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Novel Screening Approaches for the Early Detection of Gestational Diabetes Mellitus

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1. Introduction

1.1 Complications of pregnancy

Within the discipline of clinical obstetrics, our understanding of the aetiology of complications of pregnancy is lacking. This lack of understanding limits our ability to identify and implement efficacious management and intervention strategies to ameliorate any adverse effects on both the mother and her baby. This situation is further confounded by the lack of reliable screening test(s) to identify pre-symptomatic women who subsequently develop complications of pregnancy. Gestational diabetes mellitus (GDM), extremes of birth weight (intrauterine growth restriction (IUGR) and fetal macrosomia (FM)), preeclampsia toxaemia (PET) and preterm labour ((PTL), including preterm rupture of membranes) are the most important complications of pregnancy that have no effective antenatal preventative treatment.

With an incidence each of about 5-10% of all pregnancies, these complications are common, responsible for the majority of obstetric and paediatric morbidity and mortality, and can permanently impact on lifelong health. For example, extreme preterm birth, whether spontaneous, or iatrogenic to protect the mother or fetus from progressive disease, can result in perinatal death or serious permanent disability such as blindness, deafness or neurological injury (Chandiramani, et al., 2007). Very low birth weight is not only an immediate threat to the fetus, but can programme adult onset hypertension, stroke and diabetes (Barker, 2006). Gestational diabetes is associated with a range of perinatal morbidities including fetal macrosomia, hyperinsulinaemia and hypoglycaemia, but may also programme childhood obesity and adult onset cardiovascular disease and diabetes (Moore, 2010, Nolan, 2011); diseases with the greatest impact on health economics. Moreover, women who develop gestational diabetes are at greatly increased risk of developing type 2 diabetes in later life (Henry and Beischer, 1991, Lee, et al., 2007).

Early detection of disease risk and onset is the first step in implementing efficacious treatment. If such early detection tests were available they would represent a major advance and contribution to the discipline and afford the opportunity to evaluate alternate treatment

and clinical management strategies to improve health outcomes for both mother and baby. Based upon recent technological developments and studies, it is now realistic that clinically useful antenatal screening test(s) can be developed**.** Unlike diseases such as cancer where biomarkers need to be exquisitely specific, a useful antenatal screening test would ideally be highly sensitive, but not necessarily highly specific. The consequence of a false positive would be no worse than an erroneous triage to high-risk care.

1.2 The future of diagnostics

In the context of antenatal screening, the objective of proteomic approaches is to identify proteins or peptides that are informative of the risk of pre-symptomatic early pregnant women who subsequently develop complications of pregnancy. That is, how the antecedents of complications of pregnancy alter the expression of the genome and how this is manifested as altered protein and peptide expression. Informative proteins and peptides identified may be used to develop classification models (*e.g.* multiple biomarker diagnostic or prognostic tests) that assign the likelihood that an individual test sample came from a normal or "at-risk" group. Such tests (as with all *in vitro* diagnostic medical devices) inform clinical decision-making and provide an opportunity for timely and appropriate intervention. The performance of the test *(i.e.* its diagnostic efficiency) determines the quality of the information provided and ultimately patient management. The application of proteomics, thus, extends beyond mapping and comparing the protein complement of healthy and at-risk individuals and needs to be considered in the context of its contribution to the healthcare system.

Global health care is rapidly evolving, being driven by two processes - technological development and information management systems. Technological developments now provide opportunity to acquire complex information about patients. For example, rather than relying on a single measurement (*e.g.* a single biomarker diagnostic blood test) to detect disease, multiple disease markers may be simultaneously measured and combined to provide earlier and more accurate diagnosis. Information management systems are allowing such complex data to be ascribed to the individual over the course of their lifetime. The anticipated outcome of these forces is a move from the episodic, reactionary medicine of today to personalised medicine where pharmacogenetics and molecular medicine will afford the opportunity to identify predisposition to disease, risk assessment, and assign individuals to personalised, efficacious treatment/intervention groups. Both genomics and proteomics will be useful contributors to the evolving healthcare system by providing a better understanding of physiology, by defining disease risk, by enabling earlier diagnosis and by monitoring treatment responses (Figure 1).

It is now widely acknowledged that single biomarkers are unlikely to deliver the significant incremental gain in sensitivity and specificity required for the development of effective screening and classification tests requisite for the implementation of personalised medicine. New approaches based upon the measurement of multiple biomarkers of disease risk afford opportunity to increase diagnostic test sensitivity and specificity. Over the past decade, the advent and optimisation of new proteomic technologies has paved the way for new strategies for the development of such multiple biomarker diagnostic tests. Our research team has applied both candidate-based and discovery-based proteomic approaches to identify biomarkers that are informative of disease risk. The ultimate objective of these

collective efforts is the generation of multivariate, classification models that, at the very least, will allow the triage of early pregnant, asymptomatic women into low- and high-risk cohorts (Figure 2).

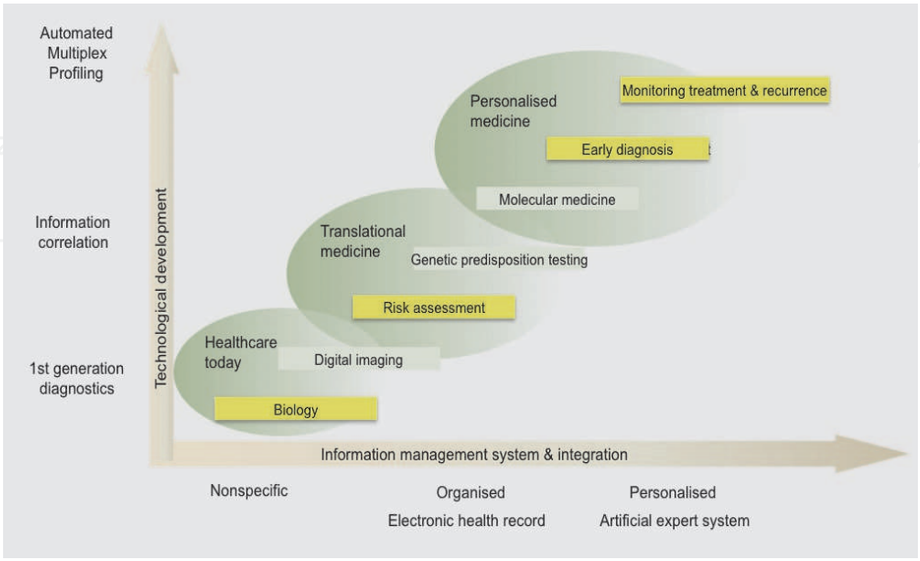

Fig. 1. The evolving healthcare system. The role of proteomics in the evolution of the healthcare system may be in the provision of multiple analyte protein and peptide profiles that facilitate risk assessment, earlier diagnosis and more effective treatment response monitoring. (Modified from "*Personalised Healthcare 2010", IBM Business Consulting Services*)

