

VIRUMILK

Biosensor for CMV Detection in Breast Milk from Lactating Women of Preterm Infants Less than 33 Weeks

S. Py¹, B. Wacogne^{1,2}, L. Pazart¹, A. Coaquette³, W. Boireau², G. Herbein^{3,4} and G. Thiriez⁵

¹INSERM-CIC 1431, Besançon University Hospital, Besançon, France

²Institute FEMTO-ST, University of Franche-Comté, Besançon, France

³Laboratory of Virology, Besançon University Hospital, Besançon, France

⁴UPRES EA4266, SFR FED 4234, Pathogens and Inflammation Laboratory, Department of Virology, University of Franche-Comté, Besançon, France

⁵Department of Neonatal Medicine, Besançon University Hospital, Besançon, France

Keywords: Cytomegalovirus, Screening, Biochips, Preterm Infants, Breastfeeding.

Abstract: Cytomegalovirus (CMV) is the leading cause of neonatal viral infection and can have a significant impact on the neurosensory development of newborns and especially preterm infants. While congenital CMV infection affects about 2-5% of very preterm infants, the risk of postnatal infection, particularly through breast milk, is much higher in this population (20%). However, infection could be considerably reduced by an early and fast screening of breast milk. Indeed, a treatment (freezing or pasteurization) of contaminated breast milk only could eliminate the virus. The idea of this position paper is that breast milk screening would help defining an appropriate and personalized feeding strategy. We explain how to develop a CMV biosensor to detect the virus in milk. It employs specific CMV antibodies grafted on a biochip surface to capture viral material and additional detection antibodies in a “sandwich assay” type system. Detection is based on optical absorption. It will be tested with a device developed previously. However, preliminary results obtained in ELISA technique with breast milk and homemade antibodies are presented in this position paper. The ulterior motive of this work is the fabrication of an autonomous and automated device that will be experimented in subsequent diagnosis strategy trial.

1 CONTEXT

Cytomegalovirus (CMV), member of the sub-family of β -herpesvirus, is only present in humans and 40 to 90% of the world population is infected. This virus, rarely dangerous for immune-competent person, is a real threat for immune-depressed people, as for example, organ transplanted or pregnant women. Following a primo-infection, CMV diffuses in the whole body and alternates latency and re-activation periods. CMV is the most frequent etiologic agent of congenital and postnatal infection of newborns and can have a significant impact on the neurosensory development of newborns and especially preterm infants (Hayashi et al. 2011). Postnatal transmission of CMV can occur during blood transfusions, while absorbing infected biological liquids like mother cervical secretions during delivery or during breastfeeding. CMV excretion in breast milk is the main source of postnatal infection.

1.1 Postnatal CMV Infection *via* Breast Milk

Breastfeeding is now clearly recognized as being superior to artificial feeding for the future of newborns and, more particularly of preterm infants. Indeed, preterm newborns are more vulnerable to digestive and neurological problems. Breast milk is better accepted and limits the risk of feared complications like necrotising enterocolitis. Its incomparable time-varying composition offers the best chances of cognitive evolution on the long term. However, breastfeeding plays a major role in the epidemiology of transmission and postnatal CMV infection. It is now well established that CMV is excreted in milk from seropositive lactating mothers, the majority of whom are asymptomatic, especially due to a reactivation of the virus. Excretion can start since the first post-partum week with a low viral

charge and reaches a maximum value 4 to 8 weeks after birth and declines steadily thereafter. Mother-to-child transmission generally occurs during the period where the virus level (DNA or viral particles) in milk is about its maximum (Hamprecht et al. 2003; Hamprecht et al. 2008).

