Serrano, Norma C; Díaz, Luis A; Páez, Maria C; Mesa, Clara M; Cifuentes, Rodrigo; Monterrosa, Alvaro; González, Adriana; Smeeth, Liam; Hingorani, Aroon D; Casas, Juan P; (2006) Angiotensin-converting enzyme I/D polymorphism and preeclampsia risk: evidence of small-study bias. PLoS medicine, 3 (12). e520-. ISSN 1549-1277 DOI: https://doi.org/10.1371/journal.pmed.0030520

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DOI: https://doi.org/10.1371/journal.pmed.0030520

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Angiotensin-Converting Enzyme I/D Polymorphism and Preeclampsia Risk: Evidence of Small-Study Bias

Norma C. Serrano1*, Luis A. Díaz1, Maria C. Páez1, Clara M. Mesa2, Rodrigo Cifuentes3, Alvaro Monterrosa4, Adriana González5, Liam Smeeth6, Aroon D. Hingorani7, Juan P. Casas6,7*

1 Universidad Autónoma de Bucaramanga, Bucaramanga, Colombia, 2 Instituto de Ciencias de la Salud, Medellín, Colombia, 3 Universidad del Valle, Cali, Colombia, 4 Universidad de Cartagena, Cartagena, Colombia, 5 Universidad Industrial de Santander, Bucaramanga, Colombia, 6 Department of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, United Kingdom, 7 Centre for Clinical Pharmacology, Department of Medicine, at British Heart Foundation laboratories at University College London, London, United Kingdom

Funding: This project was funded by a grant from Universidad Autónoma de Bucaramanga (EGEN-10) awarded to NCS. LS holds a Medical Research Council Clinician Scientist Fellowship. ADH holds a British Heart Foundation Senior Fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: University College London holds patents on the measurement of asymmetric dimethylarginine in the management of preeclampsia.

Academic Editor: Cossetta Minelli, University of Leicester, United Kingdom

Citation: Serrano NC, Díaz LA, Páez MC, Mesa CM, Cifuentes R, et al. (2006) Angiotensin-converting enzyme insertion/deletion polymorphism and preeclampsia risk: Evidence of small-study bias. PLoS Med 3(12): e520. doi:10.1371/journal.pmed.0030520

Received: June 15, 2006
Accepted: October 31, 2006
Published: December 26, 2006

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Abbreviations: ACE, angiotensin-converting enzyme; ACE-I/D, angiotensin-converting enzyme gene; CI, confidence interval; OR, odds ratio

* To whom correspondence should be addressed. E-mail: nserrano@ unab.edu.co (NCS), Juan. Pablo-Casas@lsthm.ac.uk (JPC)

ABSTRACT

Background

Inappropriate activation of the renin–angiotensin system may play a part in the development of preeclampsia. An insertion/deletion polymorphism within the angiotensin-I converting enzyme gene (ACE-I/D) has shown to be reliably associated with differences in angiotensin-converting enzyme (ACE) activity. However, previous studies of the ACE-I/D variant and preeclampsia have been individually underpowered to detect plausible genotypic risks.

Methods and Findings

A prospective case-control study was conducted in 1,711 unrelated young pregnant women (665 preeclamptic and 1,046 healthy pregnant controls) recruited from five Colombian cities. Maternal blood was obtained to genotype for the ACE-I/D polymorphism. Crude and adjusted odds ratio (OR) and 95% confidence interval (CI) using logistic regression models were obtained to evaluate the strength of the association between ACE-I/D variant and preeclampsia risk. A meta-analysis was then undertaken of all published studies to February 2006 evaluating the ACE-I/D variant in preeclampsia. An additive model (per-D-allele) revealed a null association between the ACE-I/D variant and preeclampsia risk (crude OR = 0.95 [95% CI, 0.81–1.10]) in the new case-control study. Similar results were obtained after adjusting for confounders (adjusted per-allele OR = 0.90 [95% CI, 0.77–1.06]) and using other genetic models of inheritance. A meta-analysis (2,596 cases and 3,828 controls from 22 studies) showed a per-allele OR of 1.26 (95% CI, 1.07–1.49). An analysis stratified by study size showed an attenuated OR toward the null as study size increased.

Conclusions

It is highly likely that the observed small nominal increase in risk of preeclampsia associated with the ACE-D-allele is due to small-study bias, similar to that observed in cardiovascular disease. Reliable assessment of the origins of preeclampsia using a genetic approach may require the establishment of a collaborating consortium to generate a dataset of adequate size.

The Editors’ Summary of this article follows the references.
Introduction

Preeclampsia is a maternal disease of pregnancy associated with increased blood pressure and proteinuria after 20 weeks of gestation. It is a major cause of maternal and neonatal mortality and morbidity worldwide and has particularly high incidence in Latin American and Caribbean countries, in which hypertension disorders during pregnancy account for 25.7% of maternal deaths [1,2]. Preeclampsia is thought to be the result of the interplay between important genetic components and environmental influences; however, the factors and the mechanisms that lead to preeclampsia remain elusive [3]. As a result, there is a lack of effective preventive interventions [4].

With the exception of smoking, established risk factors for cardiovascular disease, including high blood pressure, diabetes, and obesity, are also risk factors for preeclampsia [5]. In addition, women who suffer from preeclampsia have an increased risk of later cardiovascular disease, which clearly suggests a shared aetiology [6].

Inappropriate activation of the renin–angiotensin system may play a part in the development of many cardiovascular disorders, including preeclampsia [7,8]. A common insertion/deletion polymorphism within the angiotensin-I converting enzyme gene (ACE-I/D) has been reliably associated with substantial differences in the plasma and tissue angiotensin-converting enzyme (ACE) activity in a codominant (additive) fashion not only in persons of European descent, but also in other populations such as Hispanics [9–11]. Individuals carrying the D allele have higher ACE activity, which has been proposed as an intermediate phenotype of potential relevance for the development of high blood pressure and subclinical atheroma (i.e., higher intima-media thickness of the carotid artery) [10,12]. Despite the biological plausibility and the consistency of the effect of the ACE-I/D polymorphism on ACE activity, associations of the ACE-I/D polymorphism have higher ACE activity and higher measures of subclinical atheroma (i.e., higher intima-media thickness of the carotid artery) [13–16]. Moreover, systematic reviews and meta-analyses have indicated the presence of small-study bias in the published literature [13–16].