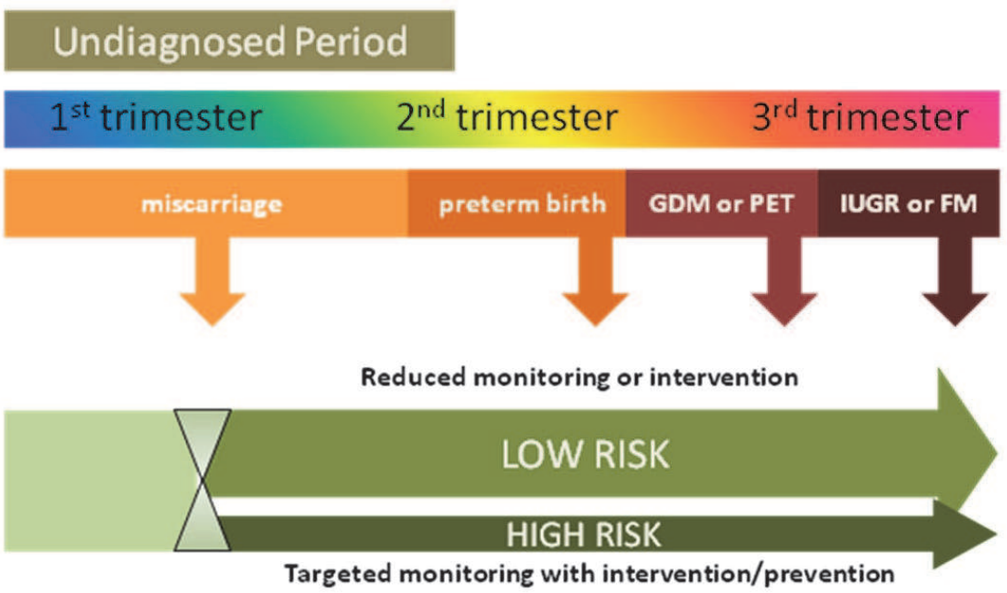

Fig. 2. Rationale for antenatal screening. The objective of implementing an antenatal screening test is to identify pre-symptomatic women who will subsequently develop complications of pregnancy and implement efficacious treatment to reduce morbidity and mortality. Currently, complications of pregnancy are not diagnosed until mid-late gestation.

2. Gestational diabetes mellitus

2.1 Current screening

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance of varying severity with onset or first recognition during pregnancy (Metzger and Coustan, 1998). GDM usually manifests in the latter half of pregnancy and is typically diagnosed by an oral glucose tolerance test. If GDM is diagnosed, women will usually be counselled and advised to adopt a healthy lifestyle for the duration of their pregnancy and where it is deemed appropriate, women may also be required to undergo pharmacologic or insulin therapy. During this time, increased surveillance of the pregnancy is undertaken to help ameliorate the consequent maternal and fetal morbidity associated with GDM. The clinical importance of this surveillance is highlighted by recent published findings that demonstrate that even mild hyperglycaemia over a prolonged period is associated with numerous adverse perinatal outcomes (Schafer-Graf, 2009) and that intensive intervention therapy can significantly reduce maternal and fetal morbidity (Crowther, et al., 2005, Landon, et al., 2009). The effects of hyperglycaemia on pregnancy outcome are underpinned by experimental studies that identify putative effector pathways by which exposure to glucose concentrations may alter placental and maternal adipose tissue phenotype and responsiveness (Coughlan, et al., 2001, Coughlan, et al., 2004, Coughlan, et al., 2004, Lappas, et al., 2004).

The current 'gold standard' for the diagnosis of GDM is the oral glucose tolerance test (OGTT) and although there is no international consensus on the diagnostic methods or the blood glucose thresholds, it has become more widely accepted that the OGTT should be performed between 24-28 weeks' gestation using a 75 g glucose load with fasting, one-hour and two-hour venous glucose determinations (Coustan, et al., 2010, Metzger, et al., 2010). Universal screening and blood glucose thresholds remain contentious and may sometimes be based on resource availability and economic factors rather than clinical factors. Often, the more 'restrictive' blood glucose limits, leading to the diagnosis of the more severe hyperglycaemic patients, is all that can be provided in some instances. By contrast, professional associations advocate more 'inclusive' diagnostic criteria, and consequently more patients being diagnosed with less severe hyperglycaemia (Agarwal, et al., 2005, Lindsay, 2011, Metzger, et al., 2010, Ryan, 2011). With the obesity epidemic well entrenched in the Western world and with more women delaying pregnancy and the associated increase in pre-pregnancy body mass index (BMI), the incidence of GDM is increasing irrespective of the diagnostic criteria used (Kerrigan and Kingdon, 2010, Wein and Beischer, 2000).

2.2 The problem with current tests

It is important to recognise that by the time GDM is diagnosed in the late second or early third trimester of pregnancy, the 'pathology' is probably established and that reversal of the potential adverse perinatal outcomes may be limited. Many health professionals advocate the need for an earlier diagnostic/predictive test for GDM while at the same time acknowledging that avenues for preventative treatment may be limited (Guedj, 2010). In fact, it is the lack of a reliable early test for GDM that has hampered the development of useful intervention therapies. Although a direct clinical benefit of the early diagnosis of GDM remains to be established conclusively, identification of women at greatest risk would allow triage of patients to an appropriate model of care and identify those who are at greatest need of glucose tolerance assessment (Caliskan, et al., 2004, Shirazian, et al., 2009).

Numerous GDM risk-factor assessments have been attempted during first trimester pregnancy and include, but not limited to, family history of GDM and/or diabetes (Savvidou, et al., 2010), maternal demographics (Alanis, et al., 2010, Phaloprakarn, et al., 2009, Shirazian, et al., 2009, Wein, et al., 1995), maternal pregnancy weight gain (Morisset, et al., 2011), fasting plasma glucose (Riskin-Mashiah, et al., 2009, Riskin-Mashiah, et al., 2010), one-hour glucose challenge test (Maegawa, et al., 2003, Nahum, et al., 2002, Punthumapol and Tekasakul, 2008), oral glucose tolerance test (Bhattacharya, 2004, Phaloprakarn and Tangjitgamol, 2008, Sacks, et al., 2003) and haemoglobin A1c levels (Maegawa, et al., 2003). Although some tests have provided a good negative predictive measure for subsequent GDM, most tests suffer from poor positive predictive values and therefore are of limited efficacy. It is evident that other metabolic markers that precede hyperglycaemia would need to be identified if GDM were to be predicted from a test in early pregnancy.

