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CNAPS VII

CIRCULATING NUCLEIC ACIDS IN PLASMA AND SERUM

24-25 October 2011

Madrid – Spain

JOURNAL OF NUCLEIC ACID INVESTIGATION 2011; VOLUME 2, SUPPLEMENT 1

Guest Editor: Damián García-Olmo, Spain

TABLE OF CONTENTS

CNAPS VII

CIRCULATING NUCLEIC ACIDS IN PLASMA AND SERUM

24-25 October 2011

Madrid – Spain

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CNAPS VII
CIRCULATING NUCLEIC ACIDS IN PLASMA AND SERUM
24-25 October 2011
Madrid – Spain
PROGRAM

24 October 2011, Monday

08:30-09:30 OPENING CEREMONY

Opening Lecture: **"The clinical utility of circulating tumor DNAs"**
Luis A. Díaz. *Johns Hopkins Hospital. Baltimore, MD, USA.*

09:30-11:00 SESSION I: "WHAT IS NEW ABOUT THE ORIGIN OF CNAPS?"

Chairpersons: Maurice Stroun, Manuel A. González

Lectures:

- **"Horizontal gene transfer: You are what you eat!"**
  Lars Holmgren. *Karolinska Institut. Stockholm, Sweden.*
- **"Transfer of functional RNA between cells via exosomes"**
  Cecilia Lässer. *University of Gothenburg. Sweden.*
- **"The biochemical composition of virtosomes"**
  Mariapia Viola-Magni. *University of Perugia. Italy.*

Free communications:

- **"Circulating DNA from tumoral origin is characterized by high fragmentation level"**
  Florent Mouliere, Bruno Robert, Celine Gongora, Anne-Sophie Guedj, Maguy Del Rio, Marc Ychou, Franck Molina, Denis Pezet, Alain R. Thierry
  Sysdiag UMR3145-CNRS/BIO-RAD; 2U896 INSERM-Université Montpellier-CRLC Montpellier IRCM-Institut Recherche en Cancérologie de Montpellier; Centre Régional de Lutte contre le Cancer Val d'Aurelle-Paul Lamarque; and Centre Hospitalier Universitaire de Clermont-Ferrand. France.

11:00-11:30 Coffee break and posters presentation

11:30-13:00 SESSION II: "THE NATURE OF CNAPS"

Chairpersons: Philippe Anker, Antonio Bernad

Lectures:

- **"CNAPS and evolution"**
  Philippe Anker. *OncoXL. Geneva, Switzerland.*
- **"Cell-free and cell-bound circulating nucleic acid complexes: mechanisms of generation, concentration and content"**
  Elena Y. Rykova. *Institute of Chemical Biology and Fundamental Medicine. Novosibirsk, Russia.*

Free communications:

- **"Profile of the circulating RNA in apparently healthy individuals obtained with massive parallel sequencing of total plasma RNA on solid platform"**
  Dmitry V. Semenov, Dmitry A. Baryakin, Evgeny V. Brenner, Alexander M. Kurilshikov, Gennady V. Vasiliev, Leonid A. Bryzgalov, Elena D. Chikova, Julia A. Fillipova, Elena V. Kuligina, Vladimir A. Richter
  Institute of Chemical Biology and Fundamental Medicine SB RAS, and Institute of Cytology and Genetics SB RAS. Novosibirsk, Russia.
- **"Circulating exosome-mediated transfer of K-ras sequences between cells"**
  Gemma Serrano-Heras, Antonio S. Valero-Liñán, Jesús Cifuentes, Damián García-Olmo, Dolores C. García-Olmo
  General University Hospital of Albacete, Universidad Autónoma de Madrid, and "La Paz" University Hospital. Spain.

13:00-14:30 Lunch

14:30-16:15 SESSION III: "CNAPS AS CLINICAL TOOLS - I"

Chairpersons: Dennis Lo, Ana Bustamante

Lectures:

- **"Prenatal diagnosis of T21 by maternal plasma DNA sequencing"**
  Dirk van den Boom. *SEQUENOM. San Diego, CA, USA.*
- **"Noninvasive fetal genomic analysis by next-generation sequencing"**
  Y.M. Dennis Lo. *The Chinese University of Hong Kong. China.*
- **"Non-invasive prenatal diagnosis: pending possibilities and moral challenges"**
  Antina de Jong. *Maastricht University. The Netherlands.*

Free communications:

- **"Further developments in the use of cell-free fetal DNA for non-invasive prenatal diagnosis of single gene disorders: Digital PCR allows diagnosis of sickle cell disease"**
  A.N. Barrett, T.C.R. McDonnell, Lynn S. Chitty
  North East Thames Regional Molecular Genetics Laboratories; Institute of Child Health; and UCLH NHS Foundation Trust. London, UK.
- **"High throughput sequencing enables non-invasive prenatal diagnosis of beta thalassaemia using SNPs"**
  Thessalia Papasavva, Wilfred van Ijcken, Chirstel Kockx, Frank G. Grosveld, Marina Kleanthous
  The Cyprus Institute of Neurology and Genetics. Nicosia, Cyprus.
  Erasmus Medical Centre. Rotterdam, Netherlands.

16:15-16:45 Coffee break and posters presentation

16:45-18:30 SESSION IV: "CNAPS AS CLINICAL TOOLS - II"

Chairpersons: David Sidransky, Félix Bonilla

Lectures:

- **"Plasma/serum-based DNA tests for stratification and monitoring of cancer patients"**
  Frank Diehl. *Johns Hopkins Kimmel Cancer Center. Baltimore, MD, USA.*
- **"DNA alterations in tumors and plasma"**
  David Sidransky. *Johns Hopkins University School of Medicine. Baltimore, MD, USA.*
- **"Detection of EGFR mutations in blood samples"**
  Miquel Tarón. *Hospital Germans Trias i Pujol. Badalona, Barcelona, Spain.*

Free communications:

- **"High levels of circulating exosomes in plasma correlate with shorter survival in human colorectal cancer in a p53/tsap6 independent way"**
  Marta Rodríguez-Moreno, Vanessa García-Barberán, Francisco Javier Silva-Leal, José Miguel García-Ruiz, Gemma Domínguez-Muñoz, Cristina Peña-Maroto, Félix Bonilla-Velasco
  Hospital Universitario Puerta de Hierro-Majadahonda. Madrid, Spain.
- **"Clinical usefulness of plasma telomerase reverse transcriptase mRNA as a tumor marker in prostate cancer"**
  Francisco Dasí, José Antonio March-Villalba, José María Martínez-Jabaloyas, María José Herrero, J. Santamaría, Manuel Gil-Salom, P. Martínez-Rodes, Salvador F. Aliño
  FIHCUV/INCLIVA; School of Medicine of the University of Valencia; and Hospital Clínico Universitario de Valencia. Spain.

20:30 Dinner

25 October 2011, Tuesday

08:30-10:15 SESSION V: "THE BIOLOGIC ROLE OF CNAPS"

Chairpersons: Peter Gahan, Gemma Serrano

Lectures:

- **"Aspects of the biology of circulating nucleic acids"**
  Peter B. Gahan. *King's College. London, UK.*
  Maurice Stroun. *OncoXL. Geneva, Switzerland.*
- **"Unlocking eoDNA, a proper approach"**
  Piet Pretorius. *North-West University. Potchefstroom. South Africa.*
- **"Circulating nucleic acids and protease activities in blood of tumor patients"**
  Heidi Schwarzenbach. *University Medical Center Hamburg-Eppendorf. Hamburg, Germany.*

Free communications:

- **"The transforming effect of cell-free nucleic acids circulating in the plasma of colorectal cancer patients remains after resection of the primary tumor"**
  Carolina Domínguez-Berzosa, Dolores C. García-Olmo, Héctor Guadalajara, Luz Vega-Clemente, Mariano García-Arranz, Damián García-Olmo
  Universidad Autónoma de Madrid, "La Paz" University Hospital, and General University Hospital of Albacete. Spain.
- **"GC-rich cell-free DNA (GC-cfDNA) activates TLR-, NFkB-, JNK/p38- and IRF-dependent signaling pathways in adipose-derived mesenchymal stem cells (ADMSC)"**
  O. Chvartatskaya, Svetlana V. Kostjuk, P. Loseva, E. Ershova, Tatiana D. Smirnova, L. Kameneva, Elena M. Malinovskaya, Ludmila N. Lyubchenko, O. Roginko, V. Kuz'min, Natalya N. Veiko
  Moscow State Pedagogical University, and Russian Academy of Sciences. Moscow, Russia.

10:15-10:45 Coffee break and poster presentation

10:45-12:30 SESSION VI: "RESEARCH APPLICATIONS USING CNAPS"

Chairpersons: Diana Bianchi, Cristóbal Belda

Lectures:

- **"Nucleic acids in amniotic fluid provide insights into fetal development"**
  Diana Bianchi. *The Mother Infant Research Institute. Tufts Medical Center. Boston, MA, USA.*
- **"Non invasive prenatal diagnosis of Down syndrome"**
  Philippos Patsalis. *The Cyprus Institute of Neurology and Genetics. Nicosia, Cyprus.*

Free communications:

- **"Detection of cell-free DNA, RNA and miRNA in exosomes: New non invasive pregnancy-associated markers?"**
  M. Coulomb, N. Bonello, C. Formizano, N. Beaufils, J. Courageot, M. Tsochandaridis, F. Dignat-George, J. Gabert, A. Levy-Mozziconacci.
  Unité Inserm 608°, Université de la Méditerranée. Marseille, France.
- **"Profiling of circulating miRNAs in maternal and umbilical cord serum together with those tissue-based miRNAs in placentas and of preeclampsia pregnant women"**
  Qi Yang, Jiafeng Lu, Hailing Li, Shenqing Wang, Jing Tu, Qinyu Ge, Zuhong Lu
  Southeast University. Nanjing, China.
- **"COLD-PCR: a novel PCR assay for noninvasive prenatal diagnosis of genetic diseases"**
  Silvia Galbiati, Angela Brisci, Faustina Lalatta, Manuela Seia, G. Mike Makrigiorgos, Maurizio Ferrari, Laura Cremonesi.
  San Raffaele Scientific Institute; Fondazione IRCCS´ Granda Ospedale Maggiore; Universitá Vita-Salute San Raffaele; and Diagnostica e Ricerca San Raffaele SpA. Milan, Italy.
  Dana-Farber/Brigham and Women´s Cancer Center, Brigham and Women´s Hospital. Boston, MA, USA.

12:30-14:00 SESSION VII: "TECHNICAL UPDATE"

Chairpersons: Ramasamyiyer Swaminathan, Mariano García-Arranz

Short lectures:

- **"Detection and characterization of circulating DNA in plasma from colorectal cancer patients by aCGH microarrays"**
  Enrique Samper. *Nimgenetics, S.L. Madrid, Spain.*
- **"Requirements for CNAPS: Pre-analytical stabilization & automated extraction [for molecular diagnostics]"**
  Martin Horlitz. *QIAGEN GmbH. Hilden, Germany.*
- **"High throughput massive parallel sequencing"**
  Ricardo Ramos-Ruiz. *Parque Científico de Madrid, Genomics Unit. Madrid, Spain.*
- **"Next generation sequencing for CNAPS based diagnostics"**
  Ekkehard Schütz. *Chronix Biomedical. San Jose, CA, USA.*
- **"From discovery to the clinic: Epi proColon, a plasma based test to detect colorectal cancer"**
  Theo de Vos. Epigenomics. *Seattle, WA, USA.*

Free communications:

- **"Nucleosome positioning can help identify DNA fragments with higher abundance in plasma"**
  Muhammed Murtaza, Y. M. Dennis Lo, Nitzan Rosenfeld
  Cancer Research UK Cambridge Research Institute, and University of Cambridge. UK.
  The Chinese University of Hong, Prince of Wales Hospital. Hong Kong, China.

14:00-15:30 Lunch

15:30-17:00 JOINT SESSION EUROGENTEST NETWORK-CNAPS RESEARCHERS.

Chairpersons: Lyn Chitty, Marta Rodríguez de Alba

Lectures:

- **"Introduction to EuroGentest, a European network for the improvement and standardization of quality in genetic testing"**
  Gert Matthijs. *Center for Human Genetics. University of Leuven. Leuven, Belgium.*
- **"Implementing non-invasive prenatal fetal sex determination using cell free fetal DNA"**
  Lyn Chitty. *Clinical Molecular Genetics Unit, UCL Institute of Child Health, London, UK.*
- **"NIPD pilot EQA organized by RAPID/UKNEQAS"**
  Rob Elles. *Central Manchester University Hospitals NHS Foundation Trust, St Mary's Hospital. Manchester, UK.*
- **"Quality Assurance of NIPD: A view from the United States"**
  Daniel B. Bellissimo. *Molecular Diagnostics, BloodCenter of Wisconsin. Milwaukee, WI, USA.*

17:00-18:30 CLOSING SESSION

Closing Lecture: **"Horizontal transmission of malignancy"**
David M. Goldenberg. *Garden State Cancer Center. Belleville, NJ, USA.*

- **Awards** for the best oral communication and for the best poster.
- **Closing remarks**
  Damián García-Olmo
- **Invitation to CNAPS VIII**
  Philippe Anker, Maurice Stroun

Session I
What is new about the origin of CNAPS?

Lectures

HORIZONTAL GENE TRANSFER: YOU ARE WHAT YOU EAT!

L. Holmgren

Karolinska Institut, Stockholm, Sweden.

Tumor formation involves the accumulation of a series of genetic alterations that are required for malignant growth. In most malignancies, genetic changes can be observed at the chromosomal level as losses or gains of whole or large portions of chromosomes. Here we provide evidence that tumor DNA may be horizontally transferred by the uptake of apoptotic bodies. Phagocytosis of apoptotic bodies derived from H-ras(V12)- and human c-myc-transfected rat fibroblasts resulted in loss of contact inhibition *in vitro* and a tumorigenic phenotype *in vivo*. Fluorescence *in situ* hybridization analysis revealed the presence of rat chromosomes or of rat and mouse fusion chromosomes in the nuclei of the recipient murine cells. The transferred DNA was propagated, provided that the transferred DNA conferred a selective advantage to the cell and that the phagocytotic host cell was p53-negative. These results suggest that lateral transfer of DNA between eukaryotic cells may result in aneuploidy and the accumulation of genetic changes that are necessary for tumor formation.

TRANSFER OF FUNCTIONAL RNA BETWEEN CELLS VIA EXOSOMES

C. Lässer

University of Gothenburg, Sweden

Exosomes are small membrane vesicles that are 40-100 nm in size, formed by cells through inward budding of the limiting membrane of the late endosome. This accumulation of internal vesicles leads to the formation of multivesicular bodies, and upon fusion with the plasma membrane the formed internal vesicles are released as exosomes into the extracellular space.

Exosomes were discovered in the early 80s as a way for reticulocytes to eradicate the transferrin receptor while maturing into erythrocytes. In the middle of the 90s, it was demonstrated that exosomes were not only used for discharging unwanted proteins; exosomes from B lymphocytes could also stimulate T lymphocytes. This finding led to renewed attention for these vesicles and since then it has been shown that a variety of cells in culture can release exosomes. Furthermore, exosomes has been detected in vivo in several body fluids such as plasma, saliva, breast milk, malignant effusions and urine.

The function of exosomes depends on the cell it is produced and released from as well as the current state of that cell. Some of the functions of exosomes are induction of the maturation of dendritic cells, induction of tolerance against allergen, induction of apoptosis in T lymphocytes, inhibition of NK cells, induction of the migration of granulocytes and eradication of established tumours in mice.

In 2007, our group showed that exosomes contain RNA, specifically mRNA and miRNA, but little or no ribosomal RNA. Furthermore, we demonstrated that the RNA was functional and could be transferred to a recipient cell and translated. This finding offered new insights into the function and potential of exosomes, with the RNA of different exosomes now being extensively investigated. One potential use of exosomal RNA is its use as a diagnostic tool. It has been shown that patients diagnosed with ovarian cancer have significantly higher levels of EpCAM-positive exosomes in their sera, which increased with the progression of disease. Furthermore, the exosomal miRNA profile strongly correlated with the tumour derived miRNA and the level of miRNA was higher in the cancer patients compared to benign disease and healthy controls. The RNA content of serum exosomes from glioblastoma patients has also been investigated for their potential role as a biomarker. This study showed that the common glioma mutated version of the EGFR mRNA, EGFRvIII, could be detected in serum exosomes from glioblastoma patients, but not in healthy controls. Furthermore, two weeks after surgery and removal of the tumour, the mRNA was no longer detected in the patients' serum, indicating that the tumour was the source of the exosomes.

The exosomal RNA is not only examined for their use in diagnostics, but also for their potential therapeutic role. As exosomes are endogenous vesicles for RNA transfer between cells, it has been hypothesised that they would be a suitable candidate vector for gene therapy. Earlier this year, it was demonstrated that siRNA loaded exosomes from dendritic cells, given intravenously to mice, could deliver the siRNA with detected effect in the recipient cells. BACE1 has been suggested as a candidate protein to inhibit in treatment for Alzheimer's, therefore siRNA against BACE1 was loaded into exosomes and administrated to mice. A knockdown effect was seen, both at the mRNA and protein level, in the cortical tissue samples. In addition, the main component of amyloid plaques, β-amyloid 1-42, was significantly decreased.

As well as the great potential of loading exosomes with therapeutic siRNA against a variety of different diseases, it has also been found that CD34+ cells release exosomes containing proangiogenic miRNA. Although it was not established that it was the miRNA that transferred the effect, the CD34+ exosomes induced angiogenic activity in endothelial cells in vitro and vessel growth in vivo in mice. The therapeutic potential of CD34+ exosomes to induce angiogenesis could improve the outcome of ischemic injuries and improve recovery.

Exosomes have been proven to present an alternative route of cell-to-cell communication by the delivery of functional RNA species from one cell to another. Further investigations of exosomal RNA will improve the understanding of this cell communicative role, as well as their potential role in therapeutics and as biomarkers.

THE BIOCHEMICAL COMPOSITION OF VIRTOSOMES

M.P. Viola-Magni

University of Perugia, Italy

Virtosomes released from chick embryo fibroblasts (CEF) can be taken up by other CEFs. Analysis of the cytosolic and released virtosomal fractions by agarose gel column chromatography shows them to be similar.
In the present research we have used isolated human lymphocytes cultured in RPMI 1640 culture medium with 15% of FCS for 3 hours. The lymphocytes were separated and gently homogenized. The cytosolic virtosome fraction was isolated by centrifuging the homogenate at 10.000 g for 10 minutes, the supernatant being further centrifuged for 1h at 120,000, the virtosomes remaining in the supernatant. The lymphocyte culture medium used, was similarly centrifuged in order to isolate the virtosomes released from the lymphocytes.
Similar amounts of DNA, RNA, proteins and PLs were isolated from the cytosolic and released virtosome fractions. The virtosomes were found to be composed of DNA ca 5%, RNA ca 30%, proteins ca 30% and phospholipids ca 35%. The main phospholipid components were phosphatidylinositol and sphingomyelin while the phosphatidylcholine level was low.
The results confirm the similarity of the cytosolic and released virtosomes.
Furthermore, the phospholipid composition was different to that expected for cellular membranes and so further supports the earlier contention that cell membrane structures are not associated with the virtosomes, especially after release from the cells.

Oral presentations

CIRCULATING DNA FROM TUMORAL ORIGIN IS CHARACTERIZED BY HIGH FRAGMENTATION LEVEL

F. Mouliere, B. Robert, C. Gongora, A.S. Guedj, M. Del Rio, M. Ychou, F. Molina, D. Pezet, A.R. Thierry

Sysdiag UMR3145-CNRS/BIO-RAD; U896 INSERM-Université Montpellier-CRLC Montpellier IRCM-Institut Recherche en Cancérologie de Montpellier; Centre Régional de Lutte contre le Cancer Val d'Aurelle-Paul Lamarque; and Centre Hospitalier Universitaire de Clermont-Ferrand, France

Circulating DNA (ctDNA) is acknowledged as a potential theranostic tool in various cancers including colorectal cancer. Many studies have been focusing on the identification of abnormal forms of DNA in plasma or serum. These reports, although promising, have led to many questions about the reliability of using abnormal ctDNA as a biomarker of cancer.
In order to rigorously analyse ctDNA as a potential theranostic tool, we thoroughly investigated ctDNA size distribution.We used a highly specific Q-PCR assay and athymic nude mice xenografted with human colon cancer cells, and we correlated our results by examining plasma from metastatic CRC patients.
Tumour-derived ctDNA was analysed by detecting various mutations such as KRAS codon 12-13 and BRAF V600E point mutations. Fragmentation and concentration of tumour-derived ctDNA is positively correlated with tumour weight. CtDNA quantification by Q-PCR depends on the amplified target length and is optimal for 60-100 bp fragments. Q-PCR analysis of plasma samples from xenografted mice and cancer patients showed that tumour-derived ctDNA exhibits a specific amount profile based on ctDNA size and significant higher ctDNA fragmentation. Metastatic colorectal patients (n=12) showed nearly 5-fold higher mean ctDNA fragmentation than healthy individuals (n=16).
Our results support the notion that ctDNA fragmentation can be accurately and easily quantified, and used to discriminate healthy subjects from cancer patients, providing new avenues for cancer diagnosis and monitoring.

Session II: The nature of CNAPS

Lectures

CNAPS AND EVOLUTION

P. Anker

OncoXL. Geneva, Switzerland

J.B. Lamarck in 1809 was the first to present a theory of evolution. He proposed it was due to the capacity of adaptationof the different species to the changes of the environment, this adaptation being acquired by the offspring. In 1868 in «The Variation of Animals and Plants under Domestication » Darwin suggested that cells are capable of excreting gemmules.These particles are able to circulate through the body and reach the gonads where they are transmitted to the next generation. One of his main arguments came from graft-hybrids. In the 50s and 60s, Russian geneticists, rejecting Neodarwinism and it's chance mutations, said that acquired characteristics were the basis of evolution.Curiously, one of the main experiments on which the Russian geneticists based their theory was the transmission of hereditary characteristics by a special technique of grafting between two varieties of plants—a mentor plant and a pupil plant. Maurice Stroun tried to repeat this kind of experiment and after three generations of grafting between two varieties of eggplant, succeeded in obtaining hereditary modifications of the pupil plants, which acquired some of the characteristics of the mentor variety.Rather than adopting the views of the Russian scientists about acquired characteristics, he suggested that DNA was circulating between the mentor and pupil plants. Hirata's group in Japan have more recently shown, 50 years later, by using molecular techniques like cloning , RFLP PCR and sequencing some genes of their graft-hybrids of pepper plants that transfer of informative molecules from the mentor to the pupli plant does exist. Nucleic acids have been found to be actively released by cells, they circulate in the body. They have been shown tobe able to transform oncogenically or to trigger antibody response but the only genetic transformation showing that Nucleic acids can go from the soma to the germen comes from graft-hybrids. This suggests that circulating nucleic acids, like Dawin's gemmules, may probably play a role in the mechanism of evolution.

CELL-FREE AND CELL-BOUND CIRCULATING NUCLEIC ACID COMPLEXES: MECHANISMS OF GENERATION, CONCENTRATION AND CONTENT

E.Y. Rykova[1], E.S. Morozkin[1], E.M. Loseva[1], A.A. Ponomaryova[2], E.V. Elistratova[1], V.H. Mileiko[1], A.V. Cherepanova[1], T.E. Skvortsova[1], O.E. Bryzgunova[1], S.N. Tamkovich[1], N.V. Cherdyntseva[2], V.V. Vlassov[1], P.P. Laktionov[1]

[1]*Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia;* [2]*Cancer Research Institute, Siberian Branch of the Russian Academy of Medical Sciences, Tomsk, Russia*

Circulating nucleic acids (cirNA) were found in blood as cell-free or being absorbed at cell surface. Cell-free nucleic acids (cfNA) are more fragmented as compared to cell-surface bound nucleic acids (csbNA) and are integrated into complexes with proteins, apoptotic bodies or nucleosomes. Whether csbNA are packed into specific structures is not clear, however certain cell-surface proteins were shown to bind "naked" NA and mediate their transport into cells. Complexes of cfNA with biopolymers from culture medium along with apoptotic bodies and nucleosomes can also be absorbed at cell surface and transported into cells. Ratio of cfNA to csbNA in the blood depends from the state of donor: main part of cirNA from healthy subjects are bound with cells, whereas cancer patients have a different distribution of cfNA and csbNA depending from tumor type. CsbDNA fraction from cancer patients contains DNA originated from cancer cells and along with cfDNA represents the valuable source of material for cancer diagnostics. Both types of cirNA could be generated by apoptosis and during the living cells activity: inhibitors of classical and nonclassical protein secretion pathways influence their concentration. The composition of cell-free DNA differs from genomic DNA of cultivated cells. Comparative study using FISH hybridization have shown overrepresentation of certain chromosome regions in the pools of cfDNA compared with genome DNA fragments from human primary and long-term cultivated cells. Massive parallel sequencing of cell-free apoptotic DNA demonstrates overrepresentation of coding DNA sequences in apoptotic DNA as compared with genome DNA indicating one of the inducing forces of cell-free DNA peculiarities.

Oral presentations

PROFILE OF THE CIRCULATING RNA IN APPARENTLY HEALTHY INDIVIDUALS OBTAINED WITH MASSIVE PARALLEL SEQUENCING OF TOTAL PLASMA RNA ON SOLID PLATFORM

D.V. Semenov, D.A. Baryakin, E.V. Brenner, A.M. Kuril'shikov, G.V. Vasiliev, L.A. Bryzgalov, E.D. Chikova, J.A. Fillipova, E.V. Kuligina, V.A. Richter

Institute of Chemical Biology and Fundamental Medicine SB RAS, and Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia

Understanding structures of circulating RNA expands fundamental knowledge of cell communications and signaling pathways as well as allows to develop new molecular diagnostic approaches. The aim of this study was to perform massive parallel sequencing of human blood plasma RNA to document profile of common and peculiar RNA species normally circulating in blood of healthy individuals.

