OBSTETRICS

Validation of metabolomic models for prediction of early-onset preeclampsia

Ray O. Bahado-Singh, MD, MBA; Argyro Syngelaki; Ranjit Akolekar, MD; Rupsari Mandal, PhD; Trent C. Bjondahl, PhD; Beomsoo Han, PhD; Edison Dong, BSc; Samuel Bauer, MD; Zeynep Alpay-Savasan, MD; Stewart Graham, PhD; Onur Turkoglu, MD; David S. Wishart, PhD; Kypros H. Nicolaides, MD

OBJECTIVE: We sought to perform validation studies of previously published and newly derived first-trimester metabolomic algorithms for prediction of early preeclampsia (PE).

STUDY DESIGN: Nuclear magnetic resonance–based metabolomic analysis was performed on first-trimester serum in 50 women who subsequently developed early PE and in 108 first-trimester controls. Random stratification and allocation was used to divide cases into a discovery group (30 early PE and 65 controls) for generation of the biomarker model(s) and a validation group (20 early PE and 43 controls) to ensure an unbiased assessment of the predictive algorithms. Cross-validation testing on the different algorithms was performed to confirm their robustness before use. Metabolites, demographic features, clinical characteristics, and uterine Doppler pulsatility index data were evaluated. Area under the receiver operator characteristic curve (AUC), 95% confidence interval (CI), sensitivity, and specificity of the biomarker models were derived.

RESULTS: Validation testing found that the metabolite-only model had an AUC of 0.835 (95% CI, 0.769–0.941) with a 75% sensitivity and 74.4% specificity and for the metabolites plus uterine Doppler pulsatility index model it was 0.916 (95% CI, 0.836–0.996), 90%, and 88.4%, respectively. Predictive metabolites included arginine and 2-hydroxybutyrate, which are known to be involved in vascular dilation and insulin resistance and impaired glucose regulation, respectively.

CONCLUSION: We found confirmatory evidence that first-trimester metabolomic biomarkers can predict future development of early PE.

Key words: ■■■

Cite this article as: Bahado-Singh RO, Syngelaki A, Akolekar R, et al. Validation of metabolomic models for prediction of early-onset preeclampsia. Am J Obstet Gynecol 2015;213:x.ex-x.ex.

A large prospective study[1] recently reported a frequency of 0.46% for early-onset preeclampsia (PE) compared to 1.6% for late-onset PE. Despite its lower frequency, early PE is of paramount importance to medical practitioners because of the strong association with adverse perinatal outcomes. A population-based study from Washington State[2] found a significantly increased adjusted odds ratios for perinatal complications including small-for-gestational-age status, fetal and neonatal death, and combined perinatal death and morbidity in early- compared to late-onset PE. A high frequency of histologic lesions consistent with placental underperfusion has been described in early PE cases[3] and points to a pathological basis for the increased rates of adverse outcomes observed in this subgroup.

Recent metaanalyses found that early aspirin prophylaxis, ie, <16 weeks' gestation, reduced the risk of subsequent PE by slightly >50% while reducing preterm delivery for PE by close to 90%.[4-6] However, after 16 weeks, aspirin prophylaxis had significantly reduced effectiveness. Developing biomarkers for the diagnosis or prediction of PE is now a priority.[7,8] Further, several national and international organizations have recommended that PE risk assessment, based largely on historical factors, be performed at initiation of prenatal care and that aspirin prophylaxis be used in appropriate high-risk cases.[9-11] Metabolomics is being extensively used as a platform for biomarker discovery in complex diseases.[12-15] Our group recently reported the feasibility of accurate first-trimester nuclear magnetic resonance (NMR)-based metabolomic prediction for both early and late PE.[16,17] It is important that the performance of the identified biomarkers be validated to reduce the risk of overfitting and overly optimistic estimates of diagnostic accuracy.[18] In this manuscript we report the results of a validation study to determine the diagnostic accuracy of the

From the Department of Obstetrics and Gynecology, William Beaumont Health, Royal Oak, MI (Drs Bahado-Singh, Bauer, Alpay-Savasan, Graham, and Turkoglu); Department of Obstetrics and Gynecology, King's College Hospital, London, United Kingdom (Ms Syngelaki and Drs Akolekar and Nicolaides); and Department of Biological Sciences, Faculty of Medicine (Drs Mandal, Bjondahl, Han, and Wishart and Mr Dong), and Department of Computing Sciences (Dr Wishart), University of Alberta, Edmonton, Alberta, Canada.

Received Jan. 14, 2015; revised May 13, 2015; accepted June 16, 2015.

Partially supported by a grant from Harris Birthright Charity.

The authors report no conflict of interest.

Corresponding author: Ray O. Bahado-Singh, MD, MBA. Ray.bahado-singh@beaumont.edu

 • http://dx.doi.org/10.1016/j.ajog.2015.06.044

metabolomic biomarkers for the first-trimester prediction of early PE.

Materials and Methods

Study population

The details of patient recruitment, and specimen collection and handling have been previously published.[16] That report consisted of 30 early PE cases and 60 healthy controls. An additional 20 early PE cases and 48 normal controls were added for the current report, resulting in a total of 50 early PE cases and 108 controls. This is part of an ongoing prospective study conducted by the Fetal Medicine Foundation, London, United Kingdom, for the first-trimester prediction of pregnancy complications including PE. The study was approved by the King's College Hospital research ethics committee. Institutional review board project no. 02-03-033 approval was obtained initially on March 14, 2003. An average-risk population of British women were prospectively screened from March 2003 through September 2009 for the prediction of pregnancy complications.[17] All patients gave written consent to participate. Pregnant women with singleton pregnancies were recruited at 11^{+0}-13^{+6} weeks' gestation. Maternal demographics and medical history were documented. First-trimester ultrasound assessment including crown-rump length and uterine artery Doppler pulsatility index (UtPI) were performed. Uterine artery Doppler screening was performed using a previously published and extensively utilized protocol.[19] To summarize, a sagittal plane of the uterus was imaged, and cervical canal and internal os were visualized. Transducer position was adjusted by tilting from side to side and using color flow Doppler. The uterine artery was identified running along the side of the uterus and cervix. The uterine artery on each side was identified and Doppler interrogation performed at the level of the internal os. UtPI was measured. To perform pulsed Doppler, a 2-mm sampling gate was placed over the point of interest and covered the uterine vessel. The angle of Doppler insonation was <30 degrees. Doppler pulsatility index (PI) was measured when 3 consecutive similar waveforms were obtained. Measurements were performed on the left and right uterine arteries. In the previously published study, the lower mean and higher UtPI were compared and the lower PI was found to have the highest screening performance. All Doppler measurements were performed by sonographers who achieved the Certificate of Competence (http://www.fetalmedicine.com). This technique of uterine Doppler measurements has been validated in a large number of patients in multiple studies. Maternal blood was obtained and immediately transferred to the laboratory within 5 minutes of collection. Specimens were left to stand for 10-15 minutes at room temperature to allow the blood to clot. The specimens were centrifuged at 3000 rpm for 10 minutes to separate serum from clots. The serum was aliquoted in 0.5 mL quantities in screw tubes. Samples were temporarily stored in a −20°C freezer and then transferred to a −80°C freezer within 24 hours.