Several studies, also usually small in size, have reported that women carrying the D allele of the ACE-I/D polymorphism have higher ACE activity and higher measures of uterine artery resistance, which is a marker for development of intrauterine growth retardation and preeclampsia [8,17]. These observations led to the proposal that the ACE-I/D polymorphism may be a good candidate in the search for a cause of preeclampsia. However, to date, studies evaluating the role of ACE-I/D polymorphism in preeclampsia have been individually underpowered to detect plausible genetic effect sizes, being much smaller than more recent studies in cardiovascular disease. We hypothesized that the published literature on the ACE-I/D polymorphism in preeclampsia might be similarly affected by small-study bias. To test this hypothesis, we conducted a new large genetic association study on the ACE-I/D polymorphism and preeclampsia in a geographical region with a high incidence of preeclampsia. We then set this study within the context of a systematic review and a meta-analysis of all studies conducted to date.

Methods

Case-Control Study Participants

A prospective case-control study was conducted in 1,711 unrelated young pregnant women (665 preeclamptic participants and 1,046 healthy pregnant controls) recruited from five Colombian cities at the time of delivery between January 2000 and December 2005. A verbal interview with a structured questionnaire was conducted by trained personnel at the time of the delivery to ascertain maternal age, gestational age, parity, smoking status during pregnancy, family history of preeclampsia, ethnic background, and socioeconomic position. On two separate occasions, the mean of two readings of blood pressure was obtained (four measurements in total) at the time of delivery. Blood pressure was measured in the right arm after a five-minute period of rest in a seated position using mercury sphygmomanometers or electronic devices calibrated against a mercury standard.

A case was defined as a primigravid woman younger than 26 years old with blood pressure 140/90 mm Hg or above, and proteinuria 0.3 grams or above in 24 hours, or a reading 2+ or above on a dipstick in a random urine determination with no evidence of urinary tract infection after 20 weeks of gestation [18]. At least one control was recruited after each case within a window of 24 hours from the same hospital that provided the cases. A control was defined as a primigravid woman younger than 26 years old without preeclampsia and in labour after 37 weeks of pregnancy. To improve the homogeneity of the phenotype under evaluation, women with a prior history of autoimmune, metabolic (including diabetes or gestational diabetes), renal, or cardiac (including chronic hypertension) diseases were excluded from the study. All participants signed the informed consent document approved by the Ethics Committee from the Universidad Autónoma de Bucaramanga, Colombia.

DNA extraction and genotyping. Blood was drawn from the antecubital vein into EDTA and samples stored at −50 °C for DNA extraction, using the QIAamp DNA blood mini-kit (QIAGEN, Hilden, Germany). The ACE-I/D polymorphism in intron 16 was detected according to the method described by Rigat, et al. [9]. PCR is known to have a tendency to preferentially amplify the short deletion (D) allele in contrast to the larger insertion (I) allele in a competitive amplification reaction when both alleles are present, as occurs in individuals with the ID genotype. This leads to mistyping of ID individuals as DD in approximately 4%–5% of the samples. To avoid any mistyping of ID as DD, a second PCR amplification using insertion-specific primers was conducted for all participants who were homozygous for the D allele [19]. For a detailed description of the genotyping methods, see Protocol S1. All available DNA samples were genotyped and included in the present report, with no exclusions. For quality control, a random sample (n = 156) was subjected to a second PCR and genotyping to minimize any possible misclassification. The Cohen’s kappa value among the samples regenotyped was equal to 0.95 (95% CI [confidence interval], 0.94–0.96), and the error rate was 2.56% (95% CI, 0.70–6.43). Genotyping was conducted blinded to the clinical status of the participants.

Statistical analysis. To evaluate the presence of differences between groups, unpaired Student’s t-, χ², or Mann-Whitney tests were used as appropriate. A test for departure from
Hardy-Weinberg equilibrium was performed by $\chi^2$ analysis. The principal a priori hypothesis was that the association between the ACE-I/D polymorphism and preeclampsia follows an additive model according to the number of D alleles. The additive “per-allele” model (in a log-scale) was based on the effect of the ACE-I/D variant on its intermediate phenotype (ACE activity), established in large studies and overviews as being a reliable association [10]. However, recessive and dominant models and multiple pairwise comparisons (II versus ID and DD versus II) were also evaluated for completeness as secondary outcomes.

Multivariate analysis using logistic regression methods was also conducted to control for potential confounders (maternal age, ethnic background, place of birth of the women, recruitment centre, socioeconomic position, urinary or vaginal infections during pregnancy, and smoking status during pregnancy).

To explore the prior hypothesis that the genotypic odds ratio (OR) is greater in women with an enriched phenotype, an analysis using an additive model was repeated for women stratified according to the presence of family history of preeclampsia (positive versus negative family history in mother or sisters), and disease severity with severe preeclampsia being defined as blood pressure above 160/110 mm Hg or proteinuria of 5 grams or more in 24 hours, eclampsia, or the HELLP syndrome. For these analyses 99% CIs were used to make some allowance for their exploratory nature. All statistical analyses were conducted using Stata, Version 9 (Stata Corporation, College Station, Texas, United States).

