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LP1

ROLE OF DNA CHIPS IN THE MOLECULAR DIAGNOSTIC LABORATORY OF THE FUTURE

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For decades, clinical laboratories have used analyzers and devices with dimensions at the meter -millimeter scale. In recent years, rapid developments have occurred in miniaturization and laboratory automation. While the latter will eventually improve on operational cost, turnaround time and number of tests performed per unit time, devices with micrometer-dimensioned components already have made considerable impact on clinical testing. A number of conventional analytical techniques including capillary electrophoresis and mass spectrometry have been adapted to microchip formats. In addition, integration of key analytical steps such as nucleic acid isolation, amplification and hybridization has been achieved at the micrometer scale. Micro-electromechanical (MEM), micro total analytical (μTAS) systems, DNA chips and microarrays now are available. These tools combined with information generated from the Human Genome Project are playing an increasing role in molecular diagnostics, and hold potential for facilitating implementation of personalized medicine and point-of-care diagnostics. Introduction of biochips in the clinical laboratory has resulted in the potential for detecting nucleic acid variation on a genome-wide scale. For example, analysis of molecular changes associated with predisposition to various cancers requires detection of specific gene mutations, detection of gene dosage changes, identification of rare fusion-gene transcripts, analysis of polymorphic repeat profiles, and/or monitoring of differentially expressed genes. Therefore, to distinguish single nucleotide differences, array-based approaches have been designed which employ ASO-based hybridization of labeled solution-phase targets, polymerase-catalyzed extension of arrayed or solution-phase oligonucleotide probes, array-based tiled sequencing strategies with overlapping probes, use of padlock probes and combination of polymerase chain reaction and ligase detection with zip-code hybridization and/or array-based hybrid capture using bar-coded array-bound oligonucleotides. In those instances in which expression profiling is required, arrays of oligonucleotides or cDNA clones are used providing data on the comparative expression of genes in normal and diseased states or for monitoring effects of drug treatment. However, despite these opportunities for biochips in the clinical laboratory, there are still important obstacles associated with their routine use including simplicity of assay design, cost for equipment and supplies, operating complexity, compatibility with automation, sensitivity, accuracy, reproducibility and validation through comparisons with standard methodologies. Developments in the world of commercial biochips, miniaturized devices and "home-brew" systems will be highlighted with an emphasis on clinical and biomedical applications. Finally, since it is anticipated that a full understanding of the pathogenesis of some diseases may require measurement of levels of proteins and their variants, implementation of proteomics-based analytical devices is anticipated to have major impact on molecular diagnostic testing. It is also clear that new tools in the nanoscale format are on the horizon: quantum dots, nanoparticles, carbon nanotubes and atomic force microscopes are now being used to directly probe DNA structure. These technologies may represent a new emerging approach promising increased throughput, sensitivity, sample processing as well as facilitate single cell and single molecule detection.

S1.1

THE ANATOMY OF THE E-LABORATORY AND ITS COMPONENT PARTS: A WORK IN PROGRESS

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The e-laboratory can be defined as a clinical laboratory with web-powered functionality. The most common example today of an e-lab is a clinical laboratory that offers its customers the opportunity to order tests and review test results using a web browser. This *web-ization* of the clinical laboratory will now provide unheard of access and ubiquity to all lab customers.
The first major LIS functions that are migrating to the web are order-entry and results-reporting (OE/RR) and the web application that provides these lab functions for both hospital and physician office personnel is the lab portal. The lab portal is a web site which supports OE/RR transactions but which can also provide lab-oriented content to physicians, patients, and healthcare consumers alike. The power of the web, as well as its ubiquity and ease of access, is illustrated by the fact that the technology has spawned a retail revolution in the normally staid clinical laboratory industry: direct access testing (DAT) whereby consumers can order laboratory tests for themselves on the web and pay for them out-of-pocket.
Decentralized testing, including point of care testing (POCT), will comprise about half of all lab testing in the very near future. The current LIS architecture is based primarily on the work flow and billing requirements of the centralized hospital or reference lab, which assumes the transportation of specimens to a high-throughput lab "factory" and which is not suitable for managing decentralized testing. We refer to this architecture as the C-LIS or centralized LIS. Systems of this type are now being augmented by a new architecture, the D-LIS (decentralized LIS), which will be based on a web infrastructure including a lab portal as a key element. The D-LIS will have a modular snap-on design with the customer selecting only the functions necessary to support his particular business needs.