The early PE cases were selected at random from our database of available stored samples. Controls were from pregnancies that delivered a phenotypically normal neonate with appropriate birthweight for gestational age at term and did not develop any hypertensive disorder of pregnancy. Each control had blood collected within 3 days of assessment of the late PE case. PE was defined as proposed by the International Society for the Study of Hypertension in Pregnancy[20] with systolic blood pressure ≥140 mm Hg or diastolic ≥90 mm Hg on ≥2 occasions 4 hours apart >20 weeks of gestation, in previously normotensive women. Proteinuria was defined as a total of 300 mg in a 24-hour urine collection or 2 readings of at least 2^{+} proteinuria on a midstream or catheterized urine specimen in the absence of a 24-hour urine collection must also have been present in addition to the hypertension. Cases diagnosed with HELLP syndrome or gestational hypertension were excluded. As previously defined in our study,[16] early PE were cases had a diagnosis that required delivery at <34 weeks.

Metabolomic analysis

The details of the NMR-based metabolomic analyses and statistical methods have been extensively described by our group[16] and are summarized below.

NMR-based metabolomic analysis

Prior to NMR analysis, serum samples were filtered through 3-kDa cut-off centrifuge filter units (Amicon Micoron YM-3; Sigma-Aldrich, St. Louis, MO) to remove blood proteins. Aliquots of each serum sample were transferred into the centrifuge filter devices and spun (10,000 rpm for 20 minutes) to remove macromolecules (primarily protein and lipoproteins) from the sample. The filtrates were checked visually for any evidence that the membrane was compromised and for these samples the filtration process was repeated with a different filter and the filtrate inspected again. The subsequent filtrates were collected and the volumes were recorded. If the total volume of the sample was <300 μL an appropriate amount from a 50-mmol/L NaH_2PO_4 buffer (pH 7) was added until the total volume of the sample was 300 μL. Any sample that had to have buffer added to bring the solution volume to 300 μL was annotated with the dilution factor and metabolite concentrations were corrected in the subsequent analysis. After this, 35 μL of D_2O and 15 μL of buffer solution containing 50 mmol/L of NaH_2PO_4 at pH 7; 11.667 mmol/L of disodium-2, 2-dimethyl-2-silceptentane-5-sulphonate; and 0.01% NaN_3 in H_2O was added to the sample.

In all, 350 μL of serum was then transferred to a microcell NMR tube (Shigemi Inc, Allison Park, PA). ^{1}H-NMR spectra were collected on a 500-MHz Inova spectrometer (Varian Inc, Palo Alto, CA) equipped with a 5-mm HCN Z-gradient PFG room-temperature probe. The singlet produced by the disodium-2, 2-dimethyl-2-silceptentane-5-sulphonate methyl groups was used as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All ^{1}H-NMR spectra were processed and analyzed using a software package (Chenomx NMR Suite Professional, Version 7.6; Chenomx Inc, Edmonton, Alberta, Canada). Each serum NMR spectrum was manually fitted to an internal spectral database of pure compounds collected under identical

FLA 5.2.0 DTD ■ YMOB10491_proof ■ 18 July 2015 ■ 8:32 pm ■ ce

TABLE 1
Demographic and clinical characteristics of early preeclampsia and control groups (combined group)

Parameter	Early PE	Control	*P* value
No. of cases	50	108	—
Maternal age, y, mean (SD)	31.0 (7.1)	31.7 (5.9)	.467
Racial origin, n (%)			.013
White	14 (28.0)	60 (55.6)	
Black	28 (56.0)	35 (32.4)	
Asian	7 (14.0)	12 (11.1)	
Mixed	1 (2.0)	1 (0.9)	
Nullipara, n (%)	23 (46.0)	45 (41.7)	.609
Weight, kg, mean (SD)	73.6 (17.3)	68.4 (14.6)	.052
Crown-rump length, mm, mean (SD)	62.3 (7.5)	64.3 (8.1)	.143
UtPI, MoM, mean (SD)	1.80 (0.69)	1.23 (0.46)	< .001

MoM, multiples of median for gestational age; *PE*, preeclampsia; *UtPI*, uterine artery Doppler pulsatility index.

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

conditions, which allowed an average of 50 compounds in each serum sample to be identified and quantified. Each spectrum was evaluated by at least 2 NMR spectroscopists to minimize errors.

Statistical analysis

Demographic and clinical data of the early PE and control groups were compared using a Student *t* test, χ^2 test, or a Fisher exact test, as appropriate.

For the comparisons of each metabolite, mean values of matched early PE and control sample populations were tested using a Student *t* test for metabolites exhibiting normal distributions or a Mann-Whitney *U* test for metabolites exhibiting nonnormal distribution. A Bonferroni corrected *P* value was calculated for multiple comparisons.

Multivariate statistical analysis was performed using log scaling to achieve the normalization of all NMR-derived metabolite concentration data. Multivariate statistical analysis was performed using principal component analysis,[21] partial least squares discriminant analysis (PLS-DA), permutation testing and variable importance in projection plot,[21,22] and stepwise logistic regression. These statistical techniques are important for analyzing metabolomic data.

