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Changes in pregnancy-related serum biomarkers early in gestation are associated with later development of preeclampsia

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1 **ABSTRACT**

2 **Background:** Placental protein expression plays a crucial biological role during normal and
3 complicated pregnancies. We hypothesized that: (1) circulating pregnancy-associated, placenta-
4 related protein levels throughout gestation reflect the uncomplicated, full-term temporal
5 progression of human gestation, and effectively estimates gestational ages (GAs); (2)
6 pregnancies with underlying placental pathology, such as preeclampsia (PE), are associated with
7 disruptions in this GA estimation in early gestation; (3) malfunctions of this GA estimation can
8 be employed to identify impending PE. In addition, to explore the underlying biology and PE
9 etiology, we set to compare protein gestational patterns of human and mouse, using pregnant
10 heme oxygenase-1 (HO-1) heterozygote (Het) mice, a mouse model reflecting PE-like
11 symptoms.

12 **Methods:** Serum levels of circulating placenta-related proteins – leptin (LEP), chorionic
13 somatomammotropin hormone like 1 (CSHL1), elabela (ELA), activin A, soluble fms-like
14 tyrosine kinase 1 (sFlt-1), and placental growth factor (PlGF)– were quantified by ELISA in
15 blood serially collected throughout human pregnancies (20 normal subjects with 66 samples, and
16 20 PE subjects with 61 samples). Linear multivariate analysis of the targeted serological protein
17 levels was performed to estimate the normal GA. Logarithmic transformed mean-squared errors
18 of GA estimations were used to identify impending PE. Then the human gestational protein
19 patterns were compared to those in the pregnant HO-1 mice.

20 **Results:** An elastic net (EN)-based gestational dating model was developed ($R^2 = 0.76$) and
21 validated ($R^2 = 0.61$) using the serum levels of the 6 proteins at various GAs from women with
22 normal uncomplicated pregnancies (n = 10 for training and n = 6 for validation). In pregnancies
23 complicated by PE (n = 14), the EN model was not ($R^2 = -0.17$) associated with GA at sampling

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24 in PE. Statistically significant deviations from the normal GA EN model estimations were
25 observed in PE-associated pregnancies between GAs of 16–30 weeks (*P* = 0.01). The EN model
26 developed with 5 proteins (ELA excluded due to the lack of robustness of the mouse ELA essay)
27 performed similarly on normal human ($R^2 = 0.68$) and WT mouse ($R^2 = 0.85$) pregnancies.
28 Disruptions of this model were observed in both human PE-associated (human: $R^2 = 0.27$) and
29 mouse HO-1 Het (mouse: $R^2 = 0.30$) pregnancies. LEP out performed sFlt-1 and PlGF in
30 differentiating impending PE at early human and late mouse gestations.
31 **Conclusions:** As revealed in both human and mouse GA EN analyses, temporal serological
32 placenta-related protein patterns are tightly regulated throughout normal human pregnancies and
33 can be significantly disrupted in pathologic PE states. LEP changes earlier during gestation than
34 the well-established late GA PE biomarkers (sFlt-1 and PlGF). Our HO-1 Het mouse analysis
35 provides direct evidence of the causative action of HO-1 deficiency in LEP upregulation in a PE-
36 like murine model. Therefore, longitudinal analyses of pregnancy-related protein patterns in sera,
37 may not only help in the exploration of underlying PE pathophysiology but also provide better
38 clinical utility in PE assessment.
39

40 **Keywords:** sFlt-1, chorionic somatomammotropin hormone like 1, placental growth factor,
41 leptin, activin A, elabela
42

43 **BACKGROUND**

44 Placental protein expression plays a crucial biological role during normal pregnancies.
45 The normal progression of a human pregnancy is associated with a precisely-timed transient
46 expression of maternal and placental proteins [1, 2]. Similarly, the placenta, an endocrine gland
47 unique to pregnancy, secretes hormones that fluctuate with respect to the gestational week of

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48 pregnancy. However, these hormones have not been useful in the development of molecular
49 metrics to estimate GA or to phenotype complicated pregnancies prior to overt clinical
50 manifestations of specific pathologic states like preeclampsia (PE) [3, 4], a pregnancy-related
51 vascular disorder affecting 5–8% of all pregnancies [5, 6].

52 PE is thought to be a multisystem disorder of pregnancy driven by alterations in placental
53 function and resolved by the delivery of the placenta and fetus [7]. A few pregnancy-associated,
54 placenta-related markers have been observed having different patterns in normal pregnancies and
55 pregnancies with PE. Chorionic somatomammotropin hormone like 1 (CSHL1; also called
56 human placental lactogen) is selectively expressed in placental villi with an important role in
57 regulating placental growth. Leptin (LEP) has been suggested to be involved in placental and
58 fetal growth [8]. The relationship between LEP and PE has been discussed in a few studies [9-
59 17]. Circulating levels of activin A, a member of the tumor growth factor protein family, can
60 increase as early as 10–15 weeks of pregnancy in women who subsequently develop PE [18].
61 Elevated placental levels of angiogenic factors (soluble fms-like tyrosine kinase or sFlt-1) and
62 decreased levels of anti-angiogenic factors (placental growth factor, PlGF) have been implicated
63 in the pathogenesis of PE [19-25]. As such, the sFlt-1/PlGF ratio has been proposed as an index
64 to diagnose and manage PE [26, 27]. A significant increase in sFlt-1 levels was also observed in
65 sera of pregnant heme oxygenase (HO)-1 heterozygote (Het, HO-1$^{+/-}$) mice, where the deficiency
66 in HO-1 results in PE-like symptoms [28]. Recent work by Ho et al showed that PE was
67 associated in mice with a deficiency in elabela (ELA), a placental hormone that enhances human
68 trophoblast invasiveness in vitro [29].

