context of those in Caucasian subjects to elucidate potential ethnic differences in the observed associations. The information obtained in this and related studies will be vital in the design of prospective, longitudinal studies designed to examine the impact of smoking on CVD biomarkers and risk, by allowing researchers to focus on clinically relevant biomarkers that have been shown to be associated with smoking.
The beneficial effects of HDL-cholesterol in the cardiovascular system have been attributed to its ability to remove cellular cholesterol via reverse cholesterol transport. It also exerts anti-inflammatory, antioxidant and antithrombotic effects, which act in concert to improve endothelial function and limit atherosclerosis progression, thereby reducing cardiovascular risk. Numerous studies have shown that smoking decreases HDL-cholesterol, especially in women (Allen et al., 1994; Eliasson et al., 2001; Moffatt et al., 2000; Ohsawa et al., 2005; Richard et al., 1997). Moreover, the effects of smoking on HDL-cholesterol occur rapidly after smoking only a few cigarettes (Unverdorben et al., 2009). HDL-cholesterol concentrations re-increase relatively quickly after stopping smoking, but decrease again upon resumption of smoking (Allen et al., 1994; Eliasson et al., 2001; Frost-Pineda et al., 2011; Moffatt et al., 2000; Richard et al., 1997; Yasue et al., 2006). The effect of smoking cessation on HDL-cholesterol was eloquently demonstrated in a meta-analysis of 45 studies with a total of 94 estimates of HDL-cholesterol based on within-subject changes. For the unweighted analysis, the overall pooled increase in HDL-C following smoking cessation was 4.13 mg/dL, while the overall pooled increase for the weighted analysis was 2.32 mg/dL (Forey et al., 2013).

In the present study, we found that the mean HDL-cholesterol concentration was significantly lower, by about 8.4%, in smokers than non-smokers, consistent with previously reported results. No differences in mean LDL-cholesterol or triglyceride concentrations were found between

| Lipid metabolism | Smokers (n=435) | Females (n=235) | All smokers (n=670) | Males (n=226) | Females (n=130) | All non-smokers (n=356) | p Value |
|------------------|----------------|----------------|--------------------|---------------|----------------|------------------------|---------|
| HDL-C (mmol/L)   |                |                |                    |               |                |                        |         |
| Mean (SD)        | 1.1 (0.3)      | 1.4 (0.4)      | 1.2 (0.4)          | 1.2 (0.4)     | 1.5 (0.4)      | 1.3 (0.4)              | <0.0001 |
| 95% CI           | 1.1–1.1        | 1.4–1.5        | 1.2–1.2            | 1.2–1.3       | 1.4–1.6        | 1.3–1.4                |         |
| Inflammation     |                |                |                    |               |                |                        |         |
| Fibrinogen (g/L) |                |                |                    |               |                |                        |         |
| Mean (SD)        | 3.0 (0.6)      | 2.9 (0.6)      | 3.0 (1.0)          | 2.8 (0.6)     | 2.8 (0.5)      | 2.8 (0.6)              | <0.0001 |
| 95% CI           | 2.9–3.0        | 2.8–2.9        | 2.9–3.0            | 2.7–2.9       | 2.7–2.9        | 2.7–2.9                |         |
| hs-CRP (mg/L)    |                |                |                    |               |                |                        |         |
| Mean (SD)        | 1.1 (2.6)      | 0.7 (1.9)      | 1.0 (2.4)          | 0.9 (3.3)     | 0.6 (2.1)      | 0.8 (3.0)              | 0.288   |
| 95% CI           | 0.9–1.4        | 0.5–0.9        | 0.8–1.1            | 0.4–1.3       | 0.3–1.0        | 0.5–1.1                |         |
| Median            | 0.3            | 0.13           | 0.2                | 0.2           | 0.1            | 0.1                    |         |
| 1st, 3rd quartile| 0.1, 1.2       | 0.1, 0.5       | 0.1, 0.9           | 0.1, 0.5      | 0.1, 0.4        | 0.1, 0.5               |         |
| WBC count (×10⁹/L) |                |                |                    |               |                |                        |         |
| Mean (SD)        | 6.7 (1.6)      | 6.2 (1.65)     | 6.5 (1.7)          | 5.4 (1.2)     | 5.0 (1.1)      | 5.3 (1.1)              | <0.0001 |
| 95% CI           | 6.5–6.8        | 6.0–6.5        | 6.4–6.7            | 5.2–5.5       | 4.9–5.2        | 5.1–5.4                |         |

