



Review

Protein Misfolding during Pregnancy: New Approaches to Preeclampsia Diagnostics

Elizaveta M. Gerasimova ^{1,2}, Sergey A. Fedotov ^{1,3} , Daniel V. Kachkin ^{1,2} ,
Elena S. Vashukova ⁴, Andrey S. Glotov ^{4,5} , Yury O. Chernoff ^{1,6} and Aleksandr A. Rubel ^{1,2,*}

¹ Laboratory of Amyloid Biology, St. Petersburg State University, 199034 St. Petersburg, Russia; elelovaya@gmail.com (E.M.G.); serg900@yandex.ru (S.A.F.); pspdaniel@mail.ru (D.V.K); yury.chernoff@biology.gatech.edu (Y.O.C.)

² Department of Genetics and Biotechnology, St. Petersburg State University, 199034 St. Petersburg, Russia

³ Pavlov Institute of Physiology, Russian Academy of Sciences, 199034 St. Petersburg, Russia

⁴ Department of Genomic Medicine, D.O. Ott Research Institute of Obstetrics, Gynecology and Reproductology, 199034 St. Petersburg, Russia; vi_lena@list.ru (E.S.V.); anglotov@mail.ru (A.S.G.)

⁵ Laboratory of Biobanking and Genomic Medicine, Institute of Translation Biomedicine, St. Petersburg State University, 199034 St. Petersburg, Russia

⁶ School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA 30332, USA

* Correspondence: a.rubel@spbu.ru; Tel.: +7-911-970-66-47

Received: 6 November 2019; Accepted: 4 December 2019; Published: 7 December 2019



Abstract: Preeclampsia (PE) is a multisystem heterogeneous complication of pregnancy remaining a leading cause of maternal and perinatal morbidity and mortality over the world. PE has a large spectrum of clinical features and symptoms, which make diagnosis challenging. Despite a long period of studying, PE etiology is still unclear and there are no reliable rapid tests for early diagnosis of this disease. During the last decade, it was shown that proteins misfolding and aggregation are associated with PE. Several proteins, including amyloid beta peptide, transthyretin, alpha-1 antitrypsin, albumin, IgG k-free light chains, and ceruloplasmin are dysregulated in PE, resulting in toxic deposition of amyloid-like aggregates in the placenta and body fluids. It is also possible that aggregated proteins induce defective trophoblast invasion, placental ischemia, ER stress, and promote PE manifestation. The fact that protein aggregation is an emerging biomarker of PE provides an opportunity to develop new diagnostic approaches based on amyloids special features, such as Congo red (CR) staining and thioflavin T (ThT) enhanced fluorescence.

Keywords: preeclampsia; amyloid; protein misfolding; diagnostic; etiology

1. Introduction

Preeclampsia (PE) is the human-specific pregnancy complication leading contributor to maternal and fetal mortality worldwide [1]. The clinical symptoms appear after 20 weeks of gestation and include new-onset hypertension, proteinuria, edema, and maternal organ dysfunction [2–4]. However, it can be difficult to distinguish PE from other pathologies that are also characterized by hypertension and proteinuria, such as chronic hypertension or glomerulonephropathy.

If left untreated, PE can progress to eclampsia, which is characterized by stroke, seizures, kidney damage, cerebrovascular accidents, microangiopathic hemolytic anemia, liver failure, pulmonary edema—all of these serious consequences of PE can result in maternal death [5,6]. The only effective treatment is the delivery of the placenta and the fetus, leading to iatrogenic prematurity [7]. This is the reason why early diagnosis is so important.

To date, a few promising biomarkers for PE prediction have been found, used alone or in combination [8,9]. They include: a) biochemical markers, such as levels of the placental growth factor

(PIGF) [10], of soluble Fms-like tyrosine kinase 1 (sFlt-1) [11,12], and the sFlt-1/PIGF ratio [13], as well as levels of the placental protein 13 (PP13) [14,15], soluble endoglin (sEng) [16,17], pregnancy-associated plasma protein A (PAPP-A) [18], and some others; and b) physiological and biophysical markers, such as mean arterial pressure and uterine artery pulsatility index [9,19]. However, non-invasive and express methods (especially for early diagnosis or prediction of PE), which would not require sophisticated equipment and complex biochemical tests and would allow preventing or starting timely and effective treatment PE before the clinical manifestation of the disease, are still lacking.

Recent studies have shown that proteins misfolding and aggregation is associated with PE. Several proteins, including amyloid beta-peptide, alpha-1 antitrypsin, albumin, IgG k-free light chains, and ceruloplasmin, are dysregulated in PE resulting in deposition of amyloid-like aggregates in the placenta and body fluids. These facts provide an opportunity to develop new diagnostic approaches owing to amyloid have special features.

This review discusses recent findings about proteins misfolding and aggregation during PE and possible diagnostic methods based on these phenomena.

2. Diagnosis of PE

Traditionally applied diagnostic criteria of PE are hypertension, appearing after 20 weeks of gestation, combined with proteinuria, that is, the concentration of total protein at the level of 300 mg or higher in a 24 h urine sample [20]. Hypertension is defined as either a systolic blood pressure at the level above 140 mm Hg, or a diastolic blood pressure (BP) at the level higher than 90 mm Hg, as detected at least at two separate occasions. If blood pressure is severe (systolic BP ≥ 160 and/or diastolic BP ≥ 110 mm Hg), the measurement should be repeated after 15 min; for less severe blood pressure, repeated measurement should be taken after 3–6 h [4].

However, according to the recommendations of the International Society for the Study of Hypertension in Pregnancy [3], and American College of Obstetricians and Gynecologists [2], proteinuria is not a necessary feature of PE. Rather, PE is diagnosed by the presence of new onset hypertension accompanied by proteinuria and/or renal insufficiency, pulmonary edema, liver involvement, hemolysis, or thrombocytopenia, neurological complications, or fetal growth restriction [21]. The reason for excluding proteinuria as a required criterion is that PE can occur before glomerular capillary endotheliosis becomes severe enough to produce proteinuria [22]. In addition, the standard cut-off for protein concentrations remains uncertain, as the typically used cut-off level of 300 mg per 24 h in urine could be too high [23,24]. Moreover, the urinary dipstick analysis, which is usually used in medical practice, demonstrates a large number of false positive results [25,26], owing to variations in protein excretion, patient diet, and time of urine sampling [24].

Hence, PE is considered a multisystemic disease with non-specific clinical features. The appearance of symptoms such as proteinuria, hypertension, liver failure, and others does not necessarily guarantee a diagnosis of PE. This uncertainty leads to serious issues, as PE requires mandatory delivery. Lack of the rapid, specific, and non-invasive method distinguishing PE from other pregnancy complications and assuring a reliable diagnosis within a short time period is a major challenge for PE research and treatment.

3. Etiology and Pathogenesis of PE

Despite numerous studies, the etiology and pathogenesis of PE are still poorly understood. The speed of PE progression is unpredictable, and the subclinical phase is long. This may lead to fetal damage and adaptive clinical manifestations, such as thrombocytopenia, oxidative stress, vascular endothelial dysfunction, systemic inflammation, altered levels of nitric oxide, and aberrant angiogenesis [27]. The central role in the pathogenesis of PE is signified by the observation that the effective treatment for this complication of pregnancy is the early delivery of the fetus and the placenta [28].

In addition, PE is a multifactorial complication of pregnancy including various subclasses. Traditionally, PE is divided into placental or early-onset PE (<34 weeks), and maternal or late-onset PE (≥34 weeks), according to gestational age at diagnosis or delivery [29,30]. These two subtypes seem to have different etiologies. In early-onset PE, abnormal placentation under hypoxic conditions with higher levels of sFlt-1 and lower levels of PlGF take place [31]. Late-onset PE seems to occur from the interaction between a presumably normal placenta and maternal factors and to be a decompensated response to the oxidative stress in the placenta by a dysfunctional maternal endothelium (one aspect of a systemic maternal inflammatory response) [32]. In addition, PE cases can also be divided into subclasses according to their severity [33]. However, these classifications still do not fully reflect the heterogeneity observed in this complication of pregnancy.

Recently, systems biology approaches identified at least three forms of PE based on placental transcriptional phenotyping by using aggregate analysis on previously published PE microarray datasets and clustering the samples based on gene expression [34,35]. The first of these subclasses may arise if the mother demonstrates cardiovascular risk factors resulting in a poor maternal response to pregnancy and development of a later-onset, less severe form of PE (so called “maternal” PE), while the fetus will likely still be normal.

Another “immunological” subclass of PE seems to depend on the presence of immunological risk factors and occur because of incompatibility between the mother and the fetus, which may evoke an immune rejection of the placenta and an immunological presentation of PE.

Finally, the third or “canonical” PE form demonstrates placental dysfunction, elevated expression of known PE markers, and genes associated with poor oxygenation. The traditional view of the pathogenetic mechanisms involved in “canonical” PE is that genetic and environmental factors contribute to the defective deep placentation. Subsequently, the ischemic placenta releases soluble factors into the maternal circulation, which are responsible for the clinical manifestations of the disease [36].

The central hypothesis explaining PE occurrence relates it to the defective trophoblastic invasion with associated uteroplacental hypoperfusion [37]. During normal pregnancy, blood flow in the uterus increases to enable perfusion of the intervillous space of the placenta and to support the growth of the fetus. Physiological transformation of the spiral arteries of the uterus, a process in which trophoblasts invade the uterus and transform the arteries from narrow-diameter to large-diameter vessels, provides the increased blood flow and adequate placenta perfusion [38]. In PE, this remodeling is impaired, the placenta is likely to be deprived of oxygen, which is thought to explain the placental ischemia [39], increased oxidative stress in placenta, and overexpression of soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble Endoglin (sEng) [40,41]. Interestingly, recent systems biology study has shifted the paradigm and shown that maternal inflammation can precede defective trophoblast invasion and shallow placentation [42]. Preexisting maternal diseases or perturbed maternal-fetal-placental immune interactions may be detected in PE earlier and upstream of placental dysfunction, not only downstream, as described before [42].

Several weeks before the appearance of PE clinical manifestations, levels of sFlt-1 and sEng are increased in the serum, and this increase exhibits a positive correlation with the disease severity [40]. sFlt-1 binds to vascular endothelial growth factor (VEGF), which is especially important for maintaining endothelial cell function in the fenestrated endothelium of the brain, liver, and renal glomerulus, and placental growth factor (PlGF), antagonizing their binding to the cell surface. High sFlt-1 and low VEGF/PlGF status contribute to the development of hypertension [43]. A similar effect on VEGF and PlGF is modulated by sVEGFR-1 (a soluble receptor of vascular endothelial growth factors). In recent years, compelling evidence has been collected to support the concept that sVEGFR-1 plays a significant role in the pathogenesis of PE, because of its inhibitory influence on VEGF and PlGF [12,44]. Serum sFlt-1/PlGF ratio has proven to be clinically useful for routine PE diagnosis [10,45,46]. Also, it should be said that automated assays for sFlt-1 and PlGF measurements in serum, plasma, or urine have been already developed [10].

If myometrial segment of the spiral arteries during pregnancy was remodeled deficiently, it can lead to intermittent hypoxia and reoxygenation, which causes oxidative stress [47]. In addition, oxidative stress can occur in a result of the increased placental mitochondrial activity and production of reactive oxygen species (ROS), overwhelming the antioxidation defense mechanisms [48]. Increased levels of ROS, which are usually observed in PE, can lead to lipid peroxidation, protein carboxylation [47], releasing of proinflammatory cytokines and chemokines in blood flow [49], and DNA oxidation. All of these processes promote deterioration of the maternal organism [48].

Placental ischemia in PE is associated with a decreased expression of anti-oxidant heme oxygenase (HO) [50], and this contributes to the increased oxidative stress and the formation of micro-emboli [51]. The HO enzyme exists in two forms, Hmox1, and Hmox2, and converts free heme, which is a source of free radicals, first into biliverdin and then into bilirubin [52]. Hmox is upregulated in hypoxia and ischemia and it produces carbon monoxide, which acts as a vasodilator and decreases perfusion pressure in the placenta [53]. Indeed, increased gene expression of Hmox decreases circulating levels of sFlt-1 [54] and leads to normal pregnancy. Furthermore, trophoblasts express Hmox during pregnancy, and it was shown that inhibition of Hmox results in defective trophoblast invasion in vitro [55]. It has been proposed that pharmacological induction of Hmox expression could relieve hypertension and reduce serum concentrations of sVEGFR-1 and oxidative stress in rodent models [56].

Thus, the imbalance between angiogenic and antiangiogenic factors leads to incomplete spiral artery remodeling, oxidative stress, placental ischemia, and releasing of soluble factors into the maternal bloodstream could contribute to clinical manifestations of PE. This view is confirmed by studies in which the injection of placental extracts of pregnant women with PE into guinea pigs elicited convulsions with liver and kidney involvement, similar to those observed in women with eclampsia [36].

However, the above-mentioned manifestations are not specific only to PE, and errors in physiological remodeling of the spiral arteries per se are not sufficient to cause PE [57], as this failure has also been observed in other obstetric syndromes, such as preterm labor [58], spontaneous abortion, fetal death, and placental abruption [59]. In addition, it should be mentioned that mechanisms responsible for the failure of the physiological transformation of the spiral arteries are not fully understood [36].

By using mRNA fingerprinting, increased levels of neurokinin B (NkB) were identified as the promising marker (and potential causative agent) associated with PE [60]. Indeed, significantly higher levels of NkB in the maternal and umbilical cord blood were observed in preeclampsia, compared to pregnancies without complications [61]. The advantage of excess NkB as a biomarker is its specificity, as elevated levels of NkB are not associated with other known hypertensive disorders [62]. It has been suggested that the defective trophoblast invasion observed in PE leads to placental ischemia and the potential release of NkB as a signal for the maternal organism to increase blood flow to the placenta. NkB acts as a dilatator in the vascular system of the placenta [63]. In addition, NkB is considered as an anti-angiogenic factor that inhibits the assembly of the vascular network of endothelial cells and angiogenesis [64]. NkB can also suppress the expression of some proteins, modulate implantation, and involve in the cellular response to hypoxia and oxidative stress [65]. Trophoblast hypoxia has been shown to stimulate the production of several proteins that are known targets of NkB suppression [66]. It is possible that excess NkB inhibits the normal cellular response to hypoxia and thus contributes to the development of PE [67]. Excess NkB could also be linked to additional clinical manifestations of PE such as thrombocytopenia, inflammation, edema, and eventually, eclampsia [65].

Systemic inflammation and overexpression of toll-like receptor 4 [43,68], as well as high levels of the heat shock protein Hsp70 in the serum [69], production of serum autoantibodies to angiotensin II receptor 1 (AT1-AA), and increased sensitivity to the effects of angiotensin II [70] were also linked to PE, however most of these traits are either not sufficiently specific or technically difficult to diagnose.

An attractive concept postulates that endothelial cell activation and/or dysfunction can be a central feature of PE [36] as vasospasm, a condition in which dysfunctional endothelium releases smaller amounts of prostacyclin and nitric oxide compared to normal and cannot induce relaxation on smooth

muscle cells, leading to a reduction in the diameter of the cerebral artery lumen, an arterial spasm, tissue ischemia, and necrosis, is a key component of this disorder, and the PE-associated proteinuria could result from the damage to the fenestrated glomerular endothelium. Indeed, levels of E-selectin and vascular cell adhesion protein 1 were higher in patients with PE than in healthy pregnant women [71], however overexpression of E-selectin is also observed in other obstetric syndromes [72].

The role of apolipoprotein E (ApoE) polymorphism in PE was proposed based on PE-like features, such as hypertension, proteinuria, and increased expression of sFlt-1, detected in the ApoE knockout mice [73,74]. Indeed, certain ApoE alleles are associated with dyslipidemia which may contribute to endothelial cell dysfunction. However, attempts to demonstrate a connection between PE and the particular allele combination of the APOR locus have failed thus far [74].

A novel and intriguing theory about PE pathogenesis is that PE is associated with protein misfolding and aggregation. At the very least, recent data implicate the high-ordered fibrous protein aggregates (amyloids) as a biomarker of PE.

4. Protein Misfolding and Amyloid Aggregation in PE

4.1. Amyloids and Amyloidogenic Diseases

More than 40 human diseases, including such neurodegenerative disorders as Alzheimer's, Parkinson's, and Huntington's diseases, and transmissible spongiform encephalopathies (TSEs), or prion diseases (such as Creutzfeldt-Jakob disease), are caused by protein misfolding, aggregation, and deposition of fibrous protein aggregates (amyloids) in tissues [75–82]. Examples of amyloidogenic proteins include amyloid β peptide (A β) and tau in Alzheimer's disease [83–85], α -synuclein in Parkinson's disease and related disorders [86,87], and prion protein (PrP) in Creutzfeldt-Jakob disease [88]. Amyloids are highly organized non-covalent cross- β protein polymers that could accumulate in the form of fibrils of 7–10 nm in diameter and are highly resistant to anti-protein agents [76]. Mechanisms of amyloid-induced damage are not yet entirely clear (and could be different in different diseases). In many cases, amyloid formation interferes with the normal protein function, although a loss of protein function per se typically is not equivalent to the respective amyloid disease manifestations. Amyloids can immobilize protein of the same sequence, present in non-amyloid form, and thus spread via the process of nucleated polymerization. Transmissible amyloids, termed prions, can even spread between organisms, causing infection diseases such as TSEs. Recent data indicate that many disease-associated amyloids possess prion properties in specific conditions. Amyloid fibrils can be detected via binding to some dyes that recognize cross- β assemblies. Examples of these dyes include a Congo red (CR) [89,90] and thioflavin T (ThT) [91–93]. Amyloids can also be detected by some amyloid-specific antibodies [94], and by electron microscopy (EM) [95,96]. Notably, the ability to form an amyloid is controlled by an amyloid protein itself, as confirmed by the observation that amyloids are formed by mammalian amyloidogenic proteins expressed in heterologous systems, such as yeast [97,98].

4.2. Amyloids in PE

Recent studies have shown that misfolded proteins accumulate in the urine, serum, and the placenta of women with PE [99–104]. Indeed, proteins are vulnerable to misfolding because of changes in genetic and environmental factors [105] (Figure 1). Hence, protein structure can be destabilized under pressures, emerging as a normal part of pregnancy. Recent studies have proven that ischemia, hypoxia, and production of proinflammatory cytokines, associated with PE, can lead to protein misfolding [106] and initiate endoplasmic reticulum (ER) stress [107,108]. Therefore, these conditions may contribute to aggregation and toxic deposition of misfolded proteins in the PE placenta and body fluids. At the same time, it is also possible that aggregated proteins deposited in trophoblasts prevent its normal invasion and induce ischemia and ER stress. It has still to be determined whether or not protein aggregation plays a causative role in PE, triggering the defects in trophoblast invasion, endothelial

cell dysfunction, oxidative stress, etc., or just represents a consequence of these aberrations. However, recent data (reviewed below) clearly point to an amyloid as an emerging biomarker of PE.

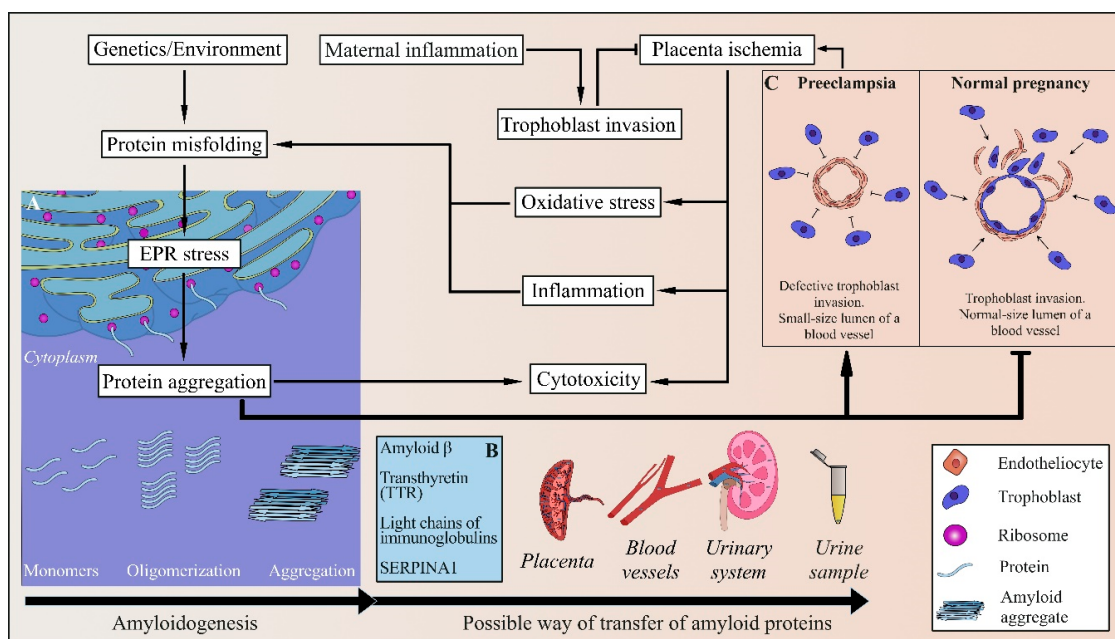


Figure 1. Pathogenesis mechanism of amyloid-based preeclampsia development. In cases of placenta ischemia that is caused by defective trophoblast invasion, oxidative stress, EPR stress, and inflammation may occur. All of these are possible causes of improper protein folding and aggregation. Amyloid aggregation of proteins can cause placenta ischemia. Amyloid aggregates can enter through the placenta into the mother's blood vessels and enter the urinary system through the bloodstream. Amyloid aggregates are found in the urine of women with preeclampsia. (A) During EPR stress, the frequency of protein misfolding increases, which leads to spontaneous aggregation and amyloidogenesis. (B) Proteins capable of amyloid aggregation in preeclampsia include amyloid β , transthyretin (TTR), immunoglobulin light chains, and alpha-1 antitrypsin. (C) In a normal pregnancy, an invasion of the trophoblasts of the embryo into the spiral arteries of the placenta occurs, which expands the lumen of the vessels and increases the flow of blood to the embryo. During PE, defective trophoblast invasion takes place, which leads to abnormal remodeling of spiral arteries and placenta ischemia.

Since kidney pathology is a hallmark of PE, and proteinuria levels usually correlate with the severity of the disease, Buhimschi et al. [99] performed the proteomic profiling of urine by using mass spectrometry and immunodetection in order to identify biomarkers that would reveal differences between PE patients, healthy women, and, most importantly, patients with proteinuria, which is not related to PE [99]. They found that women with PE (at 34–37 weeks of gestation) display a unique protein profile in their urine that can be used to predict severe PE with high accuracy and makes it possible to distinguish PE from other disorders associated with hypertension and proteinuria during pregnancy [99,101].

By using A11 and polyclonal aAPF antibodies, specifically binding to amyloid-associated epitopes [109,110], the presence of amyloid-type protofibrils and prefibrillar oligomers in the urine samples from women with PE has been detected [101]. These data were confirmed by the detection of fibrillar arborescent conformations tangled together in larger electrodense structures in the PE urine samples using transmission electron microscopy [101]. These structures were similar to images of fibrils extracted from amyloid-laden tissues [111], although the diameter of PE-associated fibrils was somewhat larger. No fibrils were detected in healthy women and patients with chronic hypertension [101].

By using tandem mass spectrometry (MS) and de novo MS-based protein sequencing, the authors identified some isoforms of alpha-1 antitrypsin and albumin as biomarkers in the urine of PE patients [99],

that were not present in the urine of non-pregnant women with proteinuria [99]. In addition, the protein component of the urine of pregnant women with PE also contained κ -free light chains of immunoglobulins (IgG), ceruloplasmin, interferon-inducible protein 6-16 (IFI6), and amyloid β [101]. Although it is still unclear which specific protein/s is/are present in the fibrillary form in the PE urine samples, all the above-mentioned proteins, with the exception of IFI6, were previously reported to undergo pathologic amyloid or amyloid-like aggregation, and are associated with some known human protein misfolding disorders [112,113]. Therefore, aggregated proteins could be used as a biomarker for predicting the onset of PE.

4.3. Alpha-1 Antitrypsin in PE

For example, increased levels of alpha-1 antitrypsin (a serine protease inhibitor, abundantly present in plasma) are detected in patients with diseases associated with an inflammatory component, such as vasculitis, certain infections, etc. **Even minor elevation in the levels of serum alpha-1 antitrypsin is accompanied by arterial hypertension [114]. Alpha-1 antitrypsin is fragmented, misfolded, and shown to aggregate in response to oxidative stress [113,115].** Supramolecular aggregates of misfolded alpha-1 antitrypsin, accumulated in hepatocytes and neurons, have recently been identified as factors in the development of serpinopathies, resulting in liver damage and encephalopathy [116]. **High frequency of liver pathologies and neurological disorders in PE women agrees with the possibility of alpha-1 antitrypsin misfolding and aggregate accumulation as one of the manifestations of PE [99].**

Moreover, in PE, high alpha-1 antitrypsin immunoreactivity was detected not only in urine but also in the serum and placenta, and significant stromal, endothelial, and intravascular deposition of misfolded alpha-1 antitrypsin aggregates has been reported, although the specific pattern of alpha-1 antitrypsin fragmentation present in urine is specific to PE [99].

4.4. Light Chains of Immunoglobulins in PE

The aggregation of immunoglobulin light-chains (λ , κ) involved in the pathogenesis of light chain (AL) amyloidosis and multiple myeloma [117–120]. AL amyloidosis is the most devastating form of systemic amyloidosis [121]. The disease is caused by deposition of amyloid fibrils, constituted by monoclonal immunoglobulin light chains, which are produced by an abnormally proliferative population of plasma cells [117–120].

Observations that AL amyloidosis most commonly affects kidney, and that patients with PE exhibit proteinuria and kidney damage, suggest that misfolding and aggregation of immunoglobulin light-chains can contribute to the PE-associated pathology.

4.5. Amyloid β in PE

Amyloid precursor protein (APP) is a ubiquitously expressed transmembrane glycosylated protein with three major isoforms (APP770, APP751, and APP695), which are produced in the result of alternative splicing of the APP gene. In the normal metabolic pathway, APP is first cleaved by α -secretase to release a soluble N-terminal fragment (sAPP α). Cleaved sAPP α is non-amyloidogenic and functions as a growth factor that promotes cell survival, proliferation, and migration [83,122]. However, in the amyloidogenic pathway, APP is cleaved by β -secretase and then γ -secretase releasing the short amyloid β (A β) peptide, which is the main component of amyloid plaques observed in the brain in Alzheimer's disease [122–124]. Due to its high propensity for oligomerization and self-assembly, A β is considered as a major factor triggering Alzheimer's disease [124–126].

MS analysis has not detected A β in the PE urine samples. However, aggregated A β could be difficult to detect by MS due to protection from proteolysis, needed for fragmentation that precedes MS analysis. Indeed, APP fragments (including A β) were found in the urine of pregnant women with PE by Western blotting [101] with specific ALZ90 monoclonal antibodies [127,128], possible dysregulation of the proteolytic cleavage of APP during PE. This agrees with increased production of α - (ADAM10) and β - (BACE2) in the placenta of women with PE [101]. Expression of one of the γ -secretase genes

(*PSEN1*) and of another gene for β -secretase (*BACE1*) was not reported as elevated at the mRNA level in the same tissues, but an increase in the products of these genes in PE trophoblasts has been demonstrated by immunohistochemistry [101]. This discrepancy between approaches is not surprising, as BACE1 expression control includes alternative splicing, post-translational modifications, cellular trafficking, and regulation of degradation [129].

Aside from its presence in the urine, A β is accumulated in the placenta of PE women [101]. Previous observations suggested that the placenta expresses APP during normal pregnancy; however, its cleavage and aggregation appear to be increased in the case of PE [104]. It was demonstrated that severe PE is associated with the deposition of amyloid-like aggregates, stained by ALZ90 antibodies [127,128] in the basal plate and villous areas of the placenta [101].

4.6. Transthyretin in PE

Recent studies have reported that transthyretin (TTR), a transporter of thyroxine and retinol, which is a known amyloidogenic protein playing a major role in the pathogenesis of familial amyloid polyneuropathy and others TTR-related amyloidosis [130], undergoes dysregulation, misfolding, and aggregation in PE [131]. TTR aggregation may lead to inflammation, oxidative stress, ER stress, and defective deep placentation [131,132]. By using specific ProteoStat dye, specifically binding to aggregated proteins, in combination with ELISA, TTR aggregates were found in the placenta and sera from patients with severe PE [131].

This remains unclear which amyloid protein is primarily responsible for the amyloid aggregation detected in PE urine, and whether or not amyloid formation triggers PE. It is possible that amyloidogenic proteins could induce aggregation of each other, so that several proteins could be present in the amyloid form at the advanced stage of the disease.

4.7. Possible Role of the Human Pregnancy Zone Protein

Some secreted proteins, known as extracellular chaperones, such as caseins [133], clusterin [134,135], haptoglobin [136], and alpha-2-macroglobulin (α 2M) [136,137], are implicated as inhibitors of protein misfolding and aggregation [133]. Pregnancy zone protein (PZP), which is very similar to α 2M, was shown to be significantly elevated in maternal plasma during pregnancy [138]. This can be revealed in serum after 3–4 weeks of gestation [139,140]. It was proposed that the high levels of PZP during pregnancy represent a maternal adaptation counteracting protein aggregation, for example via the formation of stable complexes between PZP and A β . PLZ, which is normally present as a dimer in biological fluids, is known to inhibit heat-induced protein aggregation [103] and could be a candidate for the efficient anti-aggregation chaperone similar to dimeric α 2M.

Hence, low level of production or dysregulation in the PZP chaperone function resulting in accumulation of misfolded proteins during pregnancy can lead to PE manifestation.

5. New Approaches to PE Diagnostics

Over the past twenty years, there has been significant progress in the understanding of pathophysiological mechanisms of PE and in the identification of new potential biomarkers that can be used in the diagnosis of this pregnancy complication. Some efficient approaches, such as serum sFLt1/PlGF ratio [11,141] or sEng measurement in plasma [142], have been introduced. A competing risks model, a Bayes' theorem based method, which provides an effective approach for the first-trimester prediction of preterm-PE based on maternal characteristics and medical history, biophysical (mean arterial pressure, uterine artery pulsatility index), and biochemical (placental growth factor, soluble fms-like tyrosine kinase-1) markers have been developed [9,19,143]. However, there is still a need for the cheap and specific express approach distinguishing PE and other conditions associated with hypertension and proteinuria.

One such approach could be based on MS-based identification of a unique urine proteomic fingerprint predicting PE. Such a protein profile includes alpha-1 antitrypsin, albumin, IgG k-free light

chains, ceruloplasmin, and interferon-inducible protein 6-16 [99], later these data were supplemented by α -1-antitrypsin, complement 3, haptoglobin, and trypstatin [144]. This could be complemented by detection of increased TTR levels in body fluids [71]. A disadvantage of this approach is that MS-based technology is expensive and requires a lot of time and specific equipment not readily available everywhere.

Another set of approaches employs amyloid-binding dyes, such as thioflavin T [72–74] and Congo red [89,90]. It was proposed that aggregates of misfolded protein, which are observed in urine, also circulate in the bloodstream and can be detected by using ThT-enhanced fluorescence. However, it was shown that ThT fluorescence in urine and serum was increased only in severe PE but not in mild forms of PE [100]. Another disadvantage of this method is although there were no gestation age-related changes in ThT fluorescence, enhanced ThT fluorescence was shown for late-stage severe PE (29–35 weeks) [100] when the clinical manifestation of PE has already occurred.

Buhimschi et al. designed a simpler method based on the fact that women with PE demonstrate urine congophilia—an affinity for the amyloidophilic dye CR [101]. CR staining, followed by birefringence in the polarized light, is the gold standard for the demonstration of amyloids in tissue sections [145] and in vitro (Figure 2). Although the mechanism of CR binding to amyloid fibrils is not fully understood, it is known that this phenomenon is reliant on the affinity of CR for proteins, enriched in β -sheets [90]. Only amyloids (but not any other CR binding compounds) exhibit a phenomenon of birefringence when bound to CR.

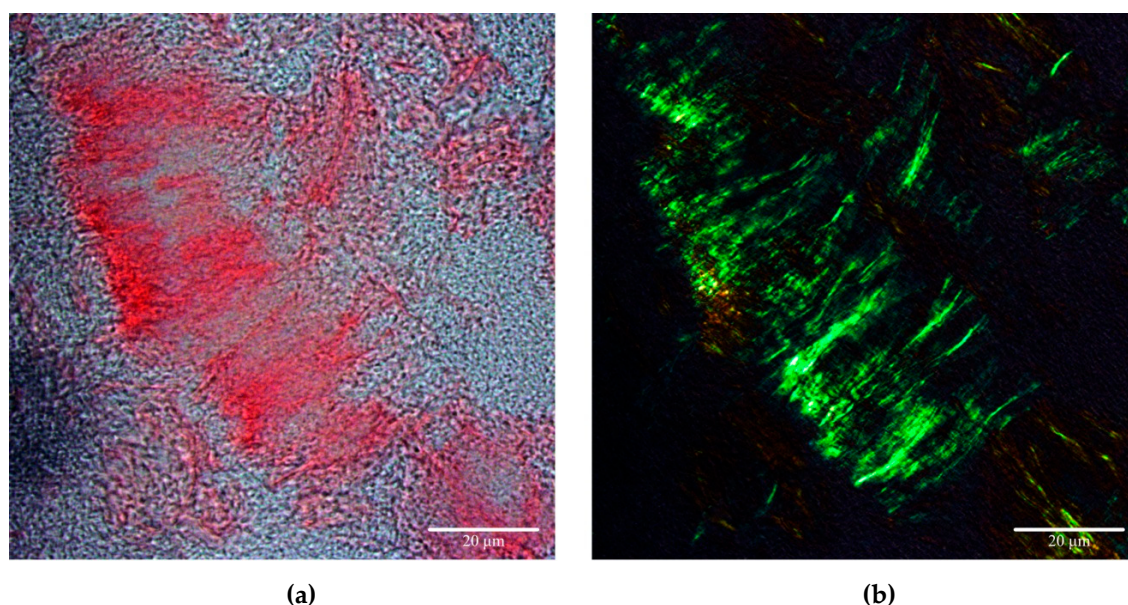


Figure 2. Congo red-stained amyloid aggregates of recombinant *S. cerevisiae* Sup35NM protein. (a) Amyloid aggregates of the yeast Sup35NM protein bind to CR; (b) CR-stained Sup35NM aggregates demonstrated yellow to apple-green birefringence under polarized light. Data are obtained by D.V. Kachkin.

Urinary congophilia (that is, the presence of urea components capable of binding CR) has previously been reported for such a “classic” human prion disease as Creutzfeldt-Jakob disease [146]. Buhimschi et al. have shown that the same approach detects amyloids by CR binding in the urine of women with severe PE. In the case of PE, congophilia develops at an early stage of the asymptomatic phase of PE (more than 10 weeks before clinical manifestation of PE) and progressively develops during pregnancy [101]. The detection approach is employing the absorption of urine proteins on the nitrocellulose filter, followed by staining with CR and washing with methanol (Figure 3). The value of the CR retention after the methanol wash (relative to the value before the wash) was proposed as a

diagnostic indicator [101]. Moreover, qualitative (visual) detection based on the presence of the red spots on the filter is also doable.

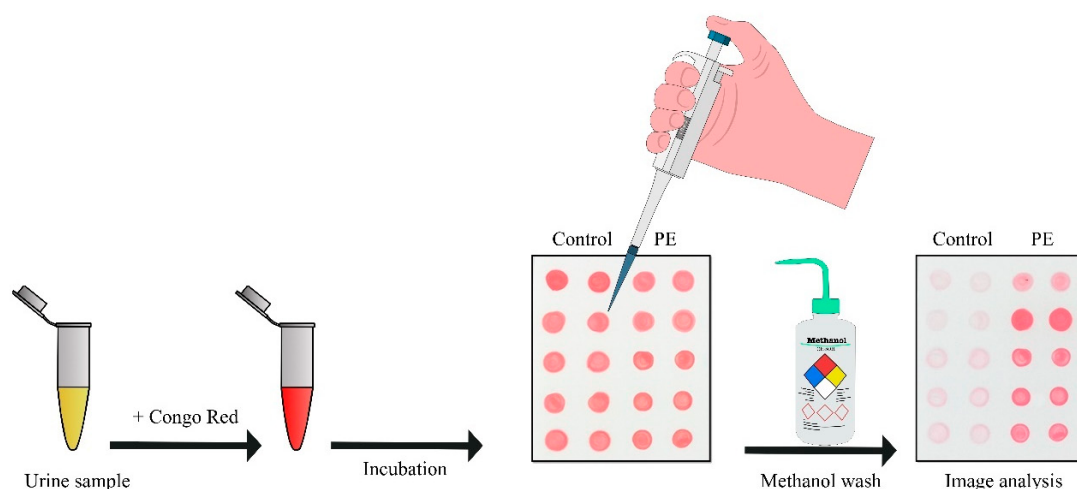


Figure 3. The scheme of the CR dot test for rapid identification of preeclampsia. Urine was mixed with a solution of CR and spotted on a strip of nitrocellulose, which was photographed before and after washing with increasing concentration of methanol. The spots corresponding to PE urine retained the red color, whereas spots of control washed away.

