Hypertension

PREECLAMPSIA

# Effective Aspirin Treatment of Women at Risk for Preeclampsia Delays the Metabolic Clock of Gestation

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**ABSTRACT:** Preeclampsia, characterized by the onset of hypertension with significant proteinuria after 20 weeks' gestation, is one of the leading causes of maternal and perinatal morbidity and mortality. Prophylactic low-dose aspirin treatment reduces the rate of preterm preeclampsia in high-risk women, but a significant proportion still develops preeclampsia. The mechanism of the prophylactic response is unknown. Here, the untargeted metabolomics analysis of 144 plasma samples from high-risk pregnant women before (11–13 weeks) and after (20–23 weeks) aspirin/placebo treatment elucidated metabolic effects of aspirin and metabolic differences potentially associated with the variation of the treatment response. We demonstrated that aspirin treatment resulted in a strong drug-associated metabolomics signature and that the preeclamptic or nonpreeclamptic outcome in response to treatment was significantly associated with the level of internal aspirin exposure ascertained from metabolomics data (*t* test, *P*=0.0083). Comparing women with and without preeclampsia after aspirin treatment, differences in 73 metabolites were detected, some of which involve pathways whose regulation is of importance in pregnancy and placental functions, such as glycerophospholipids metabolism, polyunsaturated fatty acid metabolism, and steroid hormone biosynthesis. To further examine the hypothesis that aspirin delays gestational age advancement and thus the onset of preeclampsia, we constructed a metabolic clock on pretreatment and placebo-treated samples that estimated gestational age with high accuracy and found that aspirin significantly decelerated metabolic gestational age by 1.27 weeks (95% CI, 0.66–1.88 weeks), and partially reversed one-fourth of the metabolites changed over gestational age advancement, suggesting that aspirin treatment slowed down the metabolic clock of gestation. **(*Hypertension*. 2021;78:1398–1410. DOI: 10.1161/HYPERTENSIONAHA.121.17448.)** • **Data Supplement**

**Key Words:** aspirin ■ gestational age ■ metabolomics ■ preeclampsia



Preeclampsia is a multisystem disorder of pregnancy characterized by the onset of hypertension with significant proteinuria after 20 weeks' gestation.[1] This disorder affects 2% to 5% of pregnant women and is one of the leading causes of maternal and perinatal morbidity and mortality. Globally, it is associated with 76 000 maternal and 500 000 infant deaths annually.[2] For the mother, preeclampsia causes immediate adverse effects including impairment of hepatorenal and coagulation systems.[3–5] If left untreated or in severe form, eclampsia, brain injury, and death can occur. For the baby, inadequate uteroplacental perfusion leads to fetal growth restriction and placental abruption resulting in indicated preterm birth or stillbirth.[6,7] Evidence suggests that preeclampsia can be subclassified according to gestational age at delivery, as early-onset preeclampsia requiring preterm delivery is associated with a higher incidence of perinatal and maternal complications, compared with late-onset subtype.[8] Thus, preeclampsia can be subdivided into preterm preeclampsia, with delivery at <37 weeks' gestation, and term preeclampsia, with delivery at ≥37 weeks' gestation. Term preeclampsia is less often severe but not

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## Novelty and Significance

### What Is New?

- Preeclamptic or nonpreeclamptic outcome in response to aspirin treatment was significantly associated with the level of internal aspirin exposure ascertained from metabolomics data.
- Differences in 73 metabolites were related to the development of preeclampsia despite aspirin prophylaxis, some of which involve pathways whose regulation is of importance in pregnancy and placental functions.
- Aspirin significantly decelerates metabolic gestational age by 1.27 weeks and partially reverses one-fourth of the metabolites changed over gestational age advancement.

### What Is Relevant?

- Evaluation of internal aspirin exposure ascertained directly from the metabolomics data is more predictive of subsequent development of preeclampsia than self-reported adherence to aspirin treatment.

### Summary

Aspirin reduces the risk of preeclampsia by decelerating advancement of metabolic gestational age and partially reversing a wide range of metabolic changes over advancement of gestational age. These results lend strong support to the aspirin-related delay hypothesis.

## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **AIC** | Akaike information criterion |
| **ASPRE** | Combined Multimarker Screening and Randomized Patient Treatment With Aspirin for Evidence-Based Preeclampsia Prevention |
| **LCFAs** | long-chain fatty acids |
| **LC-PUFA** | long-chain polyunsaturated fatty acids |
| **MVA** | multivariate analysis |
| **OPLS-DA** | Orthogonal Projections to Latent Structures Discriminant Analysis |
| **PIGF** | placental growth factor |
| **VIP** | Variable Importance on Projection |

without serious consequences. It constitutes 80% of all cases of preeclampsia, and therefore, is a significant burden to the health care system.[4]

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Low-dose aspirin has been proven to be beneficial in the prevention of preeclampsia when given at a daily dose of ≥100 mg before 16 weeks' gestation and when the prophylactic treatment is targeted for preterm preeclampsia.[9] Since the first evidence of potential efficacy of aspirin in 1979, numerous studies have tried to determine the effect of low-dose aspirin on the incidence of preeclampsia, with very controversial results as demonstrated in large meta-analyses including individual patient data.[10,11] Recent evidence from a multicenter, double-blind, randomized placebo-controlled trial (ASPRE trial [Combined Multimarker Screening and Randomized Patient Treatment With Aspirin for Evidence-Based Preeclampsia Prevention]) has demonstrated that aspirin prophylaxis could reduce the rates of preterm preeclampsia and all preeclampsia by 60% and 30%, respectively.[12] A secondary analysis of the ASPRE data demonstrated that the beneficial effect of aspirin was clearly associated with good self-reported adherence to treatment.[13] At ≥90% compliance, the effect size of aspirin was even higher at 76% and could reach 95% when the high-risk woman did not have a history of chronic hypertension.[13]

To date, the mechanism by which aspirin prevents preeclampsia remains unknown, which is consistent with the lack of understanding of the disease pathophysiology. Although the observations of the ASPRE trial have demonstrated that aspirin is an effective agent for the prevention of preterm preeclampsia, it is unclear as to whether aspirin prevents the occurrence of preterm preeclampsia or reduces the severity of the disorder leading to a delay in disease-onset and therefore delivery. In an exploratory analysis of data from the ASPRE trial to test the aspirin-related delay hypothesis, a reduction in the incidence of term preeclampsia in the lower-risk group (odds ratio, 0.62 [95% CI, 0.29–1.30]) was observed, whereas in the higher-risk group, there was a small increase in the incidence of term preeclampsia (odds ratio, 1.11 [95% CI, 0.71–0.75]). Within the framework of the aspirin-related delay hypothesis, the effect of aspirin was to delay the gestational age at delivery with preeclampsia by an estimated 4.4 weeks (95% CI, 1.4–7.1 weeks) for those who would be delivered at 24 weeks without aspirin treatment. The effect decreased by an estimated 0.23 weeks (95% CI, 0.021–0.40 weeks) for each week of gestation so that at $40^{+0}$ weeks, the estimated delay was 0.8 weeks (95% CI, −0.03 to 1.7 weeks). These findings imply that the aspirin-related delay in gestational age at delivery is greater for earlier than later preeclampsia.