### Table 4. Cardiovascular biomarkers.

| Biomarker                  | Smokers | Females | All smokers | Males | Females | All non-smokers | p Value |
|----------------------------|---------|---------|-------------|-------|---------|-----------------|---------|
| Lipid metabolism           |         |         |             |       |         |                 |         |
| HDL-C (mmol/L)             |         |         |             |       |         |                 |         |
| Mean (SD)                  | 1.1     | 1.4     | 1.2         | 1.2   | 1.5     | 1.3             | <0.0001 |
| 95% CI                     | 1.1–1.1 | 1.4–1.5 | 1.2–1.2     | 1.2–1.3| 1.4–1.6 | 1.3–1.4         |         |
| Inflammation               |         |         |             |       |         |                 |         |
| Fibrinogen (g/L)           |         |         |             |       |         |                 |         |
| Mean (SD)                  | 3.0     | 2.9     | 3.0         | 2.8   | 2.8     | 2.8             | <0.0001 |
| 95% CI                     | 2.9–3.0 | 2.8–2.9 | 2.9–3.0     | 2.7–2.9| 2.7–2.9 | 2.7–2.9         |         |
| hs-CRP (mg/L)              |         |         |             |       |         |                 |         |
| Mean (SD)                  | 1.1     | 0.7     | 1.0         | 0.9   | 0.6     | 0.8             | 0.288   |
| 95% CI                     | 0.9–1.4 | 0.5–0.9 | 0.8–1.1     | 0.4–1.3| 0.3–1.0 | 0.5–1.1         |         |
| Median                     | 0.3     | 0.13    | 0.2         | 0.2   | 0.1     | 0.1             |         |
| 1st, 3rd quartile          | 0.1, 1.2| 0.1, 0.5| 0.1, 0.9    | 0.1, 0.5| 0.1, 0.4| 0.1, 0.5        |         |
| WBC count (×10⁹/L)         |         |         |             |       |         |                 |         |
| Mean (SD)                  | 6.7     | 6.2     | 6.5         | 5.4   | 5.0     | 5.3             | <0.0001 |
| 95% CI                     | 6.5–6.8 | 6.0–6.5 | 6.4–6.7     | 5.2–5.5| 4.9–5.2 | 5.1–5.4         |         |

### Lipids

The beneficial effects of HDL-cholesterol in the cardiovascular system have been attributed to its ability to remove cellular cholesterol via reverse cholesterol transport. It also exerts anti-inflammatory, antioxidant and antithrombotic effects, which act in concert to improve endothelial function and limit atherosclerosis progression, thereby reducing cardiovascular risk. Numerous studies have shown that smoking decreases HDL-cholesterol, especially in women (Allen et al., 1994; Eliasson et al., 2001; Moffatt et al., 2000; Ohsawa et al., 2005; Richard et al., 1997). Moreover, the effects of smoking on HDL-cholesterol occur rapidly after smoking only a few cigarettes (Unverdorben et al., 2009). HDL-cholesterol concentrations re-increase relatively quickly after stopping smoking, but decrease again upon resumption of smoking (Allen et al., 1994; Eliasson et al., 2001; Frost-Pineda et al., 2011; Moffatt et al., 2000; Richard et al., 1997; Yasue et al., 2006). The effect of smoking cessation on HDL-cholesterol was eloquently demonstrated in a meta-analysis of 45 studies with a total of 94 estimates of HDL-cholesterol based on within-subject changes. For the unweighted analysis, the overall pooled increase in HDL-C following smoking cessation was 4.13 mg/dL, while the overall pooled increase for the weighted analysis was 2.32 mg/dL (Forey et al., 2013).

In the present study, we found that the mean HDL-cholesterol concentration was significantly lower, by about 8.4%, in smokers than non-smokers, consistent with previously reported results. No differences in mean LDL-cholesterol or triglyceride concentrations were found between
smokers and non-smokers. These results suggest that smoking perturbs lipid metabolism and might have pro-atherogenic effects by affecting HDL/LDL homeostasis, especially via reductions in anti-atherogenic HDL-cholesterol.