Total RNA was extracted from samples of blood plasma of 8 apparently healthy individuals (4 men and 4 women aged from 20 to 60 years). To obtain comprehensive cDNA libraries RNA was dephosphorylated followed by

5' phosphorylation. 5' phosphorylated total plasma RNA was ligated with adapters, reverse transcribed and 8 personalized cDNA libraries were constructed. Libraries were amplified and sequenced with SOLiD platform. The sequenced 35-nt-long reads (from 31 up to 65 millions reads per sample) were mapped to human genome, classified and quantified with two independent approaches using both BioScope® and Bowtie, Cufflinks software.
The set of rRNA fragments, mitochondrial transcripts, micro RNAs, fragments of scRNAs, snRNA and snoRNA, fragments of several mRNAs as well as the set of newly discovered transcripts were found to be permanent representatives of human blood plasma RNAs. In addition, advanced mapping allowed to identify circulating herpes virus and enterobacterial transcripts which structures can be exploited in the development of new diagnostic tests.

CIRCULATING EXOSOME-MEDIATED TRANSFER OF K-*ras* SEQUENCES BETWEEN CELLS

G. Serrano-Heras, A.S. Valero-Liñán, J. Cifuentes, D. García-Olmo, D.C. García-Olmo

General University Hospital of Albacete, Universidad Autónoma de Madrid, and "La Paz" University Hospital, Spain

Recently, investigators have begun to appreciate the fact that microvesicles are an integral part of the intercellular environment and that they play an important role as modulators of cell communication. Exosomes, small microvesicles of endocytic origin released by many cells including tumor cells, have been identified in body fluids, such as plasma, urine and ascitic fluid. It has been reported that exosomes contain nucleic acids in addition to membrane and cytosolic proteins, suggesting that they could be an important resource of cell-free nucleic acids in plasma. The aim of the present study was to analyze the DNA content of circulating exosomes and to study the ability of such small vesicles to transfer this genetic material into recipient cells. Exosomes were isolated from plasma of four patients with k-ras-mutated colon cancer as well as of three healthy subjects using filtration and ultracentrifugation techniques. Next, the identity of the purified vesicles was confirmed as exosomes by FACS analysis, which showed the presence of the surface protein CD63 and CD9, a two commonly used marker of exosomes. The analysis of exosomal DNA by real-time PCR revealed that exosomes isolated from plasma of cancer patients carried mutated and non-mutated k-ras sequences whereas those purified from plasma of healthy subjects harbored only the non-mutated sequences.
In addition, we found that incubation of NIH-3T3 fibroblasts with plasma-derived exosomes resulted in the transfer of non-mutated human k-ras to these cells.
Interestingly, mutated human k-ras sequences were also detected in the murine cells that had been cultured with exosomes obtained from plasma of colon cancer patients. These findings provide strong evidence that the horizontal transfer of oncogenes.

Session III
CNAPS as clinical tools - I

Lectures

PRENATAL DIAGNOSIS OF T21 BY MATERNAL PLASMA DNA SEQUENCING

D. van den Boom

SEQUENOM, San Diego, CA, USA

NONINVASIVE FETAL GENOMIC ANALYSIS BY NEXT-GENERATION SEQUENCING

Y.M. Dennis Lo

The Chinese University of Hong Kong, China

The presence of cell-free fetal DNA in maternal plasma has opened up possibilities for noninvasive prenatal diagnosis. The recent advent of next-generation sequencing has allowed us to analyze maternal plasma DNA with unprecedented precision. This development has enabled us to detect fetal chromosomal aneuploidies, including trisomy 21, 18 and 13, with high sensitivity and specificity. In particular, the noninvasive prenatal detection of fetal trisomy 21 has been validated in a number of independent studies. More recently, plasma DNA sequencing has been used for deducing a genomewide genetic map of the fetus. This development has the potential to greatly increase the spectrum of genetic diseases that can be analyzed using this noninvasive approach. The use of targeted sequencing is expected to further reduce the cost of this application, thus enhancing its utility in clinical scenarios.

NON-INVASIVE PRENATAL DIAGNOSIS: PENDING POSSIBILITIES AND MORAL CHALLENGES

A. de Jong

Maastricht University, The Netherlands

Non-invasive prenatal diagnosis (NIPD) has important advantages over current methods of invasive testing: the miscarriage risk attached to current methods will be avoided, testing will be easier and can be performed earlier in pregnancy. However, the scenario of pregnant women being given routine access to early, easy and risk-free prenatal diagnosis may also have morally challenging implications.
First, the anticipated introduction of NIPD for common fetal aneuploidies may lead to undermining informed decision-making about participation in prenatal screening. Although one-step NIPD seems to make counselling conceptually easier, because it avoids prior risk-assessment, the challenge will be to make women understand that having a simple blood test may lead to difficult choices about whether or not to continue the pregnancy. Furthermore, part of current testing, such as nuchal translucency (NT) measurement and invasive diagnosis for other (chromosomal) abnormalities, may remain in place in

order to avoid limiting the scope for reproductive decision-making. Thus women will be confronted with an accumulation of testing throughout the pregnancy, which may be problematic from the perspective of the balance of benefits and burdens, and in view of a risk of undermining rather than furthering autonomous reproductive choice.

Second, there is concern that NIPD may lead to trivialization of testing and selective abortion, because the availability of an early and risk-free method of detection may not only lead to a higher uptake but also to lowering the moral barrier for selective abortion. It is feared that termination of affected pregnancies comes to be regarded as a matter of course. However, higher uptake may as well be an expression of informed reproductive choice, and the fact that NIPD enables possible early termination changes the moral picture given the relatively low moral status of the embryo or fetus at this stage of development. This should affect the moral evaluation of early terminations following NIPD. Nevertheless, empirical research is needed on whether women will in fact feel more social pressures to have NIPD-testing if the iatrogenic miscarriage risk is no longer a reason for declining a prenatal diagnostic test.

Third, NIPD-testing may initially remain restricted to common aneuploidies, but if technical limitations can be overcome NIPD would appear the perfect vehicle for considerably extending the scope of prenatal screening. If so, not the one-step character of NIPD as such should be regarded as particularly morally challenging, but the conceivable convergence of this development with the introduction of new genomic technologies allowing testing for a much broader range of abnormalities than currently targeted in prenatal aneuploidy screening. The prospect of simultaneous testing for a broad range of heterogeneous fetal conditions seriously casts doubt on whether informed decision-making by pregnant women will still be possible. To the extent that this scenario effectively leads to undermining informed choice, maintaining the traditional justification of prenatal screening (in terms of enhancing reproductive autonomy) becomes problematic as well. NIPD-techniques may eventually not only be used for identifying fetal abnormalities that may lead to abortion, but also for identifying treatable conditions and for indicating pregnancy-related risk-factors such as preeclampsia. If so, screening serves two different aims: offering reproductive choice and prevention of untoward pregnancy outcomes. A simultaneous offer may blur the crucial distinction between these two goals. It will also be challenging to adequately inform women about such a compound test offer that combines tests which may lead to very different courses of action: termination/continuation of pregnancy, follow-up testing, and clinical interventions in the pregnancy.

Finally, genome-wide prenatal screening inevitably leads to blurring the line between reproductive and neonatal or childhood testing. As knowledge of predispositions for later onset disorders or susceptibility profiles for common diseases will probably not in most cases lead to a request for termination, broad prenatal screening leads to children being born with health information that may be burdensome for them and stands in the way of their right to decide for themselves whether they want to know or not.

To conclude: the introduction of NIPD in the context of prenatal screening raises ethical questions about how it will be situated in the prenatal testing cascade, how adequate counselling about its aim, scope and consequences will be ensured, and how future children's rights will be warranted.

Oral presentations

FURTHER DEVELOPMENTS IN THE USE OF CELL-FREE FETAL DNA FOR NON-INVASIVE PRENATAL DIAGNOSIS OF SINGLE GENE DISORDERS: DIGITAL PCR ALLOWS DIAGNOSIS OF SICKLE CELL DISEASE

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Background. Cell free fetal DNA (cffDNA) provides a source of fetal material for non-invasive prenatal genetic diagnosis (NIPD). Current clinical applications of NIPD rely on determining the presence or absence of a gene that has arisen de novo in the fetus, or is paternally inherited. Diagnosis of recessive conditions, where both parents carry the same abnormal allele, requires a different approach.

Objective. To explore the feasibility of NIPD for the diagnosis of sickle cell disease (SSD), caused by homozygosity for the HbS allele, using digital PCR.

Methods. Blood, taken from women at risk of SSD undergoing invasive prenatal testing (IPD), was double spun and plasma stored at -80ºC prior to extraction of cffDNA using the QIAamp Circulating Nucleic Acid kit (Quiagen), Two probes were designed, one recognizing the normal HbA allele and one detecting HbS. Digital PCR was used to test for over-representation of one allele. Results from NIPD were compared with those from IPD.

Results. Results from 21/30 samples from male bearing pregnancies were concordant. 6 discordant results contained <8% cffDNA; 3 were unclassified and had <5% cffDNA. Analysis of 30 female samples is ongoing using SNP analysis to assess fractional cffDNA concentration.

Conclusions. NIPD for SSD is possible using digital PCR. Further evaluation is required to define the lower limit of cffDNA concentration required to deliver reliable, accurate and safer prenatal diagnosis for SSD.

HIGH THROUGHPUT SEQUENCING ENABLES NON-INVASIVE PRENATAL DIAGNOSIS OF BETA THALASSAEMIA USING SNPs

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-thalassaemia is the most common autosomal recessive single gene disorder in Cyprus. Prenatal diagnosis is based on chorionic villus sampling that poses an abortion risk to the fetus. The discovery of cell-free fetal DNA in

maternal plasma has opened up new possibilities for the non-invasive prenatal diagnosis. Fetal DNA, however, represents a minor population in maternal plasma, exacerbated by the fragmentation of fetal DNA, posing a technical challenge to the researchers. With the advent of single molecule technologies and high throughput sequencing many challenges have been confronted. We used the Solexa high throughput sequencing technology for the development of a NIPD assay for β-thalassaemia based on the detection of paternally inherited fetal alleles using Single Nucleotide Polymorphisms (SNPs). This platform is based on the sequencing by synthesis (SBS) technology using four propriety fluorescently-labeled modified nucleotides to sequence the million of clusters present on the flow cell surface.

We selected 4 SNPs located on the β-globin locus that have high heterozygosity in the Cypriot population and are informative for 10 families being analyzed. Using this platform, we developed a multiplexed format and analyzed 10 maternal plasma samples for the selected SNPs obtaining an average of 1 million reads per sample.

We detected the paternally inherited alleles of the fetus in 8 out of 10 maternal plasma samples collected from at risk pregnancies. The sequencing results showed concordance with the CVS analysis of the samples.

The analytical power of this method, namely sensitivity, specificity, accuracy and precision, demonstrated by these preliminary findings, should pave the way for the clinical use of NIPD.

Session IV
CNAPS as clinical tools - II

Lectures

PLASMA/SERUM-BASED DNA TESTS FOR STRATIFICATION AND MONITORING OF CANCER PATIENTS

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The molecular characterization of tumors has shown to be important for guiding specific treatment decisions in oncology. For example, defined tumor specific somatic mutations have shown to be good predictors of response to targeted systemic therapies. The analysis of free circulating DNA isolated form plasma or serum samples can potentially extent the molecular characterization of tumors to situations were tissue testing is not available. In particular, testing options could be improved for patient stratification and monitoring of therapy response as well as recurrence. In addition, the detection of secondary resistance mutations arising during therapy could be facilitated by plasma/serum-based testing. Additional factors that make plasma/serum very attractive to oncologists are the convenience of sample collection, repeated sampling options, as well as the availability of a fresh source of genetic material.

Previous mutation detection assays have not been sufficiently specific, sensitive, and quantitative for the assessment of the clinical utility of circulating nucleic acids in oncology. Newly developed technologies based on digital PCR provide an extremely high sensitivity and at the same time allow quantification of the fraction of mutant to normal DNA molecules in a sample. One of these approaches applied by Inostics is BEAMing (Beads, Emulsion, Amplification and Magnetics). Several studies have been performed showing that advanced tumors of the breast and colon release sufficient amounts of tumor DNA for genetic testing. Newly developed multiplex BEAMing assays will be presented allowing the measurement of up to 27 mutations in 10 ampliconcs from a single plasma/serum aliquot. An update will be given on the analytical and clinical performance of these tests. In addition, we will present that quantification of circulating mutant DNA can be used to monitor tumor load in cancer patients. The rationale is similar to that employed in the care of HIV patients, in which circulating viral nucleic acids are quantitatively assessed to monitor disease and used to tailor therapy to individual patients' needs. The development of analog circulating nucleic acids tests in oncology have the potential to greatly improve patient management and as well as early detection. As sequencing technologies improve, it will become relatively simple to identify such mutations in virtually any cancer type. Indeed, such diagnostic applications are one of the major goals that we are currently focusing on.

DNA ALTERATIONS IN TUMORS AND PLASMA

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DETECTION OF EGFR MUTATIONS IN BLOOD SAMPLES

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It is well known that in Non-Small Cell Lung Cancer (NSCLC) patients, the screening of sensitizing EGFR mutations in tumor samples is becoming mandatory to treat patients with EGFR tyrosine kinase inhibitors (TKIs). However, one of the most important limitations is the lack of available tumor cells in tumor tissues (biopsies or cytologies) in around 30% of patients, and the difficulty to perform re-biopsies at baseline or during the follow-up of the patient. In this sense, the use of strategies to genotype patients using cell-free circulating DNA could be a crucial tool.

There are different studies in literature showing that plasma- and serum-based EGFR mutation testing is not only feasible but has considerable value as well. In our experience, we have analyzed paired serum and tissue samples from 164 EGFR mutated (exon 19 deletions and L858R) patients. Starting from isolated serum/plasma samples, cell-free circulating DNA was purified and analyzed for the presence of the mutations. Deletions in exon 19 were determined by length analysis after PCR amplification with the use of FAM labeled primer. Exon 21 L858R point mutations were analyzed with a 5' nuclease PCR assay (TaqMan assay) using FAM and VIC MGB labeled probes for the wild-type and the mutant sequences respectively. Both reactions were done in the presence of a protein nucleic acid (PNA) designed to inhibit the PCR amplification of the wild type allele.

Of the 164 EGFR positive patients (in tissue) in whom EGFR mutations were assessed in serum, 97 carried mutations: exon 19 deletions in 64 patients and L858R in 33 patients. Importantly, in the multivariate analysis of this study, the adjusted hazard ratios for the duration of progression-free survival were 2.94 for men ($P<0.001$); 1.92 for the presence of the L858R mutation, as compared with a deletion in exon 19 ($P = 0.02$); and 1.68 for the presence of the L858R mutation in paired serum DNA, as compared with the absence of the mutation ($P = 0.02$).

Interestingly, we found that EGFR mutation status in the serum or plasma matched that in the tumor tissue in 45% of patients with PS 0, in 57% of patients with PS 1, and in 75% of patients with PS 2 ($P=0.01$), showing the importance of tumour burden in this analysis. In addition, in our study we found a sensitivity of 60%. In previous studies, we also analyzed the specificity of our technique and we have shown a specificity higher than 98%.

In conclusion, the analysis of gene mutations at the serum or plasma level is a useful diagnostic tool that should be used to genotype and treat patients.

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Oral presentations

HIGH LEVELS OF CIRCULATING EXOSOMES IN PLASMA CORRELATE WITH SHORTER SURVIVAL IN HUMAN COLORECTAL CANCER IN A p53/tsap6 INDEPENDENT WAY

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A significant proportion of extracellular nucleic acids in plasma circulate highly protected in tumour specific exosomes but it is unclear how the liberation of exosomes is modulated in carcinogenesis. We quantified released exosomes in plasma of colorectal cancer patients to evaluate their potential as tumour biomarker. We examined the implication of TSAP6, a previously described p53-regulated gene involved in the regulation of vesicular secretion, in levels of circulating exosomes in plasma of patients as well as its potential repercussion in diagnosis and prognosis. Exosomes were isolated from plasma of 91 colorectal cancer patients and 12 controls. Exosomes were quantified by cytometry and correlated with clinical-pathological parameters of the tumours and with survival. The expression of TSAP6 mRNA was analyzed by RT-PCR and protein levels of TSAP6 and p53 were analyzed by immunohistochemistry to assess the previously described regulation of the exosome secretion TSAP6-mediated by p53. This potential regulation was also argued in colorectal cancer cell lines. The fraction of exosomes in patients was statistically higher compared with exosomes in controls. Patients with high level of exosomes in plasma had shorter overall survival than patients with low levels. Liberation of exosomes was not correlated with TSAP6 expression and regulation of TSAP6 by p53 was not shown either in tumour samples or in HCT116 p53-wt or p53-null cell lines. Levels of circulating exosomes may be used as tumour biomarker since they correlate with poor prognosis parameters and shorter survival. The p53/TSAP6 pathway was not suggested to have a determinant role in the regulation of the liberation of exosomes into the plasma of colorectal cancer patients.

CLINICAL USEFULNESS OF PLASMA TELOMERASE REVERSE TRANSCRIPTASE mRNA AS A TUMOR MARKER IN PROSTATE CANCER

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We have previously reported in a short number of patients that quantification of human telomerase reverse transcriptase (hTERT) mRNA in plasma is a very sensitive and specific method to identify prostate cancer (PCa) patients and in combination with prostate-specific antigen (PSA) a useful marker for early PCa diagnosis. This study was aimed to clarify plasma hTERT mRNA clinical significance as a biomarker for PCa. Plasma mRNA hTERT levels were determined by qRT-PCR in 105 patients showing elevated PSA levels and 68 healthy volunteers. Sensitivity and specificity in PCa diagnosis were determined using ROC curves. Correlation between plasma hTERT mRNA levels and clinical-pathological parameters was analyzed by using multivariate analysis. Plasma hTERT mRNA mean values were significantly higher ($p<0.001$) in PCa patients (Mean; IC95%: 3.35; 2.23-4.48) than in the control (0.13; 0.02-0.35), prostatitis (0.40; 0.18-0.61) and benign prostate pathologies groups (0.13; 0.01 -0.26). Sensitivity and specificity in PCa diagnosis was 85% and 90% respectively at a cut-off value of 0.45. A positive correlation between circulating hTERT mRNA levels and Gleason score ($p<0.05$), tumour stage ($p<0.001$), vascular ($p<0.001$) and perineural tumor infiltration ($p<0.001$) and patterns of spread ($p<0.001$) was observed. Most importantly, 4 out of 23 prostatitis patients that developed PCa over the next five years showed, at diagnosis, higher mean hTERT mRNA values within the prostatitis group. These results extend and confirm our previous results that plasma hTERT mRNA is a useful non-invasive tumor marker for the diagnosis of PCa and may have implications in the follow-up and minimal residual detection of the disease.

Session V
The biologic role of CNAPS

Lectures

ASPECTS OF THE BIOLOGY OF CIRCULATING NUCLEIC ACIDS

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An increasing number of examples exist showing the changed biology of cells into which CNAPS components enter.Of those biological changes caused by CNAPS components so far seen, only two – transformation from normal to cancer cells and plant homografts leading to non-epigenetic inheritance - are likely due to full integration of the components into the chromatin.In the other cases, the effect seen may be due to either transient expression or integration.
It will be shown that the mechanism by which CNAPS fragments can enter the nuclei of sperm and ova is present in animal and human systems. Furthermore, plants lack continuous germ cell lines so making them more susceptible to this process.The chromosomal integration of CNAPS sequences will result in extra copies of host sequences that may influence the biology of the individual and its progeny. However, the integration of xenogeneic fragments present in CNAPS can offer the possibility of the introduction of new sequences as has been shown through horizontal gene transfer. The implications of these possibilities for the biology of the individuals concerned and their progeny will be discussed.

UNLOCKING eoDNA, A PROPER APPROACH

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Elevated levels of circulating DNA has been shown to be associated with a vast number of disease states of various nature. Even so, there still seems to be a wide margin of error involved in circulating DNA diagnostics, even among seemingly healthy individuals. Most research in this field focuses on new links between circulating DNA and pathological states. Research on the biological function of cell free DNA has been left wanting. Understanding the origin and function of various individual forms of circulating DNA will likely lead to more effective diagnosis. The origin of circulating DNA appears to be shared between active release processes and release following apoptosis and cell damage, probably originating from various tissues and organs in the body. Active DNA release has been shown as an effective form of intercellular communication. The different actively released cell free DNA (cfDNA) forms include virtosomes, histone complexes and micro particles, while apoptosis is mainly associated with cell free DNA release in the form of apoptotic bodies and micro particles. Depending on the specificity of cfDNA associated structures, various forms may serve different roles in the body.

Various tissues may react differentlyto biological changes *in vivo*, which will likely affect DNA release. This may cause difficulties in identifying specifically related cfDNA changes. We propose that tissue and cell cultures be used for studying cfDNA release variation. Only when we better understand how cfDNA release is influenced by individual factors can we understand the large background variation in circulating DNA levels in *vivo*.

CIRCULATING NUCLEIC ACIDS AND PROTEASE ACTIVITIES IN BLOOD OF TUMOR PATIENTS

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High levels of DNA circulate predominantly in form of nucleosomes in blood of tumor patients. DNA is released into the blood circulation either by apoptotic or necrotic cells, or secreted by proliferating cells. As a late event of apoptosis intracellular endonucleases, such as caspase-activated DNases, are induced and cleave chromatin at the easily accessible internucleosomal linker regions into mono- or oligonucleosomes. Thus, the increase in nucleosome levels might be provoked by activated caspases. Caspases are cysteine proteases and belong to a large family, which consists of serine, aspartic, threonine and metallo proteases. In various cancer entities proteases are frequently overexpressed, and the prevailing acceptance that they are involved in metastases led to the development of small-molecule inhibitors for the treatment of cancer. Apart from cell-free DNA, small regulatory, non-coding RNA molecules also circulate in the blood. These microRNAs inhibit the translation of their target mRNA molecules, e.g. the transcripts of tumor suppressor genes, by binding sequence-specifically to the 3'UTR.

In our recent studies, we quantified circulating DNA and nucleosome levels as well as protease and caspase activities in blood of patients with breast, ovarian and lung cancer. Our findings demonstrated significant correlations of circulating nucleosome concentrations with DNA concentrations and caspase activities. The observed changes in apoptosis-related deregulation of proteolytic activities along with the elevated serum levels of nucleosomes and DNA in blood correlated with breast cancer progression. However, high circulating DNA concentrations in blood of breast cancer patients are no indicators of a malignant tumor, because they could not discriminate between benign and malignant lesions.

As there is an unmet need for biomarkers for the prediction and monitoring of anticancer therapies, we also carried out these analyses in serum of patients with ovarian cancer before and after first-line carboplatin/taxane-based chemotherapy. Our findings suggested that the residual tumor load after surgery may contribute to the increased DNA levels in blood of these patients. Moreover, the presence of micrometastatic cells in bone marrow was associated with increasing serum protease activities. Serum DNA levels together with bone marrow status could predict the prognosis of ovarian cancer patients undergoing platinum-based chemotherapy.

In lung tumor patients, serum DNA levels and caspase activities could also not distinguish between patients between benign disease and cancer patients. However, the levels of microRNAs (miR10b, miR141 and miR155) were significantly higher in lung cancer patients than those in patients with benign disease. Moreover, high serum miR10b values associated with lymph node metastasis.

In conclusion, our data show the diagnostic and prognostic relevance of circulating nucleic acids and proteolytic activities as blood-based markers.

Oral presentations

THE TRANSFORMING EFFECT OF CELL-FREE NUCLEIC ACIDS CIRCULATING IN THE PLASMA OF COLORECTAL CANCER PATIENTS REMAINS AFTER RESECTION OF THE PRIMARY TUMOR

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In a previous study, our group demonstrated that plasma of colon cancer patients, obtained prior to surgery, were able to oncogenically transform cultured cells.

The purpose of the present study was to examine the transforming effect of plasma of the same patients two years after resection of the primary tumor. With this purpose, we obtained blood samples from the alive patients (8/13). These samples were subjected to two consecutive centrifugations for the isolation of the cell-free plasma. By real-time PCR using FRET and PNA probes, we detected mutated human K-*ras* sequences (tumor DNA) in 4 of out the 8 plasmas analyzed. None of the 8 patients developed local recurrence but 4 had metastases when the blood samples were obtained.

We cultured NIH-3T3 cells (immortalized mouse fibroblasts) and hASCs (human Adipose Stem Cells) with the plasma of the 8 patients and of healthy volunteers (n=2). Subsequently, these cells were inoculated into immunodeficient NUDE mice.

In NIH-3T3 cells that were cultured with human plasmas, the human K-ras sequence was always detected. Mutated K-*ras* sequences were detected in the cells that were cultured with plasma containing such tumor DNA (3 of out 8 cultures). In hASCs cultures the mutated K-*ras* sequences were never detected.

The NIH-3T3 cells that had incorporated the mutated K-*ras* sequences generated tumors when were injected in mice. However, the NIH-3T3 cultures in which human tumor DNA was not detected, did not have oncogenic ability.

The plasma of the 3 patients with colorectal cancer who had tumor DNA, transfected this genetic material to NIH-3T3 cells, and produced tumors when were inoculated into NUDE mice. 2 of these 3 patients had metastases, which could explain the presence of tumor DNA in plasma. However, one of the patients had no metastasis, so

that we can conclude that the transforming ability of plasma is maintained even when the patient has no tumor. On the other hand the real-time PCR technique used in this study has proven to be highly sensitive and specific for detecting mutated K-*ras* sequences, because when tumor DNA was detected in plasma, transfection of these sequences occurred and vice versa.