A recent theory regarding the origins of preeclampsia proposes the association between premature placental aging and adverse pregnancy outcomes.[14,15]

Earlier studies have revealed profound DNA methylation changes in umbilical cord blood and placenta tissues during pregnancy and thus were used to develop molecular estimators of gestational age.[14,16–18] Such molecular clocks enabled assessment of gestational age and identified accelerated placental aging in early-onset preeclampsia cases.[14] Building an accurate molecular clock via profiling maternal metabolomics changes over gestation progression was also proven viable in healthy pregnancies recently.[19] Metabolomics profiles of maternal plasma tracked weekly during healthy pregnancies revealed a broad yet highly choreographed pattern of metabolic changes.[19] Given the possible role of aspirin in delaying placental aging and thus the onset of preeclampsia, we sought to investigate the impact of aspirin on gestational age advancement via a molecular estimator specific to high-risk pregnant women.

# METHODS

## Data Availability

All data supporting the findings of this study are included in this article and its Data Supplement.

## Sample Acquisition

This study used stored plasma samples from the ASPRE trial (URL: https://www.clinicaltrials.gov; Unique identifier: ISRCTN13633058), a double-blind, placebo-controlled aspirin trial involving pregnant women at high risk for preterm preeclampsia. Cases included were ASPRE participants from King's College Hospital, London, United Kingdom; University Lewisham Hospital, London, United Kingdom; and Medway Maritime Hospital, Kent, United Kingdom.[20] All recruited participants in the ASPRE trial provided written informed consent for the use of their clinical data and biological samples in future search studies (ethical approval by the UK National Research Ethics Committee, reference 13/LO/1479). Furthermore, ethical approval for this study was given by the Institutional Review Board (Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee, Reference Number CRE-2018.302). Screening for high-risk pregnancies among consented women was performed using an algorithm combining maternal factors, mean arterial pressure, uterine-artery pulsatility index, maternal serum pregnancy-associated plasma protein A, and PlGF (placental growth factor).[21] Screening for preeclampsia and recruitment to the ASPRE trial was during the period from July 2015 through April 2016. The trial compared aspirin at a dose of 150 mg per day with placebo from 11 to 14 until 36 weeks' gestation. Compliance was reported as the tablet count percentage difference of the total number of tablets consumed against the total number of tablets prescribed. Plasma samples were collected at 11 to 13 weeks, before commencement of either aspirin or placebo, and at 20 to 23 weeks, after commencement of either aspirin or placebo.

In this study, untargeted metabolomics profiling was performed on plasma samples of 58 participants in the aspirin group and 58 participants in the placebo group. Each treatment group contained 30 women who eventually developed preeclampsia and 28 women who did not. The samples of each of the 4 treatment/outcome combinations were matched ≈1:1:1:1 for predicted preeclampsia risk scores.

## Metabolomics Analysis and Data Processing

Sample extraction was performed on an automated liquid handling robot (Hamilton LabStar, Hamilton Robotics, Inc, Reno, NV), where 500 µL of methanol was added to 100 µL of sample to precipitate proteins. The linearity for the instrument performance standards used in the assay was documented.[22] The methanol extraction solution contained process assessment standards as previously detailed.[23] The resulting clarified supernatant extract was divided into 4 aliquots, briefly evaporated to remove the organic solvent, and stored overnight under nitrogen before preparation for analysis.

Stored plasma samples were sent to Baylor Genetics (Houston, TX) in separated batches in 2018 and 2019, respectively, and processed in collaboration with Metabolon, Inc. Samples were subject to the same untargeted metabolomics analysis as previously described.[24] Briefly, global biochemical profiling used a Waters ACQUITY ultraperformance liquid chromatography and a Q-Exactive high resolution/accurate mass spectrometer (Thermo Fisher Scientific, Waltham, MA) interfaced with a heated electrospray ionization source and Orbitrap mass analyzer operated at 35 000 mass resolution.[22]

Raw metabolite ion intensities were measured using the area under the chromatographic peak, median scaled across all participants, and log transformed. Z scores were calculated by comparing analyte log transformed, median-scaled values to the associated mean and SD found in a reference population comprised ≈400 of individuals.

## Sample Quality Assessment

As a quality control measure before analysis, we analyzed the data for the presence of outliers. For all 198 samples, 625 identified and annotated metabolites were highly represented (quantified in >80% of samples) and were included in the following analysis. Principal component analysis projection and uniform manifold approximation and projection embedding were applied to examine the overall distribution of the sample data.[25] Principal component analysis and uniform manifold approximation and projection revealed a group of 11 placebo-first outliers (Figure S2A and S2B in the Data Supplement). Further demographic information confirmed that these samples were collected from the same center (Medway Maritime Hospital, Kent, United Kingdom) and submitted within a single batch, suggesting potential source of systemic variation introduced at or before sample preparation. Thirty-eight aspirin-second samples identified as outliers via uniform manifold approximation and projection showed very low signal levels for most metabolites measured (Figure S2B and S2D). The general low intensity we observed in this group of samples (n=38) cannot be sourced to any demographic difference, for example, hospital where these samples were collected, ethnicity, or biological difference, for example, aspirin compliance, preeclampsia outcome. These samples were excluded to avoid artificial signal introduced by systemic variation. An additional 5 samples, represented by high SD in Figure S2C, were manually inspected and classified as outliers due to typical metabolic alterations of delayed sample

processing according to Jain et al.[26] Collectively, 54 samples were removed from the cohort, and the remaining 144 samples were subject to downstream analysis. For principal component analysis and uniform manifold approximation and projection, missing Z score values were imputed as the minimum Z score of the analyte in the cohort.

## Identification of Significantly Altered Metabolites

For multivariate analysis (MVA) in characterizing aspirin metabolism, Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) models of the second trimester samples were created using R package ropls.[27] Leave-2-out cross validation was performed by creating an OPLS-DA model after repeatedly removing randomly selected 2 observations to avoid potential bias. The remaining observations were subsequently fitted into this model. In the resulting model, metabolites with Variable Importance on Projection (VIP) value >1 were considered as positive contributor of the model and passed on to repeat model building process. Metabolites with VIP value >1 in the second pass were considered as putative indicators of aspirin metabolism. For univariate analysis, metabolites with 2-sided *t* test $P<0.05$ after false discovery rate correction were considered as significantly altered metabolites.