69 In this study, we chose 6 proteins as candidates of GA estimation because they are
70 associated with the placenta and reflect placental growth. Furthermore, levels of all 6 proteins

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71 differ in PE compared to a normal pregnancy. Previous studies found that LEP, CSHL1, and
72 activin A are elevated early in gestation in women who subsequently develop PE [30-32]. ELA
73 deficiency is associated with PE-like symptoms in mice [29]. The ratio of sFlt-1 and PlGF has
74 been used clinically for diagnosing PE [27].

75 We hypothesized that serum levels of placenta-related proteins, LEP, CSHL1, ELA,
76 activin A, sFlt-1, and PlGF are longitudinally regulated, and a profile of their circulating levels
77 may collectively reflect a pregnancy-associated protein panel describing the normal progression
78 of a term pregnancy. We further hypothesized that disruptions of this panel in early gestation are
79 associated with placental abnormalities and an increased risk of developing PE. We sought to
80 model the longitudinal changes in protein serum levels to estimate GA. In addition, we explored
81 whether temporal disruptions in these profiles early in gestation are harbingers of placental
82 pathology and subsequent PE. The model was first developed in human sera and then tested in
83 both human and mouse sera.

84 METHODS

85 Study design

86 The study was conducted in three phases: (1) using ELISA methods to characterize the
87 normal pattern of serum placenta-related protein levels; (2) modeling a protein-based GA
88 estimation of normal pregnancies and identifying deviations; and (3) exploration of the protein-
89 based GA estimation with a mouse PE model. Sera were collected in the first, second, or third
90 trimesters during pregnancy from women who had normal uncomplicated pregnancies or a
91 diagnosis of PE. Blood was collected at 1 to 3 time-points before week 30 of gestation and prior
92 to a confirmatory diagnosis of PE. The GA of human was determined by ultrasound
93 measurement. In the mouse, sera were collected from pregnant HO-1 Het or wild-type (WT)

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94 dams at 1 to 3 time-points between 7.5 to 18.5 days of gestation. The HO-1 Het mice have
95 elevated diastolic blood pressures and plasma sFlt-1 levels during pregnancies, mimicking the
96 PE syndrome [28]. For the human study, approval was obtained from the Stanford University
97 Institutional Review Board. Blood was collected at Stanford University Medical Center after
98 informed consent was obtained.

99 **Animal model study**

100 For the mouse study, approval was obtained from the Institutional Animal Care and Use
101 Committee at Stanford University. Mouse line maintenance, genotyping, and bleeding were as
102 previously described [28].

103 **ELISAs**

104 Sera from human subjects or mice were collected and measured using commercial kits
105 specific for the human or mouse as follows: LEP (R&D System Inc., MN, USA); CSHL1
106 (Mybiosource, San Diego, CA, USA); ELA (Peninsula Laboratories International, Inc., San
107 Carlos, CA, USA); activin A (R&D System Inc.); sFlt-1 (R&D System Inc.,); and PlGF (R&D
108 System Inc.).

109 **Statistical analyses**

110 Patient demographic data were analyzed using the "Epidemiological Calculator" (R
111 epicalc package). Hypothesis testing was performed using Mann-Whitney U-tests (two-tailed).
112 Samples collected $\geq$ 30 weeks of gestation or having any of the placenta-related protein
113 measurements out of limits on the standard curves were excluded from the cohort for modeling.
114 A 10-fold cross-validated elastic net (EN) algorithm [2, 33, 34] was used for multivariate
115 modeling of the ELISA data. The model searches for an optimum β to minimize the least squared
116 loss function with elastic net penalty:

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117 $$L(\lambda,\beta) = |y - X\beta|^2 + \lambda[\frac{(1-\alpha)|\beta|^2}{2} + \alpha|\beta|] \quad (1)$$

118 where $X=(x_1,\ldots x_6)$ is a matrix of 6 analytes, with $x_j=(x_{1j},\ldots x_{nj})^T$, where $j=1,\ldots 6$. $y = (y_1,\ldots y_n)^T$ is
119 the response (i.e., current GA). n is the number of samples in the training cohort. $|y - X\beta|^2$ is
120 the squared loss function. $\lambda[\frac{(1-\alpha)|\beta|^2}{2} + \alpha|\beta|]$ is the well-known EN penalty used for controlling
121 the model complexity. The parameters of each penalty were controlled by α and λ. α was set to 1
122 and λ was set to 0.208, which maximizes the predictive value of model measured by R^2 in the
123 cross-validation (Additional file 1). The model is thus reduced to a lasso-regularized regression.

124 The mean squared error (MSE) of the GA model was used to separate PE patients from
125 women with normal pregnancies. MSE in each woman was calculated by comparing the
126 observed GA with the model-predicted GA. Specifically, assuming a woman had estimated GA
127 of $\hat{y}_{k1},\ldots,\hat{y}_{km}$ for samples collected at the observed GA of $y_{k1},\ldots y_{km}$, the MSE of the model for
128 this woman is:
129
130 $$MSE = \frac{1}{m}\sum_{k=k1}^{km}(\hat{y}_k - y_k)^2 \quad (2)$$
131
132 where m is the number of samples. To account for the randomness of errors, only women having
133 2 or more samples collected during pregnancy (i.e., $m \geq 2$) were included for the calculations.
134 Receiver operating characteristic (ROC) curves and Mann–Whitney U-tests were used to test the
135 performance of MSE in classifying women.