Later, Rood et al. suggested the Congo Red Dot (CRD) paper test as a simple, univocal, non-invasive clinical tool for rapid PE identification [147]. This modification of the detection approach is based on the fact that CR solution spotted on paper forms hydrogen bonds with cellulose and made a tight circle. However, if in this solution (urine mixed with CR) there are aggregated proteins, they bind to CR and prevent its cellulose binding. Hence, the CR-urine solution spread on the paper forming a wide pink circle. The CRD paper test takes only about 5 minutes and demonstrates high accuracy in PE diagnosis. The authors report that the CRD paper test result can turn positive within 14 days before the clinical manifestation of PE [147]. However, the gestational age of women who took part in the research was generally between 28 and 38 weeks. Usually common PE symptoms can be detected at this stage of pregnancy [20].

Therefore, as of now, diagnostic methods based on protein misfolding during PE are proven to work in the second half of pregnancy, only a few weeks before the PE clinical manifestations. This remains to be determined if these methods are applicable to earlier stages of PE. In the case of amyloid formation playing an important role in disease development, such an applicability is likely, but requires further investigation.

Author Contributions: Conceptualization, E.M.G., Y.O.C., and A.A.R.; validation, S.A.F., A.S.G., Y.O.C., and A.A.R.; writing—original draft preparation, E.M.G.; writing—review and editing, E.M.G., S.A.F., E.S.V., A.S.G., Y.O.C., D.V.K., A.A.R.; Visualization D.V.K.; supervision, Y.O.C. and A.A.R.; project administration, A.S.G., Y.O.C., and A.A.R.; funding acquisition, A.S.G. and Y.O.C.

Funding: This study was financially supported in parts by grant 19-75-20033 from Russian Science Foundation (A.S.G., E.M.G., and E.S.V.), grant 19-34-90153 from Russian Foundation of Basic Research (Y.O.C. and D.V.K.), and by grant from St. Petersburg State University (Y.O.C., E.M.G., and A.A.R.). S.A.F. was supported by Postdoctoral Fellowship program from St. Petersburg State University.

Acknowledgments: The authors are grateful to Konstantin Yu. Kulichikhin (Laboratory of Amyloid Biology, St. Petersburg State University, Russia) for the helpful discussion. We also thank Julia V. Sopova (Laboratory of Amyloid Biology, St. Petersburg State University, Russia) for the assistance with CR staining.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

PE	Preeclampsia
CR	Congo Red
CRD	Congo Red
BP	Blood Pressure
sFlt-1	Soluble Fms-like tyrosine kinase-1
sEng	soluble Endoglin
PLGF	Placental Growth Factor
sVEGFR	Vascular Endothelial Growth Factor
VEGF	Vascular Endothelial Growth Factor
ROS	Reactive Oxygen Species
HO	Heme Oxygenase
mRNA	messenger Ribonucleic Acid
NkB	Neurokinin B
AT1-AA	Autoantibodies to Angiotensin II receptor 1
Apo E	Apolipoprotein E
TSEs	Transmissible Spongiform Encephalopathies
A β	Amyloid β peptide
EM	Electron Microscopy
ER	Endoplasmic Reticulum
TTR	Transthyretin
MS	Mass Spectrometry
igG	immunoglobulins
IFI-6	Interferon-inducible protein 6-16
APP	Amyloid Precursor Protein
sAPPa	soluble N-terminal fragment of APP
α 2M	alpha-2-macroglobulin
PZP	Pregnancy Zone Protein
ThT	Thioflavin-T

References

1. Duley, L. The Global Impact of Pre-eclampsia and Eclampsia. *Semin. Perinatol.* **2009**, *33*, 130–137. [[CrossRef](#)] [[PubMed](#)]
2. American College of Obstetricians; Task Force on Hypertension in Pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet. Gynecol.* **2013**, *122*, 1122–1131. [[CrossRef](#)]
3. Tranquilli, A.L.; Dekker, G.; Magee, L.; Roberts, J.; Sibai, B.M.; Steyn, W.; Zeeman, G.G.; Brown, M.A. The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. *Pregnancy Hypertens. Int. J. Women's Cardiovasc. Health* **2014**, *4*, 97–104. [[CrossRef](#)] [[PubMed](#)]
4. Brown, M.A.; Magee, L.A.; Kenny, L.C.; Karumanchi, S.A.; McCarthy, F.P.; Saito, S.; Hall, D.R.; Warren, C.E.; Adoyi, G.; Ishaku, S. Hypertensive disorders of pregnancy: ISSHP classification, diagnosis, and management recommendations for international practice. *Hypertension* **2018**, *72*, 24–43. [[CrossRef](#)] [[PubMed](#)]
5. Sircar, M.; Thadhani, R.; Karumanchi, S.A. Pathogenesis of preeclampsia. *Curr. Opin. Nephrol. Hypertens.* **2015**, *24*, 131–138. [[CrossRef](#)] [[PubMed](#)]
6. Ghulmiyyah, L.; Sibai, B. Maternal Mortality from Preeclampsia/Eclampsia. *Semin. Perinatol.* **2012**, *36*, 56–59. [[CrossRef](#)] [[PubMed](#)]
7. Hod, T.; Cerdeira, A.S.; Karumanchi, S.A. Molecular Mechanisms of Preeclampsia. *Cold Spring Harb. Perspect. Med.* **2015**, *5*, a023473. [[CrossRef](#)]
8. Wu, P.; van den Berg, C.; Alfirevic, Z.; O'Brien, S.; Röthlisberger, M.; Baker, P.; Kenny, L.; Kublickiene, K.; Duvetkot, J. Early Pregnancy Biomarkers in Pre-Eclampsia: A Systematic Review and Meta-Analysis. *Int. J. Mol. Sci.* **2015**, *16*, 23035–23056. [[CrossRef](#)]

9. Wright, D.; Tan, M.Y.; O’Gorman, N.; Poon, L.C.; Syngelaki, A.; Wright, A.; Nicolaides, K.H. Predictive performance of the competing risk model in screening for preeclampsia. *Am. J. Obstet. Gynecol.* **2019**, *220*, e1–e199. [[CrossRef](#)]
10. Schiettecatte, J.; Russcher, H.; Anckaert, E.; Mees, M.; Leese, B.; Tirelli, A.S.; Fiedler, G.M.; Luthe, H.; Denk, B.; Smits, J. Multicenter evaluation of the first automated Elecsys sFlt-1 and PlGF assays in normal pregnancies and preeclampsia. *Clin. Biochem.* **2010**, *43*, 768–770. [[CrossRef](#)]
11. Salahuddin, S.; Lee, Y.; Vadnais, M.; Sachs, B.P.; Karumanchi, S.A.; Lim, K.-H. Diagnostic utility of soluble fms-like tyrosine kinase 1 and soluble endoglin in hypertensive diseases of pregnancy. *Am. J. Obstet. Gynecol.* **2007**, *197*, e1–e28. [[CrossRef](#)] [[PubMed](#)]
12. Levine, R.J.; Maynard, S.E.; Qian, C.; Lim, K.-H.; England, L.J.; Yu, K.F.; Schisterman, E.F.; Thadhani, R.; Sachs, B.P.; Epstein, F.H.; et al. Circulating angiogenic factors and the risk of preeclampsia. *N. Engl. J. Med.* **2004**, *350*, 672–683. [[CrossRef](#)] [[PubMed](#)]
13. Sovio, U.; Gaccioli, F.; Cook, E.; Hund, M.; Charnock-Jones, D.S.; Smith, G.C.S. Prediction of Preeclampsia Using the Soluble fms-Like Tyrosine Kinase 1 to Placental Growth Factor Ratio: A Prospective Cohort Study of Unselected Nulliparous Women. *Hypertension* **2017**, *69*, 731–738. [[CrossRef](#)] [[PubMed](#)]
14. Wortelboer, E.; Koster, M.; Cuckle, H.; Stoutenbeek, P.; Schielen, P.; Visser, G. First-trimester placental protein 13 and placental growth factor: Markers for identification of women destined to develop early-onset pre-eclampsia. *Int. J. Obstet. Gynaecol.* **2010**, *117*, 1384–1389. [[CrossRef](#)]
15. Nicolaides, K.H.; Bindra, R.; Turan, O.M.; Chefetz, I.; Sammar, M.; Meiri, H.; Tal, J.; Cuckle, H.S. A novel approach to first-trimester screening for early pre-eclampsia combining serum PP-13 and Doppler ultrasound. *Ultrasound Obstet. Gynecol.* **2005**, *27*, 13–17. [[CrossRef](#)]
16. Venkatesha, S.; Toporsian, M.; Lam, C.; Hanai, J.; Mammoto, T.; Kim, Y.M.; Bdolah, Y.; Lim, K.-H.; Yuan, H.-T.; Libermann, T.A.; et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat. Med.* **2006**, *12*, 642–649. [[CrossRef](#)]
17. Foidart, J.-M.; Munaut, C.; Chantraine, F.; Akolekar, R.; Nicolaides, K.H. Maternal plasma soluble endoglin at 11–13 weeks’ gestation in pre-eclampsia. *Ultrasound Obstet. Gynecol.* **2010**, *35*, 680–687. [[CrossRef](#)]
18. Spencer, K.; Cowans, N.J.; Nicolaides, K.H. Low levels of maternal serum PAPP-A in the first trimester and the risk of pre-eclampsia. *Prenat. Diagn.* **2008**, *28*, 7–10. [[CrossRef](#)]
19. Poon, L.C.; Nicolaides, K.H. Early Prediction of Preeclampsia. *Obstet. Gynecol. Int.* **2014**, *2014*, 1–11. [[CrossRef](#)]
20. Diagnosis and management of preeclampsia and eclampsia. *Int. J. Gynecol. Obstet.* **2002**, *77*, 67–75. [[CrossRef](#)]
21. Lowe, S.A.; Brown, M.A.; Dekker, G.A.; Gatt, S.; McIntock, C.K.; McMahon, L.P.; Mangos, G.; Moore, M.P.; Muller, P.; Paech, M.; et al. Guidelines for the management of hypertensive disorders of pregnancy 2008. *Aust. N. Z. J. Obstet. Gynaecol.* **2009**, *49*, 242–246. [[CrossRef](#)] [[PubMed](#)]
22. Morgan, M.A.; Thurnau, G.R. Pregnancy-induced hypertension without proteinuria: Is it true preeclampsia? *South. Med. J.* **1988**, *81*, 210–213. [[CrossRef](#)] [[PubMed](#)]
23. Meyer, N.L.; Mercer, B.M.; Friedman, S.A.; Sibai, B.M. Urinary dipstick protein: A poor predictor of absent or severe proteinuria. *Am. J. Obstet. Gynecol.* **1994**, *170*, 137–141. [[CrossRef](#)]
24. Lindheimer, M.D.; Kanter, D. Interpreting abnormal proteinuria in pregnancy: The need for a more pathophysiological approach. *Obstet. Gynecol.* **2010**, *115*, 365–375. [[CrossRef](#)] [[PubMed](#)]
25. Brown, M.A.; Buddle, M.L. Inadequacy of dipstick proteinuria in hypertensive pregnancy. *Aust. N. Z. J. Obstet. Gynaecol.* **1995**, *35*, 366–369. [[CrossRef](#)] [[PubMed](#)]
26. Grauer, G.F. Proteinuria: Measurement and interpretation. *Top. Companion Anim. Med.* **2011**, *26*, 121–127. [[CrossRef](#)]
27. Shamshirsaz, A.A.; Paidas, M.; Krikun, G. Preeclampsia, Hypoxia, Thrombosis, and Inflammation. *J. Pregnancy* **2012**, *2012*, 1–6. [[CrossRef](#)]
28. Roberts, J.M.; Gammill, H.S. Preeclampsia: Recent insights. *Hypertension* **2005**, *46*, 1243–1249. [[CrossRef](#)]
29. Von Dadelszen, P.; Magee, L.A.; Roberts, J.M. Subclassification of Preeclampsia. *Hypertens. Pregnancy* **2003**, *22*, 143–148. [[CrossRef](#)]
30. Ødegård, R.A.; Vatten, L.J.; Nilsen, S.T.; Salvesen, K.; Austgulen, R. Risk factors and clinical manifestations of pre-eclampsia. *Br. J. Obstet. Gynaecol.* **2000**, *107*, 1410–1416. [[CrossRef](#)]

31. Masuyama, H.; Segawa, T.; Sumida, Y.; Masumoto, A.; Inoue, S.; Akahori, Y.; Hiramatsu, Y. Different profiles of circulating angiogenic factors and adipocytokines between early- and late-onset pre-eclampsia. *Int. J. Obstet. Gynaecol.* **2010**, *117*, 314–320. [[CrossRef](#)] [[PubMed](#)]
32. Phipps, E.; Prasanna, D.; Brima, W.; Jim, B. Preeclampsia: Updates in pathogenesis, definitions, and guidelines. *Clin. J. Am. Soc. Nephrol.* **2016**, *11*, 1102–1113. [[CrossRef](#)] [[PubMed](#)]
33. Sibai, B.M. Evaluation and management of severe preeclampsia before 34 weeks' gestation. *Am. J. Obstet. Gynecol.* **2011**, *205*, 191–198. [[CrossRef](#)] [[PubMed](#)]
34. Leavey, K.; Bainbridge, S.A. Cox Large scale aggregate microarray analysis reveals three distinct molecular subclasses of human preeclampsia. *PLoS ONE* **2015**, *10*, e0116508. [[CrossRef](#)]
35. Leavey, K.; Benton, S.J.; Grynspan, D.; Kingdom, J.C.; Bainbridge, S.A.; Cox, B.J. Unsupervised Placental Gene Expression Profiling Identifies Clinically Relevant Subclasses of Human Preeclampsia. *Hypertension* **2016**, *68*, 137–147. [[CrossRef](#)]
36. Chaiworapongsa, T.; Chaemsaihong, P.; Yeo, L.; Romero, R. Pre-eclampsia part 1: Current understanding of its pathophysiology. *Nat. Rev. Nephrol.* **2014**, *10*, 466–480. [[CrossRef](#)]
37. Gilbert, J.S.; Babcock, S.A.; Granger, J.P. Hypertension Produced by Reduced Uterine Perfusion in Pregnant Rats Is Associated With Increased Soluble Fms-Like Tyrosine Kinase-1 Expression. *Hypertension* **2007**, *50*, 1142–1147. [[CrossRef](#)]
38. Kaufmann, P.; Black, S.; Huppertz, B. Endovascular Trophoblast Invasion: Implications for the Pathogenesis of Intrauterine Growth Retardation and Preeclampsia. *Biol. Reprod.* **2003**, *69*, 1–7. [[CrossRef](#)]
39. Brosens, I.; Renaer, M. On the pathogenesis of placental infarcts in pre-eclampsia. *Int. J. Obstet. Gynaecol.* **1972**, *79*, 794–799. [[CrossRef](#)]
40. Levine, R.J.; Lam, C.; Qian, C.; Yu, K.F.; Maynard, S.E.; Sachs, B.P.; Sibai, B.M.; Epstein, F.H.; Romero, R.; Thadhani, R.; et al. Soluble Endoglin and Other Circulating Antiangiogenic Factors in Preeclampsia. *N. Engl. J. Med.* **2006**, *355*, 992–1005. [[CrossRef](#)]
41. Harihana, N.; Shoemaker, A.; Wagner, S. Pathophysiology of hypertension in preeclampsia. *Clin. Pr.* **2016**, *13*, 33–37.
42. Than, N.G.; Romero, R.; Tarca, A.L.; Kekesi, K.A.; Xu, Y.; Xu, Z.; Juhasz, K.; Bhatti, G.; Leavitt, R.J.; Gelencser, Z.; et al. Integrated Systems Biology Approach Identifies Novel Maternal and Placental Pathways of Preeclampsia. *Front. Immunol.* **2018**, *9*, 1661. [[CrossRef](#)]
43. El-Sayed, A.A.F. Preeclampsia: A review of the pathogenesis and possible management strategies based on its pathophysiological derangements. *Taiwan. J. Obstet. Gynecol.* **2017**, *56*, 593–598. [[CrossRef](#)] [[PubMed](#)]
44. Maynard, S.E.; Min, J.-Y.; Merchan, J.; Lim, K.-H.; Li, J.; Mondal, S.; Libermann, T.A.; Morgan, J.P.; Sellke, F.W.; Stillman, I.E.; et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J. Clin. Investig.* **2003**, *111*, 649–658. [[CrossRef](#)] [[PubMed](#)]
45. Ohkuchi, A.; Hirashima, C.; Suzuki, H.; Takahashi, K.; Yoshida, M.; Matsubara, S.; Suzuki, M. Evaluation of a new and automated electrochemiluminescence immunoassay for plasma sFlt-1 and PlGF levels in women with preeclampsia. *Hypertens. Res.* **2010**, *33*, 422–427. [[CrossRef](#)]
46. Knudsen, U.B.; Kronborg, C.S.; von Dadelszen, P.; Kupfer, K.; Lee, S.-W.; Vittinghus, E.; Allen, J.G.; Redman, C.W. A single rapid point-of-care placental growth factor determination as an aid in the diagnosis of preeclampsia. *Pregnancy Hypertens. Int. J. Women's Cardiovasc. Health* **2012**, *2*, 8–15. [[CrossRef](#)]
47. Burton, G.J.; Jauniaux, E. Oxidative stress. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2011**, *25*, 287–299. [[CrossRef](#)]
48. Myatt, L.; Cui, X. Oxidative stress in the placenta. *Histochem. Cell Biol.* **2004**, *122*, 369–382. [[CrossRef](#)]
49. Cindrova-Davies, T.; Spasic-Boskovic, O.; Jauniaux, E.; Charnock-Jones, D.S.; Burton, G.J. Nuclear Factor- κ B, p38, and Stress-Activated Protein Kinase Mitogen-Activated Protein Kinase Signaling Pathways Regulate Proinflammatory Cytokines and Apoptosis in Human Placental Explants in Response to Oxidative Stress. *Am. J. Pathol.* **2007**, *170*, 1511–1520. [[CrossRef](#)]
50. Zenclussen, A.C.; Lim, E.; Knoeller, S.; Knackstedt, M.; Hertwig, K.; Hagen, E.; Klapp, B.F.; Arck, P.C. Heme Oxygenases in Pregnancy II: HO-2 is Downregulated in Human Pathologic Pregnancies. *Am. J. Reprod. Immunol.* **2003**, *50*, 66–76. [[CrossRef](#)]
51. Lyall, F.; Barber, A.; Myatt, L.; Bulmer, J.N.; Robson, S.C. Hemeoxygenase expression in human placenta and placental bed implies a role in regulation of trophoblast invasion and placental function. *FASEB J.* **2000**, *14*, 208–219. [[CrossRef](#)] [[PubMed](#)]

52. George, E.M.; Granger, J.P. Heme oxygenase in pregnancy and preeclampsia. *Curr. Opin. Nephrol. Hypertens.* **2013**, *22*, 156–162. [[CrossRef](#)] [[PubMed](#)]
53. Bainbridge, S.; Farley, A.; McLaughlin, B.; Graham, C.; Marks, G.; Nakatsu, K.; Brien, J.; Smith, G. Carbon Monoxide Decreases Perfusion Pressure in Isolated Human Placenta. *Placenta* **2002**, *23*, 563–569. [[CrossRef](#)] [[PubMed](#)]
54. Cudmore, M.; Ahmad, S.; Al-Ani, B.; Fujisawa, T.; Coxall, H.; Chudasama, K.; Devey, L.R.; Wigmore, S.J.; Abbas, A.; Hewett, P.W.; et al. Negative Regulation of Soluble Flt-1 and Soluble Endoglin Release by Heme Oxygenase-1. *Circulation* **2007**, *115*, 1789–1797. [[CrossRef](#)] [[PubMed](#)]
55. McCaig, D.; Lyall, F. Inhibitors of Heme Oxygenase Reduce Invasion of Human Primary Cytotrophoblast Cells In vitro. *Placenta* **2009**, *30*, 536–538. [[CrossRef](#)]
56. Costantine, M.M.; Tamayo, E.; Lu, F.; Bytautiene, E.; Longo, M.; Hankins, G.D.V.; Saade, G.R. Using Pravastatin to Improve the Vascular Reactivity in a Mouse Model of Soluble Fms-Like Tyrosine Kinase-1-Induced Preeclampsia. *Obstet. Gynecol.* **2010**, *116*, 114–120. [[CrossRef](#)]
57. Brosens, I.; Pijnenborg, R.; Vercruysse, L.; Romero, R. The “Great Obstetrical Syndromes” are associated with disorders of deep placentation. *Am. J. Obstet. Gynecol.* **2011**, *204*, 193–201. [[CrossRef](#)]
58. Kim, Y.M.; Bujold, E.; Chaiworapongsa, T.; Gomez, R.; Yoon, B.H.; Thaler, H.T.; Rotmensch, S.; Romero, R. Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. *Am. J. Obstet. Gynecol.* **2003**, *189*, 1063–1069. [[CrossRef](#)]
59. Domisse, J.; Tiltman, A.J. Placental bed biopsies in placental abruption. *Int. J. Obstet. Gynaecol.* **1992**, *99*, 651–654. [[CrossRef](#)]
60. Page, N.; Butlin, D.; Manyonda, I.; Lowry, P. The development of a genetic profile of placental gene expression during the first trimester of pregnancy: A potential tool for identifying novel secreted markers. *Fetal Diagn. Ther.* **2000**, *15*, 237–245. [[CrossRef](#)]
61. Zulfikaroglu, E.; Ugur, M.; Taflan, S.; Ugurlu, N.; Atalay, A.; Kalyoncu, S. Neurokinin B levels in maternal and umbilical cord blood in preeclamptic and normal pregnancies. *J. Perinat. Med.* **2007**, *35*, 200–202. [[CrossRef](#)] [[PubMed](#)]
62. Page, N.M.; Kemp, C.F.; Butlin, D.J.; Lowry, P.J. Placental peptides as markers of gestational disease. *Reproduction* **2002**, *123*, 487–495. [[CrossRef](#)] [[PubMed](#)]
63. Laliberte, C.; DiMarzo, L.; Morrish, D.W.; Kaufman, S. Neurokinin B causes concentration-dependent relaxation of isolated human placental resistance vessels. *Regul. Pept.* **2004**, *117*, 123–126. [[CrossRef](#)] [[PubMed](#)]
64. Pal, S.; Wu, J.; Murray, J.K.; Gellman, S.H.; Wozniak, M.A.; Keely, P.J.; Boyer, M.E.; Gomez, T.M.; Hasso, S.M.; Fallon, J.F.; et al. An antiangiogenic neurokinin-B/thromboxane A2 regulatory axis. *J. Cell Biol.* **2006**, *174*, 1047–1058. [[CrossRef](#)]
65. Page, N.M. Neurokinin B and pre-eclampsia: A decade of discovery. *Reprod. Biol. Endocrinol.* **2010**, *8*, 4. [[CrossRef](#)]
66. Hoang, V.M.; Foulk, R.; Clauser, K.; Burlingame, A.; Gibson, B.W.; Fisher, S.J. Functional Proteomics: Examining the Effects of Hypoxia on the Cytotrophoblast Protein Repertoire. *Biochemistry* **2001**, *40*, 4077–4086. [[CrossRef](#)]
67. Sawicki, G.; Dakour, J.; Morrish, D.W. Functional proteomics of neurokinin B in the placenta indicates a novel role in regulating cytotrophoblast antioxidant defences. *Proteomics* **2003**, *3*, 2044–2051. [[CrossRef](#)]
68. Xie, F.; von Dadelszen, P.; Nadeau, J. CMV infection, TLR-2 and -4 expression, and cytokine profiles in early-onset preeclampsia with HELLP syndrome. *Am. J. Reprod. Immunol.* **2014**, *71*, 379–386. [[CrossRef](#)]
69. Molvarec, A.; Szarka, A.; Walentin, S.; Bekő, G.; Karádi, I.; Prohászka, Z.; Rigó, J., Jr. Serum leptin levels in relation to circulating cytokines, chemokines, adhesion molecules and angiogenic factors in normal pregnancy and preeclampsia. *Reprod. Biol. Endocrinol.* **2011**, *9*, 124. [[CrossRef](#)]
70. Wallukat, G.; Homuth, V.; Fischer, T.; Lindschau, C.; Horstkamp, B.; Jüpner, A.; Baur, E.; Nissen, E.; Vetter, K.; Neichel, D.; et al. Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor. *J. Clin. Invest.* **1999**, *103*, 945–952. [[CrossRef](#)]
71. Chaiworapongsa, T.; Romero, R.; Yoshimatsu, J.; Espinoza, J.; Kim, Y.M.; Park, K.; Kalache, K.; Edwin, S.; Bujold, E.; Gomez, R. Soluble adhesion molecule profile in normal pregnancy and pre-eclampsia. *J. Matern. Neonatal Med.* **2002**, *12*, 19–27. [[CrossRef](#)] [[PubMed](#)]

72. Bretelle, F.; Sabatier, F.; Blann, A.; D'Ercole, C.; Boutière, B.; Mutin, M.; Boubli, L.; Sampol, J.; Dignat-George, F. Maternal endothelial soluble cell adhesion molecules with isolated small for gestational age fetuses: Comparison with pre-eclampsia. *Br. J. Obstet. Gynaecol.* **2001**, *108*, 1277–1282.
73. Francoual, J.; Audibert, F.; Trioche, P.; Chalas, J.; Capel, L.; Lindenbaum, A.; Labrune, P.; Frydman, R. Erratum: Is a Polymorphism of the Apolipoprotein E Gene Associated with Preeclampsia? *Hypertens. Pregnancy* **2003**, *21*, 127–133. [[CrossRef](#)] [[PubMed](#)]
74. Belo, L.; Gaffney, D.; Caslake, M.; Santos-Silva, A.; Pereira-Leite, L.; Quintanilha, A.; Rebelo, I. Apolipoprotein E and cholesteryl ester transfer protein polymorphisms in normal and preeclamptic pregnancies. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2004**, *112*, 9–15. [[CrossRef](#)]
75. Aguzzi, A.; Haass, C. Games played by rogue proteins in prion disorders and Alzheimer's disease. *Science* **2003**, *302*, 814–818. [[CrossRef](#)]
76. Soto, C. Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat. Rev. Neurosci.* **2003**, *4*, 49–60. [[CrossRef](#)]
77. Labbadia, J.; Morimoto, R.I. The Biology of Proteostasis in Aging and Disease. *Annu. Rev. Biochem.* **2015**, *84*, 435–464. [[CrossRef](#)]
78. Wiggins, R.C. Prions and the Transmissible Spongiform Encephalopathies. In *Metabolic Encephalopathy*; McCandless, D., Ed.; Springer: New York, NY, USA, 2009; pp. 531–550.
79. Belay, E.D. Transmissible Spongiform Encephalopathies in Humans. *Annu. Rev. Microbiol.* **1999**, *53*, 283–314. [[CrossRef](#)]
80. Geschwind, M.D.; Legname, G. Transmissible spongiform encephalopathies. In *Protein Misfolding in Neurodegenerative Diseases: Mechanisms and Therapeutic Strategies*; CRC Press; Smith, H.J., Simons, C., Sewell, R.D.E., Eds.; Taylor and Francis Group: Abingdon, UK, 2007; pp. 221–248.
81. Iwasaki, Y. Creutzfeldt-Jakob disease. *Neuropathology* **2017**, *2*, 174–188. [[CrossRef](#)]
82. Brown, K.; Mastrianni, J.A. The Prion Diseases. *J. Geriatr. Psychiatry Neurol.* **2010**, *23*, 277–298. [[CrossRef](#)]
83. Bhadbhade, A.; Cheng, D.W. Amyloid Precursor Protein Processing in Alzheimer's Disease. *Iran. J. Child Neurol.* **2012**, *34*, 185–204.
84. Iqbal, K.; Alonso, A.; Chen, S.; Chohan, M.O.; El-Akkad, E.; Gong, C.-X.; Khatoon, S.; Li, B.; Liu, F.; Rahman, A.; et al. Tau pathology in Alzheimer disease and other tauopathies. *Biochim. Biophys. Acta Mol. Basis Dis.* **2005**, *1739*, 198–210. [[CrossRef](#)] [[PubMed](#)]
85. Iqbal, K.; Liu, F.; Gong, C.-X.; Grundke-Iqbal, I. Tau in Alzheimer Disease and Related Tauopathies. *Curr. Alzheimer Res.* **2010**, *7*, 656–664. [[CrossRef](#)] [[PubMed](#)]
86. Tysnes, O.B.; Storstein, A. Epidemiology of Parkinson's disease. *J. Neural Transm.* **2017**, *124*, 901–905. [[CrossRef](#)] [[PubMed](#)]
87. Yasuda, T.; Nakata, Y.; Mochizuki, H. α -Synuclein and neuronal cell death. *Mol. Neurobiol.* **2013**, *47*, 466–483. [[CrossRef](#)] [[PubMed](#)]
88. Imran, M.; Mahmood, S. An overview of human prion diseases. *Virol. J.* **2011**, *8*, 559. [[CrossRef](#)]
89. Wu, C.; Wang, Z.; Lei, H.; Zhang, W.; Duan, Y. Dual binding modes of Congo red to amyloid protofibril surface observed in molecular dynamics simulations. *J. Am. Chem. Soc.* **2007**, *129*, 1225–1232. [[CrossRef](#)]
90. Frid, P.; Anisimov, S.V.; Popovic, N. Congo red and protein aggregation in neurodegenerative diseases. *Brain Res. Rev.* **2007**, *53*, 135–160. [[CrossRef](#)]
91. Naiki, H.; Higuchi, K.; Hosokawa, M.; Takeda, T. Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, thioflavine T. *Anal. Biochem.* **1989**, *177*, 244–249. [[CrossRef](#)]
92. Ivancic, V.A.; Ekanayake, O.; Lazo, N.D. Binding Modes of Thioflavin T on the Surface of Amyloid Fibrils Studied by NMR. *ChemPhysChem* **2016**, *17*, 2461–2464. [[CrossRef](#)]
93. Levine, H. Thioflavine t interaction with amyloid β sheet structures. *Amyloid* **1995**, *2*, 1–6. [[CrossRef](#)]
94. Kaye, R.; Head, E.; Sarsoza, F.; Saing, T.; Cotman, C.W.; Necula, M.; Margol, L.; Wu, J.; Breydo, L.; Thompson, J.L.; et al. Fibril specific, conformation dependent antibodies recognize a generic epitope common to amyloid fibrils and fibrillar oligomers that is absent in prefibrillar oligomers. *Mol. Neurodegener.* **2007**, *2*, 18. [[CrossRef](#)] [[PubMed](#)]
95. Gremer, L.; Schölzel, D.; Schenk, C.; Reinartz, E.; Labahn, J.; Ravelli, R.B.G.; Tusche, M.; Lopez-Iglesias, C.; Hoyer, W.; Heise, H.; et al. Fibril structure of amyloid- β (1–42) by cryo-electron microscopy. *Science* **2017**, *358*, 116–119. [[CrossRef](#)] [[PubMed](#)]

96. Sunde, M.; Blake, C. The structure of amyloid fibrils by electron microscopy and x-ray diffraction. *Adv. Protein Chem.* **1997**, *50*, 123–159. [[PubMed](#)]
97. Chandramowlishwaran, P.; Sun, M.; Casey, K.L.; Romanyuk, A.V.; Grizel, A.V.; Sopova, J.V.; Rubel, A.A.; Nussbaum-Krammer, C.; Vorberg, I.M.; Chernoff, Y.O. Mammalian amyloidogenic proteins promote prion nucleation in yeast. *J. Biol. Chem.* **2018**, *293*, 3436–3450. [[CrossRef](#)] [[PubMed](#)]
98. Rubel, A.A.; Ryzhova, T.A.; Antonets, K.S.; Chernoff, Y.O.; Galkin, A.P. Identification of PrP sequences essential for the interaction between the PrP polymers and A β peptide in a yeast-based assay. *Prion* **2013**, *7*, 469–476. [[CrossRef](#)]
99. Buhimschi, I.A.; Zhao, G.; Funai, E.F.; Harris, N.; Sasson, I.E.; Bernstein, I.M.; Saade, G.R.; Buhimschi, C.S. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *Am. J. Obstet. Gynecol.* **2008**, *199*, e1–e551. [[CrossRef](#)]
100. Millen, K.R.; Buhimschi, C.S.; Zhao, G.; Rood, K.M.; Tabbah, S.; Buhimschi, I.A. Serum and Urine Thioflavin-T-Enhanced Fluorescence in Severe Preeclampsia. *Hypertension* **2018**, *71*, 1185–1192. [[CrossRef](#)]
101. Buhimschi, I.A.; Nayeri, U.A.; Zhao, G.; Shook, L.L.; Pensalfini, A.; Funai, E.F.; Bernstein, I.M.; Glabe, C.G.; Buhimschi, C.S. Protein misfolding, congophilia, oligomerization, and defective amyloid processing in preeclampsia. *Sci. Transl. Med.* **2014**, *6*, 245ra92. [[CrossRef](#)]
102. Tong, M.; Cheng, S.; Chen, Q.; DeSousa, J.; Stone, P.R.; James, J.L.; Chamley, L.W.; Sharma, S. Aggregated transthyretin is specifically packaged into placental nano-vesicles in preeclampsia. *Sci. Rep.* **2017**, *7*, 6694. [[CrossRef](#)]
103. Cater, J.H.; Kumita, J.R.; Zeineddine Abdallah, R.; Zhao, G.; Bernardo-Gancedo, A.; Henry, A.; Winata, W.; Chi, M.; Grenyer, B.S.F.; Townsend, M.L.; et al. Human pregnancy zone protein stabilizes misfolded proteins including preeclampsia- and Alzheimer's-associated amyloid beta peptide. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 6101–6110. [[CrossRef](#)] [[PubMed](#)]
104. Cheng, S.-B.; Nakashima, A.; Sharma, S. Understanding Pre-Eclampsia Using Alzheimer's Etiology: An Intriguing Viewpoint. *Am. J. Reprod. Immunol.* **2016**, *75*, 372–381. [[CrossRef](#)] [[PubMed](#)]
105. Tartaglia, G.G.; Pechmann, S.; Dobson, C.M.; Vendruscolo, M. Life on the edge: A link between gene expression levels and aggregation rates of human proteins. *Trends Biochem. Sci.* **2007**, *32*, 204–206. [[CrossRef](#)] [[PubMed](#)]
106. Mao, X.R.; Crowder, C.M. Protein Misfolding Induces Hypoxic Preconditioning via a Subset of the Unfolded Protein Response Machinery. *Mol. Cell. Biol.* **2010**, *30*, 5033–5042. [[CrossRef](#)] [[PubMed](#)]
107. Paschen, W.; Mengesdorf, T. Endoplasmic reticulum stress response and neurodegeneration. *Cell Calcium* **2005**, *38*, 409–415. [[CrossRef](#)] [[PubMed](#)]
108. Jian, B.; Hsieh, C.H.; Chen, J.; Choudhry, M.; Bland, K.; Chaudry, I.; Raju, R. Activation of endoplasmic reticulum stress response following trauma-hemorrhage. *Biochim. Biophys. Acta Mol. Basis Dis.* **2008**, *1782*, 621–626. [[CrossRef](#)]
109. Kaye, R.; Head, E.; Thompson, J.L.; McIntire, T.M.; Milton, S.C.; Cotman, C.W.; Glabe, C.G. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* **2003**, *300*, 486–489. [[CrossRef](#)]
110. Yoshiike, Y.; Kaye, R.; Milton, S.C.; Takashima, A.; Glabe, C.G. Pore-forming proteins share structural and functional homology with amyloid oligomers. *Neuromol. Med.* **2007**, *9*, 270–275. [[CrossRef](#)]
111. Shirahama, T. High-resolution electron microscopic analysis of the amyloid fibril. *J. Cell Biol.* **1967**, *33*, 679–708. [[CrossRef](#)]
112. Kumar, S.; Dispenzieri, A.; Katzmman, J.A.; Larson, D.R.; Colby, C.L.; Lacy, M.Q.; Hayman, S.R.; Buadi, F.K.; Leung, N.; Zeldenrust, S.R.; et al. Serum immunoglobulin free light-chain measurement in primary amyloidosis: Prognostic value and correlations with clinical features. *Blood* **2010**, *116*, 5126–5129. [[CrossRef](#)]
113. Huntington, J.A. Serpin structure, function and dysfunction. *J. Thromb. Haemost.* **2011**, *9*, 26–34. [[CrossRef](#)] [[PubMed](#)]
114. Engström, G.; Janzon, L.; Berglund, G.; Lind, P.; Stavenow, L.; Hedblad, B.; Lindgärde, F. Blood Pressure Increase and Incidence of Hypertension in Relation to Inflammation-Sensitive Plasma Proteins. *Arterioscler. Thromb. Vasc. Biol.* **2002**, *22*, 2054–2058. [[CrossRef](#)] [[PubMed](#)]
115. Matheson, N.R.; Wong, P.S.; Travis, J. Enzymatic inactivation of human alpha-1-proteinase inhibitor by neutrophil myeloperoxidase. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 402–409. [[CrossRef](#)]

