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Preeclamptic toxemia: a disease ripe for proteomic discovery

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"The testing of potential therapeutic agents has been hindered by the lack of a screening test capable of discriminating affected from unaffected pregnancies at a gestation time when intervention is likely to be of benefit..."

The pregnancy complication preeclampsia, characterized by hypertension, proteinuria and widespread endothelial dysfunction, is a leading cause of maternal and perinatal morbidity and mortality. To date, the only treatment has been delivery of the placenta and baby, resulting in significant iatrogenic prematurity. The testing of potential therapeutic agents has been hindered by the lack of a screening test capable of discriminating affected from unaffected pregnancies at a gestation time when intervention is likely to be of benefit, that is, before 20 weeks. Hypothesis-driven approaches have identified several candidate markers for the development of preeclampsia, although none has been shown to possess the necessary sensitivity to be useful in clinical practice. Proteomics offers the potential to identify novel markers that may be useful alone or in combination to predict this important pregnancy complication.

"In pregnancies affected by preeclampsia, evidence suggests that deficient adaptation of the maternal spiral arteries leads to a poorly perfused placenta."

In pregnancies affected by preeclampsia, evidence suggests that deficient adaptation of the maternal spiral arteries leads to a poorly perfused placenta [1]. Several *in vitro* studies [2–4] and *in vivo* observations support the hypothesis that the release of factors into the maternal circulation by a hypoperfused placenta gives rise to activation of the maternal vascular endothelium. The presence of vasoactive circulating factors in the plasma of women with preeclampsia has been demonstrated in samples taken from women at the time of disease and before onset of clinical disease [5,6], and highlights the validity of the alternative term for the disease, 'toxemia'. Characterization studies in plasma have demonstrated that these vasoactive factors are sensitive to protease digestion [7] and were retained following high-abundance protein depletion. Importantly, it was demonstrated that multiple synergistic factors were present [8]. As yet, the identity of these vasoactive circulating factors remains unknown. Several circulating proteins have been shown to be elevated in the plasma of women who subsequently developed preeclampsia [9,10]; however, none of these candidate proteins has been found to have the necessary sensitivity to be useful in a clinical setting. Use of these proteins as effective screening tools has been impeded by considerable overlap in the reference ranges of normal and diseased states. In addition, such studies presuppose the identity of potential markers based on proposed mechanisms of the disease; crucially, this excludes proteins whose role has yet to be established.

Screening for preeclampsia thus represents a significant unmet clinical need, with the presence of unknown circulating proteins prior to the onset of clinical disease. Hypothesis-generating, quantitative proteomic technologies have the potential to significantly advance both our understanding of the disease and its clinical management. Two of the advantages of proteomics over conventional protein quantification methods are that it offers the opportunity to investigate multiple proteins simultaneously

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and it facilitates an unbiased search for novel protein markers. The use of advanced mass spectrometric techniques also enables the accurate quantification of targeted proteins that can be used to validate candidate biomarkers. An early pregnancy screening test based on, as yet unidentified, circulating proteins would enable the stratification of care based on accurate risk assessment and facilitate the testing of novel treatments. Although urine and amniotic fluid samples from women with preeclampsia have yielded useful information [11–14], studying the plasma is most likely to generate an effective predictive test.

> "Hypothesis-generating, quantitative proteomic technologies have the potential to significantly advance both our understanding of the disease and its clinical management."

There are significant challenges to such a strategy; one being the huge dynamic range in the concentrations of high- and low-abundance plasma proteins. Plasma contains tens of thousands of core proteins, which exist at relatively high concentrations, and other lower abundance proteins that represent traces of physiological and pathological processes that have been encountered during constant perfusion throughout the body [15]. It is likely that biomarkers are among these lower-abundance proteins, leaked from damaged tissues and, therefore, massively diluted in the circulation [16]. Albumin, the most abundant plasma protein, has a plasma concentration of approximately 35–55 mg/ml, whereas some cytokines have a plasma concentration of 0–5 pg/ml. Albumin and IL-6, therefore, differ in plasma abundance by a factor of 10 [17], whereas unbiased mass spectrometric protein-discovery technologies have typical dynamic ranges of 10^2 to 10^4. Although this shortfall can be approached by methods that simplify the plasma proteome by the enrichment of lower-abundance proteins [18], several additional fractionation steps are necessary prior to the analysis of complex fluids such as plasma [18,19].