136 The EN model in (Eq. 1) was then adjusted using 5 analytes as inputs (ELA was excluded
137 see below). The model performance was assessed by R^2. The role of each analyte in
138 differentiating complicated from normal pregnancies was explored by analyzing the distribution
139 of the concentrations at different GAs. Comparisons were made between the human and mouse

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140 to identify the common behaviors in proteins that were associated with the outcome of PE. Loess
141 regression, Mann–Whitney U-tests, and fold changes were used for the analyses.

142 **RESULTS**

143 **Samples**

144 Forty pregnant women (20 term pregnancies, 20 with PE) receiving routine antepartum
145 care at Stanford University Medical Center were enrolled between November 2012 and May
146 2016. Patient demographics are listed in Table 1. Maternal blood was collected at one, two, or
147 three time-points during each pregnancy (at early, mid, and late-pregnancies, Fig. 1A). 10
148 women (4 normal pregnancies, 6 with PE) were excluded from the EN-based modeling because
149 samples either were not collected before 30 weeks of gestation or had at least 1 protein candidate
150 that was out of limits on its standard curve. The latter was done because outliers on the standard
151 curve might cause distortion of continuous regression analysis. There were 30 women (16
152 normal pregnancies, 14 with PE) left after exclusion. Our training cohort included 10 patients
153 who delivered at term (≥ 37 weeks GA). An independent cohort of 6 women who delivered at
154 term and 14 women diagnosed with PE were subsequently enrolled for the validation study on
155 normal pregnancy and the test on PE.

156 **Table 1. Subject demographics**

Characteristic	**Overall Normal (n = 20)**	**PE (n = 20)**
Race, No. (%)		
White	20 (100)	9 (45)
Asian	0 (0)	5 (25)
African-American	0 (0)	1 (5)
Other	0 (0)	5 (25)
Age, mean (SD), years	31.9 (4.8)	31.8 (6.0)

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GA at delivery, mean (SD), weeks	39.5 (1.2)	36.7 (3.3)

157

158 The approach was also tested with serum samples collected longitudinally from pregnant
159 WT (n = 3 with 11 samples) and HO-1 Het (n = 4 with 15 samples) mice (Additional file 2).
160 Each mouse had 3 or 4 samples collected at E7.5, E10.5, E14.5, and E18.5.

161 **A placenta-related, protein-based GA estimation of human pregnancy**

162 We hypothesized that circulating placenta-related protein expression throughout
163 pregnancy reflects the normal temporal progression of human gestation, and effectively serves to
164 estimate GA. Using an EN algorithm, we developed a 6-protein model using a training cohort of
165 10 pregnant women that was strongly associated with GA at the time of sampling ($R^2 = 0.76$, $P =$
166 $2x10^{-7}$, Fig. 1B, left panel). The EN model was prospectively tested using sera serially collected
167 from an additional 6 normal, full-term pregnant women. The EN model was found to predict GA
168 at time of sampling in this independent normal cohort ($R^2 = 0.61$, $P = 2x10^{-4}$, Fig. 1B, middle
169 panel). Univariate analyses and EN model coefficients of each protein in the model are shown in
170 Additional file 3 and 4. Together, the analyses confirmed that there is a highly-regulated
171 temporal pattern of protein levels in sera over the course of pregnancy (Fig. 1B).

172 **The placenta-related, protein-based GA estimation malfunctions in PE**

173 Based on the above findings, we hypothesized that our EN model can identify abnormal
174 phenotypes, such as in PE that may have an attendant disrupted placenta-related protein pattern.
175 In contrast to the normal cohort (training $R^2 = 0.76$ and testing $R^2 = 0.61$, Fig. 1B), the EN model
176 did not predict GA at time of sampling and yielded random data predictions in the PE cohort
177 (Fig. 1B, right panel, $R^2 = -0.17$, $P = 0.2$). These findings suggest that the protein-based GA
178 estimation is disrupted in PE.

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179 The pathogenesis of PE is complex and progresses from an asymptomatic stage,
180 characterized by placental abnormalities during the first trimester to a symptomatic stage with
181 proteinuria and hypertension in late gestation [35]. Our analyses revealed unique longitudinal
182 patterns of serum protein levels of specific biomarkers (Fig. 2): LEP, CSHL1, and ELA
183 differentiated PE from the sera of women with uncomplicated, full-term pregnancies at
184 approximately 10 weeks of gestation, indicating that the pathogenesis of PE may arise very early
185 in gestation. Differences in activin A begin to appear around 20 weeks and in sFlt-1 and PlGF
186 after 25 weeks. Examination of the pattern of protein levels revealed significant protein-specific
187 gestational windows (0–9, 10–14, 15–25, 26–33, and 27–38 weeks GA, Additional file 5 and
188 Table 2) for each biomarker. These findings are in line with our longitudinal biomarker trending
189 analyses (Fig. 2). Since there is a positive association [8] between maternal serum LEP
190 concentrations and body mass index (BMI) (and consequently, gestational weight gain) during
191 pregnancy, our analyses of LEP levels were repeated using BMI in order to normalize for serum
192 LEP abundance. Similar findings were obtained (Additional file 6). Taken together, these data
193 indicate that alterations in the pattern of serum protein levels of LEP, CSHL1, and ELA begin
194 much earlier in GA than the changes in sFlt-1 (increase) and PlGF (decrease) at late GA.
195
196 **Table 2. Comparisons of the serum levels of each protein between normal and PE**
197 **pregnancies.** Mann-Whitney U-test *P*-value was calculated. *$0.005<P<0.05$. **$P<0.005$.