116. Lomas, D.A. Polymerisation underlies alpha1-antitrypsin deficiency, dementia and other serpinopathies. *Front. Biosci.* **2004**, *9*, 2873. [[CrossRef](#)] [[PubMed](#)]
117. Ramirez-Alvarado, M. Amyloid Formation in Light Chain Amyloidosis. *Curr. Top. Med. Chem.* **2013**, *12*, 2523–2533. [[CrossRef](#)]
118. Bellotti, V.; Mangione, P.; Merlini, G. Review: Immunoglobulin Light Chain Amyloidosis—The Archetype of Structural and Pathogenic Variability. *J. Struct. Biol.* **2000**, *130*, 280–289. [[CrossRef](#)]
119. Benson, M.D.; Liepnieks, J.J.; Kluve-Beckerman, B. Hereditary systemic immunoglobulin light-chain amyloidosis. *Blood* **2015**, *125*, 3281–3286. [[CrossRef](#)]
120. Blancas-Mejia, L.M.; Misra, P.; Dick, C.J.; Cooper, S.A.; Redhage, K.R.; Bergman, M.R.; Jordan, T.L.; Maar, K.; Ramirez-Alvarado, M. Immunoglobulin light chain amyloid aggregation. *Chem. Commun.* **2018**, *54*, 10664–10674. [[CrossRef](#)]
121. Buxbaum, J.N. The systemic amyloidoses. *Curr. Opin. Rheumatol.* **2004**, *16*, 67–75. [[CrossRef](#)]
122. Nunan, J.; Small, D.H. Regulation of APP cleavage by alpha-, beta- and gamma-secretases. *FEBS Lett.* **2000**, *483*, 6–10. [[CrossRef](#)]
123. Gouras, G.K.; Olsson, T.T.; Hansson, O. β -amyloid Peptides and Amyloid Plaques in Alzheimer's Disease. *Neurotherapeutics* **2015**, *12*, 3–11. [[CrossRef](#)] [[PubMed](#)]
124. Glabe, C.G. Common mechanisms of amyloid oligomer pathogenesis in degenerative disease. *Neurobiol. Aging* **2006**, *27*, 570–575. [[CrossRef](#)] [[PubMed](#)]
125. Pearson, H.A.; Peers, C. Physiological roles for amyloid β peptides. *J. Physiol.* **2006**, *575*, 5–10. [[CrossRef](#)] [[PubMed](#)]
126. Haass, C.; Selkoe, D.J. Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. *Cell* **1993**, *75*, 1039–1042. [[CrossRef](#)]
127. Clariss, H.J.; Key, B.; Beyreuther, K.; Masters, C.L.; Small, D.H. Expression of the amyloid protein precursor of Alzheimer's disease in the developing rat olfactory system. *Brain Res. Dev. Brain Res.* **1995**, *88*, 87–95. [[CrossRef](#)]
128. Muresan, V.; Varvel, N.H.; Lamb, B.T.; Muresan, Z. The cleavage products of amyloid-beta precursor protein are sorted to distinct carrier vesicles that are independently transported within neurites. *J. Neurosci.* **2009**, *29*, 3565–3578. [[CrossRef](#)]
129. Cole, S.L.; Vassar, R. The Alzheimer's disease Beta-secretase enzyme, BACE1. *Mol. Neurodegener.* **2007**, *2*, 22. [[CrossRef](#)]
130. Ando, Y.; Coelho, T.; Berk, J.L.; Cruz, M.W.; Ericzon, B.-G.; Ikeda, S.; Lewis, W.D.; Obici, L.; Planté-Bordeneuve, V.; Rapezzi, C.; et al. Guideline of transthyretin-related hereditary amyloidosis for clinicians. *Orphanet J. Rare Dis.* **2013**, *8*, 31. [[CrossRef](#)]
131. Kalkunte, S.S.; Neubeck, S.; Norris, W.E.; Cheng, S.-B.; Kostadinov, S.; Vu Hoang, D.; Ahmed, A.; von Eggeling, F.; Shaikh, Z.; Padbury, J.; et al. Transthyretin Is Dysregulated in Preeclampsia, and Its Native Form Prevents the Onset of Disease in a Preclinical Mouse Model. *Am. J. Pathol.* **2013**, *183*, 1425–1436. [[CrossRef](#)]
132. Kalkunte, S.; Boij, R.; Norris, W.; Friedman, J.; Lai, Z.; Kurtis, J.; Lim, K.-H.; Padbury, J.F.; Matthiesen, L.; Sharma, S. Sera from Preeclampsia Patients Elicit Symptoms of Human Disease in Mice and Provide a Basis for an in Vitro Predictive Assay. *Am. J. Pathol.* **2010**, *177*, 2387–2398. [[CrossRef](#)]
133. Wyatt, A.R.; Yerbury, J.J.; Ecroyd, H.; Wilson, M.R. Extracellular Chaperones and Proteostasis. *Annu. Rev. Biochem.* **2013**, *82*, 295–322. [[CrossRef](#)] [[PubMed](#)]
134. Humphreys, D.T.; Carver, J.A.; Easterbrook-Smith, S.B.; Wilson, M.R. Clusterin Has Chaperone-like Activity Similar to That of Small Heat Shock Proteins. *J. Biol. Chem.* **1999**, *274*, 6875–6881. [[CrossRef](#)] [[PubMed](#)]
135. Yerbury, J.J.; Poon, S.; Meehan, S.; Thompson, B.; Kumita, J.R.; Dobson, C.M.; Wilson, M.R. The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures. *FASEB J.* **2007**, *21*, 2312–2322. [[CrossRef](#)] [[PubMed](#)]
136. Yerbury, J.J.; Kumita, J.R.; Meehan, S.; Dobson, C.M.; Wilson, M.R. α 2-Macroglobulin and Haptoglobin Suppress Amyloid Formation by Interacting with Prefibrillar Protein Species. *J. Biol. Chem.* **2009**, *284*, 4246–4254. [[CrossRef](#)] [[PubMed](#)]
137. Blacker, D.; Wilcox, M.A.; Laird, N.M.; Rodes, L.; Horvath, S.M.; Go, R.C.P.; Perry, R.; Watson, B.; Bassett, S.S.; McInnis, M.G.; et al. Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nat. Genet.* **1998**, *19*, 357–360. [[CrossRef](#)] [[PubMed](#)]

138. Ekelund, L.; Laurell, C.B. The pregnancy zone protein response during gestation: A metabolic challenge. *Scand. J. Clin. Lab. Investig.* **1994**, *54*, 623–629. [[CrossRef](#)]
139. Tatarinov, I.S.; Mesnianskina, N.V.; Nikulina, D.M. Immunochemical identification of beta globulin of the “pregnancy zone” in the blood serum of patients with hydatid mole and chorioepithelioma]. *Akush. Ginekolog.* **1974**, *5*, 67–68.
140. Tatarinov, I.S.; Masiukevich, V.N.; Mesnianskina, N.V.; Parfenova, L.F. Immunochemical identification of a new alpha 2 globulin in the blood serum of pregnant women]. *Akush. Ginekolog.* **1970**, *46*, 25–28.
141. Perni, U.; Sison, C.; Sharma, V.; Helseth, G.; Hawfield, A.; Suthanthiran, M.; August, P. Angiogenic Factors in Superimposed Preeclampsia. *Hypertension* **2012**, *59*, 740–746. [[CrossRef](#)]
142. Rana, S.; Cerdeira, A.S.; Wenger, J.; Salahuddin, S.; Lim, K.-H.; Ralston, S.J.; Thadhani, R.I.; Karumanchi, S.A. Plasma Concentrations of Soluble Endoglin versus Standard Evaluation in Patients with Suspected Preeclampsia. *PLoS ONE* **2012**, *7*, e48259. [[CrossRef](#)]
143. Akolekar, R.; Syngelaki, A.; Poon, L.; Wright, D.; Nicolaides, K.H. Competing risks model in early screening for preeclampsia by biophysical and biochemical markers. *Fetal Diagn. Ther.* **2013**, *33*, 8–15. [[CrossRef](#)] [[PubMed](#)]
144. Sergeeva, V.A.; Zakharova, N.V.; Bugrova, A.E.; Starodubtseva, N.L.; Indeykina, M.I.; Kononikhin, A.S.; Frankevich, V.E.; Nikolaev, E.N. The high-resolution mass spectrometry study of the protein composition of amyloid-like urine aggregates associated with preeclampsia. *Eur. J. Mass Spectrom.* **2019**, 146906671986007. [[CrossRef](#)] [[PubMed](#)]
145. Yakupova, E.I.; Bobyleva, L.G.; Vikhlyantsev, I.M.; Bobylev, A.G. Congo Red and amyloids: History and relationship. *Biosci. Rep.* **2019**, *39*, BSR20181415. [[CrossRef](#)] [[PubMed](#)]
146. Halimi, M.; Dayan-Amouyal, Y.; Kariv-Inbal, Z.; Friedman-Levi, Y.; Mayer-Sonnenfeld, T.; Gabizon, R. Prion urine comprises a glycosaminoglycan-light chain IgG complex that can be stained by Congo red. *J. Virol. Methods* **2006**, *133*, 205–210. [[CrossRef](#)]
147. Rood, K.M.; Buhimschi, C.S.; Dible, T.; Webster, S.; Zhao, G.; Samuels, P.; Buhimschi, I.A. Congo Red Dot Paper Test for Antenatal Triage and Rapid Identification of Preeclampsia. *EClinicalMedicine* **2019**, *8*, 47–56. [[CrossRef](#)]



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).



Urinary Congophilia Confirmed With the CapCord Test Is Associated With Pregnancy Outcomes in Women With Early-Onset Pre-eclampsia

Benshuo Cai¹, Xiaoying Yuan², Xingmin Li², Jun Xu² and Juan Du^{1*}

¹ Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, Shenyang, China, ² Shuwen Biotech Company Ltd., Deqing, China

OPEN ACCESS

Edited by:

Zaleha Abdullah Mahdy,
National University of
Malaysia, Malaysia

Reviewed by:

Jagidesa Moodley,
University of KwaZulu-Natal,
South Africa
Shuhaila Ahmad,
National University of
Malaysia, Malaysia

*Correspondence:

Juan Du
duj@sj-hospital.org

Specialty section:

This article was submitted to
Obstetrics and Gynecology,
a section of the journal
Frontiers in Medicine

Received: 25 April 2021

Accepted: 12 July 2021

Published: 02 August 2021

Citation:

Cai B, Yuan X, Li X, Xu J and Du J
(2021) Urinary Congophilia Confirmed
With the CapCord Test Is Associated
With Pregnancy Outcomes in Women
With Early-Onset Pre-eclampsia.
Front. Med. 8:700157.
doi: 10.3389/fmed.2021.700157

Background: The association between misfolded proteins presented in the urine of pregnant women and pregnancy outcomes associated with early-onset pre-eclampsia (PE) remains unclear. This study aimed to investigate this association to examine the predictive value of urinary congophilia in the prognostication of pregnancy outcomes in this patient group in the Chinese population.

Materials and Methods: This study included 1,397 patients, of which 46, 147, and 8 patients had gestational hypertension, PE, and chronic hypertension, respectively, and 1,196 were healthy controls undergoing the CapCord test for urinary congophilia. Patients with PE were divided into early- and late-onset groups. Patients with early-onset PE were further divided into iatrogenic prematurity and full-term delivery groups, the rates of urinary congophilia were compared between the groups; additionally, this patient group was divided into positive and negative urinary congophilia groups, clinical characteristics and pregnancy outcomes were compared between the groups. Univariate and multivariate logistic regression analyses were performed.

Results: A total of 113 (76.9%) of 147 patients in the PE group had urinary congophilia; this rate was higher than that observed in the other three groups ($\chi^2 = 780.892$, $p < 0.001$). Gestational age in the early-onset PE group at both onset and delivery was lower than that in the late-onset PE group ($p < 0.001$). The rates of iatrogenic prematurity and hemolysis, elevated liver enzymes, and low platelet count syndrome were both higher in the early-onset PE group than in the late-onset PE group ($p < 0.001$, $p < 0.05$). In addition, the rate of urinary congophilia in the early-onset PE group was higher than that in the late-onset PE group ($\chi^2 = 13.297$, $p < 0.001$). Urinary congophilia was an independent risk factor for iatrogenic prematurity among patients with early-onset PE in both univariate [odds ratio (OR) 17.143, 95% confidence interval (CI): 4.719–62.271; $p < 0.001$] and multivariate (OR 18.174; 95% CI: 4.460–74.063; $p < 0.001$) analyses. Patients with early-onset PE and urinary congophilia were more likely than their counterparts without urinary congophilia to deliver at a lower gestational age, present with iatrogenic prematurity, and have a shorter latency period between onset and delivery.

Conclusion: Urinary congophilia confirmed with the CapCord test may help predict pregnancy outcomes in patients with early-onset PE.

Keywords: congophilia, misfolded protein, early-onset pre-eclampsia, late-onset pre-eclampsia, pregnancy outcome

INTRODUCTION

Pre-eclampsia (PE) is the leading contributor to maternal and fetal mortality worldwide (1). It is defined as new-onset hypertension and proteinuria after 20 weeks of gestation (2), accounting for 17–24% of maternal deaths in low-income settings (3). Untreated PE may lead to eclampsia, renal damage, cerebrovascular accidents, microangiopathic hemolytic anemia, liver failure, and pulmonary edema, all of which increase the risk of maternal death (4, 5). Delivery is the only effective treatment for PE; however, preventing stillbirth and iatrogenic prematurity remains a challenge. Depending on the gestational age at onset, PE is classified as early- (or placental) or late-onset (or maternal) (6, 7); these subtypes appear to have different etiologies. Specifically, early-onset PE is associated with abnormal placentation; by contrast, late-onset PE is associated with an interaction between a presumably normal placenta and maternal factors such as endothelial dysfunction and microvascular damage (8, 9). The pathogenesis of PE is yet to be elucidated; however, some evidence suggests that uteroplacental hypoperfusion may lead to PE (10, 11). Meanwhile, impaired spiral artery remodeling in the uterus, which may lead to the release of antiangiogenic factors from the ischemic placenta into the maternal circulation, is a two-stage model considered central to research in the pathogenesis of PE (12).

In human cells, linear amino acid multimers can be converted into functional proteins with three-dimensional structures able to perform their function. Some diseases are associated with protein folding disturbance, which results in the formation of misfolded proteins, such as in Alzheimer's and Parkinson's diseases, and prion diseases (13–18). Recent studies have shown that misfolded proteins may accumulate in the urine, serum, and placenta of patients with PE (19–24). Urinary misfolded proteins can be detected by a point-of-care urinary Congo red test (25). Congo red is a kind of synthetic diazo dye with specific affinity for misfolded proteins (26–28). The affinity of misfolded proteins to Congo red is known as congophilia (29, 30). Buhimschi et al. proposed that urine samples of women with PE exhibited congophilia; additionally, the rate of urinary congophilia was higher among women with severe PE with indications for delivery than among their counterparts that were either healthy or diagnosed with chronic or gestational hypertension (21). However, the origin of misfolded proteins in the urine of women with PE remains unclear. Placental hypoxia and ischemia resulting from impaired placentation in PE may lead to endoplasmic reticulum stress (ERS) in the placenta (31–33). ERS may lead to chronic activation of unfolded protein response (UPR) pathways (34–36), which aim to restore endoplasmic reticulum homeostasis by removing the misfolded proteins. The activation of placental UPR occurs in early- but

not in late-onset PE or normotensive controls (33). Based on this evidence, we hypothesized that the placenta may be the main source of the misfolded proteins in the urine of patients with early-onset PE and the presence of misfolded proteins in the urine may be linked to the pathogenesis of early-onset PE but not late-onset PE. In this study, we, for the first time, compared the urinary congophilia of patients with different types of PE and normotensive controls in the Chinese population to investigate the association between the presence of misfolded proteins in the urine and early-onset PE, as well as the possible origin of the misfolded proteins in the urine in the Chinese population. Furthermore, we examined the association of urinary congophilia with the pregnancy outcomes in Chinese patients with early-onset PE to assess the predictive value of urinary congophilia in the prognostication of pregnancy outcomes in this patient group.

MATERIALS AND METHODS

Study Design

The protocol of this study was approved by the Institutional Review Board of Shengjing Hospital of China Medical University, Shenyang, Liaoning Province, China (Approval number: 2018PS195K). The need for obtaining informed consent was waived owing to the use of residual urine samples and the minimal risks involved. The study was conducted according to the principles expressed in the Helsinki Declaration.

Pregnant women aged ≥ 18 years and at the gestational age of ≥ 20 weeks, admitted to our hospital between May 2017 and August 2018 were eligible for this study. Included patients were divided into gestational hypertensive, PE, chronic hypertensive, and normotensive groups. The PE group was further subdivided into early- and late-onset groups. The patients' clinical characteristics and rates of urinary congophilia were compared among the groups. Pregnancy outcomes of the patients with early-onset PE were categorized as iatrogenic prematurity and full-term delivery. Patients with early-onset PE were further subdivided into positive and negative urinary congophilia groups.

Diagnostic Criteria

Hypertensive disorders of pregnancy were determined according to the 2018 International Society for the Study of Hypertension in Pregnancy Classification, Diagnosis, and Management Recommendations for International Practice (2).

Women were excluded from the present study if they were diagnosed with any of the followings: diabetes mellitus, respiratory disease, blood system disease, liver disease, renal disease, heart disease, fetal genetic and congenital malformation, abortion or fetal death, twin or multiple pregnancy, history

of assisted reproductive technology use, infections or pro-inflammatory states, autoimmune disease, or cancer. Women with incomplete clinical information were also excluded. Early- and late-onset PE were defined by the gestational age at onset of disease, <34 and ≥ 34 weeks, respectively (6).

Urine Sample and Clinical Data Collection

Midstream urine samples were collected from all patients for the assessment of congophilina. In patients with hypertensive disorders of pregnancy, urine samples were collected at the time of disease onset. All patients with hypertensive disorders of pregnancy were followed-up until post-delivery. Data on maternal age, gestational age at PE onset and delivery, pregnancy outcomes, and the presence of the hemolysis, elevated liver enzymes, and low platelet count syndrome (HELLP syndrome) were collected. Investigators were blinded to any personal data during congophilina measurement.

Detection of Misfolded Proteins in Urine

A point-of-care device employing the capillary tube-based slow release method (the CapCord test, available from Shuwen Biotech, Zhejiang, China) was used to detect misfolded proteins in urine samples (25). Each scorer classified the pattern of the dye into six categories relative to the reference pattern, based on the evenness of the spread and tendency of the dye to concentrate in a limited central area (Figure 1). All scorers were trained to ensure the consistency of approach.

Statistical Methods

Clinical characteristics and pregnancy outcomes were compared among the groups, using the analysis of variance or Mann-Whitney *U*-test. The difference in counts among the groups was assessed with the chi-squared test or Fisher's exact probability test. Univariate and multivariate logistic regression analyses were performed. Survival curves were compared using the Kaplan-Meier method and the log-rank test. *P*-values < 0.05 were indicative of significant findings. All statistical analyses were performed using the Statistical Product and Service Solutions (SPSS) software ver. 23.0 (IBM Corp., Armonk, NY, USA).

RESULTS

This study included 1,397 patients. The CapCord test was performed for all patients. Table 1 presents the incidence of urinary congophilina in four groups. The differences of the rates of positive urinary congophilina among different groups were significant.

Table 2 presents comparisons of the clinical characteristics and rates of urinary congophilina between early- and late-onset PE groups. The gestational age of the early-onset PE group at both PE onset and delivery was significantly lower than that of the late-onset PE group. The rates of iatrogenic prematurity and HELLP syndrome were both significantly higher in the early- than in the late-onset PE group. In addition, the rate of urinary congophilina in the early-onset PE group was significantly different from that in the late-onset PE group.

Urinary congophilina was an independent risk factor for iatrogenic prematurity in both univariate [odds ratio (OR)

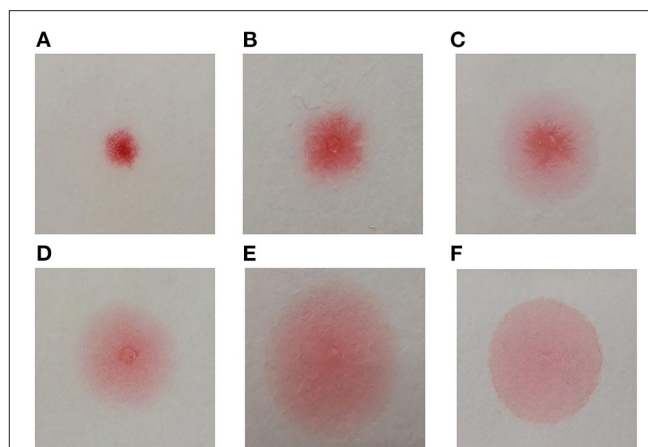


FIGURE 1 | Congo red bound to misfolded proteins in an aqueous solution migrates differentially on cellulose membrane, forming different dyeing patterns. The differences are especially apparent when the solution is slowly released into small area on the cellulose membrane through a fine-tipped capillary tube. The more Congo red is bound to misfolded proteins, the dye spreads more evenly on the membrane. The device we used included a plastic pipette to drop urine to a well-containing Congo red (0.1 mg/ml), and a capillary applicator to transfer the mixture to cellulose membrane compartment and slowly released. The test produces a result within 3 min. Classification of Congo red staining patterns (A) Small non-diffused red dot; (B) Mildly diffused dot, scarlet pseudopodium; (C) Diffused dot, scarlet pseudopodium, pink penumbra; (D) Small dot, irregular partly diffused pale red penumbra; (E) Red and scarlet dot, partly diffused pale red penumbra; (F) Large uniform pale diffused dot. The patterns (D–F) were classified as “positive” and patterns (A–C) as “negative.”

17.143; 95% confidence interval (CI) 4.719–62.271; $p = 0.000$] (Table 3) and multivariate (OR 18.174; 95% CI 4.460–74.063; $p = 0.000$) analyses.

A significant difference was found in the gestational age at delivery between patients with and without urinary congophilina in the early-onset PE group. The positive urinary congophilina group presented with a shorter latency period between onset and delivery than did the negative urinary congophilina group; finally, a significant difference was observed in the rate of iatrogenic prematurity between the groups with and without urinary congophilina within the early-onset PE group (Table 4).

In total, 86 patients with iatrogenic prematurity comprised the early-onset PE group. The Kaplan-Meier survival curves showed higher rates of iatrogenic prematurity among patients with urinary congophilina than among their counterparts without this condition ($\chi^2 = 15.976$, $p < 0.001$) (Figure 2). These findings suggest that pregnancy outcomes are poorer in early-onset PE patients with urinary congophilina than in their counterparts without this condition.

DISCUSSION

Previous studies on the presence of misfolded proteins in PE patients mostly involved Western populations (19–21, 37–39). The present study confirms that urinary congophilina occurs in Chinese women with PE. The

TABLE 1 | Incidence of positive urinary congophilina in four groups.

Variables	Gestational hypertensive (n = 46)	Pre-eclampsia (n = 147)	Chronic hypertensive (n = 8)	Normotensive (n = 1,196)	χ^2	P-value
Rate of positive urinary congophilina	1 (2.2%)	113 (76.9%) *	0 (0%)	31 (2.6%)	780.892	<0.001

Values are n and n/N (%) *p < 0.001 vs. gestational hypertensive, chronic hypertensive, and normotensive (chi-squared test or Fisher exact probability test).

TABLE 2 | Clinical characteristics and incidence of positive urinary congophilina in early- and late-onset pre-eclampsia groups.

Variables	Early-onset (n = 102)	Late-onset (n = 45)	Z	χ^2	P-value
Gestational age at onset	28.8 (26, 31.3)	37 (35.3, 38.2)	-9.653	/	<0.001
Gestational age at delivery	32.6 (30.1, 35.4)	38.1 (36.8, 38.6)	-7.584	/	<0.001
Iatrogenic prematurity	86 (84.3%)	11 (24.4%)	/	49.865	<0.001
HELLP syndrome	18 (17.6%)	1 (2.2%)	/	6.601	<0.05
CapCord test-positive	87 (85.3%)	26 (57.8%)		13.297	<0.001

Values are median (P25, P75), n or n/N (%). HELLP syndrome, hemolysis, elevated liver enzymes, and low platelet count syndrome.

TABLE 3 | Clinical characteristics of patients with early-onset PE and iatrogenic prematurity or full-term delivery.

Variables	Iatrogenic prematurity (n = 86)	Full-term (n = 16)	Univariate analysis	
			OR (95%CI)	P-value
Age (years)	30.8 ± 5.3	31.1 ± 4.5	0.989 (0.891, 1.098)	0.842
Gestational age at onset	28.1 ± 3.9	26.8 ± 5.9	1.069 (0.948, 1.205)	0.279
HELLP	17 (19.8%)	1 (6.3%)	3.696 (0.456, 29.958)	0.221
Rate of positive urinary congophilina	80/86 (93.0%)	7/16 (43.8%)	17.143 (4.719, 62.271)	<0.001

Values are mean ± standard deviation, n or n/N (%).

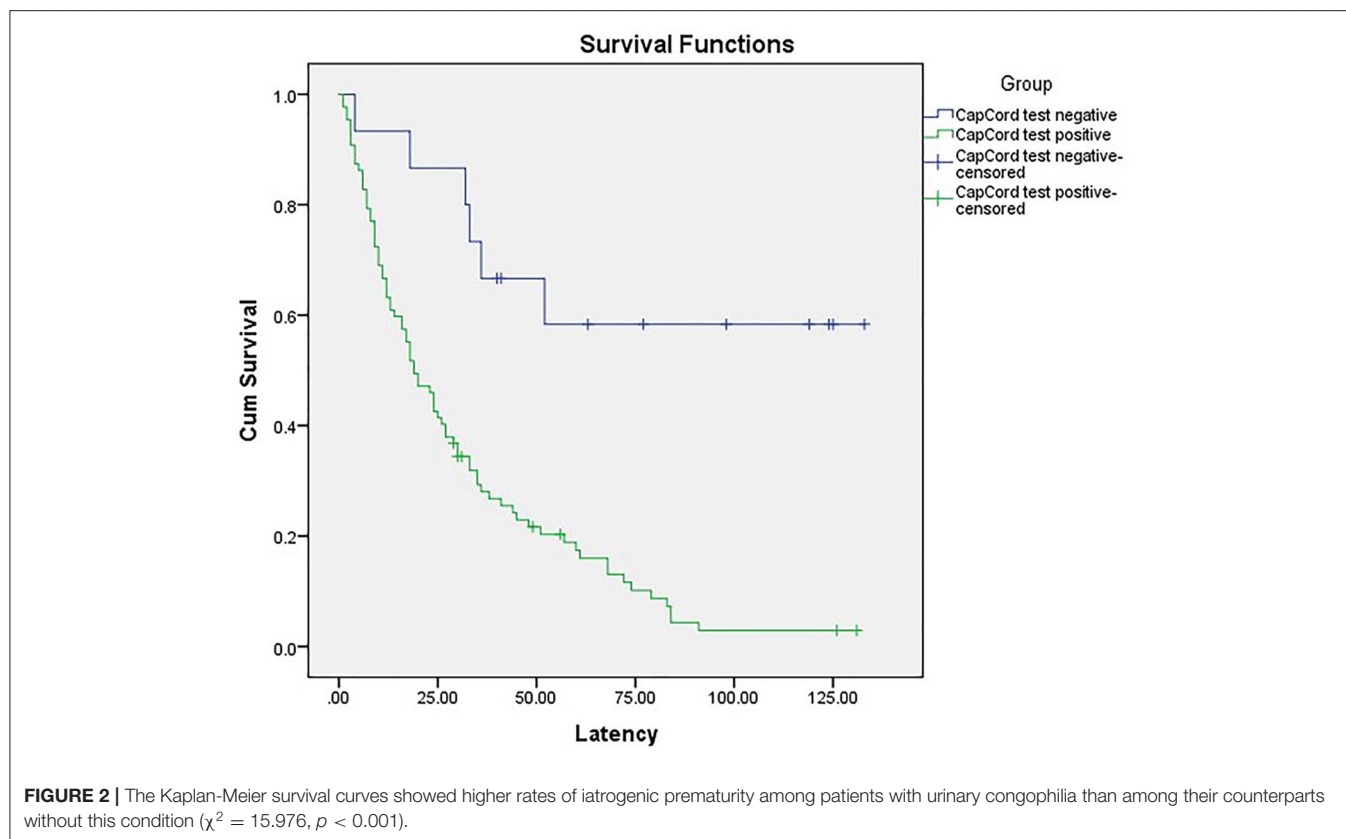
TABLE 4 | Clinical characteristics and pregnancy outcomes among patients with positive and negative urinary congophilina and early-onset pre-eclampsia.

Variables	Positive urinary congophilina (n = 87)	Negative urinary congophilina (n = 15)	F	Z	χ^2	P-value
Age (years)	30.7 ± 5.3	31.9 ± 3.6	0.635		/	0.428
Gestational age at onset (weeks)	28.1 ± 4.2	26.8 ± 4.8	1.167		/	0.283
Gestational age at delivery (weeks)	32.1 ± 3.4	36.2 ± 3.1	19.529		/	<0.001
Latency between onset and delivery (days)	19 (9, 38)	52 (33, 119)	/	-3.502	/	<0.001
HELLP syndrome (%)	17/87 (19.5%)	1/15 (6.7%)	/	/	0.708	0.400
Iatrogenic prematurity (%)	80/87 (92.0%)	6/15 (40%)	/	/	26.111	<0.001

Values are mean ± standard deviation, median (P25, P75), n or n/N (%).

present findings also support those previously reported by Buhimschi, wherein women with PE were more likely to have urinary congophilina than their counterparts with gestational hypertension, chronic hypertension, or normotension (37). Recent studies have shown that ischemia, hypoxia, and production of pro-inflammatory cytokines, all of which are associated with PE, can lead to protein misfolding (40) and initiate ERS (41, 42), which results in the chronic activation of UPRs. Yung et al. have shown that activation levels of placental UPR in patients with early-onset PE are significantly higher than

those in patients with late-onset PE or in normotensive controls, with similar values reported for the latter two groups (33). These findings provide molecular evidence that the production of placental misfolded proteins and placental ERS may contribute to early- but not to late-onset PE or normotensive controls. The present findings suggest that the rate of urinary congophilina in women with early-onset PE is significantly higher than that in the late-onset group and the normotensive group, indicating that the misfolded proteins in the urine may be linked to the pathogenesis of early-onset PE and the main source of misfolded proteins in



the urine of patients with early-onset PE may be the placenta. In addition, our finding that the rate of urinary congophilia in the late-onset PE group is significantly higher than that in the normotensive group indicates that the main source of the misfolded proteins in the urine of patients with late-onset PE is different from that of the patients in the normotensive group, and probably not the placenta. Furthermore, previous studies have demonstrated the presence of the same types of misfolded proteins in both the plasma and urine of PE patients (21, 43). Accordingly, we propose that the urinary misfolded proteins in late-onset PE may be derived from the plasma.

Studies by Buhimschi et al. have demonstrated that the assessment of urinary congophilia with the Congo red dot test is useful for the prognostication of medically indicated delivery in patients with PE, suggesting a close link between urinary congophilia and PE cases with severe maternal and fetal complications (33). In addition, previous studies have demonstrated that placental or early-onset PE is associated with a high risk of maternal and fetal complications (6, 44, 45). In the present study, the rate of urinary congophilia in the early-onset PE group was significantly higher than that in the late-onset group, suggesting an association between urinary congophilia and early-onset PE. Nevertheless, the present findings are inconsistent with those of Nagarajappa, whereby the Congo red retention value was lower in the early- than in the late-onset PE group. Furthermore, findings of this study suggest that urinary congophilia is an independent risk factor for iatrogenic prematurity in early-onset PE patients; in

fact, the present findings indicate that early-onset PE patients with urinary congophilia are at a higher risk of adverse pregnancy outcomes than are their counterparts without urinary congophilia. Based on the present findings, we propose that urinary congophilia, confirmed with the CapCord test, may support the prognostication of pregnancy outcomes in patients with early-onset PE. In other words, the urine samples of patients with early-onset PE could be collected at the time of disease onset to detect the congophilia with CapCord test, the patients with positive urinary congophilia are more likely to present adverse maternal and neonatal outcomes such as HELLP syndrome and iatrogenic prematurity, so the medically indicated delivery, as an effective treatment, should be expected as early as possible for those patients. Conversely, the patients with negative urinary congophilia probably present a relatively better maternal and neonatal outcome, so the supportive and expectant treatment should be preferred, and iatrogenic prematurity would be avoided as far as possible to extend the latency period between the onset and delivery.

In summary, results of this study suggest that the main sources of the urinary misfolded proteins in early- and late-onset PE seem different, the former may be the placenta, but the latter may be the plasma. To the best of our knowledge, this study is first to assess the association of urinary congophilia confirmed with the CapCord test with the pregnancy outcomes of Chinese patients with early-onset PE. Urinary congophilia confirmed with the CapCord test may be useful in the prognostication of pregnancy outcomes in patients with early-onset PE. However, this study

has some limitations. First, the study population was composed of Chinese patients; thus, the conclusion of this study may not be applicable to other populations. Second, the sample size is relatively small, and the error of the CapCord test and influence of gestational age on urinary congophilina are not excluded in this study. Thus, more studies that consider the influence of gestational age on urinary congophilina with CapCord test are needed to validate the present findings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Shengjing

Hospital of China Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BC: conceptualized the study and wrote the protocol and the manuscript. XY, XL, and JX: participated in the experiment and confirmed the experimental results. JD: interpreted the data and reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by Scientific Research Fund of Liaoning Provincial Education Department (CN). Award Number: FWZR2020009.