GC-RIch CELL-FREE DNA (GC-cfDNA) ACTIVATES TLR-, NFkB-, JNK/p38- AND IRF-DEPENDENT SIGNALING PATHWAYS IN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS (ADMSC)

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It is well known that GC- cfDNA accumulates in the blood plasma at disease. We have previously shown that GC-cfDNA activates TLR9-dependent signal pathway resting on the increase in TLR9 and its signal pathway adapter MyD88 expression. In this work we explored in details the influence of DNA content on the activation of genes related to TLR-dependent signal pathways. We used RT-PCR to compare the expression patterns of 86 genes in intact cells and cells exposed to the model GC-DNA for 3 hours. We have shown that GC-DNA increases the expression of TLR1, TLR9, TLR7 and TLR8, TLR5, TLR6 were up regulated to a smaller extent. TLR2, TLR4 and TLR10 expression was not affected. GC-DNA also increased the expression of TLR-dependent signal pathway adaptors and effectors: HSPD1, MYD88, TIRAP, EIF2AK2, HSPA1A, MAP2K3, SARM1, TOLLIP, IRAK1, MAP3K7IP1, TRAF6, UBE2N, TICAM2 such as NFkB-, JNK/p38- and IRF-signal pathway genes: *IL10, MAP4K4, IFNA1, IFNG, NFKBIA, REL, TNF, IL8, IFNB1, IKBKB, IL12A, IL1B, MAP3K1, NFKB1, NFKB2, NFRKB, TNFRSF1A, RELA, MAPK8, FOS, ELK1* and *IRF3*. *CLEC4E, PTGS2, NF/IL6*-signal pathway genes expression was not activated. So, ADMSC respond to changes in cfDNA GC-content by activating TLR-, NFkB-, JNK/p38- and IRF-signal pathways. Thus, ADMSC physiology can be regulated by modifications in cfDNA GC-content.

Session VI
Research applications using CNAPS

Lectures

NUCLEIC ACIDS IN AMNIOTIC FLUID PROVIDE INSIGHTS INTO FETAL DEVELOPMENT

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To date, research into cell-free fetal (cff) nucleic acids has primarily focused on maternal plasma to develop clinical applications in non-invasive prenatal diagnosis. Cff DNA and RNA are also detectable in other body fluids such as amniotic fluid (AF) (Hui and Bianchi, Hum Reprod Update 2011; 17:362-371). AF is a complex biological material that provides a unique window into the developing human. In AF supernatant, cff RNA is present in much greater concentrations than in maternal plasma; it represents a pure fetal sample that is not mixed with maternal and placental nucleic acids. The aim of my lecture will be to summarize recent findings from our laboratory with regard to gene expression in normal and abnormal fetuses, and what these findings might mean for future prenatal diagnosis and fetal treatment.

Thus far, our results in normal and abnormal AF supernatant indicate that it is feasible to isolate cff nucleic acids from small volumes of discarded AF supernatant in sufficient quality and quantity to perform microarray studies and other associated downstream analyses. Cff nucleic acids in AF represent a physiologically separate pool from cff nucleic acids in maternal plasma. The placenta is not a major source of RNA in AF. Our analyses have resulted in the development of the AF core transcriptome (AFCT), which we defineas the 470 well-annotated genes present in 12 of 12 euploid mid-trimester AF supernatant samples ($P<0.04$). Our results suggest that cff RNA in AF derives from multiple tissues and organs, not just amniocytes. Using the publicly-available Novartis Gene Expression Atlas (http://biogps.gnf.org), we found that 15 fetal transcripts within the AFCT could be categorized as highly organ-specific. Of these, nearly half were expressed by the fetal brain. More detailed studies of the nervous-system associated genes demonstrated that the AFCT also contains transcripts expressed in spinal cord, dorsal root and superior cervical ganglia.

In summary, gene transcripts that are highly expressed in the developing nervous system are consistently detectable in mid-trimester AF supernatant. These results imply that AF cff RNA may be a source of potential biomarkers in living human fetuses, and suggest future clinical applications in the study of congenital disorders of the nervous system using AF supernatant.

NON INVASIVE PRENATAL DIAGNOSIS OF DOWN SYNDROME

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The development of a Non-Invasive Prenatal Diagnostic (NIPD) test for Down syndrome (trisomy 21) has become one of the most interesting fields in the prenatal world of the last decade. Extensive investigation of epigenetic differences between the mother and the fetus has led to the identification of Differentially Methylated Regions (DMRs) which have the potential to be used in the development of NIPD test for trisomy 21 (Papageorgiou *et al.*, AJP, 2009).

In this study, we have been using a subset of our previously identified DMRs to develop a non-invasive prenatal diagnostic test for trisomy 21 using the Methylated DNA Immunoprecipitation (MeDIP) methodology in combination with Real-time QPCR. The statistical significance of the 12 selected DMRs was evaluated using the Mann-Witney U test and selection of the best DMRs was followed.

We hereby present a strategy to achieve non-invasive fetal chromosome dosage assessment through the analysis of fetal specific DMRs. We achieved non-invasive prenatal detection of trisomy 21 by determining the methylation ratio of normal and trisomy 21 cases for each tested fetal specific DMR present in maternal peripheral blood, followed by further statistical analysis. The application of the above fetal specific methylation ratio approach provided with 100% accuracy the correct diagnosis of 66 normal and 34 Down Syndrome pregnancies.

We present in this study the successful development and validation of a low cost, fast, sensitive and universal, non-invasive prenatal diagnostic test for Down Syndrome which can be potentially implemented in diagnostic laboratories.

Oral presentations

DETECTION OF CELL-FREE DNA, RNA AND miRNA IN EXOSOMES: NEW NON INVASIVE PREGNANCY-ASSOCIATED MARKERS?

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Human placenta cells release free-fetal nucleic acids which are detected in the maternal circulation during pregnancy and offering non-invasive prenatal diagnosis of the genetic status of a fetus. These cells release exosomes which are used in fetal-maternal cross-talk. Our hypothesis is that human fetal cells would secrete DNA, RNA and miRNA extracellularly via exosomes which would define new non invasive biomarkers.

To confirm theses hypothesis, microvesicles (exosomes and microparticles, MPs) were isolated using protocol developed in our hand from trophoblast cell line (JEG-3) in supernatant culture. An electronic microscopy analysis has permitted to valid this protocol. MPs were analysed by flow cytometry. Real time PCR was performed to quantify DNA (δ-globin and *SRY* gene), RNA (GAPDH and HLA G gene) and placenta-specific miRNA (23a, 96) in exosomes and microparticles. MPs and exosomes were treated with or without DNAse or Triton 0,01%. Specific plasmid calibrators were made to standardize this quantification.

Cell-free DNA and RNA were detected in both exosomes and microparticles in JEG-3 cells supernatant culture. DNAse studies reported a higher intravesicular DNA fraction in exosomes than in microparticles. MicroRNA expression levels were determined in both MPs and exosomes (miRNA 96: 180,98 vs 62,87 pg/ug of protein). miRNA secreted from human trophoblast cells via exosomes were correlated with the intracellular level of miRNAs (ratio: 10-5).

This study has demonstrated that a part of cell-free nucleic acids (DNA, RNA, and miRNA) is released extracellularly from feto/placental cells via exosomes. The "fetal" exosomes potentially could offer in a future an excellent method for non invasive prenatal diagnosis.

PROFILING OF CIRCULATING miRNAs IN MATERNAL AND UMBILICAL CORD SERUM TOGETHER WITH THOSE TISSUE-BASED miRNAs IN PLACENTAS AND OF PREECLAMPSIA PREGNANT WOMEN

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Preeclampsia is one of the leading causes of maternal and fetal/neonatal mortality and morbidity worldwide. Here, for the first time, we systemLy analyzed the expression profiles of circulating miRNAs in the maternal serum and umbilical cord serum, together with those miRNAs in their placentas of PE pregnant women, by the method of high-throughput sequencing technology, SOLiD. The samples here were collected from one normal and four preeclampsia pregnant women. Those PE related miRNAs were seperately analyzed in three types, with the rules of p-value less than 0.05, fold-change of logarithmic analysis of reads counts (log2-ratio) more than 2, and consistent dys-regulated(up-regulated or down-regulated). The reslut found that miRNAs circualting in serum were more similar but less abudant as compared to placenta, still shared the same biological features including length peak around 21-22nt, miR/miR* around 4:1, and consistent phenomena of isomiR. Moreover, plenties of novel PE-related miRNAs were found separately in all three types. In placentas, 1 miRNA were up-regulated and 10 miRNAs down-regulated. In maternal serum, 15

miRNAs were up-regulated and 7 miRNAs down-regulated. In umbilical cord serum, 2 miRNAs were up-regulated and 6 miRNAs down-regulated.
These results showed that more PE-related miRNAs were expressed in the maternal serum as compared to the other two, with one shared miRNAs in maternal and umbilical cord serum, has-miR-320c. These results indicated that those PE special miRNAs obtained in this study might be used as notable biomarkers for diagnosis and prognosis of PE, and also provided a novel and system pipeline to analyze the profile of circulating miRNA by the method of high-throughput sequencing platform.

COLD-PCR: A NOVEL PCR ASSAY FOR NONINVASIVE PRENATAL DIAGNOSIS OF GENETIC DISEASES

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Despite the advances in improving the analytical sensitivity of methods noninvasive prenatal diagnosis of genetic diseases has not yet attained a widespread clinical application. Fetal DNA in maternal plasma is diluted in a large amount of wild-type sequences of maternal origin which hamper the identification of fetal paternally inherited mutated alleles. We developed conditions for the identification of fetal alleles based on co-amplification at lower denaturation temperature-PCR (COLD-PCR), an innovative methodology which enables robust enrichment of low-abundance mutations, coupled with sequencing as downstream methodology. This strategy was applied to the identification of two prevalent mutations in the beta-globin gene. In this protocol, heteroduplexes were selectively denatured at a critical temperature and amplified allowing the enrichment of fetal mutated alleles. Full COLD-PCR enabled correct identification of fetal paternally inherited alleles in all the 4 0 couples tested. The application of COLD-PCR enables straightforward and reliable identification of inherited mutated alleles without the need of sophisticated procedures and costly equipment. Full COLD-PCR can be successfully applied to enrich DNA variants irrespective of the kind and position of the mutation thus providing a powerful tool for noninvasive prenatal diagnosis of fetal alleles in a variety of genetic diseases or in general for the identification of muted minority alleles.

Session VII
Technical update

Short lectures

DETECTION AND CHARACTERIZATION OF CIRCULATING DNA IN PLASMA FROM COLORECTAL CANCER PATIENTS BY aCGH MICROARRAYS

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REQUIREMENTS FOR CNAPS: PRE-ANALYTICAL STABILIZATION & AUTOMATED EXTRACTION [FOR MOLECULAR DIAGNOSTICS]

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Circulating, cell-free nucleic acids (ccfNA) are present in plasma, serum and other body fluids and provide targets for molecular detection of malignancies and other diseases based on a simple blood sample. Likewise, ccfNA derived from the developing fetus are present in maternal plasma and their detection enables non-invasive prenatal genetic testing. Here, the performance of a novel room temperature stabilization technology for preserving the ccfNA in whole blood and the efficiency of a new automated large volume ccfNA extraction method was evaluated.
Stabilization: Fresh whole EDTA blood was mixed with a novel stabilizing reagent immediately after blood collection to preserve the ccfNA population in whole blood as it is present at the time of venipuncture. Plasma was prepared from stabilized whole blood, followed by ccfNA extraction. Using chip-based electrophoresis of ccfNA and real-time PCR with amplicons of different lengths to track nucleic acid integrity, the circulating DNA and RNA status in plasma was shown to be preserved over 3 to 6 days compared to non-stabilized EDTA blood.
Automated extraction: ccfDNA was extracted from 3—6 mL plasma using a newly developed protocol with novel chemistry and magnetic bead technology on the QIA symphony SP instrument. In individual donor samples, the ccfDNA yield was shown to be comparable to the state-of-the-art reference (QIAamp Circulating Nucleic Acid Kit) based on real-time PCR specific for 18S rDNA targets and recovery of spiked control DNA. Our results demonstrate the feasibility and utility of a novel ccfNA pre-analytical workflow with room temperature storage of primary blood samples for up to six days until plasma separation, followed by automated large-volume ccfNA extraction compatible with real-time PCR target analysis.

HIGH THROUGHPUT MASSIVE PARALLEL SEQUENCING

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Next generation sequencing combines two innovative aspects of actual genomic technologies to reach an unprecedented level of deep-focusing into genomes. The first characteristic is a very high sequencing throughput based on simultaneous parallel reading of literally millions, to almost billions, of molecules. The basis for this ability has been the pioneer idea of clonal amplification in self-forming, single molecule-sized micro-reactors made up of an oil-in-water emulsion.[1] This system allows the parallel management of millions of different molecules that can be amplified in a single tube, but in a clonal fashion, followed by "one by one" sequencing, thus avoiding extensive, laborious and time-consuming steps in library cloning. More recently, some other systems of clonal expansion have been developed such as surface-bound "chemical" amplification or enzyme immobilization and real-time single-molecule sequencing. As a consequence, clonal expansion is performed at the molecule level and not in a live vector integrated in a bacteria or a virus, skipping the need of growing and isolating living individuals and of recovering target DNA fragments. Handling of millions of clones of DNA molecules that consist exclusively in the sequences of interest is followed by individual sequencing of each clone, which is followed by standard and well developed microscopic techniques.[2]

The second and biologically more relevant property of next gen-sequencing is the lack of previous knowledge about target DNA needed to proceed with preparation of fragment libraries. The whole process is driven by the incorporation of defined adapters to the target DNA molecules, which provide the necessary signals for amplification, enrichment and final sequencing. The binding of adapters to the target DNA molecules is driven by standard or engineered ligases and polymerases. While some bias may be observed due to posterior PCR amplification or sequencing steps that may disfavor some regions with anomalous GC-content or other special characteristics, the main advantage of this design is that it opens the gate to the sequencing of absolutely unknown genomes as long as all the process, from library-prep to clonal amplification, enrichment, sequencing and analysis do only rely in artificial sequences introduced at the beginning of the procedure, absolutely independent of the nature of DNA been studied.

While first designed as a tool to whole genome sequencing, it was immediately realized that the power of next-gen sequencing could also be dedicated to re-sequencing of reference genomes and to an unlimited number of applications. In human genomics, rapid and cost-effective genome-wide analysis of polymorphisms and structural variants, as well as RNA-seq-based transcriptome profiling, are called to substitute and improve conventional technologies in the very near future. In addition, new procedures are constantly been developed to suit different needs, such as focus-sequencing of target regions or strain-specific RNA sequencing.

In summary, working with cell-free systems in combination with procedures designed for very low represented molecules convert next-generation sequencing in an ideal tool to discover and study expression patterns associated to systems such as circulating nucleic acids.

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NEXT GENERATION SEQUENCING FOR CNAPS BASED DIAGNOSTICS

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FROM DISCOVERY TO THE CLINIC: Epi proColon, A PLASMA BASED TEST TO DETECT COLORECTAL CANCER

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Since its founding, Epigenomics has focused on discovering biomarkers based on DNA methylation patterns, and developing them into molecular diagnostic products for oncology indications. The company was amongst the earliest to embrace the concept that in cancer patients, circulating nucleic acids in blood may contain a tumor DNA fraction that could provide a new analyte for screening or diagnostics if biomarkers with the right properties could be identified. The discovery, development and commercialization of the methylated Septin9 (mSEPT9) gene, the DNA methylation biomarker at the core of our CE marked IVD kit, Epi *pro*Colon, is an excellent case study in the growing field of circulating nucleic acids.

Discovery. ^{m}SEPT9 and several hundred additional differentially methylated sequences were discoveredusing PCR based methods, and verified by differential microarray analysis using tumor and normal adjacent tissue from >50 patients.Based on these data, the company developed realtime PCR assays for>100 methylation markers, and their methylation status was determined in tissue samples from ~200 cancer patients and non disease controls, as well as in plasma pools from healthy people. From this screening effort,^{m}SEPT9 was identified as a priority candidate biomarker based on filtering for differential methylation in tumor tissue, and counter filtering for negativity in healthy plasma.

Development. The process of translating ^{m}SEPT9 from an academically interesting biomarker to a viable product required significant methodology improvements in DNA extraction from plasma, bisulfite conversion of the DNA, and the design of the ^{m}SEPT9 real time PCR assay. Assay improvements (sample processing and PCR) were assessed with analytical samples (spiked DNA) and verified with patient samples from case/control collections in a number of training/test experiments. The culmination of

this effort, following testing on >2000 subjects, was the commercial launch of the CE marked IVD kit, Epi *pro*Colonfor the measurement of mSEPT9 in plasma.To further validate the mSEPT9 biomarker for colorectal cancer screening, Epigenomics undertook a prospective collection of plasma from average risk subjects planning to undergo colonoscopy and using a modified form of the Epi *pro*Colon test, successfully identified 67%of the patients with colorectal cancer (based on colonoscopy), at a specificity of 88%, supporting our position that this test is a convenient, non-invasive assay to reach people whoare not compliant with colorectal cancer screening guidelines.

Future Development. Combining market feedback and further technology improvements, we have completed the design of the next generation Epi *pro*Colon test and in feasibility studies using case/control samples we observed a sensitivity for cancer of 91% at 87% specificity. The improved test is undergoing verification and validation for CE marking as well as safety and effectiveness testing using our prospectively collected average risk subject population in support of a PMA submission for regulatory approval to sell the test in the US.

The discovery and development of ^{m}SEPT9 illustrate the complexity and effort required to translate an interesting biomarker into a commercially viable molecular test.While compounded in part by the novelty of tests based on circulating DNA in blood andby the rarity of tests based on DNA methylation, the ^{m}SEPT9 story demonstrates the potential of using circulating nucleic acids in blood to address clinically relevant unmet needs.

Oral presentations

NUCLEOSOME POSITIONING CAN HELP IDENTIFY DNA FRAGMENTS WITH HIGHER ABUNDANCE IN PLASMA

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Measurement of circulating DNA is fast becoming a practical diagnostic tool. It would be important to identify intrinsic locus-dependent or sequence-dependent factors that can aid in the design of more accurate or sensitive assays. Recent data obtained by massively parallel paired-end sequencing of cell-free plasma DNA revealed a multi-modal size distribution of DNA fragments. The most common fragment sizes observed were between 140-170 bps, approximately the length of DNA bound in nucleosomes, indicating that nucleosome binding may inhibit degradation of circulating DNA. Nucleosome positioning across the genome is not random and is predominantly determined by DNA sequence characteristics. Hence, we hypothesized that DNA fragments with higher intrinsic nucleosome occupancy would be more prevalent in circulation as compared to those that span a low intrinsic nucleosome occupancy region. We found that nucleosome occupancy, as predicted by several models based on in-vitro and in-vivo data, correlated with physical coverage of circulating DNA fragments as measured by Illumina sequencing. Both model predictions and physical coverage show a strong GC-bias. However, even when comparing fragments of equal GC content, nucleosome occupancy estimated from a sequence-based model could help predict fragments that had higher physical coverage. Thus, nucleosome positioning models could become a useful component in design of non-invasive diagnostic assays based on circulating DNA.

Joint session EuroGentest Network-CNAPS researchers

Lectures

INTRODUCTION TO EUROGENTEST, A EUROPEAN NETWORK FOR THE IMPROVEMENT AND STANDARDIZATION OF QUALITY IN GENETIC TESTING

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Genetic testing, which includes genetic counselling, is an interactive process involving the patient and his/her family, the genetic laboratory and the referring clinician or clinical geneticist. For all genetic tests, accurate results and interpretation are essential as there may only be a single opportunity for testing (e.g. because of the risks of invasive prenatal testing). Or, some patients request presymptomatic testing for a severe familial disease, and have no clinical phenotype or symptoms at the time of testing; however, the result has huge consequences for their future.

This is not different for in non-invasive prenatal diagnostic (NIPD). But the challenges are big: the novel testingstrategies will be introduced their way very fast, and through the use of massive parallel sequencing technologies, the amount of genetic data that will be generated, will be vast.

Hence, NIPD is a major issue for EuroGentest, a European network (www.eurogentest.org) aiming to insure that all aspects of genetic testing remain of high quality and that laboratories provide accurate and reliable results for the benefit of the patients.EuroGentest is financially supported by the European Commission (FP7) and has included several activities that were formally provided by the SAFE project.

To obtain accurate results it is crucial that a genetic laboratory has reliable procedures underpinned by a robust quality management system, to minimize errors and failures. Laboratory Quality Management (QM) is a concept that covers diverse aspects of a diagnostic genetic (laboratory accreditation, external quality assessment (EQA), control materials, training, document control, etc.). EuroGentest is active in giving training workshops to aid laboratories to implement such a QM system.

High quality genetic testing is a process starting from the correct indication for the test, a fair way to fund testing and the prioritization needed for that. It also includes pretest counselling and consenting to the test, taking and sending the sample with adequate clinical information, a correctly performed test with an adequate interpreting of the result to the referring clinicians, post-test counselling and the other post test actions like informing relatives and organizing possible follow up. Thus, EuroGentest strongly aims at improving the quality of all genetic services associated with genetic testing, across Europe, and at all the stages in this process. The very rapid changes in human genetics highlight the current momentum to implement novel diagnostic tools. The participants in EuroGentest want to further contribute to guidelines and recommendations, in a concordant way and in collaboration with the professional organisations.

IMPLEMENTING NON-INVASIVE PRENATAL FETAL SEX DETERMINATION USING CELL FREE FETAL DNA

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Non-invasive prenatal determination of fetal sexusing cell free fetal DNA (ccfDNA) in maternal plasma in pregnancies at high risk of sex linked genetic disorders has been widely reported[1] and available in the UK since 2003. Access to this testing was patchy as it was initially only available on a research basis. Following early publication of a small series[2] of cases which demonstrated clear clinical utility, there was a call from genetic services in the UK to evaluate the technology formally and establish standards for more widespread implementation into the UK NHS. Here we describe this process including development of standards for extraction of cffDNA[3], a large scale audit of accuracy of testing of 528 cases performed clinically in two laboratories[4] and a detailed economic assessment[5]. Implementation of any new test into routine clinical practice not only includes development of laboratory standards but also health professional education and information for service users. Ongoing work evaluating all these aspects will be presented.

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NIPD PILOT EQA ORGANIZED BY RAPID/UKNEQAS

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Over the last 3-4 years a non invasive prenatal test for fetal sex based on detection of Y-chromosome DNA sequence in the cell free fraction of circulating maternal blood has entered service in a small number of Genetic Laboratories in the UK. This development is now being assessed by the RAPID project funded by the UK

National Institute for Health Research (NIHR) The National Genetics Reference Laboratory (Manchester) is working with RAPID and the External Quality Assessment agencies UKNEQAS the European Molecular Genetics Quality Network and EuroGentest to develop an effective and practical External Quality Assessment for laboratories offering (or planning to offer) this test.

The approach is to first pilot the feasibility of an EQA in terms of the stability and homogeneity of the biological materials presented as the EQA challenge and the logistics of distributing materials and collating and assessing returns from the laboratories. NGRL(M) and UKNEQAS distributed a set of materials representing three mock clinical cases to four participating laboratories in the UK and Netherlands in June 2010.

The materials used were pooled serum samples from women consenting to an NIPD test stored frozen for one year or more. The result (presence or absence of a detectable level Y-chromosome signal) was previously validated by two centres.

No laboratories reported an incorrect result. However two centres reported slightly lower than expected male signal for one case. We suspect that the reason for this is that the plasma samples had been archived for a number of months at -40oC and had undergone at least two freeze thaw cycles by the time they reached the participants. In our experience this can lower the SRY signal however it did not result in an incorrect result. In addition two laboratories reported a level of sample failure (inconclusive) in the plasmas from the two women without a male fetus. In all these cases the failures were due to an observed low level of SRY signal. Two participants reported sample failure (inconclusive) in the plasma samples from the two cases without a male fetus. These failures were due to an observed low level of SRY signal.

In 2010 EMQN and EuroGentest carried out a web based survey notified to approximately 800 laboratories in 42 countriesThere were 52 replies (10% response) from 27 countries (19 in the European Union). Of the respondents 17 labs currently offer NIPD and the remainder planned to develop NIPD in next 2 years. The indications listed/planned: were Sexing (43), Rhesus (25), Trisomy (10).

QUALITY ASSURANCE OF NIPD: A VIEW FROM THE UNITED STATES

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Noninvasive prenatal diagnosis (NIPD) using fetal DNA in maternal plasma has the potential to transform the practice of perinatal medicine.In hemolytic disease of the fetus and newborn (HDFN), using NIPD avoids the risks of amniocentesis including the risk of further alloimmunization.Since fetal DNA represents only a minor fraction of the total DNA in maternal plasma, molecular assays for fetal genetic markers are technically challenging.Several studies have demonstrated that the sensitivity and specificity of NIPD for the RHD gene is 96-100% but this is associated with a higher rate of false positives, false negatives and inconclusive results compared to conventional prenatal diagnostic studies. Analytic problems may be caused by the quality of DNA preparation, difficulty in controlling for appropriate input of fetal genomes, lack of adequate control fetal genetic markers, ethnic-specific variant alleles and general limitations of current technology. Therefore, NIPD brings its own unique set of quality assurance (QA) challenges for the clinical laboratory.

A robust QA program is essential for clinical labs offering NIPD testing to ensure that a clinical laboratory is maintaining a high degree of confidence in test integrity and accuracy throughout the pre-analytical, analytical and post-analytical phases. This includes sample receipt and handling, testing, result interpretation and report output. External quality assessment (EQA), accreditation, reference materials, best practice guidelines and staff training are important parts of any QA program. In the US, standards and guidelines for molecular testing from Clinical Laboratory Improvement Amendments (CLIA), American College of Medical Genetics (ACMG) and College of American Pathologists (CAP), the main accreditation organization for molecular labs, define many of the quality systems that also pertain to NIFD. In the pre-analytical phase, sample requirements must be established for gestational age, sample age, preparation and storage since sample integrity is an important aspect of NIPD. ACMG guidelines for prenatal diagnosis should be applied to NIPD, such as testing the parental samples prior to prenatal testing and having a program in place to monitor the accuracy of testing including resolution of discrepancies. For the assay method being used, laboratories should determine the appropriate input of fetal genomes and what level of maternal contamination is acceptable to ensure a correct analytic result. Finally, laboratories need a method to detect the level of contamination that leads to a diagnostic error.