S1.2

DIAGNOSTIC PROTOCOLS: QUALITY AND ROLE OF CLINICAL LABORATORIES

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Medical tests are central to modern medicine and require better characterization than has been the norm in the past. Today, laboratory specialists are increasingly asked to introduce more effective tests that allow physicians to achieve a better diagnosis, treatment and management of patients, and in fact, the advances of biomedical science have led to the discovery of many new biochemical and genetic markers. Physicians who frequently order laboratory assays outside of their field of expertise lack the knowledge base to order the optimal and rational sequence and panel of tests and to correctly interpret the results. The large menu of laboratory assays vailable today makes it increasingly difficult for the non-specialist to order all necessary tests, avoid medical errors and still contain costs. Thus, inappropriate use of laboratory tests can lead to poor case management, increased cost per patient and for the healthcare system, and, adverse medical outcomes.
Different approaches in order to achieve appropriateness in test order, interpretation and utilization have been proposed. Guidelines are viewed as a mechanism for disseminating a rapidly increasing body of knowledge. In contrast to guidelines, algorithms are intended to be simplicistic and to address a single option and therefore, they are limited and not suitable for complex decision making in which multiple choices may be more or less appropriate.
Recently, Dutch investigators introduced and evaluated a guideline-based decision support system for blood test ordering, the so-called BloodLink. Over a 1-year study period, they found that physicians modified tests recommended by guidelines in 60.9% of orders, most commonly by adding some tests. However, 52.4% of "non compliant" tests were later recommended by uptaded guidelines. It has been demonstrated that few guidelines published in the peer-reviewed medical literature completely adhere to established methodological standards, and therefore major improvements in guideline development, dissemination and implementation have been recommended.
Another important step is the need to link clinical guidelines with laboratory guidelines decribing the quality specifications and characteristics of laboratory tests recommended. Good examples of this effort are represented by the joint recommendations for the use of cardiac markers in Coronary Artery Disease, both at an international and national level, and by the guidelines for laboratory analysis in the diagnosis and management of diabetes mellitus. Linking appropriate and evidence-based test ordering with reliable quality specifications for laboratory tests, we can really promote outcomes assessment and quality improvement in modern medicine.

S1.3

ACCURACY AND TRACEABILITY: CHALLENGES FOR THE MODERN CLINICAL LABORATORY

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Providing accurate results to the patients has always been the mission of clinical laboratories. But, if the relevance of this task was great in the past, the evolution of medicine made it even bigger nowadays. The evidence based medicine, the development of decisional values, the orientation of medicine towards the well – being monitoring, together with the greater mobility of the individuals require results highly comparable and reliable over time (durable reliability) and space (horizontal reliability). To reach this goal it's necessary to produce accurate results traceable to a stated reference (vertical reliability). Accuracy can be obtained through the development of a reference measurement system, but can be transferred to the field results only via an unbroken traceability chain. There are several difficulties in defining this chain and in assuring its continuity down to the patient's result. For a quantity expressed in a unit the chain should start from the definition of the unit in the International System of Units (SI). For a chemical quantity involving amount of substance in the SI unit mole, the following step is to define a primary reference measurement procedure (e.g. gravimetry, ID-MS, coulometry) and a primary calibrator of the highest possible quality. Then there can be many further steps (definition of a secondary reference material, of a secondary reference measurement procedure, of a manufacturer master calibrator, of a manufacturer selected measurement procedure ect.) to reach eventually the measurement of the patient's sample. Each one of these steps adds a certain amount of uncertainty to the final measurement. Moreover only in a very limited number of cases the chain can start from the SI unit (that has a negligible level of uncertainty). For most of the analytes it starts from lower levels (international conventional reference measurement procedure or even manufacturer's selected measurement procedure) with much higher inherent uncertainty. The chain can then be broken at various levels by methods' unspecificity or by lack of commutability in calibration materials. There is an enormous amount of work needed to make progress in this strategic field and the ambitious goal of a global standardization can be reached only via a worldwide cooperation. International organisations like ISO, BIPM and CEN are developing the theoretical bases via a series of documents (standards). IFCC and other organizations are producing reference measurement procedures, they are preparing certified reference materials (CRM) (in collaboration with IRMM, NIST) and are activating reference laboratory networks. Manufactures should strive to develop highly specific methods, calibrators that assure traceability and commutable control materials. Finally the profession should pay more attention to this crucial aspect selecting the more accurate analytical systems, working at the implementation of more effective EQA schemes and participating to them in a more effective manner.