**Table 1. Maternal and Neonatal Characteristics of the Sample Studied**

| Category                  | Variable               | Cases ($n = 665$) | Controls ($n = 1,046$) | $p$-Value |
|---------------------------|------------------------|-------------------|------------------------|-----------|
| **Ethnic background**     | European descent       | 63 (9.5%)         | 141 (13.5%)            |           |
|                           | African American       | 117 (17.6%)       | 176 (16.3%)            |           |
|                           | Native American        | 9 (1.4%)          | 11 (1.1%)              |           |
|                           | Mixture                | 476 (71.6%)       | 718 (68.6%)            | 0.092     |
| **Socioeconomic**         | Low socioeconomic status| 585/642 (91.1%)  | 922/1,011 (91.2%)      | 0.958     |
| **Maternal**              | Maternal age (y)       | 19.0 ± 2.9        | 18.7 ± 2.6             | 0.013     |
|                           | Smoking during pregnancy| 11/660 (1.7%)    | 37/1,045 (3.5%)        | 0.023     |
|                           | Systolic blood pressure (mm Hg) | 146.1 ± 12.1    | 110.9 ± 9.2            | < 0.001   |
|                           | Diastolic blood pressure (mm Hg) | 68.1 ± 7.1    | 68.1 ± 7.1             | < 0.001   |
|                           | Multiple pregnancy     | 19/663 (2.9%)     | 4/1,038 (0.4%)         | < 0.001   |
|                           | Gestational age at delivery (wk) | 37.0 ± 3.6  | 39.2 ± 1.2             | < 0.001   |
| **Maternal ACE-I/D genotype** | DD                     | 140 (21.1%)       | 230 (22.0%)            |           |
|                           | ID                     | 382 (57.4%)       | 607 (58.0%)            | 0.72      |
|                           | II                     | 143 (21.5%)       | 209 (20.0%)            |           |
| **Allele frequency**      | D                      | 662 (49.8%)       | 1,067 (71.6%)          | 0.483     |
|                           | I                      | 668 (50.2%)       | 1,025 (28.4%)          |           |
| **Newborn characteristics** | Newborn weight (g)    | 2,626 ± 730       | 3,126 ± 434            | < 0.001   |
|                           | Newborn height (cm)    | 46.9 ± 4.6        | 49.6 ± 2.4             | < 0.001   |
|                           | Low (< 7) Apgar score at first min | 83/671 (12.4%) | 67/1,025 (6.5%)        | < 0.001   |
|                           | Low (< 7) Apgar score at fifth min | 34/671 (5.1%) | 5/1,024 (0.5%)         | < 0.001   |

Data are presented as n (%) or mean ± SD.
doi:10.1371/journal.pmed.0030520.t001

The search strategy contained both medical subject heading terms and text words as follows: “angiotensin-converting enzyme” or “ACE” or “peptidyl-dipeptidase A,” in combination with “pre-eclampsia” or “preeclampsia” or “pregnant hypertensive disorders” or “pregnancy hypertension,” and combined with “genetic” or “polymorphism(s)” or “mutation” or “genotype” or “gene(s).” No limits were used in the search strategy. We searched for any additional studies in the references of all identified publications, including previous relevant meta-analyses, and used the MEDLINE option “related articles” for all the relevant papers.

For inclusion, studies had to involve unrelated women and examine the association between the ACE-I/D polymorphism and preeclampsia. Studies published as full-length articles or letters in peer-reviewed journals in any language were included, as well as abstracts taken from reference lists of identified publications. Authors were contacted (on at least three occasions) to obtain information on the genotype frequency by case-control status and by disease severity (severe and nonsevere preeclampsia), the use of blinding of genotyping staff to clinical status, the definition of outcomes, and, in a few cases, to clarify possible overlapping of study results. A positive reply was obtained in 15 out of 21 study authors contacted.

**Data extraction.** The following information was extracted (entered into databases by two of the authors, JPC and MCP) from each study and disagreements resolved by consensus: year of publication, total cases, total controls, number of individuals by each genotype, study design, source of controls, matching variable, thresholds used to define preeclampsia, country of origin, ethnicity, $\chi^2$ goodness of fit for Hardy-Weinberg equilibrium and its p-value, use of blinding, mean age of participants, frequency of nulliparous women, and main exclusion criteria. In the few instances in which genotype frequencies provided by the investigators in tabular data differed slightly from published figures, the tabular data
were assessed by the DerSimonian and Laird Q was used to calculate the summary OR \[20\]. Heterogeneity combined estimates. The inverse variance-weighted method for individual studies, and 95% CIs were reserved for the some allowance for multiple comparisons 99% CIs were used ing ORs by logistic regression. We calculated the random model, the OR was compared between cases and controls by considered the one at risk. For the additive or per-allele 200, and \( /C21\) small-study effects \[20,21\]. To evaluate stability over time of sample size (linear-regression model) were used to evaluate linear fixed regression of the log OR against the inverse of the weighted (by the inverse of the variance of the estimate) the sample size, Egger regression asymmetry test, and a language journals). Funnel plots of the effect estimate against and publication language (English– versus non-English– others); preeclampsia definition (adequate versus unclear); European descent, Asian, Hispanic, Afro-Caribbean, and unknown); disease severity (severe versus nonsevere preeclampsia); ethnicity of women evaluated (of European descent, Asian, Hispanic, Afro-Caribbean, and others); preeclampsia definition (adequate versus unclear); and publication language (English– versus non-English–language journals). Funnel plots of the effect estimate against the sample size, Egger regression asymmetry test, and a weighted (by the inverse of the variance of the estimate) linear fixed regression of the log OR against the inverse of the sample size (linear-regression model) were used to evaluate small-study effects \[20,21\]. To evaluate stability over time of the effect estimate, cumulative meta-analysis using random effect models was conducted \[20\].

**Results**

**Case-Control Study**

Clinical and demographic data of the cases and controls are shown in Table 1. There were no significant differences in maternal age, ethnic background, and socioeconomic posi- 

| Association | Unadjusted OR (95% CI) | Model-1 OR (95% CI) | Model-2 OR (95% CI) |
|-------------|------------------------|---------------------|---------------------|
| Dominant model (D-carriers\(^a\) versus II) | 0.91 (0.71–1.17) | 0.86 (0.67–1.11) | 0.84 (0.65–1.09) |
| Recessive model (DD\(^*\) versus \(\ast\)-carriers) | 0.95 (0.75–1.21) | 0.93 (0.72–1.18) | 0.92 (0.71–1.18) |
| Additive model (per-increase in D-allele\(^a\)) | 0.95 (0.81–1.10) | 0.92 (0.78–1.07) | 0.90 (0.77–1.06) |
| Pairwise comparisons, ID\(^*\) versus II | 0.83 (0.65–1.06) | 0.87 (0.67–1.13) | 0.85 (10.65–1.12) |
| Pairwise comparisons, DD\(^*\) versus II | 0.81 (0.60–1.08) | 0.83 (0.60–1.16) | 0.82 (0.59–1.14) |

Model-1: Adjusted by maternal age, ethnicity, recruitment centre, and place of birth. Model-2: Previous variables plus socioeconomic status, infections during pregnancy (urinary or vaginal), and smoking status during pregnancy.