REFERENCES

- Duley L. The global impact of pre-eclampsia and eclampsia. *Semin Perinatol.* (2009) 33:130–7. doi: 10.1053/j.semperi.2009.02.010
- Brown MA, Magee LA, Kenny LC, Karumanchi SA, McCarthy FP, Saito S, et al. Hypertensive disorders of pregnancy: ISSHP classification, diagnosis, and management recommendations for international practice. *Hypertension.* (2018) 72:24–43. doi: 10.1161/HYPERTENSIONAHA.117.10803
- Wildman K, Bouvier-Colle MH, Group M. Maternal mortality as an indicator of obstetric care in Europe. *BJOG.* (2004) 111:164–9. doi: 10.1046/j.1471-0528.2003.00034.x-i1
- Sircar M, Thadhani R, Karumanchi SA. Pathogenesis of preeclampsia. *Curr Opin Nephrol Hypertens.* (2015) 24:131–8. doi: 10.1097/MNH.0000000000000105
- Ghulmiyyah L, Sibai B. Maternal mortality from preeclampsia/eclampsia. *Semin Perinatol.* (2012) 36:56–9. doi: 10.1053/j.semperi.2011.09.011
- von Dadelszen P, Magee LA, Roberts JM. Subclassification of preeclampsia. *Hypertens Pregnancy.* (2003) 22:143–8. doi: 10.1081/PRG-120021060
- Odegard RA, Vatten LJ, Nilsen ST, Salvesen KA, Austgulen R. Risk factors and clinical manifestations of pre-eclampsia. *BJOG.* (2000) 107:1410–6. doi: 10.1111/j.1471-0528.2000.tb11657.x
- Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med.* (2004) 350:672–83. doi: 10.1056/NEJMoa031884
- Masuyama H, Segawa T, Sumida Y, Masumoto A, Inoue S, Akahori Y, et al. Different profiles of circulating angiogenic factors and adipocytokines between early- and late-onset pre-eclampsia. *BJOG.* (2010) 117:314–20. doi: 10.1111/j.1471-0528.2009.02453.x
- Gilbert JS, Babcock SA, Granger JP. Hypertension produced by reduced uterine perfusion in pregnant rats is associated with increased soluble fms-like tyrosine kinase-1 expression. *Hypertension.* (2007) 50:1142–7. doi: 10.1161/HYPERTENSIONAHA.107.096594
- Makris A, Thornton C, Thompson J, Thomson S, Martin R, Ogle R, et al. Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1. *Kidney Int.* (2007) 71:977–84. doi: 10.1038/sj.ki.5002175
- Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. *Biol Reprod.* (2003) 69:1–7. doi: 10.1095/biolreprod.102.014977
- Aguzzi A, Haass C. Games played by rogue proteins in prion disorders and Alzheimer's disease. *Science.* (2003) 302:814–8. doi: 10.1126/science.1087348
- Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat Rev Neurosci.* (2003) 4:49–60. doi: 10.1038/nrn1007
- Labbadia J, Morimoto RI. The biology of proteostasis in aging and disease. *Annu Rev Biochem.* (2015) 84:435–64. doi: 10.1146/annurev-biochem-060614-033955
- Belay ED. Transmissible spongiform encephalopathies in humans. *Annu Rev Microbiol.* (1999) 53:283–314. doi: 10.1146/annurev.micro.53.1.283
- Iwasaki Y. Creutzfeldt-Jakob disease. *Neuropathology.* (2017) 37:174–88. doi: 10.1111/neup.12355
- Brown K, Mastrianni JA. The prion diseases. *J Geriatr Psychiatry Neurol.* (2010) 23:277–98. doi: 10.1177/0891988710383576
- Buhimschi IA, Zhao G, Funai EF, Harris N, Sasson IE, Bernstein IM, et al. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *Am J Obstet Gynecol.* (2008) 199:551 e1–16. doi: 10.1016/j.ajog.2008.07.006
- Millen KR, Buhimschi CS, Zhao G, Rood KM, Tabbah S, Buhimschi IA. Serum and urine thioflavin-T-enhanced fluorescence in severe preeclampsia. *Hypertension.* (2018) 71:1185–92. doi: 10.1161/HYPERTENSIONAHA.118.11034
- Buhimschi IA, Nayeri UA, Zhao G, Shook LL, Pensalfini A, Funai EF, et al. Protein misfolding, congophilina, oligomerization, and defective amyloid processing in preeclampsia. *Sci Transl Med.* (2014) 6:245ra92. doi: 10.1126/scitranslmed.3008808
- Tong M, Cheng SB, Chen Q, DeSousa J, Stone PR, James JL, et al. Aggregated transthyretin is specifically packaged into placental nano-vesicles in preeclampsia. *Sci Rep.* (2017) 7:6694. doi: 10.1038/s41598-017-07017-x
- Cater JH, Kumita JR, Zeineddine Abdallah R, Zhao G, Bernardo-Gancedo A, Henry A, et al. Human pregnancy zone protein stabilizes misfolded proteins including preeclampsia- and Alzheimer's-associated amyloid beta peptide. *Proc Natl Acad Sci USA.* (2019) 116:6101–10. doi: 10.1073/pnas.1817298116
- Cheng SB, Nakashima A, Sharma S. Understanding pre-eclampsia using Alzheimer's etiology: an intriguing viewpoint. *Am J Reprod Immunol.* (2016) 75:372–81. doi: 10.1111/aji.12446
- Li XM, Liu XM, Xu J, Du J, Cuckle H. Late pregnancy screening for preeclampsia with a urinary point-of-care test for misfolded proteins. *PLoS ONE.* (2020) 15:e0233214. doi: 10.1371/journal.pone.0233214
- Ashburn TT, Han H, McGuinness BF, Lansbury Jr PT. Amyloid probes based on congo red distinguish between fibrils comprising different peptides. *Chem Biol.* (1996) 3:351–8. doi: 10.1016/S1074-5521(96)90118-0
- Stopa B, Piekarska B, Konieczny L, Rybarska J, Spolnik P, Zemanek G, et al. The structure and protein binding of amyloid-specific dye reagents. *Acta Biochim Pol.* (2003) 50:1213–27. doi: 10.18388/abp.2003_3645
- Klunk WE, Pettegrew JW, Abraham DJ. Quantitative evaluation of congo red binding to amyloid-like proteins with a beta-pleated sheet conformation. *J Histochem Cytochem.* (1989) 37:1273–81. doi: 10.1177/37.8.2666510

29. Inouye H, Kirschner DA. Alzheimer's beta-amyloid: insights into fibril formation and structure from congo red binding. *Subcell Biochem.* (2005) 38:203–24. doi: 10.1007/0-387-23226-5_10
30. Buell AK, Dobson CM, Knowles TP, Welland ME. Interactions between amyloidophilic dyes and their relevance to studies of amyloid inhibitors. *Biophys J.* (2010) 99:3492–7. doi: 10.1016/j.bpj.2010.08.074
31. Burton GJ, Yung HW, Cindrova-Davies T, Charnock-Jones DS. Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. *Placenta.* (2009) 30(Suppl. A):S43–8. doi: 10.1016/j.placenta.2008.11.003
32. Mizuuchi M, Cindrova-Davies T, Olovsson M, Charnock-Jones DS, Burton GJ, Yung HW. Placental endoplasmic reticulum stress negatively regulates transcription of placental growth factor via ATF4 and ATF6beta: implications for the pathophysiology of human pregnancy complications. *J Pathol.* (2016) 238:550–61. doi: 10.1002/path.4678
33. Yung HW, Atkinson D, Campion-Smith T, Olovsson M, Charnock-Jones DS, Burton GJ. Differential activation of placental unfolded protein response pathways implies heterogeneity in causation of early- and late-onset pre-eclampsia. *J Pathol.* (2014) 234:262–76. doi: 10.1002/path.4394
34. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol.* (2012) 13:89–102. doi: 10.1038/nrm3270
35. Kaufman RJ, Scheuner D, Schroder M, Shen X, Lee K, Liu CY, et al. The unfolded protein response in nutrient sensing and differentiation. *Nat Rev Mol Cell Biol.* (2002) 3:411–21. doi: 10.1038/nrm829
36. Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature.* (2008) 454:455–62. doi: 10.1038/nature07203
37. McCarthy FP, Adetoba A, Gill C, Bramham K, Bertolaccini M, Burton GJ, et al. Urinary congophilia in women with hypertensive disorders of pregnancy and preexisting proteinuria or hypertension. *Am J Obstet Gynecol.* (2016) 215:464 e1–7. doi: 10.1016/j.ajog.2016.04.041
38. Nagarajappa C, Rangappa SS, Suryanarayana R, Balakrishna S. Urinary congophilia in preeclampsia: experience from a rural tertiary-care hospital in India. *Pregnancy Hypertens.* (2018) 13:83–6. doi: 10.1016/j.preghy.2018.05.006
39. Rood KM, Buhimschi CS, Dible T, Webster S, Zhao G, Samuels P, et al. Congo red dot paper test for antenatal triage and rapid identification of preeclampsia. *Eclin Med.* (2019) 8:47–56. doi: 10.1016/j.eclinm.2019.02.004
40. Mao XR, Crowder CM. Protein misfolding induces hypoxic preconditioning via a subset of the unfolded protein response machinery. *Mol Cell Biol.* (2010) 30:5033–42. doi: 10.1128/MCB.00922-10
41. Paschen W, Mengesdorf T. Endoplasmic reticulum stress response and neurodegeneration. *Cell Calcium.* (2005) 38:409–15. doi: 10.1016/j.ceca.2005.06.019
42. Jian B, Hsieh CH, Chen J, Choudhry M, Bland K, Chaudry I, et al. Activation of endoplasmic reticulum stress response following trauma-hemorrhage. *Biochim Biophys Acta.* (2008) 1782:621–6. doi: 10.1016/j.bbadis.2008.08.007
43. Buhimschi I, Zhao G, Saade G, Buhimschi CS. Evidence for alpha-1-antitrypsin (A1AT) polymerization in preeclampsia: a novel mechanism for endothelial cell injury. *Am J Obstet Gynecol.* (2006) 195:S150. doi: 10.1016/j.ajog.2006.10.526
44. Mongraw-Chaffin ML, Cirillo PM, Cohn BA. Preeclampsia and cardiovascular disease death: prospective evidence from the child health and development studies cohort. *Hypertension.* (2010) 56:166–71. doi: 10.1161/HYPERTENSIONAHA.110.150078
45. Paruk F, Moodley J. Maternal and neonatal outcome in early- and late-onset pre-eclampsia. *Semin Neonatol.* (2000) 5:197–207. doi: 10.1053/siny.2000.0023

Conflict of Interest: XY, XL, and JX are employed by Shuwen Biotech Company Ltd., China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Cai, Yuan, Li, Xu and Du. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Research Paper

Congo red test for identification of preeclampsia: Results of a prospective diagnostic case-control study in Bangladesh and Mexico

Hillary Bracken^{a,*}, Irina A. Buhimschi^b, Anisur Rahman^c, Patricio R. Sanhueza Smith^d, Jesmin Pervin^c, Salma Rouf^e, Manuel Bousiequez^a, Lourdes García López^f, Catalin S. Buhimschi^b, Thomas Easterling^g, Beverly Winikoff^a

^a Gynuity Health Projects, 220 East 42nd Street, Suite #710, New York, NY 10017, USA

^b Department of Obstetrics and Gynecology, University of Illinois at Chicago, College of Medicine, Chicago, IL 60612, USA

^c Maternal and Child Health Division, icddr, Dhaka, Bangladesh

^d Ministry of Health, Mexico City, Altadena 23, Nápoles, 03810 Ciudad de México, CDMX

^e Department of Obstetrics and Gynecology, Dhaka Medical College and Hospital, Dhaka, Bangladesh

^f Hospital Materno-Infantil Inguarán, Ministry of Health, Mexico City, Mexico

^g University of Washington, Seattle, WA 98195, USA

ARTICLE INFO

Article History:

Received 19 August 2020

Revised 17 November 2020

Accepted 24 November 2020

Available online 22 December 2020

ABSTRACT

Background: Misfolded proteins in the urine of women with preeclampsia bind to Congo Red dye (urine congophilia). We evaluated a beta prototype of a point-of-care test for the identification of urine congophilia in preeclamptic women.

Methods: Prospective diagnostic case-control study conducted in 409 pregnant women ($n = 204$ preeclampsia; $n = 205$ uncomplicated pregnancies) presenting for delivery in two tertiary level hospitals located in Bangladesh and Mexico. The GV-005, a beta prototype of a point-of-care test for detecting congophilia, was performed on fresh and refrigerated urine samples. The primary outcome was the prevalence of urine congophilia in each of the two groups. Secondary outcome was the likelihood of the GV-005 (index test) to confirm and rule-out preeclampsia based on an adjudicated diagnosis (reference standard).

Findings: The GV-005 was positive in 85% of clinical cases (83/98) and negative in 81% of clinical controls (79/98) in the Bangladesh cohort. In the Mexico cohort, the GV-005 test was positive in 48% of clinical cases (51/106) and negative in 77% of clinical controls (82/107). Adjudication confirmed preeclampsia in 92% of Bangladesh clinical cases (90/98) and 61% of Mexico clinical cases (65/106). The odds ratio of a urine congophilia in adjudicated cases versus controls in the Bangladesh cohort was 34.5 (14.7 – 81.1) ($p < 0.001$) compared to 4.2 (2.1 – 8.4; $p < 0.001$) in the Mexico cohort.

Interpretation: The GV-005, a beta prototype of a point-of-care test for detection of urine congophilia, is a promising tool for rapid identification of preeclampsia.

Funding: Saving Lives at Birth.

© 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

Introduction

Preeclampsia is a pregnancy-specific hypertensive disorder and a leading cause of maternal and perinatal morbidity and death worldwide: The World Health Organization (WHO) estimates that 16% of global maternal mortality (~ 63,000 maternal deaths annually) is due to preeclampsia [1]. Traditional diagnoses have relied upon clinical characteristics such as hypertension and proteinuria. However, urine dipsticks and evaluation of spot protein-to-creatinine (P:C ratio) are

known to associate with false positive and false negative results [2–4]. In high resource settings, ambiguity in diagnosis of preeclampsia generally leads to increased use of health-care resources with hospital admission for antenatal monitoring and increased early delivery for preeclampsia even when patients present with preeclampsia imitators [5]. In low-resource settings, diagnostic uncertainty may delay the receipt of appropriate care, contributing to near-miss events and high mortality [6].

Several inflammatory and placental biomarkers have been identified as potential tools for prediction or diagnosis of preeclampsia [7]. Commercially available tests for these biomarkers are laboratory-based, require a blood sample or are employed as part of complex

* Corresponding author.

E-mail address: hbracken@gynuity.org (H. Bracken).

Research in context

Evidence before this study

Preeclampsia is a pregnancy-specific hypertensive disorder and a leading cause of maternal and perinatal morbidity and mortality worldwide. Several inflammatory and placental biomarkers have been identified as potentially useful tools for the prediction or diagnosis of preeclampsia. Previous research showing that misfolded proteins in the urine of women with preeclampsia bind to Congo Red dye has been applied to develop laboratory-based techniques and a paperbased test for use at the bedside.

Added value of this study

This study reports the diagnostic characteristics of a prototype of a point-of-care test for detection of urine congophilia when used in low and middle income country setting populations. The findings suggest urine congophilia can be rapidly identified using a beta prototype of a lateral flow diagnostic device, GV-005.

Implications of all available evidence

Failure to diagnosis, misdiagnosis, delay in transfer, and receipt of unnecessary treatment contribute to high rates of maternal and neonatal morbidity and mortality associated with preeclampsia. Our study shows that a point-of-care diagnostic for detection of urine congophilia has the potential to improve the triage and diagnosis of patients with preeclampsia. A point-of-care test for the detection of urine congophilia could aid in the clinical care of preeclamptic women and reduce the morbidity and mortality associated with this disease.

algorithms making them impractical as point-of-care tests, especially for low-resource settings. A new diagnostic technology that circumvents these limitations may offer significant advantages if able to maintain the dipstick's ease of use. Women with preeclampsia excrete high amounts of misfolded protein in urine, as a consequence of increased protein misfolding load, a phenomenon more similar to Alzheimer's or prion disease [8,9]. These misfolded proteins have a propensity to bind to Congo Red (CR) dye (congophilia) [9]. Based on

this principle, our team developed and tested a urine paper-based point-of-care test for rapid screening and identification of preeclampsia, independent of clinical criteria [10,11]. The presence of congophilia among patients diagnosed with preeclampsia has been further validated by other groups in research laboratory settings using our initially reported nitrocellulose-based procedure [12–14] or a different device intended for point-of-care [15].

We hypothesized that preeclampsia is characterized by urine congophilia which can be rapidly identified using a beta prototype of a lateral flow diagnostic device, GV-005 (GestVision Inc, Groton, CT), for improved diagnosis of preeclampsia. The study objectives were: 1) to determine the prevalence of urine congophilia in tertiary facility settings in two low-resource countries (Bangladesh and Mexico) and 2) to compare the Congo Red test results to a clinical diagnosis of preeclampsia.

Methods

Study design and participants

We conducted a prospective diagnostic case-control study in the Labor and Delivery units at Dhaka Medical College in Dhaka, Bangladesh (April–July 2017) and the Hospital Materno Infantil Inguarán, Mexico City, Mexico from (July 2016 – September 2018). Both public hospitals serve as tertiary referral centers. Data collection was planned before the index test, GV-005, was performed. At both study sites, research assistants identified preeclampsia cases (with or without severe features) based on the facility's standard diagnostic criteria. For each preeclampsia case, a control patient was enrolled on the same day from women admitted for labor induction or elective caesarean section of a normal term baby. Women who agreed to provide a urine sample antepartum and were of the age eligible to consent were asked to participate. All participants provided individual written consent. The trial was approved by the Research and Ethics Committee at Dhaka Medical College and the Institutional Review Board of Mexico City's Secretariat of Health. This trial was registered as clinicaltrials.gov [NCT02381210](https://clinicaltrials.gov/NCT02381210).

Procedures

Research assistants approached eligible women immediately after confirmation of preeclampsia diagnosis or admission for delivery in

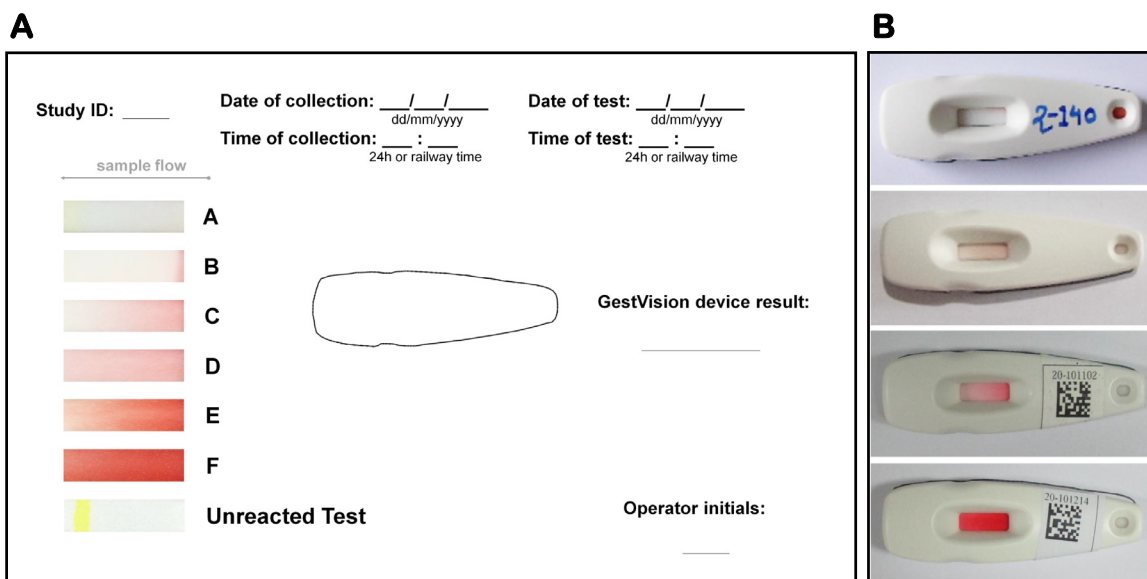


Fig. 1. Visual chromatic aid for interpretation of GV-005 and sample test devices.

the case of normal controls. After signing the consent form, the research assistant asked women to provide a urine (~10 mL) sample in a sterile 15 mL container. The research assistant labeled the sample with a unique study ID number and then transported it to a research laboratory within the same facility for immediate processing or refrigeration (contingent on time of collection and staff availability). Within 72-hours from urine collection, a laboratory technician (different than the research assistant) tested for congophilia using a research beta prototype lateral flow diagnostic device (GV-005, GestVision, USA) [16]. All laboratory staff was certified on the use of the device and interpretation of the result prior to trial initiation. The laboratory technician applied 100 microliters of urine to the test cassette using the test dropper, read the device at 3 min and scored its result on a six point scale (A-F) using a visual chromatic aid on the result recording sheet (Fig. 1). The technical performance of the GV-005 was investigated in a prior pilot study vis-à-vis the in-house paper test kit [9] and the research laboratory nitrocellulose procedure [17]. Additional information about this and the results obtained appear in Supplement A. Based on this, we considered scores A or B negative for congophilia and scores C-F positive for congophilia. Each sample was tested only once unless there was a technical issue (persistence of the blue control line) or the result was indeterminate. In this case the same sample was retested using a new device. Research laboratory staff was blinded to the clinical course of participating women and the clinicians and the research assistant were blinded to the GV-005 test results. The diagnosis of preeclampsia that resulted in the enrolment of cases and controls was left to the judgement of local providers and based on the standard diagnostic criteria at each facility. A standard proteinuria test was performed at multiple points during patient care, including at the time of diagnosis and at the time of urine collection for this trial. Similarly, clinicians undertook treatment decisions as per the local standard of care and all medical decisions were taken independent of the study protocol.

Research staff collected data on patient history, preeclampsia diagnosis, and labor and delivery outcomes transcribed from patient charts using standardized paper data collection forms. Incorrect classification of outcomes can result in the biased performance of a diagnostic test or reduced power [18]. Thus, each case and control was independently adjudicated by two obstetricians one of whom was a certified Maternal Fetal Medicine specialist. Similar approaches were employed at both sites. Both adjudicators were blinded to the congophilia test result and the enrolling provider's diagnosis. The adjudicators reviewed data collected on the trial's standard data collection forms and classified cases and controls using the American College of Obstetricians and Gynecologists (ACOG) Task Force definition of hypertensive disorders of pregnancy [19]. Details are provided in Figs. S1 and S2.

Main outcome measure

The primary outcome was the prevalence of urine congophilia in each of the two groups. Secondary outcome was the likelihood of the GV-005 (index test) to confirm and rule-out preeclampsia based on the adjudicated diagnosis (reference standard).

Statistical analysis

We based our sample size on our prior experience with identification of bio-markers specific for preeclampsia [9]. In the prior study, the Congo Red Dot test resulted in an AUC of 0.894 (95%CI: 0.866–0.918) compared to the standard proteinuria dipstick (0.825 (0.789–0.856)) (p-value<0.05) [9]. Based on these results, we projected that the study required 165 women in each arm to see a significant difference in the predictive value of the Congo Red Dot test (power: 0.8 and alpha of 5%). Additional patients were recruited to strengthen the conclusions of the analysis. Differences between the

Table 1
Characteristics of women enrolled in the case control study in Bangladesh. (n,%).

Variable	Case (n = 98)	Control (n = 98)	p-value
Maternal age (years) (SD) (range)	25.3 (5.2)(17–35)	25.5(4.8)(18–38)	0.754
Gestational age (completed weeks)			
<34 weeks	50 (51.0)	0 (0)	<0.001
34–36 weeks	32 (32.7)	0 (0)	
37–38 weeks	6 (6.1)	35 (35.7)	
39≥ weeks	10 (10.2)	63 (64.3)	
Multiple gestation	5 (10.4)	2 (3.6)	0.244
Parity (n,%)			
0	55 (56.1)	39 (39.8)	0.062
1	23 (23.5)	29 (29.6)	
2	15 (15.3)	27 (27.6)	
3+	5 (5.1)	3 (3.1)	
Pre-existing conditions before pregnancy			
diabetes	0	0	-
chronic hypertension	5 (5.1)	2 (2.0)	0.248
renal disease	0	0	-
liver disease	0	0	-
other*	2 (2.0)	4 (4.0)	0.407
Preeclampsia diagnosis by enrolling provider			
Mild preeclampsia	1 (1.0)	-	-
Severe preeclampsia	22 (22.4)	-	-
HELLP	8 (8.2)	-	-
Eclampsia	39 (39.8)	-	-
Preeclampsia uncategorized	28 (28.6)	-	-
Signs and symptoms at time of diagnosis			
Elevated BP	96 (98.0)	-	-
Proteinuria	94 (95.9)	-	-
Headache	74 (75.5)	-	-
Changes in vision	65 (66.3)	-	-
Abdominal pain	1 (1.0)	-	-
Nausea/vomiting	29 (29.6)	-	-
Seizure	39 (39.8)	-	-
Systolic BP (mmHg) at time of diagnosis			
≤139	8 (8.2)	-	-
140–159	33 (33.7)	-	-
≥160	57 (58.2)	-	-
Diastolic BP (mmHg) at time of diagnosis			
≤89	10 (10.2)	-	-
90–109	36 (36.7)	-	-
≥110	52 (53.1)	-	-
Proteinuria at time of diagnosis			
Nil or Trace	10 (10.2)	-	-
+1	10 (10.2)	-	-
+2	8 (8.2)	-	-
≥+3	70 (71.4)	-	-
Received MgSO4 ≤6 h preceding enrollment	90 (91.8)	-	-
Received antihypertensive drug ≤6 h preceding enrollment	98 (100.0)	-	-
Mode of delivery			0.115
Vaginal delivery	48 (49.0)	35 (35.7)	
Forceps or vacuum delivery	0	1 (1.0)	
C-section	50 (51.0)	62 (63.3)	
Complications of labor and delivery			
Uterine hyperstimulation	0	1 (1.0)	0.316
FHR abnormality	0	5 (5.1)	0.024
Uterine rupture	0	0	-
Placental abruption	1 (1.0)	1 (1.0)	1.00
Diagnosis of PPH (>500 ml)	1 (1.0)	0	0.316
Manual removal of placenta	34 (34.3)	14 (14.3)	0.001

(continued)

Table 1 (Continued)

Variable	Case (n = 98)	Control (n = 98)	p-value
Blood products (blood plasma, platelets or red blood cells)	0	0	-
Severe hypertension (single systolic BP $\geq$ 160 or diastolic BP $\geq$ 110) after enrolment	0	1 (1.0)	0.316
Hypovolemic shock			
Seizure after admission to labor and delivery	4 (4.0)	0	0.043
HELLP syndrome	7 (7.1)	0	0.007
Pulmonary edema	2 (2.0)	0	0.155
DIC based on clinical assessment	1 (1.0)	0	0.316
Received MgSO ₄ at time of delivery	48 (49.0)	1 (1.0)	<0.001
Received antihypertensive drugs at time of delivery	81 (81.6)	2 (2.0)	<0.001
Admission to ICU up to discharge	5 (5.1)	1 (1.0)	0.097
Indication for admission to ICU			
Uncontrolled hypertension	1 (1.0)	0	
HELLP	1 (1.0)	0	
Postpartum eclampsia	1 (1.0)	0	
DIC	1 (1.0)	0	
Cardiac arrest	1 (1.0)	0	
Postpartum cardiomyopathy	0	1 (1.0)	
Total abdominal hysterectomy/hypovolemic shock			
Maternal death	1 (1.0)	0	0.316
Delivery outcome ^a	n = 103	n = 99	<0.001
Live birth	58 (56.3)	96 (96.9)	
Stillborn	45 (43.7)	3 (3.1)	
Neonatal morbidity ^a			
Apgar <7 at 5 min	1 (1.7)	0	
Meconium-stained liquor (any grade)	2 (3.1)	0	
Neonatal convulsions	1 (1.0)	0	
Clinically diagnosed birth asphyxia	3 (3.1)	0	
Clinically diagnosed birth asphyxia	0	0	
Clinically diagnosed septicemia	1	3 (3.1)	
Other			
Baby admitted to special care nursery ^a	30 (30.6)	5 (5.2)	<0.001
Baby given oxygen ^a	24 (24.5)	4 (4.1)	<0.001
Neonatal deaths prior to discharge ^a	8 (8.1)	0	<0.001

Abbreviations: BP=Blood pressure, MgSO₄=magnesium sulfate, FHR=fetal heart rate, PPH=postpartum hemorrhage (>500 ml blood loss), DIC=Disseminated intravascular coagulation, ICU=intensive care unit. Pulmonary edema defined as oxygen saturation <90% and an abnormal chest x-ray.

^a Sample size of babies is 103 born to women in the case group and 99 babies born to women in the control group due to loss to follow-up (n = 1) and multiple pregnancies (case: 5; control: 2).

two groups were compared using chi-square (or Fischer's exact) and Student *t*-test for categorical and continuous variables, respectively using SPSS Statistics 20 (SPSS Inc. Chicago, IL) statistical software. Odds ratios or the likelihood of the GV-005 (index test) to confirm and rule-out preeclampsia based on an adjudicated diagnosis (reference standard) were calculated using MedCalc statistical software.

Role of the funding source

The funder of the study had no role in the study design, data collection or analysis. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Table 2

Characteristics of women enrolled in case control study in Mexico (n,%).

Variable	Case (n = 106)	Control (n = 107)	p-value
Mean maternal age (years) (SD) (range)	25.0(6.2)(13–39)	23.6(6.2)(14–42)	0.123
Gestational age (completed weeks)			
<34 weeks	14 (13.2)	7 (6.5)	0.016
34–36 weeks	18 (17.0)	15 (14.0)	
37–38 weeks	33 (31.1)	21 (19.6)	
39 $\geq$ weeks	41 (38.7)	64 (59.8)	
Multiple pregnancy (n,%)	1 (0.9)	0	0.187
Parity (n,%)			
0	58 (54.7)	52 (48.6)	0.003
1	37 (34.9)	23 (21.5)	
2	8 (7.5)	22 (20.6)	
3+	3 (2.8)	10 (9.3)	
Pre-existing conditions before pregnancy			
diabetes	1 (0.9)	1 (0.9)	0.995
chronic hypertension	5 (4.7)	0	0.023
renal disease	0	0	-
liver disease	0	0	-
other	6 (5.7)	7 (6.5)	0.788
Preeclampsia diagnosis at time of enrollment			
Mild preeclampsia	21 (19.8)	-	-
Severe preeclampsia	70 (66.0)	-	-
Eclampsia	2 (1.9)	-	-
Preeclampsia uncategorized	13 (12.3)	-	-
Signs and symptoms at time of diagnosis			
Elevated BP	88 (83.0)	-	-
Proteinuria	6 (5.7)	-	-
Headache	23 (21.7)	-	-
Changes in vision	2 (1.9)	-	-
Abdominal pain	11 (10.4)	-	-
Nausea/vomiting	2 (1.9)	-	-
Seizure	1 (0.9)	-	-
Systolic BP (mmHg) at time of diagnosis			
$\leq$ 139	26 (24.5)	-	-
140–159	51 (48.1)	-	-
$\geq$ 160	29 (27.4)	-	-
Diastolic BP (mmHg) at time of diagnosis			
$\leq$ 89	32 (30.2)	-	-
90–109	62 (58.5)	-	-
$\geq$ 110	12 (11.3)	-	-
Proteinuria at time of diagnosis			
Nil or Trace	26 (24.5)	-	-
+1	43 (40.6)	-	-
+2	14 (13.2)	-	-
+3	22 (20.8)	-	-
Received MgSO ₄ $\leq$ 6 h preceding enrollment (n,%)	62 (58.5)	-	-
Received antihypertensive drug $\leq$ 6 h hours preceding enrollment	19 (17.9)	-	-
Mode of delivery			
Vaginal delivery	23 (21.7)	64 (59.8)	p<0.001
Forceps or vacuum delivery	2 (1.9)	5 (4.7)	
C-section	81 (76.4)	38 (35.5)	
Complications of labor and delivery			
Uterine hyperstimulation	0	2 (1.9)	0.157
FHR abnormality	0	0	-
Uterine rupture	0	1 (0.9)	0.318
Placental abruption	1 (0.9)	1 (0.9)	0.995
Diagnosis of PPH (>500 ml)	10 (9.34)	3 (2.8)	0.043
	0	0	-
	4 (3.8)	0	0.043

(continued)

Table 2 (Continued)

Variable	Case (n = 106)	Control (n = 107)	p-value
Manual removal of placenta	4 (3.7) 6 (5.6)	0 4 (3.7)	0.043 0.507
Blood products (blood plasma, platelets or red blood cells)			
Severe hypertension (single systolic BP $\geq$ 160 or diastolic BP $\geq$ 110)			
Other			
Seizure up to discharge	2 (1.9)	0	0.153
Suspected cerebral edema or cerebral hemorrhage	0	0	–
HELLP syndrome	6 (5.7)	0	0.013
Pulmonary edema (O ₂ sat <90%, abnormal chest xray)	1 (0.9)	0	0.314
Oliguria (<25 cc/hr for 2 hr) up to 2 h after end of study period	2 (1.9)	0	0.153
DIC based on clinical assessment	0	0	–
Dialysis	0	0	–
Received MgSO ₄ at time of delivery	63 (59.4)	1 (0.9)	<0.001
Received antihypertensive drugs at time of delivery	86 (81.1)	1 (0.9)	<0.001
Admission to ICU up to discharge	32 (30.2)	2 (1.9)	<0.001
Indication for admission to ICU			
Uncontrolled hypertension	5 (4.7)	0	
PE/Eclampsia	18 (16.9)	1 (0.9)	
HELLP	4 (3.7)	0	
Other	4 (3.7)	1 (0.9)	
Maternal death	0	0	–
Outcome of delivery ^a			
Live birth	106 (99.1)	107 (100)	0.314
Stillborn	1 (0.9)	0	
Neonatal morbidity ^a			
Apgar <7 at 5 min	3 (2.9)	0	
Meconium-stained liquor (any grade)	10 (9.4)	9 (8.4)	
Neonatal convulsions	0	0	
Clinically diagnosed birth asphyxia	4 (3.8)	2 (1.9)	
Clinically diagnosed septicemia	0	0	
Other	9 (8.5)	4 (3.7)	
Baby admitted to special care nursery ^a	17 (16.0)	7 (6.5)	0.028
Baby given oxygen ^a	20 (18.9)	4 (3.7)	<0.001
Baby ventilated ^a	13 (12.3)	3 (2.8)	<0.001
Neonatal deaths prior to discharge ^a	4 (3.8)	1 (0.9)	0.171

Abbreviations: BP=Blood pressure, MgSO₄=magnesium sulfate, FHR=fetal heart rate, PPH=postpartum hemorrhage (>500 ml blood loss), DIC=Disseminated intravascular coagulation, ICU=intensive care unit. Pulmonary edema defined as oxygen saturation <90% and an abnormal chest x-ray.

^a Sample size of babies is 107 born to women in the case group and 107 babies born to women in the control group.

Results

Between July 11 2016, and Sept 25 2018, 560 women in two hospitals were enrolled in the study (Figs. 2 and 3). 144 women were excluded because the urine sample was collected postpartum. There were no missing data of the index test. Five patients recruited as controls at the Mexico site were missing clinical data necessary for adjudicators to either rule out or confirm preeclampsia and thus

excluded. Two women recruited in Bangladesh were excluded because they were lost to follow-up. In Bangladesh, 64.3% of cases (63/98) and 53.1% of controls (52/98) were tested after refrigeration ($p = 0.111$). In Mexico, 81% of cases (85/105) and 66.4% of controls were tested after refrigeration (71/107) ($p = 0.012$). Although there is no systematic data on the effect of refrigeration on CR results using the next generation GV-005 device, we do not expect the short term refrigeration to affect the results. One case from Mexico was missing data on whether the sample was tested after refrigeration. In five cases, the result of the GV-005 test was indeterminate (i.e. showed a persistent blue control line) and the laboratory technician repeated the test with a new device using the same urine sample and results included in the analysis. Clinical data was abstracted on site from maternal and neonatal medical records for 409 women. A flowchart of the study population is presented in Fig. 2 (Bangladesh) and 3 (Mexico).

Patient characteristics

Demographic and clinical characteristics of the patients enrolled in the study with complete clinical data are presented in Table 1 (Bangladesh) and Table 2 (Mexico). The Bangladesh cohort included 98 preeclampsia cases and 98 controls based on the clinical diagnosis. In Bangladesh, 55% of cases and 39% of controls were nulliparous. Approximately half (51%) of cases recruited in Bangladesh were less than 34 weeks gestation at the time of the urine sample collection. Most controls (64%) in the Bangladesh cohort were recruited at term. Among preeclampsia cases in the Bangladesh cohort, the most common signs and symptoms at the time of diagnosis were elevated blood pressure (98% or 96/98), proteinuria (96% or 94/98), headache (76% or 74/98) and changes in vision (66% or 65/98) or seizure (40% or 39/98). Most preeclampsia cases (92%) had received magnesium sulfate prior to study recruitment and urine sample collection. Over half (54%) of Bangladesh cases experienced a preeclampsia-related life threatening condition (PLTC) including seizure (44%), hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome (7%), impaired consciousness (1%), pulmonary edema (2%), disseminated intravascular coagulation (1%), abruption (1%), cardiac arrest (1%), maternal death (1%) and/or stillbirth (45%). Five cases developed 2 or more conditions. The rate of pregnancy-related complications among the control group was low and included one case of hypovolemic shock (who also underwent a hysterectomy) (1%), uterine hyperstimulation (1%), and placental abruption (1%). Most controls in the Bangladesh cohort had a live birth (97%) and all babies were alive at the time of discharge.

The Mexico cohort included 106 cases and 107 controls. Table 2 describes the demographic characteristics and delivery outcomes of the cases and controls recruited in Mexico. In the Mexico cohort, 53% of cases and 50% of controls were nulliparous. Over half of preeclampsia cases (70%) in the Mexico cohort were enrolled and delivered at 37 weeks gestation or more. The most common preeclampsia signs and symptom at the time of diagnosis among cases recruited in Mexico were elevated blood pressure (83%) or headache (22%). Slightly over half of the preeclampsia cases (57%) were treated with magnesium sulfate prior to enrolment. Approximately 11% of cases recruited in Mexico experienced a preeclampsia-related life-threatening condition including seizure (3%), HELLP syndrome (6%), liver rupture (1%), pulmonary edema (1%), abruption (1%), oliguria (2%) and stillbirth (1%). Three cases developed more than one condition. Among the Mexico control group, very few women developed pregnancy-related complications including uterine rupture (1%), placental abruption (1%) or postpartum hemorrhage (3%). All women with an uncomplicated pregnancy had a live birth at delivery; the rate of neonatal death was also low (0.9%).