3. Early screening of impending GDM

3.1 Single biomarker investigations

Recently, a number of first trimester studies have identified various biomarkers associated with subsequent development of GDM. In some cases these can be regarded as surrogate markers of inflammation such as C-reactive protein (Wolf, et al., 2003), of oxidative stress such as 8-isoPGF$_{2\alpha}$ (Rogers, et al., 2006) or of obesity such as serum triglycerides (Nolan, et al., 1995, Son, et al., 2010) and may not necessarily be specific for impending GDM. Perhaps the more exciting studies are those that have investigated serum or plasma protein biomarkers associated with early pregnancy placental function and carbohydrate/lipid metabolism. For example, it has been shown that lower sex hormone-binding globulin (Thadhani, et al., 2003), increased placental growth factor (Ong, et al., 2004), elevated leptin concentrations (Qiu, et al., 2004), reduced plasma adiponectin concentrations (Retnakaran, et al., 2004, Williams, et al., 2004) and lower follistatin-like-3 levels (Thadhani, et al., 2010) are all risk factors for subsequent development GDM. Although the associations are compelling further investigations are warranted as it appears that none of these markers alone provide adequate positive predictive values for subsequent GDM.

3.2 Multiple biomarker investigations

More recent studies have focused on multiple candidate-based profiling of blood-borne biomarkers to identify lead candidates for developing early pregnancy screening tests for gestational diabetes. For example, we measured multiple plasma biomarkers at 11 weeks' gestation in women who subsequently experienced a normal pregnancy outcome (n=14) and women who subsequently developed gestational diabetes (n=14) (Georgiou, et al., 2008). Of the biomarkers considered (insulin, adiponectin, leptin, resistin and glucose), receiver operator characteristic (ROC) curves for three biomarkers (adiponectin, insulin and random blood glucose) are presented together with a ROC curve based on the predicted posterior probability values (ppv) generated by a classification model that combined information from all three biomarkers (Figure 3). The combined model out performed individual biomarkers based upon the area under the ROC curve (combined model = 0.94; adiponectin = 0.867; insulin = 0.872 and glucose = 0.827). This simple example demonstrates the putative benefit of a multimarker approach for improving diagnostic efficiency. Similar multiple biomarker investigations in association with GDM early (Nanda, et al., 2011) or late (Bomba-Opon, et al., 2010, Lowe, et al., 2010) in pregnancy have been described.

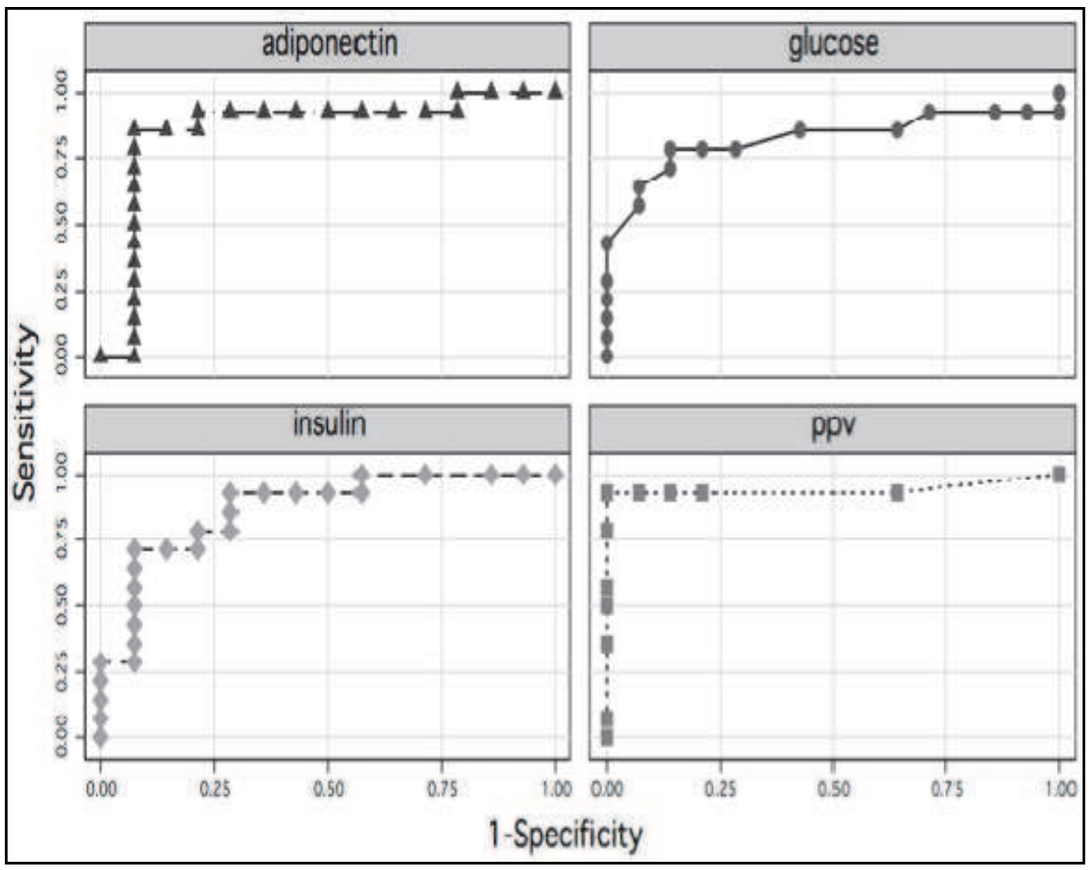

Fig. 3. The advantage of multiple biomarker screening. A comparison of ROC curves of the performance of individual biomarkers (adiponectin, insulin and glucose) and a combined model (ppv) to correctly classify women who subsequently developed gestational diabetes.

4. Proteomic approaches for early detection of GDM

4.1 Sample selection and processing

As with all analytical techniques, sample heterogeneity (*e.g.* variation from individual patients, sample collection and processing) needs to be minimised for proteomic analysis. This is particularly relevant to the collection of blood, where both the method of collection and processing may dramatically alter the peptide profile (*e.g.* clotting, temperature and time taken to process samples). For example, while the collection of serum may be suitable for some candidate-based approaches (*e.g.* protein solution array and immunoassay), the peptides generated during coagulation confound peptidomic analysis.