	0–9 weeks GA	**10–14 weeks GA**	**15–25 weeks GA**	**26–33 weeks GA**	**27–38 weeks GA**
LEP	0.02*	0.02*	$3x10^{-6}$**	0.3	0.5
CSHL1	0.4	0.01*	0.3	0.7	0.9
ELA	0.9	0.03*	0.9	0.8	0.4
Activin A	0.5	0.5	0.8	0.04*	0.2
sFlt-1	0.2	0.3	0.8	0.02*	$3x10^{-3}$**
PlGF	0.6	0.9	0.5	0.3	0.01*

198

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199 **Disruption of the protein-based GA estimation identifies impending PE**

200 Our placenta-related protein-based GA estimation characterizes the gestational progression
201 of normal term pregnancies. Significant random disruptions of this normal "term" pattern were
202 observed in women with PE. Logarithm-transformed MSE of our EN estimations were utilized to
203 define the binary classifications to identify risk for impending PE (Fig. 3 and Additional file 7).
204 For samples collected at 0–30 weeks GA, the MSE metric differentiated normal from PE (Mann-
205 Whitney U-test $P = 0.01$ on the training cohort, and $P = 0.06$ on the testing cohort) with an area
206 under the curve (AUC) of 0.88 on the training and 0.79 on the testing cohorts. An optimized
207 cutoff value calculated on the training data yielded a positive predictive value (PPV) of 0.79 with
208 a sensitivity of 1.00, and a negative predictive value (NPV) of 1.00 with a specificity of 0.50 on
209 the testing data. In contrast, in a window of 16–30 weeks of gestation, performance was
210 improved: Mann-Whitney U-test ($P = 8x10^{-3}$ on the training and $P = 0.01$ on the testing) and
211 AUC of 0.97 on the training and 1 on the testing data, PPV of 1.00 with a sensitivity of 0.88, and
212 NPV of 0.75 with a specificity of 1.00 on the testing data. These results are better than using a
213 single biomarker on the testing data in a window of 16–30 weeks of gestation (with AUCs of
214 0.53 for LEP; 0.76 for CHSL1; 0.58 for ELA; 0.53 for activin A; 0.65 for sFlt-1; and 0.65 for
215 PlGF). Thus, our results demonstrate that significant disruptions in the protein-based GA
216 estimation can be used to identify risk for impending PE.

217 **A placenta-related, protein-based GA estimation with reduced number of features**

218 Due to the lack of robustness of the mouse ELA ELISA essay, we tested the performance
219 our EN-based model excluding ELA. The model had an R^2 of 0.72 and 0.61 on the training and
220 testing cohorts, respectively (Additional file 8). Protein pattern disruptions were observed at > 30
221 weeks of gestation in women with PE ($R^2 = 0.27$, Additional file 8). Similar to the 6-protein

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222 model, the 5-protein model was still able to estimate GA during normal pregnancies.

223 **Comparative analysis of serological GA estimation between the human and a mouse model**

224 We hypothesized that similar temporal placenta-related protein expression patterns
225 should be conserved in mouse pregnancies, therefore, we explored our EN-based 5-protein
226 model to normal and pregnant HO-1 Het mice, a mouse model reflecting PE-like symptoms.
227 Model coefficients were adjusted to establish the link between GA (in days) and the targeted
228 serum protein levels. The model had an R^2 of 0.85 for pregnant WT and 0.30 for HO-1 Het mice
229 with PE-like symptoms (Fig. 4 and Additional file 9). Fold changes of protein levels of HO-1
230 Het over normal pregnancies were calculated and then compared between the human and mouse
231 in early (human: 5–26 weeks; mouse: 7.5–14.5 days) and late (human: 27–38 weeks; mouse:
232 18.5 days) gestations separately. The largest fold change was observed in LEP at late gestation of
233 mice (Additional file 9). Unlike mice, LEP levels were elevated in women with PE in early
234 gestation (Fig. 2, Additional file 5, and Table 2). Fold changes of sFlt-1 and PlGF in mice
235 increased from early to late gestation. The temporal patterns of sFlt-1 in mice were similar to
236 those in human, which decreased in early gestation and increased in late gestation of complicated
237 pregnancies (Additional file 9). In contrast, PlGF increased in mice at late gestation, but
238 decreased in human pregnancies after 27 weeks GA.

239 **DISCUSSION**

240 The placenta plays a key role in fetal development, where cell communication occurs to
241 support nutrition acquisition, immune adaption, and other functions of maternal-fetal interaction
242 [36, 37]. Placental proteins are expressed in a time-dependent manner and cross-talk with other
243 organs, such as the thyroid, pituitary, and ovary, and are necessary to ensure normal fetal
244 development. Characterization of the temporal patterns of circulating placental proteins may

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245 serve as a basis for understanding the biology underlying both normal and pathological
246 pregnancies. Our results support our hypothesis that multivariate modeling of the levels of
247 circulating placental-secreted proteins, LEP, CSHL1, ELA, activin A, sFlt-1, and PlGF can be
248 used to estimate GA during the course of a normal pregnancy, but not in women with PE. The
249 longitudinal placental-related protein pattern in sera was also observed in pregnant WT mice but
250 not in pregnant HO-1 Het mice.