\(\text{\textsuperscript{a}}\)The genotype or allele considered at risk.

doi:10.1371/journal.pmed.0030520.002

The distribution of the ACE-\(I/D\) genotypes and allelic frequencies were not significantly different according to the case-control status (Table 1). Genotype frequencies according to the ethnic group and by recruitment centre are reported in Appendix Table I in Protocol S1. Each additive model (per-D-allele) revealed a null association between the ACE-\(I/D\) variant and preeclampsia risk (crude OR = 0.95 [95% CI, 0.81–1.10]). Adjusting for maternal age, ethnicity, recruitment centre, and place of birth, aimed to minimize the effect of possible population admixture, did not change the estimate of the effect (model-1 OR = 0.92 [95% CI, 0.78–1.07]). A similar result was obtained after further adjustment for additional potential confounders such as socioeconomic position, presence of urinary or vaginal infections during pregnancy, and smoking status during pregnancy (model-2 OR = 0.90 [95% CI, 0.77–1.06]). ORs for other genetic models of inheritance also yielded a null association (Table 2). Pre-specified exploratory subgroup analyses indicated that with the exception of family history of preeclampsia (positive-history OR = 1.30 [99% CI, 0.75–2.26] versus negative-history OR = 0.86 [99% CI, 0.68–1.10]; \(p\)-value for heterogeneity equal to 0.07), no substantial heterogeneity of the genetic effect size was observed for any of the subgroups (Figure 1). Additionally, stratified analysis by recruitment centre according to the conformity with Hardy-Weinberg equilibrium yielded similar, null results (see “Appendix Table II” in Protocol S1).

**Meta-Analysis**

A total of 30 genetic association studies, including the present study, evaluating the ACE-\(I/D\) gene variant and preeclampsia risk were identified \[17,22–49\]. We excluded eight out of 30 studies for one or more of the following reasons: two in which duplication or partial overlapping of reported data were considered likely \[43,44\]; three in which the outcome evaluated was solely gestational hypertension \[45–47\]; one in which relevant data were not reported and could not be obtained from study authors \[48\]; one that only recruited women with previous preeclampsia \[17\]; and one in which the sampling frame was based on the ACE-\(I/D\) genotype \[49\]. A total of 22 genetic association studies including 2,596 cases and 3,828 controls were included in the present meta-analysis (Tables 3 and 4) \[22–42\]. Out of the 22 studies, nine were conducted with Asian participants, eight with partic-
participants of European descent, two with African Americans, two with more than one ethnic group, and one with South Asians.

The OR under an additive model for preeclampsia was 1.26 (95% CI, 1.07–1.49; \( p = 0.006 \)) (Figure 2). However, there was evidence of substantial between-study heterogeneity (\( I^2 = 75.1\% \), \( Q = 84.23, p = 0.0001 \)). Study characteristics such as blinding of genotyping staff, publication language, preeclampsia definition, and disease severity explained little of the heterogeneity (Figure 3). A stratified analysis by study size, evaluated as the number of cases in each study (<100, 100–200, and >200), showed a diminished effect as the study size increased (\( Q = 16.95, p = 0.0002 \)) (Figure 3). Analogous results were obtained when different cutoff points (<100, 100–500, and >500) for the number of cases were used (\( Q = 18.51, p = 0.0009 \)). Similarly, stratifying by ethnicity indicated that studies conducted in Asian populations tended to have a larger ORs (\( Q = 14.4, p = 0.007 \)). These findings might be explained by the fact that eight out of the nine studies conducted in Asian populations had fewer than 100 cases in each study. The funnel plot including all studies was asymmetric, and the Egger’s test (\( p = 0.06 \)) and linear-regression model test (\( p = 0.003 \)) suggested the presence of small-study bias. Furthermore, since only the published literature was included, it is possible that including unpublished studies (which more often provide evidence of negative or null effects) would have provided additional evidence of small-study bias. Taken together, these findings point to small-study bias as a potential explanation for the results observed in the meta-analysis. Discrepancy findings from large and small studies are not new in the field of genetics of complex disorders [50]. When present, discrepancy of genetic effects sizes may be due to multiple causes such as genuine heterogeneity, data manipulation and fabrication, study quality, or publication bias. A form of publication bias relevant to the current report is within-study reporting bias. Because of the facility of measuring multiple genetic markers in a study, significant positive and negative associations (sometimes arising from multiple testing) are more likely to be published early rather than late. Results from the cumulative meta-analysis support this as one possible explanation, which has been referred to as the

Discussion

The current meta-analysis, which includes new data from the largest case-control study to date, represents the most comprehensive evaluation of the ACE-I/D variant in preeclampsia. Although a pooled per-allele OR suggested evidence of an increase in the risk of preeclampsia of 1.26 (95% CI, 1.07–1.49), the robustness of this summary estimate is uncertain. First, our study found a null association of the ACE-I/D variant with preeclampsia. Moreover, there was no substantial evidence of a positive effect in any of the subgroups in the prespecified analyses. Second, the meta-analysis revealed diminishing summary risk estimate as study size increased (Figure 3), regardless of the arbitrary cutoff points used to define the categories. This result is concordant with the results of several statistical tests used to evaluate the presence of small-study bias (Egger’s test (\( p = 0.06 \)) and linear-regression model test (\( p = 0.003 \))). Furthermore, since only the published literature was included, it is possible that including unpublished studies (which more often provide evidence of negative or null effects) would have provided additional evidence of small-study bias. Taken together, these findings point to small-study bias as a potential explanation for the results observed in the meta-analysis. Discrepancy findings from large and small studies are not new in the field of genetics of complex disorders [50]. When present, discrepancy of genetic effects sizes may be due to multiple causes such as genuine heterogeneity, data manipulation and fabrication, study quality, or publication bias. A form of publication bias relevant to the current report is within-study reporting bias. Because of the facility of measuring multiple genetic markers in a study, significant positive and negative associations (sometimes arising from multiple testing) are more likely to be published early rather than late. Results from the cumulative meta-analysis support this as one possible explanation, which has been referred to as the

