Protein Profiling from Dried Serum Spots
Towards Risk Assessment of Preterm Newborns by Mass Spectrometry

Mass spectrometry-based proteome profiling provides a powerful approach to diagnose polygenic diseases like “Intrauterine Growth Restriction (IUGR)” due to its ability to characterize individual samples from newborns based on complex proteome signatures. Yet, one of the remaining challenges for making a mass spectrometry-based profiling assay of delicate samples from preterm newborns attractive for clinical use is to bridge the distance between the delivery room and the mass spectrometry laboratory. To overcome hitherto existing limitations we have developed a robust and reliable sample delivery system making use of a novel membrane-based serum / plasma storage device. Key step for success of “dried serum spot” proteome analysis is high-yield resolubilization of intact proteins and robust sample preparation for relative abundance determination by MALDI-ToF mass spectrometry, ultimately enabling clinical risk assessment based on molecular data.

Clinical Background
“Intrauterine-growth-restriction (IUGR)” is a pathological pregnancy condition in which the fetus does not reach its genetically given growth potential. IUGR affects about 3 to 8 % of pregnancies and is a risk factor for cardiovascular and metabolic diseases later in life. The mechanisms of “fetal programming” by now are unclear, but once noticeable disturbances in fetal blood composition towards an atherogenic phenotype in IUGR fetuses are found, biomarker and/or molecular profile-guided research avenues as well as optimized patient care strategies are opened. Of note, other preterm babies, categorized as “small for gestational age (SGA)”, are not associated with such developmental risks, yet at birth they are difficult to distinguish from IUGR cases by clinical means. Therefore, a robust molecular assay with only minimal invasive procedures at time of delivery was highly desired to ascertain IUGR diagnosis.

Advances in clinical sample preparation, storage, and shipment
Plasma or serum is prepared directly in the clinics immediately after blood
withdrawal from the umbilical vein of the newborn.

Blood samples are typically taken using Monovette syringes (Sarstedt, Nümbrecht, Germany). Serum/plasma is routinely prepared by centrifugation. A tiny volume of just 2 µl of serum/plasma can be deposited on the “plasma collection disc” that is mounted on the base sheet (Figure 1) of the Noviplex card (Shimadzu Europe, Duisburg, Germany).

Once deposited on the Noviplex card reservoir, serum/plasma proteins can be stored intact at room temperature for weeks. Moreover, Noviplex cards can be shipped by regular mail in a clean dry envelope without loss of quality of the adsorbed serum/plasma proteins. This novel serum/plasma handling device reduces costs for both, storage and shipping that otherwise would have to be done by freezing a serum/plasma sample at -78ºC, i.e. placing it in a freezer or keep it on dry ice. Both, storing samples at freezing temperatures and sample shipment on dry ice is demanding and can only be guaranteed by specialized birth clinics and advanced mail carriers, respectively. However, as of yet most hospitals are not prepared to store and ship frozen samples. Instead, keeping blood and other specimen and transporting such samples at room temperature is preferred routine. This typical clinical situation can now be met for analyzing intact proteins by using the Noviplex card system.

Having a reliable and robust method for serum/plasma storage and shipment at room temperature at hand places the key-step in the workflow for risk assessment of preterm newborns by MALDI ToF mass spectrometry of serum/plasma protein analysis towards facile and high-yield elution of intact proteins from the Noviplex card reservoir. To ensure high-quality analysis of intact proteins, we developed a protocol by which we were able to elute intact proteins from the “plasma collection disc” using acid-labile detergent-containing solutions (e.g. rapigest; Waters; Eschborn; Germany). To transfer resolublized serum/plasma proteins onto MALDI targets the protein solution is acidified (pH 3). While the acid-labile detergent is destroyed at this pH, solubilized serum/plasma proteins are now adsorbed onto
magnetic beads covered with hydrophobic surfaces (e.g. MagSi-proteomics C8 beads; MagnaMedics Diagnostics B.V.; Geleen; The Netherlands or Magnetic Beads MB-HIC 8; Bruker Daltonik; Bremen; Germany). Ultimately, intact serum/plasma proteins can be eluted from hydrophobic bead surfaces into salt-free and volatile buffer solutions that contain organic co-solvents from which they are finally deposited onto MALDI matrix preparations. Such prepared samples are now ready for linear MALDI-ToF MS profiling. Mass spectra after resolubilization of proteins from “dry serum spots” have been found of comparable quality to those from fresh-frozen serum/plasma samples.

**Mass spectrometric determination of proteome signatures**

Mass spectrometric profiling of severe forms of pregnancy complications has been successful with cord blood serum upon affinity enrichment of serum proteins [1-8]. Due to unsurpassed properties, such as low detection limits, multiplexing capability, rapidity, facile sample preparation, and because of unbiased protein detection likelihood which is independent from protein composition and structure, linear MALDI-ToF MS combines many advantageous features for blood protein profiling. A typical mass spectrum of such prepared samples shows ca. 50 to 70 ion signals (Figure 2) reflecting serum proteins of moderate abundance, whereas high abundant proteins such as serum albumin and immunoglobulins are depleted.

Hydrophobic surfaces on magnetic beads cause fractionation of the serum/plasma proteins and enrich for proteins, e.g. apolipoproteins, that interact with hydrophobic surfaces. Mass spectra of serum/plasma proteins are dominated by singly charged molecular ions of proteins and protein derivatives or post-translationally modified protein forms. Hence, they are readily identified through their molecular masses. Our mass spectrometry-based assay reliably determines relative abundances of serum/plasma proteins, termed in this case “IUGR signature”, which is used to identify IUGR babies and to differentiate them from other preterm, i.e. SGA newborns, upon molecular profiling of cord blood serum proteins with high confidence.

**Linear MALDI ToF MS in clinical routine**

As mentioned, MALDI-ToF MS is an automated technique that can detect hundreds of peptide and/or protein ion signals from clinically obtained body fluids in one measurement with good reproducibility and robustness. Hence, MALDI-ToF MS has enabled multiparametric analysis of the reservoir of proteins derived from semi-abundant endogenous circulating blood proteins. As a consequence, mass spectrometric measurements of the entire set of protein abundances in biological fluids allow the representation of proteome signatures for a particular pathology.
An advantage of mass spectrometry-based proteomics is that this approach does not require prior knowledge of the pathophysiological mechanisms underlying the condition of interest or even the identity of any single specific protein, as long as the signature of recorded signals remains differential. The robustness of all involved steps makes this assay attractive to clinics world-wide.

Due to the high speed and sensitivity, MALDI-ToF MS analyses can be realized within a few minutes of measurement time. The suitability of Shimadzu’s MALDI-ToF MS systems as a robust and reliable diagnostic tool is well demonstrated. Several of these analytical platforms are actually in use for the identification of microbes in the daily routine. Hence, MALDI ToF MS profiling of newborns with linear time-of-flight analyzers is envisioned to be accepted in a similar fashion in a maternity clinic in the future.

Conclusion
Proteome profiling of cord blood serum/plasma proteins enables personalized risk assessment of preterm babies by molecular differentiation between IUGR and controls (SGA). Our molecular profiling results were found to be independent from gestational age and birth weight of neonates, suggesting the profiling signature to be confirmatory to clinical surveillance with the potential to substitute clinical assessment of IUGR. Clearly, mass spectrometric profiling of intact serum proteins desorbed from “dried serum spots” enables reliable differentiation between IUGR and control samples; paving the way for future assessments in other diseases as well.

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