Metabolites with a *P* value < .3 (using univariate analysis) were selected for generating the logistic regression model. A k-fold cross-validation technique was used to ensure that the logistic regression models were robust.[18]

Two approaches were used in attempting to validate the metabolomics prediction models in an independent patient group. The performance of the previously published model[16] was evaluated in the new patient group consisting of 20 early PE cases and 48 normal controls. To perform additional validation of metabolomics algorithms, the entire data set (previously published plus new patients) was randomly split into a discovery (training) set (60%)

TABLE 2
Demographic and other characteristics early preeclampsia: discovery vs validation group

	Discovery group			Validation group		
Parameter	Early PE	Control	*P* value	Early PE	Control	*P* value
No. of cases	30	65	—	20	43	—
Maternal age, y, mean (SD)	30.6 (7.0)	31.5 (5.8)	.535	31.4 (7.4)	32.1 (6.0)	.699
Racial origin, n (%)			.42			.002
White	10 (33.3)	32 (49.2)		4 (20.0)	28 (65.1)	
Black	25 (38.5)	15 (50.0)		13 (65.0)	10 (23.3)	
Asian	4 (13.3)	7 (10.8)		3 (15.0)	5 (11.6)	
Mixed	1 (3.3)	1 (1.5)		—	—	
Nullipara, n (%)	13 (43.3)	27 (41.5)	.869	10 (50.0)	18 (41.9)	.545
Weight, kg, mean (SD)	74.2 (15.8)	69.3 (15.5)	.183	73.0 (19.7)	67.0 (13.2)	.228
Crown-rump length, mm, mean (SD)	62.4 (6.8)	64.7 (8.5)	.205	60.1 (8.6)	63.7 (7.5)	.458
UtPI, MoM, mean (SD)	1.82 (0.67)	1.25 (0.46)	< .001	1.77 (0.62)	1.20 (0.48)	.003

Discovery and validation data sets were randomly assigned to control for confounding variables.

MoM, multiples of median for gestational age; *PE*, preeclampsia; *UtPI*, uterine artery Doppler pulsatility index.

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

TABLE 3
Univariate analysis of metabolite concentrations in combined group (concentration: μmol/L)

	Combined group				
Metabolite	Early PE	Control	*P* value	Early PE/control	Fold change
No. of cases	50	108	—	—	—
2-hydroxybutyrate	23.21 (9.50)	21.39 (12.29)	.313	Up	1.08
3-hydroxybutyrate	29.77 (16.49)	39.72 (59.92)	.112	Down	−1.33
3-hydroxyisovalerate	6.46 (3.69)	5.02 (3.75)	.025	Up	1.29
Acetate	40.71 (34.29)	50.93 (39.30)	.013[a]	Down	−1.25
Acetoacetate	9.89 (7.05)	11.78 (10.80)	.191	Down	−1.19
Acetone	15.66 (5.03)	21.07 (22.19)	.018	Down	−1.35
Alanine	316.04 (91.87)	340.90 (144.19)	.193	Down	−1.08
Arginine	110.82 (32.10)	108.91 (33.73)	.738	Up	1.02
Betaine	26.18 (7.56)	24.12 (7.64)	.039[a]	Up	1.09
Carnitine	28.14 (6.45)	28.98 (12.04)	.57	Down	−1.03
Choline	24.91 (98.62)	84.87 (218.07)	< .001[a]	Down	−3.41
Citrate	86.64 (18.48)	81.25 (17.33)	.077	Up	1.07
Creatine	36.68 (14.37)	36.62 (13.75)	.979	Up	1.0
Creatinine	54.82 (11.54)	55.34 (12.55)	.804	Down	−1.01
Ethanol	30.34 (23.85)	36.71 (31.13)	.16	Down	−1.21
Formate	12.58 (4.84)	15.72 (12.12)	.022	Down	−1.25
Glucose	4397.9 (1231.4)	4014.9 (743.5)	.046	Up	1.1
Glutamine	315.37 (66.84)	315.20 (77.74)	.989	Up	1.0
Glycerol	168.72 (124.08)	322.81 (314.50)	.001[a]	Down	−1.91
Glycine	194.49 (60.87)	219.21 (88.41)	.043	Down	−1.13
Isobutyrate	6.83 (2.80)	6.20 (2.00)	.159	Up	1.1
Isoleucine	46.53 (18.66)	48.84 (18.18)	.464	Down	−1.05
Isopropanol	7.47 (6.85)	26.61 (75.71)	.011[a]	Down	−3.56
Lactate	1259.2 (509.8)	1302.6 (714.6)	.664	Down	−1.03
Leucine	82.18 (32.78)	92.99 (58.92)	.142	Down	−1.13
Malonate	14.05 (6.74)	16.02 (8.52)	.152	Down	−1.14
Methionine	20.52 (5.55)	21.60 (6.90)	.331	Down	−1.05
Ornithine	35.47 (12.70)	35.42 (14.22)	.983	Up	1.0
Phenylalanine	63.14 (13.91)	65.77 (36.03)	.028[a]	Down	−1.04
Proline	136.25 (48.15)	131.83 (53.32)	.619	Up	1.03
Propylene glycol	9.50 (4.16)	8.46 (4.27)	.039[a]	Up	1.12
Pyruvate	70.34 (35.08)	60.39 (27.43)	.059[a]	Up	1.16

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015. *(continued)*

and a validation (test) set (40%). Random stratification and allocation of patients and controls such that the proportion of cases and controls in each group was similar in terms of demographics and other potentially confounding variables. The discovery or training group was used to develop the predictive algorithm and model optimization was achieved using the cross-validation technique. The final result is a robust, optimal, and maximally parsimonious biomarker model. The predictive ability of the model was then tested independently in the validation group, which consisted of cases and control that had not been used in model generation.

FLA 5.2.0 DTD ■ YMOB10491_proof ■ 18 July 2015 ■ 8:32 pm ■ ce

TABLE 3
Univariate analysis of metabolite concentrations in combined group (concentration: μmol/L) *(continued)*

Metabolite	Combined group		*P* value	Early PE/control	Fold change
	Early PE	Control			
Serine	122.62 (33.12)	138.16 (67.37)	.054	Down	−1.13
Succinate	5.16 (3.56)	9.02 (11.12)	.001	Down	−1.75
Threonine	124.98 (29.43)	131.35 (50.76)	.322	Down	−1.05
Tyrosine	52.50 (15.54)	51.10 (19.75)	.659	Up	1.03
Valine	141.86 (45.49)	143.89 (47.10)	.799	Down	−1.01
Methylhistidine	42.60 (15.98)	40.49 (16.38)	.448	Up	1.05

P values were calculated based on *t* test.