251 Early diagnosis of PE remains a challenge in clinical settings. The traditional diagnosis of
252 PE is based on the presence of maternal hypertension and proteinuria [38]. Previous
253 transcriptomic [39-45] and proteomic [2, 46-50] profiling of normal and complicated
254 pregnancies have identified disease-specific expression patterns and signaling networks, which
255 suggest candidate biomarkers for possible early clinical diagnoses and for offering new
256 biological insights. Our findings suggest that a composite placental-related protein panel from
257 serial blood collection (for MSE calculations) may provide a diagnostic test to assess PE earlier
258 (~10 weeks of gestation) than previously suggested by sFlt-1 and PlGF (25 weeks of gestation).
259 Therefore, this model may offer a new investigational approach towards the understanding of
260 placental biology during pregnancy as well as guiding innovative methods for PE diagnosis.

261 Our findings of serum protein levels during a normal pregnancy are consistent with those
262 from previous studies. They are in line with the ranges reported in healthy pregnancies and have
263 similar patterns during the pregnancy as previous results [51-59]. We found that LEP increased
264 continuously during the first and second trimesters. Activin A remained stable between 10–20
265 weeks of gestation and increased late in the second trimester. sFlt-1 levels were also unchanged
266 before 30 weeks, while PlGF progressively rose over pregnancy. We further integrated the
267 quantitative trending information of each individual protein into a continuous regression model

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268 that expressed GA as a linear combination of the levels of proteins.

269 Current challenges in the management of PE include lack of early assessment and
270 incomplete understanding of its pathogenesis and pathophysiology at early GAs. sFlt-1 and PlGF
271 are well-established PE biomarkers [60] with clinical prognostic utilities in PE management. The
272 ratio of sFlt-1 and PlGF has been shown to effectively differentiate PE from normal term
273 pregnancies after 25 weeks of gestation [27]. Our findings that LEP, CSHL1, activin a, and ELA
274 have unique serum protein signatures, starting from early to mid-gestation, are novel and that
275 disruptions in the normal temporal placenta-related protein pattern appear at earlier GA than the
276 conventional PE biomarkers sFlt-1 or PlGF. The presence of high levels of LEP in early
277 gestation may signify the impending development of PE and thus serve as an early biomarker of
278 PE. Our analyses show that LEP can differentiate PE from normal term pregnancies at <25
279 weeks ($P = 3x10^{-6}$ at 15–25 weeks), earlier than sFlt-1, which is consistent with the previous
280 findings of other studies [61, 62]. Given that LEP is a master regulator of energy expenditure, the
281 observations suggest that placental insufficiency through energy imbalance is a precursor to PE
282 that is manifested as hypertension in mid-late gestation.

283 Our characterization of temporal patterns of protein levels in mice provided additional
284 support for our hypothesis. Applying our multivariate EN modeling on mouse sera we also found
285 an association of protein levels and GA during normal mouse pregnancies, and this relationship
286 was disrupted in pregnant HO-1 Het mice. Among the 5 proteins studied, LEP had the largest
287 fold change in PE-like (HO-1 Het) pregnancies. The main action of leptin is in the maternal
288 interface during the first stage of pregnancy regulating angiogenesis, growth and
289 immunomodulation on the placenta [63-69]. Although dysregulation of leptin levels has been
290 found correlated with the pathogenesis of various pregnancy disorders [70], including PE, the

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291 exact mechanism of action and upstream regulators remain unknown. Our characterization of the
292 pregnant HO-1 Het mouse PE model, for the first time, provides direct evidence of the causative
293 action of HO-1 deficiency in leptin upregulation in a PE-like murine model. This result, together
294 with the significant differentiating power of LEP at < 25 weeks GA in human pregnancies may
295 indicate a mechanistic role of LEP and HO-1 in the pathogenesis of PE, and deregulation of LEP
296 as an indicator of impending PE.

297 We note that placenta-related proteins have distinct temporal patterns and share common
298 characteristics between human and rodent pregnancies. sFlt-1 is upregulated in late gestation of
299 pregnant women with PE or pregnant HO-1 Het mice. Elevated sFlt-1 levels late in gestation in
300 mice are consistent with the findings of a previous study [28]. PlGF is upregulated after 14.5
301 days GA in mice while significantly down-regulated after 27 weeks GA in humans with
302 complicated pregnancies. LEP had a significant role in identifying PE or PE-like pregnancies of
303 both human and mice. The maximum differentiating power of LEP is achieved at late gestation
304 (18.5 days) in mice but in early gestation (< 25 weeks) in humans. The differences in placenta-
305 related protein patterns between humans and mice may be explained by the different placental
306 structures (e.g. a choriovitelline placenta is initially present in mice but absent in human;
307 trophoblast cell invasion is restricted in mice but deep in human) and different placental
308 endocrine functions [71-73]. Despite these differences, the similar physiological features shared
309 in human and mouse placentas, and the associations between proteins and GA in both human and
310 mice observed in our study, show that PE-related patterns found in human are preserved in mice.
311 It also demonstrates that studies on rodent models can be used to study the biology of human
312 pregnancy disorders.

313 This study has several limitations. First, the sample sizes for our human cohorts were

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314 small, and our population lacked racial heterogeneity. Second, the time intervals of blood
315 collections between two serial samples varied (3–31 weeks for normal, and 3–25 weeks for PE).
316 Most samples were collected in the first or second trimester. Only 12 normal and 9 PE patients
317 had samples collected in the third trimester. Third, serum concentrations of LEP can be
318 influenced by maternal status [74, 75]. We addressed this through the normalization to maternal
319 BMI (Additional file 6) and found the temporal pattern in LEP persisted. Fourth, variations in
320 circulating protein levels could be due to the contributions from other tissues besides the
321 placenta. Meta-analysis of PE and GA-matched uncomplicated pregnancy-associated placental
322 gene expression patterns, including the targeted analytes of this study, has revealed similar
323 expression trending along the gestations and differentiation between PE and normal controls
324 [76]. Fifth, ELA was not included in the rodent analyses due to the lack of the robustness of the
325 mouse ELISA assay. Sixth, although our protein candidates formed a panel of potential clinical
326 utility to assess impending PE, the model robustness can be greatly improved by refinements
327 using a sufficiently powered high time resolution cohort of sufficient powered sample size.

