PROJECT REVIEW

"Biosensor-based detection of hepcidin as a new biomarker of erythropoiesis stimulators abuse"

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The reliable determination of human recombinant erythropoietin (rhEpo) abuse to enhance the athletic performance is an important problem for sport and represents a continuous challenge for investigators involved in anti-doping. The analytical tests used to detect rhEpo in urines are often inadequate and suffer from many interfering factors. Hepcidin, a liver-derived peptide which is the major regulator of body iron metabolism, is an accurate indicator of changes in blood levels of Epo. Indeed, Epo administration in humans caused a marked reduction in urinary and circulating hepcidin.

Therefore, the aim of the present project is to verify whether the determination of hepcidin may represent a valid alternative method to detect an inappropriate use of rhEpo for doping purposes. Such an indirect approach could be also useful for the detection of the use of last generation pharmacological agents such as continuous erythropoietin receptor activators (CERA). The detection of hepcidin in urines and serum has been performed so far by means of immunological assays or SELDI-TOF-MS techniques, which present problems related to quantitative determination and requirement for expensive equipment and skilled personnel, respectively.

Our challenge is to provide an innovative analytical process for the evaluation of the presence of erythropoiesis stimulators abuse. The heart of the project is a new multiscreening affinity sensing platform and innovative low cost devices, for the detection of biomarkers such as hepcidin and for the doping substance itself (erythropoietin).

In this context, a flexible platform, consisting of a biochip coupled to a label free technology for simultaneous measurements in short time could represent an innovative approach for selectively detecting biomarkers of erythropoiesis stimulators abuse. The project will also consider the possibility of developing an innovative, electrochemical biosensor which can be also used in a low density array format for the simultaneous detection of several doping markers.

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Results and Conclusions

The aim of this project, which was funded as one year pilot project by WADA, was to evaluate the role of hepcidin, the major regulator of iron homeostasis, as an alternative or complementary marker to detect the abuse of rHuEpo for doping. During this period of time, we used a multidisciplinary approach combining biochemistry, molecular biology, analytical chemistry, etc., in different experimental models, and we obtained several interesting and promising results.

These results indicate that affinity-based biosensing can be an innovative approach to hepcidin detection, although the assays have to be improved in order to compete with more established analytical methods. A preliminary evaluation indicates that the developed systems, based on an antibody and a biomimetic receptor specific for hepcidin-25, have several advantages, *i.e.* they allow the direct determination of hepcidin, are reproducible, label-free, easy to achieve, quite cheap, and fast. In fact, both systems allowed hepcidin quantification in very short time (15 min), if compared to previous methods requiring several hours.

Anti-hepcidin resulted more performing than HBD: it was sensitive and reproducible in the range of physiological hepcidin levels. On the other hand, the antibody showed, compared with HBD, a short life time once immobilized on the biochip. However, this problem could be prevented by conceiving different immobilization chemistries (e.g. through Protein A/G immobilization). Finally, a general consideration should be done on the necessity to handle hepcidin (particularly standard solutions) in a way that minimizes variation and under conditions that assure reliable analytical results. Ongoing work is now devoted to exploring new antibodies and synthetic receptors which could be successfully applied to SPR for hepcidin determination, *i.e.* aptamers.

To our knowledge, the results described in this report are the first attempt to detect hepcidin, a relevant but "tricky" peptide, by affinity-based biosensors (ABBs). We are aware that there still are limitations in terms of performing features, but are currently exploring several strategies to overcome these problems. Possible approaches to enhance the analytical response by improving the detection limit could be: changing the immobilization procedures, and/or in adding suitable tails to HBD to confer more freedom to the peptide once immobilized on the biochip. Once

identified a pool of suitable bioreceptors displaying low-cost, stability, easy availability, and high sensitivity to the analytical target, we believe that affinity biosensors can be successfully applied in the near future to real samples, in both urine and serum matrix (initially flanking conventional and profiling methodologies). When validated, the biosensing approach will contribute to the development of an an innovative methods for a fast, low cost, and easy to use methods for hepcidin detection.