A review paper (Kurath et al. 2010) related to CMV transmission by breastfeeding in preterm infants shows that 87% (median value) of mothers whose CMV serology is positive for IgG (CMV⁺ mothers) excrete CMV in their milk. Among the children, about 20% (median value) are CMV positive by PCR or ELISA technique for IgM and IgG (CMV⁺ newborns) and the contamination risk increases with lactating duration (figure 1). Usually, for term babies, symptomatic infection does not exist because mothers start to transmit their antibodies during the 29th pregnancy week. Conversely, for preterm infants, the weak transmission of mother antibodies and the non-mature immune system increases the risk of symptomatic CMV infection. A small birth weight (<1.5 kg) and an early postnatal transmission constitute risk factors of symptomatic infection (Lombardi et al. 2012). Recent studies showed that postnatal CMV infection in preterm infants can lead to serious clinical consequences like respiratory distress, neutropenia, thrombocytopenia, hepatomegaly and septic syndromes, and can lead to death in rare cases (Lanzieri et al. 2013; Hamele et al. 2010; Hamprecht et al. 2008). According to Kurath *et al.*, 3.7% (median value) of the positive children develop CMV related symptomatic clinical complication with, for 0.7% (median value) of them, appearance of a severe septic syndrome (Kurath et al. 2010). Although the long-term follow-up of the neurosensory development of congenitally infected preterm infants is well documented, very few studies concern postnatal infected preterm infants and results obtained are generally controversial, in particular because of the reduce number of infants in the cohorts (Kurath et al. 2010; Bevot et al. 2012; Goelz et al. 2013).

Up to now, there is no consensus between learned societies of pediatrics concerning the attitude to be adopted and actions to be started to prevent CMV infection *via* breast milk in preterm infants less than 33 weeks.

Today, almost no national recommendations on the manipulation of the breast milk of CMV positive mothers are proposed. However, methods exist to treat breast milk and consist in heating or freezing the milk (Forsgren 2004; Hamprecht et al. 2004). Systematic pasteurization of human milk is not done

because it alters the immune components of milk which are particularly precious for very preterm infants (Chang et al. 2013). Freezing at -20 °C does not completely destroy the virus, but better preserves biological properties of human milk (Buxmann et al. 2009).

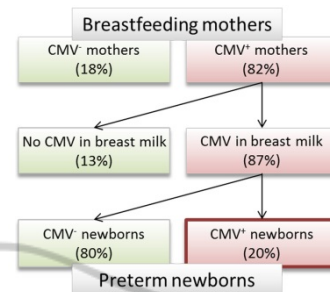


Figure 1: Simplified epidemiology of CMV infection in breastfed preterm newborns (Kurath, 2010).

For these reasons, neonatologists are squeezed in their clinical practice between the potential risk to transmit infection when breast milk is picked up to be given to the infant, and the risk to favor digestive complications or not to give the better chances of neurologic development if the breast milk is not used. In order to address this issue, the ideal solution would be to differentiate “at risk” and “non at risk” situations. In fact, treating the milk of only the “at risk” population of CMV contamination *via* the breast milk would be extremely more satisfactory than a systematic attitude.

1.2 CMV Screening Tools in Breast Milk

Most of the time in France, the presence of CMV in lactating mother’s milk is not screened and milk does not normally undergo specific pre-treatments in a breast milk bank. However, an early detection of CMV in breast milk is feasible. Studies concerning CMV transmission *via* breast milk are based on detecting the viral DNA and/or the infectious virus. Qualitative or quantitative techniques used to detect viral DNA are PCR, RT-PCR or nested PCR (Hamprecht et al. 2008). These techniques are expensive, time consuming and require the milk to be previously prepared in 2 or 3 fractions (lipidic, whey and cellular fractions) using various centrifugations. Conventional cell culture gives a result only several days after sampling and often fails because of the native mother milk cytotoxicity. This greatly reduces the detection sensitivity of the infectious virus and further requires fractionation of mother milk. Therefore, PCR and cell culture are not

adapted to rapid and early CMV detection in breast milk.

In this position paper, we show early results concerning CMV detection in breast milk and we propose the development of a CMV screening biosensor. The idea presented in this position paper is to prevent a postnatal CMV contamination for a majority of preterm newborns by using an adapted milk treatment. To do this, it is necessary to detect CMV in this liquid with a simple, fast and low-cost system.

VIRUMILK project clearly consists of bridging a clinical problem to an innovative technological solution that opens up perspectives of medical advances. It relies on previous biotechnological research studies realized during the MEDICALIP project (Mangeat et al. 2011) whose main scientific achievements are:

- Homemade production of anti-CMV antibodies, called PAbH (polyclonal human antibodies) able to trap viral material at the surface of a biosensor.
- Grafting of these antibodies on the gold surface of the biosensor while keeping their CMV capture properties. Indeed, the use of commercially available antibodies was disappointing because they lost (partially or completely) their capture properties when grafted onto the biochip. Grafting methods as well as chemical environment can, in some cases, reduce their capture efficiency. Success in producing homemade and efficient antibodies in the virology unit of Besançon University Hospital allowed continuing the project.
- Set-up of methods and opto-fluidic devices for reactive and sample flow control as well as the optical detection of the test results.