The impact of the method used to collect blood is demonstrated in Figure 4, in which paired plasma and serum samples were collected from pregnant women. Blood was either collected into EDTA or Serum Clot Activator tubes. The former was immediately centrifuged for 15 min at room temperature, while the latter was allowed to clot at room temperature for 60 min and then centrifuged for 15 min at room temperature. Following

centrifugation, the resultant plasma or serum was stored at -80° C for mass spectrometry peptide profiling. Both plasma and serum samples were subjected to protein dye binding depletion (Affi-gel Blue™) as previously described (Ahmed, et al., 2003) followed by solid phase peptide enrichment using hydrophilic-lipophilic balanced solid phase extraction sorbent in 96-well micro-elution plates, eluted and analysed using matrix assisted laser desorption ionisation – time-of-flight (MALDI-ToF) mass spectrometry (AutoFlex II, Bruker Daltonics). The resultant peptide profiles of plasma and serum while showing some concordance at m/z >4000, exhibited dramatically different peptide ion profiles at m/z < 2000 (Figure 4). It is likely that these ions represent peptides generated during the coagulation process and/or by the action of peptidases during the 60 min incubation at room temperature. Thus, for the purpose of primary peptidomic profiling of blood peptides, serum presents significant methodological challenges. Indeed, even different anticoagulant methods (*e.g.* EDTA, heparin, citrate) have been found to alter peptide ion profiles (Banks, et al., 2005).

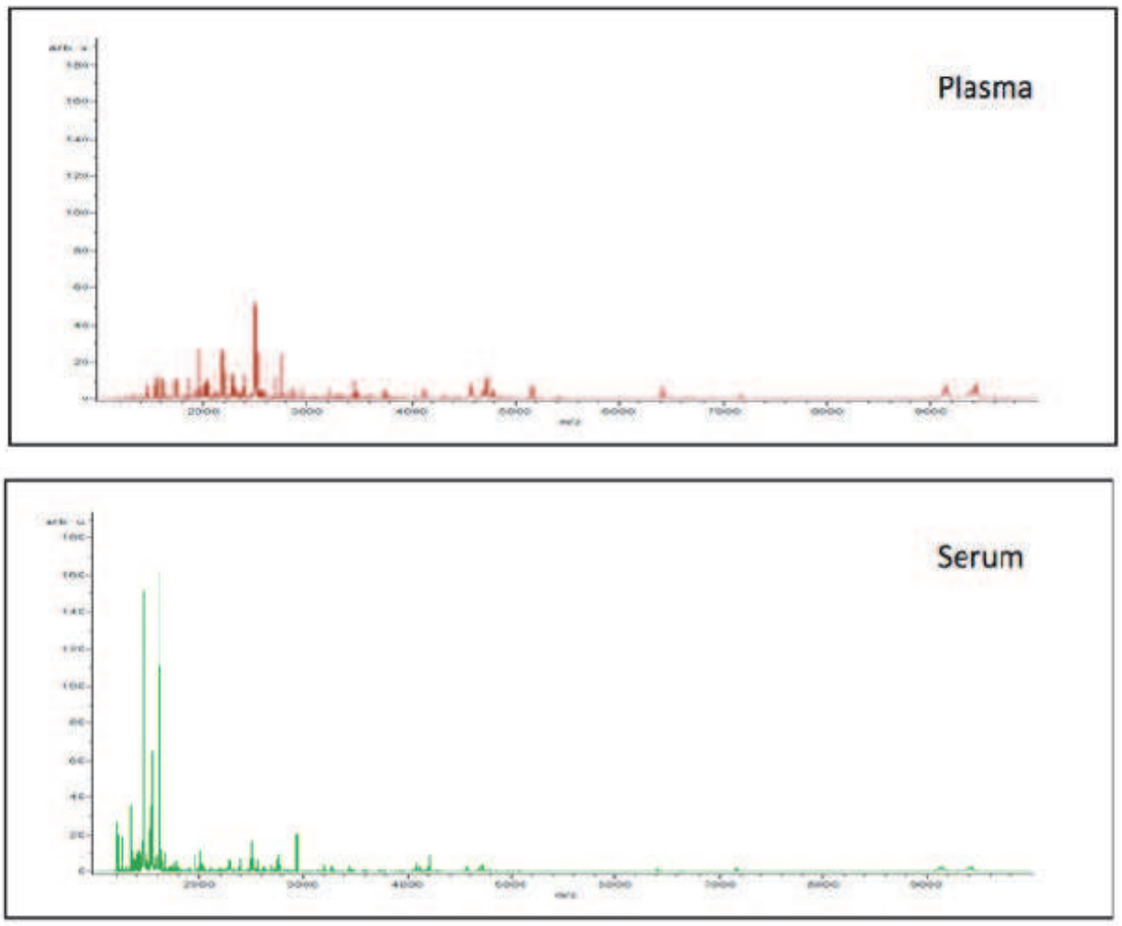

Fig. 4. Method of blood collection. Mass spectrometric peptidomic profiling of paired plasma and serum showing marked spectral differences.

4.2 Consideration of gestational change

Of particular relevance to any discussion of biomarkers for the development of antenatal screening tests is gestational variation. To be of clinical utility, any early pregnancy screening test would need to be independent of or well outside the normal early gestational changes in the subset of the proteome being interrogated. For example, in seeking to identify plasma protein biomarkers that may be of utility in identifying women at risk of developing a complication of pregnancy, it is critical to first establish the variation (both gestational and inter-patient) that occurs within the temporal window in which the test is to be applied. That is, if the objective is to identify plasma protein biomarkers using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for a test that will be applied between 6-12 weeks of gestation then the variation that occurs in the subset of proteins being screened must be established.

To assess the early-pregnancy variation that plasma proteins display by 2D electrophoresis, we completed an initial study that begins to define the gestational variation in a subset of plasma proteins. Weekly peripheral blood samples were collected from women from 6-12 weeks of pregnancy. Plasma samples were immuno-depleted of high-abundance proteins (IgY14 column, Sigma) and then labelled with fluorescent CyDyes (Cy3, Cy5) for pair-wise comparison (GE Healthcare). A pooled plasma sample was labelled with Cy2 for normalisation across gels. Labelled proteins were pooled and then separated in the first dimension (24 cm Immobiline™ Dry- Strips, pH 3-11NL) and then in the second dimension (12.5% 24 cm hand-cast acrylamide gels with low fluorescent glass). Gel were imaged using a Typhoon Trio 9100 (GE Healthcare) and then analysed using Progenesis SameSpots software (v3.2.3107.24565, Nonlinear Dynamics). The analysis focussed on spots with a greater than 1.5 fold difference. Protein spots that were common to all gels (n=89) were further investigated. Figure 5 presents a box-plot summary of the gestational variation in these proteins for one patient. To identify protein spots that varied significantly across gestation and accounting for 'false discovery rates', the combined data set was subjected to nonparametric analysis methodology using Significance of Microarray (SAM) analysis. Using a false discovery rate of 1%, 5 protein spots were identified that varied significantly during 6-12 weeks of pregnancy (Figure 6).

