Oxylipid Profile of Low-Dose Aspirin Exposure: A Pharmacometabolomics Study

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Background—While aspirin is a well-established and generally effective anti-platelet agent, considerable inter-individual variation in drug response exists, for which mechanisms are not completely understood. Metabolomics allows for extensive measurement of small molecules in biological samples, enabling detailed mapping of pathways involved in drug response.

Methods and Results—We used a mass-spectrometry-based metabolomics platform to investigate the changes in the serum oxylipid metabolome induced by an aspirin intervention (14 days, 81 mg/day) in healthy subjects (n=156). We observed a global decrease in serum oxylipids in response to aspirin (25 metabolites decreased out of 30 measured) regardless of sex. This decrease was concomitant with a significant decrease in serum linoleic acid levels (−19%, P=1.3×10−5), one of the main precursors for oxylipid synthesis. Interestingly, several linoleic acid-derived oxylipids were not significantly associated with arachidonic-induced ex vivo platelet aggregation, a widely accepted marker of aspirin response, but were significantly correlated with platelet reactivity in response to collagen.

Conclusions—Together, these results suggest that linoleic acid-derived oxylipids may contribute to the non-COX1 mediated variability in response to aspirin. Pharmacometabolomics allowed for more comprehensive interrogation of mechanisms of action of low dose aspirin and of variation in aspirin response. (J Am Heart Assoc. 2015;4:e002203 doi: 10.1161/JAHA.115.002203)

Key Words: aspirin • drugs • fatty acids • lipids • pharmacology • platelets

A cetyl salicylic acid, or aspirin, was first marketed in 1899 for reducing fever, pain, and inflammation. It was not until the 1960s that aspirin’s antiplatelet effects were realized, and aspirin is now taken by over 50 million people in the United States alone to prevent primary and secondary cardiovascular disease. The antiplatelet effects of aspirin are mediated through its ability to irreversibly inhibit cyclooxygenase-1 (COX-1), which subsequently prevents the conversion of arachidonic acid (AA) to the potent platelet activator thromboxane A2. Specifically, at low dose, aspirin acetylates platelet COX-1 in the presystemic circulation before its metabolism by the liver.1,2 Therefore, platelet function inhibition occurring at low aspirin doses is thought to result in little to no systemic effects. However, the non-COX-mediated effects of aspirin are increasingly apparent. For example, low-dose (81 mg/day) but not high-dose (650 mg/day) aspirin initiated the biosynthesis of anti-inflammatory mediators, the 15-epi-lipoxins.3 Similarly, our

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Accompanying Data S1 and Figure S1 are available at http://jaha.ahajournals.org/content/4/10/e002203/suppl/DC1

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group demonstrated that several amino acids, purine metabolites, and nonesterified fatty acids, which are not directly related to COX, were significantly altered in healthy volunteers after 81 mg/day aspirin treatment for 14 days.\(^4,5\) Understanding these non-COX-1-mediated mechanisms is critical in order to better understand the wide interindividual variability observed in aspirin response. Though aspirin use significantly reduces the risk of cardiovascular death, still \(\approx 25\%\) of high-risk patients on aspirin therapy show persistent platelet reactivity\(^6,7\) (ie, laboratory aspirin resistance) and atherothrombotic events (ie, clinical aspirin resistance) remain relatively common\(^8\) in patients on aspirin therapy. The mechanisms underlying variability in aspirin response are poorly understood. Incomplete COX-1 inhibition has been observed in several settings;\(^9,10\) however, poor response despite complete COX-1 inhibition has also been reported.\(^9,11\)

Omega-6 fatty acids such as linoleic acid (LA) (C18:2), dihomo-\(\gamma\)-linolenic acid (C20:3), or AA (C20:4) and omega-3 fatty acids such as \(\alpha\)-linolenic acid (C18:3), eicosapentaenoic acid (C20:5), or docosahexaenoic acid (C22:6) are essential polyunsaturated fatty acids. The oxidation products of polyunsaturated fatty acids, so-called oxylipids, are highly potent mediators of both pro- and anti-inflammatory processes.\(^12\) Oxylipids can be formed via COXs, lipooxygenases (LOXs), cytochrome P-450 monooxygenases (CYPs), and by nonenzymatic oxidation. Oxylipids have myriad functions that are still being elucidated. Aberrant oxylipid signaling has been shown to lead to a number of pathologies important to cardiovascular disease including hyperlipidemia, hypertension, thrombosis, and hemostasis.\(^13\) For example, coronary artery disease patients have higher plasma levels of CYP-mediated AA metabolites (namely, the oxylipid epoxyeicosatrienoic acid).\(^14\) While some studies have described the effects of aspirin on individual oxylipids,\(^15-17\) to our knowledge, the systematic effect of aspirin on oxylipid as a class has not been described. Given the complexity of the oxylipid metabolic network, a systematic approach will offer insight into how the entire oxylipid network responds to drug exposure.

Pharmacometabolomics is an emerging field that aims to use metabolomics tools to define the mechanisms of action for drugs and the biochemical basis for variation in response to treatment.\(^18,19\) Metabolic profiles integrate genetic and environmental influences and provide unique information that can help explain the drug–response phenotype.\(^20,21\) In the present investigation, we have used a robust, broad-spectrum mass spectroscopy (MS)-based metabolomics platform to measure the concentrations of multiple oxylipids in serum samples from 156 healthy volunteers of the Heredity and Phenotype Intervention (HAPI) Heart Study before and after 14 days of low-dose (81 mg/day) aspirin treatment. We aim to characterize the effect of low-dose aspirin therapy on the oxylipid metabolic pathways, to investigate sex differences in aspirin-induced oxylipid changes, and to determine whether oxylipids are associated with aspirin-induced inhibition of platelet aggregation.