Table S1 compares the profile of preeclampsia cases recruited at the two sites. A greater proportion of women with preeclampsia

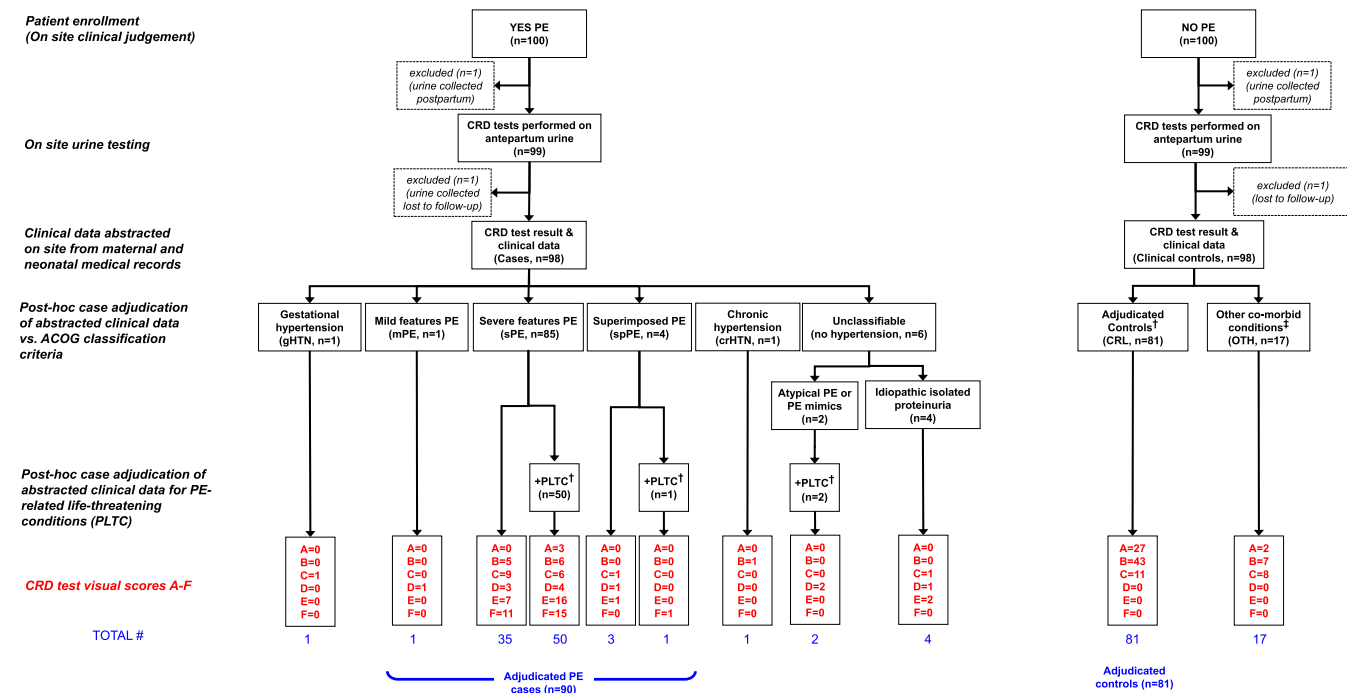


Fig. 2. Study flowchart: Bangladesh.

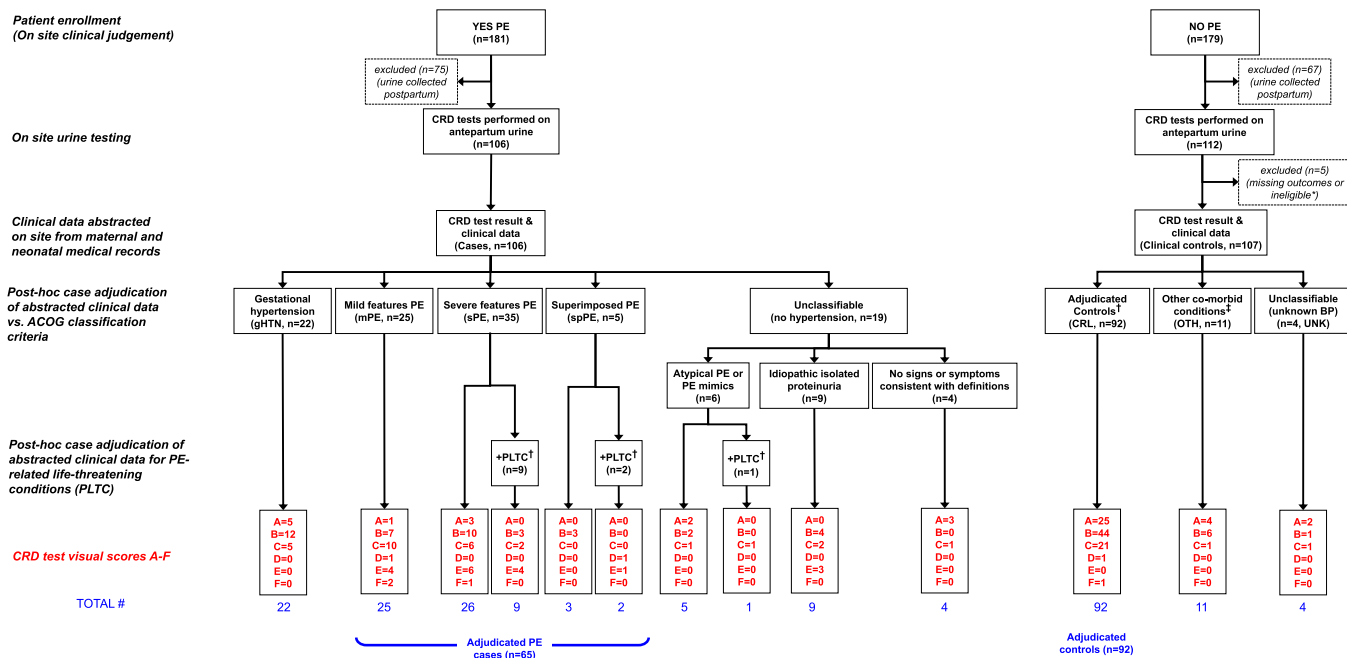


Fig. 3. Study flowchart: Mexico.

recruited at the study site in Bangladesh (40%) exhibited signs of advanced disease, i.e. seizure, compared to their Mexican counterparts (1%) ($p<0.001$). Although elevated blood pressure was listed as the most common criteria for diagnosis in both cohorts, the proportion of cases with severe hypertension (systolic ≥ 160 mmHg or diastolic ≥ 90 mmHg blood pressure) was significantly lower in Mexico

(severe systolic blood pressure: 29% or severe diastolic blood pressure: 12%) compared to cases in Bangladesh (severe systolic blood pressure: 57% or severe diastolic blood pressure: 53%) (both $p<0.001$). Women with a clinical diagnosis of preeclampsia recruited in Mexico were also more likely to be diagnosed based on less specific symptoms such as abdominal pain (10%) compared to their

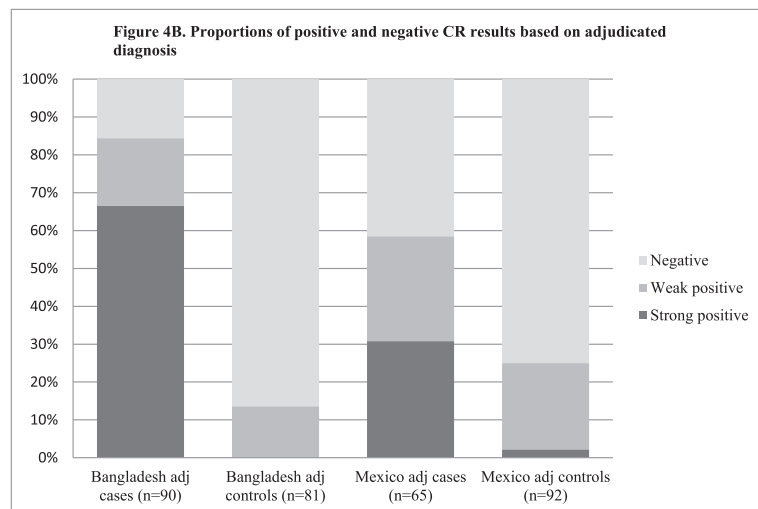
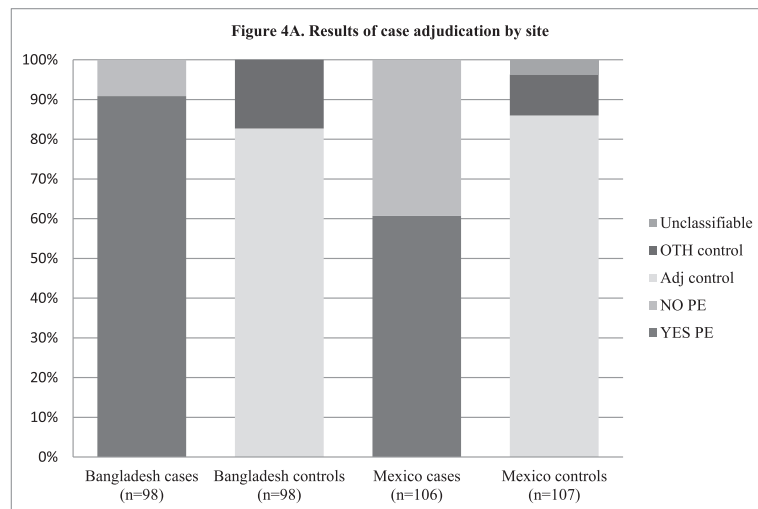
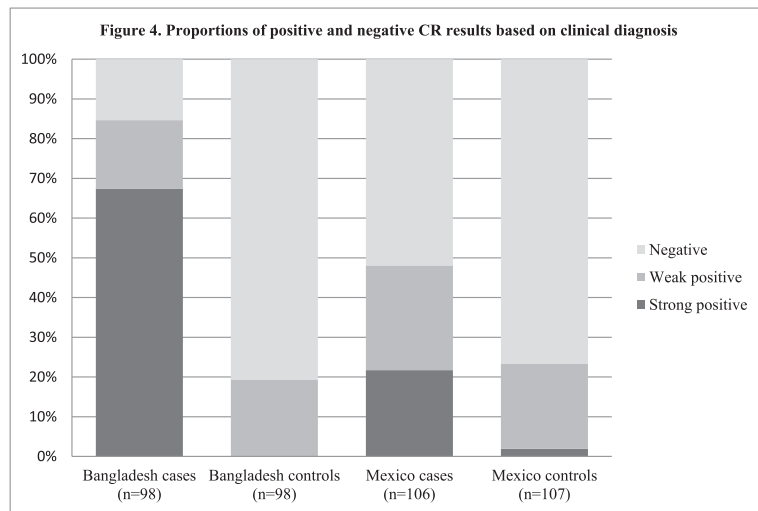


Fig. 4. Proportions of positive and negative CR results based on clinical diagnosis .
 A. Results of case adjudication by site.
 B. Proportions of positive and negative CR results based on adjudicated diagnosis.

Table 3
Diagnostic characteristics of the Congo Red test by site of recruitment.

Test Score	Odds Ratio [95%CI]	Sensitivity (%) [95%CI]	Specificity (%) [95%CI]	+LR [95%CI]	-LR [95%CI]
Bangladesh					
D – F	323.3 * [19.4 – 5393.1]	66.7 [56.9 – 76.4]	100 [100.0 – 100.0]	∞	0.33 [0.3 – 0.4]
C – F	34.5 * [14.7 – 81.1]	84.4 [77.0 – 91.9]	86.4 [79.0 – 93.9]	6.22 [3.6 – 10.8]	0.18 [0.1 – 0.3]
Mexico					
D – F	20.0 * [4.48 – 89.4]	30.8 [19.6 – 42.0]	97.8 [95.8 – 1.0]	14.2 [3.4 – 58.5]	0.7 [0.6 – 0.8]
C – F	4.2 * [2.1 – 8.4]	58.5 [46.5 – 70.4]	75.0 [66.2 – 83.9]	2.3 [1.6 – 3.5]	0.6 [0.4 – 0.8]

The reference test for cases was the diagnosis of preeclampsia at post-hoc adjudication (mild, severe or superimposed) (Bangladesh, *n* = 90 and Mexico, *n* = 65). The absence of preeclampsia was considered only in adjudicated controls (Bangladesh, *n* = 81 and Mexico, *n* = 92). The GV-005 test was evaluated as the index test. * *p* < 0.001.
Abbreviations: CI=confidence interval; +LR= positive likelihood ratio; -LR= negative likelihood ratio.

counterparts in Bangladesh (1%) (*p* = 0.005). Many cases in Mexico (25%) were diagnosed with preeclampsia in the absence of proteinuria. Although a greater proportion of cases in Bangladesh experienced a preeclampsia-related life-threatening condition, intensive care unit admissions were significantly higher among preeclampsia cases in Mexico (30%) compared to Bangladesh (2%) (*p* < 0.001).

Patient profile and adjudicated diagnosis, and GV-005 test result

The breakdown of the population for comparison of GV-005 test results, based on the final adjudicated diagnosis, is presented in Fig. 2 (Bangladesh) and Fig. 3 (Mexico). Fig. 4 describes the proportion of cases and controls at each site with a positive test result. The prevalence of a urine congophilia (primary outcome) among cases in Bangladesh was 85% (83/98) and 48% (51/106) in Mexico. Conversely, urine congophilia was not present in 81% of controls (79/98) in Bangladesh and in 77% of controls (82/107) in Mexico.

Adjudication using the ACOG criteria confirmed preeclampsia in 92% of cases in Bangladesh (90/98) and 61% of cases in Mexico (65/106) (*p* < 0.001) (secondary outcome). The diagnostic results of the CR test based on the final adjudicated diagnosis using the ACOG criteria are presented in Table 3. In Bangladesh, the odds ratio of urine congophilia in adjudicated cases versus controls was 34.5 to 1 (95% CI: 14.7–81.1) (secondary outcome). We conducted exploratory analyses and found that a better model for preeclampsia diagnosis can be achieved using the higher cut-off of D-F compared to C-F. With a higher cut-off, and only including results D-F as positive for congophilia, the odds of a positive result in adjudicated cases versus controls in the Bangladesh cohort was higher (323 to 1) (95% CI: 19.4–5393.1) (Table 3). In Mexico, the odds ratio of a positive result in adjudicated cases versus controls was 20 to 1 (95% CI: 4.5–89.4) using the cut-off of D-F and 4.2 to 1 (95% CI: 2.1–8.0, when C-F was defined as a test positive for congophilia.

Breakdown characteristics of cases, clinical diagnosis and adjudicated diagnosis by GV-005 test result

Table 4 presents the grouping of cases based on the attending team's diagnosis, final adjudicated diagnosis using ACOG criteria and result of the GV-005 test by site. In the Bangladesh control group 81 (83%) were adjudicated as non-preeclampsia and 69 of 81 (85%) adjudicated controls tested negative for congophilia. In the control group, a positive result was observed in 11 adjudicated controls (14%). Case by case analysis found that control patients who tested positive included patients with oligohydramnios, a history of hypertension and heart disease, and gestational hypertension. Amongst Bangladesh preeclampsia cases, 8 cases (8%) were adjudicated as non-

preeclampsia. In this sub-group, 1 patient had a negative test result. The CR result concurred with the adjudicated diagnosis of chronic hypertension. In the group of cases adjudicated as non-preeclampsia but with a positive CR result, 7 patients tested weak positive for congophilia including patients diagnosed with gestational hypertension, atypical preeclampsia or preeclampsia mimics and idiopathic isolated proteinuria. Amongst preeclampsia cases where the enrolling diagnosis concurred with the adjudicated diagnosis (*n* = 90), 84% had a positive GV-005 test result. Adjudicated cases who tested negative for congophilia included cases of severe preeclampsia with and without PLTC (including one death due to cardiac arrest) and 5 cases with a history of eclampsia.

Table 4 presents the attending team's diagnosis, final adjudicated diagnosis and result of the GV-005 test for the Mexico cohort. In Mexico, 92 controls (86%) were adjudicated as non-preeclampsia and of these 69 (75%) tested negative for congophilia. Case by case analysis found that adjudicated control patients with a positive test result included cases with a medically-indicated delivery for PROM, macrosomia and one case with diabetes. Among controls with other comorbid conditions (*n* = 11), the GV-005 concurred with the adjudicated diagnosis of non-preeclampsia and included patients with hypertension, oligohydramnios and abruptio. Four controls in the Mexico cohort were lacking data on the blood pressure at the time of delivery and inadequate clinical data to rule out other co-morbid conditions or preeclampsia. Amongst the pre-eclampsia cases recruited in Mexico, 41 (39%) were adjudicated as non-preeclampsia. In this sub-group, 21 (68%) tested negative for congophilia. Most of these cases had an adjudicated diagnosis of gestational hypertension, atypical preeclampsia or preeclampsia mimics or idiopathic proteinuria. Approximately half of which (41%) underwent a medically-indicated delivery for preeclampsia. Among the Mexico cases with an adjudicated diagnosis of preeclampsia (*n* = 65), 58% tested positive for congophilia while 27 (42%) had a negative GV-005 test result. This sub-group included cases of preeclampsia with and without severe features and with and without PLTC. In both the case and control groups, most false positive and false negative results occurred in the context of gestational hypertension, chronic hypertension or other preeclampsia mimics.

Discussion

This is the first study using a beta prototype of a point-of-care diagnostic device for detection of urine congophilia in a low resource setting that could potentially be manufactured at scale for commercial purposes. We also report large discrepancies in the preeclampsia diagnostic criteria between the study sites and when compared to the standard ACOG criteria.

Table 4

Breakdown of cases by final adjudicated diagnosis and Congo Red test result by site of recruitment.

Clinical Diagnosis	Adjudicated diagnosis	Congo Red test result	Case notes
Bangladesh			
CONTROL N = 98	NO PE n = 81	NO, n = 70 True negative YES, n = 11 False positive	Unanimous concordance ruling out PE. Cases in this category underwent medically indicated delivery due to prior c/s. All tested weak positive.
	Other co-morbid conditions (n = 17)	NO, n = 9 True negative YES, n = 8 False positive	CRD concurred with adjudicated diagnosis. Cases included severe oligohydramnios (n = 1), case with history of hypertension and heart disease (n = 1), gestHT without a diagnosis of PE, medically indicated delivery for reduced fetal movement (n = 1). All 8 cases tested weak positive.
CASE N = 98	NO PE n = 8	NO=1 True negative YES=7 False positive	CRD concurred with adjudicated diagnosis of chronic hypertension while managing team's call was preeclampsia, uncategorized. Case adjudications was gestational hypertension (n = 1) while managing team's diagnosis was severe preeclampsia. Case adjudication was atypical PE or PE mimics (n = 2) while managing team's diagnosis was eclampsia. Case adjudication was idiopathic isolated proteinuria (n = 4) while the managing team's diagnosis was sPE, HELLP and PE uncategorized. All 7 cases tested weak positive.
	YES PE n = 90	NO, n = 14 False negative YES, n = 76 True positive	Case adjudication was sPE no PLTC (n = 5) and sPE with PLTC (n = 9) including a maternal death due to cardiac arrest. 5 had history of eclampsia prior to admission. 6 underwent MIDPE. 7 with a GA <34 weeks. Unanimous concordance ruling in PE.
Mexico			
CONTROL N = 107	NO PE n = 92	NO, n = 69 True negative YES, n = 23 False positive	Unanimous concordance ruling out PE. Included cases with medically indicated delivery for failure to progress (n = 3), PROM (n = 1), CPD (n = 1) and short inter-pregnancy interval (n = 1). One case experienced a pph and one case with diabetes.
	Other co-morbid conditions (n = 11)	NO, n = 10 True negative YES, n = 1 False positive	CR test concurred with adjudicated diagnosis. Adjudicated diagnoses were hypertension (n = 4), oligohydramnios (n = 5) or abruption (n = 1). Case with weak positive test underwent medically indicated delivery for oligohydramnios.
	Unclassifiable (unknown BP) (n = 4)	NO, n = 3 YES, n = 1	Unknown BP at time of delivery and inadequate clinical data to rule out other co-morbid conditions or PE.
CASE N = 106	NO PE n = 41	NO, n = 28 True negative YES, n = 13 False positive	CR test concurred with adjudicated diagnosis. Adjudicated diagnosis was of gestHTN (n = 17), amPE without PLTC (n = 4), IDP (n = 4) or no PE criteria (n = 3) while managing team's call was of mPE, SPE or uncategorized PE. 17 cases underwent MIDPE. Adjudicated diagnosis included gestHTN (n = 5), amPE with PLTC (n = 1), amPE without PLTC (n = 1), IDP (n = 5) and no PE criteria (n = 1). The three cases with a strong positive result (E) had an adjudicated diagnosis of idiopathic proteinuria.
	Yes PE n = 65	NO, n = 27 False negative YES, n = 38 True positive	Cases included mPE (n = 8), SPE without PLTC (n = 13), SPE with PLTC (n = 3), spPE (n = 3). Unanimous concordance ruling in PE

*Positive test defined as score C-F. SPE = severe preeclampsia, PLTC=preeclampsia-related life threatening conditions, gestHTN=gestational hypertension, spPE=superimposed preeclampsia, amPE=atypical preeclampsia or preeclampsia mimic, MIDPE=medically indicated delivery for preeclampsia, PROM=pre-mature rupture of membranes; CPD= Cephalopelvic disproportion.

Preeclampsia is classically defined as new onset hypertension and proteinuria after 20 weeks of gestation [20]. International professional bodies have sought to refine their diagnostic criteria in recent years [21–24]. However, as our study has shown, the practice of diagnosis may vary widely depending on local clinical practice and guidelines and, in application, may result in misdiagnosis. A lack of detailed laboratory workups in both of our study settings meant that diagnoses were based often on clinical signs and symptoms resulting in some misclassification. Given this variability in diagnostic practice, we used an adjudicated diagnosis to serve as a standard reference for cases in both settings. We found more discordance between the clinical diagnosis of the treating team and the adjudicated diagnosis among preeclampsia cases recruited in Mexico compared to Bangladesh. In the Mexico sample, 39% of cases were reclassified compared to only 8% of cases in the Bangladesh ($p = 0.0001$). Bangladesh cases

were more severe and thus less likely to be clinically misclassified at enrollment. In over two-thirds of the cases misclassified, the CR test agreed with the adjudicated diagnosis. Based on these findings, we believe the CR test could be a useful tool for triaging patients in the context of a care setting such as Mexico where many patients present with existing hypertension and ruling out preeclampsia is challenging. This finding echoes results from a prior study with the paper-based CR test in a US hospital [11]. However, the GV-005 device used here had a higher false-positive rate at both centers compared with prototype tested in the USA. Possible explanations include: the device was not calibrated to the patient population; imperfect reading especially in Mexico where there was a higher turnover of staff performing the test; labor or pre-labor might induce a low degree of conophilia; and women with oligohydramnios were under-diagnosed for preeclampsia

The study was conducted in two settings with very different patient populations. Preeclampsia cases in the Bangladesh cohort presented at a more advanced stage of disease compared to Mexico and cases in Mexico were more likely to be diagnosed as preeclampsia on the basis of elevated blood pressure alone. Over half of Bangladeshi cases had preeclampsia related life-threatening conditions compared to only 11% of adjudicated cases in Mexico. The specificity and sensitivity of the test can be affected by the patient characteristics in different settings because each setting has a different mix of patients [25]. Severe cases, as in Bangladesh, are easier to detect, resulting in an increased sensitivity, while healthy controls could reduce the number of false positives resulting in an overestimate the specificity of the test. While cases in both sites were significantly more likely to have a positive test result, the odds of a positive test among cases vs controls in Bangladesh was much higher than in Mexico (Bangladesh: 323 vs Mexico: 20). The results may not be representative or applicable in other populations. As observed in our study the patient population affects the performance of the test particularly in low-resourced settings when clinical data to rule out or to rule in preeclampsia based on other laboratory tests (such as 24 h proteinuria and repeated blood work) is missing. Although these data do provide information on the suitability of the test in different subgroups of patients, a cohort study could thus provide a better estimate of test performance [26].

Laboratory technicians performing the test were trained and provided with a visual chromatic scale to guide scoring of tests. However, there was room for reader error in test interpretation and distinguishing between readings of a B vs C or C vs. D. As a result, there was the possibility of misclassification of a positive vs. a negative test. The user feedback provided during this project was applied by GestVision (USA) to develop a third generation device, the GV-010, which is intended for use at the bedside and standardized commercial manufacturing. The new test has a dichotomous (positive/negative) result and a hue guide which the operator can place by the reaction window to aid in visual interpretation of the observed pattern.

The study was powered on a combined sample size including cases and controls from both study sites. However, variation in the implementation of the study and the profile of the patient population in the two sites required us to stratify the analysis. Where possible, patients were recruited with a single case and a control recruited as a pair. In Bangladesh research staff effectively recruited cases consecutively. However, changes in staffing at the Mexico site and sampling of urine postpartum resulted in gaps and an extended recruitment period further contributing to some variation in the case definition. Our adjudicated case definition helped to address some of these inter-site differences, however, it also resulted in a reduction in the number of eligible cases in Mexico. The stratification reduced the study power resulting in wide confidence intervals in the OR at both sites, which could reduce the accuracy of the estimates.

In sum, our study found that preeclampsia is characterized by urine congophilia which can be rapidly identified using a beta prototype of a lateral flow diagnostic device, GV-005. This embodiment of the CR test could be a useful tool to aid in the diagnosis of patients with preeclampsia and identification of patients that required additional care. Delivery triage may not be very useful. Future research should explore the feasibility and acceptability of the device in settings at lower levels of the health care system in order to identify patients earlier during the antenatal period and help to ensure that patients receive appropriate care.

Data sharing statement

Data are available upon reasonable request. A member of the study team must be involved in the analysis of the data.

Contributions

IB and CB had the original idea for the study. IB, CB, TE, HB, MB, AR, JP, and BW formed the trial management team with input from other co-investigators as required. HB and MB were the study monitors. IB and CB performed the case adjudication. HB and IB conducted the main analysis and HB wrote the first draft of the clinical paper. All authors reviewed and accepted the paper prior to submission.

Declaration of Competing Interest

Dr. Irina Buhimschi reports grants from Saving Lives at Birth (SLaB) partners including USAID, Bill & Melinda Gates Foundation, Governments of Canada, Norway and UK DFID, during the conduct of the study; other from GestVision Inc. In addition, Dr. Irina Buhimschi has a patent US Patent Number 8263,342 with royalties paid by GestVision Inc, a patent US Patent Number 9229,009 with royalties paid by GestVision Inc and by Shuwen Biotech, a patent U.S. Patent Number 10,324,094 with royalties paid by GestVision Inc, and a patent European patent No 3,129,779 with royalties paid by GestVision Inc. These royalties are paid to Yale University who disburses a percentage to inventors and co-inventors. The Congo Red devices used in this study were purchased from GestVision Inc who had no input in data interpretation or decision to submit for publication.

Dr. Catalin Buhimschi reports grants from Saving Lives at Birth (SLaB) partners including USAID, Bill & Melinda Gates Foundation, Governments of Canada, Norway and UK DFID, during the conduct of the study; other from GestVision Inc. In addition, Dr. Catalin Buhimschi has a patent US Patent Number 8263,342 with royalties paid by GestVision Inc, a patent US Patent Number 9229,009 with royalties paid by GestVision Inc and by Shuwen Biotech, a patent U.S. Patent Number 10,324,094 with royalties paid by GestVision Inc, and a patent European patent No 3,129,779 with royalties paid by GestVision Inc. These royalties are paid to Yale University who disburses a percentage to inventors and co-inventors. The Congo Red devices used in this study were purchased from GestVision Inc who had no input in data interpretation or decision to submit for publication.

Dr. Easterling reports personal fees from DiabetOmics, Inc., outside the submitted work.

The other authors have no conflicts to declare.

Acknowledgments

The authors would like to thank to Dr. Kara Rood, MD, Ms. Theresa Dible, RN, Shaylyn Webster, RN and Guomao Zhao, BSc for their participation in the cross-validation of the GV-005 test against the CRD Paper Test and the CRD nitrocellulose array (Supplement A).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.eclim.2020.100678](https://doi.org/10.1016/j.eclim.2020.100678).

References

- Souza JP, Gülmezoglu AM, Vogel J, et al. Moving beyond essential interventions for reduction of maternal mortality (the WHO Multicountry Survey on Maternal and Newborn Health): a cross-sectional study. *Lancet* 2013;381:1747–55.
- Chan P, Brown M, Simpson JM, Davis G. Proteinuria in pre-eclampsia: how much matters? *BJOG* 2005;112:280–5.
- Murray N, Homer CS, Davis GK, Curtis J, Mangos G, Brown MA. The clinical utility of routine urinalysis in pregnancy: a prospective study. *Med J Aust* 2002;177:477–80.
- Kamińska J, Dymicka-Piekarska V., Tomaszewska J. et al. Diagnostic utility of protein to creatinine ratio (P/C ratio) in spot urine sample within routine clinical practice. *Crit Rev Clin Lab Sci*, 57:5, 345–64, DOI: 10.1080/10408363.2020.1723487
- Sibai BM, Stella CL. Diagnosis and management of atypical preeclampsia-eclampsia. *Am J Obstet Gynecol* 2009;200(5):481. e1–481.e4817. doi: [10.1016/j.ajog.2008.07.048](https://doi.org/10.1016/j.ajog.2008.07.048).

- 6 Thaddeus S., Maine D.. Too far to walk: maternal mortality in context. *Soc Sci Med* 1994; 38: 1091–110.
- 7 Majors CE, Smith CA, Natoli ME, Kundrod KA, Richards-Kortum R. Point-of-care diagnostics to improve maternal and neonatal health in low-resource settings. *Lab Chip* 2017;17(20):3351–3387. doi: 10.1039/c7lc00374a.
- 8 Buhimschi IA, Zhao G, Funai EF, et al. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *Am J Obstet Gynecol* 2008;199:551. [e1–16].
- 9 Buhimschi IA, Nayeri UA, Zhao G, et al. Protein misfolding, congophilia, oligomerization, and defective amyloid processing in preeclampsia. *Sci Transl Med* 2014;6:245ra92.
- 10 Buhimschi IA., Buhimschi C.S., Tagare H., Choma M., Jonas S.. Methods and compositions for detecting misfolded proteins. U.S. Patent Number 10,324,094 Filed April 10, 2014, issued June 18, 2019.
- 11 Rood KM, Buhimschi CS, Dible T, Webster S, Zhao G, Samuels P, Buhimschi IA. Congo red dot paper test for antenatal triage and rapid identification of preeclampsia. *E Clin Med* 2019;8:47–56. doi: 10.1016/j.eclinm.2019.02.004.
- 12 Sammar M, Syngelaki A, Sharabi-Nov A, Nicolaides K, Meiri H. Can staining of damaged proteins in urine effectively predict preeclampsia? *Fetal Diagn Ther* 2017;41:23–31.
- 13 Nagarajappa C, Rangappa SS, Suryanarayana R, Balakrishna S. Urinary congophilia in preeclampsia: experience from a rural tertiary-care hospital in India. *Pregnancy Hypertens* 2018;13:83–6.
- 14 McCarthy FP, Adetoba A, Gill C, et al. Urinary congophilia in women with hypertensive disorders of pregnancy and preexisting proteinuria or hypertension. *Am J Obstet Gynecol* 2016;215:464. [e1–7].
- 15 Li X-M, Liu X-M, Xu J, Du J, Cuckle H. Late pregnancy screening for preeclampsia with a urinary point-of-care test for misfolded proteins. *PLoS ONE* 2020;15(5): e0233214.
- 16 Davis, W.L., Levenson D. Device for detecting misfolded proteins and methods of use. U.S. Patent Number 10,564,153 Filed 2015, issued May 7, 2020.
- 17 Fryer HJL, Rood KM, Davis WL, Buhimschi CS, Buhimschi IA. Evaluation of a rapid urine test for preeclampsia in an antenatal triage population Abstract #7M. *Obstetrics & Gynecology*, 133. Nashville TN, USA: American College of Obstetricians And Gynecologists (ACOG) Annual Clinical and Scientific Meeting May 2019; 2019. p. 142S. doi: 10.1097/01.AOG.0000559280.75705.25.
- 18 Kahan BC, Feagan B, Jairath V. A comparison of approaches for adjudicating outcomes in clinical trials. *Trials* 2017;18:266.
- 19 American College of Obstetricians and Gynecologists. Task force on hypertension in pregnancy. Hypertension in pregnancy. Report of the American college of obstetricians and gynecologists task force on hypertension in pregnancy. *Obstet Gynecol* 2013;122:1122–3.
- 20 National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. Report of the national high blood pressure education program working group on high blood pressure in pregnancy. *Am J Obstet Gynecol* 2000;183:S1–S22.
- 21 Brown MA, Magee LA, Kenny LC, Karumanchi SA, McCarthy FP, Saito S, Hall DR, Warren CE, Adoyi G, Ishaku S. International society for the study of hypertension in pregnancy (ISSHP). The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice. *Pregnancy Hypertens* 2018 Jul;13:291–310.
- 22 Webster K, Fishburn S, Maresh M, Findlay SC, Chappell LC, Guideline Committee. Diagnosis and management of hypertension in pregnancy: summary of updated NICE guidance. *BMJ* 2019 Sep 9;366:15119.
- 23 American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Obstetrics. Gestational hypertension and preeclampsia: ACOG practice bulletin, number 222. *Obstet Gynecol* 2020 Jun;135(6):e237–60.
- 24 Poon LC, Shennan A, Hyett JA, Kapur A, Hadar E, Divakar H, McAuliffe F, da Silva Costa F, von Dadelszen P, McIntyre HD, Kihara AB, Di Renzo GC, Romero R, D'Alton M, Berghella V, Nicolaides KH, Hod M. The international federation of gynecology and obstetrics (FIGO) initiative on pre-eclampsia: a pragmatic guide for first-trimester screening and prevention. *Int J Gynaecol Obstet* 2019;145(Suppl 1):1–33 Suppl 1.
- 25 Li J, Fine JP. Assessing the dependence of sensitivity and specificity on prevalence in metaanalysis. *Biostatistics* 2011;12:710–22 [PubMed: 21525421].
- 26 Rutjes AW, Reitsma JB, Vandenbroucke JP, Glas AS, Bossuyt PM. Case-control and two-gate designs in diagnostic accuracy studies. *Clin Chem* 2005;51(8):1335–41. doi: 10.1373/clinchem.2005.048595.



Research Paper

Congo Red Dot Paper Test for Antenatal Triage and Rapid Identification of Preeclampsia

 Kara M. Rood ^{a,*}, Catalin S. Buhimschi ^{a,d}, Theresa Dible ^a, Shaylyn Webster ^a, Guomao Zhao ^{b,d}, Philip Samuels ^a, Irina A. Buhimschi ^{b,c,d}
^a Department of Obstetrics and Gynecology, The Ohio State University College of Medicine, Columbus, OH 43210, USA

^b Center for Perinatal Research, The Research Institute at Nationwide Children's Hospital, Columbus, OH 43215, USA

^c Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH 43215, USA

^d Department of Obstetrics & Gynecology, University of Illinois at Chicago College of Medicine, Chicago, Illinois, 60612, USA

ARTICLE INFO

Article history:

Received 10 August 2018

Received in revised form 12 February 2019

Accepted 15 February 2019

Available online 1 March 2019

Keywords:

Preeclampsia

Cohort study

Congophilia

Misfolded proteins

Point-of-care

ABSTRACT

Background: Proteins in the urine of women with preeclampsia (PE) bind Congo Red dye (urine congophilia). We sought to determine the diagnostic performance of a paper-based point-of-care test detecting urine congophilia for rapid triage and diagnosis of PE.

Methods: Prospective cohort study conducted in 346 consecutive pregnant women evaluated for PE in the Labour and Delivery triage unit at our institution. The Congo Red Dot (CRD) Paper Test (index test) was performed on fresh urine samples. The CRD Paper Test results were compared to an expert adjudicated diagnosis in each case. The accuracy of the CRD Paper Test was also compared to urine and serum analytes (placental growth factor and soluble fms-like tyrosine kinase-1) previously proposed as diagnostic aids for PE.

Findings: During the first triage visit, 32% (112/346) of women received a clinical diagnosis of PE. Yet, 63% (217/346) were admitted for in-patient diagnostic work-up or delivery. The CRD Paper Test was positive in 25% (86/346) of the cases. Adjudication confirmed PE in 28% (96/346) of all cases. The CRD Paper Test outperformed measured serum and urine markers (80·2% sensitivity, 89·2% specificity, 92·1% negative predictive value, 86·7% accuracy). The pre-test, positive and negative post-test probabilities were 27·7%, 74·0%, and 8·0%, respectively. Of women who were discharged undelivered, 38% (133/346) had at least one additional triage visit and the interval between the last negative and first positive CRD Paper Test was 12 (interquartile range, [5–34]) days.

Interpretation: The CRD Paper Test is a simple, non-invasive, “sample-in/answer-out” point-of-care clinical tool for rapid identification of PE.

Funding: Saving Lives at Birth Program and NICHD.

© 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Hypertensive disorders affect 15% of pregnancies and account for one quarter of the antenatal admissions [1]. Preeclampsia (PE) is a multisystem disorder specific to human pregnancy, and its incidence varies from 5 to 60% of gestations, depending on maternal co-morbidities [2,3]. The low rate of maternal deaths in Western countries is in blunt contrast with the global setting where 70,000 women are estimated to die each year from PE [4,5]. In the U.S., given the difficulty of predicting and diagnosing PE, especially in the presence of clinical confounders (e.g. hypertension, kidney disease, migraines), a significant proportion of

pregnancy-related maternal mortality and morbidity is still attributable to PE [6,7]. Hypertensive disorders are responsible for 2·6 million stillbirths occurring annually worldwide [8]. Because delivery of the baby is the only curative intervention for PE and avoidance of a stillbirth, iatrogenic prematurity will continue to be a challenge.