EQA for NIPD will be an essential component of a QA program that assesses laboratory performance using inter-laboratory comparisons.CLIA requires laboratories to participate in a program for validating the accuracy of its tests at least twice per year. Ideally, EQA results should be carefully reviewed by experts in the field and a participant summary written to address any technical or interpretive issues. Development of appropriate control reference materials is important for NIPD EQM, quality control and test validation. Access to control materials helps laboratories investigate and validate how genetic variants affect a particular assay method.

Professional practice guidelines for NIPD need to be developed to ensure that laboratories are aware of best practices, proper interpretation, limitations of techniques and technical issues. It will be crucial to educate physicians about the limitations of the test results. Finally, it is important that laboratory reports have clear interpretations, including recommendations for additional testing, if necessary, so they can monitor their patients appropriately.

Posters

1 - THE IMPACT OF LOW-FREQUENCY NOISE ON THE CONTENT OF LOW-MOLECULAR-WEIGHT DNA OF BLOOD PLASMA

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The influence of low-frequency noise is connected with both: natural phenomena (tidal waves, earthquakes, volcanic eruptions, thunders etc) and technogenic processes. Research on animals showed that the content of low-molecular-weight DNA (lmwDNA) in blood plasma of rats has increased 24 h after the single isolated exposure to noise (TBL 2225, 2000 impulses) and constituted 83 ng/mL and 84 ng/mL plasma, with the highest noise pressure level being 120dB and 150 dB (exposure time 17 min), correspondingly. The level of lmwDNA in the control group was 10 ng/mL of plasma. The increased lmwDNA content retained within 7 days after the exposure and constituted 90 ng/mL and 40 ng/mL plasma after the corresponding noise pressure levels were 120dB and 150 dB. Long-term exposure to low-frequency noise (13 weeks, 5 times a week) caused a multiple rise in the content of lmwDNA of blood plasma, which differed after the exposure to 120dB and 150 dB highest levels of noise and constituted 664 ng/mL and 395 ng/mL plasma respectively. For the first time it has been shown that exposure to low-frequency noise results in an increase of the level of circulating lmwDNA in blood. It is known, that the states linked with the increase of the apoptosis, like the exposure to ionizing radiation, are accompanied by the increase of the content of circulating lmwDNA fraction in blood. This implies that the impact of noise strengthens apoptosis. The differences in the content of lmwDNA after the impact with the levels of the noise pressure of 120 dB and 150 dB indicate that there are at least two mechanisms of the release of the lmwDNA. The impact of low-frequency noise does not only cause the stress effect, but may also have a possible direct destructive effect on one's cells.

2 - CAN PLASMA DNA MONITORING BE EMPLOYED IN PERSONALIZED CHEMOTHERAPY FOR PATIENTS WITH ADVANCED LUNG CANCER?

S. Pan, W. Xia, Q. Ding, Y. Shu, T. Xu, Y. Geng, Y. Lu, D. Chen, J. Xu, F.G Wang, C. Zhao, P. Huang, P. Huang, H. Shen, Z. Hu, Shan Lu

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Background. Personalized chemotherapy is the ideal treatment usually chosen to help improve the survival chances of patients with advanced lung cancer. However, there is no short-term evaluation protocol for predicting therapeutic efficacy. The aim of this study was to determine the prognostic value of plasma DNA for patients with advanced lung cancer.
Methods. Forty-two lung cancer patients and 200 healthy controls were included in this study. Plasma DNA was extracted from patient plasma samples with internal controls, healthy controls and quality controls by using the BILATEST DNA Kit. The quantity of plasma DNA was determined by using duplex real-time quantitative PCR.
Results. After first-line chemotherapy, plasma DNA levels of partial response patients were significantly different from those of stable disease patients or progressive disease patients, but with no statistical difference from healthy controls ($P=0.014$, $P<0.001$ and $P=0.418$, respectively). A statistically better survival time was showed in patients who had lower levels of plasma DNA after the third cycle and first-line chemotherapy ($P=0.024$ and $P=0.001$, respectively).
Conclusion. Plasma DNA level can be useful in predicting response to therapy and guiding personalized chemotherapeutic medication in patients with advanced lung cancer. Key words: plasma DNA, personalized chemotherapy, advanced lung cancer, real-time quantitative PCR.

3 - EARLY SECOND-TRIMESTER SERUM MIRNA PROFILING PREDICT GESTATIONAL DIABETES MELLITUS

C. Zhao, J. Dong, T. Jiang, Z. Shi, P. Huang, R. Huo, J. Dai, S. Pan, Z. Hu, J. Sha

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Background. Gestational diabetes mellitus (GDM) is one of the most common pregnancy complications and affects approximately 3-8% of all pregnancies. Detection of women at higher risk for GDM early in pregnancy is a desirable goal because interventions such as diet, medication, and exercise may be applied earlier and has a positive effect on maternal and fetal outcomes. The aim of this study is to systematically assess whether the serum microRNAs (miRNAs) profiling can predict GDM in advance of the measurement of blood glucose.
Methods. Serum samples collected at 16-19 gestational weeks, were obtained from 92 healthy pregnant women and 92 women who subsequently developed GDM. miRNA profiling was performed on total RNA extracted from serum obtained from 48 individuals (24 controls and 24 patients) by systematically TaqMan Low Density Array. Individual quantitative reverse transcription polymerase chain reaction assay was used to validate the profiling results in the discovery set and in a validation set of 92 controls and 92 GDM patients. Logistic regression was used to estimate the associations between miRNA expression levels and GDM risk.

Results. Two miRNAs (miR-29a and miR-222) were identified and consistently validated as efficient predictors. Taken together, women with high serum miRNA expression levels had significantly decreased risks of developing GDM for the two miRNAs (Odds ratio [OR]=0.25, 95% confidence interval [CI]=0.13-0.47 for miR-29a; and O=0.34, 95%CI=0.18-0.64 for miR-222).
Conclusions. MiR-29a and miR-222 in serum may serve as noninvasive biomarkers for predicting GDM development.

4 - EVALUATION OF BD VACUTAINER EDTA-K2 PLUS TUBES WITH AND WITHOUT POLYMER GEL FOR PLASMA DNA QUANTIFICATION

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Objective. To evaluate BD vacutainer EDTA-K2 plus tubes with and without Polymer gel for plasma DNA quantification.
Methods. A total of 20 healthy college students (10 females) were recruited with informed consent. A 6-mL blood sample was withdrawn from the antecubital vein of each individual, and equally collected into one BD vacutainer EDTA-K2 plus tube without Polymer gel (tube A) and two BD vacutainer EDTA-K2 plus tubes with Polymer gel (tube B and tube C). All blood samples were processed within 2 h of collection. After low speed (1,600 g) centrifugation step at room temperature for 10 min, the supernatant plasma samples were transferred into 1.5-mL polypropylene tubes, with particular care not to disturb the buffy coat layer. The plasma samples from tube A and B were then centrifuged at 16,000 g at 4° for 10 min to remove any remaining blood cells. The plasma samples from tube A were added into BD vacutainer EDTA-K2 plus tubes with Polymer gel (tube D) and centrifuged at 1,600 g for 10 min. Fifty thousand copies of plasmid DNA were added into 200μl cell-free plasma samples as internal controls. Thus, the plasmid DNA concentration in each plasma sample was $2.5\mu 10^5$ copies/mL. DNA was extracted from 200-μl plasma samples with an internal control using the Bilatest Viral DNA/RNA Kit (Bilatec, Viernheim, Germany), according to the manufacturer's recommendations. Duplex real-time quantitative PCR was performed for the human βactin gene and internal control plasmid DNA amplification in the same volume of 25 μl with components supplied in the TaKaRa Ex Taq™ R-PCR, version 2.1 (TaKaRa). Each reaction contained 2.5 μL of 10 μ real-time PCR buffer, 400 μM of each deoxynucleotide triphosphates, 6 mM MgCl2, 200 nM of each forward primer (F1 and F2), 700 nM reverse primer (R), 100 nM of each dual-labeled fluorescent probe (P1 and P2), 1 U TaKaRa Ex Taq HS, and 2.5 μl of extracted plasma DNA. The PCR cycling conditions were as follows: 95°C for 5 min, followed by 94°C for 30 s, 56°C for 30 s, and 72°C for 40 s, repeated for 45 cycles. DNA amplification was carried out in a 96-well reaction plate format in the Applied Biosystems 7500 Sequence Detector (Applied Biosystems, CA, USA). Fluorescence signals were detected in channels 1 and 2 for FAM- and JOE-labeled probes, respectively. The plasma DNA concentrations were calculated according to the threshold cycle (Ct) values and internal control's concentration. Paired t-test was used to detect differences of plasma DNA concentrations among tube A, B and C.
Results. The DNA concentrations of plasma samples from BD vacutainer EDTA-K2 plus tubes with Polymer gel by one-step or two-step centrifugation (tube B and C) were both lower than those of plasma samples from BD vacutainer EDTA-K2 plus tubes without Polymer gel by two-step centrifugation (tube A) (paired t-test, P=0.0064 and 0.0068). There was no statistically significant difference of DNA concentrations between plasma samples from BD vacutainer EDTA-K2 plus tubes without Polymer gel by two-step centrifugation (tube A) and plasma samples of tube A added into BD vacutainer EDTA-K2 plus tubes with Polymer gel (tube D) (paired t-test, P=0.1479).
Conclutions. The BD vacutainer EDTA-K2 plus tubes with Polymer gel was more suitable than the tubes without Polymer gel for collection of cell-free plasma samples for plasma DNA quantification.

5 - INVESTIGATION OF METHYLATION PATTERNS OF APC GENE IN LUNG CANCER WITH A NOVEL FLUORESCENCE MELTING CURVE ANALYSIS ASSAY

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Objective. To identify methylation patterns in the promoter region of APC gene in lung cancer cell lines and two lung cancer patients by fluorescence melting curve analysis assay.
Methods. After bisulfate treatment, DNA samples of lymphocytes from cord blood without and with trans-methyl treatment were amplified. The amplicons were then cloned into plasmid vector and employed as negetive and positive controls. A pair of general primes were designed to amplify the target sequence in the p APC gene romoter region comprising 21 CpG sites. DNA melting curves were acquired by measuring the fluorescence of a double-stranded DNA-binding dye (SYBR Green I) during the dissociation stage. The methylation patterns of 4 lung cancer cell lines and 2 lung cancer patients' tumor tissue cells were determined by comparison of melting temperatures (Tm) with negetive and positive controls and sequencing.
Results. Melting curve analysis showed that three of four lung cancer cell lines (NCI-H446,SPCA1,NCI-H520) displayed a melting temperature 83° as low as the unmethylated negetive control, while the other one (NCI-H460) displayed 2 melting peaks of 83° and 88° which were corresponding to the Tm of unmethylated negetive control and fully methylated positive control, respectively. Sequencing reports were all in accordance with the melting curve analysis. The Tm values of two lung can-

cer patients were both between the values of negetive and positive controls.
Conclusions. The APC promoter region methylation patterns of NCI-H446 and SPCA1 and NCI-H520 are described as unmethylated alleles, while NCI-H460 cells exhibit monoallelic methylation. Two lung cancer patients' tumor tissue cells display partial methylation in APC promoter region. Integration of PCR and fluorescence melting analysis may be useful for simple and cost-effective detection of aberrant methylation patterns.

6 - CAN MYCOBACTERIUM TUBERCULOSIS DNA BE DETECTED IN PLASMA/SERUM SAMPLES FROM TUBERCULOSIS PATIENTS?

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Background. China is one of the high burden countries of Mycobacterium tuberculosis infection. One challenge for tuberculosis (TB) control is the earlier diagnosis of M. tuberculosis. Tests based on PCR have shown promise for the detection of M. tuberculosis in different clinical samples except plasma/serum. The purpose of this study was to answer the question that whether M. tuberculosis DNA can be detected in plasma/serum samples.
Methods. A total of 43 serums and 94 plasma were collected from 124 clinical diagnosed TB patients. Four different M. tuberculosis DNA extraction methods, including phenol-chloroform method, Qiagen kit, Omega kit and magnetic bead method were compared to get higher sensitivity. One quantitative fluorescent PCR designed by this study was used for the detection of M. tuberculosis DNA.
Results. The highest DNA extraction efficiency (52.8%) and the best reproducibility (CV=26.7%) were seen in the magnetic bead method. And M. tuberculosis DNA can really be detected in some of these samples, and 39 of the 124 (31.5%) TB patients showed M. tuberculosis DNA positive in plasma or serum samples. Interestingly, 35.3% (12/34) smear negative cases demonstrate M. tuberculosis DNA positive.
Conclusion. In conclusion, this is the first study to report the existence of circulating M. tuberculosis DNA in plasma/serum from tuberculosis patients and showed that the detection of M. tuberculosis DNA may provide valuable information for the diagnosis of AFB negative TB patients.

7 - CHARACTERIZATION OF H3K9me3- AND H4K20me3-ASSOCIATED CIRCULATING NUCLEOSOMAL DNA BY HIGH-THROUGHPUT SEQUENCING IN COLORECTAL CANCER

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Background. Modified histone tails in the circulating nucleosomes bear the potential as biomarkers in cancer. Two repressive histone methylation marks, trimethylations of H3 lysine 9 (H3K9me3) and H4 lysine 20 (H4K20me3) which are hallmarks of pericentric heterochromatin, are the subject of this work. Here, we performed a chromatin immunoprecipitation (ChIP)-related high-throughput sequencing of H3K9me3- and H4K20me3-associated nucleosomal DNA in plasma of healthy subjects and the patients with colorectal cancer (CRC).
Methods. Nucleosomal DNA immunoprecipitated from 200 µl plasma were exposed to the Roche 454FLX sequencing which included a general library preparation and pyrosequencing. The generated array of sequences were compared to the human reference genome, and the repeat content was determined using the RepeatMasker software. Statistically, we compared total numbers of H3K9me3- and H4K20me3-associated sequences or the number of pericentromeric satellites such as satellite II between CRC patients and controls.
Results. For both modifications, we obtained a higher number of sequences in the healthy controls than the CRC patients where the difference was more pronounced for H3K9me3 (975 vs. 515, $p<0.01$). Sequences of satellites were highly (13-15fold) enriched in the circulation, when compared to their genome averages. The number of H3K9me3-enriched satellite II sequences was lower in CRC patients (6.7% vs. 22.6%, $p<0.01$) but this was not the case for H4K20me3 (25.6% vs. 31.7%).
Conclusions. Our findings reveal that of the two methyl marks, especially levels of H3K9me3 are lower in patients with CRC. These findings may present a basis to further work to investigate the diagnostic potential of these changes in CRC.

8 - CIRCULATING NUCLEIC ACIDS IN PLASMA FOR BIOMARKER DEVELOPMENT IN ONCOLOGY AND NEURODEGENERATIVE DISEASES

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Abstract. The technique of Methylation Detection (MethDet) has been developed for analysis of methylation in samples when only a limited amount of DNA is available (LCM, biopsies, cytological samples, plasma). It is based on digestion of DNA with a methylation-sensitive restriction enzyme and amplification of surviving fragments.
The proof-of-principle technique allows assessment of methylation in 56 promoters in each sample (MethDet-56) using cell-free circulating DNA extracted from 0.5 mL of plasma. Analysis of this DNA produced feasibility data indicating that the MethDet approach could be used to generate clinical-grade biomarkers for disease-specific detection, differential diagnosis, and treatment monitoring. This presentation will address proof-of-principle results for MethDet-based differential diagnosis of ovarian adenocarcinoma, diagnosis and detection of attacks in multiple sclerosis, and monitoring of treatment in breast cancer. Further

development of the MethDet for analysis of 12,000 fragments in each sample will be presented.

	Sensitivity (%)		Specificity (%)	
	Continuous	Fixed cutoff	Continuous	Fixed cutoff
BOD v HC	90.0 (76.7 - 100)	78.9 (64.3 - 93.5)	76.7 (66.7 - 96.7)	74.1 (58.4 - 89.8)
BOD v OvCa	73.3 (56.7 - 90.0)	82.3 (68.6 - 96.0)	80.0 (66.7 - 93.3)	71.5 (55.3 - 87.7)
OvCa vs HC	90.0 (80.0 - 100)	79.1 (64.6 - 93.6)	86.7 (66.7 - 96.7)	86.9 (74.8 - 99.0)

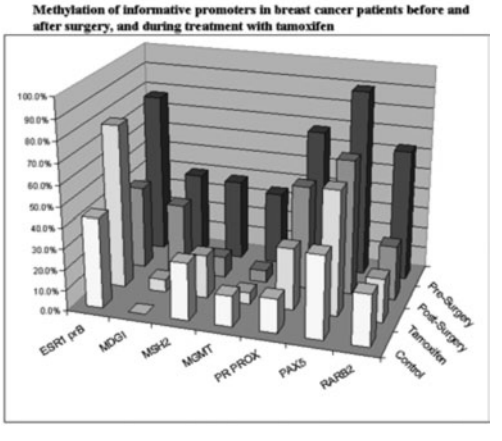
OvCa: Ovarian adenocarcinoma; BOD: Benign ovarian disease; HC: healthy control
Continuous and Fixed cutoff: different approaches for methylation comparison

Differential diagnosis of ovarian disease (expressed as sensitivity and specificity of disease detection):

	Sensitivity (%)	Specificity (%)
HC v. RRMS(r)	79.2	92.9
HC v. RRMS(e)	75.9	91.5
RRMS(r) v. RRMS(e)	71.2	70.8

HC: Healthy controls; RRMS(r): relapsing-remitting MS in remission;
RRMS(e): relapsing-remitting MS during exacerbation

Diagnosis and detection of relapses (exacerbations) in multiple sclerosis (MS) expressed as sensitivity and specificity of RRMS detection in remission and during exacerbation:



Methylation of informative promoters in plasma of breast cancer patients is influenced by treatment.

9 - THE FINAL HURDLE-WHEN IS NON-INVASIVE PRENATAL DIAGNOSIS COST-EFFECTIVE?

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Background. Scientific advances have made non-invasive prenatal diagnosis (NIPD) based on cell-free fetal DNA in maternal plasma possible. The next stage is to consider implementation in a healthcare setting. One application which has been widely promoted is the use of NIPD to target anti-D prophylaxis on "at risk" pregnancies where the fetus is RhD positive.
Method. We have undertaken the first detailed cost-effectiveness analysis of large scale introduction of NIPD testing for fetal RhD status. Two scenarios were considered.
Scenario 1 assumed that NIPD is used to target antenatal prophylaxis but that postnatal tests continue to direct post-delivery prophylaxis. In Scenario 2, NIPD also displaced postnatal testing if an RhD negative fetus had been identified.
Costs of high throughput NIPD testing; savings for prophylaxis; and estimated clinical impact (e.g. on maternal sensitisations) have been modelled. The two NIPD scenarios are compared with universal anti-D prophylaxis to estimate cost-effectiveness.
Result and Conclusion. Findings for both scenarios indicate that NIPD-targeted prophylaxis is unlikely to be cost-effective in England and Wales. However, first trimester testing and other emerging technologies will influence cost-effectiveness, as will different national contexts. The threshold royalty fee per test will also influence this conclusion.
These and other factors will be considered.

10 - ROLE OF EXTRACELLULAR DNA OXYDATIVE MODIFICATION IN RADIATION INDUCED BYSTANDER EFFECTS IN HUMAN ENDOTHELIOCYTES

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Abstract. The development of the bystander effect induced by low doses of radiation in human umbilical vein endothelial cells (HUVECs) depends on extracellular DNA (ecDNA) signaling pathway (Ermakov et al., 2011). We compared the effects of low doses of radiation (0.1Gy), as well as effects of ecDNA extracted from the culture media of irradiated cells (ecDNA-0.1Gy) and intact cells (ecDNA-0), isolated genome DNA (gDNA) and in vitro oxidized gDNA (gDNA-oxy) on intact HUVECs.We estimated the amount of ROS (using DCF-DA reagent) and NO (using CuFL reagent) in HUVECs using the flow cytometry method. We also analyzed the mRNA amount of NOX4 and eNOS genes using real-time PCR. We isolated ecDNA from culture media using the method of phenol extraction. The level

of DNA oxidation according to the presence of 8-oxy-Gua in ecDNA-0.1Gy was 2 times higher than that in ecDNA-0. EcDNA-0.1Gy, 0.1Gy and gDNA-oxy caused a lowering in mRNA eNOS amount in HUVECs, accompanied by a lowering in NO production. Besides, we detected an increase in NOX4 expression which correlated with an increase in ROS production. EcDNA-0 and gDNA did not induce such effects.NO lowering in HUVECs did not depend on the blocking of DNA-recognizing receptors (TLR9), while the effect of ROS excessive formation depended on TLR9 activity. Oxidized DNA modification due to oxidative stress is a possible cause of occurrence of stress signalization factor properties of ecDNA-0.1Gy in the radiation bystander effect in endothelial cells.

11 - QUANTIFICATION OF FREE-CIRCULATING DNA IN PLASMA OF RESPIRATORY PATIENTS: NSCLC VERSUS CHRONIC INFLAMMATION

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Background. Increased amounts of free-circulating DNA are present in peripheral blood of non-small cell lung cancer (NSCLC) patients most likely due to up-regulated cell death processes.
However, it remains questionable whether the increased release of free-circulating DNA into the bloodstream results from malignancy or chronic inflammatory response in the lungs.
Methods. Plasma samples were collected prospectively from: NSCLC patients (50 resectableI-IIIA and 35 advanced IIIB-IV), chronic inflammatory respiratory disease patients (34 COPD, 35 sarcoidosis, 32 asthma), and 40 healthy volunteers. Free-circulating DNA was extracted and quantified by real-time PCR.
Results. NSCLC group had significantly higher mean (x±SD) plasma DNA concentration with respect to patients with chronic respiratory inflammation and healthy controls (8.02±6.75 vs. 3.55±2.07 vs. 2.27±1.51 ng/mL, respectively; p<0.0000). The highest plasma DNA levels were significantly related to the early stage (p=0.0009), but not to histology of NSCLC (p=0,728). A drastic increase in plasma DNA levels up to mean 68.74±58.08 ng/mL was observed a week after the resection. NSCLC patients with no disease recurrence during 6-12 month follow-up demonstrated reduced plasma DNA levels with respect to presurgical values (p=0.0000). The plasma DNA level greater than 6.5 ng/mL was significantly associated with higher risk of lung cancer (OR=22.96, p=0.0025).
Conclusions: chronic respiratory inflammation does not contribute to elevated plasma DNA levels in NSCLC. The drastic increase in plasma DNA concentration following radical treatment is most likely due to the surgical trauma, whereas successful treatment results in reduction of DNA amount in plasma

12 - METHYLATION STATUS OF RASSF1A AND RARβ2 GENES IN THE CIRCULATING DNA FROM LUNG CANCER PATIENTS' BLOOD

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Concentration of methylated and unmethylated RASSF1A and RAR 2 tumor suppressor genes circulating in blood of 20 healthy subjects (HS), 21 patients with chronic obstructive pulmonary disease (COPD) and 50 patients with non-small cell lung cancer (NSCLC) before/after treatment was quantified by PCR for estimation of methylation index (MI). MI was calculated as % MI=100 × [copy number of methylated gene/(copy number of methylated gene + unmethylated gene)] for cell-free DNA circulating in blood plasma (cfDNA) and cell-surface-bound fraction (csb-cirDNA) of circulating DNA.
Values of RASSF1A and RAR 2 MI were significantly elevated in the cirDNA from NSCLC patients compared with HS (39% vs 19% in the csb-cirDNA, 45% vs 17% in cfDNA for RASSF1A; 36% vs 19% in csb-cirDNA, 51% vs 18% in cfDNA for RAR 2; Mann-Whitney U test, P<0.05). If at least one from RASSF1A or RAR 2 MI exceeded the cut-off values NSCLC patients were discriminated from HS with sensitivity and specificity of 90% and 82% when both cfDNA and csb-cirDNA were analyzed. Values of RAR 2 MI in the csb-cirDNA significantly increased from HS (19%) to COPD (24%) and NSCLC patients (36%) (P<0.05), but did not differ in cfDNA from COPD and HS. RASSF1 MI values of cfDNA and csb-cirDNA did not differ between COPD and HS. The association of increased value of MI with advanced stage of NSCLC was found for RAR 2 but not for RASSF1A. RAR 2 MI significantly decreased after surgical resection in the cfDNA and csb-cirDNA of 93% of NSCLC patients demonstrating usefulness of RAR 2 MI determination not only for improvement of lung cancer diagnostics but also for estimation of combined treatment efficiency. The data of RAR 2 and RASSF1A MI dinamics after combined treatment will be presented at the conference.

13 - ISOLATION AND GENETIC CHARACTERISATION OF CIRCULATING CELL-FREE FETAL DNA FOR NON-INVASIVE PRENATAL DIAGNOSIS

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Prenatal diagnosis of chromosomal anomalies is based on invasive procedures, which carry a risk of approximately 1%–2% for loss of pregnancy. An alternative to these

inherently invasive techniques is to isolate foetal DNA circulating in the pregnant mother's plasma. It is known that free circulating foetal DNA in maternal plasma is ascribed to be mainly less than 500 bp, with a majority being 300bp and can be separated by size distribution from the larger maternal DNA (>1000 bp). Separating these fragments by size facilitates an increase in the ratio of foetal to maternal DNA.
The overall objective of this study is to exploit breakthroughs at the confluences of micro, nano- and biotechnologies to create a low-cost minimally-invasive intelligent diagnosis system using a nanotechnology-based device for the separation of plasma from a maternal blood sample, isolation of circulating cell free fetal DNA and its qualitative and quantitative characterization to be used as a minimally-invasive tool for the early, primary diagnosis/monitoring of various diseases. Advances in molecular biology and biosensor technology and the integration of nanostructured functional components in macro- and microsystems will facilitate the isolation of cell free circulating fetal DNA on the basis of the size of fetal DNA fragments using chip-capillary electrophoresis followed by biosensor based genetic characterization. In this study, we will focus specifically on beta-thalassaemia, as an example of non-invasive prenatal diagnosis and screening and preeclampsia, as an example of monitoring.

