ABSTRACT SESSIONI

LP1

DNA MICROARRAYS: A REVOLUTION IN DIAGNOSTICS IN THE POST-GENOMIC ERA

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Gene expression profiling via DNA microarray chips is an emerging technology with the potential to revolutionize many aspects of clinical medicine. For instance, it opens the way to diagnostic methods which directly unveil the pathogenetic root of disease, as well as to individualized therapy which keeps into account the genetic make-up of patients and/or pathologic tissue. This new technology was made possible mainly by two factors. One is the recent advances in developing ultrasensitive methods for detecting DNA:DNA hybrids. The other is our expanding knowledge of the human genome. DNA microchips exploit assay principles of solid phase immobilization of DNA sequences, which are then used for detecting messenger RNA (mRNA) in soluble biological samples. Such principles are essentially the same as in analytical assays in which a receptor or a ligand are immobilized in solid phase, and reveal a test substance in soluble phase, e.g., immunoassays with antibodies absorbed on plastic. Solid phase assays are already disseminated in clinical and research laboratories, because they are easily executed and adapted to high-throughput screening.

To assemble DNA microchips, known sequences of expressed genes are immobilized on solid surfaces, e.g., glass, either as cDNA or, better, as synthetic oligonucleotides. A collection of genes is represented as an array of spots at variable density, between a few hundred to thousands on a single chip, in which the position of each gene sequence is predetermined and recorded for further analyses. The gene microchips are hybridized with microvolumes of fluorescence- or radioactivity-tagged test cDNA derived from cell or tissue mRNA. Once hybridization is completed, spots corresponding to expressed genes in the test sample become annealed to the tagged complementary cDNA strand, and can be detected and quantified by automated scanners, often based on confocal fluorescence. Data are analyzed with statistic methods for internal consistency, and with clustering algorithms to unveil their possible biological significance.

DNA microchips are ideal applications for oncology. Neoplasias are genetic diseases due to the accumulation of mutations in genes that control cell proliferation. Neoplasia heterogeneity is very high, even within one histologic group. Genetic alterations can vary from simple point mutations, to duplications, insertions, deletions, up to catastrophic large scale changes of the genome. These genetic mutations give rise to various altered behaviors of the neoplastic cells, which may be instrumental for diagnosis and classification. Tipically, diagnostics are based on two parameters: the appearance of the cells in histology, and their invasive behavior. These parameters have the intrinsic limitations of being poorly quantitative and inevitably rooted in subjective interpretation. Nonetheless, they are the foundations for the TNM classification of neoplasia, onto which choice of treatment is based. Several refinements have been introduced in a continuing effort for better oncologic classification. The ultimate approach, though, for full identification of a tumor remains the characterization of its genetic signature. DNA microchip technology should make it possible to implement such definitive tumor diagnosis assays. In principle, it should eventually become possible to classify tumor tissues based on their partial or complete genomic signature.

We have initiated DNA microchip studies on neoplasia of the urinary bladder (NUB), aimed at developing gene expression profiling based diagnostics. The following aspects of NUB make it an ideal model for early studies on DNA microchip application to oncology: i) high number and frequency of patients; ii) easily accessible tumor mass; iii) consistent and frequent clinical tumor sampling by endoscopy or surgery; iv) treatment choice dependent upon invasive properties of the tumor. Furthermore, there is currently no tests or marker that accurately supports NUB staging, besides histopathology which by its very nature is not quantitative. Our initial preliminary results are beginning to suggest that gene products involved in epithelial cell migration could be exploited as signatures for NUB cell clinical behavior.

In summary, several open issues remain to be addressed and solved before DNA microarrays become a part of the everyday experience of a clinical laboratory. Nonetheless, progress in this arena is ever faster and several diagnostic applications should be around the corner.

Sessione 1 - TECNOLOGIE DELLA COMUNICAZIONE
Sala A
Martedì 12 giugno, ore 10.00-11.30

S1.1

INFORMATION TECHNOLOGY AND CLINICAL LABORATORY

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The clinical laboratory end product is information. Information is also necessary to control the different phases of the analytical process, including the quality assurance programs and the validation protocols, and to make easy the management process (productivity, cost control, billing and statistical analysis). Another aspect which is becoming increasingly important is the information exchange among laboratories belonging to the same organization and between laboratories and hospitals, clinics or doctors to access or supply information in real time.
On the basis of this idea, we started two years ago the informatization project of Laboratory Dr. Echevarne throughout the following steps:
1. Building of an "intranet" (64-256 Kbd) to connect the 17 centers and the 14 emergency laboratories, belonging to the organization, distributed all over Spain and Portugal. The "intranet" is connected to Internet
2. Building of a "local intranet" (10-100 Mb) in each center, with voice and data integration to connect phones, computers, other hardware and "on-line" analyzers.
3. Hardware upgrade
4. Software development and integration with other applications (Office 2000, SPSS statistical package, etc.)
The program was developed using Object Oriented Technology, and integrates different modules in a single application at different levels:
Organization level: Analytical process, Logistics, Billing and Accounting (next Human Resources and Quality Assurance, ISO and GLPs).
Laboratory level (Departments): Anatomical and Clinical Pathology, Veterinary Pathology, Clinical Assays and Industrial Analysis.
Clinical Laboratory level (Units): Biochemistry, Hematology, Body Fluids, Blood Bank, Immunology, Allergy, Hormones, Tumor markers, Microbiology, Toxicology, Pharmacology, Citogenetics, Molecular Genetics and Molecular Pathology.
Analytical process level: pre-analytical phase, on-line connections, QC, validation (analytical and clinical), and reporting (single or cumulative), including graphic capabilities.
Conclusion: information technology is becoming as important as the analytical process in modern laboratories, to assure the quality of results, the information exchange and the management control.

S1.2

INTEGRATION BETWEEN CLINICAL LABORATORY - WARDS - TERRITORY: TECHNOLOGICAL AND ORGANIZATIONAL ASPECTS

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The analytical quality of laboratory test has improved very much in the last years thank to the improved concepts in the area on analytical quality and wider and wider use of ICT Information and Communication Technology.
Nowadays, clinical laboratories, while requested to keep the highest standard as far as the analytical quality is concerned, face the challenge of improving the service to the patient. Some aspects of this new challenge are represented by the reduction of turnaround time, of waiting lists for blood drawing and, last but not least, reduction of the many possible errors of laboratory activities (e.g. misidentification of patient-samples).
A crucial point in improving these aspects of laboratory services is represented by the need to control the preanalytical phase largely performed outside the laboratory premises and outside the control of the laboratory personnel. The recent progress of ICT makes these improvements of service much better attainable than in the past.
Is it today quite possible to electronically link clinical laboratory with ward and also with the environment external to the hospitals, until the house of the patient. This can help a lot in reducing errors and improving the turnaround time and in general improving the confort of the patient. We have elaborated in our institution original technological solutions in order to facilitate the connection between the clinical labs and its client: a fail-safe automatic identification of patient has been realised by means of a tool able to integrate also other aspects of the hospital ward activity (e.g. fail-safe administration of drugs - connection between the ward and the pharmaceutical industry").
We have also built some tools able to bring the service closer to the patient by transmitting the laboratory result everywhere they like (the kiosk project). Some technological and organizational aspects of this project will be illustrated.

S1.3

EXPLOITATION OF LABORATORY DATA BY USE OF INFORMATICS

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A database has been established at the University Department of Laboratory Medicine of the Hospital of Desio since 1976 for the population accidentally exposed to dioxin in Seveso and since 1982 for the general population of the surrounding area. Altogether personal and medical information of about 1,200,000 subjects and 40,000,000 laboratory records are stored.
Putting laboratory data, in interactive way, into the patient's information network can add (or create) high quality knowledge, real time available to the physician, but it requires an automatic access to personal, epidemiological, physio-pathological data and to databanks.
This suggests the possible role of neural networks, but also the need of more interaction between informatic, statistic, and medical expertises. In this sense we present our project.
The goal to transform the informatic system into "informative resources" requires the establishment of new methodologies and technologies (e.g. Business Intelligence, Data Warehousing).
The new informatic technologies and statistic packages moved us to explore these data and try to exploit them as further medical information. Our LIS is run by a DBMS-type application (MUMPS-Cachè) in WIN-NT configuration.
From 1999 we tried to connect our database with a system that allows analyzing data (SAS System).
The steps of Data Warehouse set-up and management by SAS/Warehouse Administrator are:

1. Data export from our database in ASCII format;
2. Data transformation, integration and validation;
3. Data availability in a Data Warehouse structure;
4. Data Mart and summary.

We present a prototype of Data Warehouse on a subset of our database and we illustrate some examples of explorative analysis of data. Steps will be: study of distribution of some analytes and correlation between them, application of neural systems and examples of data mining.

References.

1. Verrecchia F, Dell'Oro P, Segatto G. Webhouse Data Surfing. Atti del Covegno SUGItalia '99. SAS Institute Inc., Roma (1999)
2. Mocarelli P. Cure e promozione della salute: contributi possibili di laboratori immersi in reti informatiche. EsaDia 1998;1: 18-21

S1.4

PRE-LABELED TUBES: A NEW APPROACH TO SAMPLE IDENTIFICATION

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As the clinical laboratory moves toward advanced automation more and more efficient sample-handling systems are demanded. The sample identification procedure plays a key role in the whole pre-analytical and analytical processes. Usually, the Laboratory information system (LIS), often connected to the Hospital Information System (HIS), is provided with the information concerning the patient and the tests to be performed. Bar-code labels, capable of identifying the sample and the associated patient, are then printed and stuck to the appropriate tube. Mainly when run in a ward, this procedure may be affected by several drawbacks: 1. it requires bar-code label printers, 2. the printers require maintenance, paper and ink, 3. the labels must be manually stuck to the tubes, 4. the labels may be stuck improperly or incorrectly oriented. Thousand labels must be printed and stuck in our Hospital every day and the incidence of inconvenient is very high.. As an alternative, factory pre-labeled tubes may be used: a 12 figure bar-code includes information concerning the kind of tube (plain, EDTA, citrate etc.), the expire date, and an eight figures identification number which cannot be duplicated before ten year. Once the blood has been drawn the tubes are associated to the patient by simply scanning the patient bar-code (wrest bracelet, or other identification device) and the tubes themselves. Examples of different procedures and devices are shown. In conclusion the use of pre-labeled tubes seems to be capable of saving human resources and of improving the efficiency of the pre-analytical process.