4.3 Candidate-based profiling approaches (solution array workflow)

Protein and antibody arrays and multiple immunoassay methodologies represent examples of candidate or targeted proteomic approaches. The advantages of these approaches include: rapid, high throughput screening of known targets and quantitative endpoints. Multiplex protein solution array is one application that represents a generation of antibody-based detection technology that allows the simultaneous quantification of multiple analytes in a single, small volume sample. Multiplex protein solution array has a number of advantages over current analyte quantification technologies, including: measurement of many biomarkers (up to 100 different analytes) in a single sample; wider operational dynamic range; and increased sensitivity and specificity derived from multivariate modelling of combinations of biomarker analytes. This system utilises a sandwich ELISA-like protocol, in which capture antibodies are coupled to spectrally distinct polystyrene or metal beads (5-6 m diameter). Biotinylated sandwich antibody and streptavidin-phycoerytherin (PE) fluorophore are used as a reporter complex. Assays are conducted in 96-well filter-bottom

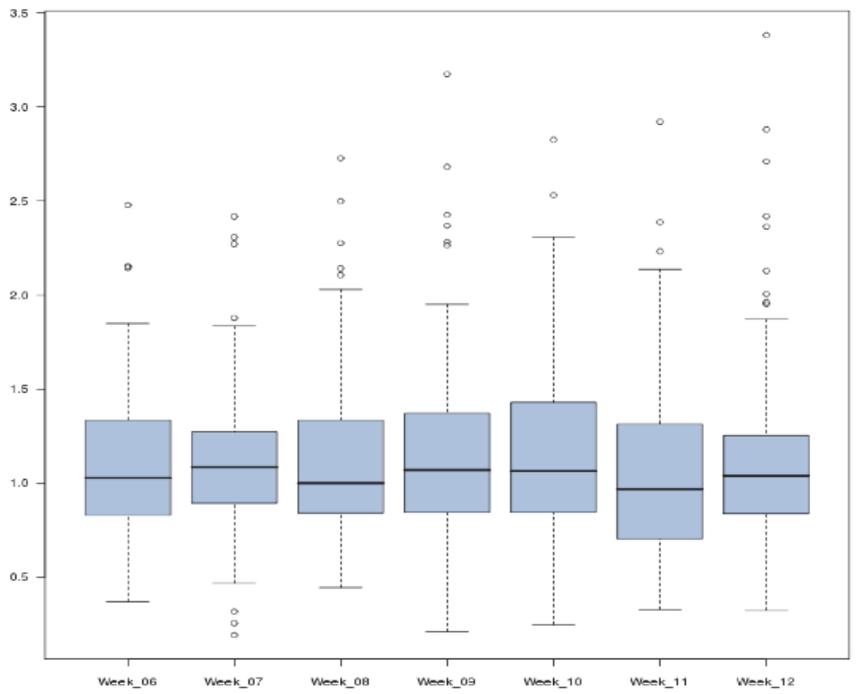

Fig. 5. Variation in plasma proteins displayed during early pregnancy (6-12 weeks of gestation). Serial peripheral blood samples were collected from women and displayed using 2D-DIGE. The data presented represent normalised spot volumes for 89 protein spot that were common to all gels.

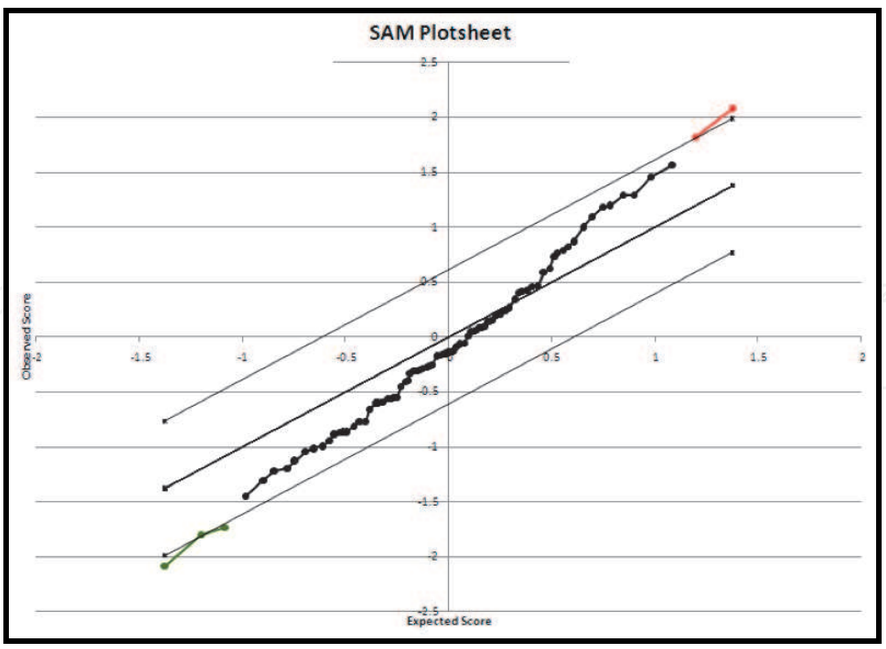

Fig. 6. Non-parametric, significance of microarray (SAM) analysis of the variation in normalised protein spot volumes during early pregnancy. Five protein spot (shown in colour) are identified as varying significantly.

plates and beads are washed by vacuum filtration. Bead identity and analyte-specific fluorescence are assessed using a flow cytometre (Luminex) fitted with dual lasers. Solution array offers excellent reproducibility (CV <10%) and analyte quantification and has the capacity to multiplex up to 100 different analytes in small sample volumes (*e.g.* 50-100 l plasma).

Various manufacturers have now produced a myriad of multiplex assay kits consisting of premixed panels of biologically related biomarkers (*e.g.* cytokines/chemokines, endocrine hormones, matrix metalloproteinases, phosphoproteins etc) or disease-related panels (*e.g.* cancer markers, autoimmunity biomarkers and more recently, diabetes biomarkers). All suppliers also offer single-plex biomarkers that can be custom mixed to produce any panel of choice. We have previously used cytokine/chemokine multiplex panels to investigate pregnancy related complications such as GDM (Georgiou, et al., 2008), intrauterine growth restriction (Georgiou, et al., 2011) and preterm prelabour rupture of membranes (Hodges, et al., 2010) as well as ovarian cancer (Edgell, et al., 2010). Although the differentially expressed biomarkers may only be associative rather than causative of disease, these and other similar studies highlight the advantages of multiple biomarker screening for improved diagnostic/predictive modelling. An important consideration for any method that utilises multiple analyte determination is the appropriate control for the false discovery rate when multiple comparisons (hypotheses) are being tested.