PE, preeclampsia.

[a] Calculated based on Mann-Whitney *U* test with nonnormal distributions. Adjusted significance level with Bonferroni correction for .05 is .0013.

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

For the selection of predictor variables in our logistic regression models, Least Absolute Shrinkage and Selection Operator[24] and stepwise variable selection were utilized for optimizing all the model components[25] via 10-fold cross-validation.

To determine the performance of each logistical regression model, area under the receiver operating characteristics (ROC) curve (AUROC or AUC) was calculated[18] as well as sensitivity and specificity values.

The MetaboAnalyst[23] was used for principal component analysis, PLS-DA and permutation analyses. All other statistical analyses were performed using the MetaboAnalyst World Wide Web server.[23] The custom programs written using the R statistical software package (http://www.r-project.org) and STATA 12.0 (http://www.stata.com) were used to perform all other statistical analyses. A more detailed description of the statistical techniques is provided in the supplementary section.

Results

Table 1 compares demographic and clinical characteristics of the combined patient group. Race/ethnicity, weight, and uterine artery Doppler values were significantly different between controls and early PE cases. Table 2 separately compares the demographic and clinical characteristics between the cases and controls in both the discovery (training) and validation subsets. There were no significant differences between early PE cases and controls in either the discovery or in the validation groups apart from the maternal race/ethnicity and UtPI values. As expected the UtPI was generally elevated in the early PE cases compared to controls. Table 3 shows the univariate comparison of metabolite concentrations in early PE cases vs controls in the combined patient groups. Metabolite concentrations are expressed in μM/L. The direction of change and fold change in metabolite concentrations are also provided in this table. Bonferroni correction (adjusted significance level of .013) was utilized. The PLS-DA analysis resulted in a good separation between the early PE and controls (Figure 1) for the combined data sets. Permutation testing demonstrated that the observed separation was statistically significant and not due to chance ($P < .001$).

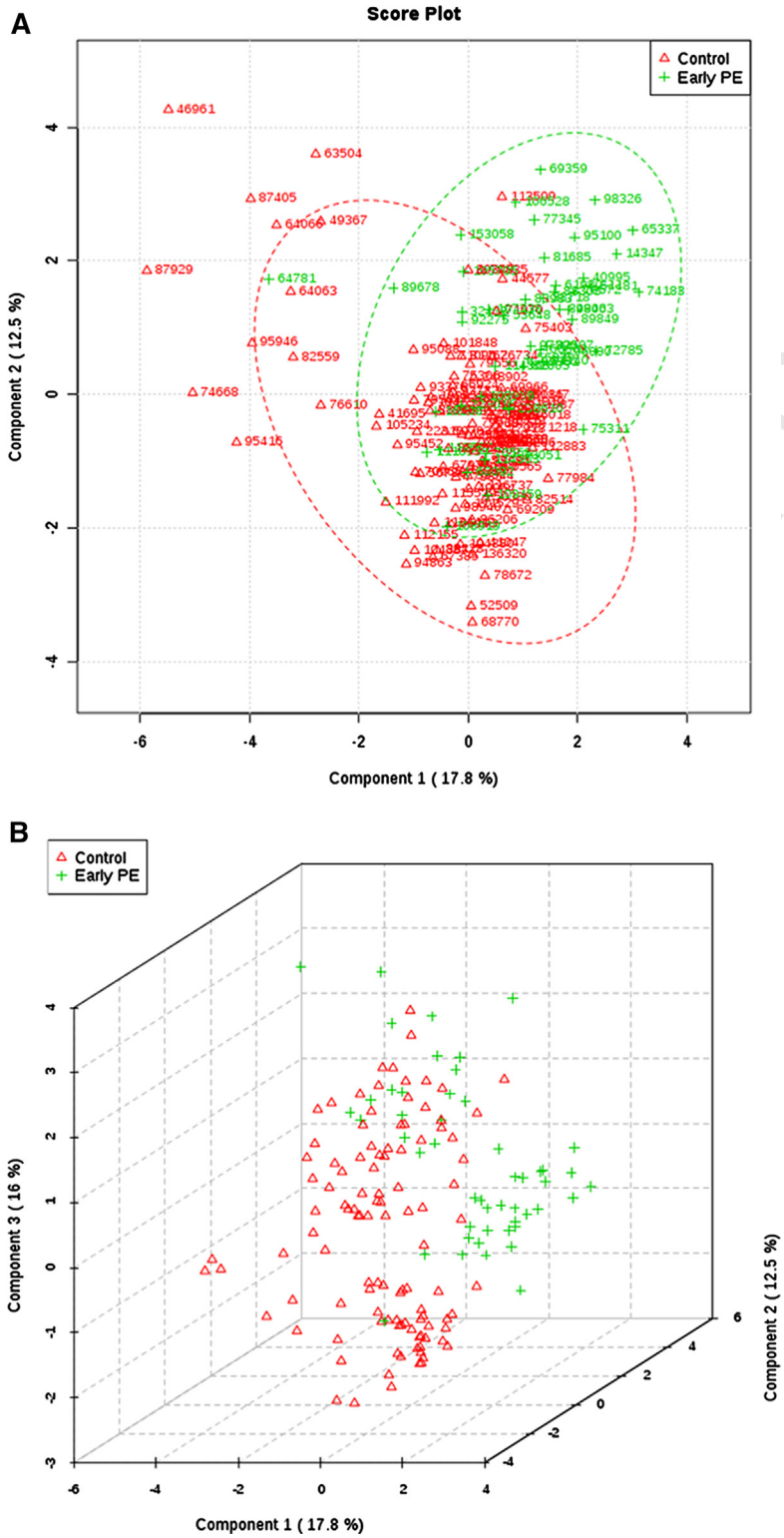
The previously published metabolite plus Doppler prediction model,[16] log (odds) = −0.008 − 0.075 acetate − 0.013 glycerol + 0.496 (3-hydroxyisovalerate) + 0.252 succinate + 0.155 crown-rump length + 8.148 UtPI multiples of median for gestational age, when tested in the new patient group (20 early PE cases and 48 normal controls) had an AUC of 0.79 (95% confidence interval, 0.65−0.93), sensitivity of 85%, and specificity of 65%. The previously published metabolite only model was not significant.