**Methods**

**HAPI Heart Study Design and Platelet Aggregation Measures**

Samples for metabolomic profiling were obtained from subjects enrolled in the HAPI Heart Study, which has been described previously.\(^22\) Briefly, participants were relatively healthy adult members of the Old Order Amish population from Lancaster County, PA. Subjects (\(N=745\)) participated in a short-term aspirin intervention where they were given 81 mg of aspirin for 14 consecutive days.\(^23\) Medication adherence was high and was monitored by pill counts. Blood samples were obtained after an overnight fast, and ex vivo platelet aggregometry was performed before aspirin therapy and again the morning after the last dose by the same technician. Aggregation was induced with collagen (2 \(\mu\)g/mL) or AA (0.5 mmol/L). More details on sample collection and ex vivo platelet testing are provided in Data S1. Blood samples for serum preparation were allowed to clot at room temperature for 15 minutes, centrifuged at \(2025\) g for 10 minutes then immediately frozen at \(-80°\text{C}\).

All study procedures were in accordance with the Declaration of Helsinki. The study was approved by the institutional review board of the University of Maryland, Baltimore and was monitored by an external data safety and monitoring board. Participants provided informed consent, including permission to contact relatives, before participation.

**Metabolomics**

In this metabolomics study, samples originated from 156 HAPI participants. Figure 1 describes the procedure used to select this subset. The 745 original HAPI participants were grouped into sex-specific quartiles of aspirin response as measured by post-aspirin collagen-stimulated platelet aggregation adjusted for participant age and preaspirin collagen-stimulated platelet aggregation. We used collagen-induced platelet aggregation to select our samples because we sought to probe non-COX-1-mediated pathways implicated in aspirin’s mechanism of action and in variation in aspirin response. Twenty-five non–first-degree relatives from each sex-specific drug–response quartile were selected for metabolic profiling. Given sample availability, samples from 156 subjects were successfully profiled both pre- and post-aspirin and were included for analysis.
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by the mean metabolite response value for the metabolite, a
and 12-HHTrE), we report metabolite response values divided
which absolute concentrations were not obtained (12-HETE
areas falling below the limits of detection and were imputed
Missing ion intensity values were assumed to result from
Statistical Analysis
R.25 Twenty-eight metabolites were successfully quanti-
ted because calibration curves failed. All metabolites
measured are listed in Table 1. AA and LA were measured
using gas chromatography–mass spectrometry as described
previously.4

Figure 1. Procedures for sample selection. Flow diagram shows
procedure for sample selection for the 156 metabolomics
substudy participants from the overall Heredity and Phenotype
Intervention (HAPI) Heart Study (n=745).

Oxylipids were extracted from 250 μL serum according to
the procedure described previously.24 Samples were analyzed
by liquid chromatography (Agilent 1260; San Jose, CA)
coupled to electrospray ionization on a triple quadrupole
mass spectrometer (Agilent 6460; San Jose, CA). Quantitation
of oxylipid response was calculated as the peak area ratios of
the target analyte to the respective internal standard. To
obtain actual concentrations, calibration samples with spiked
oxylipid levels were included in the measurement series.
Relative response ratios of oxylipids were converted into
actual concentrations (nmol/L) using the chemCal package in
R.25 Twenty-eight metabolites were successfully quantified
and 2 metabolites (12-HETE and 12-HHTrE) were semiquan-
tified because calibration curves failed. All metabolites
measured are listed in Table 1. AA and LA were measured
using gas chromatography–mass spectrometry as described
previously.4

Statistical Analysis
Missing ion intensity values were assumed to result from
areas falling below the limits of detection and were imputed
with values randomly drawn from a normal distribution with
mean close to the limit of detection. For the 2 metabolites for
which absolute concentrations were not obtained (12-HETE
and 12-HHTrE), we report metabolite response values divided
by the mean metabolite response value for the metabolite, a
scaling method that does not affect our conclusions. Metabolite response values were log-transformed before
statistical analysis and back-transformed for presentation.
We assessed the significance of the effects of aspirin exposure and sex on metabolite level using linear models
and linear mixed models in GenStat 14th edition (VSN
International, Hemel Hempstead, UK) as described in details
in Data S1.

All other statistical analyses were performed using Matlab
version R2009a (MathWorks Inc, Natick, MA) or R.25 Correla-
tions were performed using Spearman’s rank correlation. The
modulated modularity clustering algorithm26 was used to
cluster oxylipids based on their correlation coefficients.
Significant changes in correlation (post- versus pre-aspirin)
were assessed comparing the differences in correlation with a
null distribution of correlation differences. The null distribution
was generated by randomly permuting the post- and pre-aspirin
results of individuals, so that on average half of the data are
permuted. Repeating this random permutation multiple times
generated the null-distribution. For all statistical tests, each set
of P values was corrected for multiple comparisons using the
procedure described by Benjamini and Yekutieli27 in R package
multtest.28 This function computes the false discovery rate (q
value) to control the expected proportion of rejected null
hypotheses that were incorrect rejections (false discoveries)
when conducting multiple comparisons. Significance was
achieved when both P<0.05 and q<0.05.