2 THE BIOSENSOR

2.1 General Concept

The technique we proposed to detect CMV is based on antigen/antibody recognition. The biosensor consists of a gold coated biochip grafted with human polyclonal anti-CMV antibodies. CMV potentially present in the breast milk sample is captured by these antibodies. CMV detection uses a specific secondary antibody coupled to horseradish peroxidase (HRP) enzyme which subsequently recognizes the captured virus. After addition of a substrate of this enzyme, a colorimetric reaction occurs and allows transforming the substrate to a blue product. When reaction is stopped, the blue product turns into yellow. Then, the optical reading

relies on an absorbance measurement around 450 nm. A schematic representation of the biosensor is given in figure 2.

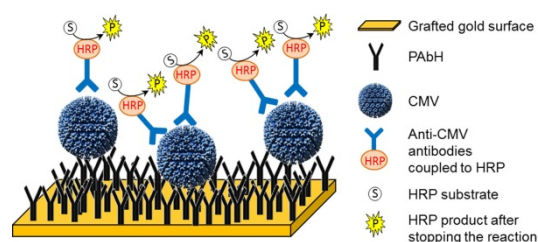


Figure 2: The CMV biosensor.

2.2 Immunosensor Engineering

Design and production of homemade chips compatible with Surface Plasmon Resonance imaging (SPRi) have been performed as previously described with the help of the MIMENTO technological platform, Besançon, France (Remy-Martin et al. 2012). Gold coated biochips are made using magnetron sputtering. Gold presents the advantage of enhancing the sensitivity, reducing the detection time and improving the specificity of the interaction under study.

A strategy of functionalization and grafting of homemade polyclonal anti-CMV antibodies was optimized to guarantee an optimum anti-CMV antibodies surface density and also to reduce non-specific interactions onto the biochip surface. This strategy is based on thiol chemistry and is well managed by the CLIPP platform (Clinical and Innovation Proteomic Platform), Besançon, France (Bruchard et al. 2013). Chips are incubated in a solution of 11-mercapto-1-undecanol/16-mercaptohexadecanoic acid (97/3 by mole) (Sigma-Aldrich) overnight at room temperature (RT). Surfaces were rinsed by ethanol and ultra-pure water. Then, 200 μ l of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at 200 mM/N-hydroxysulfosuccinimide (sulfo-NHS) at 50 mM (Amine Coupling Kit from Biacore AB, Uppsala, Sweden) are added on each surface and incubated during 30 min at RT. This step is necessary to activate C11/C16 layer. Surfaces were rinsed by ultra-pure water and different batches of PAbH directed against viral proteins from various strains (AD169 and 3 clinical strains) were spotted (0.27 μ l/spot) on the chips during 30 min at RT under sonication in a humid chamber. To ensure optimal grafting of the PAbH, antibodies are diluted in an acetate buffer at 100 μ g/mL, pH 5. Then surfaces were rinsed by ultra-pure water and a

blocking agent (Rat Serum Albumine 40 µg/mL, pH 5.2) was used to passivate the surface by incubation at RT for 30 min. Surfaces are finally rinsed with water and C11/C16 layer is deactivated using 200 µl Ethanolamine-HCl (1 M pH 8.5) during 30 min à RT. After a last rinsing by water, biochips were ready for use in SPRi experiments.

2.3 CMV Capture and Detection

Control of the grafting of homemade antibodies onto the chip surface and CMV capture were performed in a SPRi-PlexII imager (Horiba Scientific, France) equipped with an 810 nm wavelength LED and a CCD camera. Experiments were carried out at 25°C, in phosphate buffered saline (PBS) 1X. The flow rate in the chamber was 20 µl/min. CMV was commercial antigen from AD169 strain (ETI-CYTOK-M reverse plus, Diasorin). After injection (volume of 200 µl) the biochip surface was rinsed for 1 min with detergent (n-Octyl-beta-D-glucopyranoside, 40 mM) to remove unbound ligands.