328 **CONCLUSIONS**

329 Longitudinal EN analysis of the circulating pregnancy-associated, placenta-related
330 protein expression throughout pregnancy revealed patterns of the normal temporal progression of
331 human gestation which can estimate GA. The elevated MSE of the EN metric, quantifying the
332 malfunction of the estimation, offers a potential approach to identify impending PE. The protein
333 markers in sera shared by human and mouse and their significant associations with GA are
334 conserved. In addition, PE-related patterns found in human are preserved in normal and HO-1
335 Het mice. This provides direct evidence of the causative action of HO-1 deficiency in LEP
336 upregulation in a PE-like murine model. All of these demonstrate that the exploration of the

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337 temporal expression patterns of the placenta-related proteins in rodent models can be used to
338 study the biology of human pregnancy disorders like PE.
339 With our initial placental protein-based model for PE, follow-up studies with larger, high
340 time resolution cohorts of frequent samplings at different GA need to be performed to not only
341 validate our current findings but also reveal additional novel serological placental proteomics
342 patterns diagnostics of other pregnancy-related complications. Future characterization of the
343 pregnant HO-1 Het mouse PE model may shed mechanistic insights to support HO-1 causative
344 and leptin associated pathways as important predictors of diverse pregnancy disorders and the
345 therapeutic target for PE intervention.
346

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LIST OF ABBREVIATIONS

Preeclampsia: PE

Chorionic somatomammotropin hormone like 1: CSHL1

Leptin: LEP

Gestational age: GA

Soluble fms-like tyrosine kinase: sFlt-1

Placental growth factor: PlGF

Heme oxygenase-1: HO-1

Elabela: ELA

Wild-type: WT

Elastic net: EN

Mean squared error: MSE

Area under the curve: AUC

Receiver operating characteristic: ROC

Positive predictive value: PPV

Negative predictive value: NPV

Body mass index: BMI

DECLARATIONS

Ethics, consent and permissions

For the human study, approval was obtained from the Stanford University Institutional Review Board. Blood was collected at Stanford University Medical Center after informed consent was obtained. For the mouse study, approval was obtained from the Institutional Animal Care and Use Committee at Stanford University.

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Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed in this study are available upon request to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XBL, KGS, and HJC contributed to the conception and design.

JY, HZ, LC, XL, RJW, and DKS contributed to the acquisition of data.

SH, YH, HZ, LZ, LT, IM, TL, YKB, VDW, NA, BG, MSA, XZ, YML, LM, GMS, RJW, DKS, and DBM contributed to the analysis and interpretation of data.

SH and XBL drafted the manuscript.

JY, HZ, LC, YH, LZ, LT, IM, XL, TL, RJW, YKB, VDM, NA, BG, MSA, XZ, YML, LM, GMS, DKS, HJC, DBM, and KGS critically revised the manuscript.

All the authors gave final approval of the version to be submitted and agreed to be accountable for all aspects of the work.

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FIGURE LEGENDS

Fig. 1. (A) Serial blood sampling from normal term and PE subjects at different GAs. Times of sample collections, infant deliveries, suspected PE, and confirmatory PE diagnoses of individual women (denoted by each row) are represented by black circles, black squares, red unfilled triangles, and red-filled triangles, respectively. (B) The EN model, developed with serial sampling analysis of 6 placenta-related proteins, dating GAs. Left panel: training cohort using

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sera from normal term pregnancies; middle and right panels: validation cohort using sera from normal term or PE pregnancies.

Fig. 2. Maternal serum concentrations of 6 studied placenta-related proteins plotted as a function of the GA. Normal term pregnancies: green line. PE pregnancies: red line. Loess smooth function was applied. Color-coded dotted lines show the 95% confidence interval for each cohort.

Fig. 3. Mean squared error (MSE) of the EN model used to classify testing normal from PE. Mann-Whitney U-test *P*-value was calculated. The cut-off point (grey dotted line) shows the maximum value of the sum square of the sensitivity and 1-specificity on classification of training normal and PE cohorts at blood sampling at 0–30 weeks and 16–30 weeks GAs.

Fig. 4. The 5-protein EN model dating GA. Left: normal human term and PE pregnancies. Right: WT and HO-1 Het mouse pregnancies (right). RMSE: root mean square error.

SUPPORTING INFORMATION

Additional file 1. Performance of EN model with respect to α and λ in our training cohort. Left: R^2- value of the model with respect to α when λ was set to give the minimum cross-validation mean squared error (MSE). Right: Cross-validation MSE with respect to λ when $\alpha = 1$.

Additional file 2. Serial blood collection from pregnant WT (left) and HO-1 Het (right) mice at different GAs. Sample collection days and individual mice are represented by filled circles and lines, respectively.

Additional file 3. Univariate analysis of serum protein concentrations with respect to GA. Linear regression coefficients as well as 95% confidence interval and Spearman *P*-values of each protein with respect to the current GA are shown.

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Additional file 4. Coefficients of each protein analyte in the EN model. Positive and negative values indicate positive and negative correlations, respectively, between GA and the serum protein concentrations.

Additional file 5. Maternal serum concentrations of the 6 placenta-related proteins plotted at different GA intervals during pregnancy. Mann-Whitney U-test *P*-values are shown.