Using the discovery set only from the combined patient group, a series of logistic regression analyses were performed to develop biomarker models (ie, equations) for early PE prediction. Three models were developed: one consisted of UtPI only, the second used metabolites only, and the third evaluated of a combination of metabolites with clinical/demographic and Doppler data. Table 4 shows the respective logistic regression models that resulted. The performances for the discovery models in the training group and the results of 5-fold cross-validation are presented in Table 5. In the metabolite-only model the significant predictors were 2-hydroxybutyrate, 3-hydroxyisovalerate, acetone, citrate, and glycerol. The initial discovery model and the model after 5-fold cross-validation procedures for the training cohort only were compared and were found to be similar. The area under the ROC curves (AUC), sensitivity, and specificity for the 3 different models in the discovery group are shown in Table 5. The associated ROC plots in the discovery group are shown in Figure 2. The biomarker models from the discovery group were then tested on the independent validation group and their performance is shown in Table 6. The performance in the discovery (training) and validation groups were

FLA 5.2.0 DTD ■ YMOB10491_proof ■ 18 July 2015 ■ 8:32 pm ■ ce

FIGURE 1

Display of separation between early PE and controls in partial least squares discriminant analysis



A, 2- and **B**, 3-dimensional score plots.

PE, preeclampsia.

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

similar in confirming reproducibility of the algorithms. High diagnostic accuracy was achieved with the combination of metabolites plus uterine artery Doppler. These were also compared with the performance achieved by our previously published metabolite-only models.[16] The ROC plots for the models in the validation group are shown in Figure 3. [F3] The area under the curve is better for the current model compared to the previously published models.

Each of these new models was a statistically significant predictor of early PE in the validation group. The metabolite-only model had good predictive accuracy, however the combination of the UtPI and the metabolites achieved the highest predictive accuracy.

Comment

Using recommended statistical techniques[18] we have provided confirmatory data that serum metabolite biomarkers either by themselves or combined with UtPI data predict early PE in the first trimester. The metabolite-only model consisting of glycerol, 3-hydroxyisovalerate, 2-hydroxybutyrate, acetone, and citrate achieved a 75% sensitivity and 74.4% specificity in the validation group. A combined logistic regression model with glycerol, 3-hydroxyisovalerate, arginine, and UtPI data were more parsimonious while achieving a 90% sensitivity at 88.4% specificity for early PE detection. Both arginine and 2-hydroxybutyrate represent new metabolite additions to the predictive model compared to our previously published pilot data.[16] These 2 biomarkers are biologically plausible, given their known biochemistry and functions. Nitric oxide, a potent vasodilator, is a metabolic derivative of arginine. Indeed, existing evidence suggests that L-arginine supplementation reduces the rate of PE in pregnant women[26] presumably by lowering vascular tone. Two-hydroxybutyric acid or alpha-hydroxybutyrate is an organic acid derived from alpha-ketobutyrate. It is an early marker for both insulin resistance and impaired glucose regulation and its production is fueled by increased

FLA 5.2.0 DTD ■ YMOB10491_proof ■ 18 July 2015 ■ 8:32 pm ■ ce

TABLE 4

Three logistic regression models with odds ratios of each metabolite/biomarker; first is uterine artery pulsatility index only, second is metabolite only, and third is combined metabolite and uterine pulsatility index

Model[a]	Metabolite[b]	Coefficient, β	Std, β	z Value	Pr(>|z|)	Odds ratio (95% CI)	Early PE/control
UtPI only	Constant offset β_0	−3.424	0.734	−4.663	< .001	0.03 (0.01−0.13)	—
	UtPI	1.758	0.447	3.934	< .001	5.80 (2.54−14.92)	—
Metabolites only	Constant offset β_0	−5.693	8.012	−0.711	.477	0.0 (0.00−31179.47)	—
	2-hydroxybutyrate	1.692	0.654	2.589	.010	5.43 (1.62−21.87)	Up
	3-hydroxyisovalerate	1.160	0.345	3.359	.001	3.19 (1.69−6.65)	Up
	Acetone	−2.511	0.750	−3.346	.001	0.08 (0.02−0.31)	Down
	Citrate	3.592	1.292	2.780	.005	36.29 (3.50−597.11)	Up
	Glycerol	−2.371	0.594	−3.995	< .001	0.09 (0.02−0.26)	Down
Metabolites + Doppler	Constant offset β_0	−15.648	6.601	−2.370	.018	0.0 (0.00−0.03)	—
	UtPI	4.315	1.046	4.124	< .001	74.84 (12.74−846.8)	Up
	3-hydroxyisovalerate	2.566	0.652	3.937	< .001	13.01 (4.34−59.50)	Up
	Arginine	3.802	1.293	2.940	.003	44.8 (4.38−768.53)	Up
	Glycerol	−3.002	0.767	−3.916	< .001	0.05 (0.01−0.18)	Down

CI, confidence interval; *PE*, preeclampsia; *UtPI*, uterine artery Doppler pulsatility index.

[a] Formal equation of logistic regression model is written as $\log(\pi) = \beta_0 + \beta_0 X_1 + \beta_2 X_2 + \ldots + \beta_k X_k$, where π is probability of proportion of early PE case in group, and X_i is metabolite concentrations as k covariates—for example of metabolites + Doppler model, $\log(\pi) = -15.648 + 4.315$ (UtPI) + 2.566 (3-hydroxyisovalerate) + 3.802 arginine − 3.002 glycerol; [b] Metabolite concentration was generalized log-transformed for using in logistic regression model.

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

lipid oxidation and oxidative stress (http://www.hmdb.ca/).[27] Both lipid oxidation and oxidative stress have been strongly associated with PE.[28] Our results illustrate one of the important attributes of metabolomics, namely the capacity to generate credible hypotheses as to the mechanism and causation of complex disorders.