4.4 Gel-based profiling approaches

Gel-based platforms such as 1-dimensional and 2-dimensional polyacrylamide gel electrophoresis (1D or 2D-PAGE) and fluorescence 2D difference gel electrophoresis (2D-DIGE) have been used in both expression and comparative studies to define plasma protein abundance and disease-associated or treatment-induced changes. The advantage of these approaches resides in their ability to identify post-translational modified protein isoforms. The limitation of gel-based systems is their relatively low throughput, the necessity for sample processing and fractionation prior to display and limited mass range (~10-200 kDa). In addition, procedural protein losses and the overall experimental variation in estimating endpoints by 2D-PAGE may be considerable. Procedural losses of proteins during 2D-PAGE display have been reported to be as high as 80% but this can vary depending on the starting protein load (Zhou, et al., 2005). As with any other technique, variation is apportioned between technical replication, both within assay and between assay, and biologic variation (*i.e.* sample-to-sample). Estimates of the variation attributable to technical replication average 25-40%. Biological variation has been estimated to be between 24 and 70% (Molloy, et al., 2003).

4.4.1 2-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Using a traditional 2D-PAGE approach, we analysed the maternal plasma proteome from women with a normal pregnancy and compared this with women who subsequently developed GDM. Plasma samples were obtained at approximately 12 weeks' (pre-GDM) and 28 weeks' gestation (overt GDM) and gestation-matched with an equal number of normal controls. Individual plasma samples were depleted of high abundance proteins (albumin and immunoglobulins) by matrix binding centrifugation (Affi-gel Blue and Affi-gel Protein A respectively), solubilised in a multiple chaotrope buffer and focused on 11 cm, pH4-7 immobilised pH gradient strips. Second dimension electrophoresis was performed on 10% polyacrylamide gels and proteins visualised with Sypro Ruby staining. Protein spots were matched and relative abundance was determined using PD-Quest software (v7.3.1,

Bio-Rad Laboratories). Using this approach more than 600 protein spots were visualised. Of these up to 20 proteins were significantly differentially expressed in pre-symptomatic women. Some of these protein spots are unique to pre-GDM (12 weeks' gestation) while others are also differentially expressed during overt disease (28 weeks' gestation). In some cases only specific isomers of a particular protein were differentially expressed (Figure 7).
The limitations of this methodology include (i) time consuming and sometimes unreliable matching of hundreds of spots in multiple gels, (ii) problems associated with spot normalisation, (iii) limited in-built statistical capacity of software to compare protein abundance, (iv) difficulty with excision of spots especially in small gel formats, and (v) the failure to reliably characterise proteins by MALDI-ToF mass spectrometry due to low protein abundance. This necessitates the need to scale-up methods for protein characterisation (orthogonal identification).

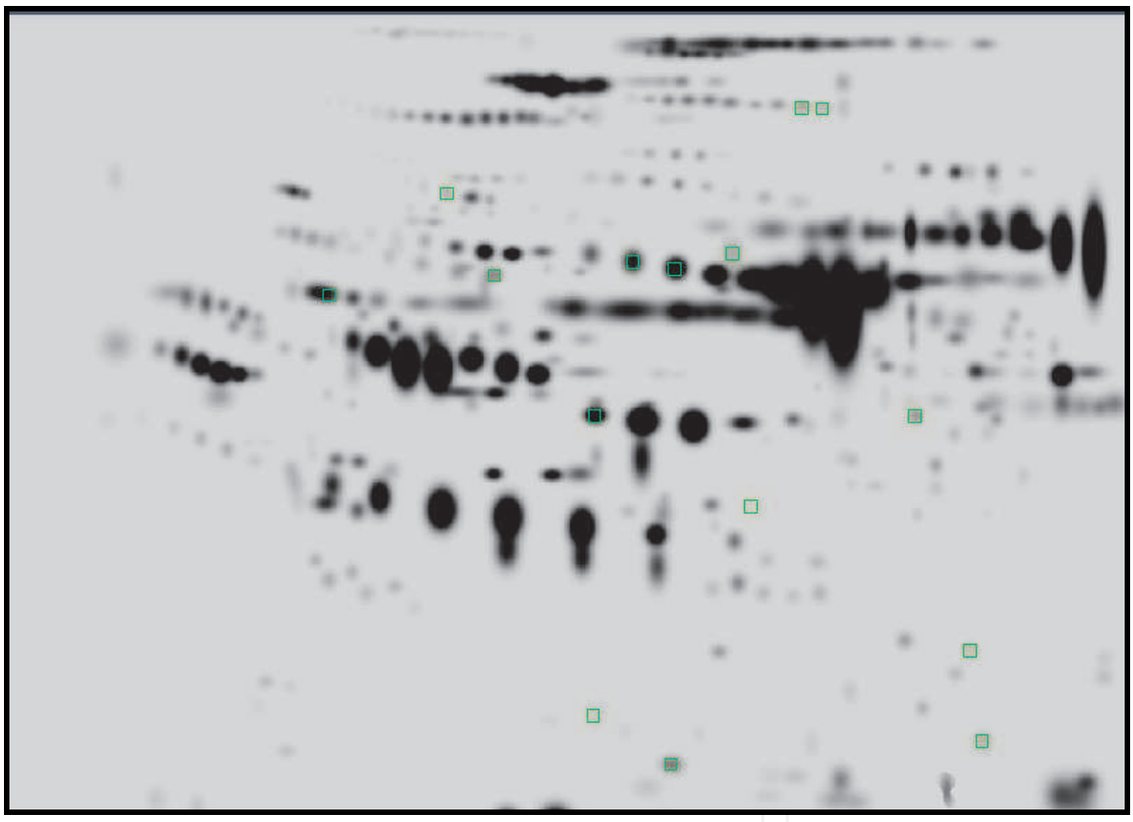

Fig. 7. 2D-PAGE Gaussian image of human plasma taken at approximately 12 weeks' gestation. Boxes indicate protein spots that were significantly differentially expressed in women who subsequently developed GDM compared to gestation-matched women who had a normal pregnancy.

4.4.2 2-Difference Gel Electrophoresis (2D-DIGE)

Some of the limitations of gel-based approaches have been overcome with the development of difference gel electrophoresis. This minimal labelling approach using fluorescent cyanine dyes increases throughput by reducing sample processing and both gel-to-gel and analytical variation by combining case and control samples into a single processing step, and by the

use of an internal standard for normalisation of data across gels (as described in 4.2 above). 2D-DIGE also delivers useful relative quantification of protein expression profiles where the dyes are purported to have sub-nanogram sensitivity and a linear response to protein concentrations of over five orders of magnitude. The dyes are also compatible with mass spectrometric analysis. With respect to analysing the plasma proteome, 2D-DIGE is still limited by the compositional complexity of plasma and similarly benefits from sample fractionation and the removal of high-abundance proteins.