For the last decade, research has focused on exploration of different inflammatory and angiogenic biomarkers for diagnosing PE [9,10,11]. Our group discovered that PE women excrete urinary misfolded proteins, raising the prospect this hypertensive condition could be a protein conformational disorder [12]. We further discovered that urine of PE women exhibits congophilia which is the affinity of misfolded proteins for the azo-dye Congo Red (CR) [13]. Based on the above premises, we found that quantification of urine congophilia carries diagnostic and prognostic potential for PE [13]. Other groups validated the clinical usefulness of assessing congophilia in pregnant women for PE diagnosis using our research laboratory protocol on nitrocellulose [14,15,16].

* Corresponding author at: The Ohio State University, Department of Obstetrics and Gynecology, Columbus, OH 43215, USA.

E-mail address: kara.rood@osumc.edu (K.M. Rood).

Research in Context

Evidence Before the Study

Preeclampsia (PE) remains a leading cause of maternal and perinatal morbidity and mortality worldwide. The World Health Organization estimates that 14% of maternal deaths in low-resource settings, approximately 341,000 cases per year, are caused by this disease. PE has a large spectrum of medical signs and symptoms resulting in a range of clinical phenotypes and outcomes, making a diagnosis on available clinical and laboratory parameters challenging. Hypertension and proteinuria are non-specific, and thus major challenges arise when differential diagnosis includes chronic hypertension, endocrine, and kidney diseases. Consequently, it is not uncommon when confronted with clinical ambiguity, particularly close to term, for physicians to indicate delivery even in the absence of a true diagnosis. During the last decade and despite major financial and research efforts, use of angiogenic factor tests for PE could not be implemented clinically considering their high costs and impracticality for point-of-care testing. Members of our group were the first to discover that PE is a protein conformational disorder, similar to Alzheimer's disease. We observed that proteins in the urine of preeclamptic women bind Congo Red dye, a feature called urine congophilia. To that end, our group reported that urine congophilia carries diagnostic potential for PE. However, previous research was focused on laboratory-based techniques and validation of congophilia. To our knowledge no studies have evaluated in a pragmatic framework the clinical utility of a simple, easy to use, non-invasive, low-cost, paper-based point-of-care test to diagnose PE within minutes, at the patient's bedside.

Added Value of the Study

This study adds value to the existing evidence by reporting for the first time in an unselected population the diagnostic characteristics of the Congo Red Dye (CRD) Paper Test for rapid diagnosis of PE, in a hospital's triage area. The CRD Paper Test has a high accuracy for diagnosis of PE and outperforms previously proposed serum and urine immunoassay tests as diagnostic aids for PE. Our analysis shows that it is not only inexpensive, easy to use, highly accepted by the nursing staff, but identifies women with PE within 3 min. If the CRD Paper Test results were available to obstetrical providers, a negative CRD Paper Test could improve wait times in obstetrical triage areas, avoid unnecessary admissions and lower the associated health care expenses. Furthermore, our findings have potential to improve accurate timing of patients' transfers to higher-acuity hospitals, and more targeted steroids and magnesium sulfate treatment in patients at risk of indicated preterm delivery from PE.

Implications of All the Available Evidence

Morbidity and mortality from PE are due to delay or misdiagnosis. Implementation of the CRD Paper Test in the triage area could be a useful tool for rapid diagnosis of PE, and avoidance of unnecessary deliveries. Further multi-center studies are warranted in high and low-income countries where CRD Paper Test has the potential to save thousands of lives.

Test turnaround times and avoiding unnecessary hospital admissions are increasingly important to clinicians faced with diagnostic uncertainty and increased health care costs. To address these gaps we modified our initial protocol for determination of urine congophilia in

a manner that allows testing at the point-of-care level. Our hypothesis was that identification of urine congophilia by using a rapid diagnostic test will have high accuracy for diagnosis of PE at the patient's bedside. We designed, developed and validated a simple bed-side, paper-based urine test kit, which we named the CR Dot (CRD) Paper Test. In this study our objective was to determine the diagnostic accuracy of the CRD Paper Test comparing it to the clinical diagnosis based on the full clinical workup of the women referred to Labour and Delivery (L&D) triage unit for evaluation of PE. The main outcome measure was Area Under the Receiver Operating Characteristic plot (AUROC) to confirm and rule out PE based on the adjudicated diagnosis.

2. Methods

2.1. Design, Study Setting, and Participants

346 consecutive pregnant patients were recruited in L&D triage unit at The Ohio State University Wexner Medical Center and followed prospectively until delivery from July 2014 to July 2015. Patients were referred from lower level healthcare facilities (local antenatal clinics or level II regional hospitals) for evaluation of hypertension and/or to rule-out PE. All women aged 18 years or older were eligible. Exclusion criteria were inability to provide informed consent and/or to establish accurate pregnancy dating based on last menstrual period confirmed by an ultrasound examination. All subjects provided written informed consent. The Human Investigation Committee of The Ohio State University Wexner Medical Center and of Nationwide Children's Hospital approved the study.

2.2. Study Protocol

Eligible, consenting women were approached for enrollment by certified research nurses immediately after their presentation to the triage area before initiation of the clinical work-up for PE. Of the approached women ($n = 353$), 98% agreed to participate. Patients were consented to provide a urine and matched venous blood sample. Refusal to provide a blood sample ($n = 107$) was not an exclusion criterion. Urine was collected in sterile containers and tested fresh without processing in the triage area. The result of the CRD Paper Test was read at 3 min. The patient and the clinical team were blinded to the results. All medical decisions were taken independent of our study protocol. Data collection was planned before initiation of the study and performance of the first CRD Paper Test. Further details about the study protocol and the processing of the remaining urine and blood samples are described in the appendix (p. 2).

Triage utilization was calculated as the time interval between patient check-in to release from the unit. Duration of hospitalization was monitored for all women admitted with uncertain PE status and discharged undelivered. The study protocol was registered using the [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02455544) (NCT02455544) system.

2.3. Case Adjudication

Because incorrect classification of outcomes can lead to reduced power and biased estimation of the diagnostic performance of a test, the ability of the CRD Paper Test and of all the other researched biomarkers was made by comparing results to the adjudication of the triage diagnostic decision [17].

Each case was adjudicated by two independent board certified Maternal Foetal Medicine specialists blinded to the results of the CRD Paper Test (KMR & CSB). The ACOG Task Force definition of hypertensive disorders of pregnancy was applied [28]. Details are provided in the appendix (p. 2).

2.4. Assessment of Urine Congophilia and Analytical Validity of the CRD Paper Test Kit

The CRD Paper Test Kits were constructed in-house using Neenah Bright premium cardstock (Neenah Paper, Inc., Alpharetta, GA). Each kit contained printed instructions, a visual chromatic scale, a sealed pipette with standardized amount of CR dye and two reaction paper surfaces affixed to the mid-portion (Fig. 1A). Details about the rationale and procedure for the CRD Paper Test Kit are provided in the appendix (pp. 2–3). Briefly, for each patient, ~150 µL of fresh urine was mixed with the CR dye placed inside the transfer pipet. After ~1 min, the mixture was dispensed into approximately equal sized drops inside the areas printed on the reaction papers. The result was read at 3 min against the visual chromatic aid (Fig. 1B). The scientific principle behind the CRD Paper Test is described in the appendix (p. 2). Training for the user procedure and evaluation of the user acceptability for the CRD Paper Test are presented in the appendix (pp. 2–3). There were no indeterminate CRD Paper Test results, and no data were excluded for the final analysis.

The analytical validity of the CRD Paper Test kit was investigated in relation to the result of the CRD nitrocellulose array that was performed as described in the appendix (p. 6). The results of the CRD nitrocellulose array were reported as %CR Retention (%CRR) as previously published [13] (appendix, p 4). Although the nitrocellulose array is a simple method for a research laboratory, it was neither intended nor designed for point-of-care testing [13]. The technical validation analysis showed no difference between the two methods (appendix p 6).

2.5. Measurement of Additional Biochemical Markers

Levels of urine and serum soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) were immunoassayed as previously described [10]. The sFlt-1/PlGF ratios were calculated and used for comparison with the results of the CRD Paper Test; details provided in the appendix (p. 5). The test performances of the urine and serum sFlt-1/PlGF ratios were calculated based on optimal and previously reported cut-offs [10,18].

2.6. Main Outcome Measure

The main outcome measure was the AUROC of the CRD Paper Test (index test) to confirm and rule-out PE based on the adjudicated

diagnosis (reference standard). There were no missing data of the index test or reference standard.

2.7. Statistical Analysis

Statistical analyses were performed with Sigma Stat, version 2.03 (SPSS Inc., Chicago, IL) and MedCalc (Broekstraat, Belgium) statistical software. Normality testing was performed using the Shapiro–Wilk test. Data were compared with Mann–Whitney Rank Sum test, 1-way ANOVA followed by Holm–Sidak tests (parametric) or Kruskal–Wallis ANOVA on ranks followed by Dunn’s tests (non-parametric). Immunoassay data was analyzed after logarithmic transformation. Spearman correlations were used to measure co-linearity between the selected independent variables. Comparisons between proportions were done with Chi-square tests.

ROC plots were used to determine optimal cut-offs for each test in our study population. Because most point-of-care tests intended for busy clinical settings have binary outcomes (positive/negative), these cut-off points were further used for calculation of diagnostic accuracy characteristics [19,20]. Test accuracy (cases correctly classified/total number of cases), Youden Index [21], sensitivity, specificity, positive and negative predictive values, and likelihood ratios (LR) were calculated from contingency tables using both the optimal cut-off in our dataset and those previously published. We took this approach to facilitate comparisons with earlier results, but also to ensure our results are applicable to our current cohort where the prevalence of the disease could be different. Confidence intervals (CI) were calculated using the bootstrapping method. Graphical representations of ROC plots for dichotomised results were visualized using LRs and the Biggerstaff method [22]. AUROC was calculated both from the continuous data output and after conversion in LR coordinates as recommended for binary data [23]. Comparison of the of the index test to comparator tests was performed on the dichotomised data output using the non-parametric method of De Long. A $p < 0.05$ was considered significant throughout all analyses.

Pre-test probability was estimated based on the prevalence of a medically indicated delivery for PE (MIDPE) in our study population. The diagnostic utility of the CRD Paper Test was investigated using LR to estimate the post-test probability based on Bayes’ theorem and Fagan’s nomogram [24]. Our initial sample size calculation was performed to detect significance for an AUROC of 0.7 with an assumption of 3:1 ratio of negative to positive cases. 140 cases were estimated as

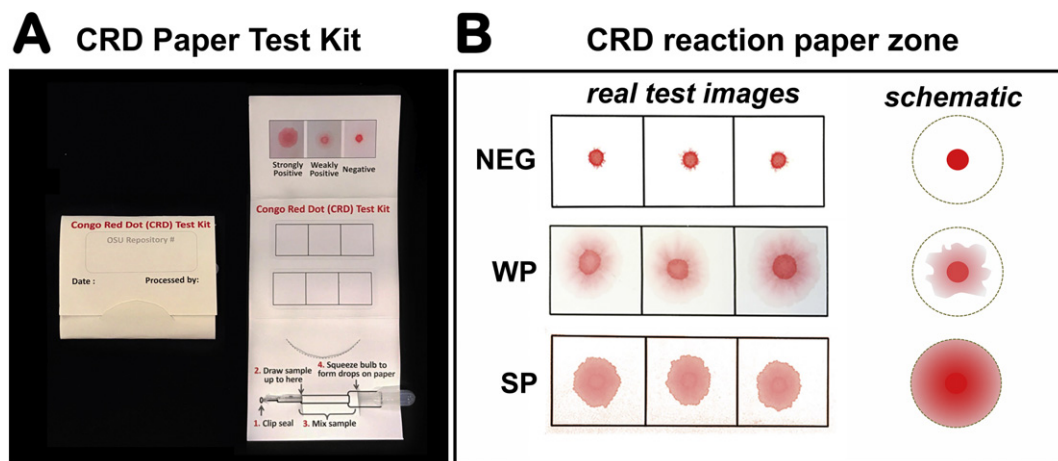


Fig. 1. Congo Red Dot (CRD) Paper Test kits. A. Kits of the CRD Paper Test were manufactured “in house” by our research staff. Each kit had two label papers incorporated, and a visual colorimetric scale marked as strongly positive (SP), weak positive (WP) and negative (NEG). The kit contained a syringe prefilled with Congo Red dye. B. Representative images from three urine samples with NEG ($n = 1$), WP ($n = 1$), and SP ($n = 1$) CRD Paper Test results. Following application of the urine over the demarcated box the operator watches the test while it develops. Although the call is made at the end of the flow (~3 min), an impression of the result may be formed within the first few seconds from applying the sample. The scientific rationale behind the CRD Paper Test is presented in the appendix (p. 2).

Table 1
Characteristics of women enrolled in the triage cohort.

Characteristics at enrollment and during gestation	n = 346
Maternal age (years)	29 [25–33]
Parity	
0	167 (48%)
1	87 (25%)
2	44 (13%)
≥3	48 (14%)
Race/ethnicity of women	
White	226 (66%)
Black/African American	101 (29%)
Hispanic	8 (2%)
Other	11 (3%)
Weight (kg)	97 [80–113]
BMI	35 [30–42]
BMI categories	
Underweight (<18.5)	0 (0%)
Normal weight (18.5–24.9)	21 (6%)
Overweight (25–29.9)	66 (19%)
Obese Class I (30–34.9)	82 (24%)
Obese Class II (35–40)	70 (20%)
Obese Class III (≥40)	97 (28%)
Not recorded	10 (3%)
Multiple gestation	14 (4%)
Gestational age at first triage visit (completed weeks)	
<20 weeks	2 (1%)
20–24 weeks	7 (2%)
25–27 weeks	16 (5%)
28–31 weeks	55 (16%)
32–33 weeks	50 (14%)
34–36 weeks	106 (31%)
37–38 weeks	78 (22%)
≥39 weeks	32 (9%)
Referral diagnoses to confirm or rule out PE in the setting of:	
Chronic hypertension (crHTN)	50 (14%)
Gestational hypertension (gestHT)	71 (21%)
Preeclampsia without severe features (mPE)	38 (11%)
Preeclampsia with severe features (sPE)	48 (14%)
Superimposed PE (spPE)	27 (8%)
Other ^a	112 (32%)
Disposition after first triage visit:	
Admitted to hospital	217 (63%)
Discharged home	129 (37%)
Number of triage visits during index pregnancy	
1 visit	212 (61%)
2 visits	91 (26%)
3–8 visits	43 (13%)
Characteristics at delivery	n = 333 ^b
Gestational age (completed weeks)	
<34 weeks	56 (17%)
34–36 weeks	72 (22%)
37–38 weeks	115 (35%)
≥39 weeks	90 (27%)
Medically indicated early delivery for preeclampsia (MIDPE) (completed weeks)	
<34 weeks	39 (12%)
34–36 weeks	43 (13%)
37–38 weeks	32 (10%)
≥39 weeks	7 (2%)
Cesarean delivery	148 (44%)

Data are median [interquartile range (IQR)] or n (%).

^a Conditions that necessitated central laboratory work-up to rule-out PE (nephropathy, lupus, fetal growth restriction [FGR], cholestasis, etc.).

^b The difference is accounted by 13 women who were lost to follow-up upon discharge from triage (n = 13, 3.75%).

necessary for error levels set at 0.05 and 260 cases for error set at 0.01. Our final sample of 346 cases was sufficient to detect significance in an AUROC of 0.630 compared to the null diagnostic value of 0.5. A $p < 0.05$ was considered significant throughout all analyses.

2.8. Role of the Funding Sources

The funding sources had no involvement in study design, data collection, analysis and interpretation, or writing the report. The corresponding author had full access to all the data and had final responsibility for the decision to submit the paper for publication.

3. Results

3.1. Patients and Associations Between Triage Diagnostic Decision, Adjudicated Diagnosis, and CRD Paper Test Result

Demographic and outcome characteristics of the patients enrolled in the study are presented in Table 1. In our cohort of women recruited in a tertiary care medical center, 48% of the enrolled patients were nulliparous, 66% were white, and 72% were obese class I or above. The distribution of GA at the first and last triage visit, and the number of triage visits per patient (range 1–8 visits) are shown in the appendix (p. 7). Women were most often referred because of co-morbidities [e.g. nephropathy, lupus, cholestasis, fetal growth restriction (FGR)] that did not allow establishment of a PE diagnosis based on clinical criteria alone. Concern for PE in the setting of chronic (crHTN) and gestational hypertension (gestHTN) followed in frequency. Overall, only 10% of the referrals were for potentially early-onset PE. The majority (n = 206, 62%) of the women in the cohort had a term delivery. The prevalence of MIDPE was 36% (121/333) and of a cesarean delivery 44% (148/333), with 70 (58%) of the women with a MIDPE delivered through cesarean.

A flowchart of the study population is presented in Fig. 2. Median triage utilization for discharged women was 191 [156–250] min. Of the patients admitted with uncertain PE status and who were discharged undelivered, the median duration of hospitalization was 2 (interquartile range [IQR] [1–3]) days. Of the patients enrolled in this study, 133 (38%) had more than one triage visit for PE evaluation.

Urine congophililia was detected in 14 (12%) patients admitted with an uncertain diagnosis and in only 59 (58%) patients admitted with a diagnosis of PE established or confirmed in triage. There were 9 (8%) instances of positive congophililia in the group of patients discharged home absent PE. The CRD Paper Test was positive in just 4 (36%) patients discharged with a diagnosis of PE. Fig. 3 illustrates comparatively, the proportion of positive CRD Paper Tests, proportion of cases adjudicated as PE, and proportion of cases with a triage diagnosis of PE or admitted for further evaluation. In our study, 35 (10%) of the patients had at least one encounter where adjudication resulted in a change in diagnosis. No adverse events resulted from performance of the CRD Paper Test. The demographic and outcome characteristics of the patients grouped by the adjudicated diagnosis of the last triage visit are presented in Table 2.

Collectively, these data suggest that in a U.S. tertiary medical center patients that are referred to hospital triage for PE evaluation spend a long time in triage unit and a large number of them are admitted for further monitoring. Not all women discharged with a diagnosis of PE to be monitored as outpatients had a positive CRD Paper Test result.

3.2. Main Outcome Measure: Diagnostic Characteristics of the CRD Paper Test Result

The breakdown of the population targeted for enrollment with the results of the CRD Paper Test based on the final adjudicated diagnosis is presented in Fig. 4. ROC analysis of the CRD Paper Test result on the ordinal scale (0–2) determined that the best model for PE diagnosis is achieved when WP = 1 and SP = 2 cases are grouped together. The main outcome measure, AUROC of the CRD Paper Test to confirm and rule-out PE based on the adjudicated diagnosis, was the highest compared to all other serum and urine biomarkers (Table 3). A positive CRD Paper Test (WP or SP) had 80.2% sensitivity, 89.2% specificity, 92.1% negative predictive value and 86.7% accuracy to correctly diagnose PE.

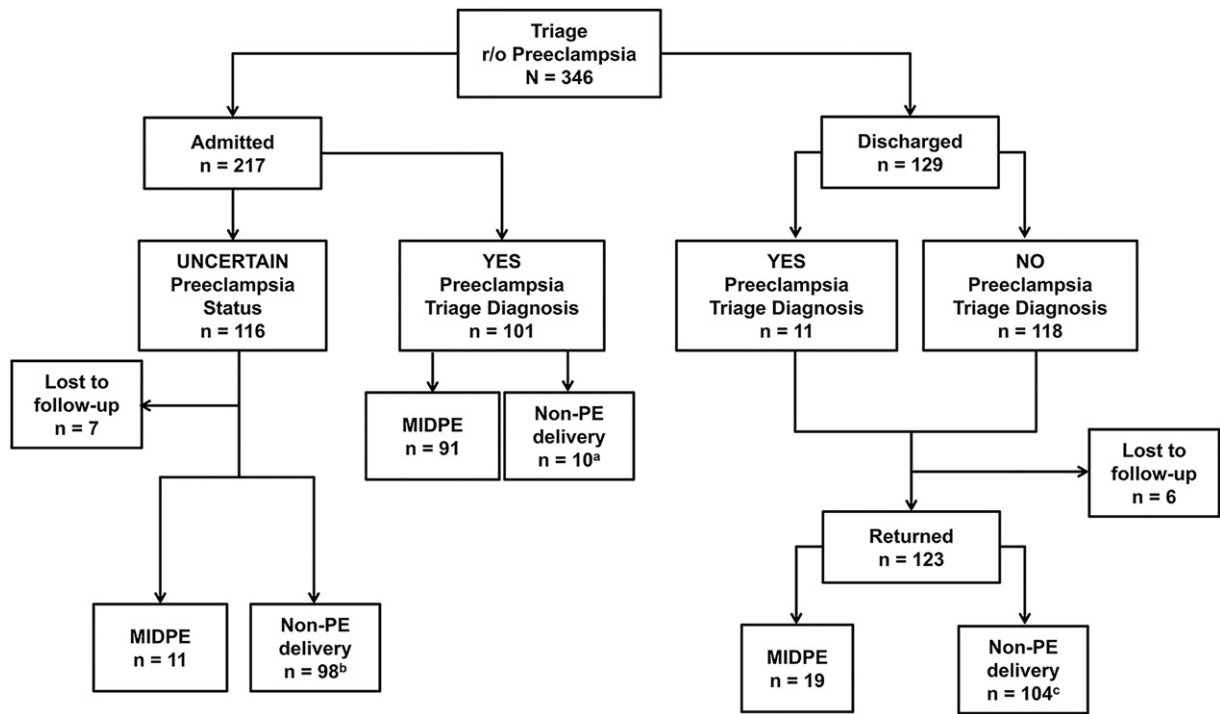


Fig. 2. Consortium diagram of patient flow. We enrolled 346 patients. Hospital admission was recommended for 217 women (63%). Preeclampsia (PE) was the primary diagnosis for admission in 101 (47%) women. Of these, 91 (90%) had a medically indicated delivery for PE (MIDPE). In the remaining ten women (10%) delivery was indicated for non-PE indications (i.e. ruptured membranes, non-reassuring fetal status). Of the 217 admitted women, PE status was uncertain in 116 (53%) patients and required hospital in-patient workup. After in-hospital clinical and laboratory evaluation, 11 (9%) patients underwent a MIDPE. Of the women initially admitted with uncertain PE status, 98 (84%) had a preterm or term delivery unrelated to PE during the first or a subsequent admission. Out of all women enrolled, 129 (37%) patients were discharged after the initial triage visit. In 118 (91%), clinical and laboratory evaluation ruled-out (r/o) PE. The remaining 11 women (9%) were discharged with a diagnosis of PE with mild features, and a recommendation for outpatient follow-up. Of all discharged women, 123 (95%) returned to the hospital for a new triage visit. In the group who returned for at least one more triage visit, 19 (15%) ultimately had a MIDPE. Of women who returned and delivered at our hospital 104 (85%) did not develop PE. ^aIndications for delivery (n = 10): non-reassuring fetal status n = 5; Preterm Premature Rupture of Membranes (PPROM) n = 2; chronic abruption n = 1; labour n = 1; cervical carcinoma n = 1. ^bIndications for delivery (n = 98): spontaneous term labour n = 15; induction of labour at term (gestational age ≥ 37 weeks) n = 8; gestational hypertension n = 34; chronic hypertension n = 13; Intrauterine fetal demise n = 1; spontaneous preterm labour n = 6; chronic abruption/vaginal bleeding n = 1; proteinuria of unknown etiology n = 2; non-reassuring fetal status n = 11; PPRM n = 1; fetal growth restriction (FGR) n = 3; elevated liver functions tests (LFTs) n = 1; diabetes n = 1; induction for oligohydramnios n = 1. ^cIndications for delivery (n = 104): spontaneous term labour n = 25; induction of labour at term (gestational age ≥ 37 weeks) n = 30; gestational hypertension n = 20; chronic hypertension n = 19; repeat cesarean for history of prior classical incision n = 3; spontaneous preterm labour n = 3; chronic abruption/vaginal bleeding n = 2; proteinuria of unknown etiology n = 1; PPRM n = 1.

LRs graphs comparing the CRD Paper Test to other urine and serum analytes dichotomised based on clinically relevant cut-offs are presented in the appendix (p. 8). For the subgroup of women where a

24-hour proteinuria was ordered and completed (n = 168), we compared the CRD Paper Test to blood pressures and total proteinuria as individual characteristics (appendix, p 9).

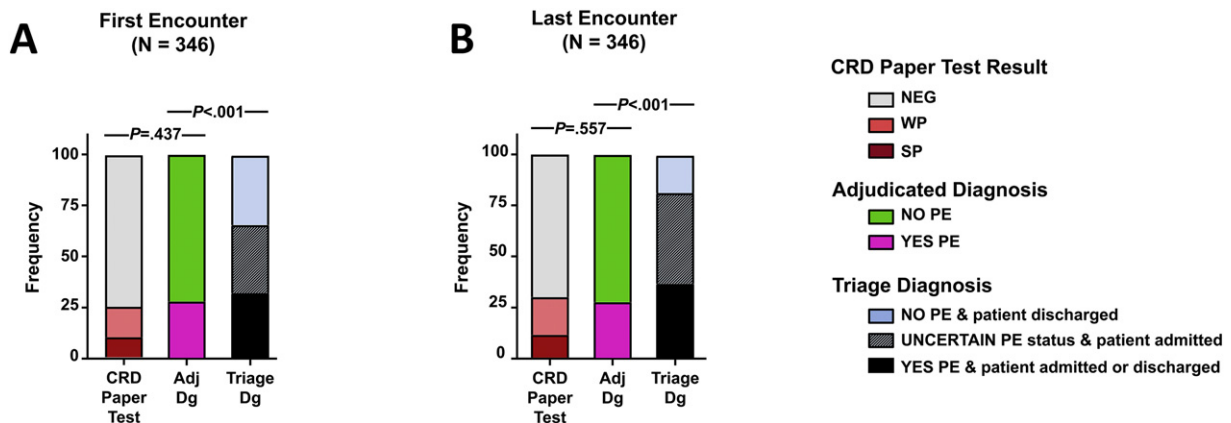


Fig. 3. Proportions of positive and negative CRD Paper Test results based on clinical triage and adjudicated diagnoses. A. After the first triage visit, based on clinical criteria alone, 112 (32%) patients had preeclampsia (PE). However, after adjudication, only 92 (26%) patients met the diagnostic criteria. The adjudication process reclassified 20 (6%) patients. Clinical criteria for PE were not met in 18 patients. In this group just one patient had a weak positive (WP) Congo Red Dot (CRD) Paper Test result. Two patients initially considered to have uncontrolled chronic hypertension, were finally adjudicated to have super-imposed PE. Both women had WP congophilia. At the first encounter, 86 (25%) patients had a positive CRD Paper Test result. The prevalence of a positive CRD Paper Test result was non-significant compared to the prevalence of PE following case adjudication. Yet, based on clinical judgment alone, only 118 (34%) patients were discharged, with the majority of them admitted for either further PE work-up or for delivery. B. Similar relationships were seen based on the analysis of the last visit data. At the last triage encounter (proximal to delivery) 128 (37%) patients had a clinical diagnosis (Dg) of PE. Yet, based on adjudicated (Adj) diagnosis at the time of the last triage visit, only 96 (29%) patients had PE. Following the last triage visit 104 (30%) patients had a positive CRD Paper Test result. The only notable exception with data analysis based on the first visit was that the proportion of women (18%, 64) discharged based on ability to rule-out PE on clinical grounds alone was lower.

Table 2
Characteristics of women grouped by adjudicated diagnosis.

Variable	NO preeclampsia adjudicated diagnosis n = 250	YES preeclampsia adjudicated diagnosis n = 96	p value
Maternal age (years)	30 [26–34]	28 [24–32]	0.041
Parity	1 [0–2]	0 [0–1]	0.114
Nulliparity	44 (46%)	52 (54%)	0.215
Multiple gestation	8 (3%)	6 (6%)	0.329
Race/ethnicity of women			0.851
White	166 (66%)	60 (63%)	
Black/African American	71 (28%)	30 (31%)	
Hispanic	6 (2%)	2 (2%)	
Other	7 (3%)	4 (4%)	
BMI (kg/m ²)	35.4 [29.9–41.2]	34.5 [29.5–42.5]	0.968
Highest systolic blood pressure (mm Hg)	148 [137–163]	161 [145–177]	<0.001
Highest diastolic blood pressure (mm Hg)	91 [82–99]	97 [86–106]	0.001
P:C ratio ordered	222 (89%)	84 (88%)	0.880
P:C ratio	0.2 [0.1–0.3]	1.0 [0.5–3.6]	<0.001
P:C ratio ≥ 0.3	48 (22%)	74 (88%)	<0.001
24 h proteinuria ordered	117 (47%)	63 (66%)	0.003
24 h proteinuria (mg/24 h)	244 [186–344]	810 [438–1923]	<0.001
24 h proteinuria ≥ 300 mg/24 h	43 (37%)	59 (94%)	<0.001
Gestational age at birth (completed weeks)*	38 [37–39]	34 [32–36]	<0.001
Birthweight (grams)*	2068 [1485–2941]	3184 [2750–3567]	<0.001
Cesarean delivery*	92 (39%)	56 (58%)	0.002
Newborn admitted to intensive care unit*	45 (19%)	60 (63%)	<0.001
Maternal and/or fetal co-morbidities†			0.912
Nephropathy	7 (3%)	2 (2%)	
Lupus	3 (1%)	1 (1%)	
Chronic hypertension	76 (30%)	27 (28%)	
History of seizures	5 (2%)	2 (2%)	
History of migraines	26 (10%)	7 (7%)	
Cholestasis	2 (1%)	2 (2%)	
Fetal growth restriction	6 (2%)	5 (5%)	

Data are presented as median [interquartile range (IQR)] or n (%). Statistical comparison performed by Mann–Whitney or Chi-square or tests, respectively. *Excludes data from the 13 women lost to follow-up. †Some cases had more than one co-morbidity. The statistical comparison between groups is in proportion of cases with at least one of the listed co-morbidities.

Using the prevalence of PE (27.7%) to estimate the pre-test probability in our population, the CRD Paper Test had a positive post-test probability of 74%, 95% CI [66–81] and a negative post-test probability of 8%, 95% CI [5–11] (appendix, p 10). The clinical interpretation of

these results is that one in 1.4 patients with a positive test has PE, while one in 1.1 patients with a negative test does not have PE. Taken together, our results indicate that CRD Paper Test is an accurate and rapid diagnostic triage test for PE.

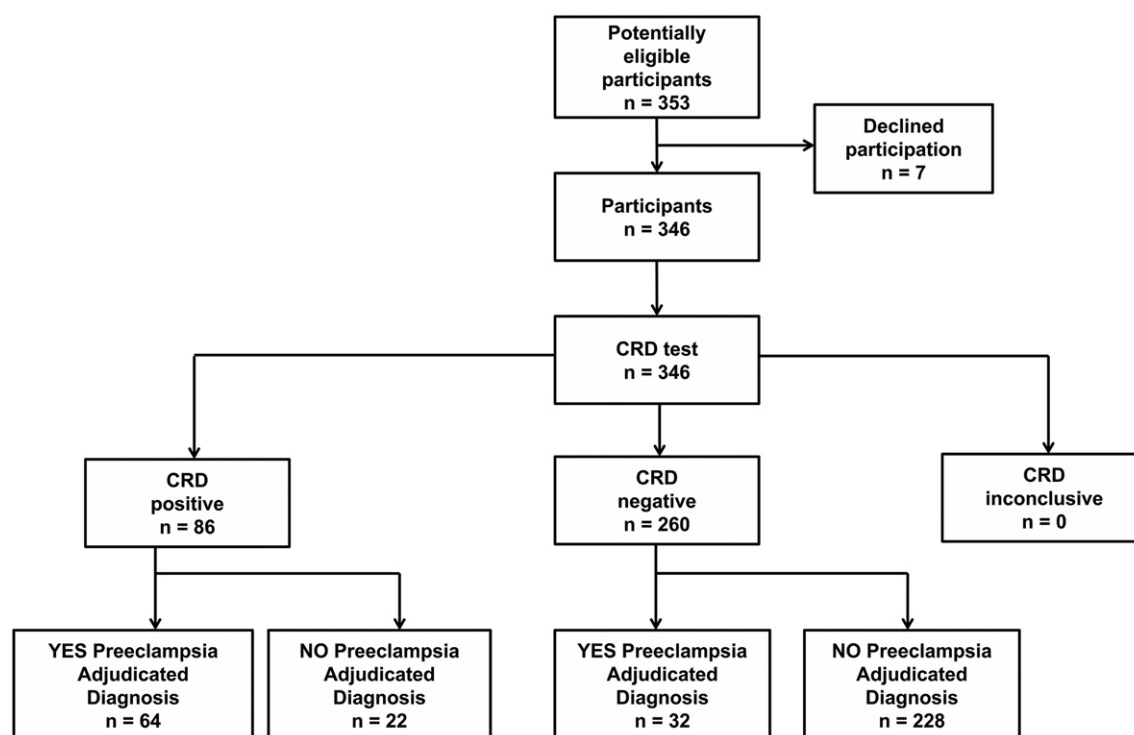


Fig. 4. STARD flow diagram for a study of 346 enrolled patients undergoing triage evaluation for preeclampsia.

Table 3
Comparative accuracy results for predicting adjudicated PE diagnosis.

Test employed, n	AUROC [95% CI]	Cut-off	AUROC-LR [95% CI]	p value vs. CRD Paper Test	Sensitivity (%) [95% CI]	Specificity (%) [95% CI]	PPV (%) [95% CI]	NPV (%) [95% CI]	+ LR [95% CI]	– LR [95% CI]	Accuracy [95% CI]	Youden Index (J)
Urine												
CRD Paper Test, n = 346	0.850 [0.808–0.886]	WP or SP†	0.847 [0.805–0.883]	NA	80.2 [70.8–87.6]	89.2 [84.7–92.8]	74.0 [66.3–80.5]	92.1 [88.7–94.6]	7.43 [5.1–10.8]	0.22 [0.1–0.3]	86.7 [83.1–90.3]	0.694
Urine sFlt-1, n = 343*	0.723 [0.673–0.770]	>29 pg/mL†	0.697 [0.645–0.745]	<0.001	55.3 [44.7–65.6]	83.5 [78.3–87.9]	55.9 [47.6–63.9]	83.2 [79.7–86.2]	3.36 [2.4–4.7]	0.53 [0.4–0.7]	75.8 [71.3–80.3]	0.389
Urine PlGF, n = 343*	0.678 [0.625–0.727]	≤29 pg/mL†	0.666 [0.613–0.716]	<0.001	64.9 [54.4–74.5]	68.3 [63.1–74.0]	43.6 [37.9–4.9]	83.7 [79.4–87.3]	2.05 [1.6–2.6]	0.51 [0.4–0.7]	67.3 [62.4–73.2]	0.336
uFP, n = 343*	0.765 [0.716–0.809]	>1.7†	0.708 [0.657–0.756]	<0.001	79.8 [70.2–87.4]	61.9 [55.5–67.9]	44.1 [39.5–48.8]	89.0 [84.3–92.5]	2.09 [1.7–2.5]	0.33 [0.2–0.5]	66.8 [61.8–71.7]	0.416
		>2.1‡	0.699 [0.647–0.747]	<0.001	60.6 [50.0–70.6]	79.1 [73.5–84.0]	52.3 [41.6–61.1]	84.2 [80.4–87.3]	2.90 [2.2–3.9]	0.50 [0.4–0.6]	74.1 [69.4–78.7]	0.398
Serum												
Serum sFlt-1, n = 239§	0.809 [0.753–0.856]	>5300 pg/mL†	0.763 [0.704–0.816]	0.030	83.9 [71.2–92.2]	69.0 [61.8–75.6]	44.7 [34.9–54.8]	93.4 [87.8–96.9]	2.70 [2.1–3.5]	0.24 [0.1–0.4]	72.4 [66.7–78.1]	0.527
Serum PlGF, n = 239§	0.747 [0.687–0.801]	≤216 pg/mL†	0.700 [0.638–0.757]	<0.001	80.0 [70.5–87.5]	52.7 [45.2–60.1]	35.6 [27.5–44.2]	93.3 [86.6–97.3]	1.85 [1.5–2.2]	0.24 [0.1–0.5]	60.7 [50.5–66.9]	0.400
Serum sFlt-1/PlGF ratio, n = 239§	0.820 [0.765–0.866]	>32.5†	0.757 [0.697–0.810]	<0.001	81.8 [69.1–90.9]	69.6 [69.4–76.1]	44.6 [34.7–54.8]	92.8 [87.1–96.5]	2.69 [2.1–3.5]	0.26 [0.1–0.5]	72.4 [66.7–78.1]	0.514
		≥85, GA 20 ^{0/7} –33 ^{6/7} ≥ 110, GA ≥ 34 ^{0/7}	0.696 [0.633–0.753]	<0.001	47.3 [33.7–61.2]	91.9 [86.9–95.4]	63.4 [46.9–77.9]	85.5 [79.6–90.0]	5.8 [3.3–10.1]	0.6 [0.4–0.7]	81.6 [76.7–86.5]	0.391

AUROC = area under the ROC plot. AUROC-LR = AUROC of the graph generated from likelihood ratio co-ordinates. PPV = positive predictive value. NPV = negative predictive value. +LR = positive likelihood ratio. –LR = negative likelihood ratio. CRD = Congo Red Dot. WP = weak positive. SP = strong positive. sFlt-1 = soluble fms-like tyrosine kinase-1. PlGF = placental growth factor. uFP = urine sFlt-1/PlGF. *Data missing for 3 patients due to insufficient sample volume. †Optimal cut-off in this population based on maximal Youden Index. ‡Previously published cut-off [10]. §Serum samples were not available for 107 women who did not agree to venipuncture. ||Previously published cut-off [18]. Accuracy characteristics were analyzed based on samples collected at the last triage encounter.

