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1 Executive summary

Neonatal sepsis is caused by bacterial pathogens that enter the blood stream and the disease remains among the major causes of infant death worldwide. Amongst other, the high rates of morbidity and mortality are also associated with short-comings in current diagnostics management of patient sepsis.

The project ASCMicroPlat aimed at developing new test methods for rapid diagnosis of neonatal sepsis, which would provide clinicians with vital information at earlier stages to reduce mortality and morbidity.

Therefore a novel Polymerase-Chain-Reaction (PCR) assay including sample preparation (DNA extraction) was developed that enables identification of a whole panel of neonatal sepsis pathogens in human serum samples within 4 hours. The assay (denominated nucleic acid assay) was successfully tested for sensitivity and specificity to enable detection of low bacterial-loads and prevent unspecific signal generation in clinically relevant samples. The nucleic acid assay was successfully integrated on LabDisk test carriers to automate the entire process flow thereby massively reducing hands-on time for testing. Hereby, microfluidic modules for each process step were developed that enable automation by simple means of a rotation frequency protocol. The only manual handling steps remained initial loading of the sample and DNA extraction reagents on the LabDisk before starting the rotational protocol. The development now enables a true `sample-to-result´ system (concept depicted in Figure 1) with ease of handling that aims at processing by untrained personnel at the point-of-care. The LabDisk test proofed to be highly sensitive and demonstrated the detection of pathogens also for extremely low bacterial loads below 10 cfu / sample. For validation of the nucleic acid assay, neonatal patient samples were collected in clinics throughout the entire duration of the project. This enabled testing the LabDisk with neonatal sepsis positive and negative samples and comparison with the diagnostic gold standard of blood culture. The tests with these clinical samples yielded promising results and demonstrated the capability of the system to conduct panel analysis of a complete set of neonatal sepsis relevant pathogens. Although the LabDisk test showed perfect agreement with the blood culture in some cases, there were several cases where contamination of skin flora bacteria, likely on the test carrier, superimposed the clinical test results. The contamination issue was identified and strategies for prevention of contamination during production were derived.

In addition to the nucleic acid assay, manual immunoassay tests (ELISA, immuno-PCR) for quantification of biomarkers C-reactive protein (CRP) and Procalcitonin (PCT) were developed. These protein-based assays were integrated on separate LabDisk test carriers for automation with rotational protocols. Hereby an automated magnetic ELISA enabled quantification of the biomarker C-reactive protein in human serum within 25 minutes in the clinically relevant range. A proof-of-principle for an automated magnetic immuno-PCR was also demonstrated for the first time that in future could provide multiplexing for analysis of both biomarkers.

Besides the assay integration, ASCMicroPlat also implemented the production of the LabDisks based on an inventive foil-technology on industrial machines. The LabDisk test carriers are fabricated by micro-thermoforming of thin polymer foils and the technology was brought from prototyping to industrial scale machines towards mass fabrication. Also a new mobile device (LabDisk player) was developed featuring fluorescence detection, PCR-thermocycling and the possibility to run predefined centrifugal protocols. This enables processing of the LabDisk at the point-of-care.