Additional file 6. Maternal serum concentrations of LEP (left) and LEP normalized to body mass index (BMI) (pg/mL/kg/m^2) (right) shown as a function of GA in normal term (red line) and PE (green line) pregnancies. Loess smooth function was applied. Color-coded dotted lines: show the 90% confidence interval for each cohort.

Additional file 7. The mean square error (MSE) of the GA model metric was used to classify training normal from PE subjects. Mann-Whitney U-test *P*-values are shown. The cutoff levels (grey dotted lines) show the maximum value of the sum square of sensitivity and 1-specificity on classification of normal pregnancies and pregnancies with PE.

Additional file 8. The EN model ($\alpha = 0.79$), developed with serial sampling analysis of 5 placenta-related proteins, used for dating GAs. Left panel: shows our training cohort (normal term sera); middle and right panels show prospective testing of our EN model using normal or PE serum from serially-collected blood samples.

Additional file 9. Serum levels of the 5 placenta-related proteins were plotted as a function of the GA during human (left) and mouse (right) pregnancies. Loess smooth function was applied. Red: PE. Green: normal. Dotted line: 95% confidence interval.

Fig. 1

A

Normal Term Samples

● Sample Collection ■ Delivery

PE Samples

● Sample Collection ■ Delivery

△ PE suspected ▲ PE diagnosed

0 10 20 30 40

Gestational Age (weeks)

0 10 20 30 40

Gestational Age (weeks)