S2.1

SCREENING FOR PROSTATE CANCER: A PRACTICAL APPROACH

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Screening for cancer must fulfil several conditions before it may be introduced in the current practice: screening for prostate cancer (PC) is still far away from this condition. Several aspects of PC screening need to be defined and most questions will be probably answered by the two randomised trials ongoing in the US (PCLO) and in Europe (ERSPC) which will provide the first endpoint (analysis) around 2008. For the time being we can only argue, and five aspects at least are worth a brief discussion.

1. screening test: digital rectal examination (too insensitive) and transrectal sonography (too costly) have been rejected in favour of PSA, which is definitely cheaper and more acceptable. Unfortunately PSA is unspecific and using a cutoff of 4 ng/ml recall rate approaches 15%, with a positive predictive value of 25% or less. An excess of useless random sextant biopsies is performed. No existing method to correct total PSA (density, free/total ratio, age aspecific range) satisfies clinical requirements. The adoption of a lower cutoff (3 ng/ml) showed a persistently high detection rate, suggesting that PSA is just an occasion for performing sextant biopsy: PSA values <10 ng/ml do not predict biopsy outcome: the feeling with studies which use PSA>3 or >4 ng/ml to prompt sextant biopsy is that we are actually selecting an unspecific sample of the population and y screening with sextant biopsy. For the moment PSA is the screening test of choice but its flaws are evident and providing that screening will turn out to be effective, the screening modality will have to be carefully reconsidered.
2. overdiagnosis: PC detection rates are 20 to 50 times higher than expected incidence. Modeling based on existing data from screening experiences suggests that for each PC which would have surfaced clinically, screening detects up to three cancers, so called "latent", which would not have surfaced as clinical in the lifetime. This finding is consistent with a model where at least 30% of subjects have latent PC (as shown by autopsy studies) and random biopsies are aggressively used.
3. overtreatment: there is presently no means to identify latent and "clinically significant" PC. This means that overdiagnosis will almost always cause overtreatment. Overdiagnosis and overtreatment are common in cancer screening, but with PC the situation is quite different from e.g. cervical cancer where most dysplasias are ovetreated as they would not have progressed to cancer, but treatment (loop resection or conization) is relatively well tolerated and has almost non side effects. On the contrary, conventional treatment of PC is heavy. Although radical radiotherapy is accepted as equally effective but with less side effects, radical prostatectomy is commonly performed, which exposes to several risks, such as perioperatory mortality (0.1-1%), severe urinary incontinence (up to 10%), sexual impotence (up to 90%). Thus the implications of overdiagnosis and overtreatment for PC screening are unique and frightening, and that's why everybody with common sense should agree that screening for PC as a current practice is unethical at the moment, at least until reliable data on screening efficacy and adverse effects will be available, allowing for a cost benefit analysis.
4. treatment: there is no agreement about the ideal treatment of early PC, commonly detected by screening. In some countries watchful waiting is a common procedure, as a measure to balance the risk of overtreatment, but although it is considered as some sort of delayed therapy, such delay might possibly flaw the advantages of early diagnosis.
5. contamination: a special issue for controlled randomised trials. Contamination by opportunistic PSA is a real menace to these studies as it dilutes the statistical power of detecting a screening effect on mortality. Opportunistic PSA is increasingly used, although it should be banned as dangerous, as it imples the same risk of overdiagnois and overtreatment even on a single individual. In a logical setting PSA should be determined in clinical practice only for the differential diagnosis of PC suspected at palpation or sonography and for the follow.up of PC patients. But logic is becoming a rare optional in clinical practice....

Screening for PC is still experimental. Nobody knows if it is useful or dangerous. Current screening is unethical as its disadvantages might be greater than its benefits. Any recommendation of current screening (AUA, ACS) is the fruit of ignorance of existing evidence and a foolish bet on a possible benefit which might end with a disaster. Something similar happened with lung cancer , a perfect example of how screening may look apparently effective at first glance and turn out ot be totally useless, but it looks like people did not pay much attention to that lesson....

S2.2

CLINICAL INDICATIONS TO BIOMARKERS

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A rise in the life expectancy, demographic changes toward older age groups and healthier conditions of men at an older age increases the impact of prostate cancer on health system as the most common cancer in men. Prostate cancer evaluation for the early diagnosis of organ-confined and potentially curable prostate cancer has dramatically changed in the past 15 years. The introduction of prostate specific antigen (PSA) testing, increased medical and public awareness for rectal digital examination (DRE) and transrectal ultrasound of the prostate (TRUS) and ultrasound-assisted needle biopsy of the prostate has been crucial for the improvement in the early diagnosis of this cancer. PSA, first identified 30 years ago, has been widely used in clinical practice in early-detection algorithm causing a significant stage shift: a marked increase of localized disease and a decline of locally advanced and metastatic prostate cancer. However, being an organ-specific and not cancer-specific marker, there have been efforts to improve the accuracy of PSA in detecting prostate cancer in the last 10 years. In fact, despite its utility, PSA is limited by a lack of specificity within the diagnostic gray zone of 4.0 to 10.0ng\ml. Approximately 25% of men with PSA value within this range harbor a malignancy, mandating a recommendation for biopsy. Moreover, even if serum PSA level of 4ng\ml or above has been considered abnormal, this cutoff point will miss approximately 21% of patients with prostate cancer who have PSA level below 4ng\ml. New methods to optimize the diagnostic value of PSA has been attempted: PSA density (PSA divided by transrectal ultrasound-determined prostate volume in cubic centimeters), PSA velocity (rate of change of PSA over time) and age-specific PSA reference ranges. PSA density with a cutoff of 0.15ng\ml\cc of prostate to decide for a prostate biopsy remains controversial due to a conflicting literature results. PSA velocity require at least 3 measurements over 2-year period and may have a clinical utility in patients with an initial negative biopsy results, who need to be followed for a further biopsy because of PSA level between 4 and 10ng\ml and a normal DRE. Age-specific PSA reference ranges improve the sensitivity for detecting prostate cancer in younger man but increasing, at same time, the number of unnecessary biopsies. In older man it increases the specificity of the test but some cancers may be missed. In the mid-ninety the molecular forms of PSA was introduced in clinical setting. Retrospective studies demonstrated that the percentage of free PSA decreases as the probability of having cancer increases, providing a better differentiation between men with and without prostate cancer when the PSA value ranges between 4 and 10ng\ml. The major clinical utility of percent free PSA has been to decrease the number of inappropriate biopsies to detect prostate cancer.

Several factors favor the use of screening for early detection of prostate cancer in men over 50 of age: absence of symptoms in early stage of this cancer, improvement in detection methods when the cancer is still confined, curability window only in the early stage. Today, even if screening is not yet standardized, case finding for prostate cancer using PSA and DRE is widely practiced. This is a process of evaluating men with symptoms or seeking medical attention through a PSA and DRE evaluation. In absence of definitive data on the improved survival obtained with cancer screening program, we believe that a screening test should be implemented in men over 45 year old within families with relatives with prostate cancer, relaying for the rest of the population on the case finding scenario.

New assays to further delineate the need for prostate biopsy among men in the diagnostic gray zone are urgently needed to reduce the potential morbidity, anxiety and medical expense currently associated with the serum PSA test.

S2.3

BIOMARKERS IN PROSTATE CANCER. THE PIVOTAL ROLE OF CLINICAL LABORATORY

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PSA is an established test in the management of prostatic cancer and, more recently, of benign prostatic hyperplasia. PSA is a paradigmatic example of the positive role of the collaboration between clinical laboratory and clinicians in improving the usefulness of a diagnostic test.
First of all, the clinical laboratory highlighted the problem of comparability between different PSA methods, pushing towards stringent standardization efforts.
Several PSA derivatives have been identified and validated, which provides clinicians with an array of diagnostic tools. Again, the role of clinical laboratory is pivotal in establishing "age adjusted reference ranges" and in setting the reliability limits of PSA velocity, according to the method used.
In addition, the increasing knowledge on basic biochemistry of PSA has lead to different tests suitable to explore the different forms of PSA and other kallicreins. The relationships between these molecular moieties provide powerful clues for cancer diagnosis. The determination of both free PSA and hK2 is still far from standardization. Differences among free PSA values obtained with different methods are clinically meaningful and both method standardization and extensive validation are needed prior to a reliable clinical use.
Several new diagnostic tools have been recently investigated. Both the determination of PSA conjugated with α2-macroglobulin and the detection of circulating cells expressing PSA mRNA seem promising diagnostic tools from a theoretical point of view. In this developing setting, the clinical laboratory has a key role in standardization, validation and quality monitoring.
Besides analytical and clinical investigations, the following two other issues have been so far barely considered and require a further investigation: the *pre-analytical phase* and the *modality of reporting results*. As concerns the former, the liability of free PSA in the biological sample is well known. In addition, how the sampling is carried out (i.e., serum *vs* plasma) has been reported to affect also total PSA. The clinical laboratory should have a role in both investigating all the pre-analytical sources of variability and in standardizing sampling procedures.
Reporting results and decision criteria are the other issues the laboratory has to deal with. Results of recent investigations suggest that post-test probability may provide more meaningful information than the mere PSA concentration with reference to the cut-off point. Moreover, proper decision criteria have been investigated in order to reliably use PSA as surrogate marker of either responsiveness or failure during endocrine therapy for advanced prostate cancer.
In conclusion, in the last ten years PSA has prompted a close integration of clinical and laboratory issues, thus contributing to highlight the increasing importance of the active role of the clinical laboratory for an increasingly favourable cost/effective use of biomarkers in oncology.

Sessione 3 - DEFINIZIONE DEI TRAGUARDI ANALITICI
Sala A
Martedì 12 giugno, ore 11.30-13.00

S3.1

THE BIOLOGICAL APPROACH

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As soon as intercomparison exercises pointed out at large interlaboratory variability and quality control programs were set up in order to assure analytical reliability, there was the need to define appropriate analytical variability limits, to guarantee clinical usefulness of laboratory results. Different strategies were followed to reach such an aim, and they were based respectively on biological, analytical and clinical aspects. Biological variation includes intra-individual variation (V_w) and inter-individual variation (V_b). V_w and V_b are considered the most reliable bases for the objective definition of allowable limits of analytical variation (V_a). As a matter of fact, V_w and V_b are intrinsic properties of the individual and of the human species respectively; they can be measured by appropriate experimental protocols, and a plenty of data are available in literature for about 300 quantities measured in human fluids for medical purposes. Taking the comparison of two subsequent measurements in the same subject as a model, allowable analytical imprecision can be chosen as a fraction of intra-individual biological variation (V_w). Integrating values of V_w and V_b, allowable limits for analytical bias can be calculated. Maximum tolerable total error of the single measurement can then be derived as a function of allowable analytical imprecision and bias. Whilst for some quantities such limits can be easily reached or even surpassed, for other quantities the same limits cannot be attained with the nowadays technology, at reasonable cost. For practical purposes, three increasing levels of performance, minimum, desirable, and optimum, may be defined, and allowable limits for analytical imprecision and bias - and for total error as well - can be accordingly calculated. The main drawback of the biological variation approach is that V_w values may differ from individual to individual, and in some pathological conditions they may be different from those measured in the healthy state. Nevertheless, this approach permits the setting of objective, scientifically based analytical goals. Such goals must not to considered as inflexible criteria of acceptability, but just as goals to be achieved with a reasonable effort. For an effective and credible implementation of the biological variation-based strategy, reliable estimates of V_w and V_b values must be available.