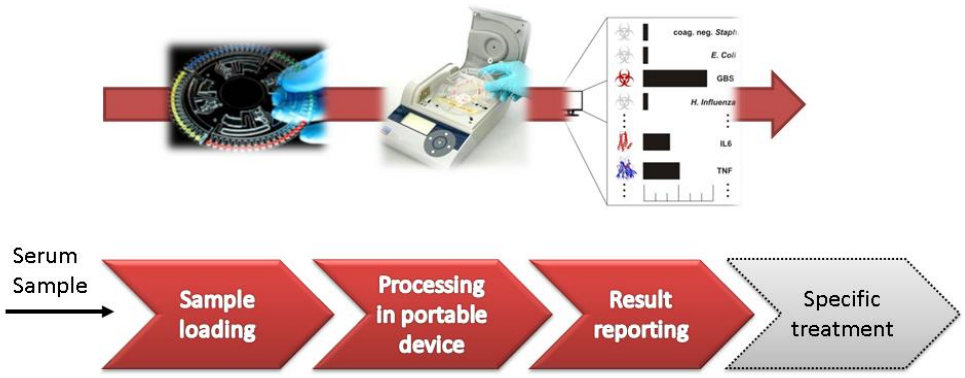

Figure 1 – Workflow of neonatal sepsis diagnostic in ASCMicroPlat

2 Description of project context and objectives

Neonatal sepsis is a potentially fatal disease characterized by a whole body inflammatory state coupled with the presence of a known or suspected infection. Mortality rates range from 1.5% in term babies to 40% in very low birth-weight babies, to up to 70% in the developing world[1,2]. Neonatal sepsis occurs when microorganism enter the bloodstream resulting in an irrepressible systemic cytokine mediated inflammatory response. This can lead to collapse of circulation and perfusion of vital organs and thus to patient death. The clinical signs of neonatal sepsis are in general nonspecific and are hard to distinguish from other medical condition such as respiratory distress syndrome or meningitis[3].

The time until initiation of antimicrobial treatment is a strong indicator of mortality rates. Administration of antibiotics within the first hour of documented hypotension is associated with a survival rate of 79.9% in adult sepsis. Each hour of delay in the administration of antibiotics was associated with an average decrease in survival of 7.6% in the following 6 hours[4]. The immune system of a neonate is relatively immature compared to adults and progression of neonatal sepsis disease can be very rapid. Sepsis is widely regarded as the most challenging problem in intensive care, where more than half of all severe cases are treated. This is a direct consequence of the complexity of the disease and its rapid progression. Current antibiotic management initiates therapy for neonates at high risk of developing sepsis. Also administration of antibiotics is initiated to all individuals that display infectious symptoms regardless if actually infected or not. This may lead to adverse drug effects as well as spreading of resistant bacterial strains.

The diagnosis of neonatal sepsis in clinics usually has two major purposes: Identification of the causative pathogenic microorganism and determination of the inflammatory immune response elicited in the patient in response to the respective microbial agent via biomarker quantification.

Quantification of diagnostic biomarkers such as C-reactive protein (CRP), interleukin-6 or tumor-necrosis-factor-alpha for determination of the inflammatory immune response is usually conducted with automated bulky, high complex workstations in the clinics or in central laboratories. CRP is an acute phase response protein and the most commonly used neonatal sepsis marker in Europe. In view of the complexity of sepsis, a combination of markers would be more effective for diagnostic purposes and the most promising of the additional markers is procalcitonin (PCT). Systemic PCT secretion is a component of the inflammatory response that appears to be relatively specific to systemic bacterial infections.

Current ´gold standard´ for investigation of sepsis and identification of pathogens is blood culture, a method that has been applied in clinics for several decades. Blood samples are cultured with specific media for growth of bacterial targets, taking 1-5 days for result reporting and identification of the causative agent. Relating to the rapidly progressing neonatal sepsis, the time to results remains one of the major drawbacks of blood culture. Also, blood culture is also associated with false negative (e.g. to previous antibiotic treatment) as well as false positive (e.g. due to contamination) results[5] that can lead to misuse of antibiotics and can worsen the prognosis of patients. To overcome the limitations of blood culture, molecular diagnostics were developed for rapid detection of specific gene sequences and identification of pathogens from neonate samples, such as whole blood, or blood serum. However, conventional molecular diagnostic tests, e.g. polymerase chain reaction (PCR) based assays are time consuming and apply complex protocols that require significant hands-on time, especially once sample pre-treatment of the sample matrix is considered as well. Therefore, usually trained personnel and high-level laboratory equipment is needed.

It is imperative to detect and diagnose neonatal sepsis as early as possible to ensure the best outcome for patients. Besides improvements in clinical specificity and sensitivity of new test methods, the state-of-the art does not provide the neonatal intensivist with a fast and accurate test, in particular at the point-of-care.