In these experiments, we collected plasma from asymptomatic pregnant women at 12-18 weeks' gestation. Pregnancies were retrospectively classified as normal (n = 10), GDM (n=5), small-for-gestational age (n=5) and large-for-gestational age (n=5). Plasma was pooled for each group and depleted of high abundance proteins (top 14) using the IgY14/Supermix system (Sigma). Samples were then concentrated and labelled with Cy2, Cy3 and Cy5 CyDye DIGE fluors (GE Healthcare) and subjected to 2D-PAGE using 13 cm, pH4-11 immobilised pH gradient strips (1st dimension) followed by 12.5% gel electrophoresis (2nd dimension) as described in section 4.2 above. The analysis focused on spots with a greater than 2-fold difference in expression. Comparison between GDM and normal yielded 10 proteins that were down-regulated and 4 proteins that were up-regulated (some being different isoforms of the same protein). Proteins associated with small or large for gestation fetal complications were also identified long before disease onset.

4.5 Mass-spectrometry based quantitative profiling approaches

There are now a number of mass spectrometry (MS)-based, relative quantification approaches currently available including: (i) Multidimensional Chromatography (*e.g.* MudPIT); (ii) Stable Isotope Labelling (*e.g.* metabolic-SILAC, enzymatic-^{18}O labelling, chemical ICPL and iTRAQ labelling); (iii) MALDI-ToF Profiling (*e.g.* SELDI™ and ClinProt™) and (iv) Label Free Quantification (*e.g.* spectral counting). Of these, stable isotope labelling is becoming the method of choice for quantitative proteomics. Stable isotope labelling has the advantages of being more sensitive and reproducible than gel-based methods. These approaches utilise either a mass tag coding strategy (*e.g.* ICPL - Isotope Coded Protein Labelling, ICAT - Isotope Coded Affinity Tag or iTRAQ - isobaric Tags for Relative and Absolute Quantification) that allow pooling of samples to reduce technical variation. Label-free quantification is an approach that holds the promise of true MudPIT-type 'shotgun' quantification but has some disadvantages in sample preparation, cost and the challenge of normalizing the data so that accurate quantification can be done across multiple samples and multiple analyses. Comparison of protein expression profiles between samples is based upon two metrics: ion peak intensities of extracted peptide signals from LC/MS profiles or spectral counting (number of times peptide precursor is selected for fragmentation) of identified proteins after MS/MS analysis (Zhu, et al., 2010).

In addition to its analytical applications, mass spectrometry affords opportunities to identify signature profiles contained within biological samples for the purpose of classification. The application of mass spectrometry is a burgeoning area within the domain of diagnostic and predictive medicine. This approach now affords the opportunity to develop disease-specific patterns or profiles based upon the presence of specific peptides in a patient sample. MS-based protein profiling relies on the presence and spatial relationships between peptide peaks to facilitate the classification of biological samples into different categories (*e.g.*

normal and disease). Based upon the analysis of a training sample set (*e.g.* disease-free patients), pattern recognition software and multivariate modelling are employed to build peptide profiles or motifs that characterise a disease-free condition. Once established, such reference profiles may be used as a template to detect variance and thus deliver a diagnosis or predictive capacity. Two of the mass spectrometry-based profiling approaches we have utilised to identify peptides that may be informative of disease risk are described.

4.5.1 MALDI-ToF peptide profiling (ClinProt™ workflow)

Matrix-affinity peptide capture coupled with mass spectrometry is a discovery-based tool for comparing peptide mass fingerprints between individual or groups of samples. Numerous 'magnetic bead capture' chemistries are available including metal affinity (Cu, Fe), cationic exchange and hydrophobic reverse phase. In a prospective study of GDM, plasma samples were collected from pregnant women at 10-14 weeks' and 26-30 weeks' gestation and retrospectively allocated to gestation-matched GDM and normal groups. Samples were analysed after removal of high abundance proteins (Affi-gel Blue/Affi-gel Protein A) following a single fractionation process. Processing of samples with magnetic beads was performed in quadruplicate using a robotic workstation. Samples were mixed with Copper Immobilised Metal Affinity Chromatography beads (IMAC-Cu, Bruker Daltonics) in 96-well plates. Unbound peptides in the supernatant were aspirated and discarded while magnetic beads were washed and bound peptides eluded. Extracted samples were then processed by traditional MALD-ToF methods and raw spectral files were analysed with ClinProTools software (v2.2, Bruker Daltonics). Based upon the analysis of peptide profiles, we were able to identify disease-specific differentially-detected peptide ion peaks (Figure 8) and to develop multivariate classification models (Support Vector Machine and Genetic Mutation Models) using ClinProtTools software that discriminated between women who subsequently experienced a normal or GDM pregnancy. For example, using a genetic mutation classification model, 5 peptides were selected that had the ability to correctly classify 100% of women to a low risk group (i.e. those women who subsequently experienced a normal pregnancy). Furthermore, the model correctly classified greater than 93% of those women who subsequently experienced a GDM pregnancy. An independent and larger cohort is now required to validate these observations.

4.5.2 Stable isotope labelling (iTRAQ workflow)

The other mass-spectrometry based approach we have used to identify disease-specific proteins is iTRAQ. This labelling method is arguably the benchmark for relative protein quantification. One significant benefit is that it allows sample multiplexing and hence the ability to perform comparative analyses of up to eight different samples. For example, seven disease conditions or treatment groups and a pooled internal control could be processed in tandem, allowing identification and quantification relative to control.

The same plasma samples described in section 4.4.2 above were subjected to iTRAQ analysis. Each pooled sample group was initially depleted of high abundance plasma proteins using the IgY14/Supermix system (Sigma). Depleted samples were digested with trypsin and each was labelled with one of four different iTRAQ reagents (ABSciex, normal - 114, IUGR - 116, GDM - 118 and FM - 121). After labelling, all 4 labelled reaction mixtures were combined and applied to a strong cation exchange (SCX) cartridge. A single fraction