Results
Participants’ Characteristics
Healthy volunteers enrolled in the HAPI Heart Study were
selected for metabolic profiling based on their ex-vivo
response to low-dose aspirin treatment. Characteristics and
platelet aggregation measures of the 156 subjects selected
for this metabolomics investigation are shown in Table 2.
Samples originated from 81 women and 75 men and sex-
specific ex-vivo response quartiles were comparable for age,
body mass index and pre-aspirin measures of collagen-
induced platelet aggregation. Women were slightly older
(P=0.02) and had a higher body mass index (P=0.02) than
men, as well as higher collagen-induced platelet aggregation
pre- and post-aspirin (P=6.0×10^{-3} and 1.0×10^{-4}, respec-
tively). However, change in collagen-induced platelet aggre-
gation upon aspirin exposure was not significantly different
between men and women (P=0.06). We also compared the
subset of 156 subjects with metabolomics profiling to the full
HAPI sample (N=745) and did not detect differences in sex
(P=0.18), age (P=0.26), body mass index (P=0.36), presence
of diabetes (P=0.59), AA-induced platelet aggregation
pre- and postaspirin (P=0.054 and 0.07, respectively) or
Table 1. Metabolites Detected

| Name       | Precursor FA | Omega 6/3 | Enzyme | Lipid Map ID       | HMDB ID  |
|------------|--------------|-----------|--------|--------------------|----------|
| 12-HETE    | AA           | n-6       | 12-LOX | LMFA03060088       | HMDB06111|
| 15-HETE    | AA           | n-6       | 15-LOX | LMFA03060001       | HMDB03876|
| 8-HETE     | AA           | n-6       | 15-LOX | LMFA03060006       | HMDB04679|
| LTB₄       | AA           | n-6       | 5-LOX  | LMFA03020001       | HMDB10185|
| 5-HETE     | AA           | n-6       | 5-LOX  | LMFA03060002       | HMDB11134|
| TXB₂       | AA           | n-6       | COX    | LMFA03030002       | HMDB03252|
| PGF₂α      | AA           | n-6       | COX    | LMFA03010002       | HMDB01139|
| 13,14-dihydro-PGF₂α | AA | n-6   | COX    | LMFA03010079       | HMDB04239|
| 12S-HHTE    | AA           | n-6       | COX    | LMFA03050002       | HMDB12535|
| 11-HETE    | AA           | n-6       | CYP    | LMFA03060028       | HMDB04682|
| 14,15-DIHETrE | AA   | n-6   | CYP    | LMFA03050010       | HMDB02265|
| 11,12-DIHETrE | AA   | n-6   | CYP    | LMFA03050008       | HMDB02314|
| 8,9-DIHETrE | AA           | n-6       | CYP    | LMFA03050006       | HMDB02311|
| 20-HETE    | AA           | n-6       | CYP    | LMFA03060009       | HMDB05998|
| 5,6-DIHETrE | AA           | n-6       | CYP    | LMFA03050004       | HMDB02343|
| 9-HOTrE    | ALA          | n-3       | 5-LOX  | LMFA02000024       | HMDB10224|
| 15(S)-HETrE | DGLA         | n-6       | 15-LOX | LMFA03050007       | HMDB05045|
| PGF₁α      | DGLA         | n-6       | COX    | LMFA03010137       | HMDB02685|
| 19,20-DHDPa | DHA          | n-3       | CYP    | LMFA04000043       | HMDB10214|
| 12(S)-HEPE | EPA          | n-3       | 12-LOX | LMFA03070008       | HMDB10202|
| 15(S)-HEPE | EPA          | n-3       | 15-LOX | LMFA03070009       | HMDB10209|
| 5(S)-HEPE  | EPA          | n-3       | 5-LOX  | LMFA03070010       | HMDB05081|
| 13-HODE    | LA           | n-6       | 15-LOX | LMFA02000022       | HMDB06939|
| 9-HODE     | LA           | n-6       | 5-LOX  | LMFA02000015       | HMDB10223|
| 9-KODE     | LA           | n-6       | 5-LOX  | LMFA02000274       | HMDB04669|
| 9,12,13-TriHOME | LA   | n-6   | 5-/15-LOX | LMFA02000014   | HMDB04708|
| 12,13-DHOME | LA           | n-6       | CYP    | LMFA02000230       | HMDB04705|
| 9,10-DHOME | LA           | n-6       | CYP    | LMFA02000229       | HMDB04704|
| 12(13)-EpOME | LA          | n-6     | CYP    | LMFA02000038       | HMDB04702|
| 9(10)-EpOME | LA           | n-6       | CYP    | LMFA02000037       | HMDB04701|

AA indicates arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYP, cytochrome P450; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid; LOX, lipoxygenase.

Collagen-induced platelet aggregation pre- and post-aspirin (P=0.58 and 0.76, respectively).

Metabolic Profiling

The metabolomic platform successfully detected 30 unique oxylipids in our samples. Besides the well-described AA metabolites, oxylipids derived from LA, dihomo-γ-linolenic acid, α-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid were also measured. Figure S1 illustrates the biochemical coverage of the platform in a pathway-specific context.