S3.2

THE "STATE OF THE ART" AS ANALYTICAL QUALITY GOAL

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Background. Management of the medical laboratory implies the statement of performance goals. An international agreement exist that proposes a hierarchical model to define quality goals for the analytical step of medical laboratory processes. Various strategies for defining goals are suggested, categorized in inverse order according to their relation with the clinical application of laboratory tests.
The "state of the art" occupies a intermediate position in this hierarchy. The goal for the individual laboratory is to reach similar performance that other laboratories using same analytical methods, for each analyte tested.. *How to know the state of the art?.* This information is, currently, acquired by participation in external quality assessment schemes. The parameter used to indicate the state of the art is inter-laboratory imprecision, expressed as coefficient of variation.
How to use the state of the art?. Individual laboratories can use this datum for two practical applications:
1) to design their internal quality control protocol, so to obtain a signal before their performance surpasses the acceptability delimited by the state of the art,
2) to evaluate their long-term imprecision comparatively with the state of the art. When the goal is not reached, an improvement process must be initiated.
The majority of external quality assessment schemes use state of the art to indicate whether the participant laboratories pass satisfactorily each survey. However, other alternatives are also used in European schemes.
Pros and cons. The main advantage of using the state of the art as quality goal is its practicability. It has also important disadvantages, such as changeability and method dependency. All these considerations will be discussed in the presentation.

S3.3

GOALS FOR IMPRECISION, BIAS AND TOTAL ANALYTICAL ERROR IN INTERNAL QUALITY CONTROL SCHEMES

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One criterion recommended for establishing the goals for analytical imprecision, bias and total analytical error is derived from data on biological variation[1].
The use of this criterion has already been questioned for various reasons. The main limitation is that its goals are often too broad or too constringent with respect to the state-of-the-art of the analytical performance.
For the limits in analytical imprecision we propose an arbitrary choice based on a compromise between biological variability and the state-of-the-art of analytical performances.
When the state-of-the-art is better than calculated by the biological variation model, we can define optimum and desirable limits at the appropriate fractile of the state-of-the-art and minimum performance at 0.75xCV of biological variation within-subject (CV_w). Inversely, when the limits are too stringent, an appropriate fractile of the state-of-the-art can be chosen as minimum performance.
For instance, with total calcium CV_w = 1.9%, minimum, desirable and optimum performance according to biological variation model is 1.4, 1.0 and 0.5 %, respectively. We propose 1.9, 1.3 and 1.1 % respectively (i.e. the 0.50, 0.20 and 0.10 fractile of the state-of-the-art of analytical variability).
Likewise, the allowable bias calculated by biological variation model is not adequate in internal quality control scheme where the bias should be zero, since the goal for the systematic error should be "to remain the same". Therefore, we propose for bias the limits estimated from statistical difference (at optimum, desirable and minimum imprecision) between the target value of internal control material and the mean resulting from a definite number of measurements.
For instance, with total cholesterol CV_w = 6.0 %, and CV_b (biological variation between-subject) = 15.2 % the desirable bias according to biological variation model is 4.1 %, while with an actual analytical CV = 3.0 % we propose for bias a limit of 2.3 %.
Therefore the maximum total analytical error (MTAE) can be calculated using our proposed values for imprecision and bias in the usual formula MTAE = k x imprecision + bias; where k is 1.65 or 2.33 for 95% or 99% acceptance.

[1]*Fraser CG, Hyltoft Petersen P, Libeer JC, Ricos C*: Proposals for setting generally applicable quality goals solely based on biology. Ann Clin Biochem 34:8-12, 1997.

S3.4

RELATIONSHIPS BETWEEN INTER-INDIVIDUAL BIOLOGICAL VARIATION AND REFERENCE VALUES

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Inter-individual biological variation (V_b) and reference intervals (RI) are basically the result of the same biological phenomenon. They differ in two aspects. One is their medical use: V_b values are used for the definition of allowable analytical accuracy and RI are used for comparison of patients' results. The other aspect is that V_b values are a rather "pure" measure of the biological phenomenon, while RI result from the sum of V_b, of intra-invidual biological variations (V_w) and of analytical variation (V_a). Applying proper calculations, V_b values can be derived from RI, if values of intra-individual biological (V_w) and analytical components (V_a) of variation are known. We applied such calculations to a set of 29 analytes included in the EQA scheme in Clinical Chemistry of the Regione Lombardia. Data for V_w were obtained from literature; V_a was assumed to be at the analytical goal level ($V_a = 0{,}5 \times V_w$); RI were those currently in use in the laboratory, or those reported in literature. The calculated V_b were then compared with the experimental V_b, as reported in literature. For more than a half of the analytes, the calculated V_b values favourably agreed with the experimental values (difference within ± 30 % of the experimental values), whilst higher differences were found for the rest of the analytes. Such a disagreement may indicate that either the V_b or the RI are not correctly estimated. The experimental values of V_b reported for some analytes as > 33 %, were already considered to be absurdely elevated, whilst in other cases errors seemed more likely to reside in the accepted RI. In any case, comparing calculated values of V_b to experimental ones can be considered a useful exercise to check the correctness of the reported values. A foundamental assumption is that both V_b and RI show a gaussian distribution, but this is not always the case. It must be stressed that in case V_b values are not reliable, care should be taken in using them to calculate analytical goals and allowable errors.

LP2

TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES: MOLECULAR, CLINICAL AND DIAGNOSTIC ASPECTS

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Transmissible Spongiform Encephalopathies (TSEs) or Prion diseases are fatal, neurodegenerative disorders of the central nervous system affecting animals and humans (i.e. scrapie in sheep and goats, BSE in cattle and Creutzfeldt-Jakob in man). They are typically but not exclusively characterized by a triad of pathological lesions within the brain: neuronal loss, hypertrophy and hyperplasia of astrocytes and spongiform degeneration. Amyloid plaque formation is a further prominent feature in some circumstances. Clinical signs of these diseases take months, years or even decades to develop and the accumulation of the pathological prion protein (PrPsc) is the hallmark of TSEs. PrPsc is the misfolded isoform of PrPc: a host-encoded glycoprotein located at the cell surface through a glycosyl-phosphatylinositol anchor (GPI). PrPc is present in a variety of tissues, mainly expressed in the central nervous system. The precise physiological function of PrPc remains enigmatic but a wealth of experimental data demonstrates its essential role in the susceptibility and in the pathogenesis of TSEs. The conversion of PrPc into a detergent insoluble and partially protease-resistant isoform (PrPsc), is the key event in the pathogenesis of TSEs. PrPc and PrPsc are identical in amino acid sequence but differ only in their conformational structure. Spectroscopic studies reveal that PrPc has a high α-helical content whereas PrPsc is rich in β-sheets. PrPsc is, according to prion theory, the sole component of the infectious particle, but an alternative hypothesis is that PrPsc serves as a shuttle substance or a receptor for a yet undiscovered virus-type agent. TSE infectious agents do not induce in the host any apparent immune response. TSEs occur either in sporadic or genetic forms. Several allelic forms of PrPc are linked to disease susceptibility in mice, sheep and man where there is a strong genetic linkage between familial TSE cases and mutations of the human PrP gene (*PRNP*). The recent epidemic outbreak of BSE in UK and the finding that BSE can also be found in many other European countries raises anxiety and concerns. Identification of variant CJD (vCJD) in 1996 was based on novel neuropathological and clinical features in a series of ten young patients. Likely, vCJD results from the ingestion of BSE infected tissues. In vCJD but not sporadic CJD patients, PrPsc accumulates in lymphoid tissues: a finding that raises concern about the possibility of infectivity in blood of vCJD patients. One hundred and one individuals have so far died of vCJD (3 in France, 1 in Ireland, 97 in UK). All analysed cases of vCJD are homozygous for methionine at codon 129 of the *PRNP* gene. We don't know if cases of vCJD in individuals who are homozygous for valine or heterozygous at the codon 129 of the *PRNP* gene will develop the disease in the next future. Continued surveillance is required to further investigate this possibility. It is not yet known how many people are incubating vCJD in the UK and elsewhere in Europe. The potential for man-to-man transmission of vCJD via blood, blood-derived products or improperly sterilized surgical instruments, is also a matter of concern. To prevent the spread of the disease and to determine how many people are infected we would need an effective, simple and sensitive diagnostic test.

S4.1

ASSESSMENT OF SPERM FERTILIZING ABILITY

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Fertilization is a multifunctional process in which the progressively motile and morphologically good human spermatozoa ascend the female genital tract, interact with the fluids present (cervical mucus, uterine, tubaric, peritoneal and follicular fluids), undergo capacitation and acrosome reaction, penetrate the cumulus oophorus, bind with the zona pellucida, pass through it, penetrate the ooplasm and finally decondense the nucleus of the head in order to form the male pronucleus that will fuse with the female pronucleus.

Many tests have been suggested in order to study, for diagnostic reasons, the different functions and properties of spermatozoa (tab 1): basic semen analysis, strict Kruger's criteria morphology, post coital test (PCT), Kremer test, sperm-cervical mucus contact test (SCMC test), *in vitro* semen preparation tests, acrosome reaction determination tests (AR), hemizona assay (HZA), zona-free hamster ova/ sperm penetration assay (SPA), nuclear decondensation, chromatine-DNA normality, chromosomic abnormalities, immunological tests, *in vitro* fertilization (IVF).

Tabella 1 - Sperm function tests

Sperm function	*Functional test*
Cervical mucus penetration	PCT, Kremer test, SCMC test
Capacitation	SPA, IVF
Acrosome reaction	AR, SPA, IVF
Zona pellucida bound	HZA, IVF
Zona pellucida penetration	IVF
Spermatozoon-oocyte fusion	SPA, IVF

The features of a test which assesses the sperm function and fertilizing abilities are the following: well-defined biological rationale which enables the creation of an experimental model "in vitro" which can be compared with the situation "in vivo"; standardization of the protocol; reproducibility of the methods; constant quality control; the predictive value on the fertilizing ability: the test should , all things considered, be able to distinguish fertile subjects from sterile subjects.