S1.4

ADVANCEMENTS IN IMMUNOASSAY STANDARDIZATION

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In clinical laboratory many analytes are presently measured by a number of different immunoassays using specific antibodies directed to the respective antigens. These immunoassays can be influenced by the nature of both the antibody and antigen. Different monoclonal antibodies may recognize different epitopes of the same antigen present in the blood. The numerical results of a measurement procedure may also be dependent on the matrix (e.g. buffer or artificial serum, instead of true human serum) in which the calibration material is dissolved. The nature and behaviour of the calibrator become in fact paramount in immunoassays where the measurement is entirely a matter of comparison of test with a calibrator. As a final consequence, analytical systems may give results which are typical for a certain method or instrument, so that different results from different assays and platforms may be obtained and this problem may cloud interpretations of reported data, creating a substantive problem for the clinical and laboratory communities. Durable standardization of quantitative measurements in Laboratory Medicine needs the consistent application of a generally accepted reference system, based on the concepts of metrological traceability, for calibration and validation of routine methods. Key elements of the system are the reference measurement procedure and different kinds of reference materials. The traceability model for quantities that are not fully physico-chemically characterized and for which results of measurements are not traceable to International System of Units, such as many analytes determined using immunoassays, emphasizes in particular the importance of the definition of the analyte ('measurand'). For instance, major standardization problems of cardiac troponin I (cTnI) measurement are associated with an insufficient definition of the entity involved. In biological samples, cTnI is present as a heterogeneous mixture of different molecular species. Intact cTnI and up to 11 modified products have been detected in the sera of patients with myocardial infarction. This microheterogeneity may only be circumvented by definition of a unique, invariant part of the molecule that is common to all components of the mixture, e.g. the epitopes that are located in the stable part of the molecule and are not affected by troponin complex formation and other 'in vivo' modifications. Owing to many complicated situations, it is clear that the progress in immunoassay standardization is slow and the corresponding problems will not be solved within the next few years. Nevertheless, a number of meaningful projects are presently underway under the auspices of IFCC and other organizations.