Effect of Sex

Given that sex differences in the metabolome and in aspirin response have been reported previously,12,23,29 we first assessed whether the effect of aspirin on oxylipids was sex dependent. We compared oxylipid levels pre- and post-aspirin in men versus women. Prior to aspirin exposure, 8 metabolites were significantly higher in women and 1 (5,6-DIHETrE) was significantly higher in men (Table 3). After aspirin exposure, 6 of these metabolites remained significantly different between men and women (Table 3). When linear mixed models were fitted to test the interaction between
Table 2. Subject Characteristics

| Response Variable | Women | Men | P Value* | Total | P value† |
|-------------------|-------|-----|----------|-------|----------|
| Quartile          | Q1    | Q2  | Q3    | Q4  | Q1 | Q2 | Q3 | Q4 | Total | P value† |
| N                 | 20    | 23  | 17    | 21  | 81 | 18 | 22 | 14 | 21 | 75 |
| Age, y            | 43±12 | 49±14 | 43±15 | 42±11 | 0.2 | 45±13 | 43±15 | 39±11 | 40±11 | 38±10 | 0.8 | 40±12 | 0.02 |
| BMI, m/kg²        | 30±6  | 29±6 | 26±5  | 26±4 | 0.07 | 28±6 | 25±3 | 27±3 | 25±3 | 26±3 | 0.4 | 26±3 | 0.02 |

Whole blood indirect COX-1 pathway: collagen-induced platelet aggregation

| Pre-aspirin, Ω  | 14±3   | 14±2   | 14±3   | 14±2   | 0.9 | 14±2   | 13±2   | 13±2   | 13±2   | 13±3   | 0.7 | 13±2   | 6×10⁻³ |
| Post-aspirin, Ω | 6±2    | 11±2   | 12±1   | 15±1   | 1×10⁻¹⁴ | 11±3   | 5±2    | 8±1    | 10±1   | 13±2   | 1×10⁻¹⁴ | 9±3 | 1×10⁻⁴ |
| Change           | −8±3   | −4±1   | −2±2   | 1±2    | 3×10⁻¹³ | −3±4   | −8±3   | −5±1   | −3±2   | 0±3    | 2×10⁻¹¹ | −4±4 | 0.06 |

Whole blood direct COX-1 pathway: arachidonic acid–induced platelet aggregation

| Pre-aspirin, Ω  | 10±2   | 11±6   | 11±2   | 11±2   | 0.6 | 11±2   | 8±4    | 8±2    | 9±3    | 9±4    | 0.72 | 8±3    | 2×10⁻⁷ |
| Post-aspirin, Ω | 0±0    | 1±3    | 2±3    | 2±3    | 0.12 | 1±3    | 0±0    | 0±1    | 1±1    | 2±3    | 8×10⁻³ | 1±2 | 0.96 |

BMI indicates body mass index; COX-1, cyclooxygenase-1.

*P values for comparing mean values of gender-specific quartiles (using Kruskal–Wallis tests).
†P values for comparing mean values in all women vs all men (using Wilcoxon tests).

Aspirin treatment and sex on oxylipid levels, no interaction remained significantly different after correction for multiple testing (8×10⁻³< P<0.92, 0.22< q<0.92), demonstrating that aspirin affected oxylipid levels similarly in men and women. Therefore, all subsequent analyses were performed in the entire cohort.

Oxylipid Signature of Aspirin Exposure

Investigating the effect of aspirin exposure on oxylipid levels in our entire cohort revealed that the levels of 26 metabolites were significantly decreased post- compared to pre-aspirin exposure, while only 1 metabolite (13,14-dihydroPGF2α) was increased (Table 4). As expected, the metabolites with the largest magnitude of decrease were known COX products, namely, TXB2 (−97%), 12-HHTrE (−87%), and 11-HETE (−85%). However, it was quite surprising to observe that aspirin also significantly decreased the levels of almost all metabolites measured, independently of their fatty acid precursor or the enzyme used for their synthesis (Figure 2). For example, aspirin strongly decreased LOX-products derived from AA (5-HETE, 12-HETE, 8-HETE, and 15-HETE), LA (9-HODE and 13-HODE), dihomo-γ-linolenic acid (15-HETE), eicosapentaenoic acid (5-HEPE, 12-HEPE, and 15-HEPE), and