Figure 1. Risk Association of ACE-I/D Variant with Preeclampsia in Selected Subgroups within the Current Study

doi:10.1371/journal.pmed.0030520.g001

Subgroups (No. of cases) | Per-allele odds ratio and 99% CI | Odds ratio (99% CI) | \( \chi^2 \) test of heterogeneity (p value)
---|---|---|---
Ethnicity | | | |
White-Hispanics (n = 63) | 0.69 (0.34, 1.42) | | |
African Americans (n = 117) | 0.93 (0.38, 1.25) | | |
“Mestizas” or Mixed (n = 476) | 0.96 (0.75, 1.23) | | |
Disease severity | | | |
Severe preeclampsia (n = 441) | 0.86 (0.67, 1.10) | | | 0.52 on 1 df (0.47) |
Non severe preeclampsia (n = 224) | 0.96 (0.71, 1.30) | | | |
Family history of preeclampsia | | | |
Positive family history (n = 156) | 1.30 (0.75, 2.26) | | | 3.12 on 1 df (0.07) |
Negative family history (n = 509) | 0.86 (0.68, 1.10) | | | |
Onset of disease | | | |
Early preeclampsia (n = 56) | 1.25 (0.68, 2.31) | | | 1.81 on 1 df (0.18) |
Late preeclampsia (n = 609) | 0.89 (0.71, 1.11) | | | |
Overall (95% CI) | | | | |
| Reference                   | Year | Total Cases | Total Controls | Total Sample Size | DD Genotype Cases, n | ID Genotype Cases, n | II Genotype Cases, n | DD Genotype Controls, n | ID Genotype Controls, n | II Genotype Controls, n | Design        |
|-----------------------------|------|-------------|----------------|-------------------|----------------------|---------------------|----------------------|------------------------|------------------------|-------------------------|----------------|
| Bai [22]                    | 2002 | 81          | 199            | 280               | 8                    | 38                  | 35                   | 31                     | 83                     | 85                      | Case-control |
| Bouba [23]                  | 2003 | 41          | 102            | 143               | 17                   | 19                  | 5                    | 29                     | 52                     | 21                      | Case-control |
| Choi H [24]                 | 2004 | 90          | 98             | 188               | 33                   | 34                  | 23                   | 14                     | 51                     | 33                      | Case-control |
| Dizon-Townson [25]          | 1995 | 124         | 200            | 324               | 49                   | 42                  | 33                   | 65                     | 98                     | 37                      | Case-control |
| Galao [26]                  | 2004 | 51          | 71             | 122               | 16                   | 23                  | 12                   | 21                     | 33                     | 17                      | Case-control |
| Gurdoll [27]                | 2004 | 95          | 89             | 184               | 47                   | 31                  | 17                   | 31                     | 37                     | 21                      | Case-control |
| Heiskanen [28]              | 2001 | 133         | 115            | 248               | 43                   | 59                  | 31                   | 31                     | 58                     | 26                      | Case-control |
| Kaur [29]                   | 2005 | 50          | 50             | 100               | 30                   | 14                  | 6                    | 15                     | 26                     | 9                       | Case-control |
| Kim [30]                    | 2004 | 98          | 110            | 208               | 26                   | 36                  | 36                   | 25                     | 52                     | 33                      | Case-control |
| Kobashi [31]                | 2005 | 122         | 291            | 413               | 19                   | 52                  | 51                   | 35                     | 136                    | 120                     | Case-control |
| Levesque [32]               | 2004 | 174         | 306            | 480               | 50                   | 92                  | 32                   | 97                     | 151                    | 58                      | Nested case-control |
| Mingwei [33]                | 1998 | 35          | 25             | 60                | 23                   | 7                   | 5                    | 2                      | 10                     | 13                      | Case-control |
| Morgan [34]                 | 1999 | 73          | 83             | 156               | 23                   | 31                  | 19                   | 25                     | 36                     | 22                      | Case-control |
| Mozgovova [35]              | 2000 | 45          | 73             | 118               | 21                   | 14                  | 10                   | 28                     | 37                     | 8                       | Case-control |
| Roberts [36]                | 2004 | 391         | 338            | 729               | 183                  | 164                 | 44                   | 152                    | 142                    | 44                      | Case-control |
| Roh [37]                    | 1997 | 36          | 115            | 151               | 4                    | 11                  | 21                   | 14                     | 62                     | 39                      | Case-control |
| Seremak-Mrozikiewicz [38]   | 2000 | 25          | 110            | 135               | 16                   | 7                   | 2                    | 34                     | 61                     | 15                      | Case-control |
| Serrano et al. (present study) | 2006 | 663         | 1046           | 1711              | 140                  | 382                 | 143                  | 230                    | 607                    | 209                     | Case-control |
| Tamura [39]                 | 1996 | 12          | 179            | 191               | 3                    | 6                   | 3                    | 63                     | 82                     | 34                      | Cohort       |
| Wang [40]                   | 2004 | 99          | 54             | 153               | 42                   | 37                  | 20                   | 14                     | 16                     | 24                      | Case-control |
| Watanabe [41]               | 2001 | 96          | 96             | 192               | 13                   | 48                  | 35                   | 10                     | 40                     | 46                      | Case-control |
| Zhou [42]                   | 1999 | 60          | 78             | 138               | 39                   | 12                  | 9                    | 8                      | 32                     | 38                      | Case-control |

*aStudies on which the reporting of the criteria to define preeclampsia was unclear. Results from these studies were compared against the others in Figure 3.

*bDefinition used from a posterior manuscript from the same authors.

BP, blood pressure; HWE, Hardy-Weinberg equilibrium.
doi:10.1371/journal.pmed.0030520.t003

Table 3. Characteristics of Published Studies of the Association between the ACE-I/D Polymorphism and Preeclampsia Included in the Meta-Analysis
### Table 3. Extended.