## Pairwise Comparison of Variable Significance

Metabolites were compared with internal aspirin exposure (predicted compliance) in pairs, evaluated by significance of information loss caused by removal of either variable in predicting intervention outcome via logistic regression. For each pair of variables, 3 such models were constructed, 2 with single variable and one with both variables. To enable comparison among models with different number of parameters, we used an information theory-based approach Akaike information criterion (AIC).[28] Briefly, AIC rewards goodness of fit of a model while adding a penalty for increasing number of parameters. Difference of AIC values tells the difference of information loss of a model relative to the other model in bits. If the AIC of model built on single variable A is d bits larger than the model built on variables A and B, we assign an upper bound of 2^(−d) to the *P* value using the Algorithmic Significance theorem[29] for information loss caused by removal of variable B. To avoid potential overfitting caused by small sample size, AIC with correction for small sample sizes was performed using AIC correction for small sample size function in R package MuMin.[30,31]

## Pathway Analysis

The aspirin-altered compounds (n=54) identified by the MVA methods mentioned above were inputted into MetaboAnalystR[32] (https://github.com/xia-lab/MetaboAnalystR) to perform the metabolic pathway analysis.[33]

## Metabolic Clock Construction

To construct a metabolic estimator of gestational age, the OPLS-DA model was trained on abundance of 625 metabolites in all aspirin-free samples using gestational age determined from the measurement of fetal crown-rump length at 11 to 13 weeks.[34] Performance of the clock was evaluated in 2 ways: (1) permutation testing performed with 50 random permutations of target (crown-rump length-determined gestational age) labels where R2 and Q2 quality metrics were used to assess degree of overfit; (2) 20-fold cross validation where Pearson correlation coefficient, mean absolute error, root mean square error were used to assess prediction accuracy.

## Code Availability

Custom code used for the analysis is accessible at https://github.com/BRL-BCM/ASPRE-analysis.

# RESULTS

A total of 144 out of 198 samples evaluated with untargeted metabolomics analysis passed the quality assessment filter (see Materials and Methods), including 33 first trimester samples from the placebo group (placebo-first), 54-second trimester samples from the placebo group (placebo-second), 37 first trimester samples (aspirin-first), and 20-second trimester samples (aspirin-second) from the aspirin group. The profiles that passed quality assessment were provided by 39 women from the aspirin group and 55 women from the placebo group (Table, Table S1).

## Metabolites and Pathways Affected by Aspirin Treatment

To identify the metabolites perturbed in response to aspirin treatment, we compared metabolomics profiles in aspirin-second samples with placebo-second samples. Univariate analysis using *t* test and false discovery rate correction for multiple testing yielded 28 significantly perturbed metabolites (Table S2).

We next performed MVA using the OPLS-DA model and reported compliance as continuous measure of aspirin administration. The analysis revealed 185 metabolites based on a VIP value >1. All the 28 compounds originally identified by univariate analysis were contained within the 185 metabolites discovered by MVA (Table S3), suggesting higher sensitivity of MVA than univariate analysis.

To further refine the list of aspirin-induced metabolomics alterations, we performed second-pass MVA using OPLS-DA and limiting the list of compounds to the previously identified 185 identified in the first pass. Results of second-pass OPLS-DA in Figure 1A and 1B showed a complete separation of placebo subjects and aspirin-treated subjects. The R2Y value of 0.807, Q2 value of 0.871 and a large separation between true and permutated model Q2 (Figure 1B) indicated an excellent model. Overall, root mean square error of cross validation and area under the curve of leave-2-out cross validation were 14.52 and 1, respectively.

The second-pass MVA narrowed the list to 54 metabolites with VIP>1 (Table S3, Figure 1C). The list included the immediate aspirin metabolites salicylate

**Table. Characteristics of Participants and Samples Collected***

| Participants characteristics | Aspirin group (n=39) | | | | Placebo group (n=55) | | | |
|---|---|---|---|---|---|---|---|---|
| | Preeclamptic (n=19) | | Normal (n=20) | | Preeclamptic (n=29) | | Normal (n=26) | |
| Reported compliance (%), range† | 63.98–100.00 | | 57.74–100.00 | | 39.47–100.00 | | 69.38–100.00 | |
| Sample characteristics | First (n=17) | Second (n=10) | First (n=20) | Second (n=10) | First (n=17) | Second (n=29) | First (n=16) | Second (n=25) |
| Gestational age at sample collection, mean (95% CI) | 12.65 (12.30–13.00) | 21.57 (21.13–22.01) | 12.82 (12.51–13.13) | 21.75 (21.29–22.21) | 12.54 (12.21–12.86) | 21.55 (21.21–21.89) | 12.55 (12.27–12.84) | 21.91 (21.60–22.22) |

*There were no significant differences in gestational age at sample collection between-group by 1-way ANOVA test (2-tailed).
†Compliance was reported as the tablet count percentage difference of the total number of tablets consumed against the total number of tablets prescribed.

(HMDB0001895) and salicylurate (HMDB0000840) as upregulated upon aspirin treatment. We observed an overall decrease of metabolites in histidine metabolism including histidine (HMDB0000167), imidazole lactate (HMDB0002320; degradation product of histidine), 1-ribosyl-imidazoleacetate (HMDB0002331; derivative compound of histamine degradation), N-acetylcarnosine (HMDB0012881; histidine-containing dipeptides), as well as a decrease of essential metabolites in glutamate metabolism including β-citrylglutamate (PubChem: 189741), glutamine (HMDB0000641), 2-pyrrolidinone (HMDB0002039), 5-oxoproline (HMDB0000267; Figure 1C). Aspirin also induced a reduction of long-chain polyunsaturated fatty acids (n3 and n6; LC-PUFAs) including dihomo-linolenate (20:3n3 or n6; HMDB0002925), and hexadecadienoate (16:2n6; HMDB0000477), as well as lysophospholipids containing LC-PUFA branches (Figure 1C, Table S3). However, several arachidonic acid-containing lipids (Figure 1C, Table S3) including 1-stearoyl-2-arachidonoyl-GPI (18:0/20:4; HMDB0009815), 1-stearoyl-2-arachidonoyl-GPC (18:0/20:4; HMDB0008048), and 2-arachidonoyl-GPE (20:4; HMDB0011487) were upregulated by aspirin. Pathway enrichment revealed several pathways significantly affected by aspirin, including (1) glutathione metabolism, (2) glycine, serine, and threonine metabolism, (3) alanine, aspartate, and glutamate metabolism, (4) glycerophospholipid metabolism, and (5) glyoxylate and dicarboxylate metabolism via pathway enrichment analysis (Figure 1D).

## Protective Effects of Aspirin Depend on Internal Exposure Ascertained From Metabolomics Data

The current study included variability in reported compliance and variability in internal exposure to aspirin ascertained directly from the metabolomics data. We next asked if the joined and individual correlation of these factors with the outcome as preeclampsia would be consistent with protective effects of aspirin. This analysis was originally prompted by informal visual observation that within the aspirin-treated group, participants who developed preeclampsia tended to be less compliant than participants without preeclampsia, although this pattern did not pass a test of statistical significance (Figure 2A and 2B).