14 - STORAGE CONDITIONS OF CDNA FROM HUMAN PLASMA SAMPLES AFFECT THE OUTPUT OF REAL-TIME PCR

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Since some years ago, the number of studies focused on cell-free circulating RNA is remarkably increasing. However, little is known about handling and storage cDNA when the objective is to quantify such RNA.
Objetive: The aim of this study was to value the output of the cDNA extracted from human plasma under different storage conditions and quantified by real-time PCR.
Methods: RNA was isolated from plasma of healthy subjects (n=11) by QIAamp UltraSens Virus kit (Qiagen®). All RNA samples were incubated with DNAse I and analized by PCR in order to verify the absence of DNA. Then, the reverse transcription of RNA was performed using Transcriptor High Fidelity cDNA Syntehsis kit (Roche®). Subsequently, the cDNA samples were divided into 2 aliquots: one of them was stored at 4ºC for 10 days, and the other aliquot was stored at –20ºC for 1 month. The concentration of plasma RNA was measured by amplification of a housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase; GAPDH) in the cDNA, using real-time PCR method with TAQMAN probes.
Results: In all samples the GADPH cDNA was successfully amplified. The mean of the quantities of this sequence detected in the samples stored at –20ºC was 0.011±0.011 fg (mean±s.d.). This mean was higher than that obtained in the samples stored at 4ºC (0.002±0.004 fg) and this difference was statistically significant (p= 0.02). Thus, the conservation of the cDNA at –20ºC gave a higher efficiency of the RNA quantitation.
In conclusion, it was determined that to obtain an efficient output of plasma RNA, reproducible, and accurate protocols are required, in which the cDNA storage conditions is an important parameter.

15 - THE ALTERED PROFILE OF SEMINAL PLASMA MICRORNAS IN INFERTILE MEN: A NOVEL TOOL IN THE MOLECULAR DIAGNOSIS OF MALE INFERTILITY

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Background. Infertility is a worldwide reproductive health problem that affects approximately 15% of couples, and half of the infertility cases are due to male factors. However, routine semen analysis has failed to distinguish accurately between fertile and infertile men, and few of biochemical parameters used in the laboratory at present has proven to be highly specific and sensitive diagnostically. So, noninvasive diagnostic methods are urgently needed for the analysis of the male reproductive system. Recently microRNAs (miRNAs) have been shown to play essential roles in spermatogenesis, little is known about seminal plasma miRNAs in infertile men. Here we sought to investigate the seminal plasma miRNA profile in infertile men to identify the miRNAs altered in infertility and to evaluate their diagnostic value.
Methods. Seminal plasma samples were taken from 289 infertile men and 168 age-matched fertile controls. The stability of the miRNAs was first assessed by time-course and freeze-thaw cycle analyses. Solexa sequencing technology was used for an initial screen of the miRNAs in samples pooled from 45 patients with nonobstructive azoospermia, 58 patients with asthenozoospermia and 100 fertile controls. A stem-loop quantitative reverse-transcription PCR (RT-qPCR) assay was conducted in the training and verification sets to confirm the altered concentrations of the miRNAs in 73 patients with nonobstructive azoospermia, 79 patients with asthenozoospermia, 34 patients with oligospermia and 68 fertile controls.
Results. Incubation of seminal plasma at room temperature for up to 24 h, at 4°C for up to 7 days, or by subjecting it to up to 8 cycles of freeze-thawing had minimal effect on levels of miRNAs. The miRNAs in seminal plasma were sufficiently stable. Solexa sequencing results showed that seminal plasma contained multiple and heterogeneous small RNA species (<30 nt in length), including miRNAs, ribosomal RNA fragments, and mRNA fragments. Compared with the control and the azoospermia, pooled seminal plasma sample from the asthenozoospermia contained a relatively larger proportion of miRNAs and a lower level of ribosomal RNA fragments. Of the 692 seminal plasma miRNAs that were scanned by Solexa sequencing, 418, 377, and 422 miRNAs were detectable in the control, azoospermia and asthenosper-

mia patients, respectively. The Solexa sequencing demonstrated 19 differently expressed miRNAs in the patient groups compared to the control group. RT-qPCR analysis in the training set from separate individuals including 30 patients with azoospermia, 30 patients with asthenozoospermia and 24 fertile controls identified 7 miRNAs (miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p, and miR-513a-5p) being significantly decreased in azoospermia but increased in asthenozoospermia ($P<0.01$). RT-qPCR analysis in the validation set of a larger cohort consisting of 43 patients with azoospermia, 49 patients with asthenozoospermia and 44 fertile controls further verified the significantly altered concentrations of the 7 miRNAs in the two case groups as compared fertile controls. The concentrations of these miRNAs in 34 patients with oligospermia were between the azoospermia group and control group, except for miR-122. The area under the ROC curve (AUC) for these miRNAs: for the azoospermia and control groups, ranged from 0.822 to 0.921; for the asthenozoospermia and control groups, ranged from 0.733 to 0.836; and greater AUC was yielded (0.963 to 0.990) for the azoospermia and asthenozoospermia groups. While the AUCs for the routine biochemical parameters (including zinc, -glucosidase acid, phosphotase and fructose) were between 0.510 and 0.622. Moreover, the concentrations of some selected miRNAs were also elevated in the sperms from the semen of the asthenozoospermia patients.
Conclusions. The profiles of seminal plasma miRNAs were specifically altered in infertile patients with azoospermia or asthenozoospermia, with the concentrations of 7 miRNAs being significantly decreased in azoospermia patients while markedly increased in asthenozoospermia patients. We establish the measurement of miRNAs in seminal plasma as a novel, noninvasive approach for diagnosing male infertility.

16 - KINETIC OF THE RELEASING OF CELL-FREE DNA AND RNA, MUTATED AND NON-MUTATED, INTO THE BLOODSTREAM DURING TUMOR PROGRESSION IN RATS

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The goal of the present study was to quantify non-tumor and tumor DNA, as well as non-tumor and tumor RNA, in the plasma of tumor-bearing rats, and to correlate those levels with tumor progression. For this purpose, we injected DHD/K12-PROb colon cancer cells subcutaneously into BD-IX rats and recorded tumor diameters weekly. Animals were distributed into six groups according to the time of the sacrifice, which ranged from the 1st to the 11th week after inoculation of cancer cells. After euthanasia, we collected plasma from each rat and quantified non-mutated and mutated DNA, and mutated and non-mutated RNA. All measurements were performed by real-time PCR using FRET and PNA probes. Overall, levels of non-mutated (non-tumor) DNA in plasma of tumor-bearing rats were significantly higher than those in healthy animals ($p= 0.009$). Levels of non-mutated RNA in tumor-bearing rats were not statistically different from those in healthy animals. The curve of the concentrations of non-mutated DNA in plasma along tumor progression appeared to follow a similar course than that of non-mutated RNA, although the former concentrations were always 2-3 magnitude orders higher. In the two curves, a peak was observed at the 3rd week after inoculation. With respect to the detection of mutated (tumor) DNA, the highest rate of positive plasmas was found at the 7th week after inoculation (50%). When such DNA was detected, the concentrations were similar to those of non-mutated DNA. Mutated RNA was not detected. As a whole, plasma levels of both non-mutated and mutated DNA, as well as of non-mutated RNA, were not significantly related to tumor size neither to metastasis. In conclusion, during tumor progression, the releasing of high quantities of non-tumor DNA appeared to be the most predictable and strong phenomenon of the studied here. Our findings support the hypothesis that the interaction between tumor and host cells is an essential phenomenon during tumor progression.

17 - NIH-3T3 FIBROBLASTS CULTURED WITH PLASMA FROM COLORECTAL CANCER PATIENTS GENERATE POORLY DIFFERENTIATED CARCINOMAS IN MICE

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It has been reported that the ability of cells to undergo cellular transitions, in particular, to switch between epithelial and mesenchymal states, might be highly advantageous during the progression of carcinoma. In the present work, we used histological and immunohistochemical methods to compare tumors generated in mice after the injection of NIH-3T3 cells that had been transformed in two different ways, namely, spontaneously and by culturing with plasma from colorectal cancer patients. The tumors generated by spontaneously transformed cells had the morphological and immunohistochemical features of poorly differentiated fusocellular sarcomas, indicating that the spontaneous transformation of NIH-3T3 fibroblasts had not induced the mesenchymal to epithelial transition of such cells.
By contrast, the injection into mice of NIH-3T3 fibroblasts that had been cultured with plasma from patients with colorectal cancer gave rise to tumors that resembled, phenotypically, tumors generated by human colon carcinoma cells. The tumors were composed of polygonal cells that were clearly positive for markers of epithelial cells but lacked the mesenchymal marker vimentin. Thus, these transformed fibroblasts generated poorly differentiated carcinomas, an observation that suggests that NIH-3T3 cells might undergo mesenchymal to epithelial conversion during plasma-induced transformation. In conclu-

sion, while spontaneous transformation of NIH-3T3 cells resulted in fusocellular sarcomas, transformation by plasma from colorectal cancer patients transformed these cells in such a way that they were able to generate tumors that phenotypically resembled the carcinomas of the original cancer patients.

18 - PLASMA CELL-FREE DNA LEVELS AFTER SEVERE TRAUMATIC BRAIN INJURY AND DURING THE NEXT 96 HOURS OF FOLLOW UP

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Plasma levels of circulating cell-free DNA are increased after trauma injury. This increase is higher during the first hours after trauma and may be related with primary outcome. The Glasgow Coma Scale (GCS) score has been frequently used as one of the most important predictors of outcome after severe traumatic brain injury (TBI). However, a difficulty of determining the initial GCS in a repeatable and reproducible manner should be also considered. Thus, a sensitive and reliable biomarker for patients at higher risk is needed to identify these patients to initiate early intervention. In this way, circulating DNA may be a possible biological marker after severe TBI. In this study, we investigated DNA plasma concentrations after severe traumatic brain injury and during the next 96 hours in the Intensive Care Unit (ICU) by real time PCR 32 patients were included and compared with 12 healthy control voluntaries. Patients suffer isolated severe TBI or severe TBI with as sociated extracranial injuries. Cell-free DNA levels were considerably higher in patients samples compared with control ones (10020.6 ±1960 versus 323 ± 118.5).
After the following four days we observed a significant fall at 24 hours (34%) being even more significant after 48 hours of admission (70%). Higher levels of circulating DNA at 24 hours was observed in non-survival patients or patients with a high damage at hospital discharged (GOS:1-3).On the other hand DNA levels at 24 hours were higher in patients with associated extracranial injuries compare with
patients with isolated TBI. In summary we found that TBI is associated with elevated DNA plasma levels. Cell-free DNA levels after 24 hours of injury should be further investigated as a possible predictor value of patient outcome.

19 - A FURTHER IMPROVEMENT IN QUANTIFYING MALE FETAL DNA IN MATERNAL PLASMA

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Background. Cell-free fetal DNA (cffDNA) in maternal plasma can be clinically useful for prenatal disorders and pregnancy monitoring. Further improvements in more sensitive, specific and quantitative detection of cffDNA in maternal plasma may expand the clinical utilities.
Methods. A real-time quantitative PCR (qPCR) assay (Y chromosome repetitive sequence (YRS) assay) was developed based on a highly repetitive sequence in the Y chromosome. Both standard qPCR and digital qPCR were performed to compare the sensitivity and specificity of this new assay versus other established male DNA specific assays.
Results. The YRS was able to detect 0.5 GE/PCR reaction when fetal DNA was present at 0.2% of total DNA. Much lower background noise than the DYS14 assay was detected for the YRS assay in analyzing plasma samples from pregnancies with female pregnancies.
Conclusion. The YRS assay is a significant further improvement in quantifying rare male fetal DNA in maternal plasma. The higher sensitivity and specificity may expand the clinical and research utilities of cffDNA.

20 - LOH PROXIMAL TO M6P/IGF2R LOCUS IN CIRCULATING DNA OF OVARIAN CANCER PATIENTS CORRELATES WITH PROGNOSIS

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We recently showed that LOH proximal to M6P/IGF2R locus (D6S1581) in primary ovarian tumors is a biomarker for disseminated tumor cells (DTC) in the bone marrow (BM). For follow-up studies of the patients, it would be desirable to discover a blood-based marker. Here, we quantified circulating DNA (cirDNA) levels in sera of 63 ovarian cancer patients before surgery and after platinum-based chemotherapy, measured incidence of LOH at four ovarian cancer relevant chromosomal loci, correlated LOH occurrence with tumor cell spread to the BM and evaluated prognostic significance of LOH. cirDNA was extracted from sera and fractionated into high- and low molecular-weight fraction (HMWF, LMWF) for LOH profiling, utilizing PCR-based fluorescence microsatellite analysis. BM aspirates were analysed for DTC by immunocytochemistry using the pan-cytokeratin antibody A45-B/B3. We observed a decline of cirDNA content in the LMWF after chemotherapy (p=0.0001) and DNA levels in HMWF were predictive for residual tumor load (p=0.017). LOH was prevalently traceable in the LMWF with an overall frequency of 67 %, only moderately ablating after therapy (45 %). Before surgery, LOH at marker D10S1765 and D13S218 correlated with FIGO stage (p=0.035, p=0.012, respectively), LOH at D10S1765 associated with tumor grading (p=0.012) and LOH at D6S1581 in both fractions predicted shorter disease-free survival (p=0.021) and overall survival (p=0.030). Allelic loss at marker D10S1765 in LMWF after therapy correlated with the occurrence of DTC in BM after therapy (p=0.017). Conclusively, we newly identified LOH at D10S1765 and D6S1581 as novel blood-based biomarkers in ovarian cancer patients, which might be of clinical relevance for monitoring studies.

21 - PROMOTORS OF IMMUNE RESPONSE GENES ARE DIFFERENTIALLY METHYLATED IN CFDNA OF HEMODIALYZED PATIENTS

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Background. Elevations of cell-free DNA(cfDNA) concentrations during hemodialysis(HD) sessions were reported in numerous studies regardless of an applied therapeutic protocol. It is generally thought that the elevated concentrations represent the consequence of apoptosis on the dialysis membranes. No data concerning the qualitative characteristics of cfDNAs in HD patients were published till today, therefore we focus on the promotormethylation status of genes involved in immune response.
Methods. We isolated cfDNA from randomLy selected patients before and after a HD session. Quantification of cfDNA in all samples was performed using Real Time PCR on GAPDH gene. The extent of promotor methylation of24 genes involved in immune response wasexamined using the Methyl Profiler DNA Methylation PCR Array System (SABiosciences, Qiagen) cluster analysis(SABiosciences, Qiagen) was used for data evaluation.
Results. We discovered significant changes in methylation status of several genes(e.g. IL13RA1, chemokine ligand 14, inhibin alpha) that were promoted in consequence of patient's blood contact with artificial surfaces of dialyzer. The character of methylation profiles provides the new tool for evaluation of actual state of immune system activity in HD patients and may be easily correlated with clinical data to bring new insights in thecomplex pathogenesis.
Conclusion. Methylation of immune response genes promotors changes during a HD procedure and the alterations can be detected using Methyl Profiler DNA Methylation PCR Array System (SABiosciences, Qiagen) at the level of cfDNA to provide the complex information about the actual state of immune response in HD patients.
Supported by the grant no.I/328 of the Ministry of Industry and Trade of the Czech Republic.

23 - CLINICAL EVALUATION OF THE MUTATIONAL STATUS, CONCENTRATION AND FRAGMENTATION INDEX WITH A NOVEL MULTIPLEX TEST FOR THE ANALYSIS OF CIRCULATING DNA IN THE CONTEXT OF THE COLORECTAL CANCER

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We developed an innovative technique for the analysis of circulating DNA (ctDNA) which simultaneously quantify tumour-derived ctDNA, determine a ctDNA fragmentation index and detect SNP. The test (IntPlex) provides multiplex analysis of ctDNA in the same sample in a quick, simple and not expensive way. IntPlex is a single-step qPCR method which involves a specifically designed primer system and is combined with a novel technique we have set up for point mutation detection based on allele-specific qPCR. Test sensitivity for point mutation detection is very high (2 mutated ctDNA fragments/µL plasma).
Evaluation and validation of our technology was examined in response to the pressing need of knowing the KRAS/BRAF mutational status before anti-EGFR therapy in colorectal cancer (CRC) patients. As a consequence the IntPlex method was adapted to detect the 6 more frequent KRAS mutations in CRC and the BRAF SNP V600E. IntPlex proof of concept was given by the full agreement concerning the KRAS mutational status of 15 samples from metastatic CRC between IntPlex and sequencing from tissue sections. Moreover, our preliminary data showed that the high sensitivity of the test revealed, in contrast to the literature, a significant disparity between the tumor-derived ctDNA concentration in healthy individuals and in CRC patients. They also indicate that ctDNA integrity index discriminates healthy subjects from cancer patients.
The objectives of our ongoing double-blinded multicentric prospective study are: i), to compare KRAS/BRAF mutational status by IntPlex to the sequencing of tumour biopsy samples; ii), to investigate whether ctDNA content or ctDNA fragmentation can readily discriminate CRC from healthy subjects.

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24 - NON-INVASIVE PRENATAL DETERMINATION OF FETAL GENDER AND SINGLE-GENE DISORDER FROM PLASMA OF PREGNANT WOMEN

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Objective. We experienced 2 cases of noninvasive prenatal diagnosis using cell-free fetal DNA (cffDNA) in maternal plasma. I will report the cases.
Case 1. We identified fetal gender for woman whose fetus was at risk of lethal X-linked disorder, L1 syndrome. We obtained maternal plasma at 10 weeks. After extracting cffDNA, real-time PCR of Y chromosome specific sequence, DYS14, was amplified. The sequence was not detected in the plasma and the fetus was determined as a female, who did not have a risk for L1 syndrome.
Case 2. The fetus had a short femur and was suspected

to be achondroplasia. The parents of the fetus did not have any features of achondroplasia.We tried to identify the point mutation of FGFR3 in maternal plasma. In 32 weeks, plasma samples were obtained and the cffDNA was amplified by the primers that included nt1138 of FGFR3 gene. The products were restricted by digestion enzyme of Sfc-I and the fragment analysis was performed. We therefore determined the point mutation of FGFR3 for achondroplasia from maternal plasma.
Conclusions. In the first case, we determined fetal sex non-invasively in at risk pregnancy for X-linked L1 syndrome. Fetal sex determination is useful in pregnancy with at risk fetus for X-linked disorders. In the second case, we directly detected a fetus-derived mutant gene in maternal plasma. This approach is useful in prenatal diagnosis of single-gene disorders caused by paternally derived or de novo mutations. We thus demonstrated clinical usefulness of noninvasive prenatal DNA analysis from maternal plasma.

25 - NON-INVASIVE PRENATAL ASSESSMENT OF FETAL SEX BY NUCLEIC ACID ANALYSIS IN MATERNAL PLASMA

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Objective. To validate a non invasive method for fetal sex assessment in the 1st trimester of pregnancy and test its clinical utility in the diagnosis of potentially affected pregnancies in female carriers of X linked disorders.
Methods. In the validation study, 100 blood samples from pregnant women at 6 -12 weeks of gestation were analysed. In the clinical study, 20 pregnancies at risk of having an affected foetus were tested. 7mL of maternal blood in EDTA were obtained and cell free DNA (cffDNA) was isolated using a commercially available kit. All women gave their informed consent.
DNA was digested using a methylation sensitive endonouclease (AciI) to remove unmethylated maternal RASSF1A. A multiplex PCR was performed for the simultaneous amplification of SRY and DYS14 sequences, along with RASSF1A and ACTB. Amplification of these loci indicates fetal gender, confirms the presence of cffDNA and allows assessment of digestion efficiency.
Results. In the validation study, Y chromosome-specific sequences were detected in 68 samples. In 32 cases, negative for Y chromosome sequences, the presence of cffDNA was ascertained through the amplification of RASFF1A in the absence of ACTB. In the clinical studies, fetal sex was correctly diagnosed in 19 pregnancies, and 1 case was reported as inconclusive.
Conclusions. Fetal sex assessment by detecting Y chromosome sequences in maternal blood can be routinely used from the 6th week of gestation. Reliable fetal sex determination from maternal blood in the 1st trimester of gestation can avoid conventional invasive methods of prenatal diagnosis.

26 - APOPTOSIS IS AN ESSENTIAL EVENT TO MEDIATE THE STRESS-SIGNALISATION FACTORS FORMATION IN IRRADIATED LYMPHOCYTES IN RADIATION-INDUCED BYSTANDER EFFECT

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EcDNA* is a signal molecule in bystander effect mediated by irradiation (Ermakov et al, 2009, 2011). Here we revealed an apoptosis of irradiated lymphocytes mediates an appearance of stress-signalization factors by ecDNA*. Lymphocytes from 23 volunteered healthy donors were used. Apoptosis level in irradiated and non-irradiated lymphocytes was identified by determination of: the concentration and size of ecDNA fragments, the nuclei morphology (microscopy), the number of haploid cells (flow cytometry), the endonuclease and caspase-3 activities. Caspase inhibitor (Biotin-DEVD-FMK) was used to proof the apoptosis role. We applied four characteristics to define the response of cells to irradiation and ecDNA* addition. We also determined a rearrangement of marker loci of chromatin in the nucleus, ribosomal DNA transcription level increase, NO and TNF- amount increase. Moreover, we showed that irradiation of lymphocytes is followed by an increase both the number of nucleosome fragments of ecDNA and haploid cells, that is correlated with caspase-3 increase and nuclease endonuclease activity increment. Blockage of apoptosis resulted in an ecDNA concentration decrease. Moreover, ecDNA isolated from medium completely loses ability to induce response in cells similar to that of mediated by irradiation. Low doses of radiation induce apoptosis in lymphocytes and appearance of new ecDNA fragments with changed properties. It is apoptotic DNA that plays a role as a stress-signalisation factor in bystander effect mediated by irradiation.

27 - IDENTIFICATION OF GASTRIC CANCER ASSOCIATED MICRORNAS IN PLASMA OF GASTRIC CANCER PATIENTS ON MICRORNAS MICROARRAY

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Background. This study was conducted to isolate gastric cancer associated miRs in plasma on a miRs microarray using pre- and post-operative paired plasma samples, except for the possible effects of the individual variability of miRs expression.
Methods. Differences of miRs expression between paired plasma samples (pre- and post-operative) of gastric cancer patients were investigated on a microarray assay. Subsequent quantitative real-time reverse-transcription PCR was performed to confirm the altered miRs expression in plasma, and investigate whether the altered expression reflect those in tumor tissue. Potential clinical applications of the plasma miRs were also assessed for

screening the disease and monitoring tumor dynamics.
Results. We identified 8 miRs that exhibited signal intensities >5 times higher in pre-operative plasma than in post-operative plasma in the same patients with gastric cancers.
The altered miRs expressions were reproducibly confirmed by RT-PCR assay. Some of miRs were found to showed higher expression in primary gastric cancer than normal mucosa. In a large-scale validation, miR-X, and -Y were found to decrease in postoperative samples compared to the levels in pre-operative samples in 85% and 90% of gastric cancer patients. The clinical application of the plasma miRs are under analyses.
Conclusions. Altered expressions of the miRs in plasma might provide potential blood-based biomarkers for gastric cancer.

28 - DIAGNOSIS BY HIGH RESOLUTION MELTING IN SERUM CELL-FREE DNA OF MEN2A: TOWARDS PRENATAL DIAGNOSIS

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At present it is possible to detect a punctual gene mutation in serum, if it is in heterozygosis, by high resolution melting (HRM) analysis with accuracy like that of the gold standard sequence technique. When the mutation is known, it is possible to use HRM assay for family studies. It is also well known that a little fraction of fetal DNA is circulating free in the pregnant serum. Its study can be a useful tool to detect a mutation pattern inherited from father and absent in mother, allowing both an early and non invasive prenatal diagnosis. Methods: We evaluated the presence of MEN2A mutation (C634Y) in serum of 4 patients with a known mutation already determined by sequencing the gene. This is a dominant autosomal punctual mutation and the diagnosis is realized by HRM in the same run that the real-time PCR. Results: HRM serum analysis revealed differences in the melting curve shapes that correlated entirely with patients diagnosed of MEN2A by blood cells sequenc ing when compared with the same number of controls. Conclusions: Cell-free DNA determination in serum samples is useful to diagnose MEN2A punctual mutations and opens the door for a non invasive prenatal diagnose in pregnant serum if mutation inheritance come from father in a safe manner for fetus and being a cheaper and faster method of diagnosis than sequencing.