Table 3. Significant Oxylipid Differences in Women Versus Men

| Metabolites | Precursor | Enzyme | Pre-aspirin | Postaspirin | P Value | Q | Women | Men | P Value | Q |
|-------------|-----------|--------|-------------|-------------|---------|---|-------|-----|---------|---|
| 5-HEPE      | EPA       | 5-LOX  | 5.3±3.4     | 3.4±2.9     | 2×10⁻⁸ | 4×10⁻⁷ | 3.6±2.3 | 2.3±1.2 | 1×10⁻⁵ | 1.8×10⁻⁴ |
| 13-HODE     | LA        | 15-LOX | 24±10.5     | 18.3±8.7    | 3×10⁻⁵ | 3×10⁻⁴ | 17.2±7.3 | 14.2±7.8 | 2×10⁻⁵ | 1.8×10⁻⁴ |
| 9-HODE      | LA        | 5-LOX  | 22.4±10.6   | 16.9±7.2    | 7×10⁻⁵ | 4×10⁻⁴ | 14.8±5.6 | 13.1±7  | 2×10⁻³ | 0.007 |
| 5,6-DiHETE  | AA        | CYP    | 3.4±1.5     | 4.4±2       | 3×10⁻⁴ | 1.2×10⁻³ | 2.5±1.1 | 3.2±1.4 | 4×10⁻⁴ | 0.002 |
| 5-HETE      | AA        | 5-LOX  | 15±6.6      | 12±5.4      | 2×10⁻³ | 6×10⁻³ | 11.5±5.7 | 9.2±4.5 | 3×10⁻³ | 0.008 |
| 9-HOTETe    | AA        | 5-LOX  | 1.1±0.6     | 0.8±0.4     | 4×10⁻³ | 0.01 | 0.7±0.4 | 0.6±0.4 | 0.25 | 0.2 |
| 9,10-EpOME  | LA        | CYP    | 3.3±1.6     | 2.7±2.6     | 2×10⁻⁴ | 0.001 | 2.6±2   | 2.3±2   | 0.2   | 0.2 |
| 12,13-DHOME  | LA       | CYP    | 12.7±5.6    | 10.7±6.3    | 3×10⁻³ | 0.008 | 11.3±6.2 | 9.3±4.9 | 3×10⁻³ | 0.008 |
| 11,12-DiHETe | AA       | CYP    | 3.7±1.3     | 3.2±1.1     | 0.01 | 0.02 | 3±0.9  | 2.8±0.9 | 0.04 | 0.06 |

Data represent mean±SD of metabolite concentrations (nmol/L) in men (n=75) and women (n=81). AA indicates arachidonic acid; ALA, α-linolenic acid; CYP, cytochrome P450; EPA, eicosapentaenoic acid; LA, linoleic acid; LOX, lipoxigenase.
Table 4. Effect of Aspirin on Oxylipids

| Name    | Precursor | Enzyme | Pre-aspirin | Postaspirin | % Change | P value | Q value |
|---------|-----------|--------|-------------|-------------|----------|---------|---------|
| TXB2    | AA        | COX    | 241.3±245.8 | 7±12.9      | -97      | 1.9×10^-26 | 3.2×10^-25 |
| 12-HHTe*| AA        | COX    | 1.8±1.8     | 0.2±0.1     | -87      | 2.3×10^-26 | 3.2×10^-25 |
| 11-HETE | AA        | COX    | 13.7±11.9   | 1.8±1.2     | -85      | 3.3×10^-26 | 3.2×10^-25 |
| 15-HETE | AA        | 15-LOX | 16.2±12.9   | 5.2±3.6     | -67      | 1.7×10^-24 | 1.2×10^-23 |
| 12-HEPE | EPA       | 12-LOX | 26.2±34.5   | 11.1±17.4   | -60      | 3.3×10^-13 | 8.2×10^-13 |
| 12-HETE*| AA        | 12-LOX | 1.3±1.4     | 0.7±1       | -53      | 8×10^-13  | 1.2×10^-12 |
| 15-HETE | DGLA      | 15-LOX | 3.2±1.5     | 1.8±0.9     | -45      | 9×10^-22  | 5.8×10^-21 |
| 15-HEPE | EPA       | 15-LOX | 1.7±1.1     | 1±0.7       | -44      | 5×10^-16  | 2.1×10^-15 |
| 8-HETE  | AA        | 15-LOX | 5.5±3.6     | 3.4±2.6     | -43      | 1.4×10^-12 | 3×10^-12  |
| PGF2α   | AA        | COX    | 5.9±3.4     | 3.9±2.3     | -31      | 7.4×10^-20 | 3.7×10^-19 |
| 9-Hote  | ALA       | 5-LOX  | 1±0.5       | 0.7±0.4     | -31      | 9.2×10^-10 | 1.5×10^-9  |
| 9-ODE   | LA        | 5-LOX  | 19.7±9.5    | 14±6.4      | -29      | 7.3×10^-14 | 2.4×10^-13 |
| 5-HEPE  | EPA       | 5-LOX  | 4±3.3       | 3±         | -28      | 2.5×10^-13 | 6.7×10^-13 |
| 5,6-DHETe| AA      | CYP    | 3.9±1.8     | 2.8±1.3     | -27      | 3.6×10^-14 | 1.3×10^-13 |
| 13-ODE  | LA        | 15-LOX | 21.3±10     | 15.8±7.7    | -27      | 6.5×10^-12 | 1.3×10^-11 |
| 5-HETE  | AA        | 5-LOX  | 13.6±6.2    | 10.4±5.3    | -26      | 2.5×10^-13 | 6.7×10^-13 |
| 12,13-DHOME| LA    | CYP    | 11.7±6      | 10.3±5.7    | -19      | 1.3×10^-4  | 1.6×10^-4  |
| 20-HETE | AA        | CYP    | 7.2±2.8     | 5.9±2.2     | -19      | 1.9×10^-8  | 2.8×10^-8  |
| 11,12-DHETe| AA   | CYP    | 3.5±1.2     | 2.9±0.9     | -16      | 9.2×10^-10 | 1.5×10^-9  |
| 9-KODE  | LA        | 5-LOX  | 2.6±1.8     | 2.2±1.6     | -15      | 1.1×10^-7  | 1.5×10^-7  |
| 9,10-DHOME| LA     | CYP    | 6±3.4       | 5.5±3.9     | -15      | 1×10^-4    | 1.3×10^-8  |
| 8,9-DHETe| AA      | CYP    | 2.3±0.7     | 2±0.6       | -14      | 1.8×10^-9  | 2.8×10^-8  |
| PGF1α   | DGLA      | COX    | 2.6±0.5     | 2.2±0.4     | -11      | 1.5×10^-11 | 2.8×10^-11 |
| 9,10-EpOME| LA     | CYP    | 3±2.1       | 2.4±2       | -11      | 1.3×10^-6  | 1.7×10^-6  |
| 12,13-EpOME| LA    | CYP    | 5±3.3       | 4.6±3       | -11      | 8.5×10^-3  | 9.4×10^-3  |
| 14,15-DHETe| AA   | CYP    | 4.8±1.2     | 4.3±1.1     | -9       | 7.8×10^-8  | 1.1×10^-7  |
| 9,12,13-TriHOME| LA | 5/15-LOX | 1.5±0.6   | 1.4±1       | -3       | 0.036    | 0.038 |
| 13,14-dihydro-PGF2α| AA | COX  | 39.9±6.5 | 41.9±6.2 | 3 | 0.004 | 0.005 |
| 19,20-DiHDP| DHA  | CYP    | 7.3±3.2     | 7.1±3       | -2       | 0.56     | 0.56 |
| LTBA    | AA        | 5-LOX  | 3.1±3.7     | 2.6±3       | 0        | 0.06     | 0.06 |