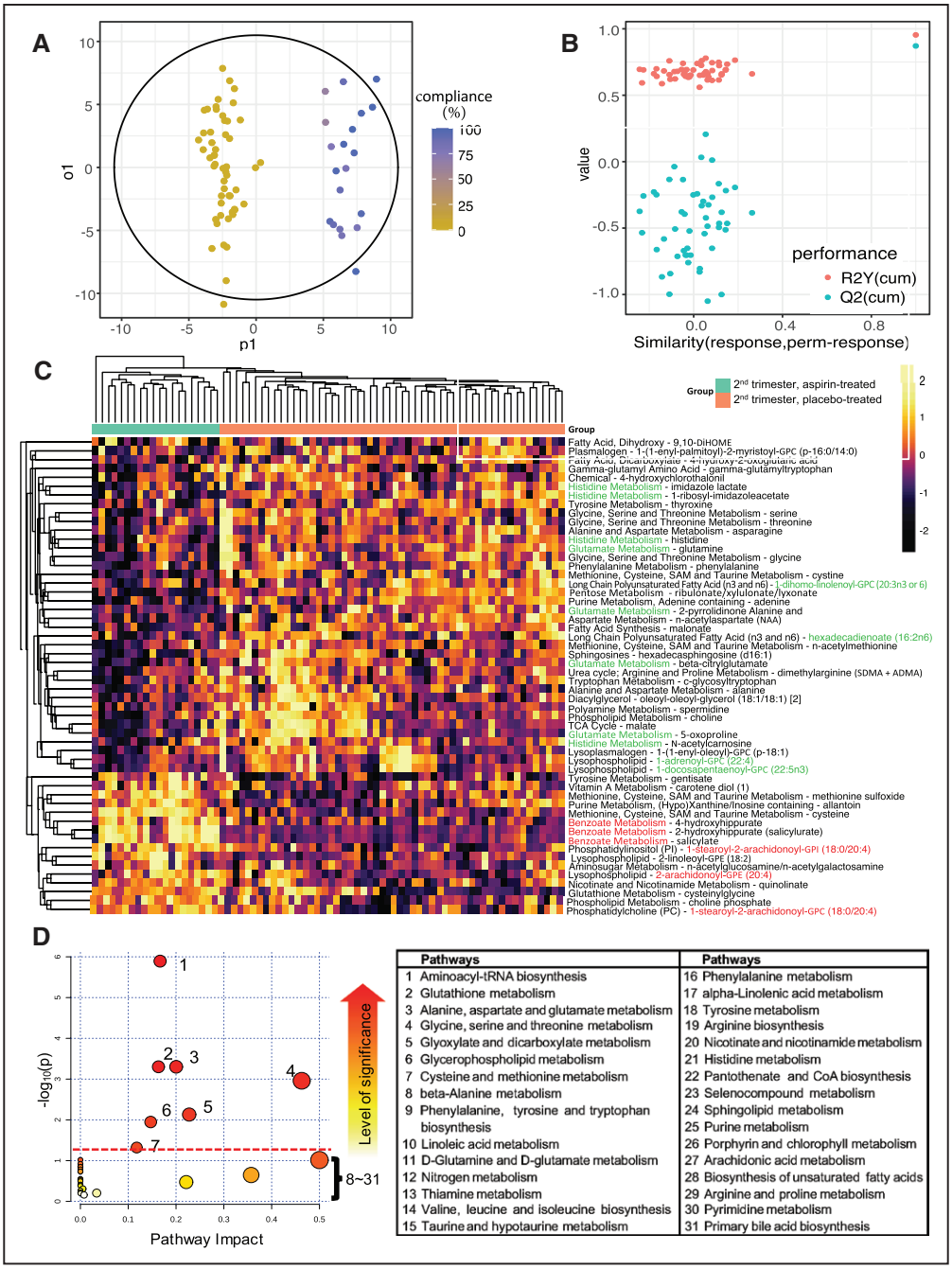
Subsequently, we asked if internal exposure to aspirin ascertained directly from the metabolomics data may be more predictive of outcomes. To this end, we constructed an OPLS-DA model to predict compliance directly from the metabolomics data. The predicted compliance, a measure of internal exposure to aspirin, indeed showed stronger association ($P$=0.0097, $t$ test; Figure 2A and 2B). We noted that the internal exposure may be a function of not only compliance but also variation in physiology and pharmacogenetics between participants.

To investigate whether the reported compliance, the level of internal aspirin exposure or both were predictive of preeclampsia, logistic regression was conducted on reported compliance, predicted compliance, and both variables in combination. Interestingly, comparison of these 2 models using AIC confirmed that internal exposure performed best in predicting the outcomes and added significant information ($P$=0.018), in contrast to reported compliance, which did not provide predictive information in addition to that provided by internal exposure (Figure 2C). These results were consistent with the model where the protective effects of aspirin were mediated by internal exposure to aspirin.

## Aspirin Treatment Slows Down the Metabolic Clock of Gestation

While the accelerated epigenetic clock of placental aging has previously been associated with high risk for preeclampsia,[14] the effect of aspirin on the pace of any molecular gestational clock (epigenetic, metabolomics, etc) has not been previously examined. To address this question, we applied a 2-stage strategy. First, we built a multivariate metabolic clock of gestation using the metabolomics profiling of the pretreatment and placebo-treated samples. Second, we used the clock to predict the gestational age in the aspirin-treated group. We hypothesized that, due to aspirin intake, a systematic shift in the aspirin-treated group may be observed.

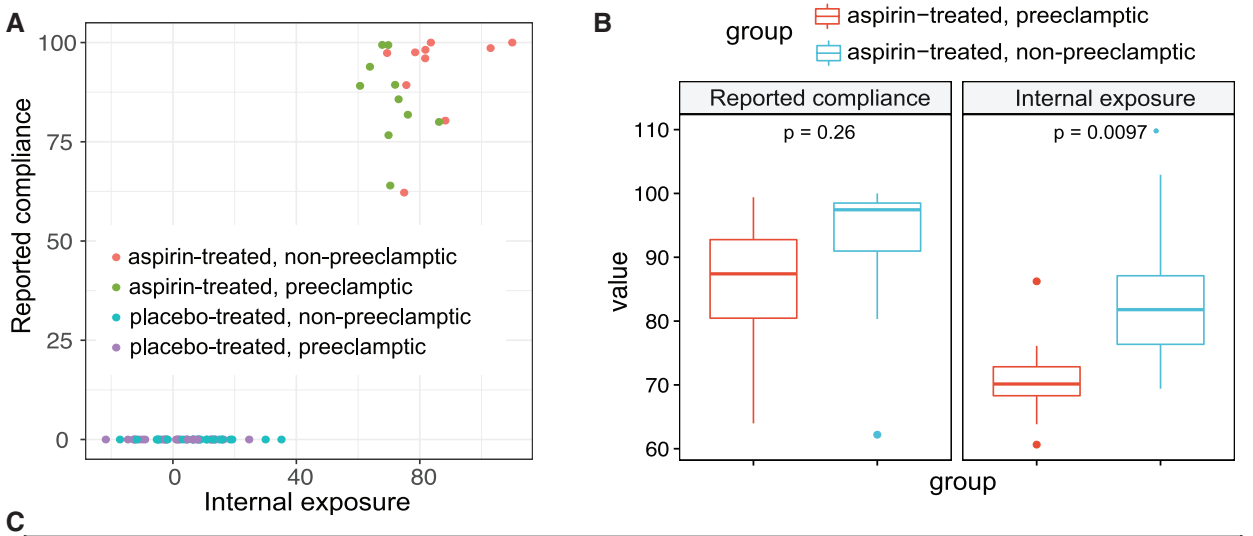
A metabolic clock of gestation was constructed as an OPLS-DA model that predicts gestational age. The model was constructed from metabolomic data of the