S1.5

ROLE OF IRMM IN THE PREPARATION OF CERTIFIED REFERENCE MATERIALS (CRM) FOR CLINICAL CHEMISTRY

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IRMM is promoting a common European measurement system in support of European Union policies.
Second largest supplier of reference materials worldwide, IRMM offers, produce, certify and distribute over 500 different BCR®[1] CRMs, in a wide range of sectors ranging from industry to agriculture, food to clinical chemistry (34 CRMs) and environmental protection, with a total stock load of some 500,000 samples.
Materials are made applying the BCR guidelines for certification of reference materials and ISO[2] guidelines. According to the stated purpose of the "In Vitro Diagnostic (IVD) Directive"[3], IRMM and IFCC[4] are working together to stimulate global collaboration for the establishment of reference systems for in vitro diagnostic measurements, and the production of CRMs in fields where IFCC and the clinical chemical community identify needs. The agreement with IFCC is understood as a co-operation on international level with the involvement of both EU and non-EU laboratories in the certification process.
The clinical relevance of the parameter to be certified and the applicability of a CRM, are the most important criteria. Five IRMM/IFCC CRMs for enzyme activity have been certified to newly developed IFCC reference method at 37°C, instead of 30 °C inconvenient for the CRM user. An IRMM/IFCC reference panel of 34 sera for cortisol has also been certified using a primary method (GC-IDMS) in two independent reference laboratories. Ongoing certification projects are for two other enzymes, myoglobin, total protein concentration in serum and for the re-certification of high levels of progesterone in human serum.
A very important aspect in the layout of certification projects is the early involvement of the CRMs users in the process: a lack of commutability of CRMs is in fact generally leading to a reduction of acceptance, limitations in use and possible gaps in the traceability chain required by the IVD directive.

[1] Bureau Communautaire des References
[2] International Standards Organization
[3] Directive 98/79/EC
[4] International Federation of Clinical Chemistry

S1.6

IFCC INTERNATIONAL PLASMA PROTEIN SURVEY: PRELIMINARY ANALYSIS OF DATA FROM ITALY

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The IFCC Committee for Plasma Proteins (C-PP) launched in February 2002 the second International Quality Assurance Project on Plasma Proteins (the first was performed in 1993) with the aims to document the uptake of BCR/IFCC/CAP CRM 470 as the prime reference material in use by commercial and other organization producing calibration materials and kits for serum protein estimations and, most importantly, to assess the degree of harmonization produced. Approximately 4400 laboratories worldwide have agreed to participate in this second survey that included this time also laboratories from Italy. The data are being collected worldwide by IFCC C-PP and the results will be available in the last quarter of 2002. Here we report a preliminary analysis of the data gathered from the Italian laboratories regarding the comparison between the results obtained using immunoturbidimetry *vs* those using immunonephelometry.
Materials and methods. Two liquid samples (A, high conc.; B, low conc.) were sent to 395 labs. Valuable data were obtained from 237 labs (60%).
Results. The results are reported in the Table. No significant differences (T test) were found between the two methods with the exception of IgM ($p<0.0001$) and IgG ($p<0.003$) for both sample A and sample B.

	IgG mg/dL	IgA mg/dL	IgM mg/dL	Tf mg/dL	AAG mg/dL	CRP mg/dL	AAT mg/dL	C3 mg/dL	C4 mg/dL	Cp mg/dL	Alb g/dL
Sample A: Immunoturbidimetry											
n	122	122	122	102	131	123	31	83	81	16	28
M	1001	198	95.1	76.9	287	3.05	127	148	27.8	28.4	4.26
CV	8.4	9.3	10.4	10.2	8.3	11.6	7.1	11.7	17.3	13.8	7.8
Sample A: Immunonephelometry											
n	103	101	101	95	82	74	66	94	94	52	40
M	1038	198	107	77.9	291	3.08	133	144	27.5	31.7	4.27
CV	6.7	7.0	9.3	7.5	6.8	10.6	8.4	10.3	12.6	14.7	7.4
Sample B: Immunoturbidimetry											
n	121	122	122	102	130	126	32	82	84	16	29
M	735	144	69.4	55.1	209	2.15	93.0	109	20.4	21.3	3.21
CV	7.4	9.0	9.9	13.7	8.4	15.9	4.8	10.6	20.3	15.0	9.3
Sample B: Immunonephelometry											
n	104	101	101	97	83	73	65	98	97	53	41
M	757	144	82.1	56.8	210	2.16	94.1	105	19.9	22.6	3.14
CV	7.9	6.3	16.5	12.2	7.4	12.0	10.7	10.4	13.0	15.1	7.7

Discussion. These results indicate that the quantification of the major plasma proteins can be equally performed by immunonephelometry or immunoturbidimetry. Further studies are warranted to investigate the significant differences found, between the two methods, for IgG and IgM.