29 - FEASIBILITY OF NON-INVASIVE FETAL RHD AND SRY DETERMINATION INTO CLINICAL ROUTINE USING A MULTIPLEX RT-PCR ASSAY FOR CELL-FREE DNA IN PREGNANT WOMEN PLASMA

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Noninvasive fetal RHD genotyping is becoming a useful tool in the screening of RHD status in order to performance antenatal Rh profilaxis in RhD negative pregnancies. This study provides information about the use of multiplex real-time polymerase chain reaction (PCR) by which RHD (Exons 5 and 7) and SRY genes are determined in a single run.
Methods. Both single and multiplex real-time PCRs targeting RHD Exons 5 and 7 and SRY were applied for the detection of fetal-specific RHD sequences and sex in maternal plasma.
A total of 1928 women were studied between 10 and 28 weeks of pregnancy, although only 134 of them were used for single TaqMan PCR studies and, till present, 828 were used for Multiplex TaqMan PCR studies. All of them were serologically typed as RhD- according to Spanish guidelines. Single and multiplex real-time PCR results were compared with postnatal serology and sex identification.
Results. There was a 100% concordance between results obtained with single and multiplex real-time PCR assays. In relation to RHD genotyping, sensitivity, specificity, and accuracy were 100, 98.6, and 99.5%, respectively by both methods.
This procedure improves the speed of the assay, avoids over-treatment among RhD negative pregnant women bearing RhD negative fetus, reduces the requirements for clinical and biological monitoring in the subset and all these data translate into a clinical benefit and costs saving >100,000 €/year.
Conclusions. The routine determination of fetal RHD status and SRY from maternal plasma, using multiplex real-time PCR, is feasible. The use of multiplex real-time PCR allows improving the response of the laboratory saving time and reagent costs, opening the door to a complete automatization of the process.

30 - CIRCULATING miRNAs AS BIOMARKERS IN OVARIAN CANCER

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A large number of miRNAs, endogenous non-coding RNAs of approximately 22 nucleotides that target mRNAs triggering their degradation or translation repression, have been found to be abnormally expressed in tumors tissues.
Microarrays, RT-qPCR and high-throughput sequencing techniques have allowed establishing characteristic miRNA expression profiles for diverse cancer types, suggesting that miRNA play a role in tumorgenesis.
The presence of tumor-derived miRNAs in easily accessible fluids, that can be obtained by non-invasive procedures, such as blood, was first reported in 2008 and has generated increasing attention.

In these last years it has been shown that alteration of serum miRNA profiles can be correlated with different types of cancers. Nevertheless there are only a limited number of studies about serum miRNAs levels in patients with ovarian cancer.
This is the most lethal gynecologic cancer due to the fact that most of the cases are diagnosed at an advanced stage. New robust and reliable biomarkers for early diagnosis and prognosis of this malignancy are essential to improve survival.
Here we review the main miRNAs which expression has been found to be altered in profiling studies of ovarian tumour tissues, serum and tumour-derived exosomes in patients with this pathology.
Most studies are based on MicroRNA microarrays and generate a large quantity of data; here we provide an ordered comparative of these data. Those miRNA that were found to be differentially expressed in several studies and showed higher changes versus normal tissues are pointed as the most promising ones.
This is the first step to study the serum level of a limited number of miRNAs with potential value as biomarkers in a large group of patients with ovarian cancer.

31 - OXIDATIVE MODIFICATIONS OF DNA ALTER ITS BIOLOGICAL ACTION ON RAT NEURONS

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In recent years, there is increasing evidence that extracellular DNA (ecDNA) has different biological actions in normal and various pathological conditions. We hypothesized that the differences may be due to DNA oxidative modifications caused by pathological processes in cells.
In this study, we compared in vitro the effects of blood plasma ecDNA of Wistar rats subjected to nembutal narcosis or brain ischemia and of native or oxidized rat genomic DNA (gDNA) in rat cerebellar granule neurons culture. Brain ischemia was induced under the narcosis by common carotid arteries occlusion.
Oxidative modifications of gDNA were performed by irradiation in the presence of methylene blue (longer than 600 nm) or H2O2 (312 nm). In vivo the effects of native and oxidized gDNA intravenous injection on morphological changes in rat brain ischemic penumbra were studied at 1st and 4th days after brain retraction at a pressure of 40 mm Hg.
We found the difference in the levels of oxidative stress (OS) induced by 24h cells incubation with 15 ng/mL ecDNA of treated rats compared to native gDNA. ecDNA from ischemic rats similar to oxidized gDNA decreased the level of OS in neurons culture. OS level induced by oxidized gDNA varied depending on the concentration and DNA oxidation technique.
Injection of native gDNA improved the ischemia-induced morphological changes. It was concluded that oxidative modifications of DNA alter its biological action.

33 - CIRCULATING NUCLEIC ACIDS: PRE-ANALYTICAL STABILIZATION IN BLOOD AND AUTOMATED LARGE-VOLUME EXTRACTION FOR DIAGNOSTIC APPLICATIONS

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Circulating, cell-free nucleic acids (ccfNA) are present in plasma, serum and other body fluids and provide targets for detection of disease states based on a simple blood sample. Here, the performance of a novel room temperature stabilization technology for preserving the ccfNA in whole blood and the efficiency of a new automated large volume ccfNA extraction method was evaluated.
Stabilization: Fresh whole EDTA blood was mixed with a novel stabilizing reagent to preserve the ccfNA population in whole blood as it is present at the time of blood draw. Plasma was prepared from stabilized whole blood, followed by ccfNA extraction. Using capillary electrophoresis of ccfNA and real-time PCR with amplicons of different lengths to track nucleic acid stability, the circulating DNA and RNA status in plasma was shown to be preserved over 3 to 6 days compared to non-stabilized EDTA blood.
Automated extraction: ccfDNA was extracted from 6 mL plasma using novel chemistry and magnetic bead technology on the QIAsymphony SP instrument. In individual donor samples, the ccfDNA yield was shown to be comparable to the state-of-the-art reference (QIAamp Circulating Nucleic Acid Kit) based on real-time PCR specific for 18S rDNA targets and recovery of spiked control DNA.
Our results demonstrate the feasibility and utility of a novel ccfNA pre-analytical workflow with room temperature storage of primary blood samples for up to six days until plasma separation, followed by automated large-volume ccfNA extraction compatible with real-time PCR target analysis.

34 - C-RICH FRAGMENTS OF CELL-FREE DNA (cfDNA) INDUCE AN ADAPTIVE RESPONSE (AR) IN HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS (MSC)

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Abstract. It is known that anti-oxidant and reparation systems activate in cells during AR. The last may be induced by low dose radiation and other toxic agents.
GC-rich fragments accumulate in human circulating DNA in pathological conditions. We have shown *in vitro* how the changes in GC-content of cfDNA in cell cultivation medium influence the expression of genes which products are involved in regulation of the oxidative stress level (NOX4, iNOS, SOD, NRF2, KEAP1), DNA repara-

tion (BRCA1) and apoptosis (BCL2). The cloned fragment of human ribosomal gene repeat (p18 plasmid) in a concentration of 50 ng/mL was used as a GC-rich DNA model. The effects were compared to those of a low dose X-ray irradiation. The ROS levels in MSC were evaluated by the fluorescence of DCFH-DA, the quantity of double-strand breaks was assessed with antibodies against ??-??2????-phosphorylated histone. MRNA levels were measured using RT-PCR. During the first 3 hours after application of p18 to the me dium the increase of ROS level and the double-strand breaks quantity can be detected. Next, after 3 hours NOX4 and iNOS mRNAs amounts decrease by 20-30% while SOD, NRF2, KEAP1, BRCA1 and BCL2 mRNAs levels undergo a 4-10-fold increase. All the effects were similar to those produced by irradiation. So, GC-rich DNA present in MSC cultivation medium induces oxidative stress and provokes certain cellular DNA damage. These events activate antioxidant and reparation systems in MSC contributing to an AR development.

35 - CELL-FREE DNA (cfDNA) FROM CANCER CELL CULTIVATION MEDIUM UPREGULATES PPARG2 EXPRESSION IN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS (ADMSC)

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We have shown previously that cfDNA may influence MSC differentiation. The total cfDNA amount is increased in the blood of cancer patients. To find out whether cancer cfDNA can influence MSCs, we explored the effects of cfDNA extracted from the cultivation medium of breast cancer cells mammospheres (mbcDNA) and from blood plasma of cancer patients (pbcDNA) on adipogenic, osteogenic and myogenic genes expression. We chose genomic DNA (gDNA) and cfDNA from blood plasma of healthy donors (dDNA) as controls. Two ADSMC cell lines were cultured for 3 days in proliferation medium containing 10 to 100 ng/mL of cfDNA. The media were then changed and the cells were cultured for 3 or 10 days without cfDNA. RT-PCR was used to analyze the expression of PPARG2, LPL, FABP4, LEP, OPG, RUNX2, SPP1, MYOD1, MYOG and MYF5. The exposure of ADMSC to mbcDNA of pbcDNA increased PPARG2 expression by a factor of 3-12, while LPL, FABP4, LEP, OPG, RUNX2 or SPP1 expression was not affected or even decreased. MYOD1 MYOG I and MYF5 expression showed a 2-3 fold increase. gDNA or dDNA did not affect these genes expression. So, mbcDNA and pbcDNA upregulate PPARG2 in ADMSC. It is known that PPARG2 activation may lead to growth inhibition, apoptosis and tumor cell differentiation. Thus, we can assume that PPARG2 activation in ADMSC, aimed at suppressing cancer progression, may be a response to cancer cell cfDNA.

36 - "FETAL" EXOSOMES: THE WAY OF NON INVASIVE PRENATAL DIAGNOSIS?

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Human placenta cells release free-fetal nucleic acids which are detected in the maternal circulation during pregnancy (3-6%) and offering non-invasive prenatal diagnosis of the genetic status of a fetus. Fetal-maternal nucleic acids chimerism contribute to limit this approach. Placenta cells release also vesicles which could be a good way to select fetal DNA in maternal plasma.
We compared cell-free DNA concentration extracted from microvesicles (microparticules and exosomes) isolated by an ultracentrifugation protocol and a exosome isolation commercial kit (Exoquick™). 27 plasmas from RhD negative women with RhD positive foetus were analysed. β-globin and RHD gene (exon 5) were analysed by a multiplex quantitative PCR using plasmid calibrators. Ultracentrifugation protocol permitted to define fetal and total DNA fractions in maternal plasma: Exosomes (15 and 462 copies/mL), microparticules (13 and 973 copies/mL) and extravesicles (210 and 4177 copies/mL). For exosome fraction, median concentration of fetal DNA is significantly higher (p=0.003) with Exoquick™ kit than ultracentrifugation protocol: 191 and 15 copies/mL respectively. Placental exosomes isolated by Exoquick™ in maternal plasma, could offer in a future an excellent method for non invasive prenatal diagnosis.

39 - QUANTIFICATION OF FETAL AND TOTAL CELL-FREE DNA IN MATERNAL CIRCULATION: RELATIONSHIPS WITH FETAL OR MATERNO-FETAL DISORDERS

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Objective. to correlate total and fetal cell-free (cf)DNA levels in maternel blood with fetal or materno-fetal pathologies, in particular with ultrasound signs.
Methods. Maternal blood samples from 74 singleton pregnancies were divided into 5 groups: intrauterine growth restriction (IUGR) associated with hypertension or preeclampsia (10), IUGR without preeclampsia (18), fetal nuchal translucency (FNT) ≥3 mm (9), second trimester triple marker (TT) ≥1/250 (23), fetal cerebral abnormalities (14). Control group consisted of 74 RhD-negative women with RhD positive fetus matched for gestational age. Fetal (SRY gene) and total (β-globin gene) cfDNA levels were quantified by real-time PCR using plasmid calibrators. Wilcoxon test was used to compare median DNA concentrations of case and control groups.

Results. Total cfDNA levels were significantly increased in groups of patients with FNT ≥3 mm (p=0,008), fetal cerebral abnormalities (p=0,019), IUGR with (p=0,007) or without preeclampsia or hypertension (p=0,016). Fetal cfDNA levels were significantly increased in groups of patients with FNT ≥3 mm (p=0,028), TT ≥1/250 (p=0,004), fetal cerebral abnormalities (p=0,005) and IUGR without preeclampsia (p=0,013).
Conclusions. This results showed a correlation between fetal and/or total cfDNA and fetal or materno-fetal disorders or risk factors: elevated FNT, elevated TT, IUGR, fetal cerebral abnormalities. However, to confirm these results obtained on small cohort, further larger studies are needed.

40 - ROUTINE FETAL RHD GENOTYPING IN MATERNAL PLASMA IN FIRST TRIMESTER: A TWO YEAR EXPERIENCE IN SOUTH OF FRANCE

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We reported a routine clinical practice of non-invasive fetal RHD genotyping from the laboratory of the south of France (2009-2010).
5523 plasmas were analysed since 7 weeks gestation with 30% (1656 samples) in first trimester, 60% (3312) trimester and 5% (276) in third trimester. DNA extraction was performed in an automated closed tube system (Easymag, Biomerieux). β-globin and RHD gene (5,7,10 exons) were analysed by a multiplex quantitative PCR using plasmid calibrators. Before 10 weeks, fetal RhD negative results were controlled two weeks later. RHD polymorphism was detected in 6% of pregnant women. All RhD polymorphism were analysed by sequencing and reported in this study. Only women carrying a weak D had an unconclusive result (0,91%). Results were compared with serological analysis of cord blood after delivery. In our hand, sensibility was 99,74%, specificity 99,24% and accuracy 99,57%. No statistical difference was observed for first, second and third trimester. Antenatal management of anti-D-alloimmunized women has been modified with this new molecular approach. This analysis would be proposed with the first-trimester screening for Trisomy 21 for all RhD negative pregnant women.

41 - MASS SPECTROSCOPIC ANALYSIS OF MICROVESICLES IN BLOOD AND URINE OF HEALTHY AND PROSTATE CANCER PATIENTS

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Since Watson and Crick studies on DNA and the deciphering of DNA bases, it was accepted that every cell contained its complement of genetic material. It is a dogma that DNA is stable without intercellular mobility. The author (Chen, 1968) postulated the hypothesis of genetic exchange; that there is a homeostatic exchange system of intercellular factors including DNA/RNA between cells and tissues. As a corollary any disturbance of homeostatic control of this exchange system can result in abnormal growth and cancer. Recent research on CNAPS and microvesicles support aspects of this hypothesis. The purpose of this study is to determine, using mass spectroscopy, if microvesicles in blood and urine of man, in healthy and diseased states, contain DNA, RNA, proteins and lipids. Blood and urine from healthy and diseased subjects, one with diabetes and prostate cancer Gleason 8-9 were examined. Results show the presence of microvesicles in blood and urine, in both healthy and diabetics, and in a diabetic prostate cancer patient, with an outer phospholipid membrane, enclosing RNA, DNA, protein and lipid molecules. These results show for first time DNA in urinary microvesicles. It may be concluded that microvesicles contain an array of intercellular factors, proteins, RNA, DNA and lipids making them possible mediators in intercellular communication, supporting Chen (1968) original hypothesis of genetic exchange.

43 - COMPARATIVE STUDY OF APOPTOTIC AND GENOMIC DNA USING MASSIVE PARALLEL SEQUENCING

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Apoptosis was shown to be the main source of cell-free DNA circulating in blood of mammals. Intracellular events mediating the apoptosis are well studied in contrast to the DNA content of apoptotic bodies. Nonequivalent representation of certain DNA sequences in genomic and apoptotic DNA was shown, demonstrating that DNA from circulating apoptotic bodies may significantly differ from parental genomic DNA.
We have compared cell-free apoptotic DNA (cf-apoDNA) ranked from 180-to 800 bp isolated from culture medium of human umbilical vein endothelial cells (HUVEC) induced to apoptosis and genomic DNA from the same normal cells using SOLID 3 platform. 49.5 and 51.5 million reads with 50 nucleotides lengths were obtained for apoptotic and genomic DNA, respectively. In total ~2.5×109 nucleotides for each of 4 DNA libraries prepared from 2 different donors were aligned to the reference genome. Close to 1× genome coverage allows to compare contents of highly repeated genomic sequences between obtained libraries.
Analysis of the data using Repbase (GIRI) demonstrates the enrichment of cf-apoDNA with Alu-repeats (the number of AluJ, AluS and AluY repeats was 2.47 (SD, 3.6%), 2.45 (SD, 5.5%) and 2.79 (SD, 6.1%) times higher in cf-apoDNA). In contrast, some of L1 elements were underrepresented in cf-apoDNA (the number of L1MA and L1ME was 1.4 (SD, 22%) and 1.45 (SD, 9%) times lower in cf-apoDNA). The number of cf-apoDNA reads mapped to exons was also 1.4 time higher than in genomic DNA libraries. These data and the location of Alu-repeats mainly in euchromatin regions demonstrate the enrichment of cf-apoDNA with gene-rich DNA sequences.

44 - DNA MICROCHIMERISM IN PERIPHERAL BLOOD OF DYZIGOTIC TWINS

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Blood chimerism in twins is probably more common than previously thought. A possible explanation for this may be that dizygotic monochorionic twins are not uncommon, but the majority of cases are not diagnosed because of limited knowledge of this as a possibility. The other reason is that the cell free nucleic acid traffic between the twins, which could lead to this phenomenon. In our study EDTA blood samples were collected form ten mature and premature newborn twin pairs discordant for gender, and six twin pairs discordant for RhD blood group also. DNA was isolated from 2 mL blood with silica adsorption method. SRY, RhD exon 7 and exon 10 specific primers were used. Real time PCR was performed. In all ten cases SRY DNA was detected in samples obtained from girl twins. RhD exon 7 and 10 was also detected in RhD negative newborns. The consequences of twin DNA microchimerism is unknown, but might have a role in the immunization of the newborn infants.

45 - CIRCULATING TUMOR-SPECIFIC DNA: A BIOMARKER FOR SCREENING AND PROGNOSTIC FACTOR IN BREAST CANCER

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Cell-free circulating DNA carries not only tumor-specific changes in its sequence but also distinctive epigenetic marks, namely DNA methylation in island CpG region promotor. Epigenetic changes are emerging as one of the most important events in carcinogenesis. Recent data suggest that benign diseases have very specific methylation patterns within cell-free circulating DNA, which are different from the pattern of a malignant tumor of the same organ.

Material and Methods. A sensitive SYBR green methylation-specific PCR quantitative technique was used to analyze the utility of circulating DNA with CpG island hypermethylation of APC, RAR- , E-Cadherin, ESR1 and 14-3-3 gene promoter regions as breast cancer biomarkers. Sera were collected in 107 operable breast cancer patients (pts) previously surgery and in 60 of those pts after treatment. Respect controls, 34 had benign breast disease and 74 with no evidence of breast disease

Results: Mean serum values of methylated ESR1 and 14-3-3 gene promoters significantly differed between breast cancer patients and healthy controls (p=0.0112 for ESR1 and p= 0.0047 for 14-3-3-). When their results were combined, it was found that hypermethylation of these two genes differentiated between breast cancer patients and healthy controls ($p \leq 0.0001$) with a sensitivity of 81% (95% confidence interval: 72–88%) and specificity of 88% (95% CI: 78–94%). Presence of methylated ESR1 in serum of breast cancer patients was associated with ER-negative phenotype (p=0.0179); and presence of methylated 14-3-3- was associated with T3-4 stage (OMS) ($p< 0.05$) and nodal positive status ($p< 0.05$). We observed lower methylated ERS1 or 14-3-3- values after surgery, respect pretreatment levels, but without an overall statistically significant difference. With a median follow up of 6 years, we found that patients with a significant decrease of sera methylated levels of both genes after surgery had better time to progression an overall survival respect patients without this observation

Conclusions. These results also suggest that this panel of genes detected in ductal lavage and blood specimens could be useful to biomarkers for early detection breast cancer. These findings cast some doubts on the utility for early cancer diagnosis of highly sensitive techniques to identify hypermethylation of specific gene promoters in DNA extracted from serum. Although numerous issues remain to be resolved, the quantitative measurement of circulating methylated DNA is a promising tool for cancer risk assessment.

47 - NOVEL DIAGNOSTIC VALUE OF CIRCULATING miR-18a IN PLASMA OF PATIENTS WITH GASTRIC CANCER

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Background and Purpose. Many studies have demonstrated the overexpression and amplification of the miR-17-92 cluster in human cancers and these contribute human carcinogenesis. MiR-18a is located in the miR-17-92 cluster and reported to be highly expressed in gastric cancer tissues. We hypothesized that miR-18a in plasma could be a potential biomarker in patients with gastric cancer.

Methods. This study was divided into three parts: 1) Confirmation of higher miR-18a levels in primary gastric cancer tissue and cell lines than normal tissues and human fibroblasts. 2) Evaluation of the plasma miR-18a assay using quantitative RT-PCR by comparing results from 51 patients with gastric cancer and 37 healthy volunteers (3) Evaluation of the assay for monitoring tumor dynamics assay in patients with gastric cancer.

Results. 1) The expression of miR-18a is higher in primary gastric cancer tissues than that in adjacent normal tissues (77.7%). Also, gastric cancer cell lines such as MKN28 and HGC27 showed extremely higher expression of miR-18a than a human fibroblast cell line. 2) The plasma concentrations of miR-18a were significantly higher in gastric cancer patients than healthy controls ($p<0.0001$). The value of the area under the receiver-operating characteristic curve (AUC) was 0.813. 3) The plasma levels of miR-18a were significantly lower in postoperative samples than preoperative samples.

Conclusion. Circulating miR-18a might provide a novel biomarker in plasma for screening gastric cancer and monitoring tumor dynamics.

50 - INVESTIGATION OF PLASMA lncRNA LEVELS IN CHRONIC LYMPHOCYTIC LEUKEMIA AND MULTIPLE MYELOMA PATIENTS

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Abstract. Background: The subject of this study is analysis of the cell-free lncRNAs (long non-coding RNAs) in blood circulation and their significance in cancer. We selected three candidate lncRNA molecules; HOTAIR which is located in the nucleus and acting either as cis- or, trans-acting epigenetic regulators of chromatin. Another lncRNA is MALAT1 which is broadly expressed in human cancers and known to be dysregulated in many cancers. The third lncRNA molecule investigated is TUG1 which has recently been shown to transcriptionally be regulated by p53 in response to DNA damage. We investigated the circulating levels of these three molecules in plasma of healthy subjects and patients with chronic myeloid leukemia (CLL), multiple myeloma (MM) and prostate cancer (PCa).

Method. 59 patients with CLL, 66 patients with MM, 20 with PCa and 40 healthy controls were enrolled in the study. Total RNA was isolated from 200µl of plasma and cDNA synthesized. We carried out real-time PCR assay using SyberGreen Chemistry (Roche) to investigate TUG1, MALAT1 and HOTAIR lncRNAs and the CT method for the analysis of expression levels of these lncRNAs.

Results. We observed significant differences in the expression level of MALAT1 in three patients groups ($p<0.001$) compared with the healthy controls. TUG1 was only significant in MM ($p=0.035$) and no difference was found for HOTAIR expression in three disease groups. Interestingly, lncRNA levels were related to the CLL and MM disease states.

Conclusion. Our results reveal that MALAT1 is differentially expressed in CLL and MM and may be a candidate biomarker molecule for diagnosis. In addition, TUG1 may be another candidate for MM diagnosis.

51 - INHIBITION OF dsRNA-INDUCED IMMUNE RESPONSE BY ODN ANALOGS OF CIRCULATING DNA

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Cell-surface-bound circulating DNAs (csbDNAs) have been shown to be more effective inhibitors of polyIC-activated secretion of pro-inflammatory cytokines than cell-free circulating or genomic DNA and are obviously enriched in sequences, responsible for this effect. 36-mer ODNs containing short sequences, responsible for binding of csbDNA with cells and possessing similar inhibiting activity, have been used for studying csbDNA-mediated immunosuppression. Twenty two single- and double-stranded (ss- and dsODNs) ODNs have been studied. All investigated ODNs did not change interleukin production in gingival fibroblasts neither when added to culture medium nor after lipofection regardless of CpG content, but both ssODNs and dsODNs inhibited polyIC-activated secretion of IL-6 and IL-8. Inhibiting activity of ODNs is sequence-specific and depends on ODN delivery route. In general free ssODNs are more efficient inhibitors, whereas dsODNs are more efficient after lipofection.

Fluorescent microscopy experiments and the data on interleukin production when free ODNs or ODN-unifectin complexes were added to cells simultaneously or few hours after polyIC demonstrate that polyIC and ODN colocalization is necessary for the effect, at that endosomal (TLR3) and cytoplasmic (RLR)-mediated pathways are mainly inhibited subject to ODN sequence.

ODN-binding proteins of living cells have been found by EMSA, the complexes with biotinilated ODNs have been isolated from cellular extract by affinity chromatography and identified by MALDI-TOF as KU70 and KU80. Whether KU complexes are directly involved in dsRNA signaling or have auxiliary functions, mediating ODN localization or polyIC-proteins interactions, remains to be investigated.

54 - ASSESSMENT OF SERUM microRNAs IN RECEPTOR-NEGATIVE AND POSITIVE BREAST CANCER PATIENTS BEFORE AND AFTER CHEMOTHERAPY

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The focus of the current study was to examine the deregulation of six microRNAs (miR10b, 17, 34a, 93, 155, 373) in breast cancer patients and to evaluate their utility as blood-based molecular markers for detection of cancer and monitoring of chemotherapy. The relative concentrations of the microRNAs were quantified in serum samples of 152 breast cancer patients before chemotherapy, 40 patients after chemotherapy, 30 patients with benign breast disease and 40 healthy women by TaqMan MicroRNA assays. Serum concentrations of miR34a ($p=0,01$), miR93 ($p=0,029$), miR373 ($p=0,0001$) and total RNA ($p=0,002$) discriminated patients with primary breast cancer from healthy controls. Increased total RNA ($p=0,05$) values associated with higher tumor grading. The serum levels of miR155 were significant lower in patients with metastatic disease than in patients with primary tumors.

These lower miR155 values correlated with lymph node metastases ($p=0,045$) and advanced tumor stages ($p=0,032$) indicating the clinical relevance of miR155 in tumor progression. In estrogen and progesterone receptor-positive patients the different expression levels of miR34a ($p=0,0001$), miR155 ($p=0,002$), miR17 ($p=0,02$), miR93 ($p=0,014$) and miR373 ($p=0,023$) correlated with chemotherapy suggesting that these miRs are suitable for monito-

ring of chemotherapy. Patients with triple negative receptor status had significantly more miR34a (p=0.0001) and miR93 (p=0.01) in their blood than patients with receptor-positive cancer. Our data show that the deregulation of miRs may discriminate breast cancer patients from healthy women but not from patients with benign breast disease. Moreover, the serum levels of miRs may be a parameter for receptor status and chemotherapy.