| Reference      | Source of Controls                                                                                   | Matching Variable                  | Systolic BP Threshold (mm Hg) | Diastolic BP Threshold (mm Hg) | Proteinuria Threshold |
|----------------|-----------------------------------------------------------------------------------------------------|------------------------------------|------------------------------|------------------------------|-----------------------|
| Bai [22]       | Normal pregnant women                                                                               | Maternal age                       | 140                          | 90                           | Not specified         |
| Bouba [23]     | Normotensive pregnant women who had undergone at least two pregnancies with no history of preeclampsia | None                               | 140                          | 90                           | >300 mg/l in a random specimen or an excretion of >300 mg per 24 h after 20 wk of gestation. |
| Choi H [24]    | Normotensive volunteers randomly recruited from the Obstetric Service at the Samsung Cheil Hospital | Maternal age                       | 140                          | 90                           | >1 g/l (or 2+ dipstick) in random urine |
| Dizon-Townson [25] | Referred to only as “controls”                                                                            | None                               | 140                          | 90                           | >500 mg of protein in 24 h or a new 3 dipstick without infectionb |
| Galao [26]     | Normotensive pregnancy under medical assistance                                                        | None                               | 140                          | 90                           | >300 mg/24 h           |
| Gurdol [27]    | 50 participants were complication-free-pregnancies. The remaining 39 women were normotensive with >2 pregnancies unaffected by preeclampsia | None                               | 140                          | 90                           | >300 mg/l in a 24-h collection |
| Heiskanen [28] | Women who delivered at Kuopio University Hospital                                                      | None                               | 140                          | 90                           | >300 mg of urinary protein in 24 h |
| Kaur [29]      | Normotensive primigravidae who were followed till delivery                                              | Gestation and maternal age         | 140                          | 90                           | >0.3 g/l              |
| Kim [30]       | Randomly selected nulliparous and parous women who were not affected by preeclampsia in the pregnancy progressing to >20 weeks’ gestation | None                               | 140                          | 90                           | >300 mg/24 h           |
| Kobashi [31]   | Randomly selected from healthy pregnant women                                                          | None                               | 140                          | 90                           | 30 mg/dl (1+ on a dipstick) or greater |
| Levesque [32]  | Normotensive nulliparous women attending a tertiary perinatal center                                   | Maternal age, gestational age, body mass index, and month of the year at delivery. Not specified | 90                           | 1+ reading on dipstick in a random urine sample |
| Mingwei [33]   | Control population                                                                                     | None                               | Not specified                | Not specified                | Not specified         |
| Morgan [34]    | Normotensive women recruited during the second half of the pregnancy                                    | None                               | 140                          | 90                           | >300 mg/l in 24 h or ≥2+ on dipstick in a random urine sample |
| Mozgovaia [35] | Pregnant women without obvious somatic pathology                                                          | None                               | 140                          | 90                           | ≥0.3 g/l in a 24 h     |
| Roberts [36]   | Healthy pregnant normotensive participants who had delivery normally beyond 37th week of gestation     | None                               | 140                          | 90                           | 1+ on dipstick after 34 wk of gestation |
| Roh [37]       | Normal population                                                                                      | None                               | 140                          | 90                           | >300 mg/24 h           |
| Seremak-Mrozikiewicz [38] | Healthy pregnant women                                                                                 | None                               | 160                          | 110                          | ≥5 g in 24 h           |
| Serrano et al. | Healthy normotensive pregnant women at delivery from the same hospitals where cases were recruited    | None                               | 140                          | 90                           | ≥0.3 g in 24 h, or ≥2+ reading on dipstick in a random sample |
| Tamura [39]    | Pregnant women enrolled in a clinical trial who did not develop preeclampsia or pregnancy induced hypertension | None                               | 140                          | 90                           | Referred to as proteinuria (not threshold reported) |
| Wang [40]      | Normal pregnant women                                                                                  | None                               | Not specified                | Not specified                | Not specified         |
| Watanabe [41]  | Healthy pregnant volunteers                                                                            | Maternal age                       | 160                          | 110                          | ≥5 g in a 24-h collection or ≥3+ on dipstick testing of two random urine samples collected at least 4 h apart |
| Zhou [42]      | Healthy pregnant women                                                                                  | None                               | Not specified                | Not specified                | Not specified         |
### Table 3. Extended.

| Reference | Reference Cited to Define Preeclampsia | Main Exclusion Criteria | Country | Ethnicity |
|-----------|----------------------------------------|--------------------------|---------|-----------|
| Bai [22]  | Authors referred only to: “rigorous definition of pre-eclampsia” | Not specified | China   | Asian     |
| Bouba [23] | Not specified | Preexisting cardiovascular or renal disease | Greece  | European |
| Choi H [24] | Cardiac disease, serum creatinine > 150 μmol/l or the presence of other systemic disease | Korea | Asian |
| Dizon-Townson [25] | Not specified | Not specified | United States | European |
| Galao [26] | History of essential or secondary hypertension, multiparous women, diabetes mellitus, renal disease | Brazil | Hispanic (European and American Indian) |
| Gurdol [27] | Preexisting hypertension | Turkey | European |
| Heiskanen [28] | Chronic hypertension and multiple pregnancy | Finland | European |
| Kaur [29] | Chronic hypertension, diabetes mellitus, hyperthyroidism, sarcoidosis, chronic renal disorders, collagen disorders | India | South Asian |
| Kim [30] | No chronic hypertension. No personal or family history of preeclampsia (only for controls) | Korea | Asian |
| Kobashi [31] | Preexisting hypertension, renal disease, diabetes mellitus, amniotic fluid abnormalities, fetal anomalies, multiple pregnancies | Japan | Asian |
| Levesque [32] | Preexisting secondary hypertension, preexisting hypertension with superimposed preeclampsia, multiple pregnancies | Canada | European |
| Mingwei [33] | Reported as standard criteria. The authors cited a Chinese textbook of obstetrics and gynecology. A | Not specified | China | Asian |
| Morgan [34] | History of essential hypertension, diabetes mellitus, or chronic renal disease | United Kingdom | European |
| Mozgovaia [35] | Not specified | Not specified | Russia | European |
| Roberts [36] | History of hypertension in a previous pregnancy (only for controls) | South Africa | Afro-Caribbean |
| Roh [37] | American College of Obstetrics and Gynecology | Not specified | Korea | Asian |
| Seremak-Mrozikiewicz [38] | Multiparous women | Poland | European |
| Serrano et al. (present study) [67] | See methods section, manuscript | Colombia | Mixed (European, Hispanic, Afro-Caribbean, and Native American) |
| Tamura [39] | Hypertension, renal disease, diabetes, or other significant medical complications, such as heart disease | United States | Afro-Caribbean |
| Wang [40] | Not specified | China | Asian |
| Watanabe [41] | History of hypertension or renal disease | Japan | Asian |
| Zhou [42] | Reported as standard criteria. The authors cited a Chinese textbook of obstetrics and gynaecology | Not specified | China | Asian |
Table 4. Characteristics of Published Studies of the Association between the ACE-I/D Polymorphism and Preeclampsia Included in the Meta-Analysis