Current proteomic investigation in reproductive medicine is limited; however, several investigators have begun to apply this technology to pregnancy complications. As reduced blood flow to the placenta is a major feature of preeclampsia and, thus, to the release of placentally derived blood-borne factors, it is possible that placental hypoxia plays an important role in the pathophysiology of the disease. 2DE and tandem mass spectrometry has been used to characterize the protein repertoire of first-trimester placental cells (cytotrophoblasts) under different oxygen tensions. Hoang *et al.* detected discrete changes in the expression of enzymes involved in glycolysis, responses to oxidative stress and 14–3–3 signaling/adapter proteins [20]. Of particular interest was the increased expression of annexin II, known to be associated with placental protein 13. Placental protein 13 has been investigated as a potential predictor of preeclampsia and has been shown to be elevated in the first trimester in women who subsequently develop the condition [21,22].

We recently used a hypoxic placental explant model to study factors present in serum-free, explant-conditioned media and their effect on endothelial cell function [23]. Factors capable of altering endothelial function were demonstrated in explant-conditioned media generated from placental explants cultured under hypoxic conditions using several different endothelial bioassays. We used a MS-based proteomics strategy in which fractionation occurs at the peptide level rather than the protein level. This 2D separation of peptides in the liquid phase (liquid chromatography) fractionation strategy yielded a much improved performance, in terms of protein identifications, than more traditional 2DE methods, as well as being more robust and reproducible. The isobaric tag for relative and absolute quantitation-based relative quantification strategy was used to examine changes in proteins liberated from placental tissue under hypoxic and normal oxygen conditions. A total of 499 distinct proteins were identified, of which 22 were found to be upregulated in the hypoxic-conditioned explant media and 41 were found to be downregulated. We were able to detect and validate changes (using ELISA measurements) in the release of IL-8 from hypoxic-conditioned placentas. To our knowledge, this is the first time that an unbiased proteomics experiment has detected and quantified changes in interleukin levels in any biological samples. In addition to the identification of cytokines, this study also recorded hypothesis-generating alterations in many extracellular matrix-remodeling proteins.

The use of 2DE gel analysis has also been applied to plasma samples in preeclampsia [24]. Plasma samples from six women with preeclampsia and six matched normotensive controls were analyzed with clusterin identified as being overexpressed in the preeclampsia samples. This finding was validated in the serum of 80 preeclamptic and 80 normal pregnant women using immunoassay measures, although there was significant overlap between the normal and preeclampsia levels. Clusterin is a multifunctional protein that may be affected by oxidative stress and has been associated with renal pathologies. Wang *et al.* used 2DE to detect differences between the plasma proteome of 11 women with established preeclampsia and matched gestational controls [25]. A significant reduction in the expression of H-ficolin (1.3-fold) and L-ficolin (3.2-fold) was found and subsequently validated on a separate cohort of samples. This study is an example of a successful, hypothesis-generation experiment, where an initial, unbiased discovery technique led to the investigation of a novel hypothesis. However, as the plasma was taken from women with established disease, it remains uncertain as to whether the altered levels of plasma ficolin and clusterin represent an etiological process or an epiphenomenon of the disease process. Studies investigating plasma at earlier gestations are needed in order to validate these findings and investigate the potential of these molecules as biomarkers for preeclampsia.

We previously assessed the use of SELDI to compare plasma taken at 26 weeks from women who subsequently developed preeclampsia (n = 8) and women who subsequently had an uncomplicated pregnancy (n = 8) [26]. Preliminary analysis demonstrated that five proteins were upregulated in samples taken from the women destined to develop preeclampsia. These proteins could not be identified using the SELDI technology. As this pilot study did not employ any prefractionation steps, these peaks are likely to represent very high-abundance plasma proteins. More recently,

we have been developing the application of the isobaric tag for relative and absolute quantitation to the analysis of plasma samples to assess the level of proteome penetration that can be achieved using this technique. This development work has been performed in pooled time-of-disease samples from women with preeclampsia and gestation-matched controls. Most of the proteins in which a significant difference in abundance was recorded were high-abundance plasma proteins, including complement cascade proteins, which exist at microgram per millimeter to milligram per millimeter levels. Several pregnancy-specific or -enriched proteins were identified as being statistically significantly higher in preeclamptic compared with control plasma. These included pregnancy-associated plasma protein-A, pregnancy-specific β-1 glycoprotein 1 and the closely related pregnancy-specific β-1 glycoprotein 9. We also identified some lower-abundance proteins that were overexpressed in preeclampsia plasma, including endoglin and placental lactogen. This observation is significant because endoglin is one of the few candidate markers for preeclampsia that has previously been identified in hypothesis-driven studies [10,27]. The increased levels of endoglin observed in preeclampsia plasma were subsequently validated in pooled and individual plasma samples using an ELISA. We are currently using the same techniques to analyze samples taken from women at 15-weeks gestation who subsequently had uncomplicated pregnancies or developed preeclampsia.