55 - TUMOR-DERIVED EXTRACELLULAR DNA AS FACTOR AFFECTING PRIMARY CELL METHYLATION

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Cell-free DNA (cfDNA) generated by tumor cells were shown to transform phenotype of normal cells but the mechanisms of cfDNA action is not known. We suggest that as far as short methylated olygonucleotides can induce of de novo methylation, aberrantly methylated cfDNA from tumor cells can interfere with genomic DNA methylation of normal cells.

Effect of cfDNA-containing microparticles from HeLa cells culture medium (cf-mpDNA) onto methylation of human primary fibroblasts (HGF) and endotheliocytes (HUVEC) genomic DNA was investigated. RARbeta2 gene fragment (Genbank X56849: 924 – 1117) methylated in HeLa and unmethylated in HGF and HUVEC was selected as a target.

HGF and HUVEC were cultivated in presence of 30% v/v of HeLa supernatants being in contact with ~100 ng/mL cf-mpDNA during 1, 3 or 6 passages (max 18 days) following with 3 passages without cf-mpDNA. After each passage methylation of RARbeta2 was evaluated by pyrosequencing (BioTage, USA) of bisulfite modified genomic, cell-surface-bound and cell-free DNA of primary cells along with measuring concentration of cfDNA in these fractions. The data of TaqMan PCR for HPV18 demonstrate that cf-mpDNA is more stable, readily bind with cell surface and penetrate into cytosol of HUVEC as compared with HGF, but HeLa's cfDNA accumulates in the nuclei of HGF 4 times better than in HUVEC. Cf-mpDNA did not induce any changes in HUVEC methylation, but after first passage of HGF without cf-mpDNA 25% methylation level of cfDNA in HGF culture medium was found. Methylation of HGF genomic DNA was not found as long as any methylation at the latter passages, considering apoptosis of HeLa's cfDNA sensitive cells. Long-term study demonstrates that cf mpDNA do not influence methylation of HGF or HUVEC genomic DNA.

56 - NOVEL DIAGNOSTIC VALUE OF CIRCULATING miR-18a IN PLASMA OF PATIENTS WITH ESOPHAGEAL SQUAMOUS CELL CARCINOMA

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Background. Several recent studies have demonstrated that microRNAs are detectable in plasma/serum. MiR-18a is located in the miR-17-92 cluster and reported to be highly expressed in esophageal squamous cell carcinoma (ESCC) tissues. We hypothesized that miR-18a in plasma could be a potential biomarker in patients with ESCC.

Methods. This study was divided into three parts: 1) Confirmation of higher miR-18a levels in primary ESCC tissues and cell lines than normal esophageal tissues and human fibroblasts. 2) Evaluation of the plasma miR-18a assay using quantitative RT-PCR by comparing from 60 patients with ESCC and 37 healthy volunteers. 3) Evaluation of the assay for monitoring tumor dynamics assay in patients with ESCC.

Results. 1) The expression of miR-18a was higher in esophageal cancer tissues and cell lines than normal tissues and fibroblasts. 2) The plasma concentrations of miR-18a were significantly higher in ESCC patients than controls ($p< 0.0001$). 3) The plasma levels miR-18a were significantly lower in postoperative samples than preoperative samples ($p< 0.0001$).

Conclusion. Circulating miR-18a might provide a novel biomarker in plasma for screening ESCC and monitoring tumor dynamics.

57 - FACILITATING IMPLEMENTATION OF NIPD: A MODIFIED PROTOCOL TO DETECT THE UNIVERSAL FETAL DNA MARKER RASSF1A

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Cell free fetal DNA (cffDNA) in maternal plasma is used for the non invasive prenatal diagnosis (NIPD) of fetal sex, RHD status in D- mothers and some single gene disorders. Failure to amplify a target sequence may be interpreted as a true negative result but could also indicate absence of cffDNA in the sample. The hypermethylated promoter of RASSF1A has been reported as a universal fetal DNA marker which can be used to confirm the presence of cffDNA. Using combinations of methylation-sensitive enzymes and a methyation-insensitive digest control we have tested maternal plasma samples from pregnant women (gestational age 5-12 weeks; n=106) for the presence of cffDNA. cfDNA was extracted from 3mL maternal plasma using the QIAamp circulating nucleic acid kit (QIAGEN). Samples were analysed in triplicate with no digestion, a methylation-sensitive digest and a methylation insensitive-digest and analysed using real-time PCR assays for RASSF1A and SRY.

RASSF1A was detectable in all undigested samples demonstrating that total cfDNA had been extracted.

99 samples (93.4%) had one or more replicates positive for hypermethylated RASSF1A indicating the presence of cffDNA. 7 samples (6.6%) had no detectable RASSF1A signal and all showed negative or inconclusive results for SRY (<5/9 replicates positive). This modified assay for

RASSF1A could be useful for laboratories undertaking NIPD of fetal sex determination and single gene disorders for use as a universal fetal DNA marker.

58 - EFFICACY OF BISULFITE MODIFICATION AND DNA RECOVERY IN CLINICAL SAMPLES WITH MINUTE AMOUNTS OF CIRCULATING DNA

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This study evaluated three commercially available bisulfite modification kits with regard to procedural DNA loss and the efficacy of cytosine conversion to uracil. The efficacy of DNA conversion was estimated by pyrosequencing of eight CpG sites of unmethylated and fully enzymatically methylated templates of RARbeta2 gene promoter region (GenBank X56849) and cirDNAs of healthy donors and prostate cancer patients. The procedural DNA loss was calculated by methyl-independent qRT-PCR. Our data demonstrate that all kits displayed similar conversion efficacy of unmethylated cytosines close to 99% independently of DNA concentration. However, when low DNA concentrations were used, the observed basic level of genomic DNA methylation increased to 11% depending on the position of CpG site. Qiagen and Chemicon kits recovered no more than 20% starting material at a high DNA input (500 ng/probe) and only 2,7-5.8% for low DNA input (10 ng/probe). EZ DNA Methylation-Gold Kit from Zymo Research provided the highest recovery regardless of the initial DNA input with average rates of no less than 86%. Thus, EZ DNA Methylation-Gold Kit from Zymo Research is the most appropriate tool for bisulfite modification of cirDNA when assaying DNA in low amounts.

59 - FREE CIRCULATING DNA: AN OBJECTIVE SEVERE PREECLAMPSIA MARKER TO MONITORIZE PATIENTS WHO COULD SUFFER HELLP SYNDROME

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Introduction. Previous studies have associated free DNA circulating with human disease and have been shown it diagnostic utility.
Preeclampsia is a multisystemic disorder that complicates 3-5% of the pregnancies and is leading cause of maternal mobility. HELLP (haemolysis, elevated liver enzyme levels, and a low platelet count) syndrome is a complicated form of severe preeclampsia with multisystem impact, characterized by the presence of microangiopathic hemolytic anaemia, hepatic dysfunction and thrombocytopenia, resulting in seizures, possible cerebral hemorrhage and subsequent maternal death. At moment, delivery of fetus and removal of the placenta is the only effective treatment.
Previous studies have indicated that preeclampsia is associated with an elevated cell free DNA, and that these elevations can occur prior to the onset of clinical symptoms.
Purpose. The aim of this study was assess the sensibility and specificity of a quantitative molecular assay in the analysis of the circulating DNA profiles in pregnancy women apparently healthy and preeclampsia to provide a useful marker for monitor the disease and previous HELLP syndrome detection.
Materials and Methods. Patients. We analyzed the free circulating DNA in a group of 10 preclamptic pregnant women and 10 healthy normotensive controls, from the Hypertension and Lipids Unit at Virgen Del Rocio University Hospital (Seville, Spain) and Institute of Biomedicine of Seville (IBIS), Spain. Informed consent was signed from all patients who had taken part of the study. *Blood Samples*. Ten mL of peripheral blood was collected to obtain serum from all patients and controls (n=20). Blood samples were centrifuged during 8 minutes at 3500 rpm and the sera were removed and stoked at -20ºC in Nunc criotubes until DNA extraction. *DNA Extraction*. Free circulating DNA was isolated from the 400 µl of serum and resuspended in a final volume of 50 µl in specific dilution buffer, using a MagnaPure instrument (Roche).*Real Time PCR*. Quantitative real time polymerase chain reaction (qPCR) of human globin gene was performed in a LightCycler® 480 instrument (Roche).
Results. In healthy controls the median concentration of circulating serum DNA was 40,6 ng/mL versus 291 ng/mL and 2.200 ng/mL in mild preeclampsia and preeclampsia with HELLP syndrome, respectively.
Conclusion. This preliminary study shows that higher levels of free circulating DNA are detected in patients with preeclampsia compared to healthy pregnant women. Patients with HELLP Syndrome had almost 55 times more free circulating DNA in sera compared to normotensive pregnancies women and about 7,2 times more compared to preeclampsia without Hellp syndrome, suggesting a new and objective analytical parameter to monitorize HELLP syndrome. Levels of free circulating DNA in serum could also be used to identify higher-risk patients for preeclampsia and prevention trials.

60 - CLINICAL USE OF NIPD FOR MONOGENIC DISORDERS: PATIENT PERSPECTIVES AND DECISION-MAKING

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Following the implementation of fetal sex and RhD determination in a clinical setting, our next aim was the incorporation of NIPD for monogenic disorders. We have currently studied 30 cases of 10 different diseases from a

research perspective (25 blind and in parallel to PD & 5 as pre-test). However, patients have begun to demand the results prior to conventional prenatal diagnosis (PD) in order to make a subsequent decision. We show our preliminary results in the clinical application of NIPD in five pregnancies at-risk of single gene disorders.
Three cases (out of the 5 pre-test) were at-risk of an autosomal dominant disorder with paternal origin (Huntington Disease-HD, Epidermollisis Bullosa-EB and Rendu Osler Weber-ROB) and two cases at-risk of a recessive disease (Leber Congenital Amaurosis-LCA and Cartilage Hair Hypoplasia-CHH) were studied in maternal plasma collected between 8-15 weeks of gestation. Minisequencing, Real-Time PCR and QF-PCR were used for the analysis of the paternal alleles in the fetal DNA.
Paternal mutation was observed in maternal plasma in the EB and the CHH cases. Paternal healthy alleles were observed in the HD and LCA cases. ROB case is under study. Confirmation of these results was only available in the CHH case in which conventional PD was performed. In EB (presenting in the family with a mild phenotype) and LCA cases, the couple decided not to undergo conventional PD and continued with gestation. HD case terminated in a spontaneous miscarriage.
We are currently using this approach as a pre-test prior to PD. Although patients are correctly informed about the research nature of these studies and the need of confirmation by conventional PD, they are beginning to consider NIPD as a real prenatal alternative.

61 - ASSESSMENT OF DIABETIC RETINOPATHY BY MEASURING RETINA SPECIFIC MRNA IN BLOOD

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Diabetic retinopathy (DR) is a common microvascular complication of diabetes mellitus. In the present study, circulating levels of three retinal cell specific mRNAs (encoding rhodopsin (Rho), retinal amine oxidase (RAO) and phosphodiesterase 6C (PDE6C)) were measured. Diabetic patients with no retinopathy (R0), background retinopathy (R1), pre-proliferative retinopathy (R2) and proliferative retinopathy (R3) were studied. Peripheral blood (2.5mL collected into PaxgeneTM blood RNA tubes, total RNA extracted and mRNA therein reverse transcribed to cDNA. The latter was measured by real time quantitative PCR using intron-spanning sequence specific primers and probes. mRNA levels were expressed as a ratio of target mRNA: -actin mRNA.
All three retina specific mRNAs were detectable in the circulation and levels of Rho and RAO mRNA, but not PDE6C were significantly higher in R2 and R3. For instance, Rho [median, (range)] in R2 [$11.15x10^{-8}$, ($4.78 x10^{-7}$)] and R3 [$23.47x10^{-8}$, ($2.16 x 10^{-7}$)] was significantly higher ($p<0.001$) than R0 [$4.82 x10^{-8}$, ($2.70x10^{-7}$)]. R3 Rho was also significantly higher ($p<0.001$) than R1 [$5.30x10^{-8}$, ($2.16x10^{-7}$)] and R2 ($p=0.02$). RAO also showed a similar trend to Rho, with R3 [$20.92x10^{-4}$, ($1.72x10^{-2}$)] levels significantly higher than R0 [$1.99x 10^{-4}$, ($5.29x10^{-2}$)]. Comparison of Rho mRNA levels in R0/R1 with R2/R3 by ROC analysis produced an area under the curve of 0.756 ($p=000$), suggesting that Rho measurement may be able to discriminate between severe and no/mild DR. Expressing the mRNA data as Rho:RAO improved the AUC to 0.823 ($p=000$) for the same comparison.
This study suggests that retina specific mRNA may be useful as a molecular marker for differentiating mild from severe DR.

62 - CIRCULATING microRNAs IN THE ASSESSMENT OF HEART FAILURE AND microRNa-133A AS A POTENTIAL BIOMARKER OF CARDIAC REMODELLING

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Objective. Histological studies of human heart tissue has shown that microRNA-133a may play a role in regulating cardiac remodelling in heart failure. Reduced levels of microRNA-133a were associated with beneficial hypertrophic growth early on in the course of heart failure. Elevated levels were associated with suppression of further hypertrophic growth which may otherwise be detrimental in the late stages of heart failure.
We aim to detect circulating microRNA-133a in blood and demonstrate its role as a non-invasive biomarker for cardiac remodelling.
Methods and Material. Circulating RNAs were extracted from whole blood of stable heart failure patients and healthy volunteers. MicroRNA-133a was amplified by reverse transcription and measured by real-time PCR. The results were normalised by RNU6B as the housekeeping gene. Additionally we measure two cardio-specific microRNAs, -208b and 499 in our study. Serum cardiac troponin T and NT-proBNP were measured in all subjects, and echocardiographic findings were available in all heart failure patients.
Results. A total of 71 patients (73.2%) and 26 healthy controls (26.8%) were recruited in the study. Ischaemic cardiomyopathy is the commonest primary cause of heart failure (n=45, 63.4%) and 88.8% (n=64) of the heart failure patients have detectable (>0.003 ng/mL) levels of serum troponin T compared to 30.8% (n=8) in the control group. The median serum NT-proBNP in the HF and control groups were 907 ng/mL vs 38 ng/mL ($p<0.001$).
All three microRNAs, 133a, 208b and 499 were detectable in circulating blood of HF patients and healthy controls. As a group, heart failure patients did not show statistically different levels of microRNA-133a compared to healthy controls ($p=0.164$). However a subgroup of mild heart failure patients belonging to the NYHA II class were found to have statistically lower microRNA-133a levels compared to controls ($p=0.032$). In contrast, patients with mild-to-moderate LV systolic dysfunction (EF>30%, n=45) were found to have significantly higher levels of microRNA-133a if they have severe symptoms compare to those with milder symptoms (NYHA III/IV vs I/II, n= 11 vs 34, $p=0.026$). We were not able to demon-

strable a role for microRNA-208b and -499 in the assessment of heart failure.
Conclusions. To our knowledge this is the first study that demonstrates the presence of circulating microRNA-133a in blood.
Circulating microRNA-133a levels appear to mirror its dualistic role as a stimulator of cardiac hypertrophy in early heart failure and inhibitor of unchecked hypertrophy in the later stages of heart failure.
Further studies are needed to evaluate the role of circulating microRNA-133a as a potential non-invasive biomarker for cardiac remodelling.

63 - CIRCULATING MICRORNA IN DIABETIC NEPHROPATHY

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Diabetic Nephropathy (DN) is a common microvascular complication of diabetes. Albumin-Creatinine Ratio (ACR) is widely used for early detection of DN.. However, ACR is associated with several known limitations. We have examined the circulating levels of 3 kidney specific microRNAs (miR-192, miR-377 and miR-215) as possible markers of DN.
In this pilot study, blood samples were taken from 9 healthy controls and 39 diabetic patients into PAXgene™ blood RNA tubes. MicroRNA was extracted, reverse transcribed to complementary DNA (cDNA) and levels quantified by real time-quantitative PCR. A comparative CT method including RNU6B as endogenous reference miRNA was used to determine differences in the levels of target miRNAs within and between groups.
In all subjects miR-192, miR-215 and RNU6B, but not miR-377, were detected. A 26.5-fold and 27.9-fold increase ($p=0.002$) in levels of miR-192 and miR-215, respectively, was observed in diabetics compared to the healthy control group. A 24.1-fold, 32.9-fold and 13.9-fold increase ($p<0.05$) in levels of miR-192 was seen in normo-, micro- and macro-albuminuria groups, respectively, compared with healthy controls. Similar observations were recorded for miR-215 which showed a 25.4-fold, 35.3-fold and 12.7-fold increase ($p<0.05$) in normo-, micro- and macro-albuminuria, respectively, compared to the healthy control group. In addition, levels of miR-192 and miR-215 were independent of confounding factors known to be associated with ACR (gender, age, duration of diabetes, BMI, etc).
This study has shown that kidney-specific miRNAs in the circulation are raised in diabetics. Further investigation may be warranted to establish these miRNAs as potential markers of diabetic kidney disease.

65 - CIRCULATING MRNA IN SALT SENSITIVITY

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Background. Aims Salt sensitivity (SS) is associated with cardiovascular morbidity and mortality. Possible mechanism of SS include reduced endothelial nitric oxide synthase (eNOS) activity, defective 11 -hydroxysterone dehydrogenase Type-II (11 -HSD2), increased aldosterone synthase (AS) activity and low-renin. The aim of this study was to investigate levels of circulating mRNA for eNOS, 11 -HSD2 and AS, in an attempt to find a biomarker for SS.
Methods. Subjects (n=79) were recruited through the REVERED STUDY. Subjects with low plasma-renin-activity (PRA), defined as <0.5 ng/mL/hr, was used to identify salt-sensitive subjects. Blood samples were collected into PAXgene™ Blood RNA Tubes. Total RNA was extracted and reverse transcribed into cDNA. Quantification was achieved by Real Time PCR.
Results. Circulating eNOS mRNA decreased with age and was significantly lower in those over 50 compared to those under 20. eNOS mRNA tended to be higher in the low-PRA group and in Black subjects. eNOS mRNA correlated positively with systolic BP, diastolic BP, cholesterol and LDL:HDL. mRNA for 11??-HSD2 and AS were undetectable in the circulation.
Conclusion. Circulating eNOS mRNA decreases with age. The tendency for eNOS mRNA to be higher with SS and other inducers of vascular injury such as BP makes it likely that eNOS mRNA is reflective of endothelial damage. However, further research is needed to determine the source of circulating eNOS mRNA and assess the clinical utility of eNOS mRNA as a biomarker of endothelial damage.

66 - ROUTINE ANTENATAL DETERMINATION OF FETAL RHESUS (RH) D STATUS USING CELL FREE FETAL DNA IN THE MATERNAL CIRCULATION IN EARLY PREGNANCY

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Fetal RHD type can be determined in D- mothers by analysis of cell free fetal DNA (cffDNA) in maternal plasma. Routine implementation may spare D– women carrying a D– fetus exposure to anti-D, with potential savings to the health service providers. Here we describe the potential for implementation in early pregnancy, evaluate effectiveness, costs and benefits, and service user opinions.
Methods. D- mothers at 7 hospitals in England had blood taken for cffDNA analysis of fetal RHD status at booking, Down's syndrome screening and the 20 week anomaly scan.
If inconclusive or a D+ fetus was predicted, anti-D was given. Mothers predicted to have a D- fetus had repeat cffDNA testing at 28 weeks when it is know to be accurate (Finning BMJ. 2008;336:816-8). Where D- status was confirmed anti-D was withheld. D type was confirmed by cord blood testing. The economic costs were eva-

luated by detailed study of anti-D usage. Health professional and patient opinions were surveyed by interviews and questionnaires.
Results. Preliminary results:

Wks	No. tests	CN	CP	FN	FP	inconclusive
5-6	28	8	7	9	0	4
7-10	728	285	342	9	3	89
11-13	762	258	437	1	7	60
14-17	515	208	261	1	4	40
18-23	663	236	368	1	7	51
24-29	1396	567	771	0	11	47

CN/CP confirmed negative/positive; FN/FP false negative/positive

Conclusions. Routine cffDNA testing for fetal RHD status in D- mothers will be possible from 11 weeks gestation. It will reduce anti-D usage by around 40%, be cost effective and favourably received by health professionals and mothers who are keen to avoid anti-D.

67 - CIRCULATING KIDNEY SPECIFIC MRNA IN DIABETIC NEPHROPATHY

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Diabetes nephropathy (DN), a common microvascular complication of diabetes. Screening for microalbuminuria is a crucial component of diabetes care to prevent progression of DN.
The potential of circulating kidney-specific nucleic acids as a non-invasive screening test for DN was examined in this study. Blood sample were taken form diabetic patients and healthy controls for the quantification of mRNA for nephrin, paracellin and nyctalopin into PAXgene blood RNA tubes.
Total RNA was extracted, reverse transcribed to complementary DNA (cDNA) and quantified by real time quantitative PCR using gene specific primers and probes.
S Nephrin and nyctalopin mRNA were detectable in all subjects, and paracellin mRNA in 53% of the subjects. ??-globin was used to normalise the marker levels.
Paracellin mRNA level was significantly higher in diabetic subjects with microalbuminuria ($8.79\pm9.99x10^{-5}$) compared to healthy subjects ($5.00\pm4.85x10^{-7}$, P=0.006), normoalbuminuria ($1.20\pm2.52x10^{-5}$, P=0.027), and macroalbuminuria ($2.78\pm6.51x10^{-5}$, P=0.016) groups.
Nyctalopin mRNA level was markedly elevated in male diabetics with normoalbuminuria (7.82±12.15) than those with microalbuminuria (1.08±2.59, P=0.024) and macroalbuminuria (0.97±2.45, P=0.030).
Furthermore, nyctalopin mRNA level was significantly increased in male diabetic controls (2.93±4.66) compared to male diabetic subjects with background diabetic retinopathy (DR) (0.83±1.88, P= 0.026), and it was also elevated in type 2 diabetics without retinopathy (2.49±4.15) than those with pre-proliferative or proliferative DR (0.08±0.07, P=0.030). The significant findings in this study confirm the potential utility of kidney-specific nucleic acids as a markers for DN.

71 - BIOLOGICAL ACTIVITY OF VIRTOSOMES RELEASED BY STIMULATED AND NON-STIMULATED LYMPHOCYTES

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Previous experiments have shown that virtosomes released from tumour cells can stimulate the synthesis of DNA in non-stimulated lymphocytes (NSL) whilst conversely, those released from non-dividing hepatocytes and NSLs can inhibit DNA synthesis in tumour cells. In order to determine if this was also true for both stimulated lymphocytes (SL) and NSLs, we used isolated human lymphocytes cultivated in RPMI 1640 medium supplemented with 15% FCS, although the serum would likely cause a low' level cell division in the NSLs.
In one experiment the lymphocytes stimulated for 72 hours with phytohaemagglutinin were washed and placed in a culture medium in which NSLs had been previously incubated for 4h to release their virtosomes. The cells were controlled daily for 72 h and the results compared with NSLs in normal culture. There was a similar, minimal increase in cell number in both cultures i.e. cell division in the SLs had been reduced.
In a second experiment, NSLs were placed in culture medium in which previously SLs had been incubated for 4h to release their virtosomes (experimental); the cells were analysed for 72h and the results compared with NSLs as a control. The increase in cell number was significantly higher (20%) in the experimental culture with respect to the control.
These results substantiate the earlier finding that the virtosomes released from non-dividing cells inhibit cell division whilst those from dividing cells stimulate cell division in non-dividing cell populations.

72 - CIRCULATING TUMOUR DNA AS A NON-INVASIVE TOOL FOR MONITORING OVARIAN CANCER DYNAMICS

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Molecular markers of response and minimal residual disease for solid tumours are urgently needed. Quantitative measurement of circulating tumour DNA (ctDNA), carrying tumour-specific sequence alterations, has been recently demonstrated as a potential tool for assessing and monitoring tumour dynamics in cancer patients. Collection of data is challenging and dynamic patterns of ctDNA have been reported for only a handful of patients to date, using specialized methods. We developed a simplified process for measuring ctDNA dynamics by integrating targeted resequencing and digital PCR, using the Fluidigm platform.
Tumour-specific mutations are detected at low cost by targeted amplification and deep sequencing of barcoded

libraries. Microfluidic digital PCR is used to quantify rare tumour DNA molecules. Quantitative assays are used as quality controls for extraction of circulating DNA. To explore the utility of this process, we collect serial plasma samples from women treated for high-grade serous ovarian cancer. We have measured ctDNA dynamics in 14 patients undergoing chemotherapy, using assays for 11 different tumour-specific mutations in the TP53 gene as personalised markers. TP53 ctDNA levels showed marked changes, with superior dynamic range and added information compared to the conventional serum marker CA-125. Our work substantially extends current knowledge on ctDNA dynamics and provides evidence of the utility of ctDNA as a non-invasive tool for monitoring ovarian cancer.