| Study              | Mean Maternal Age, Participants | Mean Maternal Age, Controls | Percentage of Nulliparous Cases | Percentage of Nulliparous Controls | Gestational Age, Cases | Gestational Age, Controls | Language of Publication | Blinding Confirmation of DD Genotype | Regenotyping of a Random Subsample | $\chi^2$ Value for HWE | Evidence for Departure from HWE (p-Value) |
|--------------------|---------------------------------|-------------------------------|---------------------------------|-----------------------------------|------------------------|--------------------------|-------------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------------|
| Bai [22]           | 28                              | 28                            | Not specified                   | Not specified                     | Not specified          | Non-English              | Unknown                 | Not reported                  | Not reported                  | 1.971                         | 0.160                                |
| Bouba [23]         | 31                              | 29                            | Not specified                   | 0                                | 34.32                  | 38.96                    | English                 | Yes                           | Yes                           | 0.069                         | 0.793                                |
| Choi [24]          | 30                              | 31                            | 69                              | 67                               | 36.2                   | 39.7                     | English                 | Yes                           | Yes                           | 0.650                         | 0.420                                |
| Dizon-Townson [25] | Not specified                    | Not specified                 | Not specified                   | Not specified                    | Not specified          | Not specified           | English                 | Unknown                       | Yes                           | Not reported                  | < 0.0001                            |
| Galao [26]         | 21                              | 25                            | 100                             | Not specified                    | 36.2                   | 39                       | English                 | No                            | Yes                           | 0.323                         | 0.569                                |
| Gurdol [27]        | 28                              | 27                            | Not specified                   | 56                               | 30-38                  | 30-38                    | English                 | Yes                           | Yes                           | 2.219                         | 0.136                                |
| Heiskanen [28]     | Not specified                    | Not specified                 | Not specified                   | Not specified                    | Not specified          | Not specified           | English                 | Yes                           | Not reported                  | 0.013                         | 0.909                                |
| Kaur [29]          | 25                              | 24                            | 100                             | 100                              | 35.9                   | 35.8                     | English                 | Yes                           | Not reported                  | 0.152                         | 0.696                                |
| Kim [30]           | 31                              | 31                            | 58.6                            | 48.2                             | 36.2                   | 39                       | English                 | Yes                           | Yes                           | 0.270                         | 0.603                                |
| Kobashi [31]       | 29                              | 29                            | 70                              | 57                               | 36.4                   | 39.1                     | English                 | Unknown                       | Yes                           | 0.140                         | 0.708                                |
| Levesque [32]      | 26                              | 26                            | 100                             | 100                              | 36.7                   | 37.8                     | English                 | Yes                           | Yes                           | 0.003                         | 0.955                                |
| Mingwei [33]       | Not specified                    | Not specified                 | 100                             | Not specified                    | Not specified          | Not specified           | Non-English             | Unknown                       | Not reported                  | 0.002                         | 0.968                                |
| Morgan [34]        | 29                              | 28                            | 84.7                            | 90                               | 35.5                   | 39.9                     | English                 | Yes                           | Yes                           | 1.433                         | 0.231                                |
| Mozgovaya [35]     | Not specified                    | Not specified                 | Not specified                   | Not specified                    | Not specified          | Not specified           | English                 | Yes                           | Not reported                  | 0.672                         | 0.412                                |
| Roberts [36]       | 26                              | 25                            | 0                               | 0                                | 34.4                   | 38.8                     | English                 | No                            | Yes                           | 1.394                         | 0.237                                |
| Roh [37]           | Not specified                    | Not specified                 | Not specified                   | Not specified                    | Not specified          | Not specified           | Non-English             | Unknown                       | Not reported                  | 1.990                         | 0.157                                |
| Seremak-Mrozikiewicz [38] | Not specified          | Not specified | Not specified | Not specified | Not specified | Not specified | Non-English | Unknown | Not reported | Not reported | 2.256 | 0.133                                |

| Serrano et al.     | 19                              | 18                            | 100                             | 100                              | 37                     | 39                       | English                 | Yes                           | Yes                           | 27.140                        | < 0.00001                           |

“Blinding” column indicates whether laboratory staff was blind or not to the case-control status. All samples were genotyped by PCR.

HWE, Hardy-Weinberg equilibrium.

doi:10.1371/journal.pmed.0030520.t004

ACE Polymorphisms and preeclampsia.
Proteus phenomenon [51]. Further evidence in support of the presence of within-study reporting bias is the fact that studies published in languages other than English, and in the Asian ethnic group, tend to have larger effects, findings consistent with other recent results [52].

Publication bias is increasingly being recognized as one of the main threats to the reliability of conclusions drawn from association studies with common disease outcomes. In the setting of cardiovascular and neurological diseases, several positive gene–disease associations, usually based on meta-analysis of small studies, have been subsequently refuted by large genetic studies [13,53]. As a result, several initiatives are
Table 5. Genotypic ORs for Preeclampsia and the ACE-I/D Variant.

| Model                                    | Comparison | ACE-I/D Variant |
|------------------------------------------|------------|-----------------|
| Additive model                           | Random, OR (95% CI), p-value | 1.26 (1.07–1.49), p = 0.006 |
|                                          | p² (p for heterogeneity) | 75.1% (p < 0.0001) |
|                                          | Egger test, p-value | p = 0.06 |
| Homozygous for rare allele versus homozygous for common allele | Random, OR (95% CI), p-value | 1.51 (1.09–2.08), p = 0.01 |
|                                          | p² (p for heterogeneity) | 71.8% (p < 0.0001) |
|                                          | Egger test, p-value | p = 0.02 |
| Heterozygous versus homozygous for common allele | Random, OR (95% CI), p-value | 0.94 (0.78–1.12), p = 0.50 |
|                                          | p² (p for heterogeneity) | 31.5% (p = 0.07) |
|                                          | Egger test, p-value | p = 0.76 |
| Recessive model                          | Random, OR (95% CI), p-value | 1.59 (1.22–2.07), p = 0.001 |
|                                          | p² (p for heterogeneity) | 74.5% (p < 0.0001) |
|                                          | Egger test, p-value | p = 0.008 |
| Dominant model                           | Random, OR (95% CI), p-value | 1.15 (0.91–1.45), p = 0.21 |
|                                          | p² (p for heterogeneity) | 63.1% (p < 0.0001) |
|                                          | Egger test, p-value | p = 0.09 |