Data represent mean±SD of metabolite concentrations (nmol/L) in all subjects (n=156) pre- and postaspirin treatment and median % change. Absolute concentrations could not be determined for metabolites with an asterisk, so data represent mean±SD of metabolite levels divided by the mean metabolite level detected in this study. AA indicates arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYP, cytochrome P450; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LOX, lipoxigenase.

α-linolenic acid (9-HotE). Although to a lesser extent, aspirin also significantly decreased levels of CYP-produced metabolites derived from LA (EpOMEs and DiHOMEs) and AA (DiHETeRs and 20-HETe).

Changes in Correlation Between Oxylipids Upon Aspirin Exposure

To further evaluate the impact of aspirin exposure on the oxylipid pathways, we conducted cluster plot analyses in order to observe correlations among oxylipid metabolites. Figure 3A displays correlations between metabolite levels pre-aspirin. Three clusters reflecting the main oxylipid biosynthetic pathways emerge from this unsupervised clustering analysis. Cluster 1 comprises AA-derived oxylipids formed through LOX or COX. Cluster 2 consists of LA-derived metabolites synthesized through LOX or CYP. Finally, AA- and docosahexaenoic acid–derived CYP products correlate with each other in cluster 3. Figure 3B displays these correlations post-aspirin treatment. Significance of change in correlation...
between metabolites post- versus pre-aspirin exposure was evaluated using permutation testing. Figure 3C displays the \( q \)-values to evaluate whether correlations between metabolites changed significantly post- versus pre-aspirin exposure. Most significant \( q \) values can be observed within Cluster 1, where correlations between COX-generated metabolites (TXB2, 12-HHTe, PGF1a, and PGF2a) and other metabolites from Cluster 1 were close to zero after aspirin treatment. This
Figure 3. Correlations between oxylipids. A, Spearman correlation coefficients for correlation between metabolite levels pre-aspirin in all 156 subjects are displayed. Metabolites were clustered based on Spearman correlation coefficients using modulated modularity clustering algorithm. B, Spearman correlation coefficients for correlation between metabolite levels post-aspirin in all 156 subjects are displayed. The clustering order is the same as in Figure 3A. C, Q values evaluating the significance of the change in correlation between metabolites post- vs pre-aspirin are displayed. The clustering order is the same as in Figure 3A.
finding was expected given the known direct COX inhibition of aspirin, which led to strong inhibition of all COX-products in our samples post-aspirin administration. Surprisingly, however, in Clusters 2 and 3, correlations between metabolites were not changed post-aspirin exposure, although the drug significantly decreased most metabolite levels. This might result from a disruption of the metabolic pathways somewhere upstream, maybe at the level of the fatty acid precursors. To evaluate this hypothesis, we measured the levels of free AA and LA, the 2 main fatty acid precursors for the oxylipid measured. Free AA levels were not significantly affected by aspirin treatment (−8%, \( P=0.42 \)), whereas those of LA were significantly decreased (−19%, \( P=1.3 \times 10^{-5} \)).

### Correlations Between Fatty Acid Precursors and Oxylipid Products

We further evaluated the causal relationship between free fatty acid precursor levels and the levels of their product oxylipids by computing the correlations between them prior to aspirin administration (Table 5 and Figure 4). We observed significant correlations between LA-derived oxylipids and LA, with correlation coefficients (\( \rho \)) ranging from 0.27 to 0.5, whereas no significant correlation was found between AA and AA-derived oxylipids (−0.13<\( \rho <0.17 \)).