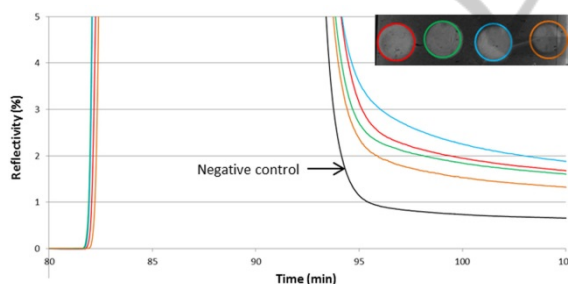


Figure 3: Sensorgram and contrast plasmon image of spots obtained with the SPRi-PlexII after injection of commercial CMV on grafted PAbH (4 spots and curves correspond to different batches of PAbH).

As seen in figure 3, a signal is observed on the four spots grafted with different batches of PAbH whereas no significant signal is observed on the non-grafted spots. The interaction is therefore specific and shows the absence of undesired non-specific binding and/or adsorption on the surface. These results are consistent with those obtained by ELISA (Enzyme-Like Immunosorbent Assays) experiments using the same batches of PAbH and the same viral material (data not shown).

In order to test whether or not similar results can be obtained with CMV positive breast milk samples, a direct sandwich ELISA experiment was performed. One CMV positive breast milk sample (volume of 5 mL) was centrifuged at 400 g during 10 min at RT in order to separate milk into 3

fractions: the cell fraction, whey and the lipidic portion which was discarded. The CMV positivity was assessed by PCR analysis and cell culture. In the meantime, remaining volumes (slightly less than 1 ml) of cell and whey fractions were stored at -80°C. When positivity is confirmed, an ELISA experiment was conducted as follows. Fetal Calf Serum (FCS) at a concentration of 1 µg/µL or two batches of PAbH (produce from two different clinical strains) at a concentration of 3.75 µg/mL, 7.5 µg/mL or 15 µg/mL in 100 µL of carbonate/bicarbonate buffer were coated overnight at 4°C in 96 wells microplates. The day after, a rinsing of wells with PBS 1X followed by a saturation step of the surface with Bovine Serum Albumin (BSA) 3% (200 µl/well) during 1 h at RT was performed. A mixture containing commercial antigen or the cell fraction (diluted at 2.5%, 7%, 22%, 50% or 66% in PBS 1X) or whey (diluted at 2%, 7%, 20%, 50% or 61% in PBS 1X) and an anti-CMV antibody conjugated to HRP diluted at 1/70 (ETI-CYTOK-M reverse plus, Diasorin) was added (100 µL/well) and incubated during 1 h at RT. Five washing (200 µL/well) were realised with a wash solution composed of PBS-Tween (ETI-CYTOK-M reverse plus, Diasorin) and the HRP substrate (hydrogen peroxide and tetramethylbenzidine) was incubated (100 µL/well) during 30 min at obscurity and at RT. The reaction was stopped with 0.2 N sulphuric acid. Absorbance of the yellow solution obtained was immediately measured around 450/630 nm. Results are presented in the histograms in figure 4.

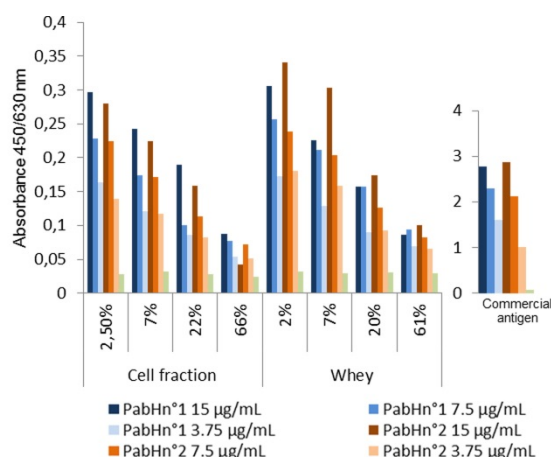


Figure 4: Absorbance values obtained by ELISA experiment using two batches of PAbH and different CMV sources (commercial antigen, breast milk cell fraction and milk whey).

A positive control is represented by the incubation with commercial antigen in which high absorbance values are obtained. Decay is observed with the increasing dilution of capture antibodies. Concerning the cell fraction and whey, values are lower overall and decay is observed with the increasing dilution of capture antibodies but also with the increasing dilution of milk fraction. As expected, incubation of CMV with FCS shows a very low signal.

SPRi and ELISA results show the capacity to capture CMV with homemade antibodies on different surfaces. Moreover, viral particles contained in a breast milk sample can also be captured and detected in ELISA experiment.