3.3. Breakdown Characteristics of Cases by MIDPE, Adjudicated Diagnosis and CRD Test Result

In Table 4 we present the grouping of cases based on final clinical outcome in real-life setting (MIDPE or no MIDPE), final adjudicated diagnosis and result of the CRD Paper Test. In the NO-MIDPE group 181 (90%) patients were adjudicated as non-PE and all had a negative CRD Paper Test. In the NO-MIDPE group, a positive CRD Paper Test was observed in 20 (10%) patients. Case by case analysis determined that these patients had a history of kidney diseases, crHTN or gestational hypertension (gestHTN). In the NO-MIDPE group 11 (5%) patients were adjudicated as PE. Of these, three (27%) patients had a negative CRD Paper Test, and were delivered for clinical indications other than PE. The other eight (73%) patients had a positive CRD Paper Test and were considered PE. However, following admission the primary indication for delivery was non-reassuring fetal status or PPRM.

In the YES-MIDPE group 36 (30%) patients were adjudicated as NO-PE. In this subgroup, 33 (92%) patients had a negative CRD Paper Test. The majority of these patients were at term or near-term (GA: 36.8 [35.5–37.5] weeks), and delivery indications were most often prompted by non-specific headache. Among the three YES-MIDPE patients adjudicated as NO-PE but with a positive CRD Paper Test, the primary indication for delivery was PE or spPE. In this scenario the adjudicated diagnosis was crHTN. In the YES-MIDPE group, the adjudicated diagnosis was in agreement with the delivery indication in 85 (70%) patients. In this subgroup, 69 (81%) patients had a positive CRD Paper Test. Yet, 16 (19%) patients with YES-MIDPE who were adjudicated as having PE had a negative CRD Paper Test. This subgroup was populated by a heterogeneous mix of cases that had crHTN, isolated features of HELLP, absent hypertension or proteinuria or a spectrum of clinical symptoms non-specific for PE in patients with complex comorbidities (i.e. headache in the context of history of migraines, epigastric pain in a patient with history of gastro-esophageal reflux). In summary, in both NO-MIDPE and YES-MIDPE groups most false positive or false negative CRD Paper Test results occurred in the context of PE imitators such as crHTN, gestHTN, or kidney disease.

3.4. Characteristics of Cases With Multiple Triage Visits

Out of all patients enrolled 133 (38%) were referred to triage more than once. Select characteristics of cases grouped by the sequence of CRD Paper Test results are presented in the appendix (p. 5). In our cohort, CRD Paper Test was consistently negative in 88 (66%) patients.

Of this group 3 (3%) patients were adjudicated as PE. Interestingly, 14 (16%) patients had a MIDPE at term. 22 (6%) patients with an initially negative CRD Paper test, displayed conghophilia at a subsequent visit. The median interval from the last negative CRD test and the first positive result was 12 [5–34] days. Women who tested consistently positive had the shortest time to delivery and the lowest GA to delivery. Only 4 patients tested negative after a prior positive (all WP) CRD Paper Test result. To summarize, the CRD Paper Test result can turn positive within 2 weeks prior to clinical manifestation of PE.

4. Discussion

In this study we examined the performance of the CRD Paper Test to diagnose PE in women who presented to L&D triage unit with clinical symptoms and signs requiring diagnostic work-up for hypertension. In comparison to previously reported urine and serum biochemical markers, the CRD Paper Test was superior in both establishing and ruling-out PE. We also determined that a significant proportion of women sent to triage to be evaluated for PE are admitted with an uncertain diagnosis. Out of this group the majority of patients are ultimately discharged undelivered. A minority of cases are discharged from triage with a diagnosis of PE to be followed-up in an outpatient setup. Interestingly, although perceived by providers as PE, some of these women did not have a positive CRD Paper Test result. For both NO-MIDPE and YES-MIDPE groups, case adjudication suggested physicians miss or over diagnose PE.

Although extensively studied for diagnosis and prediction of PE, serum and urine PIGF and sFlt-1, alone or in combination with uterine artery Doppler ultrasound, did not gain clinical momentum [25,26]. The invasive nature of blood sampling, reliance on expensive, lengthy central laboratory procedures and skilled personnel, and difficulty in interpreting results relative to GA intervals and from different platforms may have played a role [25,27]. The aforementioned factors hinder practical implementation of testing for PE in the real-world and even more so in low-resourced countries where morbidity and mortality from PE and eclampsia are the highest [4]. The operational simplicity of the CRD Paper Test fulfills the current needs for a diagnostic tool to aid in the rapid assessment and triage of women with uncertain PE diagnosis.

Traditionally, PE is defined as new-onset hypertension and proteinuria after 20 weeks of gestation [28]. Some members of the obstetrical community seem to hold firm to the view that PE is easy to diagnose. This position is difficult to support based on our pragmatic study design. We found that physicians who are unaware of the CRD Paper Test

Table 4
Breakdown of cases by outcome, final adjudicated diagnosis and Congo Red Dot (CRD) Paper Test result.

Clinical outcome ^a	Adjudicated diagnosis	Positive CRD test result	Case notes
NO MIDPE n = 212	NO PE n = 201	NO, n = 181	Unanimous concordance ruling out PE
		True negative	
		YES, n = 20	
		False positive	
	YES PE n = 11	NO, n = 3	Cases in this category were medically indicated deliveries due to worsening chronic nephropathy, worsening crHTN or gestHT without a call of PE or spPE Case adjudication was of PE w/o severe features or crHTN with spPE. All 3 cases were managed expectantly and all had medically indicated preterm deliveries for NRFHR or PPRM CRD concurred with the adjudicated diagnosis. All these cases were admitted following initial evaluation in triage (7 with a PE dg and 1 with uncertain PE status). Delivery indications were for all preterm for NRFHR or PPRM
		False negative	
		YES, n = 8	
		True positive	
YES MIDPE n = 121	NO PE n = 36	NO, n = 33	CRD concurred with the adjudicated diagnosis. Most MIDPE indications were in context of non-specific headache and the majority were near-term or at term Case adjudication was crHTN while the managing team's call was PE w/o severe features or spPE
		True negative	
		YES, n = 3	
		False positive	
	YES PE n = 85	NO, n = 16	Delivery indications and adjudication concurred as PE w/o severe features, spPE in the context of crHTN or HELLP syndrome features absent hypertension or proteinuria Unanimous concordance confirming PE
		False negative	
		YES, n = 69	
		True positive	

MIDPE = medically indicated delivery for preeclampsia. PE = preeclampsia. crHTN = chronic hypertension. gestHTN = gestational hypertension. spPE = superimposed PE. NRFHR = non-reassuring fetal heart rate. PPRM = preterm premature rupture of membranes. HELLP = hemolysis, elevated liver enzymes, low-platelet count.

^a Table does not include the 13 cases lost to follow-up.

results admit approximately a third of triaged patients due to diagnostic uncertainty. In this group, after extensive, lengthy and expensive inpatient work-up, only 9% of initially triaged women ultimately received a diagnosis of PE and had MIDPE. This is not surprising. PE is a heterogeneous unpredictable syndrome with a large spectrum of medical signs and symptoms resulting in a variety of clinical phenotypes and outcomes. In triage, clinicians take repeated blood pressure measurements and obtain information about headache, visual disturbances, chest pain, epigastric pain which none are specific to PE [25]. As it was determined from the demographic characteristics of our population the majority of the women presenting in triage are obese and already hypertensive. Because history and physical examination have limited accuracy in such population, and cannot be used alone for management decisions, the CRD Paper Test is uniquely positioned to increase the effectiveness of the triage process and possibly reduce health care costs [25]. Specifically, the CRD Paper Test has the potential to cut the need for triage referrals, decrease the turn-around time for diagnosis, and shorten the length of stay in obstetrical triage and antepartum units by eliminating unnecessary hospital admissions and/or early deliveries. A recent U.S. healthcare utilization analysis that included both maternal and neonatal costs estimated that in 2012 the incremental cost of deliveries was \$2.18 billion for the first 12 months after delivery of a mother with PE and \$1.15 billion for infants born to mothers with PE [29]. These calculations underscore the importance of an accurate test to diagnose PE.

Several groups have validated, in cohorts different than ours, that women with PE have elevated urine congophililia [14,15,16]. Importantly, all of these studies employed our previously published nitrocellulose laboratory protocol [13]. Most recently, Nagarajappa et al. concluded that urinary congophililia can be used to identify PE women from normotensive pregnant women [15]. Their study replicated our protocol in a laboratory hospital in rural India and showed that urinary congophililia was not affected by clinical variables such as GA of onset, severity, superimposition by eclampsia, fetal growth restriction or stillbirth. The authors opined that chronic kidney disorders (CKD) cannot be a major confounding factor in the clinical utility of urinary congophililia to diagnose PE as applied to the general pregnant population. In a prior study, McCarthy et al. confirmed elevated congophililia in PE, which was not present in non-pregnant women with systemic lupus erythematosus alone [16]. However, they noted some non-pregnant, advanced age women with lupus nephritis and some pregnant women with undefined CKD exhibited urine congophililia. This feature may reflect renal amyloidosis, a pre-Alzheimer state or PE in subclinical state for which they did not control or comment. Regardless, McCarthy and colleagues used urine samples from their highly selective Registry of Connective tissue diseases repository, which cannot be an accurate reflection of the general pregnant population [16]. We hope that the current study using the new CRD Paper Test will further enable and encourage other groups to perform studies for PE at point-of-care in different populations at high- and low-risk of PE.

The results of the HYPITAT-I trial changed physicians behaviour and attitude toward labour induction in women with hypertension at term [30,31]. Analysis of the trend post-HYPITAT revealed that reflex delivery of a hypertensive woman at >37 weeks led to an increase in inductions with decreased prevalence of PE [30]. Yet, what remains unknown is how many early-term (37–38 weeks) deliveries were indicated in the absence of PE, and what was the impact on neonatal outcomes, already known to be sub-optimal for early-term neonates [32]. Our adjudication process proved that ~30% of MIDPE cases did not meet full diagnostic criteria for PE. Most of these patients had a negative CRD Paper Test and were delivered at term for non-specific headaches. This approach is not unique, and emphasizes the tendency of U.S. physicians to more loosely opt for indicated delivery, especially approaching term. We believe that the CRD Paper Test has potential to shift the late preterm and early-term delivery curves to the right, and thus avoid unnecessary admissions to newborn critical care units [33]. Second, CRD Paper Test

could be a useful tool to longitudinally monitor patients across gestation. In our study, the median time interval between conversion of a negative to a positive CRD Paper Test result was 12 days. Thus, the CRD Paper Test may help guide medical decision making regarding administration of steroids and magnesium for prevention of neonatal morbidity.

Clearly, not all women presenting at the hospital or in the ambulatory centers with PE-like symptoms should be delivered as they may not actually have PE but rather PE imitators [34]. The effectiveness of hypertension and proteinuria as diagnostic “gold standard” is even further compromised when PE is superimposed on conditions, such as crHTN, liver or chronic kidney diseases. The majority of false positives and false negative cases were observed in patients with crHTN, gestHTN and kidney disorder, where an accurate diagnosis of PE cannot be made on clinical grounds alone. However, it is important that clinicians make a correct diagnosis because the management and complications from these syndromes may differ. The CRD Paper Test adds clarity to help differentiate PE from PE imitators, which should result in fewer iatrogenic preterm deliveries.

Our pragmatic study design allowed us to evaluate patient flow in real-life practice conditions. Compared to prior studies that assessed immunoassay-based diagnostic devices in PE [35], our cohort evaluated the clinical utility of a point-of-care diagnostic tool in real time, and it was executed by clinical trained nurses in the triage area. Importantly, we had the ability to observe the patients longitudinally with maximal completeness of data and minimal lost for follow-up. The likelihood of discrepant interpretation of clinical and laboratory endpoints is increased in PE considering the diagnostic subjectivity and GA at evaluation. Our adjudication process allowed us to precisely point toward the cases where availability of the CRD Paper Test result would have potentially changed the clinical practice.

This study has several limitations, including recruitment at a single site. Although other groups already confirmed the value of congophililia [16,14], this prospective cohort should be viewed as a key initial study to explore the significance of introducing the CRD Paper Test in the current clinical practice. Generalizability and cost effectiveness of using the CRD Paper Test in high and low income countries must be addressed. Such trials are currently ongoing. Reporting the results of a test modifies the clinical decision process vis-a-vis hospitalization of women with PE [36,37]. By study design the physicians in charge were unaware of the results of the CRD Paper Test. Therefore, we could not calculate the financial impact communication of the results of the CRD Paper Test that could have had on healthcare system. We believe, reporting the results of the test to the practising physicians would have eliminated unnecessary hospital admissions, and many expenses including facility fees and the costs of laboratory testing and nursing care. Based on the number of patients discharged following their initial hospital admission, we can only approximate that at least 246 inpatient care days would have potentially been saved.

In summary, CRD Paper Test is an accurate, low technology, easy to use triage diagnostic tool that allows for accurate identification of PE within minutes.

Authors' contribution

Drs. K.M. Rood, I.A. Buhimschi, and C.S. Buhimschi had full access to the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. Concept and study design: K.M. Rood, I.A. Buhimschi, C.S. Buhimschi; Acquisition, analysis, or interpretation of the data: K.M. Rood, T. Dible, S. Webster, G. Zhao, P. Samuels, I.A. Buhimschi, C.S. Buhimschi; Drafting of the manuscript: K.M. Rood, I.A. Buhimschi, C.S. Buhimschi; Critical revision of the manuscript for important intellectual content: All authors; Statistical analysis: I.A. Buhimschi, C.S. Buhimschi, K.M. Rood; Obtained funding: I.A. Buhimschi, C.S. Buhimschi.

Funding

Funded by Saving Lives at Birth (SLaB) Grant AID-OAA-A-14-00017 (IAB) from USAID, the Bill & Melinda Gates Foundation, Government of Norway, Grand Challenges Canada (Government of Canada), and the United Kingdom's Department for International Development (DFID). Additional funds were from National Institutes of Health (NIH) Eunice Kennedy Shriver National Institute of Child Health and Human Development R01 HD047321 (IAB).

Declaration of Interests

IAB and CSB are named as inventors or co-inventors on patent applications filed by Yale University on the use of protein misfolding for diagnostic and treatment purposes of PE, some which are described in the article. Both received royalties from Yale University in accordance with institutional policies for inventorship. Commercial development of the CRD Paper Test has been licensed by Yale University to GestVision Inc. and stock awarded to IAB and CSB, in accordance with institutional licensing policies. A conflict of interest mitigation plan has been set in place at The Research Institute at Nationwide Children's Hospital and The Ohio State University as required for studies sponsored by federal funds. The other authors have no conflicts to declare.

Acknowledgments

We are indebted to the nurses, fellows and residents at the Ohio State University Wexner Medical Center, Department of Obstetrics and Gynecology, and to all patients who participated in the study. IAB and CSB were supported by USAID, SLaB, and National Institutes of Health/ Eunice Kennedy Shriver National Institute of Child Health and Human Development (NIH/NICHD).

Data Availability

Following publication, the study protocol and de-identified research data can be made available upon request to either the corresponding or senior author with a signed data access agreement. Dr. Kara M. Rood had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.eclim.2019.02.004>.

References

- [1] James PR, Nelson-Piercy C. Management of hypertension before, during, and after pregnancy. *Heart* 2012;90:1499–504.
- [2] Uzan J, Carbone M, Piconne O, Asmar R, Ayoubi JM. Pre-eclampsia: pathophysiology, diagnosis, and management. *Vasc Health Risk Manag* 2011;7:467–74.
- [3] Maruotti GM, Sarno L, Napolitano R, et al. Preeclampsia in women with chronic kidney disease. *J Matern Fetal Neonatal Med* 2012;25:1367–9.
- [4] Ronsmans C, Graham WJ, Lancet Maternal Survival Series steering group. Maternal mortality: who, when, where, and why. *Lancet* 2006;368:1189–200.
- [5] World Health Organization. World health statistics 2015. Geneva, Switzerland: World Health Organization; 2015.
- [6] Khan KS, Wojdyla D, Say L, Gülmezoglu AM, Van Look PFA. WHO analysis of causes of maternal death: a systematic review. *Lancet* 2006;367:1066–74.
- [7] Hogan MC, Foreman KJ, Naghavi M, et al. Maternal mortality for 181 countries, 1980–2008: a systematic analysis of progress towards Millennium Development Goal 5. *Lancet* 2010;375:1609–23.
- [8] Lawn JE, Blencowe H, Waiswa P, et al. Stillbirths: rates, risk factors, and acceleration towards 2030. *Lancet* 2016;387:587–603.
- [9] Levine RJ, Thadhani R, Qian C, et al. Urinary placental growth factor and risk of pre-eclampsia. *JAMA* 2005;293:77–85.
- [10] Buhimschi CS, Norwitz ER, Funai E, et al. Urinary angiogenic factors cluster hypertensive disorders and identify women with severe preeclampsia. *Am J Obstet Gynecol* 2005;192:734–41.
- [11] Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004;350:672–83.
- [12] Buhimschi IA, Zhao G, Funai EF, et al. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *Am J Obstet Gynecol* 2008;199:551 [e1–16].
- [13] Buhimschi IA, Nayeri UA, Zhao G, et al. Protein misfolding, congophilin, oligomerization, and defective amyloid processing in preeclampsia. *Sci Transl Med* 2014;6:245ra92.
- [14] Sammar M, Syngelaki A, Sharabi-Nov A, Nicolaides K, Meiri H. Can staining of damaged proteins in urine effectively predict preeclampsia? *Fetal Diagn Ther* 2017;41:23–31.
- [15] Nagarajappa C, Rangappa SS, Suryanarayana R, Balakrishna S. Urinary congophilin in preeclampsia: experience from a rural tertiary-care hospital in India. *Pregnancy Hypertens* 2018;13:83–6.
- [16] McCarthy IA, Adetoba A, Gill C, et al. Urinary congophilin in women with hypertensive disorders of pregnancy and preexisting proteinuria or hypertension. *Am J Obstet Gynecol* 2016;215:464 [e1–7].
- [17] Kahan BC, Feagan B, Jairath V. A comparison of approaches for adjudicating outcomes in clinical trials. *Trials* 2017;18:266.
- [18] Verloren S, Herraiz I, Lapaire O, et al. New gestational phase-specific cutoff values for the use of the soluble fms-like tyrosine kinase-1/placental growth factor ratio as a diagnostic test for preeclampsia. *Hypertension* 2014;63:346–52.
- [19] Drain PK, Hyle EP, Noubary F, et al. Diagnostic point-of-care tests in resource-limited settings. *Lancet Infect Dis* 2014;14:239–49.
- [20] Mastrovitch TA, Bithoney WG, DeBari VA, Nina AG. Point-of-care testing for drugs of abuse in an urban emergency department. *Ann Clin Lab Sci* 2002;32:383–6.
- [21] Youden WJ. Index for rating diagnostic tests. *Cancer* 1950;3:3–35.
- [22] Biggerstaff BJ. Comparing diagnostic tests: a simple graphic using likelihood ratios. *Stat Med* 2000;19:649–63.
- [23] Johnson NP. Advantages to transforming the receiver operating characteristic (ROC) curve into likelihood ratio co-ordinates. *Stat Med* 2004;23:2257–66.
- [24] Fagan TJ. Letter: nomogram for Bayes' theorem. *N Engl J Med* 1975;293:257.
- [25] Mol BWJ, Roberts CT, Thangaratnam S, Magee LA, de Groot CJM, Hofmeyr GJ. Preeclampsia. *Lancet* 2016;387:999–1011.
- [26] O'Gorman N, Wright D, Poon LC, et al. Multicenter screening for pre-eclampsia by maternal factors and biomarkers at 11–13 weeks' gestation: comparison with NICE guidelines and ACOG recommendations. *Ultrasound Obstet Gynecol* 2017;49:756–60.
- [27] Staff AC, Burke Ó, Benton S, et al. Maternal circulating PIGF concentrations and placenta-related pregnancy complications: first results from the CoLab AngF study. *Pregnancy Hypertens* 2013;3:59.
- [28] American College of Obstetricians and Gynecologists, Task Force on Hypertension in Pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet Gynecol* 2013;122:1122–31.
- [29] Stevens W, Shih T, Incerti D, et al. Short-term costs of preeclampsia to the United States health care system. *Am J Obstet Gynecol* 2017;217:237–48 [e16].
- [30] Koopmans CM, Bijlenda D, Groen H, HYPITAT study group, et al. Induction of labour versus expectant monitoring for gestational hypertension or mild pre-eclampsia after 36 weeks' gestation (HYPITAT): a multicentre, open-label randomised controlled trial. *Lancet* 2009;374:979–88.
- [31] van der Tuuk K, Koopmans CM, Groen H, Mol BW, van Pampus MG, HYPITAT study group. Impact of the HYPITAT trial on doctors' behaviour and prevalence of eclampsia in the Netherlands. *BJOG* 2011;118:1658–60.
- [32] Helle E, Andersson S, Häkkinen U, Järvelin J, Eskelinen J, Kajantie E. Morbidity and health care costs after early term birth. *Paediatr Perinat Epidemiol* 2016;30:533–40.
- [33] Broekhuijsen K, van Baaren GJ, van Pampus MG, et al. HYPITAT-II study group. Immediate delivery versus expectant monitoring for hypertensive disorders of pregnancy between 34 and 37 weeks of gestation (HYPITAT-II): an open-label, randomised controlled trial. *Lancet* 2015;385:2492–501.
- [34] Sibai BM. Imitators of severe pre-eclampsia. *Semin Perinatol* 2009;33:196–205.
- [35] Chappell LC, Duckworth S, Seed PT, et al. Diagnostic accuracy of placental growth factor in women with suspected preeclampsia: a prospective multicenter study. *Circulation* 2013;128:2121–31.
- [36] Klein E, Schlembach D, Ramoni A, et al. Influence of the sFlt-1/PlGF ratio on clinical decision-making in women with suspected preeclampsia. *PLoS One* 2016;11:e0156013.
- [37] Redelmeier DA. Improving patient care. The cognitive psychology of missed diagnoses. *Ann Intern Med* 2005;142:115–20.

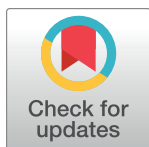
RESEARCH ARTICLE

Late pregnancy screening for preeclampsia with a urinary point-of-care test for misfolded proteins

Xing-Min Li^{1*}, Xue-Min Liu², Jun Xu¹, Juan Du², Howard Cuckle³

1 Shuwen Biotech Co. Ltd, Deqing, Zhejiang Province, China, **2** Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, Shenyang, Liaoning Province, China, **3** Department of Obstetrics and Gynecology, Columbia University Medical Center, New York City, New York, United States of America

* barclay@shuwendx.com



Abstract

The aim was to describe and assess a new late pregnancy point-of-care urinary preeclampsia screening test. Urine samples were collected from a consecutive series of 1,532 pregnant women hospitalized at 20–41 weeks gestation in a Chinese single obstetric unit. A simple disposable Congo red based device was newly developed and employed to prospectively test misfolded proteins in pregnant women's urine. A total of 140 preeclampsia cases were clinically diagnosed, 101 severe and 87 pre-term. Detection and false positive rates were similar in the training and validation subsets with combined 74% and 3.0%. The detection rate was 83% in severe, 86% in pre-term, 49% and 50% in mild and term cases ($P < 0.0001$) respectively. In conclusion, a simple point-of-care urinary test for misfolded proteins can be used to screen for preeclampsia in late pregnancy with very high screening performance. To the best of our knowledge, this is the first study to screen for preeclampsia using Congo red based device in Chinese pregnant population.

OPEN ACCESS

Citation: Li X-M, Liu X-M, Xu J, Du J, Cuckle H (2020) Late pregnancy screening for preeclampsia with a urinary point-of-care test for misfolded proteins. PLoS ONE 15(5): e0233214. <https://doi.org/10.1371/journal.pone.0233214>

Editor: Frank T. Spradley, University of Mississippi Medical Center, UNITED STATES

Received: January 22, 2020

Accepted: April 17, 2020

Published: May 20, 2020

Copyright: © 2020 Li et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: Our financial disclosure is amended as follows: This research was funded by Shuwen Biotech. The funder Shuwen Biotech provided support in the form of salaries for authors X.Li and J.X., but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Introduction

Worldwide, 2–8% of pregnant women suffer from preeclampsia, it is a major cause of maternal mortality and accounts for a large proportion of preterm deliveries [1]. Primary screening for preeclampsia in early pregnancy and low dose aspirin prophylaxis can prevent about half of preterm cases (i.e. delivering before 37 weeks gestation)—screening detection rate 77% and prevention by aspirin 62% [2]. Screening in late pregnancy is needed for women not screened earlier, and those not prevented by such screening including term preeclampsia, comprising at least two-thirds of cases. Typically, late pregnancy screening is secondary, carried out on women who present with symptoms of preeclampsia or signs of pregnancy related problems.

One approach is maternal serum screening using markers such as soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) [3]. But sFlt-1/PlGF testing requires specific equipment and is not feasible for screening in areas with limited resources. Serum PlGF and another biomarker serum pregnancy-associated plasma protein A (PAPP-A) have been

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: X.Li and J.X. are employed by Shuwen Biotech, which manufactures the CapCord test described and evaluated in this paper. H.C. is a paid consultant of PerkinElmer Life Science which has a commercial partnership with Shuwen Biotech. X.Liu and J.D. have no competing interests to declare. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

incorporated into the algorithm of the International Federation of Gynecology and Obstetrics (FIGO) for pre-eclampsia screening in first-trimester with detection rates of preterm and term preeclampsia between 75%-77% and 47%-54% at false-positive rate of 10% [4]. Another is maternal urine screening for misfolded proteins based on their affinity for the Congo red dye ('congophilia'). Initially, proteomic analysis suggested that preeclampsia is associated with misfolded proteins which are aggregated in the placenta [5]. This was then confirmed by four studies which demonstrated that in late pregnancy maternal urine samples taken at the time of preeclampsia diagnosis there was considerably more congophilia than in unaffected pregnancies [6–9].

One study followed up 28 asymptomatic women at high risk of preeclampsia and found that in seven out of nine who were subsequently shown to have the condition, the increase in congophilia occurred more than 10 weeks before diagnosis [6]. This encouraged the researchers to develop a simple point-of-care device that could be used to screen for preeclampsia. In a study of 346 women presenting to a labour and delivery triage the test had an 82% detection rate and 11% false-positive rate for preeclampsia, a performance exceeding that of serum markers tested concurrently [10].

In the current study a new point-of-care urinary Congo red test which is different from the previous method by using capillary tube-based slow release method is described and evaluated in Chinese women hospitalised in late pregnancy. The screening performance is assessed in relation to the severity of preeclampsia as well as any associations between positive test results and other factors.

Materials and methods

The protocol of this study was approved and informed consent was waived because of the study using residual sample of urinalysis and involving no more than minimal risk by the Institutional Review Board of Shengjing Hospital of China Medical University, Shenyang, Liaoning Province, China. The study was conducted according to the principles expressed in the Helsinki Declaration. All patient data were fully anonymized before accessing.

A consecutive series of hospitalized pregnant women with age ≥ 18 and gestational age ≥ 20 weeks was recruited from the No. 1 Obstetrics Ward of the Shengjing Hospital between May and December 2017. Women with infectious diseases, macroscopic urine color changes or receiving therapy in clinical trials were excluded. 1,532 women were finally included.

Mid-stream urine samples were obtained for testing misfolded proteins. Information was collected at the time of admission on maternal age and gestational age, as well as risk factors for preeclampsia including number of fetuses, family history of preeclampsia, hypertension and diabetic status. Blood pressure and proteinuria were measured, the latter using a dipstick and/or a subsequent 24 hour collection. Investigators were blinded with the misfolded proteins test results.

Congo red bound to misfolded proteins in an aqueous solution migrates differentially on cellulose membrane, forming different dyeing patterns compared with a free Congo red solution. We discovered that the differences are especially apparent when the solution is slowly released into a small area on the cellulose membrane (qualitative fast filter paper) through a fine-tipped capillary tube. The more Congo red is bound to misfolded proteins, the dye spreads more evenly on the membrane and less possible to bind with cellulose. On the basis of this finding, a point-of-care device employing the capillary tube-based slow release method (termed the CapCord test, commercially available from Shuwen Biotech) was designed and manufactured. The device includes a plastic pipette to drop urine to a well containing Congo red (0.1 mg/ml), and a capillary applicator to transfer the mixture to cellulose membrane compartment and slowly released. The test produces a result within 3 minutes.

All urine samples were tested using this device. Each scorer classified the pattern of the dye into six categories comparison with an illustrative pattern sheet considering the spreading evenness and the tendency of the dye concentrated in the limited central area (Fig 1). Prior to the study, the scorers were trained to achieve standardization avoiding artificial mis-scoring; reproducibility of scoring was assessed at that time. Results were randomized into a 'training set' of 770 samples used to group dyeing pattern categories into positive and negative and compared with the remaining 'validation set' of 762 samples. Pregnancies were followed up and diagnosis of preeclampsia and severity was made according to criteria of the American College of Obstetricians and Gynaecologists [11].

The comparison of CapCord with Congo Red Dot (CRD) test was performed in women with enough residual urine samples. CRD test was conducted as per described [6] but using different image analysis. Congo red retention (CRR) rate was calculated by the ratio of gray (Image J software, <http://imagej.nih.gov/ij/>) after and before wash-up.

SPSS 22.0 (IBM, Armonk, NY) statistical software was used for statistical analysis. Measurement data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed between affected and unaffected pregnancies using the Chi-square test; statistical

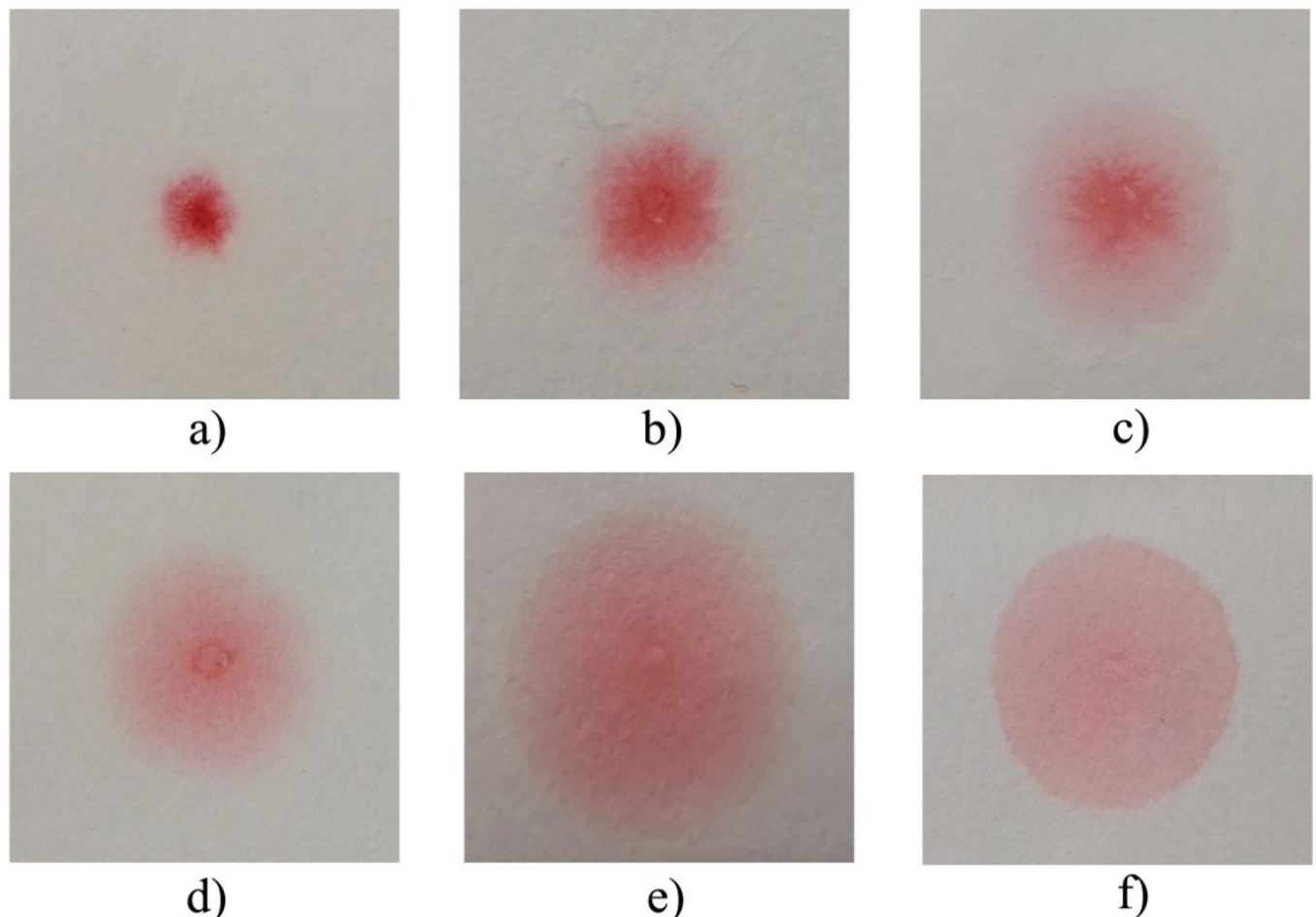


Fig 1. Classification of Congo red staining patterns (a) Small non-diffused red dot; (b) Mildly diffused dot, scarlet pseudopodium; (c) Diffused dot, scarlet pseudopodium, pink penumbra; (d) Small dot, irregular partly diffused pale red penumbra; (e) Red and scarlet dot, partly diffused pale red penumbra; (f) Large uniform pale diffused dot.

<https://doi.org/10.1371/journal.pone.0233214.g001>

significance was based on $P < 0.05$. Comparisons were made according to admission characteristics, preeclampsia risk factors and initial assessments and the detection and false-positive rates according to those which were statistically significant. Nonparametric Spearman's rank correlation was used to assess the association between spread patterns and blood pressure, urinary protein. Receiver operating characteristic (ROC) curves and area under curve (AUC) were used to quantify the performance of biomarker. Z test was used to compare AUC.

Results

Among the 1,532 consecutive pregnancies, 867 were admitted for symptoms which may or may not be associated with preeclampsia and the most common symptom is bleeding/discharge or abdominal pain 601 (39.2%) followed by elevated blood pressure 95 (6.2%) and fetal status/movement 78 (5.1%) (S1 Table). Asymptomatic cases were admitted for delivery. The mean gestational age at urine sampling is 36.6 weeks (SD = 4.2 weeks). 1,443 (94%) were tested at late pregnancy (≥ 28 weeks). A total of 140 preeclampsia cases were diagnosed (9.1%), of which 101 were severe and 87 were pre-term, delivering before 37 weeks gestation. Most of the cases were diagnosed on the day of admission and only five were diagnosed subsequently.

Table 1 shows the admission characteristics, preeclampsia risk factors and initial assessments in affected and unaffected pregnancies. As expected, there were highly statistically significant difference between those with preeclampsia and unaffected pregnancies in the gestation at admission, symptoms being included in the reason for admission, specific risk factors for preeclampsia and the initial blood pressure and proteinuria measurements.

Table 2 shows the distribution of Congo red dying patterns in affected and unaffected pregnancies included in the training subset. The best discrimination was achieved by classifying the last three patterns (d)-(f) as 'positive' and patterns (a)-(c) as 'negative'. Using that classification the detection and false-positive did not differ significantly between the training and validation subsets (Table 3). Consequently, the best estimate of performance was provided by combining the subsets with a detection rate of 74% (103/140) and false-positive rate of 3.0%

Table 1. Admission characteristics, preeclampsia risk factors and initial assessments in affected and unaffected pregnancies.