| Pathways | Pathways |
|---|---|
| 1 Aminoacyl-tRNA biosynthesis | 16 Phenylalanine metabolism |
| 2 Glutathione metabolism | 17 alpha-Linolenic acid metabolism |
| 3 Alanine, aspartate and glutamate metabolism | 18 Tyrosine metabolism |
| 4 Glycine, serine and threonine metabolism | 19 Arginine biosynthesis |
| 5 Glyoxylate and dicarboxylate metabolism | 20 Nicotinate and nicotinamide metabolism |
| 6 Glycerophospholipid metabolism | 21 Histidine metabolism |
| 7 Cysteine and methionine metabolism | 22 Pantothenate and CoA biosynthesis |
| 8 beta-Alanine metabolism | 23 Selenocompound metabolism |
| 9 Phenylalanine, tyrosine and tryptophan biosynthesis | 24 Sphingolipid metabolism |
| 10 Linoleic acid metabolism | 25 Purine metabolism |
| 11 D-Glutamine and D-glutamate metabolism | 26 Porphyrin and chlorophyll metabolism |
| 12 Nitrogen metabolism | 27 Arachidonic acid metabolism |
| 13 Thiamine metabolism | 28 Biosynthesis of unsaturated fatty acids |
| 14 Valine, leucine and isoleucine biosynthesis | 29 Arginine and proline metabolism |
| 15 Taurine and hypotaurine metabolism | 30 Pyrimidine metabolism |
| | 31 Primary bile acid biosynthesis |

**Figure 1. Metabolites and pathways affected by aspirin treatment.**

**A**, Multivariate analysis (MVA) for compliance prediction based on 185 metabolites. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot of first orthogonal component (variance not correlated to compliance) on *y* axis against predictive component on *x* axis accurately separated aspirin-treated samples (n=20) from the placebo-treated samples (n=54). Gradient color scale from yellow to blue indicates reported compliance (%) from 1 to 100. **B**, Validation plot of the OPLS-DA model obtained from 50 permutation tests. The permuted values (**left**) are lower than the original R2 and Q2 values (**right**), demonstrating low risk of overfit. **C**, Heatmap of 54 aspirin-altered metabolites (rows) identified in MVA clusters samples (columns) into groups consistent with treatment groups (aspirin, n=20; placebo, n=54). Long-chain polyunsaturated fatty acids (LC-PUFAs), lipids containing LC-PUFA branch and metabolites in histidine metabolism and glutamate metabolism pathways are downregulated (green); Aspirin metabolites, arachidonic acid-containing lipids are upregulated (red). Distance matrix: Euclidean. Hierarchical clustering method: UPGMA. **D**, Pathway enrichment analysis depicting the metabolic pathways affected by aspirin treatment. The analysis was performed on 54 aspirin-altered metabolites. The $-\log_{10}$ (*P*) value (*y* axis) represents the quantitative perturbation of pathways. The pathway impact value (*x* axis) refers to the centrality of a metabolite in the metabolic network. The node color, varying from yellow to red, is based on its *P* value, and the node radius is determined on the basis of their pathway impact values. Red dotted line indicates *P*=0.05, 2-sided hypergeometric test. Pathways are listed according to the level of significance.

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| Model | K | AUC | AICc | P-value |
|---|---|---|---|---|
| $Model_{Self\text{-}reported\ compliance}$ | 1 | 0.69 | 30.96 | 0.018 |
| $Model_{Internal\ exposure}$ | 1 | 0.85 | 23.08 | 0.24 |
| $Model_{Self\text{-}reported\ compliance\ +\ Internal\ exposure}$ | 2 | 0.85 | 25.14 | 1 |

**Figure 2. Association between internal exposure of aspirin and clinical response to aspirin treatment.**
**A**, Reported compliance plotted against internal exposure. Internal exposure is calculated as leave-2-out Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) model predicted compliance. Samples are colored differently by pregnancy outcomes and treatment groups. **B**, Distribution of reported compliance and internal exposure in aspirin-treated women with (n=10) and without (n=10) preeclampsia. Internal exposure was significantly different between preeclamptic women and nonpreeclamptic women within aspirin-treated group ($P$=0.0097, $t$ test). This difference was not statistically significant in reported compliance ($P$=0.26, $t$ test). **C**, Akaike information criteria (AIC) for the fitted logistic regression models. AICc indicates AIC with correction for small sample size; AUROC, area under the receiver operating curve; and K, number of parameters. $P$ value, algorithmic significance theorem's significance of information loss compared with model self-reported compliance + predicted compliance.

pretreatment (aspirin-first and placebo-first) and the placebo-treated (placebo-second) samples. The predictive accuracy of the model mean absolute error of 0.94 (weeks) and the actual-by-predicted linear regression $r^2$ of 0.934 indicated highly accurate prediction of gestational age (Figure 3A). The list of highly informative metabolites (with VIP>1) that showed an increase with advancing gestational age included progestin steroids hormones, specifically pregnanediol-3-glucuronide (HMDB0010318), 5α-pregnan-diol disulfate (HMDB0240582/HMDB0240581), as well as a reduction of several androgenic steroids, such as 5α-adrostan-3β,17β-diol disulfate and androstenediol (3β,17β) monosulfate (HMDB0240429; Figure 3D, Table S4). The advancing gestational age showed a correlated increase of phosphatidylethanolamines lipids and γ-glutamyl amino acids and a highly correlated decrease of metabolites in leucine, isoleucine, and valine metabolism (branched-chain amino acid metabolism) pathways.

To test whether aspirin had an impact on the metabolic clock of gestation, we applied the OPLS-DA model to predict gestational age in the aspirin-treated group. The predicted gestational age in the aspirin-treated group was significantly lower than the predicted gestational age in the placebo-treated group (Figure 3B), although the samples from both groups were collected at around the same gestational age during the second trimester. To quantify the extent of delay of the metabolic clock caused by aspirin, we adopted the concept of gestational age acceleration from the previous literature on epigenetic aging.[16,18] Specifically, we defined gestational age acceleration for each participant as the raw residual after regressing the metabolic gestational age estimate on observed gestational age. In the aspirin-treated group, gestational age acceleration significantly deviated from zero in a negative direction by 1.27 weeks on average (−0.66 to −1.88, 95% CI; Figure 3C), suggesting that aspirin significantly delayed metabolic gestational age.

## Combined Effects of Aspirin Treatment on Preeclampsia Prevention and the Gestational Age Advancement

We next examined the specific metabolites and pathways that were (1) affected by aspirin treatment; (2) predictive