The main sperm function tests available today are briefly described in this presentation, considering the control of their reability, their diagnostic usefulness, their predictive value on fertility, their correlation with the *in vitro* fertilization (IVF) results.

Sperm function is extremely complex and the fertilizing ability is correlated not to a single sperm function but to the whole process. So it is normal that there is no seminal assessment parameter or sperm function assay that alone can predict fertilization and potential of conception.

In 1991 Oheninger (Modern Andrology) had outline a diagnostic sequential scheme at many levels for the quantitative and functional assay of seminal fluid in the case of assisted reproduction (tab 2). At the first level there are the descriptive assays and the tests for the initial screening and for the correct therapeutic approach to male infertility. At the second level are the sperm function assays. At the third level there are those tests that study the sperm function directly linked to fertilization: these tests are carried out exclusively on selected semen specimens.

Tabella 2 - Diagnostic sequential scheme for the seminal assessment

1° level (initial screening)
- basic semen analysis
- strict Kruger's criteria morphology
- microbiological tests
- immunological tests
- in vitro semen preparation tests

2° level (functional tests)
- computer-assisted semen analysis (CASA)
- acrosome reaction determination tests
- biochemical determinations

3° level (functional tests directly linked to fertilization)
- hemizona assay
- zona-free hamster ova/sperm penetration assay
- nuclear decondensation
- chromatine-DNA normality
- chromosome abnormalities

Reference: Ombelet W., Vereecken A. (1995) Modern Andrology: Hum. Reprod. Volume 10 (supplement). Oxford University Press.

S4.2

HUMAN MTDNA MUTATIONS AND MALE INFERTILITY

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Untreatable subfertility due to poor semen quality accounts for 75% of male patients consulting for fertility problems. This is mainly due to low number of sperm cells (Oligozoospermia) or to impaired sperm motility (Asthenozoospermia). Spermatozoa motility depends on the ATP supplied by the mitochondrial oxidative phosphorylation (OXPHOS) system. There is some evidence that mutations in mtDNA influence the performance of mitochondrial ATP production, and consequently these mutations could be responsible for sperm dysfunction.

MtDNA is characterized by maternal inheritance, polyploidia, and high frequency of mutations. Moreover, the mutation rate varies among individuals of the same maternal lineage and between different tissues and cells of the same subject.

Mitochondrial mutation rate is 10-100 times higher than in nuclear DNA and, in addition, mitochondria lack an adequate DNA-repair mechanism, which can further increase the mutation rate.

MtDNA alteration can be analyzed in term of haplotypes and haplogroups. Such mutations accumulate within populations in relatively high number because mtDNA does not suffers selective pressure throughout the male lineage. Recent studies have examined population sample of white men having fertility problems by analysing the distribution of mtDNA haplogroups. Asthenozoospermia, but not Oligozoosperimia, resulted to be associated with mtDNA haplogroups; moreover, in the groups of Asthenozoospermia patients, a significant difference in the performance of oxidative phosphorylation system has been observed.

S4.3

PREIMPLANTATION GENETIC DIAGNOSIS

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Recent advances in molecular and fluorescent in situ hybridization (FISH) techniques have allowed the diagnosis of the most common inherited diseases from a single cell. Embrio biopsy of 1-2 cells and/or polar body biopsy have been used for the preimplantation diagnosis of single gene defects and numerical or structural chromosomal aberrations.

The polymerase chain reaction (PCR) is designed to amplify a specific DNA fragment to a level at which it can be subjected to further genetic analysis such as single stranded conformational polymorphism, heteroduplex, sequencing. These methods are subject to a variety of pitfalls: allele dropout (ADO), contamination and reduced amplification efficiency. The utilization of different DNA amplification strategies (i.e. multiplex PCR and fluorescent PCR) is discussed in order to reduce the risk of misdiagnosis associated with single cell analysis.

Preimplantation genetic diagnosis of common aneuploidies using interphase FISH analysis has been applied in human blastomeres and polar bodies with the aim of selecting against aneuploidy before embryo replacement. FISH has also been used in carriers of balanced chromosome rearrangements. FISH protocols using two rounds of hybridization and spectrally-distinct fluorescent labels permit to enumerate simultaneously on a single cell the chromosomes X,Y,13,14,15, 16,18,21 and 22, which would cover 70 per cent of the aneuploidies. This technique is not free of error: further technical improvements may be achieved, but errors due to mosaicism will not be eliminated unless more cells can be analysed (blastocyst stage). More recently combined approaches have been developed using non specific amplification of the entire genome (WGA) through PEP-PCR or DOP-PCR, and, successively, Comparative Genomic Hybridization (CGH) or Quantitative Fluorescent PCR to assess aneuploidies.

In summary the need for careful clinical evaluation of different approaches in preimplantation genetic diagnosis is urgent. Two networks of centres have been established to work together towards accumulating appropriate data: the International Working Group (IWG) and the Special Interest Group in Reproductive Genetics of the European Society for Human Reproduction and Embriology (ESHRE). The challenge is to evaluate the accuracy, safety and value of PGD as a really alternative form of prenatal diagnosis.

Sessione 6 - TECNICHE DI AMPLIFICAZIONE: LA STRADA VERSO L'AUTOMAZIONE
Sala D
Mercoledì 13 giugno, ore 11.30-13.00

S6.1

NEW SYSTEMS FOR IMPROVING QUANTITATIVE PCR

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Molecular biology laboratories need technology that is easy to use yet sufficiently flexible for a variety of applications. Real-time quantitative PCR (QPCR) allows researchers to quickly and easily quantify nucleic acids for studying gene expression, mutations analysis, disease state, and gene dosage. QPCR measures PCR product accumulation during the exponential phase of the reaction using fluorescent data and provides accurate information on initial starting copy number. PCR amplification and detection are combined in a single step, speeding the process of gathering experimental data. The QPCR process uses a single closed tube, which eliminates the need of numerous post-PCR manual steps, and reduces the possibility of introducing variability or laboratory contamination.

The application of fluorescence techniques to QPCR, together with suitable instrumentation capable of combining amplification, detection and quantification, has led to the development of kinetic QPCR methodologies that are revolutionizing the possibilities for quantitating nucleic acids. There are three competing instruments on the market; all three are run as closed-tube systems and quantification requires no post-amplification manipulation. This avoids problems of contamination, results in short turn-around times for data acquisition and analysis and minimizes hands-on time. The entire process, from PCR to full quantification, is automated , which makes these instruments ideally suited for high-throughput screening applications.

There are currently four competing techniques available that detect amplified product with about the same sensitivity. They use fluorescent dyes and combine the process of amplification and detection of a DNA or cDNA target to permit the monitoring of PCR reactions in real-time during the PCR; their high sensitivity eliminates the need for a second-round amplification, and decreases opportunities for generating false-positive results. The simplest method uses fluorescent dyes that bind specifically to double-stranded-DNA. The other three rely on the hybridization of fluorescent-labeled probes to the correct amplicon. The methods differ in their specificity, although at later amplification cycles all can show artifacts that do not correlate to specific product accumulation. As amplicon detection in the molecular beacons, hydrolysis and hybridization probe assays depends on successful hybridization of the probe, these QPCR procedures obviate the need for post-PCR Southern analysis or sequencing to confirm the identity of the amplicon.

S6.2

DETECTION OF DNA POLYMORPHISMS AND MUTATIONS BY MELTING CURVE ANALYSIS

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The imminent completion of the Human Genome Project is likely to lead to the identification of new genetic factors that contribute to human disease. In particular, the growing knowledge on DNA polymorphisms may extend our capacity to investigate the molecular bases of multifactorial tracts to define risk determinants and to determine genetic profiles in the population with respect to drug response.

Biallelic Single-Nucleotide Polymorphisms (SNPs) have become increasingly popular markers in molecular genetics because of their wide application both in evolutionary relationship studies and in the identification of susceptibility to common diseases. We have addressed the issue of SNP genotype determination by investigating variations within regions where several human Calcium channel genes are mapped in order to evaluate their role in the pathogenesis of common forms of Migraine. A new SNP scoring method, dynamic allele-specific hybridization (DASH), has been tested in order to set up a panel of SNPs markers to be applied to genetic studies. The key feature of DASH is dynamic heating and coincident monitoring of DNA denaturation. The assay is performed in a 96-wells plate compatible with automation: single strand target sequence is bound to a microtiter well and an oligonucleotide probe is hybridized in the presence of an intercalating dye. The sample is then heated and fluorescence emission is monitored over a wide temperature range in order to detect the melting temperature of the probe-target hybrid, which is indicative of their mismatch or perfect match. Experimental conditions have been set up for a total of 18 SNPs located on chromosomes 1, 3, 9, 10, 17, 19 and close to the CACNA1E, S, D, G, A, CACNB2 genes and will be applied to the study of familial Migraine.

A second goal of our work was to set up molecular testing of known genetic factors involved in the development of thrombotic disorders in humans by a real-time PCR approach on a Light Cycler system. Among these genetic factors the following have been selected: factor V/G1691A, prothrombin/G20210A, methylenetetrahydrofolate reductase (MTHFR)/C677T, cystationine beta-synthase (CBS)/844ins68. A melting curve analysis of amplycons using the hybridization probes format was performed. With this method, amplification and genotyping are performed in the same capillary, therefore minimizing the risk of contamination. Detection of mutations is based on the temperature-dependent hybridization of pair of specific probes while performing the melting curve analysis. The

assay was successfully applied to genomic DNA from whole blood. The factor V and prothrombin status of 60 patients previously analyzed by restriction analysis, was completely confirmed by this method. In addition, this assay was very fast, allowing genotyping of samples in about 30 minutes. The ability to study a large number of samples rapidly and accurately is a critical goal for the next future application of DNA analysis in routine medical diagnostics and real-time PCR seems to represent an outstanding tool to reach this objective. Also other emerging technologies such as bio-chip, real-time-sequencing, etc., together with the availability of the human draft genome sequence are opening a new era in molecular medicine.

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S6.3

ADVANCED TECHNOLOGIES FOR QUANTITATIVE EVALUATION OF MINIMAL RESIDUAL DISEASE IN HEMOPOIETIC TUMOURS

Pane F.

