Histidine-Rich Glycoprotein as an Early Biomarker of Preeclampsia

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BACKGROUND
Prediction of preeclampsia is of great interest and the coagulation system as well as the angiogenic pathway is known to be dysfunctional in preeclampsia. Histidine-rich glycoprotein (HRG) is a protein interacting with both these biological systems and the purpose of this prospective, longitudinal cohort study was to analyze whether there is a difference in circulating levels of HRG during pregnancy in women developing preeclampsia compared to normal healthy pregnancies. We furthermore wanted to evaluate whether HRG has the potential of being an early biomarker of preeclampsia.

METHODS
A cohort of healthy pregnant women (n = 469) was enrolled at gestational weeks 8–12. Plasma samples were collected at gestational weeks 10, 25, 28, 33, and 37 and analyzed with an enzyme-linked immunosorbent assay.

RESULTS
The levels of HRG decreased during pregnancy in all women, but the levels were significantly lower at gestational weeks 10, 25, and 28 in women who later developed preeclampsia than in normal pregnant women (P < 0.05, P < 0.05, and P < 0.05).

CONCLUSION
Our data indicates that HRG levels in plasma might be a possible biomarker already in gestational week 10 for prediction of later onset of preeclampsia in a low risk population.

Keywords: angiogenesis; blood pressure; histidine-rich glycoprotein; hypertension; prediction; preeclampsia

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Preeclampsia is a multiorgan pregnancy-specific disorder, which affects about 5% of all pregnancies. It is characterized by new-onset of hypertension and proteinuria after 20 weeks of gestation and is one of the major causes of maternal and infant morbidity and mortality.¹

The pathogenesis of preeclampsia is still unknown but recent theories focus on a two-stage model where the first stage is based on inappropriate trophoblast invasion of the maternal spiral arteries in early pregnancy leading to defective implantation and placentation and, as a result of this, relative hypoxia in the placenta. The second stage is associated with an imbalance of soluble placenta-derived angiogenic and antiangiogenic factors that causes endothelial cell dysfunction and increased vascular permeability.²,³ Furthermore, the activated vascular endothelium in preeclampsia is known to trigger a generalized intravascular inflammatory reaction.⁴

In normal pregnancy, the vascular endothelial cell surface is thrombo-resistant and effectively protects against clot formation. In preeclampsia however, endothelial cell dysfunction alters local anticoagulant properties, which in turn results in generally enhanced clot formation. The increased coagulability in the placental microvasculature and in the vasculature in general includes disturbances of the coagulation as well as the fibrinolytic system.⁵ Enhanced activation of platelets, elevated levels of von Willebrand factor as well as increased activity of factor VIII have been detected in preeclampsia.⁶–⁸

The coagulation system involves a number of different proteins with the enzymatic cleavage of fibrinogen to fibrin as the important end point. During normal pregnancy, the plasma level of fibrinogen increases, but in women with preeclampsia the fibrinogen level is even higher.⁹–¹⁰ Fibrinogen is known to interact with histidine-rich glycoprotein (HRG), a multidomain protein involved in homeostasis as well as in the angiogenic pathway having both angiogenic and antiangiogenic properties.¹¹,¹² HRG is present at high levels in plasma; it is synthesized by parenchymal liver cells and transported as a free protein as well as being stored in α-granules of platelets and released after thrombin stimulation.¹³ In a previous study, we have shown that both fibrinogen and HRG might be involved in the hypercoagulability and the angiogenic imbalance seen in early onset preeclampsia, which was verified by increased levels of HRG in placental tissue.¹⁰

There is no effective treatment or cure for preeclampsia except delivery. The major value of a predictor would be to identify women who will later develop preeclampsia for close antenatal surveillance, allowing early diagnosis, and timely

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delivery. We and others have studied a number of proteins interacting with angiogenesis, such as placent al growth factor, soluble fms-like tyrosine kinase-1, endoglin, and the angiopoietins. All of these factors have been identified as possible predictors of preeclampsia weeks before onset of symptoms, but not in very early pregnancy. The ultimate predictor of preeclampsia should presumably identify women with an increased risk of preeclampsia as soon as the first trimester, with the purpose of optimizing for preventive intervention. The markers identified so far are mainly second trimester markers but some have been shown to predict preeclampsia during the first trimester, although with a relatively low predictive value. 