### Association Between Oxylipids and Aspirin-Induced Inhibition of Platelet Aggregation

Finally, our study assessed whether oxylipid levels correlated with ex-vivo platelet aggregation measures. We observed significant correlations between post-aspirin oxylipid levels and collagen-induced platelet aggregation (Table 6). LA and AA levels did not correlate with collagen-induced platelet aggregation. Correlation analyses with AA-induced platelet aggregation measures were not conducted since 75% (n=117) of the subjects had a complete inhibition of AA-induced platelet aggregation postaspirin. Therefore, we evaluated differences in oxylipid, AA, and LA levels between the subjects with complete and those with incomplete AA-induced platelet aggregation postaspirin. We observed a trend towards higher levels of TXB2 in the subjects with incomplete inhibition of AA-induced platelet aggregation (\( P=0.008, q=0.1 \)). No other significant difference was found.

### Discussion

In this study, we investigated the metabolic signature of aspirin exposure in healthy volunteers using a quantitative mass spectrometry–based metabolomics platform targeted to the measurement of oxylipids. We aimed to evaluate the changes in the oxylipid metabolome in response to aspirin and to determine whether oxylipids contributed to variation in aspirin response.

The mechanisms underlying variation in response to aspirin as antiplatelet therapy are largely unknown. Several studies have reported less effective inhibition of platelet function in response to aspirin in women than in men\(^{3,30,31}\) and whether women benefit equally from aspirin therapy as men is still in debate. In the present study, we therefore first evaluated constitutive and aspirin-induced sex differences in oxylipid levels. Several previous metabolomics studies have shown considerable sex differences in the human metabolome\(^{4,5,29,32–34}\) none of them including the oxylipid metabolites. Here, we report for the first time that many oxylipid CYP products (the LA-derived 9,10 EpOME and 12,13 DiHOME and the AA-derived 5,6 DiHETrE and 11-12 DiHETrE) differ between sexes. The CYP2J and 2C families are

#### Table 5. Correlations Between Serum Oxylipid and Free Fatty Acid Levels Pre-aspirin

| Metabolite          | Enzyme | \( \rho \)  | \( P \) value |
|---------------------|--------|-------------|---------------|
| LA-derived oxylipids|        |             |               |
| 9,10-DiHOME         | CYP    | 0.39        | 1.2 \times 10^{-6} |
| 9,10-EpOME          | CYP    | 0.42        | 9 \times 10^{-8} |
| 12,13-DiHOME        | CYP    | 0.44        | 1.7 \times 10^{-8} |
| 12,13-EpOME         | CYP    | 0.36        | 6 \times 10^{-6} |
| 9-HODE              | 5 LOX  | 0.5         | 1 \times 10^{-10} |
| 9-KODE              | 5 LOX  | 0.27        | 8 \times 10^{-4} |
| 13-HODE             | 15 LOX | 0.48        | 5 \times 10^{-10} |
| AA-derived oxylipids|        |             |               |
| 11-HETE             | COX    | −0.1        | 0.35          |
| PGF\(_{2\alpha}\)    | COX    | −0.13       | 0.09          |
| 12S-HHT             | COX    | −0.11       | 0.17          |
| TXB2                | COX    | −0.09       | 0.27          |
| 20-HETE             | CYP    | 0           | 0.86          |
| 5,6-DiHETrE         | CYP    | 0           | 0.74          |
| 8,9-DiHETrE         | CYP    | 0.11        | 0.17          |
| 11,12-DiHETrE       | CYP    | 0.17        | 0.05          |
| 14,15-DiHETrE       | CYP    | 0.13        | 0.12          |
| 5-HETE              | 5 LOX  | 0.1         | 0.21          |
| 12-HETE             | 12 LOX | −0.1        | 0.36          |
| 8-HETE              | 15 LOX | 0           | 0.9           |
| 15-HETE             | 15 LOX | 0           | 0.5           |

AA indicates arachidonic acid; COX, cyclooxygenase; CYP, cytochrome P450; LA, linoleic acid; LOX, lipoxygenase.

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responsible for the synthesis of these molecules, and sex differences in human CYP activity has been extensively reported. We also observed higher levels of 5-LOX products (namely, the eicosapentaenoic acid–derived 5S-HEPE, the LA-derived 9-HODE, the AA-derived 5-HETE, and the α-linolenic acid–derived 9-HOTre) in women. This is in accordance with previous findings that androgens downregulate 5-LOX product formation. Most of these constitutive differences in oxylipid levels persisted after aspirin treatment and no sex-specific effect of aspirin on oxylipid was observed, consistent with our previous findings in this population.

Secondly, we observed that aspirin significantly decreased almost all of the oxylipids measured in our samples (26 out of 30), independently of their fatty acid precursors or their synthesizing enzymes. This finding may seem surprising, as one might have thought that inhibition of 1 enzymatic branch (COX) would divert fatty acid substrates towards other branches of the pathways, leading to higher levels of LOX- or CYP-derived products. However, oxylipid metabolism does not seem to follow a simple mass-action rule, at least when measured globally in serum, and our findings are in agreement with a previous report. In a murine inflammatory model, a single high dose of aspirin significantly lowered plasma levels of COX-, LOX-, and CYP-derived AA metabolites. The authors concluded that the COX, LOX, and CYP pathways “do not proceed in a parallel way but communicate in a dynamic manner.” In contrast, Shinde et al reported that aspirin significantly decreased TXB2 levels in plasma of healthy volunteers, but not the levels of CYP-, LOX-, or AA-derived metabolites. However, they measured oxylipids 2 hours after a single high dose of aspirin, which might explain our differing results.