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Accurate assessment of therapy efficiency is a challenge in current protocols in acute and chronic leukemias. Fusion transcripts from leukemia-associated hybrid genes, beside providing the basis for a new genetic classification of leukemias, have been proved to represent very sensitive and reliable markers for minimal residual disease (MDR) detection by PCR techniques. The clinical impact of MRD detection by classic RT PCR analysis of fusion transcripts has been clearly shown at least for chronic myeloid leukemia (CML) and acute promyelocytic leukemia patients. but the precise significance of MRD detection to assess the risk of relapse in acute leukemias patients still awaits to be fully established in most leukemias subgroups.
In recent years, real-time quantitative RT-PCR (RQ-PCR) was introduced allowing high throughput quantitative analysis of the fusion transcript levels in clinical samples. Twenty-five European laboratories from 10 countries including our institutions, have collaborated in order to establish consensus standard protocol using RQ-PCR for the main leukemia-associated fusion transcripts. The collaborative effort led to the development of an international consensus protocol based on a RQ-PCR technique to quantitate the levels of hybrid transcripts in biological samples and hence, to establish, in quantitative terms, the amount of residual leukemic cells in patients at diagnosis and during therapy follow-up. Selected primer and probes allow good sensitivity levels and the threshold of detection of 100 molecules and or 10^{-4} dilution was reached for all the fusion genes.
We monitor MRD by RQ-PCR in 32 adult B-ALL patients who were BCR/ABL-positive. As expected according to the poor prognosis associated to this genetic abnormality, we were able to observe only in 3 cases the complete clearance of BCR/ABL transcript at the end of consolidation. In this case we noted a significant correlation between the level of MRD and outcome: 14 of the 15 patients who were treated by conventional maintenance chemotherapy relapsed after a progressive increase of MRD levels, while among the 15 patients treated by bone marrow transplantation, 6 patients showing a reduction of MRD level below the detection limit, and 2 patients in which the levels of MRD were persistently close to the limit, had a long term remission (median survival >60mo). Therefore, MRD detection by RT-Q-PCR of leukemia-associated fused transcripts is a promising tool for the management of therapy in acute leukemias and for the development of appropriate patient-tailored treatment strategies.

S6.4

APO E GENOTYPING: COMPARISON BETWEEN RESTRICTION ENDONUCLEASE MAPPING AND ALLELIC DISCRIMINATION (LIGHT CYCLER)

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The molecular bases of apoE polymorphism are cysteine-arginine interchanges which determine three codominant alleles, designated ε2, ε3 and ε4. The apoE3/E3 is the most common genotype, the apoE4 protein is associated with increased total serum cholesterol and greater risk for coronary heart disease (1) and it also constitutes a major risk factor for Alzheimer's disease, the apoE2 protein seems to have a protective effect against Alzheimer's disease and might be associated with longevity. The aim of our study was to compare and evaluate two different methods for the apoE genotyping both based on the polymerase chain reaction: the restriction endonuclease mapping and the commercially available DNA assay that use rapid-cycle PCR and fluorescence resonance energy transfer (FRET) with the LightCycler. 1)The PCR was performed with the listed primers: ApoE sense: 5' TAA GCT TGG CAC GGC TGT CCA AGG A 3'; ApoE antisense:5' ACA GAA TTC GCC CCG GCC TGG TAC AC 3'.The amplification product was a 247 bp fragment, containing codons 112 and 158. ApoE amplification products were digested with *CfoI/HhaI*, overnight at 37°C, and evaluated in 12% polyacrilamide non denaturating gel. 2)In the Light Cycler ApoE Mutation Detection Kit a 265-bp fragment containing exon 4 of the apoE gene is amplified from human genomic DNA. The detection probes covering codons 112 and 158 are 5' labeled with LC-Red 640 and LC-Red 705, respectively. The corresponding anchor probes are fluorescein-labeled at their 3' ends. When a pair of hybridization probes hybridizes to the same strand internal to the unlabeled PCR primers, the probes come in close proximity, producing FRET. During FRET, the acceptor fluorophores LC-Red 640 and LC-Red 705 emit fluorescence, which is measured in exact temporal coincidence in two different channels of the optical system of the Light Cycler, using a linear arrangement of dichroic bandpass filters. We tested 86 subjects and we found a 100% concordance between the two methods. In conclusion the Light Cycler allelic discrimination method for ApoE genotyping seems to be rapid, simple and accurate.

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This study was supported partly by a grant from MURST cofin. and Ministero della Sanità-Italy

S6.5

TAQMAN™ TECHNOLOGY IN A PILOT C282Y HEMOCHROMATOSIS SCREENING IN ITALIAN NEWBORNS

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Hereditary hemochromatosis (HH, MIM#235200) is a disorder of iron metabolism that can be caused by homozygosity for the C282Y mutation in the HFE gene. Preliminary studies have estimated the frequency of this mutation at 0.5-1% in Italy, but this has not been verified on a large sample. The aim of our work was to assess the C282Y carrier frequency in a cohort of Italian newborns and to validate a new automated assay with high throughput for rapid detection of the mutation in large samples. The C282Y mutation was determined by the Taqman™ technology, a fluorogenic 5'-nuclease assay in which sequence-specific signal is generated and detected in solution during PCR, without electrophoresis or post-PCR processing (Livak KJ et al., (1995) PCR Methods Appl. 4, 357-362). DNA samples from 1331 consecutive newborns, all of Italian origin, were examined. They were obtained from dried blood spot (DBS) samples collected from the newborn screening Center. The C282Y mutation was determined by the TaqMan™ technology, which is based on the use of two fluorescent dyes (a reporter and a quencer) both attached to the probe. During PCR, the probe anneals to the target sequence between the forward and the reverse primer sites. If hybridization occurs, the probe is cleaved by the 5'-nuclease activity of the polymerase. This separates the reporter from the quencer, generating an increase in the reporter's fluorescence. Differences in fluorescence facilitates discrimination of all HFE genotypes at the 282 position. The C282Y mutation was confirmed in all positive samples assessed using an alternative method. Results Of 1331 samples examined no newborn was homozygous for C282Y, whereas 55 were C282Y heterozygotes. The overall frequency of the C282Y allele in Italy is, therefore, 2.1±0.6. The highest allele frequency was found in samples from the Northern region of Italy (2.7±1.3) and the lowest (1.7±0.9) from South-Central Italy. Discussion Our results establish the frequency of the C282Y mutation in Italy to be around 2% and confirm that the carrier frequency is lower than that found in North Europe (5-12%). The low C282Y carrier frequency recorded in Italy indicates that a molecular screening on newborns would not be cost-effective. This study also suggests that the TaqMan™ technology (with its rapidity, reliability, reproducibility and low costs, 3 EURO/sample) appears ideal for a neonatal screening program of hemochromatosis in appropriate populations.

LP3

THE ROLE OF LABORATORY MEDICINE IN EVIDENCE-BASED CLINICAL MANAGEMENT

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Diagnostic orientation, classification of signs and symptoms of the patient, critical assessment of the clinical relevance of each of them, selection and interpretation of appropriate laboratory and instrumental tests represent in summary the steps of the methodological pathway from the clinical suspect to the diagnostic confirmation. The choice and the interpretation of laboratory findings constitute an important nodal point for the global preparation of physicians. The selection of laboratory tests must be precisely addressed by the targeted clinical orientation emerged from the in-depth study of the individual patient; the same selection should be performed among the tests provided with the best diagnostic relevance, the best risk/benefit ratio, the best cost/effectiveness balance.

When the result of laboratory tests agrees with the clinical-diagnostic orientation, the last one gains a confirmation. When it is not like that, physicians have to go back to the clinical pathway so far adopted. Many and different possibilities of error have to be considered; Evidence Based Medicine (EBM) is a useful methodological guide for critical re-evaluation.

With special reference to clinical biochemistry, every single traditionally recognised phases (the pre-analytical one, the laboratory analysis and the post-analytical phase) can take advantage of the correct implementation of the method of Evidence Based Medicine.

The well-known acquisition, derived from the scientific literature, that the studies evaluating the characteristics of laboratory assays often do not satisfy methodological quality standards dealing with the independent evaluation of the tested assay, the judgement on the precision and the reproducibility of the method studied, the analysis of the correctness in the description and application of laboratory procedures, should lead to a more marked diffusion and implementation of EBM method. Moreover, this objective and appropriate scientific approach favours the interchange between clinical biochemists and clinicians.

Clinical biochemistry is a really important and promising field for the promotion of the EBM, since in the analysis laboratory many diagnostic, but also therapeutic and prognostic features of modern medicine, meet. The example of Troponins is, with reference to what so far said, paradigmatic: these biochemical components, initially considered "just" useful acute myocardial infarction (AMI) markers, turned to be also reliable prognostic indices, appropriate guides for the most appropriate therapeutic strategy in acute coronary syndromes, and now they promise to be, in the framework of Point-of-Care-Testing, indicators of the efficacy and effectiveness of best current therapeutic options in AMI.

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Sessione 8 - ANALISI DECENTRATE
Sala B
Giovedì 14 giugno, ore 10.00-11.15

S8.1

AN EXPERIENCE OF QUALITY IMPROVEMENT OF POINT-OF-CARE-TESTING

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The concept of quality is always related to the historical and organizational situation which referes to. Today, in the Laboratory Medicine the target is the final quality of the clinical results (outcomes). So the criterions and the tools of measure of the quality are recently changed.
Particularly, in the case of POCT, the idea of quality seems to assume different meanings according to the point of view: the clinicians and the health care personnel takes a "subjective" vision of the quality in terms of convenience, rapidity in the availability of results, reduction of bureaucracy.
The laboratory personnel privileges essentially a most objective vision of the quality as analytical precision and accuracy. It could appear more realistic a synthesis between this two views. This was recently proposed by Christopher Price who sets to the first place the clinical decision and with this paradigm we can revise a lot of consolidated laboratory traditions.
With these criterions the Laboratory of the University-Hospital of Padua has started a control of some decentralized tests of coagulation, bloodgas, electrolytes and whole blood glucose. In details we recommanded procedures that document the activities and the daily performances of the quality control. Regarding the electrolytes we ascertained that there were some analytical problems that we faced and resolved monitoring the systems. This fact pointed out the problem of the analytical quality. This is traditionally expected when there are new analysers autocalibrating and with good maintenance: according to some recent literature reports we suggest a better attention to the results of quality control of decentralized analyses.