The coagulation system, as well as the angiogenic pathway, is known to be dysfunctional in preeclampsia, which has drawn our attention to the importance of HRG.

The primary aim of this prospective, longitudinal cohort study was to analyze whether there is a difference in circulating levels of HRG throughout pregnancy between women developing preeclampsia and those who do not. The secondary aim was to determine whether HRG can be used as a predictor of preeclampsia in a low risk population.

METHODS

The study was approved by the regional ethics committee of the Medical Faculty of Uppsala University, and informed consent was obtained from each patient included in the study.

Study population. A cohort of healthy, pregnant women (n = 469) was enrolled in gestational weeks 8–12 at five participating prenatal centers in Värmland, Sweden during autumn 2004–spring 2007. Only women with singleton pregnancies were recruited. Women with a concurrent diagnosis such as chronic hypertension, episodes of high pressure before pregnancy, persistently elevated blood pressure before the 20th week of gestation, upper urinary tract infection, pre-existing renal disease, diabetes mellitus, and drug abuse were not included. Plasma samples were collected at gestational weeks 10, 25, 28, 33, and 37 according to the general controls for antenatal care in Sweden. Preeclampsia was defined as new-onset hypertension (140/90 mm Hg or greater) observed on at least two separate measurements 6 h or more apart, combined with proteinuria (2 or more on a dipstick or in a 24-h urine sample showing 300 mg/24 h or more). In the cohort, 20 women developed preeclampsia. Three hundred and two women in the cohort had a normal healthy pregnancy and delivered at term. From these, 44 women were randomly selected and included in the study as controls.

Sample collection. Venous blood samples were collected in tubes containing lithium/heparin. After collection the samples were put immediately into a refrigerator, where they were kept for no longer than 30 min before they were centrifuged for 10 min at 1,500g. Plasma were transferred to new tubes (Eppendorf, Hamburg, Germany) and stored at −70°C until analyzed and the mean storage time before analysis was 4 years.

Clinical and laboratory routine parameters were registered to standardize the cohort and to avoid for confounders.

Enzyme-linked immunosorbent assay. The wells of a PVC microtiter plate were coated with a polyclonal HRG capture antibody (H00003273-B01P; AbNova, Taipei, Taiwan) in a bicarbonate buffer, pH 9.6, for 1 h at 37°C, followed by two washing steps using phosphate-buffered saline. Blocking buffer containing 0.05% bovine serum albumin was added for 1 h at room temperature, followed by two more washing steps using phosphate-buffered saline. The samples were incubated for 45 min at room temperature and then washed four times. For detection, a second antibody (HRG-0119; Lena Claesson-Welsh, Department of Medical Genetics and Pathology, Uppsala University, Uppsala, Sweden), was added for 1 h at room temperature, then followed by four steps of washing. Incubation with a biotinylated anti-rabbit immunoglobulin G antibody (BA-1000; Vector Laboratories, Peterborough, UK) was performed, washed, and then streptavidin-conjugated horseradish peroxidase in phosphate-buffered saline/0.1% Tween was added. Levels of HRG were analyzed at the optical density of 450 nm after adding a TMB (3,3′,5,5′-tetramethylbenzidine) containing substrate and stopping solution (2 mol/l H2SO4).

The assay measures HRG soluble in plasma and serum (plasma without fibrinogen and clotting factors). A standard curve of purified HRG was included in each trial. The intra-assay variation was 7.7% and the interassay variation was 7.7%.

Statistics. Background variables in Table 1 are presented as mean values ± s.d. and comparisons between different
continuous variables were made with repeated measures analysis of variance. Plasma concentrations of HRG in Figure 1 and Table 2 are presented as mean values ± s.e. of the mean (s.e.m.) and comparisons between different gestational weeks were made with repeated measures analysis of variance with post-hoc Tukey test.