74 - DEVELOPMENT OF MUTATION-SPECIFIC PCR-BASED TECHNIQUES FOR DETECTION OF MINUTE QUANTITIES OF MUTANT DNA IN A HUGE BACKGROUND OF WILD-TYPE DNA WITH APPLICATION TO MUTATION HOTSPOTS IN KRAS AND BRAF GENES

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Background. Circulating nucleic acids (CNA) give us invaluable opportunities for monitoring every type of genetic chimerism. But the way of CNA-based tests from bench to bedside is much longer than anticipated, in part due to certain methodological difficulties. For example, analysis of DNA aberrations may be hampered by the challenge of detecting them in a background of wild-type DNA.
Aim of the study. The aims of the study were development of PCR-based tests for hotspot mutations in KRAS and BRAF genes able to detect minor quantities of mutant DNA and identification of the ways to ameliorate their performance with CNA samples.
Materials and Methods. Several types of wild-type blocking PCR were developed. Validation process includes testing of plasmid DNA dilutions, 80 FFPE specimens from cancer patients and comparable number of their plasma samples.
Results. Mutation-specific PCR with clamping probes in the middle of PCR product performed best in our study, with sensitivity of 15 copies. When coupled to real-time detection it showed selectivity 1:200; when coupled to sequencing, its selectivity increased to 1:2000. No false-positives or false-negatives were detected with tumour samples. Plasma testing is underway (to be presented on the conference).
Discussion. Clamping mutant-specific PCR holds much promise but has certain limitations. Real-time PCR-based assays performed good with tumour samples whereas addition of the sequencing step gives us selectivity applicable to CNA research. Future directions of our research include attempts to drop down the limit of detection, increasing selectivity by prePCR enrichment and search for most suitable sample sources and DNA extraction methods.

75 - IMPLEMENTING NON-INVASIVE PRENATAL DIAGNOSIS FOR GENETIC DISORDERS USING CELL FREE FETAL DNA INTO CLINICAL PRACTICE ACCEPTABILITY AND IMPACT ON PREGNANCY MANAGEMENT

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Background. We have offered a clinical service for fetal sex determination using cffDNA since 2006. We recently extended our work to include non-invasive prenatal diagnosis (NIPD) for some single gene disorders
Objective. To determine the impact of NIPD on management of pregnancies at high risk of genetic disorders, and determine health professional and patients opinions
Methods. cffDNA is extracted from maternal plasma using a Qiagen Kit. Mutation analysis is performed using PCR and in some cases restriction enzyme digestion. We ascertained all cases since 2007 where NIPD was performed for a single gene or sex-linked disorder. Indication, NIPD result, pregnancy management, and postnatal diagnosis were determined. Health professional and patient views were surveyed using focus groups and questionnaires
Results. NIPD was performed in more than 200 cases at risk of severe sex-linked disorders, with invasive testing required in only 45%. Invasive testing was usually avoided following NIPD for achondroplasia (n=17), thanatophoric dysplasia (n=10) and others disorders (n=8). Levels of cffDNA were too low for analysis in 3 cases. Users and providers of NIPD considered it a valuable improvement in genetic prenatal diagnosis, but raised concerns over how it should be delivered
Conclusions. NIPD is useful, robust, acceptable approach to genetic prenatal diagnosis, reducing the need for invasive testing. Care must be taken to maintain high standards of pre-test counselling and informed consent.

76 - TRANSFECTION OF NON-TUMOR HOST'S CELLS DURING TUMOR PROGRESSION. AN EXPERIMENTAL STUDY IN NUDE MICE

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It has been hypothetized that tumor growth does not occur exclusively as a result of the division of the injected cells, but also involved recruitment of host cells. The goal of the present study was to examine the ability of rat colon cancer cells to spread tumor DNA in immunosuppressed mice, as well as to transfect host's cells. Nine nude mice were subcutaneously inoculated with DHD/K12-PROb rat colon cancer cells. The growth of subcutaneous tumors was monitored in all animals and recorded weekly. Mice were sacrificed 6 weeks after injection of cells. Prior to sacrifice, samples of blood were collected in tubes with EDTA and were subjected to

centrifugation at 1,800 x g for 10 min. Then, buffy coat and plasma were separately collected and the last was subjected to a second centrifugation at 3,000 x g for 10 min. Lungs were inspected visually for the presence or absence of pulmonary metastases, since it has been reported that DHD cells injected in rats lead to lung metastasis. Samples from tumors, lungs, buffy coats and plasma were analyzed by PCR for detection of rat and mouse k-ras sequences (mutated and non-mutated sequences). No animal showed lung macrometastasis. Rat sequences (mutated and non-mutated) were detected in all tumors and in six lung samples (67%). In no mouse circulating tumor cells were detected. In two samples of plasma (22%) mutated rat DNA was found, however, this detection was not matched with the detection of non-mutated rat DNA. In other two animals (22%) mutated murine k-ras sequences were detected in lungs. This results point that during the cancer progression non-tumor host's cells might be transformed by tumor cells. Further studies are needed to value the oncogenic ability of such transformed host's cells.

77 - CIRCULATING MICRORNAS IN PLASMA OF PATIENTS WITH ESOPHAGEAL SQUAMOUS CELL CARCINOMA

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Background. Several recent studies demonstrated that microRNAs (miRNAs) are stably detectable in plasma/serum. We hypothesized that plasma miRNAs concentrations contributed to potential biomarkers in patients with esophageal squamous cell carcinoma (ESCC).
Methods. We selected three oncogenic miRNAs (miR-21, miR-184, miR-221) and one tumor suppressive miRNA (miR-375), which are frequently reported in squamous cell carcinoma, as candidate targets for this plasma miRNA assay. This study was divided into three steps: 1) Determination of appropriate plasma miRNAs in prerliminary tests. 2) Evaluation of whether the plasma miRNAs assays could monitor tumor dynamics 3) Validation study on the clinical application of plasma miRNAs assays in 50 ESCC patients and 20 healthy volunteers.
Results. 1) In preliminary tests, the plasma level of miR-21 was significantly higher ($p=0.0218$) and that of miR-375 ($p=0.0052$) was significantly lower in ESCC patients than controls. 2) The high plasma miR-21 levels reflected tumor levels in all cases (100%). The plasma level of miR-21 was significantly reduced in postoperative samples ($p=0.0058$). 3) On validation analysis, the plasma level of miR-21 tended to be higher in ESCC patients ($p=0.0649$), while that of miR-375 was significantly lower ($p<0.0001$) and the miR-21/miR-375 ratio was significantly higher ($p<0.0001$) in ESCC patients than in controls. The value of the area under the receiver-operating characteristic curve (AUC) was 0.816 for the miR-21/miR-375 ratio assay. Patients with a high plasma level of miR-21 tended to have greater vascular invasion ($p=0.1554$) and to show a high correlation with recurrence ($p=0.0164$).
Conclusion. Detection of circulating miRNAs might provide new complementary tumor markers for ESCC.

78 - NOVEL DIAGNOSTIC VALUE OF CIRCULATING miR-18a IN PLASMA OF PATIENTS WITH PANCREATIC CANCER

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Background. Several recent studies have demonstrated that microRNAs (miRNAs) are detectable in plasma/serum. We hypothesized that miR-18a in plasma is a potential biomarker in patients with pancreatic cancer.
Methods. MiR-18a is located in the miR-17-92 cluster and reported to be highly expressed in pancreatic cancer tissues. This study was divided into three parts: 1) Confirmation of higher miR-18a levels in primary pancreatic cancer tissue and cell lines than normal pancreatic tissues and human fibroblasts. 2) Evaluation of the plasma miR-18a assay using quantitative RT-PCR by comparing results from 30 patients with pancreatic cancer and 30 healthy volunteers 3) Evaluation of the assay for monitoring tumor dynamics assay in patients with pancreatic cancer.
Results. 1) The expression of miR-18a was significantly higher in pancreatic cancer tissues ($p=0.019$) and pancreatic cancer cell lines ($p=0.015$) than normal tissues and fibroblasts. 2) The plasma concentrations of miR-18a were significantly higher in pancreatic cancer patients than controls ($p<0.0001$). The value of the area under the receiver-operating characteristic curve (AUC) was 0.9222. 3) The plasma levels of miR-18a were significantly correlated with those of their corresponding tumors by Spearman's test ($r2=0.821$, $p<0.0005$).The plasma levels of miR-18a were significantly lower in postoperative samples than preoperative samples ($p<0.0001$).
Conclusion. Circulating miR-18a might provide a new complementary tumor marker for pancreatic cancer.

79 - ALTERED SERUM microRNA EXPRESSION PROFILE IN PATIENTS WITH ASTROCYTOMA: POTENTIAL BIOMARKER FOR DIAGNOSIS, CLASSIFICATION AND PROGNOSIS OF ASTROCYTOMA

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Background. Astrocytic tumors are the most common primary brain tumors. Despite the recent advances in thera-

peutic strategies, the median survival time after diagnosis of grades IV has improved only marginally and is still <1 year. At present, no serum marker has been identified for astrocytoma. Therefore, astrocytoma remains a disease for which non-invasive, clinically applicable serum biomarkers for early detection and targeted prevention are highly desirable. The involvement of circulating microRNAs (miRNAs) in cancer and their potential as biomarkers of diagnosis and prognosis are becoming increasingly appreciated, however little is known about circulating miRNA expression profiles in astrocytoma. We used genome-wide serum miRNA expression analysis to assess the potential of serum miRNAs as non-invasive biomarker of diagnosis and prognosis of astrocytoma.
Methods. A multiphase, case-control study was designed to identify serum miRNAs as potential markers for astrocytoma. In the initial biomarker-screening stage, pooled serum samples from 44 astrocytoma patients of WHO grade III-IV and 43 normal controls, respectively, underwent Solexa sequencing to select miRNAs that showed differentially expressed between the high grade astrocytoma cases and matched controls. Subsequently, confirmation analysis was performed using a TaqMan probe-based RT-qPCR assay to refine the number of serum miRNAs selected in the Solexa set. In the biomarker selection stage, 33 astrocytoma serum samples (grade III and grade IV) and 33 normal controls formed a training set, whereas an additional independent cohort of 45 astrocytoma of grade III-IV and 47 controls formed a validation set. The miRNAs whose concentrations were verified to be significantly altered by RT-qPCR were further measured in patients with low grades of astrocytoma (including 15 grade I and 55 grade II) and in 14 patients with astrocyte hyperplasia. In addition, paired serum samples before and 1 to 14 days after operation were collected from 10 of the validation phase patients to determine the effect of surgery on the miRNAs' levels.
Results. Solexa sequencing results showed that the proportion of miRNA was 51.55%% and 21.49% in two pooled serum samples from control and astrocytoma, respectively. Compared to control, astrocytoma contained a relatively lower proportion of miRNA. Among the 904 miRNAs detected, there was a difference in the expression of 50 miRNAs in comparison between astrocytoma and normal sera samples, 35 were down-regulated and 15 were up-regulated. RT-qPCR analysis in the training set from separate individuals identified 7 miRNAs (miR-15b*, miR-23a, miR-133a, miR-150*, miR-197, miR-497 and miR-548b-5p) being significantly decreased in astrocytoma of grade III-IV (P<0.001) as compared with control group. RT-qPCR analysis in the validation set of a larger cohort further verified the significantly decreased concentrations of the 7 miRNAs in high grade of astrocytoma (P<0.0001). The levels of the 7 miRNAs were also markedly decreased in low grades of astrocytoma including grade I and grade II as compared with normal controls (P<0.01). Of the selected 7 miRNAs, 6 miRNAs, except miR-497, exhibited significantly different levels between grade I and grade II (P<0.01), while no statistical difference was observed in the 7 miRNAs levels among different grade of malignant cases (grade II, III and IV). In addition, the concentrations of 4 miRNAs including miR-23a, miR-150*, miR-197 and miR-548b-5p were markedly decreased in patients with astrocyte hyperplasia as compared with normal controls (P<0.0001), while 4 miRNAs including miR-23a, miR-150*, miR-197 and miR-548b-5p were significantly higher in astrocyte hyperplasia than in astrocytoma (P<0.0001). Unsupervised clustering analyses based on the seven-serum miRNA profile showed that 100% of astrocytoma of grade I and 82.5% of control samples; 96.5% of astrocytoma of grade II and 91.3% of control samples. ROC curve analysis was performed on the selected 7 miRNAs and yielded the following AUCs: for grade I astrocytoma and normal control, AUCs ranged from 0.892-1.000; for grade II astrocytoma and normal control, AUCs ranged from 0.795-0.979. For grade I and grade II astrocytoma, AUCs of 5 miRNAs including miR-15b*, miR-23a, miR-150*, miR-197, and miR-548b-5p ranged from 0.829-0.937. Moreover, the concentrations of the 7 serum miRNAs markedly increased after operation.
Conclusions. The profiles of serum miRNAs were markedly altered in patients with astrocytoma as compared with normal controls. We determined a profile of 7 serum miRNAs as a novel blood-based biomarker for the diagnosis, classification and prognosis of astrocytoma.

80 - ROUTINE FETAL RHD GENOTYPING IN MATERNAL BLOOD

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Objective. To assess the accuracy of routine fetal RHD genotyping in maternal blood in order to avoid routine anti-D gammaglobulin in the third trimestrer, in the 40% of women carrying a RhD negative fetus.
Study Design. RhD negative pregnant women from the general population, attending one of the 6 health centers of Barcelona-Esquerra health district, were offered fetal RHD genotyping in maternal blood, at 24-26 gestational weeks, at the time of second trimester venopuncture. When the fetus was found to be RHD negative, women were counseled about avoiding routine antenatal anti-D gammaglobuline. Diagnostic accuracy of RHD genotyping was evaluated and compared to conventional RhD typing in umbilical cord blood after delivery, as well as the difficulties and barriers found in its clinical application.
Results. During the first year of the study, 201 samples from RhD negative pregnant women were obtained, and RHD genotyping revealed 125 positives (62%) and 73 negatives (36%). Concordance with convenctional RhD typing was achieved in all singleton pregnacies. Among 10 twin pregnancies, a single discrepancy with RhD typing was found in one pregnacy were a paternal variant was suspected. Genotyping was alwasys accepted when offered, and anti-D gammaglobuline was not given in none of the RHD negative fetuses, except in one pregnancy.
Conclusions. Non invasive routine RHD genotyping in

RhD negative pregnant women is an accurate method to avoid unnecessary anti-D gammaglobulin.

81 - CIRCULATING NUCLEOSOMES AND BIOMARKERS OF IMMUNOGENIC CELL DEATH AS PREDICTIVE AND PROGNOSTIC MARKERS IN CANCER PATIENTS UNDERGOING LOCAL CYTOTOXIC THERAPY

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Background. During immunogenic cell death, released products of dying cells lead to a sustained stimulation of immune cells. This effect is relevant for the persistent response to cytotoxic therapies in cancer disease.
Patients and Methods. In a prospective observation study, blood samples were taken from 49 consecutive colorectal cancer patients with extensive hepatic metastases before, 24 and 48 hours after selective internal radiation therapy (SIRT). Serum levels of circulating nucleosomes, high mobility group box 1 (HMGB1), receptor of glycation end products (RAGE) and DNAse activity that play essential roles during immunogenic cell death – along with CEA and CA 19-9 – were compared with the response to SIRT and overall survival.
Results. Serum levels of nucleosomes, HMGB1 and DNAse activity were already increased 24 hours after SIRT whereas RAGE was decreased. There was a strong correlation between nucleosomes and HMGB1. Nucleosomes and HMGB1 levels 24 hours after SIRT were significantly higher in progressive (N=35) than in non-progressive patients (N=14) while there was no difference for RAGE and DNAse. Further, high nucleosome (24h) and HMGB1 (0h, 24h) levels were associated with poor overall survival. Similarly, high CEA and CA 19-9 levels were related to non-favourable therapy response and prognosis.
Conclusion. Circulating nucleosomes and HMGB1 as biomarkers of immunogenic cell death are valuable for prediction of therapy response and prognosis in colorectal cancer patients with liver metastases undergoing SIRT.

82 - CELL-FREE DNA IN PLASMA AND SERUM INDICATES DISEASE SEVERITY AND PROGNOSIS IN TRAUMA PATIENTS

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Background. Trauma is still a major causes for mortality of people <50 years. Biomarkers are needed to estimate the severity of the condition and the patient outcome.
Methods. Cell-free (cf) DNA was determined in plasma and serum of 164 patients at time of admission. Among them were 64 patients with multiple trauma (Injury Severity Score (ISS) ≥16), 51 patients with minor trauma (ISS<16) and 49 patients with single fractures (24 femur neck and 25 ankle fractures). Disease severity was objectified by ISS and Glasgow Coma Scale (GCS).
Results. Cf-DNA levels in plasma and serum were significantly higher in patients with multiple trauma than in those with minor trauma (each p=0.002) and with single fractures (each p<0.001). Cf-DNA in plasma and serum correlated very strongly with each other (R=0.89; p<0.001) as well as with levels of alanine-aminotransferase, creatine-kinase, glucose and inversely with hemoglobin (for all R>0.30 and p<0.05). AUC in ROC curves for identification of multiple trauma patients was 76.5% and 74.3% for cfDNA in plasma and serum, respectively. Within the group of multiple trauma patients, cf-DNA levels were higher in more severely injured patients (GCS<8 versus GCS>8). 13 of the multiple injured patients died during the first week after trauma. Levels of cfDNA were significantly higher in non-surviving patients than in survivers (p<0.001). AUC in ROC curves for identification of non-surviving patients was 82.3% and 80.8% for cfDNA in plasma and serum, respectively
Conclusion. Cf-DNA is valuable for estimation of trauma severity and prognosis of trauma patients.

83 - RELEVANCE OF PLASMA DNA INTEGRITY IN BREAST CANCER PATIENTS UNDERGOING NEOADJUVANT CHEMOTHERAPY

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Background. In breast cancer patients undergoing neoadjuvant chemotherapy before surgery, biomarkers for predicting the response to the therapy are highly needed.
Patients and Methods. Concentrations of ALU115, ALU247 and DNA integrity were analyzed in prospectively collected plasma of 68 patients with localized breast cancer (UICC II and III) and of 48 patients with metastatic breast cancer and 28 healthy women as controls. In 44 patients with breast cancer who had completed the course of chemotherapy until surgery, biomarkers were evaluated concerning response to therapy (no change, NC: N=9; partial or complete remission, R: N=35)
Results. Plasma levels of ALU 115 and ALU 247 were significantly higher in patients with localized (medians 16.3 and 16.8 ng/mL) and metastasized breast cancer (22.2 and 27.8 ng/mL) than in healthy controls (1.8 and 1.9 ng/mL). However, plasma DNA integrity showed only minor differences between the diagnostic groups. Concerning therapy response, ALU 115 and 247 were elevated in non-responsive patients (21.3 and 25.5 ng/mL) as compared with responsive ones (13.6 and 15.1 ng/mL). Median DNA integrity was slightly higher in non-responsive patients, as well. While AUCs in ROC

curves for non-response were 62.2% for ALU 115 and 61.1% for DNA integrity, the combination of both yielded an AUC of 70.3%.
Conclusion. While plasma DNA levels are valuable for discrimination of breast cancer patients from controls, DNA integrity provides additive information for prediction of response to neoadjuvant therapy.

84 - ARE MESENCHYMAL STEM CELLS SUSCEPTIBLE TO BE TRANSFORMED BY TUMOR CELL-FREE NUCLEIC ACIDS?

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Mesenchymal Stem cells (MSC) are being used in a variety of clinical trials for the treatment of a broad spectrum of diseases. Specifically, human adipose-derived stem cells (hASC) have shown a high efficiency and safety for the treatment of some digestive tract diseases. These cells meet all criteria from International Scientific Society to be considered as MSC: Positive for CD 29, CD 44, CD 90, CD 105; negative for CD 34, CD 45, and CD 133. Recently it has been demonstrated that NIH-3T3 cells can be transfected and oncogenically transformed by cell-free nucleic acids. In the present study, we examine the susceptibility of cultured hASC to be transformed by such nucleic acids, as a way to value the safety of the therapeutic use of those cells. Cultures of hASC were supplemented with samples of plasma from patients with K-ras–mutated colorectal tumors or from healthy subjects. Other plates of hASC were supplemented with DNA extracted from K-ras-mutated tumors. Cultures of NIH-3T3 cells were identically treated, as positive controls. K-ras–mutated sequences were never detected in hASC. By contrast, all cultures of NIH-3T3 cells with tumor DNA and most of the cultures with plasma were positive for the detection of the mutated sequences ok human K-ras. The injection of plasma-treated hASCs into nonobese diabetic–severe combined immunodeficient mice (NOD-SCID) did not produce any oncogenic effect. By contrast, the injection of NIH-3T3 cells that had been cultured with plasma from patients with colon cancer led to the development of carcinomas. In conclusion, hASC are not susceptible to be transfected, neither transformed by tumor cell-free nucleic acids. These results support the safety of the therapeutic use of such cells, even in a neoplasic environment.

86 - MULTIPLEX MASSIVELY PARALLEL SEQUENCING FOR NONINVASIVE PRENATAL DIAGNOSIS

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The possibilities of detecting fetal aneuploidies with massively parallel sequencing using fetal DNA in plasma have been shown. We intended to validate this on the Applied Biosystems SOLiD™ 4 platform, taking into account the multiplexing possibilities.
From 37 pregnant women at various gestational ages, blood samples were drawn prior to [euploid and aneuploid fetuses: N=25] or after [aneuploid fetuses: N=12] invasive procedures. Fetal aneuploidies were trisomies 13 [N=1], 18 [N=4] and 21 [N=8], 47,XXX [N=1] and 45,X (STC) / 46,XX (LTC) [N=1]. DNA was isolated from plasma using QIAamp DNA isolation kits. Sequencing libraries were prepared individually and DNA was multiplexed after ligation of a unique sequencing barcode(4, 8 or 16 samples simultaneously). Sequence reads were mapped to the human reference genome and quantified. -scores per chromosome were calculated as previously described. Thresholds for aneuploidy were set at >+3.0 and <-3.0 for over- or under-representation, respectively, of a chromosome.
All autosomal aneuploid fetuses showed -scores >3.0 for the aberrant chromosomes, without false-positive or -negative results. The correct determination of the sex-chromosomal -scores needs optimization, but the sex-chromosomal aneuploid samples showed expected aberrant -scores for the X-chromosomes: >3.0 for the 47,XXX fetus and <-3.0 for the 45,X (STC) / 46,XX (LTC) fetus, reflecting the analysis of cytotrophoblastic DNA. Our data indicate that full-blown fetal autosomal aneuploidies can be reliably detected in maternal plasma using a SOLiD™ 4 multiplex massively parallel sequencing approach. Improvements in the multiplexing rate to reduce costs and increase efficiency are under investigation.

87 - CANCER PROGRESSION MEDIATED BY HORIZONTAL GENE TRANSFER. AN IN VITRO AND IN VIVO STUDY

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Background. The current paradigm in cancer progression is that it occurs via vertical gene transfer, it means the offspring of initiating tumor cell inherit the genetic and epigenetic alterations leading to invasion and metastases. It has been hypothetized that horizontal gene transfer may aid in this process.
Methods. Supernatants and serum from cancer cell lines and a colon cancer patient were used to prove the horizontal gene transfer to recipient immortal murine cell line NIH3T3. Nude mice and rats, and immunocompetent Wistar rats were used for in vivo experiments. Results. Murine NIH3T3 cells (cytogenetically characterized to be murine) exposed for 14 days to SW480 supernatant and serum from a colon cancer patient, but not those exposed to their own supernatant and to a healthy individual'serum showed: i) morphological transformation, ii) the ability to grow in agar, iii) to form tumors in nu/nu mice. Transformed murine cells acquired mutant human K-ras

as evaluated by PCR-sequencing and RT-PCR. To demonstrate that the transforming ability of supernatant of SW480 resides in the DNA, these experiments on "passive transfection" were repeated with supernatant pre-treated with a mix of DNAse and proteases, as expected, no transformation of recipient NIH3T3 cells was observed. Further confirmation of transformation was obtained by "active" or lipofectamine transfection to recipient cells with the genomic DNA isolated from supernatant of SW480 cells. NIH3T3 transformed cells (SWB1 cells subline) became "stable transfected" by supernatant exposure (passive transfection) after several passages FISH analyses demonstrated the presence of human repetitive sequences. Comparative Genomic Hybridization in SWB1 cells gave positive signal to hundreds of human genes. Genes MAPRE, GRB2, CADH8 and RAB30 which have demonstrated role in colon cancer were selected to confirming their presence by RT-PCR-sequencing and RT-PCR. All these genes were expressed in the SWB1 cells and were confirmed to be of human and mouse origin by sequencing. Passive transfection with the supernatant of SW480 cells was unable to transform normal human cells (fibroblasts), accordingly, intravenous daily injection of the supernatant for 1 month to nu/nu rats did not lead to histological alterations or cancer development in animals suggesting that cells need to be "initiated" in order to be transformed by supernatant or serum DNA. To prove that horizontal gene transfer leads to cancer progression, groups of 7 Westar rats were treated with the colon carcinogen dimetylhydrazine for 3 months, at month six, 10 million SW480 colon cancer cells were s.c. injected into the flank (no tumors were formed in any animal as expected) and then rats received treatment with recombinant DNAse and proteases by ip. route. Groups were as follows: i) control; ii) SW480 injection; iii) carcinogen; iv) carcinogen +SW480 injection; v) carcinogen +SW480 injection+ DNAse + proteases; vi) carcinogen+DNAse+protease. Animals were monitored with microPET-CT for tumor formation, sacrificed and autopsied. Colon adenocarcinomas were observed in: group i) 0/7; group ii) 0/7; group iii) 2/7; iv) 5/7; v) 2/7; vi) 2/7. Of note, all adenocarcinomas in groups injected with SW480 were signet-ring cell carcinomas and presented higher regional invasion and but none in the carcinogen alone or with DNAse+protease suggesting rat colon epithelium initiated by the carcinogen was fully transformed by circulating DNA originated from the SW480 cells. To confirm this, microdissected tumor cells from rat colonic tumors that received injections of SW480 cells were positive for human mutated K-ras, MAPRE, GRB2, CADH8 and RAB30 as evaluated by PCR-sequencing. Tumor cells from SW480 injected animals were FISH positive for human and rat repetitive sequences whereas carcinogen only rat tumors were negative for human and positive for rat sequences.

Conclusions. These results demonstrate that horizontal gene transfer occurs in vivo and induces tumor progression.

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Index of Authors