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S8.2

POINT OF CARE TESTING IN EMERGENCY MEDICINE

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Introduction. Point of care testing (POCT) is an alternative to central laboratory testing. POCT can be carried out at the bedside or the equipment can be in a suitable room inside a ward. Generally POCT meets the needs of a selective population such as that in the emergency department (ED), intensive care unit or operating room. In the last few years POCT has been used more and more often inside the ED, because emergency physicians need , in some circumstances, to obtain the analysis results very quickly in order to make a rapid diagnosis and be able to institute immediate therapy or change in therapy. Many emergency physicians believe that POTC could shorten the time spent in the ED, the length of stay in hospital, and the time taken to decide on the management of some patients. Although a paper published in BMJ by Kendall (1998;316:1052-1057) shows there are no differences in the amount of time spent in the department , in admission rate, in the length of stay, or mortality between those patients whose samples were tested at the point of care and those whose samples were tested by the central laboratory, there is however evidence, in the same article, that POCT produced a critical clinical time benefit for about 7% of patients and also influenced treatment in 14% of all cases.
POCT, like the laboratory, generates medical information that leads to clinical action. When the devices are used inappropriately the information can be incorrect and this is a risk to the patient. Therefore, it is important to ensure the quality of POCT . This can be done through standardized training of the operators (physicians and nurses) by using a training check list, written procedures and demonstrations. An interdisciplinary approach between central laboratory staff (physicians and technicians) and emergency department staff (physicians and nurses) is crucial for managing the quality of POTC.

The experience of POCT management in the Emergency Department of Alessandria General Hospital.

This emergency department sees about 40.000 adult people a year. About 2% of the patients are triaged as red and about 17% as yellow. Close to the emergency department there is a high dependency unit and an observation unit. Inside the observation unit there is a chest pain unit. The emergency physicians felt that given this complex organisation it was essential to be able to obtain some

analyses very quickly, because any situation that threatens a patient's life requires rapid resolution. Therefore, in 1999, it was decided, in agreement with the Hospital Manager and the Chief of the central laboratory, to establish a POCT in the ED as well as: 1) which analyses to do ; 2) the criteria for buying the instruments; 3) which operators to use and how to do the training for the management of POCT; and, 4) the necessary supervision to guarantee the POCT quality assurance. The analyses chosen were: haemoglobin measurement, pH and blood gases, carbon monoxide, electrolytes (sodium, potassium, chloride, calcium), glucose, lactate and troponin I. The criteria for buying the instruments were cost, reliability and simplicity of use. Qualified technicians trained all physicians and nurses in the management of the instruments and the time spent for every group of five people was about one hour at the beginning and another hour a month after, as refreshment. The supervision and technical assistance was and is guaranteed by a delegated physician and a central laboratory technician and inside the ED there is a written protocol and check list for the simple maintenance of devices.

Discussion. It is our opinion that the use of POCT in our emergency department is undoubtedly very important for rapid recognition of life threatening conditions (e.g. carbon monoxide poisoning, acute respiratory insufficiency, extreme hypopotassemia or hyperpotassemia), as well as for rapid intervention and stabilisation of patients (e.g. trauma patients and patients with gastrointestinal haemorrhage where the possibility to monitor haemoglobin concentration is very important).

Another field where POCT has proved very useful, is in the management of chest pain. Since the chief complaint of about 5% of the patients arriving in the emergency department is chest pain, it is important, in the absence of a diagnostic ECG, to rule out an acute myocardial infarction by monitoring troponin I as a cardiac marker inside the emergency department or inside the chest pain unit .

The only problem we have had with the management of the devices was caused by high temperatures during the Summer.

Conclusion. We believe that the availability of POCT is very useful in managing critical clinical situations in the Emergency Department, even though formal evidence is still limited and economic benefits have yet to be demonstrated.

S8.3

IS BNP, MEASURED USING A BEDSIDE ASSAY, A USEFUL TEST FOR VENTRICULAR FUNCTION ASSESSMENT IN PATIENTS WITH INFARCTION?

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Brain natriuretic peptide (BNP), a proteohormone predominantly secreted by myocardial ventricles, is a sensitive marker of changes in ventricular function. Recently, Biosite Diagnostics developed a rapid (~20 min) method for measuring BNP in whole blood on the Triage Meter, a portable fluorometer, offering the first real possibility for an easy BNP measurement in clinical practice. Here we evaluated the possible use of this marker as an outlook predictor in patients with acute myocardial infarction (AMI) admitted to coronary care unit (CCU), by determining the relation of BNP concentrations to left ventricular (LV) function (evaluated by gated SPECT imaging) and to infarct size (estimated by CK-MB peak concentrations and SPECT myocardial perfusion using Tc 99m-sestamibi). Measurements of BNP, on a single fresh EDTA whole blood sample, and SPECT were performed 2-5 days after symptom onset, i.e., in the morning on the day of CCU discharge, in 33 patients with AMI but without congestive heart failure (median time after symptom onset to admission: 4 h, range 0.5-38). SPECT was repeated approximately three months after AMI in 20 of them. Serum samples for CK-MB peak estimation were taken every 6 h throughout the first 48 h after admission. CK-MB mass concentrations were measured on the Roche Elecsys system. Blood samples for BNP assay were also obtained from 28 healthy individuals. BNP concentrations were markedly increased in AMI patients compared with normal controls (183±176 vs 9±10 pg/mL, $P<0.0001$). Reperfusion therapy (thrombolysis or angioplasty), given to 25 (76%) patients, did not significantly influence BNP concentrations (186±182 pg/mL in reperfused vs 172±165 pg/mL in non-reperfused, $P=0.85$). A significant positive correlation was found between the increase in plasma BNP concentrations and both the peak CK-MB concentration ($r=0.52$, $P=0.002$) and the perfusion defect size, expressed as % of left ventricle, at SPECT ($r=0.48$, $P=0.005$). Conversely, BNP measured in the acute phase of AMI was not related to LV ejection fraction measured both early (two to five days) and late (three months) after AMI ($r=-0.27$, $n=33$, $P=0.12$; and $r=-0.33$, $n=20$, $P=0.15$, respectively). Thus, the increase in plasma BNP during the early phase of AMI clearly reflected the infarct size but did not correlate with the amount of LV dysfunction. Further studies in an unselected AMI population are required to definitively clarify the role of BNP determination in the evaluation of LV dysfunction in these patients.

Sessione 9 - DIAGNOSTICA AVANZATA DELL'INFERTILITÀ MASCHILE
Sala D
Giovedì 14 giugno, ore 10.15-11.15

S9.1

MALE INFERTILITY AND CHROMOSOME ANOMALIES

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Human male infertility can be caused by genetic factors affecting gamete formation or function; in particular, chromosome abnormalities are often related to male subfertility as shown by their higher frequency in infertile men than in the general population.
The most significant chromosome anomalies in the infertile men constitutional karyotype are the aneuploidy for the sex chromosome, in particular the presence of an extra X chromosome (more frequent in patients with Klinefelter phenotype).
Balanced chromosome abnormalities can also be observed as X/autosome and Y/autosome translocation or autosome reciprocal translocation and robertsonian translocation, especially t(13;14).
It is still unknown how these chromosomal anomalies cause infertility.
Meiotic studies in infertile males have shown that spermatogenesis breakdown is often related to alterations in the process of chromosome synapsis.
Indeed, any condition that can interfere with X-Y bivalent formation and X chromosome inactivation is critical for the meiotic process; furthermore asynapsed regions may themselves help to eliminate spermatocytes with synaptic errors.
Recently the frequency of cromosomal numerical abnormalities of spermatozoa from infertile men with abnormal semen parameters was investigated by FISH.
The disomy frequency was correlated with the severity of oligospermia showing that during spermatogenesis of males with sperm parameters alteration a decresed frequency of meiotic chromosome pairing and crossing over may lead to spermatogenesis arrest at the meiosis stage and /or to an increase of meiotic nondisjunctions.
Meiotic arrest in some germ cells may be responsible for oligospermia and nondisjunctions in other cells for aneuploidy in mature male gametes.

S9.2

GENETIC EVALUATION OF INFERTILE MEN

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In about 40% of all infertile couples, the male partner is found to have no sperm in the ejaculate (azoospermic) or the sperm is of poor mobility or few in number (oligospermic). The reproductive prospects for such men have been enormously improved by the introduction of ICSI. Genetic macroscopic defects (detected by cytogenetic methods) and genetic microscopic defects (detected by molecular biology methods) have been identified. The search in OMIM database for "Male infertility genes" obtains at least 36 entries as result, some of which are brought back in table . These data indicate the complexity of the spermatogenesis and the consequent appraisal of the genetic causes of male sterility .In February 2001 the " Consensus Conference ", concerning the genetic tests of the infertile couples, was instituted and it has been subscribed by several national and international scientific societies so far. Genetic factors affecting male infertility are reviewed. Male infertility is classified under four general causes: spermatogenic disorder, obstruction of the seminal tract, inflammation, sexual disorders. Idiopathic spermatogenic disorder accounts for more than 50% of all of them. The causes of spermatogenic disorders are not yet completely identified. Molecular analysis of Y-chromosomal microdeletions is routinely performed in the work-up of male infertility. For the time being the molecular analysis of microdeletions of the Y chromosome is indicated in infertile patients with sperm concentration <5 x 10(6)/ ml and in men undergoing assisted reproduction techniques. Information for the control of spermatogenesis carried on the Y chromosome was first started by Tiepolo and Zuffardi (1976). Cytogenetic analysis of infertile men revealed that 0.5% had macroscopic deletions of the distal long arm of the Y chromosome (Yq). The presence of an azoospermic factor (AZF) in the long arm of the Y chromosome was proposed and further mapped to Yq11.22-23. The long arm of the Y chromosome contains more genes involved in male infertility, short stature and gonadoblastoma. The BPY2, PRY, TTY1, TTY2, and VCY genes are recently mapped within the Yq chromosome and related to male infertility. The analysis of this genes and related region of Yq chromosome allows to establish a diagnosis and to supply the genetic counselling for the couple, since such microdeletions are transmitted to the male offspring. Where male infertility is the result of congenital bilateral absence of the vas deferens (CBAVD), ICSI can be carried out with sperm recovered from the epididymis. CBAVD in some men represents a mild form of Cystic Fibrosis (CF), but in others the condition is unrelated to CF.

Furthermore, oligo- or azoospermic men show a higher incidence of CF mutations, even when they do not have CBAVD. However, most men with CBAVD are carriers of one (or sometimes two) of the hundreds of mutations of the CFTR gene associated with CF, and these have the potential to transmit CF. Genetic counselling and CFTR screening, therefore, should be offered to all couples where the man has CBAVD and is requesting ICSI. Other autosomal recessive conditions are associated with male infertility, such as the Kartagener syndrome, which results from a mutation in the Dynein gene, causing sperm to be immobile and Kallman Sindrome but these are very rare. Current biotechnology allows the study of many genes related to infertility and can clear the different causes of male sterility. In fact all these genetic tests are necessary both for the clinician and for the genetic counsellor, so that the couples can choose the better solutions and can evaluate the risks correlated to their decision.
The final decision on all questions of reproduction and genetics must be in the individual couple's hands, not in the medical practitioner's or the bureaucrat's. But in order to take such decisions responsibly, bearing in mind the social and ethical norms of the society in which they live, couples require reliable and comprehensible information. Counsellors, and especially genetic counsellors, will be more in demand than ever before: we must make sure that they are well trained, and well paid. Personal autonomy with inadequate information leads to poor decision-making, with dissatisfaction replacing the benefits that technical advance could bring.