All significance tests were two-tailed. *P values <0.05 were considered as statistically significant. A receiver operator characteristic curve was constructed to test arbitrarily chosen HRG cutoff values for predicting preeclampsia. All statistical analyses were performed by SPSS 15.0 for Windows software package.

RESULTS
Background characteristics
Table 1 shows the baseline characteristics of the cohort. The women with normal pregnancies and those who developed preeclampsia did not differ from each other according to maternal age, parity, body mass index, and smoking habit at enrolment. All women had a normal blood pressure in the first trimester although those who later developed preeclampsia had a significantly higher systolic blood pressure in gestational week 10, 117 mm Hg compared to 112 mm Hg (*P < 0.05). As expected, women with preeclampsia had a significantly higher blood pressure at delivery and 50% of them were treated during pregnancy with antihypertensive medication. They also had a significantly shorter gestational age at delivery (Table 1).

Blood samples analyzed for a number of different clinical and laboratory routine parameters indicated that hemoglobin, leukocytes, platelets, sodium, potassium, creatinine, aspartate aminotransferase, alanine amino transferase, glucose, and urate were normal in both groups at gestational week 10 (data not shown). Levels of leukocytes and potassium were, however, significantly higher in the group who developed preeclampsia compared to controls in gestational week 10 (*P < 0.05 and *P < 0.05).

Plasma levels of HRG during pregnancy
Mean levels of HRG in plasma during pregnancy are presented in Figure 1. The results indicated that HRG levels decreased as the pregnancy proceeded in all women, irrespective of whether they developed preeclampsia or not. However, in the group who developed preeclampsia the levels of HRG were significantly lower in gestational week 10, 25, and 28 compared to women with a normal healthy pregnancy (40.1 µg/ml compared to 55.9 µg/ml *P < 0.05, 30.1 µg/ml compared to 42.8 µg/ml *P < 0.05 and 25.6 µg/ml compared to 33.8 µg/ml *P < 0.05, Figures 1 and 2 and Table 2). The variability of the levels in gestational weeks 10, 25, and 28 are shown in Figure 2. A subgroup analysis was performed and three women in the cohort developed severe preeclampsia. There were no significant differences between the groups in gestational week 10, 25, and 28 (*P ≥ 0.1 in all gestational weeks, data not shown).

Cutoff values for prediction of preeclampsia
Receiver operator characteristic curves regarding the prediction of preeclampsia at arbitrarily chosen HRG cutoff values were constructed (Figure 3a–c). A cutoff value of 49.1 µg/ml for HRG at gestational week 10 showed a sensitivity of 79% and a specificity of 44% to predict preeclampsia later in pregnancy, with an accuracy of 79%. For gestational week 25 a cutoff value of 32.7 µg/ml showed a sensitivity of 79% and a specificity of 57%. At gestational week 28 a cutoff value of 27.5 µg/ml gave a sensitivity of 82% and a specificity of 64%. The accuracy of gestational weeks 25 and 28 were 84 and 89%, respectively.
DISCUSSION
Understanding the pathophysiology of preeclampsia and early risk assessment are still major challenges. In our study, where women have been included prospectively and followed longitudinally during pregnancy, we show that plasma levels of HRG are decreasing during pregnancy in general. We show furthermore that there is a difference between plasma levels in women developing preeclampsia compared to women with a normal healthy pregnancy, with significantly lower levels in gestational week 10, 25, and 28 in the group with preeclampsia. This is, to the best of our knowledge, the first publication studying levels of HRG in pregnant women collected longitudinally during pregnancy. Haukkamaa and co-workers have, though, previously studied HRG in normal pregnancies, but in a cross-sectional study where blood samples from different women at gestational weeks 27–42 were analyzed, and they noted that the levels of HRG decreased during the third trimester which is consistent with our findings. Levels of HRG in women developing preeclampsia during the third trimester have furthermore been shown to be lower compared to women with a normal pregnancy, with significantly lower levels in gestational week 10, 25, and 28 in the group with preeclampsia.