S1.7

FROZEN SERUM POOL AS CONTROL FOR CAPILLARY ELECTROPHORESIS

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By virtue of its UV-based monitoring system, capillary zone electrophoresis (CZE) permits reliable measurement of the serum protein zones. In order to fully exploit such an instrumental capability, the analytical stability of the instrument has to be checked by means of an effective QC scheme, using appropriate materials. We investigated the possibility of using frozen serum pool for assessing the stability over time of the system. A serum pool was prepared, aliquoted (1-mL portions) and stored at –80 °C. Daily, over a one-year period, one aliquot was thawed and electrophoresed on a CZE Paragon 2000 (Beckman Coulter) system. In order to assess short-time stability regressions of daily values against day (initial 26-day period) were calculated. Slope values thereby obtained showed not significantly different from 0 (P values in the range 0.15 to 0.96) for the 5 zones. For medium- long-term stability study, 12 subsequent monthly means were regressed against month number. Slope values were not significantly different from 0 for the albumin and the α-1-globulin zones (P values in the range 0.09 and 0.143 respectively). However, slopes were significantly different from 0 for the α-2-globulin zone (-0.03±0.01; P = 0.01), for the β-globulin zone (-0.04±0.01; P = 0.02) and for the γ-globulin zone (0.07±0.02; P = 0.002). Such slope values corresponded to monthly variations of -0.25%, -0.30% and +0.63%, respectively. Apparently, some deterioration of the pool occurs with ageing, becoming however evident only after the first six months of storage. Over the whole 1-year study period the monthly CV (as a measure of overall imprecision) were in the range 2.0%-8.4%, 1.7%-4.2%, 2.0%-3.7% and 1.8%-6.5% for the α-1, α-2, β and γ globulin zones, respectively, and 0.7%-1.6% for the albumin zone. Such monthly imprecision values showed a tendency to increase in the second half of the study period, particularly in the case of α-1-globulin and γ-globulin zones. As shown by others (Bossuyt X *et al.* Clin Chem 1998; 44:749-59) overall imprecision in the measurement of the five zones was roughly inversely correlated to the amount of protein in the zone, with the main exception of the γ-globulin zone whose imprecision was higher then expected on the basis of its mean amount. We conclude that frozen serum pool is a suitable material for QC of CZE. To stay on the safe side, it seems recommendable that storage is limited to a six months period.

S2.1

AUTOMATED PROTEOME SCANNER

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For several decades, DNA sequencing has progressed dramatically. In parallel, numerous genomic tools have been developed in order to study biological processes and explain physio-pathological findings in molecular terms. Therefore, after the stages of genome sequencing and gene discovery, attention must be focused on gene expression and the functions of the proteins they encode. The rate of investigation is limited by the actual methods of proteins identification and characterisation and, currently, this is the bottleneck. Techniques based on the analysis of peptides mass fingerprints with mass spectrometry such as MALDI-MS are still very powerful and may be used to increase the throughput. This involves the analysis of peptides generated by the digestion of separated proteins with residue-specific enzymes. The combination of a massively parallel proteolytic digestion method for proteins separated by bi-dimensional electrophoresis (2-DE) and a subsequent peptides mass fingerprinting analysis by MALDI-MS offers the basis for the development of an automated proteome scanner.

In 1998, at the Siena meeting call "from Geneome to Proteome", our group has presented for the first time a "parallel protein digestion during the electroblot" process. The method proposed to intercalate a membrane (Immobilon™ AV or IAV) where was covalently (IAV-trypsin) in an electrotransfer sandwich between a gel and a PVDF collecting surface (1). An improvement was achieved by operating a pre-digestion of the proteins in the gel prior to electroblotting. This "double parallel digestion" (DPD) method allowed to successfully identify proteins by measuring their peptide mass fingerprints directly on the collecting PVDF membrane from a wild range of pI and M_r using MALDI-TOF MS (2).