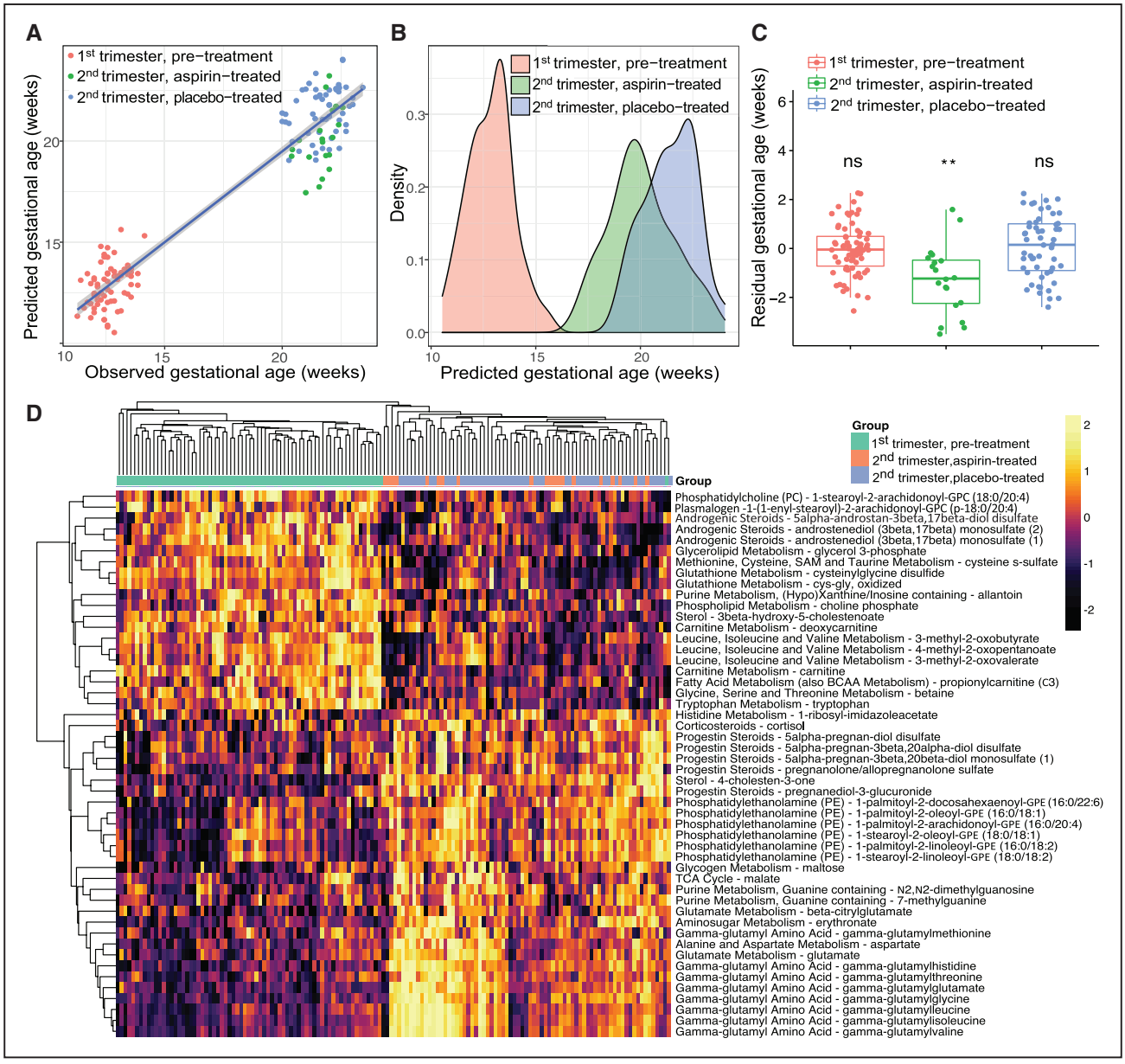


**Figure 3. Impact of aspirin treatment on the metabolic clock of gestation.**
**A**, Gestational age estimation by metabolic clock trained on samples from women not treated by aspirin accurately reflects progression at early and mid pregnancy. Observed gestational age is determined by crown-rump length. **B**, Aspirin-treated samples were estimated to have a younger gestational age then placebo-treated samples, depicted by the left shift of green peak from blue peak ($P$=0.001265; $t$ test). **C**, Gestational age acceleration is significantly deviated from 0 in aspirin-treated samples (ANOVA, $P$=6.7×10$^{-05}$; $t$ test, Bonferroni-corrected, $P$=0.0083). Gestational age acceleration is defined as raw residuals resulting from regressing the estimated gestational age on observed gestational age (regression line shown in **A**). **D**, Heatmap of top 50 metabolites for gestational age estimation. Pregnancy-related metabolites change in a highly correlated manner. Regardless of treatment, first trimester samples (pretreatment) and second trimester samples (aspirin-treated and placebo-treated) are clearly clustered separately by top metabolites in gestational age estimation. Metabolites (rows) are clustered on correlation matrix. Samples (columns) are clustered on Euclidean distance matrix. Hierarchical clustering method: UPGMA.

of clinical response to aspirin treatment; and (3) correlated with gestational age.

Within the group of 20 aspirin-treated samples (aspirin-second), 10 were collected from nonpreeclamptic women and the other 10 from preeclamptic women (Table). Comparing preeclamptic or nonpreeclamptic clinical outcomes in response to treatment, we identified 73 significantly different metabolites ($t$ test, $P$<0.05; Table S5). Metabolomics profiling of preeclamptic samples was dominated by higher levels of steroids, long-chain fatty acids (LCFAs), very LCFA, and lipids containing LCFA or very LCFA branches, with little change in short-chain or medium-chain fatty acids related compounds (Figure 4A, Table S5). Thirty-seven out of 73 significant metabolites were recognized by the Kyoto Encyclopedia of Genes and Genomes compound database and mapped to 2 major