To unravel the key points at which metabolic regulation has changed in the oxylipid pathways, we next examined aspirin-induced changes in pairwise correlations of metabolite levels. We observed that, except for the direct COX- metabolites, correlations between oxylipids did not change significantly upon aspirin administration, even though metabolite levels were altered. We postulated that this coordinated change in oxylipid levels could result from an effect of aspirin on the upstream parts of the biosynthetic pathways and found that LA levels were indeed significantly lowered by aspirin treatment. Further, we observed a high correlation between LA levels and its oxylipid products in serum. Therefore, based on our findings, we postulate that, for LA-derived oxylipids, aspirin-induced decrease in precursor availability might account for the observed decrease in circulating oxylipid levels. The correlations were poor between AA levels and its oxylipid products, suggesting that free AA is not a major source of synthesis for circulating oxylipids.

We show for the first time that low-dose aspirin therapy, such as that routinely used for prevention of cardiovascular disease, broadly decreases circulating fatty acid levels in healthy humans. We previously reported in the same popu-
lation that aspirin decreased the levels of many of the most abundant FAs, including oleic (−28%), palmitic (−10%), palmi- toleic (−31%), stearic (−15%), and lauric (−15%) acids, as well as glycerol levels (−13%). The concomitant decrease of free fatty acids and glycerol indicates a potential inhibition of lipolysis by aspirin in our subjects. The metabolic effects of aspirin are one of the least appreciated pharmaco- logical actions of this drug but have been described 125 years ago. A decrease in serum fatty acid levels has been reported following salicylate administration in rats and in healthy and diabetic humans. There is evidence that salicylates inhibit lipolysis and enhance fatty acid re-esterification in animal adipose tissue ex-vivo and downregulate the expression and activity of adipose 11β-hydroxysteroid dehydrogenase type 1, a lipolytic enzyme. However, all previous studies have used high dose of salicylates (minimum 1 g per day for 4 days). The long-term consequences of the broad decrease in fatty acid levels we present here in participants taking low-dose aspirin are unknown. In the Women’s Health Study, low-dose aspirin (100 mg every other day) did not decrease the incidence of type 2 diabetes in healthy women. However, whether daily aspirin dosing would alter or delay progression to diabetes or benefit some subgroup of patients is unknown. Based on our findings, future studies evaluating various doses of aspirin in different patient populations (men and women of varying cardiovascular risk) for its potential role in preventing diabetes or insulin resistance may be warranted.

Serum AA levels were not significantly decreased post-aspirin in our study samples. However, low correlation between adipose and circulating levels of AA have been reported in nondiabetic women under prolonged fasting, whereas LA adipose and serum levels were strongly corre- lated. These findings suggest that circulating AA levels poorly reflect adipose tissue lipolysis and might explain why AA levels were not significantly decreased upon aspirin exposure in our study. Unfortunately, n-3 fatty acid levels were not measured by our analytical platform, so whether those levels are also decreased upon low-dose aspirin treatment remains to be determined.

Finally, we investigated whether oxylipids are involved in variation in response to aspirin by correlating oxylipid levels to ex-vivo platelet aggregation measures post-aspirin. Not surprisingly, we observed higher levels of TXB2 in individuals with incomplete inhibition of AA-induced platelet aggregation. However, no other metabolite was correlated with AA-induced platelet aggregation post-aspirin exposure, suggesting that differences in oxylipid levels do not contribute appreciably to variability in traditional, COX-1-mediated measures of aspirin response. In contrast, we observed significant correlations between 4 LA-derived oxylipids (13-HODE, 9-HODE, 12,13-DiHOME, and 12,13-EpOME) and collagen-induced platelet aggregation post-aspirin. Endothelial, epidermal, polymorphonuclear cells, and platelets convert LA into 13-HODE and 9-HODE. HODEs are active mediators in hemostasis, inflammation, and cancer invasion. Low 13-HODE, 9-HODE, 12,13-DiHOME, and 12,13-EpOME in serum correlating to better non-COX1-mediated aspirin response may result from a negative feedback mechanism, although these results deserve further confirmation in other extended cohorts of healthy volunteers and/or patients with cardiovascular diseases on aspirin.

A limitation of our study that should be mentioned is the lack of a control group with oxylipid measurements taken over time without the administration of aspirin. Therefore, we are unable to separate the natural time variation of oxylipid levels from the effects of aspirin. However, based on the relatively short time period of our intervention and previous studies in which we have demonstrated minimal changes in the metabolome in placebo-treated participants, with 0 metabolites differing after 1 week of placebo and 6 of 348 measured metabolites differing significantly after 4 weeks of placebo treatment, we hypothesize that the changes in oxylipids (27 out of 30 measured) we observed after 2 weeks are likely aspirin-driven rather than lifestyle-driven.

In conclusion, our findings revealed a sex-independent, global decrease in serum oxylipids in response to low-dose aspirin treatment in healthy volunteers, as well as in oxylipids that are not synthesized by COX. We show that this global decrease in oxylipids might be related to aspirin-induced decrease in precursor nonesterified fatty acids. Finally, we observed that several LA-derived oxylipids were significantly correlated with non-COX1-mediated measures of aspirin response, thereby suggesting that these metabolites might contribute to the non-COX1-mediated variability in response to aspirin. Pharmacometabolomics provided a powerful tool to understand the mechanisms of action of aspirin and the metabolic determinants of variation of response to aspirin’s antiplatelet effects.

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Disclosures

None.

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