OMIM	*Description*
*415000	AZOOSPERMIA FACTOR 1; AZF1
400003	DELETED IN AZOOSPERMIA; DAZ
602421	CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR;CFTR
*400009	UBIQUITOUSLY TRANSCRIBED TETRATRICOPEPTIDE REPEAT GENE ON Y CHROMOSOME; UTY
*40010	DEAD/H BOX 3, Y-LINKED; DBY
480000	SEX-DETERMINING REGION Y; SRY
400005	UBIQUITIN-SPECIFIC PROTEASE 9, Y CHROMOSOME; USP9Y
*601486	DELETED IN AZOOSPERMIA-LIKE; DAZL
*179095	UBIQUITIN-CONJUGATING ENZYME E2B; UBE2B
#277180	VAS DEFERENS, CONGENITAL BILATERAL APLASIA OF; CBAVD
152780	LUTEINIZING HORMONE, BETA POLYPEPTIDE; LHB
*107910	CYTOCHROME P450, SUBFAMILY XIX; CYP19
264600	PSEUDOVAGINAL PERINEOSCROTAL HYPOSPADIAS; PPSH
*164160	LEPTIN; LEP
306970	H-Y REGULATOR; HYR
*152760	GONADOTROPIN-RELEASING HORMONE 1; GNRH1
160900	DYSTROPHIA MYOTONICA 1
*152790	LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR; LHCGR
#253300	SPINAL MUSCULAR ATROPHY I; SMA1
*313700	ANDROGEN RECEPTOR; AR
201910	ADRENAL HYPERPLASIA, CONGENITAL, DUE TO 21-HYDROXYLASE DEFICIENCY

S9.3

SEMINAL PLASMA TRANSFERRIN LEVELS AS AN INDEX OF SEMINIFEROUS TUBULAR DYSFUNCTION

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Sertoli cells play a major role in the evolution and control of spermatogenesis. The aim of this study was to investigate whether the Sertoli cell capacity to produce transferrin depends upon different relationships between germinal cells and Sertoli cells. Transferrin concentrations in seminal plasma were evaluated in 30 normozoospermic fertile men (control group) and in 83 infertile men divided into three groups: 22 normozoospermic, 36 oligospermic, and 25 azoospermic. All infertile men demonstrated the presence of autoantibodies. Cells were obtained by bilateral testicular biopsy. A qualitative and quantitative cytological evaluation was performed. Semen of each patient was collected after three days of abstinence, and examined as recommended by the WHO. Seminal plasma transferrin was measured by an automated immunonephelometric method on the BN II nephelometer (Dade Behring, Italy), based on a specific reaction with rabbit antibodies anti-human transferrin.
Transferrin median value in seminal plasma was 297 $\mu g/mL^{-1}$ (interquartile range: 142-323) in controls, 234 $\mu g/mL^{-1}$ (interquartile range: 167-399) in normospermic infertile men, 152 $\mu g/mL^{-1}$ (interquartile range: 80-187) in oligospermic infertile men, and 118 $\mu g/mL^{-1}$ (interquartile range: 58-183) in azoospermic infertile men. Transferrin values significantly differ between controls and oligospermic and azoospermic men ($p=0.05$) as well as between normospermic infertile men and oligospermic and azoospermic men ($p=0.05$). No significan difference was found between values in controls and those in normospermic infertile men. A significant correlation was found between seminal plasma transferrin and the number of spermatozoa per ejaculate ($r=0.50$; $p=0.001$) in the group of infertile men ($n=83$). No correlation was found between immature cells (spermatogons, primary and secondary spermatocytes)/Sertoli cells ratio and transferrin, while a significant correlation was found between spermatids (both overall and in the different AB and CD maturation phases) and transferrin. These results suggest that transferrin in seminal plasma contributes to the activation of seminiferous tubules and may reflect the Sertoli cell function. This is confirmed by the correlation between the concentration of seminal plasma transferrin and the number of spermatozoa in the ejaculate, suggesting that the secretion of this protein by the Sertoli cells is influenced by the presence of germinal cells, particularly spermatids.

S10.1

OXIDATIVE STRESS IN THE INDUCTION OF CELLULAR SENESCENCE

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Normal cells do not divide indefinitely, but have a finite replicative life span, due to a process termed cellular or replicative senescence. This behaviour of normal cells is in striking contrast to germline cells as well as neoplastic cells that divide indefinitely. The main features of the senescent phenotype include: i) irreversible growth arrest: cells cannot reenter the cell cycle by physiological mitogenic stimuli; ii) increased resistance to apoptotic cell death; iii) altered cellular functions, including changes in differentiation and in regulation of gene expression. Several experimental evidences suggest that replicative senescence can be considered a tumor suppressor mechanism, i.e. cells respond to potential oncogenic stimuli by adopting a senescent phenotype and, hence, it can be considered a cellular response to counteract neoplastic transformation; on the other hand cellular senescence seems to be a prerequisite for the development of aging and of age-related pathologies. Among all different causes inducing cellular senescence, telomere shortening is probably one of the most common: this process, due the repression of the telomerase activity, is very complex and its regulation in mammalian cells is multifactorial and involves telomerase gene expression, post-translational protein-protein interactions and protein phosphorylation. Several cell cycle proteins have been implicated in the genesis of cellular senescence, and these include p53, Rb, $p21^{waf1}$ and others. Recent experimental data demonstrate that the sequencial events leading to can be summarized as follows: p53 activation, $p21^{waf1}$ accumulation and the consequent cell cycle arrest due to cyclins/CDK complex inhibition by this last protein. In our laboratory in the past few years the regulation of the expression of some cell cycle regulatory genes by reactive oxygen species (ROS) has been studied in living cells (1). We demonstrated that the exposure of several cell lines to diethylmaleate (DEM), a GSH depleting agent, decreases p53-DNA binding (2), leads to $p21^{waf1}$ mRNA and protein accumulation through a p53-independent pathway (2, 3) and induces a rapid dephosphorylation of $p21^{waf1}$ protein (4) and of the retinoblastoma protein (5). These results suggest that cells can respond to a mild oxidative stress through the activation of three different processes: 1) the accumulation of $p21^{waf1}$ mRNA and protein; 2) the rapid dephosphorylation of $p21^{waf1}$ and 3) of the retinoblastoma protein. These last molecules, all involved in the cell cycle growth arrest can contribute to the induction of the senescent phenotype. Our studies are now proceeding in two different ways: 1) identification of molecular mechanisms contributing to understand more about the hypothesis of a correlation between oxidative stress, cellular senescence and organismal aging; to this aim experiments are in progress, using the PCR Real Time technique, to compare the expression of some mRNAs, whose levels change in fibroblasts derived from human samples of different ages, in different cell lines (young and senescent human fibroblasts exposed to the DEM-induced oxidative stress); 2) identification of the molecular machinery responsible for ROS production following physiological stimuli as the stimulation of cells with growth factors; a possible candidate for this role is represented by the NADPH oxidase, an enzyme machinery originally identified in phagocytes but present, as recently demonstrated, also in fibroblasts and many other eukaryotic cells. To this aim, we are studying if the inhibition of this enzyme, both by chemical inhibitors as DPI and AEBSF, or by treating the cells with antisense oligonucleotides for the catalytic subunit of this enzyme, interfere with the transduction of mitogenic stimuli.

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S10.2

DETECTION OF MUTATIONS IN THE NDUFS4 GENE OF COMPLEX I ASSOCIATED WITH FATAL NEUROLOGICAL LEIGH-LIKE SYNDROME

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In mammals complex I (NADH-ubiquinone oxidoreductase) of the respiratory chain is made up of 7 mitochondrial-encoded and 36 nuclear-encoded subunits. Papa et al. (1) have found in bovine heart mitochondria that the 18 kDa subunit, encoded by the nuclear NDUFS4 gene (chromosome 5) is phosphorylated by the cAMP-dependent protein kinase localised both in the cytosol and mitochondria (2). In human and mouse fibroblast cultures in vivo, cAMP promotes serine phosphorylation of the 18 kDa subunit and activation of complex I (3). Sequence analysis of mitochondrial and nuclear genes of complex I, in children with deficiency of this complex and exhibiting Leigh-like neurological syndrome, have revealed four different mutations of the NDUFS4 gene in four different families. A homozygous 5bp duplication in the coding sequence of NDUFS4, found in a child with fatal Leigh-Syndrome (4), destroyed the phosphorylation site and abolished cAMP activation of the complex (5). Two mutations resulted in truncated 18 kDa subunit (6), one in suppression of the expression of the subunit (7). This last non-sense mutation impaired the assembly of the complex and suppressed its activity. RFLP methods to detect heterozygous and homozygous mutations of this gene in newborns and families suspected for this defects will be described.

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S10.3

THE OXIDATIVE DAMAGE IN ERYTHROCYTE AGING

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Erythrocytes *in vivo* are targets of oxidative stress because they are exposed to high oxygen concentrations and contain iron that can auto-oxidise. The oxidative denaturation of the erythrocyte membrane has been recognized as a cause of haemolysis in senescent and pathologic cells with a shortened life span. The aim of this work was to study the oxidative damage in erythrocyte ageing as far as membrane structural integrity and metabolic capacity are concerned. The erythrocyte oxidative damage was evaluated in red cells after *in vitro* ageing and upon *in vitro* oxidative stress with tert-butylhydroperoxide (t-BHP). In order to assess the role of oxidative stress in both physiological ageing and acute haemolytic events, we studied erythrocytes from healthy subjects and from patients with G6PD-Mediterranean, a common erythrocyte defect associated with oxidative congenital haemolytic anaemia.
Cell damage was evaluated on the basis of 1) morphological alterations; 2) level of metabolic intermediates (GSH, ATP, 2,3-DPG), and enzyme activities (G6PD, GR, PK, HK); 3) level of lipid peroxidation products (TBAR); and 4) alteration of integral and cytoskeletal membrane proteins. During *in vitro* ageing the erythrocytes showed a decreased metabolic capacity with reduced ATP, 2,3-DPG and GSH concentrations and a decreased activity of enzymes, mainly PK and HK. Spherocytic eryhtrocytes were also evident, which demonstrates the presence of membrane modifications. As for structural integrity, the electrophoretic and Western blotting analyses of erythrocyte membrane proteins showed the presence of breakdown products due to the oxidative damage of the main cytoskeletal and integral membrane proteins, namely spectrin and protein band 3. Upon oxidative stress with t-BHP, erythrocytes showed an increase in TBAR, a decrease in GSH and a disco-stomatocytic deformation corresponding to extensive membrane protein degradation and the rearrangement of the cytoskeletal network. The most relevant feature was the denaturation of spectrin characterized by crosslinking products, the degradation of β spectrin into fragments between 200 and 120 kd, and the alteration of spectrin association in oligomers and tetramers. All these phenomena were more pronounced in G6PD- erythrocytes than in normal ones. Furthermore, G6PD-deficient erythrocytes showed a characteristic oxidative damage of protein band 3. In conclusion, erythrocyte physiological ageing and haemolytic events could be associated with an impairment of the antioxidant intracellular machinery, an increased susceptibility to the oxidative stress, and a progressive structural damage of cytoskeletal and integral membrane proteins.