doi:10.1371/journal.pmed.0030520.t005

now underway to help overcome problems of reporting and publication bias and to help to achieve datasets of appropriate size to detect plausible genetic effects for common disorders, which are likely to require several thousands of cases of the disease (The Wellcome Trust Case Control Consortium, http://www.wtccc.org.uk) [54]. Genetic studies in preeclampsia continue to be somewhat small in size [55,56] and are usually underpowered to detect realistic genotypic relative risks (ORs between 1.15 and 1.4) [14,57]. However, considering the low incidence of preeclampsia (2%–3% in developed countries), it is highly unlikely that a single centre will be able to amass the large number of cases required, and the development of networks of interested investigators may be essential [54]. Therefore, international collaborations, particularly among those countries with a high incidence of preeclampsia, may make recruitment more efficient and help to include participants with different cultural and genetic backgrounds, which can provide further insight into the aetiology of the disease both genetic and/or environmental.

Despite these obstacles, the investment in adequate resources to study the genetics of preeclampsia is an important priority. Observational studies and the randomised trials of interventions that have followed have been unsuccessful thus far in identifying causal pathways in preeclampsia amenable to preventive therapies, a clear example of which are the recently failed clinical trials using either antioxidant vitamins or calcium supplements [58–61]. A genetic approach that is less prone to confounding and reverse causation than nongenetic observational studies, may be more likely to identify causal pathways and may help to prioritise therapeutic targets that require evaluation in large and expensive randomised clinical trials [62,63]. The challenge is in how to make better use of the genetic approach in complex diseases such as preeclampsia, in particular to overcome random errors in risk estimates from small studies as well as publication bias. A suggested approach is to establish a collaborating consortium of investigators from existing studies in genetics of preeclampsia to reduce the multiple existing problems such as: (1) inadequate selection of candidate gene variants to be evaluated, (2) biased analyses, and selective reporting of positive results; (3) to promote access to unpublished data; (4) to overcome inadequate outcome definitions; and (5) to provide guidance for developing new large studies [54]. Until such measures are established, it will be important for both authors and journal editors to embrace the publication of both positive and negative results from “well-designed case-control” studies to diminish the problem of publication bias [64]. This approach has recently become a reality for clinical trials [65], and it might help in reducing the temptation of researchers to explore multiple hypotheses in subgroup analyses to obtain one finding of nominal statistical significance that might help acceptance of the paper.

Investigating the aetiology of preeclampsia, one of the main causes of maternal and neonatal mortality and morbidity worldwide, should be a health research priority. A genetic approach may indeed be useful, but large collaborative studies will also be needed.

Supporting Information

Figure S1. Funnel Plot of Studies of ACE-I/D Polymorphism and Preeclampsia
ORs for outcome using a per-allele model. Studies in bold are those published in non-English-language journals.
Found at doi:10.1371/journal.pmed.0030520.sg001 (38 KB PPT).

Figure S2. Cumulative Synthesis of Studies of ACE-I/D Polymorphism and Preeclampsia
OR (random effect model) for outcome using a per-allele model.
Found at doi:10.1371/journal.pmed.0030520.sg002 (36 KB PPT).

Protocol S1. ACE-I/D Polymorphism and Preeclampsia Risk: Evidence of Small-Study Bias
Found at doi:10.1371/journal.pmed.0030520.sd001 (68 KB DOC).

Alternative Language Abstract S1. Translation of the Abstract into Spanish
Translation by N. C. Serrano, Universidad Autónoma de Bucaramanga, Bucaramanga, Colombia.
Found at doi:10.1371/journal.pmed.0030520.sd002 (25 KB DOC).

Alternative Language Abstract S2. Translation of the Abstract into Chinese
Translation by D. Wang, Medical Statistics Unit, London School of Hygiene and Tropical Medicine, London, United Kingdom.
Found at doi:10.1371/journal.pmed.0030520.sd003 (33 KB DOC).
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Editors’ Summary

Background. Preeclampsia is a common condition affecting pregnant women worldwide; it is defined as the presence of increased blood pressure, together with protein in the urine. Although in many women preeclampsia may never result in symptoms, other women may experience headaches, problems with their vision, swollen ankles and feet, and other symptoms. Sometimes, preeclampsia progresses to eclampsia, in which potentially life-threatening seizures result. The causes of preeclampsia are not well understood, but several factors are known to contribute to the risk. These factors include diabetes, high blood pressure prior to pregnancy, obesity, and first pregnancy. There is also the possibility that preeclampsia has, at least in part, a genetic basis; the condition is more likely among women whose relatives have also had preeclampsia. There is evidence that women worldwide; it is defined as the presence of increased blood pressure and proteinuria. Preeclampsia may never result in symptoms, other women may experience headaches, problems with their vision, swollen ankles and feet, and other symptoms. Sometimes, preeclampsia progresses to eclampsia, in which potentially life-threatening seizures result. The causes of preeclampsia are not well understood, but several factors are known to contribute to the risk. These factors include diabetes, high blood pressure prior to pregnancy, obesity, and first pregnancy. There is also the possibility that preeclampsia has, at least in part, a genetic basis; the condition is more likely among women whose relatives have also had preeclampsia. There is also the possibility that preeclampsia has, at least in part, a genetic basis; the condition is more likely among women whose relatives have also had preeclampsia. The findings presented here suggest that “small study bias” may explain the discrepancy between the results of the case-control study and the combined analysis. That is, studies involving few participants are less reliable and more likely to produce false-positive results. Therefore, it is possible that the proposed link between ACE gene variants and preeclampsia is a spurious one. The investigators propose that in future, collaborative research networks will be needed to carry out rigorous research on the genetics of preeclampsia. Such initiatives will help to overcome the problem of bias that can arise from small studies.

What Do These Findings Mean? The findings presented here suggest that “small study bias” may explain the discrepancy between the results of the case-control study and the combined analysis. That is, studies involving few participants are less reliable and more likely to produce false-positive results. Therefore, it is possible that the proposed link between ACE gene variants and preeclampsia is a spurious one. The investigators propose that in future, collaborative research networks will be needed to carry out rigorous research on the genetics of preeclampsia. Such initiatives will help to overcome the problem of bias that can arise from small studies. Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0030250.

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