Only a few studies using metabolomics for PE prediction or detection have been published. They have, however reported significant differences in the blood or urine metabolome of pregnant women who have or are destined to develop PE.[16,17,29-32] The different biomarkers reported are partly a consequence of the different metabolomic platforms used (ie, NMR vs mass spectrometry), which tend to identify different kinds of compounds. Variation in results can also be ascribed to different specimen types, gestational age at testing, category, and indeed, definitions of PE used.

Metabolites in biological samples are in an active state of flux. Hence, the handling and storage of specimens in a standardized and relatively expeditious fashion is crucial to reducing variability in the results and to achieve optimal diagnostic accuracy. Failure to use accepted and reproducible standards for specimen collection, processing, and storage will yield only marginal results.

A potential limitation of our validation study is the modest sample size. For a proof of concept metabolomics studies 15-30 cases of equal number of controls have been considered acceptable. With

TABLE 5

Performance of regression models in discovery group and after 5-fold cross-validation maneuver

	Discovery group[b]			5-fold cross-validation		
Model[a]	AUC (95% CI)	Sensitivity	Specificity	AUC (95% CI)	Sensitivity	Specificity
UtPI	0.746 (0.692−0.801)	0.658	0.658	0.704 (0.584−0.824)	0.631	0.631
Metabolites[c]	0.896 (0.862−0.929)	0.825	0.823	0.855 (0.768−0.942)	0.800	0.785
Metabolites[d] and UtPI	0.956 (0.938−0.975)	0.908	0.908	0.917 (0.858−0.977)	0.833	0.831

AUC, area under curve; *CI*, confidence interval; *UtPI*, uterine artery Doppler pulsatility index.

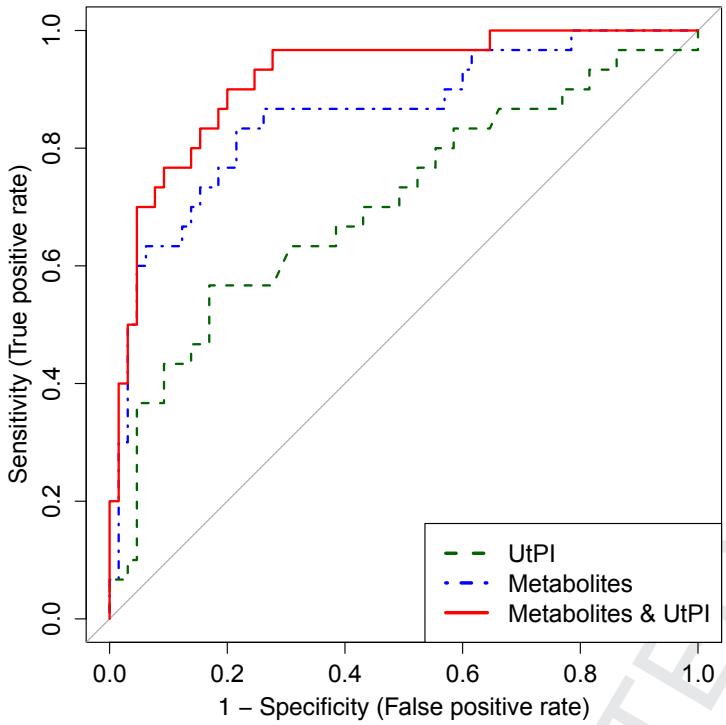
[a] See Figures 2 and 3, and Table 4 for markers and values used in respective prediction models; [b] 30 early preeclampsia cases and 65 controls; [c] 2-hydroxybutyrate, 3-hydroxyisovalerate, acetone, citrate, glycerol; [d] 3-hydroxyisovalerate, arginine, glycerol.

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

FLA 5.2.0 DTD ■ YMOB10491_proof ■ 18 July 2015 ■ 8:32 pm ■ ce

FIGURE 2

Receiver-operator characteristic curve for prediction of logistic regression models in 5-fold cross-validation of discovery group



Model	5 fold Cross-Validation of Discovery Data		
	AUC (95% CI)	Sensitivity	Specificity
Uterine artery PI	0.704 (0.584-0.824)	0.631	0.631
Metabolites[a]	0.855 (0.768-0.942)	0.800	0.785
Metabolites[b] and Uterine artery PI	0.917 (0.858-0.977)	0.833	0.831

a: 2-Hydroxybutyrate, 3-Hydroxyisovalerate, Acetone, Citrate, Glycerol

b: 3-Hydroxyisovalerate, Arginine, Glycerol

AUC, area under curve; *CI*, confidence interval; *PI*, pulsatility index; *UtPI*, uterine artery Doppler PI.

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

increasing numbers of publications such as ours that provide preliminary evidence of the value of metabolomics, studies using larger numbers of patients are likely to be presented in the future. Despite this, we confirmed that the models developed here were statistically robust and had reproducible effectiveness in an independent validation group. In particular, the combination of 3 serum metabolites and UtPI data had high predictive accuracy for early PE. Another potential limitation of our study is the inclusion of previous published[16] in one aspect of our analysis. The limitation of using the population from which the model was derived to determine sensitivity and specificity values is that this will overestimate the diagnostic performance of the model. We overcame these limitations by using 2 approaches. First, we used the conventional approach, which is to test the performance of the published model in a completely new patient group. We demonstrated that the combined metabolomics model that was previously published cases[16] significantly predicted early PE in a new patient group. The limitation of that classic approach is the small sample size, which limits study power and the chances of finding statistical significance even in biologically significant metabolites. We therefore used a second approach that randomly assigns cases and controls from the previously published and the new patient groups to a discovery group from which new algorithms are developed and a separate and independent validation group in which these algorithms are tested. This approach minimizes or eliminates biases resulting from different dates of laboratory or clinical testing (eg, different laboratory approaches, different equipment or reagents, or different personnel performing the clinical or laboratory tests). The extensive cross-validation of the model in both the discovery and validation groups yielded optimal models. The advantage of the second statistical-based approach is that it yielded stronger models with greater diagnostic accuracy, greater reproducibility, and stronger study power while minimizing the risk of bias inherent to using previously published data for assessing model performance.