Within this context, the project ASCMicroPlat aimed at realization and clinical validation of a fully integrated and automated platform for the detection of neonatal sepsis biomarkers and a panel of sepsis-causing bacteria from serum samples. Centrifugal microfluidics[6] were applied to develop an easy-to-use diagnostic test that can be applied at the point-of-care. Biomarker quantification was conducted by a novel magnetic immuno-PCR approach or by automated enzyme-linked immunosorbent assay (ELISA). Pathogen identification was based on a PCR test method that includes samples preparation (DNA extraction). The tests were integrated on a rotating test carrier, the `LabDisk´ that can be processed on a portable processing device using a specific rotational protocol. The system provides several novelties:

- Fully automated and integrated analysis from serum ("sample-to-answer") where several unit operations are integrated on the LabDisk. Major reagents of the test are pre-stored on the LabDisk.
- Multiplexed detection for a complete panel analysis of sepsis causing bacterial agents within process duration below 4 hours.
- Scalable fabrication technology based on micro-thermoforming of polymer foils, in order to achieve low-cost and high-throughput production of diagnostic kits.
- Processing in a mobile, point-of-care processing device `LabDisk player`, that provides full control of defined rotation, acceleration and temperature and conducts optical signal readout.

As part of the project a clinical study was performed using clinical samples to validate the microfluidic LabDisk technology and to investigate the interaction between presence of microbial pathogens and the host response with a view to planning patient management more appropriately. The novel system was benchmarked against the gold standards currently used in the clinical laboratories.

In the respect of providing an easy-to-use, fast and conclusive test method, the project could have significant impact and market potential in clinics and also in laboratories. In contrast to current state-of-the-art of neonatal sepsis diagnostics, the platform could very well establish timely and especially specific treatment for patients. In the long term this may also reduce the spread of multiresistant strains emerging from the unspecific use of broad-spectrum antibiotics, which is considered as one of the major clinical and public health problem within the lifetime of most people living today[7]. In the

future, possibly the LabDisk principle could be applied in other cases where timely diagnostics is of the essence (pandemics, hospital acquired infections, etc.).

The interdisciplinary consortium included a university hospital, a research institute and three industrial companies (all of which are SMEs), who have together extensive experience in all relevant fields ranging from neonatal sepsis diagnostics using PCR based assays to polymer micro fabrication techniques and microfluidics. Thus, the full supply and value chain was covered by the consortium.

3 Potential impact, main dissemination activities and exploitation results of ASCMicroPlat

3.6 Potential impact

The global frequency of neonatal sepsis is 1-21 newborns per 1000 live births with mortality rates as high as 70 %, constituting a exigent global health issue. In Europe alone, with approximately 10 million live births per year the total number of confirmed cases per year would amount close to 100,000. Currently, diagnostic test for fast identification of the causative agents of neonatal sepsis are not available. The diagnostic gold standard blood culture can take up anything from 1 to 5 days for attaining a conclusive result for clinicians. In the context of neonatal sepsis where disease progression can be extremely fast due to an underdeveloped immune system of a newborn, rapid tests are imperative for a better prognosis of patients.

The final adaptation of an integrated LabDisk test for bacteria and biomarkers will undoubtedly prove useful for diagnosis of neonatal sepsis, reducing reporting hold ups for identification of pathogens and abnormal immune responses, and improving patient outcomes in this potentially fatal disease. The current management of suspected sepsis patients involves immediate administration of broad-spectrum antibiotics in conjunction with a battery of lengthy blood tests to confirm blood infection. The clinical application of ASCMicroPlat may not eradicate the clinician's 'blind' administration of broad-spectrum antibiotics, but it provides a timely, accurate and practicable solution to generate meaningful diagnostic information within a few hours of sample collection. In this way it can be implemented into treatment regimens to help clinicians select a better course of targeted antibiotic treatment for the specific infection, improving patient survival and outcomes.

Additionally, with a targeted, specific treatment of the patient, the unnecessary use of broad-spectrum antibiotics can be reduced. This in the long run also has the potential to reduce the evolution of new resistant strains which leads to more sustainable and efficient healthcare.

The developed immuno-PCR assay for quantification of CRP and PCT (once fully validated for PCT), will offer an improvement on current gold standard biomarker detection, which typically only includes CRP. PCT is currently at the forefront of sepsis research and is proving helpful in reducing the number of false positives in the diagnosis of clinical sepsis. Moreover, the immuno-PCR assay required $\leq 5\mu l$ of sample material. This is an important factor in the setting of neonatal diagnosis. The small volumes required during immuno-PCR would undoubtedly prove very useful for sepsis diagnostics in tiny preterm babies, where the available blood volumes for diagnostic testing are limited. The proof-of-principle has also been demonstrated for quantification of biomarkers on the LabDisk.