3 A POSSIBLE DEVICE

In the frame of another research program called SmartTransfuser (Charrière et al. 2012), a laboratory prototype had been fabricated (figure 5). It consists of a fluidic system containing a biochip inserted into a cartridge. Syringes contain reagents which are driven on biochips surfaces by fluidics arrangements. The system allows controlling the fluid flows and interaction durations. Immunocapture and immunodetection of CMV are performed on a biochip. In parallel, a second biochip is used as negative control.

Preliminary results were obtained with an experimental protocol approaching as much as possible the conditions used in ELISA experiments (concentrations and incubation times of reagents are the same). Homemade functionalised biochips were designed and produced as described above and PAbH were grafted following the same protocol used for SPRi experiments. BSA was first introduced manually to the biochip followed by the mixture of commercial antigen and detection antibody conjugated to HRP at a flow rate of 100 $\mu\text{L}/\text{min}$ during 2 min. A washing was realised by 500 μL of wash solution at a flow rate of 250 $\mu\text{L}/\text{min}$. Finally, substrate was added at a flow rate of 100 $\mu\text{L}/\text{min}$ and incubated at obscurity. The excess of solution contained at the outlet of the cartridge was discarded and 100 μL of blue product was recovered in a tube and directly turned in yellow by addition of the stop solution. Negative control consists of a biochip incubated with all the reagents except the mixture antigen/antibody which is replaced by only antibody diluted at 1/70 in PBS 1X. Optical reading is performed with a spectrophotometer by measurement about 450/630 nm.

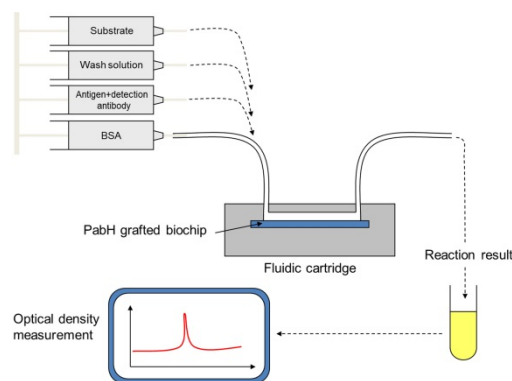


Figure 5: General concept of the CMV detection in an already available device (SmartTransfuser project). A fluidic system ensures the flow of different reagents (BSA, antigen, antibody detection coupled to HRP, wash solution and HRP substrate) on the biochip of interest.

Absorbance values obtained in first experiments were lower in comparison with ELISA results but a difference in positive and negative biochips was indeed present (factor of 5). Additional experiments are necessary to really prove the possibility to detect CMV in such a laboratory model and particularly with breast milk samples. However, this preliminary result shows that laboratory SPR and SPRi technique can be transposed to a more bulk device and that fluid flow condition in this fluidic cartridge allows specific detection of CMV.

When finalized, the biosensor validated with breast milk will be integrated into a built-in system which will be of simple use (presence or not of the virus indicated with red/green LEDs). It will allow controlling fluid flows by means of an automated management of micro-fluidic and also timing of different biochemical interactions in a similar way to what we already presented before (Mangeat et al. 2011). It will include the optical reading system of the test result which is based on an absorbance measurement. Finally, it will include a human/machine interface to use the device by a non-expert user. The finalized biodevice will then allow rapid and simplified CMV detection in breast milk of lactating mothers of preterm infants. Contact with a company was established to develop the device, but for reasons of intellectual property it is still too early to assess the cost of producing the proposed biosensor in a larger scale.

4 CONCLUSIONS

Although the risk of CMV congenital infection is

relatively low (prevalence of about 1%), the risk of postnatal contamination, in particular *via* breast milk, can be dramatic for preterm infants. Currently, the question is: should we favour a better development and take the risk of using contaminated breast milk, or should we use treated milk, even when the CMV infection is low enough to be considered safe?

To address this problem, and in the current context of breastfeeding promotion, we propose to develop a CMV biosensor based on sandwich ELISA principle. First SPRi, ELISA and assays in a laboratory model lead us to assume that a biochip CMV capture and detection is possible. However, more tests, especially with positive and negative breast milk samples, are required to validate our biosensor.

This position paper presents studies that have just started, but we think it is possible to set-up an easy to use and rapid "point-of-care" device to detect CMV in milk of lactating mothers of preterm infants. Therefore, a third answer can be proposed to the above mentioned question. The idea is to screen CMV on a routine basis and to define a personalized feeding strategy for "at risk" population only. Without such a rapid CMV test, this third solution may never exist.