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Furthermore, in another study by Tatra et al., HRG was shown to decrease during normal pregnancy. In that study a comparison with pregnant women having clinical signs of preeclampsia was performed, and no difference was detected. But in that study the women with preeclampsia were included in a cross-sectional manner, the samples were collected after symptoms of the syndrome had developed and all women were in gestational week 35 or more. We speculate about that the significant difference in HRG levels in gestational weeks 10, 25, and 28 that we have identified between normal pregnancy and pregnancies complicated by preeclampsia might be partly explained by the relative hypoxia in early pregnancy in women with preeclampsia that has been postulated to be based on defective placentation due to inappropriate trophoblast invasion of the maternal spiral arteries.

Preeclampsia is a disorder characterized by an imbalance in the angiogenic—antiangiogenic state, an excessive inflammatory response and a hypercoagulability. HRG is a protein interacting with all these processes. It has an antiangiogenic effect that has been suggested to be mediated by signal transduction targeting focal adhesions and thereby interrupting vascular endothelial growth factor-induced endothelial cell motility. It has furthermore an angiogenic effect by blocking the antiangiogenic effect of trombospondin. HRG has also been reported to act as a negative acute phase reactant and plasma levels are consequently reduced in response to tissue injury. In the regulation of the coagulation system HRG is of great importance.

The known interaction between fibrinogen and HRG is especially important in the regulation of angiogenesis and homeostasis. HRG can also be incorporated in fibrin clots, a process that decreases the amount of free HRG in plasma. Heparan sulfate-dependent binding of HRG directly to the endothelial cell surface has also been demonstrated. It has

| Table 2 | Plasma levels of HRG |
|---------|----------------------|
|         | Gestational week 10 | Gestational week 25 | Gestational week 28 | Gestational week 33 | Gestational week 37 |
| Normal  | Mean (s.e.m.)       | 55.91* (6.06)      | 42.76* (5.45)      | 33.76* (2.44)      | 29.15 (1.78)        | 28.37 (2.26)        |
|         | Preeclampsia        | 40.13 (3.71)       | 30.12 (2.26)       | 25.64 (2.33)       | 26.19 (2.00)        | 27.00 (3.07)        |

Values are presented as mean values of HRG (μg/ml). HRG, histidine-rich glycoprotein. *P < 0.05.

Figure 3 | Receiver operator characteristic curves. (a) Gestational week 10, (b) gestational week 25, and (c) gestational week 28. Area under curve is 0.650, 0.663, 0.695, respectively.
been suggested that HRG is transported in the platelets and that its release is dependent on the stimuli to which the platelets are exposed.\textsuperscript{32} The platelet degranulation response, as detected by CD63 expression, is enhanced in pregnancy, and to an even greater extent in preeclampsia.\textsuperscript{33} It is possible that the generally activated endothelium in preeclamptic women stimulates the release of these hyper-reactive granules leading to increased clot formation and adhesion of platelets to the vascular wall.

Of the previously studied markers of preeclampsia the majority have been shown to be possible predictors of preeclampsia in the second trimester\textsuperscript{15,17,18} but the purpose of an early predictive test would be to detect women who will later develop preeclampsia for optimizing close antenatal surveillance to allow early diagnosis and timely delivery.\textsuperscript{20} HRG is, to our knowledge, one of the first predictors that might be used as early as in gestational week 10, although the sensitivity and specificity is even better in gestational week 25 and 28. It has been discussed how a good predictor of preeclampsia would behave.\textsuperscript{19} We state that it is of important to minimize the numbers of false negative women rather than false positive although the ideal would be to avoid both. We have to that end chosen cutoff values in the receiver operator characteristic curve giving relatively high sensitivity and somewhat lower specificity.

One limitation with our study is the relatively small number of women included in the group developing preeclampsia and the results have to be confirmed in larger prospective studies, which we are planning for. In such studies calculations based on a number of different markers should be performed taking in consideration that preeclampsia is a syndrome with a multifactor origin. The strength of our study, on the other hand, is that the women were included prospectively and that the samples have been collected longitudinally.

Our findings indicate significantly lower levels of HRG as soon as the first trimester in women later developing preeclampsia. We conclude that HRG might have the potential for being a first trimester marker of preeclampsia in a low risk population.

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