**Inflammation**

An increased WBC count is seen as a marker of chronic, subclinical and low-grade inflammation, which is associated with an increased risk of CVD, including hypertension, atherosclerosis, 6-month mortality risk, stroke, peripheral arterial disease and mortality following myocardial infarction (Bonaterra et al., 2010). Cigarette smoking is consistently associated with an increased WBC count and increased smoking intensity with a more pronounced elevation in WBC count (Asthana et al., 2010). The mean WBC count was reported to be 7–20% higher in smokers than in non-smokers (Frost-Pineda et al., 2011; Ishizaka et al., 2007; Wannamethee et al., 2005; Woodward et al., 1999). A meta-analysis of 24 studies quantified the within-subject changes of WBC count after quitting for <13 weeks (26 estimates), 13 to <52 weeks (7 estimates) and ≥52 weeks (3 estimates). The decrease in total WBC count [10⁹/L (95% confidence interval] for these three time periods were 0.98 (0.74–1.22), 0.78 (0.58–0.98) and 0.64 (0.35–0.92), respectively (Lee et al., 2014). In the present study, we found that the mean WBC count was significantly greater (23.9%) in smokers than in non-smokers, consistent with earlier reports, supporting the use of the WBC count as a clinically relevant biomarker in smokers.

Hs-CRP is a circulating biomarker of systemic inflammation that is associated with atherosclerosis and increased CVD risk. Several observational cohort studies have shown robust associations between smoking, including the daily cigarette consumption and hs-CRP concentrations (Bazzano et al., 2003; Hansen et al., 1990; Ohsawa et al., 2005; Pearson et al., 2003; Wannamethee et al., 2005). It was also reported that hs-CRP concentrations increase rapidly, within 1 h, of smoking a cigarette, indicating the acute pro-inflammatory effects of smoking (Seet et al., 2012). Accordingly, the increased risk of CVD in smokers might involve inflammation and increase in CRP concentrations.

The present study did not find a significant association between smoking and the hs-CRP concentration. However, there may be some reasons for this. First, the hs-CRP concentrations were highly variable in smokers in this study, with a median (1st and 3rd quartiles) hs-CRP concentration of 0.2 (0.13, 0.92) mg/L. The median hs-CRP concentration was reported to be 0.43 mg/L in an earlier study of Japanese adults, much lower than the median in Western populations (1.5–2.0 mg/L) (Arima et al., 2008). Second, the hs-CRP concentration was classified as BLOQ in more than half of the subjects in our study (449 smokers and 277 non-smokers). Third, the LLOQ of our analytical method was 0.25 mg/L, which may not have been sensitive enough to determine the effect of smoking on hs-CRP concentrations in our subjects. Therefore, future studies might need a more sensitive method to measure hs-CRP and elucidate the association between smoking and hs-CRP as a marker of inflammation.

Fibrinogen is related to both endothelial dysfunction and inflammation, which are associated with CAD. Pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor, are secreted from the vascular endothelium and macrophages, and induce the production of circulatory inflammatory molecules, including hs-CRP, serum amyloid A and fibrinogen. Large cohort studies such as the Framingham study have shown that plasma fibrinogen concentrations represent an independent risk factor for myocardial infarction and stroke, and the risks of these diseases increase progressively with increasing fibrinogen concentrations (Kannel, 2005). A meta-analysis of 31 prospective studies published between 1967 and 2003 revealed that the plasma fibrinogen concentrations are greater in smokers than in non-smokers, with a greater difference in males (smokers – non-smokers: +29 mg/dL) than in females (+15 mg/dL) (Kaptoge et al., 2007). Quitting smoking decreased fibrinogen concentrations in smokers over time (Ellasson et al., 2001; Wannamethee et al., 2005).

Our results showing elevated biomarkers of inflammation are consistent with these earlier studies, and are consistent with the notion that inflammatory biomarkers, such as fibrinogen in smokers, contribute to chronic subclinical inflammation and may increase the risk of smoking-related CVD. Further studies may be needed to examine why hs-CRP was not associated with smoking in this study.