As an example to this technique, DPD treatment was applied to a 2-DE separation of an *E. Coli* sample (3). Peptides produced during these parallel transblotting processes were trapped onto a PVDF membrane. Using DPD process, all proteins of a 2-DE are first simultaneously digested proteolytically and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane. Then, the membrane was directly scanned by MALDI-TOF MS. Spectra obtained during this step were automatically treated for peak detection and calibration. The peptide masses obtained were processed by a software that makes use of the spatial correlation of data and then pipelined to a peptide mass fingerprint protein identification tool (4). The outputs were used to create 3-D MS images (5) that visualize the most important parameters of the molecular scanner data such as: pI, Mr, identification labels (SWISS-PROT or TrEMBL AC numbers, ID labels), PMF and MS intensities. By this technique, the bottleneck for protein identification is reduced as 2-DE proteins are digested during the parallel digestion followed by the scanning process where polypeptides can be identified rapidly and automatically. The resulting image can be used for automated 2-DE database update. This 2-DE MALDI-TOF MS imaging technique represents a major step towards the development of a clinical molecular scanner.

1. Bienvenut WV *et al.* From Genome to Proteome, 3rd Siena 2D Electrophoresis Meeting, Aug. 31-Sept 3, 1998
2. Bienvenut WV *et al.* (1999) *Anal.Chem. 71*, 4800-4807
3. Binz P-A *et al.* (1999) *Anal. Chem. 71*, 4981-4988
4. Gras R *et al.* (1999) *Electrophoresis 20*, 3535-3550
5. M. Müller, *et al.* (2002) *J. Am. Soc Mass Spectrom. 13*, 221-31

S2.2

PROTEOMICS OF RENAL CELL CARCINOMA

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Renal cell carcinoma (RCC) are a wide group of tumours representing about 3% of all adults cancers. Early diagnosis of these tumours is very difficult because of the lack of clinical symptoms, and they are also resistant to radio and chemotherapy. Proteomic approaches provide an important tool to identify potential protein markers to be used for diagnosis of RCC and follow-up of treatment. To this purpose, proteins of normal and tumour renal tissues were separated by 2-D PAGE. Since RCC originates in the renal cortex and is characterized by heterogeneous histological features as cell type and tumour architecture, we fitted out a method of separation of epithelial cells to obtain reproducible results. Images of 2-D PAGE gels were acquired using Melanie 3 software (GeneBio-Geneve), compared each other and matched with masters to make out proteins differentially expressed in normal and tumour samples. Spots of interest were then examined by N-terminal sequence analysis, aminoacid composition analysis and mass spectrometry (MALDI-TOF).
Four polypeptides, ubiquinol cytochrome c reductase, NADH-ubiquinone oxido- reductase complex and plasma glutathione peroxidase were not detected in RCC, while three different isoforms of the mytocondrial protein manganese superoxide dismutase (Mn-SOD) were selectively expressed in tumour tissue. In addition, higher levels of hsp27 were observed in RCC, in comparison with normal samples.
Through the integration of individual profiles, we intend to set up a reference proteome map of RCC indicating which proteins might be used as diagnostic and/or prognostic markers possibly detectable in blood and urine.

S2.3

A PROTEOMIC INVESTIGATION ON PANCREATIC ADENOCARCINOMA: DIFFERENTIAL PROTEIN EXPRESSION UPON ONSET OF CHEMORESISTANCE AND THERMOTOLERANCE