Circulating proteins are so vital to preeclampsia that the condition may provide the first demonstration of the potential of clinical proteomic technologies to translate into clinical practice. Two of the huge benefits of proteomic techniques over conventional protein analysis methods are the possibility of high-throughput analysis of thousands of proteins simultaneously and the identification of novel proteins that may stimulate new hypothesis-driven research. However, despite huge efforts, the discovery of a universally applied protein biomarker using MS techniques has yet to be achieved. Some of the reasons for this include problems with sample heterogeneity, poor instrument reproducibility, data-handling issues and an inability of current MS instruments to cope with the enormous complexity and dynamic range of biological samples [28]. Adherence to strict collection protocols, consistency of sample preparation, experimental handling and data analysis will all help to optimize results by improving technical reproducibility, while technological, quantitative and analytic advances continue to develop. From the perspective of diseases such as preeclampsia, with an incidence of 3–5%, collection of the numbers of samples necessary to perform these studies requires large collaborative efforts, such as that achieved by the Screening for Pregnancy Endpoints study [101,102]. This international consortium is currently collecting and storing blood, urine and DNA samples from women in Australia, New Zealand, Ireland and the UK, and it is making a significant contribution to this directive.

It is also important that proteins of interest that are identified in the discovery phase of a biomarker study are confirmed and validated in an independent sample set, as part of a coherent pipeline from candidate biomarker discovery to clinical implementation. It is likely that a significant number of candidate biomarkers will not be consistent between study groups as they are sensitive to changes in patient cohorts. However, the well-established use of proteins as markers for disease and physiological disturbances suggests that others may be more consistent, and it is also worth recognizing that subtle but consistent changes may be more important than larger changes identified from a small cohort of patients.

As technology improves, targeted, quantitative MS is likely to be used more frequently. This approach employs multiple reaction monitoring to isolate proteotypic peptides (easily observed in a MS platform and unique to a particular protein) for proteins of interest [29]. The relative abundance of these peptides can be compared with known amounts of synthetic peptides to provide absolute quantification. This platform is increasingly being used in an attempt to confirm potential protein-expression differences and with a greater degree of sensitivity than shotgun proteomic methods. It still enables the simultaneous quantification of multiple proteins but may also help to identify and obtain quantitative data from low-abundance proteins of interest, while at the same time preventing the time-consuming analyses of large numbers of irrelevant peptides. This approach is most likely to be of use in large cohorts, following an initial in-depth analysis of the proteome in a smaller number of samples. This strategy has yet to be proven as a high-throughput method of validating biomarkers in highly complex biofluids, such as plasma, although it has been used to successfully quantify major plasma proteins [30] and is becoming more readily accessible to nonexpert laboratories.

Another relatively untapped resource in the prediction of complex disease is the combination of existing clinical data with candidate biomarker data. Research has frequently focused on the proteins discovered without incorporating background data on established risk factors, and yet every patient sample has a wealth of existing clinical and physical data. The inclusion of measurements of known variables that can be incorporated into a systems-biology approach, to better describe the state of an individual patient and how it relates to a desired outcome (e.g., disease prediction), would be enormously beneficial.

Five-year view

Without doubt, the successful application of proteomic methodologies to complex clinical diseases remains very challenging. For example, analysis of the complete plasma proteome using MS is still not possible, and the reliable detection of very low-abundance proteins remains elusive. However, the upstream preparation techniques and available instrumentation for plasma analysis continues to expand rapidly, and new technologies and instruments boasting improved resolution and throughput times are continuously being developed. Therefore, the potential is enormous; well-designed, adequately powered studies have a real chance of delivering clinically useful biomarkers and providing novel insights into the pathogenesis of pregnancy complications such as preeclampsia. Investment in this area could be fundamental to achieving the goals towards improved detection and management of this life-threatening condition.

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