Characteristic*		Preeclampsia (n = 140)	Unaffected (n = 1392)	Statistical significance (P)
Admission characteristic				
Preterm gestation (n = 514)		108(77%)	406(29%)	<0.0001
Symptoms (n = 867)		126 (90%)	741 (53%)	<0.0001
Preeclampsia risk factor				
Maternal age over 35 (n = 391)		30 (21%)	361 (26%)	0.24
Multiple pregnancy (n = 30)		8 (5.7%)	22 (1.6%)	<0.001
Previous preeclampsia (n = 137)		86(61%)	51(3.7%)	<0.0001
Hypertension:	Chronic (n = 30)	19 (14%)	11(0.8%)	<0.0001
	Gestational (n = 29)	14 (12%)	15 (1.1%)	<0.0001
Diabetes:	Pre-gestational (n = 22)	9 (6.4%)	13 (0.9%)	<0.0001
	Gestational (n = 203)	30 (23%)	173 (13%)	<0.001
Initial assessment				
Blood pressure raised** (n = 184)		123 (88%)	61 (4.4%)	<0.0001
Proteinuria*** (n = 647)		132 (94%)	515(38%)	<0.0001

*excluded from proportions: symptoms-missing indication for admission; gestational hypertension-chronic cases; gestational diabetes-pre-gestational cases.

**systolic ≥ 140 mmHG or diastolic ≥ 90 mmHG.

***dipstick 1+ or more, or concentration ≥ 300 mg/24hr.

<https://doi.org/10.1371/journal.pone.0233214.t001>

Table 2. Training subset: Distribution of Congo red dyeing patterns in affected and unaffected pregnancies.

Pattern (see Fig 1)	Preeclampsia (n = 66)	Unaffected (n = 704)
(a) Small non-diffused red dot	2 (0.3%)	225 (32%)
(b) Mildly diffused dot, scarlet pseudopodium	9 (14%)	392 (56%)
(c) Diffused dot, scarlet pseudopodium, pink penumbra	5 (7.6%)	65 (9.2%)
(d) Small dot, irregular partly diffused pale red penumbra	1 (1.5%)	1 (0.1%)
(e) Red and scarlet dot, partly diffused pale red penumbra	10 (15%)	12 (1.7%)
(f) Large uniform pale diffused dot	39 (59%)	9 (1.3%)

<https://doi.org/10.1371/journal.pone.0233214.t002>

(42/1392). The detection rate was significantly higher in severe (83%) or preterm cases (86%) compared with mild (49%) or term (50%) cases (Table 3; $P < 0.0001$). According to gestational age at sampling, the detection rate was significantly higher before 28 weeks ($P < 0.05$) and before 34 weeks (early-onset) ($P < 0.01$). Spread patterns as ranked data were associated with systolic pressure, diastolic pressure and 24 hr urinary protein ($P < 0.0001$).

Among the 103 Congo red true-positives only one did not have proteinuria at the time of testing and in this case only a dipstick was carried out without subsequent 24 hr urine determination. Of the 42 Congo red false-positives all but seven had proteinuria at the time of testing and the discrepant cases were examined only by dipstick. Three of the false-positives had raised blood pressure at that time, but not later, and for a further nine either systolic was within 10 mmHG or diastolic within 5 mmHG of being classified as hypertension.

Table 3 also compares the detection and false-positive rates according to admission characteristics, preeclampsia risk factors and initial assessments. The detection rate was higher in cases tested preterm, as expected since this group included 91% of the severe and all of the preterm preeclampsia pregnancies. However, the detection rates of severe (88%) and mild preeclampsia (73%) were not statistically significant in preterm preeclampsia cases. There were statistically significant higher false-positive rates in multiple pregnancies and in those with a previous preeclampsia. Whilst there was a significantly lower detection rate in gestational diabetes ($P < 0.05$), this is likely to be a chance finding since many statistical comparisons were being made. The highly statistically significant increases in both detection and false-positive rates among women with proteinuria, is an expression of the expected association between misfolded and urinary protein as shown above, but the false positive rate would be 38% assuming urinary protein alone as a preeclampsia biomarker, much higher than that of misfolded protein (3%).

Of the 267 women with enough urine samples to undergo urinary CRD test, 80 were preeclampsia and 55 were severe PE. The performance of CapCord and CRD test in this cohort was similar with AUC 0.77 and 0.74 ($P = 0.41$). When cut-off of CRR was set at 17% with best Youden index, the detection rate and false positive rate of CRD test were 65% and 22.5%. The detection rate and false positive rate of CapCord test were 71.3% and 17.6%. Both methods had better detection rate for severe preeclampsia (CapCord vs. CRD test = 81.8% vs. 76.4%).

Discussion

This study confirms that a simple point-of-care urinary device for misfolded proteins can be used to screen for preeclampsia in late pregnancy in Chinese population, with a very high performance. The CapCord test is different from previous reported device [10]. The key point of the current device is using fine-tipped capillary tube to slowly release the mixture of urine and Congo red to get different spread patterns which are more distinguishable and reproducible. The overall detection and false-positive rates of 74% and 3.0% respectively compare favourably

Table 3. Congo red detection and false-positive rates according to scoring subset, type of preeclampsia, characteristics, preeclampsia risk factors and initial assessments.

	Detection rate	Statistical significance (P)	False-positive rate	Statistical significance (P)
Scoring subset				
Training	76% (50/66)	0.58	3.1% (22/704)	0.81
Validation	72% (53/74)		2.9% (20/688)	
Type of preeclampsia				
Severe	83% (84/101)	<0.0001	-	-
Mild	49% (19/39)			
Delivered pre-term	86%(75/87)	<0.0001	-	-
Term	50% (20/40)			
Late pregnancy	70.2% (87/124)	<0.05	-	-
Mid-pregnancy	100% (16/16)			
Early-onset	86.2% (56/65)	<0.01	-	-
Late-onset	62.7% (47/75)			
Admission characteristic*				
Pre-term gestation	83%(90/108)	<0.0001	3.0% (12/406)	0.93
Term	41%(13/32)		3.0% (30/986)	
Symptoms	75% (95/126)	0.14	2.8% (21/741)	0.67
Asymptomatic	57% (8/14)		3.2% (21/651)	
Preeclampsia risk factor*				
Multiple	88% (7/8)	0.36	14% (3/22)	0.003
Singleton	73% (96/132)		2.8% (39/1370)	
Previous preeclampsia	69%(59/86)	0.09	43%(22/51)	<0.0001
No previous	81%(44/54)		1.5%(20/1341)	
Chronic hypertension	79% (15/19)	0.57	0.0% (0/11)	0.56
None	73% (88/121)		3.0% (42/1381)	
Gestational hypertension	79% (11/14)	0.6	6.7% (1/15)	0.41
None	72% (77/107)		3.0% (41/1366)	
Pre-gestational diabetes	78% (7/9)	0.77	7.7% (1/13)	0.32
None	73% (96/131)		3.0% (41/1379)	
Gestational diabetes	57% (17/30)	<0.05	4.0% (7/173)	0.37
None	78% (79/101)		2.8% (34/1206)	
Initial assessment				
Blood pressure high**	76% (93/123)	0.14	4.9% (3/61)	0.37
Normal	59% (10/17)		2.9% (39/1331)	
Proteinuria***	77% (102/132)	x	6.8%(35/515)	<0.0001
Normal	13% (1/8)		0.7% (6/828)	
Overall	74% (103/140)	-	3.0% (42/1392)	-

*excluded from proportions: symptoms–missing indication for admission; gestational hypertension–chronic cases; gestational diabetes–pre-gestational cases.

**systolic ≥ 140 mmHG or diastolic ≥ 90 mmHG.

***dipstick 1+ or more, or concentration ≥ 300 mg/24hr.

<https://doi.org/10.1371/journal.pone.0233214.t003>

with rates of 67% and 8.8% in a study using a similar device [10] as well as rates of 80% and 11% using a less stringent interpretation of Congo red patterns in the latter device. The difference in detection rates between the two studies might be due to sample group, but false-positive rate 3.0% might be superior considering the sample size is 4 times more than that of previous study [10]. It is warranted for a further study to perform the both methods simultaneously.

In screening point-of-care devices are generally advantageous compared with laboratory assays due to lower cost, increased speed and less requirement for skilled technicians. Previous studies of Congo red screening for preeclampsia were carried out using the laboratory based 'retention' assay developed by Buhimschi and colleagues [6]. The sample was first assayed for total protein and standardised by concentration or dilution to achieve a given protein level. Congo red was added and the mixture spotted onto a nitrocellulose membrane, scanned for optical density, washed with increasing amounts of methanol to remove unbound colour and scanned again. The result was expressed as the percentage of staining retained after the washing step. Sammar and colleagues later showed that scanning and washing were unnecessary because of visual difference in staining pattern between affected and unaffected pregnancies [8]. The current device obviates the need for total protein measurement, already contains Congo red and is based on visually determined patterns achieving similar detection rate with CRD test [6]. The time needed for the test is at most 3 minutes and requires little operator skill.

Using the current device the detection rate was found to be higher in severe (83%) or pre-term cases (86%) compared with 49% and 50% in mild or term cases. The difference of detection rate between severe and mild preeclampsia is consistent with the correlation between spread pattern and blood pressure or urinary protein. The detection rate for pre-term preeclampsia is higher than term cases which may support the theory that these two are distinct phenotypes of preeclampsia [12]. It needs to be addressed in future study. The higher detection rate before 28 weeks and before 34 weeks (early-onset) indicates the usefulness for more intense surveillance by early detection. The other point-of-care study did not break down the results according to severity but this was done in two studies using the laboratory-based retention assay. In one study high retention was reported for 91%, 89% and 75% of those with superimposed, severe and mild preeclampsia respectively [6]. The other study reported the average retention which was 82% in severe and 61% in mild preeclampsia compared with 38% in normotensive controls [9]. As described above, the retention rate might vary in different cohorts and status of preeclampsia, which makes it difficult to set a uniform cut-off. Although previously urinary congophilia for the detection of preeclampsia has been studied in Caucasians [7], Indians [9] and Mexicans [13], this is the first urinary congophilia study of screening for preeclampsia in Chinese population.

In this paper several factors whose incidence differed in preeclampsia cases were considered. For example, about three times as many pregnancies with preeclampsia were admitted preterm compared with unaffected pregnancies but there was no difference in the Congo red false-positive rate according to gestation (Table 3). There was a statistically significantly higher detection rate among the preterm admissions but this may be attributable to most of those cases having severe preeclampsia.

The incidence of preeclampsia is about 2–3 times higher in twins than in singleton pregnancies [11,14] and there was a statistically significantly higher detection rate and false-positive rate among multiple pregnancies. This is probably due to the higher placental mass in twins and triplets [15] since it is believed that misfolded proteins aggregate in the placenta [4,6]. There was also a higher detection rate in multiple pregnancies but this did not reach statistical significance.

A previous pregnancy with preeclampsia is a risk factor in parous women. For example, in one study the incidence of preeclampsia was 42% in those with such a history compared with only 4.9% without [16]. In the current series a similar likelihood ratio was found. However, when the Congo red test performance was compared the observed false-positive rate was considerably higher in those with previous preeclampsia, a highly statistically significant difference ($P < 0.0001$). This was an unexpected finding and there is no obvious explanation although it

might be related to the high proteinuria incidence in the false-positives. Congo red is associated with proteinuria since it detects the aggregated misfolded proteins, but they are not same.

The previous point-of-care study of Congo red was carried out in the United States in a labour and delivery triage clinic among women who were mostly (66%) White but a sizable minority (29%) were African American [10]. The current study was carried out in China in a complete series of those admitted to hospital for a range of indications including a large number of delivery or symptoms such as bleeding or abdominal pain. More screening studies will be needed in different settings.

Serum PlGF is a well-recognized bio-marker for preeclampsia screening and recommended for first-trimester screening in FIGO guideline [4]. However, the performance for late-onset preeclampsia is rather compromised [17]. CapCord shows promising utility for not only early-onset but also late-onset preeclampsia.

There are at least two potential uses for a late pregnancy Congo red screening test. Firstly, it can be used in a triage situation as with the United States study [10] and in the symptomatic women included in the current study (Table 3). Secondly, it could be a routine test among asymptomatic women. In the current study, whilst the detection rate seemed lower in such women and the false-positive rate higher, these differences were not statistically significant (Table 3). Hence our results support both types of screening.

The interpretation of a non-quantitative test such as this is necessarily subjective. This is particularly so when scoring the Congo red staining pattern into six categories. However, as the study shows, it is possible to reduce the scoring to just two categories. Consequently the new commercial version of the CapCord test includes two control samples which produce positive and negative patterns in addition to the sample being tested.

In conclusion, CapCord is a simple point-of-care urinary misfolded proteins testing device for screening preeclampsia in pregnancy with gestational age after 20 weeks.

Supporting information

S1 Table. Characteristics of women enrolled in the whole cohort.
(DOCX)

S1 Data.
(XLSX)

S2 Data.
(XLSX)

Acknowledgments

We thank the staff of No. 1 Obstetrics Ward, Shengjing Hospital for their cooperation.

Author Contributions

Conceptualization: Xing-Min Li, Jun Xu, Juan Du.

Data curation: Jun Xu.

Formal analysis: Howard Cuckle.

Funding acquisition: Xing-Min Li.

Investigation: Xue-Min Liu, Jun Xu.

Methodology: Xing-Min Li, Jun Xu.

Project administration: Xue-Min Liu, Jun Xu.

Resources: Jun Xu.

Supervision: Xing-Min Li, Juan Du.

Validation: Xue-Min Liu, Jun Xu.

Visualization: Jun Xu.

Writing – original draft: Howard Cuckle.

Writing – review & editing: Xing-Min Li, Juan Du.

References

1. Duley L. The global impact of pre-eclampsia and eclampsia. *Seminars in perinatology*. 2009; 33(3):130–7. <https://doi.org/10.1053/j.semperi.2009.02.010> PMID: 19464502
2. Rolnik DL, Wright D, Poon LCY. ASPRE trial: performance of screening for preterm pre-eclampsia. *Ultrasound in obstetrics & gynecology: the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*. 2017; 50(4):492–5.
3. Zeisler H, Llurba E, Chantraine F, Vatish M, Staff AC, Sennstrom M, et al. Predictive Value of the sFlt-1: PlGF Ratio in Women with Suspected Preeclampsia. *The New England journal of medicine*. 2016; 374(1):13–22. <https://doi.org/10.1056/NEJMoa1414838> PMID: 26735990
4. Poon LC, Shennan A, Hyett JA, Kapur A, Hadar E, Divakar H, et al. The International Federation of Gynecology and Obstetrics (FIGO) initiative on pre-eclampsia: A pragmatic guide for first-trimester screening and prevention. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics*. 2019; 145 Suppl 1(Suppl 1):1–33.
5. Buhimschi IA, Zhao G, Funai EF, Harris N, Sasson IE, Bernstein IM, et al. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *American journal of obstetrics and gynecology*. 2008; 199(5):551.e1–16.
6. Buhimschi IA, Nayeri UA, Zhao G, Shook LL, Pensalfini A, Funai EF, et al. Protein misfolding, congophilin, oligomerization, and defective amyloid processing in preeclampsia. *Science translational medicine*. 2014; 6(245):245ra92.
7. McCarthy FP, Adetoba A, Gill C, Bramham K, Bertolaccini M, Burton GJ, et al. Urinary congophilin in women with hypertensive disorders of pregnancy and preexisting proteinuria or hypertension. *American journal of obstetrics and gynecology*. 2016; 215(4):464.e1–7.
8. Sammar M, Syngelaki A, Sharabi-Nov A, Nicolaides K, Meiri H. Can Staining of Damaged Proteins in Urine Effectively Predict Preeclampsia? *Fetal diagnosis and therapy*. 2017; 41(1):23–31. <https://doi.org/10.1159/000444450> PMID: 26930075
9. Nagarajappa C, Rangappa SS, Suryanarayana R, Balakrishna S. Urinary congophilin in preeclampsia: Experience from a rural tertiary-care hospital in India. *Pregnancy hypertension*. 2018; 13:83–6. <https://doi.org/10.1016/j.preghy.2018.05.006> PMID: 30177078
10. Rood KM, Buhimschi CS, Dible T, Webster S, Zhao G, Samuels P, et al. Congo Red Dot Paper Test for Antenatal Triage and Rapid Identification of Preeclampsia. *EClinicalMedicine*. 2019; 8:47–56. <https://doi.org/10.1016/j.eclinm.2019.02.004> PMID: 31193633
11. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstetrics and gynecology*. 2013; 122(5):1122–31. <https://doi.org/10.1097/01.AOG.0000437382.03963.88> PMID: 24150027
12. Phillips JK, Janowiak M, Badger GJ, Bernstein IM. Evidence for distinct preterm and term phenotypes of preeclampsia. *The journal of maternal-fetal & neonatal medicine: the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet*. 2010; 23(7):622–6.
13. JL Rodríguez Chávez EFG, Angelesvázquez MJ, Mendieta Zerón H. Evaluation of Congo Red Staining Kit to Determine Proteinuria in Preeclampsia. *Journal of Clinical and Diagnostic Research*. 2018; 12(5):QM01–QM5.
14. Laine K, Murzakanova G, Sole KB, Pay AD, Heradstveit S, Raisanen S. Prevalence and risk of pre-eclampsia and gestational hypertension in twin pregnancies: a population-based register study. *BMJ open*. 2019; 9(7):e029908. <https://doi.org/10.1136/bmjopen-2019-029908> PMID: 31278106
15. Wegrzyn P, Fabio C, Peralta A, Faro C, Borenstein M, Nicolaides KH. Placental volume in twin and triplet pregnancies measured by three-dimensional ultrasound at 11 + 0 to 13 + 6 weeks of gestation.

Ultrasound in obstetrics & gynecology: the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2006; 27(6):647–51.

16. Poon LC, Kametas NA, Chelemen T, Leal A, Nicolaides KH. Maternal risk factors for hypertensive disorders in pregnancy: a multivariate approach. *Journal of human hypertension*. 2010; 24(2):104–10. <https://doi.org/10.1038/jhh.2009.45> PMID: 19516271
17. Sonek J, Krantz D, Carmichael J, Downing C, Jessup K, Haidar Z, et al. First-trimester screening for early and late preeclampsia using maternal characteristics, biomarkers, and estimated placental volume. *American journal of obstetrics and gynecology*. 2018; 218(1):126.e1–.e13.



A Study on the Role of Urinary Congophilia in Early Detection of Preeclampsia

Anjali Rani¹ Madhu Jain¹ Debabrata Dash² Surendra P. Mishra² Milad Mohammadzadehasl¹

¹Department of Obstetrics and Gynaecology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

²Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Address for correspondence Anjali Rani, MD, Department of Obstetrics and Gynaecology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, Uttar Pradesh, India (e-mail: anjaliraniimsbhu@gmail.com).

Ann Natl Acad Med Sci (India) 2022;58:87–91.

Abstract

Introduction Preeclampsia and eclampsia are important causes of maternal morbidity. Preeclamptic women secrete misfolded proteins in the urine. Buhimschi et al had developed a new test for diagnosis of preeclampsia. This test is based on staining of misfolded protein with Congo red dye. Misfolded proteins are derived from syncytiotrophoblast microparticles (STBMs). These STBM are membrane bound vesicles and contain misfolded proteins. In preeclampsia, glomeruli of kidneys are disrupted and these damaged protein reach the urine.

Aim and Objective This study aimed to investigate the role of urinary congophilia in early prediction of preeclampsia.

Materials and Methods This test was done in 250 pregnant women attending the Gynaecological Outpatient Department. Urine sample of early morning was taken and test was done in the Department of Biochemistry. **The included pregnant women were of gestational age between 14 and 18 weeks.** The staining of urine with Congo red dye was done and washed with methanol. The retention of dye was interpreted with naked eye. The more retention of dye, the more chances of developing preeclampsia later. The patients were followed-up till delivery. The patients who developed preeclampsia later part of pregnancy were recorded. Mean arterial pressure (MAP) and past history and body mass index were also recorded.

Results Out of 250 patients, 30 developed preeclampsia later. A total of 34 patients were having positive urinary congophilia and only 20 patients developed preeclampsia later. MAP more than 90 mm Hg is abnormal but 66.7% of patients who developed preeclampsia had MAP >90 mm Hg. In 16.7% of patients, who developed preeclampsia later, had positive past history of hypertension. In 66.7% of patients, who were positive for urinary congophilia, later developed preeclampsia.

Conclusion Preeclampsia and eclampsia are important causes of maternal mortality and morbidity. So, early detection can prevent complications and timely management. Urinary congophilia is one of such test which can help in early prediction of preeclampsia. If it is combined with past maternal history and MAP, it gives more good results. The detection rate is much higher if signs and symptoms of preeclampsia are noticed timely.

Keywords

- preeclampsia
- urinary congophilia
- misfolded protein
- biomarkers
- Congo red

published online
March 9, 2022

DOI <https://doi.org/10.1055/s-0042-1743134>.
ISSN 0379-038X.

© 2022. National Academy of Medical Sciences (India). All rights reserved.

This is an open access article published by Thieme under the terms of the Creative Commons Attribution-NonDerivative-NonCommercial-License, permitting copying and reproduction so long as the original work is given appropriate credit. Contents may not be used for commercial purposes, or adapted, remixed, transformed or built upon. (<https://creativecommons.org/licenses/by-nc-nd/4.0/>)

Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

Introduction

Preeclampsia is one of the leading causes of maternal morbidity and mortality.¹ The definition of preeclampsia according to The American College of Obstetricians and Gynecologists (ACOG) is the presence of hypertension and proteinuria after 20 weeks of gestation. But in revised definition by the ACOG (2013) task force states that proteinuria may or may not present.² Preeclampsia can affect 5 to 7% of all pregnant ladies, cause 70,000 maternal deaths globally, and also 500,000 fetal deaths.^{3–5} Preeclampsia if left untreated, it can progress to eclampsia which is associated with complications like renal failure, seizures, pulmonary edema, disseminated intravascular coagulation (DIC), and can lead to death as well. The definitive treatment for severe preeclampsia and eclampsia is delivery of placenta and baby. The early prediction of preeclampsia is very important, so that timely treatment can be started and complications can be avoided or decreased.

For the last two decades, there are lots of studies to understand the pathophysiology of preeclampsia, and misfolded protein is a new concept for preeclampsia.⁶ There are various biochemical and biophysical markers which can predict preeclampsia before the clinical symptoms appear. These markers are pregnancy-associated plasma protein A (PAPP-A), placental growth factor, and placental protein 13 and others.^{7–9} An effective combine method for prediction of preeclampsia in first trimester is based on maternal history, mean arterial pressure (MAP), uterine artery pulsatility index, and biochemical markers. But still there is a need of simple, cost effective, and noninvasive method for early prediction.

It is seen in many new studies that misfolded proteins are found in various body fluids of preeclamptic women. These proteins can be found in the urine of preeclamptic women as early as 10 weeks of gestation¹⁰ as reported by Buhimschi et al.

Buhimschi et al had developed a test based on these misfolded proteins which are secreted in urine of preeclamptic women.^{11–13} Misfolded proteins bind to Congo red dye. This is known as urinary congophilia which is also used to diagnose Creutzfeldt–Jakob disease¹⁴ as described by Halimi et al.

The mechanism of binding of Congo red to amyloids is not clear. A study by Frid et al suggested that Congo red has affinity to bind proteins which are rich in β sheets.¹⁵ Rood et al¹⁶ also suggested that Congo red dot paper test is a simple, noninvasive test for diagnosis and prediction of preeclampsia.

Materials and Methods

This study was conducted at the Department of Obstetrics and Gynaecology in collaboration with the Department of Biochemistry of Banaras Hindu University. Ethical clearance was taken from the Institute Ethical Committee.

Inclusion Criteria

Patients were included with gestational age between 14 and 18 weeks. Sample size was 250.

Procedure

Early morning urine sample was collected from pregnant women attending the Gynaecological Outpatient Department.

The sample was transported to the biochemistry laboratory. Congo red was freshly prepared by taking 5 mg of dry Congo red dye and mixing it with 10 ml of distilled water. So, it became 0.5 mg/mL. It was thoroughly mixed by shaker.

Nitrocellulose membrane was taken and of size 6 cm $\times$ 4 cm. Urine sample was taken with pipette and 10 μ L was put on nitrocellulose membrane. It was dried and then staining was done with Congo red dye and incubated for 1 hour. Then to remove extra dye, it was washed with methanol (50, 70, and 90%).

The value of Congo red retention after wash with methanol is used as a diagnostic indicator. Interpretation was done by visual examination of nitrocellulose paper. Then depending on concentration, they are divided into congophilia positive or negative. Data were recorded in an excel sheet. Statistical analysis was done using the SPSS software, version 20.

Follow-up of these patient was done till delivery. Patients who had past positive history of preeclampsia were not given low-dose aspirin. Those patients who developed preeclampsia later in pregnancy were noted. Then data were correlated with the first time urinary congophilia–tested patients.

We recorded the number of patients with congophilia positive at the time of recruitment who developed preeclampsia and also the percentage of congophilia-positive patients who did not develop preeclampsia.

Results

The observations made from this study are listed in this section. A total of 250 pregnant cases of urinary congophilia were tested and among these, 30 cases developed preeclampsia later, during follow-up.

Development of Preeclampsia was seen maximum in age group 20 to 24 (36.7%) and 25 to 29 (33.3%) as presented in ► **Table 1**.

MAP >90 mm Hg is abnormal. As described in ► **Table 2**, 66.7% of patients developed preeclampsia who had MAP

Table 1 Age

Age group (y)	Development of preeclampsia	Development of preeclampsia	Total
	No	Yes	
15–19	5 (2.3%)	0 (0%)	5
20–24	73 (33.2%)	11 (36.7%)	84
25–29	88 (40%)	10 (33.3%)	98
30–35	40 (18.2%)	09 (30%)	49
>35	14 (6.4%)	0 (0%)	14
Total	220	30	250

Note: $n = 250$, $\chi^2 = 4.867$, and $p = 0.301$.

Table 2 Mean arterial pressure

Mean arterial pressure	Development of preeclampsia	Development of preeclampsia	Total
	No	Yes	
<90 mm Hg	99 (45%)	10 (33.3%)	109 (43.6%)
>90 mm Hg	121 (55%)	20 (66.7%)	141 (56.4%)
Total	220	30	250

Note: $\chi^2 = 1.461$, $p = 0.227$.

>90 mm Hg and 33.3% had MAP <90 mm Hg but still developed preeclampsia.

In ►Table 3, it is seen that 53.3% of patients who developed preeclampsia had body mass index (BMI) between 18.5 and 23 kg/m² and also, 33.3% of patients who developed preeclampsia had BMI >25 kg/m².

Table 3 Body mass index

Body mass index (kg/m ²)	Development of preeclampsia	Development of preeclampsia	Total
	No	Yes	
13–18.5	11 (5%)	1 (3.3%)	12 (4.8%)
18.5–23	86 (39.1%)	16 (53.3%)	102 (40.8%)
23–25	58 (26.45%)	03 (10%)	61 (24.4%)
>25	65 (29.55%)	10 (33.3%)	75 (30%)
Total	220	30	250

Note: $\chi^2 = 4.489$, $p = 0.213$.

Table 4 Past history of preeclampsia/eclampsia

Past h/o preeclampsia/eclampsia	Development of preeclampsia	Development of preeclampsia	Total	Values with 95% confidence interval
	No	Yes		
Absent	220 (100%)	25 (83.3%)	245 (98%)	PPV = 100 (57.8–100) NPV = 89.8% (46.7–89.8) Sensitivity = 16.7 (9.6–16.7)
Present	0	05 (16.7%)	05 (2%)	Specificity = 100 (99–100) Accuracy = 90 (88.3–90)
Total	220	30	250	

Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

Table 5 Congophilia positive and development of preeclampsia

Urinary congophilia	Development of preeclampsia	Development of preeclampsia	Total	Values with 95% CI
	No	Yes		
Negative	206 (93.6%)	10 (33.35%)	216 (86.4%)	PPV = 58.8 (45.7–69.4) NPV = 95.4% (93.3–97) Sensitivity = 66.7% (51.8–78.6)
Positive	14 (6.45%)	20 (66.7%)	34 (13.6%)	Specificity = 93.6% (91.6–95.3) Accuracy = 90.4% (86.8–93.3)
Total	220	30	250	

Abbreviations: CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

In ►Table 4, it shows that 16.7% of patients who develop preeclampsia had positive past history of hypertension. Rest 83.3% of patients who developed preeclampsia did not have past history of hypertension. The positive predictive value was 100 and negative predictive value was 89.8.

In ►Table 5, it is seen that 66.7% of patients who developed preeclampsia were congophilia positive and 33.5% of patients who were congophilia negative also developed preeclampsia. The positive predictive value was 58.8 and negative predictive value was 95.4.

►Table 6 shows binary logistic regression which described that congophilia-positive patients had 28 times more risk of developing preeclampsia. The patient with increased MAP has 1.27 times increase risk of developing preeclampsia.

Discussion

Preeclampsia and eclampsia are two of the three important causes of maternal mortality. In developed countries, maternal mortality due to preeclampsia has decreased as compared with developing countries. In developing countries, it is still high due to various factors like lack of antenatal care, poverty, lack of timely access to critical care.¹⁷ It has been seen that 10 to 15% of direct maternal deaths are due to preeclampsia and eclampsia as described by Duley.¹⁸ Preeclampsia can lead to complications like liver failure, pulmonary edema, renal failure, and DIC.

There are some points to note from this study listed below:

- In our study, 30 out of 250 patients develop preeclampsia, so the chance of developing preeclampsia was 12%.
- A previous pregnancy with preeclampsia is a risk factor in parous women. For example, in a study by Poon et al, the incidence of preeclampsia was 42% in those with such a history

Table 6 Binary logistic regression analysis of development of preeclampsia on congophilia and mean arterial pressure

Factors	Development of pre-eclampsia Yes (30) no (220)	Sig.	Odds ratio	95% confidence interval
Congophilia				
Positive	20 (66.7) 14 (6.4)	0.000	28.731	11.273 73.225
Negative	10 (33.3) 206 (93.6)			
Mean arterial pressure (mm Hg)				
> 90	121 (55.0) 20 (66.7)	0.622	1.275	0.485–3.355
≤90	99 (45.0) 10 (33.3)			

Note: $R^2 = 0.390$, $p = 0.000$.

compared with only 4.9% without previous history of pre-eclampsia.¹⁹ In our study, the incidence was 16.7% who had positive past history of preeclampsia. It has been shown by Bartsch et al²⁰ that the past history of preeclampsia increases the risk of preeclampsia in future pregnancies by eight times.

- It has been seen in the study done by Rodriguez-Chávez et al that Congo red dye has high affinity for beta-folded proteins of preeclampsia.²¹ It has also been shown that Congo red staining of misfolded proteins in preeclampsia is simple, efficient, and reproducible test, and a new line of research can be done.
- Nagarajappa et al had shown in their study that the urinary congophilia is present in Indian women with preeclampsia and it is a good screening test.²² Our study also confirms that congophilia is a good screening test and it gives better results if other parameters, like maternal history and MAP, are taken into consideration.
- According to Sammar et al, the urinary congophilia adds to the accuracy of preeclampsia prediction in first trimester.²³ The odds ratio for Congo red alone was superior than BMI and MAP.

Limitation of Study

- This kit is not available in India and also not available through online purchase.

Conclusion

Preeclampsia and eclampsia are major cause of maternal mortality. Urinary congophilia is a new screening test for preeclampsia. It has started a new research of role of misfolded proteins in preeclampsia. If it is combined with maternal history and MAP, the early detection is increased and complications can be decreased.

Funding

None.

Conflict of Interest

None declared.

Acknowledgment

Authors are thankful to Mr. Ranjeet and staff of the Biochemistry Department for their help with the samples in testing.

References

- 1 Khan KS, Wojdyla D, Say L, Gülmezoglu AM, Van Look PFA. WHO analysis of causes of maternal death: a systematic review. *Lancet* 2006;367(9516):1066–1074
- 2 American College of Obstetricians and Gynecologists Task Force on Hypertension in Pregnancy. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on hypertension in pregnancy. *Obstet Gynecol* 2013; 122(05):1122–1131
- 3 Hogan MC, Foreman KJ, Naghavi M, et al. Maternal mortality for 181 countries, 1980–2008: a systematic analysis of progress towards Millennium Development Goal 5. *Lancet* 2010;375(9726):1609–1623
- 4 Wanderer JP, Leffert LR, Mhyre JM, Kuklina EV, Callaghan WM, Bateman BT. Epidemiology of obstetric-related ICU admissions in Maryland: 1999–2008*. *Crit Care Med* 2013;41(08):1844–1852
- 5 Kuklina EV, Ayala C, Callaghan WM. Hypertensive disorders and severe obstetric morbidity in the United States. *Obstet Gynecol* 2009;113(06):1299–1306
- 6 Gerasimova EM, Fedotov SA, Kachkin DV, et al. Protein misfolding during pregnancy: new approaches to preeclampsia diagnostics. *Int J Mol Sci* 2019;20(24):6183
- 7 Schiettecatte J, Russcher H, Anckaert E, et al. Multicenter evaluation of the first automated Elecsys sFlt-1 and PlGF assays in normal pregnancies and preeclampsia. *Clin Biochem* 2010;43 (09):768–770
- 8 Nicolaides KH, Bindra R, Turan OM, et al. A novel approach to first-trimester screening for early pre-eclampsia combining serum PP-13 and Doppler ultrasound. *Ultrasound Obstet Gynecol* 2006;27(01):13–17
- 9 Spencer K, Cowans NJ, Nicolaides KH. Low levels of maternal serum PAPP-A in the first trimester and the risk of pre-eclampsia. *Prenat Diagn* 2008;28(01):7–10
- 10 Buhimschi CS, Norwitz ER, Funai E, et al. Urinary angiogenic factors cluster hypertensive disorders and identify women with severe preeclampsia. *Am J Obstet Gynecol* 2005;192(03): 734–741
- 11 Buhimschi IA, Zhao G, Funai EF, et al. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *Am J Obstet Gynecol* 2008;199(05):551.e1–551.e16
- 12 Buhimschi IA, Nayeri UA, Zhao G, et al. Protein misfolding, congophilia, oligomerization, and defective amyloid processing in preeclampsia. *Sci Transl Med* 2014;6(245):245ra92

- 13 Buhimschi I, Buhimschi CS, Choma M, Tagare H, Jonas SM. Methods and compositions for detecting misfolded proteins. US patent 10,324,094. April 10, 2014
- 14 Halimi M, Dayan-Amouyal Y, Kariv-Inbal Z, Friedman-Levi Y, Mayer-Sonnenfeld T, Gabizon R. Prion urine comprises a glycosaminoglycan-light chain IgG complex that can be stained by Congo red. *J Virol Methods* 2006;133(02):205–210
- 15 Frid P, Anisimov SV, Popovic N. Congo red and protein aggregation in neurodegenerative diseases. *Brain Res Brain Res Rev* 2007;53(01):135–160
- 16 Rood KM, Buhimschi CS, Dible T, et al. Congo red dot paper test for antenatal triage and rapid identification of preeclampsia. *EclinicalMedicine* 2019;8:47–56
- 17 Ghulmiyyah L, Sibai B. Maternal mortality from preeclampsia/eclampsia. *Semin Perinatol* 2012;36(01):56–59
- 18 Duley L. The global impact of pre-eclampsia and eclampsia. *Semin Perinatol* 2009;33(03):130–137
- 19 Poon LC, Shennan A, Hyett JA, Kapur A, Hadar E, Divakar H, et al. The International Federation of Gynecology and Obstetrics (FIGO) initiative on pre-eclampsia: a pragmatic guide for first-trimester screening and prevention. *Int J Gynaecol Obstet* 2019;145 suppl 1(suppl 1):1–33
- 20 Bartsch E, Medcalf KE, Park AL, Ray JG. High Risk of Pre-eclampsia Identification Group. Clinical risk factors for pre-eclampsia determined in early pregnancy: systematic review and meta-analysis of large cohort studies. *BMJ* 2016;353:i1753
- 21 Rodríguez-Chávez J L, Gutiérrez KF, Angelesvázquez MDe J, Zerón HM. Evaluation of Congo red staining kit to determine proteinuria in preeclampsia. *J Clin Diagn Res* 2018;12(05):QM01–QM05
- 22 Nagarajappa C, Rangappa SS, Suryanarayana R, Balakrishna S. Urinary congophilia in preeclampsia: experience from a rural tertiary-care hospital in India. *Pregnancy Hypertens* 2018;13:83–86
- 23 Sammar M, Syngelaki A, Sharabi-Nov A, Nicolaidis K, Meiri H. Can staining of damaged proteins in urine effectively predict pre-eclampsia? *Fetal Diagn Ther* 2017;41(01):23–31