**Oxidative stress**

The oxidation of lipids, proteins and nucleic acids has been implicated in the pathogenesis of many diseases, including atherosclerosis. Isoprostanes (IsoPs) are prostaglandin-like compounds formed from the peroxidation of arachidonic acid, a ubiquitous polunsaturated fatty acid. Unlike prostaglandins, which are formed by cyclooxygenase, F2-IsoPs, which include 8-epi-PGF2α, are generated by free radical-mediated peroxidation of arachidonic acid. Considering that the circulating F2-IsoP concentrations predominantly reflect its production, rather than its metabolism and excretion, it is important to measure the extent of oxidative stress in vivo (Morrow, 2005). Prior studies demonstrated that the plasma concentration and urinary excretion of F2-IsoP and its metabolites were greater in healthy smokers than in healthy non-smokers (Calapai et al., 2009; Frost-Pineda et al., 2011; Lowe et al., 2009; Morrow et al., 1995; Oguogho et al., 2000; Reilly et al., 1996). Reilly et al. reported that the average urinary excretion of 8-epi-PGF2α, as a commonly measured F2-IsoP, was 176.5 ± 30.6 pmol/mmol creatinine in heavy smokers (>30 cpd) compared with 92.7 ± 4.8 pmol/mmol creatinine in moderate smokers (15–30 cpd) and 54.11 ± 2.7 pmol/mmol creatinine in matched non-smoking control subjects (Reilly et al., 1996). Several studies have also shown that the 8-epi-PGF2α concentration decreases rapidly, within 1–2 weeks, to the concentrations observed in non-smokers (Oguogho et al., 2000; Pilz et al., 2000; Reilly et al., 1996).

In the present study, the plasma 8-epi-PGF2α concentration was significantly greater in smokers than in non-smokers (p < 0.0001). Although the association between increased oxidative stress and disease does not necessarily imply causation, F2-IsoP is elevated in atherosclerosis and other CVDs, supporting its use as a risk marker for smoking-related CVD (Davis & Roberts, 2011).
Several other biomarkers of oxidative stress were examined in this study, including ox-LDL, MDA and GPx. Ox-LDL is formed by reactions between reactive oxygen species and native LDL, and its concentrations increase rapidly during oxidative stress (Arai, 2014). Numerous studies have demonstrated that ox-LDL concentrations are associated with many forms of CVD (Ehara et al., 2001; Holvoet et al., 2003, 2007), MDA is a reactive species generated following peroxidation of polysaturated fatty acids. It is a clinically relevant molecule that can form adducts with DNA and proteins, altering their functions (Nam, 2011; Pizzimenti et al., 2013; Voulgaridou et al., 2011). Consequently, MDA and lipid peroxidation appear to contribute to the aetiology of various CVDs and neurological disorders (Cherubini et al., 2005; Dhalla et al., 1999; Muralikrishna Adibhatla & Hatcher, 2006; Siems et al., 2002; Uchida, 2000). Perhaps unexpectedly, we found no differences in the ox-LDL or MDA concentrations between smokers and non-smokers, which might be related to high inter-subject variability in these biomarkers, a low oxidative stress profile of the smokers or upregulation of some antioxidant pathways in this cohort.

Finally, we measured GPx activity. GPx is a class of antioxidant enzymes that reduces highly oxidised molecules, such as lipid hydroperoxides and hydrogen peroxide, to less harmful molecules (Winterbourn, 2013). Accordingly, changes in GPx activity are expected to affect the levels of circulating oxidised molecules, and it has been suggested that GPx could be a target for treating lung inflammation and damage in chronic obstructive pulmonary disease by clearing reactive oxygen species (Vlahos & Bozinovski, 2013). In this study, GPx activity was significantly lower in smokers than in non-smokers. Downregulation of GPx activity would be expected to lead to the accumulation of oxidative molecules. The lack of changes in MDA or ox-LDL suggests that the difference in GPx activity was not sufficient to cause excess accumulation of these oxidative molecules. It is also possible that the assays used were not sufficiently sensitive to detect small differences in values that cross-reactivity might reduce the accuracy of the assays or that GPx is not functionally related to MDA or ox-LDL.

**Endothelial function**

Adhesion molecules facilitate the adhesion and transmigration of leukocytes into the vascular endothelium and then the subendothelial space, a critical event in the initiation of atherosclerosis (Kaperonis et al., 2006). Elevated plasma sICAM-1 concentrations may reflect endothelial dysfunction and ongoing atherosclerosis (Ross, 1999). A number of epidemiologic studies have suggested that elevated sICAM-1 concentrations may be an early biomarker of atherosclerosis (Gross et al., 2012; Hsu et al., 2009). The significant associations between cell adhesion molecules and CVD risk factors highlight their usefulness as biomarkers of atherosclerosis and future coronary events. Current smoking increases the circulating concentrations of sICAM-1 by about 20–70% (Atikçan et al., 2004; Bergmann et al., 1998; Bermudez et al., 2002; Blann et al., 1997, 1998; Koundouros et al., 1996; Lavi et al., 2007; Mazzone et al., 2001; Rifai et al., 1999; Rohde et al., 1999; Scott et al., 2005; Takeuchi et al., 2002; van Tits et al., 2001; Wakelkamp et al., 2002). It was also reported that sICAM-1 concentrations are positively correlated with pack-years of smoking (Demerath et al., 2001; Miller et al., 2003) and increased in a dose-dependent manner with the amount of tobacco smoked per day (Demerath et al., 2001; Lain et al., 2006; Rohde et al., 1999; Scott et al., 2000; Takeuchi et al., 2002). These effects of smoking on sICAM-1 are reversible following smoking cessation (Bermudez et al., 2002; Halvorsen et al., 2007; Palmer et al., 2002; Scott et al., 2000; Takeuchi et al., 2002). Consistent with these earlier studies, we found that sICAM-1 concentrations were upregulated in smokers compared with non-smokers, which might reflect endothelial dysfunction and ongoing atherosclerosis in this cohort (Ross, 1999).