B

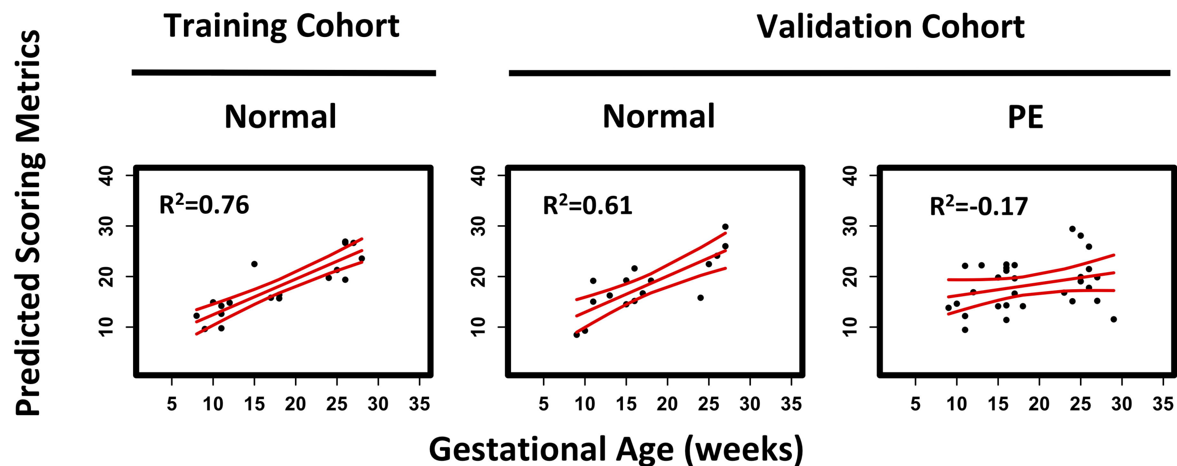

Fig. 2

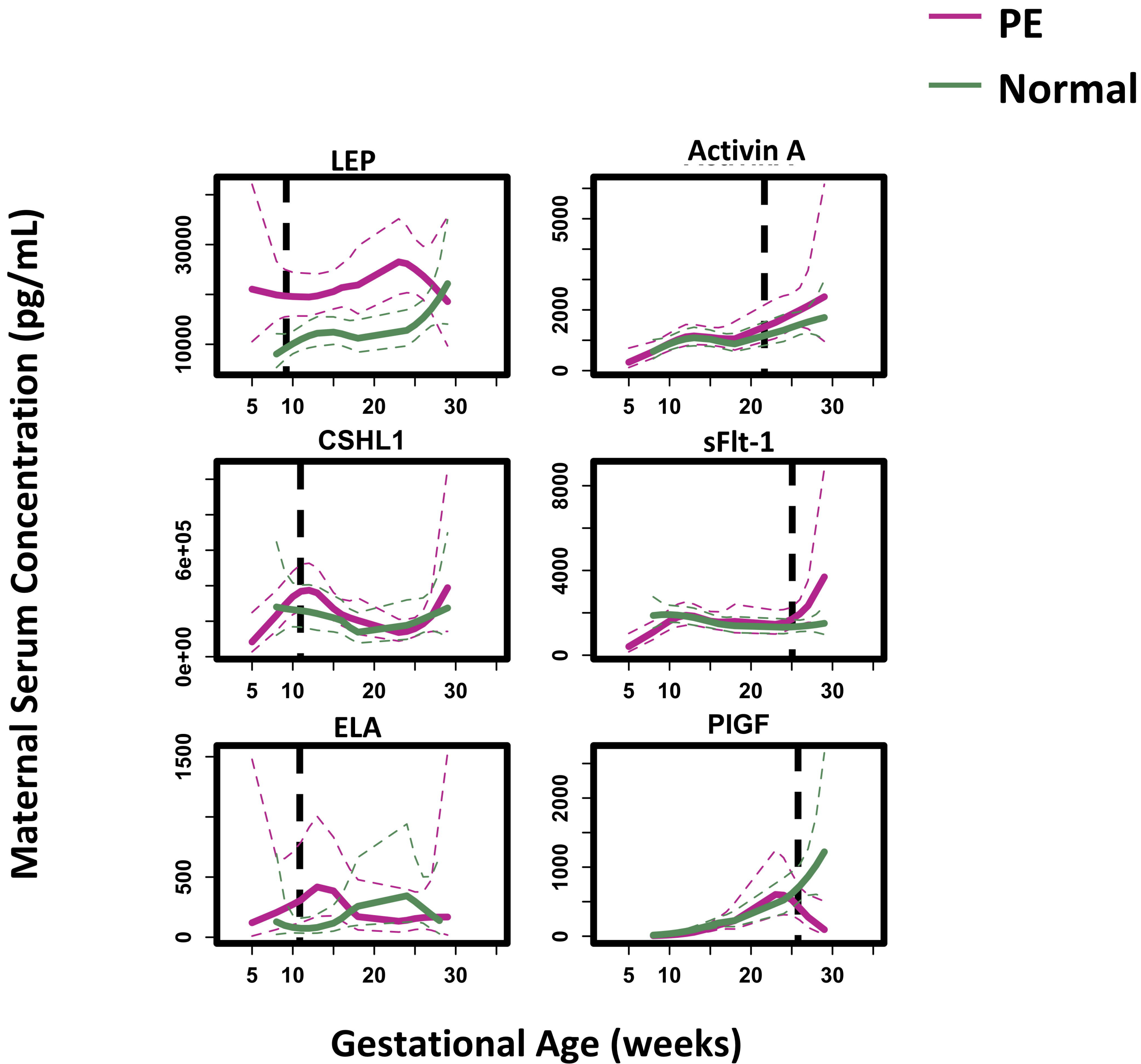

Fig. 3

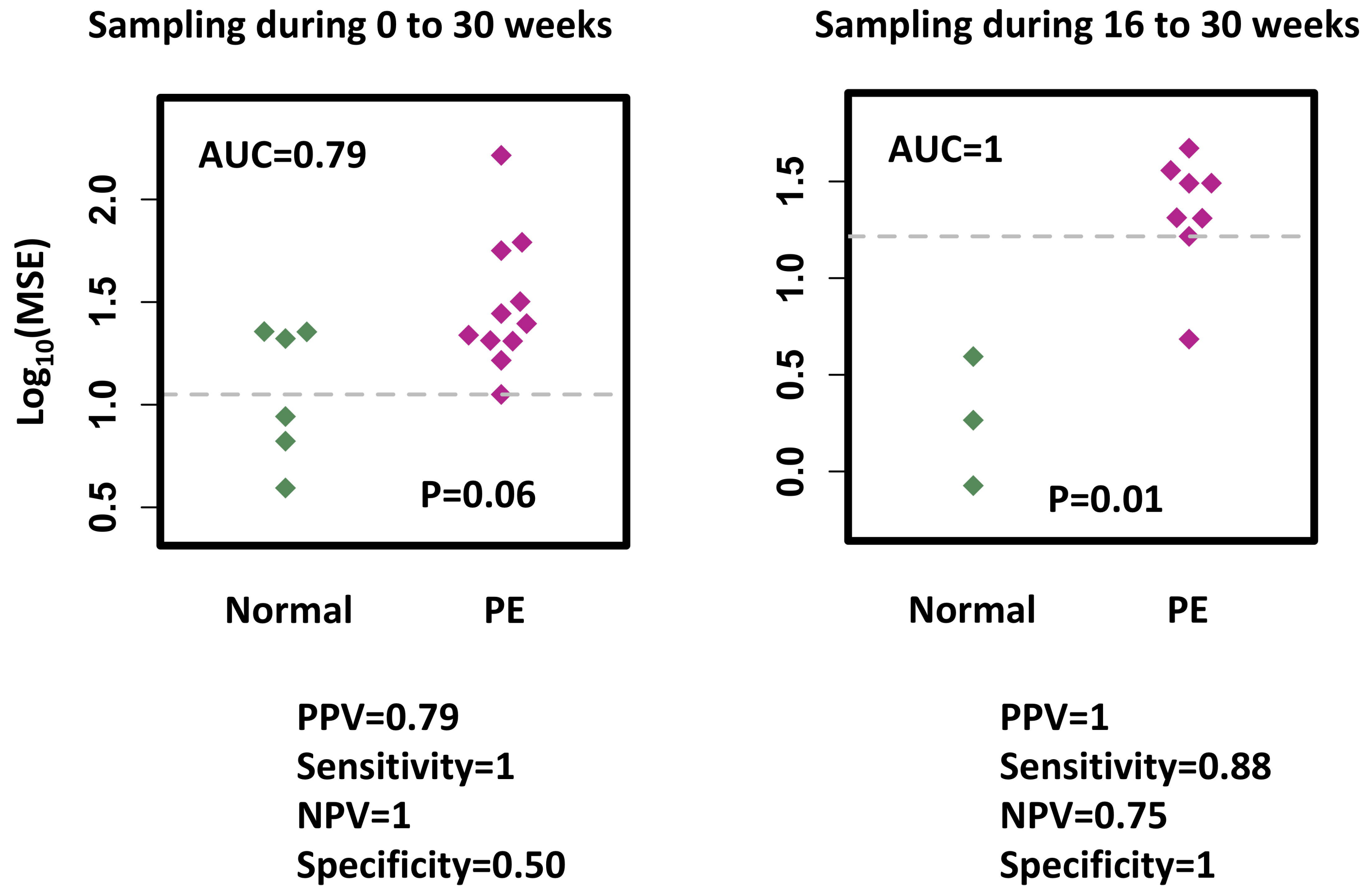

Fig. 4