Although not the intent of this study, our findings cannot claim to validate or prove the generalizability of these or other metabolomic markers to different clinical settings including different patient populations, or geographic or national areas. Large studies in these settings are a prerequisite going forward. In this study we attempted to validate previously and recently developed algorithms in a discrete clinical setting where patients are generally managed by the same physicians with the same clinical protocols. Q12

We have also noted that in the stage of marker development, metabolite

FLA 5.2.0 DTD ■ YMOB10491_proof ■ 18 July 2015 ■ 8:32 pm ■ ce

TABLE 6

Performance of logistic regression models in validation group (previously published and new models)

Model		Validation group[a] AUC (95% CI)	Sensitivity	Specificity
Publishedmodels[16]	Metabolites[b] only	0.698 (0.553–0.843)	0.65	0.651
	Metabolites,[c] UtPI, and fetal crown-rump length	0.776 (0.652–0.899)	0.70	0.721
New models	UtPI alone	0.755 (0.629–0.881)	0.65	0.674
	Metabolites only[d]	0.835 (0.769–0.941)	0.75	0.744
	Metabolites[e] and UtPI	0.916 (0.836–0.996)	0.90	0.884

AUC, area under curve; *CI*, confidence interval; *UtPI*, uterine artery Doppler pulsatility index.

[a] 20 Early preeclampsia cases and 43 controls; [b] Citrate, glycerol, 3-hydroxyisovalerate, methionine; [c] Acetate, glycerol, 3-hydroxyisovalerate; [d] 2-hydroxybutyrate, 3-hydroxyisovalerate, acetone, citrate, glycerol; [e] 3-hydroxyisovalerate, arginine, glycerol.

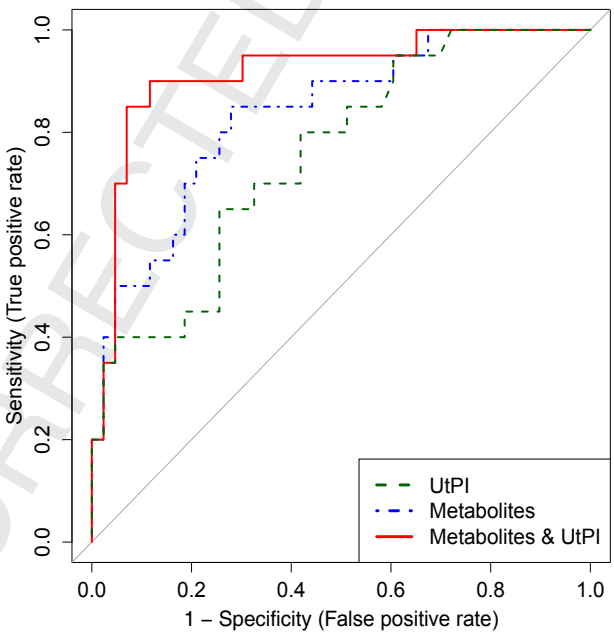
Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

samples need to be handled in a precise and reproducible manner. This is due to the large variety of metabolite classes with different physical characteristics such as half-life, reactivity, and volatility that one needs to evaluate in the discovery phase of biomarker investigation. For more practical applications such as clinical screening, one would focus on the more stable metabolites that would not significantly change concentrations in the clinical laboratory setting. In addition chemical means for quenching and stabilizing metabolites against ongoing chemical reactions or degradation outside of the body would offer another solution. A common example is the routine use of anticoagulants in blood specimens enabling testing that is not feasible after clotting. Either of these approaches could enhance the clinical practicality of a metabolomics test.

Both the American Congress of Obstetrics and Gynecologists[11] and NICE[9] recommend the use of various historical, demographic, or clinical characteristics for identifying high-risk patients that could benefit from aspirin prophylaxis. The recent report by the US Preventive Services Task Force[33] found evidence of benefit of aspirin prophylaxis for the prevention of PE. The task force emphasized the importance of accurately identifying women most likely to benefit from aspirin

FIGURE 3

Performance of algorithms in validation group: receiver-operator characteristic curves and sensitivity and specificity values



Model	Validation Data		
	AUC (95% CI)	Sensitivity	Specificity
Uterine artery PI (UtPI)	0.755 (0.629-0.881)	0.650	0.674
Metabolites[a]	0.835 (0.769-0.941)	0.750	0.744
Metabolites[b] and Uterine artery PI	0.916 (0.836-0.996)	0.900	0.884

a: 2-Hydroxybutyrate, 3-Hydroxyisovalerate, Acetone, Citrate, Glycerol

b: 3-Hydroxyisovalerate, Arginine, Glycerol

UC, area under curve; *CI*, confidence interval; *PI*, pulsatility index; *UtPI*, uterine artery Doppler PI.

Bahado-Singh. Metabolomic prediction of preeclampsia. Am J Obstet Gynecol 2015.

prophylaxis, ie, high-risk women. The "difficulty of identifying appropriate high-risk women for prophylaxis" was noted along with the fact that "suitable markers with good test performance characteristics remain elusive." In their report they emphasized that there was limited evidence of harm particularly in women at high risk for PE. Higher likelihood of harm however was noted when aspirin was given to women at low or average risk. This emphasizes the need to minimize exposure in women at low or moderate risk, the vast majority of women, and importantly the need to develop robust biomarkers with good test performance characteristics. Metabolomic markers particularly when combined with clinical and ultrasound characteristics appear to offer the possibilities of more accurate screening markers for PE and could thereby facilitate targeted deployment of prophylactic aspirin.

In conclusion, we have provided confirmatory evidence that first-trimester metabolomic biomarkers can predict the development of early PE with good to high accuracy. Metabolomic analysis can in the future contribute significantly to our understanding of the mechanism of PE. On the practical side, first-trimester early PE prediction using metabolomics may in the future have clinical value by identifying at-risk individuals to be targeted for early aspirin prophylaxis. ■

REFERENCES

1. Poon LCY, Karagiannis G, Leal A, Romero XC, Nicolaides KH. Hypertensive disorders in pregnancy: screening by uterine artery Doppler imaging and blood pressure at 11-13 weeks. Ultrasound Obstet Gynecol 2009;34:497-502.