A review of cohort studies, clinical trials and follow-up studies highlighted that elevated plasma vWF is a risk factor for CHD, venous thrombosis, ischaemia stroke, atrial fibrillation and hypertension. However, other studies failed to establish an association between vWF and endothelium-related diseases (Luo et al., 2012). In a cross-sectional study of 3585 adult smokers and 1077 non-smokers, the mean vWF concentration was 7.3% higher in smokers than in non-smokers ($p<0.0001$), and age, race, body mass index (BMI) and smoking duration had statistically significant effects on vWF concentrations in multivariate analysis (Frost-Pineda et al., 2011). In the present study of Japanese subjects, we found no significant difference in the vWF concentration between smokers and non-smokers (Table 4).

Homocysteine concentrations were slightly higher in smokers than in non-smokers, particularly in males. Elevated homocysteine is widely regarded as an independent risk factor for CVD (Cacciapuoti, 2011; Finch & Joseph, 2010); however, a meta-analysis of 12 randomised controlled trials revealed that interventions aimed at lowering homocysteine concentrations did not reduce the risk of fatal or non-fatal myocardial infarction, stroke or death from any cause compared with placebo (Martí-Carvajal et al., 2015). Therefore, the role of elevated homocysteine in the pathogenesis of CVD and the benefits of reducing homocysteine concentrations remain unclear.

**Platelet activation**

Platelet activation and enhanced coagulation are related to cardiovascular events (Davi & Patrono, 2007). Multiple cross-sectional studies have demonstrated that 11-DTXB$_2$ concentrations are 29–40% higher in smokers than in non-smokers (Calapai et al., 2009; Frost-Pineda et al., 2011). 11-DTXB$_2$ concentrations were reported to be 60% higher in people who smoked $>20$ cpd with a tar content of 10 mg (Lowe et al., 2009). In another study, Neq (in mg/24 h) was the strongest predictor of 11-DTXB$_2$ concentrations in adult smokers compared with non-smokers (Frost-Pineda et al., 2011). Another study showed that gender and daily cigarette consumption were important predictors of the 11-DTXB$_2$ concentration, and that 11-DTXB$_2$ concentrations were greater in adult smokers than in non-smokers, but were inversely correlated with smoking duration and COHb, a biomarker of exposure to carbon dioxide (Liu et al., 2011). To date, although very few studies have examined the impact of
smoking cessation on 11-DTXB₂ concentrations, it appears that its concentrations may decrease quickly after stopping smoking (Rangemark et al., 1993, Saareks et al., 2001). As in prior studies, we found that the 11-DTXB₂ concentration was about 34% greater in smokers than in non-smokers (p < 0.0001), supporting the use of 11-DTXB₂ as a potential biomarker for smoking and CVD.

**Cardiovascular biomarkers in males and females**

In our study, we observed some differences in cardiovascular biomarkers between males and females among smokers and non-smokers. In particular, HDL-cholesterol concentrations were greater in females than in males among both smokers and non-smokers, whereas hs-CRP, WBC count, 8-epi-PGF₂α, GPx, vWF and homocysteine were lower in females than in males among both smokers and non-smokers. Meanwhile, the 11-DTXB2 concentration was greater in males than in females among smokers, but not among non-smokers. These differences in CVD biomarkers between males and females may be related to a combination of genetic, lifestyle or environmental factors.

Intriguingly, a meta-analysis of 23 trials of aspirin therapy revealed that gender accounted for a substantial proportion of the variability in the efficacy of aspirin in terms of reducing the incidence of myocardial infarction, suggesting that women might be less responsive to aspirin than men are (Yerman et al., 2007). The authors speculated that resistance to aspirin, structural and physiological differences in the coronary vasculature, and lesion characteristics might explain the reduced efficacy of aspirin in women. These findings might also be supported by the differences in CVD biomarkers between men and women, as observed in our study and other studies.