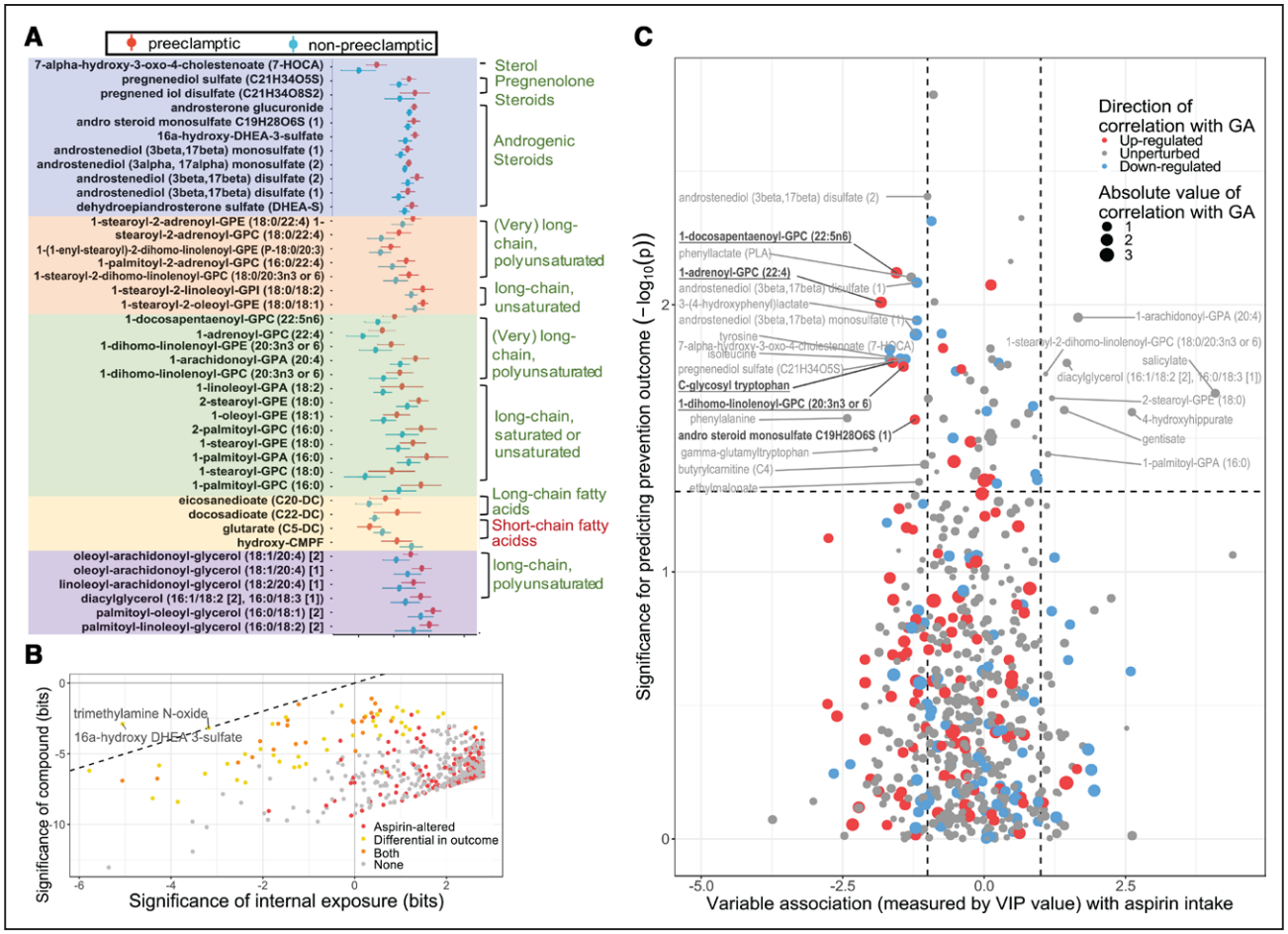


**Figure 4. Effects of aspirin on preeclampsia prevention and the gestational age advancement.**
**A**, Error-bar chart of significantly different lipids, fatty acids, and steroids in aspirin-treated participants. Dots and error bars represent means of *Z* scores and SD in preeclamptic samples (red, n=10) and nonpreeclamptic samples (blue, n=10). Background is colored according to the class of metabolites. Blue, sterol and steroids. Pink, phospholipid. Green, lysophospholipid. Yellow, fatty acid dicarboxylate. Purple, diacylglycerol. Annotations colored in green/red indicates higher/lower level in preeclamptic participants. **B**, Significance of individual metabolite in mediating intervention outcome compared with internal exposure, measured by information loss caused at the removal of the variable. When distributed on the right of line *y*=*x*, removal of internal exposure costs higher information loss than removal of the compound. **C**, Metabolites (n=625) under the impact of aspirin, pregnancy and their associations with preeclampsia prevention. Correlation with aspirin intake and gestation advancement is measured by variable importance on projection (VIP) in first-pass compliance prediction model and gestational age prediction model, respectively. Direction of correlation is indicated by positive/negative VIP value for impact of aspirin and color of the dot for gestational age. Metabolites with VIP>1 in prediction of gestational age are colored in red/blue if positively/negatively correlated with gestational age. Horizontal dashed line, *P*=0.05; Vertical dashed lines, |VIP|=1. Labeled compounds are (1) associated with aspirin intake (|VIP|>1) and (2) predictive of prevention outcome (*P*<0.05). Within labeled compounds, those changed in opposite direction over aspirin administration and gestation advancement are underlined and in black.

groups of interconnected Kyoto Encyclopedia of Genes and Genomes pathways (Figure S1). One group consisted of pathways involving glycerophospholipids metabolism, linoleate metabolism, arachidonic acid metabolism, and C21-steroid hormone biosynthesis and metabolism (Figure S1A). The other pathways involved purine metabolism, pyrimidine metabolism, tryptophan metabolism, tyrosine metabolism and urea cycle, and metabolism of arginine, proline, glutamate, aspartate, and asparagine (Figure S1B). Interestingly, in this set of differential metabolites, pairwise AIC comparison identified 16a-hydroxy DHEA 3-sulfate (HMDB0062544) and trimethylamine N-oxide (HMDB0000925) as stronger mediators of preeclampsia outcome than internal exposure signal, outperforming by 2.17 bits and 0.015 bits, respectively (Figure 4B).

Over one-third (26/73) of these differential metabolites were altered by aspirin, including aspirin's derivative metabolites salicylate and 4-hydroxyhippurate (Figure 4C). Lipids (8/26) and steroids (6/26) occupied more than half of the list (Figure 4C), reinforcing their association with pregnancy outcome and response to aspirin treatment. Changes during advancement of gestation were partially reversed by aspirin in one-fourth (48/190) of the pregnancy-related metabolites, out of which LC-PUFA containing lysolipids, 1-docosapentaenoyl-GPC (22:5n6; HMDB0010401), 1-adrenoyl-GPC

(22:4; HMDB0010401), and 1-dihomo-linolenoyl-GPC (20:3n3 or 6; HMDB0010394), an androgenic steroid and C-glycosyltryptophan (HMDB0240296) were also significantly different between samples with or without preeclampsia (Figure 4D).

## DISCUSSION

In this study, we characterized the effects of aspirin from untargeted metabolomics profiles of pregnant women at high risk of preeclampsia before and after aspirin/placebo treatment. Aspirin resulted in a strong drug-associated metabolomics signature that can accurately categorize placebo- or aspirin-treated samples. The level of internal exposure, ascertained from the metabolomics data, was found to be significantly associated with the preeclamptic or nonpreeclamptic outcome in response to treatment (*t* test, *P*=0.0097). We then identified specific differential metabolites that were predictive of clinical response, some of which are involved in pathways important in pregnancy progression, including glycerophospholipids metabolism, PUFA metabolism, and steroid hormone biosynthesis. We further constructed a metabolic clock on pretreatment and placebo-treated samples and identified that aspirin significantly decelerated metabolic gestational age, consistent with the hypothesis that aspirin delays the onset of preeclampsia.

We were able to detect many of the metabolites in aspirin-related pathways profiled in previous studies on other diseases, such as downregulated metabolites in glutamate and histidine metabolism pathways[35,36] and decreased LC-PUFA dihomo-linolenate (20:3n3 or n6; HMDB0002925) in oxylipid synthesis.[37] At the same time, we also found metabolic changes that were less often linked to aspirin, for example, an increase of arachidonic acid-containing lipids including lysophospholipid, phosphatidylcholine, and phosphatidylinositol (Figure 1C, Table S3). The increase of arachidonic acid-containing phospholipids, along with the decrease of LC-PUFAs suggest altered regulation of lipid synthesis/metabolism in aspirin administration during pregnancy at high risk of preeclampsia. This alteration could be related to aspirin's role in COX-dependent pathways. Upon aspirin administration, arachidonic acid was blocked from forming inflammatory prostaglandins via COX enzyme inhibition and redirected to produce aspirin-triggered lipoxins, which function as resolvins via COX-2.[38,39] Interestingly, higher concentrations of intermediates from omega-6 fatty acid metabolism, including arachidonic acid, linoleate, and LC-PUFAs, were reported to be associated with placental mitochondria dysfunction in preeclamptic women.[40] Increasing of 13 amino acids and their derivatives were also reported in the same study, out of which 6 were shown to be downregulated by aspirin in our analysis.[40] These results suggest that the metabolic changes in the aspirin users are very likely placenta related in terms of protection against preeclampsia.