2. Lisonkova S, Joseph KS. Incidence of preeclampsia: risk factors and outcomes. Am J Obstet Gynecol 2013;209:544.e1-12.

3. Ogge G, Chaiworapongsa T, Romero R, et al. Placental lesions associated with maternal underperfusion are more frequent in early-onset than in late-onset preeclampsia. J Perinat Med 2011;39:641-52.

4. Bujold E, Roberge S, Lacasse, et al. Prevention of preeclampsia and intrauterine growth restriction with aspirin started in early pregnancy: a meta-analysis. Obstet Gynecol 2010;116:402-14.

5. Roberge S, Villa P, Nicolaides K, et al. Early administration of low-dose aspirin for the prevention of preterm and term preeclampsia; a systematic review and meta-analysis. Fetal Diagn Ther 2012;31:141-6.

6. Roberge S, Giguere Y, Villa P, et al. Early administration of low-dose aspirin for the prevention of severe and mild preeclampsia: a systematic review and meta-analysis. Am J Perinatol 2012;29:551-6.

7. Akolekar R, Syngelaki A, Sarquis R, Zvanca M, Nicolaides KH. Prediction of early, intermediate and late pre-eclampsia from maternal factors, biophysical and biochemical markers at 11-13 weeks. Prenat Diagn 2011;31:66-74.

8. Akolekar R, Syngelaki A, Beta J, Kocylowski R, Nicolaides KH. Maternal serum placental protein 13 11-13 weeks of gestation in preeclampsia. Prenat Diagn 2009;29:103-8.

9. National Collaborative Center for Womens's and Children's Health (UK). Hypertension in pregnancy: the management of hypertensive disorders during pregnancy. London: RCOG Press; 2010.

10. World Health Organization, Department of Reproductive Health and Research, Department of Maternal, Newborn, Child and Adolescent Health, Department of Nutrition for Health and Development, WHO Recommendations for Prevention and treatment of pre-eclampsia and eclampsia, World Health Organization, 2011.

11. American College of Obstetrics and Gynecologists, Task Force on Hypertension in Pregnancy. Hypertension in pregnancy: report of the American College of Obstetrics and Gynecologists, Task Force on Hypertension in Pregnancy. Obstet Gynecol 2013;122:1122-31.

12. Stretch C, Eastman T, Mandal R, et al. Prediction of skeletal muscle and fat mass in patients with advanced cancer using a metabolomic approach. J Nutr 2012;142:14-21.

13. Walsh BH, Broadhurst DI, Mandal R, et al. The metabolomic profile of umbilical cord blood in neonatal hypoxic ischemic encephalopathy. PLoS One 2012;7:e50520.

14. Shah SH, Kraus WE, Newgard CB. Metabolomic profiling for the identification of novel biomarkers and mechanisms related to common cardiovascular diseases. Circulation 2012;126:1110-20.

15. Bogdanov M, Matson WR, Wang L, et al. Metabolomic profiling to develop blood biomarkers for Parkinson's disease. Brain 2008;131:389-96.

16. Bahado-Singh RO, Akolekar R, Mandal R, et al. Metabolomics and first-trimester prediction of early-onset preeclampsia. J Matern Fetal Neonatal Med 2012;25:1840-7.

17. Bahado-Singh RO, Akolekar R, Mandal R, et al. First-trimester metabolomic detection of late-onset preeclampsia. Am J Obstet Gynecol 2013;208:58.e1-7.

18. Xia J, Broadhurst DI, Wilson M, Wishart DS. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics 2013;9:280-99.

19. Poon LCY, Staboulidou I, Maiz N, Plassencia W, Nicolaides KH. Hypertensive disorders in pregnancy: screening by uterine artery Doppler at 11-13 weeks. Ultrasound Obstet Gynecol 2009;34:142-8.

20. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). Hypertens Pregnancy 2001;20:IX-XIV.

21. Wishart DS. Computational approaches to metabolomics. Methods Mol Biol 2010;593:283-313.

22. Xia J, Mandal R, Sineinkov IV, Broadhurst D, Wishart D. MetaboAnalyst 2.0: a comprehensive server for metabolomics data analysis. Nucleic Acids Res 2012;40:W127-33.

23. Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res 2009;37:W652-60.

24. Tibshirani R. Regression shrinkage and selection via Lasso. J R Stat Soc 1996;58:267-88.

25. Hastie T, Tibshirani R, Friedman J. The elements of statistical learning: data mining, inference, and prediction. 2nd ed, Springer Series in Statistics. Springer (NY): Springer-Verlag; 2009.

26. Dorniak-Wall T, Grivell RM, Dekker GA, Hague W, Dodd JM. The role of L-arginine in the prevention and treatment of pre-eclampsia: a systematic review of randomized trials. J Hum Hypertens 2014;28:230-23. Q13

27. Wishart DS, Jewison T, Guo AC, et al. HMDB 3.0—the human metabolome database in 2013. Nucleic Acids Res 2013;41:D801-7.

28. Gupta S, Aziz N, Sekhon L, et al. Lipid peroxidation and antioxidant status in preeclampsia: a systematic review. Obstet Gynecol Surv 2009;64:750-9.

29. Kenny LC, Bradhurst DI, Dunn W, et al. Robust early pregnancy production of later preeclampsia using metabolomic biomarkers. Hypertension 2010;56:741-9.

30. Odibo AO, Goetzinger KR, Odibo L, et al. First-trimester prediction of preeclampsia using metabolomic biomarkers: a discovery phase study. Prenat Diagn 2011;31:990-4.

31. Austdal M, Skrastad RB, Gundersen AS, Austgulen R, Iversen AC, Bathan TF. Metabolomic biomarkers in serum and urine in women with preeclampsia. PLoS One 2014;9:e99123.

32. Kuc S, Koster MPH, Pennings JLA, et al. Metabolomics profile for identification of novel potential markers in early prediction of preeclampsia. PLoS One 2014;9:e98540.

33. Henderson JT, Whitlock EP, O'Connor E, Senger CA, Thompson JH, Rowland MG. Low-dose aspirin for the prevention of mortality from preeclampsia: a systematic evidence review for the US Preventive Services Task Force. US Preventive Services Task Force Evidence Synthesis. 2014; #14-05207-EF-1.