In a study performed in Japan, HDL-cholesterol concentrations were greater in women than in men (Miwa & Fujita, 2006). The HDL-cholesterol concentrations were similar between smokers and non-smokers among both men and women in that study. In a study of 8631 women and 10,690 men aged 45–54 years in six countries (Canada, China, Israel, Poland, Russia and the USA), HDL-cholesterol was consistently greater in men than in women (Davis et al., 1996). However, the magnitude of the difference varied considerably among the six countries, being smallest in China and greatest in Canada. Adjustment for BMI, smoking, alcohol use and heart rate considerably reduced the inter-country variability, suggesting that environmental factors may be an important mediator of the difference in HDL-cholesterol and possibly other cardiovascular biomarkers between men and women.

**Cardiovascular biomarkers in Japanese and Caucasians**

In a study comparing subjects living in urban or rural Japan, Japanese Americans and Caucasian Americans (Iso et al., 1989), the mean plasma fibrinogen concentration was significantly greater in Caucasians (290 mg/dL) than in the Japanese groups (223–250 mg/dL, p < 0.001), whereas the vWF concentration was not significantly different among the ethnic groups. The mean plasma fibrinogen concentration was consistently higher in current smokers than in non-smokers within each ethnic group. The difference in the mean fibrinogen concentration remained statistically significant after adjusting for age, BMI, blood pressure, serum total cholesterol, serum triglycerides and alcohol intake. The differences in fibrinogen concentrations were probably driven by differences in environmental factors, especially diet, and genetic differences between Caucasians and Japanese. The differences in plasma fibrinogen concentrations may also explain part of the difference in CVD-related mortality among these populations.

**Limitations**

The results of this study should be interpreted with some caution, considering the limitations of this study. In particular, we could not examine the changes in cardiovascular biomarkers over time, nor could we examine whether the biomarkers examined are directly or indirectly associated with CVD. In addition, we did not examine the impact of smoking cessation on any of these cardiovascular biomarkers. Finally, the numerical difference between smokers and non-smokers was small for some cardiovascular biomarkers (e.g. HDL-cholesterol [0.1 mmol/L], fibrinogen [0.15 g/L] and 8-epi-PGF₂α [0.32 mmol/L]). Although these differences were statistically significant at p < 0.0001, this may be related to the relatively large sample size, which allowed us to detect small between-group differences. Therefore, the clinical relevance of these small differences needs to be evaluated further.

**Conclusion**

The present study provides further evidence showing that several cardiovascular biomarkers can discriminate smokers from non-smokers, and that these biomarkers could be used to evaluate the risks associated with tobacco products in future prospective, longitudinal studies.

Although the smoking rates in adult males were reported to be higher in Japan than in the US (32.4% versus 21.6%, respectively) (World Health Organisation, 2013), the age-standardised mortality rate for ischaemic heart disease (per 100,000) was reported to be three times higher in the US (129) than in Japan (38) (OECD, 2013). Meanwhile, contemporary cohort studies have suggested that the age- and confounder-adjusted CHD relative mortality risk in current versus never smokers is about 2.5 in both countries (Iso, 2011; Thun et al., 2013). These findings suggest that, while smoking has similar effects on CHD in both countries, other CHD risk factors appear to be less prevalent in Japan than in the US, or protective factors might be more prevalent in Japan.

The results of this study indicate that most of the cardiovascular biomarkers examined here were sensitive to smoking status in Japan. Our results complement those reported in a large study performed in the USA of 3585 adult smokers and 1077 non-smokers (Frost-Pineda et al., 2011). It is notable that both studies evaluated biomarkers for oxidative stress, which is implicated in cardiovascular and respiratory diseases related to the use of tobacco products. The Japanese and American smoking populations also showed increases in biomarkers related to inflammatory processes, such as the WBC count and plasma fibrinogen, although we found no difference in the mean or median hs-CRP concentrations.
between smokers and non-smokers unlike the study by Frost-Pineda et al. (2011).

In conclusion, we believe our results in a large sample of Japanese smokers and non-smokers provide further support for measuring a variety of biomarkers of lipid metabolism, inflammation, oxidative stress, endothelial function and platelet activity as cardiovascular biomarkers that could be evaluated in prospective, longitudinal studies examining the physiological effects of smoking, smoking cessation and modified-risk tobacco products.

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Declaration of interest

The authors are employees of Philip Morris Products S.A.

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