ACKNOWLEDGEMENTS

The authors would like to thank the French Agence Nationale de la Recherche, INSERM and the DGOS. This work is developed in the frame of the Biom'@x transversal axis at FEMTO-ST.

REFERENCES

- Bevot, A., et al., 2012. Long-Term Outcome in Preterm Children with Human Cytomegalovirus Infection Transmitted via Breast Milk. *Acta Paediatrica*, 101 (4), 167-172.
- Bruchard, M., et al., 2013. Chemotherapy-Triggered Cathepsin B Release in Myeloid-Derived Suppressor Cells Activates the Nlrp3 Inflammasome and Promotes Tumor Growth. *Nature Medicine*, 19 (1), 57-64.
- Buxmann, H., et al., 2009. Incidence and Clinical Outcome of Cytomegalovirus Transmission via Breast Milk in Preterm Infants ≤ 31 Weeks. *Acta Paediatrica*, 98 (2), 270-276.
- Chang, J.C., et al., 2013. Influence of Prolonged Storage Process, Pasteurization, and Heat Treatment on Biologically-Active Human Milk Proteins. *Pediatrics and Neonatology*, 54 (6), 360-366.
- Charrière, K., et al., 2012. SmarTTransfuser - A Biochip System for the Final ABO Compatibility Test, in: *SciTePress - Science and Technology Publications*, Vilamoura, Portugal, 257-262.
- Forsgren, M., 2004. Cytomegalovirus in Breast Milk: Reassessment of Pasteurization and Freeze-Thawing. *Pediatric Research*, 56 (4), 526-528.
- Goelz, R., et al., 2013. Long-Term Cognitive and Neurological Outcome of Preterm Infants with Postnatally Acquired CMV Infection through Breast Milk. *Archives of Disease in Childhood. Fetal and Neonatal Edition*, 98 (5), 430-433.
- Hamele, M., et al., 2010. Severe Morbidity and Mortality with Breast Milk Associated Cytomegalovirus Infection. *The Pediatric Infectious Disease Journal*, 29 (1), 84-86.
- Hamprecht, K., et al., 2008. Cytomegalovirus transmission to preterm infants during lactation. *Journal of Clinical Virology*, 41 (3), 198-205.
- Hamprecht, K., et al., 2004. Cytomegalovirus (CMV) Inactivation in Breast Milk: Reassessment of Pasteurization and Freeze-Thawing. *Pediatric Research*, 56 (4), 529-535.
- Hamprecht, K., et al., 2003. Rapid detection and quantification of cell free cytomegalovirus by a high-speed centrifugation-based microculture assay: comparison to longitudinally analyzed viral DNA load and pp67 late transcript during lactation. *Journal of Clinical Virology*, 28 (3), 303-316.
- Hayashi, S., et al., 2011. Transmission of cytomegalovirus via breast milk in extremely premature infants. *J Perinatol*, 31 (6), 440-445.
- Kurath, S., et al., 2010. Transmission of Cytomegalovirus via Breast Milk to the Prematurely Born Infant: A Systematic Review. *Clinical Microbiology and Infection*, 16 (8), 1172-1178.
- Lanzieri, T., et al., 2013. Breast Milk-Acquired Cytomegalovirus Infection and Disease in VLBW and Premature Infants. *Pediatrics*, 131 (6), 1937-1945.
- Lombardi, G., et al., 2012. Breast Milk-Acquired Cytomegalovirus Infection in Very Low Birth Weight Infants. *The Journal of Maternal-Fetal & Neonatal Medicine*, 25 Suppl 3, 57-62.
- Mangeat, T., et al., 2011. Detection of the cytomegalovirus: a mobile device and a disposable cartridge for detection at the patient's bed. Conference, Biodevices 2011, 26-29 January, Rome, Italy, In Proceedings of the International Conference on Biomedical Electronics and Devices, 103-108.
- Remy-Martin, F., et al., 2012. Surface Plasmon Resonance Imaging in Arrays Coupled with Mass Spectrometry (SUPRA-MS): Proof of Concept of on-Chip Characterization of a Potential Breast Cancer Marker in Human Plasma. *Analytical and Bioanalytical Chemistry*, 404 (2): 423-432.