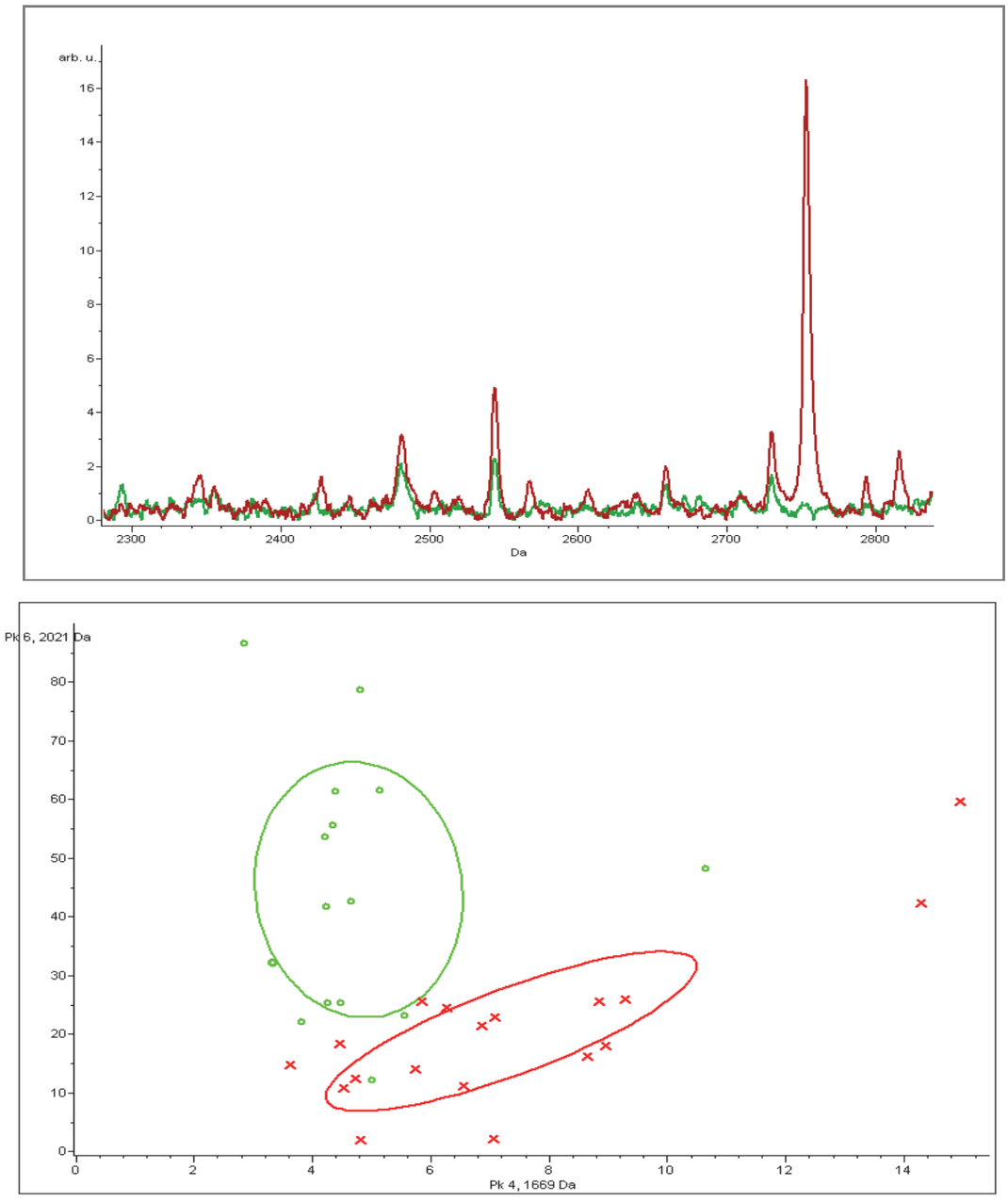

Fig. 8. MALDI-ToF peptide profile comparisons. **Top.** Example of the average peptide profiles over a limited spectral range (2300-2800 m/z) is presented to illustrate identified differences in peptide profiles between women with a normal pregnancy (red, n=19, 12 weeks) and women who subsequently developed GDM (green, n=16, 12 weeks). **Bottom.** A peptide peak cluster plot highlighting the potential for using differentially-expressed peptides to classify women into low- and high-risk groups for subsequent GDM. The plot presents the data (integrated area) of two peptide peaks (1669 vs 2021 m/z) observed in plasma obtained from women (12 weeks' gestation) who subsequently experienced a normal (red) or GDM pregnancy (green). Standard deviation envelopes are presented.

was eluted, collected, acidified and analysed by LC-MS/MS (QSTAR Elite, ABSciex) for simultaneous protein identification and peptide quantification (ProteinPilot ABSciex). Relative abundance of proteins in depleted plasma was determined by comparing the peak heights of reporter ions for each sample (m/z at 116, 118, 121) with those from the normal pregnancy (m/z at 114) pool. Using iTRAQ reagents and high resolution mass spectrometry, eight proteins that were differentially expressed (greater than 2-fold) in maternal plasma in association with GDM were unambiguously identified. Three of these proteins were up-regulated while five proteins were down-regulated. It is important to note that there was partial concordance between the identified proteins using iTRAQ and 2D-DIGE methods.

5. Conclusion

The methods we have described in this brief chapter provide proof-of-principle both technically and conceptually that biomarkers associated with disease can be reliably identified before the onset of overt disease. The challenge now remains to validate these findings in large independent cohorts to determine the predictive efficacy of these biomarkers. These emerging technologies and sophisticated modelling approaches now afford a realistic opportunity to develop and robustly evaluate the risk of asymptomatic early pregnant women developing complications of pregnancy such as GDM, IUGR, FM, PET and PTL. The development of such test(s) will provide data that better informs clinical decision-making and patient management that will not only directly benefit the immediate pregnancy, but will also help mitigate the longer-term ramifications of these conditions for both mother and baby.

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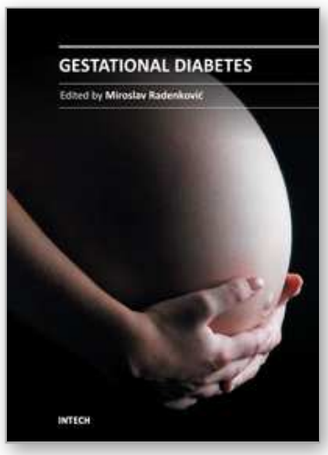

Gestational Diabetes

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Gestational diabetes mellitus is defined as hyperglycemia with onset or first recognition during pregnancy. The incidence of gestational diabetes is still increasing and this pathological condition has strong association with adverse pregnancy outcomes. Since gestational diabetes can have long-term pathological consequences for both mother and the child, it is important that it is promptly recognized and adequately managed. Treatment of gestational diabetes is aimed to maintain euglycemia and it should involve regular glucose monitoring, dietary modifications, life style changes, appropriate physical activity, and when necessary, pharmacotherapy. Adequate glycemic control throughout the pregnancy can notably reduce the occurrence of specific adverse perinatal and maternal outcomes. In a long-term prospect, in order to prevent development of diabetes later in life, as well to avoid associated complications, an adequate education on lifestyle modifications should start in pregnancy and continue postpartum.

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