Comparing aspirin-treated participants with and without preeclampsia, we have found higher levels of inosine (HMDB0000195) and xanthine (HMDB0000292) in nonresponders (Table S5), consistent with previous findings that purine pathways are associated with platelet aggregation response in healthy male and female volunteers and that poor responders show higher inosine level.[41] In addition, we have observed higher levels of 8 androgenic steroids, 2 pregnenolone steroids, and a wide range of LCFA-containing lipids in nonresponders (Table S5). These classes of metabolites are of crucial importance in maternal biological physiology during pregnancy and fetal growth. Pregnenolone is the common precursor of progesterone and glucocorticoids, both of which are significantly involved in the regulation of immune responses during pregnancy.[42] Androgenic steroids, including androstenediols, DHEA, DHEA-S (HMDB0001032), and 16a-hydroxy DHEA 3-sulfate (HMDB0062544), are mostly synthesized by fetal organs (adrenal cortex and liver) and have an impact on fetal development.[43,44] The amount of LCFA in maternal blood samples is associated with childhood metabolic health.[45] Notably, the androgenic steroid 16a-hydroxy DHEA 3-sulfate (HMDB0062544) has been identified as the strongest mediator of pregnancy outcome after treatment (Figure 4B). However, most of these steroids have not been related to preeclampsia development without aspirin treatment, as observed in placebo-treated samples. This may implicate the role of steroids in causing interindividual variability in response to aspirin intervention, independent from resistance to antithrombotic effect, and specific to its application in preeclampsia prevention.

Notably, this analysis has limitations in that a comparison between nonpreeclamptic and preeclamptic outcome is not precisely a comparison between responders and nonresponders. While we have used the best available approach currently to screen for women at high risk for preeclampsia, recruited participants might not have developed preeclampsia even without treatment. In addition, a sample size of 20 in the aspirin-treated group potentially caused the statistical analysis to be underpowered. Nonetheless, the metabolomics signature of aspirin was sufficiently strong to be identified as a significant predictor of clinical response.

One strength of our work is that we used untargeted metabolomics to establish a model to represent individual internal exposure of aspirin. The untargeted metabolomic profiles comprehensively reflect the end result of the entire chain of regulatory changes that occur in response to the level of drug intake during a period of time, permitting the monitoring of the levels of drug metabolites, as the drug's pharmacokinetic index, and the organism's response to drug, as the drug's pharmacodynamic

index.[46,47] Previous work by Wright et al[13] have demonstrated that the reported compliance affects the effect size of aspirin (odds ratio, 0.24 [95% CI, 0.09–0.65] when compliance ≥90%; odds ratio, 0.59 [95% CI, 0.23–1.53] when compliance <90%).[13] Consistent with their work, we observed that the aspirin-treated participants who developed preeclampsia tended to be less compliant than participants without preeclampsia. However, compared with the reported compliance, our data suggested that the clinical response to aspirin appeared to be more strongly dependent on the level of internal exposure, suggesting that on top of compliance, interindividual variations in pharmacokinetics (eg, absorption, distribution, metabolism, or excretion) and pharmacodynamics (eg, influence of drugs on the target, signaling downstream of the target) play a role in determination of clinical response to aspirin.

To determine aspirin's effect on gestational age advancement, we constructed a metabolic clock. To ensure a more solid model, we standardized the dating method with the measurement of fetal crown-rump length at 11 to 13 weeks instead of relying on the first date of the last menstrual period, which is not reliable and varies according to menstrual cycle length. Compared with the previous metabolic clock that was learned from 21 healthy pregnant women covering gestational age from 14 to 16 weeks to 40 weeks, our clock was trained on samples collected at 11 to 13 weeks to 20 to 24 weeks from 92 pregnant women specifically at high risk of preeclampsia.[19] The training set was selected as such because our work was originally planned to evaluate the aspirin effect for preeclampsia prevention rather than develop a molecular gestational age estimator. In the cross-validation test, our model showed an excellent prediction accuracy with a Pearson correlation coefficient ($R$) of 0.97 ($R^2$=0.94, $P<2.2\times10^{-16}$) between metabolically estimated and crown-rump length-determined gestational age, a root mean square error of 1.29, a mean absolute error of 0.94, slightly outperforms Liang et al's clock ($R^2$=0.93, $P<1\times10^{-100}$, root mean square error=2.49), and comparable to Lee et al's robust placental clock (mean absolute error=0.96, $r^2$=0.99).[18,19] Comparing to the pregnancy-related profiles reported by Liang et al, apart from confirming the increase of progestin steroid hormones including pregnanediol-3-glucuronide (HMDB0010318), 5α-pregnan-diol disulfate (HMDB0240582/HMDB0240581), our clock has also demonstrated a reduction of several androgenic steroids, such as 5α-androstan-3β,17β-diol disulfate (HMDB0000493) and androstenediol (3β,17β) monosulfate (HMDB0240429; Figure 3D, Table S4). A decrease of lipids has been observed, but phosphatidylethanolamine lipids, instead of lysophospholipids as described in Liang et al's work, are more important in gestational age prediction in our cohort (Table S4). In addition to their findings, increased γ-glutamyl amino acids in accordance with glutamate is also a major contributor to the discriminant model for gestational age estimation (Figure 3D, Table S4). All 8 γ-glutamyl amino acids ranked at the top part of variable importance list (Table S4). These findings may be specific to metabolomics changes from early to mid gestation in high-risk pregnancy.

As a matter of course, the implementation of untargeted metabolomics profiling needs to be validated due to concerns of repeatability. Despite extensive cross validation, an external validation set is needed to assess the performance of the metabolic clock and subsequently confirm the metabolic gestational age delay effect of aspirin.

Previously, Wright and Nicolaides[48] showed data from the ASPRE trial in support of the hypothesis that aspirin prevented preeclampsia by delaying the gestational age at delivery: by delaying the onset of preeclampsia, more cases of term preeclampsia were created from those who were meant to develop preterm preeclampsia, resulting in a significant reduction in the rate of per-term preeclampsia but not the rate of term preeclampsia. Via the constructed metabolic clock, we have demonstrated that aspirin significantly decreases the clock estimated gestational age. In addition, aspirin treatment partially reverses a wide range of metabolic changes over advancement of gestational age.

## PERSPECTIVES

The mechanism by which prophylactic low-dose aspirin in preventing preterm preeclampsia is unknown. This study has demonstrated that the aspirin treatment results in a strong drug-associated metabolomics signature and that the preeclamptic or nonpreeclamptic outcome in response to treatment is significantly associated with the level of internal aspirin exposure ascertained from metabolomics data. Differences in 73 metabolites are detected between women with and without preeclampsia after aspirin treatment, some of which involve pathways whose regulation is of importance in pregnancy and placental functions, such as glycerophospholipids metabolism, polyunsaturated fatty acid metabolism, and steroid hormone biosynthesis. Aspirin reduces the risk of preeclampsia by decelerating advancement of metabolic gestational age, and partially reversing a wide range of metabolic changes over advancement of gestational age. These results lend strong support to the aspirin-related delay hypothesis.

## ARTICLE INFORMATION

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