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The pancreatic adenocarcinoma is one of the most aggressive solid tumors, characterized by a strong resistance to chemotherapy and a high rate of metastasis. The best chance for survival is to diagnose the tumor at an early stage, unfortunately pancreatic cancer has a poor prognosis and it is often difficult to detect before metastasizing to other organs. Systemic chemotherapy and administration of biologically active molecules have not lead to significant improvements in the survival rate. The combination of drug treatment with radiotherapy and/or hyperthermia has been frequently employed to enhance the efficacy of such protocols. Nevertheless tumor cells tend to develop thermoresistance associated with chemoresistance, therefore strongly impairing the success of these treatments.
In order to understand the molecular basis of these phenomena we have investigated the differential protein expression profiles of human pancreas carcinoma cell lines in culture. Cell lines exhibiting drug resistance to daunorubicin, model for classical MDR profile, and mitoxantrone, model for the atypical MDR, have been selected together with their themoresistant cell counterparts. These have been compared to their original parental and parental thermoresistant lines by 2D electrophoresis, image analysis and mass spectrometry identification. Our preliminary results suggest the involvement of both enzymes and structural proteins in the onset of thermotolerance. Strikingly, glutathione S-transferase M3 isoform, an enzyme of the detoxification pathway, has been found associated in the mechanism of thermotolerance rather then in the chemoresistance onset.
Moreover we have undertaken a complete proteome classification for the protein expression profile in cell line from adenocarcinoma of the pancreas. This long-term project is aiming to the identification of potential marker proteins for this tumor. A first overview on large pH 3-10 gradient 2D-PAGE has been completed. Up to now we have identified about 350 different proteins by MALDI-TOF-MS and PSD high-energy laser induced fragmentation coupled to database search algorithm. An update description of the present results will be given during the following of the meeting.

S2.4

RECOMBINANT HUMAN ERYTHROPOIETIN (rhEPO) CHARACTERIZATION BY MEANS OF 2D-PAGE AND ESI-TOF MASS SPECTROMETRY

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Erythropoietin (EPO) is a 34 kDa glycoprotein hormone controlling red blood cell production and mainly produced by interstitial cells of the kidney cortex. EPO production is regulated by hypoxia that leads to an increase of gene transcription, as there are no preformed stores of EPO. The main targets of EPO are the colony forming unit-erythroid (CFU-E) where, after the binding to the specific receptor, it stimulates the rapid tyrosine phosphorilation of a number of proteins. As hEPO was the first hematopoietic growth factor to be cloned, rhEPO has been available as a drug since 1998 and it is used for the correction of anaemia of renal failure and chronic diseases, but the misuse of rhEPO among endurance sports athletes has been suspected for several years. Glycosilation is important for the biological activity of EPO, in fact active hEPO presents four glycosilation sites, resulting in a carbohydrate content of about 40%. Whereas the polypeptide chain is genetically controlled, the oligosaccharide chains are the result of a series of post-translational enzymatic reactions giving rise to a mixture of isoforms. The different rhEPOs can be distinguished from human due to their different carbohydrate content. Moreover Darbopoetin alfa, compared to Epoetin alfa and beta, shows an higher MW and a more basic PI as a consequence of its increased sialic acid content. The aim of the present work was to investigate the isoform pattern of different commercial rhEPOs by 2D-PAGE and to evaluate the possibility of differentiate them by mass-spectrometry. 2D-PAGE gels (n=5 each) were run for Epoetin alfa, beta and Darbopoetina alfa (kindly provided by Dompè Italy) alone and as various mixtures (n=3 each). Proteins (10 μg) were applied to 7 cm IPG strip pH 3-10 or 3-6 directly into the rehydration buffer. After 2D separation, gels were stained with Sypro Ruby Gel, the fluorescence recorded by Fluor-S System and data analyzed using PD-Quest software (Bio-Rad). The different rhEPOs were also analyzed by a Mariner ESI-TOF mass spectrometer, equipped with an Ionspray interface (Applied Biosystems). The MS instrument was interfaced to a 9012 HPLC pump from Varian (Leinì). The HPLC column was a Jupiter C5 (Phenomenex). The acquisition was performed in the mass range from 1000 to 4000 m/z at a scan rate of 3 sec/scan.
In agreement to the literature, our results suggest that it should be possible to develop a candidate reference method for rhEPOs by a combined approach of 2D-PAGE and MS.