The number of molecular diagnostic assays in clinical bacteriology has increased steadily during the last decade. However, conventional microbiology will not be replaced in the immediate future, but multiparameter identification of the most important pathogens using array-based detection technologies or rapid real-time PCR-based assays are becoming common in today's laboratories. Although a small part of the In-Vitro Diagnostics (IVD) market, molecular diagnostics market is one

of the main growth drivers of the IVD industry and currently the fastest growing of all IVD market segments. This growth is driven by clinical, social and economic pressures coupled with new technologies and IT capabilities. The ultimate aim for many molecular diagnostics companies is to develop multiparameter diagnostic assays suitable for point-of-care testing. This perfectly falls in line with the developed ASCMicroPlat LabDisk nucleic acid assay for neonatal sepsis diagnostics, yet also for other applications. The platform could be easily adapted to other applications (e.g. detection of resistance markers) with much less costs and effort. The major modules (e.g. DNA extraction) were implemented and only changes in the biochemistry would be required. This opens up opportunities for the European diagnostic industry for applications where automation and multiparameter analysis constitute major parameters for successful product development.

The centrifugal microfluidic LabDisk platform is associated with Lab-on-a-Chip and so called micro-total analysis systems (μTAS). So far, μTAS have failed to achieve a significant market impact on the huge diagnostic market. Centrifugal microfluidics is considered by several companies as a promising candidate though for truly integrated point-of-care diagnostics. At present all available commercial systems in that field either suffer from a lack of available assays (currently mostly clinical chemistry) or a small degree of integration and large foot print. In that respect, ASCMicroPlat provides several novelties with significant advancement of the state-of-the-art. It combines a high degree of integration for complex biochemical assays with small foot print for automated point-of-care analysis. It can provide European research institutions and SMEs with a platform that addresses the current shortcomings of μTAS for product development of diagnostic lab-on-a-chip applications.

The interdisciplinary ASCMicroPlat project integrated microfluidic technology, production technology, processing device technology and biochemistry into one diagnostic platform. In ASCMicroPlat, the complete value chain from development of production technologies and microfluidic design to clinical validation was covered. For production of the LabDisk test carriers, an existing blister packaging production technology was used as basis to develop a production technology for the microfluidic LabDisk disposables. This included not only the development of new tools for thermoforming but also for sealing and punching and the evaluation of flexibility in the manufacturing of different fluidic LabDisk patterns. The technology will be used in follow-up projects and may incorporate the production technology of choice for LabDisk products. The field of applications for the existing blister thermoforming technology was broadened, which may open up market opportunities for thermoforming companies in the diagnostic market.

3.7 Exploitations of results

The technical project results could be a starting point for a commercial development of a point-of-care diagnostic system for neonatal sepsis. The proof-of-principle was generated. Currently, there are expert interviews with key opinion leaders in Germany ongoing to assess the potential of such a product. Two important outcomes to this point are:

1) The number of neonatal sepsis cases are more than one order of magnitude lower than adult sepsis with about 160 deaths daily in Germany alone. An extension of the product to adult sepsis could be highly beneficial. On the other hand, adult sepsis has a largely increased list of target pathogens including fungi which would make a product development challenging.

2) The clinical process for sepsis treatment foresees an immediate administration of broad spectrum antibiotics. A diagnostic tool which could influence the initial response would have to have a time-to-result of preferrably less than 30 minutes. This is almost impossible with PCR. So an alternative based on other technologies such as Loop-mediated isothermal amplification (LAMP) or Recombinase

Polymerase Amplification (RPA) could lead to higher impact in the clinic. The current prototype requires about 4 hours for an automated microbiological determination. This is still useful for a fast de-escalation of the broad-spectrum treatment to a more specific and efficient treatment targeted to the pathogen.

A market study by HSG-IMIT to assess the requirements for a potential product is currently ongoing. If the outcome shows a clear clinical and economic benefit, this could trigger the involvement of venture capital and a spin-off company.