S11.1

PATHOGENESIS OF THROMBOSIS

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Thrombotic disorders are the most common causes of morbidity and mortality in the western world. The pathogenetic basis of thrombosis was suggested as early as in the 1860s by Rudolf Virchow, who identified three major factors (Virchow's triad) that likely contribute to thrombus formation, namely changes in vessel wall, changes in blood flow and changes in the blood constituents. Nowadays, based on the impressive advances in our knowledge on the molecular and cellular mechanisms of haemostasis, it is widely recognised that thrombosis is a multifactorial phenomenon and that the relative role of the elements of the Virchow's triad is quite different in arterial and venous thrombosis. In the former, vessel wall damage is thought to play a prominent role, as indicated by the almost invariable co-localisation of the thrombus with an atherosclerotic plaque. At the site of a pre-existing plaque, activation of the haemostatic mechanisms may be caused either by local endothelial dysfunction , i.e. loss of antithrombotic properties and induced expression of thrombogenic factors such as tissue factor, or by plaque disruption, with the consequent exposure to the blood of the thrombogenic plaque material, including platelet-activating (constituents of the extracellular matrix) and procoagulant substances (mainly tissue factor expressed by the cells in the plaque, primarily macrophages). The abrupt contact of blood with this strongly thrombogenic material is likely responsible for the initiation of thrombus formation in many circumstances, although recent evidence indicates that blood borne tissue factor may contribute to thrombus growth. Alterations of blood flow as well as a number of genetic or acquired risk factors (lipids, smoking, hypertension, diabetes, infections, biochemical alterations including increased levels of fibrinogen, factor VII/VIIa, or plasminogen activator inhibitor 1 and others) will contribute to both the formation/evolution of high risk, vulnerable plaques and to the disruption of pre-existing plaques. In venous thrombosis, morphological vascular damage does not appear to play a major role. Rather, the essential pathogenetic factor is local activation of blood coagulation in combination with stasis, as clearly inferred from the observation that thrombus formation occurs mainly in the veins of the legs, largely in the context of acquired risk factors such as surgery, underlying malignancy, pregnancy and related complications, and trauma. In these conditions, generally associated with extensive tissue damage, activation of blood coagulation may be caused by the release of tissue factor into the bloodstream. However, more recently, it has been proposed that thrombus formation in areas of stasis could be initiated by the exposure of blood to tissue factor that is made available locally, possibly as a result of endothelial activation and/or recruitment/activation of leukocytes in response to cytokines, hypoxia and other mediators released as a consequence of tissue injury and inflammation. As arterial thrombosis, venous thrombosis too will be favoured by a number of genetic or acquired predisposing factors. The most extensively studied genetic conditions include the deficiency of physiological clotting inhibitors (antithrombin, protein C and protein S), the mutations of factor V associated with resistance to activated protein C (particularly factor V Leiden), and the mutation of the prothrombin gene (prothrombin G20210A), which is associated with increased plasma levels of prothrombin. Finally, an increased risk of both arterial and venous thrombosis has been reported in subjects with high levels of homocysteine, a complex condition involving both genetic and dietary factors. Studies on numerous other haemostatic gene polymorphisms are in progress and will likely help understanding the complex interplay between genes and environment in the pathogenesis of thrombosis.

S11.2

THE D-DIMER ASSAY IN THE DIAGNOSTIC WORKOUT OF SUSPECTED VENOUS THROMBOEMBOLIC DISEASES

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D-dimer is the end product of plasmin-dependent fibrin digestion, and is composed of two moieties originally belonging to separate fibrin monomers. Elevated D-dimer levels within the circulation may result from either increased fibrin formation – and subsequent degradation - or increased fibrinolytic activity. In the latter case however, increased D-dimer levels are accompanied by increased circulating levels of fibrinogen degradation products. D-dimer is present in many forms in plasma. Plasmin digestion of fibrin gives rise to the presence of fibrin oligomers of different molecular weight, all containing a number of D-dimer moieties. Steric D-dimer presentation in such fibrin oligomers may vary, and the specific antibodies used in different assays – even with similar affinity for pure D-dimer - may have heterogeneous affinity for these D-dimer forms. Thus, D-dimer levels measured in plasma are strictly assay-dependent.

There is a large body of literature showing that D-dimer testing is cost-effective in ruling out the presence of venous thromboembolic disease in patients with the clinical suspicion of deep vein thrombosis (DVT) and/or pulmonary embolism (PE). Optimal interpretation of D-dimer results is however dependent on a number of caveats:

a) rapid, quantitative instead of qualitative (or semi-quantitative) assays should be used;
b) assay-dependent cut-off values should be adopted, which may vary depending on whether the clinical suspicion refers to DVT or to PE;
c) the age of thrombus affects the sensitivity of D-dimer assays:
d) thrombus burden affects the sensitivity of D-dimer assays, especially with the clinical suspicion of PE;
e) D-dimer sensitivity is lower for symptom-less venous thromboembolism;
f) co-morbid conditions may have a large influence on assay specificity.

Diagnosis of recurrent DVT is cumbersome and often requires venography. Interestingly, D-dimer testing may also be useful in the diagnostic workout of suspected DVT recurrence, even if patients are on oral anticoagulant treatment or are affected by cancer. However, as for the clinical suspicion of a first episode of DVT, it should be kept in mind that the diagnostic accuracy of different D-dimer assays may vary.

S11.2

ORAL ANTICOAGULANT THERAPY (OAT) TODAY: OPEN PROBLEMS

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OAT is effective to prevent and treat thromboembolic complications in patients with cardiovascular disease. Though the number of patients treated has recently increased dramatically, OAT is still under-utilised in clinical practice because of the fear of bleeding complications and of the practical difficulties associated with the therapy. The incidence of bleeding varies widely in published studies. The rates of bleeding in a recent Italian collaborative study were 0.25%, 1.1% and 6.2% patient/year (pt-y) of treatment for fatal, major and minor events, respectively, figures that were substantially lower than those reported in previous observational studies. The efficacy of OAT was also examined in the same cohort of patients The rates of fatal and major thrombotic events were 1%pt-y and 1.9%pt-y, respectively, lower than that reported in the few available studies on the same topic. The frequency of thrombotic events was, however, much higher (17.5%) when international normalized ratio (INR) levels were < 1.5, decreasing to 2.3% for INRs within the 2-2.99 category (relative risk of INRs < 2.0 vs $\geq 2 = 1.88$, $p<0.05$). These results prove that a moderate-intensity regimen (2.0-3.0 INR), associated with the careful monitoring of patients in specifically trained anticoagulation clinics, is not only safe but also effective against thrombosis.

The duration of OAT for the treatment of venous thromboembolism (VTE) is still a debated issue. The "optimal duration" of OAT after a first VTE episode is however still debated. The usual duration of treatment after a first episode of idiopathic deep-vein thrombosis is three to six months. Recent studies, however, examined the effect of longer period of treatment and showed lower recurrence rates in comparison with shorter treatment periods. The advantage of the longer treatment duration was mainly observed in patients with permanent risk factors for thrombosis. In a trial that examined for 4 year follow-up patients with a recurrent DVT episode the cumulative rate of recurrence was 20.7% in the 6 month treatment group, and only 3% in the indefinite treatment group. The present answer to the issue of "optimal duration" is therefore to carefully evaluate persistent and/or transient pro-thrombotic factors in each patient. It has been proposed to classify DVT patients into low, intermediate, and high recurrence risk groups, respectively candidates for a short, intermediate or indefinite term anticoagulant therapy. After an episode of post-operative or post-traumatic DVT, and in the absence of permanent risk factors, less than 6 months of oral anticoagulant treatment may be indicated.

From the presently available data, it can be affirmed that the duration of treatment should not depend on the results of the ecographic follow-up. More clinical trials are needed in specific categories of well characterized patients, as those with inherited or acquired thrombophilia.

It has long been known that VTE complications are frequent in patients with malignant diseases, sometimes even preceding the diagnosis of malignancy. Some reports, though not all, have outlined a higher bleeding risk, as well as more frequent VTE recurrences during OAT in these patients. In a recent evaluation of patients with cancer treated with OAT for VTE (Palareti et al., Thromb Haemost 2000), we found that these patients, when compared with patients without malignancy, had statistically significant higher rates of major (5.4% vs 0.9%) and minor (16.2% vs 3.6%) bleeding. These patients also showed a trend towards a higher rate of thrombotic complications (6.8% vs. 2.5%; p=0.058; RR 2.5 (CI 0.96-6.5)). The rate of thrombotic events was significantly higher in both cohorts when the INR was less than 2.0.

Some of the indications for OAT (e.g. venous thromboembolism and atrial fibrillation) are particularly frequent in elderly people, the fastest growing population of our society. It has long been debated whether the risk of bleeding during OAT is higher in older patients. Many reasons can be at the basis of a higher risk for bleeding complications during OAT elderly subjects: they require lower coumarin doses, are more likely to be taking interacting drugs and to have more co-morbid conditions, they have been reported to have increased vascular fragility, a factor which may increase the risk of intracranial bleeding. In a recent study (Palareti et al. Arch Intern Med 2000) we analyzed the bleeding and thrombotic events occurring during OAT in 461 patients, aged ≥ 75 y when they started OAT, and in 461 matched patients, aged <70 y. Bleeding rate was 9.9% and 6.6% pt/y in elderly patients and controls, respectively (n.s.), and 2.1% and 1.1% for major bleeding (n.s.). However, 6 and 1 events respectively were fatal (all intracranial, RR 6.4; p=0.047). Thrombosis rate was 4.2% and 2.5% pt/y in elderly patients and controls, respectively (n.s.); however 13 and 5 events were fatal (RR 2.8; p=0.041). We concluded that the risk of intracranial bleeding and fatal thrombotic events is significantly higher in elderly subjects.